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. 403851/2012-2 and 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

UNIVERSIDADE DE SÃO PAULO - BRAZIL
FACULDADE DE MEDICINA
Instituto de Medicina Tropical de São Paulo
Director: Prof. Dr. Paulo C. Cotrim

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 (1-2)

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. 2	P. 93-184	March-April, 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. 2	P. 93-184	Março-Abril, 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(2):93-95, March-April, 2014
doi: 10.1590/S0036-46652014000200001

MULTIPLEX SYBR® GREEN-REAL TIME PCR (qPCR) ASSAY FOR THE DETECTION AND DIFFERENTIATION OF *Bartonella henselae* AND *Bartonella clarridgeiae* IN CATS

Rodrigo STAGGEMEIER(1), Diogo André PILGER(2), Fernando Rosado SPILKI(1) & Vlademir Vicente CANTARELLI(1,3)

SUMMARY

A novel SYBR® green-real time polymerase chain reaction (qPCR) was developed to detect two *Bartonella* species, *B. henselae* and *B. clarridgeiae,* directly from blood samples. The test was used in blood samples obtained from cats living in animal shelters in Southern Brazil. Results were compared with those obtained by conventional PCR targeting *Bartonella* spp. Among the 47 samples analyzed, eight were positive using the conventional PCR and 12 were positive using qPCR. Importantly, the new qPCR detected the presence of both *B. henselae* and *B. clarridgeiae* in two samples. The results show that the qPCR described here may be a reliable tool for the screening and differentiation of two important *Bartonella* species.

KEYWORDS: *Bartonella;* Cat Scratch Disease; qPCR.

INTRODUCTION

Bartonella spp. are emerging infectious zoonotic agents[13,23] endemic in some South American countries[19]. *Bartonella* can infect humans as well as domestic and wild mammals[2]. There are more than 23 described species within this genus[6,8], among which, 13 are related to human diseases[15]. The most commonly reported species are *B. bacilliformis, B. quintana, B. henselae*[1] and *B. clarridgeiae*[18].

The main vectors associated with human infections are fleas (*Ctenocephalides felis*), lice (*Pediculus humanus*), sandflies (*Lutzomyia verrucarum, Lutzomyia peruensis*)[15] and ticks (*Ixodes pacificus*)[10]. The main animal reservoirs are cats, dogs and rodents[15]. The infection is generally more severe in immunocompromised hosts, but may also occur in healthy subjects[15]. *Bartonella bacilliformis* is an important cause of severe illness and death among immunocompetent adults and children[19].

B. henselae and *B. clarridgeiae* are known as the causative agents of cat-scratch disease (CSD), which is characterized by chronic lymphadenopathy[11]. Furthermore, in some cases, these bacteria may result in Parinaud oculoglandular syndrome, encephalopathy, convulsions, endocarditis, hepatosplenomegaly, glomerulonephritis, pleurisy, mediastinal adenopathy, nodules in the head of the pancreas, bacillary angiomatosis, osteomyelitis, atypical pneumonia, mammary tumours, haemolyticanaemia and eosinophilic purpura[7].

More than 4,000 cases of CSD are registered each year in the United States, resulting in over 2,000 hospitalizations in the same period, with a rate of 0.77 to 0.88 / 100,000 hospitalizations, with 55% of cases occurring in patients younger than 18 years old[14]. In the Netherlands, an estimated 2,000 cases occur per year with a rate of 12.5 cases/100 thousand inhabitants[3]. In Asian countries, reports show that the seroprevalence of *B. henselae* among the cat population ranges from 9.1% to 15.1% in Japan, 68% in the Philippines, 48% in Singapore and 54% in Indonesia[7].

Bartonella spp are difficult-to-culture bacteria, hampering laboratory diagnosis of these infections. Other diagnostic alternatives, such as Giemsa-stained blood smears[4] and serology[7], are available with limited sensitivity, which may further delay proper diagnosis.

Giemsa stained blood smears can be useful in screening blood samples, however, the presence of artifacts might be a confusing factor[24]. Serology has only a retrospective value, suggesting that the patient might have been infected during some period of their life, and hence is also of limited value. To date, no gold standard method for the detection of *Bartonella* species is available[9].

Molecular techniques are powerful tools that may be used to screen for the presence of these pathogens in clinical samples[16]. Conventional polymerase chain reaction (PCR) has been used for the detection of several *Bartonella* species, and most of those assays use gene targets located on the 16S rRNA gene[5], *ribC*gene[17], *rpo* B gene[22], 16S-23S intergenic spacer region (ITS)[20] and *glt*A gene[21]. However, real-time PCR is substituting conventional PCR in many laboratories as it is not only quicker but also more sensitive and specific than its predecessor[12].

(1) Laboratório de Microbiologia Molecular, Universidade Feevale, RS 239 nº 2755, 93352-000 Novo Hamburgo, RS, Brazil.
(2) Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Brazil.
(3) Laboratório Qualitá, Novo Hamburgo, RS, Brazil
Correspondence to: Rodrigo STAGGEMEIER, E-mail: rstaggemeier@gmail.com

The aim of this study was to evaluate a SYBR® green qPCR assay to detect and identify the presence of two *Bartonella* species directly from feline blood samples.

METHODS

Blood samples from 47 cats were collected during 2009 in two municipal animal shelters (Novo Hamburgo and São Leopoldo, south of Brazil), and have been described previously[24]. All procedures were performed under veterinary supervision and approved by the Animal Ethics Committee from Universidade Feevale, under the protocol number 2.12.03.09.1391.

DNA was extracted from blood samples with the QIAmpDNA blood mini Kit (Qiagen). Two new primer pairs were especially designed from consensus genome regions obtained from the DNA Data Bank of Japan (DDBJ). Primers targeting fragments of the Citrate synthase gene were: one generic forward primer (BART-LC-GEN-F: 5' – ATGGGTTTTGGTCATCGAGT – 3'); one specie-specific reverse *B. henselae* primer (BART-LC-HEN-R: 5' –AA ATCGACATTAGGGTAAAGTTTTT – 3'); and one specie-specific reverse *B. clarridgeiae* primer (BART-LC-CLA-R: 5'-CAAGAAGTGGATCATCTTGG – 3'). Specificity of the assay was assessed by testing known positive samples (*B. henselae, B. clarridgeiae* and *B. bacilliformis,* confirmed by DNA sequencing[24]), and several other clinical and ATCC-derived bacterial species. No false-positive reaction was noted with these tests. Apart from that, the specificity of the assay was also confirmed by *in silico* analysis of the primers, which demonstrated no cross-reaction with other *Bartonella* species. All positive results were confirmed by DNA sequencing reaction to make sure that these are true positive results and no false-positive samples were present. The results were analyzed by the DNA Data Bank of Japan (DDBJ Blast).

The reaction mix contained 2 μL of extracted DNA, 10 pM each primer, 1μL of BSA, 10 μL of SYBR green qPCR Supermix (Invitrogen) for a final volume of 20 μL. The multiplex qPCR was performed using the LightCycler®1.5 Real-Time PCR System (Roche Diagnostics) under the following conditions: 96 ºC for two min (DNA denaturation), followed by 40 cycles of 96 ºC for two sec, 60 ºC for five sec and 72 ºC for eight sec. A melting curve analysis was performed at the end of the amplification cycles. Positive reactions were recognized by typical melting temperatures (Tm ~ 82 ºC). Since both species showed the same Tm, identification of the species among positive samples was performed by a second qPCR using species-specific primers (Singleplex) under the same conditions.

RESULTS

The multiplex SYBR® green qPCR allowed the detection of *Bartonella* DNA in 25.5% (12/47) of the blood samples. In a previous study, conventional PCR analysis targeting *Bartonella* spp in the same blood specimens resulted in a detection rate of 17.02% (8/47)[24]. DNA sequencing analysis of the PCR products obtained by conventional PCR revealed the presence of only two *Bartonella* species among those samples, which consisted of *B. henselae*, present in 10.63% (5/47) of the samples, and *B. clarridgeiae* in 6.38% (3/47)[24]. When using the multiplex qPCR, additional positive samples were observed with *B. henselae* and *B. clarridgeiae* detected in 17.02% (8/47) and 12.76% (6/47) of the samples, respectively (Table 1). Moreover, this assay allowed us to observe these species co-infecting two samples, this was not previously detected using conventional PCR.

Table 1
Results by qPCR and Conventional PCR for the diagnosis of *Bartonella* infection

Samples	qPCR	PCR
1	*B. c.*	*B. c.*
2	*B. h.*	*B. h.*
3	*B. c.*	*Neg.*
4	*B. h.*	*Neg.*
5	*B. h.*	*Neg.*
6	*B. h. / B. c.*	*Neg.*
7	*B. h.*	*B. h.*
8	*B. c.*	*B. c.*
9	*B. c.*	*B. c.*
10	*B. h.*	*B. h.*
11	*B. h. / B. c.*	*B. h.*
12	*B. h.*	*B. h.*
13-47	*Neg. by both methods*	

B.h. (*Bartonella henselae*), B.c. (*Bartonella clarridgeiae*), Neg. (Negative).

DISCUSSION

Using multiplex qPCR, an additional four positive samples were found, which may represent an increase in sensitivity in comparison with the conventional PCR method. Moreover, the whole technique, including DNA extraction, could be performed within 2-3 hours, without any need of subsequent DNA sequencing for species identification. Conventional PCR, targeting consensus regions of the Citrate synthase gene may not amplify all *Bartonella* species with the same efficiency, thus explaining the differences in sensitivities obtained by our qPCR assay. Moreover, the identification of the species in positive samples depends on subsequent DNA sequencing. The advantage of the conventional PCR followed by DNA sequencing analysis would be the opportunity to identify the other or newer species that might be present in the blood samples. In this case, in a direct comparison, using the new specific primers for the detection of the two species identified by DNA sequencing, the qPCR assay was able to detect 100% of these species.

An advantage for the qPCR is the possibility of identifying the most prevalent *Bartonella* species in cat's blood samples without the need of subsequent DNA sequencing, which is laborious and, in our case, was not able to detect the presence of co-infection in two samples.

In conclusion, our results suggest that the multiplex SYBR® green qPCR may be a useful technique to detect and differentiate the two most common species of *Bartonella* directly from blood samples. With the lack of a defined gold standard method to detect the presence of different *Bartonella* species, there is a need for improved, clinically

useful methods[9] for diagnosis of these infections, and the method developed here may be a useful diagnostic tool. Furthermore, compared with conventional PCR, the multiplex SYBR® green qPCR seems to be more sensitive; however, this hypothesis needs to be further investigated using a more robust number of specimens. The real time PCR described here is faster than conventional PCR and can be adapted to detect other *Bartonella* species if necessary. The species found here are those associated with CSD, which, therefore, may pose some risk to public health, mainly for HIV-positive individuals.

RESUMO

PCR em Tempo Real (qPCR) multiplex utilizando SYBR® Green para a detecção e diferenciação de *Bartonella henselae* e *Bartonella clarridgeiae* em gatos

Um novo teste baseado na reação em cadeia da polimerase em tempo real (qPCR) com SYBR ® Green foi desenvolvido para detectar duas espécies de Bartonella, *B. henselae* e *B. clarridgeiae,* diretamente em amostras de sangue. Este teste foi utilizado em amostras de sangue obtidas de gatos que vivem em abrigos de animais do sul do Brasil. Os resultados foram comparados aos obtidos pelo PCR convencional utilizado para a detecção de *Bartonella* spp. Das 47 amostras analisadas, oito foram positivas no PCR convencional e 12 foram positivas para qPCR. A reação de qPCR, permitiu a detecção da presença simultânea de *B. henselae* e *B. clarridgeiae* em duas destas amostras. Os resultados mostram que a qPCR aqui descrita pode ser uma ferramenta confiável para a detecção e diferenciação de duas espécies importantes de *Bartonella* spp.

ACKNOWLEDGEMENTS

The authors thank the employers and volunteers of municipal shelters for providing the animals for this study. This work was supported by Universidade Feevale and Laboratório Qualitá.

REFERENCES

1. Anderson BE, Neuman MA. *Bartonella* spp. as emerging human pathogens. Clin Microbiol Rev. 1997;10:203-19.

2. Angulo FJ, Glaser CA, Juranek DD, Lappin MR, Regnery RL. Caring for pets of immunocompromised persons. J Am Vet Med Assoc. 1994;205:1711-8.

3. Bergmans AM de, Jong CM, van Amerongen G, Schot CS, Schouls LM. Prevalence of *Bartonella* species in domestic cats in The Netherlands. J Clin Microbiol. 1997;35:2256-61.

4. Billeter SA, Levy MG, Chomel BB, Breitschwerdt EB. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. Med Vet Entomol. 2008;22:1-15.

5. Birtles RJ. Differentiation of *Bartonella* species using restriction endonuclease analysis of PCR-amplified 16S rRNA genes. FEMS Microbiol Lett. 1995;129:261-5.

6. Birtles RJ, Harrison TG, Saunders NA, Molyneux DH. Proposal to unify the genera *Granhamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb.nov., *Bartonella peromysci* comb.nov., and three newspecies, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov. and *Bartonella doshia e*sp. nov. Int J Syst Bacteriol. 1995;45:1-8.

7. Boulouis HJ, Chang CC, Henn JB, Kasten RW, Chomel BB. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. Vet Res. 2005;36:383-410.

8. Brenner DJ, O'Connor SP, Winkler HH, Steigerwalt AG. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb.nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family Bartonellaceae from the order Rickettsiales. Int J Syst Bacteriol. 1993;43:777-86.

9. Caponetti GC, Pantanowitz L, Marconi S, Havens JM, Lamps LW, Otis CN. Evaluation of Immunohistochemistry in identifying *Bartonella henselae* in cat-scratch disease. Am J Clin Pathol. 2009;131:250-6.

10. Chang CC, Chomel BB, Kasten RW, Romano V, Tietze N. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. J Clin Microbiol. 2001;39:1221-6.

11. Chomel BB. Cat-scratch disease. Rev Sci Tech. 2000;19:136-50.

12. Ciervo A, Ciceroni L. Rapid detection and differentiation of *Bartonella* spp by a single-run real-time PCR. Mol Cell Probes. 2004;18:307-12.

13. Dehio C, Sander A. *Bartonella* as emerging pathogens. Trends Microbiol. 1999;7:226-8.

14. Huarcaya E, Maguiña C, Merello J, Cok J, Birtles R, Infante B, *et al.* A prospective study of cat-scratch disease in Lima-Peru. Rev Inst Med Trop Sao Paulo. 2002;44:325-30.

15. Jacomo V, Kelly PJ, Raoult D. Natural history of *Bartonella* infections (an exception to Koch's postulate). Clin Diagn Lab Immunol. 2002;9:8-18.

16. Jensen WA, Fall MZ, Rooney J, Kordick DL, Breitschwerdt EB. Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. J Clin Microbiol. 2000;38:1717-22.

17. Johnson G, Ayers M, McClure SCC, Richardson SE, Tellier R. Detection and identification of *Bartonella* species pathogenic for humans by PCR amplification targeting the riboflavin synthase gene (ribC). J Clin Microbiol. 2003;41:1069-72.

18. Kordick DL, Hilyard EJ, Hadfield TL, Wilson KH, Steigerwalt AG, Brenner DJ, *et al. Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). J Clin Microbiol. 1997;35:1813-8.

19. Maco V, Maguiña C, Tirado A, Maco C V, Vidal JE. Carrion's disease (Bartonellosis bacilliformis) confirmed by histopathology in the High Forest of Peru. Rev Inst Med Trop Sao Paulo. 2004;46:171-4.

20. Matar GM, Swaminathan B, Hunter SB, Slater LN, Welch DF. Polymerase chain reaction-based restriction fragment length polymorphism analysis of a fragment of the ribosomal operon from *Rochalimaea* species for subtyping. J Clin Microbiol. 1993;11:1730-4.

21. 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 cytrate synthase gene. J Clin Microbiol. 1995;33:1797-803.

22. 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.

23. Spach DH, Koehler JE. *Bartonella*-associated infections. Infect Dis Clin North Am. 1998;12:137-55.

24. Staggemeier R, Venker CA, Klein DH, Petry M, Spilki FR, Cantarelli VV. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in cats in the south of Brazil: a molecular study. Mem Inst Oswaldo Cruz. 2010;105:873-8.

Received: 27 November 2012
Accepted: 31 July 2013

Rev. Inst. Med. Trop. Sao Paulo
56(2):96, March-April, 2014
doi: 10.1590/S0036-46652014000200018

BOOK REVIEW*

FERREIRA, Antonio Walter & MORAES, Sandra do Lago - Diagnóstico laboratorial das principais doenças infecciosas e autoimunes. 3ª ed. Rio de Janeiro: Editora Guanabara Koogan; 2013. 477p. ilus. ISBN 978-85-277-2302-2

The laboratory diagnostic methods have increasingly incorporated fully automated and computerized procedures. In addition to the immunoassays that are available for detection of antigens, antibodies or other markers, the "methods of molecular biology and nanotechnology employed in microarrays or multiplex systems" has been having an increasing use in the clinical practice, as they have defined profiles for different clinical situations. If on one hand this large amount of information helps diagnosis, on the other hand, this diversity of methods and technologies may generate "doubts regarding the interpretation of the clinical value of the results, bewildering the professionals in relation to their initial clinical suspicion".

In this context, the book "Diagnóstico laboratorial das principais doenças infecciosas e autoimunes" ("Laboratory diagnosis of main infectious and autoimmune diseases"), third edition, edited by Antonio Walter Ferreira and Sandra do Lago Moraes offers tools and principles that are very useful for a critical view of a laboratory result. The book also allows the improvement of individual knowledge of the most important infections in the clinical practice, also including autoimmune diseases and allergies, and may be used as a reference text for courses in related areas.

The book is divided into 8 sections with 34 chapters. The first section is on "General Methodology". The aim of this section is to provide basic concepts of the methods used in immunological and molecular diagnosis. These concepts, presented in three chapters, are intended to introduce the reader to the immunological and molecular techniques that will be quoted in subsequent chapters.

Subsequent sections are on Virus, Bacteria, Protozoa, Helminths, Fungi, Allergy and Autoimmune diseases. Each of the 31 chapters makes a brief description of the disease, showing general aspects, such as the etiological agent, epidemiology, and clinical manifestations. However, the focus is on the laboratory diagnosis. The authors describe the different methods of diagnosing the disease/infection, discuss their advantages and disadvantages, their current use, and correlate the results with clinic, allowing a critical view of a laboratory result.

This third edition was being very awaited by professionals and students in the health field; they find the book a read reference, in Portuguese, in the matters addressed. The clinical and laboratory correlations presented allow professionals and students in the area to have a critical view of the methods employed.

The book was "revised and updated in light of the rapid evolution of the tests used in clinical pathology". The graphic design was more attractive with new layout and more colorful. As in previous editions, the book's editors were careful inviting experts recognized for their work in each topic covered. In this way, the book offers a great deal of information. Reading this book is a very interesting task.

MARIA CARMEN ARROYO SANCHEZ, MSc, PhD
Pesquisador Científico
Laboratório de Soroepidemiologia e Imunobiologia
Instituto de Medicina Tropical de São Paulo
E-mail: arroyo@usp.br

Editora Guanabara Koogan Ltda.
Travessa do Ouvidor, 11
20040-040 Rio de Janeiro, RJ, Brasil
E-mail: editorial.saude@grupogen.com.br
www.editoraguanabarakoogan.com.br

*This book is available at the Library of the Instituto de Medicina Tropical de São Paulo

Rev. Inst. Med. Trop. Sao Paulo
56(2):97-103, March-April, 2014
doi: 10.1590/S0036-46652014000200002

COMPARISON BETWEEN AUTOMATED SYSTEM AND PCR-BASED METHOD FOR IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF CLINICAL *Enterococcus* spp

Luciana FURLANETO-MAIA(1), Kátia Real ROCHA(2), Vera Lúcia Dias SIQUEIRA(3) & Márcia Cristina FURLANETO(2)

SUMMARY

Enterococci are increasingly responsible for nosocomial infections worldwide. This study was undertaken to compare the identification and susceptibility profile using an automated MicrosScan system, PCR-based assay and disk diffusion assay of *Enterococcus* spp. We evaluated 30 clinical isolates of *Enterococcus* spp. Isolates were identified by MicrosScan system and PCR-based assay. The detection of antibiotic resistance genes (vancomycin, gentamicin, tetracycline and erythromycin) was also determined by PCR. Antimicrobial susceptibilities to vancomycin (30 µg), gentamicin (120 µg), tetracycline (30 µg) and erythromycin (15 µg) were tested by the automated system and disk diffusion method, and were interpreted according to the criteria recommended in CLSI guidelines. Concerning *Enterococcus* identification the general agreement between data obtained by the PCR method and by the automatic system was 90.0% (27/30). For all isolates of *E. faecium* and *E. faecalis* we observed 100% agreement. Resistance frequencies were higher in *E. faecium* than *E. faecalis*. The resistance rates obtained were higher for erythromycin (86.7%), vancomycin (80.0%), tetracycline (43.35) and gentamicin (33.3%). The correlation between disk diffusion and automation revealed an agreement for the majority of the antibiotics with category agreement rates of > 80%. The PCR-based assay, the *van*(A) gene was detected in 100% of vancomycin resistant enterococci. This assay is simple to conduct and reliable in the identification of clinically relevant enterococci. The data obtained reinforced the need for an improvement of the automated system to identify some enterococci.

KEYWORDS*: Enterococcus;* MicrosScan system; PCR assay.

INTRODUCTION

Enterococci are implicated in a wide diversity of infections and are the third most common pathogen isolated from several infections worldwide[35]. According to a recent epidemiological survey conducted in Brazil, *Enterococcus* spp accounted for 4.5% of all nosocomial bloodstream infections (BSIs), resulting in 49.5% crude mortality[26].

Enterococci infections' greater mortality rates and antibiotic resistance are associated with prolonged hospitalization and increased health-care costs[1,32]. It has recently been reported that inappropriate and delayed antibiotic therapy present an independent risk factor for mortality caused by enterococcal bacteraemia[36]. Besides, the difficulty in treating enterococci infections, particularly with respect to vancomycin resistance isolates, emphasizes the need for safe and therapeutic guidance for rapid identification and effective management.

In this context, the employment of automated systems, that provide rapid identification and susceptibility testing, may lead to a significant reduction of patient morbidity, mortality and cost[3]. However, the identification and susceptibility testing of microorganisms usually takes 24-48 h after initial growth in a routine laboratory. In addition, automated systems may present problems in the identification of members of the genus *Enterococcus* in clinical laboratories[11]. Currently, several studies have compared the direct and standard methods for different automated systems[16,17,20,42].

The employment of polymerase chain reaction (PCR)-based assay in the identification of enterococci and detection of antibiotic resistance genes offered a specific and rapid alternative to standard tests, providing essential information concerning the effective management and appropriate therapy of enterococcal bacteraemia[10,14,21,41].

In this study, we compared for the first time the MicrosScan® system versus PCR-based approach for identification as well as the susceptibility profile of clinical *Enterococcus* sp.

MATERIAL AND METHODS

Isolates: A total of 30 *Enterococcus* clinical isolates were obtained from January 2008 to June 2010, from patients of the University Hospital of State University of Maringá (UEM). The origins of the isolates were

(1) Technological Federal University of Paraná, Brazil.
(2) Department of Microbiology, State University at Londrina, Paraná, Brazil.
(3) Department of Clinical Analysis, State University at Maringa, Paraná, Brazil.
Correspondence to: Luciana Furlaneto-Maia. Technological Federal University of Paraná, Av. dos Pioneiros 3131, 86036-370 Londrina, PR, Brasil. Tel.: + 55.43.99935355. E-mail: lucianamaia@utfpr.edu.br

Table 1
Primers used in this study for identification of *Enterococcus* spp. and detection of different resistance genes by PCR-based method

Gene	Nucleotide sequence (5'- 3')[a]	Ta* (ºC)	amplicon (bp)	References
tuf	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	56	112	21
vanC-1	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	56	822	10
vanC-2, *vanC-3*	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG	56	439	
$ddl_{E.faecalis}$	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	56	941	
$ddl_{E.faecium}$	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	56	550	
vanA	GTAGGCTGCGATATTCAAAGC CGATTCAATTGCGTAGTCCAA	56	231 *E. faecium* 330 *E. faecalis*	2
aac(6')- Ie- aph(2")-Ia	CAGAGCCTTGGGAAGATGAAG CCTCGTGTAATTCATGTTCTGGC	56	348	39
erm(B)	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	56	405	14
tet(L)	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCACCTAA	56	696	

Ta (ºC) = temperature of annealing/aM = A or C; R = A or G; W = A or T/ (*) with modification/gene gene *tuf*, *Enterococcus*; *vanC-1*, *E. gallinarum*; v*anC-2*, *vanC-3*, *E.casseliflavus*, *E. flavencens; tet(L)*, tetracycline; *erm(B)*, erythromycin; *aac(6')-aph(2')-Ia*, gentamicin and *vanA*, vancomycin.

urine, blood, orotracheal fluid and rectal swab. The MicrosScan® was used in the identification of enterococci and in susceptibility test by using the standard growth detection algorithms provided by the system.

Isolation of enterococcal DNA, identification and detection of resistance genes by PCR: *Enterococcus* spp. genomic DNA was extracted by the boiling method as described by MARQUES & SUZART[27]. The identification of enterococci species was performed using a polymerase chain reaction (PCR) method. PCR assay was carried out using the following species-specific primers: $ddl_{E.faecalis}$ (*E. faecalis*), $ddl_{E.faecium}$ (*E. faecium*), *vanC-1* (*E. gallinarum*), *vanC-2* (*E. casseliflavus*) and *vanC-3* (*E. flavencens*), and *tuf* for *Enterococcus* sp genus members (Table 1). The detection of resistance genes was conducted by PCR in all isolates of enterococci. The presence of gene *vanA*, *aac(6')-Ie-aph(2")-Ia*, *erm(B)* and *tet(L)*, for vancomycin, gentamicin, erythromycin and tetracycline, respectively (Table 1).

All PCR amplifications were performed in a final volume of 20 µL containing one ρmol of each primer (Forward and Reverse), 0.17 mM dNTPs, 2.5 mM $MgCl_2$, one U of Taq DNA polymerase (Invitrogen), buffer of Taq, and 10 µL template DNA. An initial cycle of denaturation (94 °C for two min), was followed by 30 cycles of denaturation (94 °C for one min), annealing at an appropriate temperature for one min and elongation (72 °C for 10 min). A Thermal Cycler (Techne-Tc3000) was used to carry out the PCR reactions. PCR products were analyzed by gel electrophoresis in 1.5% agarose stained with ethidium bromide (0.5 g.mL-1), observed under UV transillumination and photographed by L-PIX ST (LOCCUS).

Antimicrobial susceptibility testing: Susceptibility testing of four antimicrobial agents (vancomycin, 30 µg; tetracycline, 30 µg; erythromycin, 15 µg; and gentamicin 120 µg) (Laborclin) was performed by the disk diffusion assay on Muller Hinton agar plates. After 18 or 24 h of incubation at 37 °C, inhibition zone diameters around each disc were measured and the diameters of inhibition zones were interpreted according to the criteria recommended by the Clinical and Laboratory Standards Institute, 2011. *Staphlylococccus aureus* 25923 ATCC was used as a control strain. MicrosScan® system was used on the same antimicrobial agents for the antimicrobial susceptibility testing.

RESULTS

In the present study, we firstly evaluated the genetic similarities of the *Enterococcus* isolates using the RAPD-PCR analysis. The fingerprinting revealed no clonal lineage (unrelated strains) among tested isolates (data not shown).

As shown in Table 2, for 27 out of 30 (90%) isolates the identification was concordant between the automated system and the molecular method. All 20 isolates identified as *E. faecium* and seven isolates as *E. faecalis* by automation system were confirmed by PCR assay. Figure 1 illustrated the amplicon size of *Enterococcus* sp. Among the isolates tested, *E. faecium* (76.7%) had a much higher incidence rate followed by *E. faecalis* (23.3%).

The disagreement was observed in the identification of three isolates. The species classified by automation as *E. gallinarum* (isolate 817) and *E. durans/hirae* (isolate 917 and 1000) were all identified as *E. faecium* by the PCR assay.

Table 2
Identification of clinical enterococci isolates by automated systems and molecular method

Strain	origin	Identification	
		automated system	PCR-based assay
802	urine	*E. faecalis*	*E. faecalis*
817	rectal swab	*E. gallinarum*	*E. faecium*
840	blood	*E. faecalis*	*E. faecalis*
848	urine	*E. faecalis*	*E. faecalis*
872	orotracheal fluid	*E. faecalis*	*E. faecalis*
906	urine	*E. faecalis*	*E. faecalis*
917	urine	*E. durans/hirae*	*E. faecium*
924	rectal swab	*E. faecium*	*E. faecium*
925	rectal swab	*E. faecium*	*E. faecium*
928	urine	*E. faecalis*	*E. faecalis*
973	urine	*E. faecium*	*E. faecium*
1000	urine	*E. durans/hirae*	*E. faecium*
1035	rectal swab	*E. faecium*	*E. faecium*
1053	rectal swab	*E. faecium*	*E. faecium*
1062	rectal swab	*E. faecium*	*E. faecium*
1076	rectal swab	*E. faecium*	*E. faecium*
1097	rectal swab	*E. faecium*	*E. faecium*
1112	rectal swab	*E. faecium*	*E. faecium*
1114	rectal swab	*E. faecium*	*E. faecium*
1115	rectal swab	*E. faecium*	*E. faecium*
1125	rectal swab	*E. faecium*	*E. faecium*
1143	rectal swab	*E. faecium*	*E. faecium*
1211	urine	*E. faecalis*	*E. faecalis*
1215	rectal swab	*E. faecium*	*E. faecium*
1227	urine	*E. faecium*	*E. faecium*
1231	urine	*E. faecium*	*E. faecium*
1246	urine	*E. faecium*	*E. faecium*
1280	rectal swab	*E. faecium*	*E. faecium*
1295	rectal swab	*E. faecium*	*E. faecium*
1298	rectal swab	*E. faecium*	*E. faecium*

Antibiotic susceptibility phenotypes and resistance genes profile, detected by PCR, of the enterococcal isolates are shown in Table 3. The presence of resistance genes *erm(B), tet(L), vanA* and *aac(6')-Ie-aph(2")* were 86.7%, 23.3%, 80.0% and 66.7%, respectively. Several isolates harbored resistance genes to more than one antibiotic. Of significance were *tet(L)*$^+$*/erm(B)*$^+$ to *E. faecalis* (42.8%) and *erm(B)*$^+$*/aac(6')-Ie-aph(2")-Ia*$^+$*/vanA*$^+$ to *E. faecium* (69.6%).

The presence of the *vanA* gene was detected in three isolates of *E. faecalis* and twenty-two of *E. faecium*, corresponding to 42.8% and 96.6% of the isolates, respectively. The *van(A)* gene was detected in 100% of vancomycin resistant enterococci (Table 3), however, five isolates harbored the *van(A)* gene and presented vancomycin susceptibility phenotype.

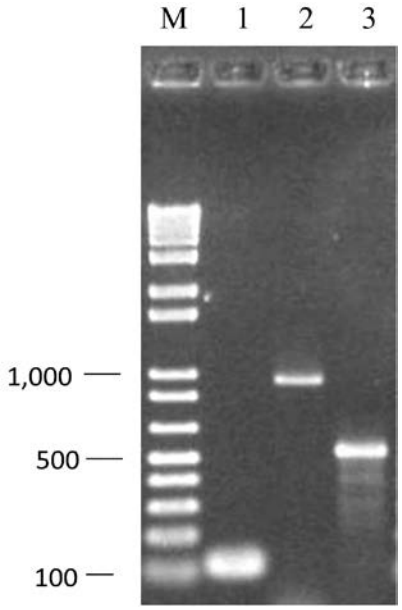


Fig. 1 - Amplification gel pictures characteristic of polymerase chain reaction (PCR) amplification of *Enterococcus* sp gene. Lanes: (1) *Enterococcus* spp. (112 pb), (2) *E. faecalis* (941 pb), (3) *E. faecium* (550 pb). M - Ladder 1kb plus (Invitrogen).

On the other hand, antimicrobial resistance phenotype was detected even in the absence of the respective resistance gene for two isolates to *erm(B),* 10 to *tet(L)* and three to *aac(6')-Ie-aph(2")-Ia* gene.

Additionally, antimicrobial susceptibilities to erythromycin, tetracyclin, vancomycin, and gentamicin were analyzed by disk diffusion. Evaluation revealed excellent agreement for all of the antibiotics with category agreement rates > 80% between automatized method and disk diffusion. Major error rates were for erythromycin, vancomycin and tetracycline with 20.7%, 7% and 16.7% respectively. Minor error rates were found as 12.1% for gentamicin.

Resistance rates obtained by disc diffusion were as follows: 86.7% for erythromycin, 80.0% for vancomycin, 43.35% for tetracycline and 33.3% for gentamicin. Resistance frequencies were higher in *E. faecium* than *E. faecalis*.

DISCUSSION

Enterococci have been implicated in severe human infections as a consequence of associated determinants of virulence and antimicrobial resistance. Accurate identification and rapid analysis of the antibiotic susceptibility pattern of the causative microbial agent leads to earlier targeting of antibiotic therapy and may be lifesaving.

In this study, we describe a comparison between automatic and PCR-based assay for identification of *Enterococcus* spp. Our results showed 90% agreement in the identification of clinically relevant enterococcal species, revealing that the PCR method is reliable and convenient for rapid identification and has potential for use in clinical microbiology laboratories.

Besides, one isolate was identified as *E. gallinarum* and two were

Table 3
PCR presence/absence assays of various antibiotic resistance genes for *Enterococcus* and antibiotic resistant phenotypes by automated systems

Isolates	Genes detected by PCR				Antibiotic resistance phenotype (MIC µg/mL)*			
	em(B)	*tet(L)*	*vanA*	*aac(6')-Ie-aph(2')-Ia*	ERY	TET	VAN	GEN
802	+	+	+	-	> 4R	> 8 R	≤ 2 S	≤ 500 S
817	-	-	+	+	≤ 0,5 S	> 8 R	8 I	≤ 500 S
840	-	-	+	-	>4 R	≤ 4 S	≤ 2 S	≤ 500 S
848	+	+	-	-	2	≤ 4 S	≤ 2 S	≤ 500 S
872	+	+	-	-	> 4 R	> 8 R	≤ 2 S	≤ 500 S
906	+	+	-	-	> 4	> 8 R	≤ 2 S	≤ 500 S
917	+	+	-	-	> 4	> 8 R	≤ 2 S	≤ 500 S
924	+	-	+	+	> 4	≤ 4 S	> 16 R	≤ 500 S
925	+	+	+	+	>4	≤ 4 S	> 16 R	≤ 500 S
928	+	-	+	+	--//--	> 8 R	≤ 2 S	--//--
973	+	-	+	+	> 4	> 8 R	≤ 2 S	--//--
1000	+	-	+	+	> 4	≤ 4 S	> 16 R	> 500 R
1035	+	-	+	+	> 4	> 8 R	> 16 R	> 500 R
1053	+	-	+	+	> 4	> 8 R	> 16 R	≤ 500 S
1062	+	-	+	+	> 4	≤ 4 S	> 16 R	> 500 R
1076	+	-	+	+	> 4	> 8 R	> 16 R	≤ 500 S
1097	-	-	+	+	> 4	> 8 R	> 16 R	≤ 500 S
1112	+	-	+	-	> 4	≤ 4 S	> 16 R	> 500R
1114	+	-	+	+	> 4	≤ 4 S	> 16 R	> 500R
1115	+	-	+	-	> 4	≤ 4 S	> 16 R	> 500 R
1125	+	+	+	+	> 4	≤ 4 S	> 16 R	> 500 R
1143	+	-	+	-	> 4	≤ 4 S	> 16 R	>500 R
1211	-	-	-	-	≤0,5	≤ 4 S	≤ 2 S	≤ 500 S
1215	+	-	+	+	> 4 R	> 8 R	> 16 R	≤ 500 S
1227	+	-	+	+	> 4	≤ 4 S	> 16 R	>500R
1231	+	-	+	+	> 4	> 8 R	> 16 R	≤ 500 S
1246	+	-	+	+	> 4	≤ 4 S	> 16 R	> 500 R
1280	+	-	+	+	> 4 R	≤ 4 S	> 16 R	> 500 R
1295	+	-	+	+	> 4 R	≤ 4 S	> 16 R	> 500 R
1298	+	-	+	+	> 4 R	> 8 R	> 16 R	≤ 500 S

MIC: minimal inhibitory concentration; ERY: erythromycin; TET: tetracycline; VAN: vancomycin; GEN: gentamicin (120 µg/mL); --//--: data not provided; S: sensible; R: resistance; I: intermediate resistance. (*) Result obtained from the automated method.

identified as *E. durans/hirae* by MicrosScan, whereas by PCR-based assay all three isolates were identified as *E. faecium*. Similar discrepancy was described by ROBREDO *et al.*[30], who compared the API20 STREP and colony hybridization for identification of enterococci obtained from several origins. According to these authors, high agreement was obtained for *E. faecalis* identification, however, for eight isolates identified as *E. durans* and *E. casseliflavus* by API20 STREP were identified as *E. faecium* according to the molecular method.

Several studies have found differences between automatic and classical or molecular bacterial identification systems. For instance, concerning Gram positive bacteria, no gram-positive cocci showed concordant identification between the direct and standard methods; other discrepancies consisted of misidentification between various species of coagulase-negative staphylococci[7].

On the other hand, some studies showed the agreement between automatic and classical or molecular bacterial identification systems[16,33]. D'AZEVEDO *et al.*[9] compared the automated Vitek system and standard methods for identification of 80 isolates belonging to different species of *Enterococcus*. The general agreement between results was 83.7%. Among isolates of *E. faecalis* and *E faecium* were observed that the automated system correctly identified 35/40 (87.5%) and 12/14 (85.7%) of the strains, respectively[9].

CEKIN *et al.*[4] demonstrated the consistency of automated systems with the conventional methods. They detected as 97.8% to identification of VRE strains using both methods.

Based on the results presented here and the previous report[39] there is a need for improvement in the automated MicrosScan system to identify enterococci.

In the present study, the genotypic basis of the resistance phenotype found in isolates of *E. faecium* and *E. faecalis* was investigated by PCR based detection of resistance genes. The majority of *Enterococcus* isolates displayed resistance to at least one antibiotic tested. Our results revealed that the *vanA* gene was predominant in *E. faecium* tests since this gene was detected in 100% of vancomycin-resistant isolates, although *Enterococcus* spp. may harbor other genes (*vanB, vanC*-1, *vanC*-2/3 and *vanD*) related to resistance[37].

Antibiotic resistance has played an essential role in the emergence of *E. faecalis* and *E. faecium* as nosocomial pathogens. Vancomycin is an important therapeutic option for the treatment of severe enterococcal infections and resistance to this type of antibiotic is concerning. Identified risk factors for vancomycin-resistant enterococci (VRE) acquisition include a prolonged hospital stay, exposure to intensive care units or residence on transplant oncology wards, prior exposure to antibiotics, and proximity to other patients infected or colonized with VRE[40].

In our study we detected the *tet(L)* gene in 23.3% (7/30) of the isolates, while four and five of these presented resistance to tetracycline in automated and disk diffusion method, respectively. Similar prevalence of *tet(L)* gene (21%) in enterococci was described by STOVCIK *et al.*[35]. In contrast, FRAZZON *et al.*[13] detected the *tet(L)* gene in only 9% of the *Enterococcus* sp isolates. Furthermore, tetracycline resistance phenotype was detected even in the absence of the *tet(L)* gene for 10 isolates. This may be explained by the fact that in enterococci two major groups of tetracycline resistance genes have been identified. One group encoding ribosomal protection proteins include *tet(M)*, *tet(O)* and *tet(S)* genes, and the another one that encodes tetracycline efflux pumps proteins include the *tet(L)* and *tet(K)* genes[18,22,28]. Similarly, erythromycin resistance was detected even in the absence of the *erm(B)*gene. This resistance may be due to the presence of *erm(A* and/or *erm(C)*) genes related to erythromycin resistance phenotype[37].

Gentamicin susceptible phenotype was detected in 36.7% of the isolates. However, 52.6% of these were detected as the *aac(6')-Ie-aph(2")-Ia* gene. Similar results were obtained by POULSEN *et al.*[29].

In our study, the MicrosScan system and disk diffusion method had an agreement of about 80%. GÜLMEZ & HASÇELIK[16] compared the Phoenix system and microdilution method and observed an excellent agreement for all of the antibiotics with category agreement rates of > 97%. In contrast, the API method was considered unreliable in detecting high levels of aminoglycoside resistance among *Enterococcus* strains compared to disc diffusion method[34].

Our data revealed high frequency of *E. faecium* and the occurrence of several multi resistance isolates. Antibiotic resistance appears to have contributed to increasing administration of inadequate antimicrobial therapy for infections, particularly enterococci nosocomial acquired infections, which is associated with greater hospital mortality rates[5,23].

Rapid and reliable identification of these antibiotic resistant organisms is crucial for patient management and infection control measures. Enterococci are intrinsically resistant to many antimicrobial agents, and their ability to acquire resistance to other agents such as aminoglycosides, β-lactams and glycopeptides (vancomycin and teicoplanin) is well known, resulting in invasive human enterococcal infections that are extremely difficult to treat.

The primary objective of the study was to determine whether molecular identification and direct antimicrobial susceptibility testing would provide results comparable to those obtained from an automated system in routine use. This study revealed that the PCR assay and disk diffusion method are in agreement with MicroScan automated system employed for identification and test susceptibility, respectively of clinical *Enterococcus* spp.

RESUMO

Comparação entre o sistema automatizado e PCR na identificação e susceptibilidade de isolados clínicos de *Enterococcus* spp

Os enterococos são cada vez mais responsáveis por infecções hospitalares em todo o mundo. Este estudo foi realizado para comparar a identificação e perfil de suscetibilidade entre o sistema automatizado MicrosScan e a técnica molecular de PCR em espécies de *Enterococcus* spp. Foram avaliados 30 isolados clínicos de *Enterococcus* spp. Os isolados foram identificados pelo sistema MicrosScan® e pela técnica de PCR. A detecção de genes de resistência a antibióticos (vancomicina, gentamicina, tetraciclina e eritromicina) foi determinada por PCR. Suscetibilidades antimicrobianas à vancomicina (30 μg), gentamicina (120 μg), tetraciclina (30 μg) e eritromicina (15 μg), foram testados

pelos métodos automatizados e pelo disco difusão, de acordo com as orientações do CLSI. No que diz respeito à identificação de *Enterococcus* em geral entre os dados obtidos pelo método de PCR e pelo sistema automático foi de 90,0% (27/30). Para todos os isolados de *E. faecium* e *E. faecalis* observamos concordância de 100%. Freqüências de resistência foi maior em *E. faecium* do que em *E. faecalis*. As taxas de resistência obtidas foi maior para eritromicina (86,7%), vancomicina (80,0%), tetraciclina (43,35%) e gentamicina (33,3%). A correlação entre a técnica de disco difusão e automação revelou-se de acordo para maioria dos antibióticos com taxas > 80%. O gene *van*(A) foi detectado em 100% dos *Enterococcus* resistentes á vancomicina. O ensaio baseado em PCR é de simples realização e de confiança para identificação de enterococos clinicamente relevantes. Os dados obtidos reforçam a necessidade de melhoria no sistema automatizado para identificar alguns enterococos.

ACKNOWLEDGEMENT

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - Brazil and Fundação Araucária – Paraná, Brazil. Kátia Real Rocha is fellowship holder of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Brazil.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES

1. Acar JF. Consequences of bacterial resistance to antibiotics in medical practice. Clin Infect Dis.1997;24(suppl 1):S17-8.

2. Bell IM, Paton JC, Turnidge J. Emergence of vancomycin-resistant enterococci in Australia: phenotypic and genotypic characteristics of isolates. J Clin Microbiol. 1998;36:2187-90.

3. Bruins MJ, Bloembergen P, Ruijs GJHM, Wolfhagen MJHM. Identification and susceptibility testing of *Enterobacteriaceae* and *Pseudomonas aeruginosa* by direct inoculation from positive BACTEC blood culture bottles into Vitek 2. J Clin Microbiol. 2004;42:7-11.

4. Cekin Y, Ozhak Baysan B, Mutlu D, Sepin Özen N, Öngut G, Dönmez L, *et al.* Comparison of Phoenix automated system, API ID 32 Strep system and lightcycler *Enterococcus* MGRADE system in the identification of clinical *Enterococcus* isolates. Mikrobiyol Bul. 2013;47:141-6

5. Chow JW, Fine MJ, Shlaes DM, Quinn JP, Hooper DC, Johnson MP, *et al*. *Enterobacter* bacteremia: clinical features and emergence of antibiotic resistance during therapy. Ann Int Med. 1991;115: 585–90.

6. CLSI-Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement Approved standard M100-S21, v. 31, 2011. Wayne; 2011.

7. Courvalin P. Vancomycin resistance in gram-positive cocci. Clin Infect Dis. 2006;42(Suppl 1):S25-S34.

8. Cueto M, Ceballos E, Martinez-Martinez L, Perea EJ, Pascua A. Use of positive blood cultures for direct identification and susceptibility testing with the Vitek 2 system. J Clin Microbiol. 2004;42:3734-8.

9. D 'Azevedo PA, Cantarelli V, Inamine E, Superti S, Dias CAG. Avaliação de um sistema automatizado na identificação de espécies de *Enterococcus.* J Bras Patol Med Lab. 2004;40;237-9.

10. Dukta-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995;33:24-7.

11. Facklam RR, Sahm D, Teixeira LM. *Enterococcus*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of Clinical Microbiology. 7. ed. Washington: ASM Press; 1999. p. 297-305.

12. Fontanals D, Salceda F, Hernandez J, Sanfeliu I, Torra DM. Evaluation of wider systems for direct identification and antimicrobial susceptibility testing of gram-negative bacilli from positive blood culture bottles. Eur J Clin Microbiol Infect Dis. 2002;21:693-5.

13. Frazzon APG, Gama BA, Hermes V, Bierhals CG, Pereira RI, Guedes AG, *et al.* Prevalence of antimicrobial resistance and molecular characterization of tetracycline resistance mediated by *tet(M)* and *tet(L)* genes in *Enterococcus* spp. isolated from food in Southern Brazil. World J Microbiol Biotechnol. 2010; 2:365-70.

14. Gevers D, Danielsen M, Huys G, Swings J. Molecular characterization of *tet(M)* genes in *Lactobacillus* isolates from different types of fermented dry sausage. Appl Environ Microbiol. 2003;69:1270-5.

15. Gholizadeh Y, Courvalin P. Acquired and intrinsic glycopeptide resistance in enterococci. Int J Antimicrob Agents. 2000;16(Suppl 1):S11-7.

16. Gülmez D, Hasçelik G. Comparison of microdilution method and Phoenix automated system for testing antimicrobial susceptibilities of *Enterococcus* strains. Mikrobiyol Bul. 2011;45:21-7.

17. Hansen DS, Jensen AG, Norskov-Lauritsen N, Skov R, Bruun B**.** Direct identification and susceptibility testing of enteric bacilli from positive blood cultures using VITEK (GNI/GNS-GA). Clin Microbiol Infect. 2002;8:38-44.

18. Huys G, D'Haene K, Collard JC, Swings J. Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. Appl Environ Microbiol. 2004;70:1555-62.

19. Jensen LB, Ahrens P, Dons L, Jones RN, Hammerum AM, Aarestrup FM. Molecular analysis of the Tn *1546* in *Enterococcus faecium* isolated from animals and humans. J Clin Microbiol. 1998;36:437-42.

20. Jin WY, Jang SJ, Lee MJ, Park G, Kim MJ, Kook JK, *et al.* Evaluation of VITEK 2, MicroScan, and Phoenix for identification of clinical isolates and reference strains. Diag Microbiol Infect Dis. 2011;70:442-7.

21. Ke D, Picard FJ, Martineau F, Ménard PHR, Ouellette M, Bergeron MG. Development of a PCR assay for rapid detection of enterococci. J Clin Microbiol. 1999;37:3497-503.

22. Kobashi Y, Hasebe A, Nishio M, Uchiyama H. Diversity of tetracycline resistance genes in bacterial isolated from various agricultural environmental. Microbes Environment. 2007;22:44-51.

23. Leibovici L, Shraga I, Drucker M, Konigsberger H, Samra Z, Pitlik SD. The benefit of appropriate empirical antibiotic treatment in patients with bloodstream infection. J Int Med. 1998;244:379-86.

24. Linden PK. Clinical implications of nosocomial Gram-positive bacteremia and superimposed antimicrobial resistance. Am J Med. 1998;104(5A):24-33.

25. Lupetti S, Barnini B, Castagna B, Nibbering PH, Campa M. Rapid identification and antimicrobial susceptibility testing of Gram-positive cocci in blood cultures by direct inoculation into the BD Phoenix system. Clin Microbiol Infect. 2010;16:986-91.

26. Marra AR, Camargo LFA, Pignatari ACC, Sukiennik T, Behar PR, Medeiros EA, *et al.* Nosocomial bloodstream infections in Brazilian Hospitals: analysis of 2,563 cases from a prospective nationwide surveillance study. J Clin Microbiol 2011;49:1866-71.

27. Marques EB, Suzart S. Occurrence of virulence-associated genes in clinical *Enterococcus faecalis* strains isolated in Londrina, Brazil. J Med Microbiol. 2004;53:1069-73.

28. Poeta P, Costa D, Sáenz N, Klibi N, Ruiz-Larrea F, Rodrigues J, *et al.* Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. J Vet Med B Infect Dis Vet Public Health. 2005;52:396-402.

29. Poulsen L, Bisgaard M, Son NT, Trung NV, An HM, Dalsgaard A. *Enterococcus* and *Streptococcus* spp. associated with chronic and self-medicated urinary tract infections in Vietnam. BMC Infect Dis. 2012,12:320.

30. Ratanasuwan W, Iwen PC, Hinrichs SH, Rupp ME. Bacteremia due to motile *Enterococcus* species: clinical features and outcomes. Clin Infect Dis. 1999;28:1175-7.

31. Robredo B, Singh KV, Baquero F, Murray BE, Torres C. Vancomycin-resistant enterococci isolated from animals and food. Int J Microbiol. 2000;54:197-204.

32. Rubin RJ, Harrington CA, Poon A, Diretrich K, Greene A, Moiduddin A. The economic impact of *Staphylococcus aureus* infection in New York city hospitals. Emerg Infect Dis. 1999;5:9-17.

33. Seo JY, Kim PW, Lee JH, Song JH, Peck KR, Chung DR, *et al*. Evaluation of PCR-based screening for vancomycin-resistant enterococci compared with a chromogenic agar-based culture method. J Med Microbiol. 2011;60:945-9.

34. Sirin MC, Adiloğlu AK. Comparison of five antimicrobial susceptibility tests in detecting high level aminoglycoside and vancomycin resistances in hospital acquired *Enterococcus* isolates. Clin Lab. 2011;57:157-62.

35. Stovcik V, Javorsky P, Pristas P. Antibiotic resistance patterns and resistance genes in enterococci isolated from sheep gastrointestinal tract in Slovakia. Bull Vet Inst Pulawy. 2008;52:53-7.

36. Suppli M, Aabenhus R, Harboe ZB, Andersen LP, Tvede M, Jensen J-US. Mortality in enterococcal bloodstream infections increases with inappropriate antimicrobial therapy. Clin Microbiol Infect. 2011;17:1078-83.

37. Torres C, Escobar S, Portillo A, Torres L, Rezusta A, Ruiz-Larrea F, *et al.* Detection of clonally related *van*B2- containing *Enterococcus faecium* strains in two Spanish hospitals. J Med Microbiol. 2006;55:1237-43.

38. Top J, Willens R, Bonten M. Emergence of CC17 *Enterococcus faecium*: from commensal to hospital-adapted pathogen. FEMS Immunol Med Microbiol. 2008;52:297-308.

39. Tritz DM, Iwen PC, Woods GL. Evaluation of MicroScan for identification of *Enterococcus* species. J Clin Microbiol. 1990;28:1477-8.

40. Usacheva EA, Ginocchio CC, Morgan M, Maglanoc G, Mehta MS, Tremblay S, *et al.* Prospective, multicenter evaluation of the BD gene Ohm vanR assay for direct, rapid detection of vancomycin- resistant *Enterococcus s*pecies in perianal and rectal specimens. Am J Clin Pathol. 2010;134:219-26.

41. Vakulenko SB, Donabedian SM, Vorkresenskiy AM, Zervos MJ, Lerner SA, Chow JW. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. Antimicrob Agents Chemother. 2003;47:1423-6.

42. Waites KB, Brookings ES, Moser SA, Zimmer DBL. Direct susceptibility testing with positive BacT/Alert blood cultures by using Micro-Scan overnight and rapid panels. J Clin Microbiol. 1998;36:2052-6.

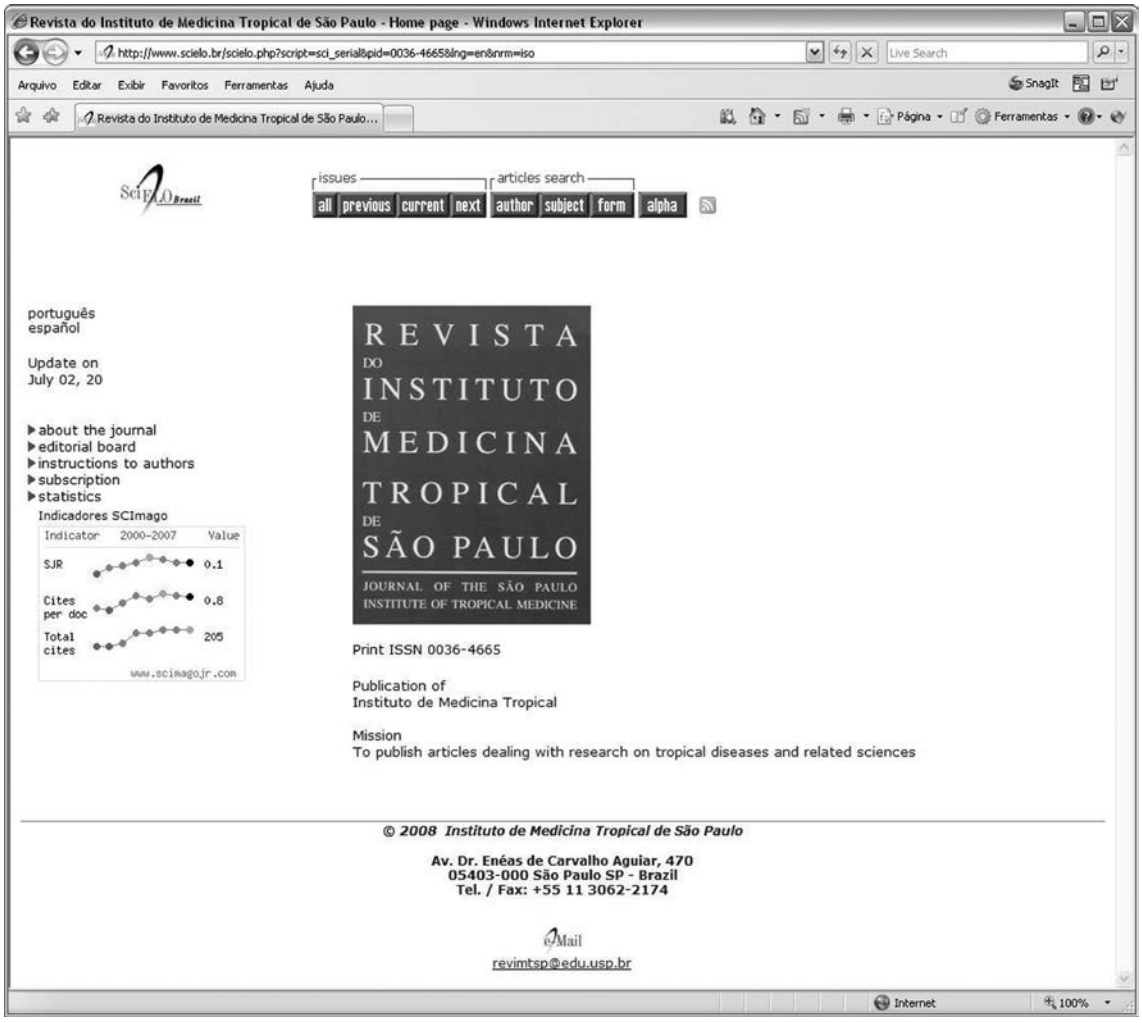
43. Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, *et al.* Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill. 2008;13:1-11.

44. Willey BM, Jones RN, McGeer A, Witte W, French G, Roberts RB, *et al.* Practical approach to the identification of clinically relevant *Enterococcus* species. Diag Microbiol Infect Dis. 1999;34:165-71.

Received: 5 November 2012
Accepted: 5 September 2013

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(2):105-109, March-April, 2014
doi: 10.1590/S0036-46652014000200003

EFFECT OF *Bifidobacterium animalis* ON MICE INFECTED WITH *Strongyloides venezuelensis*

Teresa Cristina Goulart OLIVEIRA-SEQUEIRA(1), Érica Boarato DAVID(1), Cláudia RIBEIRO(2), Semíramis GUIMARÃES(1), Ana Paula Batista MASSENO(2), Satie KATAGIRI(2) & Julio Lopes SEQUEIRA(2)

SUMMARY

The administration of viable *Bifidobacterium animalis* was tested to induce resistance against *Strongyloides venezuelensis* infection in mice. Effects on parasite burden, worm length, egg output, and intestinal mucosal histology were evaluated. The oral administration of *B. animalis,* strain 04450B, starting 14 days before the inoculation of nematode larvae significantly decreased the worm burden and egg output. In probiotic treated animals, the percent reduction of adult worms in the intestine was of 33% and the reduction of egg production was of 21%, compared with those of the control group. The duodenum villous height and villous/crypt ratio were significantly higher in probiotic-treated mice, indicating that this group could be experiencing less intestinal damage. The present findings revealed that the administration of *B. animalis* for the amelioration of host response to nematode infections is biologically plausible and could have some potential for impacting public health. Meanwhile, further study is needed to delineate the nature and identity of the factor(s) involved in these beneficial effects.

KEYWORDS: *Strongyloides venezuelensis; Bifidobacterium animalis;* Probiotics; Mice.

INTRODUCTION

In recent years, there have been remarkable advances in the understanding of the ecology, epidemiology, and morbidity related to helminthic infections, which have pointed to the need for new intervention tools, focusing especially on three main aspects: the improvement of the nutritional status of the host, the prevention or treatment of infection, and the improvement of immunocompetence[20]. Indeed, there has been a significant upsurge in research on the characterization of probiotic bacteria and the evaluation of the potential health benefits associated with the use of these microorganisms as functional food.

Probiotics are defined as live microbial food supplement microorganisms that confer health benefits when administered in adequate amounts[15]. In this way, the overall focus of probiotic research is to assess if dietary microrganisms can safely enhance immune function and gut health by improving the immune response against infectious agents.

The gastrointestinal helminth *Strongyloides stercoralis* currently infects an estimated 30-100 million people worldwide. This infection ranges from an asymptomatic clinical presentation to a severe life-threatening condition in certain population subgroups such as patients under immunosuppressive therapy and individuals with HIV/AIDS. In developing countries, where malnutrition is one of the most important causes of immunodeficiency, mainly among children, this clinical condition has been found to be associated with severe strongyloidiasis[27].

Humans are the natural host of *S. stercoralis*, and up to now, the attempts made to develop an appropriated animal model have been unsuccessful, since this parasite cannot complete its life cycle in immunologically intact mouse strains[10]. Otherwise, *Strongyloides venezuelensis*, a nematode isolated from wild rats, is considered a suitable model of strongyloidiasis, as following the infection, its larvae migrate through the lungs before establishing themselves in the duodenal mucosa, evoking innate and acquired immune responses similar to those observed in human infection by *S. stercoralis*. In human hosts and in murine models, the immune response to *Strongyloides* spp. is characterized by intraepithelial and tissue increase of eosinophils, as well as by intestinal mastocytosis and production of Th2-type cytokines[31]. Moreover, *S. venezuelensis* is safe to be used in experimental assays, being a useful biological model to investigate several aspects of host–parasite relationship, including the efficacy of therapeutic agents[24].

Lately, several probiotic microorganisms have been evaluated as single agents or combination therapies in a wide range of infectious and non-infectious diseases. Studies on probiotics have revealed that these microorganisms contribute to host defense by reinforcing non-immunological responses and stimulating both specific and non-specific host immunity[14]. Probiotic bacteria have successfully been tested in intestinal protozoan and helmintic infections such as cryptosporidiosis[29], giardiasis[6], coccidiosis[9], trichinellosis[3] and toxocariasis[3,7].

The main organisms employed as probiotics are lactic acid bacteria,

(1) Parasitology Department, Institute of Bioscience, São Paulo State University (UNESP), 18618-000 Botucatu, SP, Brazil.
(2) College of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (UNESP), 18618-000 Botucatu, SP, Brazil.
Correspondence to: Teresa Cristina Oliveira-Sequeira, Phone: + 55-14-38116239, Fax: +55-14-38153744. E-mail: sequeira@ibb.unesp.br.

especially of the genus *Lactobacillus* and *Bifidobacterium*. They occur naturally as part of the normal gut flora, having a long history of safe use in food and fermented products[14,30]. Taking into account this particular aspect and all that has been mentioned above, *Bifidobacterium animalis,* one of the main well-characterized probiotic strains that is commercially available, was used in the present study to assess their positive effects on *S. venezuelensis* infection. The *S. venezuelensis* mouse model was chosen, considering that the infection leads to a local immune response at intestine mucosal surface, and an allergic response in the lung, both proposed to be modulated by probiotic administration.

MATERIALS AND METHODS

Animals: Four-week-old male BALB/c inbred mice were purchased from the Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório (CEMIB), Universidade Estadual de Campinas, SP, Brazil. Twenty-eight animals were housed in plastic cages, fed a standard diet, and allowed free access to water. The experimental procedures were approved by the Animal Ethics Committee of College of Veterinary Medicine and Animal Science (UNESP).

Bacteria: *Bifidobacterium animalis* strain 04450B was kindly provided by Danisco Brasil Ltda. The probiotic was grown on De Man-Rogosa-Sharpe broth (Oxoid) adding NA_2CO_3 (0.02%), $CaCl_2.2H_2O$ (0.01%), and L-cysteine hydrochloride (0.05%) under anaerobic conditions at 37° C for 16 hours. Following culture, the medium was centrifuged at 3000g, and the pelleted organisms were resuspended in 10% sterile milk to give a final concentration of 2 x 10^9 CFU/mL.

Nematode: The strain of *Strongyloides venezuelensis* was isolated from a wild rat in the early '80s in Botucatu, São Paulo State, and has been maintained in the laboratory by repeated passages in Wistar rats. Filariform larvae (L_3) were obtained from fecal cultures and adjusted to the appropriate number in distilled water in order to infect the animals[23].

Experimental procedure: The animals were randomly allocated in groups of seven mice, distributed as follows: two probiotic treated groups (PB1 and PB2) and two non-treated control groups (C1 and C2). Daily, the animals of the groups PB1 and PB2 received one mL of milk containing 2 x 10^9 UFC of *B. animalis* by gavage. The control groups of mice (C1 and C2) received the same volume (one mL) of skim milk. These procedures were repeated until the animals had been killed.

Fourteen days after the beginning of the experiment, 2,000 infective larvae (L3) of *S. venezuelensis* were subcutaneously inoculated in all animals of the four groups, according to AMARANTE & OLIVEIRA-SEQUEIRA[1]. For collection of fecal samples, the animals of probiotic treated (PB1 and PB2) and control (C1 and C2) groups were allocated in boxes where they stayed for three hours. The feces obtained from each group constituted a sample for fecal egg counts (FEC). Fecal egg counts were daily assessed by the modified McMaster technique[16] in which, each egg counted represents 100 eggs per gram of feces (EPG).

Six days after the inoculation of the nematode infective larvae, the mice of PB1 and C1 groups were killed. The small intestine (SI) was removed and a segment of one cm was sectioned (2-6 cm from the pyloric ring) and immersed in Boin's fixative for histological analysis. For the recovery and counting of adult worms, the remaining small intestine was inverted over a thin wire support, placed in 20 mL tubes containing phosphate-buffered saline (PBS), and incubated for four hours at 37 ºC[1]. The wire supports with intestines were then vigorously shaken and removed from tubes. Supernatants were discharged and the sediment containing the worms was preserved in formaldehyde 5%. The nematodes were counted under stereoscopic microscopy. To determine the worms' length, 20 partenogenetic females recovered from each animal were measured using a computerized image analysis system (QWin Lite 2.5, Leica), adapted in DMLB light photomicroscope (Leica).

The fecal egg counts were carried out for the remaining animals of groups PB2 and C2 until the 14th day, when they were killed. At the necropsy, the small intestine was removed and processed as previously described.

Histological analysis: The fixed fragments of duodenum tissues were embedded in paraffin, and cut into 3-5 µm thick sections. The sections stained with hematoxylin-eosin were used for histopathological analysis and for villous height and crypt depth measurements. Villous height was measured from the tip to the crypt junction, and the crypt depth was defined as the depth of the invagination between adjacent villi. The ratio of villous/crypt length (v/c) was also calculated. The villous and crypt lengths measurements were performed in 10-crypt units of each sample, as recommended by IERNA *et al.*[19]. These results were expressed as a mean of each group.

Statistical analysis: The two-tailed Student's *t*-test was employed to evaluated group differences in relation to parasitological parameters (worm burden and female length), and the differences related to histological parameters (crypt and villous length) were analyzed by Mann-Whitney *U* test. p values < 0.05 were considered statistically significant.

RESULTS

Parasitological parameters: Kinetics of fecal egg counts (FEC) in *S. venezuelensis* infected mice (probiotic-treated and control groups) are shown in Fig. 1. In both animal groups, eggs were first detected in feces five days after larval subcutaneous inoculation. In probiotic-treated animals, egg output peaked on day 7 when it reached 25,500 EPG. In the control group, the peak occurred on day 6, reaching 40,400 EPG. The patent period of infection was seven and eight days, respectively, for control and probiotic-treated groups. The total FEC at the end of the patent period was 60,367 and 76,433 EPG in probiotic treated and control groups, respectively.

On day 6 after infection, the worm burden assessment (Fig. 2) revealed that the number of partenogenetic females (5,235) recovered from all seven animals of *B. animalis*-treated group was lower than that from the control one (7,819). The mean number of females recovered from probiotic-treated mice was 654 (± 137) while in the control group it was 977 (± 183). In *B. animalis*-treated mice the minimum (216) and maximum (608) numbers of parasitic females recovered were lower than those from control animals, which ranged from 608 to 1,388 worms. All these differences were statistically significant ($p < 0.05$).

The female length recovered from probiotic-treated mice ranged from 1,860 to 4,082 µm (2,943 ± 128 µm), whereas those from control mice

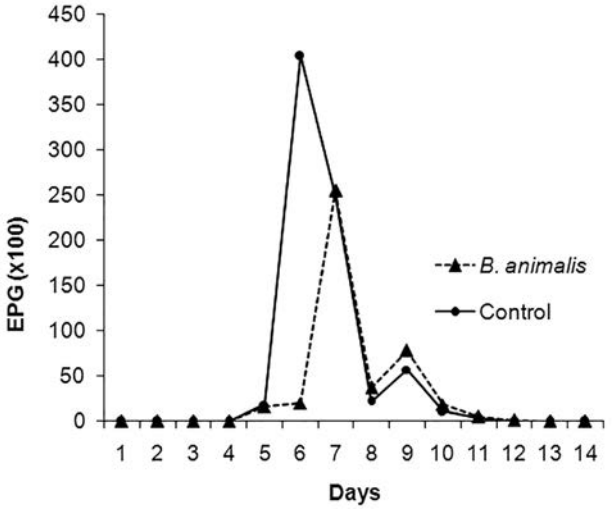


Fig. 1 - Kinetics of FEC in *S. venezuelensis* infected mice probiotic-treated and control groups. 177x133 mm (100 x 100 DPI).

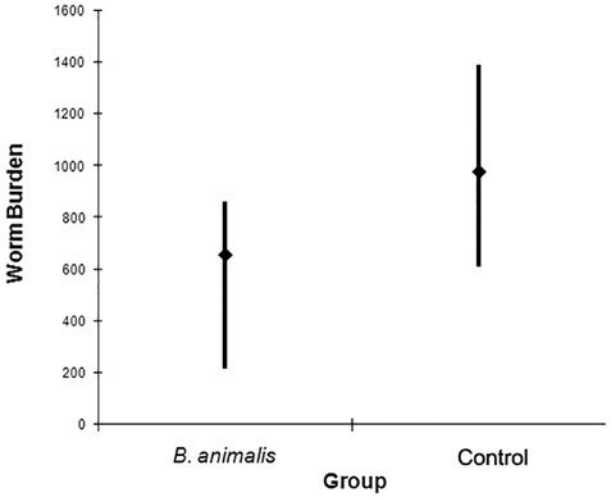


Fig. 2 - Mean, minimum and maximum numbers of *S. venezuelensis* partenogenetic females recovered from probiotic-treated and control groups of mice. 163x133 mm (100 x 100 DPI).

ranged from 2,319 to 3,781 µm (3,025 ± 151 µm), showing no statistical differences ($p = 0.25$).

Histological analysis: In the sections of duodenum collected at the 6th day after infection (dpi), mature worms were found in the lumina of the gut, lodged in spaces between mucosal epithelial cells and into the lamina propria (LP) of the villi. At this time, in mice of both PB1 and C1 groups, the presence of worms was accompanied by a diffuse cellular infiltration consisting of polimorphonuclear eosinophils and mononuclear cells. These inflammatory cells were sparsely distributed in the basal zone of LP, while at tip of the villi, these cells were clustered. Blood vessels at the tip of the villi showed some degree of congestion together with interstitial edema. Besides, the epithelial cells of the top of the villi showed a slight level of vacuolar degeneration. In the mice killed at 14th dpi, no worms were seen in the intestine, either in probiotic-treated or in control mice, but the same kind of cellular infiltration observed at 6th dpi was still present.

The villous height, crypt depth, and villous/crypt ratio in the upper small intestine of control and probiotic-treated mice on the 6th (PB1 and C1) and 14th (PB2 and C2) days after infection with *S. venezuelensis* are shown in Table1. Probiotic administration did not affect crypt depth, but the associated villous height and villous/crypt ratio were significantly greater in probiotic-treated mice killed at 6th dpi (PB1) when compared to its respective control group (C1) and to probiotic-treated (PB2) and control (C2) groups, killed at 14th dpi.

Table 1
Effect of probiotic treatment on villous height and crypt depth (µm), and villous/crypt ratio in mice infected with *S. venezuelensis*

Groups	Villous height (Mean ± SEM)	Crypt depth (Mean ± SEM)	Villous/crypt ratio
PB1	477.1(86.4)[a]	236.8(33.2)	2.01 [a]
PB2	358.7(42.7)[b]	254.6(58.0)	1.41[b]
C1	387.9(69.3)[b]	264.3(71.4)	1.47[b]
C2	374.1(57.5)[b]	279.0(60.5)	1.34[b]

Values followed by different letters in columns are significantly different ($p < 0.05$)

DISCUSSION

Helminth infections are among the most common infections in humans. In developing countries, this infection is frequently associated with malnutrition, representing a significant cause of child developmental retardation. On the other hand, in most developing countries, there are public school feeding programs designed to avoid malnutrition focusing on complementary nutrition, child intestinal parasite control or both[35]. Therefore, the present study was an attempt to investigate the effect of probiotic supplementation on the modulation of helminth infection using a murine model.

Several clinical studies have demonstrated the therapeutic and/or prophylactic efficacy of specific probiotics against acute viral gastroenteritis and antibiotic-associated diarrhea. The studies on the protection afforded probiotics have been performed mainly with bacterial enteropathogens, but it has been suggested that they may also influence the degree of parasitic infection such as those due to *Giardia*[5,18], *Eimeria*[9,22] and *Cryptosporidium*[29].

In the present study, the administration of *B. animalis* to *S. venezuelensis* infected mice triggered a protective effect, characterized by a reduced number of worms and also by a reduced egg output. The percentage of adult worm reduction in the intestine, six days after *S. venezuelensis* infection, was 33% of mice treated with *B. animalis*. In the same way, the reduction of egg production in the probiotic-treated mice was 21%. Interestingly, the reduction of egg output was especially significant in the peak of elimination, when besides the reduction of 37%, peak delayed one day in probiotic treated mice in relation to control mice. Female length and pre-patent period were not affected by the probiotic administration, but the patent period was one day longer in probiotic-treated mice.

In relation to probiotic effects on helminth infections, the few available studies provide conflicting results. Reduction of worm burden

was reported in mice infected with *Trichinella spiralis*[3,4,25] and *Toxocara canis*[2,7]. The failure of probiotics in affecting parasite load was reported in rats[12] and mice[33] infected with *T. spiralis*, and finally, an enhancement of mice susceptibility to *Trichuris muris* was attributed to *Lactobacillus casei* administration[11].

The overall evaluation of the effect of probiotic administration on the worm burden revealed that is difficult to compare results, because of differences in study design, animal model, probiotic dose and strain, and administration route. Nevertheless, most of the available studies revealed promising results, encouraging further investigations in order to confirm the real role of probiotics in host response to nematode infections.

Worm burden is the most frequently investigated aspect in terms of host response to helminth infections, but this response may also interfere with some of the physiological functions of the parasite, including reduction of parasite fertility, limited growth and structural damage[21]. Our data revealed no effect of probiotic administration on the growth of female recovery on the 6th day after infection ($p = 0.26$). So, instead of being related to poor female growth, the observed reduction of egg output may be related to worm burden, because fecal egg counts are correlate highly with parasite load[26]. This is an important observation because egg production is the main means by which the parasite is known to spread. Reduction of fecal shed forms like cysts and oocysts associated with probiotic administration was previously reported in *Giardia*[32] and *Eimeria acervulina* infections[8,9,22].

The beneficial claims of probiotics activities are poorly understood, needing scientific validation that can be addressed by assessing probiotic strains under controlled experimental conditions. In order to understand the immunomodulatory mechanism by which probiotic administration improves the host response against *S. venezuelensis* infection, some components of the intestinal response considered relevant against nematode infection were investigated.

In the present work, eosinophils and mononuclear were the main cells infiltrating the intestinal mucosa of mice, showing similar patterns of distribution in probiotic-treated mice and control group, either after six or fourteen days after the inoculation of the *S. venezuelensis* larvae. Increased eosinophils and mononuclear cell numbers are commonly associated with helminth infections, and in *S. stercoralis* infection of mice, their role as antigen-present cell for the induction of the primary and expansion of secondary Th2 immune response has been characterized[28]. According to the data obtained here, *B. animalis* administration appears to make no difference in patterns of effector cells infiltration and spatial distribution. Therefore, the effect of the probiotic administration in reducing the worm burden and the egg output in *B. animalis* treated mice cannot be attributed to a modulation of these inflammatory cells, at least at the intestinal level. It would be of great interest to know whether *B. animalis* has an effect upon these cells during lung migrating stages, since *S. venezuelensis* infection also increases eosinophil and mononuclear cell numbers in blood and bronchoalveolar fluid[23]. Besides, it appears that activated eosinophils are relevant for the destruction of migrating larvae, rather than for the elimination of adult worms[13].

Villous atrophy and/or crypt hyperplasia are occasionally induced by nematode infection[17] and high apoptotic rates concomitant with low cell proliferation was found associated with a *S. stercoralis* infection in humans[34]. On the other hand, it has been demonstrated that the administration of either *Bifidobacterium adolescentis* or *Bifidobacterium longum* increase the height of duodenum villi[36]. Thus, in the present work, the measurement of villous height and crypt depth was performed in order to verify if probiotic administration has some effect in repairing the intestinal epithelium during *S. venezuelensis* infection.

Data obtained here revealed that the duodenum villous height and villous/crypt ratio were significantly higher in probiotic-treated mice killed six days after *S. venezuelensis* infection, indicating that this group could be experiencing less intestinal damage. Fourteen days after infection, when most of the worms were expelled, no difference in villous length or villous/crypt ratio was found in probiotic-treated and control mice. A time declining effect of the administration of *Bifidobacterium longum* upon the mice's villous length was previously reported[36].

Taken together, the present findings suggest that the administration of *B. animalis* to improve immunity to nematode infections is biologically plausible and could have some impact in public health. Considering that intestinal parasitism and malnutrition share a similar geographical distribution, studies with probiotics should focus on the development of functional foods for the treatment of both diseases.

RESUMO

Efeito da administração de *Bifidobacterium animalis* sobre a infecção por *Stongyloides venezuelensis* em camundongos

Os efeitos da administração de *Bifidobacterium animalis* viáveis sobre a infecção por *Strongyloides venezuelensis* foram avaliados em camundongos experimentalmente infectados. Os parâmetros analisados incluíram a carga parasitária, o comprimento dos vermes, a quantidade de ovos eliminados e a histologia da mucosa intestinal. A administração oral da cepa 04450B de *B. animalis,* iniciada 14 dias antes da inoculação de larvas do nematódeo, foi acompanhada de uma redução significativa do número de vermes que se estabeleceu no intestino e do número de ovos eliminados nas fezes. Nos animais tratados com o probiótico, o percentual de redução de vermes adultos no intestino foi de 33% e da produção de ovos foi de 21%, em comparação com os do grupo controle. O comprimento das vilosidades do duodeno e a relação vilus/cripta foram significativamente maiores nos animais tratados, indicando que nestes animais as lesões intestinais foram mais leves. Os resultados do presente trabalho revelaram que a administração de *B. animalis* com o propósito de modular a resposta do hospedeiro contra infecções por nematódeos é uma possibilidade biologicamente plausível com impacto potencial em saúde pública. No entanto, são ainda necessários mais estudos para esclarecer os mecanismos de ação destes microrganismos e identificar os fatores envolvidos na produção dos efeitos benéficos.

REFERENCES

1. Amarante AFT, Oliveira-Sequeira TCG. *Strongyloides venezuelensis* infection susceptibility of seven inbred strains of mice. Arq Bras Med Vet Zootec. 2002;54:273-8.

2. Basualdo J, Sparo M, Chiodo P, Ciarmela M, Minvielle M. Oral treatment with a potential probiotic (*Enterococcus faecalis* CECT 7121) appears to reduce the parasite burden of mice infected with *Toxocara canis*. Ann Trop Med Parasitol. 2007;101:559-62.

3. Bautista-Garfias CR, Ixta-Rodríguez O, Martínez-Gómez F, López MG, Aguilar-Figueroa BR. Effect of viable or dead *Lactobacillus casei* organisms administered orally to mice on resistance against *Trichinella spiralis* infection. Parasite. 2001;8(2 Suppl):S226-8.

4. Bautista-Garfias CR, Ixta O, Orduña M, Martínez F, Aguilar B. Cortés, A. Enhancement of resistance in mice treated with *Lactobacillus casei*: effect on *Trichinella spiralis* infection. Vet Parasitol. 1999;80:251-60.

5. Benyacoub J, Pérez PF, Rochat F, Saudan KY, Reuteler G, Antille N, *et al. Enterococcus faecium* SF68 enhances the immune response to *Giardia intestinalis* in mice. J Nutr. 2005;135:1171-6.

6. Besirbellioglu BA, Ulcay A, Can M, Erdem H, Tanyuksel M, Avci IY, *et al. Saccharomyces boulardii* and infection due to *Giardia lamblia*. Scand J Infect Dis. 2006;38:479-81.

7. Chiodo PG, Sparo MD, Pezzani BC, Minvielle MC, Basualdo JA. *In vitro* and *in vivo* effects of *Enterococcus faecalis* CECT7121 on *Toxocara canis*. Mem Inst Oswaldo Cruz. 2010;105:615-20.

8. Dalloul RA, Lillehoj HS, Tamim NM, Shellem TA, Doerr JA. Induction of local protective immunity to *Eimeria acervulina* by a *Lactobacillus*-based probiotic. Comp Immunol Microbiol Infect Dis. 2005;28:351-61.

9. Dalloul RA, Lillehoj HS, Shellem TA, Doerr JA. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. Poult Sci. 2003;82:62-6.

10. Dawkins HJ, Grove DI. Attempts to establish infections with *Strongyloides stercoralis* in mice and laboratory animals. J Helminthol. 1982;56:23-6.

11. Dea-Ayuela MA, Rama-Iñiguez S, Bolás-Fernandez F. Enhanced susceptibility to *Trichuris muris* infection of B10Br mice treated with the probiotic *Lactobacillus casei*. Int Immunopharmacol. 2008;8:28-35.

12. de Waard R, Garssen J, Snel J, Bokken GC, Sako T, Veld JH, *et al*. Enhanced antigen-specific delayed-type hypersensitivity and immunoglobulin G2b responses after oral administration of viable *Lactobacillus casei* YIT9029 in Wistar and Brown Norway rats. Clin and Diagn Lab Immunol. 2001;8:762-7.

13. El-Malky M, Maruyama H, Hirabayashi Y, Shimada S, Yoshida A, Amano T, *et al.* Intraepithelial infiltration of eosinophils and their contribution to the elimination of adult intestinal nematode *Strongyloides venezuelensis* in mice. Parasitol Int. 2003;52:71-9.

14. Gill HS. Probiotics to enhance anti-infective defences in the gastrointestinal tract. Best Pract Res Clin Gastroenterol. 2003;17:755-73.

15. Guarner F, Schaafsma GJ. Probiotics. Int J Food Microbiol.1998;39:237-8.

16. Gordon HM, Whitlock HV. A new technique for counting nematode eggs in sheep faeces. J Counc Sci Ind Res. 1939;12:50-2.

17. Hashimoto K, Uchikawa R, Tegoshi T, Takeda K, Yamada M, Arizono N. Depleted intestinal goblet cells and severe pathological changes in SCID mice infected with *Heligmosomoides polygyrus*. Parasite Immunol. 2009;31:457-65.

18. Humen MA, De Antoni GL, Benyacoub J, Costas ME, Cardozo MI, Kozubsky L, *et al*. *Lactobacillus johnsonii* La1 antagonizes *Giardia intestinalis in vivo*. Infect Immun. 2005;73:1265-9.

19. Ierna MX, Scales HE, Mueller C, Lawrence CE. Transmembrane tumor necrosis factor alpha is required for enteropathy and is sufficient to promote parasite expulsion in gastrointestinal helminth infection. Infect Immun. 2009;77:3879-85.

20. Koski KG, Scott ME. Gastrointestinal nematodes, nutrition and immunity: breaking the negative spiral. Annu Rev Nutr. 2001;21:297-321.

21. Krupp IM. Effects of crowding and superinfection on habitat selections and egg production in *Ancylostoma caninum*. J Parasitol. 1961;47:957-61.

22. Lee SH, Lillehoj HS, Dalloul RA, Park DW, Hong YH, Lin JJ. Influence of *Pediococcus*-based probiotic on coccidiosis in broiler chickens. Poult Sci. 2007;86:63-6.

23. Machado ER, Carlos D, Lourenço EV, Sorgi CA, Silva EV, Ramos SG, *et al*. Counterregulation of Th2 immunity by interleukin 12 reduces host defenses against *Strongyloides venezuelensis* infection. Microbes Infect. 2009;11:571-8.

24. Machado ER, Ueta MT, Gonçalves-Pires MR, de Oliveira JB, Faccioli LH, Costa-Cruz JM. Diagnosis of human strongyloidiasis using particulate antigen of two strains of *Strongyloides venezuelensis* in indirect immunofluorescence antibody test. Exp Parasitol. 2001;99:52-5.

25. Martínez-Gómez F, Santiago-Rosales R, Bautista-Garfias CR. Effect of *Lactobacillus casei* Shirota strain intraperitoneal administration in CD1 mice on the establishment of *Trichinella spiralis* adult worms and on IgA anti-*T. spiralis* production. Vet Parasitol. 2009;162:171-5.

26. Oliveira-Sequeira TCG, Amarante AFT. Dynamics of *Strongyloides venezuelensis* infection and relationship between fecal egg counts and parasite burden in Swiss mice. Rev Bras Med Vet. 2001;23:99-102.

27. Olsen A, van Lieshout L, Marti H, Polderman T, Polman K, Steinmann P, *et al*. Strongyloidiasis: the most neglected of the neglected tropical diseases? Trans R Soc Trop Med Hyg. 2009;103:967-72.

28. Padigel UM, Hess JA, Lee JJ, Lok JB, Nolan TJ, Schad GA, *et al*. Eosinophils act as antigen-presenting cells to induce immunity to *Strongyloides stercoralis* in mice. J Infect Dis. 2007;196:1844-51.

29. Pickerd N, Tuthill D. Resolution of cryptosporidiosis with probiotic treatment. Postgrad Med J. 2004;80:112–3.

30. Reid G, Jass J, Sebulsky MT, McCormick JK. Potential uses of probiotics in clinical practice. Clin Microbiol Rev. 2003;16:658-72.

31. Rodrigues RM, Silva NM, Gonçalves AL, Cardoso CR, Alves R, Gonçalves FA, *et al*. Major histocompatibility complex (MHC) class II but not MHC class I molecules are required for efficient control of *Strongyloides venezuelensis* infection in mice. Immunology. 2009;128:432-41.

32. Shukla G, Devi P, Sehgal R. Effect of *Lactobacillus casei* as a probiotic on modulation of giardiasis. Dig Dis Sci. 2008;53:2671-9.

33. Verdú EF, Bercík P, Bergonzelli GE, Huang XX, Blennerhasset P, Rochat F, *et al*. *Lactobacillus paracasei* normalizes muscle hypercontractility in a murine model of postinfective gut dysfunction. Gastroenterology. 2004;127:826-37.

34. Werneck-Silva AL, Alvares EP, Gama P, Damião AO, Osaki LH, Ogias D, *et al.* Intestinal damage in strongyloidiasis: the imbalance between cell death and proliferation. Dig Dis Sci. 2006;51:1063-9.

35. Worku N, Erko B, Torben W, Belay M, Kasssu A, Fetene T, *et al*. Malnutrition and intestinal parasitic infections in school children of Gondar, North West Ethiopia. Ethiop Med J. 2009;47:9-16.

36. Yang H, Liu A, Zhang M, Ibrahim SA, Pang Z, Leng X, *et al*. Oral administration of live *Bifidobacterium* substrains isolated from centenarians enhances intestinal function in mice. Curr Microbiol. 2009;59:439-45.

Received: 14 May 2013
Accepted: 23 July 2013.

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(2):111-114, March-April, 2014
doi: 10.1590/S0036-46652014000200004

PREVALENCE OF INTESTINAL PARASITES AMONG FOOD HANDLERS IN WESTERN IRAN

Farnaz KHEIRANDISH(1) Mohammad Javad TARAHI(2) & Behrouz EZATPOUR(3)

SUMMARY

Parasitic infection is one of the problems that affect human health, especially in developing countries. In this study, all of the fast food shops, restaurants, and roast meat outlets of Khorramabad (Western Iran) and all the staff employed by them, some 210 people, were selected through a census and their stools were examined for the presence of parasites. The parasitological tests of direct wet-mount, Lugol's iodine staining, formaldehyde-ether sedimentation and Trichrome staining techniques were performed on the samples. The data was analyzed with a chi-square test and logistic regression was selected as the analytical model. The results showed 19 (9%) stool specimens were positive for different intestinal parasites. These intestinal parasites included *Giardia lamblia* 2.9%, *Entamoeba coli* 4.3%, *Blastocystis* sp. 1.4%, and *Hymenolepis nana* 0.5%. There was a significant difference between the presence of a valid health card, awareness of transmission of intestinal parasites, participation in training courses in environmental health with intestinal parasites ($p < 0.05$). No statistically significant difference was found between the rate of literacy and gender among patients infected with intestinal parasites ($p > 0.05$). To control parasitic infection in food handlers, several strategies are recommended such as stool examinations every three months, public education, application of health regulations, controlling the validity of health cards and training on parasitic infection transmission. In this regard, the findings of the present study can be used as a basis to develop preventive programs targeting food handlers because the spread of disease via them is a common problem worldwide.

KEYWORDS: Intestinal parasite; Food handler; Prevalence; Western Iran.

INTRODUCTION

Humans have always been in contact with different parasites and one of the hygiene indices of the health of each society is the occurrence of parasites in a population[30,31]. Africa, Asia and the Americas are more affected by these debilitating diseases than other parts of the world[31]. Therefore, contamination with intestinal parasites is a worldwide health problem.

Despite the fact that some parasites are well tolerated in healthy people, other factors debilitate the body through their repetitive damages. For example, *Giardia lamblia* disturbs the fat absorption process, some worms create anemia, and other parasites (each with special mechanisms, such as sensitivity reactions simultaneously with other diseases) make treatment more difficult and diagnosis more complicated[7].

With regard to the social, economic and geographical conditions of Iran and its population changes, this country is an appropriate place for the growth and reproduction of all kinds of parasites[28]. In spite of all considerable actions to harness and control parasitical diseases and reduce their occurrences in recent years, contamination with intestinal parasites is one of the problems in this country[3,6].

The reasons for the incidence of parasites in some parts of the country are: the special climate of the region local traditions and customs, and the use of human and animal fertilizers in agriculture and vegetable planting[6]. In some parasites, infection can be transmitted directly from one infected person to another, and is indirectly transferred through the exchange of tools[12]. Another mode of transmission can be through the contamination of farmlands by human feces due to the use of raw sewage and plant feeding, especially of raw vegetables. This is one of the most important factors of contamination[7]. The other ways are contamination of water by sewage and also the transmission through some insects such as flies and beetles[7,16]. The spread of disease via food handlers is a common and persistent problem worldwide[2,27].

If food handlers are contaminated with parasites which have the potential to be directly transmitted from one person to another, they can transmit contamination to food, dishes and finally to the people who use them[5]. It is necessary to note that infected persons act as carriers after imperfect treatment; therefore parasites can be transferred from these persons to others[5].

Food sold in markets may be contaminated by hands that have not been washed after defecation or from flies that land on both food and

(1) Department of Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran. Tel/Fax: +98 661 6200133, P.O. Box: 381351698. E-mail: kheirandish81@yahoo.com
(2) Department of Epidemiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, Tel/Fax: +98 21 88989127, P.O. Box: 1471613151. E-mail: tarrahi_mj@yahoo.com
(3) Department of Parasitology and Mycology, Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran. Tel/Fax: +98 661 6200133, P.O. Box: 381351698. E-mail: bezatpour@gmail.com
Correspondence to: Farnaz Kheirandish, Department of Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran, Tel/Fax: +98 661 6200133, P.O. Box: 381351698. E-mail: Kheirandish81@yahoo.com

feces hence increasing the risk of transmission of intestinal parasites for consumers[19]. Healthy preparation, transportation, preservation and distribution of food can help prevent food contamination[10,11]. Food handlers with poor personal hygiene and inadequate knowledge on food safety could be the source of food borne pathogens[14].

Regarding the patients and complications of parasitic diseases, the importance of their eradication or control becomes more evident. This is possible by identification and detailed study of the geographical distribution as well as ratio of contamination and effects of different cultural, social, and geographical factors. The aim of this study was to assess the prevalence of parasitic infections and make the association of positivity with some epidemiological factors.

METHODS

Study area: Khorramabad, located in Western Iran, has a population of 487,187 inhabitants, and 210 staff of fast food shops, roasted meat shops, and restaurants, according census in 2011.

Feces collection and analysis: All food handlers in Khorramabad were investigated. Out of the 210 individuals included in this study, 184 (87.6%) were men and 26 (12.4%) women. The median age of the patients was 31 with a range of 16 to 60 years. To select the samples, a census method was used so that the stools of all the mentioned staff were taken.

The questionnaire was prepared on the basis of gender, age, literacy rate, awareness of intestinal parasitical disease transmission, validity of health cards and participating in training courses in environmental health.

People who constantly work in the deli have a health card, which is issued by the health deputy of the university of medical sciences and should be checking for infectious and contagious diseases every six months. If a person is infected, immediately take them to a doctor and until they are fully cured, they are not allowed to work. But people who work as temporary workers do not have this card and if they have a parasitic infection, this can be hazardous for others.

Completion of the questionnaires and the collecting of the samples were performed by people who have been trained for this purpose. They attended the places, completed the questionnaires, delivered the stool containers to the staff, collected the containers from them the following morning, and delivered them immediately to the research laboratory of Lorestan University of Medical Sciences.

The parasitological test of direct wet-mount, Lugol's iodine staining, formaldehyde-ether sedimentation and Trichrome staining techniques were performed on the samples[9]. The guide for diagnosis of intestinal parasites was used as an identification reference[32].

Statistical analysis: The data was analyzed with chi-square test and logistic regression was selected as the analytical model. R software was used to calculate ORs, 95% confidence intervals (CI) and associated p values[22]. Univariate analyses were performed to assess the effect of individual factors on contamination, then a multivariate model was fitted to identify independent predictors of poor outcomes. Backwards elimination procedures were performed within each domain, with explanatory variables carried forward from each domain that were either significant ($p < 0.05$) or borderline significant ($0.05 < p < 0.2$). The final regression model included only those variables that were independently significant at the 5% level.

RESULTS

The prevalence of intestinal parasites in 210 stool samples was 9%. The results of our study showed that contamination with intestinal worms and protozoa were 0.5% and 8.5%, respectively. Also occurrence of pathogenic and nonpathogenic parasites was 4.8% and 4.2%, respectively.

Intestinal parasites included *Giardia lamblia* 2.9%, *Entamoeba coli* 4.3%, *Blastocystis* sp. 1.4%, and *Hymenolepis nana* 0.5%.

Out of the 210 food handlers, 190 (90.47%) had a health card and 10 (9.53%) did not. Of the 190 health card holders, 164 (86.32%) had valid dates and 26 (13.68%) did not have valid dates.

Out of 210 food handlers, 209 of them answered the question about awareness of intestinal parasites transmission. There was a significant difference between validity date of health card, awareness of intestinal parasites transmission, participation in training courses in environmental health with intestinal parasites ($p < 0.05$). No statistically significant difference was found between rate of literacy and gender among patients infected with intestinal parasites ($p > 0.05$). These variables were entered in the regression model. The results of univariate conditional logistic regression analyses were shown in Table 1.

Awareness of the transmission of intestinal parasitical diseases was due to the influence of participating in training courses and also the observation of the collinearity between the two. The variable of participating in training courses was not used in the logistic regression model. Odds ratio of having a valid health card and the awareness of intestinal parasites transmission was 0.08 and 0.07, respectively (Table 1).

DISCUSSION

Parasitic infection is one of the problems that affect human health, especially in developing countries[30,31]. Different studies have been conducted in the field of intestinal parasite prevalence in food handlers[19,29]. In this study, the low prevalence of intestinal parasites found in the food handlers is different from what was reported by other authors in different parts of the world[19,29].

The study was conducted in Khorramabad on 816 bakery workers and the results showed 96 (11.9%) stool specimens were positive for different intestinal parasites[15]. The prevalence of intestinal parasites in bakery workers and food handler were almost identical. It may be due to the similarity of conditions.

The results of our study showed that contamination with protozoa was greater than that by helminthes. Similar studies about the prevalence of intestinal parasites were conducted on food handlers, in which some results are the same, and showed protozoan infections were more common than worm infections[3,8,24,26,29]. One reason for this is that transfer of protozoa is much easier than the transfer of the eggs or larvae of worms[13,25]. In this study, the rates of infection with pathogenic and non-pathogenic parasites were similar (4.8% and 4.2% respectively).

Table 1
Prevalence of intestinal parasites according to epidemiological factors observed in staff of fast food shops, roasted meat shops, and restaurants in Khorramabad city, West of Iran, in 2011

Variable	No. of samples	Frequency of contamination	Odds Ratio (95% CI)	*p*-value
Gender				
Male	184	9.24% (17/184)	Ref.	
Female	26	7.69% (2/26)	0.85 (0.08-4.1)	0.797
Level of education				
Low literate	104	7.69% (8/104)	Ref.	
Diploma	93	10/75% (10/93)	1 (0.11-8.7)	1
Academic degree	13	7.69% (1/13)	1.45 (0.17-12.32)	0.74
Age group (years)				
> 25	59	3.39% (2/59)	Ref.	
25-40	114	11.4% (13/114)	0.29 (0.05-1.67)	0.165
40-70	37	10.81% (4/37)	1.06 (0.32-3.48)	0.921
Awareness of diseases transmission				
No	34	38.23% (13/34)	Ref.	
Yes	175	3.43% (6/175)	0.07 (0.2-0.23)	0.001
Holding valid health card				
No	26	30.77% (8/26)	Ref.	
Yes	164	3.05% (5/164)	0.08 (0.02-0.32)	0.001

This may be an indicator of poor health care and this makes possible the transmission of pathogenic parasites. There are different reports on the prevalence of intestinal parasitic infection in men and women. Some of them are more common in men and by contrast, some are more common in women[1,3,20,21,23]. But in this study no significant difference was found between the sexes among patients infected with intestinal parasites. In some studies that were conducted in other areas, several effective issues were reported in the reduction of parasitic infections such as mechanization of soil, high use of antiparasitics, easy access to public health facilities, and sanitation conditions[3,4,17,18,29]. In the present study, the low prevalence observed may be because most of those investigated possessed valid health cards and had some awareness of the transmission of diseases.

Therefore, in order to further reduce intestinal parasitic infections in food handlers, several strategies are recommended such as stool examinations with concentration methods every three months, public education, the application of health regulations, controlling the validity of health cards and training on parasitic infection transmission.

Also, since participation in training courses in environmental health increased awareness of the transmission of diseases ($p < 0.05$), it is recommended that further training courses will be held and food handlers are required to attend the courses. Participants infected by parasites were given advice on how to treat the infections. The findings of the present study can be used as a basis to develop preventive programs targeting food handlers because the spread of disease via them is a common problem worldwide. In this regard, the authors intend to use the findings of this study to support public health decision-making in the area of control strategies for intestinal parasitic infections.

RESUMEN

La prevalencia de parásitos intestinales entre los manipuladores de alimentos en el oeste de Irán

Infección parasitaria es uno de los problemas de salud humana, especialmente en los países en desarrollo. En este estudio, todas las tiendas de comida rápida, restaurantes, y tiendas de carne asada en Khorramabad (oeste de Irán) y todo el personal que trabaja en ellos, incluyendo 210 personas fueron seleccionadas a través de los censos y las heces fueron examinadas para detectar la presencia de parásitos. La prueba parasitológica directa de mojado de montaje, la tinción de Lugol's yodo, sedimentación formaldehído-éter y tricrómicas técnicas de tinción se realizaron sobre las muestras. Los datos fueron analizados con la prueba de chi-cuadrado y regresión logística seleccionado como el modelo analítico. Los resultados mostraron 19 (9%) muestras de heces fueron positivos para diferentes parásitos intestinales. Los parásitos intestinales incluyen *Giardia lamblia* 2.9%, *Entamoeba coli* 4.3%, *Blastocystis* sp. 1.4%, *Hymenolepis nana* 0.5%. Hubo diferencia significativa entre la presencia de la tarjeta sanitaria válida, el conocimiento de la transmisión de los parásitos intestinales, la participación en cursos de formación en materia de salud ambiental con parásitos intestinales ($p < 0.05$). No hubo diferencia estadísticamente significativa entre la tasa de alfabetización y el sexo entre los pacientes infectados con parásitos intestinales ($p > 0.05$). Para controlar la infección parasitaria en los manipuladores de alimentos se recomiendan varias estrategias, tales como examen de heces cada tres meses, la educación pública, se aplican las normas sanitarias, el control de validez de la tarjeta sanitaria y la transmisión formación infección parasitaria. En este sentido, los resultados del presente estudio se pueden utilizar como una base para desarrollar programas de prevención

dirigidos a los manipuladores de alimentos debido a la propagación de enfermedades a través de ellos es un problema común en todo el mundo.

ACKNOWLEDGEMENTS

The authors hereby thank the Deputy of Research and Technology and co-researchers of Lorestan University of Medical Sciences, as well as all the people who helped us in this research; their contributions are sincerely appreciated. The authors declare that no conflict of interests exists.

REFERENCES

1. Al-Zain B, Al-Hindi A. Distribution of *Strongyloides stercoralis* and other intestinal parasites in household in Beit-Lahia city, Gaza Strip, Palestine. Ann Alquds Med. 2005;1:48-52.

2. Andargie G, Kassu A, Moges F, Tiruneh M, Huruy K. Prevalence of bacteria and intestinal parasites among food-handlers in Gondar town, northwest Ethiopia. J Health Popul Nutr. 2008;26:451-5.

3. Arani AS, Alaghehbandan R, Akhlaghi L, Shahi M, Lari AR. Prevalence of intestinal parasites in a population in south of Tehran, Iran. Rev Inst Med Trop Sao Paulo. 2008;50:145-9.

4. Ashtiani MT, Monajemzadeh M, Saghi B, Shams S, Mortazavi SH, Khaki S, *et al*. Prevalence of intestinal parasites among children referred to Children's Medical Center during 18 years (1991-2008), Tehran, Iran. Ann Trop Med Parasitol. 2011;105:507-12.

5. Babiker MA, Ali MSM, Ahmed ES. Frequency of intestinal parasites among food-handlers in Khartoum, Sudan. East Med Health J. 2009;15:1098-104.

6. Daryani A, Ettehad GH, Sharif M, Ghorbani L, Ziaei H. Prevalence of intestinal parasites in vegetables consumed in Ardabil, Iran. Food Control. 2008;19:790-4.

7. David TJ, William AP. Markell and Voge's Medical Parasitology. Ninth Ed. Toronto: Saunders Elsevier; 2006. Chapter 1.

8. Diaz E, Mondragon J, Ramirez E, Bernal R. Epidemiology and control of intestinal parasites with nitazoxanide in children in Mexico. Am J Trop Med Hyg. 2003;68:384-5.

9. Garcia LS. Diagnostic medical parasitology. 5th ed. Washington: ASM Press; 2007.

10. Green L, Selman C, Banerjee A, Marcus R, Medus C, Angulo FJ, *et al*. Food service workers self reported food preparation practices: an EHS-Net study. Int J Hyg Environ Health. 2005;208:27-35.

11. Hennessy TW, Cheng LH, Kassenborg H, Ahuja SD, Mohle-Boetani J, Marcus R, *et al*. Egg consumption is the principal risk factor for sporadic *Salmonella* serotype Heidelberg infection: a case-control study in food net sites. Clin Infect Dis. 2004;38(Suppl 3):S237-43.

12. Idowu OA, Rowland SA. Oral fecal parasites and personal hygiene of food handlers in Abeokuta, Nigeria. Afr Health Sci. 2006;6:160-4.

13. Jacobsen KH, Ribeiro PS, Quist BK, Rydbeck BV. Prevalence of intestinal parasites in young Quichua children in the highlands of rural Ecuador. J Health Popul Nutr. 2007;25:399-405.

14. Kaferstein FK. Food safety as public health issue for developing countries. Geneva: WHO; 2003.

15. Kheirandish F, Tarahi MJ, Haghighi A, Nazemalhosseini- Mojarad E, Kheirandish M. Prevalence of intestinal parasites in bakery workers in Khorramabad, Lorestan Iran. Iran J Parasitol. 2011;6:76-83.

16. Monzon RB, Sanchez AR, Tadiaman BM, Najos OA, Valencia EG, Rueda RR, *et al*. A comparison of the role of *Musca domestica* (Linnaeus) and *Chrysomya megacephala* (Fabricius) as mechanical vectors of helminthic parasites in a typical slum area of metropolitan Manila. Southeast Asian J Trop Med Public Health. 1991;22:222-8.

17. Mortean ECM, Falavigna DLM, Janeiro V, Falavigna-Guilherme AL, Gomes ML. Low intestinal parasites as an health indicator in a municipality of southern Brazil with intensive agricultural mechanization. SaBios: Rev Saúde Biol. 2012;7:23-9.

18. Nasiri V, Esmailnia K, Karim G, Nasir M, Akhavan O. Intestinal parasitic infections among inhabitants of Karaj City, Tehran province, Iran in 2006-2008. Korean J Parasitol. 2009;47:265-8.

19. Nyarango RM, Aloo PA, Kadiru EW, Nyanchongi BO. The risk of pathogenic intestinal parasite infections in Kisii Municipality, Kenya. BMC Public Health. 2008;8:237.

20. Okyay P, Ertug S, Gultekin B, Onen O, Beser E. Intestinal parasites prevalence and related factors in school children, a western city sample -Turkey. BMC Publ Health. 2004;4:64.

21. Quihui L, Valencia ME, Crompton DW, Phillips S, Hagan P, Morales G, *et al*. Role of the employment status and education of mothers in the prevalence of intestinal parasitic infections in Mexican rural schoolchildren. BMC Publ Health. 2006;6:225.

22. R Development Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2012.

23. Saab BR, Musharrafieh U, Nassar NT, Khogali M, Araj GF. Intestinal parasites among presumably healthy individuals in Lebanon. Saudi Med J. 2004;25:34-7.

24. Saeed HA, Hamid HH. Bacteriological and parasitological assessment of food handlers in the Omdurman area of Sudan. J Microbiol Immunol Infect. 2010;43:70-3.

25. Saygi G, Ozcelik S, Poyraz O. A survey of intestinal parasites in student of adults educational center in Sivas, Turkey. J Egypt Soc Parasitol. 1995;25:303-10.

26. Sayyari AA, Imanzadeh F, Bagheri-Yazdi SA, Karami H, Yaghoobi M. Prevalence of intestinal parasitic infections in the Islamic Republic of Iran. East Mediterr Health J. 2005;11:377-83.

27. Scott E. Food safety and foodborns diseases in 21th century homes. Can J Infect Dis. 2003;14:277-80.

28. Tappeh KhH, Mohammadzadeh H, Rahim RN, Barazesh A, Khashaveh Sh, Taherkhani H. Prevalence of intestinal parasitic infections among mentally disabled children and adults of Urmia, Iran. Iran J Parasitol. 2010;5:60-4.

29. Takizawa MG, Falavigna DL, Gomes ML. Enteroparasitosis and their ethnographic relationship to food handlers in a tourist and economic center in Parana, Southern Brazil. Rev Inst Med Trop Sao Paulo. 2009;51:31-5.

30. World Health Organization. Report on global surveillance of epidemic-prone infectious diseases-leishmaniasis. 2006. Available from: http://www.who. int/csr/resources/publications/CSR_ISR_2000_1leish/en/index.html

31. World Health Organization. Control of leishmaniasis. Report by the secretariat. 2007. Available from: http://apps.who.int/gb/ebwha/pdf_files/WHA6 0/A60_10-en.pdf

32. World Health Organization. Medios auxiliaries para el diagnostico de las parasitosis intestinales. Geneva: WHO; 1994.

Received: 4 November 2012
Accepted: 25 June 2013

Rev. Inst. Med. Trop. Sao Paulo
56(2):115-119, March-April, 2014
doi: 10.1590/S0036-46652014000200005

PREHISTORICAL *Pediculus humanus capitis* INFESTATION: QUANTITATIVE DATA AND LOW VACUUM SCANNING MICROSCOPY

Juliana M.F. DUTRA(1), Arthur Daniel ALVES(1), Thaila PESSANHA(1), Rachel RACHID(2) ,Wanderley de SOUZA(2), Pedro Marcos LINARDI(3) , Luiz Fernando FERREIRA(1), Sheila Mendonça de SOUZA(1) & Adauto ARAUJO(1).

SUMMARY

A pre-Columbian Peruvian scalp was examined decades ago by a researcher from the Oswaldo Cruz Foundation. Professor Olympio da Fonseca Filho described nits and adult lice attached to hair shafts and commented about the origin of head lice infestations on mankind. This same scalp was sent to our laboratory and is the subject of the present paper. Analysis showed a massive infestation with nine eggs/cm^2 and an impressive number of very well preserved adult lice. The infestation age was roughly estimated as nine months before death based on the distance of nits from the hair root and the medium rate of hair growth. A small traditional textile was associated with the scalp, possibly part of the funerary belongings. Other morphological aspects visualized by low-vacuum scanning electron microscopy are also presented here for adults and nits.

KEYWORDS: *Pediculus humanus;* Head lice; Paleoparasitology; Human hair; Parasitism; Evolution.

INTRODUCTION

The family Pediculidae includes two types of lice that uniquely infest humans and are generally site-specific: *Pediculus humanus capitis* (the head louse) and *Pediculus humanus humanus* (the body louse), also considered by some authors as distinct species, in spite of molecular studies evidenced that they are conspecific[15,16]. Fertilized eggs of sucking lice are referred to as nits and are firmly cemented to the hair shaft. Subsequently, eggs develop through three nymph instars before achieving adulthood[5,9].

Lice infestation is dated from 25 million years ago (MYA) in primates[22]. At 6 MYA mark humans and monkeys are supposed to take different evolutionary ways[21,22]. Lice infestation is mentioned in the Bible as the third plague punishing the Egyptians when the Pharaoh denied the request of Moses to set the Israelites free - *"when all the dust throughout the land of Egypt became lice"*.

Evidence of louse infestation in archeological samples came with RUFFER[26] studies in Egyptian mummies from the Royal dynasty. The dynamics of infestation and transmission of head and body louse in the past is very well documented in many publications on the issue[1,3,8,11,14,21,24]. Hair samples, hair attached to the skull, in the form of scalp or fragments found in urns, carry lice and nits that have not yet been dated in the timeline of man's presence in the Americas. The world's oldest known direct head-louse association - a nit on a human hair - was found at a 10,000 year old archaeological site in northeastern Brazil[1].

Lice infestation in ancient populations is associated to different groups, economic conditions and periods; hygiene and cultural habits determine the prevalence and frequency of infestation in human groups. A double infestation by *Pediculus capitis* and *Pthirus pubis* (pubic louse) was described by FORNACIARI *et al.*[11] , in the mummy of the King of Naples, Ferdinand II of Aragon (1467-1496). Another relevant episode of body lice infestation including parts of the abdomen or the dorsum of five lice evolved in Napoleon's soldiers in Vilnius, when Napoleon Bonaparte besieged the city in 1812, during the Russian Campaign[20]. Evidence of ancient lice infestation in America was improved by the studies of mummies from the Chiribayan culture[23], Chinchorro culture[25] and others from northern Chile[3].

Studying nits in mummified bodies is quite simple, since they keep attached to the hairs even after death. If no grooming or other strategy help to get rid of them, the dead nits can persist for a long time firmly attached, being preserved in the mummified bodies and loose hairs. LINARDI *et al.*[17] proposed that nit persistence on hair shafts could help to estimate the time lapse of the infestation, using the distance between the nit and the scalp as a reference. On the other hand, the finding of adult lice is more surprising, because they are expected to abandon the body of the deceased soon after death, because of the cooling of the corpse.

Here, we reviewed a case of louse infestation in a scalp of Peruvian origin. A previous description of this scalp was presented by FONSECA FILHO[10] which mentioned only the finding of nits and lice in the sample. From that, the elevated number of nits and lice called our attention and

(1) Laboratório de Paleoparasitologia, Escola Nacional de Saúde Pública Sérgio Arouca, Fundação Oswaldo Cruz, R. Leopoldo Bulhões 1480, Manguinhos, 21041-210 Rio de Janeiro, RJ, Brazil. Tel.: +55 (21) 2598-2566.

(2) Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho s/n, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil.

(3) Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. Av. Antônio Carlos 6627, Caixa Postal 486, 30161-970 Belo Horizonte, MG, Brazil.
Correspondence to: Juliana M.F. DUTRA, E-mail: jsantiago@ensp.fiocruz.br

a more detailed investigation was carried out using new methods for estimating the infestation and low vacuum scanning electron microscopy for parasite documentation.

MATERIAL AND METHODS

Human scalp and hair sample: In 1981, Dr. Dorath Pinto Uchoa, archaeologist of the Museu de Arqueologia e Etnologia da Universidade de São Paulo, Brazil, sent to the Laboratory of Paleoparasitology of ENSP/FIOCRUZ the present scalp for paleoparasitological examination. The scalp was said to belong to the private collection of Paulo Duarte, a famous Brazilian anthropologist, and had been previously examined by professor Olympio da Fonseca Filho, probably in 1960's.

The material consists of fragments of different sizes of a human scalp and some loose hair shafts containing lice and nits. The fragments suggest that the piece was rotten and the skin/hairs were cut in some areas, possibly to provide minor samples for analysis. The hair was partially lost and was cut into different segments, a signal of destructive bioactivity. In the mummified scalp the skin is thin and elastic but most of the hairs still have their roots firmly inserted in site. The dark brown hairs are flexible and there is no signal of discoloration. Putting the different fragments together and respecting the orientation of the hairs it is possible to confirm that it was originally a bigger scalp, possibly from the top and back parts of the head.

Quantitative analysis for nit size: The methodology follows REINHARD & BUIKSTRA[23].

After the first examination, four different fragments of the scalp, identified as A, B, C and D respectively, were selected for quantitative studies. The biggest fragment measured 6.7 x 4.4 cm (length x width) and three smaller ones sized between 2 and 5 cm were used for counting nits and adults. For counting nits a 2 x 2 cm square window was cut into a cardboard paper and placed above different regions of each fragment. The nits inside each of those delimited areas were counted. A total of three windows were counted.

For bright field microscopy analysis, fifty nits were separated randomly under a stereomicroscope. After 72 hours of rehydration in trisodium phosphate 0.5% aqueous solution[6] the nits were mounted on glass slides for observation. All were measured and photographed under a Primo Star microscopy associated with Zen® software (Carl Zeiss), in a final magnification of 100x.

Infestation profile: In order to analyze the infestation profile, ten hair shafts with nits were separated randomly from each fragment and measured. Hair shaft length and the distance between nit position and the scalp were measured using a millimeter scale, following LINARDI *et al.*[17]. The maximum number of nits per shaft was also calculated.

Scanning electron microscopy: Nits and adults were observed under low-vacuum scanning electron microscopy (SEM). For SEM preparation, four nits were rehydrated as previously described at item 2, then washed in distillated water and dried at room temperature. Hair shafts were mounted on stubs with double side carbon tape (TED PELLA Inc.) and examined in a low-vacuum SEM at QUANTA 250 (FEI Company). Adults were not rehydrated before SEM observation.

Fifty nits were randomly selected and prepared for light microscopy after rehydration and measured in length and width. The morphological aspect of nits was also evaluated (Fig. 1). For inside nit exposition, the hair shaft containing nits was mounted on a stub with a double side carbon tape. In the sequence, adhesive tape was stuck on the top to remove the superficial layer of the sample. Both halves were observed under low-vacuum SEM as described above.

RESULTS

The results presented here were conclusive for heavy infestation determined by the high density of nits in the scalp, as summarized in Table I. All the fragments of the scalp showed a high-density value of nits, ranging from 3.5 nits/cm^2 to 9.5 nits/cm^2.

The morphological characteristics of the nits as well as adults, and the maximum width (average: 469.38 ± 100 μm) and length (average: 1,126.92 ± 221 μm) of the nit size showed no differences when compared with modern nits. The good state of preservation of nits/adults allowed a morphologically detailed description by light and scanning electron microscopy, helping to confirm its morphological characteristics and to compare with other descriptions in the literature (Fig. 1 and 2). Nymphs inside the eggs could be identified after the eggshell was removed with adhesive tape (Fig. 2B-2D).

Fig. 1 - Eggs/nits under bright field microscopy. After the rehydration process, it is possible to visualize the embryonic stage inside the eggshell. 50 nits were measured: the size ranged between 1,126.92 μm (length) and 469.38 μm (width). Scale bar = 200 μm.

The nit's position was useful to determine the time of infestation, and the distance from the hair root measured between 0.2 cm and 9.5 cm (Table I). The longest hair shaft measured in the scalp (fragment A) also showed the major distance from nits to skin.

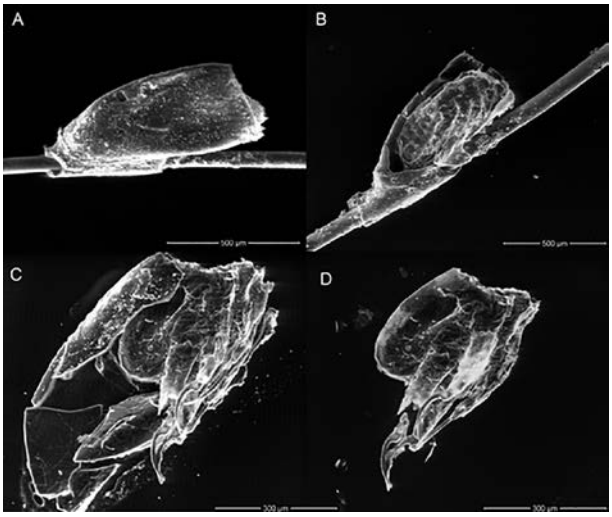


Fig. 2 - Low-vacuum scanning microscopy of eggs/nits attached to a hairshaft. The eggshell was removed with adhesive tape and showed the presence of a larvae hatching. Scale bar = 500 μm.

Table 1
Nits density and position on hair shaft

	Fragments			
	A	B	C	D
Hair shaft length				
Longest*	18	26	20	14
Shorter	5	5	4	5
Max. nits per area**	9.5	6	3.5	3.6
Distance of nits from scalp				
Min.	2.3	0.5	0.6	0.2
Max.	9.5†	4.0	3.3	2.6
Max. nits per hair shaft	4	1	1	1

*centimeter scale; ** 2x2 cm square; †nit on the longest hair shaft found (18 cm).

Empty nits positioned 9.5 cm away from scalp points for a period of nine months before death as a possible infestation date. Other viable nits (Fig. 2B-2D) situated at 0.2 cm from the base of the scalp could signalize a minimum infestation age of 5-7 days before death, in accordance with the emergence of the first nymph instar[5,13].

Low-vacuum SEM was used for morphological analysis in adults and nits. Adults showed a very well preserved state, with a flattened and desiccated body from the aging process (Fig. 3). It was possible to visualize lateral spiracles (Fig. 3A and C - arrows) and the genital aperture from females (Fig. 3D - asterisk). One female in particular that had lost a pair of antenna and the first pair of clawed legs, although the eyes were preserved (Fig. 3B).

DISCUSSION

Recent observations done by REINHARD AND BUIKSTRA[23] also

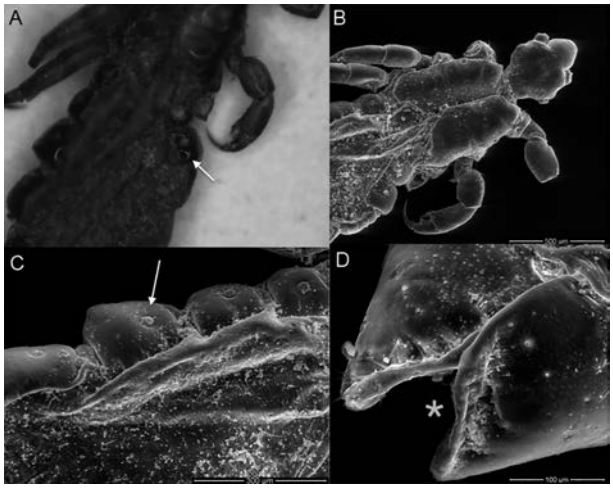


Fig. 3 - Adults seen under a stereomicroscope and by Low-vacuum scanning microscopy. It can be noted that the lateral spiracles were well preserved (Fig. 3A and C - arrows) and the genital aperture from females (Fig. 3D - asterisk).

showed an expressive infestation in Chiribaya people, an Andean group living around 900 and 1350 AD in southern Peru. In that study the mean maximum louse density was 8.9 nits/cm^2 in cotton samples, but the value for all other sites studied was 2.28 nits/cm^2. As stated by those authors, a mean maximum density ranging from five nits/cm^2 to 22 nits/cm^2 represent heavy infestation, making the value found in the present case, of nine nits/cm^2, very representative. The finding of a heavy infestation seems not to be the rule even in people of the past of the Andean area, and special conditions must be considered here that may justify the present results. The unknown origin of the scalps prevents further detailed interpretation but one possible explanation is the individual condition, as also inferred by the papers cited above where we can find differences in individual infestation values, especially because one ill or handicapped people could be more susceptible to infestation. As in any culture in the past, age groups, sex and social conditions could all affect the possibility of regular hygiene practices/grooming and their benefits could be available to some individuals and not to others in the same group. The second possibility is that the scalp examined may come from one group more affected than others by the infestation[23], and this could be associated with some special conditions such as periods of violence, crowding conditions, scarcity periods, imprisonment, and others moments of social disruption that would certainly affect the possibility of keeping traditional practices. In America, the contact period after the XVI century, as well as the troubled periods under the pressure of expanding dominant groups like Wari, Tiwaknaku or Inka could represent such situations[19].

Adults and nits, as demonstrated by low-vacuum SEM pictures here, were an interesting finding. As stated before, although nits are usually found attached to hairs and scalps, the finding of adults, in considerable numbers, is not expected in such mummified samples, because the free adults try to abandon the corpse after death, maximizing their chances of survival and reproduction.

Following LINARDI *et al.*[18], nits, nymphs and adults can be found alive 24 hours after removal from the host. Nits can still be attached to hair shafts even after death. A cement substance produced in the accessory glands of the louse is laid by females together with eggs; it prevents the

egg from becoming unstuck from the hair shaft, even after the egg has hatched, and they migrate far from the scalp following hair growth[17].

The dynamics of hair growth differ between ethnic groups, and vary with age, gender, body site and environmental changes. Physiological or pathological states such as pregnancy, malnutrition or disease can also modulate hair growth[7]. Assuming one cm/month as a reasonable medium rate of growth for humans we can estimate the infestation time in this case. Otherwise, infestation time cannot be precisely estimated because many factors can change the equation, including the fact that human hair growth is variable in the same head[12]. Taking the heavy density of nits found and the multiple presences in a single hair shaft, this possible period of infestation does not seem so unlikely, even though it is impossible to determine other parameters that could impair hair growth in this individual. ARRIAZA *et al.*[3], describing a Maita Chiribaya mummy lice infestation, also found a heavy infestation, confirming numerous specimens in different stages of development. The results were associated with the life style of the group. For REINHARD & BUIKSTRA[23] who first found a similar heavy infestation in the population of Chiribaya, the prevalence in men, instead of women and children, could point to differences in social attributes. They also described the combs used by those people. The practice of combing the hair is very well known for different native groups, and the special hairdressings using tresses and other special fashions among Andean groups inspired even modern stylists. The combing practices among women probably also helped to remove adults and minimize the symptoms of intense itching caused by *P. h. capitis*. Although ARRIAZA *et al.*[2] and ARRIAZA & STANDEN[4] maintain that lice infestations should be endemic in the Andes region, because of the archaeological findings of fine combs possibly used for nits or adults removal, we must keep in mind that the existence of very sophisticated combing and dressing patterns in the Andean region goes beyond the possible functional lice-related practices. On the other hand, the endemicity of *P. h. capitis* is confirmed by the study of different samples as described in the literature. It was not possible to compare the total counting of 17 adult lice in this scalp with the finding of other authors because the present sample was fragmented, but it is possible to maintain that the individual had an active infestation when he/she died. The practice of wrapping corpses in order to prepare the funerary bundle may explain the persistence of the adults in the scalp after death, preventing them from abandoning the head. This makes us consider that differences in funerary practices certainly have an impact on the possibility of recovering or not, adult lice, and consequently, vary interpretations of final results in archaeological samples.

CONCLUSION

Lice infestation, one of the most antique host-parasite interactions was present in Andean groups, and the finding of well preserved adult forms, eggs and nits helps in their study over time and space. In the scalp we examined, the use of SEM techniques helped to confirm the good preservation of the parasites and to detail the description of the different stages (adult to nits) of the parasite present in the hairs or free in the scalp. The application of quantitative methods to estimate the number of nits per area of scalp, and the distance between the nits and the scalp, helped to compare the severity and antiquity of the infestation with other similar studies in South America. The infestation in the present case was considered intense, lasting for about nine months before death, being no doubt, active in the moment the individual died.

Finally, results proved to be a good opportunity to call attention to the beginnings of paleoparasitology in our country, as professor Olympio da Fonseca Filho was a pioneer on this matter describing parasites in archaeological material. As far as we know, he had the opportunity of examining this scalp years before he published his book in the early 1970's[10]. The essence of paleoparasitology, while discussing the origins and evolution of host-parasite relationships, is present in his book.

RESUMO

Infestação pré-histórica por *Pediculus humanus capitis:* análise quantitativa e por microscopia de varredura de baixo vácuo

Há décadas um escalpo peruano, datado do período pré-colombiano, foi examinado por um pesquisador da Fundação Oswaldo Cruz. O Professor Olympio da Fonseca Filho descreveu lêndeas e adultos fixos a fios de cabelos e fez comentários sobre a origem da infecção por piolhos na espécie humana. Este mesmo escalpo foi enviado ao nosso laboratório e é objeto deste artigo. Sua análise mostrou maciça infestação, com nove lêndeas/cm^2 em impressionante número de adultos muito bem preservados. O tempo de infestação foi estimado em cerca de nove meses antes da morte, baseado na maior distância entre lêndeas e o couro cabeludo, levando em consideração taxa média de crescimento capilar de 1 cm por mês. Um pequeno pedaço de tecido tradicional peruano foi encontrado associado ao escalpo, provavelmente pertencente ao conjunto de peças usado no ritual funerário. Aqui, apresentamos alguns aspectos morfológicos de adultos e lêndeas vizualizados por microscopia eletrônica de varredura de baixo vácuo.

ACKNOWLEDGMENTS

We would like to thank Dr. Dorath Pinto Uchôa for giving us the opportunity to examine such precious material. Also, we would like to thank Dr. Silvia Maranca and Dr. Veronica Wesolowski for helping us identify the origin of the archaeological material.

PML, AA, WS are research fellows of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil). Fundings by CNPq, FAPERJ (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

REFERENCES

1. Araujo A, Ferreira LF, Guidon N, Maues da Serra-Freire N, Reinhard K, Dittmar K. Ten thousand years of head lice infestation. Parasitol Today. 2000;16:269.

2. Arriaza B, Allison M, Standen V, Focacci G, Chacama J. Peinados precolombinos en momias de Arica. Chungara Rev Antropol Chilena. 1986;(16-17):353-75.

3. Arriaza B, Orellana NC, Barbosa HS, Menna-Barreto RF, Araújo A, Standen V. Severe head lice infestation in an Andean mummy of Arica, Chile. J Parasitol. 2012;98:433-6.

4. Arriaza B, Standen V. Bioarqueología: historia biocultural de los antiguos pobladores del extremo norte de Chile. Santiago de Chile: Editorial Universitaria; 2008.

5. Busvine JR. Insects and hygiene. London: Methuen; 1966.

6. Callen E, Cameron TWM. A prehistoric diet revealed in coprolites. New Scientist. 1960;8:35-40.

7. Chamberlain AJ, Dawber RP. Methods of evaluating hair growth. Australas J Dermatol. 2003;44:10-8.

8. Ewing HE. Lice from human mummies. Science. 1924;60(1556):389-90.

9. Ferris GF. The sucking lice. Mem Pacific Coast Entomol Soc. 1951;1:1-320.

10. Fonseca Filho O. Parasitismo e migrações humanas pré-históricas. Rio de Janeiro: Mauro Familiar; 1972.

11. Fornaciari G, Giuffra V, Marinozzi S, Picchi MS, Masetti M. "Royal" pediculosis in Renaissance Italy: lice in the mummy of the king of Naples Ferdinand II of Aragon (1467-1496). Mem Inst Oswaldo Cruz. 2009;104:671-2.

12. Harkey R. Anatomy and physiology of hair. Forensic Sci Int. 1993;63:9-18.

13. Hopkins GHE. The host-association of lice of mammals. Proc Zool Soc London. 1949;119:387-604.

14. Horne P. Head lice from an Aleutian mummy. Paleopathol Newsl. 1979;25:7-8.

15. Leo NP, Campbell NJM, Yang X, Muncuoglu K, Barker SC. Evidence from mitochondrial DNA that head and body lice of humans (Phthiraptera: Pediculidae) are conspecific. J Med Entomol. 2002;39:662-6.

16. Light JE, Toups MA, Reed DL. What's in a name: the taxonomic status of human head and body lice. Mol Phylogenet Evol. 2008;47:1203-16.

17. Linardi PM, Botelho JR, de Maria M, Cunha HC. O significado do sítio de aderência das lêndeas de *Pediculus capitis* em amostras de cabelos recolhidos do chão de barbearias. Rev Soc Bras Med Trop. 1987;20:209-12.

18. Linardi PM, De Maria M, Botelho JR, Cunha HC, Ferreira JB. Prevalence of nits and lice in samples of cut hair from floors of barbershops and beauty parlors in Belo Horizonte, Minas Gerais State, Brazil. Mem Inst Oswaldo Cruz. 1988;83:471-4.

19. Owen BD. A model of multiethnicity: state collapse, competition, and social complexity from Tiwanaku to Chiribaya in the Osmore Valley, Peru. [PhD thesis]. Los Angeles: University of California; 1993.

20. Raoult D, Dutour O, Houhamdi L, Jankauskas R, Fournier PE, Ardagna Y, *et al*. Evidence for louse-transmitted diseases in soldiers of Napoleon's Grand Army in Vilnius. J Infect Dis. 2006;193:112-20.

21. Raoult D, Reed DL, Dittmar K, Kirchman JJ, Rolain JM, Guillen S, *et al*. Molecular identification of lice from pre-Columbian mummies. J Infect Dis. 2008;197:535-43.

22. Reed DL, Light JE, Allen JM, Kirchman JJ. Pair of lice lost or parasites regained: the evolutionary history of anthropoid primate lice. BMC Biol. 2007;7:5-7.

23. Reinhard KJ, Buikstra J. Louse infestation of the Chiribaya culture, southern Peru: variation in prevalence by age and sex. Mem Inst Oswaldo Cruz. 2003;98(Suppl 1):173-9.

24. Rick FM, Rocha GC, Dittmar K, Coimbra CEA Jr, Reinhard K, Bouchet F, *et al*. Crab louse infestation in pre-Columbian America. J Parasitol. 2002;88:1266-7.

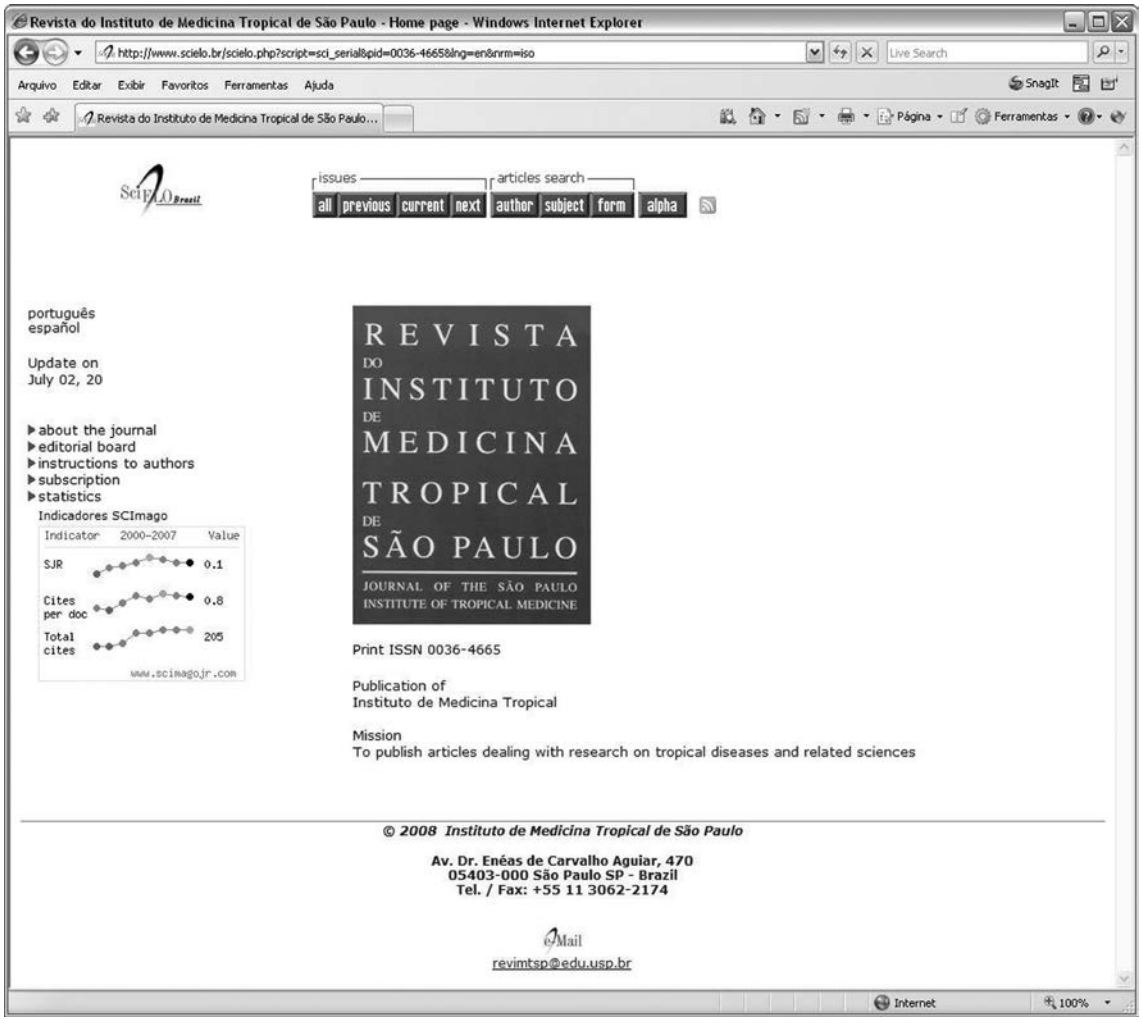
25. Rivera MA, Mumcuoglu KY, Matheny RT, Matheny DG. Huevecillos de *Anthropophthirus capitis*, en momias de la tradición Chinchorro Camarones 15-d, norte de Chile. Chungara Rev Antropol Chilena. 2008;40:31-9.

26. Ruffer MA. Studies in the paleopathology of Egypt. Chicago: The University of Chicago Press; 1921.

Received: 18 February 2013
Accepted: 26 June 2013

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(2):121-125, March-April, 2014
doi: 10.1590/S0036-46652014000200006

DECREASING PREVALENCE OF THE ACUTE/SUBACUTE CLINICAL FORM OF PARACOCCIDIOIDOMYCOSIS IN MATO GROSSO DO SUL STATE, BRAZIL

Larissa Rodrigues FABRIS(1), Úrsulla Vilella ANDRADE(1), Aline FERREIRA DOS SANTOS(1), Ana Paula da Costa MARQUES(2), Sandra Maria do Valle Leone de OLIVEIRA(1), Rinaldo Pôncio MENDES(3) & Anamaria Mello Miranda PANIAGO(1)

SUMMARY

With the objective to evaluate the behavior of paracoccidioidomycosis in the last three decades, clinical and epidemiological data of 595 patients admitted to clinical services of the Federal University of Mato Grosso do Sul from 1980 to 2009 were investigated. Gender, age distribution, clinical form, comorbidity with tuberculosis or AIDS, and mortality were compared by decades of clinical admission. It was shown that during the three decades there was a decrease in women percentage, and the same manner occurred a reduction in participants in the age group of 20 to 39 years. Moreover, the acute/subacute forms have been diminished in the period. These fluctuations are closely related and can be simultaneously analyzed. Increased AIDS co-infection prevalence from the first to the second decade was also revealed, coinciding with the appearance of the retroviral epidemic and stabilizing during the third decade. No change in the tuberculosis co-infection rate was observed (overall = 6.9%). It reinforces the importance of this co-morbidity. The overall mortality rate remained steady at 6.7%, not varying significantly from one decade to another. The persistent mortality rate calls attention to the importance of this neglected disease.

KEYWORDS: Paracoccidioidomycosis; Geographic area; Age group.

INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the thermal dimorphic fungus *Paracoccidioides brasiliensis* and is endemic in Latin America. Brazil presents the highest number of PCM cases of the Latin countries, with PCM predominantly occurring in the southern, southeastern and midwestern regions of Brazil[6,12,24].

Infection occurs through the inhalation of conidia and mycelia fragments from the soil and is predominant in rural workers. Once inhaled, the conidia advance to the pulmonary alveoli, where they settle, advance into the yeast phase and constitute the primary complex. Thereafter, lymphohematogenous dissemination can spread the fungi to any site in the organism. Individuals with appropriate cell mediated immunity show regression of these foci; however, viable fungi can remain in a latent stage, which can reactivate causing a disease with the chronic form. Individuals with compromised cellular immunity can develop the disease immediately after the infection, usually presented as the acute/subacute form[14].

PCM demonstrates some puzzling regional differences, such as the rarity of acute/subacute cases in Uruguay[11], Paraguay[28] and Argentina[15]. In Brazil, the prevalence of the acute/subacute clinical form varies by state and region as well, being rare in Rio Grande do Sul[10] and more frequent in the states of Maranhão[20,38], Pará[13], Minas Gerais[23], São Paulo[6], Mato Grosso do Sul[24], and Rio de Janeiro[37].

However, as transversal analyses of PCM in the region are scarce, this study was proposed to verify if modifications in the behavior of PCM have occurred over the past three decades in the endemic area of Mato Grosso do Sul.

PATIENTS AND METHODS

Study design. Case series observational epidemiological study.

Location and study period. Research was conducted at the Department of Infectious and Parasitic Diseases (IPD) at the Federal University of Mato Grosso do Sul University Hospital (NHU-UFMS), located in Campo Grande - MS. Data for this study were collected as part of systemic mycoses clinical services and included cases treated from 1980 to 2009.

Study participants. All cases of paracoccidioidomycosis confirmed by identification of the etiologic agent by direct mycological examination, culture or histopathology from clinical specimens were included in this study.

(1) Faculdade de Medicina, Universidade Federal de Mato Grosso do Sul (UFMS), Campo Grande, MS, Brazil. E-mails: larissafabris@ibest.com.br, ursulla1@gmail.com, alineafds@yahoo.com.br, sandrinhaleone@gmail.com, anapaniago@yahoo.com.br
(2) Centro de Ciências Biológicas e da Saúde, Universidade Federal de Mato Grosso do Sul, Campo Grande (UFMS), MS, Brazil. E-mail: apcmarques@hotmail.com
(3) Faculdade de Medicina, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil. E-mail: tietemendes@terra.com.br
Correspondence to: Anamaria Mello Miranda Paniago. E- mail: anapaniago@yahoo.com.br

Data collection. The clinical and epidemiological data of patients admitted between 1980 and 1998 were obtained by retrospective analysis of medical records. From 1999 onwards, the study became prospective and utilized a collection protocol developed for this purpose. Patients were divided into three groups according to decade of diagnosis: 1980 to 1989, 1990 to 1999 and 2000 to 2009.

Classification of clinical forms and outcomes. Patients were classified by acute/subacute or chronic clinical form according to specifications set forth by MENDES[21].

Cure was defined as the disappearance of the clinical manifestations previously presented by every patient, associated with disappearance of the pulmonary lesions of active disease on chest radiography or its substitution by fibrotic scars, normalization of the erythrocyte sedimentation rate (or C-reactive protein serum levels), and a regression to negative of the antibody serum levels determined by the double agar gel immunodiffusion test, independently of casual relapse and re-treatment.

Deaths were considered only those occurring in patients with active disease clinically attributable to paracoccidioidomycosis and/or confirmed by postmortem examination.

Statistical analysis. Comparison of frequencies was performed by chi-square test or Fisher's exact test. When three groups were compared, analysis was completed with the Goodman test. Continuous variables were presented as mean and standard deviation. The comparison between two means was performed by Student's t test. Statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, North Carolina). Differences were considered significant when $p \leq 0.05$.

Ethical considerations. The project was approved by the Federal University of Mato Grosso do Sul Ethics Committee on Human Research, under protocol No. 1620 on March 4th, 2010.

RESULTS

During the study period, 595 patients ranging in age from four to 94 years (45.2 ± 14.2) were included and 549 (92.3%) of these patients were male. Of these patients, 73 (12.3%) presented the juvenile form. Association with HIV was observed in 16 (2.7%) patients and association with tuberculosis was found in 41 (6.9%) patients. There were 40 (6.7%) deaths among the 595 patients evaluated during the period from 1980 to 2009.

The first decade (1980 - 1989) revealed a higher prevalence of female patients and a lower prevalence of male patients than in the third decade (2000 - 2009), as shown in Table 1. The prevalence of patients up to 39 years of age was lower in the third decade (2000 - 2009) than in the other two decades; however, patients aged 40 to 59 years old were more prevalent in the third decade (2000 - 2009) than in the other two decades (Table 1). The first decade (1980 - 1989) showed a higher prevalence of acute/subacute cases and a lower prevalence of chronic cases than in other decades (Table 1). Finally, coinfection with HIV increased with time, but remained stable for the last two decades, whereas coinfection with tuberculosis remained stable throughout the three decades (Table 1).

The average age increased from the first to the third decade of study: 41.8 ± 15.1 years in the first decade, 45.0 ± 13.3 years in the second decade and 49.5 ± 13.1 years in the third decade ($p < 0.01$).

Mortality remained stable throughout the three decades and showed no difference in clinical form, except a tendency for more acute-subacute cases than chronic cases in the second decade ($p = 0.062$), as shown in Table 2.

Regarding treatment, cotrimoxazole was taken by 90.3% of patients

Table 1
Distribution of 595 paracoccidioidomycosis patients by period of admission at the Infectious and Parasitic Diseases - University Hospital of the Universidade Federal do Mato Grosso do Sul, and clinical and epidemiological (demographic) findings

	1980-1989 n=206		1990-1999 n=216		2000-2009 n=173		*p* value
	N	%	N	%	N	%	
Gender							<0.03
Female	24	11.7	14	6.5	8	4.6	
Male	182	88.3	202	93.5	165	95.4	
Age group (years)							<0.01
0-19	18	8.7	12	5.6	4	2.3	
20-39	69	33.5	62	28.7	27	15.6	
40-59	91	44.2	112	51.9	108	62.4	
≥60	28	13.6	30	13.9	34	19.7	
Clinical Form							<0.01
Acute/Subacute	44	21.4	21	9.7	8	4.6	
Chronic	162	78.6	195	90.3	165	95.4	
HIV co-infection	0	0.0	9	4.2	7	4.0	<0.01
Tuberculosis co-infection	13	6.3	10	4.6	18	9.8	0.12

Table 2
Distribution of 595 paracoccidioidomycosis patients by period of admission at the service, clinical form and outcome

	Decade			Total	*p* value
	1980-1989	1990-1999	2000-2009		
Acute/subacute form (AF)					0.70
Death	5 (11.4)	4 (19.0)	1 (12.5)	10	
Cured	39 (88.6)	17 (81.0)	7 (87.5)	63	
Chronic Form (CF)					0.49
Death	9 (5.6)	14 (7.2)	7 (4.2)	30	
Cured	153 (94.4)	181 (92.8)	158 (95.8)	492	
Total					
Death	14 (6.8)	18 (8.3)	8 (4.6)	40	0.35
Cured	192 (93.2)	198 (91.7)	165 (95.4)	555	

in the first decade, 90.0% in the second decade and 89.6% in the third decade.

DISCUSSION

The prevalence of different clinical forms of PCM varies depending on the geographic area in which it occurs. The acute-subacute form, also called the juvenile form, is less prevalent than the chronic form, or the adult form. However, PCM prevalence was higher in the states of Pará, Maranhão, Minas Gerais, Goiás, São Paulo and Rio de Janeiro[4,7,14,20,23,37,38], than observed in the state of Rio Grande do Sul and in neighboring countries like Argentina and Paraguay, where the disease occurs almost exclusively in adult men[10,15,18,28].

These differences have not been explained and may be related to multiple factors. Deforestation in agricultural frontiers, such as occurs in the Amazon region in the states of Pará, Maranhão and Tocantins, could result in a greater risk of presenting the acute/subacute form due to an higher exposure rate to the inoculum and/or by reaching a population whose previous exposure to fungus never occurred or was uncommon. Occupational exposure of children and young adults to *P. brasiliensis* during farm work may be greater in these locations.

Furthermore, the discovery of differences between pathogenic strains of *P. brasiliensis*, which culminated in the description of a new species named *Paracoccidioides lutzii*[35], could explain these clinical differences, which are still unknown for PCM but demonstrated in classical and African histoplasmosis and *neoformans* e *gattii* cryptococcosis[33,34]. Studies on the geographical distribution of *P. lutzii* are still preliminary[36].

The reduction in acute/subacute cases observed during the study period may have been due to the significant increase in the age group affected by the disease related to the lower exposure of children and adolescents to the fungus as a result of intense public policy aimed at reducing child labor in the plantations of the country[8].

The prevalence and incidence of the acute/subacute form also depends on environmental conditions. A cluster of this clinical form was observed in the region of Botucatu and was related to the El Niño Southern Oscillation (1982 - 1983). This climatic event increased the soil water storage with consequent production of conidia and increased moisture in the air in 1984, leading to this temporal cluster in 1985[3]. Interestingly, in this study, the prevalence of the acute/subacute form was higher during the period 1980 - 1989, the same decade in which the cluster was observed in the region of Botucatu (SP). If this explanation is applied to the findings of the State of Mato Grosso do Sul, the first decade of this study would have shown an increase in acute/subacute cases. Thus, a study of climatic variables and soil conditions could contribute to a more concrete explanation of the observed variation.

The prevalence of female cases also decreased. In the prepubertal age, PCM has been known to occur in similar proportions between boys and girls, with a gender ratio of 1.7:1.0[13]. After menarche, women become less susceptible to PCM due to the presence of estrogen, which slows or inhibits the transformation of filamentous phase, the infective, pathogenic yeast phase[31]. In this way, the chronic form is much more prevalent in men, with a gender ratio of 15:1[24].

In this study, there were no cases of PCM co-infection with AIDS in the first decade, a period in which there occurred fewer AIDS cases in the state and AIDS was prevalent in homosexual men and/or residents of the most populous cities[7]. In the next two decades, the prevalence of this co-infection remained stable at 4.2% and 4.0%, respectively.

Since *P. brasiliensis* is an intracellular parasite and resistance to PCM depends on cell mediated immunity, a significant increase of PCM associated with HIV infection was expected. This association has been observed less frequently than with other endemic systemic mycoses, such as histoplasmosis[5,9,22,25]. The usage of low doses of trimethoprim-sulfamethoxazole for prophylaxis of *Pneumocystis jirovecci* by patients in the study population could be a possible explanation for the relatively low incidence of PCM-AIDS co-infection. This medicine would also have protected against *P. brasiliensis* infection.

Another possible explanation could be less exposure of HIV-infected patients, who generally live in urban areas, to *P. brasiliensis*, which is reserved in rural agricultural areas. A study of HIV-infected individuals[29], in the same study center showed a 12.5% prevalence of *P. brasiliensis* infection by assessing intra-dermal reactions with gp43. The dosage used was 0.1mL of gp43 at a concentration of 60 µL/mL, which is the

same as used in other studies with non-HIV infected individual[19]. In that study, regarding the levels of CD4+ cells, 23.3% of patients presented less than 200 cells/mm^3, 22.2% presented between 200 and 350 cells/mm^3, and 54.5% presented more than 350 cells/mm^3. The possible anergy of HIV-positive patients is important to consider, especially those with lower CD4+. No control group was used for comparison. However in the same region in a population of farm workers with no HIV-infected individuals, the prevalence was found to be 45.8%[19].

PCM-tuberculosis coinfection has been observed in 5.5% to 19% of patients with this mycosis[17,24,26] and constitutes an important public health issue, not only for the high prevalence of tuberculosis in Brazil, but also because they are involved in the differential diagnosis of many cases[16].

Throughout the study period, 6.9% of PCM patients showed active pulmonary tuberculosis during the course of this mycosis. The prevalence of PCM-tuberculosis co-infection remained stable throughout the study period. Interestingly, tuberculosis is observed in poorly ventilated urban areas and PCM predominates in rural environments[27]. The migration of rural workers to urban areas is increasing, which could explain the increased exposure of the patient with PCM to *Mycobacterium tuberculosis*.

Data on the mortality of the disease are rare. Deaths by PCM have been observed in 4.3% to 14.0% of cases, and the most frequent cause of death of patients with the chronic form are respiratory failure and Addison's syndrome, while the acute/subacute cases are intensely disseminated and involve multiple organs[1,2,37]. In the present study, 6.7% of patients died, with no noticeable reduction in this rate from the first to the third decade. A potential explanation for this finding could be the difficulty of PCM treatment compliance observed in the study population[24].

Treatment was the same (cotrimoxazole) throughout the study period; however, the dosage and period of treatment could not be ascertained during the retrospective phase of the study (1980 to 1998).

For the same reason, other data, as such sequel and relapse rates and time between onset of symptoms and treatment initiation, could not be compared and was a limitation of the study design

The main change in the epidemiology of the disease observed over the three decades studied in Mato Grosso do Sul was the increased age of cases affected by paracoccidioidomycosis which was accompanied by a reduction in the prevalence of female cases and acute/subacute cases.

RESUMO

Redução na prevalência da forma aguda/subaguda da paracoccidioidomicose em Mato Grosso do Sul, Brasil

Com o objetivo de avaliar o comportamento da paracoccidioidomicose nas últimas três décadas, dados clínicos e epidemiológicos de 595 pacientes atendidos dentre 1980 a 2009 no Hospital da Universidade Federal de Mato Grosso do Sul foram estudados. Sexo, faixa etária, forma clínica, associação com tuberculose ou AIDS e mortalidade foram comparados por década em que a doença foi diagnosticada. Observou-se, nas três décadas do estudo, uma redução do percentual de mulheres, de pacientes do grupo de 20 a 39 anos, assim como de casos com a forma aguda/subaguda. Estas alterações estão intimamente relacionadas e podem ser analisadas simultaneamente. Houve aumento de casos de coinfecção com AIDS da primeira para segunda década, coincidindo com o surgimento da epidemia, e manteve-se estável durante a década seguinte. Não houve alteração da taxa de coinfecção com tuberculose, que no geral foi de 6,9% o que reforça a importância desta comorbidade. A taxa geral de mortalidade foi de 6,7% e também não variou entre as décadas estudadas. A manutenção da taxa de óbitos chama a atenção para a relevância dessa doença negligenciada**.**

REFERENCES

1. Andrade ALSS. Paracoccidioidomicose linfático-abdominal: contribuição ao seu estudo**.** Rev Patol Trop. 1983;12:165-256.

2. Azevedo JF, Lisboa CSG. Paracoccidioidomicose - estudo de 106 casos. J. Pneumol. 1980;6:30-3.

3. Barrozo LV, Benard G, Silva MES, Bagagli E, Marques SA, Mendes RP. First description of a cluster of acute/subacute paracoccidioidomycosis cases and its association with a climatic anomaly. Plos Negl Trop Dis. 2010;4(3):e 643.

4. Bellisimo-Rodrigues F, Machado AA, Martinez 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.

5. Benard G, Duarte AJS. Paracoccidioidomycosis: a model for the evaluation of the effects of HIV infection in the natural history of endemic tropical diseases. Clin Infect Dis. 2002;31:1032-9.

6. Blotta MHSL, Mamoni RL, Oliveira SJ, Noier SA, Papaiordanou PM, Goveia A. Endemic regions of paracoccidioidomycosis in Brazil: a clinical and epidemiologic study of 584 cases in the southeast region. Am J Trop Med Hyg**.** 1999;61:390-4.

7. Brasil. Boletim Epidemiológico AIDS. Julho a setembro de 2001. [Cited: 2012 Sept 05]. Available from: http://www.aids.gov.br/sites/default/files/Boletim_Epidemiologico_2001_III_Aids_0.pdf

8. Brasil. Decreto Legislativo n° 178, de 14 de dezembro de 1999, e promulgada pelo Decreto no 3.597, de 12 de setembro de 2000. Estatuto da Criança e do Adolescente [Internet]. Ministério da Saúde. 2008 nov. 14 [Cited: 2012 Aug 15]. Available from: http://bvsms.saude.gov.br/bvs/publicacoes/estatuto_crianca_adolescente_3ed.pdf.

9. Chang MR, Taira CL, Paniago AMM, Taira DL, Cunha RV, Wanke B. Study of 30 cases of histoplasmosis observed in the Mato Grosso do Sul State, Brazil. Rev Inst Med Trop Sao Paulo. 2007;49:37-9.

10. Colares SM, Marcantônio S, Zambonato S, Severo LC. Acute/subacute disseminated paracoccidioidomycosis. First case in Rio Grande do Sul State, Brazil. Rev Soc Bras Med Trop. 1998;31:563-7.

11. Conti-Díaz IA, Callegari LF. Paracoccidioidomicosis en Uruguay: su estado y problematica actuales. Bol Of Sanit Panam. 1979;86:219-27.

12. Coutinho ZF, Silva D, Lazera M, Petri V, Oliveira RM, Sabroza PC, *et al*. Paracoccidioidomycosis mortality in Brazil. Cad Saúde Pública. 2002;18:1441-54.

13. Fonseca ERS, Pardal PPO, Severo LC. Paracoccidioidomicose em crianças em Belém do Pará. Rev Soc Bras Med Trop. 1999;32:31-3.

14. Franco M, Peraçoli MT, Soares A, Montenegro MR, Mendes RP, Meira DA. Host-parasite relationship in paracoccidioidomycosis. Curr Top Med Mycol. 1993;5:115-49.

15. Gimenez MM, Storni LP, Gimenez MF. Paracoccidioidomicosis. Nuestra experiencia. Rev Argent Dermatol. 1984;65:273-9.

16. Hijjar MA, Oliveira MJPR, Teixeira GM. A tuberculose no Brasil e no mundo. Bol Pneumol Sanit. 2001;9(2):9-16.

17. Leão RC, Mendes E. Paracoccidioidomycosis, neoplasia and associated infections. Allergol Immunopathol (Madr). 1980;8:185-8.

18. Londero AT, Ramos CD. Paracoccidioidomicose. Estudo clínico e micológico de 260 casos observados no Estado do Rio Grande do Sul. J Pneumol. 1990;16:124-8.

19. Marques APC, Oliveira SMVL, Rezende GR, Melo DA, Fernandes-Fitts SM, Pontes ERJC, *et al.* Evaluation of *Paracoccidioides brasiliensis* infection by gp 43 intradermal test in rural settlements in Central-West Brazil. Mycopathologia. 2013,175:3-4.

20. Matos WB, Dos Santos GM, Silva VEB, Rosario Gonçalves EG, Silva AR. Paracoccidioidomycosis in the state of Maranhão, Brazil: geographical and clinical aspects. Rev Soc Bras Med Trop. 2012;45:385-9.

21. Mendes RP. The gamut of clinical manifestations. *In*: Franco M, Lacaz CS, Restrepo-Moreno A, Del Negro G. Paracoccidioidomycosis. Boca Raton: CRC; 1994. p. 233-58.

22. Morejón KML, Machado AA, Martinez R. Paracoccidioidomycosis in patients infected with and not infected with human immunodeficiency virus: a case-control study. Am J Trop Med Hyg**.** 2009;80:359-66.

23. Nogueira MGS, Andrade GMQ, Tonelli E, Diniz SN, Goes AM, Cisalpino PS. Aspectos laboratoriais evolutivos de crianças em tratamento de paracoccidioidomicose. Rev Soc Bras Med Trop. 2006;39:478-83.

24. Paniago AMM, Aguiar JIA, Aguiar ESA, Cunha RV, Pereira GROL, Londero AT, *et al*. Paracoccidioidomicose: estudo clínico e epidemiológico de 422 casos observados no estado de Mato Grosso do Sul. Rev Soc Bras Med Trop. 2003;36:455-9.

25. Paniago AMM, Freitas ACC, Aguiar ASA, Aguiar JIA, Cunha RV, Castro ARCM, *et al.* Paracoccidioidomycosis in patients with human immunodeficiency virus: review of 12 cases observed in an endemic region in Brazil. J Infect. 2005;51:248-52.

26. Quagliato-Júnior R, Grangeia TAG, Massucio RAC, Capitani EM, Rezende SM, Balthazar AB. Association between paracoccidioidomycosis and tuberculosis: reality and misdiagnosis. J Bras Pneumol. 2007;33:295-300.

27. Rodrigues CC. Avaliação da infecção por *Histoplasma capsulatum* por meio de reações intra-dérmicas em moradores da zona urbana e rural do Município de Pratânia (SP). [tese]. Botucatu: Universidade Estadual Paulista, Faculdade de Medicina; 2004.

28. Rolon PA. Paracoccidioidomicosis - Epidemiologia en La República Del Paraguay, Centro Sud América. Mycopathologia. 1976;59:67-80.

29. Sarti ECFB, Oliveira SMVL, Santos LFS, Camargo ZP, Paniago AMM. Paracoccidioidal infection in HIV patients at an endemic area of paracoccidioidomycosis in Brazil. Mycopathologia. 2012;73:145-9.

30. Severo LC, Geyer GR, Londero AT, Porto MS, Rizzon CFC. The primary pulmonary lymph node complex in paracoccidioidomycosis. Mycopathologia. 1979;67:115-8.

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

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

33. Singh S, Kalra R, Chhabra S, Agarwal R, Garg S, Mathur SK. Variable clinical presentations of histoplasmosis: a report of six cases. Trop Doct. 2012;42:32-4.

34. Speed B, Dunt D. Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans.* Clin Infect Dis. 1995;21:28-34.

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

36. Theodoro RC, Teixeira MM, Felipe MSS, Paduan KS, Ribolla PM, San-Blas G, *et al.* Genus *Paracoccidioides*. Species recognition and biogeographic aspects. Plos One. 2012;7(5):e37694.

37. Valle ACF, Wanke B, Wanke NCF, Peixoto TC, Perez M. Tratamento da paracoccidioidomicose: estudo retrospectivo de 500 casos: análise clínica, laboratorial e epidemiológica. An Bras Dermatol. 1992;67:251-4.

38. Veras KN. Paracoccidioidomicose. Estudo epidemiológico e clínico de pacientes internados no Hospital de Doenças Infecto-Contagiosas (HDIC) em Teresina, Piauí. Identificação de reservâreas nos Estados do Pará e Maranhão. [dissertação]. Rio de Janeiro: Fundação Oswaldo Cruz; 1995.

Received: 28 January 2013
Accepted: 5 August 2013

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(2):127-132, March-April, 2014
doi: 10.1590/S0036-46652014000200007

ASSOCIATION BETWEEN SMOKING, CRACK COCAINE ABUSE AND THE DISCONTINUATION OF COMBINATION ANTIRETROVIRAL THERAPY IN RECIFE, PERNAMBUCO, BRAZIL

Joanna d'Arc Lyra BATISTA(1), Maria de Fátima Pessoa Militão de ALBUQUERQUE(2), Marcela Lopes SANTOS(2), Demócrito de Barros MIRANDA-FILHO(3), Heloísa Ramos LACERDA(1,3), Magda MARUZA(4), Libia Vilela MOURA(1), Isabella COIMBRA(3) & Ricardo Arraes de Alencar XIMENES(1,3)

SUMMARY

Despite the effectiveness of combination antiretroviral therapy in the treatment of people living with HIV/AIDS (PLWHA), nonadherence to medication has become a major threat to its effectiveness. This study aimed to estimate the prevalence of self-reported irregular use of antiretroviral therapy and the factors associated with such an irregularity in PLWHA. A cross-sectional study of PLWHA who attended two referral centers in the city of Recife, in Northeastern Brazil, between June 2007 and October 2009 was carried out. The study analyzed socioeconomic factors, social service support and personal habits associated with nonadherence to antiretroviral therapy, adjusted by multivariable logistic regression analysis. The prevalence of PLWHA who reported irregular use of combination antiretroviral therapy (cART) was 25.7%. In the final multivariate model, the irregular use of cART was associated with the following variables: being aged less than 40 years (OR = 1.66, 95%-CI: 1.29-2.13), current smokers (OR = 1.76, 95%-CI: 1.31-2.37) or former smokers (OR = 1.43, 95%-CI: 1.05-1.95), and crack cocaine users (OR = 2.79, 95%-CI: 1.24-6.32). Special measures should be directed towards each of the following groups: individuals aged less than 40 years, smokers, former smokers and crack cocaine users. Measures for giving up smoking and crack cocaine should be incorporated into HIV-control programs in order to promote greater adherence to antiretroviral drugs and thus improve the quality of life and prolong life expectancy.

KEYWORDS: AIDS; Adherence; Antiretroviral therapy; Associated factors.

INTRODUCTION

Although the number of new HIV infections has declined globally by 19% over the past decade and the access to antiretroviral therapy in low- and middle-income countries has increased, HIV infection rates are increasing in several countries in Eastern Europe and central Asia, notably among injecting drug users and their sexual networks[29]. Access to antiretroviral treatment (ART) has been primarily responsible for prolonging and improving the lives of people living with HIV/AIDS (PLWHA), with Brazil, being the first developing country to implement the universal distribution of these drugs[8,18]. Consequently, nonadherence to the use of antiretroviral drugs is the greatest risk for a non-effective response to treatment and the possibility of spreading resistant viruses[25,27]. Moreover, poor adherence is one of the factors that may lead to a lower CD4 cell count, higher plasma viral RNA levels and delayed immune recovery, with progression of the disease that can lead to death[11].

In the treatment of chronic disease, the degree of adherence is always influenced by a number of different factors: characteristics of the individual, characteristics of the disease, social support network (family and friends) and factors related to the health service, such as difficulties in accessing it, and the relationship of the health team/individual[12]. Amongst the characteristics of the individual, habits such as alcoholism, drug abuse and irregular use of antiretrovirals[21]place the individual at further risk of secondary infections. Furthermore, studies have shown that the quantity of pills is also an important factor that influences the rate of adherence[8,11].

For the treatment of HIV infection to be effective, several authors recommend an adherence level of at least 95% of the prescribed drugs to decrease the chance of viral resistance[6]. However, recent studies indicate that for boosted protease inhibitors an adherence level greater than 80% would be equally effective[16].

Different methods have been employed in order to measure adherence to antiretrovirals, without a consensus gold standard. Due to their simplicity and the low costs involved, methods that use self-reports are widely used, and studies have demonstrated an association between the measure of self-reported adherence to antiretrovirals and HIV plasma viral load (with odds ratios and hazard ratios on the order of 2.0), making it possible to use this strategy in the conduct of PLWHA[26,30].

The aim of this study was to identify the prevalence of the factors

(1) Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil.
(2) Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Recife, Pernambuco, Brazil.
(3) Universidade de Pernambuco, Recife, Pernambuco, Brazil.
(4) Hospital Correia Picanço, Secretaria de Saúde, Recife, Pernambuco, Brazil
Correspondence to: Joanna L. Batista, Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Av. Moraes Rego s/n, Campus da Universidade Federal de Pernambuco, Cidade Universitária, 50670-420 Recife, Pernambuco, Brasil. Fax: 55 81 2101-2614. E-mail joannalyra@gmail.com.br

associated with the irregular use of antiretroviral drugs in people living with HIV, with special emphasis on socioeconomic factors and life habits, intending thereby to identify the groups at greatest risk so as to develop new strategies that set out to minimize the problem.

METHODS

The study was conducted in Recife, a city in northeastern Brazil, with an estimated population of 1,561,659 inhabitants. This cross-sectional study was carried out in June 2007 and October 2009, with people living with HIV/AIDS aged 18 years and over, who attended two HIV referral centers in the state of Pernambuco (Hospital Correia Picanço and Hospital Universitário Oswaldo Cruz), which attend around 60% of the state's patients. Individuals were invited to participate in the study either during routine consultations and/or hospitalization. Those who agreed to take part gave informed consent and were then interviewed by trained professionals who used a questionnaire developed specifically for this study. Hospitalized patients who could not answer the questionnaire due to their clinical condition were excluded from the study.

The dependent variable was the self-reported irregular use of combined antiretroviral therapy (cART), categorized in 'yes' - when patients reported a discontinuation of treatment at some point on their own - and 'no'.

The independent variables were classified as: demographic (age and race), socioeconomics (marital status, social service support, head of household's income), how long the patient has been aware of being HIV positive, life habits (alcoholism and drug abuse). Information regarding the use of cART was collected from medical records.

Users of illicit drugs (crack cocaine and cocaine) fell into the following categories: non-users; intermittent (intermittent users who had abstained during the past year) and current users (who had been using during the past year).

The criteria adopted for alcohol consumption was based on the number of drinks per day, according to the definition of alcohol consumption patterns approved by the Centers for Disease Control and Prevention (CDC)[7]. Patients were questioned about their alcohol consumption in the previous three months. Patients were classified as abstainers or light drinkers (non-drinkers or less than two drinks per day for men and one drink for women), and heavy drinkers (more than two drinks per day for men and more than one drink per day for women).

The Student's t-test was used to compare the mean scores of the independent samples and variance was tested with Levene's test. All variables associated with the irregular use of cART in the univariate analysis with a *p*-value of less than 0.20 were included in the multivariate analysis, using logistic regression and Odds Ratio (OR) with a confidence interval of 95%. The variables whose association with the event was statistically significant ($p < 0.05$) remained in the final multivariate model. The software used was Stata 11.2 (Stata-Corp LP, College Station, TX). The study is part of a cohort, which has been carried out in two research centers, and was approved by the Ethics and Research Committee of the Centro de Ciências da Saúde da Universidade Federal de Pernambuco.

RESULTS

In the period from June 2007 to October 2009, 1815 PLWHA were interviewed, of which 1432 (78.9%) were taking cART. Of these, 52 (3.6%) did not answer the question referring to the irregular use of treatment, and were therefore excluded from the study. The study sample was made up of 1380 patients with a mean age of 40.6 years (18-80) and a median age of 40.0 years; 64.1% were male and 79.1% had a monthly family income of less than two minimum wages. The majority (84.7%) lived in the metropolitan region of Recife. The prevalence of people who reported irregular use of cART was 25.7%. Among people who reported irregular use of cART, 42% had stopped taking the pills in the two weeks preceding the study interview.

A comparison of the mean ages, CD4 cell count and HIV viral load at the time of the interview revealed an association between self-reported irregular use of cART, being under 40 years old and a lower CD4 cell count (Table 1).

Table 1 shows the frequency distribution of the studied factors and the

Table 1
Frequencies and univariate analysis of the association between characteristics of people living with HIV/AIDS and the irregular use of antiretroviral treatment, Recife, Pernambuco, Brazil, 2009

	Irregular use of ART (case)		Regular use of ART (control)		Crude OR (CI)	*p*
	n	%	n	%		
Age						
Years (mean)	38.8		41.3			**0.001***
Age						
≥ 40 years	157	21.39	577	78.61	1.0	
< 40 years	198	30.65	448	69.35	1.62 (1.27 - 2.07)	**0.000**
Total	**355**	25.72	**1025**	74.28		
Sex						
Female	138	27.88	357	72.12	1.0	
Male	217	24.52	668	75.48	0.84 (0.65 - 1.08)	**0.171**
Total	**355**	25.72	**1025**	74.28		

Table 1 (continuation)

	Irregular use of ART (case)		Regular use of ART (control)		Crude OR (CI)	*p*
	n	%	n	%		
Skin color						
White	92	25.34	271	74.66	1.0	
Non-white	263	25.86	754	74.14	1.03 (0.78 - 1.35)	**0.847**
Total	**355**	25.72	**1025**	74.28		
Alcohol intake						
Heavy drinker	225	23.61	728	76.39	1.0	
Light drinker or abstainer	130	30.44	297	69.56	1.42 (1.10 - 1.83)	**0.007**
Total	**355**	25.72	**1025**	74.28		
Smoking status						
Never smoked	133	21.28	492	78.72	1.0	
Former smoker	97	26.08	275	73.92	1.30 (0.97 - 1.76)	**0.083**
Current smoker	125	32.64	258	67.36	1.79 (1.34 - 2.39)	**0.000**
Total	**355**	25.72	**1025**	74.28		
Use of cocaine						
Never used	311	25.04	931	74.96	1.0	
Intermittent users**	37	29.84	87	70.16	1.27 (0.85 - 1.91)	**0.243**
Current users	7	58.33	5	41.67	4.19 (1.32 - 13.3)	**0.015**
Total	**355**	25.76	**1023**	74.24		
Use of crack						
Never used	319	24.79	968	75.21	1.0	
Intermittent users**	22	32.84	45	67.16	1.48 (0.88 - 2.51)	**0.141**
Current users	14	56.00	11	44.00	3.86 (1.73 - 8.59)	**0.001**
Total	**355**	25.74	**1024**	74.26		
Can read and write						
Yes	317	26.05	900	73.95	1.0	
No	37	24.03	117	75.97	0.90 (0.61 - 1.33)	**0.589**
Total	**354**	25.82	**1017**	74.18		
Monthly income of head of family						
≥ 2 minimum wages	55	22.36	191	77.64	1.0	
< 2 minimum wages	251	26.96	680	73.04	1.28 (0.92 - 1.79)	**0.144**
Total	**306**	26.00	**871**	74.00		
Marital status						
Married	64	22.38	222	77.62	1.0	
Other***	291	26.60	803	73.40	1.26 (0.92 - 1.71)	**0.146**
Total	**355**	25.72	**1025**	74.28		
Social service support						
Lives with family or partner	282	25.59	820	74.41	1.0	
Lives alone or in a shelter	73	26.26	205	73.74	1.03 (0.77 - 1.39)	**0.820**
Total	**355**	25.72	**1025**	74.28		
City of residence						
Recife	148	26.86	403	73.14	1.0	
Other	204	24.97	613	75.03	0.91 (0.71 - 1.16)	**0.433**
Total	**352**	25.73	**1016**	74.27		
CD4 baseline						
cells/mm^3 (mean)	383.9		446.1			**0.002***
Viral load baseline						
copies/mL (mean)	29,501.7		18,114.7			**0.198***

* Student's t test; ** Users who have abstained during the past year; *** Separated, divorced, widowed or single.

Table 2
Multivariate analysis of the association between characteristics of people living with HIV/AIDS and the irregular use of antiretroviral treatment, Recife, Pernambuco, Brazil, 2009

	Crude OR		OR adjusted by the multivariate	
	OR (CI)	*p*	OR (CI)	*p*
Age				
≥ 40 years	1.0		1.0	
< 40 years	1.62 (1.27 - 2.07)	**0.000**	1.66 (1.29 - 2.13)	**0.000**
Smoking				
Never smoked	1.0		1.0	
Former smoker	1.30 (0.97 - 1.76)	**0.083**	1.43 (1.05 - 1.95)	**0.023**
Current smoker	1.79 (1.34 - 2.39)	**0.000**	1.76 (1.31 - 2.37)	**0.000**
Use of crack				
Never used	1.0		1.0	
Intermittent users*	1.48 (0.88 - 2.51)	**0.141**	1.20 (0.70 - 2.06)	**0.501**
Current users	3.86 (1.73 - 8.59)	**0.001**	2.79 (1.24 - 6.32)	**0.013**

* Users who have abstained during the past year.

result of the univariate analysis of the association with the self-reported irregular use of cART.

Regarding the habits of the study population (Table 1), 69.1% were considered abstainers or light drinkers of alcoholic beverages; most participants had never used cocaine or crack cocaine (90.1% and 93.3% respectively); in relation to smoking, most individuals (54.7%) were current or former smokers, the majority (84.2%) having consumed cigarettes for 10 years or more.

In the multivariate analysis (Table 2), associations with the irregular use of cART in a statistically significant manner were, an age lower than or equal to 39 ($p < 0.001$), a former smoker ($p = 0.023$), currently smoking ($p < 0.001$) and currently using crack cocaine ($p = 0.013$).

DISCUSSION

After adjusting the socioeconomic and social service support variables, the discontinuation of combination antiretroviral therapy was associated with the following variables: being aged less than 40 years, a former smoker or a current smoker and a crack cocaine user.

The prevalence of people who reported irregular use of cART was 25.7%. This prevalence was similar to the prevalence of non-adherence to treatment encountered in a number of studies in Brazil[4,6,23,25]. Most studies conclude that taking less than 90-95% of prescription drugs is an indication of non-adherence, unlike the present study, which evaluated individual patient responses to questions regarding the discontinuation of treatment at some point in time. Studies also show the importance of including additional resources to self-reporting, such as testing tablets, to provide a better evaluation of adherence[22]. Even without a consensus concerning the method of measuring this adherence, studies have indicated similar results of prevalence, demonstrating that the most appropriate method of certifying adherence to treatment was to question the patient directly. The strategy which uses, direct patient questioning, was chosen because it is easy to employ in everyday health care. We are aware that adherence may be overestimated due to a fear of displeasing the interviewer, the health professional or physician (false negative response); however those who actually report poor adherence are probably expressing the truth (decrease in false positive response) and are an important group to intervene with. The option for the question on abandonment of treatment at some point, without the restriction to a length time before and after the interview, aimed to expand the knowledge of the problem. We recognize that in the systematic care to the patient, the definition of a time span would be crucial in the evaluation of the patient's response to treatment and in the selection of the best intervention to be adopted to change the patient's behavior. It would also minimize recall bias.

Most of the patients from this study were male and had a low monthly family income. These factors were related to poor adherence to antiretroviral treatment in another study[12]. Low socioeconomic status may be a proxy of several factors associated to nonadherence, such as difficulties in accessing the health system, difficulties involved in understanding the individual regarding his/her health situation, etc.[26]. Since the research involved a very low socioeconomic population, the study of the association of characteristics such as lifestyle, with the irregular use of cART, may be more specific to define the individual at risk.

Research has indicated that older patients have a better adherence to antiretroviral treatment[15], which also reinforces the findings of this study. It is possible that, due to a better perception of their own health, older people tend to better assimilate the importance of treatment and the consequences of interrupting it. This data was also encountered in another study undertaken in Brazil, where being younger was related to low adherence to the treatment[5].

Being a former smoker or a current smoker was associated with the discontinuation of combination antiretroviral therapy. Cigarette smoking has been associated with sexual behavior and drug abuse[1,13] and with a lower inclination to develop healthy behaviors related to

diet[17,20] and physical activity[17]. The same mechanism that predisposes all these behaviors may also lie behind, associated or not with these factors, a behavior less prone to adhere to medication. A study carried out by our group, in a cohort of people living with HIV/AIDS and under tuberculosis treatment, showed an association between smoking and the evasion of tuberculosis treatment[19]. The present study corroborates the findings of other authors who found an association between smoking and lower adherence to antiretrovirals[4,24], either smoking alone or as part of a risky behavior profile. Depression has been thought of as a possible mediator between smoking and adherence to antiretrovirals[28] but this is not a finding common to all authors[4].

People who use drugs tend to be more socially vulnerable and have a chaotic, unstable lifestyle that influences the adherence to any type of chronic treatment[8]. The use of illicit drugs has been associated with both nonadherence and a decrease in the viral and immunologic responses to antiretrovirals[18], the former also being a finding in the present study. Research has demonstrated that cocaine (including the crack variation) has an influence over the pathogenesis of AIDS, accelerating its progression and the risk of mortality, morbidity and an increased viral load[9,10]. For this group, an increase in the viral load may be related to nonadherence to the treatment, which may lead to viral resistance and subsequently to an immunological decline, thus favoring the HIV virus and its replication.

Studies show that direct administered antiretroviral therapy (DAART) in people living with HIV/AIDS who use drugs may improve adherence and thus improve the immune status of the individual[2,3]. Beyond DAART, other interventions such as counseling by motivational interviewing or video information, which is feasible in health care centers, improve adherence to antiretroviral treatment and maintain their efficacy three months later[14].

Once adherence to treatment has been promoted through individual approaches and discussions by health professionals in the majority of the specialized services for treating PLWHA, differentiated attention towards smokers and crack users, as well as interaction with specific control programs, are central to good adherence to antiretroviral treatment and the consequent reduction of viral resistance.

This study has some constraints. We are aware that the strategy used to measure the use of illicit substances may not be very accurate as a participant who has experienced crack cocaine once in the previous 12 months, an occasional user and a drug dependent (who used crack cocaine in the same time span), are all classified as current users of crack cocaine, although usage patterns and possibly behavior differ. The constraint posed to the interpretation of our findings by the discontinuation of combination antiretroviral therapy being measured has already been mentioned. Finally, a major limitation of this study, because of its cross-sectional design, is that it is not possible to establish the temporal sequence between exposure and outcome and differentiate cause and effect. Nevertheless the results pointed to groups which should be monitored differentially because of their association with discontinuation of combination antiretroviral therapy.

Further studies, using a longitudinal design and more restricted definitions, could complement our findings.

RESUMO

Associação entre tabagismo e o uso de crack com a descontinuidade da terapia antirretroviral combinada em Recife, Pernambuco, Brasil

Apesar da eficácia da terapêutica antirretroviral combinada para o tratamento de pessoas vivendo com HIV/Aids, a não adesão aos medicamentos tem se tornado uma das maiores ameaças à efetividade dessa terapêutica. O objetivo desse estudo foi estimar a prevalência de uso irregular autorreferido da terapia antirretroviral e os fatores associados com essa irregularidade em pessoas vivendo com HIV. Foi realizado um estudo seccional de pessoas vivendo com HIV/Aids atendidas em dois centros de referência no Recife, Nordeste do Brasil, entre junho 2007 e outubro de 2009. Foram analisados os fatores socioeconômicos, de apoio social e de hábitos do indivíduo, ajustados através de análise de regressão logística multivariada. A prevalência de pessoas vivendo com HIV/Aids que relataram o uso irregular da terapia antirretroviral combinada (TARC) foi de 25,7%. No modelo multivariado final, o uso irregular da TARC esteve associado às seguintes variáveis: ter menos de 40 anos (OR = 1,66, IC95%: 1,29-2,13), fumantes (OR = 1,76, IC95%: 1,31-2,37) ou ex-fumantes (OR = 1,43, IC95%: 1,05-1,95) e ser usuário de crack (OR = 2,79, IC95%: 1,24-6,32). Medidas especiais devem ser direcionadas para cada um dos seguintes grupos: adultos com menos de 40 anos, fumantes, ex-fumantes e usuários de crack. Ações voltadas para a cessação do tabagismo e do crack devem ser incorporadas ao programa de controle dos infectados pelo HIV, visando promover a maior adesão aos antirretrovirais e, consequentemente, aumentar a expectativa e a qualidade de vida.

COMPETING INTERESTS

The authors declared that there are no competing interests.

FUNDING

Ministério da Saúde/Programa DST/AIDS/UNESCO (CSV 182/06 - Projeto "Estudo Clínico-Epidemiológico da co-infecção HIV/Tuberculose em Recife"). The authors received partial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (scholarship 150425/2012-0 to J.D.L.B., 300917/2006-6 to R.A.A.X. and 301779/2009-0 to M.F.P.M.).

REFERENCES

1. Anteghini M, Fonseca H, Ireland M, Blum R. Health risk behaviors and associated risk and protective factors among Brazilian adolescents in Santos, Brazil. J Adolesc Health. 2001;28:295-302.

2. Babudieri S, Dorrucci M, Boschini A, Carbonara S, Longo B, Monarca R, *et al.* Targeting candidates for directly administered highly active antiretroviral therapy: benefits observed in HIV-infected injecting drug users in residential drug-rehabilitation facilities. AIDS Patient Care STDS. 2011;25:359-64.

3. Berg KM, Litwin A, Li X, Heo M, Arnsten JH. Directly observed antiretroviral maintenance clinics: a randomized controlled trial. Drug Alcohol Depend. 2011;113:192-9.

4. Bonolo PF, Machado CJ, Cesar CC, Ceccato MG, Guimaraes MD. Vulnerability and non-adherence to antiretroviral therapy among HIV patients, Minas Gerais State, Brazil. Cad Saude Publica. 2008;24:2603-13.

5. Carvalho CV, Duarte DB, Merchán-Hamann E, Bicudo E, Laguardia J. Determinantes da aderência à terapia anti-retroviral combinada em Brasília, Distrito Federal, Brasil, 1999-2000. Cad Saude Publica. 2003;19:593-604.

6. Carvalho CV, Merchán-Hamann E, Matsushita R. Determinantes da adesão ao tratamento anti-retroviral em Brasília, DF: um estudo de caso-controle. Rev Soc Bras Med Trop. 2007;40:555-65.

7. Centers for Disease Control and Prevention. Fact sheets in alcohol and public health. [cited 2011 October 7]. Available from: http://www.cdc.gov/alcohol/fact-sheets/binge-drinking.htm

8. Colombrini MR, Lopes MHBM, de Figueiredo RM. Adesão à terapia antiretroviral para HIV/AIDS. Rev Esc Enferm USP. 2006;40:576-81.

9. Cook JA. Associations between use of crack cocaine and HIV-1 disease progression: research findings and implications for mother-to-infant transmission. Life Sci. 2011;88(21-22):931-9.

10. Cook JA, Burke-Miller JK, Cohen MH, Cook RL, Vlahov D, Wilson TE, *et al*. Crack cocaine, disease progression, and mortality in a multicenter cohort of HIV-1 positive women. AIDS. 2008;22:1355-63.

11. Do NT, Phiri K, Bussmann H, Gaolathe T, Marlink RG, Wester CW. Psychosocial factors affecting medication adherence among HIV-1 infected adults receiving combination antiretroviral therapy (cART) in Botswana. AIDS Res Hum Retroviruses. 2010;26:685-91.

12. Gir E, Vaichulonis CG, Oliveira MD. Adesão à terapêutica anti-retroviral por indivíduos com HIV/AIDS assistidos em uma instituição do interior paulista. Rev Lat Am Enfermagem. 2005;13:634-41.

13. Hershberger SL, Fisher DG, Reynolds GL, Klahn JA, Wood MM. Nicotine dependence and HIV risk behaviors among illicit drug users. Addict Behav. 2004;29:623-5.

14. Ingersoll KS, Farrell-Carnahan L, Cohen-Filipic J, Heckman CJ, Ceperich SD, Hettema J, *et al.* A pilot randomized clinical trial of two medication adherence and drug use interventions for HIV+ crack cocaine users. Drug Alcohol Depend. 2011;116:177-87.

15. Kastrissios H, Suarez JR, Katzenstein D, Girard P, Sheiner LB, Blaschke TF. Characterizing patterns of drug-taking behavior with a multiple drug regimen in an AIDS clinical trial. AIDS. 1998;12:2295-303.

16. Kobin AB, Sheth NU. Levels of adherence required for virologic suppression among newer antiretroviral medications. Ann Pharmacother. 2011;45:372-9.

17. Fine LJ, Philogene GS, Gramling R, Coups EJ, Sinha S. Prevalence of multiple chronic disease risk factors: 2001 National Health Interview Survey. Am J Prev Med. 2004;27(2 Suppl):18-24.

18. Lucas GM, Cheever LW, Chaisson RE, Moore RD. Detrimental effects of continued illicit drug use on the treatment of HIV-1 infection. J Acquir Immune Defic Synd. 2001;27:251-9.

19. Maruza M, Albuquerque MFPM, Coimbra I, Moura LV, Montarroyos UR, Miranda-Filho DB, *et al*. Risk factors for default from tuberculosis treatment in HIV-infected individuals in the state of Pernambuco, Brazil: a prospective cohort study. BMC Infect Dis. 2011;11:351.

20. Morabia A, Curtin F, Bernstein MS. Effects of smoking and smoking cessation on dietary habits of a Swiss urban population. Eur J Clin Nutr. 1999;53:239-43.

21. Pence BW, Thielman NM, Whetten K, Ostermann J, Kumar V, Mugavero MJ. Coping strategies and patterns of alcohol and drug use among HIV-infected patients in the United States Southeast. AIDS Patient Care STDS. 2008;22:869-77.

22. Polejack L, Seidl EMF. Monitoramento e avaliação da adesão ao tratamento antirretroviral para HIV/aids: desafios e possibilidades. Cien Saude Colet. 2010;15(Suppl 1):1201-8.

23. Seidl EMF, Melchíades A, Farias V, Brito A. Pessoas vivendo com HIV/AIDS: variáveis associadas à adesão ao tratamento anti-retroviral. Cad Saude Publica. 2007;23:2305-16.

24. Shuter J, Bernstein SL. Cigarette smoking is an independent predictor of nonadherence in HIV-infected individuals receiving highly active antiretroviral therapy. Nicotine Tob Res. 2008;10:731-6.

25. Silva MC, Ximenes RA, Miranda Filho DB, Arraes LW, Mendes M, Melo AC, *et al*. Risk-factors for non-adherence to antiretroviral therapy. Rev Inst Med Trop Sao Paulo. 2009;51:135-9.

26. Simoni JM, Kurth AE, Pearson CR, Pantalone DW, Merrill JO, Frick PA. Self-report measures of antiretroviral therapy adherence: a review with recommendations for HIV research and clinical management. AIDS Behav. 2006;10:227-45.

27. Venkatesh KK, Srikrishnan AK, Mayer KH, Kumarasamy N, Raminani S, Thamburaj E, *et al*. Predictors of nonadherence to highly active antiretroviral therapy among HIV-infected South Indians in clinical care: implications for developing adherence interventions in resource-limited settings. AIDS Patient Care STDS. 2010;24:795-803.

28. Webb MS, Vanable PA, Carey MP, Blair DC. Medication adherence in HIV-infected smokers: the mediating role of depressive symptoms. AIDS Educ Prev. 2009;21(3 Suppl):94-105.

29. World Health Organization. Global health sector strategy on HIV/AIDS 2011-2015. Geneva: WHO; 2011.

30. Wilson IB, Carter AE, Berg KM. Improving the self-report of HIV antiretroviral medication adherence: is the glass half full or half empty? Curr HIV/AIDS Rep. 2009;6:177-86.

Received: 14 August 2012
Accepted: 5 August 2013

Rev. Inst. Med. Trop. Sao Paulo
56(2):133-138, March-April, 2014
doi: 10.1590/S0036-46652014000200008

RISK FACTORS OF HIV-1 VERTICAL TRANSMISSION (VT) AND THE INFLUENCE OF ANTIRETROVIRAL THERAPY (ART) IN PREGNANCY OUTCOME

Maria F.M. BARRAL, Gisele R. de OLIVEIRA, Rubens C. LOBATO, Raul A. MENDOZA-SASSI, Ana M.B. MARTÍNEZ & Carla V. GONÇALVES

SUMMARY

In the absence of intervention, the rate of vertical transmission of HIV can range from 15-45%. With the inclusion of antiretroviral drugs during pregnancy and the choice of delivery route this amounts to less than 2%. However ARV use during pregnancy has generated several questions regarding the adverse effects of the gestational and neonatal outcome. This study aims to analyze the risk factors for vertical transmission of HIV-1 seropositive pregnant women living in Rio Grande and the influence of the use of ARVs in pregnancy outcome. Among the 262 pregnant women studied the rate of vertical transmission of HIV was found to be 3.8%. Regarding the VT, there was a lower risk of transmission when antiretroviral drugs were used and prenatal care was conducted at the referral service. However, the use of ART did not influence the outcome of pregnancy. However, initiation of prenatal care after the first trimester had an influence on low birth weight, as well as performance of less than six visits increased the risk of prematurity. Therefore, the risk factors analyzed in this study appear to be related to the realization of inadequate pre-natal and maternal behavior.

KEYWORDS: HIV; Infectious Disease Transmission; Vertical; Antiretroviral Therapy; Pregnancy outcomes.

INTRODUCTION

According to UNAIDS, 34 million people are infected with HIV worldwide. Among them, 30 million are adults and 16.8 million women[27]. In Brazil, from 1980 to June 2011 608,230 cases of Aids were reported and over the past 12 years a stabilization of the incidence rates has been observed[3]. In 2010, the sex ratio was 1.7 new cases in men to one case in women, 71% of infected women are of reproductive age, with a prevalence of 0.4% of infected pregnant women[2,3].

In the absence of intervention, the rates of HIV transmission during pregnancy, labor or breastfeeding vary from 15-45%. Related to maternal-to-child transmission (MTCT), 35% occur during pregnancy, 65% in the peripartum and the risk of transmission through breastfeeding varies from 7% to 22%. Besides obstetric and neonatal variables, maternal variables such as viral load, advanced disease, co-infections, delay or non-use of antiretroviral (ARV) at the beginning or during pregnancy influence risk of HIV MTCT[5,18].

Vertical HIV transmission (VT) rates can be reduced to levels below 2% if women living with HIV have a suitable attendance during pregnancy with the use of ARV and reduce of the viral load (VL)[6]. In Brazil between 1980 and 2011 there were 14,127 cases of Aids in children under five. Over the last 12 years a 49.1% reduction in the absolute number of cases and 40.7% in the incidence rate[3] was observed. However, a multicenter study conducted by the Brazilian Society of Pediatrics in 2004 showed that the rate of HIV MTCT in Brazil was 6.8%[8].

There are still several barriers to effective prevention of perinatal transmission of HIV both related to female behavioral characteristics, as well as the quality of clinical services provided to them[8,10,24]. Although the main and the most effective way to reduce to VL, use of ART during pregnancy has generated conflicting data about possible adverse effects to the neonate[7], such as: preterm delivery[1,17], low birth weight[22], a decrease in neurological function[28], and low Apgar scores[1]. However, to date, there is no consensus on the association between antiretroviral drugs used by women during pregnancy and their consequences. Until now there is no sufficient evidence to support that the combined use of ARV has a negative association with pregnancy outcome[1,7,8,10,17,22,28].

The current study aimed to analyze the risk factors associated with vertical transmission of pregnant HIV-infected women in the city of Rio Grande and the influence of ART in pregnancy outcome such as prematurity, Apgar score and low birth weight.

MATERIAL AND METHODS

This study was conducted in Rio Grande, located in the southern coastal plain of Rio Grande do Sul, Brazil. The primary health care system is composed of 32 primary care units (PCU), and two general hospitals.

Faculdade de Medicina, Universidade Federal de Rio Grande, Rio Grande, RS, Brazil.
Correspondence to: Carla V. Gonçalves, Faculdade de Medicina da Universidade Federal do Rio Grande/FURG. R. General Osório S/Nº, 96200-190 Rio Grande, Rio Grande do Sul, Brasil. Phone: + 55.53.3233-8842. E-mail: carlavg@brturbo.com.br

Health care for people living with HIV is offered by a specialized unit at the University Hospital Miguel Riet Correa Junior (HU-FURG). In 2003, a specific unit for seropositive pregnant women was created. Every patient has a medical record with information from ongoing prenatal, delivery and HIV MTCT. This cohort study included all pregnant women that attended the HU-FURG between July 2003 and July 2007. This endpoint date was chosen because until this period there were 95 women using Biovir® and Nelfinavir® and the same number using Biovir® and Kaletra®. The study was submitted and approved by the Ethical Committee of FURG (23116001368/2003-44 protocol). All procedures were carried out in accordance with the guidelines of the Helsinki Declaration.

The independent variables measured were: maternal age, years of schooling, number of pregnancies, parity, trimester of prenatal care initiation, number of prenatal visits, location of prenatal care (HU-FURG, other facilities, or no prenatal care), type of delivery, fasting glucose in the first or third trimester (≥ 86 mg/dL), CD4 + ≥ 350 count and viral load of the mother (≥ 1000 copies).

An aliquot of blood was collected from all pregnant women in the thirty fourth week of gestation for viral load quantification and CD4+T lymphocytes count. The use of ARV was categorized by time in two groups (cutoff 9/10 weeks). The type of ARV exposure was classified as: no use, use of Biovir® and Nelfinavir®, Kaletra® and Biovir®, other ARV. Outcomes studied were: birth weight ≤ 2500 g, fifth minute Apgar score ≤ 7; delivery at less than 37 weeks of gestation and HIV VT.

Status of viral infection of the newborn and time of transmission was determined by the technique of Polymerase Chain Reaction (PCR) from pro-viral DNA extracted (commercial kit ® Illustra blood genomic Prep Mini Spin, GE Healthcare ®) from the first blood collection (24/48 hours), and at 30 and 60 days after birth.

Multivariate analysis was based on an analysis model using Poisson regression with robust adjustment of variance. After calculating prevalence ratios (PR) and their respective confidence intervals of 95% (95% CI), variables that had a *p*-value ≤ 0.20 in the bivariate analysis were included in the model. For the birth weight ≤ 2500g outcome the variables included were: number of births, start of prenatal care trimester, and time of use of ARV. Adjusted analysis of Apgar score ≤ 7 at the fifth minute included: maternal age and schooling years, number of prenatal care visits, site of prenatal care, fasting glucose, viral load, ARV use and time of ART. For pre-term birth ≤ 37 weeks the following variables were analyzed: number of prenatal care visits, fasting glucose, and use of ARV. VT outcome was adjusted for: location of prenatal care, delivery type, CD4, viral load, time and use of ARV. The Wald test was used for statistical analysis and a *p*-value equal to or less than 0.05 of a two-tailed test was considered as significant.

RESULTS

The study included a total of 262 women with a mean age of 27 years (SD ± 6.24). 68.7% had four or less years of schooling and the number of pregnancies was on average 2.58 (SD ± 2.21) with a parity of 2.09 (SD ± 1.82).

The location of prenatal care was HU- FURG in 83.5% of cases, onset of prenatal care was on average 16 weeks of pregnancy, carrying around five visits each. Type of delivery was cesarean section in 61.5% of cases. Average weight of newborns was 2894g and Apgar score at five minutes was 9. Delivery was on average at 40 weeks of gestation. Regarding to the HIV status, the mean of CD4 + T cells was 574 cel/uL, the viral load was 10,603 copies/mL at 34 weeks of pregnancy and time of use of ARV was 16 weeks (SD ± 2.42).

Table 1 shows the results of the prevalence ratio (PR) and their Confidence Interval (95%CI). None of the studied factors showed significant association with the outcomes low birth weight and preterm birth. For the outcome fifth minute Apgar ≤ 7, women who had less than five visits had a PR 2.18 (1.08 to 4.40) and those with fasting glucose ≥ 86 mg/dL had PR of 2.42 (1.26 to 4.63). On the other hand, a lower risk of low Apgar score was observed (PR 0.47; CI: 0.26-1.66) in infants born from pregnant women with longer use of ARV (≥ 10 weeks).

From the 262 pregnant women, 10 (3.8%) transmitted HIV to the newborns. In the bivariate analysis those with three or more pregnancies had an increased risk of virus transmission (p = 0.05). The prenatal location also showed a significant association with the risk of MTCT, when held in a location other than the University Hospital (PR 9.37; 95% CI: 2.51 to 34.9) and in case of lack of prenatal (PR 2.90; CI: 0.59-14.24). Furthermore, maternal viral load ≥ 1000 was associated with a higher prevalence ratio (PR: 4.98; CI: 1.32-18.7) of HIV transmission to the newborn. The non-use of ARV was also related to increased risk of transmission (RP 12.9 p = 0.003), the use of Biovir ® and Nelfinavir® had a prevalence rate increased by three times (PR 3.0 p = 0.003) compared to the use of Biovir ® and Kaletra ®.

In the multivariate analysis (Table 2), after adjustment for selected variables from the bivariate analysis, it was observed that low birth weight (≤ 2500 g) had a higher probability of occurring when the start of prenatal care occurred in the 3rd trimester (PR 1.07; CI: 1.01-1.14). Regarding the pre-term birth (≤ 37 weeks), five or less visits had a higher probability of outcome (PR1.08; CI: 1.02-1.15). Finally, association of HIV VT with the use of ARV remained significant, with use of Biovir® and Kaletra® featuring a lower likelihood of outcome (PR 0.09; CI: 0.01-0.73), and prenatal location losing statistical significance.

DISCUSSION

The present study showed that undesirable pregnancy outcomes in women with HIV was associated to variables related to prenatal care and that VT was lower with use of Biovir® and Kaletra® and when prenatal care was taken under a specialized center. Among possible limitations is the fact that although HU-FURG is the only referral center for pregnant HIV-infected women of Rio Grande, some cases of prenatal care and delivery might have happened outside this service. Thus, pregnant women who had labor outside HU-FURG were lost in this study. Data from women who had their pregnancy monitoring outside HU-FURG, but delivery performed in this hospital, were registered in our system retroactively to childbirth.

Currently, Aids is a major public health problem worldwide. Clinical status of pregnant HIV-infected women has improved considerably with the introduction of ART. This resulted in decreased rates of HIV MTCT[6,10,23,25]. Nonetheless, studies have reported that despite the benefits of ART during pregnancy, some women exposed

Table 1
Adverse pregnancy outcome factors associated with HIV-infected women

Variable	Weight ≤ 2500g		Apgar ≤ 7		Delivery before ≤ 37 weeks		HIV vertical transmission	
	N (%)	PR ($_{95\%CI}$)	N (%)	PR ($_{95\%CI}$)	N (%)	PR ($_{95\%CI}$)	N (%)	PR ($_{95\%CI}$)
Age (years)		***p* = 0.29**		***p* = 0.14**		***p* = 0.65**		***p* = 0.52**
30 or more	15 (19.5)	1.0	16 (24.6)	1.0	19 (24.7)	1.0	4 (4.9)	1.0
20 to 29	26 (19.1)	0.37 (0.09-1.50)	16 (13.9)	0.57 (0.30-1.05)	26 (19.3)	0.78 (0.46-1.31)	4 (2.8)	0.56 (0.14-2.17)
19 or less	2 (7.1)	0.98 (0.55-1.74)	6 (25.0)	1.02 (0.45-2.29)	6 (21.4)	0.07 (0.39-1.95)	2 (6.5)	1.31 (0.25-6.78)
Schooling (years)		***p* = 0.98**		***p* = 0.10**		***p* = 0.75**		***p* = 0.88**
5 years or more	17 (13.9)	1.0	4 (8.2)	1.0	11 (19.3)	1.0	7 (5.5)	1.0
4 years or less	8 (14.0)	0.99 (0.46-2.16)	18 (18.4)	2.25 (0.81-6.29)	21 (17.4)	0.90 (0.47-1.74)	3 (5.0)	1.10 (0.30-4.12)
Pregnancies		***p* = 0.57**		***p* = 0.82**		***p* = 0.95**		***p* = 0.05**
One	6 (12.5)	1.0	6 (13.3)	1.0	10 (20.8)	1.0	0 (0.0)	1.0
Two	10 (18.5)	1.48 (0.58-3.77)	8 (18.2)	1.36 (0.52-3.61)	10 (18.5)	0.89 (0.41-1.95)	2 (3.6)	----
3 or more	17 (19.5)	1.56 (0.66-3.70)	11 (15.9)	1.20 (0.48-3.00)	17 (19.8)	0.95 (0.47-1.90)	8 (8.9)	-----
Parity		***p* = 0.15**		***p* = 0.70**		***p* = 0.29**		***p* = 0.60**
Pimiparous	8 (11.9)	1.0	10 (18.2)	1.0	11 (16.4)	1.0	3 (4.1)	1.0
Multiparous	24 (20.2)	1.69 (0.00-3.55)	16 (15.8)	0.87 (0.42-1.79)	27 (22.9)	1.39 (0.74-2.63)	7 (5.7)	1.42 (0.38-5.31)
Start of prenatal care		***p* = 0.07**		***p* = 0.71**		***p* = 0.41**		***p* = 0.54**
1° trimester	17 (19.5)	1.0	11 (13.8)	1.0	17 (19.5)	1.0	4 (4.3)	1.0
2° trimester	18 (22.0)	1.12 (0.62-2.03)	12 (16.4)	1.20 (0.56-2.54)	21 (25.6)	1.31 (0.75-2.30)	3 (3.3)	0.77 (0.18-3.37)
3° trimester	2 (5.3)	0.27 (0.07-1.11)	6 (20.0)	1.45 (0.59-3.58)	6 (15.8)	0.81 (0.35-1.89)	3 (7.7)	1.79 (0.42-7.62)
Number of visits		***p* = 0.26**		***p* = 0.02**		***p* = 0.16**		***p* = 0.54**
≥ 6 visits	16(15.0)	1.0	10 (10.3)	1.0	15 (14.0)	1.0	4 (3.6)	1.0
≤ 5 visits	22 (20.8)	0.99 (0.46-2.16)	20 (22.5)	2.18 (1.08-4.40)	29 (27.4)	1.95 (1.11-3.43)	6 (5.2)	1.46 (0.42-5.04)
Prenatal care location		***p* = 0.98**		***p* = 0.07**		***p* = 0.61**		***p* = 0.001**
University Hospital	35 (17.4)	1.0	31 (17.0)	1.0	43 (21.4)	1.0	5 (2.5)	1.0
Other facilities	3 (30.0)	1.72 (0.64-4.65)	0 (0.0)	---	1 (10.0)	0.47 (0.07-3.06)	3 (23.1)	9.37 (2.51-34.9)
No prenatal care	4 (16.0)	0.92 (0.36-2.37)	8 (47.1)	2.76 (1.52-5.02)	6 (25.0)	1.17 (0.56-2.45)	2 (7.1)	2.90(0.59-14.24)
Type of delivery		***p* = 0.87**		***p* = 0.22**		***p* = 0.42**		***p* = 0.16**
Cesarean section	26 (17.4)	1.0	22 (16.7)	1.0	34 (22.8)	1.0	4 (2.7)	1.0
Vaginal	17 (18.3)	1.05 (0.60-1.82)	17 (23.6)	1.42 (0.81-2.49)	17 (18.5)	0.81 (0.48-1.36)	6 (6.3)	1.32 (0.69-8.17)
Fasting glucose		***p* = 0.48**		***p* = 0.04**		***p* = 0.10**		***p* = 0.43**
≤ 85 mg/dL	31 (18.9)	1.0	20 (13.8)	1.0	32 (19.5)	1.0	7 (4.0)	1.0
≥ 86 mg/dL	6 (14.3)	0.76 (0.34-1.65)	10 (27.8)	2.42 (1.26-4.63)	13 (31.0)	1.59 (0.92-2.74)	3 (6.7)	1.68 (0.45-6.23)
CD4		***p* = 0.98**		***p* = 0.79**		***p* = 0.41**		***p* = 0.16**
≥ 350	30 (17.8)	1.0	25 (16.9)	1.0	36 (21.4)	1.0	7 (3.9)	1.0
≤ 349	10 (18.2)	1.02 (0.54-1.96)	8 (18.6)	1.10 (0.54-2.26)	9 (16.4)	1.10 (0.54-2.26)	3 (5.2)	1.32 (0.35-4.95)
Viral load		***p* = 0.23**		***p* = 0.17**		***p* = 0.39**		***p* = 0.008**
≤ 999	24 (15.9)	1.0	19 (14.4)	1.0	33 (16.9)	1.0	3 (1.9)	1.0
≥ 1000	16 (22.5)	1.42 (0.00-2.50)	13 (22.4)	1.56 (0.83-2.94)	12 (21.9)	0.77 (0.43-1.41)	7 (9.3)	4.98 (1.32-18.7)
Antirretroviral		***p* = 0.29**		***p* = 0.09**		***p* = 0.13**		***p* = 0.003**
No ARV	7 (16.7)	1.0	10 (32.3)	1.0	10 (24.4)	1.0	6 (13.6)	12.9 (1.61-104.3)
Biovir e Nelfinavir	12 (12.8)	0.77 (0.32-1.81)	9 (12.2)	0.38 (0.17-0.84)	13 (13.8)	0.57 (0.27-1.19)	3 (3.2)	3.00 (0.32-28.3)
Biovir e Kaletra	20 (23.8)	1.43 (0.60-3.11)	18 (22.0)	0.68 (0.35-1.31)	22 (26.2)	1.07 (0.56-2.05)	1 (1.1)	1.0
Other ARV	3 (18.8)	1.13 (0.33-3.83)	2 (12.3)	0.41 (0.10-1.66)	5 (31.3)	1.28 (0.52-3.17)	0 (0.0)	----
Time of use of ARV		***p* = 0.18**		***p* = 0.01**		***p* = 0.99**		***p* = 0.001**
≤ 9 weeks	8 (12.3)	1.0	16 (22.5)	1.0	14 (21.9)	1.0	10 (15.2)	1.0
≥ 10 weeks	29 (19.9)	1.61 (0.78-3.34)	19 (15.9)	0.47 (0.26-1.66)	32 (21.9)	1.0 (0.58-1.75)	0 (0.0)	----

Table 2
Adjusted analysis for unfavorable pregnancy outcome factors associated with HIV-infected women

Outcome	Variable	**Prevalence Ratio** ($_{95\%}$CI)	***p* -value**
Low birth weight (≤ 2500g)	**Start of prenatal care**		0.005
	1st trimester	1.0	
	2nd trimester	0.98 (0.92-1.05)	
	3rd trimester	1.07 (1.01-1.14)	
Delivery ≤ 37 weeks	**Number of visits**		0.013
	≥ 6 visits	1.0	
	≤ 5 visits	1.08 (1.02 - 1.15)	
HIV Vertical Transmission	**Prenatal care location**		0.06
	University Hospital	1.0	
	Other facilities	7.61 (1.04 - 55.57)	
	No prenatal care	2.31 (0.21 - 24.75)	
	Antirretroviral		0.041
	No ARV	1.0	
	Biovir and Nelfinavir	0.21 (0.05 - 1.00)	
	Biovir and Kaletra	0.09 (0.01 - 0.73)	
	Other ARV		

to ARV showed anemia, nausea, vomiting, hyperglycemia and elevated aminotransferase[1,18].

Most studies showed that the use of Biovir ®during pregnancy has not been associated with an increased risk of teratogenicity[4,26], low birth weight, smaller head circumference, cognitive dysfunction and preterm delivery[1,4,17,22,28]. However, research shows that the use of protease inhibitors (Nelfinavir® and Kaletra®) could be associated to an increased risk of preterm birth[11,19], although no association with low birth weight and lower Apgar score was found[18,26,27,28 ,29].

In the bivariate analysis no significance was found between low birth weight and preterm birth and the other variables. After multivariate analysis, women who started prenatal care in the third trimester had a 7% higher risk of having infants with low birth weight. Likewise, pregnant women who had five or less visits had an increased risk of 8% for delivering before 37 weeks of gestation. In accordance to other published studies[6,1,12,18,23] this research found no negative association of ART and low birth weight or prematurity.

Highest prevalence of preterm delivery and low birth weight has been associated with elective cesarean section[9,12,13,20]. Studies show that the current indication for elective cesarean, although decreasing the risk of transmission, may increase the prevalence of preterm births when performed before 38 weeks[11,13,14,22]. In our study the prevalence of delivery with less than 37 weeks was 22.8% among women undergoing cesarean section and 18.5% among women who gave birth vaginally. The high prevalence of caesarean section in this study is related to maternal viral load greater than or equal to 1000 copies/mL (32.1%) or your ignorance after the 34th week (11.1%). We emphasize the importance of accurate assessment of gestational age (GA) with a first trimester ultrasound and date of last menstrual period compatible (LMP), when it is necessary to indicate elective cesarean section in this group.

In this study, the use of ART showed no association with a low score in the 5th minute Apgar. YOSHIMOTO *et al.* (2005)[29], investigating the clinical and laboratory evolution of neonates from HIV-infected mothers, reported no relation between Apgar score and presence of HIV. Other studies in Brazil showed that the Apgar score in newborns of mothers with HIV was higher than seven in the first and fifth minute, not being related with the HIV infection. Values less than seven are associated with unfavorable outcomes during childbirth, especially neonatal asphyxia[16,24,30].

HIV MTCT rate in this study was 3.8%. Among the factors that influenced HIV transmission are prenatal care outside the HIV service and non-use of combined ARV. After multivariate analysis, a 7-fold risk of transmitting HIV to the newborns was observed when women received prenatal care outside the reference service (HU-FURG). Probably the women who received prenatal care outside the reference service (HU-FURG) did not use or started using ARVs late. A fact that may have influenced a higher viral load in these women at greater risk and consequent VT. The Brazilian government has guidelines for the management of pregnant women with HIV, which emphasize the importance of early referral of pregnant women to a reference service[2]. Another factor that influenced MTCT was the ART, women who used Biovir® and Kaletra® during pregnancy had 91% less risk of transmitting HIV to the neonates when compared with women who did not use ARV. Already using Biovir® and Nelfinavir® reduced the risk of MTCT by 88% compared to not using ARVs. TORNATORE *et al.* (2010)[23] in the same city demonstrated that the use of ARV during pregnancy lowers the risk of VT by 68%.

The association between quality of prenatal care and pregnancy outcome is well established[21]. This study demonstrated that pregnant women with HIV, having adequate care, are susceptible to the very same factors that affect pregnancy outcomes in non-HIV women. Therefore, a

proper prenatal care and initiation in the first trimester of pregnancy are crucial for avoiding risk associated with an unfavorable outcome. The rate of mother to child transmission of HIV becomes almost zero when these appropriate measures are taken. Despite the possible risks of using ARV by the mother during pregnancy, the benefits to the newborn are compensatory and its use did not influence the outcome of pregnancy. Undesirable outcomes (birth weight ≤ 2500g, Apgar score ≤ 7 in the fifth minute, delivery ≤ 37 weeks and HIV MTCT) seem to be related to the realization of inadequate prenatal care and maternal behavior. More research on the subject is essential, including studies analyzing long term effects in children who were exposed to ARV during pregnancy.

RESUMO

Fatores de risco para a transmissão vertical do HIV-1 e a influência da terapia antirretroviral (ARV) no desfecho gestacional

Na ausência de intervenção, as taxas de transmissão vertical do HIV podem variar de 15-45%. Com a inserção dos antirretrovirais durante a gestação e a escolha da via de parto estas taxas chegam a menos de 2%. No entanto o uso de ARV na gestação tem gerado várias duvidas quanto aos efeitos adversos causados ao desfecho gestacional e ao neonato. Este estudo objetiva analisar os fatores de risco da transmissão vertical do HIV-1 em gestantes soropositivas atendidas na cidade do Rio Grande e a influência do uso do ARV no desfecho gestacional. Entre as 262 gestantes estudadas a taxa de transmissão vertical do HIV encontrada foi de 3,8%. Em relação à TV, foi observado menor risco de transmissão quando esta havia feito uso de antirretrovirais e o pré-natal era realizado no serviço de referência. Entretanto, o uso de ARV não influenciou negativamente o desfecho gestacional. No entanto, o inicio do pré-natal após o primeiro trimestre teve influencia sobre o baixo peso ao nascer, assim como a realização de menos de seis consultas aumentou o risco de prematuridade. Portanto, os fatores de risco analisados neste estudo parecem estar relacionados à realização não adequada do pré-natal e ao comportamento materno.

REFERENCES

1. Areechokchai D, Bowonwatanuwong C, Phonrat B, Pitisuttithum P, Maeka-A-Nantawat W. Pregnancy outcomes among HIV-infected women undergoing antiretroviral therapy. Open AIDS J. 2009;3:8-13.

2. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Programa Nacional de DST e Aids. Recomendações para profilaxia da transmissão vertical do HIV e terapia antirretroviral em gestantes. Brasília: Ministério da Saúde; 2010.

3. Brasil. Ministério da Saúde. Boletim epidemiológico AIDS e DST. Brasília: Ministério da Saúde; 2011.

4. Brogly S, Abzug M, Watts DH, Cunningham CK, Williams PL, Oleske J, *et al*. Birth defects among children born to HIV-infected women: pediatric AIDS clinical trials protocols 219 and 219C. Pediatr Infect Dis J. 2010;29:721-7.

5. Cecchini D, Martinez M, Astarita V, Nieto C, Giesolauro R, Rodriguez C. Prevención de la transmisión vertical del VIH-1 en un hospital público de complejidad terciaria de Buenos Aires, Argentina. Rev Panam Salud Publica. 2011;30:189-95.

6. Cooper E, Charurat M, Mofenson L, Hanson IC, Pitt J, Diaz C, *et al*. Combination antiretroviral strategies for the treatment of pregnant HIV-1-infected women and prevention of perinatal HIV-1 transmission. J Acquir Immune Defic Syndr. 2002;29:484-94.

7. El Beitune P, Duarte G, Quintana S, Figueiró-Filho E, Marcolin AC, Abduch R. Antiretroviral therapy during pregnancy and early neonatal life: consequences for HIV-exposed, uninfected children. Braz J Infect Dis. 2004;8:140-50.

8. Ferreira C, Ribeiro D, Oliveira EC, Barbosa MJ, Simão MB, Pinto VM. O desafio da redução da transmissão vertical do HIV e da sífilis no Brasil. DST – J Bras Doenças Sex Transm. 2007;19:184-6.

9. Fuchs K, Wapner R. Elective cesarean section and induction and their impact on late preterm births. Clin Perinatol. 2006;33:793-801.

10. Kissin DM, Mandel MG, Akatova N, Belyakov NA, Rakhmanova AG, Voronin EE, *et al*. Five-year trends in epidemiology and prevention of mother-to-child HIV transmission, S. Petersburg, Russia: results from perinatal HIV surveillance. BMC Infect Dis. 2011;11:292.

11. Kourtis AP, Schmid CH, Jamieson DJ, Lau J. Use of antiretroviral therapy in pregnant HIV-infected women and the risk of premature delivery: a meta-analysis. AIDS. 2007;21:607-15.

12. Lisonkova S, Hutcheon JA, Joseph KS. Temporal trends in neonatal outcomes following iatrogenic preterm delivery. BMC Pregnancy Childbirth. 2011;25:11-39.

13. Lopez M, Figueras F, Hernandez S, Lonca M, Garcia R, Palacio M, *et al*. Association of HIV infection with spontaneous and iatrogenic preterm delivery: effect of HAART. AIDS. 2012;26:37-43.

14. Machado LS. Cesarean section in morbidly obese parturients: practical implications and complications. N Am J Med Sci. 2012;4:13-8.

15. Marazzi MC, Palombi L, Nielsen-Saines K, Haswell J, Zimba I, Magid N, *et al*. Extended antenatal use of triple antiretroviral therapy for prevention of mother-to-child transmission of HIV-1 correlates with favorable pregnancy outcomes. AIDS. 2011;25:1611-8.

16. Oliveira MIV, Bezerra MGA, Bezerra Filho JG, Bezerra JP, Oliveira R, Feitosa RFG. Puérperas com vírus humano da imunodeficiência positivo (HIV+) e as condições de nascimento de seus recém-nascidos. Enfermería Global. 2012;28:439-52.

17. Patel K, Shapiro DE, Brogly SB, Livingston EG, Stek AM, Bardeguez AD, *et al*. Prenatal protease inhibitor use and risk of preterm birth among HIV-infected women initiating antiretrovirals drugs during pregnancy. J Infect Dis. 2010;201:1035-44.

18. Pilotto JH, Velasque L, Freidman RK, Moreira RI, Veloso VG, Grinsztejn B, *et al*. Maternal outcomes after highly active antiretroviral therapy for the prevention of mother-to-child transmission in HIV-infected women in Brazil. Antivir Ther. 2011;16:349-56.

19. Ravizza M, Martinelli P, Bucceri A, Fiore S, Alberico S, Tamburrini E, *et al*. Treatment with protease inhibitors and coinfection with hepatitis C virus are independent predictors of preterm delivery in HIV-infected pregnant women. J Infect Dis. 2007;195:913-4.

20. Reddy UM, Ko CN, Raju TN, Willinger M. Delivery indications at late-preterm gestations and infant mortality rates in the United States. Pediatrics. 2009;124:234-40.

21. Santos IS, Matijasevich A, Silveira MF, Sclowitz IK, Barros AJ, Victora CG, *et al.* Associated factors and consequences of late preterm births: results from the 2004 Pelotas birth cohort. Paediatr Perinat Epidemiol. 2008;22:350-9.

22. Schulte J, Dominguez K, Sukalac T, Bohannon B, Fowler M. Declines in low birth weight and preterm birth among infants who were born to HIV-infected women during an era of increased use of maternal antiretroviral drugs: pediatric spectrum of HIV disease, 1989-2004. Pediatrics. 2007;119:900-6.

23. Tornatore M, Gonçalves CV, Mendoza-Sassi RA, Silveira JM, D'Avila NE, Maas CG, *et al*. HIV-1 vertical transmission in Rio Grande, Southern Brazil. Int J STD AIDS. 2010;21:351-5.

24. Vasconcelos AL, Hamann EM. Por que o Brasil ainda registra elevados coeficientes de transmissão vertical do HIV? Uma avaliação da qualidade da assistência prestada a gestantes/parturientes infectadas pelo HIV e seus recém-nascidos. Rev Bras Saúde Mater Infant. 2005;5:483-92.

25. Volmink J, Siegfried N, van der Merwe L, Brocklehurst P. Antiretrovirals for reducing the risk of mother-to-child transmission of HIV infection. Cochrane Database Syst Rev. 2007;(1):CD003510.

26. Watts DH, Huang S, Culnane M, Kaiser KA, Scheuerle A, Mofenson L, *et al*. Birth defects among a cohort of infants born to HIV-infected women on antiretroviral medication. J Perinat Med. 2011;39:163-70.

27. WHO - World Health Organization. Mother-to-child transmission of HIV. (cited 2012 Nov). Available from http://www.who.int/hiv/topics/mtct/en/

28. Williams PL, Marino M, Malee K, Brogly S, Hughes MD, Mofenson LM. Neurodevelopment and in utero antiretroviral exposure of HIV-exposed uninfected infants. Pediatrics. 2010;125:250-60.

29. Yoshimoto C, Diniz EM, Vaz FA. Evolução clínica e laboratorial de recém-nascidos de mães HIV positivas. Rev Assoc Med Bras. 2005;51:100-5.

30. Zorzi PM, Madi JM, Rombaldi RL, Araújo BF, Zatti H, Madi SR, *et al*. Fatores perinatais associados a recém-nascidos de termo com pH<7,1 na artéria umbilical e índice de Apgar <7,0 no 5º minuto. Rev Bras Ginecol Obstet. 2012;34:381-5.

Received: 20 May 2013
Accepted: 2 September 2013

Rev. Inst. Med. Trop. Sao Paulo
56(2):139-142, March-April, 2014
doi: 10.1590/S0036-46652014000200009

QUANTITATIVE REAL-TIME PCR (Q-PCR) FOR SPUTUM SMEAR DIAGNOSIS OF PULMONARY TUBERCULOSIS AMONG PEOPLE WITH HIV/AIDS

Yvana Maria Maia de ALBUQUERQUE(1,3), Ana Luiza Magalhães de Andrade LIMA(5), Ana Kelly LINS(2), Marcelo MAGALHÃES(2) & Vera MAGALHÃES(1,2,4)

SUMMARY

Objective: To assess quantitative real-time polymerase chain reaction (q-PCR) for the sputum smear diagnosis of pulmonary tuberculosis (PTB) in patients living with HIV/AIDS with a clinical suspicion of PTB. **Method:** This is a prospective study to assess the accuracy of a diagnostic test, conducted on 140 sputum specimens from 140 patients living with HIV/AIDS with a clinical suspicion of PTB, attended at two referral hospitals for people living with HIV/AIDS in the city of Recife, Pernambuco, Brazil. A Löwenstein-Jensen medium culture and 7H9 broth were used as gold standard. **Results:** Of the 140 sputum samples, 47 (33.6%) were positive with the gold standard. q-PCR was positive in 42 (30%) of the 140 patients. Only one (0.71%) did not correspond to the culture. The sensitivity, specificity and accuracy of the q-PCR were 87.2%, 98.9% and 95% respectively. In 39 (93%) of the 42 q-PCR positive cases, the CT (threshold cycle) was equal to or less than 37. **Conclusion:** q-PCR performed on sputum smears from patients living with HIV/AIDS demonstrated satisfactory sensitivity, specificity and accuracy, and may therefore be recommended as a method for diagnosing PTB.

KEYWORDS: HIV/AIDS/tuberculosis co-infection; Diagnosis; Real time PCR.

INTRODUCTION

Infection by the human immunodeficiency virus (HIV) is an important risk factor in the development of tuberculosis (TB). HIV increases not only the risk of reactivating latent *Mycobacterium tuberculosis* (MTB) but also the re-infection of the disease[7]. The annual risk of progressing to TB amongst coinfected patients varies between five and 15%, depending on the degree of immunosuppression, against 0.5% and 1% in non-coinfected patients[12].

In most cases, pulmonary tuberculosis (PTB) in those living with HIV/AIDS presents itself in an atypical clinical form, and from a clinical or radiological viewpoint is very often indistinguishable from other opportunistic infections[7].

Conventional laboratory techniques used for diagnosing PTB, such as the sputum smear test by Ziehl-Neelsen staining, which despite being inexpensive, presents low sensitivity since most coinfected patients have paucibacillary diseases[9,16,17]. Although the culture has a greater sensitivity of between 19 and 96%, and a specificity of 100%, and is the gold standard, it takes between four and eight weeks to obtain results[13,15,16].

In daily practice, it is common to prescribe anti-TB drugs for patients living with HIV/AIDS, without any confirmation of TB-disease, due to diagnostic difficulties and severity of symptoms. This conduct frequently leads to complications, not only due to the toxicity of anti-TB drugs, but also because of the interaction between these drugs and antiretroviral therapy (ART).

Studies have suggested that the inclusion of quantitative real-time polymerase chain reaction (q-PCR) is a method that may assist in diagnosing a variety of infections, including that caused by MTB[5,6,8,10]. q-PCR eliminates the gel electrophoresis steps in order to assess the results. Thus, it is a quicker, more sensitive technique, which also presents a lower risk of causing environmental contamination[6,8].

The aim of the present study is to assess q-PCR's effectiveness in confirming a diagnosis of PTB in sputum from patients living with HIV/AIDS and with a clinical suspicion of PTB.

METHODOLOGY

This was a prospective study to assess the accuracy of a diagnostic test, conducted between August 2009 and January 2012. A total of 140 patients were included in the study who were aged 18 and over, HIV-infected and with a clinical suspicion of PTB, and who were attending two referral hospitals for HIV/AIDS in the city of Recife, Pernambuco, Brazil. Patients were excluded from the study if they were

Study conducted at Hospital das Clínicas-UFPE and Hospital Correia Picanço, Recife-Pernambuco, Brazil.
(1) Tropical Medicine Post-graduation, Universidade Federal de Pernambuco, Recife-PE, Brazil.
(2) Laboratório Marcelo Magalhães, Recife-PE, Brazil.
(3) Correia Picanço Hospital- State Secretariat of Health, Recife-PE, Brazil.
(4) Clínicas Hospital, Universidade Federal de Pernambuco-UFPE, Recife-PE, Brazil.
(5) Faculdade Pernambucana de Saúde, Recife-PE, Brazil.
Correspondence to: Yvana Maria Maia de Albuquerque. Rua João Cardoso Aires 267/202, Boa Viagem, 51130-300 Recife, PE, Brasil. E-mail: yvana.albuquerque@terra.com

taking anti-TB drugs or were unable to provide sputum samples for the study.

All patients tested HIV-positive, which was conducted by enzyme immunoassay (ELISA, Abbott Laboratories) and confirmed by immunofluorescence or Western blot, as required by the Ministry of Health (Ordinance No. 59).

A sputum sample was collected from each patient. With patients who were unable to produce sputum spontaneously, sputum induction was performed with nebulized 3% hypertonic saline, for 20 minutes, with the aim of obtaining suitable material for the tests[2,14]. Collected data was stored in the study's database.

Sputum decontamination was undertaken with the NaOH**-**N**-**acetyl cysteine method. With the obtained sediment, slides were prepared for direct testing, performed with the Ziehl-Neelsen staining technique, and seeded in Löwenstein-Jensen solid medium (LJ (Difco-USA)) and 7H9 broth (Becton-Dickinson Co. MD-USA). The remaining sediment was maintained at -80 ºC until the q-PCR was conducted to identify the DNA of the MTB complex.

The cultivated material was examined twice per week during the first two weeks and once per week over the course of eight weeks. The culture, gold standard, was considered positive when at least one of the media presented mycobacterial growth. Furthermore, to confirm the identity of the species *M. tuberculosis* within the MTB complex, a commercial niacin accumulation test was performed (Becton, Dickinson).

The q-PCR methodology used was previously published by LEMAITRE *et al.*[10] and involved:

Step 1: Extraction of DNA from sputum: Tissue Protocol using the QIAamp DNA mini kit, following manufacturer's recommendations, manufactured by Qiagen, Hilden, Germany.

Step 2: DNA Amplification: In summary: Primers and probes were used for IS6110 (Gene Bank No. X52471), designed from Primer Express Software, 2.0 (BIOSYTEMS), obtained from Applied Biosystems, Warrington, UK. The nucleotide sequences of the primers were: 5'- CCGAGGCAGGCATCCA-3' (position 1062 to 1077) and 5'-GATCGTCTCGGCTAGTGCATT-3' (position 1112 to 1132). The sequence of the probe was 5'-FAM-TCGGAAGCTCCTATGAC-MGB-3' (position 1095 to 1111).

PCR amplification was performed in triplicate with a total volume of 25 µL containing the TaqMan Universal PCR master mix 2X (Applied Biosytems), with 300 nM of each primer, 200 nM probe and 5 µL of extracted DNA. A BioRad iCycler IQ 5 Thermal Cycler was used, with the following conditions: two minutes at 50 ºC, 10 minutes at 95ºC and 50 cycles at 95 ºC for 15 seconds and 60 ºC for one minute. The q-PCR was analyzed with Bio-Rad iQ5 1.0 software. For each reaction, positive and negative controls were used.

All positive culture tests for mycobacteria and q-PCR negative tests for MTB were submitted to the q-PCR test to identify MTB complexes, *M.avium-intracellulare, M.chelonae/abscessus* and *M.kansasi,* using primers and probes designed by LEUNG *et al.*[11]

Patients were assessed at the moment of collecting the material, reassessed after the culture results, observing the response to anti-TB treatment.

In order to undertake data analysis, rates were obtained for sensitivity, specificity, positive predictive values, negative predictive values and accuracy. The Kappa index was used to compare the LJ and 7H9 media. The Pearson's chi-squared test was used to assess the association between categorical variables; a 5% margin of error. The program used for typing in all data and for obtaining the calculations was SPSS 17.

The present study was approved by the Ethics Committee at the Centro de Ciências da Saúde at Universidade Federal de Pernambuco (UFPE), Protocol No. 01.470.172.000-09

RESULTS

A total of 140 sputum specimens from 140 patients living with HIV/AIDS with a clinical suspicion of PTB were analyzed. Of these, 47 (33.6%) were confirmed by culture, and 78 (55.7%) were male. Ages ranged from 19 to 64 years, a mean age of 37.13 years, a median of 36 years and a standard deviation of 9.86 years.

In 37 (26.4%) of the 140 patients, the direct sputum test with Ziehl-Neelsen staining was positive. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the direct test was 78.7%, 100%, 100%, 90.3% and 92.8%, respectively (Table 1).

q-PCR was positive in 42 (30%) of the 140 patients. Sensitivity, specificity, PPV, NPV and accuracy of the q-PCR were respectively: 87.2%, 98.9%, 97.6%, 93.9% and 95% (Table 1).

Of the 42 patients with positive q-PCR results, 39 (93%) presented a threshold cycle (Ct) equal to or less than 37. The sensitivity of the q-PCR, considering this Ct value as indicative of positivity, was 92.3%, the PPV was 97.5% and the accuracy was 92.9%. It was not possible to determine the specificity and NPV due to the low frequency (one case) of negative results, when compared to the culture, the gold standard method.

The CD4 T-cell count varied between two and 1301 cells/mm^3 presenting a median value of 148.50. No statistically significant difference was observed between the q-PCR results and the value of the CD4 cells, $p = 0.952$ (Pearson chi-squared test).

DISCUSSION

Of the 140 patients studied with a clinical suspicion of PTB, only 47 (33.6%) confirmed PTB with culture, the gold standard method. However, anti-TB treatment was initiated in 75 (53.6%) patients, 28 (20%) of whom initiated treatment without diagnostic confirmation. According to the reviewed medical records a therapeutic response to anti-TB treatment was observed in just eight (5.7%) of these patients and they were considered PTB cases with a negative culture. Due to the high frequency and severity of this coinfection, as well as the difficulty of arriving at a diagnosis with culture empirical treatment for TB is not uncommon in patients living with HIV/AIDS and respiratory symptoms. However, this conduct does not always prove beneficial, since coinfected patients present more adverse reactions to anti-TB drugs[3]. Moreover, there may be an interaction

Table 1
Comparison between qPCR and the direct test with the results of the culture (gold standard): sensitivity (S), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and accuracy (A)

Test	Gold standard		Total	Percentage measurements				
	Positive	Negative		S	Sp	PPV	NPV	A
• PCR-1								
Positive	41	1	42	87.2	98.9	97.6	93.9	95.0
Negative	6	92	98					
Total	47	93	140					
• BAAR								
Positive	37	-	37	78.7	100.0	100.0	90.3	92.8
Negative	10	93	103					
Total	47	93	140					

(1): PCR was only positive in 42 patients.

between the anti-TB drugs and antiretroviral therapy, which may impose limitations on the therapeutic response of these patients. These facts reinforce the importance of a rapid, safe diagnostic test, so as to reduce the number of these unnecessary treatments.

The sensitivity and specificity of the direct test were 78.7% and 100% respectively, where sensitivity was higher and specificity similar to other studies. In the direct test, KIBIKI *et al.* 2007 encountered a sensitivity of 66%, while REWATA *et al*. 2009, encountered 66.7% and SCHERER *et al.* 2011, 60%. The PPV and NPV of the direct test were 100% and 90.3% respectively. The PPV was similar and the NPV was higher to that encountered by REWATA *et al.* 2009, who obtained a NPV of 83.3%. The high sensitivity of the direct test in the present study is probably related to the method of obtaining the sputum samples. The sputum induction technique was used for 26 (18.6%) patients who were complaining of a dry cough. Induced sputum samples are of good quality, similar to those obtained with bronchoalveolar lavage (BAL) obtained by bronchoscopy[2,14]. Furthermore, the direct test was conducted after sputum decontamination and subsequent centrifugation, which may have contributed to the test's greater sensitivity.

The q-PCR demonstrated a sensitivity and specificity of 87.2% and 98.9%, respectively. These findings were similar to those of KIBIKI *et al.* 2007, who performed q-PCR with the BAL of patients living with HIV/AIDS. In KIBIKI's study, the results for sensitivity were 85.7% and 96.4% and for specificity were 52.3% and 90.9% with Ct values of 32 and 40. It is important to emphasize that the present study used sputum in the q-PCR assessment, while KIBIKI *et al.* 2007, used BAL. Since similar results were obtained, this would suggest that sputum induction using a nebulizer is equivalent to BAL, demonstrating how this procedure could be useful in regions where it is difficult to perform a bronchoscopy.

Of the 42 positive q-PCRs, only one was not confirmed by culture, this patient supplied a relatively substantial amount of *M. tuberculosis* DNA (Ct = 35) and a satisfactory response to anti-TB treatment, suggesting a failure of the culture. In fact, the sensitivity of the culture, despite being gold standard, may not be so high in paucibacillary patients. The sensitivity of the culture in coinfected patients varies between 19 and 96%[15].

The population levels of CD4 T-cells cannot be related to the results of the q-PCR. This demonstrates the usefulness of this test for diagnosing PTB at all stages of HIV infection. In patients with advanced AIDS, with low CD4 counts, PTB appears in an atypical form, and may be confused with several other infections[12]. The early, effective diagnosis of PTB is essential in order to ensure proper patient care, as well as reducing the transmission of MTB[1].

It may be concluded that q-PCR, performed on sputum samples obtained either spontaneously or after induction, yields satisfactory levels of sensitivity, specificity and accuracy. Thus, given that q-PCR is a quick technique, it is recommended for routine use in the management of patients living with HIV/AIDS.

RESÚMEN

Reacción en cadena de polimerasa en tiempo real cuantitativa (qPCR) para el diagnóstico de tuberculosis pulmonar en esputo de pacientes con VIH/sida

Objetivo: Evaluar la Reacción en Cadena de Polimerasa en tiempo real cuantitativa (qPCR) para el diagnóstico de tuberculosis pulmonar (TBP) en esputo de pacientes con sida y sospecha clínica de TBP. **Método:** Se trata de un estudio prospectivo para evaluación de precisión de prueba diagnóstica, realizado en 140 muestras de esputo provenientes de 140 pacientes con sida y sospecha clínica de TBP atendidos en dos hospitales de referencia para atención VIH/sida en Recife-PE, Brasil. Se utilizó el cultivo en medios Löwenstein-Jensen y 7H9 como estándar de oro. **Resultados:** De las 140 muestras de esputo, 47 (33,6%) fueron positivas por el estándar de oro. La qPCR fue positiva en 42 (30%) de los pacientes. En apenas un (0.71%) caso no correspondió con el cultivo. La sensibilidad, especificidad y precisión de la qPCR fueron 87,2%, 98,9% y 95% respectivamente. De las 42 qPCR positivas en 39 (93%) el CT (threshold cycle) fue igual o inferior a 37. **Conclusión:** La qPCR realizada en muestra de esputo de pacientes con sida demostró sensibilidad, especificidad y precisión satisfactoria, pudiendo ser recomendada como método de diagnóstico de TBP.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Laboratório Marcelo Magalhães for their support in performing the sputum smear tests.

The authors declare that they have no conflicts of interest.

REFERENCES

1. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, *et al.* Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med. 2010;363:1010-5.

2. Conde MB, Soares SLM, Mello FCQ, Resende VM, Almeida LL, Reingold AL, *et al*. Comparison of sputum induction with fiberoptic bronchoscopy in the diagnosis of tuberculosis: experience at an acquired imumune deficiency syndrome reference center in Rio de Janeiro, Brazil. Am J Respir Crit Care Med. 2000**;**162:2238-40.

3. De Lima MFS, Melo HRL. Hepatotoxicity induced by antituberculosis drugs among patients coinfect with HIV and tuberculosis. Cad Saude Publica. 2012;28:698-708.

4. El-Sadr WM, Tsiouris SJ. HIV-associated tuberculosis: diagnostic and treatment challenges. Semin Respir Crit Care Med. 2008;29:525-31.

5. Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, *et al*. Real-time PCR in clinical microbiology: applications for routine. Clin Microbiology Rev. 2006;19:165-256.

6. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods. 2001;25:386-401.

7. Kibiki GS, Mulder B, Ven AJAM, Sam N, Boeree MJ, Zanden A, *et al*. Laboratory diagnosis of pulmonary tuberculosis in TB and HIV endemic settings and the contribution of real time PCR for *M. tuberculosis* in bronchoalveolar lavage fluid. Trop Med Int Health. 2007;12:1210-7.

8. Kim K, Lee H, Lee M, Lee S, Shim T, Lim SY, *et al*. Development and application of multiprobe real-time PCR method targeting the *hsp*65 gene for differentiation of *Mycobacterium* species from isolates and sputum specimens. J Clin Microbiol. 2010;48:3073-80.

9. Kivihya-Ndugga L, van Cleeff M, Juma E, Kimwomi J, Githui W, Oskam L, *et al*. Comparison of PCR with the routine procedure for diagnosis of tuberculosis in population with high prevalences of tuberculosis and human immunodeficiency virus. J Clin Microbiol. 2004;42:1012-5.

10. Lemaitre N, Armand S, Vachée A, Capilliez O, Dumoulin C, Courcol RJ. Comparison of the real time PCR method and the Gen-Probe amplified *Mycobacterium tuberculosis* direct test for detection of *Mycobacterium tuberculosis* in pulmonary and nonpulmonary specimens. J Clin Microbiol. 2004;42:4307-9.

11. Leung KI, Yip CW, Cheung WF, Lo ACT, Ko WM, Kam KM. Development of a simple and a low-cost real-time PCR method for the identification of commonly encountered mycobacteria in a high throughput laboratory. J Appl Microbiol. 2009;107:1364-77.

12. Melo FAF, Afiune JB, Hijja MA, Gomes M, Rodrigues DSS, Klautau GB, *et al*. Coinfect tuberculosis and HIVAIDS. In: Veronesi R, Focaccia R, editors Treaty of infectology. 4th ed. São Paulo: Atheneu; 2009. p. 1313-21.

13. Padmapriyadarsini C, Narendran G, Swaminathan S. Diagnosis and treatment of tuberculosis in HIV co-infected patients. Indian J Med Res. 2011;134**:**850-65.

14. Parry CM, Kamoto O, Harries AD, Wirima JJ, Nyirenda CM, Nyangulu DS, *et al*. The use of sputum induction for establishing a diagnosis in patients with suspected pulmonary tuberculosis in Malawi. Tuber Lung Dis.1995;76:72-6.

15. Rewata L, Rutherford M, Apriani L, Janssen W, Rahmadi A, Parwati I, *et al*. Improving diagnosis of pulmonary tuberculosis among HIV/AIDS patients: literature review and experience in teaching hospital in Indonesia. Acta Med Indones. 2009;41(Suppl 1):57-64.

16. Scherer LC, Sperhacke RD, Jaraziwski C, Cafrune PI, Michelon CT, Ruperthal R, *et al*. Comparison of two laboratory-developed PCR methods for the diagnosis of pulmonary tuberculosis in Brazilian patients with and without HIV infection. BMC Pulm Med. 2011;11:15**.** http//www.biomedcentral.com/1471-2466/11/15

17. van Cleeff M, Kivihya-Ndugga L, Githnui W, Ng'ang'a L, Kibuga D, Odhiambo J, *et al*. Cost-effectiveness of polymerase chain reaction versus Ziehl-Neelsen smear microscopy for diagnosis of tuberculosis in Kenya. Int J Tuberc Lung Dis. 2005;9:877-83.

Received: 16 November 2012
Accepted: 1 August 2013

Rev. Inst. Med. Trop. Sao Paulo
56(2):143-149, March-April, 2014
doi: 10.1590/S0036-46652014000200010

ASPECTS OF THE ECOLOGY OF PHLEBOTOMINES (Diptera: Psychodidae: Phlebotominae) IN AN AREA OF CUTANEOUS LEISHMANIASIS OCCURRENCE, MUNICIPALITY OF ANGRA DOS REIS, COAST OF RIO DE JANEIRO STATE, BRAZIL

Gustavo Marins de AGUIAR(1), Alfredo Carlos Rodrigues de AZEVEDO(2), Wagner Muniz de MEDEIROS(3), João Ricardo Carreira ALVES(1) & Vanessa RENDEIRO(1)

SUMMARY

Over a complete two-year period, phlebotomine specimens were caught in an area of cutaneous leishmaniasis occurrence in the municipality of Angra dos Reis. A manual suction tube was used to catch phlebotomines on house walls, and also light traps in domestic and peridomestic settings and in the forest. This yielded 14,170 specimens of 13 species: two in the genus *Brumptomyia* and eleven in the genus *Lutzomyia*. *L. intermedia* predominantly in domestic and peridomestic settings, with little presence in the forest, with the same trend being found in relation to *L. migonei*, thus proving that these species have adapted to the human environment. *L. fischeri* appeared to be eclectic regarding location, but was seen to be proportionally more endophilic. *L. intermedia* and *L. migonei* were more numerous in peridomestic settings, throughout the year, while *L. fischeri* was more numerous in domestic settings except in March, April, May and September. From the prevalence of *L. intermedia*, its proven anthropophily and findings of this species naturally infected with *Leishmania* (*Viannia*) *braziliensis*, it can be incriminated as the main vector for this agent of cutaneous leishmaniasis in the study area, especially in the peridomestic environment. *L. fischeri* may be a coadjuvant in carrying the parasite.

KEYWORDS: Phlebotomines; Cutaneous Leishmaniasis; Angra dos Reis.

INTRODUCTION

The present study is the first of an intended series on the ecology of phlebotomines in areas of cutaneous leishmaniasis occurrence in the Serra do Mar, i.e. the coastal mountain range of the states of Rio de Janeiro and São Paulo.

The traditional communities present in the study area are characterized by their development of subsistence agriculture, non-mechanized fishing and a variety of differentiated economic activities that came to be introduced into their day-to-day lives after the opening of the Rio-Santos highway (BR 101), which facilitated growth of tourism and real-estate speculation. Consequently, these communities present low income and schooling levels, minimal access to the official healthcare network and a particular absence of adequate participation in the local economy[4].

Land occupation has been driven by individuals coming in from more highly valued parts of the region and has generally taken place in a non-harmonious manner. Thus, over recent decades, this has favored proliferation of areas at risk of *Leishmania* infection. Consequently, phlebotomine species that have already adapted to domestic and peridomestic environments can transmit the parasite to domestic animals, which serve as reservoirs[4].

Changes to the environment in many regions of Brazil have modified the epidemiological profile of leishmaniasis. Thus, wild mammals that are reservoirs for *Leishmania* have been able to invade peridomestic areas that are populated by phlebotomine species that have become adapted to environments modified by humans. Maintenance of cutaneous leishmaniasis in these ecologically altered areas clearly indicates that a cycle of secondary transmission in the peridomestic environment has evolved[19,23].

Currently, cutaneous leishmaniasis presents three characteristic epidemiological patterns: wild, occupational and leisure. In addition, it has rural and periurban characteristics relating to the migratory process, in which hill slopes have been occupied, with transformation of forested land to secondary and residual forests. In the states of Rio de Janeiro and São Paulo, *L. intermedia s. lat*. has been the vector, especially in settings of intense human action, affecting individuals of both sexes in the domestic environment[31].

In view of the diversity of phlebotomine species and the peculiarities of the areas involved, with or without transmission of cutaneous leishmaniasis, studies on the bioecology of species that have been shown to be vectors or are suspected of being vectors can be expected to provide useful information for constructing indicators that contribute towards

(1) Laboratório de Diptera, Instituto Oswaldo Cruz/Fiocruz, Av. Brasil 4365, 21040-360 Rio de Janeiro, RJ, Brazil.
(2) Laboratório de Transmissores de Leishmanioses, Instituto Oswaldo Cruz/Fiocruz, Rio de Janeiro, RJ, Brazil.
(3) Secretaria de Estado de Saúde.
Correspondence to: Gustavo Marins de Aguiar. E-mail: aguiar@ioc.fiocruz.br

risk assessment, thereby giving rise to prevention and control measures that are more effective.

With the aim of learning more about the habits of phlebotomines, a series of captures was undertaken. The objectives were to determine the local phlebotomine fauna, the behavior of the main species (endophilic or exophilic), their monthly frequencies and occurrence in domestic and forested environments.

MATERIAL AND METHODS

Study area: The municipality of Angra dos Reis is situated in the Rio de Janeiro State (23° 59' 27" S and 44º 15' 52" W) and is 155 km from the state capital. It is one of the oldest settled areas on the coast of the state of Rio de Janeiro and on the entire Atlantic coastline of Brazil, occupying a narrow strip of land between the bay of Ilha Grande and the escarpment of the Serra do Mar (the coastal mountain range). Angra is a word in Portuguese meaning "wide-open cove or small bay that appears where the land rises high above the shoreline" (Fig. 1).

The climate is hot and humid, with a rainy season in the summer. The mean annual rainfall is 2,279 mm, but this is not evenly distributed over the year[24].

The peninsula of Angra dos Reis was discovered in 1502 and because of its natural beauty and strategic position, it became a privileged and sought-after space, both as the state capital and as the private enterprise capital. Within this context, three major projects were implemented: the nuclear power complex, the terminal of the bay of Ilha Grande (TEBIG) and the Rio-Santos coastal highway (BR 101). Of these, the construction of the highway, which crosses the entire length of the municipality, was the greatest cause of the transformations, both at a social and at an environmental level[26].

A house in the Camorim district of the municipality of Angra dos Reis was chosen to serve as a vector capture station. This house was selected because two cases of cutaneous leishmaniasis had been found in the family living there, and because it was suitable for systematized phlebotomine capture, given that it had outhouses for domestic animals and a banana plantation, and was relatively close to the forest.

Every month from March 1996 to February 1998, the investigators spent two nights in the study area. Phlebotomines that landed on both the internal and the external walls of the house were caught using manual suction tubes between the following hours: 18:00 to 20:00, 21:00 to 23:00 and 00:00 to 02:00. A total of 288 hours were spent on this activity. The CDC light traps (a total of three), were also installed inside the home, in the peridomestic area (next to the chicken coop) and in the forest (around 300 meters from the house), always at the same sites and same times (18:00 to 06:00 the next morning) and one m above the ground. The traps were in position for a total of 576 hours at each collection site.

To analyze the monthly frequencies of the most numerous species at the three collection sites, the Williams mean (X_w) was calculated as described by HADDOW[17,18]. The numbers of phlebotomines caught inside the house (internal walls and light traps) in the peridomestic area (external walls and light traps) and in the forest (light traps) were summed.

RESULTS

For two consecutive years, by summing up the results each month, 14,170 phlebotomines of 13 species were caught. There were two species of the genus *Brumptomyia* França & Parrot 1921 and eleven of the genus *Lutzomyia* França 1924 as listed in the following, in order of frequency, using the designations of YOUNG & DUNCAN[34]:

Lutzomyia (*Nyssomyia*) *intermedia* (Lutz & Neiva, 1912)
Lutzomyia (*Pintomyia*) *fischeri* (Pinto, 1926)
Lutzomyia migonei (França, 1920)
Lutzomyia (*Nyssomyia*) *whitmani* (Antunes & Coutinho, 1939)
Lutzomyia (*Pintomyia*) *pessoai* (Coutinho & Barretto, 1940)
Lutzomyia monticola (Costa Lima, 1932)
Lutzomyia (*Psychodopygus*) *ayrozai* (Barretto & Coutinho, 1940)

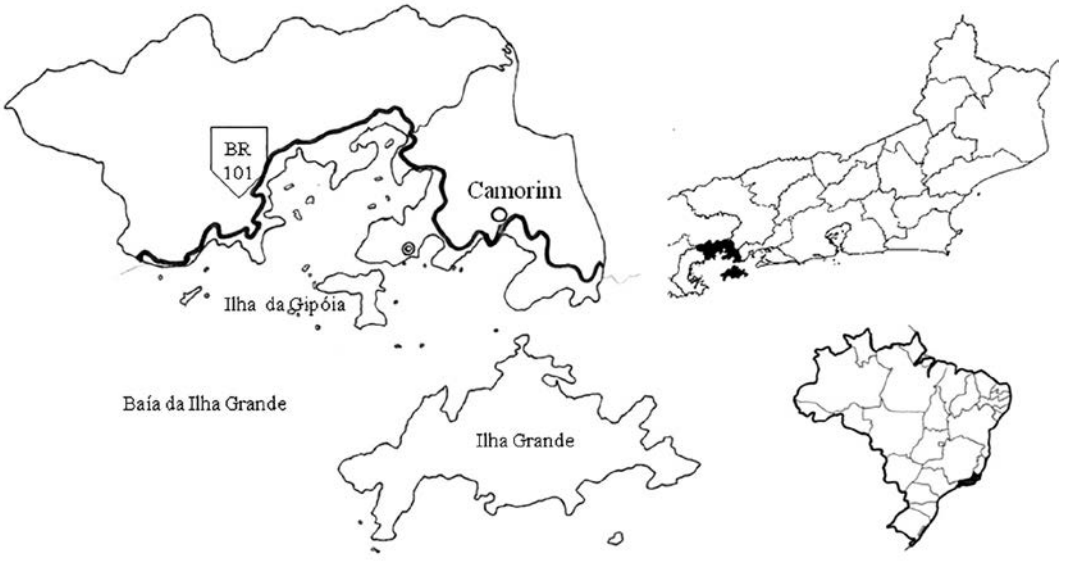


Fig. 1 - Map showing the location of the study area, in the Camorim district of the municipality of Angra dos Reis, state of Rio de Janeiro, Brazil.

Lutzomyia (*Psathyromyia*) *shannoni* (Dyar, 1929)
Lutzomyia edwardsi (Mangabeira, 1941)
Lutzomyia barrettoi (Mangabeira, 1942)
Brumptomyia avellari (Costa Lima, 1932)
Lutzomyia aragaoi (Costa Lima, 1932)
Brumptomyia guimaraesi (Coutinho & Barretto, 1941)

Table 1 presents the monthly frequencies of the species *L. intermedia*, *L. fischeri*, *L. migonei* and *L. whitmani*, which together accounted for 99.6% of the total number of specimens caught. The number of females was overall greater than the number of males: 57% versus 43%. *L. intermedia* showed balanced division, with only slightly more females; *L. migonei* showed a significantly greater number of males, while *L. fischeri* showed a predominance of females. Regarding the specimens caught when they landed on the internal and external walls of the house, *L. intermedia* was seen to be proportionally more exophilic, such that 70% of the examples were collected from the external walls, while *L. fischeri* was more endophilic, such that 72% of its specimens were caught on the internal walls of the house. *L. migonei* was seen to be clearly exophilic, such that 97% were on the external walls.

Among the phlebotomines caught using light traps, the predominance of *L. intermedia* was again greatest in the peridomestic area, with smaller numbers inside the house and a few specimens in the forest. *L. fischeri* presented similar numbers inside and outside the house. In the forest, it had much smaller number, but nonetheless it was the predominant species. At this collection site, the presence of *L. whitmani* was also noteworthy.

Also in relation to Table I and equally in Fig. 2, it was noted that the four most important species had higher mean numbers during the hot and humid period of the year, i.e. between October and January, with maximum peaks in December for *L. intermedia* and January for *L. fischeri* and *L. migonei*. The predominance of *L. intermedia* was clear in all months, both inside the house and in the peridomestic area. In the cooler and drier part of the year, from May to August, it presented a certain balance with *L. fischeri*, but from August onwards inside the house and from September onwards in the peridomestic area, the means gradually increased until reaching their maximum peak in December. Even though the means for *L. fischeri* were much lower than those of *L. intermedia*, *L. fischeri* showed significant presence inside the house, with means that gradually increased up to the maximum peak, in January. In the peridomestic area, the means were more balanced over the year, such that the presence of *L. fischeri* was usually slightly greater than that of *L. migonei*, except in the hot and humid period, when the latter predominated. In the forest, *L. fischeri* predominated except between April and July, when there was a balance with *L. whitmani*, which registered more significant means between December and February.

DISCUSSION

In the Americas, American cutaneous leishmaniasis is widespread, affecting all countries except for Uruguay and Chile[16]. In Brazil, this disease extends across all states of the federation, with a tendency towards urban areas. Its extent is especially related to environmental changes introduced through human action, in areas of continuous population flow, especially in areas with high levels of tourism such as the region of the Green Coast, i.e. the coastline of the states of Rio de Janeiro and São Paulo[3].

In the southeastern region of Brazil, a process of geographical expansion of the endemic area of cutaneous leishmaniasis has been witnessed. This has probably taken place as a result of the introduction of the parasite into new areas by means of migration of infected people and domestic animals[4,12,22,27,28].

In most areas with *Leishmania* (*Viannia*) *braziliensis* transmission in the southeastern and northeastern regions of Brazil, there is absolute predominance of *L. intermedia* or *L. whitmani*, followed by *L. fischeri* and *L. migonei*, in domestic environments[10,13,29,30,33].

FORATTINI & OLIVEIRA[14] raised the hypothesis that *L. intermedia s. lat.* (*intermedia* complex formed by *L. intermedia s. str.* and *L. neivai*) was the main transmitter of *Leishmania braziliensis*, while GOMES *et al.*[15] corroborated the suspicions of FORATTINI *et al.*[13] that this species carried the parasite in periurban areas.

Lutzomyia intermedia (Lutz & Neiva 1912) and *L. neivai* (Pinto 1926) are captured in various parts of Brazil and show a remarkable intraspecific and intrapopulational variation gradient[7]. *L. neivai* was considered synonymous junior of *L. intermedia*[21], both with allopatric distribution in regions west of the Serra do Mar, in the states of São Paulo and Paraná and sympatric speciation in the Serra do Mar, in the state of São Paulo[6,20].

American cutaneous leishmaniasis acquired epidemic characteristics on the northern coastline of the State of São Paulo beginning in the 1990s. From secondary data, a descriptive study of the disease in the four municipalities making up this region over the period from 1993 to 2005 was conducted. The frequency of phlebotomine capture in the probable transmission locations was analyzed. Among the 2,758 phlebotomines captured, *Lutzomyia intermedia s. lat.* predominated (80.4%) inside homes and in areas surrounding them[11].

In 1978, ARAÚJO FILHO[8] studied an outbreak of the disease in Ilha Grande, state of Rio de Janeiro and showed that *L. intermedia* predominated over the other species. On that occasion, this author reported that *L. flaviscutelatta*, the vector for *Leishmania amazonensis*, was present.

AGUIAR *et al.*[4] studied the phlebotomine fauna of Paraty, a municipality on the coast of the state of Rio de Janeiro and took the view that *L. intermedia* could be considered to be a potential vector for *Leishmania braziliensis*, given its high prevalence, anthropophilic nature and high degree of eclectic behavior, as well as because this species had already been incriminated in other areas of the southeastern region of Brazil. The insignificant presence of this species in wild environments and its adaptation to environments modified by humans led these authors to conclude that *L. intermedia* was transmitting the parasite in domestic and peridomestic settings.

AZEREDO-COUTINHO *et al.*[9] reported on an unusual case of human infection by *Leishmania amazonensis* that occurred in the historical city of Paraty. This species, which typically occurs in Amazônia, had already been identified in the states of Goiás, Mato Grosso, Bahia, Minas Gerais, São Paulo, Paraná and Santa Catarina, and it was also recently held responsible for the development of an autochthonous case of diffuse cutaneous leishmaniasis, which is a rare and severe form of the disease[9,31].

Table 1

Monthly averages of phlebotomines caught when they landed on internal (Int) or external (Ext) walls of the house, or caught in CDC traps installed inside the house (Dom), in the peridomestic area (Per) or in the forest (For), added the monthly results from March 1996 to February 1998; Angra dos Reis, state of Rio de Janeiro

Spe	Local		Sex	Years/Months												Total
				1996 - 97										1997 - 98		
				Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	
L. intermedia	Wall	Int	F	76	24	16	19	34	27	62	65	79	134	191	125	838
			M	11	8	-	-	-	1	3	7	15	23	13	18	113
		Ext	F	267	75	62	56	44	59	66	76	128	203	239	182	1,457
			M	127	33	19	17	24	28	30	32	84	126	168	172	860
	CDC	Dom	F	115	45	35	23	44	46	97	93	144	194	135	146	1,117
			M	12	4	3	1	1	7	21	47	63	94	78	53	384
		Per	F	122	39	21	27	32	37	43	201	297	338	401	299	1,857
			M	289	114	81	89	62	68	93	321	524	708	416	326	3,091
		For	F	-	-	-	-	1	1	-	-	-	-	-	-	2
			M	-	-	1	-	1	-	-	-	-	2	-	-	4
L. fischeri	Wall	Int	F	11	24	14	22	35	46	35	59	48	84	65	54	497
			M	-	-	-	-	1	1	-	-	-	1	2	-	5
		Ext	F	7	10	9	4	15	10	26	10	19	32	29	20	191
			M	1	-	-	-	2	2	3	5	4	4	3	5	29
	CDC	Dom	F	34	16	5	12	4	9	38	81	114	147	283	181	924
			M	2	-	-	1	-	-	6	3	8	7	3	8	38
		Per	F	84	40	36	18	16	31	56	71	79	83	88	53	655
			M	13	3	2	3	-	5	29	26	35	44	51	38	249
		For	F	23	3	-	2	1	2	9	17	20	34	21	18	150
			M	29	4	1	3	1	4	13	19	21	42	31	26	194
L. migonei	Wall	Int	F	-	-	-	1	-	1	-	-	3	1	1	-	7
			M	-	-	-	-	-	-	-	-	-	-	1	1	2
		Ext	F	6	2	2	1	-	2	7	4	16	32	14	24	110
			M	21	10	7	4	3	6	15	12	22	44	20	29	193
	CDC	Dom	F	6	2	-	1	-	2	4	4	7	9	5	6	46
			M	3	1	-	-	-	-	2	1	4	5	3	7	26
		Per	F	11	2	-	3	2	6	8	17	28	43	40	29	189
			M	38	7	5	16	8	11	20	47	79	155	185	124	695
		For	F	-	-	-	-	-	-	1	-	-	-	-	-	1
			M	2	-	-	-	-	-	-	-	1	1	-	-	4
L. whitmani	Wall	Int	F	-	-	-	-	-	-	-	-	-	-	-	-	-
			M	-	-	-	-	-	-	-	-	-	-	-	-	-
		Ext	F	-	-	-	-	-	-	-	-	-	-	-	-	-
			M	-	-	-	-	-	-	-	-	-	-	-	-	-
	CDC	Dom	F	-	-	-	-	-	-	-	-	-	-	-	-	-
			M	-	-	-	-	-	-	-	-	-	-	-	-	-
		Per	F	1	-	-	-	-	-	1	2	-	3	1	1	9
			M	-	-	-	-	-	-	2	-	1	-	-	1	6
		For	F	7	5	3	-	-	-	1	4	5	16	12	10	63
			M	4	2	1	1	3	3	4	9	12	19	24	31	113

L. = *Lutzomyia* Spe = species.

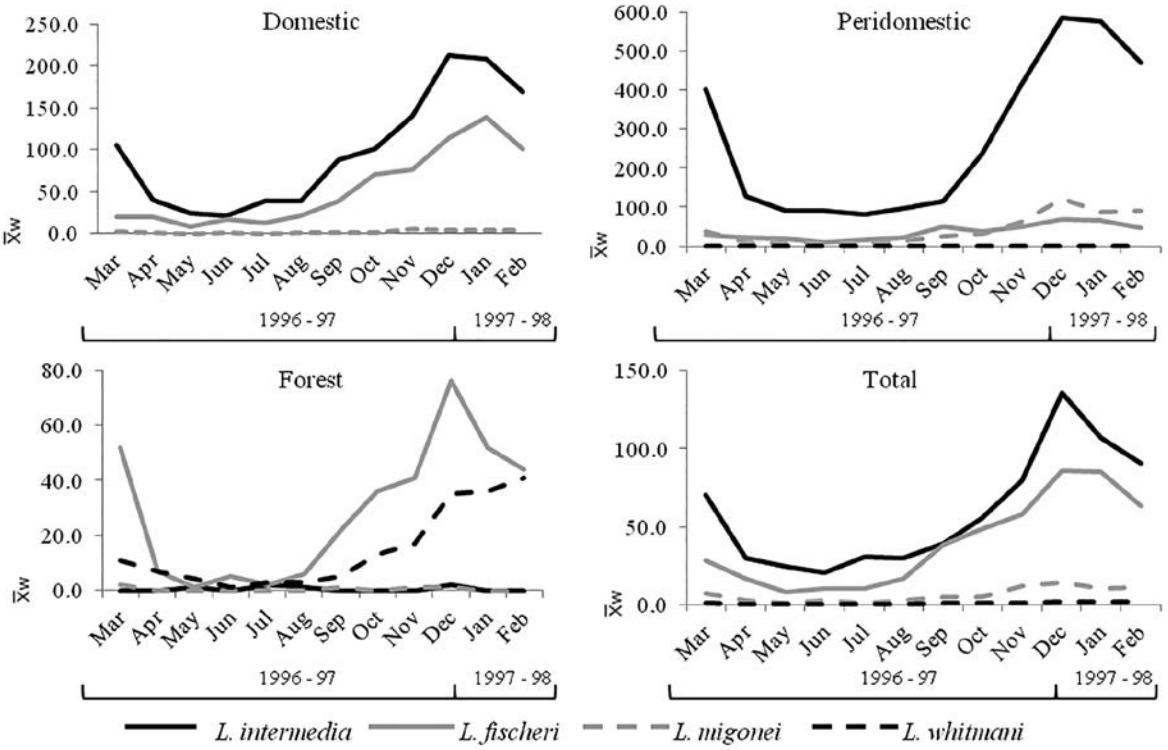


Fig. 2 - Monthly averages ($\overline{X}w$) of phlebotomines caught inside the house, in the peridomestic area or in the forest added to the monthly results from March 1996 to February 1998; Angra dos Reis, Rio de Janeiro State.

It is important to remember that in Brazil, human migrations to new agricultural areas and to urban centers have contributed towards propagating various diseases. Leishmaniasis is no different in this respect, given that large outbreaks of this disease have occurred especially in recently colonized areas. Thus, the changes to the natural environment caused by humans have had a huge impact on the behavior of the phlebotomine fauna, such that some species have disappeared while others have adapted to the human environment. Situations like this can be demonstrated in many foci of cutaneous leishmaniasis in the states of Rio de Janeiro, São Paulo and Paraná. In these regions that have been occupied for longer times, the environment changes were accompanied by adaptation of the components of the transmission cycle. In this manner, the changes induced selection of the phlebotomine species and reservoirs, which became better-fitted for survival under the new conditions represented by residual forests close to human homes in rural zones or on the periphery of urban zones with or without rural characteristics[3, 27, 32].

As a consequence of the drastic changes to the environment caused by human interference, some wild mammals that are reservoirs for *Leishmania* invaded domestic areas in which some phlebotomine species with eclectic feeding habits could be found. Through this, a transmission cycle with the potential to affect humans was established[28,32].

From research carried out so far in the southeastern and southern regions of Brazil, it can be said that *L. neivai* and *L. migonei* are the phlebotomine species with the greatest capacity for adaptation to human environments and therefore have the greatest possibility of adaptation to the domestic environment. The proof of this is the ever smaller numbers of specimens found in forested areas. Another important factor is the male/female ratio in the peridomestic environment, which suggests that the natural shelters and breeding sites of these species are nearby, given that, as is well known, the males do not have much capacity for flight and appear in large numbers in the peridomestic area motivated by mating[5]. However, in the district of Posse, in a rural zone of the municipality of Petrópolis, at a distance of 112 km from the city of Rio de Janeiro, and in the municipality of Mesquita, a periurban region on the Gericinó massif, state of Rio de Janeiro, investigations demonstrated that *L. intermedia* also occurred in significant numbers in the residual forests. In Mesquita, a hypothesis of three transmission cycles was also formulated (domestic, extradomestic and wild). The authors highlighted the sloth as a possible reservoir for *Leishmania braziliensis*, which would act as a link between the wild and peridomestic environments, while dogs and horses would participate in the domestic cycle, with *L. intermedia* as the main vector[23,32]. However, in all the locations where this species predominates, the numbers reveal that there is greater activity in the domestic and peridomestic environments, thus proving the trend towards domestic adaptation of this species[2].

Over the years, in Angra dos Reis, increasing devastation of the forest and growth of banana plantations (where the inhabitants frequently construct their houses, following a habit that started with the irregular division of the land into plots in the 1960s) have led to decreases in the phlebotomine and forest animal fauna. However, the density of *L. intermedia* in the domestic and peridomestic environments has increased: in these settings, survival of the species has been ensured through the close feeding relationship with humans and domestic and synanthropic animals.

In the study area, out of the 13 species obtained, six species (*L. intermedia*, *L. migonei*, *L. fischeri, L. whitmani*, *L. pessoai* and *L. ayrozai*) had already been found naturally infected with *Leishmania braziliensis* and *Leishmania naiffi*[25,31]. It has also been suggested that all of these species are transmitters in the epidemiological chain of cutaneous leishmaniasis and that they frequent both the domestic environment and remnant forest[1,27,28].

The high prevalence of *L. intermedia* has also been proven. Even with negative data from investigation of natural infection, the adaptive capacity of *L. intermedia* to environments modified by humans and its proven anthropophily allows it to be suggested that this species can be incriminated as the main vector for *Leishmania* (*Viannia*) *braziliensis* and that the transmission takes place in the domestic/peridomestic environment.

In relation to monthly frequency, *L. intermedia* presented great predominance in all the months of the year, both in the domestic and in the peridomestic environment. There was a gradual increase in frequency over the hotter and more humid period (between October and January, with mean temperatures ranging from 26 to 29 °C and relative air humidity from 84 to 87%. The maximum peak of activity was in December, whereas for *L. fischeri* and *L. migonei*, the highest peaks were in January.

The presence of *L. fischeri* in domiciliary environments, represented by much higher numbers of females, leads to the supposition that this species is not yet undergoing a process of domestic adaptation. Thus, it still maintains its natural shelters and breeding sites in the residual forest, as proven by the balance between the sexes at this study location, with slightly higher numbers of males. It can therefore be said that this species is eclectic regarding its blood meal location. Considering also that this species presented significant numbers at all three collection sites and that the distance from the house to the forest was around 300 meters, the data suggests that this is a species with greater dispersion.

Recently, in the municipality of Porto Alegre, in the state of Rio Grande do Sul, PITA-PEREIRA *et al.*[25] found naturally infected *L. fischeri* specimens in a periurban area by means of the PCR technique. In the present study area, even though there were no observations of natural infection due to *Leishmania* sp., important epidemiological factors relating to *L. fischeri* were observed, especially its high anthropophily, degree of eclecticism and endophilia, along with levels of occurrence in foci of cutaneous leishmaniasis in the Brazilian Southeastern region that have always been notable. Hence, the results suggest that this species may act as a secondary vector for *Leishmania braziliensis* in the domestic/peridomestic environment. Since its population remains predominantly in the forest, it may participate in transmission within its natural enzootic cycle.

In relation to *L. migonei*, it was found that this species had greater adaptation to the peridomestic area, particularly in outhouses where domestic animals are kept. This was corroborated by the small number of specimens found in the forest, along with the greater presence of males in the peridomestic area. These factors demonstrate that this species is well adapted to environments that have been subjected to human influence.

Even with a small number of specimens, the presence of *L. whitmani* stood out. This species was only caught in light traps, and in more significant numbers in the forest. It has been recorded in the state of Rio de Janeiro at low rates, but in studies conducted in the municipality of Posse, state of Rio de Janeiro, SOUZA *et al*.[32] the hypothesis that *L. whitmani* may be exerting pressure on the ecological niche of *L. intermedia* was raised.

RESUMO

Aspectos da ecologia dos flebotomíneos (Diptera: Psychodidae: Phlebotominae) em área de ocorrência de leishmaniose tegumentar, Município de Angra dos Reis, orla marítima do estado do Rio de Janeiro, Brasil

Durante dois anos completos foram feitas capturas de flebotomíneos em área de leishmaniose tegumentar no município de Angra dos Reis. Utilizou-se tubo de sucção manual, para as capturas dos flebotomíneos pousados nas paredes da casa, além de armadilhas luminosas, no domicílio, peridomicílio e na mata. Foram obtidos 14.170 exemplares, de treze espécies, duas do gênero *Brumptomyia* França & Parrot 1921 e onze do gênero *Lutzomyia* França 1924. *L. intermedia* teve supremacia no peridomicílio e no domicílio, com pouca presença na mata, o mesmo ocorreu com *L. migonei*, comprovando a adaptação dessas espécies ao ambiente humano. *L. fischeri* aparece com característica eclética quanto ao local, mostrando-se proporcionalmente mais endófila. *L. intermedia* e *L. migonei* foram mais numerosas no peridomicílio, durante todos os meses do ano, enquanto *L. fischeri*, excetuando os meses de março, abril, maio e setembro, foi mais numerosa no domicílio. Pela prevalência, comprovada antropofilia e por ter sido encontrada infectada naturalmente por *Leishmania* (*Viannia*) *braziliensis*, *L. intermedia* pode ser incriminada como o principal vetor desse agente da leishmaniose tegumentar na área de estudo, sobretudo no ambiente peridomiciliar. *L. fischeri*, pelas características apresentadas, pode ser um coadjuvante na veiculação do parasita.

REFERENCES

1. Aguiar GM, Rendeiro V. Flebotomíneos (Diptera: Phlebotominae) em área de foco ativo de leishmaniose tegumentar, orla marítima do Estado do Rio de Janeiro, Brasil. In: 21º Congresso Brasileiro de Entomologia; 2006 ago 6-11; Recife. **Resumos.** p. ID: 1231-1.

2. Aguiar GM, Medeiros WM. Distribuição regional e habitats das espécies de flebotomíneos do Brasil. In: Rangel EF, Lainson R, editores. Flebotomíneos do Brasil. Rio de Janeiro: Fiocruz; 2003. p. 207-55.

3. Aguiar GM, Medeiros WM, De Marco TS, Santos SC, Gambardella S. Ecologia dos flebotomíneos da Serra do Mar, Itaguaí, Estado do Rio de Janeiro, Brasil. I. A fauna flebotomínica e prevalência pelo local e tipo de captura (Diptera, Psychodidae, Phlebotominae). Cad Saude Publica. 1996;12:195-206.

4. Aguiar GM, Medeiros WM, Santos TG, Klein AFL, Ferreira VA. Ecology of sandflies in a recent focus of cutaneous leishmaniasis in Paraty, littoral of Rio de Janeiro State (Diptera, Psychodidae, Phlebotominae). Mem Inst Oswaldo Cruz. 1993;88:339-40.

5. Aguiar GM, Vilela ML. Aspects of the ecology of sandflies at the Serra dos Órgãos National Park, State of Rio de Janeiro. VI. Shelters and breeding places (Diptera, Psychodidae, Phlebotominae). Mem Inst Oswaldo Cruz. 1987;82:585-6.

6. Andrade Filho JD, Galati EAB, Falcão AL. *Nyssomyia intermedia* (Lutz & Neiva, 1912) and *Nyssomyia neivai* (Pinto, 1926) (Diptera: Psychodidae: Phlebotominae) geographical distribution and epidemiological importance. Mem Inst Oswaldo Cruz. 2007;102:481-7.

7. Andrade Filho JD, Galati EAB, Falcão AL. Polymorphism, inter-population and inter-specific variation in *Nyssomyia intermedia* (Lutz & Neiva) and *Nyssomyia neivai* (Pinto) (Diptera, Psychodidae, Phlebotominae). Rev Bras Entomol. 2006;50:385-93.

8. Araujo Filho NA. Epidemiologia da leishmaniose tegumentar americana na Ilha Grande, Estado do Rio de Janeiro, estudo sobre a infecção humana, reservatórios e transmissores. [dissertação]. Rio de Janeiro: Faculdade de Medicina Universidade Federal do Rio de Janeiro; 1978.

9. Azeredo-Coutinho RBG, Conceição-Silva F, Schubach A, Cupolillo E, Quintella LP, Madeira MF, *et al*. First report of diffuse cutaneous leishmaniasis and *Leishmania amazonensis* infection in Rio de Janeiro State, Brazil. Trans R Soc Trop Med Hyg. 2007;101:735-7.

10. Azevedo ACR, Rangel EF. A study of sandfly species (Diptera: Psychodidae: Phlebotominae) in a focus of cutaneous leishmaniasis in the municipality of Baturité, Ceará, Brazil. Mem Inst Oswaldo Cruz. 1991;86:405-10.

11. Condino MLF, Galati EAB, Holcman MM, Salum MRB, Silva DC, Novaes Júnior RA. Leishmaniose tegumentar americana no litoral norte paulista, período 1993 a 2005. Rev Soc Bras Med Trop. 2008;4:635-42.

12. Falqueto A. Especificidade alimentar de flebotomíneos em duas áreas endêmicas de leishmaniose tegumentar no Estado do Espírito Santo. [tese]. Rio de Janeiro: Instituto Oswaldo Cruz/Fundação Oswaldo Cruz; 1995.

13. Forattini OP, Rabello EX, Serra OP, Cotrim MD, Galati EAB, Barata JMS. Observações sobre a transmissão de leishmaniose tegumentar no Estado de São Paulo, Brasil. Rev Saude Publica. 1976;10:31-43.

14. Forattini OP, Oliveira DE. Um foco de leishmaniose tegumentar na zona sul do Estado de São Paulo, Brasil. Arq Fac Hig São Paulo. 1957;11:23-4.

15. Gomes AC, Rabello EX, Santos JLF, Galati EAB. Aspectos ecológicos da leishmaniose tegumentar americana. I. Estudo experimental da frequência de flebotomíneos a ecótopos artificiais com frequência especial a *Psychodopygus intermedius.* Rev Saude Publica. 1980;14:540-56.

16. Gontijo CMF, Melo MN. Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. Rev Bras Epidemiol. 2004;7:338-49.

17. Haddow AJ. Studies on the biting habits and medical importance of east African mosquitoes in the genus Aedes. I. Subgenera *Aedimorphus*, *Banksinella* and *Nunnius*. Bull Entomol Res. 1960;50:759-79.

18. Haddow AJ. Studies on the biting habits of African mosquitoes: an appraisal of methods employed, with special reference to the twenty-four-hour catch. Bull Entomol Res. 1954;45:199-242.

19. Lainson R, Shaw JJ. New world leishmaniasis. The Neotropical *Leishmania* species. In: Cox FEG, Kreier JP, Wakelin D, editors. Topley & Wilson's microbiology and microbial infections. 9th ed. London: Hodder Arnold; 1998. vol. 5. Parasitology. p. 242-66.

20. Marcondes CB, Lozovei AL, Vilela JH. Distribuição geográfica de flebotomíneos do complexo *Lutzomyia intermedia* (Lutz & Neiva, 1912) (Diptera, Psychodidae). Rev Soc Bras Med Trop. 1998,31:51-8.

21. Marcondes CB. A redescription of *Lutzomyia* (*Nyssomyia*) *intermedia* (Lutz & Neiva, 1912), and ressurection of *L. neivai* (Pinto, 1926) (Diptera, Psychodidae, Phlebotominae). Mem Inst Oswaldo Cruz. 1996;91:457-62.

22. Marzochi MCA, Souza WJS, Coutinho SG, Toledo LM, Grimaldi Filho G, Momen H. Evolution of diagnostic criteria in human and canine mucocutaneous leishmaniasis in a Rio de Janeiro district where *Leishmania braziliensis braziliensis* occurs. In: 9º Reunião Anual de Pesquisa Básica em Doença de Chagas; 1982 nov 8-10; Caxambu. Abstract 46. p. 63.

23. Menezes CRV, Azevedo ACR, Costa SM, Costa WA, Rangel EF. Ecology of American cutaneous leishmaniasis in the State of Rio de Janeiro, Brazil. J Vector Ecol. 2002;27:207-14.

24. Nimer E. Climatologia da Região Sudeste: introdução à climatologia dinâmica. Rev Bras Geogr. 1972;34:3-48.

25. Pita-Pereira D, Souza GD, Pereira TA, Zwetsch A, Britto C, Rangel EF. *Lutzomyia* (*Pintomyia*) *fischeri* (Diptera: Psychodidae: Phlebotominae), a probable vector of American cutaneous leishmaniasis: detection of natural infection by *Leishmania* (*Viannia*) DNA in specimens from the municipality of Porto Alegre (RS), Brazil, using multiplex PCR assay. Acta Trop. 2011;120:273-5.

26. Prefeitura de Angra dos Reis [homepage on the internet]. História do município [cited: 2012 Mar 16]. Available from: http://www.angra.rj.gov.br/asp/municipio/muni_historia.asp

27. 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.

28. Rangel EF, Lainson R. Ecologia das leishmanioses: transmissores de leishmaniose tegumentar americana. In: Rangel EF, Lainson R, editores. Flebotomíneos do Brasil. Rio de Janeiro: Fiocruz; 2003. p. 291-310.

29. Rangel EF, Barbosa AF, Andrade CA, Sousa NA, Wermelinger ED. Development of *Leishmania (Viannia) braziliensis* (Vianna, 1911) in *Lutzomyia intermedia* (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) under experimental conditions. Mem Inst Oswaldo Cruz. 1992;87:235-8.

30. Rangel EF, Azevedo AC, Andrade CA, Souza NA, Wermelinger ED. Studies on sandfly fauna (Diptera: Psychodidae) in a foci of cutaneous leshmaniasis in Mesquita, Rio de Janeiro State, Brazil. Mem Inst Oswaldo Cruz. 1990;85:39-45.

31. Secretaria de Vigilância em Saúde. Ministério da Saúde. Manual de vigilância da leishmaniose tegumentar americana. 2nd ed. Brasília: Ministério da Saúde; 2007.

32. Souza NA, Andrade-Coelho CA, Vilela ML, Peixoto AA, Rangel EF. Seasonality of *Lutzomyia intermedia* and *Lutzomyia whitmani* (Diptera: Psychodidae: Phlebotominae), occurring sympatrically in area of cutaneous leishmaniasis in the State of Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2002;97:759-65.

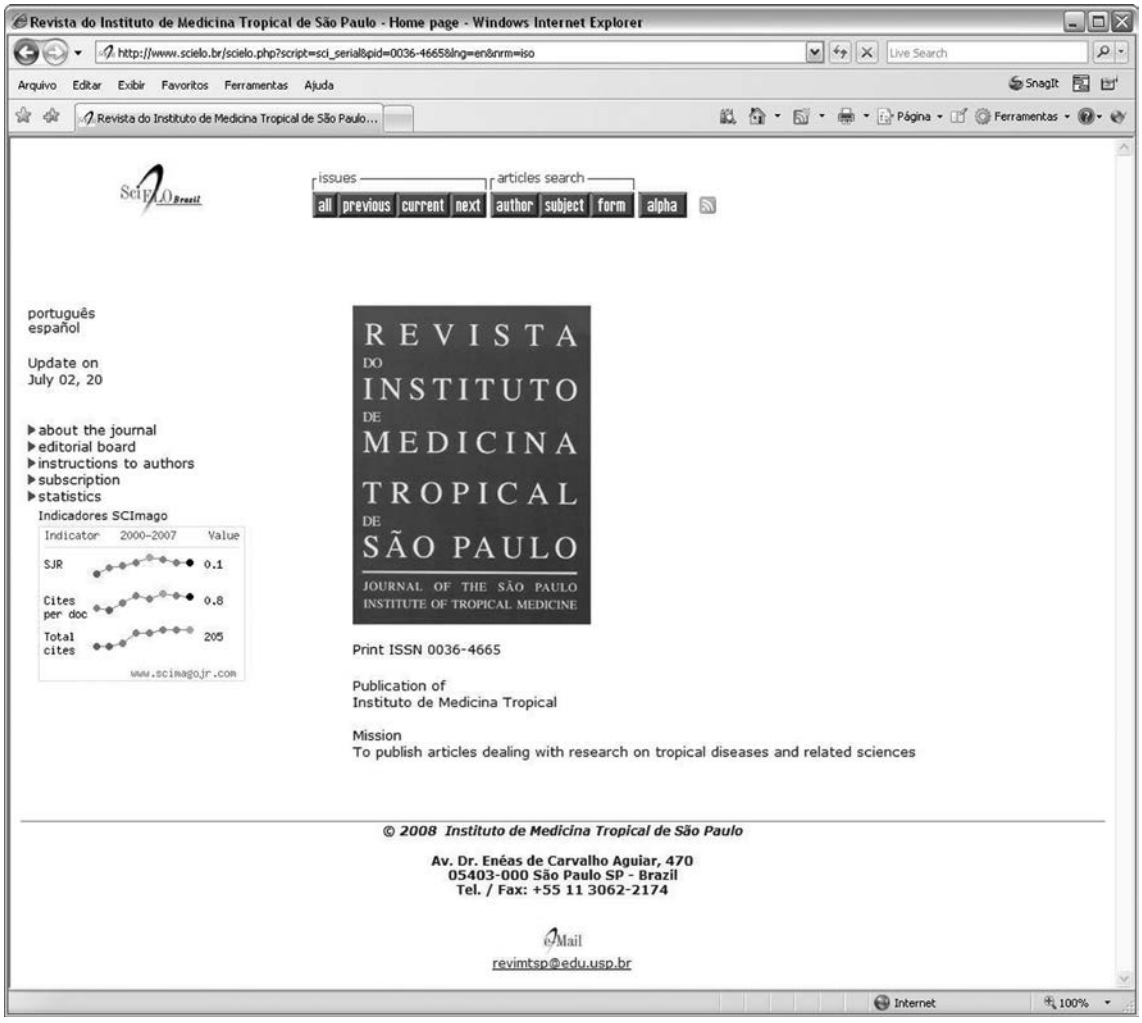
33. Souza NA, Andrade-Coelho CA, Vilela ML, Rangel EF. The phlebotominae sand fly (Diptera: Psychodidae) fauna of two Atlantic rain forest reserves in the State of Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2001;96:319-24.

34. 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). Gainesville: Associated Publishers American Entomological Institute; 1994. (Mem. Amer. Entomol. Inst. no 54). Available from: http://www.dtic.mil/cgi-bin/GetTRDoc?Location=U2&doc=GetTRDoc.pdf&AD=ADA285737

Received: 1 March 2013
Accepted: 20 August 2013

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(2):151-155, March-April, 2014
doi: 10.1590/S0036-46652014000200011

TEMPORAL TRENDS IN THE DETECTION RATES OF HEPATITIS B IN THE SANTA CATARINA STATE, BRAZIL

Chaiana Esmeraldino Mendes MARCON(1), Ione Jayce Ceola SCHNEIDER(2) & Jefferson TRAEBERT(3)

SUMMARY

Hepatitis B is a serious public health problem. The state of Santa Catarina presents areas of high endemicity. The aim of this study was to describe temporal trends in detection rates of hepatitis B in the period from 2002 to 2009 in Santa Catarina and in its regions. A time series study was carried out. Crude rates were calculated and standardized by age using the direct method. Annual variation percentages were estimated by Joinpoint regression. There were two distinct and significant trends in Santa Catarina. From 2002 to 2006 a significant increase of 5.9% per year was observed. From 2006, there was a significant decrease of 6.4% per year. In this same period the southern and far-western regions had significant increases of 15.9% and 4.6% and significant decreases of 7.5% and 4.8%, respectively. Greater Florianópolis and Northeast also showed significant increases until 2006, of 15.4% and 17.4%, respectively. In the following period, non-significant decreases of 5.8% and 9.8% respectively were observed. Foz do Rio Itajaí and Planalto Serrano showed non-significant increases up to half of the studied period of 21.1% and 12.0%, respectively and after, significant decreases of 21.5% and 18.0%, respectively. Vale do Itajaí showed a significant decrease of 9.7%; Planalto Norte showed a non-significant decrease of 0.6% and Midwest a non-significant increase of 2.7% per year, in the period from 2002 to 2009.

KEYWORDS: Hepatitis B; Epidemiology; Temporal distribution.

INTRODUCTION

Hepatitis B is considered a serious public health problem all over the world. The disease affects both genders, but infections in females require special attention due to the possibility of vertical transmission. The presence of HBV in the semen and in vaginal secretion means that transmission is enabled by sexual intercourse. The contamination also may occur by sharing syringes, occupational exposure, contaminated blood transfusion and through family life habits[3,12,13].

Approximately two billion people are contaminated by HBV and 350 million, or 5% of the world population, have developed chronic forms of hepatitis B[19]. According to the World Health Organization (WHO), China and the rest of Asia are areas of high endemicity. The Amazon Region and Central Europe have high rates of chronic disease. The WHO estimates that 2% to 5% of the general population of the Middle East and the Indian Subcontinent are chronically infected with hepatitis B[19]. The Brazilian Health Ministry estimates that 15% of the Brazilian population has already had contact with HBV and about 1% of the population has developed a chronic form of the disease[10,11]. A greater prevalence is found in the Northern region, however studies performed in 1980 and 1990 showed a growing trend in the South, in the Amazonian Region and in some towns from Espírito Santo[5].

A population-based cross-sectional study carried out in 26 of the Brazilian state capitals and in the Federal District between 2005 and 2009 showed 7.4% seropositivity for anti-HBc infection in the age group 10-69 years old, indicating low endemicity. The region had a prevalence of 9.59% and a higher detection rate in 2010, that reached 14.3/100 thousand inhabitants; Florianópolis was the capital with the highest detection rate in the region with 21.1/100 thousand inhabitants[4]. The west region of Santa Catarina state presents high endemicity[5,7] mainly in the regions of Chapecó, São Miguel do Oeste and Concórdia where the detection rates have varied between 67.77/100 thousand habitants in 2006 and 54.81/100 thousand habitants in 2010[17].

The aim of this study was to describe the temporal trend of detection rates of hepatitis B in the period from 2002 to 2009 in the state of Santa Catarina and in its health regions.

MATERIAL AND METHODS

A time series study was carried out using notified cases of hepatitis B in the state of Santa Catarina from 2002 to 2009. Notification data was obtained through the Brazilian National Case Registry Database of Brazilian Health Ministry.

(1) Master of Science Program in Health Sciences. Universidade do Sul de Santa Catarina, Tubarão/SC, Brazil.
(2) School of Medicine. Universidade do Sul de Santa Catarina, Palhoça/SC, Brazil.
(3) Master of Science Program in Health Sciences and School of Medicine. Universidade do Sul de Santa Catarina, Tubarão/SC, Brazil.
Correspondence to: Chaiana E. M. Marcon, Av. José Acácio Moreira 787, Dehon, Tubarão, Santa Catarina. E-mail: chaianaa@hotmail.com

Estimates regarding the resident population of the state and its health regions were obtained from the censuses or inter-censuses dataset and were used as denominators for the detection rate calculation. Initially, crude rates of notification of hepatitis B were calculated. Next, they were standardized by age using the direct method and the population of Santa Catarina in 2010 as the standard.

The standard rates were used in the trend analysis through an estimation of regression models. For the modeling process, standard rates (y) were the dependent variable, and the years of the period in the study were the independent variable (x).

After analyzing the historical series and the oscillation due to the small number of cases in some periods, the decision was made to calculate the moving mean based on three terms. During this process, the coefficient of each year was determined by the arithmetic mean of coefficients of the previous year, of the year itself and of the next year. This resulted in the presentation of data of years from 2002 to 2009, although data from 2001 to 2010 had been used.

The software Joinpoint (Statistical Research and Applications Branch, National Cancer Institute, Rockville, MD, USA) version 3.5.4 was used, to compute the annual variation of notifications in the period from 2002 to 2009. The software executes a segmented linear regression (joinpoint regression) to estimate the percentage of annual variation and identify points where there is modification of a trend. The models in which a different number of trend modification "points" from 0 (case in which the trend is represented by a single line segment) were assumed and adjusted successively until the maximum number was two. The model chosen was the one with the highest number of points in which the statistical significance ($p < 0.05$) was maintained. The annual variation in percentage (AVP) was calculated from the estimated inclination for each line segment (regression coefficient). Its statistical significance was estimated by the method of least-squares through a generalized linear model, assuming that rates follow a distribution of Poisson, since the rates variation were not constant along the periods. For each line segment 95% confidence intervals (95% CI) were calculated.

RESULTS

In the studied period, 9,421 cases of hepatitis B were detected in Santa Catarina. The number of detected cases by health regions is shown in Table 1.

Two significant trends were observed in the state of Santa Catarina in the studied period. In the first one, from 2002 to 2006, a significant increase of 5.9% per year (95% CI 3.6; 8.3) was observed in the notification rates. From 2006, there was an inversion of the trend with a significant decrease of 6.4% per year (95% CI -9.7; -3.1). The same behavior was observed in the southern and far-western health regions. In the period as a whole, (2002 to 2009) only in the southern region a significant increase of 5.2% per year (95% CI 4.2; 6.2) was observed (Table 2 and Fig. 1).

In the health regions of North-east and Greater Florianópolis significant increases in the years from 2002 to 2006 were observed, but a decrease in rates in the subsequent period proved to be insignificant. Two distinct trends were also observed in the health region of Foz do Rio Itajaí, with a significant decrease in the period from 2004 to 2009, but with no significant statistical trend in the anterior period of study. The same occurred in the Planalto Serrano. However, the period of significant decrease was from 2005 to 2009, which was enough to result in a significant decrease of 6.3% per year (95% CI -11.9; -0.3) in the period from 2002 to 2009 (Table 2 and Fig. 1).

Table 1
Number of detected cases of hepatitis B by health regions in the 2002 to 2009 period

HEALTH REGION	DETECTED CASES
Far West	3,492
Foz do Rio Itajaí	74
Planalto Serrano	81
Northeat	508
Great Florianópolis	1,180
South	848
Planalto Norte	1,221
Mid West	1,162
Vale do Itajaí	855
Santa Catarina	9,421

In the Planalto Norte, Midwest and Vale do Itajaí health regions only one trend was observed. Nevertheless, only the latter presented a significant trend with a decrease of 9.7% per year (95% CI -12.9; -6.4) (Table 2 and Fig. 1).

DISCUSSION

It was possible to observe that the Santa Catarina State and six of its nine health regions presented distinct trends in relation to the detection of hepatitis B. The first trend was a significant increase of rates in the early years of study, occurred in the state as a whole and in the far-western, northeastern, Greater Florianópolis and southern regions. In the southern region, the increase in the period from 2002 to 2006 was of a great enough magnitude (15.9% per year) to generate a significant increase in the period of study of 5.2% per year. The second trend was a significant decrease of rates that occurred in the state as a whole, and in Foz do Rio Itajaí, Planalto Serrano and far-western and southern regions. It is worth highlighting the fact that the regions of Foz do Rio Itajaí and Planalto Serrano had great increases in annual variations (21.1% and 12.0%, respectively) in the first period of the study, but such increases were not statistically significant. This fact may have been influenced by the small number of reported cases in these regions (74 and 81, respectively). The magnitude of decrease in the Planalto Serrano from 2005 to 2009 (18.0% per year) was enough to generate a significant decrease of 6.3% per year in the whole period of study. In the Vale do Rio Itajaí a single trend of decrease of 9.7% per year was observed.

Although this study does not have an explanatory methodological design, it is possible to deduce that the decrease in the rates of detection could be a reflection of programs of immunization, as well as more effective, vigilant actions towards the prevention of hepatitis B. These

Table 2
Variation of annual percentage in the period from 2002 to 2009 of detection rates of hepatitis B in Santa Catarina and in its health regions

State and health regions	Period	Variation of annual percentage (IC 95%)	Variation of percentage from 2002 to 2009 (IC 95%)
Santa Catarina	2002 to 2006	5.9* (3.6; 8.3)	0.4 (-0.8; 1.6)
	2006 to 2009	-6.4* (-9.7; -3.1)	
Far West	2002 to 2006	4.6* (2.1; 7.2)	0.5 (-0.8; 1.8)
	2006 to 2009	-4.8* (-8.3; -1.1)	
Foz do Rio Itajaí	2002 to 2004	21.1 (-33.1; 119.0)	-11.2 (-21.8; 0.9)
	2004 to 2009	-21.5* (-33.5; -7.3)	
Planalto Serrano	2002 to 2005	12.0 (-5.4; 32.7)	-6.3* (-11.9; -0.3)
	2005 to 2009	-18.0* (-27.3; -7.6)	
Northeast	2002 to 2006	17.4* (6.0; 30.1)	4.9 (-0.6; 10.7)
	2006 to 2009	-9.8 (-22.3; 4.8)	
Great Florianópolis	2002 to 2006	15.4* (2.2; 30.4)	5.8 (-0.6; 12.6)
	2006 to 2009	-5.8 (-20.8; 12.0)	
South	2002 to 2006	15.9* (13.8; 17.9)	5.2* (4.2; 6.2)
	2006 to 2009	-7.5* (-9.8; -5.2)	
Planalto Norte	2002 to 2009	-0.6 (-2.1; 0.8)	-0.6 (-2.1; 0.8)
Mid West	2002 to 2009	2.7 (-3.1; 8.7)	2.7 (-3.1; 8.7)
Vale do Itajaí	2002 to 2009	-9.7* (-12.9; -6.4)	-9.7* (-12.9; -6.4)

* *p*- value < 0.05.

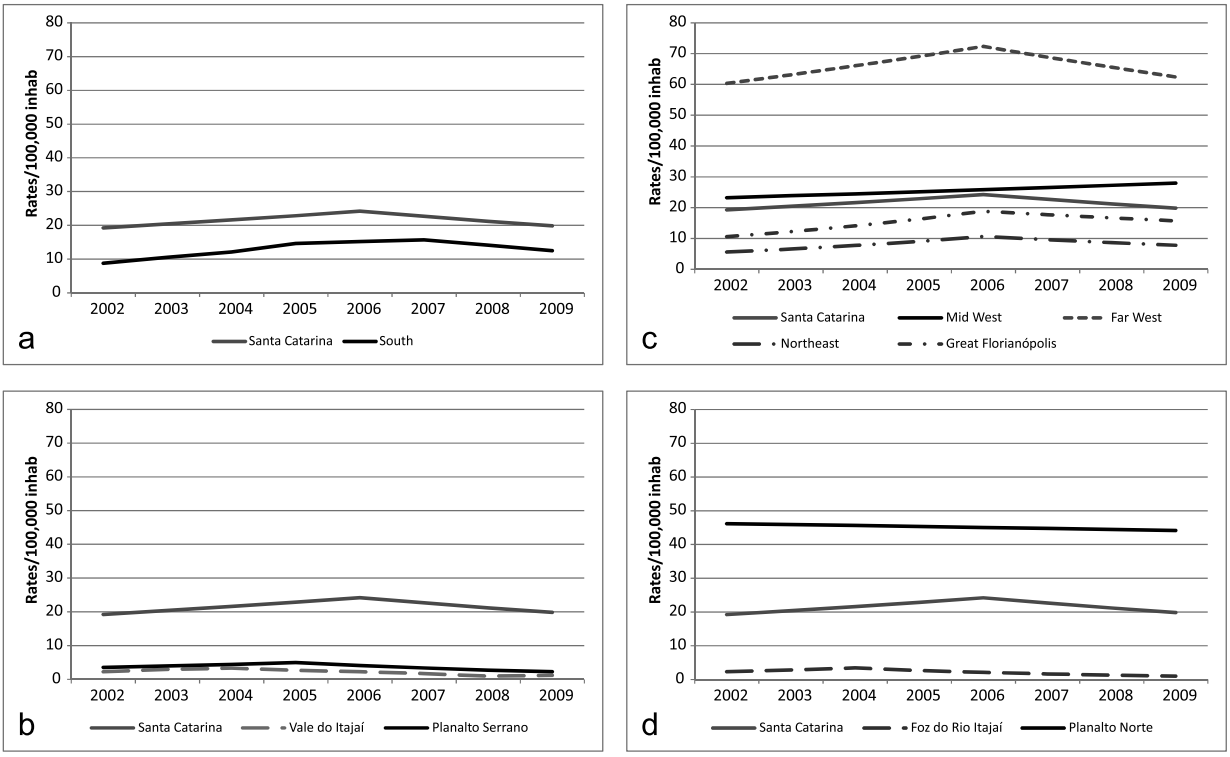


Fig. 1 - Trend of incidence rates/100 thousand inhabitants by hepatitis B in the period from 2002 to 2009 in *Santa Catarina* and in its health regions. a. Health region with significant increase; b. Health region with significant decrease; c. Health region with no significant increase; d. Health region with no significant decrease.

aspects provide a plausible hypothesis that explains the growing trend of rates of notification in early years of study, followed by a decreasing trend. This fact has been observed in many countries. A significant decrease of infection by hepatitis B was attributed to children who have been vaccinated since 1991 in Egypt[20] and since 1993 in Pakistan[1] and in Iran for all neonates and high-risk groups[2]. Data reported in Italian studies indicated that the implementation of the vaccination had a great impact on the control and prevention of hepatitis B in Italy[8,9,16]. There was an overall decline in the HBsAg positivity rate and an overall increase in the population immunity to hepatitis B in Singapore after the implementation of the national childhood immunization programme[14]. The universal implementation of vaccination against the hepatitis B virus at birth has reduced its occurrence in adolescents and young adults in Taiwan[18].

The immunization against HBV aims to prevent the disease and to avoid the chronification and evolution of liver cirrhosis and hepatocellular carcinoma. In Brazil the inclusion of the vaccination against hepatitis B in the public vaccination schedule has developed gradually since 1989. Due to the high prevalence of hepatitis B in western Amazonia, the vaccination campaigns against hepatitis B were introduced in the municipalities of Purus, Boca do Acre and Lábrea. In 1991 the vaccine became part of the public vaccination schedule in the state of Amazonas. In 1992 it was made available in the states of the Legal Amazon, Paraná, Espírito Santo, Santa Catarina and the Federal District for children younger than five years old. In 1994 the vaccine was made available to healthcare professionals from the private sector, firefighters, police, military and students of medicine, dentistry, nursing and biochemistry. In 1998 the vaccine was made available to all children in the country who were less than one year old. In 2001, the vaccine was made available to people who were under 20 years old. In 2011 the age increased to 20 to 24 years. In 2012 the vaccine was made available to people aged 25-29 years and in 2013 the vaccine was extended to the age group of 30-49 years old and also for individuals over 50[6].

A study carried out by the Brazilian Ministry of Health discovered that the number of confirmed cases in the country in 1996 was 8,512, with a vaccine covering 13% of children who were under one year old. In 2002, the number of confirmed cases decreased to 3,160 with a vaccine covering 89%[6]. In Santa Catarina, a study carried out in Itajaí demonstrated that the vaccine covering in adolescents was of 97.5% and the prevalence of HBsAg was of 0.6%[9].

Among different effective measures, the prevention of vertical transmission of HBV should be an important aim because the proportion of chronification is very high when hepatitis B is acquired in early years of life. In a study carried out in the state of São Paulo it was observed that the administration of immunoglobulin in neonates, often does not occur in the stipulated period of 12 hours after birth, which is a great failure in the duty of care of the newborn[15].

The significant increase in the detection rates in the health region of the South highlights the importance of more effective preventative measures. More effective measures with regards to immunization should be put in place: a reduction in substance abuse, an education on the importance of the practice of safe sex, better care should be taken during the administration of blood transfusions, adequate care should be provided for babies born from HBV-infected mothers and informative campaigns about the disease should be developed. These actions should also aim to establish a wider vaccine covering, especially in adolescents and adults.

The results of this descriptive study should be viewed with caution since its design does not allow for cause-effect relationship, but rather describes the rates of detection of hepatitis B during a limited period of years. It can be concluded that the state of Santa Catarina presented two significant trends in the studied period. The first, from 2002 to 2006, was that there was a significant increase in the detection rate of 5.9% per year. From 2006 there was an inversion of the trend, with a significant decrease of 6.4% per year. This same pattern was observed in the southern and far-western health regions but with different rates. The health regions of Greater Florianópolis and the North-east also showed significant increases until 2006, but after that year, the reversal of trends was not significant. The health regions of Foz do Rio Itajaí and Planalto Serrano showed no significant increases up to the half of the studied period, with significant trend reversals. Three health regions showed a single trend. The Vale do Itajaí showed a significant decrease, the Planalto Norte had a non-significant decline and the Midwest had a non-significant increase.

RESUMO

Tendência temporal da taxa de detecção de hepatite B no estado de Santa Catarina, Brasil

A hepatite B é um grave problema de saúde pública. O estado de Santa Catarina apresenta áreas de alta endemicidade. O objetivo deste estudo foi descrever a tendência temporal da taxa de detecção de hepatite B no período de 2002 a 2009 em Santa Catarina e em suas macrorregiões. Foi realizado um estudo de séries temporais com dados de notificação. As taxas brutas foram calculadas e padronizadas por idade pelo método direto. Estimou-se a variação anual por intermédio de regressão linear segmentada. O estado apresentou duas tendências significativas distintas. De 2002 a 2006, observou-se aumento significativo de 5,9% ao ano. A partir de 2006, queda significativa de 6,4% ao ano. Também nesses períodos, as macrorregiões Sul e Extremo-Oeste apresentaram aumentos significativos de 15,9% e 4,6% e quedas significativas de 7,5% e 4,8%, respectivamente. A Grande Florianópolis e o Nordeste também apresentaram aumentos significativos até 2006, de 15,4% e 17,4% respectivamente. No período seguinte, ocorreram quedas não significativas de 5,8% e 9,8%, respectivamente. A Foz do Rio Itajaí e o Planalto Serrrano apresentaram aumentos não significativos até a metade do período estudado de 21,1% e 12,0%, respectivamente, e depois, quedas significativas de 21,5% e 18,0%, respectivamente. O Vale do Itajaí mostrou queda significativa de 9,7%; o Planalto Norte, queda não significativa de 0,6% e o Meio-Oeste, aumento não significativo de 2,7% por ano, no período de 2002 a 2009.

REFERENCES

1. Ahmed W, Qureshi H, Arif A, Alam SE. Changing trend of viral hepatitis. A twenty one year report from Pakistan Medical Research Council Research Centre, Jinnah Postgraduate Medical Centre, Karachi. J Pak Med Assoc. 2010;60:86-9.

2. Alavian SM, Fallahian F, Lankarani KB. The changing epidemiology of viral hepatites B in Iran. J Gastrointestin Liver Dis. 2007;16:403-6.

3. Aquino JA, Pegado KA, Barros LP, Machado LFA. Soroprevalência de infecções por vírus da hepatite B e vírus da hepatite C em indivíduos do estado do Pará. Rev Soc Bras Med Trop. 2008;4:334-7.

4. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de DST, Aids e Hepatites Virais. Boletim Epidemiológico. Brasília, 2012. [cited: 2013 Jun 14]. Available from: http://www.aids.gov.br/sites/default/files/anexos/publicacao/2012/51820/boletim_epidemiol_gico_hepatites_virais_2012_ve_12026.pdf

5. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Caderno 6. 7 ed. Brasília: Ministério da Saúde; 2005.

6. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância das Doenças Transmissíveis. Nota Técnica Conjunta N 02/2013/ CGPNI/ DEVEP e CGDHRV/DST- AIDS/SVS/MS. Brasília: Ministério da Saúde; 2013.

7. Chávez JH, Campana SG, Haas P. Panorama da hepatite B no Brasil e no estado de Santa Catarina. Rev Panam Salud Publica. 2003;14:91-6.

8. Da Villa G, Romanò L, Sepe A, Iorio R, Paribello N, Zappa A, *et al.* Impact of hepatitis B vaccination in a highly endemic area of south Italy and long-term duration of anti-HBs antibody in two cohorts of vaccinated individuals. Vaccine. 2007;25:3133-6.

9. De Paschale M, Manco MT, Belvisi L, Brando B, Latella S, Agrappi C, *et al*. Prevalence of markers of hepatitis B virus infection or vaccination in HBsAg-negative subjects. Blood Transfus. 2012;10:344-50.

10. Ferreira CT, Silveira TR. Hepatites virais: aspectos da epidemiologia e da prevenção. Rev Bras Epidemiol. 2004;7:473-87.

11. Ferreira CT, Silveira TR. Viral hepatitis prevention by immunization. J Pediatr (Rio J). 2006;82(3 Suppl):S55-66.

12. Ferreira MS. Diagnóstico e tratamento da hepatite B. Rev Soc Bras Med Trop. 2000;33:389-400.

13. Figueiredo NC, Page-Shafer K, Pereira FEL, Miranda AE. Marcadores sorológicos do vírus da hepatite B em mulheres jovens atendidas pelo Programa de Saúde da Família em Vitória, estado do Espírito Santo, 2006. Rev Soc Bras Med Trop. 2008;41:590-5.

14. Hong WW, Ang LW, Cutter J, James L, Chew SK, Goh KT. Changing seroprevalence of hepatitis B virus markers of adults in Singapore. Ann Acad Med Singapore. 2010;39:591-8.

15. Perim EB, Passos ADC. Hepatite B em gestantes atendidas pelo Programa do Pré- Natal da secretaria municipal de saúde de Ribeirão Preto, Brasil: prevalência da infecção e cuidados prestados aos recém- nascidos. Rev Bras Epidemiol. 2005;8:272-81.

16. Romanò L, Paladini S, Tagliacarne C, Zappa A, Zanetti AR. The changing face of the epidemiology of type A, B, and D viral hepatitis in Italy, following the implementation of vaccination. Vaccine. 2009;27:3439-42.

17. Santa Catarina. Secretaria de Estado da Saúde. Plano Estadual de Saúde 2012-2015. Florianópolis, 2011. [cited: 2013 Jun 14]. Available from: http://www.saude. sc.gov.br/materiais/ PES_2012_CES.pdf

18. Su W, Liu C, Liu D, Chen S, Huang J, Chan M. Effect of age on the incidence of acute hepatitis B after 25 years of universal newborn hepatitis B immunization program in Taiwan. J Infect Dis. 2012;205:757-61.

19. World Health Organization. Hepatite B. Geneva: World Health Organization; 2012. Available from: www.who.int/mediacenter /factsheets/fs204/ES/

20. Zakaria S, Fouad R, Shaker O, Zaki S, Hashem A, El-Kamary SS, *et al*. Changing patterns of acute viral hepatitis at a major urban referral Center in Egypt. Clin Infect Dis. 2007;44:e30-6.

Received: 15 April 2013
Accepted: 14 August 2013

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(2):157-163, March-April, 2014
doi: 10.1590/S0036-46652014000200012

OCCUPATIONAL EXPOSURES TO BODY FLUIDS AND BEHAVIORS REGARDING THEIR PREVENTION AND POST-EXPOSURE AMONG MEDICAL AND NURSING STUDENTS AT A BRAZILIAN PUBLIC UNIVERSITY

Fernanda Ribeiro Fagundes de SOUZA-BORGES(1), Larissa Araújo RIBEIRO(2) & Luiz Carlos Marques de OLIVEIRA(1,3)

SUMMARY

A cross-sectional study was conducted to assess the frequencies and characteristics of occupational exposures among medical and nursing students at a Brazilian public university, in addition to their prevention and post-exposure behavior. During the second semester of 2010, a self-administered semi-structured questionnaire was completed by 253/320 (79.1%) medical students of the clinical course and 149/200 (74.5%) nursing students who were already performing practical activities. Among medical students, 53 (20.9%) suffered 73 injuries, which mainly occurred while performing extra-curricular activities (32.9%), with cutting and piercing objects (56.2%), in the emergency room (39.7%), and as a result of lack of technical preparation or distraction (54.8%). Among nursing students, 27 (18.1%) suffered 37 injuries, which mainly occurred with hollow needles (67.6%) in the operating room or wards (72.2%), and as a result of lack of technical preparation or distraction (62.1%). Among medical and nursing students, respectively, 96.4% and 48% were dissatisfied with the instructions on previously received exposure prevention; 48% and 18% did not always use personal protective equipment; 67.6% and 16.8% recapped used needles; 49.3% and 35.1% did not bother to find out the source patient's serological results post-exposure; and 1.4% and 18.9% officially reported injuries. In conclusion, this study found high frequencies of exposures among the assessed students, inadequate practices in prevention and post-exposure, and, consequently, the need for training in "standard precautions" to prevent such exposures.

KEYWORDS: Occupational exposure; Medical students; Nursing students; Needlestick injuries; Sharp injuries.

INTRODUCTION

While performing their activities, healthcare workers (HCWs) are at risk of occupational exposures, which can be caused by needle stick injuries (NSIs), sharp object injuries and body fluids being in contact with mucous membranes or non-intact skin. In the United States, it is estimated that 503,000 cutting and piercing injuries (385,000 of which were in hospitals) and 146,000 mucocutaneous exposures occurred among HCWs in 2000[21]. In addition, among these professionals, it is estimated that 100,000 sharp injuries (SIs) occur in the United Kingdom[39] and 500,000 NSIs occur in Germany hospitals[28] every year, where nearly 160 HCWs retire annually as a result of infections acquired through occupational transmission[8]. However, it is estimated that more than 90% of occupational exposures which occur worldwide occur in developing countries[49].

Approximately 60 pathogens can be transmitted through occupational exposures, including viruses, bacteria, parasites and yeasts, and hepatitis B (HBV), hepatitis C (HCV) and human immunodeficiency (HIV) viruses account for the majority of cases worldwide[38]. After parenteral exposure with a contaminated needle or sharp object infected with HIV, HCV or HBV, the risk of infection is 0.3%, 1.8% and approximately 30%, respectively, among susceptible individuals[5]. It was estimated that, as a result of occupational exposures, 16,000 HCV infections, 66,000 HBV infections and 1,000 HIV infections occurred worldwide in 2000[23]. Due to the risk of acquiring infections that can be transmitted by body fluids, the occupational exposures can result in social stigmas[30], in addition to psychological stress, with negative repercussions on family, social and professional relationships.

Medical and nursing students develop their skills by performing procedures in which they need to handle cutting and piercing objects with possible accidental contact with body fluids. In some studies, it was reported that medical[28] and nursing students[47] have higher risks of suffering occupational exposures than graduate professionals. There are several factors that can contribute to this greater vulnerability among students, such as lack of experience, skills or knowledge about how to handle certain instruments; anxiety; tiredness; lack of tutorial support; lack of care of oneself or other professionals, among others[24,25,27,30].

The frequencies of the occupational exposures among medical

(1) Postgraduate Program in Health Sciences of the Federal University of Uberlândia Medical School (FAMED/UFU). Uberlândia, MG, Brazil.
(2) Undergraduate medical student of the FAMED/UFU. Uberlândia, MG, Brazil.
(3) Associate professor of the Department of Internal Medicine, FAMED/UFU. Uberlândia, MG, Brazil.
Correspondence to: Dr. Luiz Carlos Marques de Oliveira, Departamento de Clínica Médica, Faculdade de Medicina da Universidade Federal de Uberlândia. Av. Pará 1720, Bloco 2H, Campus Umuarama, 38405-320 Uberlândia, MG, Brasil. Phone/fax: +55.34.3218-2246. E-mail: oliveiralcm@ufu.br

and nursing students described in several studies vary according to the geographical area of the world where the study was conducted and according to the methodology used during data collection. Some studies took into consideration all injuries occurring among clinical course students, whereas others only considered the injuries that occurred in the last year or last semester of the undergraduate course or only those that had been officially reported. Additionally, certain studies took into consideration only NSIs and/or SIs. Thus, frequencies of occupational exposures varied between 11% and 74.4% among medical students[1,6,8,15,16,20,26-29,31,42,43,45] and between 8.8% and 61.5% among nursing students[7,10,32,34,37,41,46,47,49].

In Brazil, there are few studies that assess the frequencies and characteristics of occupational exposures among medical[24] and nursing students[3,17,25]; in some studies, the officially reported exposures were evaluated[33,35]. Knowing the characteristics of such injuries can contribute to the implementation of strategies for their prevention. The present study aimed to assess the following among medical and nursing students of a Brazilian public university: 1) the frequencies of occupational exposures; 2) some characteristics of these exposures and the post-exposure actions taken 3) whether the students had received appropriate orientation about their behavior in relation to the prevention of such exposures; and 4) whether they had knowledge about the major risks involved in these exposures and the post-exposure actions to be taken.

MATERIALS AND METHODS

Setting: This cross-sectional study was performed at the end of the second semester of 2010, in the *Faculdade de Medicina da Universidade Federal de Uberlândia* (FAMED/UFU), in the city of Uberlândia, state of Minas Gerais, Southeastern Brazil. The FAMED/UFU offers three courses: medicine, nursing and nutrition. The curriculum of the undergraduate medical course is comprised of 12 semesters, the first four of which are pre-clinical and the other eight are clinical. The internship corresponds to the period between the 10th and 12th semesters. Medical students can also participate in extracurricular training programs, which are recognized by the FAMED/UFU and where practical activities are developed. The curriculum of the undergraduate nursing course was comprised of eight semesters at the time of data collection; in the following year, two more semesters were included. From the 4th semester on, nursing students perform practical activities, and the period between the 6th and 8th semesters corresponded to the curricular training program, which mainly included practical activities. Medical and nursing students enrolled in the course semesters, described in the present study, perform practical activities in the different levels of the Health Care Network, including the Clinical Hospital of the Federal University of Uberlândia (HC-UFU in the Portuguese acronym).

Subjects and data collection: During the second semester of 2010, there were 320 undergraduate medical students enrolled in the clinical period of their course and 200 nursing students enrolled in the 4th semester or higher, and all of them were invited to participate in this study. During these periods, medical and nursing students are susceptible to the occurrence of occupational exposures, as they participate in practical activities where they handle cutting and piercing objects or where they may be in contact with body fluids. Students were approached in classrooms or at the HC-UFU, between activities.

First, the study objectives were explained and participants' anonymity was guaranteed. Next, a self-administered semi-structured questionnaire was given to each student, including 26 questions to collect socio-demographic data (sex, age, course, semester of enrollment) and several characteristics of possible occupational exposures. The students were asked to report any occupational exposure that had occurred since the beginning of their course. With regard to potentially contaminating injuries, the following data was collected: their frequencies, the material involved (cutting and/or piercing injuries with used hollow needles, suture needles or sharp material, or body fluids being in contact with mucous membranes), affected body area, whether the student was performing or assisting the procedure, hospital site where the injury occurred, frequency of use of personal protective equipment (PPE) and whether there was tutorial support at the moment of exposure.

In addition, data were collected about the reasons attributed to the occurrence of exposures, and on the actions taken after the exposure, such as informing a preceptor and reporting to the Specialized Safety Engineering and Occupational Medicine Service of the HC-UFU (SESMT in the Portuguese acronym). Also, they were asked whether post-exposure the student's and patient's serological tests for HBV, HCV and HIV were performed, whether the students bothered to find out the results of such tests, whether the tests of any source patient was positive for some of these viruses (yes or no), and whether chemoprophylaxis was necessary.

All the students were also asked whether previous instructions on occupational exposure prevention had been satisfactorily received, in addition to instructions on how to act in case of any exposure, what viruses can be transmitted through such injuries and the actions to be taken in case of contact with body fluids from patients infected with these viruses. With regard to knowledge about actions to be taken after biological exposure, knowledge about reporting the injury, about the need for the patient's and student's serological tests, and about the possible need for chemoprophylaxis was taken into consideration.

Data analysis: The information collected was tabulated and stored in the database of the Software Statistical Package for Social Sciences (SPSS version 17.0, IBM Inc., Chicago, IL, USA, 2008). A descriptive analysis was performed for all study variables and the odds ratio with its 95% confidence interval was calculated to compare the frequency of exposures between sexes. p-values < 0.05 were considered to be significant.

Ethical issues: The present research project was approved by the Institutional Human Research Ethics Committee, official expert opinion 330/10, and a written consent was obtained from each student participating in this study.

RESULTS

Medical students: A total of 253/320 (79.1%) medical students participated in the present study, of which 104 (41.1%) were males and 149 (58.9%) were females, with a mean age (standard deviation) of 23.4 ± 3.1 years (19 to 35 years). Among the 253 medical students, 53 (20.9%) suffered 73 biological exposures, i.e. 35 suffered one injury each, 16 suffered two injuries, and two suffered three injuries. Among students enrolled in each academic semester, the following reported at least one exposure: 0/28 students of the 5th semester, 0/29 students of the

6th semester, 2/30 (6.7%) students of the 7th semester, 5/34 (14.7%) of the 8th semester, 13/39 (33.3%) of the 9th semester, 10/30 (33.3%) of the 10th semester, 13/33 (39.4%) of the 11th semester, and 10/30 (33.3%) of the 12th semester. The frequencies of such injuries were similar (OR = 1.0; 95%CI 0.6 to 1.9) among men [22 (21.2%)] and women [31 (20.8%)]. The frequencies of injuries according to academic semester of occurrence are shown in Table 1. The frequencies of cutting and/or piercing injuries and of body fluids being in contact with mucous membranes, the hospital sites of occurrence and the reasons attributed to the occurrence of exposures are listed in Table 2.

Table 1
Frequency of occupational exposures among medical (N = 73) and nursing (N = 37) students according to academic semester of occurrence

Semester of occurrence	Occupational exposures			
	Medical students		Nursing students	
	N	%	N	%
4th	NA		6	16.2
5th	1	1.4	10	27.0
6th	4	5.5	8	21.6
7th	24	32.9	2	5.4
8th	9	12.3	3	8.1
9th	3	4.1	NA	
10th	19	26.0	NA	
11th	12	16.4	NA	
12th	1	1.4	NA	
Others[a]	0		8	21.6
Total	73	100	37	100

N (%): number and percentage. [a] Three (8.1%) injuries occurred in the 1st semester and five (13.5%) in the 3rd semester. NA = not applicable.

Occupational exposures occurred more frequently while students performed procedures [62 (84.9%)] than while they assisted/observed procedures [11 (15.1%)]; additionally, these injuries were more frequent when students had tutorial support [46 (63%) *vs.* 27 (37%)]. The body parts affected were the hands [42 (57.5%)] and the ocular and oral mucous membranes [26 (35.6%)], whereas five injuries (6.8%) were associated with other unspecified parts. Students were not wearing gloves in 8/73 (11%) injuries.

A total of 55 injuries (75.3%) were reported to a preceptor and one (1.4%) was also reported to the SESMT. In 12 out of the 18 (24.7%) exposures that were not reported to the preceptor or SESMT, students informed the reasons for not reporting: little or no perception of risk [6 (50%)], belief that it was not necessary [4 (33.3%)], fear and embarrassment [1 (8.3%)], and lack of orientation about reporting [1 (8.3%)]; this information could not be obtained from four exposures. Students had post-exposure serological tests in 36 (49.3%) injuries. Among the 73 source patients, four (5.5%) had positive serological tests for HBV, HCV or HIV, 33 (45.2%) had negative serological tests for these viruses, and students were not concerned about the results of the patients' tests in 36 (49.3%) exposures. Additionally, students underwent chemoprophylaxis in 2/73 (2.7%) exposures.

Table 2
Frequency of occupational exposures among medical (N = 73) and nursing (N = 37) students according to type of injury, site of occurrence and reasons attributed to the occurrence of exposures

Variables	Occupational exposures			
	Medical students		Nursing students	
	N	%	N	%
Cutting and piercing injuries	41	56.2	27	73.0
Suture needles	25	34.2	0	
Hollow needles	10	13.7	25	67.6
Blades	6	8.2	2	5.4
Body fluids	32	43.8	10	27.0
Blood	26	35.6	4	10.8
Others	6[a]	8.2	6[b]	16.2
Site of occurrence[c]				
Emergency room	29	39.7	2	5.6
Operating room	23	31.5	10	27.8
Obstetric center	13	17.8	0	
Medical clinic ward	1	1.4	9	25.0
Pediatric clinic ward	1	1.4	7	19.4
Others	6[d]	8.2	8[e]	22.2
Reason for exposure[f]				
Lack of technical preparation	20	27.4	7	18.9
Distraction	20	27.4	16	43.2
Tiredness	9	12.3	6	16.2
Lack of tutorial support	9	12.3	1	2.7
Lack of PPE	5	6.8	2	5.4
Lack of care from others	4	5.5	0	
Others	10[g]	13.7	6[h]	16.2

[a] Nasal secretion (n = 1), amniotic liquid (n = 1), synovial liquid (n = 1), not informed (n = 3). [b] nasal secretion (n = 4), not informed (n = 2). [c] percentages calculated considering the valid responses. [d] outpatient surgery (n = 4), meeting room (n = 1), maternity ward (n = 1). [e] Intensive care unit (n = 4); blood bank (n = 1); infectious diseases ward (n = 1), vaccination (n = 1), maternity ward (n = 1). [f] student could choose more than one alternative. [g] patient movements (n = 2); defects in the equipment (n = 1); not specified (n = 7). [h] nervousness (n = 1); haste (n = 1); peer pressure (n = 1); not specified (n = 3).

Among medical students who participated in this study, nine (3.6%) said that they had satisfactorily received previous instructions on biological exposure prevention, 56 (22.1%) considered these instructions to be insufficient, and 188 (74.3%) said that they had not received any instructions. Recapping used needles was performed by 156 (61.7%) students, 15 (5.9%) reported that they recapped used needles with one

hand and using a protective shield and 82 (32.4%) said that they did not do this. Among 236/253 (93.3%) students who gave information on the use of PPE, 123 (52.1%) reported that they always used PPE, 108 (45.8%) used it sporadically, and five (2.1%) never used it.

When asked about what viruses could be transmitted through occupational exposures, 202 (79.8%) students mentioned the HCV; 206 (81.4%), the HBV; 217 (85.8%), the HIV; and 36 (14.2%) did not mention any viruses. The actions to be taken after any biological exposures were unknown or partially known by 212 (83.8%) medical students. The actions to be taken after being exposed to body fluids from infected patients were unknown or partially known by 246 (97.2%) students.

Nursing students: A total of 149/200 (74.5%) nursing students participated in this study, of which 22 (14.8%) were males and 127 (85.2%) were females, with a mean age of 22.1 ± 3.7 years (18 to 42 years). Among the 149 nursing students, 27 (18.1%) suffered 37 occupational exposures, i.e. 19 students suffered one injury each, six suffered two injuries, and two suffered three injuries. Among students enrolled in each academic semester, the following reported at least one exposure: 5/31 (16.1%) students enrolled in the 4th semester; 1/31 (3.2%) in the fifth semester; 7/29 (24.1%) in the 6th semester; 4/28 (14.3%) in the 7th semester; and 10/30 (33.3%) in the 8th semester. The frequencies of occupational exposures were similar (OR = 1.5; 95%CI 0.4 a 5.4) among men [3 (13.6%)] and women [24 (18.9%)]. The frequencies of injuries according to the academic semester of occurrences are shown in Table 1. The frequencies of cutting and piercing injuries and of body fluids being in contact with mucous membranes, the hospital sites of occurrence and the reasons for such occurrences are listed in Table 2.

Occupational exposures occurred more frequently while students performed procedures [30 (81.1%)] than while they assisted/observed procedures [7 (18.9%)]; additionally, exposures were more frequent when there was tutorial support [23 (62.2%) *vs.* 14 (37.8%)]. The body parts affected were the hands [29/36 (80.6%)] and ocular and oral mucous membranes [7/36 (19.4%)]; one student did not give this information. Students were not wearing gloves in 5/37 (13.5%) injuries.

A total of 25 injuries (67.6%) were reported to a preceptor and seven (18.9%) were also reported to the SESMT. In eight out of the 12 (32.4%) exposures that were not reported to the preceptor or SESMT, students informed the reasons for not reporting: negligence [3 (37.5%)], little or no perception of risk [1 (12.5%)], belief that it was not necessary [1 (12.5%)], fear of warnings [1 (12.5%)], embarrassment [1 (12.5%)], and unwillingness to report [1 (12.5%)]; this information could not be obtained from four exposures. Students had post-exposure serological tests in 16 (43.2%) injuries. Among the 37 source patients, three (8.1%) had positive serological tests for HBV, HCV or HIV, 21 (56.8%) had negative serological tests for these viruses, and students were not concerned about the results of the patients' tests in 13 (35.1%) exposures. Additionally, students underwent chemoprophylaxis in 4/37 (10.8%) exposures.

Among nursing students who participated in this study, 78 (52.3%) said that they had satisfactorily received previous instructions on exposure prevention, whereas 60 (40.3%) considered these instructions to be insufficient, and 11 (7.4%) said that they had not received them. Recapping used needles was performed by 22 (14.8%) students, three (2%) reported that they recapped used needles with one hand and using a protective shield and 124 (83.2%) said that they did not do this. Among 134/149 (89.9%) students who gave information on the use of PPE, 110 (82.1%) reported that they always used PPE, whereas 24 (17.9%) used it sporadically.

When asked about what viruses could be transmitted through occupational exposures, 99 (66.4%) students mentioned HCV; 119 (79.9%), HBV; 127 (85.2%), HIV; and 21 (14.1%) did not mention any of them. The actions to be taken after any biological exposure were unknown or partly known by 127 (85.2%) students. The actions to be taken after being exposed to body fluids from infected patients were unknown or partly known by 128 (85.9%) students.

DISCUSSION

The present study revealed high frequencies of occupational exposures among medical and nursing students. In other studies conducted in Brazil, high frequencies of exposures were also found among medical students of the Federal University of Minas Gerais (34.2%)[24] and among nursing students of universities located in the interior of the state of São Paulo (12.4%[3] and 40%[25]). There were no differences in frequencies of exposures between women and men, as observed in Malaysia[16], in the USA[30] and in Austria, Germany and the United Kingdom[27]. There was an increase in the number of medical students who reported injuries as the course advanced, as observed in other studies[8,24,26]. This results from the increasing number of procedures that they must perform during their training to acquire the skills required, which leads to a more frequent risk of exposure. However, it should be emphasized that, among medical students, one-third of the injuries occurred in the 7th academic semester. In this semester, there are no disciplines in the medical course that puts students at risk of injuries. However, medical students may take an extra-curricular training program where they perform invasive procedures, such as sutures and local anesthesia, and where they can assist major procedures. On the other hand, the low frequency of exposures in the 12th semester results from the fact that medical students only perform outpatient procedures with low risk of exposure in this semester.

Studies conducted in several parts of the world have found an increase in the incidence of injuries among nursing students as the course advances[7,34,41,49], as a result of the higher frequency of contact with patients and handling of cutting and piercing objects. However, the present study showed a reduction in the occurrence of occupational exposures among nursing students as the course advanced. A similar fact was observed in an Italian study that found that the probability of occupational exposure among nursing students was significantly lower with the increase in clinical skills and in knowledge about exposure prevention and the risks involved with injuries[22]. In addition, it should be emphasized that one-fifth of the exposures involving nursing students occurred in the 1st and 3rd course semesters, which could only take place in extra-curricular activities.

Medical students more frequently suffered injuries with suture needles, whereas nursing students more frequently suffered injuries with hollow needles, as a result of the characteristics of the activities performed by them. In the hospital where the present study was conducted, suture procedures are exclusively performed by physicians or medical students. In other studies conducted in Brazil[24] and in the USA[30], medical students

also suffered more frequent injuries with suture needles as well, whereas nursing students were injured mostly by hollow needles[37,47].

Among medical students, the hospital sites with the highest occurrence of exposures were the emergency room, operating room and obstetric center. In such locations, they have a greater chance of handling cutting and piercing objects, which may contribute to the occurrence of injuries, in addition to their inexperience, anxiety in performing a procedure, rushing, and the patient's suffering and/or pressure. Among nursing students, injuries mainly occurred in the operating room and wards of the medical and pediatric clinics, the three sites where they most frequently develop their skills.

Medical and nursing students reported that lack of technical preparation, distraction and tiredness were the main reasons for the occurrence of exposures, as similarly observed in other studies with medical students[24,27,30] and nursing students[7]. However, while some students attributed injuries to the lack of tutorial support, paradoxically, these injuries most frequently occurred when there was tutorial support. In these cases, the students' anxiety in performing a procedure associated with feelings of being watched, assessed or hurried could contribute to the occurrence of such injuries. In this study, lack of care from other professionals was the reason attributed by medical students for 5.5% of exposures, a lower frequency than that found among medical students in Toronto, Canada (48%)[6].

This study showed that many students, especially medical students, have a habit of recapping used needles. Since 1987, when "universal precautions" were published to prevent the transmission of infections through exposures involving body fluids, it was determined that needles should never be recapped, purposely bent or broken, or removed from disposable syringes[4]. Despite these instructions, worldwide, medical students[1,9,26,29,36,43] and nursing students[7,37,47] frequently recap used needles and many believe that this procedure is safe and reduces the risk of exposures[9]. Recapping needles with one hand while using a protective shield or tweezers is an action performed by several students, although this way of recapping needles does not eliminate the risk of occupational exposure[13].

Injuries most frequently affected the hands and ocular and oral mucous membranes, as observed in other studies with medical students[24,31] and nursing students[25,41]. This fact shows the importance of emphasizing the use of gloves, masks and goggles when students are performing or assisting procedures.

Slightly more than two-thirds of exposures were informed to a preceptor and official reports were rarely made. These reports enable the hospital committee to know the reality and thus take action to minimize the occurrence of such exposures. However, despite all biological exposures causing suffering and anguish from the fear of acquiring infections, not reporting exposures is the rule rather than the exception worldwide. Similarly to what was observed in this study, the reasons for medical students[15,16,26,28,45] and nursing students[7,10,32,34,37] not reporting injuries are similar, including little or no perception of risk, embarrassment, lack of time, fear of the consequences of reporting (avoidance of the inconvenience of a report and follow-up, the negative effect on their professional career and the stigmatization by other students, residents and professionals), reluctance to admit lack of knowledge about how to handle instruments, not knowing how and where to report, among others.

Many students who suffered injuries were not concerned about finding out the results of patients' serological tests and did not know what viruses can be transmitted through occupational exposure. Additionally, 85% of them did not know what actions should be taken after any biological exposure. These results show that several students were not aware of the risks to which they were exposed when performing the procedures. Additionally, they reveal the need for greater efforts to teach standard precautions before the beginning of their practical activities.

Exposure prevention among students must be an institutional concern, although every preceptor and student must become aware of their responsibility for this prevention. Some studies revealed that knowledge about "universal precautions" alone is not sufficient to implement safe practices[11,19]. In the present study, based on the information obtained, nursing students seem to receive more and better instructions on occupational exposure prevention than medical students, as observed in South Korea[12]. Medical students reported not receiving instructions on occupational exposure prevention, recapping used needles and not using PPE more frequently than nursing students. However, the frequencies of occupational exposures and not wearing gloves at the time of exposure were similar between medical and nursing students. Students need to receive not only information, but also training for "standard prevention"[22]. In a study conducted in Seattle, USA, medical students reported that the teaching on the use of universal precautions was inadequate and that preceptors were poor role models when they ignored these precautions[40]. On the other hand, prospective studies with medical students[14] and nursing students[44,48] have showed that structured training for occupational exposure prevention improves knowledge and behavior and reduces the number of exposures, in addition to a possible development of a better professional behavior.

Students also need to receive information about the main pathogens that can be transmitted through biological exposure, how to prevent these infections, how to behave in case of exposure, and how to report occupational exposures. A previous study revealed that medical students of the FAMED/UFU had high rates of vaccination against hepatitis B[18] and that this vaccine must be made available without cost to all students in the area of health prior to their practical activities. No vaccines against HIV or HCV have been developed until now, however, chemoprophylaxis can be effective against HIV, although it must start in the first hours after exposure[2].

STUDY LIMITATIONS

In this cross-sectional study, students were required to remember whether they had suffered occupational exposure or not, in addition to several characteristics of such exposures and, for this reason, memory bias may have occurred. Nonetheless, occupational exposures are stressful events that are not easily forgotten, especially potentially contaminating ones. As the present study was conducted in one university exclusively, the results cannot be extended to the entire country. Further studies should be conducted to find out whether the results of the present study can represent what occurs in other Brazilian universities. Occupational exposures considered to be low risk were not assessed, such as those occurring when uncapping new needles, breaking vials or being injured by unused objects.

In some studies conducted with nursing students it was observed that accidents of this nature may represent more than 80% of the total and the injuries caused by them, especially in the hands, can lead to contamination after exposure to biological material[25,34]. Additionally, researchers of this study did not investigate the number of procedures performed by each student, thus hindering the calculation of the risk per procedure.

CONCLUSIONS

The present study showed high frequencies of potentially contaminating occupational exposures among medical and nursing students. Moreover, there were many reports of insufficient or inexistent instructions on biological exposure prevention. This could justify students' frequent neglect for the use of PPE, the frequent high-risk procedures such as not wearing gloves or recapping used needles, not reporting exposures to official institutions, and the low level of knowledge about post-exposure actions. Additionally, the high frequency of exposures in extra-curricular activities stood out. The results of this study will be sent to the proper authorities of the FAMED/UFU for the continuous development and improvement of strategies to protect students against such exposures.

RESUMO

Exposições ocupacionais a fluídos corporais e comportamentos em relação à sua prevenção e pós-exposição entre estudantes de medicina e de enfermagem de universidade pública brasileira

Estudo transversal foi realizado para verificar, entre estudantes de medicina e de enfermagem de universidade pública brasileira, as frequências e características de exposições ocupacionais e seus comportamentos na prevenção e pós-exposição. Durante o segundo semestre de 2010, questionário autoaplicável e semiestruturado foi completado por 253/320 (79,1%) estudantes de medicina do curso clínico e por 149/200 (74,5%) estudantes de enfermagem que já exerciam atividades práticas. Entre os estudantes de medicina, 53 (20,9%) sofreram 73 acidentes, que ocorreram principalmente em atividades extracurriculares (32,9%), com objetos pérfuro-cortantes (56,2%), na sala de emergência (39,7%) e em decorrência de despreparo técnico ou distração (54,8%). Entre os alunos de enfermagem, 27 (18,1%) sofreram 37 acidentes, que ocorreram principalmente com agulhas ocas (67,6%), no centro cirúrgico ou enfermarias (72,2%) e em decorrência de despreparo técnico ou distração (62,1%). Entre os alunos de medicina e de enfermagem, respectivamente, 96,4% e 48% estavam insatisfeitos com orientações previamente recebidas de prevenção de acidentes, 48% e 18% nem sempre utilizam equipamento de proteção individual, 67,6% e 16,8% reencapam agulhas usadas, 49,3% e 35,1% não se preocuparam em conhecer os exames sorológicos do paciente-fonte pós-exposição e 1,4% e 18,9% relataram o acidente oficialmente. Em conclusão, neste estudo verificaram-se altas frequências de exposições entre os estudantes avaliados, práticas inadequadas na prevenção e pós-exposição e, consequentemente, a necessidade de treinamento nas "precauções padrão" para prevenção de tais exposições.

ACKNOWLEDGEMENTS

Authors would like to thank all medical students and nursing students who participated in this study.

AUTHORS' CONTRIBUTIONS

FRFSB participated in the data collection and analysis and manuscript preparation. LAR participated in the study design, data collection and analysis and manuscript preparation. LCMO participated in the study design, data analysis and manuscript preparation. All authors read and approved the final manuscript.

REFERENCES

1. Al-Dabbas M, Abu-Rmeileh NME. Needlestick injury among interns and medical students in the Occupied Palestinian Territory. East Mediterr Health J. 2012;18:700-6.

2. Brasil. Ministério da Saúde. Secretaria de Estado de Saúde de Minas Gerais. Atendimento ao acidentado com material biológico. 2004. Available from: http://www.saude.mg.gov.br/politicas_de_saude/dst-aids/encontro-de-referencia-dst_aids/vi-encontro-de-referencias-em-dst_aids/Protocolo%20Biosseguranca.pdf

3. Canalli RTC, Moriya TM, Hayashida M. Acidentes com material biológico entre estudantes de enfermagem. Rev Enferm UERJ. 2010;18:259-64.

4. Centers for Disease Control and Prevention. Perspectives in disease prevention and health promotion. Update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other bloodborne pathogens in health-care settings. MMWR. 1988;37:377-88.

5. 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. U.S. Public Health Service. MMWR Recomm Rep. 2001;50:1-42.

6. Cervini P, Bell C. Brief report: needlestick injury and inadequate post-exposure practice in medical students. J Gen Intern Med. 2005;20:419-21.

7. Cheung K, Ching SSY, Chang KKP, Ho SC. Prevalence of and risk factors for needlestick and sharps injuries among nursing students in Hong Kong. Am J Infect Control. 2012;40:997-1001.

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

9. Elliott SKF, Keeton A, Holt A. Medical students' knowledge of sharps injuries. J Hosp Infect. 2005;60:374-7.

10. Irmak Z, Baybuga MS. Needlestick and sharps injuries among Turkish nursing students: a cross-sectional study. Int J Nurs Pract. 2011;17:151-7.

11. Karim J, Al-Saraji M, Al-Mousawi F, Al-Haddad Z, Al-Sharaf D, Marwan Y, *et al*. Knowledge and self-reported practice of universal precautions among Kuwait university medical students in their clinical years. Med Princ Pract. 2012;21:328-33.

12. Kim KM, Kim MA, Chung YS, Kim NC. Knowledge and performance of the universal precautions by nursing and medical students in Korea. Am J Infect Control. 2001;29:295-300.

13. Marziale MHP, Rodrigues CM. A produção científica sobre os acidentes de trabalho com material perfurocortante entre trabalhadores de enfermagem. Rev Lat Am Enfermagem. 2002;10:571-7.

14. Merlin JS, Morrison G, Gluckman S, Lipschik G, Linkin DR, Lyon S, *et al.* Blood and body fluid exposures among US medical students in Botswana. J Gen Intern Med. 2011;26:561-4.

15. Moon CS, Hwang JH, Lee CS, Park KH, Kim ES. Exposure to blood and body fluid among medical students in Korea. Am J Infect Control. 2010;38:582-3.

16. Norsayani MY, Hassim IN. Study on incidence of needle stick injury and factors associated with this problem among medical students. J Occup Health. 2003;45:172-8.

17. Oliveira AC, Gonçalves JA. Incidência de acidentes com material perfurocortante entre alunos de graduação em ciências da saúde. Ci Cuid Saude. 2009;8:385-92.

18. Oliveira LCM, Pontes JPJ. 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.

19. Osborn EHS, Papadakis MA, Gerberding JL. Occupational exposures to body fluids among medical students: a seven-year longitudinal study. Ann Intern Med. 1999;130:45-51.

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

21. Perry J, Jagger J. Healthcare worker blood exposure risks: correcting some outdated statistics. Adv Exp Prev. 2003;6:28-31.

22. Petrucci C, Alvaro R, Cicolini G, Cerone MP, Lancia L. Percutaneous and mucocutaneous exposures in nursing students: an Italian observational study. J Nurs Scholarsh. 2009;41:337-43.

23. Prüss-Ustün A, Rapiti E, Hutin Y. Estimation of the global burden of disease attributable to contaminated sharps injuries among health-care workers. Am J Ind Med. 2005;48:482-90.

24. 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.

25. Reis RK, Gir E, Canini SRMS. Accidents with biological material among undergraduate nursing students in a public Brazilian university. Braz J Infect Dis. 2004;8:18-24.

26. Rosenthal E, Pradier C, Keita-Perse O, Altare J, Dellamonica P, Cassuto JP. Needlestick injuries among French medical students. JAMA. 1999;281:1660.

27. Salzer HJF, Hoenigl M, Kessler HH, Stigler FL, Raggam RB, Rippel KE, *et al.* Lack of risk-awareness and reporting behavior towards HIV infection through needlestick injury among European medical students. Int J Hyg Environ Health. 2011;214:407-10.

28. Schmid K, Schwager C, Drexler H. Needlestick injuries and other occupational exposures to body fluids amongst employees and medical students of a German university: incidence and follow-up. J Hosp Infect. 2007;65:124-30.

29. Shariati B, Shahidzadeh-Mahani A, Oveysi T, Akhlaghi H. Accidental exposure to blood in medical interns of Tehran University of Medical Sciences. J Occup Health. 2007;49:317-21.

30. Sharma GK, Gilson MM, Nathan H, Makary MA. Needlestick injuries among medical students: incidence and implications. Acad Med. 2009;84:1815-21.

31. Shen C, Jagger J, Pearson RD. Risk of needle stick and sharp object injuries among medical students. Am J Infect Control. 1999;27:435-7.

32. Shiao JSC, McLaws ML, Huang KY, Guo YL. Student nurses in Taiwan at high risk for needlestick injuries. Ann Epidemiol. 2002;12:197-201.

33. Shimizu HE, Ribeiro EJG. Ocorrência de acidente de trabalho por materiais perfurocortantes e fluídos biológicos em estudantes e trabalhadores da saúde de um hospital escola de Brasília. Rev Esc Enferm USP. 2002;36:367-75.

34. Smith DR, Leggat PA. Needlestick and sharps injuries among nursing students. J Adv Nurs. 2005;51:449-55.

35. Souza RT, Bica CG, Mondadori CS, Ranzi AD. Avaliação de acidentes de trabalho com materiais biológicos em médicos residentes, acadêmicos e estagiários de um hospital-escola de Porto Alegre. Rev Bras Educ Med. 2012;36:118-24.

36. Sullivan M, Masters O, Venkatesan P. Needlestick injuries amongst medical students in Birmingham, UK. J Hosp Infect. 2000;44:240-1.

37. Talas MS. Occupational exposure to blood and body fluids among Turkish nursing students during clinical practice training: frequency of needlestick/sharp injuries and hepatitis B immunisation. J Clin Nurs. 2009;18:1394-403.

38. Tarantola A, Abiteboul D, Rachline A. Infection risks following accidental exposure to blood or body fluids in health care workers: a review of pathogens transmitted in published cases. Am J Infect Control. 2006;34:367-75.

39. Trim JC, Elliott TSJ. A review of sharps injuries and preventative strategies. J Hosp Infect. 2003;53:237-42.

40. Tucker A, Phillips WR. Medical students and infection control: risks and precautions. Tokai J Exp Clin Med. 1999;24:169-76.

41. Unver V, Tastan S, Coskun H. The frequency and causes of occupational injuries among nursing students in Turkey. Arch Environ Occup Health. 2012;67:72-7.

42. Varma M, Mehta G. Needle stick injuries among medical students. J Indian Med Assoc. 2000;98:436-8.

43. Varsou O, Lemon JS, Dick FD. Sharps injuries among medical students. Occup Med. 2009;59:509-11.

44. Wang H, Fennie K, He G, Burgess J, Williams AB. A training programme for prevention of occupational exposure to bloodborne pathogens: impact on knowledge, behaviour and incidence of needle stick injuries among student nurses in Changsha, People's Republic of China. J Adv Nurs. 2003;41:187-94.

45. Wicker S, Nurnberger F, Schulze JB, Rabenau HF. Needlestick injuries among German medical students: time to take a different approach? Med Educ. 2008;42:742-5.

46. Yamazhan T, Durusoy R, Tasbakan MI, Tokem Y, Pullukcu H, Sipahi OR, *et al*. Nursing students' immunisation status and knowledge about viral hepatitis in Turkey: a multi-centre cross-sectional study. Int Nurs Rev. 2011;58:181-5.

47. Yang YH, Wu MT, Ho CK, Chuang HY, Chen L, Yang CY, *et al.* Needlestick/sharps injuries among vocational school nursing students in southern Taiwan. Am J Infect Control. 2004;32:431-5.

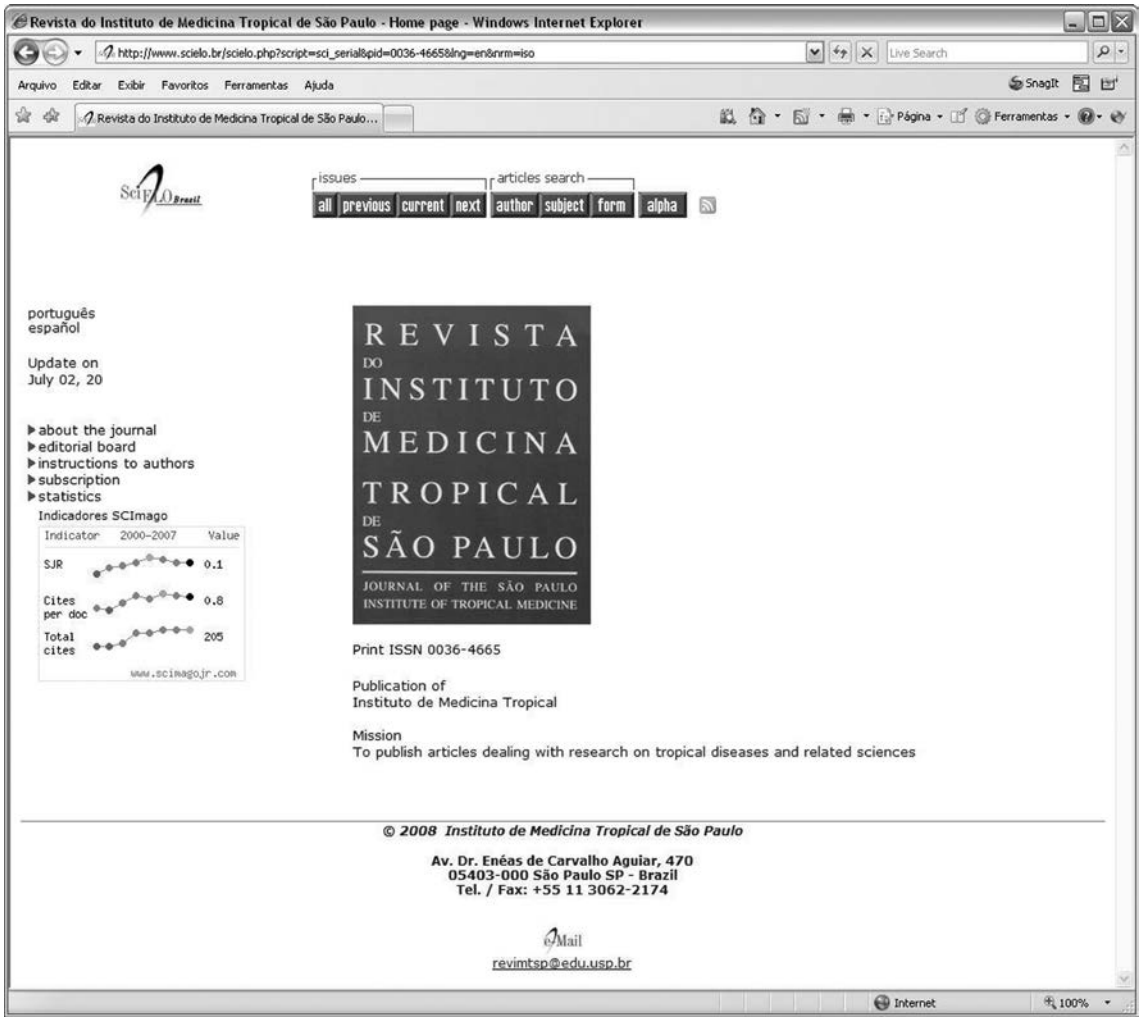
48. Yao WX, Wu YL, Yang B, Zhang LY, Yao C, Huang CH, *et al.* Occupational safety training and education for needlestick injuries among nursing students in China: intervention study. Nurse Educ Today. 2013;33:834-7.

49. Zhang Z, Moji K, Cai G, Ikemoto J, Kuroiwa C. Risk of sharps exposure among health science students in northeast China. Biosci Trends. 2008;2:105-11.

Received: 27 January 2013
Accepted: 18 July 2013

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(2):165-167, March-April, 2014
doi: 10.1590/S0036-46652014000200013

VERTICAL TRANSMISSION OF DENGUE VIRUS IN *Aedes aegypti* COLLECTED IN PUERTO IGUAZÚ, MISIONES, ARGENTINA

Manuel ESPINOSA(1), Sergio GIAMPERETTI(2), Marcelo ABRIL(1) & Alfredo SEIJO(2)

SUMMARY

A finding of vertical transmission of the DEN 3 virus in male specimens of *Aedes aegypti*, collected in the 2009 fall-winter period, in Puerto Iguazú city, Misiones, Argentina, using the RT-PCR technique in a 15-specimen pool is reported. This result is analyzed within the context of the epidemiological situation of Argentina's northeast border.

KEYWORDS: Dengue; *Aedes aegypti;* Vertical transmission; Argentina.

INTRODUCTION

Transovarial transmission of the dengue virus, which was found in non-hematophagous male specimens, or in the aquatic stage of larvae and pupae, was reported early in the 1950s by ALBERT SABIN[21], in a pioneering piece of work on different aspects of the disease and subsequently, in the 1980s, on *Aedes aegypti* and *Aedes albopictus* by different researchers[9,11,19,20]. In recent decades, research has increased, some of it originating in clinical laboratory tests in which mosquitoes were infected[3,10], and others originating in field studies which accounted for natural transmission. The latter observations come both from researchers from the Americas[8,9,14], especially Brazil[4,6,15,16,23,24], India[1,2] and South East Asia[5,12,17]. The four dengue virus serotypes were found with natural vertical transmission. In India, higher transovarial transmission rates were found during winter for *A. albopictus*, whereas, *A. aegypti* showed higher rates in warm and rainy seasons in arid or semi-arid areas[1]. In another work, simultaneous vertical transmission for DEN 2 and DEN 3 was found in *A. albopictus*[16].

This form of transmission could be relevant to keep viral circulation running during interepidemic periods. However, the role that the occurrence of outbreaks can have is unknown, with the typical form of transmission being: person to viremia- vector- susceptible person, being the most important one.

MATERIALS AND METHODS

The collections were made in Puerto Iguazú city, located at 25º 36' south latitude and 54º 35' west longitude, in the province of Misiones, Argentina. With an overall surface of 760 km^2 and a population of 82,227 inhabitants (2010 census), the city is set up in the tri-border area, opposite Foz do Iguaçú city in Brazil and Ciudad del Este in Paraguay (Fig. 1).

Fig. 1 - Georeferencing of Puerto Iguazú city, Misiones, Argentina.

During the period ranging from April to September 2009, adult mosquitoes were collected in the urban area of the city, using BG-Sentinel® traps. Specimens were classified by genus and species and separated by sex to constitute 10 pools of 15 to 20 mosquitoes. The ratio between the number of positive pools and the total number of mosquitoes studied, multiplied by one thousand, is the minimum infection rate (MIR).

RNA extraction was made using the Trisol LS Reagent® method (Invitrogen™) according to the manufacturer's protocol. RT-PCR was performed on the mosquito pool according to LANCIOTTI *et al.* protocol[13]. The primers used in the first round were:

D1 (5'-TCAATATGCTGAAACGCGCGAGAAACCG-3') and
D2 (5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3') of Operon™.

(1) Fundación Mundo Sano. Buenos Aires, Argentina.
(2) Servicio de Zoonosis, Hospital F.J. Muñiz, GCBA. Buenos Aires, Argentina.
Correspondence to: Manuel Osvaldo Espinosa, Fundación Mundo Sano, Programas y Proyectos, Paraguay 1535, CABA, Buenos Aires1061, Argentina. Phone: 54-11-4872-1333/1334. E-mail: mespinosa@mundosano.org

A nested-PCR was performed in the second round with D1 primer and those specific for each dengue serotype (TS1, TS2, TS3 and TS4) which amplify the genC-prM regions in: 482, 119, 290 and 392 base pairs of DEN 1, DEN 2, DEN 3 and DEN 4 respectively. Detection of amplified fragments was done with 1.5% agarose gels revealed with GelRed®. The amplicon obtained was purified and sequenced by the company Macrogen™, Seoul, Korea. The nucleic acid sequence was compared with sequences of the GenBank BLAST, using the Clustal W (Megalign Software) alignment program.

RESULTS

For male specimens of *Aedes aegypti,* only one 15-mosquito pool could be constituted, which proved positive for DEN 3. Sequencing of nucleic acids (Fig. 2) compared with sequences of the GenBank yielded 100% compatibility with sequences (ID): EF546774.2, EF546773.2, FJ373306.1, FJ182005.1, AB038479.1 and resulted in the following reports:

Lineage Report

Dengue virus

Dengue virus 3. 55 295 hits [viruses] Dengue virus type 3 isolate INDI06DEN13 polyprotein gene, p.

Dengue virus 2. 55 2 hits [viruses] Dengue virus type 2 isolate 152/ BRAZ/99 nonfunctional polyp.

Taxonomy Report

Dengue virus. 297 hits, 2 orgs [root; Viruses; ssRNA viruses; ssRNA positive-strand viruses; Flaviviridae; Flavivirus; Dengue virus group]

Dengue virus 3. 295 hits, 1 orgs.

Dengue virus 2. 2 hits, 1 orgs.

Taking into account that the sample was a very small one, MIR was not calculated.

ATGCTTCGACATGGTGGTGGACTCAGGATGCTCTGTCTCATGAT
GATGTTAAAAATGCCTCCCTACACGGACGTCTATGATGCGACCT
CCCTCCATCGTTAATCCTCCTGACTGACCTATACTGAACCAGTA
CCTTGAGGGAAAGGCGAAAAGAACCCCGGCGAGGGGAGTGAA
ACAGAACCTGAAGACATTGACTGTTGGTGCAAGCTCTGTCTCA
TGATGATGTTAAAGAAGGAAAAAAATTACGCCCATGCCCAAGG
GAAAACCCCACTTTAAAGTATCCCTCTCGTCGGAAGGCGAATAT
GCTGCCGAAAAATTGCTTTTTGCTTCCCAAAGATCCCGCTTCTG
TACTCTGCAGAGATGTGCATGTGGACAACGTGTTGTAGGGGGA
TGGTCTGTCAACACCCCCCACCTCGTGAGGCACCTCCCGCCAT

Fig. 2 - Nucleotide sequence of the isolated fragment. ADN transcript (434 pb).

DISCUSSION

This is the first communication of vertical transmission of the dengue virus in *Aedes aegypti* in Argentina. Although there is a lack of knowledge regarding the frequency with which it can occur, the degree of importance of the genesis and evolution after an outbreak, or whether it can also originate it *per se*, it is important to consider that it has been detected in a region with intense circulation of people due to the high levels of tourist traffic that occurs there throughout the year. DEN 3 serotype was the cause of an outbreak of serious magnitude in Paraguay, between December 2006 and May 2007, and which had an impact on Argentine cities near border areas. In 2000, the northeast region had presented DEN 1 cases associated to the Paraguay outbreak (December 1999 to May 2000). On the other hand, in 2009, dengue showed very low activity in the northeast border; this was due to DEN 2. In the same year, Argentina had the highest record of cases in history as a result of the epidemy, which originated in the northeast and which also affected big cities, including Buenos Aires, due to DEN 1[22]. As of 2011 and until this article was written (January 2013), the dengue patients assisted by our service and who came from Paraguay correspond to DEN 2, the serotype that had not had any epidemic circulation in the region until said year.

The finding of DEN 3 with vertical transmission in *Aedes aegypti*, in situations in which other serotypes are circulating, could constitute a natural reservoir, also taking into account that the population's immunity against DEN 3 should be high, as a result of the cases that occurred in 2007.

An important aspect, which has recently been reported, is the finding of *Aedes albopictus*[6] in Puerto Iguazú, Misiones, which is the other dengue vector with proven capacity for vertical transmission.

In this work, mosquitoes were collected during the fall-winter months, which do not record occurrences of cases. This fact can strengthen the hypothesis in which vertical transmission would keep viral circulation running during interepidemic periods, but other studies relate it to the pre-epidemic period[1,14].

Due to the fact that the sample was reduced, no indices could be established or statistical analyses carried out; however, it is surprising that only one pool with 15 specimens studied has been positive. On the other hand, a study carried out in Colombia[18] did not demonstrate vertical transmission in one sample of 1,400 male *A. aegypti* specimens, when the MIR in over two thousand female specimens was 11.6%.

Circulation of the three serotypes, the presence of two *Aedes* species with transmission capacity and the finding of vertical transmission make the Argentine northeast an area of high risk for dengue occurrence.

Within this line of investigation, it is necessary to increase the number of collections at throughout the year, which will improve sensitivity to entomological surveillance, enable estimation of temporal-spatial variations and improve knowledge about vertical transmission in each species.

RESUMEN

Transmisión vertical de virus dengue en *Aedes aegypti*, capturados en Puerto Iguazú, Misiones, Argentina

Se comunica el hallazgo de transmisión vertical de virus DEN 3 en ejemplares machos de *Aedes aegypti*, capturados en otoño-invierno de 2009, en la ciudad de Puerto Iguazú, Misiones, Argentina, utilizando la técnica de RT-PCR en un pool de 15 ejemplares. Se analiza este resultado en el contexto de la situación epidemiológica de la frontera nordeste de Argentina.

ACKNOWLEDGEMENTS

To Drs. Delia Enría and Alejandra Morales of Instituto Nacional de Enfermedades Virales (INEVH), J Maiztegui, Pergamino, Buenos Aires, Argentina, for their constant support and advice.

REFERENCES

1. Angel B, Joshi V. Distribution and seasonality of vertically transmitted dengue viruses in *Aedes* mosquitoes in arid and semi-arid areas of Rajasthan, India. J Vector Borne Dis. 2008;45:56-9.

2. Arunachalam N, Tewari SC, Thenmozhi V, Rajendran R, Paramasivan R, Manavalan R, *et al.* Natural vertical transmission of dengue viruses by *Aedes aegypti* in Chennai, Tamil Nadu, India. Indian J Med Res. 2008;127:395-7.

3. Castro MG, Nogueira RM, Schatzmayr HG, Miagostovich MP, Lourenço-de-Oliveira R. Dengue virus detection by using reverse transcription-polymerase chain reaction in saliva and progeny of experimentally infected *Aedes albopictus* from Brazil. Mem Inst Oswaldo Cruz. 2004;99:809-14.

4. Cecílio, AB, Campanelli ES, Souza KPR, Figueiredo LB, Resende, MC. Natural vertical transmission by *Stegomyia albopicta* as dengue vector in Brazil. Braz J Biol. 2009;69:123-7.

5. Chye JK, Lim CT, Ng KB, Lim JMH, George R, Lam SK. Vertical transmission of dengue. Clin Infect Dis. 1997;25:1374-7.

6. de Figueiredo MLG, de C Gomes A, Amarilla AA, de S Leandro A, de S Orrico A, de Araujo RF, *et al.* Mosquitoes infected with dengue viruses in Brazil. Virol J. 2010;**7**:152-7.

7. Espinosa M, Weinberg D, Gómez A, Abril M. Primer registro de *Aedes albopictus* (Skuse) (Diptera: Culicidae) en la Ciudad de Puerto Iguazú, Misiones, Argentina. Rev Arg Zoonosis Enf Infec Emerg. 2012;7:24-6.

8. Günther J, Martínez-Muñoz JP, Pérez-Ishiwara DG, Salas-Benito J. Evidence of vertical transmission of dengue virus in two endemic localities in the State of Oaxaca, Mexico. Intervirology. 2007;50:347-52.

9. Hull B, Tikasingh E, Souza M, Martinez R. Natural transovarial transmission of dengue 4 virus in *Aedes aegypti* in Trinidad. Am J Trop Med Hyg. 1984;33:1248-50.

10. Joshi V, Mourya DT, Sharma RC. Persistence of dengue-3 virus through transovarial transmission passage in successive generations of *Aedes aegypti* mosquitoes. Am J Trop Med Hyg. 2002;67:158-61.

11. Khin MM, Than KA. Transovarial transmission of dengue 2 virus by *Aedes aegypti* in nature. Am J Trop Med Hyg. 1983;32:590-4.

12. Kow CY, Koon LL, Yin PF. Detection of dengue viruses in field caught male *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Singapore by Type-Specific PCR. J Med Entomol. 2001;38:475-9.

13. Lanciotti RS, Calisher CH, Gübler DJ, Chan NG, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reversed transcriptase-polymerase chain reaction. J Clin Microbiol. 1992;30:545-51.

14. Le Goff G, Revollo J, Guerra M, Cruz M, Barja Simon Z, Roca Y, *et al.* Natural vertical transmission of dengue viruses by *Aedes aegypti* in Bolivia. Parasite. 2011;18:277-80.

15. Lourenço de Oliveira R, Honório NA, Castro MG, Schatzmayr HG, Miagostovich MP, Alves JC, *et al.* Dengue virus type 3 isolation from *Aedes aegypti* in the municipality of Nova Iguaçu, State of Rio de Janeiro. Mem Inst Oswaldo Cruz. 2002;97:799-800.

16. Martins VE, Alencar CH, Kamimura MT, de Carvalho Araújo FM, De Simone SG, Dutra RF, *et al.* Occurrence of natural vertical transmission of dengue-2 and dengue-3 viruses in *Aedes aegypti* and *Aedes albopictus* in Fortaleza, Ceará, Brazil. PLoS One. 2012;7(7):e41386. doi: 10.1371/journal.pone.0041386.

17. Mulyatno KC, Yamanaka A, Yotopranoto S, Konishi E. Vertical transmission of dengue virus in *Aedes aegypti* collected in Surabaya, Indonesia, during 2008-2011. Jpn J Infect Dis. 2012;65:274-6.

18. Romero-Vivas CM, Leake CJ, Falconar AK. Determination of dengue virus serotypes in individual *Aedes aegypti* mosquitoes in Colombia. Med Vet Entomol. 1998;12(3):284-8.

19. Rosen L, Shroyer DA, Tesh RB, Freier JE, Lien JC. Transovarial transmission of dengue viruses by mosquitoes: *Aedes albopictus* and *Aedes aegypti.* Am J Trop Med Hyg. 1983;32:1108-19.

20. Rosen L. Mechanism of vertical transmission of the dengue virus in mosquitoes. CR Acad Sci III. 1987;304:347-50.

21. Sabin AB. Research on dengue during World War II. Am J Trop Med Hyg. 1952;1:30-50.

22. Seijo A, Romer Y, Espinosa M, Monroig J, Giamperetti S, Ameri D, *et al.* Brote de dengue autóctono en el Área Metropolitana Buenos Aires. Experiencia del Hospital de Enfermedades Infecciosas F. J. Muñiz. Medicina (B Aires). 2009;69:593-600.

23. Vilela APP, Figueiredo LB, dos Santos JR, Eiras AE, Bonjardim CA, Ferreira PCP, *et al.* Dengue virus 3 genotype I in *Aedes aegypti* mosquitoes and eggs, Brazil, 2005-2006. Emerg Infect Dis. 2010;16:989-92.

24. Zeidler JD, Acosta PO, Barrêto PP, Cordeiro JS. Dengue virus in *Aedes aegypti* larvae and infestation dynamics in Roraima, Brazil. Rev Saúde Pública. 2008;42:986-91.

Received: 29 April 2013
Accepted: 19 July 2013

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(2):169-174, March-April, 2014
doi: 10.1590/S0036-46652014000200014

SEROPREVALENCE OF *T. cruzi* INFECTION IN BLOOD DONORS AND CHAGAS CARDIOMYOPATHY IN PATIENTS FROM THE COAL MINING REGION OF COAHUILA, MEXICO

José Gerardo MARTÍNEZ-TOVAR(1), Eduardo A. REBOLLAR-TÉLLEZ(2) & Ildefonso FERNÁNDEZ SALAS(2)

SUMMARY

Context and Objective: Chagas disease is considered a worldwide emerging disease; it is endemic in Mexico and the state of Coahuila and is considered of little relevance. The objective of this study was to determine the seroprevalence of *T. cruzi* infection in blood donors and Chagas cardiomyopathy in patients from the coal mining region of Coahuila, Mexico. **Design and Setting:** Epidemiological, exploratory and prospective study in a general hospital during the period January to June 2011. **Methods:** We performed laboratory tests ELISA and indirect hemagglutination in three groups of individuals: 1) asymptomatic voluntary blood donors, 2) patients hospitalized in the cardiology department and 3) patients with dilated cardiomyopathy. **Results:** There were three levels of seroprevalence: 0.31% in asymptomatic individuals, 1.25% in cardiac patients and in patients with dilated cardiomyopathy in 21.14%. **Conclusions:** In spite of having detected autochthonous cases of Chagas disease, its importance to local public health remains to be established as well as the details of the dynamics of transmission so that the study is still in progress.

KEYWORDS: Chagas disease; American trypanosomiasis; *Trypanosoma cruzi;* Chagasic cardiomyopathy; Coal mining region; Serology; Seroprevalence, ELISA.

INTRODUCTION

Chagas disease or American trypanosomiasis is a neglected public health problem in Latin America[33]. The causative agent of the disease is the protozoan *Trypanosoma cruzi* (Chagas 1909) (Kinetoplastida: Trypanosomatidae), which is a flagellate haemoparasite. The life cycle of the pathogen involves two hosts corresponding to an insect and to a vertebrate. Parasites are transmitted by haematophagous bugs. The vector is an insect of the family Reduviidae, and the subfamily Triatominae. The main route of infection of *T. cruzi* to humans is during defecation after blood-feeding. Nevertheless, other mechanisms for transmission have been documented e.g. blood transfusions from *T. cruzi*-infected individuals[37], transplacental route[5,27], organ transplantation[20,40], breast feeding[11,12], laboratory accidents[15,18], skinning wild animals[14] and eating undercooked parasitized meat or consuming drink contaminated with triatomine feces[44].

The importance of Chagas disease in Mexico was highlighted in the national seroprevalence studies reported by VELASCO-CASTREJON in 1992[46] and more recently by NOVELO-GARZA in 2010[32]. In both studies it was shown that central and southern Mexico had the highest prevalence rates for the presence of positive antibodies against *T. cruzi*. The states with the highest frequencies were Chiapas and Oaxaca with 5.0% to 4.5% respectively, whereas the mean national seroprevalence was estimated in 1.6%.

For the state of Coahuila, the seroprevalence rate, was found to range from 0.1% to 0.6%[46]. Knowledge of Chagas Disease in Coahuila is virtually nonexistent and data on prevalence, incidence, transmission or vector species in the region have been rarely been referenced[8].

Because very little is known about Chagas disease in the north of Mexico and especially in the state of Coahuila, in January 2011 we established a research protocol. Its main aim was to provide evidence about the prevalence of infection, and to determine the population and potential risk factors, as well as to conduct entomological investigations to identify potential vectors. During the first phase of this research protocol, we established a primary objective which was to determine the prevalence of antibodies against *Trypanosoma cruzi* that occurs in the coal mining region of Coahuila. The studied population was divided into three groups: (1) asymptomatic blood donors, (2) patients admitted to the cardiology department and (3) patients with dilated cardiomyopathy.

METHODS

The work was carried out with blood donors and patients of the Hospital General de Zona No. 24 of the Mexican Institute of Social Security in Nueva Rosita, Coahuila. This hospital is the main centre of medical services in the coal mining region of Coahuila and serves a

(1) Doctoral Student. Hospital General de Zona No 24, Instituto Mexicano del Seguro Social, Nueva Rosita, Coahuila, México.
(2) Medical Entomologist. Universidad Autónoma de Nuevo León. Facultad de Ciencias Biológicas. Laboratorio de Entomología Médica.
This work was conducted at Hospital General de Zona No 24 del Instituto Mexicano del Seguro Social in Nueva Rosita, Coahuila, México.
Correspondence to: Eduardo A. Rebollar Téllez. Ph.D. Universidad Autónoma de Nuevo León. Facultad de Ciencias Biológicas. Laboratorio de Entomología Médica. Av. Universidad s/n, Cd. Universitaria, San Nicolás de los Garza Nuevo León. CP 66400. Tel.: +52 81 8332 4714. E-mail: eduardo.rebollartl@uanl.eud.mx

population of 140,000 inhabitants. Medical facilities possess 82 hospital beds and an average of 6,000 discharges per year.

The coal mining region of Coahuila is made up of five municipalities which are: Sabinas, San Juan de Sabinas, Múzquiz, Juárez and Progreso. The coal mining region is located between latitude 27°51'36'' - 28º59'24'' N and longitude 101º07'12-101º14'24'' W and 380 meters above the sea. It has a semi-arid climate which means that it is very hot in summer and cold in winter[17].

From January through June of 2011 samples were taken in three groups of individuals to determine the presence of antibodies to *Trypanosoma cruzi*: (1) blood donors, (2) patients admitted to the cardiology department and (3) patients with dilated cardiomyopathy. We included all persons who attended as volunteer blood donors, those who were admitted to the cardiology hospital department and those reported in the clinical diagnosis as dilated cardiomyopathy patients (I42.0 key of the International Classification of Diseases tenth edition)[34] in the period indicated. The only exclusion criterion was refusal to participate in the study. The samples of each individual of these groups were analyzed to determine the presence of antibodies to *T. cruzi*. The positive cases underwent an epidemiological study that included blood samples taken from house cohabitants. Informed consent was requested. The study was approved by the Local Bioethics Committee under the registration number 2012- 506-25.

The blood donor group included 1615 asymptomatic individuals who came voluntarily to donate blood to the blood bank of the same hospital and covered eligibility criteria according to the corresponding Mexican Official Standard (Norma Oficial Mexicana NOM-003-SSA2-1993, "Provision of human blood and blood components for therapeutic purposes").[30]

The second group was composed of patients admitted to the cardiology hospital department in the same period, these included a total of 160 people with various diseases requiring hospital treatment.

The third group was made up of patients with a diagnosis of dilated cardiomyopathy. Through the clinical file we found 14 patients with this diagnosis. These patients were visited at their homes, and in coordination and support of a public health team, blood samples were taken from those individuals.

Blood samples were obtained from these patients by a puncture in peripheral blood, serum separated by centrifugation at 1200 xg for 10 minutes, aliquoted in Eppendorf tubes and frozen at -20 °C until analysis.

Two tests were used for the determination of antibodies:

1. Enzyme linked immunoassay (Biokit - ELISA ChagasWerfen, Barcelona, Spain) is an immunoassay method in which microtiter wells are coated with four recombinant antigens representing immunodominant epitopes of *T. cruzi*. This test is based on the detection of antibody responses to four complementary immunodominant epitopes that were discovered by serologic expression cloning, by using sera from infected patients. These epitopes are expressed as a single recombinant protein, called Therapeuticf, consisting of 101 amino acids, including the amino acid hexahistidine tag used for purification. This protein is expressed in an *E. coli* expression vector and is purified to a single band on SDS page gels[36]. This procedure was carried out according to Biokit's specifications. The study has a sensitivity of 100% and a specificity of 99.24% according to the manufacturer[13, 35].

2. Indirect haemagglutination (HI), also known as reverse passive hemagglutination with Chagatest R (Wiener Laboratory, Rosario, Argentina), based on the property of producing antibodies specific agglutination in the presence of red blood cells sensitized with the corresponding antigens. The procedure was carried out according to the manufacturer's specifications. Titers of 1:16 were considered positive[29,45].

The enzyme-linked immunoassay was used as a screening test. Those samples that were positive in the first instance were subsequently analyzed by indirect hemagglutination as a confirmatory test. In accordance with the actual guidelines[31], the confirmation of the diagnosis of Chagas disease is established by at least two different positive serologic tests. No other tests were carried out.

In the cases that were found positive samples with both tests, we requested them to answer a questionnaire to elaborate on an epidemiological study that included medical history, history of blood transfusion, travel to endemic areas, chest radiographs, electrocardiogram, housing data, risk activities, photographic identification of triatomines and blood sampling from cohabitants. The medical history included questions on alimentary habits.

In positive cases, a search of triatomines at their home premises was conducted. Triatomine bugs were sought within and around the houses.

RESULTS

The study population had the following characteristics: a total of 1615 volunteer donors whose ages ranged between 18 and 65 years old. It was found that 88% were men (n = 1421) and the remaining 12% were women (n = 194), the average age of the sample was 37 years. Patients hospitalized in the cardiology department were 56 women (35%) and 104 men (65%), with a mean age of 69.4 years old. In the group of patients with dilated cardiomyopathy there were 14 cases, including eight women (54%) and six men (46%) with a mean age of 60.9 years old. Only one of the sampled cohabitants was found as positive, a five year old girl. All positive cases were reactive for both ELISA and HAI.

The results of this study are summarized in Table 1. A total of 1615 asymptomatic individuals were analyzed as potential blood donors, five were positive. Out of 160 patients admitted to the cardiology department we found two positive cases. Finally, in the third group of 14 patients with dilated cardiomyopathy, we found that three of them were positive. There was a positive sample derived from the study of co-inhabitants. Seroprevalence levels were found to be 0.31%, 1.25% and 21.14% in asymptomatic individuals, cardiac patients and in patients with dilated cardiomyopathy, respectively. Table 2 shows the individual characteristics of positive cases. Additionally, the geographic distribution of cases in the study area is shown in Figure 1.

The epidemiological survey of positive cases revealed that none had been born outside the studied area nor had any traveled to an endemic

Table 1
Seroprevalence results found in the coal mining region of Coahuila in relation with the different groups analyzed

Origin	Samples	Positive samples	Seroprevalence (%)
Blood tranfusion center	1615	5	0.31
Cardiology Hospital Service	160	2	1.25
Cardiomyopathy	14	3	21.14

area of American trypanosomiasis. The houses of individuals who tested positive were built of block and cement. The questionnaire that included a question on alimentary habits did not reveal any answers that may indicate oral contamination. At the time of our entomological survey, no triatomines were found inside or around the home premises. Only one of the positive cases of dilated cardiomyopathy had had a blood transfusion two years before this study and corresponded to a 60-year-old female who has lived in this area all her life. The transfusion was conditioned by anemia secondary to metrorrhagia.

Socioeconomic status is about average lower middle class, none were considered to be living in a state of poverty. Positive cases were questioned about risk activities such as going to camps, sleeping outdoors, as well as the consumption of wild animals and so forth and the responses were all negative. People were also shown actual-size pictures of triatomines for identification and we recorded that none of them were able to recognize the vector correctly.

DISCUSSION

The northeast region of Mexico (states of Coahuila, Nuevo León and Tamaulipas) is usually not considered as part of the endemic area of Chagas disease in Mexico and for these reasons the disease has largely been neglected by the health sector. In a study carried out by GALAVÍZ-SILVA *et al.* (2009)[13] a seroprevalence of 2.8 % antibodies against *T. cruzi* was found in blood donors in a hospital of the state of Nuevo León. Regarding the state of Coahuila, there has been virtually no diagnosis in Chagas for the past 20 years. The national study of VELASCO-CASTREJON *et al.*

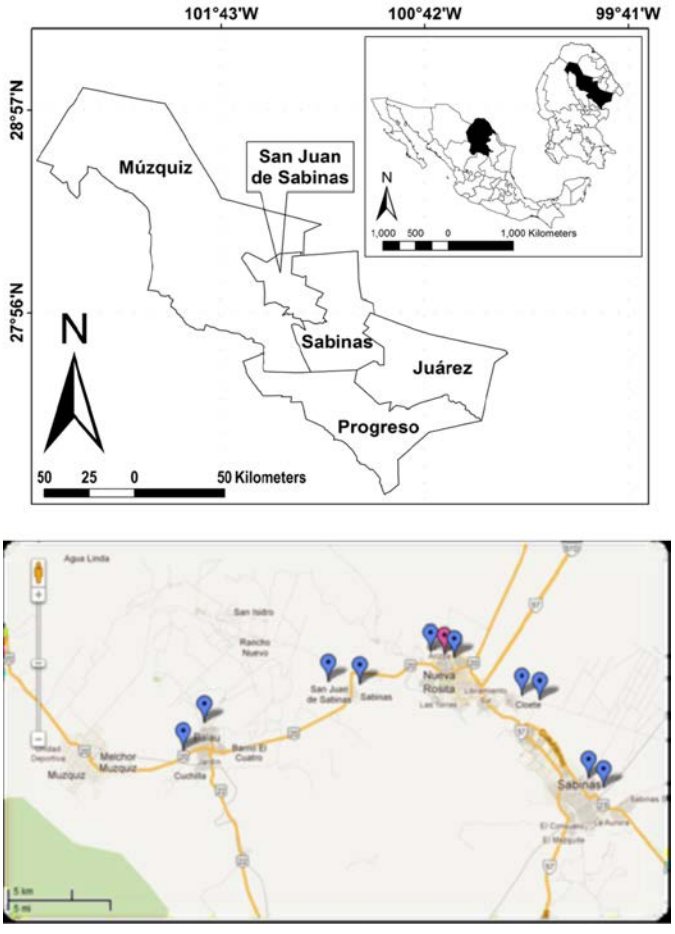


Fig. 1 - Above, a map showing the five municipalities that comprised the coal mining regions in the state of Coahuila. The insert in the upper right corner, represents (black filled shapes) the location of the state of Coahuila in Mexico, as well as the proportion of the coal mining regions in the state of Coahuila. Below, geographical location of Chagas disease cases in the coal mining region of Coahuila in the period January to June 2011. The image was taken from Google Earth®. The blue balloons correspond to the location of the cases. The red balloon represents a positive case found during the epidemiological study.

Table 2
Main epidemiological characteristics of positive cases with *T. cruzi* positive serological results Coahuila coal mining region

Progressive number	Age	Sex	Detection	Diagnosis	Previous blood transfusions	Travel to endemic zones	Recognition of triatomines
1	42	M	Transfusion center	Asymptomatic	Negative	Negative	Negative
2	53	M	Transfusion center	Asymptomatic	Negative	Negative	Negative
3	35	M	Transfusion center	Asymptomatic	Negative	Negative	Negative
4	26	M	Transfusion center	Asymptomatic	Negative	Negative	Negative
5	25	M	Transfusion center	Asymptomatic	Negative	Negative	Negative
6	76	M	Hospital	Cardiac Failure	Negative	Negative	Negative
7	60	F	Hospital	Cardiac Failure	Negative	Negative	Negative
8	76	M	Home	Dilated Cardiomyopathy	Negative	Negative	Negative
9	60	F	Home	Dilated Cardiomyopathy	Positive 2 years ago	Negative	Negative
10	61	F	Home	Dilated Cardiomyopathy	Negative	Negative	Negative
11	5	F	Home	Asymptomatic	Negative	Negative	Negative

in 1992 in a sample of 1976 people, found a prevalence of 0.1 to 1:32 dilution using hemagglutination and indirect immunofluorescence[46]. To the best of our knowledge, no other study has been carried out in the state of Coahuila, and therefore this paper represents the first evidence that there is a suggested risk of infection of *T. cruzi* among the human population. The work of NOVELO-GARZA, in 2010 revised the responses to antibodies against *T. cruzi* using the ELISA test carried out among blood donors in the Mexican Social Security Institute and reported that in a population of 230,074 they found a seroprevalence of 0.406 %. For the state of Coahuila that National survey included 4611 persons of which 10 were positive for an overall rate of 0.217 in the state. If the state is divided into north and south regions, leaving the towns of Monclova, Nueva Rosita and Piedras Negras as north, then it can be seen that this estimate increases to 0.37 %, which would be a similar estimate to that found in this study. In the city Nueva Rosita which is the center of the coal mining region, the seroprevalence was found to be 0.77 %, and this corresponds to a single case in a sample of only 129 people[32].

The laboratory diagnosis of Chagas disease depends on the clinical stage. It is known that in acute cases it is only possible to identify the etiologic agent, whereas an indeterminate and chronic diagnosis is based on the presence of antibodies against the parasite protozoon *T. cruzi* in sera of infected individuals. These antibodies are mostly detected using different serological tests, the most widely used are the indirect hemagglutination (IHA), enzyme-linked immunosorbentassay (ELISA) and indirect immunofluorescence (IIF), due to the easy implementation, low cost and good results in terms of specificity and sensitivity. Based on several protocols, it is considered to be a case of Chagas disease when the blood samples of an individual give positive results from at least two different serologic tests[21,38,43].

Most recently the polymerase chain reaction has been used to detect *T. cruzi* DNA in tissues samples from necropsies and additionally, with this technique it has been possible to determine the number of copies as a way to establish the parasite load in these patients[22]. The use of molecular techniques to confirm the diagnosis of Chagas disease in a daily clinical practice is still out of reach of most health institutions in Mexico[6]. In our study, confirmation of positive samples by molecular techniques was not considered at this stage because the main objective was to detect seroprevalences in a particular population using the resources provided by the hospital of Instituto Mexicano del Seguro Social (IMSS).

In the present study we found a seroprevalence of 0.31%, 1.25% and 21.14% for the blood donor (asymptomatic) group, hospitalized cardiac patients and patients with dilated cardiomyopathy respectively. These figures show that the coal mining region of Coahuila is an area in which there is circulation of *T. cruzi* although traditionally it was considered non-endemic region. This data also suggests that Chagas disease may represent a cause for cardiomyopathy. It is likely that many cases are not recognized by the health institutions. Nonetheless, our entomological surveys were limited; we found that in those communities there is basically no knowledge about the vectors and/or transmission mechanisms. Lack of knowledge on vectors or transmission mechanisms represents a shortcoming in the implementation of any prevention program. As was previously mentioned in the results, none of the positive cases were able to identify the insect vector and we did not found triatomine bugs during the search inside and around their houses. The failure to find triatomines in the houses does not necessarily indicate their absence. It is possible that insects might not have been properly detected during searches or that perhaps they have been conducted at a time of the year when their presence is scarce. It is also possible to consider that there is a transmission cycle well outside houses, which may be conditioned by the migration of vectors between suburban and wild environments[24]. We suggest that more systematic and thorough entomological studies are required to evaluate the transmission and risk potential of triatomine vectors occurring in the region.

An extradomiciliary cycle of *T. cruzi* can be carried out and maintained in vertebrates other than man, including domestic animals such as pet dogs[3]. In the United States of America (USA), and particularly in the state of Texas, several vertebrate species (armadillos, coyotes, raccoons, opossums and rats of the genus *Neotoma*) have been documented as having tested positive for the infection with *T. cruzi*[42]. Until recently, in the USA only seven indigenous cases of Chagas disease have been reported (four in Texas, one in Tennessee, one in California and one in Louisiana)[4,10]. In addition to the above cases, CANTLEY *et al.,* cited a study "The United Sates *Trypanosoma cruzi* Infection Study" (USTC), which made possible infection screening of blood donors and found that an initial sample of 29 million, 1084 tested positive for antibodies *T. cruzi* and after performing exclusion criteria for a follow-up study, it was determined that 15 indigenous cases were confirmed by tests conducted by the Centers for Disease Control (CDC) and also added a case from the state of Mississippi. In the above-mentioned study there were 15 new autochthonous Chagas cases[7]. These new cases certainly indicate that the prevalence of infection with *T. cruzi* in the USA may in fact be an underestimation of the actual disease prevalence and we suggest that something similar may well be happening in northern Mexico, where Chagas disease is still considered unimportant by national health programs.

It should be noted that the U.S.-Mexico border region, shares some socio-cultural aspects, such as significant migratory movement and the presence of some common parasitic diseases, including Chagas disease[16]. Furthermore, it has been reported that in Texas there have been seven major species of triatomine[19] of which *Triatoma gerstaeckeri*, *Triatoma lecticularia* and *Triatoma sanguisuga* are considered fairly common and have extensive geographic distributions including many northern states in northeastern Mexico[42]. Many Texas counties that are bordering Mexico have records of the presence of bugs infected with *T. cruzi*[19]. It remains to be seen which other triatomine species exist in the state of Coahuila and what the population abundance, seasonality, infection rate and vectorial capacity are. Studies conducted in Nuevo Leon, a Mexican State next to Coahuila, reported *T. cruzi* infection in collected domestic and wild *Triatoma gerstaeckeri* made in municipality of General Teran[23,26]. A recent study carried out by our research group, reported the presence of native triatomines *T. gerstaeckeri* and *T. rubida* in the north of Coahuila near to the coal region, which may represent another risk factor for local Chagas disease infection[25].

Regarding the disease, at this stage it can be said that there are several elements that indicate the presence of autochthonous cases of Chagas. First of all, we have the confirmation of eleven positive samples by two different serological tests and their clinical histories. Secondly, the fact that none of the individuals who tested positive had travelled to high endemicity areas, restricts the possibility of acquiring the infection elsewhere. Thirdly, we have shown in another publication the presence of two triatomine bugs in Coahuila (i.e. *T. gerstaeckeri* and *T. rubida*),

which have been recognized as species of medical importance in the USA. Fourthly, there are some paleoparasitological reports[2,39] that demonstrate that ancient mummies (aged *circa* 1,000 years) found by the Rio Grande (Rio Bravo) border between Coahuila and Texas were indeed infected with *T. cruzi*. All the above evidence led us to believe that there is an as yet unraveled transmission cycle of *T. cruzi* in the region and that therefore, more detailed studies are required to fully assess the impact and magnitude of risk to the human population.

Recognizing the problem of Chagas disease by health authorities and inhabitants of this particular region is a very important aim to be achieved and nowadays much work has yet to be carried out in these areas. There is also the problem of the treatment since there is no consensus about the correct or optimal management of indeterminate and chronic cases[1]. Until now there is no effective treatment for the indeterminate and chronic cases. Although there are studies on developing alternative autologous stem cell treatment and experimental drugs, its management has been limited to the treatment of clinical presentation, e.g. heart failure or arrhythmias[28]. It is important at the time of detection of indeterminate cases the decision of treatment in order to prevent the chronic condition leading to high costs and mortality in health[47].

CONCLUSION

In this study, we report the presence of autochthonous cases of Chagas disease in the coal zone of Coahuila, which highlights the importance of screening studies and finding cases more frequently and with greater geographic coverage. It is very important to disseminate information about the disease, the parasite and its insect vector between inhabitants living in risk areas. With extensive preventative actions the epidemiological costs will be lower and patient expectations about treatment will be better. Therefore we stress the urgent need to continue with more studies to determine the extent of the Chagas problem in the state and in the northeastern region of the country.

RESUMO

Soroprevalência da infecção pelo *T. cruzi* em doadores de sangue e cardiomiopatia chagásica em pacientes da região carbonífera de Coahuila, México

Contexto e Objetivo: A doença de Chagas é mundialmente considerada uma doença emergente, é endêmica no México e no estado de Coahuila e considerada de pouca relevância. O objetivo do estudo foi determinar a soroprevalência da infecção pelo *T. cruzi* em doadores de sangue e cardiomiopatia chagásica em pacientes da região carbonífera de Coahuila, México. **Desenho e Local:** Estudo epidemiológico, exploratório e prospectivo em um hospital geral no período de janeiro a junho de 2011. **Métodos:** Foram realizados testes de laboratório ELISA e hemoglutinação indireta em três grupos de indivíduos: 1) doadores de sangue voluntários assintomáticos, 2) pacientes internados na área de cardiologia e 3) pacientes com cardiomiopatia dilatada. **Resultados:** Foram achados três níveis de soroprevalência: 0,31% em indivíduos doadores de sangue assintomáticos, 1,25% em pacientes cardiopatas e, em pacientes com cardiomiopatia dilatada 21,14%. **Conclusão:** Detectamos casos autóctones de doença de Chagas em área considerada não endêmica. Deve ser determinada sua importância na saúde pública regional e local, para estabelecer os detalhes do mecanismo de transmissão. O estudo ainda está em desenvolvimento.

ACKNOWLEDGEMENTS

The first author is grateful for the support given to CONACyT through a scholarship for doctoral studies # 392195. We would also like to thank the staff of the General Hospital No. 24 of the Mexican Social Security Institute in Nueva Rosita, Coahuila for the facilities granted.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Amunárriz M, Quito S, Tandazo V, Lopez M. Seroprevalencia de la enfermedad de Chagas en el cantón Aguarico, Amazonia ecuatoriana. Rev Panam Salud Pública. 2010;28:25-9.

2. Araujo A, Jansen AM, Reinhard K, Ferreira LF. Paleoparasitology of Chagas disease: a review. Mem Inst Oswaldo Cruz. 2009;104(Suppl I):9-16.

3. Bear CB, Pye G, Steurer JF, Rodríguez R, Campman R, Peterson AT, *et al.* Chagas disease in a domestic transmission cycle in Southern Texas, USA. Emerg Infect Dis. 2003;9:103-5.

4. Bern C, Montgomery SP. An estimate of the burden of Chagas disease in the United States. Clin Infect Dis. 2009; 49(5):e52-4.

5. Bern C, Verastegui M, Gilman RH, Lafuente C, Galdos-Cardenas G, Calderon M, *et al.* Congenital *Trypanosoma cruzi* transmission in Santa Cruz Bolivia. Clin Infect Dis. 2009;49:1167-74.

6. Brasil PE, De Castro L, Hasslocher-Moreno AM, Sangenis LHC, Braga JU, ELISA versus PCR for diagnosis of chronic Chagas disease: systematic review and meta-analysis. BMC Infect Dis. 2010;10:337.

7. Cantley PT, Stramer SL, Townsend RL, Kamel H, Ofafa K, Todd CW, *et al*. The United States *Trypanosoma cruzi* infection study: evidence for vector-borne transmission of the parasite that causes Chagas disease among United States blood donors. Transfusion. 2012;52:1922-30.

8. Cruz-Reyes A, Pickering-López JM. Chagas disease in Mexico: an analysis of geographical distribution during the past 76 years: a review. Mem Inst Oswaldo Cruz. 2006;101:345-54.

9. Diaz JH. Recognizing and reducing the risks of Chagas disease (American Trypanosomiasis) in travelers. J Travel Med. 2008;15:184-95.

10. Dorn P, Perniciario L, Yabsley MJ, Roelling DM, Balsamo G, Diaz J, *et al.* Autochthonous transmission of *Trypanosoma cruzi*, Louisiana. Emerg Infect Dis. 2007;13:605-7.

11. Ferreira CS, Martinho PC, Amato-Neto V, Cruz RR. Pasteurization of human milk to prevent transmission of Chagas disease. Rev Inst Med Trop Sao Paulo. 2001;43:161-2.

12. Ferreira CS, Amato-Neto V, Gakiya E, Bezerra RC, Rodriguez-Alarcon RS. Microwave treatment of human milk to prevent transmission of Chagas disease. Rev Inst Med Trop Sao Paulo. 2003;45:41-2.

13. Galavíz-Silva L, Molina-Garza DP, Gonzalez-Santos MA, Mercado-Hernandez R, González-Galaviz JR, Rosales-Encina JL, *et al.* Update on seroprevalence of anti-*Trypanosoma cruzi* antibodies among blood donors in northeast Mexico. Am J Trop Med Hyg. 2009;81:404-6.

14. Hanford EJ, Zhan FB, Lui Y, Giordano A. Chagas disease in Texas: recognizing the significance and implications of evidence on the literature. Soc Sci Med. 2007; 65:60-79.

15. Herwaldt, BL. Laboratory acquired parasitic infection from accidental exposures. Clin Microbiol Rev. 2001;14:659-88.

16. Hotez P, Bottazzi ME, Dumonteil E, Valenzuela JG, Kamhawi S, Ortega J, *et al*. Texas and Mexico: sharing a legacy of poverty and neglected tropical diseases. PLoS Negl Trop Dis. 2012;6(3):e1497. doi:10.1371/journal.pntd.0001497.

17. Instituto Nacional de Estadística y Geografía. Mexico en cifras. Available from: http://www.inegi.org.mx/sistemas/mexicocifras/default.aspx

18. Kinoshita-Yanaga AT, Toledo MJO, Araujo SM, Vier BP, Gomes ML. Accidental infection by *Trypanosoma cruzi* follow up by the polymerase chain reaction: case report. Rev Inst Med Trop Sao Paulo. 2009;51:295-8.

19. Kjos SA, Snowden KF, Olson JG. Biogeography and *Trypanosoma cruzi* infection prevalence of Chagas disease vectors in Texas, USA. Vector Borne Zoonotic Dis. 2009;9:41-9. doi:10.1089/vbz.2008.0026.

20. Kun H, Moore A, Mascola L, Stevrer F, Lawrence G, Kubak B, *et al*. Transmission of *Trypanosoma cruzi* by heart transplantation. Clin Infect Dis. 2009;48:1534-40.

21. López-Antuñano JF, Rangel-Flores H, Ramos C. Diagnosis of Chagas' disease. Rev Latinoam Microbiol. 2000;42:121-9.

22. Marcon GEB, Albuquerque DM, Batista AM, Andrade PD, Almeida EA, Guariento ME, *et al. Trypanosoma cruzi*: parasite persistence in tissues in chronic chagasic Brazilian patients. Mem Inst Oswaldo Cruz. 2011;106:85-91.

23. Martinez-Ibarra JA, Galaviz-Silva L, Lara-Campos C, Trujillo-Garcia C. Distribución de los triatominos asociados al domicilio humano en el municipio de General Terán, Nuevo León, Mexico. Southwest Entomol. 1992;17:261-5.

24. Martinez-Ibarra JA, Grant-Guillen Y, Morales-Corona ZY, Haro-Rodriguez S, Ventura-Rodriguez LV, Nogueda-Torres B, *et al*. Importance of species of Triatominae (Heteroptera: Reduviidae) in risk of transmission of *Trypanosoma cruzi* in Western Mexico. J Med Entomol. 2008;45:476-82.

25. Martínez-Tovar JG, Rodríguez-Rojas JJ, Arque-Chunga W, Lozano-Rendon JA, Ibarra-Juárez LA, Dávila-Barboza A, *et al.* Nuevos registros geográficos y notas de infección de *Triatoma gerstaeckeri* (Stål) y *Triatoma rubida* (Uhler) (Hemiptera: Reduviidae: Triatominae) en Nuevo Leon y Coahuila, México. Acta Zool Mex. 2013;29:227-33.

26. Molina-Garza ZJ, Rosales-Encina JL, Galaviz-Silva L, Molina-Garza D. Prevalencia de *Trypanosoma cruzi* en triatominos silvestres de Nuevo León. Salud Pública Mex. 2007;49:37-44.

27. Muñoz J, Portús M, Cortachan M, Fumadó V, Gascon J. Congenital *Trypanosoma cruzi* infection in a non-endemic area. Trans R Soc Trop Med Hyg. 2007;101:1161-2

28. Murator C, Baranchuk A. Current and emerging therapeutic options for the treatment of chronic chagasic cardiomyopathy. Vasc Health Risk Manag. 2010;6:593-601.

29. Neal RA, Miles RA. Indirect haemagglutination test for Chagas' disease, with a simple method for survey work. Rev Inst Med Trop Sao Paulo. 1970;12:325-32.

30. Norma Oficial Mexicana NOM-003-SSA2-1993, "Para la disposición de sangre humana y sus componentes con fines terapéuticos". Available from: http://www.salud.gob.mx/unidades/cdi/nom/003ssa23.html

31. Noma Oficial Mexicana NOM-032-SSA2-2012, Para la vigilancia epidemiológica, prevención y control de las enfermedades transmitidas por vector. Available from: http://www.salud.gob.mx/unidades/cdi/nom/032ssa202.html

32. Novelo-Garza B, Benitez-Arvizu G, Pena-Benitez A, Galvan-Cervantes J, Morales Rojas A. Detección de *Trypanosoma cruzi* en donadores de sangre. Rev Med Inst Mex Seguro Soc. 2010;48:139-44.

33. Organización Mundial de la Salud. 2005. Reporte del grupo de trabajo científico sobre la enfermedad de Chagas. Copyright © World Health Organization on behalf of the Special Programme for Research and Training in Tropical Diseases. Actualización 2007. Available from: http://whqlibdoc.who.int/hq/2007/TDR_SWG_09_spa.pdf

34. Organización Panamericana de la Salud. Clasificación estadística internacional de enfermedades y problemas relacionados con la salud. 10ª rev. Washington: OPS; 1995. v. 3. (Publ Cient. no. 554).

35. Otani MM, Vinelli E, Kirchhoff LV, del Pozo A, Sands A, Veracauteren G, *et al.* WHO comparative evaluation of serologic assays for Chagas disease. Transfusion. 2009;49:1076-82.

36. Persing D. Update on testing for Chagas disease. In: 74th Meeting Blood Products Advisory Committee. At Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research. Silver Spring, Maryland; 2002 September 12. p. 262-5. Available from: http://www.fda.gov/OHRMS/DOCKETS/ac/02/transcripts/3892t1.doc

37. Ponce C. Transfusion transmission of Chagas disease in Honduras and other Central American countries. Medicina (B Aires). 1999;59(Suppl 2):135-7.

38. Ramos-Ligonio A, Ramirez-Sánchez ME, Gonzalez-Hernandez JC, Rosales-Encina JL, Lopez-Monteon A. Prevalencia de anticuerpos contra *Trypanosoma cruzi* en donadores de sangre del IMSS, Orizaba, Veracruz, México. Salud Pública Méx. 2006;48:13-21.

39. Reinhard K, Fink TM, Skiles J. A case of megacolon in Rio Grande Valley as a possible case of Chagas Disease. Mem Inst Oswaldo Cruz. 2003;98(Suppl 1):165-72.

40. Riarte A, Luna C, Sabatiello R, Sinagra A, Schiavelli R, De Rissio A, *et al*. Chagas' disease in patients with kidney traansplants: 7 years of experience, 1989-1996. Clin Infect Dis. 1999;29:561-7.

41. Sanchez-Guillen MC, Bernabe C, Guegan JF. Tibayrenc M, Velazquez-Rojas M, Martinez-Munguia J, *et al*. High prevalence anti-*Trypanosoma cruzi* antibodies, among blood donors in the state of Puebla, a non-endemic area of Mexico. Mem Inst Oswaldo Cruz. 2002;97:947-52.

42. Sarkar S, Strutz SE, Frank DM, Rivaldi CL, Sissel B, Sanchez-Cordero V. Chagas disease risk in Texas. PLoS Negl Trop Dis. 2010;4(10):e836. doi:10.1371/journal.pntd.0000836.

43. Sosa-Jurado F, Zumaquero-Ríos JL, Reyes PA, Cruz-Garcia A, Guzman-Bracho C, Monteon VM. Factores bióticos y abióticos que determinan la seroprevalencia de anticuerpos contra *Trypanosoma cruzi* en el municipio de Palmar de Bravo, Puebla, México. Salud Pública Mex. 2004;46:39-48.

44. Toso AM, Vial FU, Galanti N. Transmisión de la enfermedad de Chagas por vía oral. Rev Med Chil. 2011;139:258-66.

45. Vega-Chirinos S, Naquira-Velarde C. Manual de procedimientos de laboratorio para el diagnóstico de la tripanosomiasis americana (enfermedad de Chagas). Lima: Instituto Nacional de Salud; 2006. (Serie de Normas Tecnicas 26). ISBN 9972-857-30-1.

46. Velasco-Castrejon O, Valdespino JL, Tapia-Conyer R, Salvatierra B, Guzman-Bracho C, Magos C, *et al.* Seroepidemiología de la enfermedad de Chagas en México. Salud Pública Mex. 1992;34:186-96.

47. Viotti R, Vigliano C, Lococo B, Bertocchi G, Petti M, Alvarez MG, *et al.* Long-term cardiac outcomes of treating chronic Chagas disease with Benznidazole versus no treatment. Ann Intern Med. 2006;144:724-34.

Received: 20 May 2013
Accepted: 10 September 2013

Rev. Inst. Med. Trop. Sao Paulo
56(2):175-177, March-April, 2014
doi: 10.1590/S0036-46652014000200015

BRIEF COMMUNICATION

TWO NEW RECORDS OF *Isomyia paurogonita* FANG AND FAN, 1986 AND *Sumatria latifrons* Malloch, 1926 (DIPTERA: CALLIPHORIDAE) FROM NORTHERN THAILAND, WITH REVISED KEY TO THE SPECIES OF *Isomyia*

Nophawan BUNCHU(1,2), Kittikhun MOOPHAYAK(3), Sangob SANIT(4), Kabkaew L. SUKONTASON(4), Kom SUKONTASON(4) & Hiromu KURAHASHI(5)

SUMMARY

During the annual fly survey at Doi Nang Kaew in Doi Saket District, Chiang Mai Province of Thailand in 2011, *Isomyia paurogonita* Fang & Fan, 1986 (Diptera: Calliphoridae) and *Sumatria latifrons* Malloch, 1926 (Diptera: Calliphoridae) were collected for the first time in Thailand. They are the rare species of the subfamily Rhiniinae (tribe Cosminini). Prior to this finding, fifteen species of *Isomyia* and two species of *Sumatria* were recorded from Thailand. Therefore, 96 blow fly species have been found in this country. These new locality records of both flies are very important for further research on their biology and ecology in Thailand.

KEYWORDS: Blow fly; *Isomyia paurogonita; Sumatria latifrons;* Key; Thailand.

During fly surveys conducted in forested and mountainous areas in 2011, a large number of blow flies were collected from the mountain, namely Doi Nang Kaew, in Doi Saket District of Chiang Mai Province, northern Thailand. The specimens were then pinned, labeled, and identified by the first and last authors. The identification revealed that some of the blow flies collected are *Isomyia paurogonita* Fang and Fan, 1986 (Fig. 1 A, B) and *Sumatria latifrons* Malloch, 1926 (Fig. 2 A, B). The voucher specimens were deposited in the collection of the International Department of Dipterology, Japan.

Recently, 94 blow fly species were found and recorded from Thailand[1,2]. They were classified to nine subfamilies; i.e., Ameniinae, Calliphorinae, Luciliinae, Phumosiinae, Polleniinae, Bengaliinae, Auchmeromyiinae, Chrysomyinae, and Rhiniinae[1]. The genera *Isomyia* and *Sumatria* are included in the tribe Cosminini of the subfamily Rhiininae. Fifteen species of *Isomyia* have been previously recorded from Thailand including *Isomyia borneensis* (Perris, 1951), *Isomyia cupreoviridis* (Malloch, 1928), *Isomyia dotata* (Walker, 1856), *Isomyia facialis* James, 1970, *Isomyia hetauda* Kurahashi & Thapa, 1994, *Isomyia lugubris* James, 1970, *Isomyia oestracea* (Séguy, 1934), *Isomyia pichoni* (Séguy, 1934), *Isomyia pictifacies* (Bigot, 1877), *Isomyia pseudonepalana* (Senior-White, Aubertin & Smart, 1940), *Isomyia pseudoviridana* (Peris, 1952), *Isomyia singhi* Kurahashi & Thapa, 1994, *Isomyia versicolor* (Bigot, 1877), *Isomyia viridaurea* (Wiedemann, 1819), and *Isomyia watanasaki* Kurahashi & Bunchu, 2011[1,2]. Meanwhile, only two species of *Sumatria*, *Sumatria chiekoae* Kurahashi & Tumrasavin, 1992 and *Sumatria brevis* James, 1966 have already been recorded from Thailand. By adding two new species to the existing records, 96 blow fly species have been listed in Thailand.

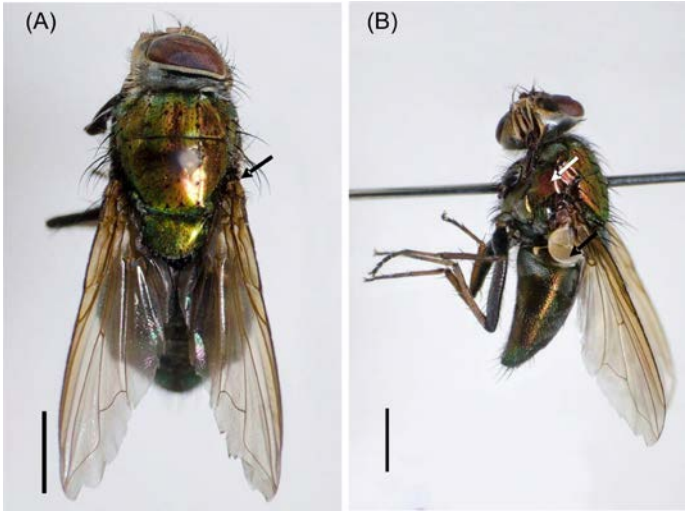


Fig. 1 - *Isomyia paurogonita* Fang and Fan, 1986, female (A) Habitus, dorsal view (bar = 2 mm), Arrow indicates a fuscous basicosta ; (B) Habitus, lateral view (bar = 2 mm), White arrow indicates a mesopleuron largely clothed with yellow hairs; while black arrow points a tongue-shape thoracic squama.

(1) Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Muang District, Phitsanulok 65000, Thailand.
(2) Centre of Excellence in Medical Biotechnology, Faculty of Medical Science, Naresuan University, Muang District, Phitsanulok 65000, Thailand.
(3) Mahidol University, Nakhonsawan Campus, Nakhonsawan 60130, Thailand.
(4) Department of Parasitology, Faculty of Medicine, Chiang Mai University, Muang District, Chiang Mai 50200, Thailand.
(5) International Department of Dipterology, Hikawadai 1-2-21, Higashikurume City, Tokyo 203-0004, Japan.
Correspondence: Dr. Nophawan Bunchu, Faculty of Medical Science, Naresuan University, Muang District, Phitsanulok 65000, Thailand. E-mail: bunchu_n@hotmail.com

As for *Isomyia paurogonita*, it was firstly described in the Yunnan Province, China and hitherto was known in Malaysia (Borneo, Malaya)[3]. However, the knowledge of its bionomics is limited. HEO *et al.*[3] reported the adult morphology and collection places as well. This fly species was found in forested and rural areas in peninsular Malaysia at various altitudes. The key to Oriental *Isomyia* species of the Viridurea has been previously reported by JAMES[4]. To update the taxonomic key of the genus *Isomyia* of Thailand, the latest edition key of KURAHASHI & BUNCHU[6] is revised and provided in this report.

Key to the species of *Isomyia*

1. Subcostal sclerite (Sc) next to humeral cross vein (h) bare below 2
- Sc next to h setulose below 17

2. Body very stout; thoracic squama strongly lobulate 3
- Body usually slender; thoracic squama not lobulate, like tongue-shape 11

3. Lunule with several short black setulae 4
- Lunule bare 5

4. Body entirely black, with purplish tinge; mesonotum and abdomen with cinereous pollinosity which is rather uniform in density except on posterior margins of tergites ..... *I. lugubris* James
- Body entirely or largely metallic green, blue or purple in ground color; mesonotum more or less pollinose and tergite 5 green dorsally, with dense, ashy pollinosity which, at least from posterior view, largely conceals background in striking contrast to the dark blue to purple on tergites ..... 1+2-4 [No record from Thailand] ..... *I. chalconotum* James

5. Both mesopleural hairs and hairs of other pleural areas, soft and yellow to golden, except for usual black setulae just below notopleural suture 6
- Mesopleural hairs more extensively black than indicated above, with some soft black hairs on mesopleuron, sometimes remote from notopleural suture, and on sternopleuron 9

6. Basicosta bright yellow; epaulet yellowish 7
- Basicosta black; epaulet black 8

7. Pleura and abdomen densely pollinose in male, less so in female, but dorsum of tergite 5, when viewed laterally at angle, with tessellated pattern of pollinosity; black lateral bristles on tergite 1+2 surrounded, at least on three sides, by pale yellow hairs.... *I. viridaurea* (Wiedemann)
- Pleura and abdomen less densely pollinose, without tessellated pattern; black lateral bristles on tergite 1+2 surrounded by black hairs . ..... *I. pichoni* (Séguy)

8. Tergites 3-4 distinctly marginal banded, with median stripe in male, broadly metallic banded with copper tinge in female; wing hyaline in male and female, with distinct fuscous cloud apically in female; parafrontal in female about 1/2 as wide as frontal stripe at middle of frons; hind tibia with 1 *av* in male ..... *I. borneensis* James
- Tergites 3-4 without any marginal band and median stripe; wing hyaline, clear in male and with apical unclear/indistinct fuscous in female; parafrontal in female subequal to frontal stripe at middle of frons; hind tibia without *av* in male [No record from Thailand] ..... *I. electa* (Villeneuve)

9. Alar and thoracic squamae wholly dark brown to black ..... *I. oestracea* (Séguy)
- Alar and thoracic squamae white, at least at base 10

10. Alar squama, in both sexes, white on basal 1/2 or more; thoracic squama white at base; larger species, 10.0-11.0 mm in length ..... *I. pictifacies* (Bigot)
- Alar and thoracic squamae wholly white in female; alar one white only anteroventrally in male; smaller species, 7.0-9.0 mm in length ..... *I. facialis* James

11. Basicosta yellow; male sternite 5 with normal shaped lateral lobes ..... *I. pseudonepalana* (Senior-White, Aubertin & Smart)
- Basicosta fuscous to black 12

12. Mesopleuron entirely or largely covered with black hairs except for several yellow ones on lower margin 13
- Mesopleuron largely clothed with yellow hairs on lower 1/3 to 1/2 and along posterior margin before row of yellow pilosity 15

13. Mesopleuron entirely covered with black hairs, with row of long black pilose hairs along posterior margin 14
- Mesopleuron largely clothed with black hairs, yellow hairs present on lower small part, with row of yellow pilosity ..... *I. versicolor* (Bigot)

14. Wing hyaline; lunule blackish setulose; AS3 largely fuscous; gena black; body metallic green; tergites 3--4 with fine black longitudinal stripe; hypopygium prominent, metallic green ..... *I. pseudoviridana* Peris
- Wing smoked on apical 1/2; lunule bare; antennae entirely yellowish orange; gena reddish on anterior 2/3; body dark olivious green, with bronzy tinge; tergites 3-4 with triangular blackish spot; hypopygium normal, with GS1 dark olivious green ..... *I. singhi* Kurahashi & Thapa

15. Lunule distinctly with black setulae .... *I. paurogonita* Fang & Fan
- Lunule bare 16

16. Parafacial in profile narrower than width of AS3; parafacial setulae fine, white, shorted than width of AS3 in lateral view [No record from Thailand] ..... *I. fulvicornis* (Bigot)
- Parafacial in profile 1.5-2.0 x as width of AS3: parafacial setulae usually black, if partially white, the longest black ones exceeding width of AS3 ..... *I. hetauda* Kurahashi

17. Thorax rather densely pollinose, with three distinct broad black stripes [No record from Thailand] ..... *I. malayensis* (Townsend)
- Thorax inconspicuously pollinose, with indistinct dark spots, but no stripes 18

18. Thoracic squama lobulate inwards; setulae on ventral surface of sc yellowish, fine; mesopleural and pteropleural hairs yellow ..... *I. cupreoviridis* (Malloch)
- Thoracic squama not lobulate; setulae of sc on ventral surface of wing black; mesopleural and pteropleural hairs black to fuscous 19

19. Head holoptic in male; male cercus free from one another ..... *I. dotata* (Walker)
- Head subholoptic in male; male cercus fused with one another .....*I. watanasaki* Kurahashi & Bunchu

Sumatria latifrons, whose type locality is Sumatra, Indonesia, was previously recorded from Malaysia (Borneo, Malaya) and Indonesia (Sumatra)[7]. Few reports related to its bionomics can be found. The previous report only indicated that adults were found on decaying animals in mountainous areas[5]. This species was already included in the key of KURAHASHI & BUNCHU, but had not yet been recorded in Thailand at that time[6]. These last authors mentioned that this species will be most probably found by future surveys in the southern part. Interestingly, we found it in Chiang Mai Province in the northern part.

Adults of *S. latifrons* can be identified by the following three important characteristics: 1) arista shortly pilose on ventral side, 2) thorax wholly brownish black, pollinose stripes limited to presutural area and gradually disappearing, and 3) femora partly testaceous or bright orange (Fig. 2).

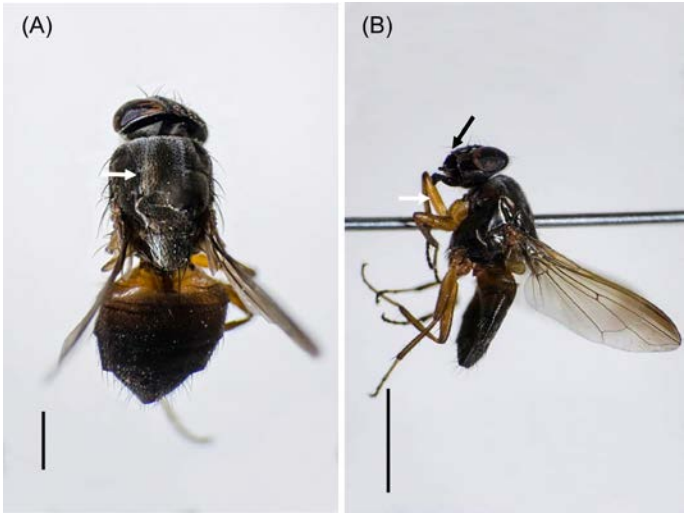


Fig. 2 - *Sumatria latifrons* Malloch, 1926, female (A) Habitus, dorsal view (bar = 1 mm), Arrow indicates a pollinose stripe on brownish black thorax; (B) Habitus, lateral view (bar = 2 mm), Black arrow indicates a shortly pilose arista (Only ventral side); while white arrow points a bright orange femur.

Note on specimens, localities, altitudes, collection dates and collectors:

Isomyia paurogonita Fang and Fan, 1986

Specimens examined. THAILAND: 1 ♀, Chiang Mai Province, Doi Saket District, Doi Nang Kaew, 1,016 m, 10.iii.2011, K. Moophayak; 4 ♂, same locality, 1,142 m, 13.xi.2011, H. Kurahashi.

Sumatria latifrons Malloch, 1926

Specimens examined. THAILAND: 1 ♀, Chiang Mai Province, Doi Saket District, Doi Nang Kaew, 1,142m, 13.xi.2011, H. Kurahashi; 1♀, same locality, 1,016m, 13.xi.2011, H. Kurahashi.

ACKNOWLEDGEMENTS

Financial support was provided by the Thailand Research Fund (RSA5580010). We thank the Division of Research Administration, Naresuan University, for defraying the publication cost.

RESUMO

Dois novos registros de *Isomyia paurogonita* Fang e Fan, 1986 e *Sumatria latifrons*, Malloch, 1926 (Diptera: Calliphoridae) do norte da Tailândia, com chave revisada para as espécies de *Isomyia*

Durante a pesquisa anual de moscas em Doi Nang Kaew no Distrito de Doi, Província de Chiang Mai, Thailandia, em 2011, *Isomyia paurogonita* Fang e Fan, 1986 (Diptera: Calliphoridae) e *Sumatria latifrons,* Malloch, 1926 (Diptera: Calliphoridae) foram coletados pela primeira vez na Tailândia. São espécies raras da sub-família Rhiniinae (tribo Cosminini). Antes deste achado, 15 espécies de *Isomyia* e duas de *Sumatria* foram relatadas na Tailândia. Portanto, 96 espécies de ”blow flies” foram encontradas neste país. Estes achados locais de ambas as moscas são bastante importantes para a posterior pesquisa de sua biologia e ecologia na Tailândia.

REFERENCES

1. Bunchu N. Blow fly (Diptera: Calliphoridae) in Thailand: distribution, morphological identification and medical importance appraisals. Int J Parasitol Res. 2012;4:57-64.

2. Bunchu N, Sukontason K, Sanit S, Chidburee P, Kurahashi H, Sukontason KL. Occurrence of blow fly species (Diptera: Calliphoridae) in Phitsanulok Province, Northern Thailand. Trop Biomed. 2012;29:532-43.

3. Heo CC, Aisha S, Kurahashi H, Omar B. New locality record of *Isomyia paurogonita* Fang & Fan, 1986 (Diptera: Calliphoridae) from peninsular Malaysia and Borneo. Trop Biomed. 2013;30:159-63.

4. James MT. A partial revision of the Oriental *Isomyia* of the Viridaurea group (Diptera: Calliphoridae). Smithsonian Contr Zool. 1970;67:1-20.

5. Kurahashi H, Benjaphong N, Omar B. Blow flies (Insecta: Diptera: Calliphoridae) of Malaysia and Singapore. Raffles B Zool. 1997(Suppl 5):74-82.

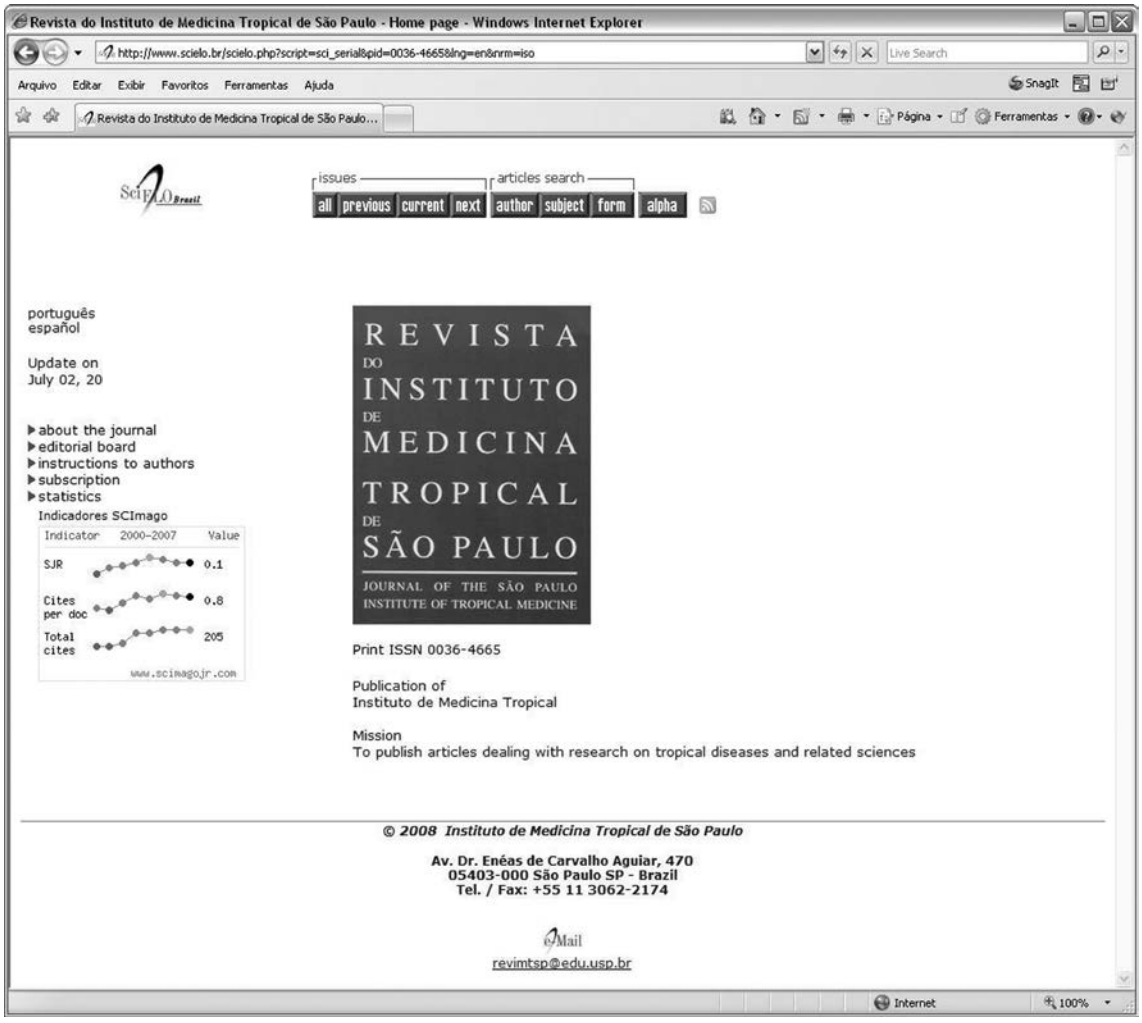
6. Kurahashi H, Bunchu N. The blow flies recorded from Thailand, with the description of a new species of *Isomyia* Walker (Diptera: Calliphoridae). Jpn J Syst Entomol. 2011;17:237-78.

7. Kurahashi H, Leh MU. The blow flies from Sarawak, East Malaysia (Diptera Calliphoridae), with practical keys and a checklist. Sarawak Mus J. 2009;87:299-300.

Received: 26 April 2013
Accepted: 25 June 2013

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(2):179-182, March-April, 2014
doi: 10.1590/S0036-46652014000200016

BRIEF COMMUNICATION

SPECIES COMPOSITION OF CARRION BLOW FLIES IN NORTHERN THAILAND: ALTITUDE APPRAISAL

Kittikhun MOOPHAYAK(1), Tunwadee KLONG-KLAEW(2), Kom SUKONTASON(2), Hiromu KURAHASHI(3), Jeffery K. TOMBERLIN(4) & Kabkaew L. SUKONTASON (2)

SUMMARY

Distribution and occurrence of blow flies of forensic importance was performed during 2007 and 2008 in Chiang Mai and Lampang Provinces, northern Thailand. Surveys were conducted in forested areas for 30 minutes using a sweep net to collected flies attracted to a bait. A total of 2,115 blow flies belonging to six genera and 14 species were collected; *Chrysomya megacephala* (Fabricius) (44.7%), *C. pinguis* (Walker) (15.1%), *C. chani* Kurahashi (9.3%), *C. thanomthini* Kurahashi & Tumrasvin (0.3%); *Achoetandrus rufifacies* (Macquart) (10.5%), *A. villeneuvi* (Patton) (2.2%); *Lucilia papuensis* Macquart (2.2%), *L. porphyrina* (Walker) (12.4%), *L. sinensis* Aubertin (0.7%); *Hemipyrellia ligurriens* (Wiedemann) (1.3%), *H. pulchra* (Wiedemann) (0.1%); *Hypopygiopsis infumata* (Bigot) (0.6%), *Hy. tumrasvini* Kurahashi (0.2%) and *Ceylonomyia nigripes* Aubertin (0.4%). Among them, *C. megacephala* was the predominant species collected, particularly in the summer. The species likely to prevail in highland areas are *C. pinguis, C. thanomthini, Hy. tumrasvini, L. papuensis* and *L. porphyrina*.

KEYWORDS: Blow flies; Carrion flies; Forensic entomology; Altitude; Thailand.

Blow flies (Diptera: Calliphoaridae) represent a key group of insects used as entomological evidence in forensic investigations throughout the world including, but not limited to, North America[1,5,6], Europe [2] and Asia[10,13]. A review of forensic entomology cases occurring in northern Thailand between 2000 and 2006 was conducted[13]. For 30 cases, 30 cadavers from various places of death (*e.g*., forested, suburban and urban areas) were investigated, with forested areas being the most common places of death. However, currently, little is known about the environmental factors regulating the distribution of blow flies, particularly those of forensic importance, such as altitude. Up until now, studies of carrion-frequenting blow flies have been limited in Thailand[3,11].

This study was conducted to elucidate species composition of blow flies in northern Thailand and determine whether blow fly species distribution could be related to altitude activity. This information will provide a database on blow flies from a specific location in northern Thailand, particularly in shaded forested areas, where deaths occur most commonly. Such information could prove vital when determining whether remains have been moved between regions.

The study periods were July 2007 for the rainy season, December 2007 for the winter and March 2008 for the summer. Adult fly collections were performed in the forested areas of Chiang Mai and Lampang provinces in northern Thailand. Such areas comprised of mixed deciduous forest. Five sites were selected for each period. Four sites were selected from Chiang Mai, *i.e*., Huey Tueng Tao reservoir (Mueang district); Siridhon observatory at Doi Suthep-Pui Mt. (Mueang district); Headquarters at Doi Suthep-Pui Mt. (Mueang district); and Doi Nang Kaew (Doi Saket district). One site was selected from a mountainous area in Lampang province (Doi Khun Tan, Mae Ta district). Table 1 and Figures 1 and 2 display the collection sites for assessing the distribution of carrion blow flies.

Adult flies attracted to baited meat were caught with a sweep net at different times of the day between 10.00 a.m. and 3.00 p.m., based on the flight activity and feeding time of flies in this group (HK, personal observation). The one-day tainted beef (500 g placed on a plastic plate) was used as bait, which was placed on the ground. The altitudes were coordinated using a handheld e-Trex® Garmin GPS. The collection period was performed in 30 min. The captured flies were transferred to a transparent jar containing ethyl acetate. The dead flies were then transferred to a transparent tube for identification in the laboratory of the Department of Parasitology, Faculty of Medicine, Chiang Mai University. All flies collected were pinned individually, and subsequently identified

(1) Mahidol University Nakhon Sawan Campus, Nakhon Sawan, Thailand.
(2) Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
(3) Department of Medical Entomology, National Institute of Infectious Diseases, Tokyo, Japan.
(4) Department of Entomology, Texas A&M University, College Station, Texas, USA.
Correspondence to: Dr. Kabkaew L. Sukontason, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand. E-mail: kabkaew.s@cmu.ac.th

Table 1
Collection sites of carrion blow flies in northern Thailand during 2007 and 2008

Altitude (asl)	Site	Location (District, Province)	Latitude (North)	Longtidute (East)
300-450 m	A	Huey Tueng Tao (Mueang, Chiang Mai)	18° 51' 52"	98° 56' 16"
	B	Samoeng-Hang Dong Rd. (Hang Dong, Chiang Mai)	18° 45' 14"	98° 52' 54"
451-600 m	C	Doi Khun Tarn (Mae Ta, Lampang)	18° 23' 31"	99° 12' 55"
751-900 m	D	Sirindhorn observatory (Mueang, Chiang Mai)	18° 47' 19"	98° 55' 17"
	E	Headquaters Suthep-Pui (Mueang, Chiang Mai)	18° 48' 40"	98° 55' 00"
901-1,050 m	F	Doi Nang Kaew (Doi Saket, Chiang Mai)	19° 03' 46"	99° 22' 37"

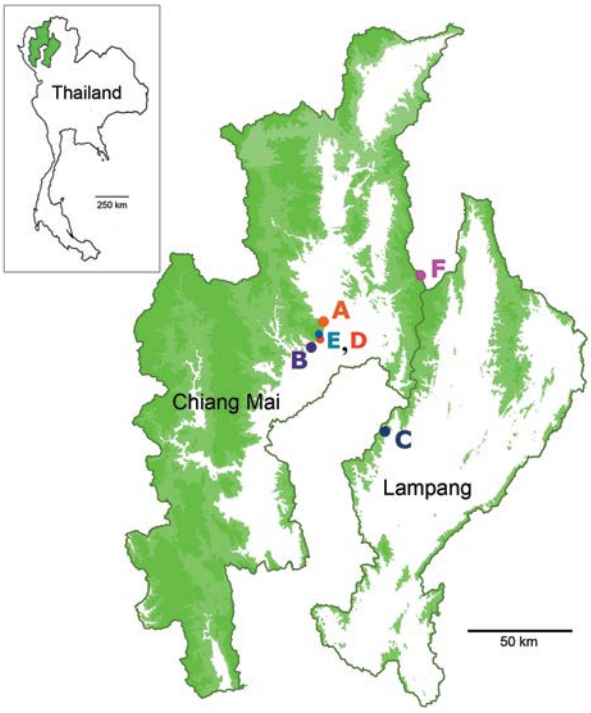


Fig. 1 - Carrion blow fly collection sites in northern Thailand. A (Huey Tueng Tao, Mueang, Chiang Mai); B (Samoeng-Hang Dong Rd., Hang Dong, Chiang Mai) at 300-450 m; C (Doi Khun Tarn, Mae Ta, Lampang) at 451-600 m; D (Sirindhorn observatory, Mueang, Chiang Mai); E (Headquarters Suthep-Pui, Mueang, Chiang Mai) at 751-900 m; F (Doi Nang Kaew, Doi Saket, Chiang Mai) at 901-1,050 m.

Fig. 2 - General topography of the blow fly collections in Northern Thailand. Upper display of site C in Fig. 1 (Doi Khun Tarn, Mae Ta, Lampang) at 451-600 m; with lower display of site F in Fig. 1 (Doi Nang Kaew, Doi Saket, Chiang Mai) at 901-1,050 m.

from external morphology using the key of KURAHASHI *et al*.[7] and the aid of a binocular microscope.

A total of 2,115 carrion blow flies belonging to 14 species of six genera were collected in this study. They were *Chrysomya chani* Kurahashi, *C. megacephala* (Fabricius), *C. pinguis* (Walker), *C. thanomthini* Kurahashi & Tumrasvin; *Ceylonomyia nigripes* Aubertin; *Achoetandrus rufifacies* (Macquart), *A. villeneuvi* (Patton); *Hemipyrellia ligurriens* (Wiedemann), *H. pulchra* (Wiedemann); *Hypopygiopsis infumata* (Bigot), *Hy. tumrasvini* Kurahashi; *Lucilia papuensis* Macquart, *L. porphyrina* (Walker) and *L. sinensis* Aubertin. Among them, *C. megacephala* was the predominant species collected, particularly in the summer (Table 2) which is similar to results found in previous investigations such as those in Chiang Mai[12,14], Phitsanulok in the north[3], and Ubon Ratchathani in the northeastern region of Thailand[4]. Approximately 945 of this fly species were attracted to the bait after its placement. Their frequency of occurrence was consistent throughout the season with its populations peaking during the summer, as previously reported in Thailand[12].

Data from this study indicate an altitude of distribution for each blow fly species in mixed deciduous forests (*e.g*., Huey Tueng Tao reservoir, Doi Suthep-Pui Mt., Doi Nang Kaew and Doi Khun Tan). On the basis of restricted distribution at various altitudes, some species, such as *C. pinguis, C. thanomthini, Hy. tumrasvini, L. papuensis,* and *L. porphyrina* are likely to prevail in highland areas. This observation correlates with

Table 2
Species composition of blow flies collected in northern Thailand during 2007 and 2008

Species	Altitude (metre above sea level)				Total
	300-450	451-600	751-900	901-1,050	
Achoetandrus rufifacies	191	8	3	1	203
	10	5	0	1	16
	3	1	0	0	4
A. villeneuvi	8	4	3	3	18
	0	8	2	2	12
	0	10	0	6	16
Chrysomya chani	2	24	3	3	32
	0	66	4	11	81
	0	79	3	1	83
C. pinguis	0	5	37	192	234
	0	19	2	10	31
	0	23	4	28	55
C. megacephala	342	43	87	161	633
	57	25	28	15	125
	40	100	16	31	187
C. thanomthini	0	0	0	5	5
	0	0	0	0	0
	0	0	0	1	1
Ceylonomyia nigripes	0	0	0	0	0
	0	5	0	0	5
	0	4	0	0	4
Hemipyrellia ligurriens	2	0	0	0	2
	11	0	0	0	11
	14	0	0	0	14
H. pulchra	0	0	0	0	0
	0	0	0	1	1
	0	0	0	0	0
Hypopygiopsis infumata	1	7	1	0	9
	0	1	0	0	1
	1	1	0	0	2
Hy. tumrasvini	0	0	0	0	0
	0	0	0	4	4
	0	0	0	0	0
Lucilia papuensis	0	0	11	14	25
	8	3	0	7	18
	0	2	0	1	3
L. porphyrina	0	5	24	71	100
	0	0	0	27	27
	2	84	13	37	136
L. sinensis	0	0	0	1	1
	0	0	0	0	0
	0	15	1	0	16

Numbers in the yellow line represent summer collection (March 2008), those in the blue line represent rainy season collection (July 2007) and those in the orange line represent winter season collection (December 2007).

data from previous investigations conducted in southeast Asian countries, *e.g*., Thailand, Malaysia, Singapore, Cambodia, Laos, Vietnam, and the Philippines[3,7-9]. Interestingly, *C. megacephala* is widespread as it was commonly collected from lowland to highland areas. A previous study revealed that *C. megacephala* appears to spread up to the highest peak of Inthanon mountain in Chiang Mai province (2,667 meters above sea level) (KL Sukontason, unpublished data), suggesting strong adaptation to a man-made environment at various altitudes from lowland to highland fauna (Fig. 3).

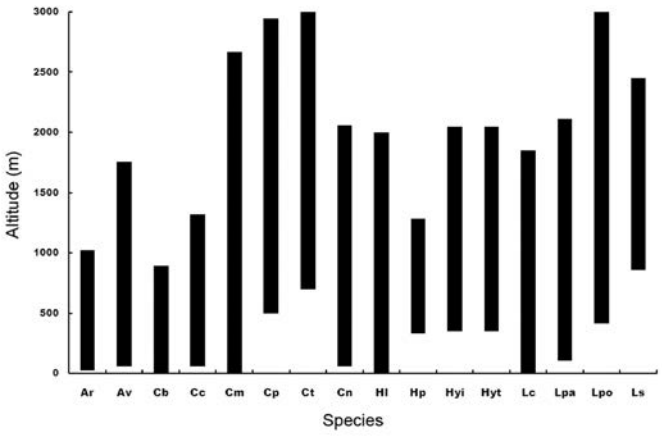


Fig. 3 - Altitudinal distribution of carrion blow flies. Data gathered from Thailand [this study, recent survey in 2013 and TUMRASVIN *et al.*[14]], Malaysia and Singapore[7], Cambodia, Laos and Vietnam[8] and the Philippines[9]. Abbreviations: Ar, *Achoetandrus rufifacies*; Av, *A. villeneuvi*; Cb, *Chrysomya bezziana*; Cc, *Chrysomya chani*; Cm, *Chrysomya megacephala*; Cp, *Chrysomya pinguis*; Ct, *Chrysomya thanomthini*; Cn, *Ceylonomyia nigripes*; Hl, *Hemipyrellia ligurriens*; Hp, *Hemipyrellia pulchra*; Hyi, *Hypopygiopsis infumata*; Hyt, *Hypopygiopsis tumrasvini*; Lc, *Lucilia cuprina*; Lpa, *Lucilia papuensis*; Lpo, *Lucilia porphyrina*; Ls, *Lucilia sinensis*.

On the other hand, no specimen of *Lucilia cuprina* (Wiedemann), which is another forensically important blow fly, was captured in this study. A possible explanation for this may be the tendency of this species to prevail more in lowland and residential areas, which were not included in this study. *L. cuprina* was collected in and around human habitations, such as restaurants, garbage piles, school cafeterias and paddy fields in the urbanized areas of Ubon Ratchathani[4], as similarly observed in Phitsanulok province[3]. However, a study conducted by TUMRASVIN *et al.*[14] in 1978 managed to collect this species at high altitudes up to 1,400 meter, but in very low numbers. It should be noted that the altitudinal distribution of fly fauna varies, based on seasonal changes, even in northern Thailand[14].

In conclusion, the data obtained from this study provides species composition of forensically important blow flies as related to altitude. Based on the fact that several synanthropic species are present at a wide range of altitudes, their application for explaining the re-location of corpses is limited, especially when the immature specimens of these flies are found to be associated. However, in the case of some feral species having unique foci of altitude, such data are crucial for detecting movement of corpses post-mortem, particularly when moved from a high altitude in forested areas to lowland residential areas.

RESUMO

Composição das espécies de moscas-varejeiras do lixo no norte da Tailândia: avaliação da altitude

Distribuição e ocorrência de moscas-varejeiras de importância forense foi realizada durante 2007 e 2008 nas províncias de Chiang Mai e Lampang, norte da Tailândia. Os levantamentos foram feitos em áreas de florestas, durante 30 minutos usando rede de varredura para coletar as moscas atraídas por iscas. Um total de 2115 moscas-varejeiras pertencentes a seis gêneros e 14 espécies foram coletados; *Chrysomya megacephala* (Fabricius) (44,7%), *C. pinguis* (Walker) (15,1%), *C. chani* Kurahashi (9,3%), *C. thanomthini* Kurahashi & Tumrasvin (0,3%); *Achoetandrus rufifacies* (Macquart) (10,5%), *A. villeneuvi* (Patton) (2,2%); *Lucilia papuensis* Macquart (2,2%), *L. porphyrina* (Walker) (12,4%), *L. sinensis* Aubertin (0,7%); *Hemipyrellia ligurriens* (Wiedemann) (1,3%), *H. pulchra* (Wiedemann) (0,1%); *Hypopygiopsis infumata* (Bigot) (0,6%), *Hy. tumrasvini* Kurahashi (0,2%) e *Ceylonomyia nigripes* Aubertin (0,4%). Dentre elas a *C. megacephala* foi a espécie predominante coletada particularmente no verão. As espécies capazes de predominar nas áreas altas são: *C. pinguis, C. thanomthini, Hy. tumrasvini, L. papuensis* e *L. porphyrina*.

ACKNOWLEDGMENTS

The work was supported by the Thailand Research Fund and Royal Golden Jubilee Ph.D. Program (PHD/0203/2548 and PHD/0246/2550). The authors of this study are grateful to the Faculty of Medicine and Chiang Mai University for defraying the publication cost.

REFERENCES

1. Anderson GS. Wildlife forensic entomology: determining time of death in two illegally killed black bear cubs. J Forensic Sci. 1999;44:856-9.

2. Benecke M. Six forensic entomology cases: description and commentary. J Forensic Sci. 1998;43:797-805.

3. Bunchu N, Sukontason K, Sanit S, Chidburee P, Kurahashi H, Sukontason KL. Occurrence of blow flies species (Diptera: Calliphoridae) in Phitsanulok province, northern Thailand. Trop Biomed. 2012;29:532-43.

4. Chaiwong T, Srivoramas T, Sukontason K, Sanford M, Moophayak K, Sukontason KL. Survey of the synanthropic flies associated with human habitations in Ubon Ratchathani province of northeast Thailand. J Parasitol Res. 2012;2012:613132. doi:10.1155/2012/613132.

5. Goff ML, Odom CB. Forensic entomology in the Hawaiian Islands. Three case studies. Am J Forensic Med Pathol. 1987;8:45-50.

6. Greenberg B, Kunich JC. Entomology and the law: flies as forensic indicators. Cambridge: Cambridge University Press; 2002.

7. Kurahashi H, Benjaphong N, Omar B. Blow flies (Insecta: Diptera: Calliphoridae) of Malaysia and Singapore. Raffles Bull Zool. 1997;(Suppl 5):1-88.

8. Kurahashi H, Chowanadisai L. Blow flies (Insecta: Diptera: Calliphoridae) from Indochina. Species Divers. 2001;6:185-242.

9. Kurahashi H, Magpayo FR. Blow flies (Insecta: Diptera: Calliphoridae) of the Philippines. Raffles Bull Zool. 2000;(Suppl 9):1-78.

10. Lee HL, Krishnasamy M, Abdullah AG, Jeffery J. Review of forensically important entomological specimens in the period of 1972-2002. Trop Biomed. 2004;21:69-75.

11. Lertthamnongtham S, Sukontason KL, Sukontason K, Piangjai S, Choochote W, Vogtsberger RC, *et al.* Seasonal fluctuations in populations of the two most forensically important fly species in northern Thailand. Ann Trop Med Parasitol. 2003;97:87-91.

12. Ngoen-klan R, Moophayak K, Klong-klaew T, Irvine KN, Sukontason KL, Prangkio C, *et al.* Do climatic and physical factors affect populations of the blow fly *Chrysomya megacephala* and house fly *Musca domestica*? Parasitol Res. 2011;109:1279-92.

13. Sukontason K, Narongchai P, Kanchai C, Vichairat K, Sribanditmongkol P, Bhoopat T, *et al.* Forensic entomology cases in Thailand: a review of cases from 2000 to 2006. Parasitol Res. 2007;101:1417-23.

14. Tumrasvin W, Sucharit S, Kano R. Studies on medically important flies in Thailand. IV. Altitudinal distribution of flies belonging to Muscidae and Calliphoridae in Doi Indhanondh Mountain, Chiengmai, in early summer season. Bull Tokyo Med Dent Univ. 1978;25:77-81.

Received: 12 June 2013
Accepted: 18 July 2013

Rev. Inst. Med. Trop. Sao Paulo
56(2):183-184, March-April, 2014
doi: 10.1590/S0036-46652014000200017

LETTER TO THE EDITOR

CLINICAL MANIFESTATIONS SEEMED MORE SEVERE AMONG PATIENTS WITH ANTIBODY 4-FOLD OR A GREATER INCREASE IN TITER BOTH AGAINST pH1N1 AND AGAINST SEASONAL INFLUENZA THAN THOSE WHOSE ANTIBODY 4-FOLD OR GREATER INCREASE IN TITER WAS ONLY AGAINST ONE TYPE OF SEASONAL INFLUENZA

June 24, 2013

To the editor

In 2009, after an emergence of pandemic influenza A(H1N1) (pH1N1), one of our serological investigations showed 23 students whose paired serum antibodies simultaneously presented 4-fold or greater increase in titer both against pH1N1 and against seasonal influenza (SI). This caught our attention and in order to further understand clinical features of these cases, a case control study was conducted. From October 2009 to September 2012, we collected the acute and convalescent phase serum samples from patients whose throat swabs were positive for pH1N1 or SI by real-time reverse transcription polymerase chain reaction (RT-PCR)[5]. The patients' information, including clinical symptoms, self-protect measures, and social activities after illness, was also collected through inspecting medical records, and interviewing face to face or by telephone.

Paired blood samples were used to test antibodies against 2009 pandemic A(H1N1) influenza virus and four kinds of seasonal influenza subtype virus (H3N2, H1N1, By, and Bv), which were detected by Hemagglutination inhibition (HI) assays. The influenza viruses used were A/GuangdongLiwan/SWL1538/2009 (H1N1), A/TianjinJinnan/15/2009 (H1N1), A/FujianTongan/196/2009 (H3N2), B/JiangxiXiushui/32/2009 Victoria, and B/Guangdong Xindong/134/2009 Yamagata. The HI assay was performed using a standard technique[2]. Serum samples were treated with receptor destroying enzyme to remove nonspecific hemagglutination. Serum samples were diluted in serial two-fold dilutions from 1:10 to 1:640 and then mixed with chicken red blood cells and the virus strain. HI titer was determined as the highest dilution of serum which showed hemagglutination inhibition.

During the study period, a total of 2,079 paired blood samples were collected, of which 68 cases (3.27%) whose antibodies were simultaneous 4-fold or greater increase in titer against pH1N1 and SI. Among the 68 cases, the sex distribution was 61.76% (N = 42) male and 39.14% (N = 26) female, the age ranged from five to 81 years old (median age = 20), the patients had fevers ranging from 37.5 °C to 40.6 °C, the disease course lasted from two to 11 days. Primary clinical symptoms were a sore throat (65/68, 95.59%), a cough (37/68, 54.41%), and a headache (31/68, 45.59%). The proportion of patients who had arthralgia, nausea, vomiting and diarrhea was 8.82% (6/68), 7.35% (5/68), 4.41% (3/68), and 4.41% (3/68), respectively.

Of those whose antibodies presented 4-fold or greater increase in titer only against one subtype of SI, 136 cases were selected into a control group as a 1:2 match according to the following matching criteria: onset date (+/– 20 days), age (+/– 4 years), and sex. Compared to the control group (136 cases), the proportion of patients with a fever ≥ 38.5 °C, disease course ≥ 5 days, and clinical symptoms ≥ three episodes were significantly higher ($p < 0.05$) among the case group (68 cases) (Table 1).

Table 1
Comparing the clinical characteristics between case group (68 cases) and control group (136 cases)

Clinical symptoms	Case group		Control group		x^2	p
	No.	%	No.	%		
Fever ≥ 38.5 °C	26	38.24	33	24.27	4.30	0.04*
Disease course ≥ 5 days	19	27.94	14	10.29	10.41	0.00*
Cough	37	54.41	66	48.53	0.63	0.43
Sore throat	65	95.59	129	94.85	0.05	0.82
Headache	31	45.59	55	40.44	0.49	0.48
Nasal congestion	23	33.82	40	29.41	0.41	0.52
Rhino rhea	17	25.00	32	23.53	0.05	0.82
Sputum production	14	20.59	25	18.38	0.14	0.70
Fatigue	23	33.82	41	30.15	0.29	0.59
Myalgia	9	13.24	16	11.77	0.09	0.76
Chills	11	16.18	15	11.03	1.08	0.30
Arthralgia	6	8.82	15	11.03	0.24	0.63
Nausea	5	7.35	7	5.15	0.40	0.53
Vomiting	3	4.41	5	3.68	0.07	0.80
Diarrhea	3	4.41	2	1.47	1.64	0.20
Clinical symptoms ≥ 3 episodes above	32	47.06	43	31.62	4.65	0.03*

*$p < 0.05$

Taken together, we reported that of the 2079 influenza patients, 68 cases were found to have simultaneous 4-fold or greater increase in serum antibody titers against pH1N1 and SI, these cases appeared to have more severe clinical pictures, including higher fever, longer disease course and more episodes of clinical symptoms. A possible explanation for this might be that these cases presented with a co-infection. Before emergence of pH1N1, co-infection has been proved to exist in seasonal

influenza. For example, NISHIKAWA *et al.* found a patient who was simultaneously infected with seasonal influenza A(H1N1) and A(H3N2) during the epidemic of 1981[3]. In 2006, TODA *et al.* isolated the A/H3 and B viruses from an influenza patient[4]. In addition, GOKA *et al.* also found co-infection was associated with higher risk of admission to ICU/death[1]. Due to virus isolation not being conducted among our samples, more evidence regarding pH1N1 and SI needs to benefit from molecular virology research in future.

Tiegang LI
Ming WANG
Correspondence to: Ming Wang, M.D,
Guangzhou Center for Disease Control and Prevention,
Guangdong Province, 510440, China.
E-mails: wangming@gzcdc.org.cn
tiegang1977@126.com

REFFERENCES

1. Goka E, Vallely P, Mutton K, Klapper P. Influenza A viruses dual and multiple infections with other respiratory viruses and risk of hospitalisation and mortality. Influenza Other Respi Viruses. 2013;7:1079-87.

2. Kendal AP, MacDonald NE. Influenza pandemic planning and performance in Canada, 2009. Can J Public Health. 2010;101:447-53.

3. Nishikawa F, Sugiyama T. Direct isolation of H1N2 recombinant virus from a throat swab of a patient simultaneously infected with H1N1 and H3N2 influenza A viruses. J Clin Microbiol. 1983;18:425-7.

4. Toda S, Okamoto R, Nishida T, Nakao T, Yoshikawa M, Suzuki E, *et al*. Isolation of influenza A/H3 and B viruses from an influenza patient: confirmation of co-infection by two influenza viruses. Jpn J Infect Dis. 2006;59:142-3.

5. Wu W, Kang X, Bai Z, Liu L, Li J, Wu X, *et al.* Detection of pandemic influenza A/H1N1/2009 virus by real-time reverse transcription polymerase chain reaction. J Virol Methods. 2010;165:294-6.