REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

Established: 1959.

The year 2014 is the 56th anniversary of continuous publication

ISSN 0036-4665
ISSN 1678-9946 on line

The Revista do Instituto de Medicina Tropical de São Paulo is abstracted and/or indexed in: Index Medicus, Biological Abstracts, EMBASE/Excerpta Medica, Hepatology/Rapid Literature Review, Tropical Diseases Bulletin, Referativnyi Zhurnal: All-Russian Institute of Scientific and Technical Information (VINITI), Periódica - Índice de Revistas Latinoamericanas en Ciencias, Helminthological Abstracts, Protozoological Abstracts, Review of Medical and Veterinary Mycology, PubMed, UnCover, HealthGate, OVID, LILACS, MEDLINE, New Jour, ExtraMED, Free Medical Journals, ISI (Institute for Scientific Information), BIOSIS Previews, Scopus, Science Citation Index Expanded (SciSearch), Journal Citation Reports/Science Edition, Current Contents®/Clinical Medicine and Index Copernicus.

ON LINE ACCESS - http://www.imt.usp.br/portal/ - FREE PDF ACCESS TO ALL PAST ISSUES, from 1959 on (Financial support by "Alves de Queiroz Family Fund for Research).

http://www.scielo.br/rimtsp - FULL TEXT, SINCE 1984. E-mail: revimtsp@edu.usp.br

Reprints may be obtained from Pro Quest Inf. and Learning, 300 North Zeeb Road, Ann Arbor, Michigan 48106-1346 - USA.

The Revista do Instituto de Medicina Tropical de São Paulo is supported by: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Universidade de São Paulo and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

This issue was financed by: CNPq Proc. 405008/2013-9.

Desktop Publishing by: *Hermano* - e-mail: hermano@nextis.com. Phone: 55.11.5571-8937. - **Printed by:** *Elyon Indústria Gráfica,* Phone: 55.11.3783-6527. **English Revision:** global@globaltranslations.com.br

The purpose of the "Revista do Instituto de Medicina Tropical de São Paulo" (Journal of the São Paulo Institute of Tropical Medicine) is to publish the results of researches which contribute significantly to knowledge of all transmissible diseases.

REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO (JOURNAL OF THE S. PAULO INSTITUTE OF TROPICAL MEDICINE).
São Paulo, SP-Brasil, 1959 -
v. ilust. 28 cm

1959-2013, 1-55
1973-2002 (supl. 1-12)
2003 (supl. 13 - on-line only)
2005-2012 (supl. 14-18)
2014, 56 (1-4)

ISSN 0036-4665
ISSN 1678-9946 on line

REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

Impact Factor: 0.959

ISSN 0036-4665
ISSN 1678-9946 on line

Rev. Inst. Med. Trop. Sao Paulo	Vol. 56	No. 4	P. 277-368	July-August, 2014

CONTENTS

ADDRESS
INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO
Av. Dr. Enéas de Carvalho Aguiar, 470
05403-000 São Paulo, SP - Brazil
Phone/Fax: 55.11.3062.2174; 55.11.3061-7005
e-mail: revimtsp@edu.usp.br

SUBSCRIPTIONS
FOREIGN COUNTRIES
One year (six issues) ..... U$ 200.00
Single issue ..... U$ 50.00

REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

Impact Factor: 0.959

ISSN 0036-4665
ISSN 1678-9946 on line

Rev. Inst. Med. Trop. Sao Paulo	Vol. 56	No. 4	P. 277-368	Julho-Agosto, 2014

CONTEÚDO

ENDEREÇO
INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO
Av. Dr. Enéas de Carvalho Aguiar, 470
05403-000 São Paulo, SP - Brasil
Fone/Fax: 55.11.3062.2174; 55.11.3061-7005
e-mail: revimtsp@edu.usp.br

Rev. Inst. Med. Trop. Sao Paulo
56(4):277-279, July-August, 2014
doi: 10.1590/S0036-46652014000400001

SEROLOGIC SURVEY ON HANTAVIRUS IN BLOOD DONORS FROM THE STATE OF SANTA CATARINA, BRAZIL

Caio Maurício Mendes de CORDOVA(1) & Luiz Tadeu Moraes FIGUEIREDO(2)

SUMMARY

Emergent diseases such as Hantavirus Cardio-pulmonary Syndrome (HCPS) are able to create a significant impact on human populations due to their seriousness and high fatality rate. Santa Catarina, located in the South of Brazil, is the leading state for HCPS with 267 reported cases from 1999 to 2011. We present here a serological survey on hantavirus in blood donors from different cities of the state of Santa Catarina, with an IgG-ELISA using a recombinant nucleocapsid protein from Araraquara hantavirus as an antigen. In total, 314 donors from blood banks participated in the study, geographically covering the whole state. Among these, 14 individuals (4.4%) had antibodies to hantavirus: four of 50 (8% positivity) from Blumenau, four of 52 (7.6%) from Joinville, three of 50 (6%) from Florianópolis, two of 50 (4%) from Chapecó and one of 35 (2.8%) from Joaçaba. It is possible that hantaviruses are circulating across almost the whole state, with important epidemiological implications. Considering that the seropositive blood donors are healthy individuals, it is possible that hantaviruses may be causing unrecognized infections, which are either asymptomatic or clinically nonspecific, in addition to HCPS. It is also possible that more than one hantavirus type could be circulating in this region, causing mostly benign infections.

KEYWORDS: Hantavirus; Seroprevalence; Blood Donors; Hantavirus Cardio-pulmonary Syndrome.

INTRODUCTION

Emergent diseases that are commonly caused by zoonotic microbes, such as Hantavirus Cardio-Pulmonary Syndrome (HCPS), cause a significant impact on human populations due to their seriousness and high case fatality rates. Hantaviruses are 100 to 120 nm in diameter tri-segmented RNA viruses of the Bunyaviridae family. The large (L) RNA segment codes for a RNA polymerase, the medium (M) segment encodes the glycoproteins of viral surface (Gn and Gc), and the small segment (S) codes for the nucleocapsid (N) protein, which is an important viral antigen and induces antibody production after infection[3]. Small mammals, especially rodents, are the natural reservoirs of hantavirus. The transmission of these viruses to man occurs by the inhalation of contaminated aerosols containing excreta of infected rodents. In the Americas, hantaviruses are the cause of HCPS, which has been recognized since 1993[5]. In Brazil, about 1600 HCPS cases were reported from 1993 to 2013 with a 40% case fatality rate (Source: SVC/MS). Five hantaviruses are known to cause HCPS in Brazil: Anajatuba, Araraquara (ARAV), Castelo dos Sonhos (CSV), Juquitiba (JUQV), and Laguna Negra-like virus[3]. Some factors could be highlighted as contributors to the emergence of hantaviruses, including ecological degradation with deforestation for poorly planned urban expansion, intensive agriculture and cattle raising.

In recent years, a recombinant N protein of ARAV was developed, which has been used as an antigen in an ELISA for the diagnosis of hantavirus infections[4]. This ELISA is considered an important weapon for serologic surveillance of hantavirus infections in humans and rodents in Brazil and has been used in many epidemiological studies.

Santa Catarina, located in the South of Brazil, is the leading state on HCPS reported cases (Source: SVC/MS). However, the prevalence and other factors related to hantavirus infection and HCPS are not well known in the state. We present here a serologic survey of hantavirus in blood donors from different cities of the state of Santa Catarina, by using an IgG-ELISA with the N recombinant protein of ARAV.

MATERIAL AND METHODS

Participants and serum samples: In total, 317 blood donors from both genders (101 men and 216 women), from ages of 18 to 68 years participated in this serological survey. From February through March 2012, these participants donated blood and those that signed a consent form were enrolled in the study. In order to geographically represent the whole state of Santa Catarina, 40 to 50 participants from each of the blood banks in the state's larger cities were included in the study: Blumenau city in the Itajaí Valley; Joinville, in the north of the state; Florianópolis city,

(1) Universidade de Blumenau-FURB, Depto. de Ciências Farmacêuticas, Blumenau, SC, Brazil.
(2) Universidade de São Paulo-USP-RP, Faculdade de Medicina, Centro de Pesquisa em Virologia. Ribeirão Preto, SP, Brazil.
Correspondence to: Prof. Dr. Caio M.M. de Cordova, Universidade de Blumenau, Depto. de Ciências Farmacêuticas, Rua São Paulo 2171, Campus III, 89030-000 Blumenau, SC, Brasil. Tel: +55 47 3321 7318. E-mail: cmcordova@furb.br

in the eastern coast; Criciúma city, in the south of the state; Lages city, in the central mountains plateau; and Chapecó city and Joaçaba city, in the west of the state. A 5 mL blood sample was collected in Vacutainer™ (Becton Dickinson, Franklin Lakes, NJ, USA) from each participant by an officer of the blood bank, together with samples used for routine serologic tests. The blood was centrifuged at 3,000 rpm for five min; the serum was separated, carried to the laboratory on ice and stored at -20 ºC until use. This Research Project was approved by the Ethics Committee for Human Research of the Regional University of Blumenau (Protocol nº 155/10), in accordance with the Helsinki Declaration of 1975 as revised in 1983 and the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS/OMS 1982 & 1993).

IgG-ELISA for hantavirus: The serum samples were tested in duplicate by an anti-hantavirus IgG-ELISA using the N recombinant protein of ARAV as antigen. Tests were performed as previously described[4]. Briefly, polystyrene microtiter plates (polystyrene high biding, Corning) were coated overnight in a wet chamber at 4 °C with 2 µg/mL of ARAV N protein, or control antigen. All incubations were conducted at 37 °C for one hour and plates were washed six times with a wash buffer (phosphate-buffered saline [PBS]–0.1% Tween 20) between each step. Firstly, 150 µL of a blocking solution containing 10% skimmed milk in powder in [PBS]–0.1% Tween 20 was added to the wells. All serum samples were diluted 1:100 in dilution buffer (5% skimmed milk powder in PBS-Tween-20), as were the positive and the negative control sera, and 50 µL was added to the antigen-coated wells. Peroxidase-labeled affinity-purified goat anti-human IgG Fc antibody (50 µL/well) was added and specific antibody binding was detected by the addition of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (100 µL/well), with the absorbance measured at 405 nm. Values were expressed as the optical density (OD) obtained with ARAV N antigen minus the OD values for the control antigens. The cut-off value of the test was determined by the mean plus three standard deviations of the ODs obtained for at least four negative-control serum samples, plus three standard deviations of the mean.

RESULTS AND DISCUSSION

This serologic survey of the hantavirus included 314 participants from blood banks geographically covering the whole state of Santa Catarina (Fig. 1). Of these, 14 individuals (4.4%) had antibodies to hantavirus: four of 50 (8% positivity) from Blumenau city, four of 52 (7.6%) from Joinville city, three of 50 (6%) from Florianópolis city, two of 50 (4%) from Chapecó city and one of 35 (2.8%) from Joaçaba city. No seropositive results were observed among the 42 participants of Criciúma city or 35 participants from Lages city.

The levels of antibody to hantavirus in blood donors in any state could provide information on the circulation of these viruses in the entire state, including geographical areas where HCPS has not been reported.

Hantavirus infections causing HCPS are well known in the state of Santa Catarina, as 267 confirmed cases were reported from 1999 through December 2011. Curiously, only a few HCPS cases have been reported on the eastern coast in the state Capital Florianópolis, and in the north-eastern region of Joinville[2]. In the present study, more than 50% of individuals that were seropositive for hantavirus were from these regions. Antibodies to hantavirus were not detected in participants from the South

Fig. 1 - Map of the South of Brazil showing the city location of blood banks of the state of Santa Catarina that provided participants for the Hantavirus serological survey. Source: http://maps.google.com/

of Santa Catarina (Criciúma city). However, a study in a rural population next to Criciúma city showed a 2.3% seroprevalence to hantavirus and all seropositive individuals referred a previous severe pneumonia that could have been HCPS[6].

This study shows that 4.4% of blood donors are infected by hantavirus in most of the Santa Catarina territory. Considering that blood donors are commonly local inhabitants, it is possible that hantaviruses are circulating across practically all of the state. Of note, a study including 340 individuals inhabiting two cities in the western border of the state of Santa Catarina with Argentina, found 3.5% of individuals to be seropositive for hantavirus[7]. It might be worth noting that hantavirus infection is usually acquired by contact with wild rodents, often in the rural areas, and not in the urban environment. Blood Banks as the ones evaluated in the present study usually receive donors from the whole region they serve, or even from abroad. Additional studies evaluating the precise origin of the seropositive donors might be of interest to determine the possible site of origin of hantavirus exposure.

Considering that the seropositive blood donors are healthy individuals, it is possible that hantaviruses may be causing unrecognized infections, which are either asymptomatic or clinically nonspecific, in addition to HCPS. It is also possible that more than one hantavirus type could be circulating in this region, causing mostly benign infections. Once immune activation has been associated with HCPS pathogenesis, it is possible that some polymorphisms in genes involved in immune response may affect the development of HCPS[1]. Further studies are necessary in order to explain unrecognized infections by hantaviruses.

RESUMO

Inquérito sorológico para hantavírus em doadores de sangue no Estado de Santa Catarina, Brasil

Doenças emergentes como a Síndrome Cárdio-Pulmonar por Hantavírus (SCPH) são capazes de promover um grande impacto nas populações humanas devido a sua severidade e alta letalidade. Santa Catarina, localizada ao Sul do Brasil, é o estado com o maior número

de relatos de SCPH, com 267 casos desde 1999 a 2011. Apresentamos aqui um inquérito sorológico para hantavírus em doadores de sangue de diferentes cidades do Estado de Santa Catarina, usando um teste IgG-ELISA com proteína recombinante de nucleocapsídeo do hantavírus Araraquara como antígeno. No total, 314 doadores de banco de sangue participaram do estudo, cobrindo geograficamente todo o Estado. Dentre estes, 14 indivíduos (4,4%) apresentaram anticorpos para hantavírus: quatro de 50 (8% de positividade) provenientes de Blumenau, quatro de 52 (7,6%) de Joinville, três de 50 (6%) de Florianópolis, dois de 50 (4%) de Chapecó e um de 35 (2,8%) de Joaçaba. É possível que hantavírus estejam circulando praticamente em todo o Estado, com implicações epidemiológicas importantes. Considerando que os doadores de sangue soropositivos são indivíduos saudáveis, é possível que hantavírus possam estar causando infecções não diagnosticadas, sejam elas assintomáticas ou clinicamente inespecíficas, além da SCPH. É possível ainda que mais de um tipo de hantavírus possa estar circulando na região, causando infecções mais benignas.

ACKNOWLEDGEMENTS

The authors acknowledge all blood donors that kindly agree to participate of this study. The authors also acknowledge the directors and officers of the blood bank of the state of Santa Catarina (HEMOSC) that participated in the study.

AUTHORS CONTRIBUTIONS

Cordova CMM was responsible for sample collection, experimental procedures and writing the manuscript, and collaborated in study design; Figueiredo LTM was responsible for the study design, and collaborated in the experimental procedures and writing the manuscript.

FINANCIAL SUPPORT

Ministry of Health, Brazil; Ministry of Science and Technology; State Secretary of Health of Santa Catarina; Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina; University of Blumenau; MS/CNPq/FAPESC/SES PPSUS 003/2010 research grant nº 3.747-2011-8.

REFERENCES

1. Borges AA, Donadi EA, Campos GM, Moreli ML, de Sousa RL, Saggioro FP, *et al*. Association of -308G/A polymorphism of tumor necrosis factor-α gene promoter with susceptibility to development of hantavirus cardiopulmonary syndrome in the Ribeirão Preto region, Brazil. Arch Virol. 2010;155:971-5.

2. DIVE-SES-SC. Diretoria de Vigilância Epidemiológica, Secretaria de Estado da Saúde de Santa Catarina 2012. A hantavirose no Estado de Santa Catarina, 2000-2011. Florianópolis: Secretaria de Estado da Saúde. Sistema Único de Saúde. Superintendência de Vigilância em Saúde. Diretoria de Vigilância Epidemiológica. [cited 2013 Jan 28]. Available from: http://www.dive.sc.gov.br/conteudos/zoonoses/RoedoresAves/Hantavirose/Hantavirose_no_estado_SC.pdf

3. Figueiredo GG, Borges AA, Campos GM, Machado AM, Saggioro FP, Sabino Júnior GS, *et al*. Diagnosis of hantavirus infection in humans and rodents in Ribeirão Preto, State of São Paulo, Brazil. Rev Soc Bras Med Trop. 2010;43:348-54.

4. Figueiredo LT, Moreli ML, Borges AA, de Figueiredo GG, Badra SJ, Bisord I, *et al*. Evaluation of an enzyme-linked immunosorbent assay based on Araraquara virus recombinant nucleocapsid protein. Am J Trop Med Hyg. 2009;81:273-6.

5. Nichol ST, Spiropoulou CF, Morzunov S, Rollin PE, Ksiazek TG, Feldmann H, *et al*. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science. 1993;262(5135):914-7.

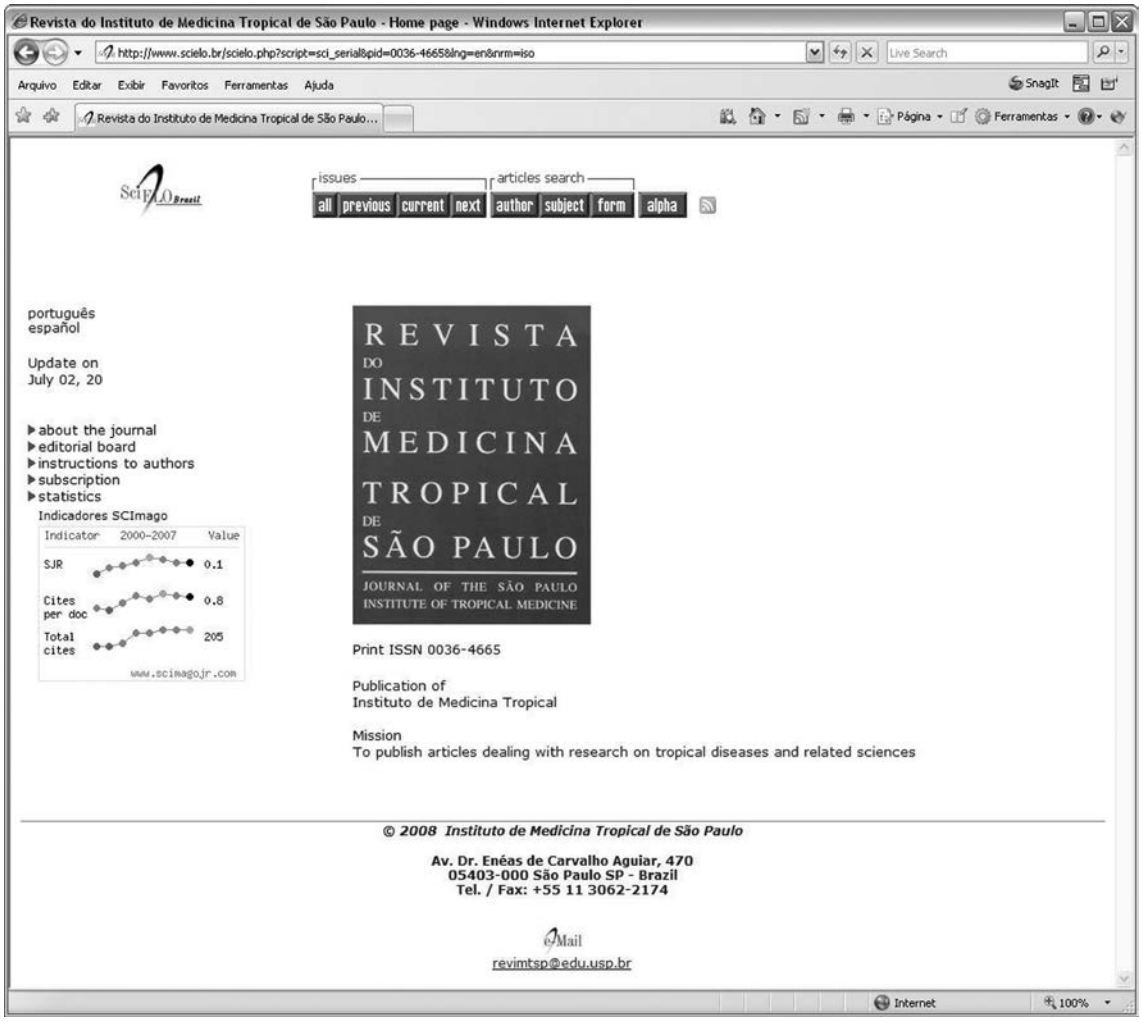
6. Pereira GW, Teixeira AM, Souza MS, Braga AD, Santos Junior GS, Figueiredo GG, *et al*. Prevalence of serum antibodies to hantavirus in a rural population from the southern state of Santa Catarina, Brazil. Rev Soc Bras Med Trop. 2012;45:117-9.

7. Souza WM, Machado AM, Disner GR, Boff E, Machado ARSR, Pádua M, *et al*. Antibody levels to hantavirus in inhabitants of western Santa Catarina State, Brazil. Rev Inst Med Trop Sao Paulo. 2012;54:193-6.

Received: 27 March 2013
Accepted: 8 January 2014

Revista do Instituto de Medicina Tropical de São Paulo
on line.

Publications from 1984 to the present data are now available on:
http://www.scielo.br/rimtsp
PAST ISSUES FROM 1959 ON (PDF)
www.imt.usp.br/portal/



SciELO – The Scientific Electronic Library OnLine - SciELO is an electronic virtual covering a selected collection of Brazilian scientific journals.

The library is an integral part of a project being developed by FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo, in partnership with BIREME – the Latin American and Caribbean Center on Health Sciences Information.

SciELO interface provides access to its serials collection via an alphabetic list of titles or a subject index or a search by word of serial titles, publisher names, city of publication and subject.

The interface also provides access to the full text of articles via author index or subject index or a search form on article elements such as author names, words from title, subject and words from full text.

FAPESP/BIREME Project on Scientific Electronic Publications
Latin American and Caribbean Center on Health Sciences Information
Rua Botucatu 862 – 04023-901 São Paulo, SP – Brazil
Tel. (011) 5576-9863
scielo@bireme.br

Rev. Inst. Med. Trop. Sao Paulo
56(4):281-285, July-August, 2014
doi: 10.1590/S0036-46652014000400002

PREVALENCE OF PARACOCCIDIOIDOMYCOSIS INFECTION BY INTRADERMAL REACTION IN RURAL AREAS IN ALFENAS, MINAS GERAIS, BRAZIL

Evandro Monteiro de Sá MAGALHÃES(1), Carla de Fátima RIBEIRO(1), Carla Silva DÂMASO(1), Luiz Felipe Leomil COELHO(1), Roberta Ribeiro SILVA(2), Eric Batista FERREIRA(3), Maria Rita RODRIGUES(4), Zoilo Pires de CAMARGO(5), Tânia Regina Grão VELLOSO(6) & Luiz Cosme Cotta MALAQUIAS(1)

SUMMARY

This study aimed to estimate the prevalence of paracoccidioidal infection by intradermal reaction (Delayed-Type Hypersensitivity, DTH) to *Paracoccidioides brasiliensis* in rural areas in Alfenas, Southern Minas Gerais (MG) State, Brazil, and to assess risk factors (gender, occupation, age, alcohol intake and smoking) associated with infection. We conducted a population-based cross-sectional study using intradermal tests with gp 43 paracoccidioidin in 542 participants, who were previously contacted by local health agents and so spontaneously attended the test. Participants underwent an interview by filling out a registration form with epidemiological data and were tested with an intradermal administration of 0.1 mL of paracoccidioidin in the left forearm. The test was read 48 hours after injection and was considered positive if induration was greater than or equal to 5 mm. Out of 542 participants, 46.67% were positive to the skin test. Prevalence increased in accordance with an increase of age. There was statistical significance only for males. Occupation, alcohol intake and smoking habits were not significantly associated with the risk of paracoccidioidomycosis infection. There is relevance of paracoccidioidomycosis infection in such rural areas, which suggests that further epidemiological and clinical studies on this mycosis should be done in the southern part of Minas Gerais State.

KEYWORDS: Paracoccidioidomycosis; Epidemiology; Health surveys; Intradermal tests.

INTRODUCTION

Paracoccidioidomycosis is a systemic mycosis, endemic and limited to Latin America. It is caused by the dimorphic species of fungus *Paracoccidioides brasiliensis* or *Paracoccidioides lutzii* which can exist as a mycelial stage and yeast[26,36]. The mycelial form is found in nature at temperatures between 18 °C and 25 °C and produces spores or yeast-like conidia which may cause infections. Spores inhaled by susceptible hosts are converted into yeast in the tissues. By inhalation spores target the lungs and later on reach any systemic structure through the lymphatic or the blood stream, especially affecting skin, mucous membranes, lymphatic tissue and adrenal glands[13,33].

P. brasiliensis, or *P. lutzii,* lives in argillaceous or sandy soil with adequate humidity. This fact points out the possibility of saprophytic life in soil, rich in organic matter, humid and protected against sunlight[27].

Paracoccidioidomycosis distribution is heterogeneous showing high and low endemicity in different areas, in accordance with the climate and the agricultural conditions in the region. In Brazil, which is responsible for more than 80% of paracoccidioidomycosis cases in the world, asymptomatic infection is revealed by a cutaneous (Delayed-Type Hypersensitivity) test using the antigen paracoccidioidin. This disease has higher prevalence in the Southeast, South and Central West regions[20]. It is believed that about 10% of the population may have been infected by the fungus, which represents a more impressive percentage than that related to other neglected diseases, e.g., schistomiasis and leishmaniasis[14,20]. There are also reports of cases in non endemic areas, related to people who had lived in or visited Latin America before the beginning of signs and symptoms of the disease. Under this condition paracoccidioidomycosis is considered a traveler's disease[18,25].

The disease is mainly found among men who work in rural areas and it affects them between the ages of 30 to 50 years old when they are supposed to produce at their most productive age. For this reason, this disease has an important impact on the production chain and on public health[2,3,19]. Recently, it was included among the neglected diseases, that have a significant impact on public health and that are not sufficiently quantified due to the lack of available information, that paracoccidioidomycosis is not a disease of compulsory notification[10,19].

With this in mind, the goal of this study is to quantify the prevalence of people prone to *P. brasiliensis* in rural areas, in Alfenas - Southern

List of Abbreviations and Acronyms: IDR**:** intradermal reaction; CI: Confidence Interval.

(1) Instituto de Ciências Biomédicas, Universidade Federal de Alfenas, MG, Brazil.
(2) Faculdade de Nutrição, Universidade Federal de Alfenas, MG, Brazil.
(3) Instituto de Ciências Exatas, Universidade Federal de Alfenas, MG, Brazil.
(4) Faculdade de Ciências Farmacêuticas, Universidade Federal de Alfenas, MG, Brazil.
(5) Departamento de Biologia Celular, Universidade Federal do Estado de São Paulo, SP, Brazil.
(6) Departamento de Clínica Odontológica, Universidade Federal do Espírito Santo, ES, Brazil.
Correspondence to: Luiz Cosme Cotta Malaquias, Phone: + 55.35.3299-1305, Fax: +55.35.3299-1063. E-mail: luizcosme@hotmail.com

MG, as well as to evaluate the influence of age, gender, alcohol intake, smoking and occupation associated with infection.

MATERIALS AND METHODS

Population studied: This study was carried out in the rural area, in Alfenas-MG, Brazil, from May to December 2009. The selected population to be studied consisted of 542 people, who lived in rural area. The selection was carried out on spontaneous demand. The participants were previously contacted by health agents. On the scheduled day, participants in the study (male and female, over 10 years old) were submitted to an interview, filling out a registration form, which presented epidemiologic data. Pregnant women were excluded. The written informed consent was obtained from each participant, taking into consideration the resolutions of the Brazilian National Health Council No. 196/1996 and 347/2005.

Intradermal tests The antigen used in the present work for the intradermal test was the exoantigen of *P. brasiliensis* (strain B-339), rich of gp43 (minimum concentration of 70%) and was referred to as gp43 paracoccidioidin. The purified glycoprotein of 43 kD from *P. brasiliensis* (gp43) was tested as paracoccidioidin, in DTH tests, in both experimental animals and patients with paracoccidioidomycosis, and compared with the traditional polysaccharide Fava Netto antigen.

The use of gp43 as paracoccidioidin in humans showed that this molecule can be used to evaluate the DTH response in patients with PCM. They found that patients who were responsive to *P. brasiliensis* antigen, 92.3% reacted against gp43 and 53.8% reacted against Fava Netto antigen. Gp43 skin test responses were significantly higher than those obtained with Fava Netto antigen, demonstrating the efficacy of this preparation[30].

The intradermal reaction tests were done by injecting 0.1 mL of exoantigen in the left forearm and readings of possible intradermal reaction were taken 48 hours after the injection. Induration equal to 5 mm or over was interpreted as a positive result.

Clinical, radiological and serological evaluation: Blood samples were taken, in order to perform hemogram and double agar gel immune diffusion tests. All IDR positive individuals were submitted to chest x-rays and a medical examination (one examining doctor) to evaluate and exclude possible clinical disease in activity and/or sequelae.

Statistical tests: The data were submitted to statistical analysis by means of software R[24]. Analysis of frequency distribution of the main variables was performed, in order to characterize the population of the present study. The existence of association among the qualitative variables was evaluated by the chi-square test and the level of significance was 5%. The logistic regression analysis was performed to confirm and inform the *odds ratio*, in relation to the factors that were significant, expressing the protectoral or potentializing effect on the studied disease.

RESULTS

A total of 557 intradermal reaction tests were performed but 15 individuals did not return for the results and were excluded from the research. Of the remaining 542 studied individuals, positive reaction to intradermal injection of paracoccidioidin was observed in 46.67% (Confidence Interval 95% 0.42 to 0.51) of participants, which corresponds to 253 individuals. Of the individuals tested, 270 (49.82%) belonged to the male gender and 272 (50.18%) to the female gender. The age varied from 11 to 86 years old and the average age was 43 years. In relation to occupation, most participants reported regular activity in rural areas (53.50%) revealing major exposure to *P. brasiliensis*. In relation to habits, 29.33% were addicted to smoking and 20.67% to alcohol (Table 1). None of the participants assessed presented clinical signs, radiological and serological findings indicative of disease activity. Table 2 shows the distribution of the intradermal reaction adjusted for age, gender, occupation, tobacco and alcohol use. Among subjects with positive IDR, 56.30% were men (Confidence Interval 95% 0.50 to 0.62) and 37.13% women (Confidence Interval 95% to 0.43) ($p < 0.001$). The prevalence rate of sensitization grew progressively, in accordance with increasing age. From 11 to 29 years old, the prevalence rate was 35.37%; from 30 to 49, it was 49.56% ($p < 0.01$). Over 50 years old, the rate was 52.66% ($p < 0.01$), being the highest one.

Table 1
Frequency distribution of intradermal test, gender, occupation, smoking and alcohol intake of the residents of an endemic area for paracoccidioidomycosis in 2009 in Alfenas, MG, Brazil

Variants		Number of individuals	%
*IDR			
	Negative	289	53.33
	Positive	253	46.67
	Total	542	100.0
Gender			
	Male	270	49.82
	Female	272	50.18
	Total	542	100.0
Occupation			
	Farm work	290	53.50
	Others	252	46.50
	Total	542	100.0
Smoking			
	Yes	159	29.33
	No	383	70.67
	Total	542	100.0
Alcohol intake			
	Yes	112	20.67
	No	430	79.33
	Total	542	100.0

*IDR = Intradermal reaction.

In relation to occupation (work in farming), 48.28% were positive to the IDR test. People addicted to tobacco were 52.2% positive; people addicted to alcohol were 55.36% positive. On the other hand, there was no statistical significant association between positive intradermal reaction (sensitization) and professional activity and a smoking habit ($p > 0.05$). However, addiction to alcohol revealed an influence to the level of positive

Table 2
Intradermal results distributed by gender, profession, age range, smoking and alcoholism variants

Variants		Intradermal reaction						*p*-value*
		Positive		Negative		Total		
		***N (253)	%	N (289)	%	N (542)	%	
Age range	11-29	52	35.37	95	64.63	147	27.12	0.004674
	30-49	112	49.56	114	50.44	226	41.70	
	> 50	89	52.66	80	47.34	169	31.18	
Gender	Female	101	37.13	171	62.87	272	50.18	0.00001
	Male	152	56.30	118	43.70	270	49.82	
Occupation	Farm work*	140	48.28	150	51.72	290	53.51	0.4758
	Nonfarm work	113	44.84	139	55.16	252	46.49	
Smoking	Yes**	83	52.20	76	47.80	159	29.34	0.1174
	No	170	44.39	213	55.61	407	70.66	
Alcohol intake	Yes**	62	55.36	50	44.64	112	20.66	0.04994
	No	191	44.42	239	55.58	430	79.33	

*It refers to chi-square test (5% of significance). **This group contains the remaining individuals and the formers. ***N = number of individuals.

Table 3
Parameters estimation of logistic regression, odds ratio and its confidence interval of 95% for infection prevalence by *Paracoccidioides brasiliensis* (intradermal positive reaction) only for significant variants

Variables	Estimation	*Odds ratio*	*CI95%	*p*-value
Male gender	0.768424	2.16	1.53 to 3.05	1.33×10^{-5}
11 to 29 years	-0.68334	0.50	0.32 to 0.81	0.00372
30 to 49 years	-0.03918	0.96	0.63 to 1.46	0.85085

*CI = Confidence Interval.

intradermal reaction ($p < 0.05$), which was not observed by the logistic regression analysis (Table 3).

DISCUSSION

Paracoccidioidomycosis is the commonest systemic mycosis in Latin America, predominantly affecting individuals who have frequent and close contact with soil. It mainly affects people who are in their most productive stage of life[2,26]. Epidemiological surveys have been used to evaluate paracoccidioidomycosis-infection prevalence in Brazil and in other countries in Latin America[2,8]. However, there is an impressive variability in relation to results that can be related to the characteristics of the studied population and to the nature or concentration of the used antigen. It should also take into consideration the chemical compounds and presence of fungus in soil[22,34].

Classically, paracoccidioidin, a polysaccharide extracted from different strains of *P. brasiliensis*, was the most used antigen for epidemiologic survey studies. More recently, it has been valued using purified antigens in intradermal tests with promising results using the exoantigen gp 43 as paracoccidioidin considered immunodominant and specific component[9,11,18,30,31]. In Brazil, the paracoccidioidin Fava Netto antigen was used in epidemiological surveys, in order to assess the prevalence of PCM infection. Results are not homogeneous, with positivity rates ranging from 2% among children from different areas of Rio de Janeiro, to 82% in Cachoeira do Sul[8]. Using the same antigen in Colombia, the prevalence of sensitization to *P. brasiliensis* can reach up to 77% of the population in certain rural areas[4], while in Venezuela, the prevalence rates of infection in recent years ranged from 10.2%[33] to 19.7%[6]. Argentina has lower prevalence rates of PCM compared to other Latin American countries, a variation being observed in prevalence between 1.6%[16] to 10.2%[38]. Some authors have currently used exoantigen purified gp43 in epidemiological surveys for the delimitation of endemic areas, also noting variable results (4-45%)[9,11,17,30,31]. In this study, we found a prevalence of 46.67% of positive tests in rural areas of Alfenas, MG, Brazil using gp43 paracoccidioidin (Table 1). Similar prevalence (49.5%) was also observed in other rural areas (Ibiá, MG) using Fava Netto paracoccidioidin[34] and in the northwest of Paraná State (43.0 %) using gp43 paracoccidioidin[9].

On the other hand, such antigen may induce cross-reaction in relation to other fungi antigens, e.g., *Histoplasma capsulatum*, *Coccidioides immitis* and *Sporothrix schenckii*[13,31,34]. Simultaneous positive tests for histoplasmin and paracoccidioidin do not necessarily imply a dependence relation among them, taking into consideration the coexistence of fungi in the same area. Meanwhile, this result indicates that it is not possible to state that there is no cross-reaction[5,34].

Despite the fact that all participants lived in rural areas, only 53.50% reported to be farm workers (Table 1). And 48.28% had positive intradermal reaction to paracoccidioidin. This was not statistically significant as a risk variant for positive intradermal reaction (Table 2). This could be explained by the fact that they lived in an endemic area and had worked sporadically in farming such as coffee harvest.

Gender differences are one of the most important characteristics of this systemic fungal disease. The incidence and progression of PCM in endemic areas is much higher in adult men than women. In relation to gender and positivity to paracoccidioidin test, we found a significant

statistical difference among men and women ($p < 0.01$) (Table 2). The multiparametric statistical analysis confirmed these results (Table 3). Prevalence of cases in male gender observed in this study is in accordance with numerous other studies[5,11,16] although recently MARQUES *et al* (2013)[17] showed different results.

The differences observed as to the prevalence between men compared to women are not due solely to less exposure or social conditions. Epidemiological data indicate that hormonal factors can have a strong role in the pathogenesis of the disease[23,28,32]. Experimental studies in animal tests demonstrate the protective effect of feminine hormones, providing support for the role of 17 β-estradiol in the innate resistance of females to the PCM[29,35].

Moreover, the ability of the hormone to modulate the production of cytokines may be associated with improvement of immune response[23,32]. Although women can be infected early, estrogen appears to affect the transition yeast and mycelial increase the secretion of IFN-γ and Th1 cell levels and lower levels of IL-10 as demonstrated in experimental models[12,15,21,23]. Furthermore, confirmation of subclinical infection in healthy and asymptomatic, skin tests for delayed hypersensitivity, the paracoccidioidin reveals heterogeneity of response in relation to gender[16,32]. One should take into account that the agent-host relationship factors such as higher or lower environmental exposure, nutritional status, comorbidities, alcoholism and smoking among others, probably interfere with the immune response favoring the progression of infection to disease[13,26].

Any individual exposed to this fungus is susceptible to infection. However, infection rates increase accordingly with age, as it was observed both in our present study and by SILVA-VERGARA & MARTINEZ (1998)[34]. This may be more likely related to a higher possibility of sensitization to the fungus, in relation to people living in an endemic area. Prevalence from 30 to 50 years old was 49.56%, the average age being 43 years old.

On the other hand, some life habits may be related to either acquisition of infection or development of the disease. Among other triggering factors, smoking and alcohol addiction have long been associated with the disease. A smoking habit is more likely to influence and/or facilitate the development of this infection due to some possible factors such as change of the mucociliary activity, diminishing of immunity and defects of immunological response of macrophages. In relation to alcohol addiction, it is thought that this drug is a risk cofactor associated with smoking[1,7]. In the present study, 29.33% of the individuals reported to be addicted to tobacco and 20.67% to alcohol. Among the tobacco addicted, 52.20% were positive to a skin test and among alcohol addicted 55.36% were positive. Meanwhile, such variants were not statistically significant in accordance with the regression analysis (Table 3).

The intradermal test convinced us that it was an important way of identifying if someone was or was not sensitized by the antigen. This makes it easier for patient follow-up, in order to facilitate a future diagnosis and early treatment.

In addition to this, after the intradermal test, it is possible to detect paracoccidioidomycosis endemicity in rural areas. Our results showed a high prevalence of sensitization to *P. brasiliensis* antigen in rural areas, in Alfenas - in Southern MG State. In our view, this data points out the necessity of public health policies to deal with mycosis in this region.

RESUMO

Prevalência da paracoccidioidomicose por intradermorreação em áreas rurais de Alfenas, Minas Gerais, Brasil

Este estudo teve como objetivo estimar a prevalência de sensibilização da pele pelo *Paracoccidioides brasiliensis* em áreas rurais em Alfenas, MG, Brasil, e avaliar os fatores de risco associados à infecção. Foi realizado um estudo transversal de base populacional utilizando testes intradérmicos com paracoccidioidina em 542 indivíduos selecionados por demanda espontânea. Os participantes foram submetidos a uma entrevista através do preenchimento de um formulário de inscrição com os dados epidemiológicos e os testes com a administração intradérmica de 0,1 mL de paracoccidioidina no antebraço esquerdo. O teste foi lido 48 h após a injeção e foi considerado positivo se enduramento era maior ou igual a 5 mm. De 542, 46,67% participantes foram positivos ao teste de pele. Prevalência aumentou de acordo com o aumento da idade. Houve significância estatística apenas para o sexo masculino. Profissão, alcoolismo e tabagismo não foram significativamente associados com o risco de infecção paracoccidioidomicose. Há relevância da infecção paracoccidioidomicose em áreas rurais, o que sugere mais estudos epidemiológicos e clínicos sobre esta micose no sul do estado de Minas Gerais.

ACKNOWLEDGMENTS

We would like to thank Nurse Sonia Maria Paz Lima de Souza for helping us with IDR and Dr Adelino Moreira de Carvalho from the School of Medicine at Universidade José do Rosario Vellano, Alfenas-MG, with the paper translation and Dr Eva Burger for her critical review of the manuscript.

FINANCIAL SUPPORT

FAPEMIG APQ-01125-11.

REFERENCES

1. Abreu e Silva MA, Salum FG, Fiqueiredo MA, Cherubini K. Important aspects of oral paracoccidioidomycosis: a literature review. Mycoses. 2013;56:189-99.

2. Bellisimo-Rodrigues F, Machado AA, Martins R. Paracoccidioidomycosis epidemiological features of a 1,000-cases series from a hyperendemic area on the southeast of Brazil. Am J Trop Med Hyg. 2011;85:546-50.

3. Cano LE, González A, Lopera D, Naranjo TW, Restrepo A. Pulmonary paracoccidioidomycosis: clinical, immunological and histopathological aspects. *In:* Irusen EM, editor. Lung diseases-selected state of art reviews. Rijeka: Intech-Europe; 2012. p. 359-91. Available from: http://intechopen.com

4. Cadavid D, Restrepo A. Factors associated with *Paracoccidiodes brasiliensis* infection among permanent residents of three endemic areas in Colombia. Epidemiol Infect. 1993;111:121-33.

5. Cermeño J, Cermeño J, Godoy G, Hernández I, Orellán Y, Blanco Y, *et al*. Epidemiological study of paracoccidioidomycosis and histoplasmosis in a suburb of San Félix city, Bolívar State, Venezuela. Invest Clin. 2009;50:2013-20.

6. Cermeño JR, Cermeño JJ, Hernández I, Godoy G, Cermeño JJ, Cabello I, *et al*. Histoplasmine and paracoccidiodine epidemiological study in Upata, Bolívar State, Venezuela. Trop Med Int Health. 2005;10:216-9.

7. dos Santos WA, da Silva BM, Passos ED, Zandonade E, Falqueto A. Associação entre tabagismo e paracoccidioidomicose: um estudo de caso-controle no Estado do Espírito Santo, Brasil. Cad Saúde Pública. 2003;19:245-53.

8. Fava SC, Fava Netto C. Epidemiologic surveys of histoplasmin and paracoccidioidin sensitivity in Brazil. Rev Inst Med Trop Sao Paulo. 1998;40:155-64.

9. Fornajeiro N, Maluf MLF, Takahachi G, Svidzinski TIE. Inquérito epidemiológico sobre a paracoccidioidomicose utilizando a gp43 em dois municípios do noroeste do Paraná, Brasil. Rev Soc Bras Med Trop. 2005;38:191-3.

10. Hotez PJ, Bottazzi ME, Franco-Paredes C, Ault SK, Periago MR. The neglected tropical diseases of Latin America and the Caribbean: a review of disease burden and distribution and a road map for control and elimination. PLoS Negl Trop Dis. 2008;2(9):e300.

11. Kalmar EM, Alencar FE, Alves FP, Pang LW, Del Negro GM, Camargo ZP, *et al*. Paracoccidioidomycosis: an epidemiologic survey in a pediatric population from the Brazilian Amazon using skin tests. Am J Trop Med Hyg. 2004;71:82-6.

12. Karhawi AS, Colombo AL, Salomão R. Production of IFN-gamma is impaired in patients with paracoccidioidomycosis during active disease and is restored after clinical remission. Med Mycol. 2000;38:225-9.

13. Lacaz CS, Porto E, Martins JEC, Heins-Vaccari E, de Melo NT. Paracoccidioidomicose. *In:* Lacaz CS, Porto E, Martins JEC, Heins-Vaccari EM, Melo NT, editores. Tratado de micologia médica Lacaz. 9ª ed. São Paulo: Sarvier; 2002. p. 639-729.

14. Liese B, Rosenberg M, Schratz A. Programmes, partnerships, and governance for elimination and control of neglected tropical diseases. Lancet. 2010;375:67-76.

15. Mamoni RL, Blotta MH. Kinetics of cytokines and chemokines gene expression distinguishes *Paracoccidioides brasiliensis* infection from disease. Cytokine. 2005;32:20-9.

16. Mangiaterra ML, Giusiano GE, Alonso JM, Gorodner JO. *Paracoccidioides brasiliensis* infection in a subtropical region with important environmental changes. Bull Soc Pathol Exot. 1999;92:173-6.

17. Marques AP, Oliveira SM, Rezende GR, Melo DA, Fernandes-Fitts SM, Pontes ER, *et al.* Evaluation of *Paracoccidioides brasiliensis* infection by gp 43 intradermal test in rural settlements in Central-West Brazil. Mycopathologia. 2013;176:41-7.

18. Marques SA. Paracoccidioidomycosis. Clin Dermatol. 2012;30:610-15.

19. Martinez R. Paracoccidioidomycosis: the dimension of the problem of a neglected disease. Rev Soc Bras Med Trop. 2010;43:480.

20. Moreira APV. Paracoccidioidomicose: histórico, etiologia, epidemiologia, patogênese, formas clínicas, diagnóstico laboratorial e antígenos. Bol Epidemiol Paul. 2008;5:11-24.

21. Oliveira SJ, Mamoni RL, Musatti CC, Papaiordanou PM, Blotta MH. Cytokines and lymphocyte proliferation in juvenile and adult forms of paracoccidioidomycosis: comparison with infected and non-infected controls. Microbes Infect. 2002;4:139-44.

22. Ono MA, Itano EN, Mizuno LT, Mizuno EH, Camargo ZP. Inhibition of *Paracoccidioides brasiliensis* by pesticides: is this a partial explanation for the difficulty in isolating this fungus from the soil? Med Mycol. 2002;40:493-9.

23. Pinzan CF, Ruas LP, Casabona-Fortunato AS, Carvalho FC, Roque-Barreira MC. Immunological basis for the gender differences in murine *Paracoccidioides brasiliensis* infection. PLoS One. 2010;5:e10757.

24. R CORE TEAM. The R Project for Statistical Computing. Vienna: R Foundation for Statistical Computing; 2004. p. 20-2 updated on 2011 August 16-18. [cited 2011 Nov 12]. Available from: http://www.R-project.org/

25. Restrepo A, Gómez BL, Tobón A. Paracoccidioidomycosis: Latin America's own fungal disorder. Curr Fungal Infect Rep. 2012;6:303-11.

26. Restrepo A, Gonzalez A, Agudelo CA. Paracoccidioidomycosis. *In:* Kauffman CA, Pappas PG, Sobel JD, Dismukes WE, editors. Essentials of clinicals mycology. 2nd ed. New York: Springer; 2011. p. 367-85.

27. Restrepo A, McEwen JG, Castañeda E. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? Med Mycol. 2001;39:233-41.

28. Restrepo A, Salazar ME, Cano LE, Stover EP, Feldman D, Stevens DA. Estrogens inhibit mycelium-to-yeast transformation in the fungus *Paracoccidioides brasiliensis*: implications for resistance of females to paracoccidioidomycosis. Infect Immun. 1984;46:346-53.

29. Salazar ME, Restrepo A, Stevens DA. Inhibition by estrogens of conidium-to-yeast conversion in the fungus *Paracoccidioides brasiliensis*. Infect Immun. 1988;56:711-3.

30. Saraiva EC, Altemani A, Franco MF, Unterkircher CS, Camargo ZP. *Paracoccidioides brasiliensis*-gp43 used as paracoccidioidin. J Med Vet Mycol. 1996;34:155-61.

31. Sarti EC, de Oliveira SM, dos Santos LF, de Camargo ZP, Paniago AM. Paracoccidioidal infection in HIV patients at an endemic area of paracoccidioidomycosis in Brazil. Mycopathologia. 2012;173:145-9.

32. Shankar J, Restrepo A, Clemons KV, Stevens DA. Hormones and the resistance of women to paracoccidioidomycosis. Clin Microbiol Rev. 2011;24:296-313.

33. Shikanai-Yasuda MA, Telles Filho FQ, Mendes RP, Colombo AL, Moretti ML. Consenso em paracoccidioidomicose. Rev Soc Bras Med Trop. 2006;39:297-310.

34. Silva-Vergara ML, Martinez R. Inquérito epidemiológico com paracoccidioidina e histoplasmina em área agrícola de café em Ibiá, Minas Gerais, Brasil. Rev Iberoam Micol. 1998;15:294-7.

35. Stover EP, Schär G, Clemons KV, Stevens DA, Feldman D. Estradiol-binding proteins from mycelial and yeast-form cultures of *Paracoccidioides brasiliensis*. Infect Immun. 1986;51:199-203.

36. Teixeira MM, Theodoro RC, de Carvalho MJ, Fernandes L, Paes HC, Hahn RC, *et al.* Phylogenetic analysis reveals a high level of speciation in the *Paracoccidioides* genus. Mol Phylogenet Evol. 2009;52:273-83.

37. van Gelderen de Komaid A, Durán E, Borges de Kestelman I. Histoplasmosis and paracoccidioidomycosis in northwestern Argentina. III. Epidemiological survey in Vipos, La Toma, and Choromoro - Trancas, Tucumán, Argentina. Eur J Epidemiol. 1999;15:383-8.

Received: 16 July 2013
Accepted: 28 January 2014

LIBRARY OF THE
SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

Website: www.imt.usp.br/portal
Address: Biblioteca do Instituto de Medicina Tropical de São Paulo da Universidade de São Paulo
Av. Dr. Enéas de Carvalho Aguiar, 470. Prédio 1 – Andar térreo.
05403-000 São Paulo, SP, Brazil.
Telephone: 5511 3061-7003 - **Fax**: 5511 3062-2174

The Library of the São Paulo Institute of Tropical Medicine (IMTSP Library) was created on January 15, 1959 in order to serve all those who are interested in tropical diseases. To reach this objective, we select and acquire by donation and / or exchange appropriate material to be used by researchers and we maintain interchange between Institutions thorough the Journal of the São Paulo Institute of Tropical Medicine, since the Library has no funds to build its own patrimony.

The IMTSP Library has a patrimony consisting of books, theses, annals of congresses, journals, and reference works.

The collection fo journals existing in the Library can be verified through the USP – Bibliographic Database – OPAC – DEDALUS http://dedalus.usp.br:4500/ALEPH/eng/USP/USP/DEDALUS/start of the USP network.

Rev. Inst. Med. Trop. Sao Paulo
56(4):287-290, July-August, 2014
doi: 10.1590/S0036-46652014000400003

DETERMINATION OF VIRAL TROPISM BY GENOTYPING AND PHENOTYPING ASSAYS IN BRAZILIAN HIV-1-INFECTED PATIENTS

Liã Bárbara ARRUDA(1,2), Marilia Ladeira de ARAÚJO(2), Maira Luccia MARTINEZ(2), Claudio Roberto GONSALEZ(3), Alberto José da Silva DUARTE(2), Eoin COAKLEY(4), Yolanda LIE(4) & Jorge CASSEB(1,2)

SUMMARY

The clinical application of CCR5 antagonists involves first determining the coreceptor usage by the infecting viral strain. Bioinformatics programs that predict coreceptor usage could provide an alternative method to screen candidates for treatment with CCR5 antagonists, particularly in countries with limited financial resources. Thus, the present study aims to identify the best approach using bioinformatics tools for determining HIV-1 coreceptor usage in clinical practice. Proviral DNA sequences and Trofile results from 99 HIV-1-infected subjects under clinical monitoring were analyzed in this study. Based on the Trofile results, the viral variants present were 81.1% R5, 21.4% R5X4 and 1.8% X4. Determination of tropism using a Geno2pheno$_{[coreceptor]}$ analysis with a false positive rate of 10% gave the most suitable performance in this sampling: the R5 and X4 strains were found at frequencies of 78.5% and 28.4%, respectively, and there was 78.6% concordance between the phenotypic and genotypic results. Further studies are needed to clarify how genetic diversity amongst virus strains affects bioinformatics-driven approaches for determining tropism. Although this strategy could be useful for screening patients in developing countries, some limitations remain that restrict the wider application of coreceptor usage tests in clinical practice.

KEYWORDS: HIV-1; Tropism; Brazil; V3 loop.

INTRODUCTION

Maraviroc, which blocks virus entry by binding to the CCR5 coreceptor, was the first CCR5 antagonist approved for clinical use. This molecule has been widely recommended for controlling HIV infection with a low level of drug resistance[6,14,28]. However, the use of this drug class in clinical practice requires the assessment of the coreceptor usage before and during the treatment[20]. Studies comparing the efficiency of phenotypic and genotypic assays, in order to develop widely applicable strategies useful in developing countries, have raised several issues regarding the determination of HIV-1 tropism[7,18].

Although phenotypic tropism tests (PTT) are considered the gold standard for determining coreceptor usage, their wide application in clinical practice is limited mainly by their high cost and the logistical restrictions of the procedures[1,7]. In this context, programs able to predict coreceptor usage based on V3 sequences have provided a suitable alternative for screening candidates for CCR5 antagonist therapy[23].

Several bioinformatics protocols for tropism prediction have been published. They assess tropism based on amino acids sequences from the V3 loop, which is known to be an important binding region on the gp120 envelope protein[19,23]. Because the use of phenotypic assays is still limited, the European Guidelines have encouraged the application of bioinformatics programs in coreceptor usage determination[27]. However, due to the intrinsic differences of each predictive system, divergent outputs are expected and remain a reason for concern in the wider application of this approach[9,15,19,23-24].

The aim of this study was to examine the use of the Geno2pheno$_{[coreceptor]}$ program for tropism prediction and determine its usefulness as an alternative method in clinical practice for screening CCR5 antagonist therapy candidates for the first time in Brazil.

MATERIAL AND METHODS

Sampling: Sample collection occurred between July 2009 and October 2010, from the HIV outpatient clinic ADEE3002/HCFMUSP. Only 99 from this cohort showed the minimum plasmatic RNA viral load necessary to qualify for the Trofile assay (> 1000 copies/mL, as recommended by Monogram Biosciences, San Francisco, CA). The written informed consent for collecting blood samples and the protocol for this study were approved by the Ethical Research Board of the

(1) Institute of Tropical Medicine of Sao Paulo, University of Sao Paulo, Dr. Eneas de Carvalho Aguiar Avenue 470, Building II, Sao Paulo, SP. Brazil.
(2) Laboratory of Investigation in Dermatology and Immunodeficiencies, LIM56, Department of Dermatology School of Medicine at University of Sao Paulo, Dr. Eneas de Carvalho Aguiar Avenue 470, Building II, 3rd floor, Sao Paulo, SP, Brazil.
(3) HIV Out-clinic, Ambulatory of Secondary Immunodeficiencies, ADEE3002, Department of Dermatology, Hospital of Clinics at School of Medicine at University of Sao Paulo.
(4) Monogram Biosciences, Inc., 345 Oyster Point Blvd. South San Francisco, CA 94080-1913, USA.
Correspondence to: Jorge Casseb, Laboratório de Investigação em Dermatologia e Imunodeficiências, LIM56, Av. Dr. Eneas de Carvalho Aguiar 470, 3º andar, Prédio 2, 05403-000 São Paulo, SP, Brasil. Tel.: +55 11 3061 7194. Fax: +55 11 3081 7190. E-mail: jcasseb10@gmail.com

Hospital das Clinicas, Faculdade de Medicina da Universidade de São Paulo (Cappesq - HC/FMUSP, number 08/0108).

Subjects: A total of 99 patients under clinical monitoring were included in this study, of whom 29 (29.3%) were females and 70 (70.7%) were males. With respect to antiretroviral therapy, 62 (62.6%) patients were drug-naïve, and 37 (37.4%) were under HAART. Only subjects being treated with nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI) and protease inhibitors (PI) were studied.

After signing the informed consent form, 15 mL of venous blood was collected in EDTA-treated tubes from each patient. Plasma samples were separated and stored at -70 °C until their shipment to Monogram Biosciences to perform the Trofile assay. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll-Hypaque density gradient centrifugation and stored at -70 °C until use. DNA was extracted from PBMCs using the GFX Genomic Blood DNA Purification Kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

The HIV-1 plasmatic viral load was measured using the Versant HIV RNA 3.0 Assay (bDNA) (Siemens Healthcare Diagnostics, San Francisco, USA), and CD4+/CD8+ T cell counts were determined by flow cytometry. These data were collected during routine clinical monitoring of patients and were obtained from the laboratory database.

In addition, 185 sequences containing known coreceptor sequence variations were obtained from the HIV Sequence Database (www.hiv-web.lanl.gov) to assess the application of genotypic tropism test (GTT) in identifying different HIV-1 variants. Only V3 sequences with 35 amino acids were evaluated; sequences with 34 or 36 amino acids were excluded. All deposited sequences originating in Brazil were included, and at least 5% of the sequences for each group were selected from other countries of origin (Table 1).

Table 1
Distribution of HIV database sequences by subtype and phenotypic tropism

Subtype	R5	R5X4	X4	Total
B	115	18	13	146
C	23	7	5	35
F1	2	1	1	4
Total	140	26	19	**185**

V3 region amplification and sequencing: The V3 region was amplified by nested polymerase chain reaction (PCR) using the previously described outer primers ED5/ED12 and inner primers ED31/ED33[4]. The PCR using the outer primers was performed with the following steps: one cycle of 94 °C for one min; 35 cycles of 94 °C for 45 sec, 55 °C for 45 sec, and 72 °C for two min; and a final extension cycle of 72 °C for 10 min. The PCR using the inner primers was performed with the following steps: one cycle of 94 °C for one min; 35 cycles of 94 °C for one min, 55 °C for one min and 72 °C for one min and 30 sec; and a final extension cycle of 72 °C for 10 min. The 50-µL reaction mixtures contained 2 mM $MgCl_2$, 2 µM of each primer, 0.2 mM of each dNTP, 1 U Taq DNA polymerase and 5 µL of extracted DNA for the outer PCR or 5 µL of PCR product for the inner PCR.

The inner PCR products were purified using the QIAquick PCR fragment purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The sequencing reaction was carried out using the ABI Prism Big Dye Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA), adding 20-100 ng of amplified and purified product and each inner PCR primer at 1 µM. The reaction was carried out according to the manufacturer's instructions, and the sequencing was performed using the ABI Prism 3100 system (Applied Biosystems, Foster City, USA). The resulting nucleotide sequences were edited using SeqScape Software v.2.6 (Applied Biosystems).

HIV-1 envelope subtyping was performed by manual phylogenetic analysis using the 2008 Alamos reference dataset (available at http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html). Sequences were aligned and edited, and a tree was constructed with the Neighbor-Joining method and the Maximum Composite Likelihood model with 1000 bootstrap replicates using the MEGA v.5 package[25].

Tropism determination: Coreceptor usage was determined using the $Geno2pheno_{[coreceptor]}$ bioinformatics tool (G2P)[10]. False positive rates (FPR) were 10% for the proviral DNA sequences obtained in this study or 20% for clonal and clinical data.

The Trofile Coreceptor Tropism Assay (Monogram Biosciences, San Francisco, USA) was performed on plasma samples from patients showing viral load > 1000 copies/mL.

RESULTS

Comparison between PTT and GTT: From the 99 samples sent for analysis with the Trofile assay, 55 (55.6%) were identified as R5 viruses, 22 (22.2%) were R5X4, and only one (1%) sample showed tropism to the CXCR4 coreceptor. The Trofile assay was unable to generate results for 21 (21.2%) samples. Therefore, the coreceptor prevalence within the assayable sample population was 70.5% (55/78) R5, 28.3% (22/78) R5X4 and 1.3% (1/78) X4 strains.

Readable sequences were obtained from 70 proviral DNA sequences, resulting in the generation of both Trofile and G2P results for 56 samples. These matched sample measures allowed for sensitivity and specificity testing, which showed that a FPR of 10% gave the highest sensitivity and specificity (Table 2).

Table 2
Sensitivity and specificity test for $Geno2pheno_{[coreceptor]}$ false positive rates in prediction of CXCR4 coreceptor usage

G2P FPR	Sensitivity (%)	Specificity (%)
10%	54.5	84.4
20% clinical data	50.0	82.6
20% clonal data	34.8	68.1

Note: G2P FPR: $Geno2pheno_{[coreceptor]}$.

Based on the G2P FPR of 10%, 55 (78.5%) and 15 (21.4%) of the 70 sequences obtained in this study were predicted to be R5 and X4 strains, respectively. There were no differences between the Trofile

results (R5 = 70.5%; X4 and R5X4 = 29.5%) and G2P results found for this population.

Envelope subtyping was performed on the sequences obtained in this study. Only B (n = 46), C (n = 2) and F1 (n = 8) subtypes were found, and it was not possible to identify any recombinant forms. Tropism prediction showed good accuracy regardless of the subtype, generating concordances between the Trofile and G2P results of 76.1% for subtype B samples, 100% for subtype C and 87.5% for subtype F1. Due to the small number of clinical samples in our study, a dataset containing 185 sequences from the Los Alamos HIV-1 sequence database was also analyzed. The prediction accuracy for the samples in this dataset was represented by concordances between the Trofile and G2P results of 88.3% for subtype B, 88.6% for subtype C and 100% for subtype F1 both sequences available in this database.

DISCUSSION

Phenotypic tropism tests (PTT) still represent the gold standard in tropism determination, but high cost and logistical restrictions are significant barriers preventing the wide application of these assays, particularly in developing countries[7,23]. Thus, the adoption of bioinformatics tools for determining coreceptor usage could provide a more accessible alternative for screening candidates for CCR5 antagonist therapy[19,23].

The phenotyping test has some limitations in the clinical setting. While the commercially available Trofile assay is the most widely used phenotypic test[1,7] and provides accurate determination of HIV tropism, it was unable to generate results for 21% of the samples in this study. An additional important limitation of the Trofile assay is the requirement that samples have a minimum plasmaviral load of 1000 copies/mL. Based on studies that identified similar tropism behavior in 75% to 90% of sequences from plasma and PBMCs, using proviral DNA as the source of viral genetic material could be the most reliable option for obtaining V3 sequences. Moreover, X4 viruses were detected more frequently in DNA samples from PBMCs than in RNA samples from plasma[13,20,28]. In fact, the Trofile test is mostly used in clinical trials and, in the meantime, has been replaced in the clinical setting by genotyping tests (at least in Europe). Furthermore, the Trofile test is not an entirely phenotypic assay, as a library of V3 loop sequences of HIV-RNA from the test sample is prepared by PCR amplification. There is bias in this PCR amplification, as is in any PCR. Only the read out of the Trofile test is phenotypic. In contrast, the MT2 assay is entirely phenotypic. Bias is also present in population based genotypic testing by the PCRs and the sequencing reactions used.

Bulk sequencing of proviral DNA is an intrinsically limited technique, because it produces a consensus sequence from dominant strains within the viral quasispecies[7,19]. Although it has been suggested that this problem could be mitigated by increasing the number of replicate sequences produced from each sample, this modification also increases the method's costs and time required and could further limit its use, particularly in countries with restricted financial resources[7,11]. The present study aimed to develop an accurate and cost-effective strategy for applying tropism testing in clinical practice. Although the European Guidelines recommend performing G2P with an FPR of 10% for triplicate samples and 20% for samples with a single sequence[27], the present study's sensitivity and specificity tests showed that a G2P FPR = 10% is the best significance level, even for single sequences (Table 2).

This study showed that the concordance between PPT and GTT was also high for different subtypes (mean of concordance = 88%), and this finding was confirmed by testing a large dataset from the Los Alamos Sequence Database (mean of concordance = 92%). These results corroborate previous studies that showed 80-90% agreement between phenotypic and genotypic tests for determining tropism[2,16-17,26]. Despite the high concordance between the phenotypic and genotypic assays, there has been disagreement between the results generated by different bioinformatics-based assays, and several approaches to improving these predictions have been proposed[2-3,21-22,26]. Additional studies are needed to determine if viral sequences in the Brazilian patient population might be so distinct as to invalidate their analysis with available bioinformatics tools, which derive their datasets mainly from sequences found in patients from the USA and European countries.

Furthermore, while the determination of HIV-1 tropism has focused on the V3 loop, other features of gp120 could influence virus affinity for a particular coreceptor, including N-glycosylation sites and variations in extensions of the V1/V2 region[8,12]. Information on the three-dimensional structure of the V3 loop and clinical and laboratory data from patients, such as T cells counts and viral load, could also substantially improve tropism predictions[19].

CONCLUSIONS

The application of bioinformatics tools in coreceptor usage determination has been widely studied and could provide an alternative approach in clinical practice for screening candidates for CCR5 antagonist therapy, especially in cases where confirmatory assays for coreceptor usage determination are unavailable. This study showed that G2P analysis of sequences from PBMCs with an FPR of 10% could be the most suitable alternative approach for determining tropism, although this approach should be tested in a nationwide study to determine how HIV genetic diversity influences the findings.

RESUMO

Determinação do tropismo viral por ensaios genotípicos e fenotípicos em pacientes brasileiros infectados por HIV-1

A aplicação clínica dos antagonistas de CCR5 envolve em primeiro lugar determinar o uso de co-receptor pela cepa viral infectante. Programas de bioinformática que prevêem o uso co-receptor poderiam fornecer um método alternativo para selecionar candidatos para o tratamento com os antagonistas do CCR5, particularmente em países com poucos recursos financeiros. Assim, o presente estudo teve por objetivo identificar a melhor abordagem utilizando ferramentas de bioinformática para determinar qual o tipo de co-receptor do HIV-1 que poderia ser usado na prática clínica. Sequências de DNA proviral e Trofile resultados a partir de 99 pacientes infectados pelo HIV-1 sob monitorização clínica foram avaliadas. Com base nos resultados do Teste Trofile, as variantes virais presentes eram R5 (81,1%), R5X4 (21,4%) e X4 (1,8%). Determinação do tropismo pela análise do Geno2pheno, com taxa de falso positivos de 10% apresentou desempenho mais adequado para esta amostragem: as cepas R5 e X4 foram encontradas

em frequências de 78,5% e 28,4%, respectivamente, e foi de 78,6% a concordância entre os resultados fenotípicos e genotípicos. Mais estudos são necessários para esclarecer como a diversidade genética entre as cepas do vírus afeta abordagens baseadas na determinação do tropismo pelas ferramentas de bioinformática. Embora esta estratégia possa ser útil para o rastreio de pacientes em países em desenvolvimento, permanecem algumas limitações que restringem a aplicação mais ampla para utilização de testes de co-receptor na prática clínica.

ACKNOWLEDGEMENTS

We thank all patients who participated in this study. We also thank Rosangela Araujo and Noemia Orii for performing the flow cytometry experiments, José Eduardo Martins for the determination of HIV-1 viral load and the financial support from FAPESP (08/58138-0; 08/51265-6; 10/00222-5); FFM; CNPq.

REFERENCES

1. Braun P, Wiesmann F. Phenotypic assays for the determination of coreceptor tropism in HIV-1 infected individuals. Eur J Med Res. 2007;12:463-72.

2. Chueca N, Garrido C, Alvarez M, Poveda E, de Dios Luna J, Zahonero N, *et al*. Improvement in the determination of HIV-1 tropism using the V3 gene sequence and a combination of bioinformatic tools. J Med Virol. 2009;81:763-7.

3. Delobel P, Nugeyre MT, Cazabat M, Pasquier C, Marchou B, Massip P, *et al*. Population-based sequencing of the V3 region of env for predicting the coreceptor usage of human immunodeficiency virus type 1 quasispecies. J Clin Microbiol. 2007;45:1572-80.

4. Delwart EL, Herring B, Rodrigo AG, Mullins JI. Genetic subtyping of human immunodeficiency virus using a heteroduplex mobility assay. PCR Methods Appl. 1995;4:S202-16.

5. Dybowski JN, Heider D, Hoffmann D. Prediction of co-receptor usage of HIV-1 from genotype. PLoS Comput Biol. 2010;6:e1000743.

6. Emmelkamp JM, Rockstroh JK. CCR5 antagonists: comparison of efficacy, side effects, pharmacokinetics and interactions--review of the literature. Eur J Med Res. 2007;12:409-17.

7. Harrigan PR, Geretti AM. Genotypic tropism testing: evidence-based or leap of faith? AIDS. 2011;25:257-64.

8. Hughes A, Nelson M. HIV entry: new insights and implications for patient management. Curr Opin Infect Dis. 2009;22:35-42.

9. Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, He HX, *et al*. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. J Virol. 2003;77:13376-88.

10. Lengauer T, Sander O, Sierra S, Thielen A, Kaiser R. Bioinformatics prediction of HIV coreceptor usage. Nat Biotechnol. 2007;25:1407-10.

11. McGovern RA, Harrigan PR, Swenson LC. Genotypic inference of HIV-1 tropism using population-based sequencing of V3. J Vis Exp. 2010(46).pii:2531. doi: 10.3791/2531.

12. Mild M, Kvist A, Esbjörnsson J, Karlsson I, Fenyo EM, Medstrand P. Differences in molecular evolution between switch (R5 to R5X4/X4-tropic) and non-switch (R5-tropic only) HIV-1 populations during infection. Infect Genet Evol. 2010;10:356-64.

13. Paar C, Geit M, Stekel H, Berg J. Genotypic prediction of human immunodeficiency virus type 1 tropism from plasma and peripheral blood mononuclear cells in the routine clinical laboratory. J Clin Microbiol. 2011;49:2697-9.

14. Parra J, Portilla J, Pulido F, Sanchez-de la Rosa R, Alonso-Villaverde C, Berenguer J, *et al*. Clinical utility of maraviroc. Clin Drug Investig. 2011;31:527-42.

15. Pillai S, Good B, Richman D, Corbeil J. A new perspective on V3 phenotype prediction. AIDS Res Hum Retroviruses. 2003;19:145-9.

16. Prosperi MC, Fanti I, Ulivi G, Micarelli A, De Luca A, Zazzi M. Robust supervised and unsupervised statistical learning for HIV type 1 coreceptor usage analysis. AIDS Res Hum Retroviruses. 2009;25:305-14.

17. Raymond S, Delobel P, Mavigner M, Cazabat M, Souyris C, Sandres-Saune K, *et al*. Correlation between genotypic predictions based on V3 sequences and phenotypic determination of HIV-1 tropism. AIDS. 2008;22:F11-6.

18. Recordon-Pinson P, Soulie C, Flandre P, Descamps D, Lazrek M, Charpentier C, *et al*. Evaluation of the genotypic prediction of HIV-1 coreceptor use versus a phenotypic assay and correlation with the virological response to maraviroc: the ANRS GenoTropism study. Antimicrob Agents Chemother. 2010;54:3335-40.

19. Sander O, Sing T, Sommer I, Low AJ, Cheung PK, Harrigan PR, *et al*. Structural descriptors of gp120 V3 loop for the prediction of HIV-1 coreceptor usage. PLoS Comput Biol. 2007;3(3):e58.

20. Saracino A, Monno L, Cibelli DC, Punzi G, Brindicci G, Ladisa N, *et al*. Co-receptor switch during HAART is independent of virological success. J Med Virol. 2009;81:2036-44.

21. Seclén E, Garrido C, González M del M, González-Lahoz J, de Mendoza C, Soriano V, *et al*. High sensitivity of specific genotypic tools for detection of X4 variants in antiretroviral-experienced patients suitable to be treated with CCR5 antagonists. J Antimicrob Chemother. 2010;65:1486-92.

22. Seclén E, Soriano V, González MM, Gómez S, Thielen A, Poveda E. High concordance between the position-specific scoring matrix and geno2pheno algorithms for genotypic interpretation of HIV-1 tropism: V3 length as the major cause of disagreement. J Clin Microbiol. 2011;49:3380-2.

23. Sierra S, Kaiser R, Thielen A, Lengauer T. Genotypic coreceptor analysis. Eur J Med Res. 2007;12:453-62.

24. Sing T, Low AJ, Beerenwinkel N, Sander O, Cheung PK, Domingues FS, *et al*. Predicting HIV coreceptor usage on the basis of genetic and clinical covariates. Antivir Ther. 2007;12:1097-106.

25. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731-9.

26. Trabaud MA, Icard V, Scholtes C, Perpoint T, Koffi J, Cotte L, *et al*. Discordance in HIV-1 co-receptor use prediction by different genotypic algorithms and phenotype assay: intermediate profile in relation to concordant predictions. J Med Virol. 2012;84:402-13.

27. Vandekerckhove LP, Wensing AM, Kaiser R, Brun-Vezinet F, Clotet B, De Luca A, *et al*. European guidelines on the clinical management of HIV-1 tropism testing. Lancet Infect Dis. 2011;11:394-407.

28. Verhofstede C, Vandekerckhove L, Eygen VV, Demecheleer E, Vandenbroucke I, Winters B, *et al*. CXCR4-using HIV type 1 variants are more commonly found in peripheral blood mononuclear cell DNA than in plasma RNA. J Acquir Immune Defic Syndr. 2009;50:126-36.

Received: 28 July 2013
Accepted: 30 January 2014

Rev. Inst. Med. Trop. Sao Paulo
56(4):291-296, July-August, 2014
doi: 10.1590/S0036-46652014000400004

FACTORS ASSOCIATED TO ADHERENCE TO DIFFERENT TREATMENT SCHEMES WITH MEGLUMINE ANTIMONIATE IN A CLINICAL TRIAL FOR CUTANEOUS LEISHMANIASIS

Madelon Novato RIBEIRO(1), Maria Inês Fernandes PIMENTEL(1), Armando de Oliveira SCHUBACH(1), Raquel de Vasconcellos Carvalhães de OLIVEIRA(1), José Liporage TEIXEIRA(1), Madson Pedro da Silva LEITE, Monique FONSECA(1), Ginelza Peres Lima dos SANTOS(1), Mariza Matos SALGUEIRO(1), Erica de Camargo Ferreira e VASCONCELLOS(1), Marcelo Rosandiski LYRA(1), Mauricio Naoto SAHEKI(1) & Claudia Maria VALETE-ROSALINO(1,2)

SUMMARY

The favorable outcome of the treatment of a disease is influenced by the adherence to therapy. Our objective was to assess factors associated with adherence to treatment of patients included in a clinical trial of equivalence between the standard and alternative treatment schemes with meglumine antimoniate (MA) in the treatment of cutaneous leishmaniasis (CL), in the state of Rio de Janeiro. Between 2008 and 2011, 57 patients with CL were interviewed using a questionnaire to collect socioeconomic data. The following methods were used for adherence monitoring: counting of vial surplus, monitoring card, Morisky test and modified Morisky test (without the question regarding the schedule); we observed 82.1% (vial return), 86.0% (monitoring card), 66.7% (Morisky test) and 86.0% (modified Morisky test) adherence. There was a strong correlation between the method of vial counting and the monitoring card and modified Morisky test. A significant association was observed between greater adherence to treatment and low dose of MA, as well as with a lower number of people sleeping in the same room. We recommend the use of the modified Morisky test to assess adherence to treatment of CL with MA, because it is a simple method and with a good performance, when compared to other methods.

KEYWORDS: Cutaneous leishmaniasis; Pentavalent antimonials; Adherence; Therapy; Treatment.

INTRODUCTION

Cutaneous Leishmaniasis (CL) is a disease caused by a protozoan of the *Leishmania* genus and transmitted by the bites of female Phlebotomus (Dipteran, Psychodidae, Phlebotominae). In the state of Rio de Janeiro, Brazil, CL is caused mainly by *L. (Viannia) braziliensis*[1,28].

Pentavalent antimonials have been used for decades and are still the first drug of choice in the treatment of leishmaniasis, despite its known toxicity, difficult handling and not fully understood mechanism of action[9,10].

Meglumine antimoniate (MA) is supplied in 5 mL vials containing 1.5g N-methylglucamine, equivalent to 405 mg of pentavalent antimony (Sb^{5+})[34]. It can be administered parentally (IM or IV). The therapeutic schemes may vary in duration, total dose and daily dose. Poor therapeutic response has been described[29].

In Brazil, the Ministry of Health[18] recommends a dose of 10-20 mg Sb^{5+}/kg/day, over 20 days, taking into consideration a maximum limit of three vials per day, to treat CL patients with MA.

Clinical studies in Rio de Janeiro, with long-term patient monitoring, have suggested that both, the use of regular schemes with low doses (5 mg Sb^{5+}/kg/day) administered systemically and the intralesional therapy with MA[18,22,30,31], can be effective schemes, achieving cure rates similar to those obtained with higher doses, although with lower toxicity, greater ease of implementation and lower cost[27]. Schemes with intermittent doses of 15 mg Sb^{5+} obtained greater adherence and effectiveness, when compared with continuous schemes[2].

According to the World Health Organization (WHO)[35], adherence may be defined as the degree of a person's acceptance of the recommendations of the practitioner or other health care providers, which include taking drugs, following a diet, behavioral changes and attending appointments previously made. However, since it demands the participation of the patient, a good patient - health provider relationship should be established[24].

The methods adopted to assess adherence to treatment may be classified as direct or indirect methods, but no method is considered the gold standard for this evaluation[6]. The direct methods quantify the drug or the metabolite in biological fluids and/or test specimens[11,33]. The indirect methods assess adherence through interviews with patients and counting the drugs that the patient still has remaining, which tends to be a method more prone to error caused by the patient[11,33].

(1) Instituto de Pesquisas Evandro Chagas, IPEC/FIOCRUZ, Rio de Janeiro. RJ, Brazil.
(2) Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, RJ, Brazil.
Correspondence to: Ms. Madelon Novato Ribeiro, Instituto de Pesquisas Evandro Chagas, FIOCRUZ, Departamento Vigilância em Leishmaniose, Av. Brasil 4365, 21040-360 Rio de Janeiro, RJ, Brasil. Phone: 55.21.3865-2121. E-mail: mjrfarmacia@yahoo.com.br, nutballet@hotmail

Adherence to therapy is a phenomenon subject to many factors that directly affect the patient[24,31]. Adherence questionnaires are methods used to assess results. The structured interview is one of the most used methods, because of its low cost and accessible implementation[24]. Some forms used in adherence interviews are found in literature, such as the Morisky test[19,20].

The WHO[35] mentions several factors that can influence adherence to therapy: socioeconomic and cultural factors, those related to health providers and services, factors in relation to treatment and the patient.

The objective of the present study is to assess factors associated with adherence to patients included in a clinical trial with MA, in the treatment of cutaneous leishmaniasis in the state of Rio de Janeiro (RJ).

METHODS

Between 2008 and 2011, 60 patients with CL were observed. They were all over 13 years, participants in a controlled clinical trial, blind and in phase III of equivalence between the standard treatment scheme (20 mg Sb^{5+}/kg/day for 20 consecutive days) and other MA alternative schemes, ongoing at the Evandro Chagas Research Institute - IPEC, Oswaldo Cruz Foundation, Rio de Janeiro. The test was registered at the site http://clinicaltrials.gov - Identifier: NCT01301924, and approved by the Ethics Committee on Research/IPEC under the number 0055.0.0009.000-07.

Initially, sixty individuals were randomly distributed, 15 in each of the treatment groups by intramuscular administration of MA. From the initial 60 CL patients, three were excluded, because they did not make the adherence evaluation: two of them refused to participate in the adherence evaluation, and the other one died. The 57 remaining patients were allocated as follows: 20 mg Sb^{5+}/kg/day for 20 consecutive days (n = 13), 20 mg Sb^{5+}/kg/day in two intermittent series of 10 days interspersed by a 10-day interval (n = 14), 5 mg Sb^{5+}/kg/day for 30 consecutive days (n = 15) and 5 mg Sb^{5+}/kg/day in three intermittent series interspersed by 10-day intervals (n = 15). For analysis purposes, the patients were grouped in high (20 mg Sb^{5+}/kg/day) and low (5 mg Sb^{5+}/kg/day) doses; and consecutive and intermittent doses.

At the time of medicine administration, the patients were interviewed with the aid of a standardized questionnaire for socioeconomic data collection (gender, age, coming from an endemic area or not, people co-habiting in the same house, people sleeping in the same room, marital condition, level of education, personal and family monthly income, smoking habits, alcohol and drug consumption, who administers the medicine and their instruction level, whether the medicine affects the daily life) and assessment of satisfaction with treatment, IPEC and the healthcare team.

Clinical healing was defined as lesion scarring with no recurrence within a year after treatment. Monitoring abandonment was defined as the patient not returning to the appointments provided during the first year after the beginning of the treatment.

Adherence to therapy was evaluated according to four criteria (counting of vial surplus, monitoring card, Morisky test and modified Morisky test).

Counting of vial surplus: The vials for the treatment were supplied with a small surplus, and the patient was advised to return the vials that were not used at the next appointment. Patients were considered adherent when they delivered vials in equal or lower number than expected and non-adherent when they delivered a greater number of vials.

Monitoring card: Patients registered date and time of each dose in the monitoring card and requested the signature of the person who applied it. The patients that returned the card completely filled out, with intervals between doses not greater than three days, were considered adherent.

Morisky test: it was applied at the end of the treatment and comprised four dichotomous questions (each negative answer received one point); and a patient was considered adherent if they got four points and non-adherent when they got any other lower value[19,20]. The four questions of the Morisky test are: 1 - Have you ever forgotten to take your medicine? 2 - Sometimes do you forget to take your medicine? 3 - When you feel better, do you sometimes stop taking your medicine? 4 - At times, if you feel worse when you take your medicine, do you stop taking them?

Modified Morisky test: in the modified version, the 2nd question regarding the schedule of taking the medicine was not considered, and a patient was considered adherent when they got three points and non-adherent, any lower value.

In the exploratory analysis, the simple frequencies of the qualitative variables and the median with interquartile range of the quantitative variables were calculated. The association of adherence (vial counting, Morisky test, modified Morisky test and monitoring card) with the socio-demographic and clinical variables and the treatment groups (high doses - 20 mg Sb^{5+}/kg/day - or low - 5 mg Sb^{5+}/kg/day; consecutive or intermittent doses) was evaluated by the Fisher's exact test. The Mann-Whitney test was used to assess the distribution difference of the quantitative variables in relation to the adherence variable.

The degree of concordance between the methods of adherence determination was evaluated by the simple Kappa index and categorized according to LANDIS & KOCH[12] as $\kappa: < 0$ (non concordance), κ: 0-0.19 (poor concordance), κ: 0.20-0.39 (fair concordance), κ: 0.40-0.59 (moderate concordance), κ: 0.60-0.79 (substantial concordance), κ: 0.80-1.00 (perfect concordance). The adopted level of significance for all the statistical tests was 5%. The Statistical Package for the Social Sciences (SPSS) version 16.0 was used for data analysis.

RESULTS

The median age was 40 years (minimum of 15 and maximum of 71), predominantly men (68.4%), white (61.4%), single or widowed (54.4%), education to 1st grade school (57.9%), monthly family income between one and five minimum wages (each minimum wage corresponding to US$ 321.77)[3] and resident in RJ endemic areas (86%). There was a median of three family members, ranging from one to eight members, with a median of two people sleeping in the same room (minimum one and maximum six). Regarding habits, it was found that the patients were 84.1% non-smokers, 93.0 non-users of illicit drugs and 57.9 % non-users of alcohol.

MA administration was done by health care providers in 71.9% of the cases, 68.4% of them with a college degree. The distance between

Table 1
Comparison between adherence to high dose (20 mg Sb^{5+}/kg/day) and low dose (5 mg Sb^{5+}/kg/day) schemes; and consecutive or intermittent administration

Adherence	Dose					Type of administration				
	20 mg Sb^{5+}/kg/day		5 mg Sb^{5+}/kg/day		*p*	Consecutive		Intermittent		*p*
	n	%	n	%		n	%	n	%	
Surplus vial Counting	20	74.1	26	90	0.171	22	78.6	24	86	0.485
Morisky test	17	63	21	70	0.574	18	64.3	20	69	0.708
Modified Morisky test	20	74.1	29	97	**0.021**	24	85.7	25	86	1.000
Monitoring Card	23	85.2	26	87	1.000	23	82.1	26	90	0.470

n = number of patients adherent to treatment. *p* = *p*-value. **In bold**, *p*-significant value.

Table 2
Assessment of the distribution difference of the quantitative variables, in relation to the adherence variable by the Mann-Whitney test

Adherence methods	Family members sleeping in the same room						*p*-value
	Adherence			Non-adherence			
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Surplus vial counting	2.00	1	4	3.00	2	6	0.001
Morisky test	2.00	1	4	2.00	1	6	0.037
Modified Morisky test	2.00	1	4	3.00	2	6	0.001
Monitoring card	2.00	1	6	2.50	2	4	0.012

home and the administration site was less than 10 km in 82.5% of cases, and 63.2 % of the patients reported that the treatment did not affect their daily life.

All patients reported satisfaction with treatment, IPEC and the medical staff.

88.2% of the patients were healed. We observed 82.1% adherence to treatment by controlling returned vials, 86.0% by the monitoring card, 66.7% through the Morisky test and 86.0% through the modified Morisky test.

There was a greater adherence of the group that received low doses when evaluated by the modified Morisky test (*p*-value = 0.021). The comparisons between adherence assessment methods and high and low dose schemes, and between consecutive and intermittent doses are shown in Table 1.

We did not observe a significant correlation between the qualitative variables and adherence to treatment. We observed that there were a significantly greater number of persons sleeping in the same room among those that did not adhere to treatment, by the four adherence evaluation methods (Table 2). No significant correlation was observed between adherence to treatment and the other quantitative variables assessed: age and number of family members (Table 2).

The degree of concordance among the adherence evaluation criteria measured by the Kappa index was considered satisfactory (substantial and perfect concordance), for most criteria, except between the Morisky test and the monitoring card, which presented poor concordance (Table 3).

Table 3
Concordance among adherence to treatment assessment methods in each treatment scheme in the clinical trial

Adherence	Kappa Index (κ)	Degree of Concordance*
Surplus vial counting and Monitoring card	0.736	Substantial Concordance
Surplus vial counting and Modified Morisky test	0.736	Substantial Concordance
Modified Morisky test and Monitoring card	0.418	Moderate Concordance
Surplus vial counting and Morisky test	0.351	Fair Concordance
Morisky test and Monitoring card	0.123	Poor Concordance

Note: *Classification according to Landis JR, Kock GG. 1977.

DISCUSSION

Factors associated with adherence of patients to different treatment schemes were evaluated during a clinical trial for CL treatment with MA, using a socioeconomic and evaluation of satisfaction with treatment questionnaire, return of non-used medicine vials, monitoring card, Morisky test and modified Morisky test. We did not find similar studies

in literature, comparing adherence to CL treatment with MA measured using different assessment methods.

Similar to our results, the variable gender has not shown, in the literature, a significant relationship with adherence to therapy[33]. Regarding the socioeconomic factors, the correlation found between adherence assessed by all the methods studied and a higher number of family members sleeping in the same room may be related to a more precarious economic condition of the patients. The correlation between adherence and socioeconomic characteristics of the patients has been widely studied in other infectious diseases, and contradictory results that may vary with the methods used have been found[5,15,25].

Although the use of medication did not alter the daily life of most patients, this occurred in 40% of them, which is understandable, considering that it is a parental medication that needs a qualified health professional to administer it, forcing the patient to leave home daily. It has been reported that patients present higher adherence levels to treatments when the administration is simple and the recommendations are easy to understand, they are short-term treatments and the patients do not significantly alter their daily lives[7]. On the other hand, adherence studies through intravenous (IV) administration of MA would require hospitalization or a day-hospital base, a condition that we cannot afford for all CL patients in Rio de Janeiro. We could, however, assume that adherence to treatment would be higher with IV MA, due to the discomfort associated with the use of intramuscular medication, and to the hospitalization itself[21,27].

The high adherence level observed in the present study can be partially explained by the voluntary participation in the clinical trial and the good relationship between patient and health care providers at IPEC, as proved in the assessment questionnaire of satisfaction with care and the hospital. The good relationship between health care providers and patients is increasingly recognized as a determinant for adherence to therapy[16,24].

A study of cultural and socioeconomic conditions showed that, despite the lower education and income of the population treated for ATL, the abandonment percentage was lower (1.6%), when compared to percentages previously reported for the Metropolitan Region of Belo Horizonte (25%)[25]. This result is compliant with the good adherence results of the present study, where the good relationship between patients and the health care team, as well as the voluntary participation, helped to obtain it.

Higher abandonment frequency in patients with a continuous treatment scheme, when compared to intermittent schemes of MA, has already been described[2]. Although our study did not present correlation between adherence and intermittent or continuous schemes, a significantly higher adherence was observed in the group that received low doses, when compared to the group that received high doses, when assessed by the modified Morisky test. The patients who received low doses may have presented better adherence, because the administration was simpler, there were fewer side effects and consequently lower modification of the daily life[7]. Especially for the elderly, the use of lower doses is recommended since the toxic effects of antimony are less pronounced and, consequently, the adhesion to therapy is greater[23]. Besides, it can be assumed that the refusal of two patients to respond to adherence assessment methods suggests that poor adherence is associated with a high dose.

It is believed that it is possible to monitor adherence to the prescribed treatment by counting the medication, as previously described in tuberculosis[15]. The medicine vial counting allows determination of the number of vials used, however, it is a method that requires collaboration and is subject to manipulation by the patient. It may also overestimate adherence, because the fact of returning the correct number of surplus vials, or less, does not necessarily ensure that the medication was correctly administered by the patient.

The monitoring card is a method that tends to underestimate adherence, because it needs the collaboration of the patient and the administrator: if, for some reason, it does not contain the signature of the administrator or if it is not completely filled out, the patient is considered non-adherent, even if they received the correct medication.

The Morisky test[19,20] tends to overestimate non-adherence, as opposed to what is expected from indirect methods and does not give the patient the opportunity to express their difficulties and understand the treatment[13]. In the Morisky test, carelessness regarding medication time has been the answer most frequently given by the patients. Being a deposit drug, in which the therapeutic effect of antimony seems to be supplied by the fraction accumulated in the tissues[4,8,17,26,32] carelessness, regarding the time when the medication is taken, it would not significantly affect adherence, if the daily administrations followed the schedule prescribed for the patient. When the modified Morisky test was applied, a significant correlation between adherence and low dose of meglumine antimoniate was obtained.

A study using the Morisky test showed low adherence level in leprosy treatment, indicating that patients did not have adequate knowledge of the principles of multidrug therapy, despite claiming to be familiar with leprosy and its therapy[14]. The Morisky test helps to identify the main reasons why patients do not adhere properly to treatment, which can help health care providers to find efficient solutions to solve adherence problems.

A study that assessed adherence to tuberculosis treatment by providing medication kits and the monitoring card revealed that counting the daily kits allowed assessing adherence to treatment[15]. In the present study, among the various methods, there was greater concordance between the vial counting and the monitoring card, although the first overestimates and the second underestimates adherence to treatment. When we applied the modified Morisky test, we observed a substantial concordance with the method of vial return and moderate with the monitoring card, thus suggesting that the first was adequate and simple to assess adherence of this group of patients.

We found a correlation between non-adherence to CL treatment with MA, assessed by various methods, and a higher number of family members sleeping in the same room. The assessment by the modified Morisky test showed better adherence in the group that received low doses of MA, than in the group that received high doses. Due to the good performance and simplicity of the method, when compared to other tests, we suggest the use of the modified Morisky test to assess adherence to CL treatment with MA.

RESUMO

Fatores associados à adesão a diferentes esquemas de tratamento com antimoniato de meglumina em ensaio clínico para leishmaniose cutânea

O desfecho favorável ao tratamento de uma enfermidade é influenciado pela adesão à terapia. Objetivamos avaliar fatores associados à adesão ao tratamento dos pacientes incluídos em ensaio clínico de equivalência entre o esquema de tratamento padrão e alternativos com antimoniato de meglumina (AM) no tratamento da leishmaniose cutânea (LC) no estado do Rio de Janeiro. Entre 2008 e 2011, 57 pacientes com LC foram entrevistados através de questionário para coleta de dados socioeconômicos. Para monitorização da adesão foram utilizados os seguintes métodos: contagem de ampolas excedentes, cartão de acompanhamento, teste de Morisky e teste de Morisky modificado (sem a pergunta referente ao horário). Observou-se adesão de 82,1% (devolução de ampolas), 86,0% (cartão de acompanhamento), 66,7% (teste de Morisky) e 86,0% (teste de Morisky modificado). Houve forte concordância entre o método contagem de ampolas e cartão de acompanhamento, bem como teste de Morisky modificado. Verificou-se associação significativa entre maior adesão ao tratamento e baixa dose de AM, bem como com menor número de pessoas dormindo no mesmo quarto. Recomendamos a utilização do teste de Morisky modificado na avaliação da adesão ao tratamento da LC com AM por ser método simples e com bom desempenho quando comparado aos outros testes.

ACKNOWLEDGEMENTS

Cláudia Maria Valete-Rosalino, Armando de Oliveira Schubach and Madson Pedro da Silva Leite were responsible for the design of the study. Madelon Novato Ribeiro, José Liporage Teixeira and Monique Reis da Fonseca collected data. All authors were responsible for conducting the study and managing data; Madelon Novato Ribeiro, Cláudia Maria Valete-Rosalino, Raquel de Vasconcellos Carvalhaes de Oliveira, Maria Inês Fernandes Pimentel and Armando de Oliveira Schubach analyzed and interpreted data, and prepared manuscript; all authors reviewed and approved manuscript.

We are grateful to Jacline Novato Ribeiro, Margareth de Araújo Silva, Michele Aparecida Ferreira Moreira de Oliveira, Fátima Peres Lima Dantas and Felipe Maia Maquieira da Silva for their help with the patients before the interviews. We also thank Dr. Sandro Javier Bedoya-Pacheco for help with the database.

This study was funded by PAPES-FIOCRUZ, National Council for Scientific and Technological Development (CNPq) and Carlos Chagas Filho Foundation for Research Support in the State of Rio de Janeiro (FAPERJ). Funding agencies had no interference in the design and conduct of the study; collection, management, analysis and interpretation of the data; and preparation, review, or approval of the manuscript.

REFERENCES

1. Ashford RW. The leishmaniasis as emerging and reemerging zoonoses. Int J Parasitol. 2000;30:1269-81.

2. Azeredo-Coutinho RB, Mendonca SC. An intermittent schedule is better than continuous regimen of antimonial therapy for cutaneous leishmaniasis in the municipality of Rio de Janeiro, Brazil. Rev Soc Bras Med Trop. 2002;35:477-81.

3. Brazilian Central Bank. Currency conversion. [cited 2012 January 01]. Available from: http://www4.bcb.gov.br/pec/conversao/conversao.asp

4. Chulay JD, Fleckenstein L, Smith DH. Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate. Trans R Soc Trop Med Hyg. 1988;82:69-72.

5. Conrad P. The meaning of medication: another look at compliance. Soc Sci Med. 1985;20:29-37.

6. Cramer JA, Mattson RH, Prevey ML, Scheyer RD, Ouellette VL. How often is medication taken as prescribed? A novel assessment techinique. JAMA. 1989;261:3273-7.

7. Goldberg AL, Cohen G, Rubin AH. Physician assessments of patient compliance with medical treatment. Soc Sci Med. 1998;47:1873-6.

8. Goodwin LG, Page JE. A study of the excretion of organic antimonials using a polarographic procedure. Biochem J. 1943;37:198-209.

9. Goodwin LG. Pentostam (sodium stibogluconate): a 50-year personal reminiscence. Trans R Soc Trop Med Hyg. 1995;89:339-41.

10. Herwaldt BL. Leishmaniasis. Lancet. 1999;354(9185):1191-9.

11. Haynes RB, Taylor DW, Sackett DL, editors. Compliance in health care. Baltimore: Johns Hopkins University Press; 1979.

12. Landis JR, Kock GG. The measurement of observer agreement for categorical data. Biometrics. 1977;33:159-74.

13. Leite SN, Vasconcellos MPC. Adesão à terapêutica medicamentosa: elementos para a discussão de conceitos e pressupostos adotados na literatura. Cienc Saúde Colet. 2003;8:775-82.

14. Lira KB, Leite JJG, Maia DCBSC, Freitas RMF, Feijão AR. Knowledge of the patients regarding leprosy and adherence to treatment. Braz J Infect Dis. 2012;16:472-5.

15. Maciel ELN, Silva AP, Meireles W, Fiorotti K, Hadad DJ, Dietze R. Tratamento supervisionado em pacientes portadores de tuberculose utilizando supervisores domiciliares em Vitória, Brasil. J Bras Pneumol. 2008;34:506-13.

16. Marinker M, Shaw J. Not to be taken as directed: putting concordance for taking medicines into practice. BMJ. 2003;326:348-9.

17. Miekeley N, Mortari SR, Schubach AO. Monitoring of total antimony and its species by ICP-MS and on-line ion chromatography in biological samples from patients treated for leishmaniasis. Anal Bioanal Chem. 2002;372:495-502.

18. Ministerio da Saude. Secretaria de Vigilancia em Saude. Manual de vigilancia da Leishmaniose Tegumentar Americana. 2. ed. atual. Brasilia: Ministerio da Saúde; 2010.

19. Morisky DE, Levine DM, Green LW, Smith CR. Health education program effects on the management of hypertension in the elderly. Arch Int Med. 1982;142:1835-8.

20. Morisky DE, Green LW, Levine DM. Concurrent and predictive validity of a self-reported measure of medication adherence. Med Care. 1986;24:67-74.

21. Oliveira-Neto MP, Schubach A, Mattos M, Gonçalves-Costa SC, Pirmez C. Treatment of American cutaneous leishmaniasis: a comparison between low dosage (5 mg/kg/day) and high dosage (20 mg/kg/day) antimony regimens. Path Biol. 1997;45:496-9.

22. Oliveira-Neto MP, Schubach A, Mattos M, da Costa SC, Pirmez C. Intralesional therapy of American cutaneous leishmaniasis with pentavalent antimony in Rio de Janeiro, Brazil - an area of *Leishmania (V.) braziliensis* transmission. Int J Dermatol. 1997A;36:463-8.

23. Oliveira-Neto MP, Mattos M, Pirmez C, Fernandes O, Gonçalves-Costa SC, Souza CFS, *et al*. Mucosal leishmaniasis ("espundia") responsive to low dose of N-methyl glucamine (Glucantime ®) in Rio de Janeiro, Brazil. Rev Inst Med Trop Sao Paulo. 2000;42:321-5.

24. Osterberg L, Blaschke T. Adherence to medication. N Engl J Med. 2005;353:487-97.

25. Passos VMA, Barreto SM, Romanha AJ, Krettli AU, Volpini AC, Gontijo CM, *et al*. Leishmaniose tegumentar na região metropolitana de Belo Horizonte: aspectos clínicos, laboratoriais, terapêuticos e evolutivos (1989-1995). Rev Soc Bras Med Trop. 2001;34:5-12.

26. Roberts WL, Berman JD, Rainey PM. *In vitro* antileishmanial properties of tri- and pentavalent antimonial preparations. Antimicrob Agents Chemother. 1995;39:1234-9.

27. Schubach AO, Marzochi KBF, Moreira JS, Schubach TM, Araujo ML, Vale AC, *et al*. Restrospective study of 151 patients with cutaneous leishmaniasis treated with meglumine antimoniate. Rev Soc Bras Med Trop. 2005;38:213-7.

28. Shaw JJ. Taxonomy of genus *Leishmania*: present and future trends and their implications. Mem Inst Oswaldo Cruz. 1994;89:471-8.

29. Valencia C, Arévalo J, Dujardin JC, Llanos-Cuentas A, Chappuis F, Zimic M. Prediction score for antimony treatment failure in patients with ulcerative leishmaniasis lesions. PLoS Negl Trop Dis. 2012;6:e1656. doi: 10.1371/journal.pntd.0001656. Epub 2012 Jun 12.

30. Vasconcellos EFC, Schubach AO, Valete-Rosalino CM, Coutinho RS, Conceição-Silva F, Salgueiro MM, *et al*. American tegumentary leishmaniasis in older adults: 44 cases treated with an intermittent low-dose antimonial schedule in Rio de Janeiro, Brazil. J Am Geriatr Soc. 2010;58:614-6.

31. Vasconcellos EC, Pimentel MIF, Schubach AO, de Oliveira RV, Azeredo-Coutinho RB, Silva FC, *et al*. Intralesional meglumine antimoniate for treatment of cutaneous leishmaniasis patients with contraindication to systemic therapy from Rio de Janeiro (2000 to 2006). Am J Trop Med Hyg. 2012;87:257-60.

32. Váquez L, Scorza Dagert JV, Scorza JV, Vicuña-Fernández N, Petit de Peña Y, López S, *et al*. Pharmacokinetics of experimental pentavalent antimony after intramuscular administration in adult volunteers. Curr Ther Res Clin Exp. 2006;67:193-203.

33. Vermeire E, Hearnshaw H, Van Royen P, Denekens J. Patient adherence to treatment: three decades of research. A comprehensive rewiew. J Clin Pharm Ther. 2001;26:331-42.

34. World Health Organization. Letter to Aventis pharma drug regulatory affairs, Europe. Essential drugs and medicines policy. Geneva: WHO; 2001.

35. World Health Organization. Adherence to long-term therapies: evidence for action. Geneva: WHO; 2003.

Received: 22 July 2013
Accepted: 10 January 2014

Rev. Inst. Med. Trop. Sao Paulo
56(4):297-300, July-August, 2014
doi: 10.1590/S0036-46652014000400005

PARTICIPATION OF TICKS IN THE INFECTIOUS CYCLE OF CANINE VISCERAL LEISHMANIASIS, IN TERESINA, PIAUÍ, BRAZIL

José Henrique Furtado CAMPOS(1) & Francisco Assis Lima COSTA(2)

SUMMARY

In this study, we detected *Leishmania* spp. infection in *R. sanguineus* collected from dogs that were naturally infected with *L. (L.) infantum*. We examined 35 dogs of both sexes and unknown ages. The infected dogs were serologically positive by the immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and Quick Test-DPP (Dual Path Platform), as well as parasitological examination of a positive skin biopsy or sternal bone marrow aspiration. Ten negative dogs were included as controls. The ticks that infested these dogs were collected in pools of 10 adult females per animal. The PCR was performed with specific primers for *Leishmania* spp., which amplified a 720-bp fragment. Of the 35 analyzed samples, a product was observed in eight samples (8/35; 22.9%). We conclude that the presence of parasite DNA suggests that ticks participate in the zoonotic cycle of canine visceral leishmaniasis, in the city of Teresina, Piauí.

KEYWORDS: *Rhipicephalus sanguineus; Canis familiaris; Leishmania* spp.

INTRODUCTION

Canine visceral leishmaniasis (CVL) is a disease of great zoonotic potential[5]. It is caused by the protozoan *L. (L.) infantum* in the Americas[19]. The major means of transmission of the parasite to humans and other vertebrate hosts is through the bite of infected female sand flies[25]. Currently, *Lutzomyia (Lutzomyia) longipalpis* is considered the primary transmitter of the species *L. (L.) infantum* in Brazil. However, *Lutzomyia cruzi* in the state of Mato Grosso do Sul and *Lutzomyia migonei* and *Lutzomyia firmatoi* in Rio de Janeiro are also considered vectors of visceral leishmaniasis (VL)[26,29].

The high incidence of CVL in Brazil and the occurrence of autochthonous cases in the absence of phlebotomine vectors suggest the possibility that *R. sanguineus* participates in the transmission of infection in dogs. The importance of *R. sanguineus* in the transmission of pathogens to humans and dogs has been substantiated by recent reviews[9,10,11,21,22].

R. sanguineus is the most frequent ectoparasite of dogs in Brazil. In a study of dogs in Belo Horizonte and neighboring counties, the rate of infestation by *R. sanguineus* was found to be 37.9%. In Juiz de Fora, the index was found to be 60.6% in shelter dogs parasitized by this tick species[10,16,24].

The likely involvement of *R. sanguineus* in the transmission of *L. (L.) infantum* in dogs was demonstrated in the 1930s in France, where the ability of the tick to mechanically transmit the protozoan to rodents was observed[4]. In Brazil, the presence of morphologically similar forms of *Leishmania* spp. to "Leptomonas" in ticks collected from dogs with visceral leishmaniasis (VL) has been reported[23]. Other studies have found ticks to be naturally infected with *L. (L.) infantum*[7,11]. The presence of *L. (L.) infantum* in the salivary glands of ticks and the presence of *Leishmania* spp. RNA were recently reported[6,11].

The possible participation of *R. sanguineus* in the transmission of VL, particularly in areas where autochthonous cases of CVL occur without the presence of *Lutzomyia (Lutzomyia) longipalpis*, should be the focus of further studies[8]. Similarly, in areas of high endemicity for CVL, where the euthanasia of dogs with VL shows no positive correlation with a decrease in the incidence of the disease[14] the possible involvement of other likely vectors should be considered[29]. In this context, the objective of this study was to detect *Leishmania* spp. in *R. sanguineus* fed directly to dogs that were naturally infected by *L. (L.) infantum* to determine the possible participation of the tick in the cycle of visceral leishmaniasis in the endemic area of Teresina, Piauí.

MATERIALS AND METHODS

This study was conducted with 65 dogs of both sexes and mixed breed that were from a Brazilian endemic area (Teresina, state of Piauí) and were infested with ticks. However, only 35 dogs had a positive parasitological result upon skin biopsy or aspiration of sternal bone marrow and a positive

(1) Pós-Graduando da Universidade Federal do Piauí (UFPI).
(2) Professor Associado da Universidade Federal do Piauí/CCA/DCCV, Campus da Socopo, 64049-550 Teresina, Piauí, Brazil.
Correspondence to: José Henrique Furtado Campos. E-mail: jhfcampos@gmail.com

Table 1
Examination performed on 35 dogs naturally infected by *L. (L.) infantum* and pooled ticks collected from these animals. Teresina, Piauí, 2012

Tests Dogs	Serology			Parasitological			PCR/Ticks		
	P	N	%	P	N	%	P	N	%
Infected	35	0	100.0	35	0	100.0	08	27	22.9
Uninfected	0	10	100.0	0	10	100.0	0	10	100.0

P = positive; N = Negative; PCR = Polymerase chain reaction.

serological diagnosis by indirect immunofluorescence techniques (Bio-Manguinhos, Fiocruz), ELISA (Bio-Manguinhos, Fiocruz) and Quick Test-DPP (Dual Path Platform) for the detection of anti-*Leishmania* antibodies. The serological diagnosis and PCR were performed in the Laboratory of Parasitology of the Center for Health Sciences, Federal University of Piauí. This project was approved by the ethical commission of the Federal University of Piauí as number 007/12.

Collection of ticks: Ten adult female engorged ticks were collected per animal. Ticks were placed in vials with 70% alcohol and taken to the laboratory. The identification of ticks was performed according to the classification key of ARAGÃO & FONSECA (1961)[2].

In the laboratory, ticks were washed with distilled water and dried on paper towels. With the aid of a scalpel blade, the ticks were sectioned at the side for the complete extraction of material ingested during its blood meal. The extracted material was placed in an Eppendorf tube and stored in a freezer at -70 ºC until the DNA extraction procedure was performed.

PCR: DNA was extracted from samples taken from ticks and the sternal bone marrow of dogs using a commercial kit from Promega (Cat. # A1120, Wizard Genomic DNA Purification Kit), according to the manufacturer's recommendations. The PCR reaction mixture was prepared at a final volume of 25 µL using SuperMix from Invitrogen, which contains 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 mM of each dNTP (deoxynucleotide triphosphate), and 22 U of recombinant Taq DNA polymerase/mL). The primers used were Lin R4 (5 '- GGG GTA GTT GGT AAA TAG GG - 3') and Lin 19 (5 'CAG AAC GCC ACC CCT CG - 3'), which were originally developed by ARANSAY *et al.* (2000)[3] and are specific to the minicircle region of *Leishmania* spp. The mixture for the PCR reaction included 22 µL of SuperMix, 1 µL of each primer, and 1 µL of the DNA sample. Amplification was performed in a thermocycler (Eppendorf Mastercycler Gradient) for a total of 35 cycles, with an initial cycle of 94 °C for five minutes, followed by 34 cycles of 30 seconds at 95 °C, 30 seconds at 58 °C, and one minute at 72 °C, with a final cycle of seven min at 72 °C. The PCR product was run on a 1.5% agarose gel, stained with ethidium bromide (1.0 µL/10 mL), and viewed in a UV transilluminator (Bioagency). DNA extracts from cultures of *Leishmania* spp. were used as positive controls. As a negative control, we used a mixture of the reactants used in the PCR reaction without the initial template DNA. Comparative data analyses were performed using Fischer's nonparametric statistical test. The level of significance was set at $p < 0.05$.

RESULTS

Of the 65 dogs examined, all were parasitized by ticks, but only 35 naturally infected dogs (G1) used in this study were reactive in all serological tests (IFAT, ELISA, and RT - DPP) and parasitological examinations performed on a skin biopsy and/or a sternal bone marrow smear[20]. The IFAT showed high titers of antibodies to canine IgG in most of the evaluated samples (Tables 1 and 2). Dogs that were uninfected (G2) were all non-reactive in the exams.

Table 2
The results of PCR performed on 35 pooled samples of *R. sanguineus* collected from dogs naturally infected with *L. (L.) infantum*. Teresina, Piauí, 2012

Clinical status	PCR					
	P	%	N	%	T	%
Infected	08	22.9	27	77.1	35	100.0
Uninfected	0	0.0	10	100.0	10	100.0

In the 35 naturally infected dogs (G1), the most frequent clinical manifestations were increased superficial lymph node size, weight loss, onychogryphosis, skin lesions, conjunctivitis, ulceration of the skin, alopecia, coryza, desquamation of the skin, keratitis, depigmentation of the nose, pale mucous, apathy, hyperkeratosis and edema as found in other study[1].

Most uninfected dogs (Group 2) showed no clinical signs of CVL, with the exception of four dogs that had only one clinical sign, either alopecia, weight loss, onychogryphosis, or hyperkeratosis.

The PCR results of pooled samples of *R. sanguineus* collected from eight dogs were positive (8/35-22.9%). In the group of uninfected dogs, no DNA amplification was observed in the collected *R. sanguineus* or bone marrow samples.

DISCUSSION

In this study, PCR performed on material extracted from pools of ten engorged adult female ticks from each of the dogs that were naturally infected with *L. (L.) infantum* revealed that 22.9% of the ticks were infected. It is important to note that we used only adult female ticks that were engorged by spending time feeding on the host. COUTINHO *et al.* (2005)[7] analyzed ticks collected in various life cycle stages from 21 dogs with symptoms of CVL from the endemic area of Minas Gerais and identified DNA from *Leishmania* spp. in six ticks (15.4%). Another study of 128 ticks collected from dogs from an endemic area of VL in Italy found DNA from *Leishmania* spp. in 13 ticks (10.1%)[28]. The results of the present study, conducted in the endemic area of Teresina, present a higher percentage

of ticks infected by *L. (L.) infantum*, which is significant and alongside other epidemiological factors may be related to participation of ticks in the infectious cycle of the disease, as has been previously suggested[7,11,15]. However, amastigote forms of the parasite were not detected in ticks in this study. Nonetheless, transmission has been demonstrated experimentally, and flagellated forms, such as the infective form of the parasite, have been observed in material extracted from ticks[7,23,27].

The involvement of ticks in the epidemiology of CVL requires further study. However, many studies have detected DNA and RNA from *Leishmania* spp. in ticks, with an infection rate that is higher than the natural rate of infection in *Lutzomyia longipalpis*[7,11,18]. Notably, RNA from *Leishmania* spp. has been detected in *R. sanguineus* maintained after blood feeding in a greenhouse under appropriate temperature and humidity conditions for ten days. Additional factors support the participation of the tick in the life cycle of CVL, including its extremely common contact with dogs, its longevity, its ability to adapt to the environment, the process and durability of blood feeding and the locomotion capacity of males seeking a new host[6,9,10,28].

In addition to these considerations, one very important fact is that DNA found in adult females has also been detected under experimental conditions in eggs and in the subsequent life stages of the tick[13]. When feeding on the host, *R. sanguineus* excretes a substantial amount of potentially contaminated saliva. A study reported the presence of *L. (L.) infantum* DNA in the salivary glands of ticks[11].

Although it has not been proven that ticks can transmit infections to dogs, dogs can transmit infections to ticks. One study demonstrated this by placing healthy ticks maintained in a laboratory on dogs with VL. After a few days, *Leishmania* spp. DNA was detected in the respective ticks, and this infection was present in subsequent stages of tick development [23].

In the slides examined in our study, which were created with material extracted from ticks, no evolutionary form of *Leishmania* spp. or any similarly flagellated forms were observed. However, specimens of *R. sanguineus* infected with shapes similar to promastigotes of *L. (L.) infantum* have been reported, but this result should be viewed with some caution because some monogenetic trypanosomatids found in *R. sanguineus* can be easily confused with promastigotes of *Leishmania* spp.[17,23,27].

The possibility of transmission of *L. (L.) infantum* by *R. sanguineus* implies that new measures are necessary for the control of VL due to the difficulty of controlling this ectoparasite, which is widespread in Brazil[10]. New experimental studies for adapting a proper method of xenodiagnostic testing using ticks and demonstrating the transmission of infection from an infected tick to a dog will be needed to definitively prove the role of *R. sanguineus* in the transmission of *L. (L.) infantum* to dogs.

Based on the results presented herein, we conclude that the presence of *Leishmania* spp. in ticks collected from parasitologically positive dogs from areas in which CVL is endemic demonstrates that this arthropod is infected and may have epidemiological importance, particularly given the large number of positive dogs and the low incidence of natural infection in sandflies (less than 1.0%)[18]. This possibility should be corroborated with various autochthonous VL cases in which the presence of *Lutzomyia longipalpis* is not detected.

RESUMO

Participação do *Rhipicephalus sanguineus* no ciclo infeccioso da leishmaniose visceral canina em Teresina, Piauí, Brasil

Neste estudo foi detectada infecção por *Leishmania* spp. em *Rhipicephalus sanguineus* (*R. sanguineus)* de cães naturalmente infectados por *Leishmania (Leishmania) infantum* = *L. (L.) infantum*. Foram utilizados 35 cães de ambos os sexos e idades desconhecidas, sorologicamente positivos pelas técnicas de reação de imunofluorescência indireta (RIFI), enzyme-linked immunosorbent assay (ELISA) e Quick Test-DPP (Dual Path Plataform), e com exame parasitológico positivo em biópsia de pele ou punção de medula óssea esternal e 10 cães domiciliados negativos, como controle. Os carrapatos que infestavam esses cães foram coletados em pool de 10 fêmeas adultas por animal. A Reação em Cadeia pela Polimerase (PCR = Polymerase Chain Reaction) foi realizada com primers para *Leishmania* spp., que amplificaram 720pb. O resultado das 35 amostras processadas revelou a amplificação de oito amostras (8/35 - 22,9%). Conclui-se que a presença do DNA do parasita sugere que carrapatos podem estar participando do ciclo zoonótico da leishmaniose visceral canina na cidade de Teresina, Piauí.

REFERENCES

1. Almeida MAO, Jesus EEV, Sousa-Atta MLB, Alves LC, Berne MEA, Atta AM. Clinical and serological aspects of visceral leishmaniasis in Northeast Brazilian dogs naturally infected with *Leishmania chagasi.* Vet Parasitol. 2005;127:227-32.

2. Aragão HB, Fonseca F. Notas de ixodologia. VIII. Lista e chave para os representantes da fauna ixodológica brasileira. Mem Inst Oswaldo Cruz. 1961;59:115-29.

3. Aransay AM, Scoulica E, Tselentis Y. Detection and identification of *Leishmania* DNA with naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. Appl Environ Microbiol. 2000;66:1933-8.

4. Blanc G, Caminopetros J. La transmission du Kala-azar méditerranéen par une tique: *Rhipicephalus sanguineus*. C R Acad Sci. 1930;191:1162-4.

5. Camargo JB, Troncarelli MZ, Ribeiro MG, Langoni H. Leishmaniose visceral canina: aspectos de saúde pública e controle. Clín Vet. 2007;71:86-92.

6. Colombo FA, Odorizzi RMFN, Laurenti MD, Galati EAB, Canavez F, Pereira-Chioccola VL. Detection of *Leishmania (Leishmania) infantum* RNA in fleas and ticks collected from naturally infected dogs. Parasitol Res. 2011;109:267-74.

7. Coutinho MTZ, Bueno LL, Sterzik A, Fujiwara RT, Botelho JR, De Maria M, *et al*. Participation of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the epidemiology of canine visceral leishmaniasis. Vet Parasitol. 2005;128:149-55.

8. Dantas-Torres F, Brandão-Filho SP. Visceral leishmaniasis in Brazil: revisiting paradigms of epidemiology and control. Rev Inst Med Trop Sao Paulo. 2006;48:151-6.

9. Dantas-Torres F. Canine vector-borne diseases in Brazil. Parasit Vectors. 2008a;1:25.

10. Dantas-Torres F. The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): from taxonomy to control. Vet Parasitol. 2008b;152:173-85.

11. Dantas-Torres F, Lorusso V, Testini G, de Paiva-Cavalcanti M, Figueredo LA, Stanneck D, *et al*. Detection of *Leishmania infantum* in *Rhipicephalus sanguineus* ticks from Brazil and Italy. Parasitol Res. 2010a;106:857-60.

12. Dantas-Torres F, Martins TF, de Paiva-Cavalcanti M, Figueredo LA, Lima BS, Brandão-Filho SP. Transovarial passage of *Leishmania infantum* kDNA in artificially infected *Rhipicephalus sanguineus*. Exp Parasitol. 2010b;125:184-5.

13. Dantas-Torres F, Latrofa MS, Otranto D. Quantification of *Leishmania infantum* DNA in females, eggs and larvae of *Rhipicephalus sanguineus*. Parasit Vectors. 2011;4:56.

14. Dietze R, Barros GB, Teixeira L, Harris J, Michelson K, Falqueto A, *et al*. Effect of eliminating seropositive canines on the transmission of visceral leishmaniasis in Brazil. Clin Infect Dis. 1997;25:1240-2.

15. Gezoon. Gerência de Zoonoses. Teresina: Fundação Municipal de Saúde; 2009. Available from: http://saude.teresina.pi.gov.br/zoonoses

16. Linardi PM, Nagem, RL. Pulicídeos e outros ectoparasitos de cães de Belo Horizonte e municípios vizinhos. Rev Bras Biol. 1973;33:529-38.

17. Machattie C, Chadwick CR. Notes on a trypanosome infection of the dog tick *Rhipicephalus sanguineus* in Iraq. Trans R Soc Trop Med Hyg. 1930;23:417-20.

18. Martín-Sánchez J, Gállego M, Barón S, Castillejo S, Morillas-Marquez F. Pool screen PCR for estimating the prevalence of *Leishmania infantum* infection in sandflies (Diptera: Nematocera, Phlebotomidae). Trans R Soc Trop Med Hyg. 2006;100:527-32.

19. Mauricio IL, Howard MK, Stothard JR, Miles MA. Genomic diversity in the *Leishmania donovani* complex. Parasitology. 1999;119:237-46.

20. Meinkoth JH, Cowell RL, Tyler RD, Morton RJ. Coleta e preparo de amostras. In: Cowell RL, Tyler RD, Meinkoth JH, DeNicola DB, editores. Diagnóstico citológico e hematologia de cães e gatos. São Paulo: Medvet; 2009. p. 1-8.

21. Otranto D, Dantas-Torres F, Breitschwerdt EB. Managing canine vector-borne diseases of zoonotic concern: part one. Trends Parasitol. 2009a;25:157-63.

22. Otranto D, Dantas-Torres F, Breitschwerdt EB. Managing canine vector-borne diseases of zoonotic concern: part two. Trends Parasitol. 2009b;25:228-35.

23. Paz GF, Ribeiro MFB, Michalsky EM, da Rocha Lima AC, França-Silva JC, Barata RA, *et al*. Evaluation of the vectorial capacity of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the transmission of canine visceral leishmaniasis. Parasitol Res. 2010;106:523-8.

24. Rodrigues AFSF, Daemon E, D'Agosto M. Investigação sobre alguns ectoparasitos em cães de rua no município de Juiz de Fora, Minas Gerais. Rev Bras Parasitol Vet. 2001;10:13-9.

25. Santa Rosa ICA, Oliveira ICS. Leishmaniose visceral: breve revisão sobre uma zoonoze reemergente. Clín Vet. 1997;2:24-8.

26. Santos SO, Arias J, Ribeiro AA, de Paiva Hoffmann M, de Freitas RA, Malacco MA. Incrimination of *Lutzomyia cruzi* as a vector of American visceral leishmaniasis. Med Vet Entomol. 1998;12:315-7.

27. Sherlock I. Notas sobre a transmissão da leishmaniose visceral no Brasil. Rev Bras Malariol D Trop. 1964;16:19-26.

28. Solano-Gallego L, Rossi L, Scroccaro AM, Montarsi F, Caldin M, Furlanello T, *et al*. Detection of *Leishmania infantum* DNA mainly in *Rhipicephalus sanguineus* male ticks removed from dogs living in endemic areas of canine leishmaniosis. Parasit Vectors. 2012;5:98.

29. Souza MB, Marzochi MCA, Carvalho RW, Ribeiro PC, Pontes CS, Caetano JM, *et al*. Ausência da *Lutzomyia longipalpis* em algumas áreas de ocorrência de leishmaniose visceral no município do Rio de Janeiro. Cad Saúde Pública. 2003;19:1881-5.

Received: 3 January 2013
Accepted: 27 January 2014

Rev. Inst. Med. Trop. Sao Paulo
56(4):301-305, July-August, 2014
doi: 10.1590/S0036-46652014000400006

FUNGEMIA CAUSED BY *Candida* SPECIES IN A CHILDREN'S PUBLIC HOSPITAL IN THE CITY OF SÃO PAULO, BRAZIL: STUDY IN THE PERIOD 2007-2010

Vanessa Kummer Perinazzo OLIVEIRA(1), Luciana da Silva RUIZ(1), Nélio Alessandro Jesus OLIVEIRA(2), Débora MOREIRA(1), Rosane Christine HAHN(3), Analy Salles de Azevedo MELO(4), Angela Satie NISHIKAKU(4) & Claudete Rodrigues PAULA(5)

SUMMARY

Candidemia remains a major cause of morbidity and mortality in the health care environment. The epidemiology of *Candida* infection is changing, mainly in relation to the number of episodes caused by species *C.* non-*albicans*. The overall objective of this study was to evaluate the frequency of yeasts of the genus *Candida*, in a four-year period, isolated from blood of pediatric patients hospitalized in a public hospital of the city of São Paulo, Brazil. In this period, yeasts from blood of 104 patients were isolated and, the identified species of *Candida* by phenotypic and genotypic methods were: *C. albicans* (39/104), *C. tropicalis* (25/104), *C. parapsilosis* (23/104), *Pichia anomala* (6/104), *C. guilliermondii* (5/104), *C. krusei* (3/104), *C. glabrata* (2/104) and *C. pararugosa* (1/104). During the period of the study, a higher frequency of isolates of *C.* non-*albicans* (63.55%) ($p = 0.0286$) was verified. In this study we verified the increase of the non-*albicans* species throughout the years (mainly in 2009 and 2010). Thus, considering the peculiarities presented by *Candida* species, a correct identification of species is recommended to lead to a faster diagnosis and an efficient treatment.

KEYWORDS: *Candida*; Candidemia; Pediatric.

INTRODUCTION

Candidemia is an important concern in the clinical medicine related to the public health[20], mainly because of the high mortality rates in children and adults, 30% and 50% respectively[38]. The risk factors for the acquisition of this infection are similar in both ages and include a long-term hospitalization in an Intensive Care Unit, previous bacterial infection, central venous catheter use, parenteral nutrition and immunosuppression. The yeasts of the genus *Candida* represent the third/fourth pathogens responsible for the bloodstream infections[10,11,24] and their epidemiology has been well studied in the United States and Europe, but not in Latin America. In Brazil, the incidence rates are fragmented when considering data from all regions of the country[10,27].

Although *C. albicans* remains the most frequent species of yeast isolated from bloodstream infections, over the last years the number of candidemias caused by non-*albicans* species has been increasing. Being that, some studies have reported that from 40% to 50% of the infections are caused by other species of *Candida*[16,31,33]. Moreover, invasive infections caused by non-*albicans* species are more difficult to be treated due to its eventual innate or acquired resistance to antifungal agents. Therefore the treatment administration should be based on the species-level identification[23].

Many epidemiological studies have described the distribution of the species, antifungal susceptibility and risk factors in the adult population[10,33]. However, there are few studies about candidemia in the pediatric population[30,34]. Some studies, done in hospitals from different geographic regions, report that the most frequent species that cause bloodstream infections in pediatric patients are *C. albicans* and *C. parapsilosis*[16,28]. In adults, the most frequent isolated species are *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei*[28].

In Brazil, the most commonly isolated species in pediatric patients are C. *albicans, C. parapsilosis* and *C. tropicalis.* In adult patients the most common species are the same as in pediatric patients, except because of the species *C. tropicalis* that is the second most frequent species in adults[10]. This high isolation of *C. parapsilosis* in pediatric patients could be explained by the fact that this species is related to prematurity, presence of central venous catheters and the use of total parenteral nutrition[30].

Anatomical and physiological differences between the pediatric and adult patients change the susceptibility to infections caused by different species of *Candida*, which therefore influences the antifungal treatment approach, including issues related to the toxicity of the drugs, pharmacokinetic and dosage[41].

Considering the existence of a restricted amount of data to guide the clinical decisions to child patients with invasive fungal infections when

(1) Department of Microbiology, Institute of Biomedical Science II, University of São Paulo (USP), São Paulo, SP, Brazil.
(2) Lillehei Heart Institute, Dept. of Medicine, University of Minnesota, Minneapolis-MN, USA.
(3) Division of Infectious and Tropical Diseases, Federal University of Mato Grosso, MT, Brazil.
(4) Special Laboratory of Mycology, Federal University of São Paulo (UNIFESP).
(5) Department de Stomatology, School of Dentistry, University of São Paulo (USP). São Paulo, SP, Brazil.
Correspondence to: Claudete Rodrigues Paula, PhD, Departmento de Estomatologia, Faculdade de Odontologia, Universidade de São Paulo (USP), Av. Prof. Lineu Prestes 2277, 05508-000 São Paulo, SP, Brasil. Phone/fax: 55.11.30917883. E-mail: crpmicol@uol.com.br

compared to adults with similar infections, this study had as its objective to evaluate the frequency of the yeast species isolated from blood during four years in a pediatric hospital in São Paulo, Brazil.

PATIENTS

From 2007 to 2010, yeasts isolated from blood of 104 patients, represented by children from five to 11 years old, were studied. Being that, each isolated represented a different episode of candidemia. The patients were hospitalized in a tertiary infant hospital with 90 hospital beds located in the city of São Paulo, Brazil.

MATERIAL AND METHODS

Isolate Process: The blood was seeded in a biphasic culture medium BACTEC (Difco^{tm}, USA). Once the fungal growth was detected, the medium was seeded in Sabouraud dextrose agar added to chloramphenicol to the yeasts isolation, after that it was seeded in a CHROmagar® Candida Chromogenic Medium to ensure the purity of the isolates and then phenotypically identified.

Phenotypic identification: The conventional identification of all the isolates was performed according to the protocol of KURTZMAN *et al.*[20], using the germ tube test, microculture, auxanogram and zymogram. The phenotypic identification between *C. albicans* and *C. dubliniensis* was performed evaluating the colony color in a CHROmagar® medium, the thermotolerance[34] and the growth in a hypertonic medium[3].

Genotypic identification: The molecular technique was used to the complex *C. parapsilosis* and for the differentiation of *C. albicans* and *C. dubliniensis*.

DNA extraction: The DNA extraction from the culture was carried out using the *PrepMan^{TM} Ultra Sample Preparation reagent Quick Reference Card kit* (Applied Biosystems, USA), according to the commercial protocol.

Differentiation of the complex C. parapsilosis (C. parapsilosis sensu stricto, C. orthopsilosis, C. metapsilosis): To the differentiation of the complex *C. parapsilosis* (*C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis*) a Ribosomal DNA ITS sequencing was performed[37]. The amplification of the ITS-rDNA region was accomplished using the universal primers V9G / LS266[13] and the Big Dye terminator 3.1 kit (Applied Biosystems, USA). The edition of the nucleotide sequences was performed and analyzed by comparing them with the sequences available in the GenBank using the BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Differentiation between C. albicans and C. dubliniensis: It was carried out according to MANNARELLI & KURTZMAN[22]. The PCR was performed with two pairs of specific primers: one pair for *C. dubliniensis* (sense CDU2 and anti-sense NL4CAL) and another pair for *C. albicans* (sense CAL5 and anti-sense: NL4CAL). The presence or not of amplified fragments was visualized by using the agarose gel electrophoresis.

Quality control: To control the quality of the phenotypic and genotypic methods, the strain patterns used were: ATCC 64548 (*C. albicans*), ATCC 777 (*C. dubliniensis*), *C. parapsilosis* (ATCC 22019), *C. orthopsilosis* (ATCC 96141) and *C. metapsilosis* (ATCC 96143).

Statistical analysis: Quantitative variables were analyzed by the unpaired Student's t test or Mann-Whitney (Prism version 5.0, GraphPad Software Inc., La Jolla, CA, USA). A p value < 0.05 was considered to be statistically significant.

RESULTS

During the period of the study, a total of 104 yeast strains were isolated from hemocultures. Figure 1 represents the total of yeasts isolated from blood per year.

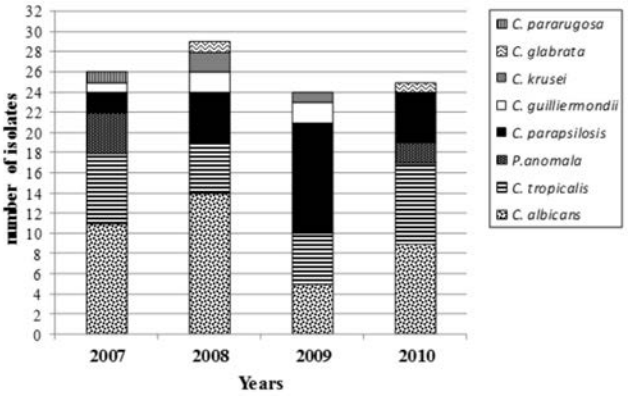


Fig. 1 - Number of strains and species isolated from blood of 104 pediatric patients, hospitalized in a children´s public hospital, from 2007 to 2010.

The isolation of *C. albicans* in the children's public hospital studied was kept in the first two years, with the frequency of 42.3% in 2007 and 48.3% in 2008. In 2009 we had a decrease in the isolation rate (21.7%) followed by an increase in 2010 (36%). The isolation of *C. non-albicans* has always been higher than the one verified for *C. albicans* ($p = 0.0286$) and differently from these species, we had a considerable increase in the isolation rate for non-*albicans* species in the last two periods. The change in the numbers of isolates of *C. albicans* and non-*albicans* from 2007 to 2010 can be observed in the Figure 2.

In the four years of this study, the total frequency of identified species

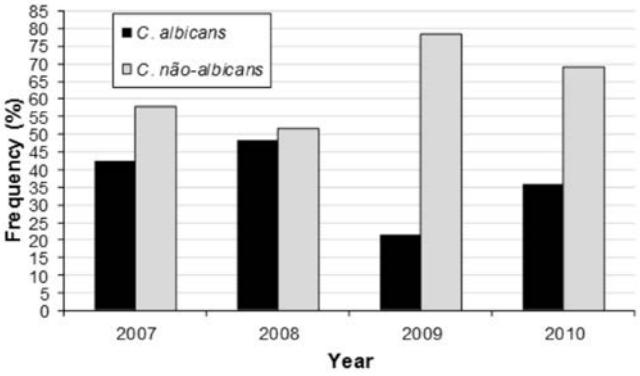


Fig. 2 - Frequency of isolates of *C. albicans* and non-*abicans* per year in a children's public hospital.

of *Candida* were: 37.5% of *C. albicans* (39/104), 24.03% of *C. tropicalis* (25/104), 22.11% of *C. parapsilosis* (23/104), 5.8% of *Pichia anomala* (6/104), 4.8% of *C. guilliermondii* (5/104), 2.88% of *C. krusei* (3/104), 1.92% of *C. glabrata* (2/104) and 0.96% of *C. pararugosa* (1/104). In the genotypic testing for differentiation molecular of *C. albicans* and *C. dubliniensis* and *C. parapsilosis* complex, the result was consistent with *C. albicans* and C. *parapsilosis* in 100% of the samples. The frequency of the species per year can be observed in Figure 1.

DISCUSSION

Candida albicans is an etiologic agent in about 40-60% of the bloodstream infections in pediatric patients[14,28]. Studies have reported an increase in the rate of infections caused by non-*albicans* species[6]. In research, done in a pediatric hospital in the USA which had 97 patients studied, species of *Candida* (being the most frequent *C. albicans* and *C. parapsilosis*) were the most commonly isolated among fungemic patients, present in 91% of the cases. The species of *Candida* more commonly isolated from blood have changed along the time. In general, *C. albicans* represents 60% of the cases of isolates from blood between 1991 and 1996 and 48% of the isolates between 1997 and 2001, what was in accordance in the groups of ages[1]. We emphasize in our study that 104 children with fungemia were observed in a shorter period than the study previously mentioned.

In another study performed in adults and pediatric patients with candidemia in a hospital in Porto Rico, the non-*albicans* species represented around 83% of the isolates; meanwhile *C. albicans* was responsible for 17% of the cases[12]. In our study, *C.* non-*albicans* (62.5%) were also more isolated than *C. albicans* (37.5%). We noticed that in 2007 *C. albicans* represented 42.3% of the isolates, in 2008 we had a small increase of isolates of *C. albicans* (48.3%), but in 2009 and 2010 we had a decrease of isolates of these species, 21.7% and 36% respectively. The increase of the isolation rates of non-*albicans* species in patients with candidemia in Brazilian hospitals has been reported for a while[9,31]. Different authors have also reported an increase in the incidence of candidemia caused by non-*albicans* species, represented by *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. tropicalis*. These authors affirmed that, however in the last years *C. albicans* has been responsible for more than a half of the cases of candidemias, the rates of infections caused by non-*albicans* species have slowly increased[18,25,41]. In epidemiology, this could be associated with severe immunosuppression or illness, prematurity, exposure to broad-spectrum antibiotics, older patients and selective pressure exerted by prophylactic antifungals in patients at high risk of developing invasive fungal infections[35].

Recent studies have reported that *C. tropicalis* is one of the species that most frequently causes urinary tract infections and candidemia, especially in patients with cancer[26].

Recent studies evinced that the increase of *C. parapsilosis* incidence may be explained by some relevant factors such as parenteral nutrition and the use of central venous catheters, and this is explained by the fact that this species has the facility to form biofilm[2,19]. However, possible sources of infections and routes of transmission of infections caused by *C. parapsilosis* are not always easily identified and in some cases the epidemiology of the infections remains undercover[5]. Therefore, the epidemiological relevance of this study should be highlighted considering that *C. parapsilosis* was one of the most frequently isolated species and had our special attention.

Comparing nowadays with the 1980's, we can notice that, at present, there is a higher frequency of species *albicans* causing bloodstream infections as relevant pathogens like *C. glabrata* in the USA. Meanwhile, in Europe, Canada and Latin America, *C. parapsilosis* and *C. tropicalis* are the most frequent species responsible for causing bloodstream infections[28].

The increase of fungemias caused by non-*albicans* species like *C. glabrata* and *C. krusei,* is really relevant to the therapy. *C. krusei* has an inherent resistance to fluconazole and *C. glabrata* is relatively resistant to fluconazole, but fluconazole is the most frequently chosen antifungal drug to be used in this cases.

C. guilliermondii has also been isolated in our study. However, infections caused by *C. guilliermondii* are not common. They occurred in patients with cancer, hematological neoplasias as nosocomial infections by the use of central venous catheter having a high mortality rate[40].

Blood, skin, nails, tissues and, less frequent urine and genital tract, represent the most common places where the fungi have been more frequently isolated as a possible pathogen[36].

Regarding the *P. anomala* strains, it is highlighted in a previous study that it had already been isolated from a child patient in a pediatric hospital[30]. PASQUALOTTO *et al*.[32], in another study carried out in a pediatric hospital in Brazil, from October 2002 to December 2004, an outbreak of *P. anomala* had been detected. Seventeen patients had developed fungemia due to this species and molecular studies demonstrated that this outbreak was caused by just one strain. This yeast was not isolated, neither from the health professionals' hands nor from the environment.

In India, a hospital outbreak caused by *P. anomala* in a 23-month period (from April 1996 to February 1998), that had the spread from the fungi on the health professionals' hands, led to the commission of the hospital infection control to have a strict and educational control to improve the hand washing. Analyzing the potential risk factors to infections by *P. anomala* in children, we verified that some of these factors are: prematurity, very low-weight, a long-term hospitalization[8], the use of central venous catheters, total parenteral nutrition, lipid emulsion, and the previous use of antimicrobial and other invasive procedures[4].

Based on the finding of this study and the literature, we could notice the relevance of studying nosocomial infections cases by yeasts, especially from the genus *Candida*. We verified the increase of the non-*albicans* species throughout the years (mainly in 2009 and 2010). The non-*albicans* species cases have been increasing year after year in these nosocomial infection cases and concerning all these data, it is highlighted a correct species-level identification to lead to a fast diagnosis and efficient treatment.

RESUMO

Fungemia por espécies de *Candida* em Hospital Pediátrico da cidade de São Paulo, Brasil: estudo no período de 2007 a 2010

Candidemia permance como a maior causa de morbidade e mortalidade em ambiente hospitalar. A epidemiologia de infecções por

Candida vem se alterando, principalmente em relação ao número de episódios causados por espécies não-*albicans*. Este estudo teve como objetivo avaliar a frequência, em um período de quatro anos, de leveduras do gênero *Candida* isoladas de sangue de pacientes pediátricos internados em hospital público da cidade de São Paulo, Brasil. Neste período foram isoladas leveduras de sangue de 104 pacientes, e as espécies de *Candida* identificadas, por métodos fenotípicos e genotípicos, foram: *C. albicans* (39/104), *C. tropicalis* (25/104), *C. parapsilosis* (23/104), *Pichia anomala* (6/104), *C. guilliermondii* (5/104), *C. krusei* (3/104), *C. glabrata* (2/104) e *C. pararugosa* (1/104). Em todo período do estudo foi observada maior frequência de isolamento de *C.* não-*albicans* (63,55%) (p = 0,0286). Neste estudo verificou-se aumento das espécies não-*albicans* ao longo dos anos (principalmente em 2009 e 2010), assim, ressalta-se que correta identificação em nível de espécie é recomendável, para que isso acarrete diagnóstico rápido e tratamento eficaz.

ACKNOWLEDGMENTS

The authors express their thanks to PhD Marina Korte for his revision of the English text, to CNPQ and FAPESP for their financial support and to Dr. Arnaldo Lopes Colombo for support in the molecular study.

REFERENCES

1. Abelson JA, Moore T, Bruckner D, Deville J, Nielsen K. Frequency of fungemia in hospitalized pediatric inpatients over 11 years at a tertiary care institution. Pediatrics. 2005;116:61-7.

2. Almirante B, Rodríguez D, Cuenca-Estrella M, Almela M, Sanchez F, Ayats J, *et al.* Epidemiology, risk factors, and prognosis of *Candida parapsilosis* bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003. J Clin Microbiol. 2006;44:1681-5.

3. Alves SH, Milan EP, de Laet Sant'ana P, Oliveira LO, Santurio JM, Colombo AL. Hypertonic Sabouraud broth as a simple and powerful test for *Candida dubliniensis* screening. Diagn Microbiol Infect Dis. 2002;43:85-6.

4. Aragão PA, Oshiro ICV, Manrique EI, Gomes CC, Matsuo LL, Leone C, *et al. Pichia anomala* outbreak in a nursery: exogenous source? Pediatr Infect Dis J. 2001;20:843-8.

5. Asbeck ECV, Huang YC, Markham AN, Clemons NV, Stevens DA. *Candida parapsilosis* fungemia in neonates: genotyping results suggest healthcare workers hands as source, and review of published studies. Mycopathologia. 2007;164:287-93.

6. Awasthi AK, Jain A, Awasthi S, Ambast A, Singh K, Mishra V. Epidemiology and microbiology of nosocomial pediatric candidemia at a northern Indian tertiary care hospital. Mycopathologia. 2011;172:269-77.

7. Cantón E, Pemán J, Quindós G, Eraso E, Miranda-Zapico I, Álvarez M, *et al*. Prospective multicenter study of the epidemiology, molecular identification, and antifungal susceptibility of *Candida parapsilosis, Candida orthopsilosis*, and *Candida metapsilosis* isolated from patients with candidemia. Antimicrob Agents Chemother. 2011;55:5590-6.

8. Chakrabarti A, Singh K, Narang A, Singhi S, Batra R, Rao KLN, *et al.* Outbreak of *Pichia anomala* infection in the pediatric service of a tertiary-care center in Northern India. J Clin Microbiol. 2001;39:1702-6.

9. Colombo AL, Nucci M, Salomão R, Branchini ML, Richtmann R, Derossi A, *et al.* High rate of non-*albicans* candidemia in Brazilian tertiary care hospitals. Diagn Microbiol Infect Dis. 1999;34:281-6.

10. Colombo AL, Nucci M, Park BJ, Nouér SA, Arthington-Skaggs B, Matta DA, *et al.* Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. J Clin Microbiol. 2006;44:2816-23.

11. Colombo AL, Guimarães T, Silva LR, de Almeida Monfardini LP, Cunha AK, Rady P, *et al*. Prospective observational study of candidemia in São Paulo, Brazil: incidence rate, epidemiology, and predictors of mortality. Infect Control Hosp Epidemiol. 2007;28:570-6.

12. Conde-Rosa A, Amador R, Perez-Torres D, Colón E, Sánchez-Rivera C, Nieves-Plaza M, *et al.* Candidemia distribution, associated risk factors, and attributed mortality at a university-based medical center. ProHealth Sci. 2010;29:26-9.

13. De Hoog GS, Gerrits van den Ende AHG. Molecular diagnostics of clinical strains of filamentous basidiomycetes. Mycoses. 1998;41:183-9.

14. Festekjian A, Neely M. Incidence and predictors of invasive candidiasis associated with candidemia in children. Mycoses. 2011;54:146-53.

15. Garcia-Effron G, Canton E, Pemán J, Dilger A, Romá E, Perlin DS. Epidemiology and echinocandin susceptibility of *Candida parapsilosis sensu lato* species isolated from bloodstream infections at a Spanish university hospital. J Antimicrob Chemother. 2012;67:2739-48.

16. García-Rodríguez J, Cantón E, Pemán J, Alvarez M, Ezpeleta G, Gómez-Nieto A, *et al.* Incidencia etaria y geográfica y patrón de sensibilidad a los antifúngicos de las especies de *Candida* causantes de candidemia en la población pediatrica española. Enferm Infecc Microbiol Clin. 2013;31:363-8.

17. Gonçalves SS, Amorim CS, Nucci M, Padovan ACB, Briones MRS, Melo ASA, *et al*. Prevalence rates and antifungal susceptibility profiles of the *C. parapsilosis* species complex, results from a nationwide surveillance of candidaemia in Brazil. Clin Microbiol Infect. 2010;16:885-7.

18. Hinrichsen SL, Falcão E, Vilella TAS, Rêgo L, Lira C, Almeida L, *et al. Candida* isolates in tertiary hospitals in northeastern Brazil. Braz J Microbiol. 2009;40:325-8.

19. Horasan ES, Ersöz G, Göksu M, Otag F, Kurt AO, Karaçorlu S, *et al.* Increase in *Candida parapsilosis* fungemia in critical care units: a 6-years study. Mycopathologia. 2010;170:263-8.

20. Kurtzman CP, Fell JW, Boekhout T. The yeasts, a taxonomic study. Burlington: Elsevier Science; 2011.

21. Lortholary O, Desnos-Ollivier M, Sitbon K, Fontanet A, Bretagne S, Dromer, F, *et al.* Recent exposure to caspofungin or fluconazole influences the epidemiology of candidemia: a prospective multicenter study involving 2,441 patients. Antimicrob Agents Chemother. 2011;55:532-8.

22. Mannarelli BM, Kurtzman CP. Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR. J Clin Microbiol. 1998;36:1634-41.

23. Morace G, Borghi E, Iatta R, Amato G, Andreoni S, Brigante G, *et al.* Antifungal susceptibility of invasive yeast isolates in Italy: the GISIA3 study in critically ill patients. BMC Infect Dis. 2011;11:130.

24. Motta AL, Almeida GMD, Almeida Jr JN, Burattini MN, Rossi F. Candidemia epidemiology and susceptibility profile in the largest Brazilian teaching hospital complex. Braz J Infect Dis. 2010;14:441-8.

25. Muñoz P, Giannella M, Fanciulli C, Guinea J, Valerio M, Rojas L, *et al. Candida tropicalis* fungemia: incidence, risk factors, and mortality in a general hospital. Clin Microbiol Infect. 2011;17:1538-45.

26. Negri M, Martins M, Henriques M, Svidzinski TIE, Azeredo J, Oliveira R. Examination of potential virulence factors of *Candida tropicalis* clinical isolates from hospitalized patients. Mycopathologia. 2009;169:175-82.

27. Nucci M, Queiroz-Telles F, Tobón AM, Restrepo A, Colombo AL. Epidemiology of opportunistic fungal infections in Latin America. Clin Infect Dis. 2010;51:561-70.

28. Nucci M, Queiroz-Telles F, Alvarado-Matute T, Tiraboschi IN, Corte J, Zurita J, *et al.* Epidemiology of candidemia in Latin America: a laboratory-based survey. Plos One. 2013;8:e59373.

29. Oeser C, Lamagni T, Heath PT, Sharland M, Ladhani S. The epidemiology of neonatal and pediatric candidemia in England and Wales, 2000-2009. Pediatr Infect Dis J. 2013;32:23-6.

30. Paula CR, Krebs VLJ, Auler ME, Ruiz LS, Matsumoto FE, Silva EH, *et al*. Nosocomial infection in newborns by *Pichia anomala* in a Brazilian intensive care unit. Med Mycol. 2006;44:479-84.

31. Paula CR, Montelli AC, Ruiz LS, Batista GCM, Matsumoto FE, Volperarnoni M, *et al.* Infecção hospitalar fúngica: experiência em hospitais públicos de São Paulo. Prática Hosp. 2007;52:63-6.

32. Pasqualotto AC, Sukiennik TC, Severo LC, De Amorim CS, Colombo AL. An outbreak of *Pichia anomala* fungemia in a Brazilian pediatric intensive care unit. Infect Control Hosp Epidemiol. 2005;26:553-8.

33. Ruiz LS, Sugizaki MF, Montelli AC, Matsumoto FE, Pires MFC, Da Silva BCM, *et al.* Fungemia by yeasts in Brazil: occurrence and phenotypic study of strains isolated at the public hospital, Botucatu, São Paulo. J Mycol Med. 2005;15:13-21.

34. Ruiz LS, Khouri S, Hahn RC, da Silva EG, de Oliveira VK, Gandra RF, *et al.* Candidemia by species of the *Candida parapsilosis* complex in children's hospital: prevalence, biofilm production and antifungal susceptibility. Mycopathologia. 2013;175:231-9.

35. Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJS. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol. 2013;62:10-24.

36. Savini V, Catavitello C, Di Marzio I, Masciarelli G, Astolfi D, Balbinot A, *et al.* Pan-azole-resistant *Candida guilliermondii* from a leukemia patient's silent funguria. Mycopathologia. 2010;169:457-9.

37. Sullivan DJ, Westerneng TJ, Haynes KA, Bennet DE, Coleman DC. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology. 1995;141(Pt7):1507-21.

38. Tavanti A, Davidson AD, Gow NAR, Maiden MCJ, Odds FC. *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis g*roups II and III. J Clin Microbiol. 2005;43:284-92.

39. Viudes A, Pemán J, Cantón E, Úbeda P, López-Ribot JL, Gobernado M. Candidemia at a tertiary-care hospital: epidemiology, treatment, clinical outcome and risk factors for death. Eur J Clin Microbiol Infect Dis. 2002;21:767-74.

40. Yamamura M, Makimura K, Fujisaki R, Satoh K, Kawakami S, Ota HNY. Polymerase chain reaction assay for specific identification of *Candida guilliermondii* (*Pichia guilliermondii*). J Infect Chemother. 2009;15:214-8.

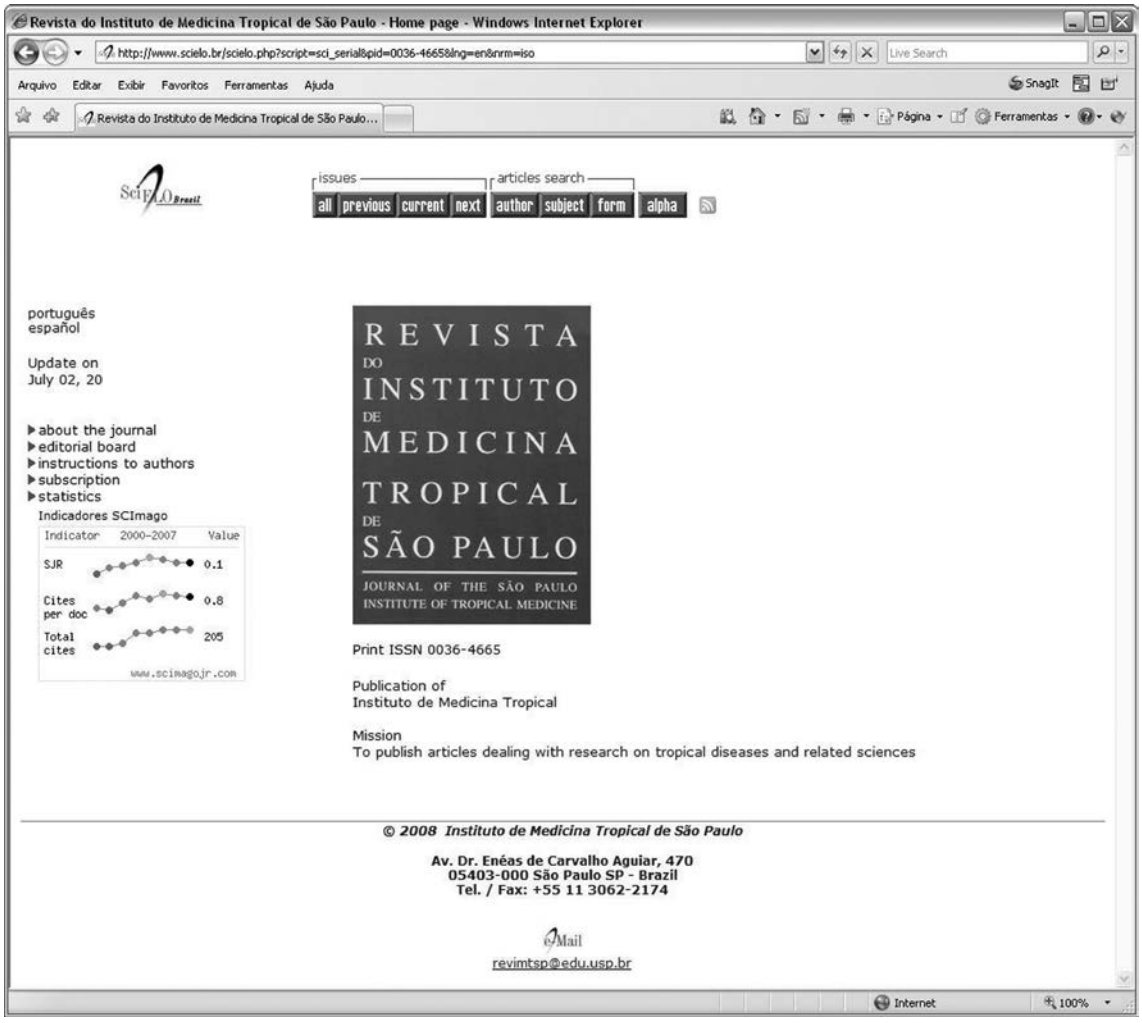
41. Yapar N, Pullukcu H, Avkan-Oguz V, Sayin-Kutlu S, Ertugrul B, Sacar S, *et al.* Evaluation of species distribution and risk factors of candidemia: a multicenter case-control study. Med Mycol. 2011;49:26-31.

42. Zaoutis TE, Prasad PA, Localio AR, Coffin SE, Bell LM, Walsh TJ, *et al*. Risk factors and predictors for candidemia in pediatric intensive care unit patients: implications for prevention. Clin Infect Dis. 2010;51:38-45.

Received: 3 July 2013
Accepted: 10 January 2014

Revista do Instituto de Medicina Tropical de São Paulo
on line.

Publications from 1984 to the present data are now available on:
http://www.scielo.br/rimtsp
PAST ISSUES FROM 1959 ON (PDF)
www.imt.usp.br/portal/



SciELO – The Scientific Electronic Library OnLine - SciELO is an electronic virtual covering a selected collection of Brazilian scientific journals.

The library is an integral part of a project being developed by FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo, in partnership with BIREME – the Latin American and Caribbean Center on Health Sciences Information.

SciELO interface provides access to its serials collection via an alphabetic list of titles or a subject index or a search by word of serial titles, publisher names, city of publication and subject.

The interface also provides access to the full text of articles via author index or subject index or a search form on article elements such as author names, words from title, subject and words from full text.

FAPESP/BIREME Project on Scientific Electronic Publications
Latin American and Caribbean Center on Health Sciences Information
Rua Botucatu 862 – 04023-901 São Paulo, SP – Brazil
Tel. (011) 5576-9863
scielo@bireme.br

Rev. Inst. Med. Trop. Sao Paulo
56(4):307-311, July-August, 2014
doi: 10.1590/S0036-46652014000400007

HEPATITIS B VACCINATION COVERAGE AND POSTVACCINATION SEROLOGIC TESTING AMONG MEDICAL STUDENTS AT A PUBLIC UNIVERSITY IN BRAZIL

Eduardo Pernambuco de SOUZA(1) & Marcelo de Souza TEIXEIRA(2)

SUMMARY

The aim of this cross-sectional study was to determine the hepatitis B vaccination coverage among medical students at a public university in Rio de Janeiro, Brazil, and their compliance with the postvaccination serologic testing recommendations. Of the total of 858 students, 675 (78.7%) participated in the study. Among the participants, 48.9% (95% CI: 45.1% to 52.7%) were vaccinated against hepatitis B (received ≥ 3 doses of the vaccine), 31.6% were not (received 0, 1 or 2 doses), and 19.6% did not know their vaccination status. Hepatitis B vaccination coverage increased from 26.0% among first-year students to 70.6% among sixth-year students while the prevalence of unknown vaccination status decreased from 39.7% among first-year students to 2.4% among sixth-year students. The frequency of unvaccinated students ranged from 23.7% among fifth-year students to 34.4% among first-year students. Only 34.8% of the vaccinated students performed the anti-HBs testing after vaccination. Among these medical students, we found a low adherence to the hepatitis B vaccination and to the postvaccination serologic testing. A comprehensive hepatitis B immunization program should be offered to students at this medical school.

KEYWORDS: Vaccination; Hepatitis B; Medical students.

INTRODUCTION

The hepatitis B virus infection can cause severe chronic liver disease, and is a public health problem in several countries[31]. In Brazil, approximately 14,000 cases of hepatitis B virus infection are reported every year and 120,343 cases were reported from 1999 to 2011[3].

The hepatitis B virus can be transmitted through percutaneous or mucosal exposure to infected blood or body fluids, and health-care personnel are at risk of acquiring the disease from needle stick injuries or other types of occupational exposures[9]. This risk may be higher during the professional training period[11,14,24,25]. Therefore, the hepatitis B vaccination is strongly recommended for health-care personnel, including medical students and students of other health professions, and these individuals should perform the hepatitis B surface antibody (anti-HBs) testing 1-2 months after vaccination to evaluate the protective antibody response to the vaccine[4,11].

In Brazil, students currently entering medical school were born before the implementation of universal hepatitis B vaccination of infants in 1998[5]. Vaccination of adolescents was recommended in 2001[5], but this group is difficult to be reached by immunization programs, and studies have reported low vaccination coverage among Brazilian adolescents[6,27]. Consequently, students may enter medical school still unvaccinated against hepatitis B, and immunization programs at medical schools could contribute to vaccination of susceptible students. However, not all Brazilian medical schools have implemented such programs or student vaccination requirements. Therefore, it cannot be assumed *a priori* that most medical students are properly protected against hepatitis B. In fact, inadequate hepatitis B vaccination coverage has been observed among medical students and students of other health professions in Brazil[7,8,12,29].

Information on vaccination coverage among medical students regarding the vaccines that are recommended for health-care personnel can contribute to the implementation of appropriate immunization efforts at medical schools. In this context, the aim of this study was to determine the hepatitis B vaccination coverage among medical students at a public university in Brazil, and their compliance with the postvaccination serologic testing recommendations.

MATERIALS AND METHODS

This cross-sectional study was conducted at the *Universidade Federal do Estado do Rio de Janeiro* Medical School. This medical school is one of the three public medical schools located in the city of Rio de Janeiro, Brazil, and offers a six-year course in medicine. Approximately one hundred and forty students are admitted each year. Students begin to have contact with patients and to perform

(1) Professor Adjunto de Pediatria, Escola de Medicina e Cirurgia, Universidade Federal do Estado do Rio de Janeiro (UNIRIO).
(2) Aluno do Curso de Graduação em Medicina, Escola de Medicina e Cirurgia. Universidade Federal do Estado do Rio de Janeiro (UNIRIO).
Correspondence to: Prof. Eduardo Pernambuco de Souza. Escola de Medicina e Cirurgia. Hospital Gaffrée e Guinle. Rua Mariz e Barros 775. Tijuca. 20270-004 Rio de Janeiro, RJ, Brasil. Tel: 21 22644159. E-mail: edupsz1@gmail.com

some procedures, with risk of occupational exposures, in the second year of medical school. All medical students enrolled at this school in the second semester of 2010 were eligible to participate in this study. The invitation to participate was made during mandatory classes for students on different occasions.

A self-administered questionnaire was used to collect the following data: age, sex, and the student's current year in medical school; whether the student had been vaccinated against hepatitis B or not; the number of vaccine doses received by the student; whether the student had performed the anti-HBs testing after vaccination, and the approximate interval between the last vaccine dose and the anti-HBs testing.

In the present study, students were considered "vaccinated" against hepatitis B when they had received three or more doses of the vaccine, and were considered "unvaccinated" when they had not received the vaccine or had received only one or two vaccine doses. Vaccination status was considered "unknown" when students reported that they did not know if they had received the vaccine or they did not know the number of doses received.

The data were entered into an electronic database using double data entry, and a descriptive analysis was performed for all study variables. The 95% confidence interval was calculated for the frequency of students who were "vaccinated" against hepatitis B. Vaccination status was compared across gender using the chi-square test. Analyses were performed with Epi Info (version 3.5.3 - 2011).

This study was approved by the ethics review committee of the Gaffrée e Guinle University Hospital at the *Universidade Federal do Estado do Rio de Janeiro*, and all students signed an informed consent before entering the study.

RESULTS

In the second half of 2010, 858 students were enrolled at the *Universidade Federal do Estado do Rio de Janeiro* Medical School, and 675 (78.7%) responded to the survey questionnaire. Response rate ranged from 63.0% among sixth-year students to 92.4% among fourth-year students (Table 1). The mean age of the students was 22.8 years ± 2.8 (range: 17 to 49 years): 59.7% were female students, and 40.3% were male students. Information on age was missing for eight students.

Of the total of 675 students, 330 students (48.9%; 95% CI: 45.1% to 52.7%) were vaccinated against hepatitis B (received ≥ 3 doses of the vaccine), 213 (31.6%) were unvaccinated (received zero, one or two doses of the vaccine), and 132 students (19.6%) did not know their vaccination status (did not know if they had received the vaccine or did not know the number of vaccine doses received). Among the 213 students who were considered unvaccinated against hepatitis B, 80 students (37.6%) did not receive any dose of the vaccine, 51 students (23.9%) received one dose, and 82 students (38.5%) received two doses.

The ≥ 3-dose hepatitis B vaccination coverage increased from 26.0% among first-year students to 70.6% among sixth-year students. On the other hand, the prevalence of unknown vaccination status decreased from 39.7% among first-year students to 2.4% among sixth-year students. The frequency of unvaccinated students ranged from 23.7% among fifth-year students to 34.4% among first-year students (Table 1).

Among male students (n = 272), 47.8% were vaccinated against hepatitis B, 32.4% were not, and 19.9% did not know their vaccination status. Among female students (n = 403), 49.6% were vaccinated, 31.0% were not, and 19.4% did not know their vaccination status ($p = 0.89$).

Of the total of 330 fully vaccinated students, 115 students (34.8%) performed the anti-HBs testing after vaccination. Additionally, 15 students who had received one or two doses of the vaccine, and eight students who did not know the number of doses of the vaccine they had received also performed the anti-HBs testing after vaccination. Accordingly, a total of 138 students did the postvaccination serologic testing. Most students performed the anti-HBs testing one or more years after vaccination or did not know this interval (Table 2).

DISCUSSION

In this study we observed a low uptake of the hepatitis B vaccine among a group of Brazilian medical students since 31.6% of them were not fully vaccinated. The proportion of unvaccinated students may have even been underestimated because approximately 20% of the students

Table 1
Response rate and hepatitis B vaccination status according to year of medical school among Brazilian medical students

Year of medical school	Response rate (n = 858)		Vaccination status (n = 675)					
			Vaccinated		Unknown		Unvaccinated	
	n/N	%	n	%	n	%	n	%
First	131/149	87.9	34	26.0	52	39.7	45	34.4
Second	102/145	70.3	31	30.4	36	35.3	35	34.3
Third	114/147	77.6	58	50.9	19	16.7	37	32.5
Fourth	146/158	92.4	81	55.5	15	10.3	50	34.2
Fifth	97/124	78.2	66	68.0	8	8.2	23	23.7
Sixth	85/135	63.0	60	70.6	2	2.4	23	27.1
Total	675/858	78.7	330	48.9	132	19.6	213	31.6

Table 2
Approximate interval between the last dose of the hepatitis B vaccine and the anti-HBs testing among Brazilian medical students (n = 138)

Approximate interval between the last dose of the vaccine and the anti-HBs testing	Students	
	n	%
< 1 year	23	16.7
1-4 years	34	24.6
≥ 5 years	19	13.8
Unknown	58	42.0
Missing	4	2.9

did not know their vaccination status, and some of these students could be unvaccinated. In addition and perhaps more importantly, among students attending the final year of medical school, hepatitis B vaccination coverage was only 70.6%, and in this group 2.4% of the students did not know their vaccination status.

Few studies have investigated the hepatitis B vaccination coverage among medical students in Brazil[1,7,12,24]. One study reported a vaccination coverage of 71.3% among fifth-year medical students[12] and this result was similar to that observed among our students who were in the same year of medical school (68.0%). In another study, a higher vaccination coverage and of 86.8% was observed among second- to sixth-year medical students[24]. At a medical school in the state of Santa Catarina, Brazil only 53.8% of the students were vaccinated against hepatitis B, but in this study the number of vaccine doses was not determined, and the vaccination status was unknown for approximately 43% of the students[1].

Other studies conducted in Brazil assessed the hepatitis B vaccination coverage among different groups of health-care personnel, with varying results. As among our students, vaccine uptake was suboptimal in some of these groups[2,8,17,28-30]. Vaccination coverage of less than 80% has been reported among physicians in two Brazilian cities[17,28], residents in pediatrics[30], dental students[8,29], and health-care workers in the city of Belo Horizonte[2]. Among health-care students, including medical students, who were exposed to biological material in São Paulo-Brazil, only 77.1% of them had received three or more doses of the vaccine[18]. On the other hand, vaccination coverage of more than 90% was observed among dentists[16] and physicians[2,15] in some Brazilian cities. However, one must be careful to compare the results of these studies because different methods were used to assess the vaccination status (self-reported vaccination status or vaccination records), and in some studies it is unclear whether vaccinated individuals were only those who had received the 3-dose vaccine series or were individuals who had received at least one dose of the vaccine. In other countries, including the US, optimal hepatitis B vaccination coverage among health-care personnel has not been achieved either[22].

Interestingly, we observed that hepatitis B vaccination coverage increased progressively from 26.0% among first-year students to 70.6% among sixth-year students while the prevalence of unknown vaccination status decreased from 39.7% to 2.4%. Since this study was not prospective and we did not investigate when students were vaccinated, whether before or during the medical course, we cannot explain these findings with certainty. Perhaps, students attending different years of the medical course had already different vaccination status when they were admitted to medical school. However, during the medical course students have several opportunities to learn about the importance of being vaccinated against hepatitis B and of knowing their vaccination status. The acquisition of this knowledge might have contributed to a higher vaccination coverage and a lower prevalence of unknown vaccination status among students attending the last years of the medical course. Nevertheless, in each year of this medical course, approximately a quarter to a third of the students had not received the 3-dose hepatitis B vaccine series.

In Brazil, the hepatitis B vaccine is available free of charge to health-care personal, including medical students, at a large network of health-care facilities. In the present study, we did not investigate the reasons that could eventually explain why students were not vaccinated. However, the medical school where this study was conducted does not have a student immunization program or vaccination requirements, and this might have contributed to the low hepatitis B vaccine uptake among our study group. In this regard, a higher hepatitis B vaccination coverage was reported among Brazilian medical students to whom an immunization program was offered at their medical school[24].

Exposure to blood or body fluids potentially contaminated with hepatitis B virus is common among medical students[12,14,18,24-26]. Moreover, in Brazil, the number of confirmed cases of hepatitis B infection is higher among young adults than among other age groups, and most cases of the disease are probably acquired through sexual contact[3]. The majority of medical students are young adults, and thus unvaccinated medical students may be at risk of acquiring the disease not only through occupational exposures but also by sexual contact.

In the present study, only 34.8% of the vaccinated students performed the anti-HBs testing after vaccination to determine their antibody response to the vaccine. However, based on this result we cannot conclude whether or not the students complied with the postvaccination serologic testing recommendations for health-care personnel because we did not investigate when students were vaccinated, and the anti-HBs testing was not mandatory for those students vaccinated in the past, before entering medical school[11]. In fact, different recommendations would have been acceptable for students vaccinated before entering medical school: not to perform the anti-HBs testing unless they were exposed to blood or body fluids[11] or to perform the testing and eventually administer a booster dose of the vaccine to those with anti-HBs concentrations of less than 10 mIU/mL[4,10]. On the other hand, students vaccinated during the medical course should have performed the anti-HBs testing 1-2 months after the last dose of the hepatitis B vaccine series[4,11]. Nevertheless, we can conclude that the response to the vaccine was unknown for most of the vaccinated students, and this can be an issue in some circumstances. When the response to the vaccine is unknown, the management of health-care personnel potentially exposed to hepatitis B virus is more complex, and in this context the anti-HBs testing should be performed immediately. However, in some settings in Brazil, the anti-HBs testing or its results may not be readily available. Other studies conducted in Brazil also reported that a significant proportion (approximately 32% to 48%) of health-care personnel who were vaccinated against hepatitis B did not perform the postvaccination serologic testing[15,24,28].

In addition, most students performed the anti-HBs testing one or more years after the last dose of the vaccine or did not know how long

after vaccination the testing had been performed. The anti-HBs testing performed years after vaccination is not adequate for evaluating the response to the vaccine because anti-HBs concentrations decline over time. However, individuals who responded to the 3-dose vaccine series remain protected even if their anti-HBs concentration declines to below 10 mIU/mL due to immune memory[23]. In this respect, among health-care students in Taiwan, who were vaccinated during infancy with a recombinant hepatitis B vaccine, a study reported that after 16 years only 33% had protective levels of anti-HBs, but 95.9% of the anti-HBs negative students achieved protective levels of anti-HBs after a booster dose of the vaccine, indicating the presence of immune memory[20]. Perhaps, in the present study, students performed the anti-HBs testing years after vaccination because they were vaccinated in the past and desire to know whether they were protected against hepatitis B or not. In this context, to perform the anti-HBs testing is an acceptable option as far as the test results are correctly interpreted and the students are properly counseled.

In Brazil, universal hepatitis B vaccination of infants was implemented in 1998, and vaccination of adolescents younger than 20 years of age was recommended in 2001[5]. A study conducted among Brazilian medical students in 2009 reported that approximately 60% of the students were vaccinated against hepatitis B before entering medical school[24]. In the future, more students will possibly enter medical school already vaccinated against hepatitis B and medical schools will have to decide whether to recommend the anti-HBs testing for all students or only for those who have an occupational exposure.

This study has some limitations. Assessment of vaccine uptake was based on student self-reported vaccination status, and misclassifications might have occurred. However, the use of vaccination records to assess vaccination status can also lead to misclassification of individuals with missing vaccination records. Among some groups, missing or unavailable vaccination records are not uncommon[15,30], and a study conducted among Brazilian nursing and medical students reported that, after several attempts, only 11% of the students provided their vaccination cards[7]. Furthermore, we could have had more accurate and complete information on the postvaccination serologic testing if we had determined when students were vaccinated, whether at medical school or before, and if we had reviewed the laboratory reports for the anti-HBs testing. However, laboratory reports are not easily available. Some fifth- and sixth-year students answered the study questionnaire later than the other students. Consequently, they had more time to clarify their vaccination status, and to complete their hepatitis B vaccination schedule, perhaps influenced by their knowledge about the survey. This might have contributed to increase the vaccination coverage in this group. On the other hand, there are few studies reporting on hepatitis B vaccination coverage among Brazilian medical students, and the present study, despite its limitations, can provide additional information regarding health-care students compliance with the recommended vaccines in Brazil.

In conclusion, in the present study we found a low uptake of the hepatitis B vaccine among a group of Brazilian medical students, despite the wide availability of the vaccine. In addition, most vaccinated students did not perform the postvaccination serologic testing and thus, their response to the vaccine was unknown. It is well recognized that medical students have a high risk of being exposed to blood and body fluids potentially contaminated with hepatitis B virus, and hepatitis B vaccination is mandatory or recommended for these students in several countries[11,13,19,21] . Accordingly, this medical school should urgently consider to implement a comprehensive student immunization program against hepatitis B, which may contribute to improve students' adherence to vaccination. This program should be offered early in the medical course and provide not only vaccination but also screening and follow up. Furthermore, education on prevention and management of occupational exposures should be also provided to these students, and counseling should be immediately available in case of exposure. Finally, continuous surveillance of hepatitis B vaccination coverage among medical students should be undertaken in Brazil, especially at those medical schools which do not have student vaccination requirements.

RESUMO

Cobertura vacinal para a hepatite B e teste sorológico pós-vacinal entre estudantes de medicina de universidade pública no Brasil

O objetivo deste estudo transversal foi determinar a cobertura vacinal para a hepatite B entre estudantes de medicina de uma universidade pública no Rio de Janeiro, Brasil e a sua adesão às recomendações sobre o teste sorológico pós-vacinal. De um total de 858 estudantes, 675 (78,7%) participaram do estudo. Entre os participantes, 48,9% (IC de 95%: 45,1% a 52,7%) eram vacinados contra a hepatite B (receberam ≥ 3 doses da vacina), 31,6% não eram (receberam zero, uma ou duas doses) e 19,6% não sabiam o seu status vacinal. A cobertura vacinal para a hepatite B aumentou de 26,0% entre os estudantes do primeiro ano para 70,6% entre os estudantes do sexto ano, enquanto a prevalência de status vacinal ignorado diminuiu de 39,7% entre os estudantes do primeiro ano para 2,4% entre os estudantes do sexto ano. A frequência de estudantes não vacinados variou de 23,7% entre os estudantes do quinto ano a 34,4% entre os estudantes do primeiro ano. Apenas 34,8% dos estudantes vacinados realizaram o teste anti-HBs após a vacinação. Entre esses estudantes de medicina encontramos uma baixa adesão à vacinação contra a hepatite B e ao teste sorológico pós-vacinal. Um amplo programa de imunização contra a hepatite B deveria ser oferecido aos estudantes, nessa escola médica.

AUTHOR CONTRIBUTIONS

Both authors participated in all study phases. EPS coordinated the study.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

1. Arent PM, Cunha L, Freitas PF. Situação vacinal dos estudantes de medicina da Universidade do Sul de Santa Catarina no período prévio ao internato. Rev Ciênc Méd. 2009;18:13-20.

2. Assunção AA, Araújo TM, Ribeiro RB, Oliveira SV. Hepatitis B vaccination and occupation exposure in the healthcare sector in Belo Horizonte, Minas Gerais. Rev Saúde Pública. 2012;46:665-73.

3. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Boletim Epidemiológico Hepatites Virais (versão preliminar). 2012; Ano III (nº 01). [cited 2012 Sep 12]. Available from: http://www.aids.gov.br/publicacao/2012/boletim_epidemiologico_de_hepatites_virais_2012

4. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Manual dos Centros de Referência para imunobiológicos especiais. 3ª ed. 2006. [cited 2011 Nov 19].Available from: http://portal.saude.gov.br/portal/arquivos/pdf/livro_cries_3ed.pdf

5. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Programa Nacional de Imunizações - 30 anos. 2003. [cited 2012 Oct 9]. Available from: http://portal.saude.gov.br/portal/arquivos/pdf/livro_30_anos_pni.pdf

6. Bueno MM, Matijasevich A. Avaliação da cobertura vacinal contra hepatite B nos menores de 20 anos em municípios do Estado do Rio Grande do Sul, Brasil. Epidemiol Serv Saúde. 2011;20:345-54.

7. Cabrera EMS, Merege CES. Inquérito vacinal de alunos da graduação em medicina e enfermagem da Faculdade de Medicina de São José do Rio Preto (SP, Brasil) nos anos de 2006 e 2007 e suas possíveis implicações na atuação discente. Ciênc Saúde Coletiva. 2011;16:547-52.

8. Carneiro GGVS, Cangussu MCT. Prevalência presumível, cobertura vacinal, conhecimentos e atitudes relativos à hepatite B em graduandos de Odontologia da Universidade Federal da Bahia. Rev Odontol UNESP. 2009;38:7-13.

9. Centers for Disease Control and Prevention. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. MMWR Recomm Rep. 2001;50(RR-11):1-52.

10. Centers for Disease Control and Prevention (CDC). Ask the Experts: Hepatitis B. [cited 2012 Oct 10]. Available from: http://www.cdc.gov/vaccines/vpd-vac/hepb/default.htm

11. Centers for Disease Control and Prevention (CDC). Immunization of health-care personnel: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2011;60(RR-7):1-45.

12. Chehuen Neto JA, Sirimarco MT, Leite ICG, Gonçalves MPC, Delgado AAA, Camilo GB, *et al.* Situação vacinal dos discentes da Faculdade de Medicina da UFJF-MG. Rev Bras Educ Med. 2010;34:270-7.

13. De Schryver A, Claesen B, Meheus A, van Sprundel M, Francois G. European survey of hepatitis B vaccination policies for healthcare workers. Eur J Public Health. 2011;21:338-43.

14. Deisenhammer S, Radon K, Nowak D, Reichert J. Needlestick injuries during medical training. J Hosp Infect. 2006;63:263-7.

15. Dinelli MI, Moreira TD, Paulino ER, da Rocha MC, Graciani FB, de Moraes-Pinto MI. Immune status and risk perception of acquisition of vaccine preventable diseases among health care workers. Am J Infect Control. 2009;37:858-60.

16. Ferreira RC, Guimaraes AL, Pereira RD, Andrade RM, Xavier RP, Martins AM. Hepatitis B vaccination and associated factors among dentists. Rev Bras Epidemiol. 2012;15:315-23.

17. Garcia LP, Facchini LA. Vacinação contra a hepatite B entre trabalhadores da atenção básica à saúde. Cad Saude Publica. 2008;24:1130-40.

18. Gir E, Netto JC, Malaguti SE, Canini SR, Hayashida M, Machado AA. Accidents with biological material and immunization against hepatitis B among students from the health area. Rev Lat Am Enfermagem. 2008;16:401-6.

19. Government of South Australia, Immunisation Section. Communicable Disease Control Branch. Immunisation Guidelines for Health Care Workers in South Australia. 2010. [cited 2013 Mar 17]. Available from: http://www.health.sa.gov.au/pehs/Immunisation/hcwguidelines-pehs-sahealth-1004.pdf

20. Lin CC, Yang CY, Shih CT, Chen BH, Huang YL. Waning immunity and booster responses in nursing and medical technology students who had received plasma-derived or recombinant hepatitis B vaccine during infancy. Am J Infect Control. 2011;39:408-14.

21. Lindley MC, Lorick SA, Spinner JR, Krull AR, Mootrey GT, Ahmed F, *et al.* Student vaccination requirements of U.S. health professional schools: a survey. Ann Intern Med. 2011;154:391-400.

22. Lu PJ, Byrd KK, Murphy TV, Weinbaum C. Hepatitis B vaccination coverage among high-risk adults 18-49 years, U.S., 2009. Vaccine. 2011;29:7049-57.

23. Mast EE, Weinbaum CM, Fiore AE, Alter MJ, Bell BP, Finelli L, *et al.* A comprehensive immunization strategy to eliminate transmission of hepatitis B virus infection in the United States: recommendations of the Advisory Committee on Immunization Practices (ACIP). Part II: immunization of adults. MMWR Recomm Rep. 2006;55(RR-16):1-33.

24. Oliveira LC, Pontes JP. Frequency of hepatitis B immunity and occupational exposures to body fluids among Brazilian medical students at a public university. Rev Inst Med Trop Sao Paulo. 2010;52:247-52.

25. Patterson JM, Novak CB, Mackinnon SE, Ellis RA. Needlestick injuries among medical students. Am J Infect Control. 2003;31:226-30.

26. Reis JMB, Lamounier Filho A, Rampinelli CA, Soares ECS, Prado RS, Pedroso ERP. Training-related accidents during teacher-student-assistance activities of medical students. Rev Soc Bras Med Trop. 2004;37:405-8.

27. Riente KBC, Tsuguta EN, Barbosa SRBS, Zaparoli MA. Avaliação da cobertura vacinal contra Hepatite B em 15 municípios da região metropolitana oeste de São Paulo. BE CVE. 2012;2:162-73. [cited 2012 Oct 9]. Available from: http://www.cve.saude.sp.gov.br/boletim/pdf/bol1012.pdf

28. Rossato EM, Ferreira J. Acidentes com perfurocortantes e cobertura vacinal contra hepatite B entre trabalhadores da Saúde no Município de Santa Rosa, Estado do Rio Grande do Sul, Brasil, 2008. Epidemiol Serv Saúde. 2012;21:487-96.

29. Silva FAG, Guedes EA, Miasato JM. Prevalência da vacinação contra hepatite B de graduandos em Odontologia do UNIFESO/RJ. Arq Odontol. 2009;45:117-21.

30. Silveira MB, Perez DA, Yamaguti A, Saraiva EZ, Borges MG, de Moraes-Pinto MI. Immunization status of residents in pediatrics at the Federal University of Sao Paulo, Brazil. Rev Inst Med Trop Sao Paulo. 2011;53:73-6.

31. World Health Organization. Prevention & Control of Viral Hepatitis Infection: Framework for Global Action. 2012:1-28. [cited 2012 Sep 12]. Available from: http://www.who.int/csr/disease/hepatitis/Framework/en/index.html

Received: 28 January 2013
Accepted: 13 January 2014

Rev. Inst. Med. Trop. Sao Paulo
56(4):312, July-August, 2014
doi: 10.1590/S0036-46652014000400008

CORRESPONDENCE

ACUTE TUBULAR NECROSIS AND DENGUE

February 24th, 2014

Sir,

The recent article on "acute tubular necrosis and dengue" is very interesting[2]. REPIZO *et al.* concluded that "serum creatine phosphokinase should be monitored in DF patients to allow for an early diagnosis of rhabdomyolysis and the institution of renal protective measures[2]". In fact, the renal complication of dengue is not rare and it should be kept in mind for all practitioners[5]. The renal problem in dengue can be seen in either hemorrhagic or non-hemorrhagic cases[1]. The underlying etiology of renal failure is believed to be due to the poor fluid management of the infected cases[4]. The strong clinical relationship with fatality can be seen in such cases. The immunopathology seems to have little role in induction of acute tubular necrosis[3]. The patient with a previous renal problem has a greater risk to develop renal failure. A careful follow-up of the fluid replacement therapy is the key factor for success in control and prevention of acute renal episode in dengue patients.

Viroj WIWANITKIT
Visiting professor, Hainan Medical University, China;
Visiting professor, Faculty of Medicine, University of Nis, Serbia;
Adjunct professor, Joseph Ayobabalola University, Nigeria;
Professor, senior expert, Surin Rajabhat University, Surin province, Thailand.
Correspondence to:
Prof. Viroj Wiwanitkit
Wiwanitkit House, Bangkhae, Bangkok Thailand
Email: wviroj@yahoo.com

REFERENCES

1. Mohsin N, Mohamed E, Gaber M, Obaidani I, Budruddin M, Al Busaidy S. Acute tubular necrosis associated with non-hemorrhagic dengue fever: a case report. Ren Fail. 2009;31:736-9.

2. Repizo LP, Malheiros DM, Yu L, Barros RT, Burdmann EA. Biopsy proven acute tubular necrosis due to rhabdomyolysis in a dengue fever patient: a case report and review of literature. Rev Inst Med Trop Sao Paulo. 2014;56:85-8.

3. Wiwanitkit V. Immune complex: does it have a role in pathogenesis of renal failure in dengue infection? Ren Fail. 2005;27:803-4.

4. Wiwanitkit V. Acute renal failure in the fatal cases of dengue hemorrhagic fever, a summary in Thai death cases. Ren Fail. 2005;27:647.

5. Wiwanitkit V. Dengue fever: diagnosis and treatment. Expert Rev Anti Infect Ther. 2010;8:841-5.

Rev. Inst. Med. Trop. Sao Paulo
56(4):313-324, July-August, 2014
doi: 10.1590/S0036-46652014000400009

SUSCEPTIBILITY TO ANTIBIOTICS IN URINARY TRACT INFECTIONS IN A SECONDARY CARE SETTING FROM 2005-2006 AND 2010-2011, IN SÃO PAULO, BRAZIL: DATA FROM 11,943 URINE CULTURES

Érique José PEIXOTO DE MIRANDA(1), Gerson Sobrinho SALVADOR DE OLIVEIRA(1), Felício Lopes ROQUE(2), Sílvia Regina DOS SANTOS(3), Rodrigo Diaz OLMOS(4) & Paulo Andrade LOTUFO(5)

SUMMARY

Introduction: Urinary tract infection (UTI) has a high incidence and recurrence, therefore, treatment is empirical in the majority of cases. **Objectives:** The aim of this study was to analyze the urine cultures performed at a secondary hospital, during two periods, 2005-2006 and 2010-2011, and to estimate the microbial resistance. **Patients and methods:** We analyzed 11,943 aerobic urine cultures according to basic demographic data and susceptibility to antibiotics in accordance with the Clinical and Laboratory Standards Institute (CLSI) for Vitek 1 and 2. **Results:** Most of our cohort consisted of young adult females that were seen at the Emergency Department. *E. coli* was the most frequent (70.2%) among the 75 species isolated. Resistance of all isolates was ≥ 20% for trimethoprim/sulfamethoxazole (TMP/SMX), norfloxacin, nitrofurantoin, cefazolin and nalidixic acid. Although *E. coli* was more susceptible (resistance ≥ 20% for TMP/SMX and nalidixic acid) among all of the isolates, when classified by the number and percentage of antibiotic resistance. Global resistance to fluoroquinolones was approximately 12%. Risk factors for *E. coli* were female gender and an age less than 65 years. Men and patients older than 65 years of age, presented more resistant isolates. Extended spectrum beta-lactamases (ESBL) were identified in 173 out of 5,722 Gram-negative isolates (3.0%) between 2010 and 2011. **Conclusion:** *E. coli* was the most frequent microbe isolated in the urine cultures analyzed in this study. There was a significant evolution of bacterial resistance between the two periods studied. In particular, the rise of bacterial resistance to fluoroquinolones was concerning.

KEYWORDS: Urinary tract infection; Bacteria; Susceptibility test; Urine culture; Brazil.

INTRODUCTION

Urinary tract infection (UTI) is one of the most common infections diagnosed in patients, ranking first in the United States of America (USA). UTIs are characterized by high incidence, high recurrence and a mild severity of evolution in most cases. Because of the uncomfortable symptoms they cause, they often force physicians to introduce antibiotic treatment prior to obtaining the culture results[14,15]. The resolution of between 25% and 42% of cystitis UTIs in the placebo arms of randomized controlled trials, with little incidence of evolution to complicated infections and a proportion of one case of pyelonefritis per every 28 cases of cystitis, confirms the mild character of UTIs[15]. Current guidelines establish that lower UTIs in young women should be treated empirically with short-term antibiotics. Therefore, it is important that the antimicrobial susceptibility data of major uropathogens are known. In particular, given that it is an infection that is most often treated empirically, data on major uropathogens should be systematically and regularly analyzed. However, in Brazil, there is a paucity of such data.

Despite the guidelines, requests for urinalysis and microscopic examination of urine (pyuria, leukocyte esterase, nitrite, and the presence of bacteria) as well as requests for urine culture for diagnosis of uncomplicated UTIs, is common among physicians in Brazil[14,23]. As a result, many cases that should be treated in a primary setting care are unnecessarily referred to the emergency department of secondary hospitals, resulting in increased health care costs. In clinical practice, physicians and their patients commonly insist that urine tests are necessary for the diagnosis of uncomplicated UTIs. The greatest proof of these assertions is evident from retrospective studies in several

(1) Infectious diseases and attending physician in the Internal Medicine Division of the University Hospital of the University of São Paulo, University of São Paulo (USP), Brazil. E-mails: eriquemiranda@gmail.com; gersonsalvador@gmail.com.
(2) Nephrologist and attending physician in the Internal Medicine Division of the University Hospital of the University of São Paulo, University of São Paulo (USP), Brazil. E-mail: felicioroque@uol.com.br.
(3) Pharmacist, chief of the Microbiology section of the Clinic Laboratory of the University Hospital of the University of São Paulo, University of São Paulo (USP), Brazil. E-mail: silviados@uol.com.br.
(4) Professor, Department of Internal Medicine, University of São Paulo School of Medicine (FMUSP), São Paulo, Brazil. Chief of the Internal Medicine Division of the University Hospital of the University of São Paulo, University of São Paulo (USP), Brazil. E-mail: olmos.rodrigo@gmail.com.
(5) Titular professor, Department of Internal Medicine, University of São Paulo School of Medicine (FMUSP), São Paulo, Brazil. Coordinator of the Internal Medicine Division and Research Mayor of the University Hospital of the University of São Paulo, University of São Paulo (USP), Brazil. E-mail: palotufo@hu.usp.br.
Sponsorship: None.
Correspondence to: Érique José Peixoto de Miranda, Hospital Universitário (HU-USP), Universidade de São Paulo, Av. Prof. Lineu Prestes 2565, Butantã, 05508-000 São Paulo, SP, Brasil. Tel.: +55 11 3091-9275. E-mail: eriquemiranda@gmail.com.

regions of Brazil, which were only possible because of this "breach of protocol"[2,4,5,9,10,19,20,22,24,25,26].

We hypothesized that, in recent years in our environment, the microbiological profile of isolated uropathogens has become increasingly worse. Recent literature[15] postulates a fluoroquinolone sparing protocol; however, if more than 20% of uropathogens are resistant to a drug, it cannot be used empirically. Therefore, the aim of this study is to retrospectively analyze the bacterial susceptibility data of uropathogens isolated from patients at a university community hospital in the last decade and to establish a new treatment protocol in this healthcare setting.

MATERIALS AND METHODS

The University Hospital of São Paulo is a 246-bed secondary care setting, one of the teaching hospitals of the University of São Paulo School of Medicine, and serves a population of approximately 500,000 inhabitants in the western area of the city of São Paulo. The hospital Emergency Department serves approximately 800 patients a day.

This is a retrospective study, which included positive urine cultures from patients who were collected during the periods from 2005 to 2006 and from 2010 and 2011, at the University Hospital in the Emergency Department, Outpatient Clinics, and from external patients. The aim of the study was to compare resistance to antimicrobial agents during two time periods, regardless of whether more than one urine culture per patient was collected during those time periods. The samples were processed and analyzed at the Section of Microbiology of the Clinical Laboratory of the University Hospital.

Only aerobic bacterial infections were analyzed in this study. Anaerobic bacterial infection, fungal infection and negative cultures were excluded from this study. Positive urine cultures were obtained by consulting Apolo software (Oracle Corporation, Redwood City, California, USA), without consulting the medical records of the patients, except for information about patients with urine culture with count < 100,000 colony forming units (CFU) per milliliter of urine.

Urine collected by all methods during these time periods was included, although the standardized laboratory method is to collect the specimen urine midstream.

Urine cultures were taken quantitatively distributed on blood agar and MacConkey agar plates with a calibrated loop with a volume of 10 μL (references 35095 and 35092, respectively, bioMérieux, Rio de Janeiro, Brazil). CPS ID2 chromogenic agar (bioMérieux, Rio de Janeiro, Brazil) has been used in the laboratory since 2012. Urine cultures with a count ≥ 100,000 colony forming units (CFU) per milliliter of urine were considered positive after incubation in a bacteriologic incubator at 35 °C for at least 24 hours[27]. This study also included as positive counts > 1,000 CFU and < 100,000 CFU of less than or equal to two infectious agents isolated (only pure or mixed culture) in the analysis because the patients presented symptoms of a UTI, which was documented in their chart[16]. Only one the most significant germs isolated between two agents from each agar plate was considered in the analysis.

Identification of bacterial species and an antibiogram was performed by Vitek 1 (from 2005 to 2006) and by Vitek 2 (from 2010 to 2011) (bioMérieux, St. Louis, Missouri, USA). Card N104 was used in Vitek 2. Detection of **e**xtended spectrum beta-lactamases (ESBL) was performed on the samples from 2010 to 2011 by Vitek 2.

The standardization of the antibiogram followed the standards and recommendations of the Clinical and Laboratory Standards Institute (CLSI), documents M100-S15 (2005)[5], M100-S16 (2006)[6], M100-S20 (2010)[7] and M100-S21 (2011)[8]. Antibiograms of nonfermenting Gram-negative agents were confirmed by the E-test® (AB Biodisk, Solna, Sweden) and/or Kirby-Bauer disk diffusion[18] methods, according to the same standards. No value of the minimum inhibitory concentration was computed in the analysis due to the lack of reliability of the automated antibiogram, especially the values obtained by Vitek 1. ATCC (American Type Culture Collection) strains of *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* ESBL (ATCC 700063), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and *Shigella sonnei* (ATCC 25931) were used for quality control of Vitek analysis. For analysis purposes, we considered the frequency of susceptibility rather than resistance, which is different from most studies in the literature because the semiquantitative antibiogram also measures intermediate susceptibility. For the purposes of the analysis, intermediate susceptibility was included as resistance, as occurs in clinical practice.

A review of the literature was performed in MEDLINE with the terms "outpatient UTI" and "resistance to antibiotics" and "Brazil", as well as LILACS and SciELO (Scientific Electronic Library Online), which are Latin American scientific databases.

ETHICS

This study was approved by the Research Ethics Committee (Advisory Board) of the University Hospital of the University of São Paulo.

STATISTICAL ANALYSIS

Demographic data (sex, age and setting of the healthcare entrance at the hospital) and periods were submitted to exploratory data analysis and the assumption of normality. Numerical data were presented as the medians and interquartile ranges (IQR: Q1 and Q3) because the data were non-normal. Categorical data (sex, setting and susceptibility to antibiotics) were presented as the number and percentage. The Mann-Whitney U test was used for comparison of numerical non-normal variables. The Pearson uncorrected chi-square (χ^2) or Fisher's exact tests were used for comparison of susceptibility between the two periods (2005-2006 vs. 2010-2011). A crude (unadjusted or univariate) odds ratio, 95% confidence interval and p-value were used to assess risk factors for infection by the most frequently isolated bacteria. All tests were two-sided. Statistical significance was considered at $p < 0.05$. Statistical analysis was performed using the statistical software SPSS 10.0 for Windows (IBM, Chicago, IL, USA).

RESULTS

In both periods analyzed, 11,943 aerobic urine cultures were obtained, 5,755 (48.2%) from the period between 2005 and 2006 and 6,188 (51.8%) from the period between 2010 and 2011. This is an average of

7.88 positive urine cultures per day during the first period and 8.40 in the second. Only 849 (7.1%) cultures presented counts less than 100,000 UFC in the first period (2005-2006).

The median age of patients was 36 years old (IQR: 20-65 years), ranging from zero to 100 years of age. There was a statistically significant difference between the median age of men and women in the study: 57.5 years old (IQR: 14.25-76 years) vs. 32 years old (IQR: 21-59 years), respectively; $p < 0.0001$.

By age group, among patients 0 to 14 years old, the frequency of positive urine cultures was 2,007 (16.8%); 15 to 64 years, 6,834 (57.2%); and ≥ 65 years, 3,102 (26.0%). The female-to-male ratio per age strata was the following: 0.73 (206/283) for < 2 years old; 3.60 (267/74) for ≥ 2-5 years; 5.20 (229/44) for >5-14 years; 6.25 (2,794/447) for ≥14-64 years; and 1.64 (877/534) for > 65 years ($p < 0.0001$, Pearson chi-square).

The frequency of females was 77.6%, with females between the ages of 14 and 64 representing 23.4% of the total. Among the patients studied, the majority of samples were collected in the Emergency Department, 8,931 (74.8%). Other samples, 1,944 (16.3%), were collected from the outpatient Clinic, and others, 1,059 (8.9%), were considered "external" (patients from primary care units in the western region of São Paulo).

It was not possible to calculate the positivity rate of the urine cultures. Of all of the isolates, 10,891 (91%) were Gram-negative, of which, 10,704 were Enterobacteriaceae (98.3%) and 187 (1.7%) were nonfermenting Gram-negative bacteria (manly *Pseudomonas aeruginosa*, 132; *Acinetobacter baumannii*, 36; and *Stenotrophomonas maltophilia*, 7). Seventy-five different bacterial species were isolated from the urine cultures (Table 1), but the most frequently observed species were *Escherichia coli* (8,376, 70.2%). The order of frequency of bacteria was statistically significant and varied between men and women ($p < 0.0001$) (Table 2).

The main risk factor for *E. coli and Staphylococcus saprophyticus* infection was female sex (Table 2) [crude odds ratio (OR) = 3.60, 95% CI (confidence interval): 3.30 to 3.90; $p < 0.0001$ and OR = 10.37, 95% CI: 3.84-28.0; $p < 0.0001$, respectively]. Both bacteria were also more common in younger patients. *E. coli* was more common in patients less than 65 years old [(OR) = 1.98 (95% CI: 1.81 to 2.16); $p = 0.033$], and *S. saprophyticus* was more prevalent in patients aged 14 to 65 years [OR = 8.62 (95% CI: 4.77-15.58); $p < 0.0001$]. For *Proteus mirabilis*, the main risk factor was male sex, even though an age of less than 65 years (OR = 1.20, 95% CI: 1.01 to 1.44; $p = 0.033$) was also a risk factor. In the stratum of ages 0-14, we observed the largest male-to-female ratio (1.7), whereas in the 15-64 year stratum it was 0.15, and in the older age stratum (≥ 65 years) it was 0.61.

Male sex was also a risk factor for the following less frequent bacteria: *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Enterobacter aerogenes*, *P. aeruginosa*, *Enterobacter cloacae*, *Citrobacter freundii* and *Morganella morganii* (Table 2). The crosstabulation between stratum of age and gender resulted in a male-to-female rate in the age range of 65 years or greater divided per bacteria: 0.32 (*E. coli*), 1.023 (*E. faecalis*), 1.65 (*E. cloacae*), 1.625 (*C. freundii*), 3.25 (*Staphylococcus epidermidis*), 1.81 (*Klebsiella oxytoca*), 2.22 (*E. aerogenes*), 2.26 (*S. aureus*), and 1.875 (*Citrobacter koseri*).

Table 1
Frequency of species among 11,943 bacteria isolated from urine cultures collected between 2005-2006, and 2010- 2011 at the University Hospital of the University of São Paulo (HU-USP)

Order	Bacteria isolated in urine culture	Frequency (n)	Percentage (%)
1º	*Escherichia coli*	8,376	70.2%
2º	*Proteus mirabilis*	795	6.8%
3º	*Klebsiella pneumoniae*	692	5.9%
4º	*Enterococcus faecalis*	322	2.8%
5º	*Enterobacter aerogenes*	187	1.7%
6º	*Staphylococcus saprophyticus*	148	1.2%
7º	*Pseudomonas aeruginosa*	132	1.1%
8º	*Enterobacter cloacae*	126	1.1%
9º	*Citrobacter freundii*	122	1.0%
10º	*Morganella morganii*	104	0.9%
11º	*Staphylococcus aureus*	98	0.8%
12º	*Staphylococcus epidermidis*	85	0.7%
13º	*Citrobacter koseri*	82	0.7%
14º	*Staphylococcus* sp. DNAse negative	65	0.5%
15º	*Staphylococcus warneri*	62	0.5%
16º	*Serratia marcescens*	62	0.5%
17º	*Klebsiella oxytoca*	58	0.5%
18º	*Streptococcus agalactiae*	43	0.4%
19º	*Staphylococcus hominis*	38	0.3%
20º	*Acinetobacter baumannii*	36	0.3%
21º	Others	310	2.1%
	Total	11,943	100.0%

Notes: 1: neg.: negative, or *Staphylococcus* spp., neither classified as *S. aureus* (DNAse positive) nor had a specie discrimination, due to lack of discriminatory power by automated method (Vitek 1 or 2).
Appendix: Another isolates, in order of frequency, from the twenty-first: *Staphylococcus haemolyticus, Enterococcus faecium, Streptococcus viridans, Streptococcus mitis, Proteus vulgaris, Staphylococcus simulans, Providencia stuartii, Providencia rettgeri, Staphylococcus sciuri, Salmonella* spp., *Stenotrophomonas maltophilia, Staphylococcus auricularis, Staphylococcus capitis, Citrobacter amalonaticus, Proteus penneri, Raoultella ornithinolytica, Raoultella planticola, Staphylococcus xylosus, Enterobacter asburiae, Enterococcus* spp., *Serratia fonticola, Serratia liquefaciens, Streptococcus anginosus, Streptococcus sanguinis, Aeromonas* spp., Gram-negative (not discriminated), *Burkholderia cepacia, Citrobacter braakii, Citrobacter* spp., *Enterobacter gergoviae, Enterobacter* spp., *Pseudomonas* spp., coagulase-negative *Staphylococcus* spp., *Staphylococcus intermedius, Staphylococcus lentus, Staphylococcus lugdunensis, Streptococcus bovis, Streptococcus* spp. *gallolyticus* var. *gallolyticus, Acinetobacter haemolyticus, Acinetobacter lwoffii, Acinetobacter* spp., nonfermenting Gram-negative bacteria, *Enterobacter sakazaki, Enterococcus hirae, Klebsiella ornithinolytica, Pantoea* spp., *Proteus* spp., *Providencia* spp., *Pseudomonas fluorescens, Pseudomonas putida, Staphylococcus cohnii* spp. *cohnii, Streptococcus gordonii, Streptococcus parasanguinis, Streptococcus pyogenes,* beta-hemolytic group G *Streptococcus* spp.

Extended spectrum beta-lactamases (ESBL) were identified in 173 of the 5,722 Gram-negative isolates (3.0%) that were collected between 2010 and 2011 and analyzed by the Vitek 2. Of these, 108 were

Table 2
Frequency by gender of species among 11,943 bacteria isolated from urine cultures collected between 2005-2006, and 2010- 2011 at the University Hospital of the University of São Paulo (HU-USP)

Order	Male, n = 2,673	Female, n = 9,270	Odds ratio*	CI 95%	*p*
1º	*Escherichia coli*, 1,271 (47.5%)	*Escherichia coli*, 7,103 (76.6%)	3.60 (F)	3.30-3.90	<0.0001
2º	*Proteus mirabilis,* 302 (11.3%).	*Proteus mirabilis,* 493 (5.31%)	2.27 (M)	1.94-2.64	<0.0001
3º	*Klebsiella pneumoniae,* 215 (8.04%)	*Klebsiella pneumoniae,* 477 (5.1%)	1.61 (M)	1.36-1.91	<0.0001
4º	*Enterococcus faecalis,* 151 (5.65%)	*Enterococcus faecalis,* 171 (1.8%)	3.19 (M)	2.53-4.01	<0.0001
5º	*Morganella morganni,* 77 (2.88%)	*Staphylococcus saprophyticus,* 144 (1.5%)	–	–	–
6º	*Pseudomonas aeruginosa,* 76 (2.84%)	*Enterobacter aerogenes,* 131 (1.4%)	–	–	–
7º	*Enterobacter cloacae*, 59 (2.20%)	*Enterobacter cloacae*, 67 (0.72%)	3.09 (M)	2.17-4.41	<0.0001
8º	*Citrobacter freundii*, 56 (2.10%)	*Citrobacter freundii*, 66 (0.71%)	2.98 (M)	2.08-4.27	<0.0001
9º	*Enterobacter aerogenes*, 56 (2.10%)	*Staphylococcus warneri,* 59 (0.63%)	–	–	–
10º	*Staphylococcus aureus*, 52 (1.94%)	*Pseudomonas aeruginosa*, 56 (0.60%)	–	–	–

Notes: *(F): female, reference is male; (M): male, reference is female (reference is the counterpart of the gender indicated in parentheses). *Morganella morganni:* OR = 10.15 (95% CI: 6.53-15.76), $p < 0.0001$ (male); *Pseudomonas aeruginosa:* OR = 4.81 (95% CI: 3.40-6.82), $p < 0.0001$ (male); *Staphylococcus saprophyticus:* OR = 10.37 (95% CI: 3.84-28.0), $p < 0.0001$ (female); *Enterobacter aerogenes*: OR = 1.48 (95% CI: 1.08-2.02), $p = 0.012$ (male).

Escherichia coli (1.3% of total per specie), 59 were *K. pneumoniae* (8.5%), four were *K. oxytoca* (6.9%), and two were *P. mirabilis* (0.25%). Of these, 4.6% (n = 8) of patients were 0-14 years old; 38.7% (n = 67) were 15-64 years; 56.7% (n = 98) were ≥ 65 years old; 60.1% (n = 104) were female; and 74% (n = 128) were from the Emergency Department. Of the patients ≥ 65 years old, 55.1% were female and 44.9% were male.

Although it was neither carried out using the Hodge test nor another method for detecting carbapenemases, including KPC (*K. pneumoniae* carbapenemase), the susceptibility to ertapenem was high among isolates of Enterobacteriaceae (*E. coli*, 100%; *Proteus mirabilis*, 99.5%; *K. pneumoniae*, 98.5%; and *E. aerogenes*, 100%) during the period between 2010 and 2011. Global analysis of the isolates demonstrated a susceptibility of 99.8% to ertapenem (2010-2011). Susceptibility to vancomycin among isolates of *E. faecalis* was 98.6% and 97.3% ($p = 0.699$), in the 2005-2006 and 2010-2011 periods, respectively.

SUSCEPTIBILITY

In an attempt to select an appropriate antibiotic for empirical treatment, we performed a comprehensive analysis of the antibiograms of 11,943 isolates from urine cultures from the two periods studied, despite differences in interpretation of susceptibility, particularly among Gram-positive and Gram-negative bacteria**.** Global susceptibility was 83.4% (67,864 out of 81,406), in 2005-2006 vs. 83.5% (72,442 out of 86,770) in 2010-2011, $p = 0.499$. Only clindamycin, cefazolin, erythromycin, gentamicin (high level resistance, tested for *Enterococcus* spp. infection), linezolid, penicillin G (parenteral), tetracycline and vancomycin had no statistically significant differences in antimicrobial susceptibility in both periods. All of these antimicrobial agents are used for covering Gram-positive bacterial infections. All of the following antimicrobial agents were highly statistically significant different in the two periods ($p < 0.0001$): nalidixic acid (88.7 vs. 78.1), amikacin (98.4 vs. 99.5), amoxicillin/clavulanate (98.4 vs. 99.5), ampicillin/sulbactam (21.9 vs. 43.6), cephalothin (77.9 vs. 64.7), cefepime (98.0 vs. 95.4), ceftriaxone (98.2 vs. 95.9), ciprofloxacin (90.1 vs. 83.4), gentamicin (94.4 vs. 93.3), imipenem (99.8 vs. 93.1), meropenem (89.0 vs. 99.8), nitrofurantoin (86.4 vs. 77.4), norfloxacin (91.3 vs. 75.6) and piperacillin/tazobactam (72.9 vs. 94.4). The difference was minor in the following cases: ampicillin (45.1 vs. 44.0, $p = 0.037$), aztreonam 57 (48.3 vs. 61.5, $p = 0.048$), cefuroxime axetil (92.1 vs. 86.9, $p = 0.004$), oxacillin (22.4 vs. 35.6, $p = 0.001$), rifampin (96.4 vs. 97.7, $p = 0.002$) and trimethoprim/sulfamethoxazole (TMP/SMX) (68.0 vs. 65.9, $p = 0.035$). Global resistance to fluoroquinolones (levofloxacin, norfloxacin and ciprofloxacin) was 12%.

Among *E. coli*, the most commonly isolated agent, only TMP/SMX and nalidixic acid showed a resistance rate of ≥ 20%, in the period from 2010 to 2011. The global susceptibility was 88.3% (50,142/56,764) vs. 86.1% (55,436/64,400); $p < 0.0001$. Among *P. mirabilis*, except for colistin and nitrofurantoin, which were antibiotics against which this species has intrinsic resistance (0% susceptibility in this study) and TMP/SMX (susceptibility of 77.8% and 74.5% in the first and second periods, respectively), all other antibiotics had < 20% resistance in both periods. *K. pneumoniae* had a global susceptibility of 80.8% (3,242/4,013) vs. 76.8% (4,719/6,150); $p < 0.0001$. Various antibiotics showed a resistance rate higher than 20%, but norfloxacin was a good option in both periods that were studied (89.7% vs. 82.6%). Among *E. faecalis*, the global susceptibility was 77.2% (880/1,141) vs. 82.2% (1,328/1,616); $p = 0.001$, which indicated an improvement in susceptibility during the two periods.

The most resistant species was *E. aerogenes*, with a global susceptibility of 70.9% (878/1,237) vs. 68.9% (1,012/1,468); $p = 0.674$. In contrast to the aforementioned bacteria, *S. saprophyticus* presented a favorable profile with an improvement in global susceptibility during the two periods, 61.1% (1,249/2,043) vs. 88.2% (15/17); $p = 0.042$. The reason that the number of samples that were obtained during the second period was small is because the laboratory is no longer disclosing the antibiogram for *S. saprophyticus,* as recommended by the CLSI. Among *P. aeruginosa***,** the global susceptibility was 48.3% (532/1,102) in 2005-2006 vs. 85.6% (640/748) in 2010-2011; $p < 0.0001$.

The global frequency of intermediate susceptibility was the following: 1.7% (2005-2006) and 2.7% (2010-2011) (global); 1.6%

Table 3

Susceptibility of species among 11,943 bacteria isolated from urine cultures collected between 2005-2006, and 2010-2011 at the University Hospital of the University of São Paulo (HU-USP)

Antibiotic	*Escherichia coli*			*Proteus mirabilis*			*Klebsiella pneumoniae*			*Enterococcus faecalis*			*Enterobacter aerogenes*			*S. saprophyticus*			*Pseudomonas aeruginosa*		
	2005-2006	2010-2011	*p*	2005-2006	2010-2011	*p*	2005-2006	2010-2011	*p*	2005-2006	2010-2011	*p*	2005-2006	2010-2011	*p*	2005-2006	2010-2011	*P*	2005–2006	2010–2011	*P*
Nalidixic acid	90.1%	78.1%	<0.0001	91.6%	85.9%	0.011	87.1%	74.9%	<0.0001				86.2%	90%	0.422				42.9%		
Amikacin	99.8%	99.8%	0.783	98.8%	99.7%	0.316	96.7%	99.7%	0.006				95.3%	96.9%	0.932				78.6%	95.5%	0.004
Amoxicillin/clavulanate	83.5%	86.8%	<0.0001	91.9%	78.6%	<0.0001	90.4%	84.9%	0.034				5.7%	1%	0.155				28.6%		
Ampicillin										100%	90.2%	0.004									
Cefazolin	95.2%	91.9%	0.040	95.6%	76.2%	0.023	95.3%	79.2%	0.054				17.4%	0%	0.888						
Cefepime	99.6%	97.2%	<0.0001	98.2%	95.4%	0.035	94.8%	84.2%	<0.0001				95.3%	97.9%	0.643				80.3%	92.4%	0.076
Ceftazidime				100%	95.4%	1.000	50%	85.5%	0.073					95%					75.8%	92.4%	0.017
Ceftriaxone	99.6%	97.8%	<0.0001	98.5%	81%	<0.0001	95.9%	91.7%	0.328				93%	100%	1.000						
Cefuroxime axetil	96.8%	92.3%	<0.0001	96.9%	81%	0.003	88.6%	87.5%	1.000												
Ciprofloxacin	92.9%	84.9%	<0.0001	95.1%	87.9%	<0.0001	89.9%	78.1%	<0.0001	73.9%	74.8%	0.859	92%	92%	0.991	100%	100%	1.000	68.2%	89.4%	0.003
Clindamycin																99.3%					
Colistin		100%			0%			66.7%													
Erythromycin																79.5%					
Ertapenem		100%			99.5%			98.5%						100%							
Streptomycin										68.8%											
Streptomycin (high level resistance)										78.8%											
Gentamicin	96.6%	94.3%	<0.0001	95.8%	91.5%	0.013	94.9%	90.1%	0.021	100%			96.6%	97%	1.000				61.5%	89.4%	<0.0001
Gentamicin (high level resistance)										78.8%	79.8%	0.206									
Imipenem	100%	100%	1.000	100%	100%	1.000	100%	100%	1.000				100%						89.4%	91.2%	0.762
Levofloxacin		100%						100%													
Linezolid										97.5%	98.9%	1.000									
Meropenem	100%	100%	1.000	100%	100%	1.000		100%						100%					85%	92.4%	
Minocycline																					
Nitrofurantoin	98.4%	92.3%	<0.0001	1%	0%	0.147	63%	32.7%	<0.0001	100%	97.3%	0.478	47.1%	5%	<0.0001	99.3%			28.6%		
Norfloxacin	92.7%	85.7%	<0.0001	94.8%	100%	0.695	89.7%	82.6%	0.479				92%	100%	1.000				67.9%	86.7%	0.037
Oxacillin																	100%				
Penicillin G										89.8%	85.9%	0.294									
Piperacillin/tazobactam	100%	96.9%	1.000	100%	96.2%	1.000	16.7%	88.7%	<0.0001					87.5%					89.4%	94%	0.588
Rifampin																99.3%	100%	1.000			
Tetracycline										26.8%	18.9%	0.326				81.5%					
Trimethoprim/sulfamethoxazole	65%	62.9%	0.023	77.8%	74.5%	0.268	82.7%	77.9%	<0.0001				92%	94%	0.583	95.2%			3.1%		
Vancomycin										98.6%	97.3%	0.699				100%	100%	1.000			

Note: * $p < 0.05$ compared between gender within each stratum of age indicated (Pearson uncorrected chi-square test or Fisher's exact test when values ≤ 5).

and 2.7% (*E. coli*); 0.8% and 2.5% (*Proteus mirabilis*); 3.0% and 3.5% (*K. pneumoniae*); 0.5% and 2.0% (*E. faecalis*); 3.6% and 3.7% (*E. aerogenes*); 0.5% and 0% (*S. saprophyticus*); and 4.6% and 5.3% (*P. aeruginosa*).

The comparison among the age strata, 0-14, 15-64 and ≥ 65 years old, versus gender, among the 45 antibiotics that were tested (Table 4), showed statistically significant differences for 25 of the antibiotics between males and females, with greater rates of resistance in men and in patients older than 65 years. The antibiotics demonstrating these differences were the following: nalidixic acid, amikacin, amoxicillin/clavulanate, ampicillin, ampicillin/sulbactam, aztreonam, cephalothin, cefazolin, cefepime, cefotaxime, ceftazidime, ceftriaxone, cefuroxime axetil, ciprofloxacin, carbenicillin, ertapenem, streptomycin (high level resistance), gentamicin, imipenem, meropenem, nitrofurantoin, piperacillin/tazobactam, ticarcillin, TMP/SMX and vancomycin.

DISCUSSION

This is the third largest study of susceptibility to antibiotics in urinary tract infections in Brazil (Table 5). All studies of this type in Brazil, except for one[10], are retrospective and exclude many demographic variables, except gender and age, as well as clinical, radiological or laboratory variables, which does not allow multivariate analysis, and thus, greatly reduces the impact of these publications[1,2,4,9,19,20,25,26].

Initially, this study had the goal of guiding the implementation of a protocol for the treatment of UTIs; however, the lack of clinical data (mainly regarding the uncomplicated infections) and the large number of isolates, preclude such an achievement.

Considering that $5.30 US dollars was paid for each urine culture and antibiogram according to the table of Brazilian Unified Health System, the "estimated cost" of this study was $63,297.90[27]. Taking into account that in the same period approximately 60,000 urine cultures were requested, of which, only 20% were positive, the total cost to the health system was about $183,000.00 just in the University Hospital of the University of São Paulo, which is one of 6,753 hospitals in Brazil, of which only 30% are public. This justifies conducting a prospective study to address the clinical characteristics of patients in Brazil.

The biggest (multicenter) impact study involving Brazilian cases of UTI isolates was the SENTRY[13], which was published a decade ago and involved four centers in the country but only included inpatients. The methodology included the use of the gold standard procedure of microdilution for the preparation of the antibiograms and involved 1,961 urine isolates. *E. coli* was the most frequent pathogen that was isolated followed by *Klebsiella* spp., *P. aeruginosa*, and *P. mirabilis*. At that time, the amount of detected resistance was > 45% to TMP/SMX and a resistance to fluoroquinolones of 17.5-18.9% was detected. Furthermore, the susceptibility to broad-spectrum antibiotics (91.0-100.0%) and nitrofurantoin (87%) remained high, including piperacillin/tazobactam, aztreonam, extended-spectrum cephalosporins (third and fourth generations), carbapenems and amikacin. At that time, the biggest problems in terms of resistance was carbapenem-resistant *P. aeruginosa*, ciprofloxacin-resistant *E. coli* and ESBL-producing *K. pneumoniae*. The Enterobacteriaecae producing carbapenemases were not even mentioned in that study[13].

The data found in this study are similar to those found in previous literature, with some minor peculiarities. The proportion of females (77.6% vs. 69%-88.8% in studies), the female-to-male ratio by age group, the age group most affected (15-64 years: 57.2%) and the first isolated agent (*E. coli*, 70.2%) and frequency is very similar to that found in many published studies[2,4,9,10,15,19,20,25,26]. The percentage of *E. coli* that is isolated from UTIs, according to international literature, is 75% to 95%[15]. In Brazil, the frequency varied from 48.2%[9] to 71.6%[19] in São Paulo. The proportion of Gram-negative isolates varied from 84.4% to 87.3%[4,19,24].

The second most frequent agent presented a variation in different studies in Brazil. Except in studies by COSTA *et al.*[9] and KOCH *et al.*[20], in which *Proteus mirabilis* was the second most frequently isolated uropathogen, the second most commonly isolated pathogen was *Enterococcus* spp.[25], *K. oxytoca* and *S. agalactiae*[22], *Enterobacter* spp.[2], and *K. pneumoniae*[4,11,19,22].

In the one Brazilian study to date[11] about ESBL-producing Enterobacteriaceae in community-acquired UTIs, which evaluated 78,964 urine cultures in Juiz de Fora, State of Minas Gerais, Southeastern of Brazil, from 2001 to 2009, detected a frequency of 0.66% (n = 512) over nine years in the majority of patients older than 65 years (female, 52.6% and male, 48.1%), which is similarly to the results of this study. The percentage reported in the literature varied from 0.2% to 3.5%[11], which is close to the value of 3.0% among Gram-negative tested between 2010 and 2011, in this study. Although the most frequently isolated bacterium that produces ESBL is *Klebsiella* spp. (24 to 60%), DIAS *et al.* also detected *E. coli* (72.4%), followed by *Klebsiella* spp. (19.8%), which is similar to this study in which *E. coli* (62.4%), *Klebsiella* spp. (36.4%) and *Proteus* spp. (1.2%) were isolated. This study did not perform a confirmatory test for ESBL, which can result in false positives[12].

Global resistance was ≥ 20% for TMP/SMX, tetracycline, norfloxacin, nitrofurantoin, erythromycin, cefazolin and nalidixic acid, especially leveraged by other bacteria than *E. coli*. Global resistance was ≥ 20% for TMP/SMX, tetracycline, norfloxacin, nitrofurantoin, erythromycin, cefazolin and nalidixic acid in the isolated bacteria other than *E. coli*. There was significant difference between virtually all antibiotics in the two periods studied, which was commonly highly significant ($p < 0.0001$); however, no difference was observed between the antibiotics used for coverage of Gram-positive bacteria, except for rifampin and oxacillin. The fact that there was no statistically significant difference between the two periods in the overall susceptibility to key antibiotics by Gram-positive bacteria reveals the minor importance of these bacteria in the pathogenesis of UTIs in the study population, due to the reduced presence of selective pressure, which occurs with antibiotics that are used to treat Gram-negative bacteria.

The study by KOCH *et al.*[20] assessed the temporal trends of antimicrobial resistance, from 2000 to 2004 of gentamicin ($p = 0.98$), cephalothin ($p = 0.29$), cefoxitin ($p = 0.16$), TMP/SMX ($p = 0.40$), nalidixic acid ($p = 0.02$), ceftriaxone ($p = 0.12$) and ciprofloxacin ($p = 0.07$). Therefore, unlike this study, only nalidixic acid had statistical significance and ciprofloxacin had a statistical trend, which demonstrates the selective pressure that was exerted by quinolones in Rio Grande do Sul, southern Brazil. The same study also showed that older patients showed increased resistance to nalidixic acid, ciprofloxacin and

Table 4

Susceptibility to antibiotics of all 11,943 bacteria isolated from urine cultures, between 2005 and 2006, and 2010 and 2011 at the University Hospital of the University of São Paulo (HU-USP), stratified by gender and stratum of age

Antibiotic, susceptible, n(%)	Male gender			Female gender		
	0-14 years	15-64 years	≥65 years	0-14 years	15-64 years	≥65 years
Nalidixic acid	603 (97.3)*	532 (74.2)*	506 (59.3)*	1.198 (94.1)*	4.794 (88.1)*	1.220 (69.8)*
Amikacin	528 (100)	635 (96.1)*	794 (95.0)*	1.107 (100)	4.740 (99.8)*	1.569 (99.2)*
Amoxicillin/clavulanate	454 (73.2)*	490 (68.3)*	530 (62.1)*	1.050 (82.5)*	4.637 (85.2)*	1.395 (79.8)*
Ampicillin	253 (38.8)	304 (37.7)*	318 (31.3)*	539 (41.2)	2.802 (48.6)*	880 (46.5)*
Ampicillin/sulbactam	3 (16.7)	17 (29.8)*	40 (44.9)*	2 (11.1)	39 (17.1)*	14 (23.3)*
Aztreonam	9 (100)	25 (49.0)	31 (41.9)*	18 (85.7)	11 (45.8)	27 (64.3)*
Cephalothin	457 (73.2)*	448 (60.3)*	443 (49.4)*	964 (75.4)*	4.154 (76.2)*	1.185 (66.9)*
Cefazolin	87 (77.0)*	102 (68.5)*	106 (70.7)*	178 (87.7)*	741 (73.0)*	217 (83.8)*
Cefepime	527 (99.8)	604 (91.4)*	725 (86.5)*	1.095 (98.9)*	4.692 (98.7)*	1.499 (94.8)*
Cefotaxime	230 (95.0)	296 (81.8)*	374 (73.3)*	549 (95.8)	2.717 (97.2)*	854 (88.5)*
Cefoxitin	0	4 (14.8)*	3 (6.8)	5 (45.5)	17 (65.4)*	4 (16.0)
Ceftazidime	254 (98.8)	349 (87.5)*	444 (81.0)*	609 (97.9)	2.902 (97.7)*	1.383 (87.7)*
Ceftriaxone	383 (98.7)*	374 (91.9)*	382 (91.0)*	705 (100)*	2.677 (99.5)*	807 (97.6)*
Cefuroxime axetil	272 (93.2)*	243 (77.9)*	227 (70.7)*	528 (97.8)*	1.954 (96.1)*	557 (88.8)*
Ciprofloxacin	657 (98.8)	641 (76.2)*	692 (63.0)*	1.308 (98.2)	5.519 (92.5)*	1.446 (74.7)*
Clindamycin	12 (100)	21 (63.6)*	41 (75.9)	14 (100)	263 (98.9)*	25 (58.1)
Chloramphenicol	–	–	1 (100)	–	2 (100)	–
Carbenicillin	32 (37.6)	52 (61.9)	32 (45.7)*	59 (39.6)	306 (55.8)	97 (60.2)*
Colistin	–	3 (75.0)	2 (100)	1 (100)	4 (80.0)	4 (100)
Erythromycin	9 (75.0)	16 (48.5)*	31 (57.4)	13 (86.7)	216 (81.2)*	23 (53.5)
Ertapenem	231 (100)	318 (99.4)	446 (98.9)*	563 (100)	2778 (99.9)	933 (99.9)*
Streptomycin	7 (58.3)	10 (62.5)	38 (73.1)	5 (55.6)	14 (63.6)	30 (62.5)
Streptomycin (high level resistance)	10 (83.3)	15 (57.7)	39 (76.5)*	8 (100)	35 (72.9)	18 (34.0)*
Gentamicin	623 (97.0)	702 (86.8)*	855 (84.5)*	1.271 (96.6)	5.617 (96.4)*	1.680 (91.0)*
Gentamicin (high level resistance)	21 (87.5)	27 (64.3)*	80 (77.7)	17 (100)	64 (91.4)*	70 (70.0)
Imipenem	296 (100)	338 (98.5)*	383 (98.0)*	545 (100)	1.975 (100)*	646 (99.5)*
Levofloxacin	–	–	–	–	7 (100)	3 (100)
Linezolid	24 (100)	55 (100)	117 (99.2)	22 (100)	205 (98.6)	96 (99.0)
Meropenem	241 (100)	362 (98.4)*	509 (97.7)*	582 (100)	2.800 (100)*	968 (99.7)*
Minocycline	50 (58.8)*	58 (69.0)	47 (67.1)	115 (77.2)*	393 (71.7)	115 (71.4)
Nitrofurantoin	389 (60.2)*	584 (74.8)*	660 (67.7)*	1.119 (86.2)*	5.010 (86.8)*	1.515 (81.7)*
Norfloxacin	400 (98.0)	363 (80.6)*	338 (65.6)*	733 (98.4)	2.664 (94.7)*	712 (79.3)*
Oxacillin	5 (29.4)	16 (39.0)*	43 (51.8)	7 (31.8)	67 (18.6)*	21 (37.5)
Penicillin G	23 (54.8)	38 (44.2)*	89 (47.1)	19 (49.6)	112 (23.9)*	95 (57.6)
Piperacillin/tazobactam	131 (97.8)	184 (84.4)*	258 (81.9)*	306 (96.5)	1.326 (96.4)*	503 (93.8)*
Polymyxin B	2 (100)	15 (100)	23 (100)	13 (100)	4 (100)	11 (100)
Quinupristin/dalfopristin	3 (50.0)	11 (68.8)	17 (56.7)	1 (33.3)	28 (93.3)	10 (45.5)
Rifampin	17 (100)	35 (85.4)*	78 (94.0)	22 (100)	361 (99.7)*	51 (89.5)
Teicoplanin	16 (100)	27 (100)	59 (89.4)	17 (100)	135 (99.3)	61 (98.4)
Tetracycline	20 (47.6)*	50 (54.9)*	81 (54.4)	45 (71.4)*	350 (66.5)*	78 (48.4)
Ticarcillin	73 (81.1)*	86 (78.9)*	82 (73.2)*	141 (91.0)*	520 (93.4)*	162 (89.5)*
Tigecycline	–	3 (75.0)	2 (100)	1 (100)	4 (80.0)	3 (100)
Tobramycin	85 (100)	83 (98.8)	71 (98.6)	147 (98.7)	547 (99.5)	159 (98.8)
Trimethoprim/sulfamethoxazole	415 (65.5)	485 (63.0)*	564 (59.2)*	807 (62.4)	4.007 (70.6)*	1.176 (65.1)*
Vancomycin	43 (100)	93 (98.9)	190 (94.1)*	43 (100)	496 (100)	170 (98.8)*

Note: * $p < 0.05$ compared between gender within each stratum of age indicated (Pearson uncorrected chi-square test or Fisher's exact test when values ≤ 5).

Table 5
Brazilian retrospective surveillance studies of community-acquired urinary tract infections

	Rocha JL *et al.*[25]	Costa LC *et al.*[9]	Braoios A *et al.*[4]	Koch CR *et al.*[20]	Kiffer CR *et al.*[19]	Santo E *et al.*[26]	Dias RC *et al.*[10]	Bail L *et al.*[2]	Dias VC *et al.*[11]	Andrade SS *et al.*[1]
Positive urine cultures (n)	9,798	566	1,252	957	37,261	78	186; 118 *E. coli*	106	78,964	611
Setting of study	Curitiba, Southern of Brazil	Campina Grande, Northeastern of Brazil	Presidente Prudente, Southeastern of Brazil	Porto Alegre, Southern of Brazil	São Paulo, Southeastern of Brazil	Ribeirão Preto, Southeastern of Brazil	Rio de Janeiro, Southeastern of Brazil	Ponta Grossa, Southern of Brazil	Juiz de Fora, Southeastern of Brazil	Brasília, Florianópolis, Porto Alegre, São Paulo, Brazil
Period of study	May to Dec 2009	Jan 2006 to Jun 2008	Jan 2006 to Dec 2007	Jan 2000 to Dec 2004	Jan 2000 to Dec 2003	Jul 2000 and Jul 2003.	Mar 2005 to Nov 2006	Jan to Dec 2001	2001 to 2009	Jan to Dec 2013
Year of publication	2012	2010	2009	2008	2007	2007	2009	2006	2012	2006
Antibiogram method	Vitek 2	disk diffusion	disk diffusion	disk diffusion	disk diffusion, Vitek 1	disk diffusion	disk diffusion and E-test	disk diffusion	disk diffusion	microdilution
Standardization	CLSI	CLSI	CLSI	CLSI	CLSI	CLSI	CLSI	CLSI	CLSI	CLSI
Females (%)	88%	85.2%	69.01%	81.3%	88.8%	not available	100%	70%	69.8%	75.6%
Most frequent age strata	21-40 years (36.9%)	> 60 years (36.9%)	20-49 years (52.95%)	not available	13-60 years (60.0%)	not available	not available	not available	not available	not available
Gram-negatives (%)	not available	not available	84.42%	not available	87.3%	100%	100%	92.5%	100%	not available
E. coli (%)	66.1%	48.2%	65.97%	66.2%	71.6%	100%	100%	58.6%	72.4%	66%
Other frequent agents (%)	*Enterococcus spp (8.1), Klebsiella spp. (5.9), S.saprophyticus 381 (3.9), Proteus mirabilis (3.9), Enterobacter spp. (2.7), Pseudomonas aeruginosa (1.2)*	P.mirabilis (11), K. pneumoniae (9.9), S.saprophyticus (5.5), Enterobacter sp. (5.3), S. epidermidis (5.1), S.aureus (3.5), K.oxytoca (3.5). P.aeruginosa (2.5)	Klebsiella pneumoniae (6.07), Proteus mirabilis (5.51), Enterobacter sp (2.88), Pantoea agglomerans (1.92), Citrobacter sp (1.36).	Proteus mirabillis (8.4), Klebsiella sp (5.6)	K. pneumoniae (6.4), P. mirabilis (6.1), E. faecalis (4.8), P. aeruginosa (1.8), S. saprophyticus (1.6), E. aerogenes (1.6), E. cloacae (1.1).	No	No	Enterobacter sp. (11.8), Proteus sp.(4.4),Serratia sp (2.9),Klebsiella sp.(2.9).	Klebsiella pneumoniae (17.4%), Morganella morganii (2.5%), Proteus mirabilis (1.9%), Klebsiella oxytoca (1.3%), Klebsiella ozaenae (1.1%).	Klebsiella spp. (7%), P. mirabilis (6.4%), Enterococcus spp. (5.6%), P. aeruginosa (4.6%).
Clinical variables present	no	no	no	no	no	no	yes	no	no	no
E. coli susceptibility	Ampicillin (55.9), nitrofurantoin (96.0), TMP-SMX (65.8), fluoroquinolones[1] (82.2), extended-spectrum cepholosporins[2] (96.8), Gentamicin (93.1).	Ciprofloxacin (78.8), norfloxacin (77), nitrofuratoin (93), TMP/SMX (54.3)	Amoxicillin/clavulanate (94), ampicillin (48), cephalotin (59), cefuroxime (86), ceftriaxone (90), ceftazidime (97), cefepime (97), ciprofloxacin (86), gentamicin (96), nitrofurantoin (96), TMP/SMX (62)	Gentamicin (88), cephalotin (55), nitrofurantoin (86), ceftriaxone (98), ciprofloxacin (87), TMP/SMX (54).	Ampicillin (57), cephalotin (86), ceftriaxone (99), ciprofloxacin (88), gentamicin (97), nitrofurantoin (97), norfloxacin (88), TMP/SMX (66), tetracycline (69)	Ampicillin (63), cephalotin (92), cefuroxime (99), ceftriaxone (99), gentamicin (100), nitrofurantoin (97), norfloxacin (88), TMP/SMX (71), tetracycline (69)	Ampicillin (29), TMP/SMX (52), amoxicilline/clavulanate (81), floroquinolones[3] (94), cefuroxime (96), fosfomycin (97), nitrofurantoin (97), gentamicin (97), cefotaxime (98)	Ampicillin (49), cephalotin (74), TMP/SMX (56), nitrofurantoin (95), norfloxacin (88), gentamicin (88)	2009: Amikacin (94), ciprofloxacin (24), gentamicin (69), nitrofurantoin (88), TMP/SMX (24), imipenem (100)	Ampicillin (50), amoxicillin/ clavulanate (89), cefuroxime (99), ciprofloxacin (90), levofloxacin (91), nitrofurantoin (98), TMP/SMX (58).
Proteus sp. susceptibility	–	Ciprofloxacin (91.2), norfloxacin (91.2), nitrofurantoin (58.1), TMP/SMX (58.1)	Amoxicillin/clavulanate (96), ampicillin (5), cephalotin (92), cefuroxime (85), ceftriaxone (82), ceftazidime (93), cefepime (95), ciprofloxacin (83), gentamicin (93), nitrofurantoin (83), TMP/SMX (82)	Gentamicin (91), cephalotin (44), nitrofurantoin (40), ceftriaxone (91), ciprofloxacin (91), TMP/SMX (47).	Ampicillin (81), cephalotin (97), ceftriaxone (99), ciprofloxacin (96), gentamicin (98), nitrofurantoin (0), norfloxacin (96), TMP/SMX (78), tetracycline (0)	–	–	–	2009: Amikacin (100), ciprofloxacin (100), gentamicin (0), nitrofurantoin (0), TMP/SMX (50), imipenem (100)	–

Table 5
Brazilian retrospective surveillance studies of community-acquired urinary tract infections (cont.)

	Rocha JL *et al.*[25]	Costa LC *et al.*[9]	Braoios A *et al.*[4]	Koch CR *et al.*[20]	Kiffer CR *et al.*[19]	Santo E *et al.*[26]	Dias RC *et al.*[10]	Bail L *et al.*[2]	Dias VC *et al.*[11]	Andrade SS *et al.*[1]
Klebsiella sp. susceptibility	–	Ciprofloxacin (82.1), norfloxacin (78.6), nitrofurantoin (76.8), TMP/SMX (71.4)	Amoxicillin/clavulanate (90), ampicillin (42), cephalotin (80), cefuroxime (84), ceftriaxone (88), ceftazidime (94), cefepime (97), ciprofloxacin (94), gentamicin (94), nitrofurantoin (12), TMP/SMX (96)	Gentamicin (85), cephalotin (43), nitrofurantoin (42), ceftriaxone (94), ciprofloxacin (79), TMP/SMX (53).	Ampicillin (0), cephalotin (92), ceftriaxone (98), ciprofloxacin (94), gentamicin (97), nitrofurantoin (79), norfloxacin (91), TMP/SMX (82), tetracycline (80)	–	–	–	2009: Amikacin (94), ciprofloxacin (13), gentamicin (63), nitrofurantoin (44), TMP/SMX (24), imipenem (100)	–
E. faecalis susceptibility	–	–	–	–	Ampicillin (99), ciprofloxacin (84), nitrofurantoin (99), tetracycline (40)	–	–	Nitrofurantoin (100), norfloxacin (100), gentamicin (100)	–	–
E. aerogenes susceptibility	–	Ciprofloxacin (85.7), norfloxacin (81), nitrofurantoin (66.7), TMP/SMX (47.6)	–	–	Ampicillin (0), cephalotin (0), ceftriaxone (98), ciprofloxacin (95), gentamicin (98), nitrofurantoin (79), norfloxacin (95), TMP/SMX (92), tetracycline (93)	–	–	–	2009: Amikacin (76), ciprofloxacin (50), gentamicin (75), nitrofurantoin (0), TMP/SMX (50), imipenem (100)	–
S. saprophyticus susceptibility	–	Ciprofloxacin (73.3), norfloxacin (72.4), nitrofurantoin (86.7), TMP/SMX (39.3)	Amoxicillin/clavulanate (94), cephalotin (86), clindamicin (94), oxacillin (91), ciprofloxacin (94), gentamicin (97), nitrofurantoin (97), norfloxacin (97), TMP/SMX (94), vancomycin (100).	–	Ciprofloxacin (99), nitrofurantoin (99), tetracycline (84).	–	–	–	–	–
P. aeruginosa susceptibility	–	–	–	–	Ceftriaxone (11), ciprofloxacin (37), gentamicin (52), norfloxacin (38).	–	–	Norfloxacin (33), gentamicin (33)	–	–
Temporal trend of resistance	–	–	–	Ciprofloxacin (p=0.07), nalidixic acid (P=0.02)	Ciprofloxacin, norfloxacin, tetracycline, nalidixic acid (all: p< 0.0001).	–	–	–	–	–

Notes: 1: floroquinolones are ciprofloxacin and levofloxacin; 2: extended-spectrum cephalosporins includes ceftriaxone and cefotaxime; 3: includeds norfloxacin, ciprofloxacin and levofloxacin.

nitrofurantoin, and in men, for many of the antibiotics that were tested, with the exception of TMP/SMX[20]. KIFFER *et al.*[19] demonstrated a temporal trend of a decreased susceptibility to quinolones from 2000 to 2003 including ciprofloxacin, norfloxacin, tetracycline, and nalidixic acid ($p < 0.0001$). An earlier study, that was conducted two decades ago in Salvador, Northeastern Brazil, showed a reported resistance to norfloxacin that reached 9.1% and to ciprofloxacin of 16.5%[21].

The study by DIAS *et al.*[11] evaluated the temporal trend of resistance among ESBL-producing bacteria and showed a peak of occurrence in 2005 (2000-2009) for all antibiotics close to 80% (amikacin, ciprofloxacin, gentamicin, nitrofurantoin, TMP/SMX and imipenem) and a second peak in 2008 (ciprofloxacin and TMP/SMX were close to 80% and nitrofurantoin was close to 40%).

The susceptibility of *E. coli* in 10 Brazilian studies of community-acquired UTIs showed that only TMP/SMX, cephalothin and norfloxacin presented < 80% susceptibility[1,2,4,9,10,11,19,20,25,26]. In addition to nitrofurantoin, to which *P. mirabilis* presents intrinsic resistance, TMP/SMX and cephalothin were not good options for treatment of UTIs. According to other studies in Brazil, *K. pneumoniae* retains relative susceptibility to fluoroquinolones, as shown in this study, and only TMP/SMX and nitrofurantoin were not good options for treatment of UTIs. The profile found in the literature for *E. aerogenes* is similar to that found in our study, although the literature data indicate a decreased susceptibility to TMP/SMX[9,11,19].

Except for lower levels of susceptibility to tetracycline and ciprofloxacin, the data regarding *E. faecalis* from this study resembles that found in the study by KIFFER *et al.*[19]. Data regarding *S. saprophyticus* demonstrated distinct profiles of susceptibility, and are similar to the data found in this study. Finally, there is a notable discrepancy between the data regarding the susceptibility of *P. aeruginosa* in this study and that of KIFFER *et al.*[19], the latter being characterized by bacteria with more resistance.

A preliminary conclusion of the risk factors could indicate a predominance of *E. coli* in young women and a predominance of other more resistant isolates, particularly of *Proteus* spp. in men, which, according to literature, are most affected by urinary infections when they develop benign prostatic hyperplasia (BPH), even though the odds ratio is statistically significant for patients younger than 65[14,15]. Data from a higher incidence of resistance in elderly men are corroborated by three other Brazilian studies[19,20,25]. ROCHA *et al.*[25] also demonstrate a statistically significant difference of the frequency of bacteria between men and women. They found a predominance of *Enterococcus* spp., *Enterobacter* spp. and *Pseudomonas* spp., but not *Proteus* spp. in men and a predominance of *S. saprophyticus* and *E. coli* in women. The same study also demonstrated a highly statistically significant susceptibility to antibiotics by *E. coli* and others, which corroborates the data from this study.

The favorable resistance profile of *E. coli* results in an expanded list of oral therapeutic options for cystitis treatment in young women, including pregnant women, such as fluoroquinolones for not pregnant patients, and cephalexin, cefuroxime axetil and amoxicillin/clavulanate for pregnant patients. However, a policy of sparing fluoroquinolones in such cases is urgently needed. Although it was previously a good option for young women, nitrofurantoin had low levels of susceptibility by the bacteria that were frequently isolated, such as *Proteus* spp. (intrinsically resistant), *Klebsiella* spp., *Enterobacter* spp. and *Pseudomonas* spp., as well as *M. morganii* and *S. marcescens* (both intrinsically resistant).

Therefore, the biggest problem with making recommendations for empiric antibiotics according to this casuist is men and the elderly. *P. mirabilis*, a urease-producing bacteria that is capable of causing struvite urolithiasis and was the second most frequent isolate, presents intrinsic resistance to several antibiotics, such as cefazolin, tigecycline, polymyxins and nitrofurantoin. Therefore, any empirical recommendation for men and elderly people, cannot dispense with fluoroquinolones or a reassessment based on early urine cultures.

For complicated infections and pyelonephritis, fluoroquinolones, which have renal penetration, such as ciprofloxacin, moxifloxacin and levofloxacin (although the latter two are reserved for respiratory infections because of their expanded spectrum for Gram-positive bacteria and atypical bacteria) are also good choices, although third and fourth generation cephalosporins, as well as aminoglycosides, are a second option and are preferred in cases of severe sepsis and septic shock. Amoxicillin/clavulanate is a good oral treatment option, especially after a loading dose of extended-spectrum cephalosporins or aminoglycosides.

Fosfomycin, although not evaluated by the antibiograms in this study, had a good susceptibility profile in a small Brazilian study (97.0% of susceptibility, n = 118)[10] and is an option for multidrug-resistant infections, especially for ESBL- and carbapenemase-producing bacteria.

An elegant study from the viewpoint of mathematical modeling in the city of São Paulo, Brazil, by KIFFER *et al.*[18] in 2002, demonstrated the correlation between the probability of resistance of strains of *E. coli* in urine and the "density the ciprofloxacin usage for the previous three months" (defined as D_DDDA), $p = 0.023$. Additionally, ciprofloxacin D_DDDA was found to be related to clusters[18]: the western region of São Paulo was deemed a hot spot for the use of ciprofloxacin (average probability of 0.8808). This study is an elegant demonstration of how the indiscriminate use of ciprofloxacin against uropathogens may result in the loss of a medication to treat a broad spectrum of uropathogens. Furthermore, taking into account that approximately 30% to 45% of ciprofloxacin ingested is excreted in a non-metabolized form in the urine, widespread use can have environmental consequences due to presence of the antibiotic in the sewage and exposure of Enterobacteriaceae to the antibiotic in that environment. This finding only reinforces the urgent need, based on studies, for an efficient policy of reducing the use of fluoroquinolones and broader-spectrum antibiotics.

A recent study in the United States[17] showed a higher risk of levofloxacin-resistant *E. coli* related to previous use of this antibiotic (adjusted OR = 7.6, 9% CI: 2.1-27.5, $p = 0.002$) and previous hospitalization (OR = 2.0, 95% CI: 1.0-3.9, $p = 0.04$) as well as a statistically significant risk to resistance to other antibiotics in isolates resistant to this fluoroquinolone. Another interesting finding was increased rates of resistance to TMP/SMX despite a 50% decrease in the selective pressure, which is most likely due to multidrug resistance plasmids or multidrug efflux pumps.

This study has several limitations, even though it has the merit of

being one of the few and largest of its type in Brazil: there is one small study of the antimicrobial susceptibility profile according to clinical presentation. In our analysis, we do not separate nosocomial infections from those acquired in the community. The samples were collected not only from midstream but also by other techniques, such as indwelling catheter, which can increase the chance of isolating hospital germs. Despite this, our resistance rates are similar to studies that included only community isolates. Our analysis was based on retrospective data and focused on microbiological aspects. Reproducibility problems can arise due to differences in interpretation of the minimum inhibitory concentration (MIC) between CLSI and EUCAST (European Committee on Antimicrobial Susceptibility Testing) documents.

Studies assessing other demographic, clinical, radiological and laboratory risk factors (including multivariate analysis to control for confounding factors) for infections by germs other than *E. coli*, such as *Proteus* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Klebsiella* spp., are urgently needed, to make treatment recommendations that can save broad-spectrum drugs, such as fluoroquinolones, and third and fourth generations of cephalosporins, carbapenems, fosfomycin and aminoglycosides.

CONCLUSIONS

The most favorable scenario is the treatment of *E. coli*. TMP/SMX and nalidixic acid were only two whose resistance exceeds the threshold of 20%, which contraindicates their use according to the guidelines, in our study. The rise of resistance to fluoroquinolones is concerning and averaged 12%. Furthermore, the 20% threshold resistance was exceeded by norfloxacin in the overall analysis, mainly at the expense of bacteria other than *E. coli*. In this study, a high frequency of community-acquired ESBL bacteria was found, which is greater than that found in a study in Brazil, but within the range described in the literature. Most isolates were observed in the elderly. As in other studies, the main problem of resistance was noted in men and in the elderly, which limits most conservative treatment recommendations that save broad-spectrum antibiotics such as fluoroquinolones, aminoglycosides and third and fourth generations cephalosporins. Well-controlled prospective studies that evaluate the risk factors for UTIs by bacteria other than *E. coli* and resistance in Brazil are greatly needed.

RESUMO

Suscetibilidade a antibióticos em infecções de trato urinário em um hospital secundário, 2005-2006 e 2010-2011, em São Paulo, Brasil: dados de 11.943 uroculturas

Introdução: A infecção do trato urinário (ITU) tem alta incidência e recorrência, e o tratamento é empírico na maioria dos casos. **Objetivos:** O objetivo deste estudo foi analisar as culturas de urina realizadas em um hospital secundário, durante dois períodos: 2005-2006 e 2010-2011, para estimar a resistência microbiana. **Pacientes e métodos:** Foram analisadas 11.943 culturas aeróbicas de urina de acordo com um conjunto de dados demográficos básicos e susceptibilidade aos antibióticos, obedecendo às normas do *Clinical and Laboratory Standards Institute* (CLSI) para Vitek 1 e 2. **Resultados:** A maioria dos participantes era adulta e jovem atendida no Serviço de Emergência. *E. coli* foi a mais freqüente (70,2%) entre as 75 espécies isoladas. Resistência de todos os isolados foi ≥ 20% para sulfametoxazol/trimetoprim (SMX/TMP), norfloxacina, nitrofurantoína, cefazolina e ácido nalidíxico, apesar de *E. coli* ter sido mais suscetível (resistência ≥ 20% apenas para SMX/TMP e ácido nalidíxico) entre todos os isolados, levando em conta a porcentagem de resistência e o número de antibióticos testados. Resistência às fluoroquinolonas foi de 12%. Fatores de risco para *E. coli*: sexo feminino e idade < 65 anos. Homens e pacientes com mais de 65 anos apresentaram isolados mais resistentes. Beta-lactamases de espectro estendido (ESBL) foram identificadas em 173 de 5.722 isolados Gram-negativos (3,0%), 2010-2011. **Conclusões:** *E. coli* foi o isolado mais sensível a antibióticos. Houve uma evolução significativa da resistência antimicrobiana entre os dois períodos. Foi preocupante o aumento da resistência às fluoroquinolonas.

ACKNOWLEDGEMENTS

We would like to express gratitude to Valéria Cassettari, MD, MSc who is an infectious diseases physician and chief of the Infection Control Department at the University Hospital of the University of São Paulo (USP), whose work and dedication resulted in the database analyzed in this study under her authorization. Although she did not want to be part of writing this manuscript, her initiative was critical to this study and was only motivated by the spirit of the rational use of antibiotics and good clinical practice.

CONFLICTS OF INTEREST (DISCLOSURES)

No potential conflict of interest are declared for this paper.

REFERENCES

1. Andrade SS, Sader HS, Jones RN, Pereira AS, Pignatari AC, Gales AC. Increased resistance to first-line agents among bacterial pathogens isolated from urinary tract infections in Latin America: time for local guidelines? Mem Inst Oswaldo Cruz. 2006;101:741-8.

2. Bail L, Ito CAS, Esmerino LA. Infecção do trato urinário: comparação entre o perfil de susceptibilidade e a terapia empírica com antimicrobianos. Rev Bras Anal Clin. 2006;38:51-6.

3. Bauer AW, Perry DM, Kirby WMM. Single disc antibiotic sensitivity testing of staphylococci; an analysis of technique and results. Arch Intern Med. 1959;104:208-16.

4. Braoios A, Turatti TF, Meredija LCS, Campos TRS, Denadai FHM. Infecções do trato urinário em pacientes não hospitalizados: etiologia e padrão de resistência aos antimicrobianos. J Bras Patol Med Lab. 2009;45:449-56.

5. Clinical Laboratory Standards Institute. Normas de desempenho para teste de sensibilidade antimicrobiana: 15° suplemento informativo. CLSI document M100-S15. Vol. 25 (1). Clinical Laboratory Standards Institute, Wayne, PA.

6. Clinical Laboratory Standards Institute. CLSI document M100-S16. Clinical Laboratory Standards Institute, Wayne, PA.

7. Clinical Laboratory Standards Institute. CLSI document M100-S20. Clinical Laboratory Standards Institute, Wayne, PA.

8. Clinical Laboratory Standards Institute. CLSI document M100-S21. Clinical Laboratory Standards Institute, Wayne, PA.

9. Costa LC, Belém LF, Freitas e Silva PM, Pereira HS, Silva Júnior ED, Leite TR, *et al*. Infecções urinárias em pacientes ambulatoriais: prevalência e perfil de resistência aos antimicrobianos. Rev Bras Anal Clin. 2010;42:175-80.

10. Dias RC, Marangoni DV, Smith SP, Alves EM, Pellegrino FL, Riley LW, *et al*. Clonal composition of *Escherichia coli* causing community-acquired urinary tract infections in the State of Rio de Janeiro, Brazil. Microb Drug Resist. 2009;15:303-8.

11. Dias VC, da Silva VL, Firmo Ede O, Bastos LQ, Bastos AN, Bastos RV, *et al*. Distribution of ESBL-producing enterobacteria associated to community-acquired monomicrobial urinary tract infections and antimicrobial susceptibility trends over a 9-year period. J Chemother. 2012;24:178-81.

12. Espinar MJ, Rocha R, Ribeiro M, Gonçalves Rodrigues A, Pina-Vaz C. Extended-spectrum β-lactamases of *Escherichia coli* and *Klebsiella pneumoniae* screened by the VITEK 2 system. J Med Microbiol. 2011;60:756-60.

13. Gales AC, Sader HS, Jones RN, SENTRY Participants Group (Latin America). Urinary tract infection trends in Latin American hospitals: report from the SENTRY antimicrobial surveillance program (1997-2000). Diagn Microbiol Infect Dis. 2002;44:289-99.

14. Gupta K, Hooton TM, Naber KG, Wullt B, Colgan R, Miller LG, *et al*. International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: a 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. Clin Infect Dis. 2011;52:e103-20.

15. Hooton T. Clinical practice. Uncomplicated urinary tract infection. N Engl J Med. 2012;366:1028-37.

16. Hooton TM. The current management strategies for community-acquired urinary tract infection. Infect Dis Clin North Am. 2003;17:303-32.

17. Johnson L, Sabel A, Burman WJ, Everhart RM, Rome M, MacKenzie TD, *et al*. Emergence of fluoroquinolone resistance in outpatient urinary *Escherichia coli* isolates. Am J Med. 2008;121:876-84.

18. Kiffer CR, Camargo EC, Shimakura SE, Ribeiro PJ Jr, Bailey TC, Pignatari AC, *et al*. A spatial approach for the epidemiology of antibiotic use and resistance in community-based studies: the emergence of urban clusters of *Escherichia coli* quinolone resistance in São Paulo, Brazil. Int J Health Geogr. 2011;10:17.

19. Kiffer CR, Mendes C, Oplustil CP, Sampaio JL. Antibiotic resistance and trend of urinary pathogens in general patients from a major urban city. Int Braz J Urol. 2007;33:42-9.

20. Koch CR, Ribeiro JC, Schnor OH, Zimmermann BS, Müller FM, D' Agostin J, *et al*. Antimicrobial resistance of uropathogens among outpatients, 2000-2004. Rev Soc Bras Med Trop. 2008;41:277-81.

21. Lopes AA, Salgado K, Martinelli R, Rocha H. Aumento da freqüência de resistência à norfloxacina e ciprofloxacina em bactérias isoladas em uroculturas. Rev Assoc Med Bras. 1998;44:196-200.

22. Mora JF, Menezes I, Requia MK, Sardiglia CU. Perfil dos pacientes com infecções do trato urinário diagnosticados no município de Flor do Sertão - SC. Rev Bras Anal Clin. 2008;40:321-3.

23. Nostrand JD, Junkins AD, Bartholdi RK. Poor predictive ability of urinalysis and microscopic examination to detect urinary tract infection. Am J Clin Pathol. 2000;113:709-13.

24. Poletto KQ, Reis C. Suscetibilidade antimicrobiana de uropatógenos em pacientes ambulatoriais na Cidade de Goiânia, GO. Rev Soc Bras Med Trop. 2005;38:416-20.

25. Rocha JL, Tuon FF, Johnson JR. Sex, drugs, bugs, and age: rational selection of empirical therapy for outpatient urinary tract infection in an era of extensive antimicrobial resistance. Braz J Infect Dis. 2012;16:115-21.

26. Santo E, Salvador MM, Marin JM. Antimicrobial resistance among urinary tract *Escherichia coli* isolates from inpatients and outpatients in a tertiary care center in São Paulo, Brazil. Int J Infect Dis. 2007;11:558-9.

27. SIGTAP. Sistema de Gerenciamento da Tabela de Procedimentos. Available from: sigtap.datasus.gov.br/

28. Stamm WE, Counts GW, Running KR, Fihn S, Turck M, Holmes KK. Diagnosis of coliform infection in acutely dysuric women. N Engl J Med. 1982;307:463-8.

Received: 15 August 2013
Accepted 13 November 2013

Rev. Inst. Med. Trop. Sao Paulo
56(4):325-331, July-August, 2014
doi: 10.1590/S0036-46652014000400010

INCIDENCE OF DIARRHEA BY *Clostridium difficile* IN HEMATOLOGIC PATIENTS AND HEMATOPOIETIC STEM CELL TRANSPLANTATION PATIENTS: RISK FACTORS FOR SEVERE FORMS AND DEATH

Fernanda SPADÃO(1), Juliana GERHARDT(1), Thais GUIMARÃES(1), Frederico DULLEY(2), João Nóbrega de ALMEIDA JUNIOR(3), Marjorie Vieira BATISTA(4), Maria Aparecida SHIKANAI-YASUDA(4), Anna Sara LEVIN(4) & Silvia Figueiredo COSTA(4)

SUMMARY

We describe the rate of incidence of *Clostridium difficile*-associated diarrhea (CDAD) in hematologic and patients undergone stem cell transplant (HSCT) at HC-FMUSP, from January 2007 to June 2011, using two denominators 1,000 patient and 1,000 days of neutropenia and the risk factors associated with the severe form of the disease and death. The ELISA method (Ridascreen-Biopharm, Germany) for the detections of toxins A/B was used to identify *C. difficile*. A multivariate analysis was performed to evaluate potential factors associated with severe CDAD and death within 14 days after the diagnosis of CDAD, using multiple logistic regression. Sixty-six episodes were identified in 64 patients among 439 patients with diarrhea during the study period. CDA rate of incidence varied from 0.78 to 5.45 per 1,000 days of neutropenia and from 0.65 to 5.45 per 1,000 patient-days. The most common underlying disease was acute myeloid leukemia 30/64 (44%), 32/64 (46%) patients were neutropenic, 31/64 (45%) undergone allogeneic HSCT, 61/64 (88%) had previously used antibiotics and 9/64 (13%) have severe CDAD. Most of the patients (89%) received treatment with oral metronidazole and 19/64 (26%) died. The independent risk factors associated with death were the severe form of CDAD, and use of linezolid.

KEYWORDS: Hematology; Bone marrow transplant; *C. difficile.*

INTRODUCTION

Diarrhea is a common complication in patients who receive high doses of chemotherapy and in those undergone hematopoietic stem cell transplant (HSCT)[3,13,21,23]. *Clostridium difficile* has frequently been identified as cause of infectious diarrhea in hospital setting. Its incidence rates range from 4.8% to 9% in patients with acute myelogenous leukemia, from 4.9% to 7.5% in patients undergoing autologous and from 14% to 30.4% in those undergoing allogeneic HSCT[1,2,4,16,25]. The frequent and prolonged use of antibiotics may increase even further the risk of *C. difficile* associated diarrhea (CDAD) in this population of patients[23]. Despite these risk factors being frequent in these patients, data of CDAD in haematologic patients are still scarce.

Therefore, studies that evaluate CDAD in this population of patients can be useful for delineating measures of control and prevention of dissemination of this agent.

OBJECTIVE

To describe the rate of incidence and treatment of CDAD in hematological and HSCT patients, and the risk factors associated with the severe form of the CDAD and death.

PATIENTS, MATERIAL, METHODS

Study setting: The hematology and bone marrow transplant wards have 20 beds, are located in the *Central Institute of Hospital das Clinicas* (ICHC – FMUSP), Brazil, a teaching hospital with 1,000 beds. The bone marrow ward has four rooms, totalizing eight beds, and one room for contact isolation. All suspected cases of CDAD were placed in a contact isolation room, and remained in isolation until resolution of symptoms.

Study design: It is a retrospective study, the clinical records of all the patients in the hematology and bone marrow transplant wards during the period from January 2007 to June 2011 that performed *C. difficile* toxins A/B were analyzed. Its study has been approved by the Hospital das Clinicas of University of São Paulo, Brazil, Ethics Committee.

Definition of a case of CDAD: Hematologic patient with diarrhea (three or more soft stools within 24 hours) and positive for toxins A/B, who received treatment for CDAD.

(1) Nosocomial Infection Control Team of Hospital das Clinicas of University of São Paulo, São Paulo, SP, Brazil.
(2) Bone Marrow Transplant Unit, Hospital das Clinicas of University of São Paulo, São Paulo, SP, Brazil.
(3) Laboratory of Microbiology of Hospital das Clinicas of University of São Paulo, São Paulo, SP, Brazil
(4) Infectious Diseases Department of Unversity of São Paulo, Brazil.
Correspondence to: Silvia Figueiredo Costa, Departamento de Doenças Infecciosas da Universidade de São Paulo, Av. Dr. Arnaldo 455, Cerqueira Cesar, 01246-000 São Paulo, SP, Brasil. Fax: +55.11.30617043. E-mail: costasilviaf@ig.com.br

Patients considered suspect were those who presented diarrhea and collected stools for the investigation of *C. difficile* toxins A/B.

Definition of severe disease: Patients presented with one or more of the following variables during the treatment of diarrhea: hypotension; shock, renal insufficiency (50% decreases in creatinine clearance), toxic megacolon; colectomy and death within up to 30 days of onset of clinical symptoms.

The incidence rates of CDAD were calculated using two denominators: 1,000 patient-days and 1,000 days of neutropenia.

Data collection: Data on the number of hematologic and HSCT patients who sent stool samples for toxins A/B investigation were provided by the Information and Hospital Management System (SIGH) of the Central Laboratory Department (DLC). Data with positive toxins A/B are stored in a database of the sub-commission of hospital infection control of the Central Institute of Hospital das Clinicas of University of São Paulo.

The following variables were evaluated: age, gender, underlying disease, type of autologous and allogeneic HSCT (related or unrelated), time of transplant until the onset of diarrhea, presence of neutropenia, neutropenia/day, mucositis (presence and degree of mucositis as per the WHO), graft versus host disease (GVHD), the antimicrobials used; immunosuppressant drugs and chemotherapy, prior use of an antibiotic (up until 30 days before the development of diarrhea), and if the patients stayed in the same room. Other causes of diarrhea (rotavirus, parasites, GVHD, or neutropenic colitis), reactivation of cytomegalovirus (PCR and/or antigenemia positive in the blood), Vancomycin-Resistant Enterococci (VRE) colonization, performance of colonoscopy, and presence of pseudomembrane. The surveillance culture for identification of (VRE) is carried out weekly by rectal swab and/or stool culture and seeded in a selective medium with 6 μg/mL of vancomycin from all patients in the hematology and bone marrow transplant wards. Antigenemia and real-time polymerase chain reaction for Cytomegalovirus are performed twice a week in all HSCT patients from the moment of marrow infusion to one hundred days after transplantation.

The room and period of inpatient stay of the patients were evaluated to verify how many patients were contacts of a positive case of *C. difficile*, and how many developed CDAD.

The following data relative to the treatment of CDAD were evaluated: type of antibiotic used (oral/venous metronidazole or vancomycin), dosage, time, change in clinical picture. Clinical response in seven days: symptom resolution; partial response (50% reduction in frequency of diarrhea episodes); no response; worsening (megacolon and/or perforation). Response at the end of treatment (last day of antibiotic specific for the treatment of CDAD) was evaluated: symptom resolution; partial response (50% reduction in the frequency of diarrhea episodes); no response; worsening; and if there is relapse within 30 days.

Deaths during the treatment of CDAD were evaluated, considering death until 14 days after the onset of treatment, and death during hospitalization.

Microbiological procedures: The ELISA method (Ridascreen-Biopharm, Germany) was used to identify *C. difficile* toxins A/B.

Statistical analysis: The information was filed in a computerized database utilizing the Epi Info 6.04b program. A descriptive analysis was made of patient characteristics; continuous variables were expressed as mean, standard deviation, median, and interval, and compared by Wilcoxon's test, and the categorical variables by Chi-squared and Fisher's Exact tests. The outcomes studied were severity of the clinical symptoms and death within 14 days after the diagnosis of CDAD. The level of significance adopted for comparison of the variables in the bivariate was the value of $p < 0.05$. A multivariate analysis was performed to evaluate potential factors associated with severe CDAD and death within 14 days after the diagnosis of CDAD, using multiple logistic regression. The variables with $p < 0.10$ in the bivariate analysis and biological plausibility were tested in the multivariate analysis by stepwise forward. Chi-squared for tendency was used to evaluate the distribution of suspected and confirmed cases of CDAD and the incidence of cases confirmed during the study period from 2007 to 2011.

RESULTS

During the study period (January 2007 to June 2011), 983 hematology and 1136 HSCT inpatients were followed, a total of 25,312 patients-day and 9,902 days of neutropenia. Four hundred thirty-nine patients with diarrhea performed *C. difficile* toxins A/B tests. A total of 66 episodes of CDAD in 64 patients were positive and the records of these patients were reviewed. During the study period the cumulative incidence of CDAD was 3.1%. The rate of incidence of CDAD per 1,000 days of neutropenia varied from 0.78 to 5.45 and per 1,000 patient-days varied from 0.78 to 10.24 during the study period. The Chi-squared for tendency showed that the number of suspected cases of CDAD ($p = 0.4180$) and severe form of diseases ($p = 0.69$) remained stable, in contrast with the increase in number of confirmed cases ($p = 0.0006$).

The clinical and demographic data of 64 patients with CDAD over the study period are shown on Table 1. Most (68%) of the patients were males, the age varied from 12 to 65 years old, and the mean age was 38.9; median 38.5 and mode 38.0 years old. The most common underlying disease was acute myeloid leukemia 30/64 (44%), and of these, 32/64 (46%) were neutropenic, 31/64 (45%) undergone allogeneic HSCT, and 61/64 (88%) of the patients had received antibiotics. *C. difficile*-associated diarrhea was more frequent in HSCT patients 46/64 (69%), of which 31/46 (67%) were allogeneic transplants, than in hematologic patients 18/64 (31%). The overall mortality was 24/64 (35%) and the mortality within 14 days of diagnosis was 19/64 (26%).

At the onset of diarrhea, the use of metronidazole was introduced after collecting the stool before the result of test for CDAD. The average number of days to perform the toxins test until the use of metronidazole was one day. Most (89%) of the patients were treated with metronidazole; four patients initiated oral treatment and then switched to intravenous; three patients were hospitalized in ICU and received metronidazole intravenously. After seven days of treatment with metronidazole, 37% of the patients showed a partial response, and 57% had symptoms resolution with 14 days of treatment. Three patients experienced relapse of the disease in less than 30 days. Of the 43% that did not show symptom resolution at the end of treatment (14 days), 24% died during hospitalization, 3% had coinfection, and 3% GVHD. The severe form of

Table 1
Demographic and clinical data of hematologic and HSCT patients with diarrhea due to *C. difficile*, HC-FMUSP, 2007 to June 2011

	N: 64 patients	%
Age	Mean age 38.5 (12-65 years old)	
Underlying Disease		
Aplasia	3	4
Acute Lymphoid Leukemia	8	12
Acute Myeloid Leukemia	30	44
Chronic Myeloid Leukemia	4	6
Lymphoma	9	13
Multiple Myeloma	5	7
Others	7	10
Hospitalization Unit		
Hematology	23	33
Bone Marrow	46	67
Type of HSCT		
Allogeneic	31	45
Unrelated	4	6
Autologous	14	20
Days after HSCT until diarrhea	Mean 11 (1-407)	
Days of hospitalization until diarrhea	Mean 9 (1-105)	
Days of neutropenia until diarrhea	Mean 13 (3-36)	
Prior use of antibiotic	61	88
Sulfamethoxazole+trimethoprim	20	29
Carbapenems	37	54
Cefepime	18	26
Colistin	13	19
Linezolid	7	10
Quinolone	9	13
Piperacillin/Tazobactam	16	23
Hospitalization in the same room with case positive for *C. difficile*	18	26
GVHD	17	25
ERV	30	44
Co-infection		
CMV	14	20
Parasites	3	5
Severe forms	9	13
Mortality within 14 days	19	26
General mortality	24	35
Neutropenia	32	46
Days of hospitalization. until diarrhea, mean (variation)	Mean 9 (1-105 days)	
Days of diarrhea until toxin collection (variation)	Mean 2 (1-20 days)	
Toxin collection until initiation of treatment (variation)	Mean 1(0-20 days)	
Megacolon	2	3
Severe forms	9	13
TREATMENT		
Metronidazole IV	7	10
Metronidazole PO	61	88
Vancomycin IV	0	
Vancomycin PO	0	
Partial Response	24*	37
End of treatment	39•	57.4
Relapse	3	4.3
Patients did not finish treatment	20	24.6

GVHD: Graft versus host disease; VRE: Vancomycin-resistant enterococci, CMV: cytomegalovirus; ATB: antibiotic. *number of patients with partial response in the first 7 days of treatment, •number of patients who finished treatment and experienced symptom resolution, IV: intravenous; PO: oral

the disease was found in only nine patients (14%), and all of them died; seven of them (80%) died within 14 days.

The bivariate analysis of the risk factors associated with the severe form of CDAD identified allogeneic HSCT, use of glycopeptides and cyclophosphamide as risk factors; however, the multivariate analysis did not identify any independent risk factor (Table 2).

The bivariate analysis of risk factors associated with death within 14 days of the diagnosis of CDAD identified allogeneic transplant, severe form of the disease, linezolid and cyclophosphamide use and hospitalization as risk factors for death and lymphoma as protector. Two models of multivariate analysis were conducted to evaluate the death outcome, one with and the other without the age variable. In spite of not showing $p < 0.1$, age was included in the model because it had been described in various studies as a risk factor associated with mortality. On the other hand, ICU stay is a collinear variable of severity and was not included in the model (Table 3).

DISCUSSION

During the period studied, the cumulative incidence of CDAD was 3.1%, it rose without increasing in clinical suspicion and severe forms of diseases. These data are interesting and in accordance with many countries that have been showing the increasing in CDAD incidence. Different denominators have been used to calculate the incidence of infection by *C. difficile*[1,2,3,4,10,16,25]. The guideline of CDAD recommends the using as denominator 100 thousand patients[11]. In our study, we opted to use two denominators, namely, patient-days and days of neutropenia, because of the population studied. The presence and duration of the neutropenia are important risk factors for healthcare associated infections in this population of patients, and has been used as a denominator by other authors[14,17]. One limitation of our study is precisely the method "ELISA" used to diagnosis infection by *C. difficile* that is not very sensitive, sensitivity varying from 63 to 94%[11].

We identified more CDAD in HSCT patients (69%), of which 31/46 (67%) were allogeneic transplants, than in hematologic patients (31%). The mean of days of HSCT until the onset of diarrhea was 11 days in the present study. TOMBLYN *et al*. 2002[23] described a mean of one day after HSCT (varied from three to seven days). A greater frequency of *C. difficile* infection is expected during the first month after HSCT, a period when the patients are exposed to chemotherapy and antibiotics, important risk factors associated with this agent. Nine of sixty-four (13%) patients presented the severe form of CDAD, higher than the 5% previously described by WILLENS *et al*. 2012[27].

Previous use of antimicrobials is cited by various authors[1,2,4] as an important risk factor for the development of CDAD. Most of the patients of this study received antibiotics before the onset of the diarrhea; the most commonly used was carbapenem. However, 12% did not; this finding may be a result of crossover transmission of the agent, or exposure to other risk factors.

New drugs to treat CDAD are now available[18], despite this, data have been showing that metronidazole is a safe option to treat *C. difficile* infection in hematologic and HCST patients, with few cases of relapse[13,16,27]. In our study 57% of patients had symptoms ending with

Table 2
Risk factors associated with the severe form of diarrhea caused by *C. difficile* in hematologic and HSCT patients

Variable	Severe form		Bivariate analysis	
	Yes N = 9	No N = 57	OR (95% CI)	*p* value*
Age	21-57 (39)			0.5
Male	5(11%)	39(89%)	0.6(0.15-3.1)	0.41
Underlying disease				
Lymphoma	0(0)	9(100%)	0	0.26
AML	4(13%)	26(87%)	1(0.22-4.5)	0.61
ALL	2(25%)	6(75%)	2.5(0.3-14.9)	0.27
BML	1(33%)	3(67%)	0	0.43
Medullary aplasia	0(0)	3(100%)	2.3(0-24.8)	0.65
Multiple myeloma	0(0)	5(100%)	0	0.48
Allogeneic HSCT	**7(23%)**	**24(77%)**	**5.1(1-38.6)**	**0.03**
Autologous HSCT	0(0)	14(100%)	0	0.11
Prior use				
Quinolone	2(22%)	7(78%)	2.1(0.25-12.1)	0.33
Sulfamethoxole +trimethoprim	1(5%)	19(95%)	0.27(0.01-1.88)	0.19
Cefepime	1(6%)	17(94%)	0.32(0.01-2.22)	0.25
Carbapenems	4(11%)	33(89%)	0.65(0.14-2.84)	0.4
Colistin	1(8%)	12(92%)	0.5(0.02-3.61)	0.45
Glycopeptides	**2(5%)**	**35(95%)**	**0.2(0.02-1.02)**	**0.04**
Piperacillin-Tazobactam	2(12%)	14(88%)	0.9(0.12-4.8)	0.65
VRE	2(7%)	28(93%)	0.3(0.04-1.62)	0.15
CMV	2(14%)	12(88%)	1.1(0.14-5.93)	0.58
GVHD	3(18%)	14(82%)	1.6(0.29-7.46)	0.38
Days hospitalized for diarrhea, mean(variation)	1(1-22)	10(1-105)		0.16
Days for diarrhea collection toxins, mean	1(1-20)	1(1-18)		0.23
Collection of toxins/onset of treatment, mean	2(1-6)	1(0-20)		0.94
Chemotherapy	2(6%)	30(94%)	0.29(0.03-1.42)	0.11
Cyclophosphamide	**4(31%)**	**9(69%)**	**4.3(0.89-20.85)**	**0.05**
Steroids	2(29%)	5(71%)	3(0.35-19.13)	0.22
Immunosuppression	7(15%)	41(85%)	1.6(0.32-12.24)	0.44
Same room as a positive case for *C. difficile*	0(0)	18(100%)	0	0.05

GVHD: Graft versus host disease; AML: Acute Myeloid Leukemia, ALL; Acute Lymphoid Leukemia, CML: Chronic Myeloid Leukemia; HSCT VRE: Vancomycin-resistant Enterococci; CMV: cytomegalovirus.

14 days of treatment. Coinfection and the presence o GVHD may be the reasons for the non-resolution of symptoms. The literature shows that, until the moment, there is a lack of association of resistance to metronidazole with non-response or relapse[5,6,18,19,22,26]. In Brazil, two studies that evaluated *C. difficile* susceptibility to metronidazole showed that all strains were susceptible to metronidazole and did not identified ribotype 027 in the country[8,9]. In the present study, it was not possible to evaluate the impact of sensitivity of *C. difficile* isolates on therapeutic response.

Quinolone is frequently used as bacterial prophylaxis during periods of neutropenia; it has been recently associated with outbreaks of community infection and severe form of CDAD[4,11]. Therefore, we assessed the association of previous use of quinolone with severe forms of disease. However, bivariate analysis showed that the risk factors for the severe form of CDAD were allogeneic HSCT, and use of glycopeptides and cyclophosphamide, risk factors directly associated with the severity of the patient. Sharing the same room was a protective factor for the development of the severe form of CDAD. Nevertheless, in the multivariate analysis it was not possible to identify any independent risk factor associated with the severe form of CDAD, probably due to the size of the population evaluated.

The mortality in the present study was not greater than previously described[1,10]. Overall mortality was 37.5%, 34% in hematologic and 66% in HSCT patients, 58% of them allogeneic transplanted, and death within

Tabela 3
Multivariate analysis of risk factors associated with the severe form of diarrhea caused by *C. difficile* in hematologic and HSCT patients

Variable	Multivariate analysis	
	OR (95% CI)	*p* value**
Allogeneic HSCT	2.5(0.2-23.9)	0.40
Glycopeptides	0.25(0.03-1.7)	0.16
Cyclophosphamide	4.9(0.5-43)	0.14
Same room as a positive case for *C. difficile*	0(0-1.0)	0.97

GVHD: Graft versus host disease; AML: Acute Myeloid Leukemia, ALL; Acute Lymphoid Leukemia, CML: Chronic Myeloid Leukemia; HSCT VRE: Vancomycin-resistant Enterococci; CMV: cytomegalovirus.

14 days was 19/64 (26%). On the other hand, the mortality among the severe form of CDAD was unusually high, 80% of patients died within 14 days. The severe forms of CDAD were treated with metronidazole in our study what can have been impacted in the high mortality. The IDSA guideline recommends the use of vancomycin orally for severe forms of CDAD, but this presentation is not yet available in Brazil[11].

Age is one of the primary risk factors associated with death in patients with CDAD[20]. The mean age of the patients studied in our cases was 38.9 years old, a young population probably due to the type of patient evaluated, 75% of patients of less than 50 years old and three (5%) patients of 12 years old. Other risk factors described as associated with death in hematologic patients are colonization by VRE [28], GVHD[12] and severe form of diseases[15,16]. We found that the severe form of diseases, ICU stay, allogeneic HSCT, and use of cyclophosphamide and linezolid were risk factors for death within 14 days, and lymphoma was protector.

Table 4
Bivariate analysis of risk factors associated with death within 14 days of diagnosis of diarrhea by *C. difficile* in hematologic and HSCT patients

Variable	**Death in 14 days N = 19**	**Survival N = 45**	**Bivariate Analysis**	
			OR (95% CI)	***p* value**
Age, mean years old	39(19-59)	38(12-65)		0.76
Gender				
Male	13(29%)	31(71%)	1.3(0.43-4.35)	0.41
Underlying disease				
Lymphoma	**0(0)**	**9(100%)**	**0**	**0.04**
AML	9(30%)	21(70%)	1.2(0.41-3.66)	0.44
ALL	3(37%)	5(63%)	1.6(0.29-8)	0.38
CML	0(0)	4(100%)	0	0.26
Medullary aplasia	1(33%)	2(67%)	1.3(0-18.36)	0.62
Multiple myeloma	1(20%)	4(80%)	0.6(0.02-5.52)	0.57
Allogeneic HSCT	**12(39%)**	**19(61%)**	**2.7(0.92-8.67)**	**0.05**
Autologous HSCT	2(14%)	12(86%)	0.3(0.05-1.7)	0.18
Prior use antibiotic				
Quinolone	3(38%)	5(62%)	0.5(0.11-3.42)	0.26
Sulfa+trimethoprim	7(35%)	13(65%)	1.6(0.51-5.15)	0.27
Cefepime	5(28%)	13(72%)	1(0.3-3.37)	0.60
Carbapenem	13(35%)	24(65%)	2.3(0.76-7.15)	0.10
Colistin	6(46%)	7(54%)	2.8(0.8-9.94)	0.09
Glycopeptides	10(27%)	27(73%)	0.9(0.32-2.72)	0.56
Linezolid	**4(57%)**	**3(43%)**	**4.1(0.83-20.8)**	**0.08**
Piperacillin/Tazobactam	3(19%)	13(81)	0.5(0.13-2.13)	0.28
Febrile neutropenia	7(22%)	25(78%)	0.5(0.18-1.74)	0.24
CMV	4(29%)	10(71%)	1(0.25-3.89)	0.58
VRE	6(20%)	24(80%)	0.5(0.15-1.53)	0.16
Other parasites	0(0)	5(100%)	0	0.18
Severe form	**7(79%)**	**2(22%)**	**13.2(2.6-103.59)**	**0.001**
Immunosuppressor	16(33%)	32(67%)	2.9(0.8-14.22)	0.08
Chemotherapy	7(22%)	25(78%)	0.5(0.18-1.74)	0.24
Steroids	3(43%)	4(57%)	2.1(0.36-11.39)	0.29
Cyclophosphamide	6(46%)	7(54%)	3(0.83-11.37)	0.09
ICU stay	**11(52%)**	**10(48%)**	**5.3(1.69-17.63)**	**0.003**

AML: Acute Myeloid Leukemia; ALL: Acute Lymphoid Leukemia; CML: Chronic Myeloid Leukemia; HSCT: hematopoietic stem cells transplant; VRE: Vancomycin-resistant enterococci; CMV: cytomegalovirus; ICU: Intensive Care Unit.

Table 5
Mutivariate analysis of risk factors associated with death within 14 days of diagnosis of diarrhea by *C. difficile* in hematologic and HSCT patients

Variable	Multivariate Analysis	
	OR (95% CI)	*p* value
Lymphoma	0 (0.0-1.0)	0.96
Allogeneic HSCT	2.14 (0.65-7.21)	0.22
Linezolid	**5.02(1-25.0)**	**0.050**
Severe form	**8.90 (1.92-41.0)**	**0.015**
Cyclophosphamide	2.513(0.61-10.0)	0.19

No patient with lymphoma died. In the multivariate analysis, however, the variables that remained as independent risk factors for death were severe form of the disease, and the use of linezolid. The risk factors identified in the present study are merely a marker of severity. VRE has been previously described as risk factor for CDAD; however, it was not identified as risk factor in our study. On the other hand, linezolid that has been used in our hospital to treat patients colonized by VRE, with persistent febrile neutropenia, or in sepsis and septic shock was a risk factor, unlike two previous studies that showed a potential benefit of linezolid in CDAD, an "in vitro" study that showed action of linezolid against *C. difficile* using a gut model and a epidemiologic study that demonstrated that linezolid protected patients with ventilator-associated pneumonia to develop CDAD[7,24].

CONCLUSIONS

The incidence of CDAD increased significantly over the study period; however, the severe form of diseases remained stable. The independent risk factors associated with death in hematologic and HSCT patients in 14-days of onset of CDAD were severe form of the disease, and the use of linezolid.

RESUMO

Diarreia por *Clostridium difficile* em pacientes hematológicos e transplantados de células tronco hematopoiéticas: fatores de risco da forma grave e morte

Descrevemos a taxa de incidência de diarreia associada a *Clostridium difficile* (CDAD) em pacientes hematológicos e submetidos a transplante de células-tronco hematopoiéticas (TCTH) internados no HC-FMUSP no período de janeiro de 2007 a junho de 2011 usando dois denominadores 1.000 paciente e 1.000 dias de neutropenia e os fatores de risco associados à forma grave da doença e morte. O método de ELISA (Ridascreen-Biopharm, Germany) de detecção de toxinas A/B foi utilizado para o diagnóstico de *C. difficile*. Análise multivariada usando regressão logística múltipla foi conduzida para avaliar os potenciais fatores de risco associados com forma grave de CDAD e morte em até 14 dias do diagnóstico. Sessenta e seis episódios foram identificados em 64 pacientes entre 439 pacientes que apresentaram diarreia durante o período do estudo. A taxa de incidência de CDAD variou de 0,78 a 5,45 por 1.000 dias de neutropenia e de 0,65 para 5,45 por 1.000 pacientes-dias. A doença de base mais comum foi leucemia mielóide aguda 30/64(44%), 32/64 (46%) pacientes estavam neutropênicos, 31/64 (45%) foram submetidos à TCTH alogênico, 61/64 (88%) usaram antibióticos previamente e 9/64 (13%) apresentaram forma grave da doença. A maioria dos pacientes (89%) utilizou metronidazol oral no tratamento da CDAD e 19/64 (26%) evoluiram para óbito. Os fatores de risco independentes associados à morte foram forma grave da doença e uso de linezolida.

ACKNOWLEDGMENTS SECTION

There is no conflict of interest. This study had no financial funding.

REFERENCES

1. Alonso CD, Treadway SB, Hanna DB, Huff CA, Neofytos D, Carroll KC, *et al*. Epidemiology and outcomes of *Clostridium difficile* infections in hematopoietic stem cell transplant recipients. Clin Infect Dis. 2012;54:1053-63.

2. Altclas J, Requejo A, Jaimovich G, Milovic V, Feldman L. *Clostridium difficile* infection in patients with neutropenia. Clin Infect Dis. 2001;5:723.

3. Arango JI, Restrepo A, Schneider DL, Callander NS, Ochoa-Bayona JL, Restrepo MI, *et al*. Incidence of *Clostridium difficile* associated diarrhea before and after autologous peripheral blood stem cell transplantation for lymphoma and multiple myeloma. Bone Marrow Transplant**.** 2006**;**37:517-21.

4. Avery R, Pohlman B, Adal K, Bolwell B, Goldman M, Kalaycio M, *et al*. High prevalence of diarrhea but infrequency of documented *Clostridium difficile* in autologous peripheral blood progenitor cell transplant recipients. Bone Marrow Transplant. 2000;25:67-9.

5. Arvand M, Hauri AM, Zaiss NH, Witte W, Bettge-Weller G. *Clostridium difficile* ribotypes 001, 017 and 027 are associated with lethal *C. difficile* infection in Hesse, Germany. Euro Surveill. 2009;14(45):pii=19403.

6. Bacci S, Molback K, Kjeldsen MK, Olsen KEP. Binary toxin and death after *Clostridium difficile* infection. Emerging Infect Dis. 2011;17:976-82.

7. Baines SD, Noel AR, Huscroft GS, Todhunter SL, O'Connor R, Hobbs JK, *et al*. Evaluation of linezolid for the treatment of *Clostridium difficile* infection caused by epidemic strains using an *in vitro* human gut model. J Antimicrob Chemother. 2011;7:1537-46.

8. Balassiano IT, Miranda KR, Boente RF, Pauer H, Oliveira IC, Santos-Filho J, *et al*. Characterization of *Clostridium difficile* strains isolated from immunosuppressed inpatients in a hospital in Rio de Janeiro, Brazil. Anaerobe. 2009;15:61-4.

9. Balassiano IT, dos Santos-Filho J, Vital-Brazil JM, Nouér SA, Souza CR, Brazier JS, *et al*. Detection of cross-infection associated to a Brazilian PCR-ribotype of *Clostridium difficile* in a university hospital in Rio de Janeiro, Brazil. Antonie Van Leeuwenhoek. 2011;99:249-55.

10. Bilgrami S, Feingold JM, Dorsky D, Edwards RL, Bona RD, Khan AM, *et al*. Incidence and outcome of *Clostridium difficile* infection following autologous peripheral blood stem cell transplantation. Bone Marrow Transplant. 1999;23:1039-42.

11. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, *et al.* Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infect Control Hosp Epidemiol. 2010;31:431-55.

12. Chakrabarti S, Lees A, Jones SG, Milligan DW. *Clostridium difficile* infection in allogeneic stem cell transplant recipients is associated with severe graft-versus-host disease and non-relapse mortality. Bone Marrow Transplant. 2000;26:871-6.

13. Chopra T, Chandrasekar P, Salimnia H, Heilbrun LK, Smith D, Alangaden GJ. Recent epidemiology of *Clostridium difficile* infection during hematopoietic stem cell transplantation. Clin Transplant. 2011;25:82-7.

14. Dettenkofer M, Wenzler-Röttele S, Babikir R, Bertz H, Ebner W, Meyer E, *et al*. Surveillance of nosocomial sepsis and pneumonia in patients with a bone marrow or peripheral blood stem cell transplant: a multicenter project. Clin Infect Dis. 2005;40:926-31.

15. Dubberke ER, Sadhu J, Gatti R, Reske KA, DiPersio JF, Devine SM, *et al*. Severity of *Clostridium difficile*-associated disease (CDAD) in allogeneic stem cell transplant recipients: evaluation of a CDAD severity grading system. Infect Control Hosp Epidemiol. 2007;28:208-11.

16. Leung S, Metzger BS, Currie BP. Incidence of *Clostridium difficile* infection in patients with acute leukemia and lymphoma after allogeneic hematopoietic stem cell transplantation. Infect Control Hosp Epidemiol. 2010;31:313-5.

17. Mendes ET, Dulley F, Basso M, Batista MV, Coracin F, Guimarães T, *et al*. Healthcare-associated infection in hematopoietic stem cell transplantation patients: risk factors and impact on outcome. Int J Infect Dis. 2012;16:424-8.

18. Musgrave CR, Bookstaver PB, Sutton SS, Miller AD. Use of alternative or adjuvant pharmacologic treatment strategies in the prevention and treatment of *Clostridium difficile* infection. Int J Infect Dis. 2011**;**15:438-48.

19. Oka K, Osaki T, Hanawa T, Kurata S, Okazaki M, Manzoku T, *et al*. Molecular and microbiological characterization of *Clostridium difficile* isolates from single, relapse, and reinfection cases. J Clin Microbiol. 2012;3:915-21.

20. Ram R, Gafter-Gvili A, Raanani P, Yeshurun M, Shpilberg O, Dreyer J, *et al*. Surveillance of infectious complications in hemato-oncological patients. Isr Med Assoc J. 2009;3:133-7.

21. Schalk E, Bohr UR, Konig B, Scheinpflug K, Mohren M. *Clostridium difficile*-associated diarrhoea, a frequent complication in patients with acute myeloid leukaemia. Ann Hematol. 2010;89:9-14.

22. Tenover FC, Tickler IA, Persing DH. Antimicrobial-resistant strains of *Clostridium difficile* from North America. Antimicrob Agents Chemother. 2012;56:2929-32.

23. Tomblyn M, Gordon L, Singhal S, Tallman M, Williams S, Winter J, *et al*. Rarity of toxigenic *Clostridium difficile* infections after hematopoietic stem cell transplantation: implications for symptomatic management of diarrhea. Bone Marrow Transplant. 2002;30:517-9.

24. Valerio M, Pedromingo M, Muñoz P, Alcalá L, Marin M, Peláez T, *et al*. Potential protective role of linezolid against *Clostridium difficile* infection. Int J Antimicrob Agents. 2012;5:414-9.

25. Vehreschild MJ, Meissner AM, Cornely OA, Maschmeyer G, Neumann S, von Lilienfeld Toal M, *et al*. Clinically defined chemotherapy-associated bowel syndrome predicts severe complications and death in cancer patients. Haematologica. 2011;96:1855-60.

26. Venugopal AA, Riederer K, Patel SM, Szpunar S, Jahamy H, Valenti S, *et al*. Lack of association of outcomes with treatment duration and microbiologic susceptibility data in *Clostridium difficile* infections in a non-NAP1/BI/027 setting. Scand J Infect Dis. 2012;44:243-9.

27. Willems L, Porcher R, Lafaurie M, Casin I, Robin M, Xhaard A, *et al*. *Clostridium difficile* infection after allogeneic hematopoietic stem cell transplantation: incidence, risk factors, and outcome. Biol Blood Marrow Transplant. 2012;18:1295-301.

28. Zirakzadeh A, Gastineau DA, Mandrekar JN, Burke JP, Johnston PB, Patel R. Vancomycin-resistant enterococcal colonization appears associated with increased mortality among allogeneic hematopoietic stem cell transplant recipients. Bone Marrow Transplant. 2008;41:385-92.

Received: 18 April 2013
Accepted: 7 January 2014

LIBRARY OF THE
SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

Website: www.imt.usp.br/portal
Address: Biblioteca do Instituto de Medicina Tropical de São Paulo da Universidade de São Paulo
Av. Dr. Enéas de Carvalho Aguiar, 470. Prédio 1 – Andar térreo.
05403-000 São Paulo, SP, Brazil.
Telephone: 5511 3061-7003 - **Fax**: 5511 3062-2174

The Library of the São Paulo Institute of Tropical Medicine (IMTSP Library) was created on January 15, 1959 in order to serve all those who are interested in tropical diseases. To reach this objective, we select and acquire by donation and / or exchange appropriate material to be used by researchers and we maintain interchange between Institutions thorough the Journal of the São Paulo Institute of Tropical Medicine, since the Library has no funds to build its own patrimony.

The IMTSP Library has a patrimony consisting of books, theses, annals of congresses, journals, and reference works.

The collection fo journals existing in the Library can be verified through the USP – Bibliographic Database – OPAC – DEDALUS http://dedalus.usp.br:4500/ALEPH/eng/USP/USP/DEDALUS/start of the USP network.

Rev. Inst. Med. Trop. Sao Paulo
56(4):333-340, July-August, 2014
doi: 10.1590/S0036-46652014000400011

In vitro SCREENING ANTIBACTERIAL ACTIVITY OF *Bidens pilosa* LINNÉ AND *Annona crassiflora* MART. AGAINST OXACILLIN RESISTANT *Staphylococcus aureus* (ORSA) FROM THE AERIAL ENVIRONMENT AT THE DENTAL CLINIC

Jeferson Junior da SILVA(1), Cláudio Daniel CERDEIRA(1), Juliana Moscardini CHAVASCO(1), Ana Beatriz Pugina CINTRA(1), Carla Brigagão Pacheco da SILVA(1), Andreia Natan de MENDONÇA(1), Tati ISHIKAWA(1), Marcelo Fabiano Gomes BORIOLLO(2) & Jorge Kleber CHAVASCO(1)

SUMMARY

Currently multiresistant *Staphylococcus aureus* is one common cause of infections with high rates of morbidity and mortality worldwide, which directs scientific endeavors in search for novel antimicrobials. In this study, nine extracts from *Bidens pilosa* (root, stem, flower and leaves) and *Annona crassiflora* (rind fruit, stem, leaves, seed and pulp) were obtained with ethanol: water (7:3, v/v) and their *in vitro* antibacterial activity evaluated through both the agar diffusion and broth microdilution methods against 60 Oxacillin Resistant *S. aureus* (ORSA) strains and against *S. aureus* ATCC 6538. The extracts from *B. pilosa* and *A. crassiflora* inhibited the growth of the ORSA isolates in both methods. Leaves of *B. pilosa* presented mean of the inhibition zone diameters significantly higher than chlorexidine 0.12% against ORSA, and the extracts were more active against *S. aureus* ATCC ($p < 0.05$). Parallel, toxicity testing by using MTT method and phytochemical screening were assessed, and three extracts (*B. pilosa,* root and leaf, and *A. crassiflora,* seed) did not evidence toxicity. On the other hand, the cytotoxic concentrations (CC_{50} and CC_{90}) for other extracts ranged from 2.06 to 10.77 mg/mL. The presence of variable alkaloids, flavonoids, tannins and saponins was observed, even though there was a total absence of anthraquinones. Thus, the extracts from the leaves of *B. pilosa* revealed good anti-ORSA activity and did not exhibit toxicity.

KEYWORDS: Plant extracts; Products with antimicrobial action; *Staphylococcus aureus*; ORSA; Toxicity tests; Microbial sensitivity tests.

INTRODUCTION

Staphylococcus aureus is found in the human microbiota and may become pathogenic under certain conditions. It is a human pathogen distinguished by its ability to cause infection in virtually every tissue and organ system of the body, leading to serious illnesses[24,41]. The use of methicillin and other synthetic penicillins, such as oxacillin started in 1959, and represented a significant step in antistaphylococcal therapy worldwide. However, the identification of strains of Methicillin-resistant *S. aureus* (MRSA) was recorded in 1962 (hereafter called Oxacillin-resistant *S. aureus* [ORSA]), thus spreading fast around the world in subsequent years. From the 1980s, the occurrence of ORSA infections increased considerably in several countries, and strains of ORSA have assumed increasing importance internationally. In addition, at present, besides having established itself as an important hospital pathogen, it is now beginning to prevail in the wider community as well, becoming one common cause of infections with high rates of morbidity and mortality throughout the world[3,6,60].

Resistance can be intrinsic to a bacterium, or acquired through mutations or by the incorporation of new genes. The increasing prevalence of nosocomial infections caused by ORSA/MRSA during the last two decades throughout the world has been associated with the widespread occurrence of specific strains of ORSA/MRSA international[1]. Based on the genotyping techniques of electrophoresis in pulsed-field gel (EPFG), SCC*mec* typing and multilocus typing sequence, several pandemic MRSA clones were identified, including the Iberian (ST247-SCC*mec*IA), Brazilian (ST239-III), New York/Japan (ST5-II), Pediatric (ST5-IV), EMRSA-16 (ST36-II), EMRSA-15 (ST22-IV), and Berlin (ST45-IV) clones[1,19,35]. Studies from Brazil showed the predominance of BEC, a multi-resistant clone first described in Brazil, in 1992, which accounted for 70-80% of total isolates of ORSA/MRSA in Brazilian hospitals[20,58].

Dissemination of ORSA strains is one of the major health issues faced by a great number of countries and the phenomenon of the multiresistance is becoming alarming. Until today, it has been observed there are an increasing number of studies related to the epidemiology of this microorganism in centers or in intensive care units which include immunocompromised patients who have undergone surgical intervention, who have been transplanted or who are suffering from severe burns highlighting the

(1) Microbiology and Immunology Department, Biomedical Science Institute, Federal University of Alfenas, Alfenas, MG, Brazil.
(2) Research Laboratory of Genetics and Molecular Biology, University José do Rosário Vellano, Alfenas, MG, Brazil.
Correspondence to: Jeferson Junior da Silva, Universidade Federal de Alfenas, R. Gabriel Monteiro da Silva 700, 37130-000 Alfenas, MG, Brazil. Tel. +55.35.99681667. E-mail: jefersonbiomed@hotmail.com

need for the development of drugs that are effective in treating these infections[6,33,39]. To overcome the problem of the multiresistance of the microorganisms, scientific efforts have been made. Nevertheless, for implementation in medical particle, little has been achieved.

The study of antimicrobial agents on plants has great importance and is crucial in several sectors of pharmaceutical sciences and cosmetics. Furthermore, plants are strong candidates in studies of bio-prospecting and the use of data from such studies as a first step to the discovery of the pharmacological activity for new antimicrobial agents is extremely important, especially in a country like Brazil that offers a huge biodiversity to be analyzed, but only a few of these have been scientifically investigated[5,31,32,37].

Bidens pilosa Linné (family Asteraceae), commonly called "picão-preto" is a medicinal plant native to South America that nowadays is distributed all over the world, mainly in tropical and subtropical regions[16]. It is a small erect annual herb growing up to 1.5 m high. Considered as a weed in many places, it has bright green leaves with serrated prickly edges and produces small yellow flowers[42]. This plant is widely used either in traditional medicine or folk medicine by indigenous people to treat a variety of illnesses including pain, fever, angina, diabetes, edema (water retention), infections, inflammation, anti-flu and in gastroenteritis treatment[10,29]. Extensive researches over the past decades have shown that *B. pilosa* has activities: antiviral[11], antifungal[18,40] and antibacterial[23,28,48]. Some classes of compounds, such as flavonoids and polyacetylenes, were isolated from the *B. pilosa* and are generally associated in the pharmacological activities of this plant[16,22,44,51].

Annona crassiflora Mart., 1841, popularly known as "Araticum" or "marolo" or "field araticum", is a member of the Annonaceae family found in the Brazilian Cerrado, with socio-economic and medicinal importance. The traditional use of this plant includes the treatment of wounds, venereal diseases, snakebites, louses and, furthermore, it can act as an antimicrobial, antidiarrheal and antirheumatic[61]. SANTOS *et al.*[50] isolated and determined the structure of a new acetogeninase, a prototype substance from the ethanol extract seeds, which was named crassiflorina or araticulin. There have been many studies done to elucidate the biological actions of acetogeninases: RUPPRECHET *et al.*[49] and ZAFRA-POLO *et al.*[64] relate them to cytotoxic, antimitotic, antimicrobial and antimalarial; MORRE *et al.*[38] and OBERLIES *et al.*[43] reported antitumor activity in *in vitro* and *in vivo* with different strains of cancer cells, and some acetogeninases were effective in the inactivation of multiresistant cell strains to certain drugs without causing toxicity in other human cells.

Because of an increase in the microbial resistance to antimicrobials available, as well as the limited therapeutic alternatives, there is a need to search for novel substances with antimicrobial activity. In this context, this study had as its aim to evaluate the *in vitro* antibacterial activity of hydroethanolic extracts from medicinal plants found in the Cerrado of Minas Gerais State (*Bidens pilosa* Linné and *Annona crassiflora* Mart.*)* against ORSA strains, isolates from the environmental sources (dental clinic) previously characterized and genotyped, besides the evaluation of the resistant profile of the strains to antibiotics utilized in medical particles. In addition, the toxicity and phytochemistry profile of the extracts were assessed.

MATERIALS AND METHODS

Obtaining the ORSA samples: A total of 60 ORSA samples (n = 60) from an aerial environment have been isolated at the Integrated Dental Clinic, characterized and maintained at the Research Laboratory of Genetics and Molecular Biology, both at the University José do Rosário Vellano in Alfenas-MG. These samples obtained were collected using Petri plates (90 mm ± 15 mm) containing 20 mL of Mannitol salt agar (Merck), exposed for two hours during two shifts, from two hours after the beginning of each shift, and strategically placed in Dental Clinics. The identity of *Staphylococcus aureus* species was obtained using the classical microbiology methods. Additionally, a confirmatory test was carried out to evaluate the resistance to oxacillin, suggestive of the *mec*A gene presence by screening the Mueller-Hinton Agar supplemented culture with 684 mM NaCl (4% w/v) and oxacillin (6 μg mL^{-1}) similar to the method described previously by NCCLS, current Clinical and Laboratory Standards Institute (CLSI) in the document M7-A6[12], in which the standard strain of *S. aureus* ATCC 25923 oxacillin-susceptible was also used to ensure reproducibility and accuracy. These isolates were kept in the permanent culture -70 ºC. At the time of analysis when utilized against the plant extracts, it was reactivated.

Collecting of the plants: Chart 1 shows the coordinates where the samples of *B. pilosa* and *A. crassiflora* were collected. After collecting the plants used in this work, they were identified, registered and filed at the Herbarium of the UALF in the Federal University of Alfenas by Prof. Dr. Marcelo Polo.

Preparation of the extracts: The plants' parts from *B. pilosa* (root, stem and flower) and *A. crassiflora* (rind fruit, stem, seed, pulp and leaf) were cleaned and cut manually, then were mixed at a concentration of 20% weight/volume of ethyl alcohol 70%. Next, they were macerated for seven days and kept in the dark with daily shaking. After maceration, they received filtration through "nylon" and again through a paper filter. Subsequently, the extracts were subjected to evaporation and concentration under the negative pressure of 500 mmHg and 60 ºC. They were then distributed in five mL bottles, frozen and lyophilized, whereby yielding dried crude hidroalcoholic extracts. The powders were stored at 20 °C and finally, before testing, were solubilized in water.

Chart 1
Data of collection places and identification of *Bidens pilosa* and *Annona crassiflora* analyzed

Family	Specie	Collection place	Coordinates	Collection date	Exsicata number
Asteraceae	*Bidens pilosa* Linné	Pouso Alegre city, MG	22° 27' 75"S 18° 45' 90"W	January, 2011	1745
Annonaceae	*Annona crassiflora* Mart. (1841)	Alterosa city, MG	21° 850' 18"S 46° 513' 06"W	March, 2011	1401

Screening of antibacterial activity: *In vitro* microbiological tests were performed at the Laboratory of Microbiology and Immunology of the Federal University of Alfenas. Firstly, the isolates were inoculated in specific Vogel Johnson agar (with potassium tellurite), submitted to a confirmation of the *S. aureus* specie by tests of biochemistry screening (tests of catalase, coagulase and DNase) and method of Gram. Susceptibility testing to antibiotics was conducted by using a method described previously by BAUER and KIRBY[4]. The antibacterial activity of the extracts was evaluated through agar diffusion following the document CLSI[12] with modifications according to SILVA *et al.*[56] (Fig. 1). Suspensions of overnight-cultured ORSA isolates were prepared in saline solution (0.9% NaCl) with a turbidity corresponding to a 0.5 tube of Mac-Farland scale and inoculated on the surface of culture medium Mueller-Hinton Agar with the help of swabs. Forty µL of these extracts (at a concentration of 100 mg/mL) were placed in wells of four mm diameter made in the culture medium. The plates were incubated at 37 °C for 18 hours. After incubation, the reading of growth inhibition zone diameters was performed, by using chlorhexidine solution at 0.12% as a positive control, and distilled water as a negative control. The standard strain of *S. aureus* ATCC 6538 susceptible to the oxacillin was also used with the aim of making a comparison.

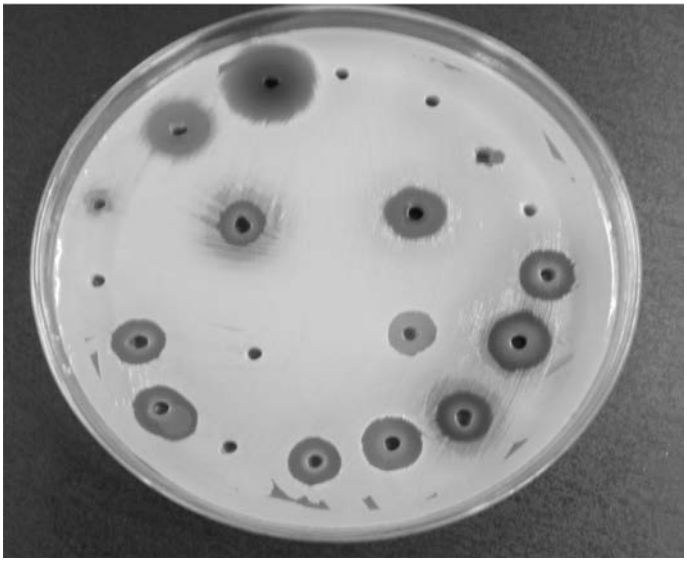


Fig. 1 - Determination of the antimicrobial activity of plant extracts according to the methodology of SILVA *et al.*, 2010.

All extracts were submitted to the minimum inhibitory concentration (MIC) by using broth microdilution methodology as proposed in document M7A6[12]. This methodology was used to evaluate the susceptibility of the 60 ORSA samples. The test was carried out in sterile microdilution plates with 96 flat bottomed wells, composed of eight series identified from A to H, each one with twelve wells. Firstly, 100 µL of Mueller Hinton broth medium was dispensed into the wells on a microplate. Afterwards the extracts were diluted consecutively into the wells of the plate with a concentration ranging from 50 to 0.09 mg/mL. The suspensions of overnight-cultured of ORSA isolates were suspended in saline solution with the turbidity adjusted. They were then dispensed into the wells at a volume of 10 µL per well. The columns 11 and 12 were reserved as controls. The control of the growth promotion was composed of 100 µL broth plus 10 µL of the inoculums, the control of sterility was composed of 100 µL of the culture medium, and the control of extract was composed of 100 µL of the culture medium plus 100 µL of extract. The plates were incubated at 37 ºC for 24 hours. The readings were made visually comparing the growth of the microorganisms in the wells numbered one to ten with the controls as recognized by CLSI[12]. The lowest concentration that produced a significant inhibition of the growth was established as the MIC value.

Cytotoxicity screening - MTT method: The cytotoxicity was assessed by using the MTT method (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenytetrazoliumbromide). Briefly, in accordance with ARAUJO *et al.*[2], in this test, $1x10^4$ cells (derived from *Aedes albopictus* mosquito larvae) were seeded per well in 96-well tissue culture plates containing 0.1 mL of L-15 medium supplemented with 1% of fetal bovine serum and with decreasing dilutions from these extracts (5 to 0.039 mg/mL). For cell control, only the medium was added. After incubation, 10 µL of MTT was added to the wells and incubated for four hours at room temperature, in order to incorporate the MTT for the formation of the formazan crystals. Spectrophotometric analysis was performed by using a reading at a wavelength of 600 nm. The percentage of cytotoxicity was calculated using the formula [(AB) / AX100], where A and B are values of optical densities of treated and controlled cells, respectively. So, the 50% cytotoxic (CC_{50}) and 90% cytotoxic (CC_{90}) concentrations were calculated and defined as the concentration of the extract that reduced the absorbance of treated cells in 50% and 90% respectively, when compared with those of the cell control.

Evaluation of phytochemical profile of the extracts: The qualitative analyses of chemical substances of the extracts analyzed were determined by colorimetric methods and/or precipitator, according to COSTA[13]. These methodologies were selected to determine the presence of alkaloids, anthraquinones, flavonoids, tannins, and saponins.

Statistical Analysis: The agar diffusion tests were realized in triplicate and the results were statistically analyzed using Sisvar Software Version 5.3. With the aim of comparing the means, the ANOVA analysis followed by SCOTT & KNOTT[55] as a post test were performed, and the means were statistically different when $p < 0.05$.

RESULTS

ORSA samples: All the 60 samples analyzed in this experiment were properly confirmed as Oxacillin Resistant *Staphylococcus aureus* (ORSA) through a method recognized for definition of resistance to oxicillin. Furthermore, the susceptibility profile of the samples to the different groups of antibiotics, commonly used in the clinic, was investigated by an antibiogram. In Figure 2 it can be observed its heterogeneous multidrug resistance to several classes of the antibiotics analyzed. Resistance to vancomycin, Azithromycin, and amoxicillin with clavulanic acid were presented for 40, 53.3, and 40% of 60 samples respectively. It was observed there was a significant intermediate resistance of the isolated to ciprofloxacin and clindamycin.

Screening of antibacterial activity of plant extracts through agar diffusion technique: In the reading of the growth inhibition zones (IZ) diameters (Table 1), the extracts from the stem and root of *B. pilosa* were inactive, and the extracts from the flower and leaf of *B.*

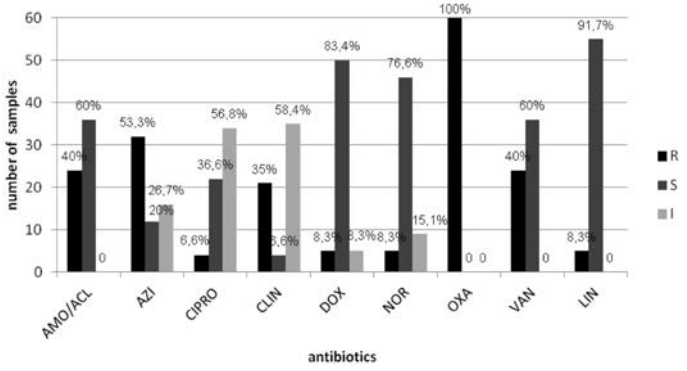


Fig. 2 - Profile of the sensitivity from the 60 samples isolated from aerial environment at the dental care clinic to antibiotics commonly utilized for treating *S. aureus* infections. AMO/ACL: amoxicillin with clavulanic acid (30); AZI: Azithromycin (15); CIPRO: Ciprofloxacin (05); CLIN: Clindamycin (02); DOX: doxycycline (30); NOR: Norfloxacin (10), OXA: Oxacillin (10); VAN: Vancomycin (30); LIN: Linezolid (30). R: Resistant ; S: Sensitive; I: Intermediate.

pilosa were effective against the 60 samples analyzed with an interval of IZ diameters ranging from eight to 23 millimeters (mm). For *A. crassiflora*, extracts from the stems, pulp, rind fruit and leaf were actives against ORSA with IZ diameters that ranged from five to 15 mm. It was verified for the extracts from the *B. pilosa* and *A. crassiflora* there were better activities through the agar diffusion method as against *S. aureus* ATCC than on ORSA samples. The extract from the leaf of *B. pilosa* had the highest mean of the inhibition zone (IZ) diameters (17 mm) against ORSA samples used in the experiment (n = 60), followed by extract from the flower of *B. pilosa* (14 mm) (statistically significant with $p < 0.05$). Moreover, extract from the leaf of *B. pilosa* was the most active against *S. aureus* ATCC, and on ORSA as well, although the mean of the IZ diameters values for this extract on ATCC (26 mm) was statistically different from that presented against ORSA (17 mm) ($p < 0.05$). Against ORSA, only extracts from the leaf of *B. pilosa* demonstrated inhibitory activity significantly better than those presented for chlorhexidine 0.12% (positive control), which had a mean of the IZ diameters values of 15 mm. Otherwise, against standard strain, the extracts from the leaf and flower of *B. pilosa* (26 and 22 mm) and rind fruit of *A. crassiflora* (18 mm) were more active than positive control (16 mm) ($p < 0.05$). In general, when comparing the activities of all extracts against *S. aureus* ATCC and against the isolates of ORSA, the overall mean of IZ diameters against ATCC was higher than against ORSA, so the extracts were more active against the standard strain than on the isolates ($p < 0.05$).

Determination of Minimum Inhibitory Concentration (MIC): The obtained results of MIC values are shown in Table 1, where the extracts from the stem of *B. pilosa* high MIC values (50 mg/mL) against ORSA can be observed. On the other hand, for the extracts from the leaf and flower, the inhibition of the ORSA isolates with MIC values of 6.25 and 12.5 respectively was verified. For the extracts from the leaf and flower of *B. pilosa*, the MIC values were 25 and 1.56 respectively against *S. aureus* ATCC. For *A. crassiflora*, all extracts presented MIC values close to the maximum concentration used (50 mg/mL). The extracts from

Table 1
Interval and mean of the growth inhibition zone (IZ) diameters, MIC values, assessment of toxicity, and qualitative results of the phytochemical analysis of hidroethanolics extracts from the *Bidens pilosa* Linné and *Annona crassiflora* Mart

Species	Extract	Interval and mean[1,2,3] of the IZ diameters (mm)		MIC (mg/mL)		Assessment of toxicity		Selectivity Index (SI)		Secondary metabolites				
		ORSA[4]	*S. aureus* ATCC 6538	ORSA	*S. aureus* ATCC 6538	CC_{50} (mg/mL)	CC_{90} (mg/mL)	ORSA	*S. aureus* ATCC 6538	Al	An	Fl	Ta	Sa
Bidens pilosa	Root	0 (0)[a]	0 (0)[a]	N	N	NT	NT	NA	NA	-	-	-	+	+
	Stem	0 (0)[a]	0 (0)[a]	50	25	3.91	7.27	0.078	0.156	+	-	-	+	+
	Flower	8 – 20 (14)[e]	21 -23 (22)[i]	12.5	1.56	2.82	5.41	0.226	1.808	+	-	+	+	+
	Leaf	8 -23 (17)[g]	25 -28 (26)[j]	6.25	25	NT	NT	NA	NA	+	-	+	+	+
Anonna crassiflora	Rind fruit	6 – 15 (12)[d]	16 -20 (18)[h]	50	6. 25	2.06	9.38	0.041	0.330	+	-	-	+	-
	Stem	5 – 15 (9)[b]	10 – 14 (12)[d]	25	1.56	5.86	10.77	0.234	3.756	-	-	-	+	-
	Seed	0 (0)[a]	0 (0)[a]	50	N	NT	NT	NA	NA	-	-	-	-	-
	Pulp	6 – 15 (9)[b]	14 -18 (16)[f]	25	12.5	2.68	5.98	0.107	0.214	+	-	-	-	+
	Leaf	6 – 14 (10)[c]	10 -14 (12)[d]	25	25	3.86	8.03	0.154	0.154	+	-	+	-	-
Controls	Chlorexidine 0.12	10 – 26 (15)[f]	15 -18 (16)[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Distilled water	0 (0)[a]	0 (0)[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[1]Between parenthesis; [2]means followed by same letter show no statistic difference each other according to Scott & Knott test at 5% of significance (α=0.05); [3]All tests by agar diffusion with the extracts at a concentration of 100 mg/mL; [4]n=60 samples; MIC: Minimum inhibitory concentration; CC_{50}: 50% cytotoxic concentration; CC_{90}: 90% cytotoxic concentration; SI= CC_{50} / MIC; N= absence of inhibition at the maximal concentration used; NT= Non-toxic at the concentrations used; NA = Not applicable to this analysis; Al: Alkaloids; An: Anthraquinones; Fl: Flavonoids; Ta: Tannins; Sa: Saponins; + : positive reaction; - : negative reaction.

the stem of *A. crassiflora* and flower of *B. pilosa* presented the lowest MIC values against *S. aureus* ATCC (1.56 mg/mL).

Phytochemical evaluation of plant extracts: The phytochemical profiles of the extracts (Table 1) revealed that all extracts from the *B. pilosa* were positive for tannins and saponins, and almost all were positive for alkaloids (three in four extracts). For *A. crassiflora*, the variable presence of alkaloids, flavonoids, tannins and saponins was evidenced. All extracts (from the *B. pilosa* and *A. crassiflora*) were negative for anthraquinones.

Evaluation of cytotoxic activity of extracts on cell culture: Parallel analysis was held for evaluation of cytotoxicity of the extracts. In the examination carried out on cell culture, in accordance with Table 1, the extracts from the leaf and root of *B. pilosa* showed no toxicity in the concentrations tested on cell culture. On the other hand, the 50% cytotoxic concentration (CC_{50}) ranged from 1.30 mg to 9.56 mg/mL for all others extracts. The 90% cytotoxic concentration (CC_{90}) ranged from 2.55 to 19.00 mg/mL. For *A. crassiflora*, the CC_{50} and CC_{90} ranged from 2.06 to 5.86 and from 5.93 to 10.77 respectively. For the extract from the seed, it was verified there was no toxicity in the concentration tested. According to PROTOPOPOVA *et al.*[47] the selectivity index (SI) is calculated as the ratio between the CC_{50} and the MIC. The values found were lower in the analysis against ORSA. For the IS values against the standard strain, the most satisfactory was 3.756 for the extract from the stem of *A. crasssiflora*.

DISCUSSION

The ORSA samples analyzed in this experiment are from the environment at the dental care clinic (air), where the transmission of pathogens is intense due to the instrumentation used, such as high speed instruments. These factors make the dental clinical environment as prone to cross-contamination and/or cross-transmission for *S. aureus*, as in a hospital environment[46,62]. It can lead to contamination and infection of patients. The profile of the sensitivity (Fig. 1) for the ORSA samples analyzed strengthens previous studies, which indicate a massive dissemination of the multiresistant *S. aureus*, in which most strains of ORSA exhibit high level resistance to commercially available antimicrobial agents. Furthermore, *S. aureus* remains a major cause of nosocomial disease[15] and recent reports have indicated that the epidemiology of ORSA may be undergoing a change through the emergence of community-acquired infections[27]. Until recently, vancomycin has been used to treat ORSA infections, and the failure in the treatment with vancomycin and other antimicrobial agents has been reported, raising concerns that treatment of ORSA infections has become even more problematic and has led to higher rates of morbidity and mortality throughout the world[1,15,59]. Therefore, there is a need to search for new therapies. Previous studies have related the importance of plants as a source of compounds with antimicrobial activity or provide a viable therapeutic strategy as phytomedicines[36,48,61,63].

In this work, the anti-ORSA activity of the extracts from the different parts of *B. pilosa* and *A. crassiflora* were evaluated through the agar diffusion method and broth microdilution. In the evaluation through the agar diffusion method, it has shown a ranging interval of the IZ diameters for each extract against the 60 ORSA isolates (Table 1). This may be at least partly explained as a result of morphophysiological differences, derived from the specific genetics of the samples[1,3,19,20]. Despite the reproducibility of the results through the diffusion method in the Mueller-Hinton Agar[12], nowadays it is known that in the evaluation of the antimicrobial activity of plant extracts, which are complex blends, through agar diffusion, several factors can influence the IZ diameters values and the reproducibility remains controversial[45]. In addition, because of the extracts, depletion or destruction of the compounds of the culture medium can occur, inhibiting the microorganisms; therefore, perhaps the antimicrobial action did not result from a direct interaction between the extracts and the microorganisms.

Regarding the diffusion agar method, there are a large number of scientific studies highlighting the antibacterial properties of plants[14]. MATTANA *et al.*[34] observed resistant *S. aureus* to methicillin highly susceptible to ethyl acetate and ethanol extracts of *Acacia aroma*, through the agar diffusion method. It also observed that ethanol extracts showed greater antimicrobial activity compared to extracts which had only water as a solvent. The extracts used in this study have undergone maceration through ethyl alcohol 70% and an excellent antibacterial performance by agar diffusion was observed, thus confirming the assertion described by MATTANA *et al.*[34], probably due to the alcohol that extracts greater numbers of polar and nonpolar substances. Additionally, the difference of solubility for each extract can be an influence during the diffusion in agar, where extracts with the greatest concentrations of more polar substances show better diffusion than those richer in non-polar substances. In our study, the extracts were more active against the standard strain of *S. aureus* than against the ORSA isolates, and the extract from the leaf of *B. pilosa* was the most active by agar diffusion ($p < 0.05$).

In the determination of MIC (Table 1) by using broth microdilution, it was found that the extract from the stem of *B. pilosa* showed MIC value of 50 mg/mL and, in this sense, the antibacterial activity of this extract should be taken into account with the cytotoxic activity, aiming either studies for bioassay-guided fractionation of novel compounds or likewise to studies of the quality, effectiveness and safety of herbal medicinal preparations[63].

The extracts from the leaf and flower of *B. pilosa* showed inhibition results at lower concentrations. FABRI *et al*.[21] reported the antimicrobial activity of members of the family Asteraceae, to which *B. pilosa* belongs. Despite the extracts from the stem of *B. pilosa* and the seed of *A. crassiflora* at a concentration of 100 mg/mL no evidenced antibacterial activity through the agar diffusion method was observed; MIC values of 50 mg/mL were verified, in both cases. In accordance with HADECEK & GREGER[25], it is acknowledged that the broth microdilution presents higher sensitivity than the agar diffusion method, by allowing direct contact between the compound, the medium and the microorganisms, so that the inactivity of the extracts through the agar diffusion parallel the activity by broth microdilution, probably occurring due to this.

MATTANA *et al.*[34] related the activity of organic extracts of *Acacia aroma* against ORSA with MICs that ranged from 2.5 to 10 mg/mL. The MIC values for *Hyptis martiusii* on MRSA and MSSA (methicillin sensitive *S. aureus*) developed by COUTINHO *et al.*[14] ranged from 128 to ≥ 1024 mg/mL, values greater than that found in our study. Analyzing the results of MIC in Table 1, in general, the extracts evaluated showed higher MIC values against ORSA when compared with the values found against the standard strain sensitive to oxacillin *S. aureus.* Only the extract

from the leaf of *B. pilosa* had low MIC values on ORSA (6.25 mg/mL) when comparing those presented against a standard strain (25 mg/mL).

As for the phytochemical profile, it was observed that, among extracts, there was a total absence of anthraquinones and the variable presence of alkaloids, flavonoids, tannins, and saponins. It confirms previous studies in which such compounds are commonly isolated from these plants[7,8,30].

A. crassiflora extracts showed the presence of alkaloids in rind fruit, pulp and leaf extracts. In 1982, LEBOEUF *et al.*[30] published a review about the Annonaceae phytochemical family, where the predominance of aporphine and oxoaporphine alkaloids among secondary metabolites isolated from species belonging to the family was reported. It was observed that the extracts from the stem and fruit bark presented tannins. Saponins were observed only in the pulp extract and flavonoids only in leaf extract. Besides alkaloids, constituents like polyphenols, essential oils, terpenes and aromatic substances are also found in family representatives[30]. Regarding the phenolic compounds in the Annonaceae family the most frequent ones are flavonoids[57].

Analysis of *B. pilosa* revealed that the majority of the extracts showed positive for alkaloids, flavonoids, tannins and saponins. This result confirms previous studies which state that the main compounds already isolated from the plant are poliacetylenic and flavonoid substances[8]. BORGES[7] also determined the presence of phenolic compounds such as tannins and flavonoids, and also showed positive results for saponins and alkaloids, which are results observed in this study. In addition, researches have reported that alkaloids, flavonoids, tannins and saponins are implicated in the antimicrobial action[9,17,53,54]. So, the activity found in our study for the plants evaluated could be associated with the presence of these compounds[26].

Concerning the inactivity or antimicrobial activity of the extracts and the presence of metabolites mentioned, the complexity of plant extracts should not be overlooked, being the final response (antibacterial activity) result of synergistic, antagonistic and other interactive effects among their bioactive components[61]. Previous studies have reported the antimicrobial activity for the metabolite isolated, and in the case of the study with crude extracts, interactions between the compounds must be considered possible. Notwithstanding this, the antimicrobial activity presented could be mediated at least partly by the compounds from the extracts, which could affect the morphophysiology of the microorganism.

With the results of the assessment of toxicity (Table 1), it can be observed that extract from the leaf of *B. pilosa* showed a desirable characteristic among the extracts because it showed no toxic at the concentration utilized, besides presenting a lower MIC (6.25 mg/mL) against ORSA, with a good chance of application in further studies by bio-prospection. The extract from the stem of *A. crassiflora* proved to be the more toxic. As to the selectivity, extract from the stem of *A. crassiflora* showed the better selectivity index (SI) against ORSA (0.234), and on standard strain (3.756).

In summary, the extracts from the flower and leaf of *Bidens pilosa;* and rind fruit, pulp, stem and leaf of *A. crassiflora* inhibited the growth of the ORSA isolates by both methods tested; however, extracts from the root of *B. pilosa* and seed of *A. crassiflora* displayed no antimicrobial activity. As for the toxicity, some extracts did not show toxicity. Therefore, a good extract due to the fact that it showed lower MIC and no toxic effect was the extract from the leaf of *B. pilosa.* Further studies must be realized, in order to identify and isolate compounds responsible for this activity and understand how they exercise this effect on the *Staphylococcus* species.

RESUMO

Triagem *in vitro* da atividade antibacteriana de *Bidens pilosa* Linné e *Annona crassiflora* Mart. contra *Staphylococcus aureus* resistente à oxacilina (ORSA) provenientes do ambiente aéreo na clínica odontológica

Atualmente *Staphylococcus aureus* multirresistente é causa comum de infecções com altas taxas de morbidade e mortalidade mundialmente, o que direciona esforços científicos na busca de novos antimicrobianos. Neste estudo, nove extratos de *Bidens pilosa* (raiz, caule, flor e folhas) e de *Annona crassiflora* (casca do fruto, caule, folha, semente e polpa) foram obtidos com etanol:água (7:3, v/v) e suas atividades antibacteriana *in vitro* avaliadas através de difusão em agar e microdiluição em caldo contra 60 cepas de Oxacillin Resistant *S. aureus* (ORSA) e contra *S. aureus* ATCC 6538. Os extratos de *B. pilosa* e *A. crassiflora* inibiram o crescimento dos isolados ORSA em ambos os métodos. O extrato da folha de *B. pilosa* apresentou média dos diâmetros dos halos de inibição significativamente maior que a clorexidina 0,12%, contra os isolados ORSA, e os extratos foram mais ativos contra *S. aureus* ATCC ($p <$ 0,05). Paralelamente, teste de toxicidade pelo método MTT e triagem fitoquímica foram avaliadas, e três extratos (raiz e folha de *B. pilosa* e semente de *A. crassiflora*) não apresentaram toxicidade. Por outro lado, as concentrações citotóxicas (CC_{50} e CC_{90}) para os outros extratos variaram de 2,06 a 10,77 mg/mL. Observou-se variável presença de alcalóides, flavonóides, taninos e saponinas, apesar de total ausência de antraquinonas. Portanto, os extratos das folhas de *B. pilosa* revelaram boa atividade anti-ORSA e não exibiram toxicidade.

ACKNOWLEDGEMENTS

The authors acknowledge Dr. Marcelo Polo for the identification of the plant material. This research was supported by FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais - APQ-3897-4.03/07).

REFERENCES

1. Aires de Sousa M, de Lencastre H. Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. FEMS Immunol Med Microbiol. 2004;40:101-11.

2. Araújo SAC, Teixeira MFS, Dantas TVM, Miranda AM, Lima FES, Melo VSP, *et al*. Avaliação *in vitro* da atividade citotóxica de drogas antivirais em fibroblastos caprinos. Cienc Anim. 2008;18:25-31.

3. Ayliffe GAJ. The progressive intercontinental spread of methicillin resistant *Staphylococcus aureus*. Clin Infect Dis. 1997;24(Suppl 1):S74-S9.

4. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45:493-6.

5. Barbosa-Filho JM, Nascimento-Júnior FA, Tomaz ACA, Athayde-Filho PF, Silva MS, Cunha EVL, *et al*. Natural products with antileprotic activity. Rev Bras Farmacogn. 2007;17:141-8.

6. Bell JM, Turnidge JD. High prevalence of oxacillin-resistant *Staphylococcus aureus* isolates from hospitalized patients in Asia-Pacific and South Africa: results from SENTRY Antimicrobial Surveillance Program, 1998-1999. Antimicrob Agents Chemother. 2002;46:879-81.

7. Borges CC. Análise farmacognóstica de *Bidens pilosa* (l.) (Asteraceae) [monografia]. Criciúma: Universidade do Extremo Sul Catarinense, Faculdade de Farmácia; 2009.

8. Brandão MGL, Krettli AU, Soares LSR, Nery CGC, Marinuzzi HC. Antimalarial activity of extracts and fractions from *Bidens pilosa* and other *Bidens* species (Asteraceae) correlated with the presence of acetylene and flavonoid compounds. J Ethnopharmacol. 1997;57:131-8.

9. Bruneton J. Pharmacognosy, phytochemistry, medicinal plants. 2nd ed. Paris: Lavoisier Publishing/Intercept; 1999.

10. Chang JS, Chiang LC, Chen CC, Liu LT, Wang KC, Lin CC. Antileukemic activity of *Bidens pilosa* L. var. *minor* (Blume) Sherff and *Houttuynia cordata* Thunb. Am J Chin Med. 2001;29:303-12.

11. Chiang LC, Cheng HY, Liu MC, Chiang W, Lin CC. *In vitro* anti-herpes simplex viruses and anti-adenoviruses activity of twelve traditionally used medicinal plants in Taiwan. Biol Pharm Bull. 2003;26:1600-4.

12. Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6. 6th ed. Wayne: NCCLS; 2003.

13. Costa AF. Isolamento e identificação dos constituintes vegetais. In: Farmacognosia. Lisboa: Fundação Calouste Gulbenkian; 1982. vol. 3. p. 926-62.

14. Coutinho HDM, Costa JGM, Siqueira-Júnior JP, Lima EO. *In vitro* anti-staphylococcal activity of *Hyptis martiusii* Benth against methicillin-resistant *Staphylococcus aureus*-MRSA strains. Rev Bras Farmacogn. 2008;18(Suppl):670-5.

15. Cordeiro JCR, Reis AO, Miranda EA, Sader HS. *In vitro* antimicrobial activity of the aminoglycoside arbekacin tested against oxacillin-resistant *Staphylococcus aureus* isolated in Brazilian hospitals. Braz J Infect Dis. 2001;5:130-5.

16. Cortés-Rojas DF, Chagas-Paula DA, da Costa FB, Souza CRF, Oliveira WP. Bioactive compounds in *Bidens pilosa* L. populations: a key step in the standardization of phytopharmaceutical preparations. Rev Bras Farmacogn. 2013;23:28-35.

17. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999;12: 564-82.

18. Deba F, Xuan TD, Yasuda M, Tawata S. Chemical composition and antioxidant, antibacterial and antifungal activities of the essential oils from *Bidens pilosa* Linn. var. radiata. Food Control. 2008;19:346-52.

19. Deurenberg RH, Vink C, Kalenic S, Friedrich AW, Bruggeman CA, Stobberingh EE. The molecular evolution of methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect. 2007;13:222-35.

20. Dos Santos Soares MJ, da Silva-Carvalho MC, Ferreira-Carvalho BT, Figueiredo AM. Spread of methicillin-resistant *Staphylococcus aureus* belonging to the Brazilian epidemic clone in a general hospital and emergence of heterogenous resistance to glycopeptide antibiotics among these isolates. J Hosp Infect. 2000;44:301-8.

21. Fabri RL, Nogueira MS, Dutra LB, Bouzada MLM, Scio E. Potencial antioxidante e antimicrobiano de espécies da família Asteraceae. Rev Bras Pl Med. 2011;13:183-9.

22. Galati G, O'Brien PJ. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radical Biol Med. 2004;37:287-303.

23. Geissberger P, Séquin U. Constituents of *Bidens pilosa* L.: do the components found so far explain the use of this plant in traditional medicine? Acta Trop. 1991;48:251-61.

24. Gelatti LC, Bonamigo RR, Becker AP, D'Azevedo PA. *Staphylococcus aureus* resistentes a meticilina: disseminação emergente na comunidade. An Bras Dermatol. 2009;84:501-6.

25. Hadecek F, Greger H. Testing of antifungal natural products: methodologies, comparability of results and assay of choice. Phytochem Anal. 2000;11:137-47.

26. Ho KY, Tsai CC, Huang HS, Chen CP, Lin TC, Lin CC. Antimicrobial activity of tannin components from *Vaccinium vitis-idaea* L. J Pharm Pharmacol. 2001;53:187-91.

27. Hou Z, Meng JR, Zhao JR, Hu BQ, Liu J, Yan XJ, *et al*. Inhibition of β-lactamase-mediated oxacillin resistance in *Staphylococcus aureus* by a deoxyribozyme. Acta Pharmacol Sin. 2007;28:1775–82.

28. Khan MR, Kihara M, Omoloso AD. Antimicrobial activity of *Bidens pilosa*, *Bischofia javanica*, *Elmerillia papuana* and *Sigesbekia orientalis*. Fitoterapia. 2001;72:662-5.

29. Kviecinski MR, Felipe KB, Schoenfelder T, de Lemos Wiese LP, Rossi MH, Gonçalvez E, *et al*. Study of the antitumor potential of *Bidens pilosa* (Asteraceae) used in Brazilian folk medicine. J Ethnopharmacol. 2008;117:69-75.

30. Leboeuf M, Cavé A, Bhaumik PK, Mukherjee B, Mukherjee R. The phytochemistry of the Annonaceae. Phytochemistry. 1982; 21:2783-813.

31. Leitão SG, Castro O, Fonseca EN, Julião LS, Tavares ES, Leo RRT, *et al*. Screening of Central and South American plant extracts for antimycobacterial activity by the Alamar Blue test. Rev Bras Farmacogn. 2006;16:6-11.

32. Lima IO, Oliveira RAG, Lima EO, Farias NMP, Souza EL. Atividade antifúngica de óleos essenciais sobre espécies de *Candida.* Rev Bras Farmacogn. 2006;16:197-201.

33. Masunari A, Tavares LC. Aplicação de estudos de QSAR-2D em derivados 5-nitro-2-tiofilidênicos com atividade antimicrobiana frente a *Staphylococcus aureus* multiresistente (MRSA). Rev Bras Cienc Farm. 2006;42:203-14.

34. Mattana CM, Satorres SE, Sosa A, Fusco M, Alcará LE. Antibacterial activity of extracts of *Acacia aroma* against methicillin-resistant and methicillin-sensitive *Staphylococcus.* Braz J Microbiol. 2010;41:581-7.

35. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol. 2003;41:5113-20.

36. Meng JC, Zhu QX, Than RX. New antimicrobial mono and sesquiterpenes from *Soroseris hookeriana* subsp. *erysimoides*. Planta Med. 2000;66:541-4.

37. Michelin DC, Moreschi PE, Lima AC, Nascimento GGF, Paganelli MO, Chaud MV. Avaliação da atividade antimicrobiana de extratos vegetais. Rev Bras Farmacogn. 2005;15:316-20.

38. Morre DJ, de Cabo R, Farley C, Oberlies NH, McLaughlin JL. Mode of action of bullatacin, a potent antitumor acetogenin: inhibition of NADH oxidase activity of HELa and HL-60, but not liver, plasma membranes. Life Sci. 1995;56:343-8.

39. Moreira MR, Cardoso RL, Almeida AB, Gontijo Filho PP. Risk factors and evolution of ventilator-associated pneumonia by *Staphylococcus aureus* sensitive or resistant to oxacillin in patients at the intensive care unit of a Brazilian university hospital. Braz J Infect Dis. 2008;12:499-503.

40. Motsei ML, Lindsey KL, Van Staden J, Jäger AK. Screening of traditionally used South African plants for antifungal activity against *Candida albicans.* J Ethnopharmacol. 2003;86:235-41.

41. Nadarajah J, Lee MJ, Louie L, Jacob L, Simor AE, Louie M, *et al*. Identification of different clonal complexes and diverse amino acid substitutions in penicillin-binding protein 2 (PBP2) associated with borderline oxacillin resistance in Canadian *Staphylococcus aureus* isolates. J Med Microbiol. 2006;55:1675-83.

42. Nguelefack TB, Dimo T, Mbuyo EP, Tan PV, Rakotonirina SV, Kamanyi A. Relaxant effects of the neutral extract of the leaves of *Bidens pilosa* Linn on isolated rat vascular smooth muscle. Phytother Res. 2005;19:207-10.

43. Oberlies NH, Chang CJ, McLaughlin JL. Structure activity relationships of diverse Annonaceous acetogenins against multidrug resistant human mammary adenocarcinoma (MCF-7/Adr) cells. J Med Chem. 1997;40:2102-6.

44. Oliveira FQ, Andrade-Neto V, Krettli AU, Brandão MGL. New evidencies of antimalarial activity of *Bidens pilosa* roots extracts correlated with polyacetylene and flavonoids. J Ethnopharmacol. 2004;93:39-42.

45. Ostrosky EA, Mizumoto MK, Lima MEL, Kaneko TM, Nishikawa SO, Freitas BR. Métodos para avaliação da atividade antimicrobiana e determinação da concentração mínima inibitória (CMI) de plantas medicinais. Rev Bras Farmacogn. 2008;18:301-7.

46. Powell JP, Wenzel RP. Antibiotic options for treating community acquired MRSA. Expert Rev Anti Infect Ther. 2008;6:299-307.

47. Protopopova M, Hanrahan C, Nikonenko B, Samala R, Chen P, Gearhart J, *et al*. Identification of a new antitubercular drug candidate, SQ 109, from a combinatorial library of 1,2-ethylenediamines. J Antimicrob Chemother. 2005;56:968-74.

48. Rojas JJ, Ochoa VJ, Ocampo SA, Muñoz JF. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. BMC Complement Altern Med. 2006;6:2.

49. Rupprechet JK, Hui YH, McLaughlin JL. Annonaceous acetogenins: a review. J Nat Prod. 1990;53:237-78.

50. Santos LP, Boaventura MAD, Sun NJ, Cassady MJ, Oliveira AB. Araticunlin, a *bis*-tetrahydrofuran polyketide from *Annona crassiflora* seeds. Phytochemistry. 1996;42:705-7.

51. Santos DY, Salantino ML. Foliar flavonoids of Annonaceae from Brazil: taxonomic significance. Phytochemistry. 2000;55:567-73.

52. Saúde-Guimarães DA, Faria AR. Substâncias da natureza com atividade anti-*Trypanosoma cruzi*. Rev Bras Farmacogn. 2007;17:455-65.

53. Scalbert A. Antimicrobial properties of tannins. Phytochemistry. 1991;30:3875-83.

54. Schenkel, EP, Gosmann, G, Athayde, ML. Saponinas. In: Simões, CM, Schenkel, EP, Gosmann, G, Mello, JCP, Mentz, LA, Petrovick, PR. Farmacognosia: da planta ao medicamento. 3. ed. Porto Alegre: Ed. UFRGS/Ed. UFSC; 2001. p. 27, p. 597- 619.

55. Scott AJ, Knott M. Cluster analysis method for grouping means in the analysis of variance. Biometrics. 1974;30:507-12.

56. Silva RB, Almeida CR, Chavasco JM, Chavasco JK. Antimycobacterial activity evaluation and MIC determination of liophilizated hydroalcoholic extracts *Bixa orellana* L., Bixaceae. Rev Bras Farmacogn. 2010;20:171-4.

57. Simões CMO, Schenkel EP. Farmacognosia: da planta ao medicamento. Florianópolis: Ed. da UFSC; 2004.

58. Teixeira LA, Resende CA, Ormonde LR, Rosenbaum R, Figueiredo AM, de Lencastre H, *et al* .Geographic spread of epidemic multiresistant *Staphylococcus aureus* clone in Brazil. J Clin Microbiol. 1995;33:2400-4.

59. Tenover FC, Lancaster MV, Hill BC, Steward CD, Stocker SA, Hancock GA, *et al*. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. J Clin Microbiol. 1998;36:1020-7.

60. Velasco D, del Mar Tomas M, Cartelle M, Beceiro A, Perez A, Molina F, *et al*. Evaluation of different methods for detecting methicillin (oxacillin) resistance in *Staphylococcus aureus*. J Antimicrob Chemother. 2005;55:379-82.

61. Vilar JB, Ferreira FL, Ferri PH, Guillo LA, Chen Chen L. Assessment of the mutagenic, antimutagenic and cytotoxic activities of ethanolic extract of araticum (*Annona crassiflora* Mart. 1841) by micronucleus test in mice. Braz J Biol. 2008;68: 141-7.

62. Wallin TR, Hern HG, Frazee BW. Community-associated methicillin resistant *Staphylococcus aureus*. Emerg Med Clin North Am. 2008;26:431-55.

63. World Health Organization. Traditional medicine strategy 2002-2005. Geneva: WHO; 2002. [cited 2013 Mar 10]. Available from: http://whqlibdoc.who.int/hq/2002/who_edm_trm_2002.1.pdf

64. Zafra-Polo MC, Figadère B, Gallardo T, Tormo JR, Cortes D. Natural acetogenins from Annonaceae, synthesis and mechanisms of action. Phytochemistry. 1998; 48:1087-117.

Received: 11 April 2013
Accepted: 27 January 2014

Rev. Inst. Med. Trop. Sao Paulo
56(4):341-346, July-August, 2014
doi: 10.1590/S0036-46652014000400012

ANTIMICROBIAL DRUG RESISTANCE IN STRAINS OF *Escherichia coli* ISOLATED FROM FOOD SOURCES

Mohammed Uddin RASHEED(1), Nooruddin THAJUDDIN(2), Parveez AHAMED(2), Zelalem TEKLEMARIAM(3) & Kaiser JAMIL(1)

SUMMARY

A variety of foods and environmental sources harbor bacteria that are resistant to one or more antimicrobial drugs used in medicine and agriculture. Antibiotic resistance in *Escherichia coli* is of particular concern because it is the most common Gram-negative pathogen in humans. Hence this study was conducted to determine the antibiotic sensitivity pattern of *E. coli* isolated from different types of food items collected randomly from twelve localities of Hyderabad, India. A total of 150 samples comprising; vegetable salad, raw egg-surface, raw chicken, unpasteurized milk, and raw meat were processed microbiologically to isolate *E. coli* and to study their antibiotic susceptibility pattern by the Kirby-Bauer method. The highest percentages of drug resistance in isolates of *E. coli* were detected from raw chicken (23.3%) followed by vegetable salad (20%), raw meat (13.3%), raw egg-surface (10%) and unpasteurized milk (6.7%). The overall incidence of drug resistant *E. coli* was 14.7%. A total of six (4%) Extended Spectrum β-Lactamase (ESBL) producers were detected, two each from vegetable salads and raw chicken, and one each from raw egg-surface and raw meat. Multidrug resistant strains of *E. coli* are a matter of concern as resistance genes are easily transferable to other strains. Pathogen cycling through food is very common and might pose a potential health risk to the consumer. Therefore, in order to avoid this, good hygienic practices are necessary in the abattoirs to prevent contamination of cattle and poultry products with intestinal content as well as forbidding the use of untreated sewage in irrigating vegetables.

KEYWORDS: *E. coli;* Multidrug resistant; ESBL; Agriculture; Medicine.

BACKGROUND

Escherichia coli is the most prevalent facultative anaerobic species in the gastrointestinal tract of human and animals, usually a harmless microbe, but it is also a medically important bacteria causing a number of significant illnesses[14].

Vegetables may be contaminated through insufficiently-treated water and fertilizers or may be compromised by the use of biocides during cultivation[7]. Similarly, animals can also become infected from water or food contaminated with wastes of human or animal origin or with human carrier workers. One of the possible ways of entry of various microbes could be the handling of meat and meat products by adopting improper hygienic measures during handling and processing[20].

Raw meat and vegetables are particularly likely to carry large numbers of bacteria. The same *E. coli* and *Klebsiella* spp. serotypes have been found in food and in the patients who consumed it[11,12]. A sterile diet was shown to lower the number of *E. coli* serotypes found in the feces of test persons[6]. Bacteria escaping alive through the digestive tract to the colon are often transient[13], the resident flora having a protective effect against intruders. The transfer of drug resistance within the gastrointestinal tract is still possible; thus, if our food contains substantial numbers of resistant bacteria, it could be an important source of resistance in fecal flora.

It has been suggested that resistance in bacterial populations may spread from one ecosystem to another[18]. The wild dissemination of antimicrobial resistance among bacterial populations is an increasing problem worldwide.

Antibiotics are often used for therapy of infected humans and animals as well as for prophylaxis and growth promotion of food producing animals. Many findings suggest that inadequate selection and abuse of antimicrobials may lead to resistance in various bacteria and make the treatment of bacterial infections more difficult[21]. Antimicrobial resistance in *E. coli* has been reported worldwide. Treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents[32]. Over the years, resistance to cephalosporins among members of enterobacteriaceae has increased mainly due to the spreading of Extended-spectrum β-Lactamases (ESBL)[40].

(1) Centre for Biotechnology & Bioinformatics, Jawaharlal Nehru Institute of Advanced Studies, Secunderabad, India.
(2) Department of Microbiology, Bharathidasan University, Tiruchirappalli, India.
(3) Medical Laboratory Sciences, College of Health and Medical Sciences, Haramaya University, Harar, Ethiopia.
Correspondence to: Rasheed M.U. E-mail: hmzrshd@yahoo.co.in

As commensal bacteria constitute a reservoir of resistance genes for (potentially) pathogenic bacteria, their level of resistance is considered to be a good indicator for selection pressure by antibiotic use and for resistance problems to be expected in pathogens[24]. Hence the aim of this study was to determine the antibiotic sensitivity pattern of *E. coli* isolated from different types of food items collected from in and around the Hyderabad city of Andhra Pradesh, India. The result of this study demonstrated that organisms harboring Extended Spectrum β-Lactamase (ESBL) enzymes are multi-drug resistant showing resistant to 12 or more drugs tested and thus, could pose serious challenge to the public health.

METHODS

A total of 150 samples each comprising 30 numbers; vegetable salad (carrot, cucumber, cabbage, tomatoes, spinach, lettuce, beet root and radish), raw egg-surface, raw chicken, unpasteurized milk of buffalo, and fresh raw meat of sheep were collected randomly from twelve different localities of Hyderabad. All samples were aseptically collected and then packaged in sterile polythene zip bags and carried to the laboratory in aseptic conditions in a cold box within two hours from the time of purchase. Duplicate samples were obtained whenever possible. All samples were analyzed within 2-4 hours after their arrival to the laboratory. A sharp sterile knife was used to cut samples from surfaces in sterile trays.

To isolate bacteria, a 25-g portion of samples (in case of eggs, each egg separately) was placed into sterile 225 mL Tryptic Soy Broth (TSB) for 6-8 h at 37 ºC.

Culture in TSB was streaked onto MacConkey's agar (MAC) plates and incubated for 18-24 hours at 35+2 °C. Lactose fermenting colonies were picked and identified[8] by gram stain, motility and standard biochemical tests, *viz*., catalase, oxidase, fermentation of lactose and glucose using triple sugar iron agar, production of indole, methyl red test, voges proskauer test, urease test and utilization of citrate.

Samples were also processed to isolate other medically important food borne pathogens like *Salmonella* spp, *Staphylococcus aureus* and *Bacillus cereus.* Biochemically confirmed isolates of *E. coli* were subjected to antimicrobial sensitivity testing.

Antimicrobial Susceptibility Testing: Susceptibility tests were performed using the Kirby-Bauer method on Mueller-Hinton agar in accordance with Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (NCCLS 2002)[26] and using 19 antibacterial agents: Ampicillin (10 mcg), Amoxycillin (25 mcg), Amoxyclav (20/10 mcg (30 mcg)), Aztreonam (30 mcg), Cefotaxime (30 mcg), Ceftazidime (30 mcg), Ceftriaxone (30 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (5 mcg), Colistin (10 mcg), Co-trimoxazole (1.25/23.75 mcg), Gentamicin (10 mcg), Imipenem (10 mcg), Meropenem (10 mcg), Ofloxacin (5 mcg), Piperacillin+tazaobactum (100/10 mcg), Streptomycin (10 mcg), Tetracycline (30mcg), and Tigecycline (15 mcg).

The *E. coli* isolates were inoculated in nutrient broth and incubated at 35+2 °C for five h. The broth was diluted in normal saline solution to a density of 0.5 McFarland turbidity standard. Cotton swabs were used for streaking the diluted broth onto Mueller-Hinton agar plates. After air drying, antibiotic discs were placed 30 mm apart and 10 mm away from the edge of the plate. Plates were inverted and incubated aerobically at 35+2 °C for 16 to 18 hours. The zone of inhibition and resistance was measured, recorded, and interpreted according to the recommendation of the CLSI (NCCLS 2002). The ATCC strain of *E. coli* 25922 was used as a control strain. All the bacteriological media and antimicrobial disks were purchased from HiMedia Laboratories, Mumbai, India. Isolates with resistance or with decreased susceptibility to any of the 3rd Generation Cephalosporin (3GC) were selected for further study.

Extended Spectrum β-Lactamase (ESBL) Confirmatory Tests

Double Disc Synergy Test (DDST)[17]: The isolated colonies were inoculated in nutrient broth at 35+2 °C for five h. The turbidity was adjusted to 0.5 McFarland standard and lawn culture was made on Mueller-Hinton agar using sterile swab. An Augmentin disc (20/10 mcg) was placed in the center of plate. Both sides of the Augmentin disc, a disc of cefotaxime (30 mcg) and ceftazidime (30 mcg), were placed with center to center distance of 15 mm to the centrally placed disc. The plate was incubated at 35+2 °C overnight. ESBL production was interpreted as the 3rd-generation cephalosporin disc, inhibition was increased towards the Augmentin disc or if neither discs were inhibitory alone but bacterial growth was inhibited where the two antibiotics were diffused together.

Phenotypic Confirmatory Disc Diffusion Test (PCDDT) for ESBL[25]: ESBL production was confirmed among potential ESBL-producing isolates by phenotypic tests. Lawn culture of the organism was made and a 3rd-generation cephalosporins ceftazidime (30 mcg) disc and ceftazidime + clavulanic acid (30 mcg + 10 mcg) disc was placed with 25 mm apart. An increase of ≥ 5 mm in zone of inhibition for ceftazidime + clavulanic acid compared to ceftazidime was confirmed as ESBL producers.

95% confidence interval (CI) was calculated for incidence, drug resistance and ESBL production in *E. coli* strains. The difference in resistant and susceptibility pattern between ESBL and non-ESBL producers of *E. coli* strains results was analyzed statistically using X^2 testing and p value of ≤ 0.05 was regarded as significant.

RESULTS

A total of 99 (66%) biochemically confirmed isolates of *E. coli* were isolated from a total of 150 different food items as listed in Table 1. All 99 isolates of *E. coli* tested for their antibiotic profile against 19 different antimicrobial agents.

Resistance to one or more antimicrobial agents was found in 22 (14.7%) isolates of *E. coli* detected from the total of 150 samples and a pattern of multiple drug resistance was observed (Table 2). The dominant type of resistance was to ampicillin and amoxicillin identically detected in 20 (13.3%) isolates, followed by tetracycline in 19 (12.6%), co-trimoxazole in 17 (11.3%), streptomycin in 12 (8%), ciprofloxacin and ofloxacin in 10 (6.6%) each, cefotaxime in 8 (5.3%), and gentamicin, chloramphenicol, and amoxyclave in 7 (4.6%) of each isolates. Twenty two *E. coli* isolates elicited 18 different patterns of antibiotic resistance to the agents used in this study (Table 2). None of the isolate was found resistant to imipenem, tigecycline and colisitin.

Of the 22 isolates of *E.coli*, eight were screened according to CLSI guidelines and selected for conformational tests of ESBL, namely, DDST

Table 1
Incidence of drug resistant *E. coli* from food items

S. No.	Type of food	No. of samples	Incidence of *E. coli* (%), 95%CI	Incidence of drug resistant *E. coli* (%), 95%CI	Incidence of ESBL Producers-*E. coli* (%), 95%CI
1	Vegetables salad	30	23 (76.7) 57.71-90.06	6 (20) 7.71-38.56	2 (6.7) 0.81-22.07
2	Raw egg - surface	30	18 (60) 40.60-77.34	3 (10) 2.11-26.52	1 (3.3) 0.08-17.21
3	Raw chicken	30	25 (83.3) 65.27-94.35	7 (23.3) 9.93-42.28	2 (6.7) 0.81-22.07
4	Unpasteurized milk	30	13 (43.3) 25.46-62.57	2 (6.7) 0.81-22.07	0 (0)
5	Raw meat	30	20 (66.7) 47.18-82.71	4 (13.3) 3.75-30.72	1 (3.3) 0.08-17.21
Total No. of Samples		150	99 (66) 57.82-73.52	22 (14.7) 9.42-21.35	6 (4) 1.48-8.50

Table 2
Antibiotic resistance profile of isolates of *E. coli*

Non-ESBL Producers	No. of isolates	Source of food
T	1	V
Co	1	RM
A, Amx, T	1	V
A, Amx, Co, T	4	C *2n*, UM, RM
A, Amx, Co, S	1	E
A, Amx C, G,S	1	V
A, Amx, Co, S,T	2	C, RM
A, Amx, Cip, Co, G, S, T,	1	V
A, Amx, AC, Ctx, Cip, Ofx, T	1	E
A, Amx, Cip, Co, G, Ofx, S, T	1	C
A, Amx, C, Cip, Co, G, Ofx, S, T	1	C
A, Amx, Ac, Ctx, C, Cip, Ofx, S, T	1	UM
ESBL Producers		
A, Amx, Ac, At, Ctx, Caz, Ctr, C, Cip, Co, Ofx, T	1	C
A, Amx, Ac, At, Ctx, Caz, Ctr, Cip, Co, G, Ofx, S, T	1	RM
A, Amx, At, Ctx, Caz, Ctr, C, Cip, Co, G, Ofx, S, T	1	E
A, Amx, Ac, At, Ctx, Caz, Ctr, Co, G, Ofx, Pit, S, T	1	V
A, Amx, Ac, At, Ctx, Caz, Ctr, C, Cip, Co, Mrp, Ofx, T	1	V
A, Amx, Ac, At, Ctx, Caz, Ctr, C, Cip, Co, G, Ofx, S, T	1	C

V: Vegetables salad, E: Raw egg - surface, C: Raw chicken, UM: Unpasteurized milk, RM: Raw meat. A: Ampicillin, Amx: Amoxycillin, AC: Amoxyclav, At: Aztreonam, Ctx: Cefotaxime, Caz: Ceftazidime, Ctr: Ceftriaxone, C: Chloramphenicol, Cip: Ciprofloxacin, Cl: Colistin, Co: Co-trimoxazole, G: Gentamicin, Ipm: Imipenem, Mrp: Meropenem, Ofx: ofloxacin, Pit: Piperacillin-tazaobactum, S: Streptomycin, T: Tetracycline, Tgc: Tigecycline.

and PCDDT. Of these eight isolates, six isolates found ESBL-positive by the DDST were also ESBL-positive by the PCDDT and the remaining two isolates found ESBL-negative by both the techniques. Thus, a 100 per cent concurrence was noted in the results obtained by the DDST and PCDDT for the eight isolates tested retrospectively. All the six strains of ESBL-producing *E. coli*, showed enhanced susceptibility to ceftazidime and/or cefotaxime in the presence of clavulanic acid, a typical finding for an ESBL producer.

A significant difference in resistant and susceptibility pattern was found between ESBL and non-ESBL producers of *E. coli* strains (Table 2). In all the cases p value was < 0.05. Among non-ESBL producers only two isolates showed resistant to 3rd generation cephalosporin, cefotaxime. Whereas all six ESBL producers were resistant to all 3GC tested and also to aztreonam. Moreover, only ESBL producers have exhibited resistance to meropenem and piperacillin+tazobactum. Among six ESBL producers one strain was resistant to meropenem and another to piperacillin+tazobactam. All the six ESBLs were sensitive only to imipenem, meropenem, piperacillin + tazaobactum, tigecycline, colistin, chloramphenicol, gentamicin, streptomycin, ciprofloxacin and amoxyclav in different patterns.

The number of antibiotics against which each isolate showed resistance ranged between one and 14. Among the non-ESBL producers, two were exhibited resistant to two different single antibiotics (Tetracycline and Co-trimoxazole respectively), six were found to be resistant to less than five antibiotics, five showed resistance to 5-7 antibiotics and three showed resistance to 8-9 antibiotics. In case of ESBL producers, one isolate was resistant to 12 antibiotics, four to 13 antibiotics and one to 14 antibiotics.

DISCUSSION

Antimicrobial resistance has been recognized as an emerging worldwide problem in human and veterinary medicine[2,10] both in developed and developing countries. It is also well documented that widespread use of antibiotics in agriculture and medicine is accepted as a major selective force in the high incidence of antibiotic resistance among gram-negative bacteria[23]. A variety of foods and environmental sources

harbor bacteria that are resistant to one or more antimicrobial drugs used in human or veterinary medicine and in food-animal production[3,5].

Several studies have documented the drug resistant *E. coli* and other coliforms in vegetables[29], poultry[18], egg[4], milk[9] and raw meat[35].

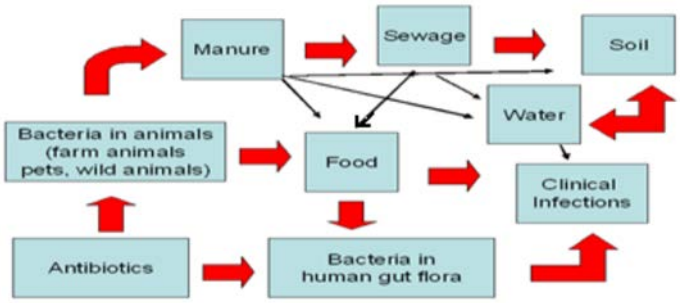


Fig. 1 - Antibiotic resistance flow chart in bacteria and the environment[36].

In this study, the highest percentages of drug resistance in isolates of *E.coli* were detected from raw chicken (23.3%) followed by vegetable salad (20%), raw meat (13.3%), raw egg-surface (10%) and unpasteurized milk (6.7%). The overall incidence of drug resistant *E. coli* was 14.7%.

Antibiotic resistance in *E. coli* is of particular concern because it is the most common Gram-negative pathogen in humans, the most common cause of urinary tract infections, a common cause of both community and hospital-acquired bacteraemia[33] as well as a cause of diarrhea[19]. In addition, resistant *E. coli* strains have the ability to transfer antibiotic resistance determinants not only to other strains of *E.coli*, but also to other bacteria within the gastrointestinal tract and to acquire resistance from other organisms[28].

Different use patterns of antimicrobial agents are expected to have some impact on the distribution of antimicrobial resistance phenotypes[1,22] and possibly of resistant determinants. The result of the antibiotic resistance analysis revealed that among 16 non-ESBLs, only four and two isolates had similar antibiotic patterns to four and five drugs respectively. All the six ESBLs had different patterns of drug resistance.

Current work revealed that all the co-trimoxazole resistant isolates except one were multi-drug resistant. In *E. coli*, trimethoprim-sulfamethoxazole resistance often correlates with the presence of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes in integrons[15,39]. Multiple antibiotic resistance may be acquired through mobile genetic elements such as plasmids, transposons, and class 1 integrons[34].

The number of studies describing the prevalence of ESBL-producing Enterobacteriaceae has increased rapidly around the world[36]. A total of six (4%) ESBL producers were detected in this study, two each from vegetable salads and raw chicken, and one each from raw egg-surface and raw meat. No ESBL producer was detected in the unpasteurized milk.

The result of this investigation shows that organisms harboring Extended Spectrum β-Lactamase enzymes are multi-drug resistant showing resistant to 12 or more drugs tested and thus, could pose serious challenge to the public health. ESBLs are often encoded by genes located on large plasmids, and these also carry genes for resistance to other antimicrobial agents[31].

In recent years, ESBL-producing Enterobacteriaceae isolates have shifted from the hospital to the community and the environment[27]. ESBL-producing Enterobacteriaceae have been recovered from different sources in the community, including cattle, chickens, pigs, raw milk, and lettuce[16,30,37], and a recent study from India reported that a substantial number of tap water samples were contaminated with carbapenemase bla_{NDM-1} producing organisms[38]. Most of the studies on this subject have been conducted in developed countries, but the major epicenters of ESBL-expressing bacteria are located in Asia, Africa, and the Middle East[36].

CONCLUSIONS

Even though the incidence of multidrug resistant and ESBL producers were not high in our study but still it is a matter of concern, since there is a reservoir of antibiotic resistant genes within the community, and that the resistance genes and plasmid-encoded virulent genes are easily transferable to other strains. Pathogen cycling through food is very common and might pose a potential health risk to the consumer.

Therefore, cautions are necessary to decrease the incidence of multi-drug resistant strains of *E. coli* in animals and people. In order to achieve this, good hygienic practices are necessary from the farm to the family table especially in the abattoirs to prevent contamination of cattle and poultry products and abattoir environment with intestinal content. Health authorities should focus on implementing the legislation that forbids irrigation with untreated sewage water of both root and leafy vegetables.

Furthermore, there is a need to emphasize the rational use of antimicrobials and strictly adhere to the concept of "reserve drugs" to minimize the misuse of available antimicrobials in agriculture and medicine. In addition, regular antimicrobial susceptibility surveillance is essential.

RESUMO

Resistência microbiana a drogas em linhagens de *Escherichia coli* isoladas de fontes alimentares

Variedade de alimentos e fontes ambientais contem bactérias resistentes a uma ou mais drogas antimicrobianas usadas em medicina e agricultura. Resistência antibiótica pela *Escherichia coli* é particularmente preocupante porque ela é o patógeno mais comum Gram negativo em humanos. Portanto este estudo foi conduzido para determinar o aspecto de sensibilidade antibiótica da *E. coli* isolados de diferentes tipos de alimentos obtidos ao acaso de 12 localidades de Hyderabad, India. Um total de 150 amostras compreendendo saladas, vegetais, superfícies de ovos crus, galinhas cruas, leite não pasteurizado e carne crua foram processados microbiologicamente para isolar *E. coli* e estudar o quadro de sensibilidade antibiótica pelo método de Kirby-Bauer. A maior percentagem de resistência à droga foi isolada de *E. coli* obtidos de galinha crua (23,3%) seguido de saladas e vegetais (20%), carne crua (13,3%), superfície do ovo cru (10%) e leite não pasteurizado (6,7%). Incidência total de *E. coli* resistente foi de 14,7%. Um total de seis (4%) Extended Spectrum β-Lactamase (ESBL) produtores foram detectados, dois cada de salada de vegetais e galinha crua e um cada de superfície de ovo cru e carne crua. Espécies resistentes a múltiplas

drogas de *E. coli* são matéria de preocupação uma vez que os genes de resistência podem facilmente ser transferidos para outras linhagens. O ciclo do patógeno é muito comum nos alimentos e pode ser risco potencial para a saúde do consumidor. Portanto, para evitar isto boas práticas de higiene são necessárias nos abatedouros para prevenir a contaminação de gado e aves com conteúdo intestinal assim como proibir o uso de águas de esgoto não tratadas para irrigar vegetais.

REFERENCES

1. Aarestrup FM. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Int J Antimicrob Agents. 1999;12:279-85.

2. Amundsen D, Lindholm C, Goyal SM, Robinson RA. Microbial pollution of well water in southeastern Minnesota. J Environ Sci Health. 1988;23:453-68.

3. Anderson AD, Nelson JM, Rossiter S, Angulo FJ. Public health consequences of use of antimicrobial agents in food animals in the United States. Microb Drug Resist. 2003;9:373-9.

4. Arathy DS, Vanpee G, Belot G, Mathew V, DeAllie C, Sharma R. Antimicrobial drug resistance in *Escherichia coli* isolated from commercial chicken eggs in Grenada, West Indies. West Indian Med J. 2011;60:53-6.

5. Bager F, Helmuth R. Epidemiology of resistance to quinolones in *Salmonella.* Vet Res. 2001;32:285-90.

6. Bettelheim KA, Cooke, EM, O' Farrell S, Shooter RA. The effect of diet on intestinal *Escherichia coli.* J Hyg (Lond). 1977;79:43-5.

7. Beuchat LR. Pathogenic microorganisms associated with fresh produce. J Food Prot. 1996;59:204-16.

8. Cheesbrough M. Microbiology. In: Medical laboratory manual for tropical countries. Oxford: ELBS Butterworth-Heinemann Ltd; 1993. vol. II.

9. Cızek A, Dolejska M, Novotna R, Haas D, Vyskocil M. Survey of Shiga toxigenic *Escherichia coli* O157 and drug-resistant coliform bacteria from in-line milk filters on dairy farms in the Czech Republic. J Appl Microbiol. 2008;104:852-60.

10. Cohen ML. Changing patterns of infectious disease. Nature. 2000;406:762-7.

11. Cooke EM, Sazegar T, Edmonson AS, Brayson JC, Hall D. *Klebsiella* species in hospital food and kitchens: a source of organisms in the bowel of patients. J Hyg (Lond). 1980;84:97-101.

12. Cooke EM, Kumar PJ, Shooter RA, Rousseau SA, Foulkes AL. Hospital food as a possible source of *Escherichia coli* in patients. Lancet. 1970;1(7644)**:**436-7.

13. Corpet DE. Antibiotic resistance from food. N Eng J Med.1988;318:1206-7.

14. Friedman ND, Kaye KS, Stout JE, McGarry SA, Trivette SL, Briggs JP, *et al.* Health care-associated bloodstream infections in adults: a reason to change the accepted definition of community-acquired infections. Ann Intern Med. 2002;137:791-7.

15. Huovinen P, Sundström L, Swedberg G, Sköld O. Trimethoprim and sulfonamide resistance. Antimicrob Agents Chemother. 1995;39:279-89.

16. Jakobsen L, Kurbasic A, Skjot-Rasmussen L, Ejrnaes K, Porsbo LJ, Pedersen K, *et al. Escherichia coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs share phylogroups and antimicrobial resistance with community-dwelling humans and patients with urinary tract infection. Foodborne Pathog Dis. 2010;7:537-47.

17. Jarlier V, Nicolas MH, Fournier G, Philippon A. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev Infect Dis.1998;10:867–78.

18. Johnson JR, Sannes MR, Croy C, Johnston B, Clabots C, Kuskowski MA, *et al.* Antimicrobial drug-resistant *Escherichia coli* from humans and poultry products, Minnesota and Wisconsin, 2002–2004. Emerg Infect Dis.2007;13:838-46.

19. Kaper JB, Nataro JP, Mobley HLT. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2:123-40.

20. Kiranmayi CB, Krishnaiah N, Subhashini N, Amaravathi P, Maheswari M, Ramya P. PCR analysis of mutton and chicken samples for the presence of Shiga toxigenic *E.coli.* Arch Clin Microbiol. 2011;2:2-4. Available from: [http://imedpub.com/ojs/index.php/acmicrob/article/view/272/260]

21. Kolár M, Urbánek K, Látal T. Antibiotic selective pressure and development of bacterial resistance. Int J Antimicrob Agents. 2001;17:357-63.

22. Kulkarni RK, Das GP, Dharmi S, Jamil K. Evaluation of antibiotic sensitivity and resistance of *Klebsiella* species from humans respiratory and urinary tract infections. Res J BioTechnol. 2007; 2(4):29-33.

23. McKeon DM, Calabrese JP, Bissonnette GK. Antibiotic resistant gram-negative bacteria in rural groundwater supplies. Water Res. 1995;29:1902-8.

24. Murray BE. Problems and dilemmas of antimicrobial resistance. Pharmacotherapy.1992;12(6 Pt 2):86S-93S.

25. NCCLS. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5 and informational supplement M100-S10. Wayne: NCCLS; 2000.

26. NCCLS. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard M31-A2. Wayne, NCCLS; 2002.

27. Okeke IN, Laxminarayan R, Bhutta ZA, Duse AG, Jenkins P, O'Brien TF, *et al*. Antimicrobial resistance in developing countries. Part I: recent trends and current status. Lancet Infect Dis. 2005;5:481-93.

28. Österblad M, Hadanen A, Manninen R, Leistevuo T, Peltonen R, Meurman O, *et al.* A between-species comparison of antimicrobial resistance in enterobacteria in fecal flora. J Antimicrob Chemother.2000;44:1479-84.

29. Österblad M, Pensala O, Peterzéns M, Heleniusc H, Huovinen P. Antimicrobial susceptibility of Enterobacteriaceae isolated from vegetables. J Antimicrob Chemother. 1999;43:503-9.

30. Ramchandani M, Manges AR, DebRoy C, Smith SP, Johnson JR, Riley LW. Possible animal origin of human-associated, multidrug-resistant, uropathogenic *Escherichia coli*. Clin Infect Dis. 2005;40:251-7.

31. Rawat D, Nair D. Extended-spectrum β-lactamases in Gram negative bacteria. J Glob Infect Dis.2010;2:263-74.

32. Sabaté M, Prats G, Moreno E, Ballesté E, Blanch AR, Andreu A. Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater. Res Microbiol. 2008;159:288-93.

33. Salvadori M, Coleman BL, Louie M, McEwen S, McGeer A. Consumption of antimicrobial-resistant *Escherichia coli*-contaminated well water: human health impact. PSI Clin Res. 2004;6-25. Available from: [http://microbiology.mtsinai.on.ca/research/wellwaterstudy/PSI_doc_2004.pdf]

34. Singh R, Schroeder CM, Meng J, White DG, McDermott PF, Wagner DD, *et al.* Identification of antimicrobial resistance and class 1 integrons in Shiga toxin producing *Escherichia coli* recovered from humans and food animals. J Antimicrob Chemother. 2005;56:216-9.

35. Srinivasa Rao T, Gill JPS, Ravi Kumar GVVPS, Ghatak S. Multi drug resistance patterns of Shiga toxin - producing *Escherichia coli* (STEC) and non - STEC isolates from meats, RTE meat foods, drinking water and human diarrhoeic samples of Punjab, India. Arch Clin Microbiol. 2011;2:1-12. Available from: [http://imedpub.com/ojs/index.php/acmicrob/article/view/256]

36. Tham J. Extended-spectrum beta-lactamase-producing enterobacteriaceae: epidemiology, risk factors, and duration of carriage. Sweden: Department of Clinical Sciences/Malmö Infectious Disease Research Unit/Lund University; 2012. Available from: [http://lup.lub.lu.se/luur/download?func=downloadFile&recordOId=3045564 &fileOId=3045665]

37. Vincent C, Boerlin P, Daignault D, Dozois CM, Dutil L, Galanakis C, *et al.* Food reservoir for *Escherichia coli* causing urinary tract infections. Emerg Infect Dis. 2010;16:88-95.

38. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. Lancet Infect Dis. 2011;11:355-62.

39. White PA, McIver CJ, Rawlinson WD. Integrons and gene cassettes in the Enterobacteriaceae. Antimicrob Agents Chemother. 2001;45:2658-61.

40. Yusha´u M, Umar MI, Suleiman K. Indigenous commercial drinks as potential sources of extended spectrum β-lactamases (ESBLS) producing organisms in Kano, Nigeria. Int J Biomed Health Sci. 2010;6:103-8.

Received: 5 August 2013
Accepted: 8 January 2014

Rev. Inst. Med. Trop. Sao Paulo
56(4):347-352, July-August, 2014
doi: 10.1590/S0036-46652014000400013

EXPERIMENTAL INFECTION WITH *Toxocara cati* IN PIGS: MIGRATORY PATTERN AND PATHOLOGICAL RESPONSE IN EARLY PHASE

Irma Estela SOMMERFELT(1), Adriana DUCHENE(2), Betina DAPRATO(1), Clara María LOPEZ(1), Natalia CARDILLO(1) & Aníbal Juan FRANCO(1)

SUMMARY

Experimental inoculations of approximately 100,000 infective *Toxocara cati* larval eggs were done in twelve pigs. The *T. cati* eggs used for inoculation were collected from cat's feces. Another group of three pigs served as an uninfected control. Groups of infected pigs were euthanized at seven, 14, 21, and 28 days post-inoculation (dpi). Tissue samples were taken for digestion and histopathology changes in early phase. The number of larvae recovered from the lungs peaked at seven and 14 dpi and were also present at 21, and 28 dpi. Larvae of *T. cati* were present in the lymph nodes of the small and large intestine at seven, 14, and 28 dpi and at seven, 14, 21, and 28 dpi respectively. In other studied tissues, no larvae or less than one larva per gram was detected. The pathological response observed in the liver and lungs at seven and 14 dpi, showed white spots on the liver surface and areas of consolidation were observed in the lungs. The lungs showed an inflammatory reaction with larvae in center at 28 dpi. In the liver we observed periportal and perilobular hepatitis. The lymph nodes of the intestines displayed eosinophil lymphadenitis with reactive centers containing parasitic forms in some of them. The granulomatous reaction was not observed in any tissues. The role of the other examined tissues had less significance. The relevance of this parasite as an etiological agent that leads to disease in paratenic hosts is evident.

KEYWORDS: Animal model; Larval recovery; *Toxocara cati*; Tissue lesions; Infected pig; Toxocariasis.

INTRODUCTION

Toxocara canis and *Toxocara cati* are common parasites of dogs and cats, respectively. Both are zoonotic agents that can cause significant disease in humans as well. Human infection with larval *Toxocara* produces classical visceral larva migrans; incomplete visceral larva migrans; compartmentalized forms: ocular and neurological toxocariasis; covert toxocariasis; and asymptomatic toxocariasis[14].

T. cati infects both domestic and wild felines. Undeveloped eggs deposited by adult parasites are excreted in the feces of the host but are not infective in this first stage. Infective forms develop after some time in the environment. Cats can be infected by ingesting embryonated *T. cati* eggs from the soil or larvae in paratenic hosts and play a fundamental role in the dispersion of the parasite[5,6,13,22]. The presence of *T. cati*-infected felines in public spaces, their defecation habits, and their direct contact with humans constitute significant factors that promote the transmission of this zoonosis. A number of studies taking place worldwide have described the extent of environmental contamination with this parasite. The cat population lacks sanitary control, reproduces freely, and transforms open spaces into permanent sources of infection, thus increasing the risk of infection for the humans that spend time in these areas[21].

Despite the use of several animal models and strategies to analyze the pathological tissue alterations and behavior of migrating of *Toxocara* spp. larvae in mammals, several controversies persist. The results of multiple studies vary based on the animal model chosen, the parasite species inoculated, and the larval dose(s) used for infection. Experimental infections with *T. canis* and *T. cati* have been described in different animal models, including mice, Mongolian gerbils, rats, and chickens[2,3,5,8,11,12,15,18,22, 24,25].

Pigs are paratenic hosts of the *T. cati* parasite and they have many physiological similarities to humans. Pigs have been used as an animal model for the study of the Toxocariasis due to *T. canis.* Pigs experimentally infected with *T. canis* were previously used to study the migratory pattern of the parasite as well as to analyze the pathological changes, immunological response, and hematological disturbances triggered by infection[3,9,15,19,20,24]. *T. canis* is better recognized as the main cause of the toxocariasis. *T. cati* has been little studied as an etiological agent for human toxocariasis. STRUBE *et al.*[23] expressed that "No experimental infections of pigs with *T. cati* could be found in the literature; there for the migratory route of this species in this particular host remains to be determined". In this work we use pigs for studying toxocariasis due to this parasite. The objectives of this work were to study

(1) Cátedra de Salud Pública. Facultad de Ciencias Veterinarias, Universidad de Buenos Aires. Av. Chorroarín 280, 1427, Buenos Aires, Argentina.
(2) Cátedra de Patología Clínica. Facultad de Ciencias Veterinarias, Universidad de Buenos Aires. Av. Chorroarín 280, 1427, Buenos Aires, Argentina.
Correspondence to Irma Estela Sommerfelt, Cátedra de Salud Pública, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires. Av. Chorroarín 280, 1427, Buenos Aires, Argentina. Tel: 54 11 4524 8453; Fax: 54 11 4524 8491. E-mail: isom@fvet.uba.ar

the migratory pattern of *Toxocara cati* larvae in the early phase of the infection in pigs and the pathological response of the tissues.

MATERIALS AND METHODS

The inoculums were prepared with *T. cati* eggs obtained from the feces of naturally infected cats. *T. cati* eggs were collected by brine flotation from the stools and washed in tap water. Each 2 g of tissue was incubated in 0.5% formaldehyde with 0.03 mL of iodopovidona 10% during 35 days at 28 ºC. They were shaken daily for eight hours. The embryonation of eggs followed weekly by light microscopy[20]. To evaluate the infectivity of the embryonated *T. cati* eggs, three mice were inoculated and then euthanized at one day post-inoculation (dpi). Animals were provided by the Faculty of Veterinary Sciences of the University of Buenos Aires and were housed according to the accepted standards of laboratory animal management[1]. The liver and lungs were processed by the acid digestion technique as previously described[9]. Embryonated eggs were concentrated using Benbrook's flotation technique with a saturated sugar solution. The supernatant was extracted and washed three times, with each wash followed by successive sedimentation steps by centrifugation[7]. Each inoculum contained approximately 100,000 eggs in one mL of saline solution (0.9 N NaCl). The experimental protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals, Faculty of Veterinary Science, University of Buenos Aires.

Fifteen 40-day-old male Yorkshire pigs weighing 18 kg each were obtained from a swine production facility with excellent health conditions. Four groups of three animals were randomly chosen to receive the inoculums and the remaining group was designated as the uninfected control. Prior to inoculation, pigs were fasted for 12 hours. Each one mL inoculum was administered orally using a syringe. The syringe was then washed with two mL of saline solution, which was also given to the animal. Control animals received one mL of saline solution in a similar manner. All animals were examined clinically once a week. The experimental protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals, Faculty of Veterinary Science, University of Buenos Aires, and animals were maintained according to accepted standards[1].

Groups of three infected pigs were euthanized by standard methods[1] at seven, 14, 21, and 28 dpi and were subsequently analyzed by necropsy. The control group was euthanized at 28 dpi. Each organ and tissue was weighed, and 100 g samples were randomly selected and divided into two halves. Organs and tissues including liver, lungs, kidneys, heart, eyes, brain, tongue, skeletal muscles and lymph nodes of the small and large intestines were collected. Eyes of each pig were sent one for pathological observation and the other for artificial digestion.

Macroscopic analysis was performed at necropsy. Microscopic evaluation of the selected organs and tissues was performed according to the technique previously described[20]. Each 50 g sample of tissue had been submerged in 10% formaldehyde and then processed for histopathology. One eye from each animal was entirely analyzed.

The artificial digestion method previously described[9] was used to recover *T. cati* larvae from each 50 g sample of tissues. One eye from each animal was completely digested. A volume of 0.3 mL of concentrated material was observed under a light microscope (10 x), and the total number of larvae was recorded. The number of larvae per gram of tissue in each sample and the total weight of each organ was used to estimate the number of larvae present in each organ.

The recovery of larvae from tissues at different time points was analyzed using a non-parametric test (Kruskal-Wallis chi-square approximation). In cases in which the time effect was significant, ANOVA (analysis of variance) and polynomial contrasts were used. The level of significance was set at 5% (p-values < 0.05). InfoStat software was used for all statistical analyses.

RESULTS

T. cati larvae were recovered from tissues of all the inoculated pigs (Table 1). An evaluation of the number of larvae recovered from the lungs showed a significant difference among seven, 14, 21, and 28 dpi ($X^2 = 8.3497$; $p = 0.0393$) that can be explained by a significant linear trend ($p = 0.002$) that describes the greater recovered at seven and 14 dpi. Larva recovery from the lymph nodes of the small intestine peaked at 14 dpi, and significant differences among seven, 14, 21, and 28 dpi were detected ($X^2 = 9.0702$, $p = 0.0284$), also explained by a significant linear trend ($p = 0.0009$). Larva recovery from the lymph nodes of the large intestine peaked at seven dpi, and statistical analysis detected significant differences among seven, 14, 21, and 28 dpi ($X^2 = 8.5556$, $p = 0.0358$) with a significant linear trend ($p = 0.0013$). An evaluation of the number of larvae recovered from the liver, brain, kidneys, heart, muscle, and eyes showed no significant differences among seven, 14, 21, and 28 dpi. No larvae were recovered from any tissue of the control animals.

Macroscopic lesions on the lungs with areas of consolidation over the entire surface were visible at seven and 14 dpi, but these lesions decreased at 21 and 28 dpi. Microscopic lesions were observed throughout the experiment. At seven dpi, the lungs displayed an inflammatory reaction consisting of a mixture of mononuclear and polymorphonuclear cells (lymphocytes, plasmocytes, and eosinophils). We detected multifocal nodular pneumonia and an accumulation of eosinophils at 14 dpi. Non-suppurative diffuse pneumonia with a lymphoid hyperplasia focus containing lymphocytes, plasmocytes, and eosinophils was observed at 21 and 28 dpi. Larvae were present at the center of the lesion at 28 dpi (Fig. 1).

At seven and 14 dpi, macroscopic lesions on the liver had small white spots 2-3 mm in diameter on its surface; very few spots were detected at 21 or 28 dpi. Microscopically we observed periportal and perilobular hepatitis with a predominance of leukocytes, lymphocytes, and plasmocytes in infected pigs, along with a smaller proportion of eosinophils at seven and 14 dpi (Fig. 2).

Macroscopically, the lymph nodes of the small and large intestines were slightly edematous at seven and 14 dpi but were normal at the other time points. Microscopically, a granulomatous reaction with larvae in the pericapsular external zone of the lymph nodes was visible at seven and 14 dpi (Fig. 3), and giant cells were also present. Such lesions were not detected at 21 or 28 dpi. The Splendore-Hoeppli phenomenon, observed in some fungal and parasitic diseases as the result of a local antigen-antibody complex formation, was detected in lymph nodes and was characterized by the deposition of amorphous eosinophilic hyaline material around the larvae (Fig. 4).

Table 1
Mean number of *T. cati* larvae recovered per gram of tissues at euthanized time points, in tissues of pigs experimentally infected with 100,000 *T. cati* eggs

Tissues	Days post-inoculation			
	7	14	21	28
Lung	18.98 ± 7.20[a] (11-25)	19.77 ± 5.34[a] (14.32-25)	0.91 ± 0.81[a] (0.07-1.71)	3.17 ± 4.19[a] (0.65-8.02)
Lymph nodes (SI)*	6.30 ± 5.08[a] (0.78-10.8)	13.25 ± 17.28[a] (2.76-33.2)	0	0.16 ± 0.27[a] (0-0.48)
Lymph nodes (LI)**	15.88 ± 8.11[a] (7-22.9)	10.25 ± 13.71[a] (0.93-26)	0.21 ± 0.36[a] (0-0.63)	0.29 ± 0.29[a] (0-0.59)
Liver	0	0.05 ± 0.05[a] (0-0.10)	0.13 ± 0.23[a] (0-0.40)	0.01 ± 0.02[a] (0-0.03)
Brain	0.0 ± 0.01[a] (0-0.02)	0.02 ± 0.04[a] (0-0.08)	0.01 ± 0.02[a] (0-0.05)	0.02 ± 0.03[a] (0-0.06)
Kidney	0.09 ± 0.01[a] (0.08-0.11)	0.15 ± 0.25[a] (0-0.45)	0	0
Heart	0.02 ± 0.02[a] (0-0.04)	0	0	0
Muscle	0.40 ± 0.33[a] (0.02-0.64)	0.99 ± 1.20[a] (0.18-2.37)	0.05 ± 0.04[a] (0.01-0.10)	0.59 ± 0.35[a] (0.37-1.0)
Eyes	0	0	0	0.02 ± 0.02[a] (0.01-0.05)

*: small intestine; **: large intestine. [a]Range (± S.E.).

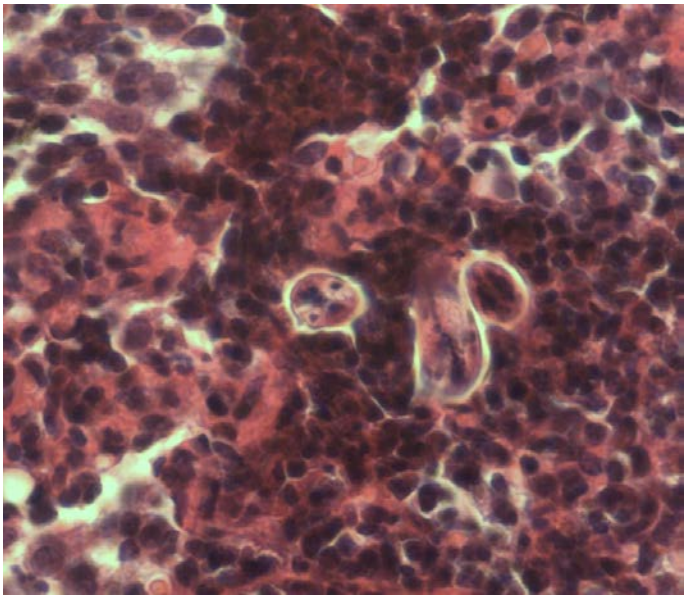


Fig. 1 - Lung tissue from a pig experimentally infected with 100,000 *T. cati* eggs, showing infiltration of eosinophils with larvae in the center (E/H 40x).

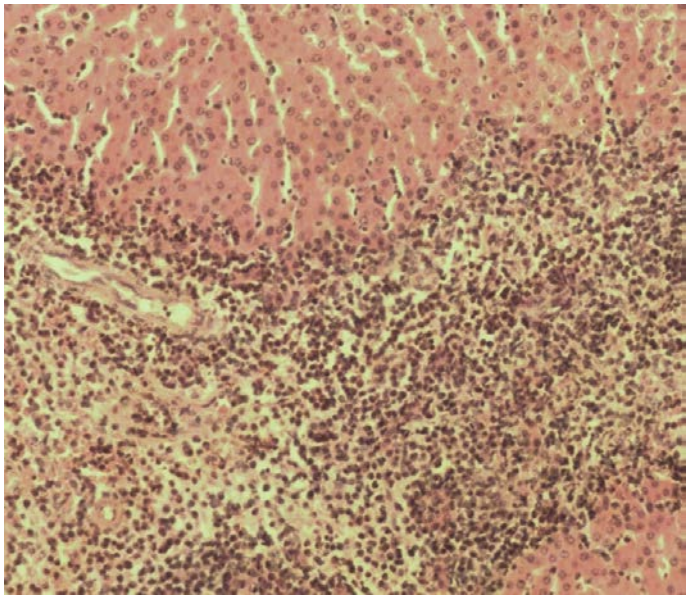


Fig. 2 - Liver tissue from a pig experimentally infected with 100,000 *T. cati* eggs, showing infiltration of lymphocytes, plasmocytes, and a small number of eosinophils (E/H 10x).

No macroscopic lesions were observed in the tongue. Tongues from infected pigs displayed microscopic lesions with non-suppurative and segmental myositis accompanied by leukocytes, lymphocytes, plasmocytes, and a small number of eosinophil cells at 14 and 28 dpi.

No macroscopic lesions were observed in the kidneys. Microscopic images of the kidneys showed focal non-suppurative interstitial nephritis at 14, 21 and 28 dpi.

Muscle did not present macroscopic alterations. Microscopically,

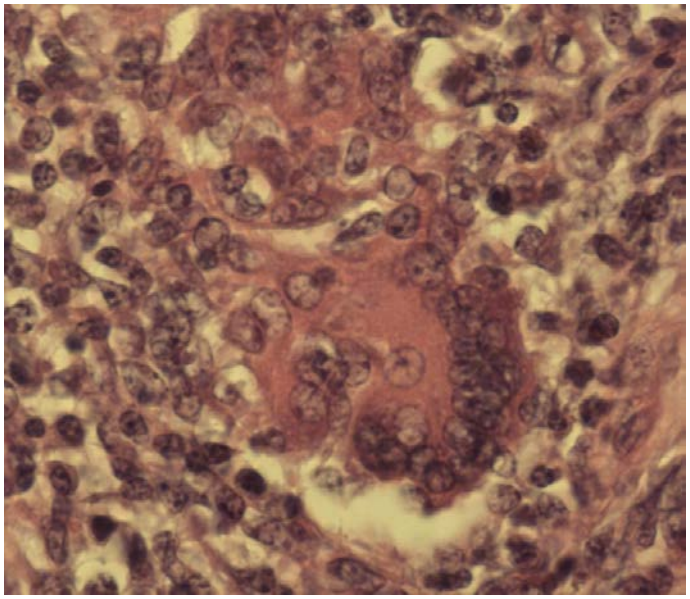


Fig. 3 -Lymph node tissue from a pig experimentally infected with 100,000 *T. cati* eggs, showing a reactive center in the follicle lymphoid with giant cells (E/H 40x).

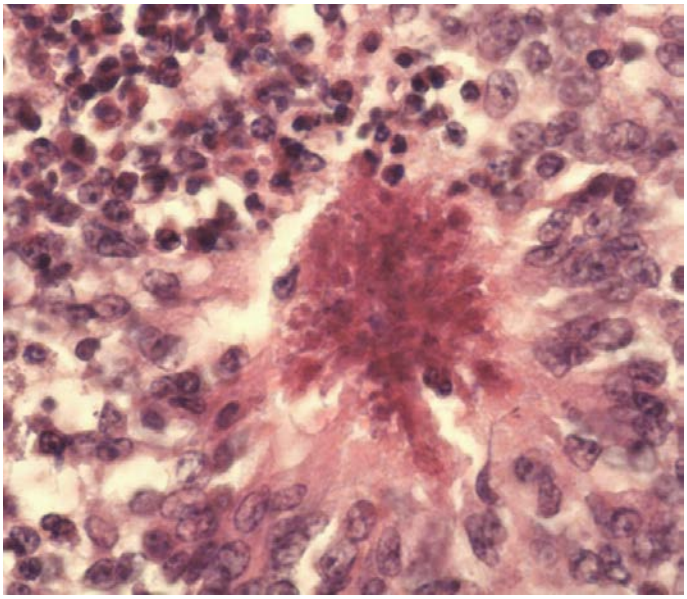


Fig. 4 - Lymph node tissue from a pig experimentally infected with 100,000 *T. cati* eggs, showing the Splendore-Hoeppli phenomenon (E/H 40x).

several myositis foci with accumulated lymphocytes and eosinophils were observed at 14 dpi.

Pathological lesions were not detected in the brain or eyes.

In any tissue of the pigs infected with *T. cati,* the typical granulomatous reaction composed of a mixture of eosinophils, lymphoid cells, and epithelial cells surrounding a central necrotic core containing visible larvae was not observed.

No pathological lesions were observed in the control animals.

DISCUSSION

This study is the first to quantify larvae recovered from *T. cati*-infected pig tissues by using digestion techniques. The histological observations previously reported by RONÉUS[16] demonstrated the presence of *T. cati* larvae in inoculated pigs but did not quantify the larvae present in various tissues.

Here, the highest numbers of larvae in the early stages of infection were recovered from the lungs and the lymph nodes of the large and small intestines (seven and 14 dpi). The remarkably high persistence of larvae in the lungs throughout the study indicates a predilection of *T. cati* larvae to migrate to the lungs. The lymph nodes also appeared to be another preferred site in the migratory pattern of the parasite. We also observed the persistence of *T. cati* larvae in pig muscles. *T. cati* larvae have been shown to accumulate and persist in diverse organs, depending on the animal model used to study its migratory patterns[2,3,5,8,11,12,15,18,22,25].

In pigs, DONE *et al.*[4] reported that *T. canis* larvae have been previously shown to migrate primarily to the liver and lungs; HELWIGH *et al*.[10] and TAIRA *et al*.[24], to the lungs and lymph nodes; SOMMERFELT *et at*.[20] observed to the lymph nodes, liver, and lungs. Therefore, lungs and lymph nodes are the most frequent sites for both *T. canis* and *T. cati* parasites during migration through the host. We found macroscopic lesions in the liver and lungs, in agreement with RONÉUS[16]. However, previous study also described changes in the kidneys[16] that we did not observe in the present study. In the lungs, microscopic lesions were detected early in the infection and persisted throughout the study. This observation is in contrast with RONÉUS[16], who reported that lung lesions appeared, later in the infection. However, the results of our study agree with those of RONÉUS[16] in regard to the liver lesions; both studies showed periportal and perilobular hepatitis and demonstrated that the severity of the liver lesions depends on the time at which they were examined.

The data from this study and previous reports suggest a type of larval migratory pattern in which the larvae reach the lungs and the liver by migrating through the mesenteric lymph nodes. Previous reports of macroscopic or microscopic lesions produced by *T. cati* in other animal models are somewhat in agreement with our observations of lesions in lungs, liver, kidneys, and muscle[2,3,5,11,22]. Our results also agree with previous observations of macroscopic lesions in pigs inoculated with *T. canis*[4,10,17,20]. White spots on the liver were also observed by TAIRA *et al.*[24]. One notable difference between our study and previous research that inoculated *T. canis,* was the absence of the typical granulomatous reaction in our sample.

In conclusion, the results of this study show that the *T. cati* parasite is able to migrate through diverse tissues of the pig. Therefore, the relevance of this parasite as an etiological agent that leads to disease in paratenic hosts is evident. In our animal model, the pig, the parasite primarily migrated to the lungs and the lymph nodes. The migration of the larvae to the eyes observed in our research suggests its participation in the production of ocular larva migrans. The absence of the typical granulomatous reaction in tissues should be studied further to evaluate its influence on the immune response to *T. cati* infection and compare with the response of *T. canis* where the granulomatous reaction was present[19,20].

Our study contributes to the knowledge of the migratory behavior of *T. cati* larvae in pigs that could help for the comprehension of this parasite distribution on other paratenic host, as humans and to elucidate relevance of the host-parasite relationships especially in human toxocariasis.

ETHICAL STANDARDS

The experimental protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals, Faculty of Veterinary Science, University of Buenos Aires. Argentina.

CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

RESUMEN

Infección experimental en cerdos con *Toxocara cati*: patrón migratorio y respuesta patológica en etapa temprana

Se realizó la infección experimental de doce cerdos con aproximadamente 100.000 huevos infectivos de *Toxocara cati*. Los huevos de *T. cati* utilizados en la inoculación fueron recolectados de heces felinas. Otro grupo de tres cerdos no infectados se utilizó como control. Grupos de cerdos infectados se eutanaciaron a los 7,14,21 y 28 días posinoculación (pi). Se tomaron muestras de tejidos para digestión y evaluación de cambios histopatológicos en la etapa temprana de la infección. El número de larvas recuperadas de los pulmones se incrementó en los días 7 y 14 pi, recuperándose también los días 21 y 28 pi. Se encontraron larvas de *T. cati* en los linfonódulos del intestino delgado y grueso los días 7,14 y 28 pi y los días 7,14,21 y 28 pi respectivamente. En los restantes tejidos estudiados o no se recuperaron larvas o los valores fueron menores a una larva por gramo de tejido. La respuesta patológica observada en el hígado y los pulmones a los 7 y 14 días posinoculación, mostró en la superficie del hígado manchas blancas y en los pulmones áreas de consolidación. Los pulmones presentaron una reacción inflamatoria con presencia de larva en el centro en el día 28 pi. En el hígado se observó una hepatitis periportal y perilobular. Los linfonódulos del intestino presentaron una linfoadenitis eosinofílica con un centro reactivo conteniendo formas parasitarias en algunos de ello. En ninguno de los tejidos se observó la típica reacción granulomatosa. El rol de los restantes tejidos examinados fue de menor significancia. Queda evidenciada la importancia de éste parasito como un agente etiológico que desarrolla la enfermedad en hospederos paraténicos.

ACKNOWLEDGEMENTS AND FINANCIAL SUPPORT

This research was supported by the University of Buenos Aires, Secretary of Science and Technique. Argentina (Research number 20020100100387).

AUTHORS CONTRIBUTIONS

Conceived and designed the experiments: I E Sommerfelt, A J Franco. Performed the experiments: I E Sommerfelt, A Duchene, B Daprato, C M López, N Cardillo, A J Franco. Analyzed the data: C M López , I E Sommerfelt. Contributed reagents/materials/analysis tools: A Duchene, N Cardillo, B Daprato, I E Sommerfelt, C M López, A J Franco. Wrote the paper: I E Sommerfelt.

REFERENCES

1. American Veterinary Medical Association. 1993 report of the AVMA panel on euthanasia. J Am Vet Med Assoc. 1993;202:229-49.

2. Azizi S, Oryan A, Sadjjadi SM, Zibaeri M. Histopathologic changes and larval recovery of *Toxocara cati* in experimentally infected chickens. Parasitol. Res. 2007;102:47-52.

3. Cardillo N, Rosa A, Ribicich M, López C, Sommerfelt I. Experimental infection with *Toxocara cati* in BALB/c mice, migratory behaviour and pathological changes. Zoonoses Public Health. 2008;56:198-205.

4. Done JT, Richardson MD, Gibson TE. Experimental visceral larva migrans in the pigs. Res Vet Sci. 1960;1:133-51.

5. Dubey JP. Migration of *Toxocara cati* larvae in mice. Trop Geogr Med. 1968;20:172-6.

6. Dubinský P, Havasiová-Reiterová K, Petko B, Hovorka I, Tomasovicocá O. Role of small mammals in the epidemiology of toxocariasis. Parasitology. 1995;110(Pt2):187-93.

7. Fan CK, Lin YH, Du WY, Su KE. Infectivity and pathogenicity of 14-month-cultured embryonated eggs of *Toxocara canis* in mice. Vet Parasitol. 2003;113:145-55.

8. Fisher M. *Toxocara cati*: an underestimated zoonotic agent. Trends Parasitol. 2003;1:167-70.

9. Gamble HR, Bessonov AS, Cuperlovic K, Gajadhar AA, van Knapen F, Noerckler F, *et al*. International commission on trichinellosis: recommendations on methods for the control of *Thichinella* in domestic and wild animals intended for human consumption. Vet Parasitol. 2000;93:393-408.

10. Helwigh AB, Lind P, Nansen P. Visceral larva migrans: migratory pattern of *Toxocara canis* in pigs. Int J Parasitol. 1999;29:559-65.

11. Hrckova G, Velebný S, Tomasovicová O, Medvedóvá M, Pajerský A. Pathomorphological changes in mice infected with *Toxocara cati* following administration of fenbendazole and glucan. Acta Parasitol. 2000;46:313-20.

12. Okoshi S, Usui M. Experimental studies on *Toxascaris leonina.* VI. Experimental infection on mice, chickens and earthworms with *Toxascaris leonina*, *Toxocara canis* and *Toxocara cati*. Nihon Juigaku Zasshi. 1968;30:151-66.

13. Parsons JC. Ascarid infections of cats and dogs. Vet Clin North Am Small Anim Pract. 1987;17:1307-39.

14. Pawlowski Z. Toxocariasis in humans: clinical expression and treatment dilemma. J Helminthol. 2001;75:299-305.

15. Prokopic J, Figallová SV. Migration of some roundworm species in experimentally infected white mice. Folia Parasitol (Praha). 1982;29:309-13.

16. Ronéus O. Parasitic liver lesions in swine, experimentally produced by visceral larva migrans of *Toxocara cati.* Acta Vet Scand. 1963;4:170-96.

17. Ronéus O. Studies on the aetiology and pathogenesis of white pots in the liver of pigs. Acta Vet Scand. 1966;7(Suppl. 16):1-112.

18. Schön J, Stoye M. Prenatal and galactogenic infections with *Toxocara* mystax Zeder, 1800 (Anisakidae) in the mouse. Zentralbl Veterinamed B. 1986;33:397-412.

19. Sommerfelt IE, Santillán G, López C, Ribicich M, Franco A**.** Immunological and hematological response in experimental *Toxocara canis*-infected pigs. Vet Parasitol. 2001;96:127-34.

20. Sommerfelt IE, Rosa A, Duchene A, Degregorio O, López C, Pisanú A, *et al*. *Toxocara canis* in experimentally infected pigs: migratory pattern and tissue lesions. Vet Parasitol. 2004;125:323-34.

21. Sommerfelt IE, Cardillo N, López C, Ribicich M, Gallo C, Franco A. Prevalence of *Toxocara cati* and other parasites in cats' faeces collected from the open spaces of public institutions: Buenos Aires, Argentina. Vet Parasitol. 2006;140:296-301.

22. Sprent JFA. The life history and development of *Toxocara cati* (Schrank 1788) in the domestic cat. Parasitology. 1956;46:54-78.

23. Strube C, Heuer L, Janecek E. *Toxocara* spp.infections in paratenic hosts. Vet Parasitol. 2013;193:375-89.

24. Taira K, Saeed I, Lind P, Murrell KD, Kapel CMO. Population dynamics of *Toxocara canis* in pigs receiving a single or multiple infection. Parasitology. 2003;127(Pt 6):593-602.

25. Zibaei M, Sadjjadi SM, Uga S. Experimental *Toxocara cati* infection in gerbils and rats. Korean J Parasitol. 2010;48:331-3.

Received: 20 September 2013
Accepted: 21 November 2013

Rev. Inst. Med. Trop. Sao Paulo
56(4):353-356, July-August, 2014
doi: 10.1590/S0036-46652014000400014

BEHAVIORAL AND MEMORY CHANGES IN *Mus musculus* COINFECTED BY *Toxocara canis* AND *Toxoplasma gondii*

Flávia Motta CORRÊA(1), Pedro Paulo CHIEFFI(1,2), Susana A. Zevallos LESCANO(1,2) & Sergio Vieira dos SANTOS(1,2)

SUMMARY

Several researchers have stated that parasites can alter the behavior of their hosts, in order to increase the transmission rate, principally when prey-predator relationships are a reliable way of infection transmission. The aim of this study was to verify the occurrence of changes in anxiety and short-term memory patterns in experimentally infected *Mus musculus* by *Toxocara canis* and/or *Toxoplasma gondii*. Forty male *Mus musculus* (Balb/c) eight-week-old were divided into four groups of 10 mice each. One group was infected with 300 eggs of *Toxocara canis*; a second group was submitted to infection with 10 cysts of *Toxoplasma gondii*; a third group was concomitantly infected with both parasites with the same inoculums and the last group was maintained without infection. The anxiety levels were evaluated using an elevated plus maze and an actometer; the short-term memory was determined by a two-way active avoidance equipment. The determination of anxiety levels were conducted 40 and 70 days after infection and the short-term memory was evaluated 140 days after infection. Mice chronically infected by *Toxoplasma gondii* showed impaired learning and short-term memory, but no significant differences were found in mice infected by *Toxocara canis* or concomitantly infected by *Toxocara canis* and *Toxoplasma gondii* when compared to non infected mice.

KEYWORDS: *Mus musculus; Toxocara canis; Toxoplasma gondii;* Concomitant infections; Behavior alterations.

INTRODUCTION

According to the manipulation hypothesis, a parasite can alter the behavior of their hosts specifically to increase the transmission. This hypothesis requires that the change of behavior is a sophisticated product resulting in the parasite manipulation of the host, rather than a by-product of other physiological activities of the parasite[3,32].

There are many examples in the literature of behavioral changes in insects, crustaceans, and fish acting as intermediate hosts in the life cycle of several species of parasites; however, little is known about behavioral changes in mammals[1-3,5,21,36].

Some studies show evidence of behavioral changes in rodents in experimental protocols with single infections; however, in natural conditions, the occurrence of co-infection or multiple parasite infections of the same host should be common[4,8,26,33,35,40,42].

Toxoplasma gondii is a protozoan parasite, whose definitive hosts are felines, but other warm-blooded vertebrates may act as intermediate hosts. *Toxocara canis* is a nematode parasite of dogs that eventually infects small mammals, which do not reach maturity. These mammals act as paratenic hosts, because they maintain the larvae in their organs for a long time and have an important role in the transmission of the parasite through the predator-prey relationship. Both parasites can cross the blood-brain barrier, settling in areas of the central nervous system of rodents related to the control of anxiety and locomotion[9,16,22].

QUEIROZ *et al.*[33] investigated the behavior of *Rattus norvegicus* infected by *Toxocara canis* and/or *Toxoplasma gondii*, and concluded that both parasites influenced rodent behavior. However, when the rats were concomitantly infected, a behavior modulation was observed, resulting in slight absence of behavioral alterations.

Due to the high frequency of *Mus musculus* infection by both parasites in natural conditions and the importance of these rodents as paratenic hosts, the aim of the present study was to verify the occurrence of changes in anxiety and short-term memory patterns in experimentally infected *Mus musculus* by *Toxocara canis* and/or *Toxoplasma gondii.*

MATERIALS AND METHODS

Forty male *Mus musculus* (Balb/c) eight-week-old were obtained from the Central Animal Laboratory of the Faculty of Medicine, University of São Paulo. The eggs of *T. canis* were obtained by dissecting female worms, recovered from naturally infected dogs captured by the Center for Zoonosis

(1) Faculdade de Ciências Médicas da Santa Casa de São Paulo. São Paulo, SP, Brazil. E-mail: pchieffi@usp.br
(2) Instituto de Medicina Tropical de São Paulo (LIM-06). São Paulo, SP, Brazil.
Correspondence to: Sergio Vieira dos Santos. E-mail: sergio.bio@usp.br

Control of Guarulhos (CCZ/GRU). The cysts of *T. gondii* (ME49 strain cystogenic referring to genotype II) were provided by the Laboratory of Protozoology of the Institute of Tropical Medicine of São Paulo.

The mice were divided into four groups, namely, *Toxocara*: 10 mice infected with 300 eggs of *Toxocara canis*, *Toxoplasma*: 10 mice infected with 10 cysts of *Toxoplasma gondii*; Concomitant infection: 10 mice infected with 300 eggs of *T. canis* and 10 cysts of *T. gondii*, and Control: 10 mice without infection.

The behavior of the mice was assessed by testing in the elevated plus maze, to determine the levels of anxiety, using the technique described by PELLOW & FILE[30]. The motor activity, another behavioral parameter, was measured using the technique of determination of motor activity in the open field, using an Actometer, as described by SILVA *et al.*[34], NASELLO *et al.*[29] and GUARALDO *et al.*[18].

The performance evaluation was conducted on two occasions: 40 and 70 days after infection.

To assess learning and memory consolidation, a two-way active avoidance equipment (Ugo Basile, Comerio, Italy) was used on two occasions, as described by KORTE & DE BOER[28], 140 days after infection.

At the end of the experiment, all the rats were euthanized and the carcasses were submitted to digestion with HCl 0.5% (XI & JIN, 1998)[44] and the central nervous system was macerated in saline solution 0.9% for the recovery of *Toxocara canis* larvae and *Toxoplasma gondii* cysts, respectively.

The data were expressed as the mean ± standard deviation. Statistical comparisons were performed using the Two-way ANOVA for the behavioral variables and the t-Student test for the evaluation of short-term memory. Only probability values which were (p) smaller than 0.05 were considered as statistically significant.

All care procedures were performed strictly according to the guidelines for animal experimentation, as stipulated in the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication Number 86–23, Bethesda, MD). The experimental protocol was approved by the Research Ethics Committee on Animal Experiments of the São Paulo Institute of Tropical Medicine (process no. 2011/098).

RESULTS

The analysis of the variables in the elevated plus maze showed a significant difference ($p < 0.05$) between the group infected by *Toxoplasma gondii* and the control group at 40 dpi; on the other hand, the group with concomitant infections did not show any significant difference in comparison to the non-infected control group (Fig. 1).

On evaluation of the frequency of entries into the closed arms of the elevated plus maze, there was a significant difference ($p < 0.05$) between the groups infected with *T. canis* and *T. gondii*, but not with the group with concomitant infections, in comparison to the control group (Fig. 2).

The same variables observed in an Elevated Plus Maze at 70 days post-infection showed no significant difference among all the groups.

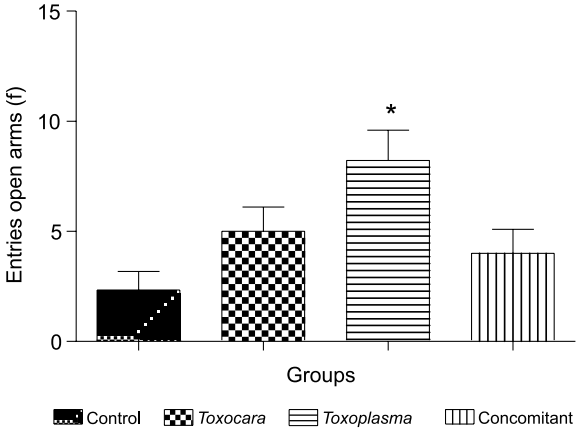


Fig. 1 - Frequency of entries into the open arms of the Elevated Plus Maze *Mus musculus* infected with *Toxocara canis* and/or *Toxoplasma gondii* at 40 dpi. *$p < 0.05$ related to the control.

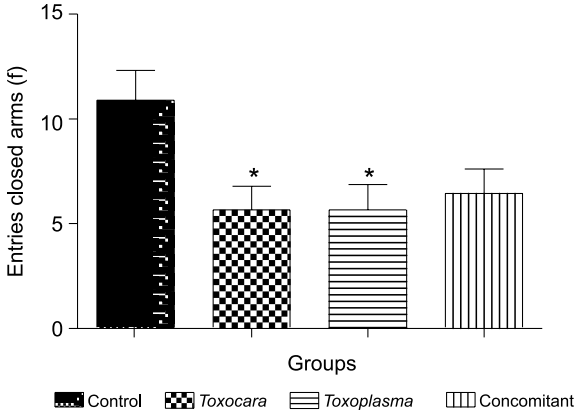


Fig. 2 - Frequency of entries into the closed arms of the Elevated Plus Maze *Mus musculus* infected with *Toxocara canis* and/or *Toxoplasma gondii* at 40 dpi. *$p < 0.05$ related to the control.

In aversive avoidance, there was no significant difference between the groups, however, only the group infected with *T. gondii* showed a difference between the first and second test (Fig. 3).

All mice of the infected groups showed, at least, a *Toxocara canis* larvae and/or *Toxoplasma gondii* cyst into the brain.

DISCUSSION

Since the 70's, interest from researchers has been increasing on the behavioral changes shown by infected rodents[4,8,15,19,25,27].

Several studies show changes in the behavior of rodents infected with *Toxocara canis* and *Toxoplasma gondii*. These changes can probably be considered as a means of facilitating the transmission of both parasites to their paratenic hosts by behavioral manipulation[4,7,10,12-14,20,39].

Mus musculus plays an important role in the life cycle of *Toxoplasma gondii* and *Toxocara canis*, because it can harbor cysts and larvae,

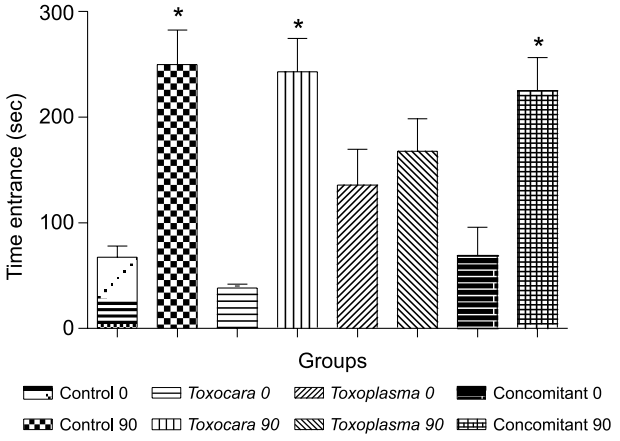


Fig. 3 - Time difference between training and test *Mus musculus* infected with *Toxocara canis* and/or *Toxoplasma gondii* in aversive avoidance test. * = difference between training and control test, *T. canis*, *T. gondii* and concomitant groups. $p < 0.05$.

respectively, in the muscles and other organs, like the central nervous system, for a long time. Moreover, these rodents are part of the food chain of definitive hosts of both parasites[23,41] and can be transmitted to their definitive host through prey-predator relationships.

COX & HOLLAND[11] suggest that the decreased levels of aggression linked to decreased levels of anxiety and lack of inhibition to open environments increases the risk of predation of these rodents and, consequently, could facilitate the transmission of parasites through a prey-predator relationship, a possible way of *Toxocara canis* and *Toxoplasma gondii* transmission, respectively, to dogs and cats.

In the present study, mice infected with *T. gondii* evaluated in the elevated plus maze were less anxious due to higher input frequency in the open arms. However, the same was neither observed in the group infected with *T. canis*, nor in the group concomitantly infected with both parasites. One hypothesis regarding the lack of significance in the behavioral data of the group infected with *T. canis* is the quantity of eggs used for the mice infection. However, QUEIROZ *et al*.[33] had already found similar results in *Rattus norvegicus* concomitantly infected by *Toxocara canis* and *Toxoplasma gondii*, suggesting occurrence of a modulation in behavioral changes when rats were concomitantly infected. On the other hand, COX & HOLLAND[12,13] found that mice with large amount of *T. canis* larvae in the brain (inoculums of 3,000, embryonated) present increased behavioral alterations than mice infected by smaller quantities of larvae.

Infection with *T. canis* and *T. gondii* can also influence the memory in rodents[24]. Several surveys conducted on rodents report the presence of *T. canis* larvae in the telencephalon and cerebellum, and cysts of *T. gondii* distributed in various brain regions, but with a higher incidence in the region of the amygdale, areas related to learning, memory, coordination and control of voluntary movements[17,35,38].

In this study, no significant differences were observed in the groups infected with *T. canis* and with concomitant infection when the short-term memory was evaluated by aversive avoidance. However, the animals infected with *T. gondii* showed a significant difference in this test. These results support the hypothesis that animals chronically infected with *T. gondii* have impaired learning and memory[31,43] and reinforces the hypothesis of QUEIROZ *et al*.[33] concerning the occurrence of modulation in the behavioral response when rodents were co-infected by both parasites.

RESUMO

Alterações comportamentais e na memória de *Mus musculus* coinfectado por *Toxocara canis* e *Toxoplasma gondii*

Pesquisadores afirmam que parasitos podem alterar o comportamento de seus hospedeiros a fim de aumentar a sua taxa de transmissão. O objetivo deste estudo foi verificar a ocorrência de alterações na ansiedade e padrões de memória de curta duração em *Mus musculus* experimentalmente infectados por *Toxocara canis* e/ou *Toxoplasma gondii*. Utilizaram-se 40 camundongos da espécie *Mus musculus* machos (Balb/c) com oito semanas de idade, divididos em quatro grupos de 10 ratos cada. Um grupo foi infectado com 300 ovos de *Toxocara canis*, um segundo grupo foi submetido à infecção com 10 cistos de *T. gondii*, um terceiro grupo foi infectado concomitantemente com ambos os parasitas e o último grupo foi mantido sem infecção. Os níveis de ansiedade foram avaliados por meio de labirinto em cruz elevado e actômetro, a memória de curta duração foi determinada por esquiva aversiva. A determinação dos níveis de ansiedade foi realizada 40 e 70 dias após infecção e a memória de curto prazo foi avaliada 140 dias após a infecção. Camundongos cronicamente infectados por *Toxoplasma gondii* mostraram deficiência de aprendizagem e memória de curto prazo, mas não foram encontradas diferenças significantes em camundongos infectados por *Toxocara canis* ou concomitantemente infectados por *Toxocara canis* e *Toxoplasma gondii* quando comparados com camundongos não infectados.

ACKNOWLEDGMENTS

Funding for this study was provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

REFERENCES

1. Adamo SA, Shocmaker KL. The effects of parasitism on the octopaminergic system of *Manduca sexta*: a possible mechanism underlying host behavioral change. Can J Zool. 2000;78:1-8.

2. Adamo, SA. Modulating the modulators: parasites, neuromodulators and host behavioral change. Brain Bahav Evol. 2002;60:370-7.

3. Barnard CJ. Parasitic relationships. In: Barnard CJ, Behnke JM, editors. Parasitism and host behaviour. London: Taylor and Francis; 1990. p. 1-33.

4. Berdoy M, Webster JP, Macdonald DW. Fatal attraction in rats infected with *Toxoplasma gondii*. Proc Biol Sci. 2000;267:1591-4.

5. Biron DG, Maché L, Ponton F, Loxdale HD, Galéotti N, Renault L, *et al*. Behavioural manipulation in a grasshopper harbouring hairworm: a proteomics approach. Proc Biol Sci. 2005;272:2117-26.

6. Burren CH. The distribution of *Toxocara canis* larvae in the central nervous system of the mouse. Trans R Soc Trop Med Hyg. 1971;65:450-3.

7. Burright RG, Donovick PJ, Dolinsky Z, Hurd Y. Behavioral changes in mice infected with *Toxocara canis*. J Toxicol Environ Health. 1982;10:621-6.

8. Chieffi PP, Aquino RTR, Paschoalotti MA, Ribeiro MC, Nasello AG. Muscular strength decrease in *Rattus norvegicus* experimentally infected by *Toxocara canis.* Rev Inst Med Trop Sao Paulo. 2009;51:73-5.

9. Chieffi PP, Aquino RTR, Paschoalotti MA, Ribeiro MCSA, Nasello AG. Behavioral change in *Rattus norvegicus* experimentally infected by *Toxocara canis* larvae. Rev Inst Med Trop Sao Paulo. 2010;52:243-6.

10. Chieffi PP, Santos SV, Queiroz ML, Lescano, SAZ. Human toxocariasis: contribution by Brazilian researchers. Rev Inst Med Trop Sao Paulo 2009;51:301-8.

11. Cox DM, Holland CV. Relationship between three intensity levels of *Toxocara canis* larvae in the brain effects on exploration, anxiety, learning and memory in the murine host. J. Helminthol. 2001;75:33-41.

12. Cox DM, Holland CV. The influence of mouse strain, infective dose and larval burden in the brain on activity in *Toxocara*-infected mice. J Helminthol. 2001;75:23-32.

13. Cox DM, Holland CV. The relationship between numbers of larvae recovered from the brain of *Toxocara canis*-infected mice and social behaviour and anxiety in the host. Parasitology. 1998;116:579-94.

14. Dolinsky ZS, Burright RG, Donovick PJ, Glickman LT, Babish J, Summer B, *et al*. Behavioral effects of lead and *Toxocara canis* in mice. Science. 1981;213(4512):1142-4.

15. Donovick PJ, Burright RG. The consequences of parasitic infection for the behavior of the mammalian host. Environ Health Perspect. 1987;73:247-50.

16. Dubey JP. Toxoplasmosis: a waterborne zoonosis. Vet Parasitol. 2004;126:57-72.

17. Good B, Holland CV, Stafford P. The influence of inoculum size and time post-infection on the number and position of *Toxocara canis* larvae recovered from the brains of outbred CD1 mice. J Helminthol. 2001;75:175-81.

18. Guaraldo L, Chagas DA, Konno AC, Kom GP, Pfiffer T, Nasello AG. Hydroalcoholic extract and fractions of *Davilla rugosa* Poiret: effects on spontaneous motor activity and elevated plus-maze behavior. J Ethnopharmacol. 2000;72:61-7.

19. Havlícek J, Gasová ZG, Smith AP, Zvára K, Flegr J. Decrease of psychomotor performance in subjects with latent "asymptomatic" toxoplasmosis. Parasitology. 2001;122:515-20.

20. Hay J, Arnott MA, Aitken PP, Kendall AT. Experimental toxocariasis and hyperactivity in mice. Z Parasitenkd. 1986;72:115-20.

21. Helluy S, Holmes JC. Serotonin, octopamine and the clinging behavior induced by the parasite *Polymorphus paradoxus* (Acanthocephala) in *Gammarus lacustris* (Crustacea). Can J Zool. 1990;68:1214-20.

22. Henriquez SA, Brett R, Alexander J, Pratt J, Roberts CW. Neuropsychiatric disease and *Toxoplasma gondii* infection. Neuroimmunomodulation. 2009;16:122-33.

23. Holland CV, Cox DM. *Toxocara* in the mouse: a model for parasite-altered host behaviour? J Helminthol. 2001;75:125-35.

24. Holland CV, Hamilton CM. The significance of cerebral toxocariasis: a model system for exploting the link between brain involvement, behaviour and the immune response. J Exp Biol. 2013; 216(PT 1):78-83.

25. Holmes JC, Bethel WM. Modification of intermediate host behaviour by parasites. *In:* Canning EU, Wright CA, editors. Behavioural aspects of parasite transmission. London: Academic Press; 1972. p. 123-49.

26. House PK, Vyas A, Sapolsky R. Predator cat odors activate sexual arousal pathways in brains of *Toxoplasma gondii* infected rats. PLoS ONE. 2011;6:e23277. doi:10.1371/journal.pone.0023277.

27. Keymer AE, Read AF. Behavioral ecology: the impact of parasitism. *In:* Toft CA, Aeschlimann A, Bolis L, editors. Parasite-host associations: coexistence or conflict? Oxford: Oxford University Press; 1991. p. 37-61.

28. Korte SM, De Boer SF. A robust animal model of state anxiety: fear-potentiated behaviour in the elevated plus-maze. Eur J Pharmacol. 2003;463:163-75.

29. Nasello AG, Machado C, Bastos JF, Felício LF. Sudden darkness induces a high activity-low anxiety state in male and female rats. Phisiol Behav. 1998;63:451-4.

30. Pellow S, File SE. Anxiolytic and anxiogenic drug effects on exploratory activity in elevated plus-maze: a novel test of anxiety in the rat. Pharmacol Biochem Behav. 1986;24:525-9.

31. Piekarski G, Zippelius HM, Witting PA. Auswirkungen einer letenten *Toxoplasma* infection auf das Lernvermogen von weiben Laboratoriumsratten und mausen. Z Parasitenkd. 1978;57:1-15.

32. Poulin R. The evolution of parasite manipulation of host behaviour: a theoretical analysis. Parasitology. 1994;109(Suppl):S109-18.

33. Queiroz ML, Viel, TA, Papa CHG, Lescano SAZ, Chieffi PP. Behavioral changes in *Rattus norvegicus* coinfected by *Toxocara canis* and *Toxoplasma gondii*. Rev Inst Med Trop Sao Paulo. 2013;55:51-3.

34. Silva MRP, Bernardi MM, Nasello AG, Felício LF. Influence of lactation on motor activity and elevated plus-maze behavior. Braz J Med Bio Res. 1997;30:241-4.

35. Skallová A, Kodym P, Frynta D, Flegr J. The role of dopamine in *Toxoplasma*-induced behavioural alterations in mice: an ethological and ethopharmacological study. Parasitology. 2006;133:525-35.

36. Thomas F, Schimidt-Rhaesa A, Martin G, Manu C, Durand P, Renaud F. Do hairworms (Nematomorpha) manipulate the water seeking behaviour of their terrestrial host? J Evol Biol. 2002;15:356-61.

37. Thomas F, Ulitsky P, Augier R, Dusticier N, Samuel D, Strambi C, *et al*. Biochemical and histological changes in the brain of the criket *Nemobius sylvestris* infected by the manipulative parasite *Paragordius tricuspidatus* (Nematomorpha). Int J Parasitol. 2003;33:435-43.

38. Vyas A, Pillai AG, Chattarji S. Recovery after chronic stress fails to reverse amygdaloid neuronal hypertrophy and enhanced anxiety-like behavior. Neuroscience. 2004;128:667-73.

39. Vyas A, Kim SK, Giacomini N, Boothroyd JC, Sapolsky RM. Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. Proc Natl Acad Sci USA. 2007;104:6442-7.

40. Vyas A, Sapolsky R. Manipulation of host behaviour by *Toxoplasma gondii*: what is the minimum a proposed proximate mechanism should explain? Folia Parasitol (Praha). 2010;57:88-94.

41. Webster JP. Rats, cats, people and parasites: the impact of latent toxoplasmosis on behaviour. Microbes Infect. 2001;3:1037-45.

42. Webster JP. The effect of *Toxoplasma gondii* on animal behavior: playing cat and mouse. Schizophr Bull. 2007;33:752-6.

43. Witting PA. Learning capacity and memory of normal and *Toxoplasma*-infected laboratory rats and mice. Z Parasitenkd. 1979;61:29-51.

44. Xi WG, Jin LZ. A novel method for the recovery of *Toxocara canis* in mice. J Helminthol. 1998;72:183-4.

Received: 27 November 2013
Accepted: 14 March 2014

Rev. Inst. Med. Trop. Sao Paulo
56(4):357-360, July-August, 2014
doi: 10.1590/S0036-46652014000400015

BRIEF COMMUNICATION

MOLECULAR DETECTION OF *Leishmania* IN PHLEBOTOMINE SAND FLIES IN A CUTANEOUS AND VISCERAL LEISHMANIASIS ENDEMIC AREA IN NORTHEASTERN BRAZIL

Vanessa Cristina Fitipaldi Veloso GUIMARÃES(1), Pietra Lemos COSTA(1), Fernando José da SILVA(1), Fábio Lopes de MELO(2), Filipe DANTAS-TORRES(1,3), Eduardo Henrique Gomes RODRIGUES(1) & Sinval Pinto BRANDÃO-FILHO(1)

SUMMARY

Several phlebotomine sand fly species have been regarded as putative or proven vectors of parasites of the genus *Leishmania* in Brazil, but data for the northeastern region remains incipient. In this study, a total of 600 phlebotomine sand flies were grouped in pools of 10 specimens each and tested by a *Leishmania* genus-specific PCR and by a PCR targeting *Leishmania* (*Leishmania*) *infantum*. Fourteen out of 60 pools were positive by the genus-specific PCR, being five pools of *L. migonei*, seven of *L. complexa*, one of *L. sordellii* and one of *L. naftalekatzi*, which correspond to a minimal infection rate of 2.3% (14/600). Our results, associated with their known anthropophily and their abundance, suggest the participation of *L. migonei* and *L. complexa* as vectors of *Leishmania* in northeastern Brazil. Remarkably, this is the first time in this country that the detection of *Leishmania* DNA in *L. sordellii* and *L. naftalekatzi* has been reported, but future studies are necessary to better understand the significance of these findings.

KEYWORDS: Phlebotomine sand flies; *Leishmania*; Transmission.

Leishmania parasites are the causative agents of leishmaniasis, a group of diseases with a broad clinical spectrum, which may be present in cutaneous and visceral forms, according to the parasite species involved and the host response to infection. Brazil represents one of the largest foci of leishmaniasis from a worldwide perspective[2], with several thousand cases notified every year and sporadic outbreaks reported in both rural and urban centers.

The most important causative agents of American cutaneous leishmaniasis (ACL) and zoonotic visceral leishmaniasis (VL) are *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *infantum* (syn. *L. chagasi*), respectively. Their transmission occurs as the result of the bite of phlebotomine sand fly females, such as *Lutzomyia whitmani*, *L. intermedia*, *L. migonei*, *L. wellcomei* and *L. complexa* for *L.* (*V.*) *braziliensis* and *L. longipalpis* for *L.* (*L.*) *infantum*[13]. Indeed, several phlebotomine sand flies have been regarded as putative or proven vectors of *L.* (*V.*) *braziliensis* in Brazil[11,13], but studies have been limited to particular regions of the country and data from northeastern Brazil remains incipient.

ACL is the most prevalent form of leishmaniasis in Pernambuco State, northeastern Brazil[9]. Since the 1980s, the number of VL cases has been on the rise and a 5-fold increase in the number of municipalities that report one or more VL cases was recorded between 1990 and 2001[6]. It means that both forms of leishmaniasis are widespread in Pernambuco, with overlapping distribution in some areas, which provides us with the opportunity to study the diversity of potential vectors for both *L.* (*V.*) *braziliensis* and *L.* (*L.*) *infantum* in sympatric areas. In this perspective, we have recently conducted an entomological survey to study the fauna of phlebotomine sand flies in an area where ACL and VL occur in sympatry in northeastern Brazil[9]. Herein, we assessed the infection by *Leishmania* spp. in phlebotomine sand flies collected in the framework of our previous study.

The study was carried out in a rural community located in the municipality of São Vicente Férrer (07°35'27"S, 35°29'27"'W) in the northern rainforest area of Pernambuco, where both visceral and cutaneous leishmaniasis are endemic. The municipality has an area of 110,489 sq km and an estimated population of 17,000 inhabitants. The climate is tropical with a mean average annual temperature of 23 °C. Throughout the years, this area has been affected by an intense process of deforestation and the primary vegetation was largely substituted by banana tree plantations and rural properties, in which the presence of animal shelters (e.g., chicken coops and stables) near remnants of Atlantic rainforest is commonly observed.

Insects were captured monthly from September 2009 to September 2010 using CDC light traps (33 traps per month on average), from 18:00

(1) Department of Immunology, Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz (Fiocruz), Recife, Pernambuco, Brazil. E-mails: vanessa@cpqam.fiocruz.br, lemos.pietra@gmail.com, fs@cpqam.fiocruz.br, filipe.vet@globo.com, eduardoh@cpqam.fiocruz.br, sinval@cpqam.fiocruz.br
(2) Department of Parasitology, Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz (Fiocruz), Recife, Pernambuco, Brazil. E-mail: fabio@cpqam.fiocruz.br
(3) Department of Veterinary Medicine, University of Bari, Valenzano, Bari, Italy.
Correspondence to: Dr. Sinval Pinto Brandão Filho. Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Caixa Postal 7472, Av. Moraes Rego s/n, 50670-420 Recife, PE, Brasil. Phone: 55 81 2101-2562. E-mail: sinval@cpqam.fiocruz.br

to 6:00, during four consecutive nights. Traps were placed either in the peridomicile or forest remnants, and the specimens captured were identified morphologically[16]. In total, 600 unfed female phlebotomine sand flies belonging to four species (*L. migonei*, *L. complexa*, *L. sordellii* and *L. naftalekatzi*) were grouped in pools of 10 specimens each and stored at -70 °C until DNA extraction.

DNA extraction was effected as described elsewhere[1] with some modifications. Pools of insects were macerated in 1.5 mL tubes containing one mL of PBS, which were then centrifuged at 12000 *g* for two min. After removing the supernatant, 100 µL of lysis solution type 1 (GenomicPrep Cells and Tissue DNA isolation kits, GE Healthcare, Piscataway, NJ, USA) were added and the tubes shaken vigorously for 15 s. After adding 10 µL of proteinase K (30 mg/mL) the tubes were shaken again for 15 s and incubated at 56 ºC for one hour. Then, the suspension was once again incubated at 70 °C for 10 min and then centrifuged at 6000 *g* for 10 min. The supernatants were transferred to 1.5 mL tubes and frozen in absolute ethanol (in a volume twice as much the supernatant recovered). The material was stored at -20 ºC for 18 h and, after that, the tubes were centrifuged at 6500 *g* for five min and after discharging the supernatants, the sediments were dried at room temperature. The extracted DNA was re-suspended in 50 µL 0.1 × TE buffer (pH 8.0) at 70 °C and stored at -20 ºC. The concentration and purity of DNA samples were assessed using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech).

The quality of DNA samples was assessed by PCR using the primers 5Llcac (5'-GTGGCCGAACATAATGTTAG-3') and 3Llcac (5'-CCACGAACAAGTTCAACATC-3')[10], which amplify a 220 bp fragment of the cacophony gene of *Lutzomyia* spp[3]. PCR products were resolved on 1.5% agarose gel and visualized by ethidium bromide staining.

For *Leishmania* spp. detection, we used the primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3')[7], which amplifies the internal transcription spacer 1 (ITS-1), a noncoding region placed at SSUrRNA, bounded by the genes 18S and 5.8S, which produce a 300-350 bp fragment of *Leishmania* spp. Amplification reactions were performed in a 50 µL volume containing 100 mM Tris-HCl, 250 mM KCl, 2.5 mM $MgCl_2$, 250 µM dNTPs, 50 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 2 µL of the template DNA. The amplification conditions consisted of 35 cycles of 95 °C for 40 s, 53 °C for 45 s and 72 °C for one min, which were preceded of initial denaturation of 95 °C for three min DNA extracted from *L.* (*V.*) *braziliensis* promastigotes (MHOM/BR/75/M2903) was used as positive control. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining, using 100 bp ladder DNA (GibcoBRL-Life Technologies) as a molecular marker.

All samples were also tested by a PCR targeting the kDNA of *L.* (*L.*) *infantum* using the primers Linf 1B 23F (5'-TCCCAAACTTTTCTGGTCCT-3') and Linf 1B 154R (5'-TTACACCAACCCCCAGTTTC-3')[12]. The reaction was carried out in a 25 µL final volume containing 10 mM Tris-HCl, 50 nM KCl, 1.5 $MgCl_2$ 0.2 mM dNTPs, 5 pmol of each primer, 2.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 2 µL of the template DNA. Amplification conditions consisted of 35 cycles of 94 °C for 30 s, 67 °C for one min and 72 °C for 30 s, which were preceded by initial denaturation of 94 °C for one min. DNA extracted from *L.* (*L.*) *infantum* promastigotes (MHOM/BR/1974/PP75) was used as positive control. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining, using 100 bp ladder DNA (GibcoBRL-Life Technologies) as a molecular marker.

All 60 pools of phlebotomine sand flies belonging to four species (*Lutzomyia migonei*, *Lutzomyia complexa*, *Lutzomyia sordellii*, and *Lutzomyia naftalekatzi*) were analyzed and all samples were positive for the fragment of 220 bp corresponding to cacophony gene, confirming the quality of the DNA obtained (Fig. 1). A total of 14 pools were positive by *Leishmania* genus-specific PCR, being five pools of *L. migonei*, seven of *L. complexa*, one of *L. sordellii* and one of *L. naftalekatzi* (Fig. 2), which corresponds to a minimal infection rate of 2.3% (14/600) (Table 1).

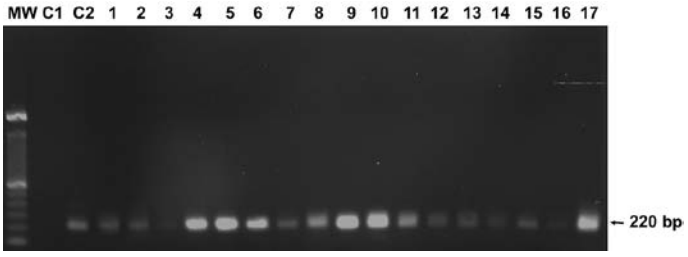


Fig. 1 - Agarose gel electrophoresis showing PCR amplification of cacophony gene IVS6 region of phlebotomine sand flies. MW: molecular weight marker (100 bp DNA Ladder); C_1: no DNA; C_2: pools of phlebotomine sand flies (positive control); lanes 1-17: positive samples of phlebotomine sand flies.

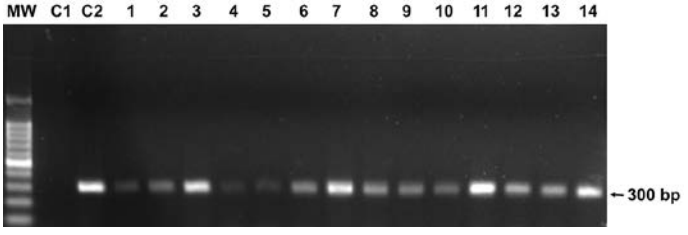


Fig. 2 - Agarose gel electrophoresis stained showing PCR amplification of *Leishmania* spp. DNA from phlebotomine sand flies. MW: molecular weight marker (100 bp DNA Ladder); C_1: no DNA; C_2: *L.* (*V.*) *braziliensis* DNA (positive control); lanes 1-5: *L. migonei* samples; lane 6-12: *L. complexa*; lane 13: *L. sordellii*; lane 14: *L. naftalekatzi*.

Table 1
Positivity of *Leishmania* DNA in phlebotomine sand flies species tested by the *Leishmania* genus-specific polymerase chain reaction (PCR), Northeastern Brazil

Species	Infected specimens/ total specimens tested	Minimum infection rate (%)[a]	Collection site
Lutzomyia migonei	5/190	2.63	Animal shelters
Lutzomyia complexa	7/370	1.89	Forest remnant
Lutzomyia sordellii	1/20	5.0	Forest remnant
Lutzomyia naftalekatzi	1/20	5.0	Forest remnant
Total	14/600	2.33	

[a]Minimum infection rate (MIR) = [(minimum number of infected specimens/total specimens tested) x 100].

Lutzomyia migonei has been implicated as a vector of *L.* (*V.*) *braziliensis* in different Brazilian regions[13]. This phlebotomine sand fly is widespread in Brazil, including in northeastern Brazil. In our study, *L. migonei* was the most abundant species (71.8%), being found both indoors and in the peridomicile, especially in animal shelters (chicken pen and stables)[9]. In southeastern Brazil, it has also been found naturally infected by *L.* (*V.*) *braziliensis* and regarded as a possible secondary vector[13]. *Lutzomyia migonei* has also been considered to be a putative vector of *L.* (*L.*) *infantum*[11], including in the area where the present study was carried out, where the primary vector (*L. longipalpis*) is absent[5]. In a study conducted in Rio de Janeiro near 10 years ago, the absence of *L. longipalpis* in six areas where VL is endemic provided circumstantial evidence for the participation of *L. migonei* as a vector of *L.* (*L.*) *infantum*[14]. In the present study, all specimens of *L. migonei* were negative for *L.* (*L.*) *infantum*, which strongly indicates that they were infected by *L.* (*V.*) *braziliensis*.

The positivity of *L. complexa* to *Leishmania* sp. and negativity to *L.* (*L.*) *infantum* is in line with previous studies and indicates the participation of this species as a vector of *L.* (*V.*) *braziliensis* in forested environments in different Brazilian regions[13]. In fact, this species predominates in areas of the Atlantic rainforest and it displays strong anthropophilic behavior. On the other hand, the detection of *Leishmania* DNA in pools of *L. sordellii* and *L. naftalekatzi* has been reported, for the first time, in Brazil. Recently, females of *L. sordellii* were dissected and flagellates were seen in two of them, but an attempt to isolate the parasite failed[4]. *L. sordellii* is widespread in the country being found in many habitats, such as tree trunks, rock crevices, caves, animal shelters (e.g. chicken coops, pigsties, corrals) and households of different areas of Brazil[9]. However, *L. sordellii* is known to feed on cold-blooded rather than warm-blooded vertebrates[15], which probably indicates that it plays no role in the transmission of *Leishmania* parasites. Conversely, *L. naftalekatzi* apparently has a more restricted distribution[8] and there is limited information on its biology, including feeding habits, but so far there is no evidence suggesting its participation in the transmission of *Leishmania* parasites. In this regard, further studies with a larger number of specimens would be interesting to assess the actual prevalence of *Leishmania* infection in these phlebotomine sand flies, but also to identify the parasite species they carry.

The high degree of anthropophily of *L. migonei* and its overlapping distribution with ACL cases may suggest its participation in the zoonotic transmission cycle of ACL in the study area. In the same way, our data suggests the involvement of *L. complexa* in the enzootic cycle of *L.* (*V.*) *braziliensis*, mainly considering its close association with forested environments. For future studies, it will be important to focus on the isolation and characterization of *Leishmania* parasites in the aforementioned phlebotomine species, as well as to assess their feeding source, as this information might be of great epidemiological relevance.

RESUMO

Detecção molecular de *Leishmania* spp. em flebotomíneos em área endêmica para leishmaniose tegumentar e visceral no nordeste do Brasil

Vários flebotomíneos têm sido considerados como possíveis vetores suspeitos ou comprovados de parasitas do gênero *Leishmania* no Brasil, mas os dados para região nordeste continuam incipientes. Neste estudo, 600 flebotomíneos foram agrupados em grupos de 10 espécimes e testados por uma PCR específica para o gênero *Leishmania* e por outra PCR para *Leishmania* (*Leishmania*) *infantum*. Quatorze dos 60 grupos foram positivos por PCR gênero-específica, sendo cinco de *L. migonei*, sete *L. complexa*, um de *L. sordellii* e um de *L. naftalekatzi*, o que corresponde a uma taxa mínima de infecção de 2,3% (14/600). Nossos resultados, associados à antropofilia e abundância dessas espécies, sugerem a participação de *L. migonei* e *L. complexa* como vetores de *Leishmania* no nordeste do Brasil. Notavelmente, a detecção de DNA de *Leishmania* em *L. sordellii* e *L. naftalekatzi* é relatada pela primeira vez no Brasil, mas futuros estudos são necessários para compreender melhor o significado desses achados.

ACKNOWLEDGMENTS

Thanks to Kyldman Thais da Silva, Kamila Gaudêncio da Silva and Rafael Acioli Medeiros for their support with some laboratory work.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This research was supported by Fundação de Amparo à Pesquisa de Pernambuco (FACEPE), APQ-0630-2.13/08.

REFERENCES

1. Adamson RE, Ward RD, Feliciangeli MD, Maingon R. The application of random amplified polymorphic DNA for sandfly species identification. Med Vet Entomol. 1993;7:203-7.

2. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, *et al*. Leishmaniasis worldwide and global estimates of its incidence. PLos One. 2012;7:1-12.

3. Bottecchia M, Oliveira SG, Bauzer LG, Souza NA, Ward RD, Garner KJ, *et al*. Genetic divergence in the *cacophony* IVS6 intron among five Brazilian populations of *Lutzomyia longipalpis*. J Mol Evol. 2004;58:754-61.

4. Carvalho GML, Brazil RP, Saraiva L, Quaresma PF, Botelho HA, Ramos MCNF, *et al*. Hourly activity and natural infection of sandflies (Diptera: Psychodidae) captured from the aphotic zone of a cave, Minas Gerais State, Brazil. Plos One. 2012;7:1-6.

5. Carvalho MR, Valença HF, Silva FJ, Pita-Pereira D, Pereira TA, Britto C, *et al*. Natural *Leishmania infantum* infection in *Migonemyia migonei* (Franca, 1920) (Diptera: Psychodidae: Phlebotominae) the putative vector of visceral leishmaniasis in Pernambuco State, Brazil. Acta Trop. 2010;116:108-10.

6. Dantas-Torres F, Brandão-Filho SP. Expansão geográfica da leishmaniose visceral no estado de Pernambuco. Rev Soc Bras Med Trop. 2006;39:352-6.

7. El Tai NO, El Fari M, Mauricio I, Miles MA, Oskam L, El Safi S, *et al*. *Leishmania donovani*: intraspecific polymorphisms of Sudanese isolates revealed by PCR based analyses and DNA sequencing. Exp Parasitol. 2001;97:35-44.

8. Falcão AL, Andrade-Filho JD, Almeida FA, Brandão-Filho SP. *Lutzomyia naftalekatzi*, a new species of phlebotomine sand fly (Diptera: Psychodidae) from Zona da Mata region, Pernambuco, Brazil. Mem Inst Oswaldo Cruz. 2000;95:843-8.

9. Guimarães VCFV, Costa PL, Silva FJ, Silva KT, Silva KG, Araújo AIF, *et al*. Phlebotomine sand flies (Diptera: Psychodidae) in São Vicente Férrer, a sympatric area to cutaneous and visceral leishmaniasis in Pernambuco, Brazil. Rev Soc Bras Med Trop. 2012;45:66-70.

10. Lins RM, Oliveira SG, Souza NA, de Queiroz RG, Justiniano SC, Ward RD, *et al*. Molecular evolution of the cacophony IVS6 region in sandflies. Insect Mol Biol. 2002;11:117-22.

11. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Med Vet Entomol. 2013;27:123-47.

12. Paiva-Cavalcanti M, Felinto de Brito ME, de Souza WV, de Miranda Gomes Y, Abath FG. The development of a real-time PCR assay for the quantification of *Leishmania infantum* DNA in canine blood. Vet J. 2009;189:356-8.

13. Rangel EF, Lainson R. Proven and putative vectors of American cutaneous leishmaniasis in Brazil: aspects of their biology and vectorial competence. Mem Inst Oswaldo Cruz. 2009;104:937-54.

14. Souza MB, Marzochi MCA, Carvalho RW, Ribeiro PC, Pontes CS, Caetano JM, *et al*. Ausência da *Lutzomyia longipalpis* em algumas áreas de ocorrência de leishmaniose visceral no município do Rio de Janeiro. Cad Saúde Pública. 2003;19:1881-5.

15. Tesh RB, Chaniotis BN, Aronson MD, Johnson KM. Natural host preferences of Panamanian phlebotomine sandflies as determined by precipitin test. Am J Trop Med Hyg. 1971;20:150-6.

16. Young DG, Duncan MA. Guide to the identification and geographic distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). Mem Am Entomol Inst. 1994;54:1-881.

Received: 13 August 2013
Accepted: 10 February 2014

Rev. Inst. Med. Trop. Sao Paulo
56(4):361-362, July-August, 2014
doi: 10.1590/S0036-46652014000400016

CASE REPORT

RESOLUTION OF CUTANEOUS LEISHMANIASIS AFTER ACUTE ECZEMA DUE TO INTRALESIONAL MEGLUMINE ANTIMONIATE

Erica de Camargo Ferreira e VASCONCELLOS(1), Maria Inês Fernandes PIMENTEL(1), Cláudia Maria VALETE-ROSALINO(1,2), Maria de Fátima MADEIRA(1) & Armando de Oliveira SCHUBACH(1,3)

SUMMARY

We report a case of a 42 year-old female, who came to a leishmaniasis reference center in Rio de Janeiro, Brazil, presenting a cutaneous leishmaniasis lesion in the right forearm. Treatment with low-dose intramuscular meglumine antimoniate (MA) (5 mg Sb^{5+}/kg/day) was initiated, with improvement after 28 days, although with the development of generalized eczema. After 87 days, the lesion worsened. Patient refused treatment with amphotericin B. MA was then infiltrated in the lesion, in two sessions, resulting in local eczema, with bullae formation; however, twenty days after, both the ulcer and eczema receded. Intralesional administration of MA should be used carefully when previous cutaneous hypersensitivity is detected.

KEYWORDS: Cutaneous leishmaniasis; Therapy; Intralesional; Meglumine antimoniate; Eczema.

CASE REPORT

American tegumentary leishmaniasis (ATL) is an infectious disease caused by protozoa of *Leishmania* genus, transmitted by female sandflies (Phlebotominae). Few medications are efficient in its treatment, among them meglumine antimoniate (MA). Due to the difficulties linked to the administration of MA, less toxic alternative therapies with low doses or intralesional injections (IL) of MA have been studied[5,6]. We report a case of a patient who presented resolution of cutaneous leishmaniasis (CL) after development of acute eczema in the site of the injection of MA.

A 42 year-old white female, who signed a free informed consent form, came to the Leishmaniasis Surveillance Laboratory, Evandro Chagas Clinical Research Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, presenting a CL lesion in the right forearm for 45 days. Imprint of the lesion showed amastigotes parasites, and *Leishmania (Viannia) braziliensis* was identified from the culture of a fragment of the lesion obtained through biopsy. Mucosal lesions were not seen in fiber optic otorhinolaryngological examination of the upper airways and digestive tract. Treatment with low-dose intramuscular (IM) MA - 5 mg Sb^{5+} per kilogram per day - was initiated (Aventis, São Paulo, Brazil). After a 28-day treatment, she improved. However, she presented generalized eczema, treated with oral dexclorpheniramine and dexametasone ointment. After 87 days, the ulcer worsened (Fig. 1A). The second choice drug, amphotericin B desoxycolate, was offered to the patient, but she refused to be hospitalized and reported difficulties in going to the hospital three times a week to receive the medication in a day-hospital regimen. Alternative drugs such as pentamidine and liposomal amphotericin B were not available. Treatment was started with 7 mL of MA, without any diluents, injected into the lesion edge until its whole base got infiltrated. She developed moderate local eczema. Oral dexchlorpheniramine was prescribed and the ulcer improved. After 13 days, a second IL MA injection was made, with 5 mL of this drug, and the eczema worsened, with bullae formation (Fig. 1B); however, twenty days after, both ulcer and eczema receded (Fig. 1C).

IL way of administration is a viable alternative to systemic MA in older people or in conditions in which systemic treatment is not tolerated, due to its efficacy and infrequent, slight to moderate adverse events[5,6]. Local therapy was considered unsuitable for the treatment of New World cutaneous leishmaniasis caused by *L. (V.) braziliensis* or *L. (V.) panamensis* because of the potential risk of mucosal metastasis; however, as systemic treatment does not guarantee prevention of later mucocutaneous leishmaniasis, which is found in less than 5% of the cases, local treatments should be explored. It is now considered acceptable to use local therapy in selected cases of New World cutaneous leishmaniasis. The World Health Organization states that in patients with mild disease or with comorbid conditions, treatments safer than systemic therapy should be preferred, even if the level of evidence for efficacy is weak[7].

(1) Leishmaniasis Surveillance Laboratory, Evandro Chagas Clinical Research Institute, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil.
(2) Otorhinolaryngology and Ophthalmology Department, Rio de Janeiro Federal University, Rio de Janeiro, RJ, Brazil
(3) Productivity scholarship from Scientific and Technology National Council (CNPq), Brasília, Brazil; and Our State Scientist from Carlos Chagas Filho Foundation for the Research Support in Rio de Janeiro State (FAPERJ).
Correspondence to: Érica de Camargo Ferreira e Vasconcellos, Av. Brasil 4365, Manguinhos, 21040-360 Rio de Janeiro, RJ, Brasil. Phone: +55 21 3865-9541. E-mail: erica.vasconcellos@ipec.fiocruz.br

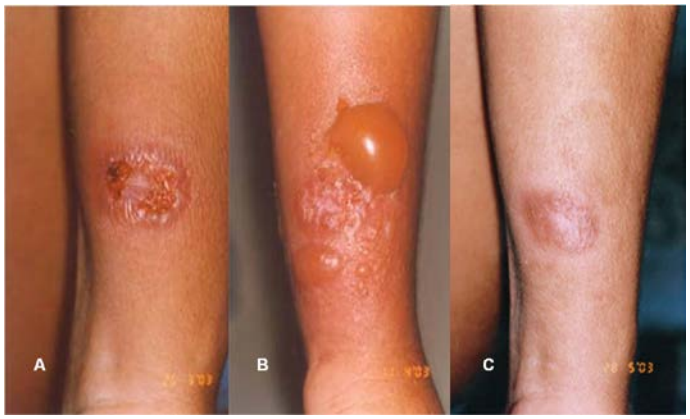


Fig. 1 - Lesion's follow up: **A -** Ulcerated lesion, before the first intralesional infiltration, measuring 20 x 10 mm, with infiltrated erythematous borders, partially covered by crusts and with an epithelialized center of about 5 mm. **B** - Edema of the forearm and hand, after the second intralesional infiltration of the lesion with meglumine antimoniate. Infiltration, erythema and coalescing vesicles, turning into larger bubbles of up to 35 mm. **C** - Atrophic scar, with erythema, residual hyperpigmentation on the spot where the biggest bubble was located (49 days after the second intralesional meglumine antimoniate).

ASTE *et al.* (1998) had a successful experience with this local therapy, with 11% of the patients presenting mild reactions, such as erythema or pruritus, without systemic adverse effects. Patients didn't need to interrupt the treatment[1].

CORDOBA *et al.* (2012) described a series of 70 patients with cutaneous leishmaniasis, treated with weekly intralesional infiltrations of MA (Glucantime®). Nine of them developed infiltrated itchy erythematous and vesiculous plaques at the injection sites. After cutaneous tests, they concluded that type IV hypersensitivity could be involved in the mechanism of the cutaneous reaction[3].

Some other diseases have been occasionally treated with local drugs that induce hypersensitivity reactions; resolution of warts with dinitrochlorobenzene (DNCB) is well documented[4]. In an animal model with guinea pigs, DNCB inhibited the development of leishmaniasis cutaneous lesions, but only when applied in the infection sites[2].

Probably, hypersensitivity to MA was involved in the resolution of the lesion in our patient. Although the response to treatment in the present case was excellent, the great intensity of the eczematous local cutaneous reaction is indicative that this way of administration should be used carefully as an alternative to systemic MA treatment, when previous cutaneous hypersensitivity is detected.

RESUMO

Resolução de leishmaniose cutânea após eczema agudo devido a antimoniato de meglumina intralesional

Relatamos caso de paciente de 42 anos atendida em centro de referência em leishmanioses no Rio de Janeiro, Brasil, apresentando lesão de leishmaniose cutânea no antebraço direito. Iniciado tratamento com baixa dose de antimoniato de meglumina (AM) intramuscular (5 mg Sb^{5+}/kg/dia), houve melhora após 28 dias, porém com desenvolvimento de eczema generalizado. Após 87 dias, notou-se piora da lesão. A paciente recusou o tratamento com anfotericina B. Infiltrou-se AM na lesão em duas sessões, resultando em eczema local com bolhas. Entretanto, 20 dias depois, tanto a úlcera quanto o eczema regrediram. A administração intralesional do AM deve ser utilizada com cautela em pacientes com hipersensibilidade cutânea a este fármaco.

ACKNOWLEDGMENTS

This study was supported by IPEC/FIOCRUZ.

CONFLICT OF INTEREST

There is no conflict of interest regarding any of the authors.

AUTHORS AND CONTRIBUTORS

Érica de Camargo Ferreira e Vasconcellos: literature search, data collection, figures, writing. Maria Inês Fernandes Pimentel: literature search, data collection, figures, writing. Cláudia Maria Valete-Rosalino: patient assistance (otorhinolaryngological), writing. Maria de Fátima Madeira: patient assistance (laboratorial diagnosis and species characterization), writing. Armando de Oliveira Schubach: literature search, figures, patient assistance (dermatological), writing.

REFERENCES

1. Aste N, Pau M, Ferreli C, Biggio P. Intralesional treatment of cutaneous leishmaniasis with meglumine antimoniate. Br J Dermatol. 1998;138:370-1.

2. Behin R, Mauel J, Rowe DS. Mechanisms of protective immunity in experimental cutaneous leishmaniasis of the guinea-pig. III. Inhibition of leishmanial lesion in the guinea-pig by delayed hypersensitivity reaction to unrelated antigens. Clin Exp Immunol. 1977;29:320-5.

3. Córdoba S, Gandolfo Cano M, Aguado M, Huerta-Brogera M, Romero A, Martínez-Morán C, *et al.* Delayed allergic skin reactions due to intralesional meglumine antimoniate therapy for cutaneous leishmaniasis. Allergy. 2012;67:1609-11.

4. Kwok CS, Gibbs S, Bennett C, Holland R, Abbott R. Topical treatments for cutaneous warts. Cochrane Database Syst Rev. 2012;9:CD001781.

5. Oliveira-Neto MP, Schubach A, Mattos M, da Costa SC, Pirmez C. Intralesional therapy of American cutaneous leishmaniasis with pentavalent antimony in Rio de Janeiro, Brazil - an area of *Leishmania (V.) braziliensis* transmission. Int J Dermatol. 1997;36:463-8.

6. Vasconcellos EC, Pimentel MI, Schubach AO, de Oliveira RV, Azeredo-Coutinho RB, Silva FC, *et al.* Intralesional meglumine antimoniate for treatment of cutaneous leishmaniasis patients with contraindication to systemic therapy from Rio de Janeiro (2000 to 2006). Am J Trop Med Hyg. 2012;87:257-60.

7. World Health Organization. Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010. Geneva: WHO; 2010. (WHO Technical Report Series; no. 949)

Received: 2 August 2013
Accepted: 14 November 2013

Rev. Inst. Med. Trop. Sao Paulo
56(4):363-365, July-August, 2014
doi: 10.1590/S0036-46652014000400017

CASE REPORT

MOLECULAR IDENTIFICATION OF *Bartonella henselae* IN A SERONEGATIVE CAT SCRATCH DISEASE PATIENT WITH AIDS IN RIO DE JANEIRO, BRAZIL

Alexsandra R.M. FAVACHO(1), Isabelle ROGER(2), Amanda K. AKEMI(3), Adonai A. PESSOA JR.(1), Andrea G. VARON(2), Raphael GOMES(1), Daniela T. GODOY(1), Sandro PEREIRA(3) & Elba R.S. LEMOS(1)

SUMMARY

Bartonella henselae is associated with a wide spectrum of clinical manifestations, including cat scratch disease, endocarditis and meningoencephalitis, in immunocompetent and immunocompromised patients. We report the first molecularly confirmed case of *B. henselae* infection in an AIDS patient in state of Rio de Janeiro, Brazil. Although DNA sequence of *B. henselae* has been detected by polymerase chain reaction in a lymph node biopsy, acute and convalescent sera were nonreactive.

KEYWORDS: *Bartonella henselae*; Cat scratch disease; Human immunodeficiency virus; Molecular diagnosis; Rio de Janeiro; Brazil.

INTRODUCTION

Bartonella species are small, fastidious, Gram-negative, rod-shaped bacteria that are associated with infections in immunocompetent and immunocompromised patients. There are more than 22 species so far described in the *Bartonella* genus, with *Bartonella henselae*, *B. bacilliformis*, and *B. quintana* accounting for the majority of human cases. *Bartonella henselae*, the main agent of the cat scratch disease (CSD), is also associated with a wide variability in clinical manifestations, including Parinaud's oculoglandular syndrome, endocarditis, meningoencephalitis, disseminated disease, and fever of unknown origin[2,9]. Immunocompromised hosts, particularly severely immunocompromised patients infected with HIV, are more susceptible to infection from *B. henselae*, and the clinical course may be more severe[9]. The transmission of *B. henselae* from cats to humans frequently occurs through direct or indirect scratches, bites, or licks from infected cats and probably from the bite of an arthropod vector, *Ctenocephalides felis*, the cat flea[2].

We report the diagnosis of *B. henselae* infection using molecular methods in an HIV-positive patient who was negative to the gold standard serologic tests.

CASE REPORT

A 34-year-old man infected with human immunodeficiency virus (HIV) from the Municipality of Rio de Janeiro, Brazil, was admitted to hospital in August 2011. Upon arrival, the patient was uncomfortable and febrile (39.5 °C) with a cluster of warm, red, enlarged, tender unilateral lymph nodes on the right epitrochanteric (> 10 cm), axillar, supraclavicular, periauricular, and posterior cervical chain (Fig. 1). A discrete nonpruritic rash was noted on the torso and abdomen. The patient had been scratched on the abdomen and bitten on the thumb by his 7-month-old kitten seven days before. He also mentioned numerous fleabites prior to the start of his symptoms and that his kitten spent most of the time outdoors in contact with roaming cats.

Diagnosis of HIV seropositivity had been made two years previously. The CD4 count was 244 cells/mm^3 with a viral load of 215,857 copies/mm^3 at admission. Other laboratory data revealed biochemical panel and blood count within the normal range, elevated C-reactive protein, cultures from tissue biopsy and multiple blood cultures were sterile for aerobic and anaerobic bacteria, mycobacteria and fungi. The patient was positive for toxoplasmosis (IgG), but the serology studies for viral hepatitis, cytomegalovirus, and syphilis were negative.

CSD was suspected, but serum samples collected on days 11 and 19 of the illness were nonreactive to *B. henselae,* using a commercial kit of indirect immunofluorescence assay (IFA) for class-specific IgG antibody (Bion®, USA). In addition, the serum from the patient's cat was submitted for IFA and was *B. henselae* reactive (titer of 64). Histopathological examination of the posterior cervical node revealed a granulomatous inflammation and neutrophilic microabscesses. No organisms were observed using Gram, Ziehl-Neelson, and Warthin-Starry silver stains.

(1) Laboratório de Hantaviroses e Rickettsioses, Instituto Oswaldo Cruz (IOC)/FIOCRUZ.
(2) Instituto de Pesquisa Clínica Evandro Chagas (IPEC).
(3) Laboratório de Pesquisa Clínica em Dermatozoonoses em Animais Domésticos (LAPCLIN-DERMZOO), Instituto de Pesquisa Evandro Chagas (IPEC); FIOCRUZ, Rio de Janeiro, RJ, Brasil.
Correspondence to: Alexsandra R.M Favacho, Laboratório de Hantaviroses e Rickettsioses (LHR), Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, Manguinhos, 21040-900 Rio de Janeiro, RJ, Brasil. Tel: +55-2125621727. Fax: +55-2125621897. E-mail: afavacho@ioc.fiocruz.br

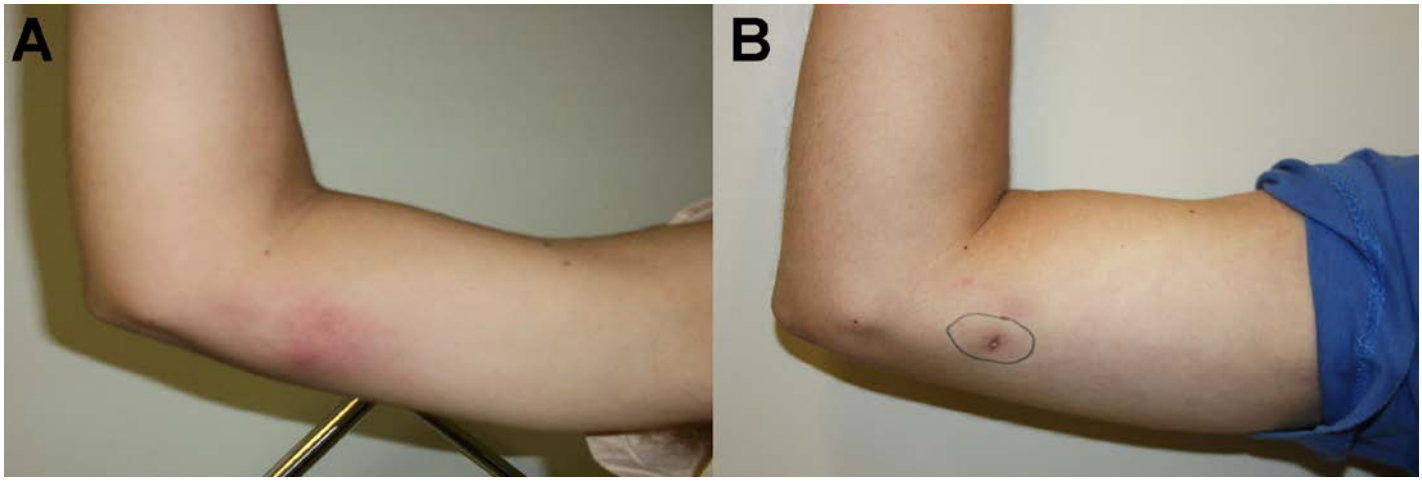


Fig. 1 - (A) Photograph of the right epitrochlear regions showing a diffuse erythematous skin rash. **(B)** Total recovery from lymph node lesions after antibiotic treatment (azithromycin).

Abdominal ultrasound revealed a slightly enlarged spleen; the chest radiograph and transthoracic echocardiography were otherwise normal.

Several days later, the patient maintained moderate fever associated with an episodic dull abdominal pain and worsening of the epitrochanteric lesion. Abdominal ultrasound and computed tomography of the abdomen on day 19 of the illness revealed an enlarged spleen with multiple discrete nodules and intra-abdominal lymphadenopathy. A lymph node ultrasound detected a large nonhomogeneous subcutaneous collection on the anterior epitrochlear region that was collected prior to antibiotic therapy and evaluated using culture and molecular analysis.

Intravenous amoxicillin-clavulanate (1 g) and oral azithromycin (500 mg) were started empirically to cover bacterial infections associated with a cat bite and were stopped after eight days. Trimethoprim-sulfamethoxazole 160/800 mg and HAART (tenofovir 300 mg, lamivudine 300 mg, and efavirenz 600 mg) were subsequently initiated on the 20th day of hospital stay with a progressive and steady rise in CD4 cell count.

DNA isolated from patient and cat serum and lymph node tissue/aspirates patient samples were used as templates for the polymerase chain reaction (PCR) assays using genus-specific primers targeting the 16S-23S rRNA intergenic region[6], riboflavin synthase C gene[4], the citrate synthase gene (*gltA*)[7], and β subunit of RNA polymerase gene (*rpoB*)[8]. A second step was performed using species-specific primers targeting the heat shock protein gene (*hrtA*)[1] following reported protocols.

B. henselae DNA sequences were only detected on the histological (lymph node tissue) sample, while the detection in cat serum samples was not confirmed. The amplification products were purified, and the sequencing was performed using an ABI PRISM BigDye terminator v.3.1 cycle sequencing kit (Applied Biosystems, CA, USA). The resulting sequences (GenBank accession no. JX028199.1) were submitted to BLASTn, and the nucleotide sequence generated from the lymph node sample clearly demonstrated 100% identity with the homologous gene fragment of the *hrtA* gene from *B. henselae* Houston-1 strain, complete genome. The diagnosis of *B. henselae* lymphadenitis and splenitis was confirmed; the patient's condition improved with the treatment, and he was discharged after 31 days of hospitalization. Azithromycin was maintained for two months. The patient remained asymptomatic and without evidence of recurrence of disease one year after hospital discharge.

DISCUSSION

Bartonella spp. have worldwide distribution and are zoonotic agents of public health with importance in both human and veterinary medicine[2,5]. Although bartonellosis are not notifiable diseases in Brazil, the number of cases has been rising in the last two decades (National Rickettsial Reference Laboratory 2012, unpublished data).

This study presents the first report of molecular identification of *B. henselae* in lymph node tissue specimen from a patient who is HIV positive and seronegative to *Bartonella* infection in Rio de Janeiro, Brazil. Although IFA assay is considered the gold standard technique for the diagnosis *Bartonella*, this patient failed to develop normal antibody response to this agent. Serologic sensibility varies from one laboratory to another ranging from nearly 100% to < 30%, depending on the antigen used, the cut-off chosen, and the test procedures. In addition, serological assays exhibit low specificity and cross-reactivity can occur between *Bartonella* spp. and agents such as Epstein-Barr virus, cytomegalovirus, *Toxoplasma gondii* and *Streptococcus pyogenes*[10]. Therefore, the detection of *B. henselae* DNA by PCR and nucleotide sequencing in the lymph node specimen proved to be useful in the diagnosis of infection.

The possibility that the patient's cat might have been involved in zoonotic transmission was reinforced; the patient's cat was seroreactive to *B. henselae* antigens. As cats may have persistent and asymptomatic bacteremia, they represent a potential danger of exposure for people with immunosuppressive conditions[5].

The suspicion of CSD and the appropriate empiric antibiotic treatment, even before the molecular diagnostics, improved the clinical status of the patient. Although no follow-up PCR was performed, the patient remained entirely asymptomatic 14 months after the illness. There are no controlled trials of treatment for bartonellosis in HIV-infected individuals, but antibiotic treatment for at least three months has been

recommended[3]. In conclusion, given the low sensitivity of the serological assays, PCR diagnosis for *B. henselae* should be considered for patients with suspected CSD even after a negative result in serology test. This way, more frequent and appropriate diagnosis will be made, leading to a better treatment and cure.

AUTHORS' CONTRIBUTIONS

ARMF conceived and designed the study, performed analysis and interpretation of these data, and prepared the manuscript; DTG, SAP and ERSL participated in interpretation of data and manuscript revision; AAP, AAK and RG carried out the laboratory tests; IR, AV and ERSL carried out the clinical assessment; all authors read and approved the final manuscript. ARMF and ERSL are guarantors of the paper.

RESUMO

Identificação molecular de *Bartonella henselae* em paciente com SIDA soronegativo para doença da arranhadura do gato no Rio de Janeiro, Brasil

Bartonella henselae está associada a um amplo espectro de manifestações clínicas, incluindo a doença da arranhadura de gato, endocardite, e meningoencefalite, em pacientes imunocompetentes e imunocomprometidos. Relatamos o primeiro caso confirmado por método molecular de *B. henselae* em um paciente com SIDA no estado do Rio de Janeiro, Brasil. Apesar da sequência de DNA de *B. henselae* ser detectada pela reação em cadeia da polimerase em uma biópsia do linfonodo, soros das fases aguda e convalescente foram não reativos.

ACKNOWLEDGMENTS

We are thankful to Dr. Jairo Dias Barreira at Universidade do Rio de Janeiro (UNIRIO) for the taxonomic identification of the cat's arthropod. We are also grateful for the technical assistance of Endiá Barros de Almeida at Hantavirosis and Rickettsiosis Laboratory, on the serological techniques. We would like to thank CNPq for student fellowships.

FUNDING

None.

COMPETING INTERESTS

None declared.

ETHICAL APPROVAL

This study was approved by the Oswaldo Cruz Foundation/ FIOCRUZ, the Comissão de Ética em Pesquisa - CEP (CEP authorization no. 559/10).

REFERENCES

1. Anderson B, Sims K, Regnery R, Robinson L, Schmidt MJ, Goral S, *et al.* Detection of *Rochalimaea henselae* DNA in specimens from cat scratch disease patients by PCR. J Clin Microbiol. 1994;32:942-8.

2. Breitschwerdt EB, Maggi RG, Chomel BB, Lappin MR. Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. J Vet Emerg Crit Care (San Antonio). 2010;20:8-30.

3. Dutta A, Schwarzwald HL, Edwards MS. Disseminated bartonellosis presenting as neuroretinitis in a young adult with human immunodeficiency virus infection. Pediatr Infect Dis J. 2010;29:675-7.

4. Johnson G. Ayers M, McClure SC, Richardson SE, Tellier R. Detection and identification of *Bartonella s*pecies pathogenic for humans by PCR amplification targeting the riboflavin synthase gene (*ribC*). J Clin Microbiol. 2003;41:1069-72.

5. Lamas CC, Mares-Guia MA, Rozental T, Moreira N, Favacho ARM, Barreira J, *et al. Bartonella* spp. infection in HIV positive individuals, their pets and ectoparasites in Rio de Janeiro, Brazil: serological and molecular study. Acta Trop. 2010;115:137-41.

6. Maggi RG, Breitschwerdt EB. Potential limitations of the 16S-23S rRNA intergenic region for molecular detection of *Bartonella* species. J Clin Microbiol. 2005;43:1171-6.

7. Norman AF, Regnery R, Jameson P, Greene C, Krause DC. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J Clin Microbiol. 1995;33:1797-803.

8. Renesto P, Gouvernet J, Drancourt M, Roux V, Raoult D. Use of *rpoB* gene analysis for detection and identification of *Bartonella* species. J Clin Microbiol. 2001;39:430-7.

9. Resto-Ruiz S, Burgess A, Anderson BE. The role of host immune response in pathogenesis of *Bartonella henselae*. DNA Cell Biol. 2003;22:431-40.

10. Vermeulen MJ, Verbakel H, Notermans DW, Reimerink JH, Peeters MF. Evaluation of sensitivity, specificity and cross-reactivity in *Bartonella henselae* serology. J Med Microbiol. 2010;59:743-5.

Received: 8 August 2013
Accepted: 28 November 2013

Rev. Inst. Med. Trop. Sao Paulo
56(4):366, July-August, 2014
doi: 10.1590/S0036-46652014000400018

LETTER TO THE EDITOR

GENETIC DIVERSITY OF MEASLES VIRUS - RESURGENCE OF NEW GENOTYPE D8 IN SAO PAULO, BRAZIL

Sao Paulo, April 28, 2014

Dear Sir:

Measles virus (MV) has been under control in Brazil because of the high coverage rates of immunization. Emphasized by the measles control global program at the Pan American Health Organization, with the use of several strategies of vaccination programs to reduce the number of circulating chains of transmission of the virus, these programs have successfully decreased the incidence of measles on a global scale. However, measles outbreaks still occasionally occur in areas with high vaccine coverage as a result of imported transmission[1,2].

For the goal of MV elimination, genotypes characterization of circulating wild-type MV is useful to document the interruption of transmission of endemic measles infection. In a meeting organized by the World Health Organization (1998), a nomenclature of MV genotypes and the targets for molecular epidemiological studies were established. Currently, 24 genotypes of MV have been identified and they have been distributed by geographical patterns[6,7].

The Epidemiologic Surveillance Center and Adolfo Lutz Institute in São Paulo are consistent in the investigation and continuous analysis of the activity of the measles control program. Retrospective studies of the genetic diversity of MV in São Paulo indicated that genotypes D5 had been registered in 1995 and 1996 and that the circulation of genotypes D6 was responsible for the 1997 epidemic[5]. After this period, the MV was interrupted with the absence of endemic genotypes. However, cases of imported virus were registered - genotype D5 in 2000, 2001 and 2005, genotype D4 in 2011 and genotype D8 in 2012 and 2013[2,3,4].

A resurgence of indigenous MV cases has occurred in Sao Paulo in 2014. A total of 90 patients suspected of MV infection were analyzed for measles-specific immunoglobulin (IgM) by Elisa and Real-time quantitative PCR. Of these patients, MV infections were confirmed in seven cases. They did not receive the vaccine nor had any record of vaccination for MV; regarding age, patients were between eight month-old and 34 years-old.

The genetic analysis was carried out from the seven confirmed measles cases. The sequences of N gene were determined by means of comparison with those from the GenBank reference strains. The results in five cases showed the presence of genotypes D8, a new genotype circulating in Sao Paulo. This genotype had been previously reported with an imported case in 2012 and 2013. In one of these cases, the patient had traveled to Fortaleza (Brazil), where MV genotype D8 had been circulating, and returned to Sao Paulo.

In addition, we conducted the analysis of two imported cases, which the surveillance had reported as patients who had traveled to Europe and returned to Sao Paulo presenting symptoms. The phylogenetic analysis of one of these cases showed that MVs were genotype B3 and that the other case was confirmed only by IgM and 4-fold increase in the IgG antibody titer in acute and convalescent serum specimens.

The government has been making great efforts to achieve the goal of measles elimination adopting surveillance programs to implement investigation strategies in susceptible populations and others to improve supplemental immunization.

Maria Isabel de OLIVEIRA(1)
Ana Maria Sardinha AFONSO(1)
Cristina Figueiredo ADELAIDE(1)
Xênia Rawena Medeiros Romeu LEMOS(2)
Jalusy ALMEIDA (2)
Ana Lucia FRUGIS YU (3)
Suely Pires CURTI (1)
(1)Virology Center, Adolfo Lutz Institute, São Paulo, SP, Brazil
(2)Oswaldo Cruz Institute, Rio de Janeiro, RJ, Brazil
(3)Epidemiologic Surveillance Center of the State of São Paulo, São Paulo, SP, Brazil
Correspondence to: Maria Isabel de Oliveira
Centro de Virologia, Instituto Adolfo Lutz
Av. Dr Arnaldo 355, 01246-902 São Paulo, SP, Brasil
Phone: +55 11 30682994, Fax: +55 11 30883753
E-mail: olive40@uol.com.br

REFERENCES

1. Bellini WJ, Rota PA. Biological feasibility of measles eradication. Virus Res. 2011;162:72-9.

2. Ceará. Secretaria da Saúde. Boletim Epidemiológico: sarampo. Fortaleza: Secretaria da Saúde; 2014. Available from: file://C:/Users/biblioteca/Downloads/boletim_epid_sarampo_12_05_2014.pdf

3. Oliveira MI, Curti SP, Figueiredo CA, Afonso AM, Sallum MA, Durigon EL. Molecular analysis of a measles virus isolate from Brazil: a case originating in Japan. Acta Virol. 2004;48:9-14.

4. Oliveira MI, Afonso AM, Figueiredo CA, Yu AL, Curti SP. Molecular surveillance of an imported measles virus infection in Sao Paulo, Brazil. Rev Inst Adolfo Lutz. 2008;67:83-6.

5. Oliveira MI, Rota PA, Curti SP, Figueiredo CA, Afonso AM, Theobaldo M, *et al*. Genetic homogeneity of measles viruses associated with a measles outbreak, Sao Paulo, Brazil,1997. Emerg Infect Dis. 2002;8:808-13.

6. Rota PA, Brown K, Mankertz A, Santibanez S, Shulga S, Muller CP, *et al.* Global distribution of measles genotypes and measles molecular epidemiology. J Infect Dis. 2011;204:S514-23.

7. WHO. Standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses. Wkly Epidemiol Rec.1998;73:265-72. Available from: http://www.who.int/docstore/wer/pdf/1998/wer7335.pdf

Rev. Inst. Med. Trop. Sao Paulo
56(4):367-368, July-August, 2014
doi: 10.1590/S0036-46652014000400019

LETTER TO THE EDITOR

HUMAN INFECTION WITH AVIAN INFLUENZA A (H7N9) VIRUS

Shanghai, February 24th, 2014

Dear Editor,

On March 31st, 2013, the National Health and Family Planning Commission (NHFPC) of China announced that three fatal cases of rapidly progressive pneumonia, respiratory failure and acute respiratory distress syndrome (ARDS) were confirmed to be infected with a novel reassortant avian-origin influenza A (H7N9) virus[9]. As of February 18th, 2014, a total of 347 laboratory-confirmed cases and 109 deaths had been reported in mainland China[7]. The novel avian influenza virus has caused global concern as a potential pandemic threat.

According to recent studies, the elderly have increased risk for H7N9 virus infection[2,3]. Furthermore, patients with underlying diseases are significantly associated with the infection[1-3]. It is reported that the median age of 111 patients with H7N9 virus infection was 61 and 42.3% of them were 65 years of age or older[3]. A total of 61.3% of the patients had one or more underlying medical conditions, such as hypertension, diabetes, coronary heart disease, chronic obstructive pulmonary disease (COPD) and so on[3].

Human infection with H7N9 virus has been reported sporadically and is mainly associated with exposures to poultry[1,4]. However, no history of recent close contact with poultry was found in some cases[2,8]. Indeed, H7N9 virus had been detected among live poultry at local markets in some areas of China[8,11]. Therefore, the most likely source of H7N9 virus in these cases seems to be from the environments contaminated with the novel avian influenza virus. Epidemiologically, the elderly patients predominate in the H7N9 avian influenza outbreak, for the reason that retirees have more opportunities to shop in the live animal markets and are, therefore, more likely to be exposed to the environments that are contaminated with H7N9 virus[3].

The clinical features of H7N9 virus infection are broadly similar to those of H5N1 virus infection[3,8]. The laboratory findings including leukopenia, lymphocytopenia, thrombocytopenia and increased levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK) and C-reactive protein (CRP) are also commonly seen in patients with H7N9 virus infection[3,8]. Furthermore, the disease is characterized by bilateral ground-glass opacities and consolidation[3,8], as seen in Fig. 1.

Human infection with H7N9 virus shows a case-fatality rate of 31% (109/347), which is not as high as that of H5N1 virus infection (59%)[6]. The clinical outcome, on the other hand, is inconsistent with that of previous reports on avian influenza A (H7) virus infection, which is usually associated with poultry outbreaks, but causes mild or moderate illness in humans[4]. It is estimated that the fatality risk is 36% (95% CI 26-45) on admission to hospital for H7N9 virus infection[10]. Increasing age along with a history of smoking, chronic lung disease, immunosuppression, chronic drug use and delayed antiviral treatment are considered as risk factors which might contribute to the fatal outcome[5]. According to statistics, the median time from onset of symptoms to initiation of antiviral therapy is 7.4 and 4.6 days in the fatal and non-fatal cases, respectively[5]. Importantly, the relatively good clinical outcome may be attributed to early diagnosis and antiviral treatment, which are the most effective strategies for managing H7N9 virus infection.

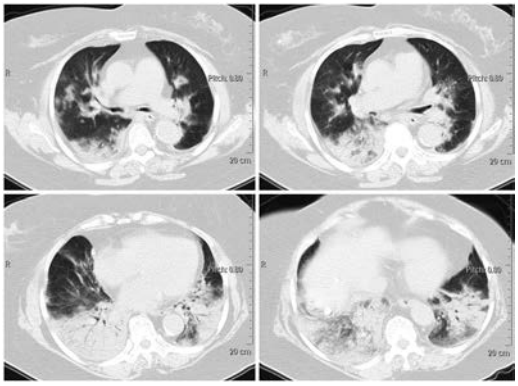


Fig. 1 - Computed tomography (CT) scan of the chest in a 74-year-old female infected with avian influenza A (H7N9) virus showing a mixed pattern of ground-glass opacities and consolidation with bilateral pleural effusions.

Yi ZHANG
Yong-Sheng YU
Zheng-Hao TANG
Xiao-Hua CHEN
Guo-Qing ZANG
Department of Infectious Diseases,
Shanghai Jiao Tong University Affiliated Sixth People's Hospital,
Shanghai, P. R. China
Correspondence to: Guo-Qing Zang,
Department of Infectious Diseases,
Shanghai Jiao Tong University Affiliated Sixth People's Hospital,
600 Yishan Road, 200233 Shanghai, P. R. China
Phone: + 86 21 64369181-58673; Fax: + 86 21 24058384
E-mail: zangguoqin@126.com

ACKNOWLEDGEMENTS

This work was partly supported by the Medical Education Research Project of Shanghai Jiao Tong University School of Medicine (No. YB130910).

REFERENCES

1. Ai J, Huang Y, Xu K, Ren D, Qi X, Ji H, *et al.* Case-control study of risk factors for human infection with influenza A (H7N9) virus in Jiangsu Province, China, 2013. Euro Surveill. 2013;18:20510.

2. Chen X, Yang Z, Lu Y, Xu Q, Wang Q, Chen L. Clinical features and factors associated with outcomes of patients infected with a Novel Influenza A (H7N9) virus: a preliminary study. PLoS One. 2013;8:e73362.

3. Gao HN, Lu HZ, Cao B, Du B, Shang H, Gan JH, *et al.* Clinical findings in 111 cases of influenza A (H7N9) virus infection. N Engl J Med. 2013;368:2277-85.

4. Li Q, Zhou L, Zhou M, Chen Z, Li F, Wu H, *et al.* Epidemiology of human infections with avian influenza A (H7N9) virus in China. N Engl J Med. 2014;370:520-32.

5. Liu S, Sun J, Cai J, Miao Z, Lu M, Qin S, *et al.* Epidemiological, clinical and viral characteristics of fatal cases of human avian influenza A (H7N9) virus in Zhejiang Province, China. J Infect. 2013;67:595-605.

6. Mei Z, Lu S, Wu X, Shao L, Hui Y, Wang J, *et al.* Avian influenza A (H7N9) virus infections, Shanghai, China. Emerg Infect Dis. 2013;19:1179-81.

7. Ministry of Agriculture of the People's Republic of China. National poultry H7N9 influenza eradication program (Chinese). [Cited: Feb 22, 2014]. Available from: http://www.moa.gov.cn/zwllm/tzgg/tfw/201402/t20140220_3791429.htm

8. Shi J, Xie J, He Z, Hu Y, He Y, Huang Q, *et al.* A detailed epidemiological and clinical description of 6 human cases of avian-origin influenza A (H7N9) virus infection in Shanghai. PLoS One. 2013;8:e77651.

9. World Health Organization. Human infection with influenza A (H7N9) virus in China. Global Alert and Response (GAR). [Cited: Feb 10, 2014]. Available from: http://www.who.int/csr/don/2013_04_01/en/

10. Yu H, Cowling BJ, Feng L, Lau EH, Liao Q, Tsang TK, *et al.* Human infection with avian influenza A H7N9 virus: an assessment of clinical severity. Lancet. 2013;382:138-45.

11. Yu H, Wu JT, Cowling BJ, Liao Q, Fang VJ, Zhou S, *et al.* Effect of closure of live poultry markets on poultry-to-person transmission of avian influenza A H7N9 virus: an ecological study. Lancet. 2014;383:541-8.