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REVIEW

TOXOPLASMOSIS IN MEXICO: EPIDEMIOLOGICAL SITUATION IN HUMANS AND ANIMALS

Ivonne HERNÁNDEZ-CORTAZAR(1,2), Karla Y. ACOSTA-VIANA(1), Antonio ORTEGA-PACHECO(2), Eugenia del S. GUZMAN-MARIN(1), Armando J. AGUILAR-CABALLERO(2) & Matilde JIMÉNEZ-COELLO(1)

SUMMARY

Toxoplasmosis is a parasitic disease widely distributed throughout the world, infecting a wide variety of animal species including humans. In Mexico, this parasite has been detected in different parts of the country, particularly in the tropical areas where the parasite can remain infective for long periods of time due to the environmental conditions (i.e. high temperature and humidity over the whole year). Several epidemiological studies have been conducted in both human and animal populations, but despite the wide distribution of the agent in the country, there is a significant lack of knowledge on the parasite transmission, treatment alternatives and control measures. The lack of feral cat populations and control measures in sites of meat production for human consumption are playing a role that has led to the wide spread of the disease in the country, particularly in tropical areas of Southeastern Mexico. For these reasons, this manuscript aims to review the published information on relevant epidemiological aspects of infection with *T. gondii* in humans and animals from Mexico.

KEYWORDS: Toxoplasmosis; Neglected disease; Mexico; Endemic; Zoonosis.

INTRODUCTION

Toxoplasmosis is a worldwide parasitic zoonotic disease produced by the protozoan *Toxoplasma gondii* (*T. gondii*). This intracellular parasite can infect all warm-blooded animals including humans, marine mammals and birds[33,49,68,87]. Animals from the *Felidae* family play an important role in the epidemiology and maintenance of the disease since they can complete the life cycle of this parasite; they are the definitive hosts that can excrete thousands of environmentally resistant oocysts[47]. Since its first description in rodents (*Ctenodactylus gondii*) in North Africa by NICOLLE & MANCEAUX in 1908, the parasite has been recognized as a zoonotic agent[77]. At that time, the detection of *T. gondii* appeared to be only of academic interest, but when WOLF *et al.* found an associated encephalomyelitis in infants during 1939[98], *T. gondii* was identified as a cause of congenital disease. Currently, *T. gondii* is recognized as an important opportunistic pathogen of fetuses, newborns and patients with a variety of primary and secondary immunodeficiencies[81]. The most common source of infection for humans is through the ingestion of tissue cysts from undercooked meat, by eating food or drinking water contaminated with sporulated oocysts and congenital transmission[83].

Infection with the parasite can result in a broad range of clinical signs depending on the host animal species. *T. gondii* can be fatal in some species of marine mammals and marsupials[68]. However, only a small percentage of exposed humans or other animals develop clinical signs of the disease[47]. The most dangerous complications of toxoplasmosis are found in patients whose immunity has been depressed by malignancies and anti-tumor therapy, those with acquired immunodeficiency syndrome (AIDS)[52], or with immunosuppressive drugs following organ transplantation. Toxoplasmosis ranks high on the list of diseases that lead to the death of patients with AIDS[75]. Additionally, in case of maternal infection acquired during pregnancy, Toxoplasma can infect the fetus with variable severity, depending on the trimester at which the pregnant woman acquired the infection, and on the efficacy of the placental barrier[89]. The risk of congenital infection is lower when maternal infection occurs during the first trimester (10-15%) and higher when the infection occurs during the third trimester (60-90%)[84]. However, congenital infection usually leads to more severe disease when it occurs during the first trimester[67].

The seroprevalence of human toxoplasmosis can range from 10 to 50% in temperate developed countries to over 80% in developing countries of the tropics[73]. Mexico is among the developing countries where the infection is common due to environmental exposure. Sources of infection may vary greatly among different ethnic groups and geographical locations[94]. Infective oocysts are everywhere and can contaminate water, soil, fruits or vegetables[53]. Undercooked meat contaminated with tissue cysts may also be an important source of infection[94]. In Mexico, the seroprevalence ranges from 15 to 50% among the general population[62]. Mexico is a large country with a human

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population in 2010 of more than 112 million, spread all over the country, and with very different ecological regions including subtropical areas, arid regions, temperate regions (mountains), and a very large coastal area of the Atlantic and Pacific Oceans. Areas with the highest prevalence are wet coastal regions of the Gulf of Mexico and the Pacific (64%), while the arid region scored the lowest prevalence (13%)[97]. In many developing tropical countries, the presence of extensive or semi-extensive animal production systems (grazing animals) is very common, which increases the risk of contact with the agent[70]. The wide differences of seroprevalence among geographical regions may be related to several factors, such as dietary habits and climate variations. The latter has a significant influence on the presence and persistence of infective oocysts, especially in tropical conditions where the temperature and precipitation can maintain the soil moisture, so that the oocysts remain viable in the environment for long periods[76,80].

In studies conducted on different populations of animals in Mexico, a wide distribution of the parasite has been found; reports exist on family pets (dogs and cats)[15,31,39,63,74,85,92], as well as animal species raised for food production (poultry, goats, sheep and pigs)[11,34,50,60], and wildlife species.

The aim of this paper was to review the published literature about the current status of relevant epidemiological aspects of *T. gondii* infection in humans and animals from Mexico.

HUMAN STUDIES IN MEXICO

Adults: The toxoplasmosis situation in the adult population from Mexico has been largely explored (Table 1). In a study in Merida, Yucatán, Southeast Mexico, a significant association between 100 cases of spontaneous abortion and infection with *T. gondii* has been reported. Antibodies to *T. gondii* were found in 47% of the studied population, using a Sabin-Feldman test with titers of 1:64 and 1:128[99]. In another study conducted in Oaxaca (a state located in Southern Mexico), the general seroprevalence was 3.8% (124/3229), with a slight variation depending on the rural community sampled (range 1.3%-8.9%). All sampled communities were grouped in eight areas (I to VIII) based on altitude, longitude and topography. The highest prevalence rate was found in the Tehuantepec zone with 8.9% (zone VI), this area being where the highest seroprevalence was registered, whereas the coastal region of Oaxaca (with an altitude of 0 to 400 m) showed prevalence rates of 2.4, 4.6 and 5%. In the central and northern regions of Oaxaca, rates of 1.3, 2.0 and 2.7% were recorded. This large variation in the seroprevalence found in the same state is highly dependent on the geography and microclimates (humidity, altitude and temperature) of each particular region. However, the lower prevalence was associated to the almost total absence of cats and the lack of meat in their diet[64]. In 1992, from 29,279 blood samples evaluated from people in all the 32 states of the Mexican Republic, the highest prevalence rate was found in coastal areas (40-65%), in people with low socioeconomic status and also in women of reproductive age[97].

In another study, 350 women with high-risk pregnancies were studied. From them, 122 (34.9%) were seropositive to IgG and 76 (20.7%) to IgM *T. gondii* specific antibodies. In the same study, a group of women with recurrent spontaneous abortions were serologically evaluated and presented a seropositivity of 44.9% for IgG and 33.3% for IgM *T. gondii* antibodies[57]. Moreover, in the town of Comitan Chiapas, a southern Mexican state, a prevalence of 5% of *T. gondii* antibodies was detected in the general population and in 18% of women at risk of abortion[90].

The presence of antibodies to *T. gondii* in 59 cat owners through the indirect ELISA was investigated; 38 cases (64%) were positive to IgG and 70.8% of their cats were positive to IgG, 8.3% to IgM and 62.5% to IgA. Cohabitation with *T. gondii* infected cats, feeding them with leftovers and raw viscera, and lack of control over how to manage their feces were identified as important risk factors associated with humans becoming infected with the parasite[56].

In a public hospital in Durango, Northern Mexico, which is characterized by arid and dry environmental conditions, 343 pregnant women were evaluated for *T. gondii*. From them, 21 (6.1%) showed IgG antibodies but none (0%) IgM antibodies; a multivariate analysis showed that infection was associated with living in houses with earthen floors, residing outside the state of Durango, and consumption of turkey meat[24].

In another report from the same region, a comparison of the seroprevalence of IgG and IgM *T. gondii* antibodies among patients at a psychiatric hospital and a population of blood donors as a control group was performed. IgG antibodies were found in 25 (18.2%) of 137 psychiatric patients and 16 (8.9%) of 180 controls. Regarding IgM antibodies, psychiatric patients showed 4.4% compared with 2.2% in the control group[1]. In the same region (Durango), a prevalence of IgG antibodies in 32 (7.4%) of 432 healthy blood donors was reported, with 8 (1.8%) of them being also positive to IgM. The infection with *T. gondii* was associated with contact with cats. It was also determined that prevalence increases with age and decreases with a higher education level[19].

The prevalence of infection with *T. gondii* was investigated in two populations exposed to solid waste using an ELISA test. Seropositivity of IgG was 21.1% out of 90 scavengers and 8.4% out of 83 waste workers. Regarding IgM antibodies, 2.2% were presented in the waste collectors, but none (0%) in the waste workers. The seroprevalence was associated with the consumption of food found in the garbage and lack of education[16]. The epidemiology of infection with *T. gondii* was studied in people from three rural regions from the state of Durango, using an ELISA test. It was found that 110 (23.8%) of 463 individuals evaluated had IgG antibodies and 10 (2.2%) of these were also positive for IgM. The high prevalence of infection was observed in participants older than 70 years of age and those with good conditions in their homes. Infection was also associated with the consumption of meat from turkeys and squirrels[2]. In another study, the usefulness of filter paper-embedded blood (FPEB) for the diagnosis of *T. gondii* in pregnant woman was evaluated; IgM, IgG and IgG avidity was determined. IgM detection in FPEB was 92% sensitive and 100% specific. The results indicated that FPEB is useful to infer the infection phase, and thus to speed clinical decisions in congenital toxoplasmosis management[38]. In a further study conducted in nine rural communities in the state of Durango, 439 pregnant women were evaluated through a commercial ELISA. In total, 36 (8.2%) women had IgG antibodies and 10 (2.3%) were also seropositive to IgM. All IgM positive sera showed high values of IgG avidity, suggesting a chronic infection. The seroprevalence was significantly higher in women of low socioeconomic status (14%) than in those with a higher socioeconomic status (6.6%). Multivariate analysis showed that infection with *T. gondii* had an association with dwellings with earthen floors[28].

Table1
Seroprevalence of *Toxoplasma gondii* in human adults from Mexico

Year	Region	State	Test	Category	Prevalence (%)	Patients tested	Reference
1989	Southeast	Yucatán	SF	Women with miscarriages	47	100	Zavala-Velázquez *et al.,* 1989 (99)
1991	Southeast	Oaxaca	IHA	General population	5.1	3229	Goldsmith *et al*., 1991 (64)
1992	Southeast Central Northern	Tabasco Estado de México Baja California Sur	IFA	General population	67.5 32.1 17.4	29 279	Velasco-Castrejón *et al.,* 1992 (97)
1995	Central	Mexico	ELISA	Pregnant women Women with Miscarriages	34.9 IgG 20.7 IgM 44.9 IgG 33.3 IgM	350	Galvan-Ramirez *et al.,* 1995 (57)
1998	Southeast	Chiapas	IFA	General Population Pregnant women	5 18	50	Romero-Cabello *et al.,* 1998 (90)
1999	Central	Jalisco	ELISA	General population owners cats	64 IgG	59	Galvan-Ramirez *et al.,* 1999 (56)
2006	Northern	Durango	ELISA	Pregnant women	6.1 IgG	343	Alvarado-Esquivel *et al.,* 2006 (24)
2006	Northern	Durango	ELISA	Mentally-ill patients Blood donors	18.2 IgG 4.4 IgM 8.9 IgG 2.2 IgM	137 180	Alvarado-Esquivel *et al.,* 2006 (1)
2007	Northern	Durango	ELISA	Blood donors	7.4 IgG 1.8 IgM	432	Alvarado-Esquivel *et al.,* 2007 (19)
2008	Northern	Durango	ELISA	Waste pickers Waste workers	21.1 IgG 2.2 IgM 8.4 IgG	90 83	Alvarado-Esquivel *et al.,* 2008 (16)
2008	Northern	Durango	ELISA	Rural population	23.8 IgG 2.2 IgM	463	Alvarado-Esquivel *et al.,* 2008 (2)
2009	Northern	Durango	ELISA	Pregnant women	8.2 IgG 2.3 IgM	439	Alvarado-Esquivel *et al.,* 2009 (28)
2010	Northern	Durango	ELISA	Rural population (Mennonites)	30.3 IgG 3.3 IgM	152	Alvarado-Esquivel *et al.,* 2010 (22)
2010	Northern	Durango	ELISA	Plumbers Construction workers Gardeners	6.6 IgG 8.4 IgG 1.4 IgM 6 IgG 2.4 IgM	61 203 168	Alvarado-Esquivel *et al.,* 2010 (17)
2011	Southeast	Yucatán	ELISA	General population without cats contact	25 IgG 37 IgM	80	Jiménez-Coello *et al.*, 2011 (69)
2011	Northern	Durango	ELISA	Mentally-ill patients General population	20 IgG 5.3 IgG	50 150	Alvarado-Esquivel *et al.*, 2011 (29)
2011	Northern	Durango	ELISA	General population	6.1 IgG 2.1 IgM	974	Alvarado-Esquivel *et al.,* 2011 (7)
2011	Northern	Durango	ELISA	Butchers General population	7 IgG 4 IgM 9 IgG 2 IgM	124 248	Alvarado-Esquivel *et al.,* 2011 (14)
2011	Northern	Durango	ELISA	Patients with liver disease General population	13.3 IgG 2.7 IgM 10.7 IgG 3.3 IgM	75 150	Alvarado-Esquivel *et al.,* 2011 (26)
2011	Northern	Durango	ELISA	Fruit and vegetable workers General population	7.5 IgG 1 IgM 7.8 IgG 2.8 IgM	200 400	Alvarado-Esquivel *et al.,* 2011 (6)
2012	Northern	Durango	ELISA	Rural population	22.4 IgG 9.6 IgM	156	Alvarado-Esquivel *et al.,* 2012 (5)
2012	Northern	Durango	ELISA	Patients with accidents at work General population	8.3 IgG 0.8 IgM 5.3 IgG 2.3 IgM	133 266	Alvarado-Esquivel *et al.,* 2012 (27)
2012	Northern	Durango	ELISA	Elderly people	12 IgG 2.9 IgM	483	Alvarado-Esquivel *et al.,* 2012 (13)
2012	Southeast	Yucatán	ELISA PCR	Women with miscarriages	55 IgG 20 IgM 19 PCR	100	Vado-Solís *et al.,* 2012 (95)

ELISA: Enzyme-Linked ImmunoSorbent Assay. **PCR**: Polymerase Chain Reaction. **SF**: Sabin Feldman Technique. **IFA**: Indirect Fluorescent Antibody technique. **IHA**: Indirect Haemagglutination test.

In a community of Mennonites (ethnic German descent established in rural communities) from the state of Durango, it was found that 46 (30.3%) of 152 people had IgG antibodies to *T. gondii* and five (3.3%) also showed IgM seropositivity. The infection with *T. gondii* was associated with the presence of cats in homes, the consumption of livestock and pigeon meat, and the drinking of untreated water[22].

There have been studies on workers occupationally exposed to water, wastewater and soil in the state of Durango. IgG antibodies were reported in 4 (6.6%) of 61 plumbers, 17 (8.4%) of 203 construction workers and 10 (6.0%) of 168 gardeners; IgM antibodies were also found in three (1.5%) construction workers and four (2.4%) gardeners, but none (0%) in the plumbers. The multivariate analysis showed that infection with *T. gondii* was positively associated with eating unwashed fruits and the meat of farm animals[17].

In a cross-sectional study of 80 individuals with no history of previous contact with cats, seropositivity for ELISA IgG and IgM was found in

29 (37%) and 20 (25%) of the cases respectively. From positive cases, 14 (18%) were positive to both antibodies. There was a significant association of seropositivity to IgM in individuals consuming pork and the meat of wild animals. Although participants did not have prior contact with cats, the infection was present in them[69].

In a case-control study, the association between 50 schizophrenic patients and a control group of 150 individuals was made using an ELISA test. A high seroprevalence of IgG antibodies to *T. gondii* was found in 10 (20%) of 50 patients with schizophrenia compared with the eight (5.3%) of 150 control subjects. IgG levels greater than 150 IU/mL were more frequently found in schizophrenic patients than in controls (10% *vs.* 2%, respectively). In the same study, two patients were recently diagnosed with schizophrenia and one showed IgM antibodies. The seroprevalence in this study was significantly higher in patients with a history of cleaning cat feces and suffering from simple schizophrenia[29].

Also in the state of Durango, *T. gondii* was monitored in the general population, finding 6.1% IgG positive cases and 2.1% of IgM antibodies in 974 individuals. IgG levels from 13-99, 100-150 and > 150 IU/mL were found in 14 (23.7%), three (5.1%) and 42 (71.2%) positive cases respectively. The infection was significantly associated with the consumption of pork and squirrel meat[7]. Considering that raw meat represents a high risk for infection with *T. gondii* cysts, a case-control study evaluating 124 butchers and 248 subjects from the general population as a control group was conducted. IgG antibodies were found in eight (7%) butchers and 22 (9%) of the control subjects using an ELISA test. IgG levels were > 150 IU/mL in seven (6%) of the butchers and in 14 (6%) of the controls. IgM antibodies were found in five (4%) of the butchers and four (2%) of the controls. No statistically significant differences were found between groups[14].

In order to find an association between infection with *T. gondii* and patients with liver disease, a case-control study using an ELISA was conducted. IgG antibodies were found in 10 (13.3%) of 75 patients and in 16 (10.7%) of 150 control subjects. IgM antibodies were found in two (2.7%) patients and five (3.3%) controls. In this study seropositivity to *T. gondii* did not show any association with the diagnosis of liver disease. However, seropositivity in patients was associated with consumption of deer, quail and lamb meat[26]. In a retrospective study, a significant association between the presence of IgG antibodies and an age ≥ 50 years was reported[8].

Recent reports showed that 7.5% of workers exposed to raw and unwashed fruits and vegetables exhibit a significant association with seropositivity towards *T. gondii*[6]. The epidemiology of *T. gondii* has been studied in Tepehuan people (indigenous ethnic group from Northern Mexico) finding IgG seropositivity in 35 (22.4%) of 156 people. Furthermore, 15 (9.6%) showed IgM antibodies using ELISA[5].

In another case-control study, patients with recent accidents at work showed an IgG seropositivity of 8.3% while the controls had a seroprevalence of 5.3%. Seroprevalence was significantly higher in patients who have accidents at work and low socioeconomic status; a positive association with consumption of pork was also found[27]. In senior individuals (≥ 60 years of age), the seropositivity of IgG and IgM was 12% and 2.9% respectively. The presence of cats in the neighborhood, the consumption of pork, pigeon, iguana and armadillo meat showed a positive association with infection with *T. gondii*[13]. In a study conducted in the state of Yucatán (Southeastern Mexico) in 100 women with spontaneous abortion, it was found that 58 had antibodies to *T. gondii*; 32 were positive to IgG; two to IgM; five to both IgG and IgM; six were positive to IgG and PCR; one to IgM and PCR; and 12 to IgG, IgM and PCR. Therefore, 55% of women were seropositive to at least IgG, 20% to IgM and 19% to PCR[95].

The Mexican national seroprevalence of *T. gondii* reported from two national bank sera was 60.1% to 62.6%. Coastal states and children showed the highest prevalences, while the midwest region showed a lower number of positive cases. A positive correlation of positive cases was associated with environmental temperature from 21 states where the prevalence was higher[36]. In a recent meta-analysis of *Toxoplasma gondii* infection among the Mexican population from 1951 until 2012, the average prevalence was 27.97% and the weighted prevalence 19.27%. The weighted prevalence was higher in women with spontaneous abortions (35.13%), immunocompromised patients (28.54%) and mental patients (38.52%). The infection with *T. gondii* among the Mexican population showed a downward trend of 0.1%/year over a period of six years, which represents a decrease in the prevalence of 5.8%[62].

Immunodeficient patients: In a retrospective study from 1988 to 1993 in 177 patients with AIDS in Mexico City, nine (5.09%) of them developed toxoplasmosis. In two patients, the initial manifestation of HIV infection was toxoplasmic meningoencephalitis and the remaining seven had been diagnosed with AIDS, with an average of ten months between the first event and the diagnosis of toxoplasmosis. In the same study, the count of TCD4+ cells was performed simultaneously to the diagnosis of toxoplasmosis, finding a mean of 78 cells/μL. These findings are in agreement with the common features of cerebral toxoplasmosis in HIV-infected patients with CD4+ cells below 100/μL[43]. In another study on 92 patients infected with HIV, 46 (50%) were IgG seropositive to *T. gondii* by ELISA test and only one case (1%) was IgM seropositive. Of the 92 patients, 53 were HIV positive and 39 had AIDS. These findings highlight the importance of monitoring patients with HIV antibodies due to the high risk of cerebral toxoplasmosis, which is the second leading cause of death in these patients[58].

In the state of Yucatán, an IgG antibodies prevalence of 47% was found in 95 patients with HIV type 1. In the same study, 69% of 100 HIV-negative blood donors were used as a control group. The high prevalence in both groups suggested that toxoplasmosis was an endemic zoonosis in the Southeast Mexico[66].

A seroprevalence from 85 patients with hearing impairments in Durango, Mexico associated with *Toxoplasma gondii* IgG antibodies was studied, finding 8.2% positive cases. There were also positive cases in 10% of 50 patients undergoing hemodialysis, in 12.9% of 234 patients with visual impairments, and 6.8% of 103 persons at risk of immunosuppression. In total, 47 (10.0%) of 472 subjects had IgG antibodies to *T. gondii* and six (1.3%) of them also showed IgM antibodies. The infection with *T. gondii* was significantly associated with the consumption of undercooked meat, ingestion of raw cow's milk, the presence of cats in the household, and raising animals[18].

Infants: The earliest report of *T. gondii* in children in Mexico dates from 1976, when five cases of fatal toxoplasmosis were reported from

the "Hospital Infantil de Mexico". In all cases, the parasite was identified in brain tissue and in some cases in the liver and lungs. Considering the early beginning of the neurological manifestations and severity of brain injuries reported, it was assumed that the five cases were of prenatal toxoplasmosis[91]. Likewise, in another early survey from the central region of Mexico, 667 children were reported with the presence of specific antibodies of *T. gondii*[65]. Further investigations demonstrated a positive relationship between the indirect immunofluorescence (IFI) to *T. gondii* and clinical findings in a population of 328 children with cerebral palsy[90].

In a screening study conducted with 1003 newborn infants, two asymptomatic cases positive to ELISA IgM and IgG were found demonstrating that the ratio is about two cases of congenital toxoplasmosis per 1,000 newborns in Mexico City[96]. A toxoplasmosis case in a 7-year-old child who received a liver transplant was described as demonstrating seroconversion to *T. gondii* antibodies after surgery; six months later, lesions compatible with *T. gondii* and CMV chorioretinitis appeared. The patient was treated and clinical manifestations improved and remained stable for 12 months until the recurrence of new lesions in the retina. These data concluded the feasibility of *T. gondii* transmission after organ transplantation[55]. In another study IgG subclasses against *T. gondii* were detected in mother/newborn pairs. Antibodies of the IgG2 subclasses were more frequent among congenitally infected newborns than in non-infected. Active fetal antibody synthesis of the 4 subclasses was also demonstrated, IgG3 and IgG4 being related to clinical problems and IgG1 to protection from damage. The identification of IgG2, IgG3 and IgG4 in newborns and the comparison with the response of their mothers might be helpful to diagnose congenital transmission early after birth[37]. This method of early diagnosis is consistent with previous studies[82,88]. In the case of congenital transmission, *T. gondii* strain I was identified in four mothers and their newborns. These results provide information regarding the strains present in humans, but found no relationship between parasite load and genotype of *T. gondii* with vertical transmission. Two congenital cases of newborns showed severe disease, one fatal soon after birth and the other was born asymptomatic but developed a mild problem later on[86]. In a recent systematic review database, the prevalence of infection with *T. gondii* was 0.61% from 4833 asymptomatic newborns, whereas in 895 symptomatic newborns a prevalence of 3.0% was found[59]. There are few studies concerning the genotype of *T. gondii* circulating in Mexico. No data on frequency in other regions about infecting genotypes is available.

STUDIES IN ANIMALS

Several studies on toxoplasmosis have been conducted in the different animal populations in Mexico, where a wide distribution of the parasite has been observed (Table 2).

Poultry: The prevalence of *T. gondii* in free breeding chickens (*Gallus domesticus*) is a good indicator of the presence of the parasite in the environment because chickens feed from the ground. In the first report made on free breeding chickens in Mexico, the presence of antibodies specific to *T. gondii* was found in 13 (6.2%) of 208 birds using a modified agglutination test. Likewise, *T. gondii* was isolated from six chickens and the genotyping of five isolates were type III and one was an isolate of type I[48].

Recent studies have reported antibodies to *T. gondii* in wild birds, using the modified agglutination test. Seropositivity was found in 17 (2.6%) of 653 birds, which included one out of two "Curve-billed thrashers" (*Toxostoma curvirostre*), two out of four ducks (one *Anas platyrhynchos* and one *Anas diazi*), one out of two eagles (*Aquila* sp), five (27.8%) out of 18 Mexican grackles (*Quiscalus mexicanus*), seven (1.3%) of 521 pigeons (*Columba livia*) and one (14.3%) out of seven quails (*Coturnix coturnix*). *T. gondii* was isolated in one out of six seropositive pigeons. The DNA obtained revealed the presence of an atypical genotype[20]. In another study, antibodies to *T. gondii* were found in 36 (6.9%) of 519 chickens (*Gallus domesticus*) using the modified agglutination test. The seroprevalence of *T. gondii* was significantly higher in chickens raised in backyards (25.5%) than those raised on farms (4.9%)[12].

Cats: Domestic cats and wild felines are involved in the full cycle of *T. gondii,* because they can host sexually mature parasites in their gastrointestinal tract and excrete oocysts in their feces. In a study conducted between 1984 and 1999 in North, Central and South America, antibodies to *T. gondii* was found in 22.4% of 438 pumas (*Felis concolor*) and 51.7% of 58 bobcats (*Lynx rufus*)[74]. In the state of Durango, a prevalence of 21% against *T. gondii* was found in 105 cats using the modified agglutination test. In this study, cats over one year of age had a significantly higher frequency of infection than younger cats (less than six months of age)[15].

In another study of cats in the state of Colima (west coast of Mexico), *T. gondii* antibodies were found in 28.8% of 80 domestic cats using an indirect ELISA-IgG. Prevalence among cats fed with homemade food was higher than in cats fed commercial pellets[63]. In Mexico City, 37 (21.8%) of 169 cats were seropositive for anti-*T. gondii* IgG using an indirect ELISA. The increase in frequency was related to age. The main risk factors were female gender, feeding cats with raw meat and infrequent cleaning of the litter box[31].

In Northeastern Mexico, a study of *T. gondii* on wild cats revealed a prevalence of 69% in 26 ocelots (*Leopardus pardalis*)[85].

In the state of Yucatán, Mexico, the seroprevalence of *T. gondii* found in domestic cats was 75.5% (166/220) of IgM antibodies and 91.8% (202/220) to IgG. From the 220 studied cats, 79% (173/220) were PCR positive. The number of cats per household and low body condition is associated with reactivation of chronic infection[39].

Rabbits: In domestic rabbits, an isolated study reported the prevalence of antibodies to *T. gondii* as 26.9% from 77 out of 286 animals from three different farms, using an ELISA test[54].

Dogs: In dogs, antibodies to *T. gondii* were found in 52 (51.5%) of 101 dogs in the state of Durango, using the modified agglutination test[45]. Frequencies have also been reported in 61.7% of stray dogs from Oaxaca, using indirect ELISA[40], although this species is not considered relevant in the epidemiology of the parasite, and these animals are considered as incidental hosts.

Pigs: The transmission of *T. gondii* to humans has usually been attributed to the ingestion of raw or undercooked meat. Pork meat in particular is considered as one of the most common sources of infection. A study conducted on 48 samples of pork meat from butchers in the state of Jalisco, Mexico revealed that all samples studied were negative following histopathology, and were PCR negative. However, IgG and IgM

Table 2
Seroprevalence of *Toxoplasma gondii* in animals from Mexico

Year	State	Animal	Specie	Prevalence (%)	Test	Reference
2004	State of Mexico	Chickens	*Gallus domesticus*	6.2	MAT	48
2011	Durango	Wild birds	*Toxostoma curvirostre* *Anas platyrhynchos* *Anas diazi* *Aquila* sp *Quiscalus mexicanus* *Columba livia* *Coturnix coturnix*	2.6	MAT	20
2012	Durango	Chickens	*Gallus domesticus* Raised in backyards Raised on farms	 25.5 4.9	MAT	12
2004	Mexico	Pumas Lynxes	*Felis concolor* *Lynx rufus*	16.7 66.6	IHA ILAT	74
2007	Durango	Domestic cats	*Felis silvestris catus*	21	MAT	15
2007	Colima	Domestic cats	*Felis silvestris catus*	28.8 IgG	ELISA	63
2008	Mexico City	Domestic cats	*Felis silvestris catus*	21.8 IgG	ELISA	31
2012	Tamaulipas	Ocelots	*Leopardus pardalis*	69	ILAT	85
2012	Yucatan	Domestic cats	*Felis silvestris catus*	75.5 IgM 91.8 IgG	ELISA	39
2006	State of Mexico	Rabbits	*Oryctolagus cuniculus*	26.9 IgG	ELISA	54
2007	Durango	Dogs	*Canis familiaris*	51.5	MAT	45
2012	Oaxaca	Stray dogs	*Canis familiaris*	61.7 IgG	ELISA	40
2011	Yucatan	Pigs	*Sus scrofa*	100 IgG	ELISA	79
2011	Durango	Pigs	*Sus scrofa*	32.1	MAT	9
2012	Oaxaca	Pigs	Raised in backyards Raised on farms	17.2 0.5	MAT	4
2008	Puebla-Veracruz	Sheep	*Ovis aries*	77-84 IgG	ELISA	35
2008	Colima	Sheep	*Ovis aries*	29.1 IgG	ELISA	34
2012	Durango	Sheep	*Ovis aries*	15.1	MAT	10
2013	Oaxaca	Sheep	*Ovis aries*	23.1	MAT	3
2011	Durango	Goats	*Capra hircus*	31	MAT	11
2013	Michoacan	Dairy Goats	*Capra hircus*	15.2	MAT	25
2012	Durango	Horses	*Equus caballus*	6.1	MAT	21
2012	Coahuila Nuevo Leon Tamaulipas	Deer	*Odocoileus virginianus*	13.9 IgG	ELISA	78
2012	Quintana Roo Mexico City	Dolphins Sea lions	*Tursiops truncatus truncatus* *Tursiops truncatus gillii* *Zalophus californianus*	87.3	MAT	23
2005	Mexico City	Opossums Ringtail cat Spotted skunks Weasel Rock squirrels Gray squirrels Feral cats Feral dogs	*Didelphis virginianus* *Bassariscus astutus* *Spilogale gracilis* *Mustela frenata* *Spermophilus variegatus* *Sciurus aureogaster* *Felis catus* *Canis familiaris*	23.9	CF	92
2009	Durango	Dogs Cats Opossums Rats Mice	*Canis familaris* *Felis catus* *Didelphis virginianus* *Rattus* spp. *Mus musculus*	45.3 9.3 16.6 0.8 3.1	MAT	50

ILAT: Latex agglutination test. **MAT**: Modified agglutination test. **IHA**: Indirect agglutination test. **CF**: Complement fixation. **ELISA**: Enzyme-Linked ImmunoSorbent Assay.

antibodies were found in one of 48 mice inoculated through an ELISA. The frequency in the pork was 2.1%, which was lower than expected but similar to that found in other regions[60].

In a cohort study conducted in 64 newly weaned pigs, from two farms with different densities of cats, on the farm with higher cat density (FA), 97.5% of 31 pigs were seroconverted in the second sampling and 100% in the third sample, while in the second farm (FB) (with lower cat density) all pigs were seroconverted in the fourth sampling, using an indirect ELISA. The true incidence rate (TIR) was 0.67 and 0.43 for FA and FB respectively, during the first four weeks at risk. The relative risk (RR) was 1.5. Animals from FA had a higher risk of infection compared to FB; however, in the end, all pigs showed antibodies against *T. gondii.* These results suggested a major environmental contamination with oocysts, since the study area is located in an endemic area[79].

In another study, 136 (12.7%) of 1074 evaluated pigs from Durango State showed antibodies against *T. gondii,* using the modified agglutination test. In this study, the seroprevalence varied according to the geographic regions in which they were bred; in the mountainous region a significantly higher seroprevalence (32.1%) was found compared to those raised in the valley (13.0%) and semi-desert regions (14.0%)[9]. In pigs reared in backyards, the seropositivity was 37.9% from 337 evaluated animals, while only one (0.5%) of 188 pigs raised on farms showed antibodies against *T. gondii*. The higher seroprevalence of *T. gondii* from the backyard pigs was found in animals ≥ 9 months of age (40%), in females (40%), in pigs from the isthmus region (33.3%), and those raised in tropical climates (65%). The results confirm that the management system (outdoor *vs.* interior with biosecurity) is a key factor in the epidemiology of swine toxoplasmosis[4].

Sheep: In a study located in Eastern Mexico, a frequency range of 77-84% of anti-*T. gondii* antibodies was found in 103 sera of sheep, using an indirect ELISA. The higher prevalence of toxoplasmosis in environments with warm and humid climates was attributed to the high viability of the *T. gondii* oocysts[35]. In another study conducted in the coastal, mountainous and hill regions in the state of Colima, serum samples of 351 sheep were tested using a previously standardized indirect ELISA. The frequency of IgG antibodies depended on the altitude, being higher than 1200 meters above sea level, and the size of the flock, with a higher frequency being found in the largest[34]. In Northern Mexico, a *T. gondii* seropositivity of 15.1% was found in 511 animals tested using a modified agglutination test. Seroprevalence significantly increased with age, indicating transmission soon after birth[10]. In Southern Mexico, a seroprevalence of 23.1% was found in sheep, using the modified agglutination test. The seroprevalence was significantly higher in sheep reared under semi-intensive (grazing cultivated pasture and hay) conditions than those reared under semi-extensive (grazing lands with natural grass) conditions. Additionally, the seroprevalence of *T. gondii* was higher in mixed breed sheep than in purebreds[3].

Goats: Few studies have been conducted on the seroprevalence of *T. gondii* in goats. Antibody seropositivity evaluated from 562 domestic goats, using the modified agglutination test, showed a prevalence of 31%. In this study, seroprevalence was widely distributed in the geographic region studied, and increases with age and race. Goats reared in the semi-desert (Nubian) had a significantly higher seroprevalence (32.7%) than those raised in the mountains (18.6%) (mixed breeds). Positivity was found in all 12 (100%) farms sampled[11]. In dairy goats, the seropositivity found was 15.2% from 341 examined animals. An increase in seroprevalence was found in goats 13-24 and 49-86 months of age (25% and 22.9% respectively). Additionally, goats from warm-humid climates and at 1,700 meters above sea level had higher seroprevalence (62.1%)[25].

Horses: In horses from the Northwest Mexico, seroprevalence to *T. gondii* was reported in 30 (6.1%) of 495 animals, using the modified agglutination test (MAT). Seroprevalence was higher in horses from rural areas (7.8%) compared to those living in urban areas (0%)[21].

Monkeys: Toxoplasmosis causes a fatal, multi-systemic disease in New World primates, such as impaired respiratory and multifocal necrotic lesions. Two fatal cases of acute toxoplasmosis in squirrel monkeys (*Saimiri sciureus*) were found; the main pathological findings included pulmonary edema, interstitial pneumonia, hepatitis, and necrotizing lymphadenitis. In addition, structures similar to tachyzoites of *T. gondii* by histopathology in these organs were described. The diagnosis was confirmed by immunohistochemistry, transmission electron microscopy, and real time PCR. Quantification of the parasite load was < 14 and 23 parasites/mg tissue and genotyping was similar to the reference strain type I[41].

Deer: In white-tailed deer from Northern Mexico, a seroprevalence of 13.9% (74/532) was found using an ELISA test. These results were associated with management factors on farms, such as the number of deer per hectare and the geographic location[78].

Marine mammals: Toxoplasmosis in marine mammals is of great importance since these animals serve as sentinel organisms for the level of oocyst pollution in the seas. A study in Central and Southern Mexico was conducted on 75 marine mammals in captivity through the modified agglutination test. Antibodies to *T. gondii* were found in 55 (87.3%) of 63 Atlantic bottlenose dolphins (*Tursiops truncatus truncatus*), in three Pacific bottlenose dolphins (*Tursiops truncatus gillii*), in two out of four California sea lions (*Zalophus californianus*), but not in three West Indian manatees (*Trichechus manatus*) or two Patagonian sea lions (*Otaria flavescens*). All marine animals sampled were found in healthy conditions and have had no records of cases of clinical toxoplasmosis in at least the last 15 years[23].

Other species: In wild mammals such as opossums (*Didelphis virginiana*), ring-tailed cats (*Bassariscus astutus*), spotted skunks (*Spilogale gracilis*), weasels (*Mustela frenata*), rock squirrels (*Spermophilus variegatus*), gray squirrels (*Sciurus aureogaster*), feral cats (*Felis catus*), and feral dogs (*Canis familiaris),* a 23.9% seroprevalence of *T. gondii* has been reported[92]. Later studies conducted in the state of Durango showed antibodies to *T. gondii* in 11 (16.6%) of 66 opossums (*Didelphis virginianus*), two (0.8%) of 249 rats (*Rattus* spp.), four (3.1%) of 127 mice (*Mus musculus*) and 0 (0%) of 69 squirrels (*Spermophilus variegatus*). Additionally, viable *T. gondii* cysts were isolated from tissue (brain and heart) in three of 28 seropositive dogs and five of eight seropositive cats but no isolations were obtained in other animal species studied[50].

Genotyping of Toxoplasma gondii in animals from Mexico: Very few reports reveal predominant genotypes of *T. gondii* present in animals. In 2004, *T. gondii* was isolated from six chickens and all six isolates were avirulent for mice. Genotyping of chicken isolates of *T. gondii* using

the SAG2 locus indicated that five were type III and one was type I[48]. In another study, viable *T. gondii* was isolated in tissues from three of 28 seropositive dogs and five of eight seropositive cats and four isolates from free-range chickens from Mexico, previously isolated. The PCR-RFLP identified five genotypes. One genotype (the four chicken isolates) belongs to the clonal Type III lineage, three genotypes were reported in previous reports, and are different from Type I, II and III lineages that predominate in North America and Europe, and one genotype is unique[50]. Two cases of lethal acute toxoplasmosis in squirrel monkeys (*Saimiri sciureus*) from Mexico City were studied, where digestion of the *SAG3* gene amplicon showed similar bands to type I reference strains[41]. Recent studies found a new atypical genotype of *T. gondii* in a wild puma (*Felis concolor*) that was virulent for mice, this is the only study from wildlife in Mexico[44].

Analysis: The highest seroprevalence of toxoplasmosis found in human adults from the southeastern states of Mexico such as Yucatán, Tabasco and Jalisco, could be due to the climatic conditions found in those areas, characterized by their tropical climates (high temperature and humidity) which promote the survival of the parasite oocysts in the environment[56,64,97,99]. However, the low seroprevalence reported in some areas, like in Oaxaca State, may be related to a low density of cats and low consumption of meat in the diet of the general population[64]. Even when located in a tropical region, the humidity, temperature and presence of cats play an important role in the transmission of this parasite. The seroprevalence found in the northern regions of Mexico (i.e. Durango state) is lower than that from other regions of Mexico; the differences between the two regions could be explained by the dry climate and relatively high altitude in the region and probably the characteristics of the studied populations. Seroprevalence, particularly in breeding animals (pigs and sheep), is much higher in tropical areas, which is also related to the climatic conditions. It is noteworthy that Yucatán state is an endemic region where animals and humans are constantly exposed to the parasite due to the high contamination of the environment with infective oocysts[39].

CONCLUSION

Reports of *T. gondii* in Mexico indicate that this neglected disease is widely distributed in humans and domestic and wild animals throughout the country, with increased prevalences in tropical regions. A high seroprevalence is commonly found in farm animals in the tropical areas of Mexico where *T. gondii* is endemic; in these endemic areas *T. gondii* is maintained viable for a long time in the soil and water, which are critical for its transmission to animals. This agent is usually found in meat produced for human consumption, which could be a major source of infection. Fetuses of susceptible pregnant women (i.e. those having not been previously exposed to the parasite) and immunocompromised patients are at high risk of developing clinical signs associated with the agent such as abortion, malformations and fatal cases of meningoencephalitis respectively. Efforts in the field of education should be oriented towards the importance of hygiene when handling and preparing food. It is vital to implement control and surveillance programs for the prevention and control of toxoplasmosis in Mexico at the farm level for meat production, reducing the environmental oocyst load and maintaining a low incidence of positive cases in animal production species for meat consumption and, consequently, reducing the spread to the human population.

RESUMEN

Toxoplasmosis en México: situación epidemiológica en humanos y animales

La toxoplasmosis es una enfermedad parasitaria ampliamente distribuida en todo el mundo y puede infectar a una gran diversidad de especies animales y a los humanos. En México, esta parasitosis ha sido detectada en diferentes partes del país, en particular en las zonas tropicales en donde debido a las condiciones ambientales (ej. alta temperatura y humedad a lo largo todo el año), el parásito puede mantenerse infectante por largos períodos de tiempo en el medio ambiente. Se han realizado diversos estudios epidemiológicos tanto en poblaciones humanas como en animales y se observa que a pesar de la amplia distribución y buen establecimiento del agente en el país, existe un importante desconocimiento desde la epidemiologia, tratamientos alternativos o las medidas de control. La falta de control de las poblaciones ferales de felinos así como el establecimiento de medidas de control en unidades de producción de carne de diferentes especies para consumo humano están jugando un papel primordial que ha favorecido la amplia diseminación de la enfermedad en el país, principalmente en las zonas tropicales del sureste mexicano. Por lo que este manuscrito tiene como objetivo revisar la información publicada hasta ahora que nos describe aspectos epidemiológicos relevantes de la infección por *T. gondii* en humanos y animales de México.

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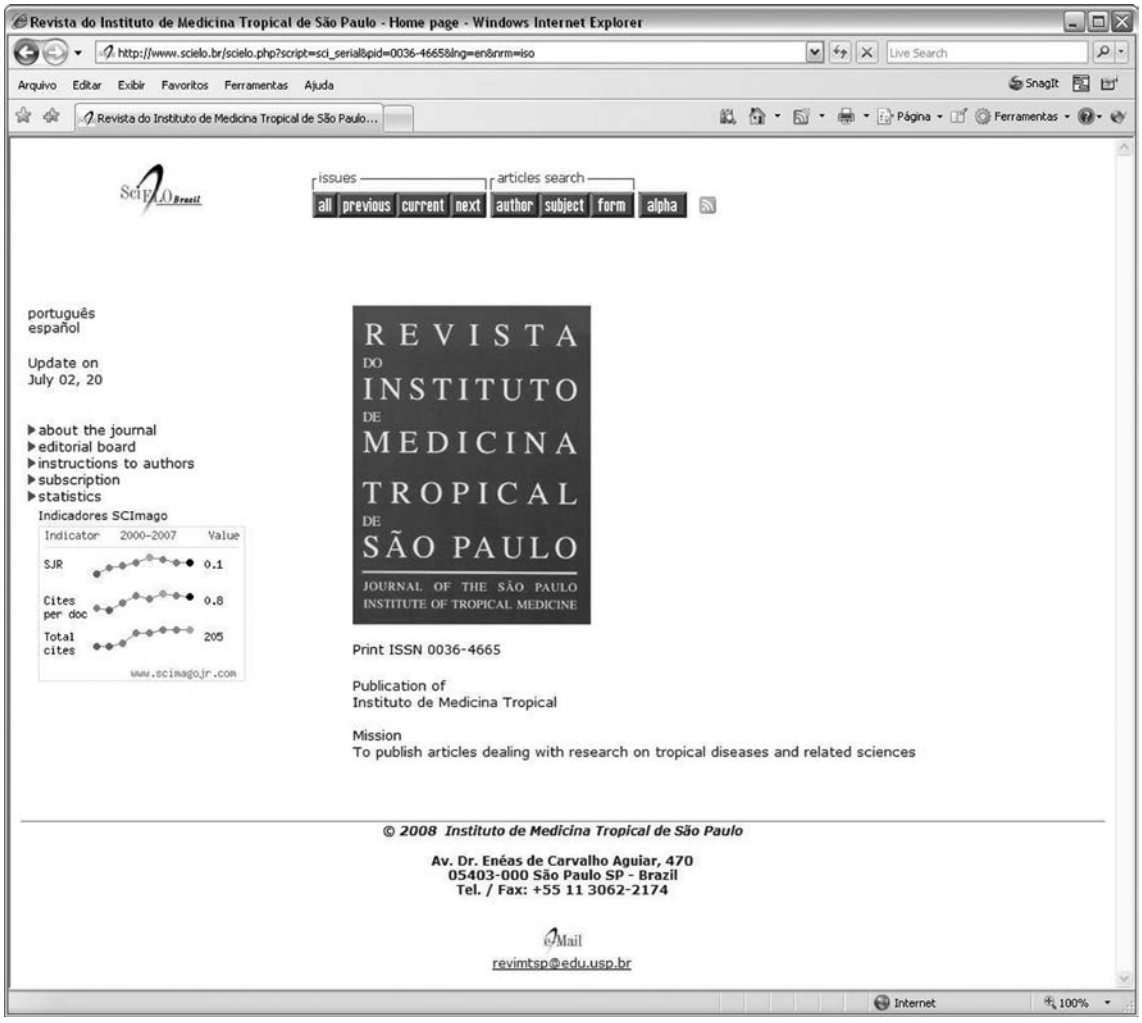

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DISCORDANCE BETWEEN BODY MASS INDEX AND ANTHROPOMETRIC MEASUREMENTS AMONG HIV-1-INFECTED PATIENTS ON ANTIRETROVIRAL THERAPY AND WITH LIPOATROPHY/LIPOHYPERTROPHY SYNDROME

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SUMMARY

Introduction: Highly Active Antiretroviral Therapy (HAART) has improved and extended the lives of thousands of people living with HIV/AIDS around the world. However, this treatment can lead to the development of adverse reactions such as lipoatrophy/lipohypertrophy syndrome (LLS) and its associated risks. **Objective:** This study was designed to assess the prevalence of self-reported lipodystrophy and nutritional status by anthropometric measurements in patients with HIV/AIDS. **Methods:** An observational study of 227 adult patients in the Secondary Immunodeficiencies Outpatient Department of Dermatology, Hospital das Clínicas, Faculty of Medicine, University of São Paulo (3002 ADEE-HCFMUSP). The sample was divided into three groups; Group 1 = 92 patients on HAART and with self-reported lipodystrophy, Group 2 = 70 patients on HAART without self-reported lipodystrophy and Group 3 = 65 patients not taking HAART. The nutritional status of individuals in the study sample was determined by body mass index (BMI) and percentage of body fat (% BF). The cardiovascular risk and diseases associated with abdominal obesity were determined by waist/hip ratio (WHR) and waist circumference (WC). **Results:** The prevalence of self-reported lipoatrophy/lipohypertrophy syndrome was 33% among women and 59% among men. Anthropometry showed depletion of fat mass in the evaluation of the triceps (TSF) in the treatment groups with HAART and was statistically independent of gender; for men $p = 0.001$, and for women $p = 0.007$. Similar results were found in the measurement of skin folds of the upper and lower body ($p = 0.001$ and $p = 0.003$ respectively). In assessing the nutritional status of groups by BMI and % BF, excess weight and body fat were more prevalent among women compared to men ($p = 0.726$). The WHR and WC revealed risks for cardiovascular and other diseases associated with abdominal obesity for women on HAART and with self-reported LLS ($p = 0.005$) and ($p = 0.011$). **Conclusions:** Anthropometric measurements were useful in the confirmation of the prevalence of LLS. BMI alone does not appear to be a good parameter for assessing the nutritional status of HIV-infected patients on HAART and with LLS. Other anthropometric measurements are needed to evaluate patients with the lipoatrophy/lipohypertrophy syndrome.

KEYWORDS: HIV; Lipoatrophy/lipohypertrophy syndrome; Nutrition; Anthropometry; Brazil.

INTRODUCTION

The widespread use of Highly Active Antiretroviral Therapy (HAART) has promoted a sustained reduction in both morbidity and mortality associated with HIV-1[20,33]. Although the curve of the HIV epidemic has been showing signs of flattening around the world, HIV infection remains almost invariably fatal if the individuals are not treated with HAART[6,33,38].

Despite the increases in the availability of HAART, other challenges related to the management of patients with HIV are just beginning to surface. For example, HAART itself can cause a variety of adverse effects such as the lipoatrophy/lipohypertrophy syndrome[3,4,8,9]. Such an occurrence may contribute to a decrease in adherence to antiretroviral therapy, adding to the difficulties inherent to prolonged treatment with combinations of drugs which characterize HAART[14]. Moreover, both the changes in the composition and distribution of body fat may negatively affect self-image, thus interfering with treatment adherence and contributing to possible therapeutic failure[2,9].

Although there is no consensus on the prevention or treatment of the lipoatrophy/lipohypertrophy syndrome, the World Health Organization has indicated that nutrition should be a part of all programs for control and treatment of HIV/AIDS, since diet and adequate nutritional status can improve adherence and effectiveness of treating patients on HAART[10,36]. The identification of early morphological changes for these patients

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through the assessment and diagnosis of their nutritional status can help to establish effective interventions for the treatment of metabolic and morphological reactions[14,31]. Identification and appropriate treatment for these conditions can both improve patients' long-term care and help provide a better outlook for their quality of life[2,19,21].

According to the American Dietetic Association, anthropometry has been widely used to assess the health and nutritional status of individuals, communities or populations, not only due to its simplicity and low cost, but also because it can provide an approximation of body composition and distribution of body fat[1,15,18]. The aim of this study was to assess the prevalence of self-reported lipoatrophy/lipohypertrophy and nutritional status by anthropometric measurements in patients with HIV/AIDS in a cohort of outpatient HIV-infected persons in São Paulo, Brazil.

MATERIAL AND METHODS

This observational study was based on data collected between September 2006 and July 2008 from a referral center for the treatment of HIV infection in São Paulo, Brazil, in the Secondary Immunodeficiencies Outpatient Department of Dermatology, Hospital das Clínicas, Faculty of Medicine, University of São Paulo (ADEE 3002-HCFMUSP). The study was approved by the Research Ethics Committee of the University of São Paulo Medical School (Research Protocol No. 0221/07).

This open cohort consists of 320 HIV-1-positive patients who have been followed since 1989. The sample consisted of 227 subjects (71% of the cohort) divided into three groups:

Group 1 = 92 HIV-1-positive patients on HAART and with self-reported lipoatrophy/lipohypertrophy syndrome (LLS); Group 2 = 70 HIV-1-positive patients on HAART without self-reported LLS; Group 3 = 65 HIV-1-positive patients without HAART.

The characterization of groups in terms of self-reported lipodystrophy was conducted through a questionnaire to identify socio-demographic, clinical characteristics, as well as a specific question for the patient to determine if he/she had observed bodily changes consistent with LLS after the initiation of HAART. Study inclusion criteria were HIV infection, willingness to sign the consent form and no constraint for the test performance of bioimpedance (BIA). Exclusion criteria were pregnancy, known cardiovascular disease or previous heart surgery and refusal to sign the consent form. All the patients were interviewed by the first Author using an adapted questionnaire to identify morphological body modifications after the initiation of HAART.

Anthropometric methods, which included body mass index (BMI), waist/hip ratio (WHR), waist circumference (WC) as well as an estimate of body fat percentage (BF%), were used to evaluate the nutritional status of patients and possible changes in the deposition of fat in specific areas of the body. In addition, bioelectrical impedance (BIA) was also performed[8,11,13].

BMI was calculated as the ratio of the current weight (kg) by height (m) squared[16,17,39]. BF% was calculated as the sum of skinfolds of upper and lower limbs. The nutritional status was classified according to WHO recommendations for adults[37].

The assumed value of triceps skinfold (TSF), (mm^2), was the average of three measurements and percentages[16], by sex and age. The nutritional status classification was determined according to the recommendations of BLACKBURN and THORNTON[5]. Skinfold measurements were performed on the triceps, biceps, subscapular, abdominal, iliac, thigh and calf. These were obtained with the aid of the caliper Cescorf® (Cescorf, Porto Alegre, Brazil) and rounded to the nearest 0.5 mm and made in duplicate for each type of measurement[16]. Classification of % BF was based on recommendations made by GALLAGHER *et al.* by means of prediction equations[17].

The percentage of body fat (% BF) was determined both as above described and by electrical impedance analysis (BIA) (Systems Inc. 101Q, RJL Systems, Clinton Township, MI). For examination of the BIA, the measurements were made on the right side of the body and patients were asked about restrictions for the performance of these tests[12]. Measurements were performed according to the instructions in the user manual from CompCorp. The values of resistance and inductance were interpreted by software from Vcorp[11]. Using the IDF classification, WC cutoffs were used for the diagnosis of metabolic syndrome[25,26]. All measurements were done by one of the Authors (LS).

In addition to anthropometric data, socio-demographic characteristics were recorded as categorical and continuous variables: gender, age, education, social interaction, marital status, housing conditions, income and employment status, total duration of HAART, CD4 T-cell count, length of time from the first positive HIV test, the current HIV viral load and the presence of self-reported lipoatrophy/lipohypertrophy.

Statistical analyses were performed using the software Minitab® 15m to verify that changes in nutritional status and body fat distribution were related to gender, use of HAART and presence of lipoatrophy/lipohypertrophy[30]. These were performed by descriptive analysis. To evaluate the association between categorical variables, the chi-square test was used. For the relationship between quantitative and categorical variables with normal distribution, the Student t-test (for two variables) was used. An association was made between variables of the study (anthropometrics and body fat distribution) according to defined groups (G1, G2, G3) and analyzed by ANOVA (used for three variables), with a confidence level of 95% and 5% significance according to the Tukey test for comparison.

RESULTS

The average age of the 227 study subjects was 42.5 years with 75 (33%) being female. Average length of education was 11 years and most subjects shared a residence with three or more persons. The majority of men were single individuals. Over half of the patients own their own homes with proper sanitation and the per capita income was one to five times the minimum wage. There was no statistically significant difference by gender for these variables. Self-reported prevalence of the LLS was 33% among women and 59% among men (Table 1).

In Table 2, it is noted that this group had, on average, longer use of HAART, a higher T CD4 cell nadir, and more years of clinical follow-up compared with those without self-reported lipodystrophy and those not taking HAART. Analysis of triceps skinfold (TSF) found a depletion

Table 1
Socio-demographic characteristics of the 227 HIV-1-infected individuals by gender

Variable	Males (152)	Females (75)	*p* value
Age (Mean ± SD), years	43 ± 8	42 ± 9	NS
Years of schooling			NS
Unknown	13 (8.55)	5 (6.66)	
None	1 (0.65)	1 (1.33)	
≤ 8 years	41 (26.97)	22 (29.33)	
9-11 years	62 (40.78)	39 (52)	
>11 years	38 (25)	7 (9.33)	
Home sharing			NS
Unknown	12 (7.89)	5 (6.66)	
No	34 (22.36)	4 (5.33)	
With 2 persons	31 (20.39)	13 (17.33)	
With more than 2 persons	74 (48.68)	52 (69.33)	
Marital status			NS
No answer	15 (9.86)	6 (8)	
Single	83 (54.60)	20 (26.66)	
Married	24 (15.78)	20 (26.66)	
Divorced	9 (5.92)	17 (22.66)	
Home ownership			NS
No answer	15 (9.86)	8 (10.66)	
Owner	82 (53.94)	42 (56)	
Tenant	44 (28.94)	20 (26.66)	
Other	11 (7.23)	4 (5.33)	
Employment condition			NS
No answer	10 (6.57)	6 (8)	
Employed	103 (67.76)	42 (56)	
Unemployed	39 (25.65)	27 (36)	
Income (minimum wage)			NS
None	10 (6.57)	12 (16)	
< 1	11 (7.23)	13 (17.33)	
1-5	97 (63.81)	36 (48)	
6-10	16 (10.52)	6 (8)	
> 10	4 (2.63)	-	
No answer	13 (8.55)	7 (9.33)	
Self reported body changes	59 (38.81%)	33 (44%)	

NS: Not statistically significant when *p* value > 0.05; Income: 1 minimum wage = ~ U$300.00.

of fat mass in groups undergoing treatment with HAART and this result was statistically significant when compared to the group without HAART (for men $p = 0.001$, for women $p = 0.007$). A similar result was determined in the analysis of skin folds of the upper and lower limbs, where groups using HAART had greater loss of fat tissue which was statistically significant compared to Group 3 ($p = 0.001$) ($p = 0.003$). In the assessment of nutritional status by BMI, the majority of men were eutrophic. However this result was not statistically significant between groups ($p = 0.72$). Similarly, measurement of the sum of skinfolds revealed excess body fat tissue in all groups. Men on HAART and with self-reported lipodystrophy were identified by WHR measurements as having statistically significant higher risk for cardiovascular diseases when compared to those without LLS ($p = 0.0001$). However, when the comparison refers to CA ≥ 90 cm no significant difference was found between men in the three groups.

For women under HAART and with lipodystrophy, the measurements for WHR and CA showed a higher risk for cardiovascular diseases ($p = 0.005$ and $p = 0.01$, respectively).

DISCUSSION

The strengthening of social support, adherence to treatment and the establishment of life goals are important factors in the quality of life for those living with HIV[39]. The socio-demographic characteristics of this study reflect the epidemiological trends of HIV in Brazil. With a ratio of two men to one woman, an increasing number of women were observed, mainly due to heterosexual transmission of HIV being observed more recently[6]. Most patients in this study have, on average, some level of school education, and most are employed with reasonably stable incomes and good social status, unlike other groups of patients with HIV in Brazil who predominantly have a low level of education and poor social conditions.

Overall, body changes have been reported in 20-80% of patients on HAART, consisting primarily of Caucasian males, which roughly agrees with the results of this study[7,22]. When subcutaneous fat of the upper and lower limbs was estimated by TSF, a depletion of fat mass was found in groups undergoing treatment by HAART. These results support the hypothesis that morphological changes associated with lipoatrophy of the upper and lower limbs are common among HIV-infected individuals undergoing HAART. There are no standardized criteria for the diagnosis of LLS and such changes are clinically evident approximately six to 24 months after HAART[28,29,33].

To identify changes in the distribution of body fat, monitoring of skinfolds has been recommended[5,23,24]. According to HEYWARD & STOLARCKZYK, these anthropometric measurements have been used in studies focusing on HIV/AIDS in developed countries, yet currently there is little published data using the sum of skinfolds for the distribution of fat per body segment in adults with HIV infection[10,26,33].

A study by SANCHES[36] also found that 57% of their sample showed accumulation of abdominal fat, 55% had decreased peripheral fat with loss of subcutaneous tissue in the arms, legs and buttocks, and 33% stated that if they knew their physical appearance would acquire such a form, they would have opted not to begin treatment despite being aware of the risks to their health.

Table 2
Cardiovascular risk according to sex, anthropometric variables and self-reported groups of lipoatrophy/lipohypertrophy syndrome (LLS)

Variables	Males				Females			
	Group 1	Group 2	Group 3	*p* value	Group 1	Group 2	Group 3	*p* value
Subjects	59	52	41		33	18	24	
Years under HAART	7 ± 4	5 ± 4	-	0.02	7 ± 4	4 ± 5		0.01
Nadir TCD4 cells (cels/mm³) Median (Q1-Q3)	542 (335-732)	359 (187-503)	431 (287-628)	0.01	427 (310-607)	342 (267-468)	474 (396-804)	0.04
Time of HIV infection since diagnosis	9 ± 5	5 ± 4	2 ± 3	0.001	9 ± 5	5 ± 5	5 ± 6	0.003
HIV viral load (copies/Ml)	400 (400-1053)	400 (400-3309)	15000 (1993-65675)	0.000	436 (400-6178)	400 (138-1576)	4410 (1632-14350)	0.009
TSF				0.001				0.007
Depletion	50 (84.74%)	42 (80.76%)	20 (48.78%)		26 (78.78%)	15 (83.33%)	12 (50%)	
Eutrophic	4 (6.77%)	6 (11.53%)	3 (7.31%)		6 (18.18%)	3 (16.66%)	4 (16.66%)	
Excess	5 (8.47%)	4 (7.69%)	17 (41.46%)		1 (3.03%)	-	8 (33.33%)	
% folds members Mean ±SD	32 ± 7	35 ± 9	38 ± 7	0.001	39 ± 9	45 ± 8	46 ± 7	0.003
BMI				0.72				0.05
Depletion	-	-	-					
Eutrophic	32 (54.23%)	28 (53.84%)	20 (48.78%)		-	1 (5.55%)	-	
Excess	27 (45.76%)	24 (46.15%)	20 (48.78%)		12 (36.36%)	13 (72.22%)	12 (50%)	
% fat by Durnin				0.03				0.014
Depletion	-	-	1 (2.43%)		-	-	-	
Eutrophic	4 (6.77%)	16 (30.76%)	6 (11.53%)		-	-	1 (4.16%)	
Excess	53 (89.83%)	33 (63.46%)	31 (75.60%)		33 (100%)	18 (100%)	23 (95.83%)	
% fat by BIA				0.13				0.19
Depletion	4 (6.77%)	-	3 (7.31%)		-	-	-	
Eutrophic	22 (37.28%)	26 (50%)	9 (21.95%)		4 (12.12%)	5 (27.77%)	2(8.33%)	
Excess	33 (55.93%)	25 (48.07%)	27 (65.85%)					
Cardiovascular risk assessed by waist/hip ratio				0.001				0.005
Low and Intermediate	27 (45.76%)	32 (61.53%)	28 (68.29%)		2 (6.06%)	4 (22.22%)	5 (20.83%)	
High and very high	32 (54.23%)	20 (38.46%)	12 (29.26%)		31 (93.93%)	14 (77.77%)	19 (79.16%)	
Cardiovascular risk assessed by waist circumference according to WHO				0.63				0.01
Low and intermediate	52 (88.13%)	49 (94.23%)	35 (85.36%)		11 (33.33%)	11 (61.11)	13 (54.16%)	
High	5 (8.47%)	3 (5.76%)	5 (12.19%)		22 (66.66%)	7 (38.88%)	5 (20.83%)	
Cardiovascular risk assessed by waist circumference - according to IDF				0.63				0.01
Men ≥ 90 and women ≥ 80	30 (50.84%)	24 (46.15%)	18 (43.90%)		30 (90.90%)	10 (55.55%)	19 (79.16%)	

SD = Standard Deviation. Group 1 = Self-reported lipoatrophy/lipohypertrophy and HAART. Group 2 = HAART and no lipoatrophy/lipohypertrophy and Group 3 = Not on HAART.
For relationships between categorical and quantitative variables with normal distribution the t student's test was used (for two variables). The association between the study variables (anthropometry and body fat distribution) was performed according to defined groups (G1, G2 and G3) and analyzed by ANOVA (used for 3 variables), with a confidence level of 95% and significance of 5%, according to the comparison test of Tukey.

The cardiovascular risk associated with obesity has been defined by observational evidence, especially in cohort and case-control studies such as the Nurses' Health Study, which showed a relative risk of death of 1.6 to 2.2 associated with a BMI between 27 and 32, compared to the group with BMI < 19. The NHANES Study - National Health and Nutrition Examination Survey- reported a 1.5 relative risk for CVD later in life for women with BMI > 29, compared to a reference population with BMI < 21[40].

Although BMI is employed in most population studies because of ease of use, it does not provide enough data to accurately assess body composition. Thus, there is an increased interest in measurements that best describe the amount of body fat and its relationship with obesity. BMI in itself provides only an incomplete picture of cardiovascular risk since it does not identify individuals with abdominal obesity[18,32,34].

This study found a high prevalence of increased body fat (BF) and

of individuals described as overweight when assessed by BMI and by their percentage of BF, particularly women undergoing HAART and with self-reported lipodystrophy. Additionally for this group, high values of WHR and of Abdominal Circumference point to a high cardiovascular risk.

Among 223 HIV-infected individuals aged 20-59 from the city of São Paulo, the prevalence of central obesity was 45.7%, with women having a higher prevalence of abdominal obesity and overweight status when compared to men, coinciding with the results of this study[7,27]. The author also cites previous studies with patients treated using protease inhibitors showing that the accumulation of fat is often higher among women[12,15]. Also, the development of male body patterns has been reported in most women with LLS[15, 20]. Such results agree with the findings of the present research.

Despite the limitations of this study, such as sample size, study design and cohort effect, the results indicate that other anthropometric measurements in addition to BMI are important for assessing the nutritional status of patients with LLS undergoing HAART. Insights regarding the relative value of using multiple measurements to assess the nutritional status of HIV-infected individuals were obtained. Thus, the analysis of skinfold thickness, which should be a practice adopted by all multidisciplinary teams, can help identify individuals at high risk for lipoatrophy/lipohypertrophy syndrome and for concurrent metabolic alterations.

CONCLUSIONS

- Anthropometric assessment through skinfold measurements proved to be a good method for determining loss of fatty tissue in the upper and lower limbs, regardless of gender, in HIV-infected patients undergoing HAART.

- The BMI alone does not seem to be an adequate parameter for assessing the nutritional status of HIV-infected patients undergoing HAART and the lipoatrophy/lipohypertrophy syndrome. Other anthropometric measurements are needed to evaluate patients with the syndrome.

- There was a strong association between self-reported lipoatrophy/ lipohypertrophy syndrome, HAART, body composition, and cardiovascular disease risk associated with abdominal obesity.

- These studies have identified the need to perform a variety of measurements to assess the nutritional status of persons living with HIV/AIDS in clinical practice.

AUTHOR'S CONTRIBUTIONS

All authors read and contributed to this manuscript. All authors have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data: LRS, JC; DCS, LAMF, AJDS; FGP. (2) drafting the article or revising critically important intellectual content: LRS, JC. (3) final approval of the version to be submitted: LRS, JC; DCS, AJDS; CRG; FGP, LAMF. The authors declare not to have any conflict of interest.

RESUMO

Discordância entre o índice de massa corporal e outras medidas antropométricas em pacientes infectados pelo HIV com a síndrome de lipoatrofia/lipohipertrofia em uso de medicação antirretroviral

Objetivos: A terapia antirretroviral altamente ativa (HAART) tem melhorado e aumentado a vida de milhares de pessoas que vivem com a infecção pelo HIV/AIDS em todo o mundo. No entanto, este tratamento pode levar ao desenvolvimento da síndrome da lipodistrofia (LDS). Este estudo foi desenvolvido para avaliar a prevalência de auto-relato de LDS, perfil nutricional e medidas antropométricas de pacientes com HIV/AIDS. **Métodos:** Estudo observacional de 227 pacientes adultos, divididos em: Grupo 1: 92 pacientes em HAART e com LDS; Grupo 2: 70 pacientes em tratamento com HAART e sem LDS e Grupo 3: 65 pacientes que não tomam HAART. O estado nutricional foi avaliado pelo índice de massa corporal (IMC) e o percentual de gordura corporal (%GC) por meio de medidas antropométricas. **Resultados:** A prevalência de auto-relato de LDS foi de 44% entre as mulheres e 39% entre os homens. DC do tríceps (PCT) apresentou-se mais elevada no grupo HAART e LDS (homens $p <$ 0,001; mulheres $p <$ 0,007) em comparação com aqueles sem HAART, respectivamente. IMC revelou excesso de peso para a maioria dos indivíduos. **Conclusões:** As medidas antropométricas foram úteis para confirmar a prevalência de auto-relato da síndrome da lipodistrofia. A avaliação das dobras dos braços e pernas revelou-se um bom método para avaliação antropométrica de lipoatrofia de membro, independentemente do sexo. Estes resultados permitiram o estabelecimento de estratégias para o diagnóstico precoce da LDS na prática clínica, em pessoas vivendo com HIV / AIDS.

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PRETERM BIRTH AND FETAL GROWTH RESTRICTION IN HIV-INFECTED BRAZILIAN PREGNANT WOMEN

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SUMMARY

Introduction: Maternal HIV infection and related co-morbidities may have two outstanding consequences to fetal health: mother-to-child transmission (MTCT) and adverse perinatal outcomes. After Brazilian success in reducing MTCT, the attention must now be diverted to the potentially increased risk for preterm birth (PTB) and intrauterine fetal growth restriction (IUGR). **Objective**: To determine the prevalence of PTB and IUGR in low income, antiretroviral users, publicly assisted, HIV-infected women and to verify its relation to the HIV infection stage. **Patients and Methods**: Out of 250 deliveries from HIV-infected mothers that delivered at a tertiary public university hospital in the city of Vitória, state of Espírito Santo, Southeastern Brazil, from November 2001 to May 2012, 74 single pregnancies were selected for study, with ultrasound validated gestational age (GA) and data on birth dimensions: fetal weight (FW), birth length (BL), head and abdominal circumferences (HC, AC). The data were extracted from clinical and pathological records, and the outcomes summarized as proportions of preterm birth (PTB, < 37 weeks), low birth weight (LBW, < 2500g) and small (SGA), adequate (AGA) and large (LGA) for GA, defined as having a value below, between or beyond the ±1.28 z/GA score, the usual clinical cut-off to demarcate the 10th and 90th percentiles. **Results**: PTB was observed in 17.5%, LBW in 20.2% and SGA FW, BL, HC and AC in 16.2%, 19.1%, 13.8%, and 17.4% respectively. The proportions in HIV-only and AIDS cases were: PTB: 5.9 versus 27.5%, LBW: 14.7% versus 25.0%, SGA BW: 17.6% versus 15.0%, BL: 6.0% versus 30.0%, HC: 9.0% versus 17.9%, and AC: 13.3% versus 21.2%; only SGA BL attained a significant difference. Out of 15 cases of LBW, eight (53.3%) were preterm only, four (26.7%) were SGA only, and three (20.0%) were both PTB and SGA cases. A concomitant presence of, at least, two SGA dimensions in the same fetus was frequent. **Conclusions**: The proportions of preterm birth and low birth weight were higher than the local and Brazilian prevalence and a trend was observed for higher proportions of SGA fetal dimensions than the expected population distribution in this small casuistry of newborn from the HIV-infected, low income, antiretroviral users, and publicly assisted pregnant women. A trend for higher prevalence of PTB, LBW and SGA fetal dimensions was also observed in infants born to mothers with AIDS compared to HIV-infected mothers without AIDS.

KEYWORDS: Pregnancy; HIV; Infant; Preterm birth; Fetal weight; Low birth weight; Small for gestational age.

INTRODUCTION

The main consequences of maternal HIV infection to fetal health are mother-to-child transmission (MTCT) and the potentially increased risk of adverse pregnancy outcomes. The timely diagnosis and the provision of free antiretroviral therapy (ART) reduced MTCT[12] in Brazil. Attention must now be paid to the potentially increased risk for preterm birth (PTB) and intrauterine fetal growth restriction (IUGR).

PTB and IUGR are the most determining factors of perinatal morbidity and mortality all over the world[13,33], which are also more related to neonatal complications[50,59], poor postnatal growth[5,22,23], and diseases whose consequences may extend to late adulthood[3], with the burden of the high cost of prolonged surveillance and intervention[18].

The relationship between these adverse outcomes and the intrauterine exposure to HIV and to antiretroviral treatment remains controversial and unresolved. It is unknown if PTB and IUGR result from a direct effect of the virus, from the immunosuppression, from associated co-morbidities or from non-HIV associated factors[4]. Antiretroviral therapy use has also been suggested as a possible risk factor[19,38].

The reported prevalence of PTB and IUGR in infants born to HIV-infected mothers is variable and inconsistent[29,46]. This may be due to several reasons, including inappropriate assessment of gestational age (GA), lack of a standardized definition and assessment of IUGR, and the absence of adjustment for confounding factors[4,13]. The difficulty of obtaining precise information on GA has resulted in the use of low birth weight (LBW) as a proxy for IUGR in some studies[46]. In many places, the

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same social and behavioral risk factors related to HIV-infection, preterm birth and IUGR may be shared by the same women, resulting in a high prevalence of these adverse outcomes[50].

In this scenario, it is necessary to know the local reality in order to face the challenge. The city of Vitória has a population of about 327,081 with a 0.856 human development index, the lowest infant mortality rate in Brazil (7.93 : 1000) with a perinatal component of 65.7%[56] and a 63.0% fetal death proportion[39]. However, as in other places, there is high income distribution disparity and limited access to high quality healthcare[41]. From 1997 to 2001, the prevalence rate of HIV-infection in pregnant women was 0.41%[41], which was similar to the mean Brazilian rate[52]. The 3.1% MTCT rate was also similar to the 2.7 to 8.6% prevalence rates reported for Brazil[38,47,52].

Most of the published Brazilian studies have focused on gestational HIV-infection prevalence and on the MTCT rate with less attention paid to PTB and IUGR prevalence and associated outcomes. Therefore, the aim of this study was to determine the prevalence of PTB and of fetal growth disturbances in a public tertiary referral maternity unit for low income, HIV-infected women who are taking ART, as well as to verify its relation to the HIV infection stage.

PATIENTS AND METHODS

This is a case series observational and analytic study conducted at Cassiano Antonio Moraes University Hospital (HUCAM), a 314-bed tertiary public hospital in the city of Vitória, state of Espírito Santo, Southeastern Brazil. The HUCAM is one of the three regional referral centers for the treatment and care of HIV-infected patients, with an annual mean of 1500 deliveries.

Data were retrieved from HUCAM records for all 250 deliveries of HIV-infected pregnant women that occurred between November 2001 and May 2012. From this data, gestations that met all the following criteria were selected for study: known maternal HIV-infection diagnosis, singleton pregnancy, a minimum of 22 gestational weeks confirmed by an ultrasound examination before 22 weeks, and an adequate set of maternal and fetal biometrical data suitable for fetal growth analysis. One twin pregnancy was excluded, as were cases with missing ultrasound data or unavailable clinical records. In the studied period, one woman had two gestations and the remaining women had only one, totaling 74 gestations from 73 women for the study.

The study protocol was approved by the Institutional Review Board. As a retrospective survey of records, informed signed consent was not required, but access to patients' records was obtained from institutional and Public Health Authorities. All women and their children received the standard obstetrical and neonatal care. Strict confidentiality was adhered to regarding any data collected throughout the study.

Data on maternal, pregnancy and neonatal characteristics including infant HIV status during pediatric follow-up were collected from medical, obstetrical, neonatal and laboratory institutional records as well as the State Health Secretariat public reports. The data were extracted in a structured form, entered and analyzed in an Excel sheet (Microsoft Office 2010, Microsoft Corporation, USA) formatted to calculate the GA and to analyze the fetal dimensions and growth.

The diagnosis of HIV infection was based on the Brazilian National AIDS Program guidelines, with the following considered as positive indicators: a positive sample by ELISA screening, with confirmation by Western blot or indirect fluorescence reaction, and HIV infection diagnosis by Rapid Tests[11]. An HIV infection was classified as AIDS according to the current modified Centers for Disease Control and Prevention (CDC) criteria and the Rio de Janeiro-Caracas (1992) criteria and/or a documented medical AIDS diagnosis[11]. Children exposed to HIV were followed and those with two positive HIV DNA polymerase chain reaction (PCR) tests were classified as HIV-infected; a child with at least one negative DNA PCR test after the age of two months or a negative HIV-antibody test after the age of 18 months was classified as uninfected[11]. The medication type, duration, and beginning of use were combined to describe the multiclass ART used by each woman, during each week of gestation. A medication was considered as taken if used for at least five uninterrupted weeks. The beginning of use was classified according to the critical fetal growth period as either before 16 weeks (including before pregnancy), between 16 and 28 weeks or after 28 weeks of gestation.

Maternal BMI was classified according to WHO criteria[58] and HIV-associated co-morbidity was ascertained according to the Rio de Janeiro-Caracas criteria[11].

The GA at birth was expressed in exact days of gestation, calculated from the last menstrual period if confirmed by ultrasound examination before 22 weeks, using the last menstrual period estimate in cases with a discrepancy of up to seven days; otherwise the ultrasound estimate was used[43,44]. In cases of fetal deaths, the GA at death was calculated by subtraction of the intrauterine retention time estimated by anatomic-pathological criteria[28] from the calculated GA at birth. PTB was defined as delivery at less than 37 weeks of gestation, and very PTB as less than 34 weeks of gestation. Major fetal congenital malformation includes potentially lethal abnormalities or those that will require immediate postnatal surgery[57].

Neonatal dimensions (birth weight (BW), birth length (BL), head (HC) and abdominal (AC) circumferences) were measured within 24 hours of birth by neonatal doctors or nurses. In fetal death cases, the autopsy dimensions were used. Low birth weight (LBW) was defined as less than 2,500 grams, macrosomia as more than 3,999 grams and normal weight as anything between these two values.

To compare the various fetal dimensions, regardless of the GA at birth, all fetal dimensions were converted to z-scores for gestational age (z/GA score), using published fetal normative reference values for weight[25], BL[17], HC[15], and AC[16], re-expressed as proportional growth curves[25,26,27] and adjusted for location, term of pregnancy, neonatal measurement, and population median value at 40 weeks as has been recently recommended[7,26,32,40]. For birth length, the fetal length was estimated by femur diaphyseal length using the Hadlock formula[30] and the proportional growth curve was elaborated with the Chitty fetal femur length growth curve[17]. For the local values, the birth weight (3458 ± 415g), length (495 ± 23mm), head (350 ± 15mm) and abdominal circumference (325 ± 20mm) values at 277 to 284 days (40 weeks) from 273 singleton (male and female), non-malformed, live births in the city of Vitória from middle and high-income women between 2008 and 2013 were taken. The reference values for fetal weight (FW) were further adjusted for maternal race, weight, height, parity and fetal gender[25] and

BL[30] and HC for fetal sex only[48]. Each fetal dimension was categorized as small (SGA), adequate (AGA) and large (LGA) for GA, defined as having a z/GA value below, between or beyond the ±1.28 z/GA score, the usual clinical cut-off to demarcate the 10th and 90th percentiles. Venn diagrams were used to analyze the combining distribution of the PTB, LBW and categorized fetal growth assessments.

Statistical analysis. Cases with missing data for a variable were excluded from the analysis of that specific variable. The available data from excluded cases were compared with the included cases. For categorical data, frequency distributions and corresponding 95% confidence intervals (95% CI) were calculated. For continuous variables, a standard descriptive statistical analysis was performed, including the calculation of the arithmetic mean and standard deviation, median, quartiles and range as well as the distribution depicted as box-plots[31]. The proportions of observed growth disturbances categories (SGA, AGA, and LGA), were compared with the expected values (10%, 80%, and 10%), taking the reference value distribution as a control group[14]. The quantitative contrasts between groups' proportions were analyzed by direct increment[24], and by chi-square test statistics with a bicaudal 0.05 significance value; the Fisher exact test was used if the expected count for a cell table was less than 5.

RESULTS

Casuistry, maternal, gestational and perinatal characteristics, and outcome. The 74 cases studied amount to 26.6 % of HUCAM and 6.6% of all Espírito Santo State maternal HIV-infected deliveries in the studied period. Maternal demographic, clinical, obstetrical and perinatal details and outcomes are shown in Tables 1 and 2.

Table 1
Sociodemographic, behavioral, and medical characteristics

	Included cases		Excluded cases
	HIV	AIDS	All
	F/N (%)[a]	F/N (%)	F/N (%)
Demographic			
Maternal age - less than 20 years	3/34 (8.8%)	1/40 (2.5%)	18/172 (10.4%)
Maternal age - more than 35 years	3/34 (8.8%)	7/40 (17.5%)	13/172 (7.5%)
Ethnicity - non-white	28/34 (82.3%)	28/40 (70.0%)	115/150 (76.6%)
Instruction - illiterate or less than 5 years	9/34 (26.4%)	10/40 (25.0%)	15/35 (42.8%)
Instruction - 5 to 8 years	11/34 (32.3%)	10/40 (25.0%)	13/35 (37.1%)
Occupation - housewife	20/33 (60.6%)	23/40 (57.5%)	84/105 (80.0%)[b]
Marital status - unmarried	3/31 (9.6%)	8/39 (20.5%)	14/32 (43.7%)[b]
Residency outside Vitoria City	25/34 (73.5%)	27/40 (67.5%)	90/160 (56.2%)[b]
Obstetrical history			
Primiparity	10/34 (29.4%)	8/40 (20.0%)	8/34 (23.5%)
Multiparity - 3 or more	10/34 (29.4%)	10/40 (25.0%)	10/34 (29.4%)
Previous abortion	11/34 (32.3%)	13/40 (32.5%)	8/34 (23.5%)
Previous perinatal death	4/34 (11.7%)	3/40 (7.5%)	1/34 (2.9%)
Current pregnancy			
Prenatal care - < 6 attendances	7/33 (21.2%)	10/40 (25.0%)	37/63 (58.7%)[b]
Prenatal care - not in HUCAM	21/34 (61.7%)	19/40 (47.5%)	35/61 (57.3%)
Body mass index - < 18.5	3/34 (8.8%)	4/38 (10.5%)	4/31 (12.9%)
Body mass index - 25 < BMI < 30	6/34 (17.6%)	9/40 (22.5%)	6/31 (19.3%)
Body mass index - BMI > 29.9	4/34 (11.7%)	1/40 (2.5%)	2/31 (6.4%)
Smoking - any	4/34 (11.7%)	7/39 (17.9%)	16/34 (47.0%)[b]
Alcohol abuse	2/34 (5.8%)	0/40 (0.0%)	7/35 (20.0%)[b]
Illicit drug use	2/34 (5.8%)	1/40 (2.5%)	7/35 (20.0%)[b]
Maternal anemia	6/32 (18.7%)	5/38 (13.1%)	10/31 (32.2%)
Hypertension (any type)	4/33 (12.1 %)	5/40 (12.5%)	3/32 (9.3%)
Urinary tract infection	7/33 (21.2%)	6/40 (15.0%)	5/31 (16.1%)
Diabetes mellitus	1/33 (3.0%)	0/40 (0.0%)	0/34 (0.0%)
Syphilis	1/33 (3.0%)	1/40 (2.5%)	5/31 (16.1%)[b]
HSV infection	0/33 (0.0%)	2/38 (5.2%)	1/34 (2.9%)
HPV infection (condiloma acuminatum)	2/32 (6.2%)	3/38 (7.8%)	3/33 (9.0%)

a: number refers to absolute frequency (F) of category, number of cases with data (N) and relative frequency (%); **b**: $p < 0.05$ on chi square or Fisher exact test between included versus excluded cases; **c**: $p < 0.05$ on chi square or Fisher exact test between HIV versus AIDS cases; non-significant contrast not flagged.

Table 2
Labor, perinatal outcome and HIV infections details

	Included cases		Excluded cases
	HIV	AIDS	All
	F/N (%)[a]	F/N (%)	F/N (%)
Labor and perinatal outcome			
Labour - spontaneous	12/34 (35.2%)	13/40 (32.5%)	16/34 (47.0%)
Labour - none	19/34 (55.8%)	26/40 (65.0%)	18/34 (52.9%)
Rupture of Membranes - spontaneous	6/33 (18.1%)	8/39 (20.5%)	9/34 (26.4%)
Caesarian delivery	26/34 (76.4%)	37/40 (92.5%)[c]	109/155 (70.3%)[b]
Fetal sex - female	22/34 (64.7%)	19/40 (47.5%)	77/154 (50.0%)
Gestational age - mean ± s (days)	267 ± 19	262 ± 24	263 ± 25
Gestational age - median (IQR)	271 (266 - 275)	271 (263 - 273)	271 (260 - 278)
Preterm birth - less than 37 weeks	2/34 (5.9%)	11/40 (27.5%)[c]	22/92 (23.9%)
Birth weight - mean ± sd (grams)	2939 ± 715	2717 ± 751	2840 ± 684
Birth weight - median (IQR) (grams)	2950 (2669 - 3339)	2893 (2519 - 3169)	2900 (2550 - 3280)
Low birth weight - less than 2500 grams	5/34 (14.7%)	10/40 (25.0%)	36/157 (22.9%)
Apgar score - less than 7 in first minute	1/33 (3.0%)	1/39 (2.5%)	13/150 (8.6%)
Need for intensive neonatal care	7/32 (21.8%)	11/39 (28.2%)	5/32 (15.6%)
Major congenital anomalies	0/34 (0.0 %)	2/40 (5.0%)	0/35 (0.0%)
ToRCH infection - syphilis	1/74 (2.9%)	1/40 (2.5%)	-
ToRCH infection - toxoplasmosis	0/74 (0.0%)	1/40 (2.5%)	-
Fetal death	1/34 (2.9%)	1/40 (2.5%)	2/172 (1.1%)
Neonatal death	0/34 (0.0%)	1/40 (2.5%)	3/172 (1.7%)
HIV infection details			
HIV diagnosis prior to current pregnancy	15/34 (44.1%)	36/41 (87.8%)[c]	22/35 (62.8%)
HIV status of sexual partner - positive	13/23 (56.5%)	13/27 (48.1%)	12/20 (60.0%)
Cases with AIDS defining features	-	40/74 (54.1%)	70/119 (58.8%)
Viral load - 1000 to 10000 copies	8/24 (33.3%)	6/39 (15.3%)	6/25 (24.0%)
Viral load - more than 10000 copies	4/24 (16.6%)	8/39 (20.5%)	6/25 (24.0%)

a: number refers to absolute frequency (F) of category, number of cases with data (N) and relative frequency (%); **b**: $p < 0.05$ on chi square or Fisher exact test between included versus excluded cases; **c**: $p < 0.05$ on chi square or Fisher exact test between HIV versus AIDS cases; non-significant contrast not flagged.

HIV infection. HIV infection was diagnosed before the current pregnancy in 68.9% of cases and during antenatal care in 31.1% of cases. AIDS cases amounted to 40 (54.1%) of the cases. Among cases with data, 28.8% (19/66) had a CD4 count of less than 350 cells/mm^3 (10.6% less than 200 cells/mm^3), and 19.0% (12/63) had higher than 10,000 viral load copies/mL. Out of 54 children with at least 18 months of postnatal follow-up, there was no MTCT of HIV; no data were available about the 20 other children.

Perinatal outcome. The cesarean delivery rate was 85.1%, without variation in the studied period. There was one neonatal death and two major fetal congenital malformations, all three from AIDS cases.

Antiretroviral therapy. According to ART use criteria (being used for at least five uninterrupted weeks of gestation), the lack of use of any ART during gestation was observed in five of the 74 cases (6.8%). Of the 69 gestations in which ART was used, 24 (34.8%) started ART prior to the current gestation, 11 (15.9%) started between one and 15 weeks, 30 (43.5%), between 16 and 28 weeks, and four (5.8%) between 29 weeks and delivery; intrapartal zidovudine was used in 95.7% cases. One-drug ART was used in six (8.7%) of the cases, two-drug therapy in one (1.4%), and a three or more drug therapy (HAART) in 62 (89.9%); 56 (90.3%) out of the 62 HAART users took a protease inhibitor, of which 19 (30.6%) took the protease inhibitor drug during the whole gestation. Therapy drug switch was observed in nine (13.0%) of the 69 cases.

Gestational age. The mean GA for all cases was 264 (37 weeks and 6 days) ± 22 days, but was five days lower for the AIDS cases. The overall PTB rate was 17.5% (95% CI: 0 to 38.3%) (Table 3), higher in AIDS cases; the very preterm birth (less than 34 weeks) rate was 10.8% without significant difference between AIDS and HIV-only cases; no post-term delivery was observed. A comparison between the included and the excluded cases regarding PTB revealed similarity, but there was a significant difference between the AIDS and HIV-only cases (Table 2).

Fetal growth disturbances. The distribution of fetal growth disturbances in all cases and according to HIV infection status (HIV-only and AIDS) is shown in Figure 1 and in Table 3. The median birth

Table 3
Distributions of gestational age, absolute and z score value of fetal dimensions, and proportions of preterm births, low birth weight and small for gestational age

Variable	All **cases**	HIV **cases**	AIDS **cases**	
Gestational age (days) - n	**74**	**34**	**40**	
mean ± sd	264 ± 22	267 ± 20	262 ± 23	
median (interquartile range)	271 (265 - 275)	271 (266 - 277)	271 (263 - 274)	
<34 weeks - F (%)[a]	8 (10.8%)	2 (5.8%)	6 (15.0%)	ns
<37 weeks - F (%)	13 (17.5%)	3 (8.8%)	10 (25.0%)	ns
Fetal weight (grams) - n	**74**	**34**	**40**	
mean ± sd	2819 ± 738	2939 ± 715	2717 ± 751	
median (interquartile range)	2915 (2595 - 3232)	2950 (2669 - 3339)	2893 (2518 - 3169)	
z/GA score - mean ± sd	-0.40 ± 1.37	-0.23 ± 1.49	-0.55 ± 1.26	
< 2500 grams	15 (20.2%)	5 (14.7%)	10 (25.0%)	ns
SGA - F (%)	12 (16.2%)	6 (17.6%)	6 (15.0%)	ns
Fetal length (mm) - n	**73**	**33**	**40**	
mean ± sd	466 ± 40	478 ± 26	456 ± 47	
median	470 (460 - 485)	475 (460 - 490)	470 (444 - 485)	
z/GA score - mean ± sd	-0.55 ± 1.28	-0.19 ± 1.00	-0.86 ± 1.14	
SGA - F (%)	14 (19.1%)	2 (6.0%)	12 (30.0%)	*
Head circumference (mm) -n	**72**	**33**	**39**	
mean ± sd	335 ± 30	339 ± 21	330 ± 36	
median	340 (330 - 350)	340 (335 - 350)	340 (330 - 348)	
z/GA score - mean ± sd	-0.28 ± 1.32	-0.20 ± 1.00	-0.35 ± 1.54	
SGA - F (%)	10 (13.8%)	3 (9.0%)	7 (17.9%)	ns
Abdominal circumference (mm) - n	**63**	**30**	**33**	
mean ± sd	303 ± 40	307 ± 27	300 ± 49	
median	310 (295 - 330)	308 (300 - 320)	310 (290 - 330)	
z/GA score - mean ± sd	-0.40 ± 2.03	-0.41 ± 1.13	-0.39 ± 2.62	
SGA - F (%)	11 (17.4%)	4 (13.3%)	7 (21.2%)	ns

[a]: number refers to absolute frequency (F) of category, number of cases with data (N) and relative frequency (%). *: $p < 0.05$ on chi square test between HIV versus AIDS cases; non-significant contrast not flagged.

weight was 57 grams higher in HIV-only cases compared to AIDS cases. LBW was observed in 20.2% (95% CI: 11.1 to 29.5%) of cases, with a higher non-significant proportion (25.0% versus 14.7%) in AIDS than in HIV-only cases. Fetal macrosomia was observed in one HIV-only case.

The mean BW z/GA score was -0.40 ± 1.37 (all cases), -0.55 (AIDS), and -0.23 (HIV-only) cases. SGA BW was found in 16.2% (95% CI: 7.8 to 24.6%) of cases without significant difference between HIV-only and AIDS cases (17.6% versus 15.0%); among the 12 SGA birth weight three (25.0%) were preterm. LGA BW was found in 6.8% (95% CI: 1.1 to 12.5%) of cases, occurring in all but one of the HIV-only cases. LBW and SGA BW were not significantly more frequent in cases with mothers that were smokers compared to those that were non-smokers (9.1% versus 9.7% and 9.1% versus 17.7%, respectively).

The mean BL z/GA score was -0.55 ± 1.27 (all cases), -0.19 (HIV-only), and -0.86 (AIDS). The median birth length was 5 mm lower in AIDS than HIV-only cases (Table 3) but a SGA BL was found in 19.1% (95% CI: 10.0 to 27.8) of cases, with a higher significant rate in AIDS cases (30.0% versus 6.0%). LGA BL was found in 4.1% of cases (95% CI: 0 to 8.6%), all in HIV-only cases.

The mean head circumference z/GA score was slightly reduced with a mean of -0.28 ± 1.32 (all cases), -0.20 (HIV-only), and -0.35 (AIDS). A SGA HC was observed in 13.8% (95% CI: 8.9 to 26.3%) of cases with a higher, but not significantly, rate in AIDS than in HIV-only cases (17.9% versus 9.0%). LGA HC was observed in nine (12.2%, 95% CI: 4.7 to 19.7%) cases, five in AIDS cases. The mean abdominal circumference z/GA score was also slightly reduced with a mean of -0.40 ± 2.03 without significant difference between HIV-only and AIDS cases. A SGA AC was observed in 11 (17.4%, 95% CI: 0 to 40.0%) cases and a LGA AC in five (6.8%, 95% CI: 1.1 to 12.5%) cases. Neither the SGA nor LGA AC proportion differed between AIDS and HIV cases.

Combining distribution of PTB and growth restriction. Out of 15 cases of LBW, eight (53.3%) were preterm only, four (26.7%) were SGA only, and three (20.0%) were both PTB and SGA cases. Out of the 63 cases with all four fetal dimensions available, the combined

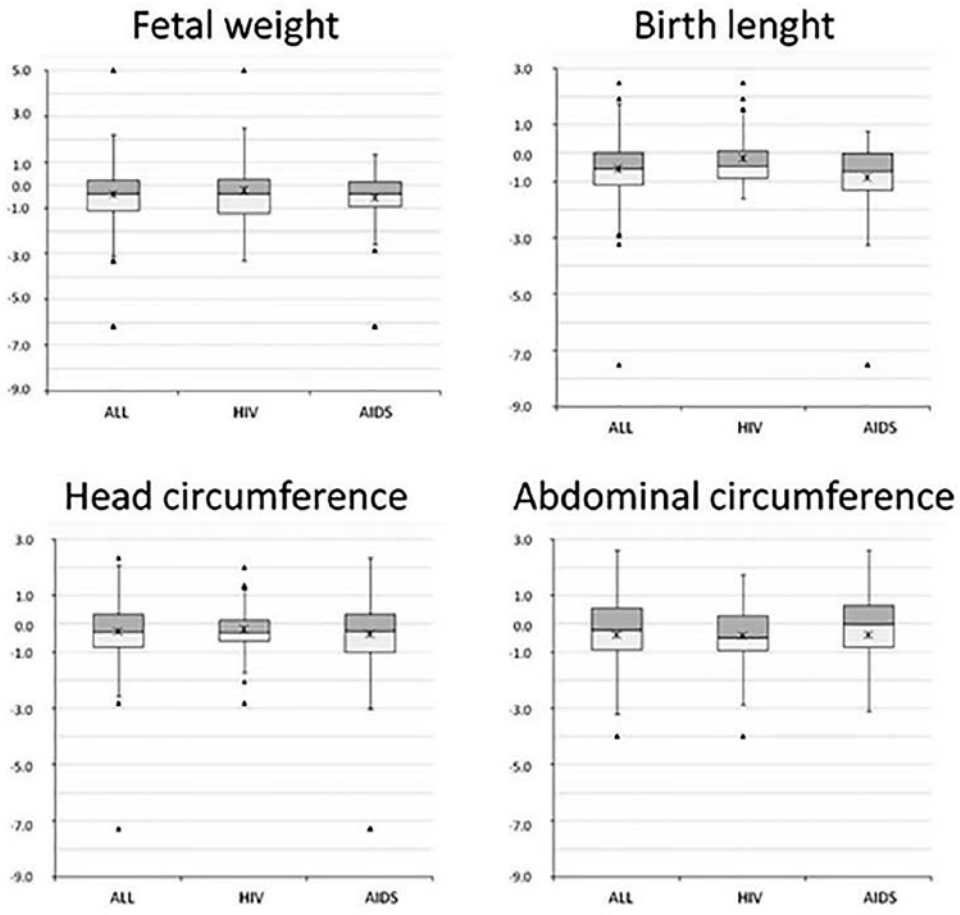

Fig. 1 - Distribution of z-score for gestational age of fetal dimensions.

distribution was analyzed by Venn diagram (not shown) and revealed that 24 (38.1%) cases had, at least, one SGA dimension, 11 (17.5%) at least two, 13 (20.6%) only one, seven (11.1%) only two, and two (3.2%) all four SGA fetal dimensions. Out of the 12 SGA BW, 50% were also SGA BL and SGA AC.

DISCUSSION

The HIV-infected women assisted at HUCAM come from the local community, mainly from outside the metropolitan area of the city of Vitória. Due to the study design, case selection was made from a tertiary hospital and there was a strict requirement for ultrasound validated GA estimation, the representativeness of casuistry cannot be assured. A comparison between included and excluded cases regarding the available demographic and clinical details (Tables 1 and 2) reveals similarity in maternal age, ethnicity, instruction, parity, previous abortion, AIDS, PTB birth and LBW proportions, but the included cases have significantly more women residing outside the area of the city of Vitória and significantly fewer cases of housewives, unmarried women, alcohol, illegal drug and tobacco users, low antenatal attendance, syphilis in current pregnancy, and cesarean delivery so a selection bias could not be excluded from cases with a lower risk of adverse outcomes. However, HIV-only cases did not differ significantly from AIDS cases regarding any demographics and behavioral and medical characteristics (Table 2). Moreover, the demographics and clinical characteristics of the studied cases were similar to other local studies[41] and likely represent the poor pregnant women of the large metropolitan areas of Brazil. The fact that 68.9% of the mothers knew their HIV status before pregnancy highlights the need for education in order to minimize the disease's burden.

The 17.5% PTB rate observed in this study was higher than that reported for the general population of developed countries (5.0 to 7.0%)[35], Brazil (3.4 to 15.0%)[51], and the state of Espírito Santo (4.8%)[10]. Furthermore, it was similar to the prevalence reported in HIV-infected pregnancies in Europe (14.7 to 16.0%)[9,53], Africa (13.3 to 21,8%)[46,54] and Brazil (11.5 to 18.2%)[36,32], as well as the prevalence reported for the USA (17.0 to 19.9%)[49,19]. A non-significant trend for a higher proportion of PTB in AIDS cases, compared with HIV-only cases was observed.

In this study, compared to the expected population distribution (10%), the BW z/GA score had a shift towards lower values and the proportion of SGA BW was higher, clearly indicating some fetal growth restriction in the children of HIV-infected women. The results of this investigation are similar to those reported by FAUSTO *et al*.[23], in a cohort of 130 full-term

infants in Belo Horizonte, state of Minas Gerais: mean BW z/GA score of -0.52 ± 0.88 for the non-infected and -0.83 ± 1.11 for the infected infants, without statistically significant differences between both groups. Although they do not report the SGA proportion, there is a clear shift towards lower values. The finding of an increased proportion of SGA BW in infants of HIV-positive mothers in this investigation accords with previous studies in Africa[46,54], but was lower than the 31.2% reported by AARON *et al.* in the USA[1].

Likewise, the observed 20.2% prevalence of LBW was higher than the 10 to 18% for developed countries[19], the 9.0% for population-based Brazilian studies[51], and for the 6.6% of SINASC-ES[10]. However, it was similar to the reported proportion in Europe[9], the USA[7], Africa[54], and Brazilian HIV-infected women[37]. LBW may be due to PTB, SGA or both[22]. In developed countries, where LBW rates are low, it is caused mainly by PTB whereas in developing countries, where the prevalence is high, LBW is often due to IUGR[6,34]. In the casuistry of this study, 26.7% of LBW were due to SGA alone, 53.3% to PTB alone and 20.0% to both PTB and SGA BW. Previous reports of more LBW in AIDS than in non-AIDS cases[21, 36] could not be confirmed.

Little attention has been paid to the temporal and the compartmental frames of fetal growth, that is, the timing at which each fetal organ has its maximum growth and is more vulnerable to growth disturbance. As the various risk factors may act at different moments of intrauterine life they may have different effects on different fetal organs[20]. The distribution of SGA affecting fetal compartments other than BW has rarely been reported. The observed proportion of SGA BL in babies born to HIV-infected mothers agrees with some[5,45] but not all studies[29]. HC is an important parameter to assess, because the brain is the last and least affected organ in fetal deprivation[15]; a reduced HC was reported in some studies[2,45], but not confirmed in others[29]. Conversely, the AC, reflecting the fetal liver and abdominal subcutaneous mass,[16] is the most sensitive and early affected dimension in IUGR; a report of AC analysis in infants of HIV-infected mothers could not be found.

Despite the presence of only 11 (17.5%) cases with at least two concomitant SGA fetal dimensions, it was found that 50% of the 12 cases with SGA BW also have SGA BL or SGA AC, pointing to a split distribution of symmetric and asymmetric types of BW growth restriction. Only one report pointing to an early effect of HIV infection on fetal growth[21] could be found.

The relative contribution of HIV and non-HIV-infection risk factors to adverse perinatal outcomes has not been well defined. Maternal poverty, lack of social support, risk behaviors such as tobacco and illegal drug use, medical and gestational diseases such as anemia, diabetes, hypertension, among others, are well-known risk factors for PTB and IUGR in both non-HIV-infected and HIV-infected women[18]. Such factors could add to HIV-infection and related conditions (such as high viral load, immunosuppression, HIV-associated infections and ART), increasing the risk of PTB and poor fetal growth. Therefore, it is difficult to separate the independent roles of the non-HIV associated factors from those more directly related to HIV in PTB and IUGR determination[8]. In this study, the following tendency was observed: a higher prevalence of PTB, LBW and SGA BW, BL, HC, and AC in infants born to HIV-infected mothers with AIDS compared with those born to non-AIDS mothers. However, due to the small casuistry, it was not possible to explore the role of the severity of HIV infection or its complex interaction with non-HIV comorbidity and specific ART in PTB and fetal growth determination. Larger studies are necessary to solve this issue[13].

There are many reasons for the discrepancy in reported HIV-infection related prevalence of PTB and IUGR. Firstly, few studies have used adequate estimates of GA[13,19,44]. A systematic review of Brazilian PTB prevalence found only one out of twelve population-based studies using an early ultrasound validated GA estimate[51]. The compliance with that requirement in casuistry of low income publicly assisted women may explain the huge case exclusion in this study.

Secondly, the mean is not an adequate indicator of growth disorders. Besides, comparing the observed mean with a local control group could be misleading because such a control group may not adequately reflect a normal population. However, even without a difference between HIV-exposed and non-exposed fetuses (or in HIV-only versus AIDS cases, as shown in the present study), a significant difference in SGA proportions of growth disturbances could emerge from comparison between the study group and reference distribution, the true "control group"[14].

Thirdly, the lack of a standardized definition of IUGR[13], and the use of a non-representative reference norm[7,40,43,55] limit comparability between studies[40,7]. As the references for birth dimensions underestimate the preterm growth compared with a fetal reference[32], a hybrid fetal and neonatal growth curve adjusted to local median term neonatal dimensions should be used[7,25,40]. Furthermore, any available population reference does not account for within-population variations attributable to maternal height, weight, parity and fetal gender, known factors that may affect fetal growth[26,27] for which the customized or individualized reference has been proposed[26,27,40]. The decision of what reference to adopt affects the growth categorization and the prevalence of growth disorders[1]. There is no published growth study either employing that approach in Brazil, or using fetal rather than birth weight reference standard in HIV-infected women.

Lastly, it is difficult to discriminate the role of HIV-infection on fetal growth from the numerous and geographically variable proportion of others risk factors[13,50]. To add complexity, the association of these risk factors leads to the existence of multiple possible groups, which would demand a larger number of cases to allow a detailed analysis.

Limitations of this study. The exclusion of many cases due to missing records and data accounts for an inaccurate representation of the overall local or national HIV-infected mothers. This is a typical issue of the health care contradiction of low-income people in Brazil: regardless of the open access to healthcare and the high proportion of prenatal attendance and hospital delivery, there is evidence of poor quality care[42] such as many instances of non-compliance with standard care, lack of access to and delay in delivering complementary exam results. Besides, the poor quality, loss of, and hindrance in accessing patients' records explain, for instance, the lack of explicit records of cesarean delivery. However, in spite of the huge exclusion, no significant difference regarding PBW or LBW rate was observed.

Due to the small casuistry, a multivariate analysis and adjustment for specific ARV regimen and for important risk factors such as smoking, hypertension and anemia in the PTB and IUGR determination could not be performed. Also, it was not possible to address the potential effects of

different ARV schemes in gestation, with or without protease inhibitor (PI), that are not clearly established[9,19,49,54]. However, as the majority of patients were already using ARV, the longer exposure to ARV could explain the higher proportion of adverse fetal outcomes in this study.

Finally, a serial fetal growth assessment, as recommended and clinically employed with symphysis-fundal height[26] and ultrasound measurements[44], was not made. Indeed, a true growth study demands demonstration of a reduced growth velocity along with the gestation. However, the study was limited to a few adequate sets of timely ultrasound measurements of fetal dimensions.

Larger and multicenter natural history studies of HIV-infected cohorts of pregnant women are necessary to provide more definite answers regarding the relationship between HIV infection, co-morbidity, ART use, and preterm birth and fetal growth disorders. Despite the small sample size and these limitations, the authors believe the results of this study are important because of the scarcity of Brazilian reported studies on PTB and IUGR prevalence in HIV-infected women. Moreover, they emphasize the need for prenatal, at birth and postnatal growth monitoring to identify infants with suboptimal growth and to ensure appropriate care and treatment, in order to improve their clinical course, quality of life and prospects.

CONCLUSION

This study found a high prevalence of preterm birth, low birth weight and small gestational age, fetal weight, length, head and abdominal circumferences associated with HIV-infected, ART using, low-income and publicly assisted Brazilian women. A trend for higher prevalence of PTB, LBW and SGA fetal dimensions in infants born to AIDS compared with non-AIDS mothers was observed.

CONFLICTS OF INTEREST

The authors declare none.

RESUMO

Nascimento pré-termo e restrição de crescimento fetal em gestantes brasileiras infectadas pelo HIV

Introdução: A infecção materna pelo HIV e comorbidades associadas podem ter duas consequências para a saúde fetal, a transmissão vertical e o desfecho perinatal adverso. Após o sucesso em reduzir a transmissão vertical, deve-se dar atenção ao risco potencial de nascimento pretermo (PRT) e de restrição de crescimento fetal (RCF). **Objetivo**: Determinar a prevalência de PRT e RCF em gestantes de baixa renda, infectadas pelo HIV, usuárias de terapia antirretroviral atendidas em hospital público terciário e verificar sua relação com o estágio da infecção viral. **Casuística e métodos**: Dentre os 250 partos de gestantes infectadas pelo HIV, ocorridos em um hospital universitário na cidade de Vitória, estado do Espírito Santo, Sudeste do Brasil, entre novembro de 2001 e maio de 2012, foram selecionadas 74 gestações não-gemelares, com idade gestacional confirmada por ultrassonografia e as dimensões neonatais: peso ao nascer (PN), comprimento (CN) e perímetros cefálico (PC) e abdominal (PA). Os dados foram extraídos dos prontuários clínicos e laboratoriais e o desfecho sumarizado como nascimento pretermo (PRT < 37 semanas), baixo peso ao nascer (BPN < 2500g) e como pequeno (PIG), adequado (AIG) e grande (GIG) para a IG, definido como tendo um menor valor, entre e maior que ± 1.28 z/IG escore, o critério clínico usual para demarcar os percentis 10 e 90. **Resultados**: PRT foi observado em 17,5%, BPN em 20,2% e PN, CN, PC e PA PIG em 16,2%, 19,1%, 13,8% e 17,4%, respectivamente. As respectivas proporções observadas nos casos de HIV e AIDS foram: PRT: 5,9 versus 27,5%, BPN: 14,7% versus 25,0%, PFN PIG: 17,6% versus 15,0%, CN: 6,0% versus 30,0%, PC: 9,0% versus 17,9% e PA: 13,3% versus 21,2%; somente a diferença de CN PIG foi estatisticamente significativa. Dentre 15 neonatos com BPN, oito (53,3%) eram somente PRT, quatro (26,7%) PIG somente e três (20,0%) PRT e PIG. Concomitância no mesmo caso de pelo menos duas dimensões PIG foi observada frequentemente. **Conclusão**: A proporção de baixo peso ao nascer foi maior que a prevalência local e brasileira e foi observada uma tendência para maior proporção de dimensões fetais PIG que a distribuição populacional esperada nesta pequena casuística de filhos de gestantes infectadas pelo HIV, usuárias de antirretrovirais, de baixa renda e assistidas em hospital público terciário. Observou-se também tendência para maior prevalência de PTR, BPN e dimensões fetais PIG em recém-nascidos de mães com AIDS comparados com os de mães infectadas por HIV sem AIDS.

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AUTHORS' ROLES

Merçon de Vargas PR and Reis HLB participated in the conception and design of the study, as well as the drafting of the article. Merçon de Vargas PR, Reis HLB, Rosato DP, Rocha DR, Araujo KS and Ribeiro LP participated in the acquisition, analysis and interpretation of data. Merçon de Vargas PR, Reis HLB and Passos MRL contributed in the critical revisions of important intellectual content and final approval of the version to be published; all authors gave constructive comments during the writing and interpretation of results and substantially contributed to and approved the final manuscript.

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Enterobacteriaceae ISOLATES FROM THE ORAL CAVITY OF WORKERS IN A BRAZILIAN ONCOLOGY HOSPITAL

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SUMMARY

The evaluation of workers as potential reservoirs and disseminators of pathogenic bacteria has been described as a strategy for the prevention and control of healthcare-associated infections (HAIs). The aim of this study was to evaluate the presence of *Enterobacteriaceae* in the oral cavity of workers at an oncology hospital in the Midwest region of Brazil, as well as to characterize the phenotypic profile of the isolates. Saliva samples of 294 workers from the hospital's healthcare and support teams were collected. Microbiological procedures were performed according to standard techniques. Among the participants, 55 (18.7%) were colonized by *Enterobacteriaceae* in the oral cavity. A total of 64 bacteria were isolated, including potentially pathogenic species. The most prevalent species was *Enterobacter gergoviae* (17.2%). The highest rates of resistance were observed for β-lactams, and 48.4% of the isolates were considered multiresistant. Regarding the enterobacteria isolated, the production of ESBL and KPC was negative. Nevertheless, among the 43 isolates of the CESP group, 51.2% were considered AmpC β-lactamase producers by induction, and 48.8% were hyper-producing mutants. The significant prevalence of carriers of *Enterobacteriaceae* and the phenotypic profile of the isolates represents a concern, especially due to the multiresistance and production of AmpC β-lactamases.

KEYWORDS: Carriers; *Enterobacteriaceae*; Multidrug-resistant; Beta-lactamases.

INTRODUCTION

Healthcare-associated infections (HAIs) are transmissible and result from the interaction of multiple factors which work differently in the infection chain[9]. In this context, workers have been indicated as possible disseminators of pathogenic microorganisms in and out of the hospital environment[17, 29].

In their daily work, these workers are exposed to various health risks, such as areas of insalubrity, contact with sick people and various biological agents. These factors, associated with the time spent in the institution, type of care provided and lack of adherence to biosecurity measures, make these workers susceptible to colonization by different microorganisms, including enteric bacteria, such as *Enterobacteriaceae*[9,32].

Once colonized, the carrier condition is established, and these individuals begin to work directly in the transmission chain of HAIs, both as reservoirs and sources of infectious agents. The dissemination of microorganisms into the environment and into susceptible hosts increases the risk of infection and the occurrence of outbreaks[9,29].

The investigation of workers as carriers of pathogenic and multiresistant bacteria has been cited as a strategy to prevent and control HAIs. The majority of studies available on the topic report that HAIs are primarily associated with microbial transmission through the hands and nasal cavities of the workers[4,12,19]. Yet the colonization of other anatomical sites also contributes to the spread of pathogens[9,15,35].

The mouth is an important location for investigation, since its anatomical and physiological characteristics make it a favorable location for microbial proliferation[13,21]. Microorganisms can spread from the mouth via aspiration through oropharyngeal secretions or transmission via saliva droplets from speaking, coughing, sneezing or breathing[9,13].

According to literature, *Staphylococcus aureus* is the most commonly studied colonization agent among workers at healthcare institutions[4,12,29]. Studies on colonization by gram-negative bacteria, especially *Enterobacteriaceae,* are rare, yet it is important to learn more regarding carriers in public health settings.

Enterobacteriaceae is a family of gram-negative rods (GNR) that have stood out in the healthcare environment due to the variety of severe infections they can cause, and their high rates of antimicrobial resistance[35]. One aggravating factor in this scenario has been the

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emergence of β-lactamase-producing strains, which constitute the most important mechanism of resistance to β-lactam antimicrobials[35].

In light of the lack of studies on the theme, this study was designed to increase knowledge regarding the colonization of the oral cavity of workers by *Enterobacteriaceae.* The objective of this study was to analyze the presence of *Enterobacteriaceae* in the oral cavity of workers in an oncology hospital, as well as to characterize the phenotypic profile of the isolates.

MATERIAL & METHODS

Study type and location: This was a cross-sectional, descriptive epidemiological study, performed with workers in a large oncology hospital that is a reference for cancer treatment. On average, this hospital treats 28,000 patients every month, who are predominantly users of the Brazilian Unified Health System (SUS, as per its acronym in Portuguese), and performs 67,000 procedures, including: consultations, hospitalizations, surgeries, treatments and exams, among others.

The present study was carried out between May 2009 and November 2010, and is part of a larger surveillance study on the colonization of workers by pathogenic microorganisms. The research proposal was approved by the hospital's Ethics Research Committee (Protocol-CEPACCG/040/08). All the workers were informed regarding the objectives of the study, as well as the collection procedures. The workers who agreed to participate in the study signed the Free and Informed Consent Form (FICF).

Study population: The study population consisted of 294 workers, including 149 members of the healthcare team and 145 workers from the support sector. The healthcare team was comprised of physicians, nurses, nursing technicians and assistants, pharmacists, physicists, physical therapists, nutritionists and psychologists. These professionals worked in the following departments: Surgery Center, Hospital Infection Control, Dressing, Endoscopy, Nursing Stations, First Aid, Adult and Child Chemotherapy, Radiotherapy, Rehabilitation and Physiotherapy, Intensive Therapy and Bone Marrow Transplant.

The support team was comprised of workers from the following departments: Materials and Sterilization, Sterilization and Cleaning, Nutrition and Diet and Clothes/Materials Reprocessing. The participants were listed and codified based on the information obtained at the institution.

These workers were chosen because they were considered to be directly responsible for patient healthcare, removal of dirt and contamination from the environment, preparation and distribution of food, as well as reprocessing of materials and clothing used in the hospital.

The following inclusion criteria were observed: belonging to one of the professional categories cited above and working in one of the chosen sectors during the study period. Workers who were using antimicrobials, or who had collected specimens seven days prior to data collection for this study, were excluded from participating in the study.

Collection procedures: The study had three phases: the first entailed inviting the workers to participate, clarifications regarding the research, and signing the FICF; the second phase entailed application of a questionnaire for collection of sociodemographic, professional, disease/infection and behavioral characteristics of the workers; and the third phase entailed collection of unstimulated saliva samples[18]. All three phases were performed on the same day.

Collection and processing of the samples: One unstimulated saliva sample (0.7 to 1.0 mL) was collected from each participant and each was placed in a disposable and sterilized plastic (polypropylene) container, totaling 294 samples. Collection was done by the worker, and supervised by the study researchers and their assistants[18]. The samples were homogenized (vortex) and 20 μL aliquots were sowed in a selective culture of MacConkey agar, followed by incubation at 35 ºC for 24-48 h[35].

Isolation and identification of the *Enterobacteriaceae*: The microbiological procedures for the isolation and identification of microorganisms were performed according to standardized and countersigned techniques[35]. Standard strains from the American TypeCulture Collection (*Escherichia coli* ATCC® 2592 and *Klebsiella pneumoniae* ATCC® 700603) were used as quality control for the tests performed.

The bacteria isolates in MacConkey agar were previously identified, according to the macroscopic and microscopic characteristics (Gram staining) of the colonies. Differentiation of the species was done through a series of biochemical screening (carbohydrate fermentation in Kligler Iron agar and cytochrome oxidase production) and classification tests (indole production; presence of motility; ornithine, arginine and lysine decarboxylation; citrate utilization; phenylalanine deaminase production; urease production; hydrogen sulfide production and methyl red test).

Profile of susceptibility to anti-infective agents: The susceptibility profile of the isolates was evaluated using the method of disc diffusion in agar (antibiogram), according to recommendations of the Clinical and Laboratory Standards Institute, state of Pennsylvania, USA[5]. The microorganisms that were simultaneously resistant to two or more different classes of antimicrobials were considered multiresistant[28].

In total, 16 antimicrobial agents were evaluated: amoxicillin/clavulanic acid, aztreonam, cefepime, cefotaxime, cefoxitin, cefpodoxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, piperacillin-tazobactam, tetracycline and trimethoprim-sulfamethoxazole[5].

Phenotypic detection of β-lactamases production: The three types of β-lactamases studied were: inducible chromosomal AmpC β-lactamase, Extended Spectrum β-lactamase (ESBL) and *Klebsiella pneumoniae* Carbapenemase (KPC). Phenotypic tests were performed in two stages, screening and confirmation, according to standardized techniques.

The screening for the resistance phenotypes was performed with an antibiogram by disc diffusion, through the use of tracer drugs[5]. Production of AmpC and ESBL was confirmed by the disc approximation test[22] and the production of KPC by the modified Hodge test[5].

AmpC: Isolates belonging to the CESP group (*Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., *Providencia* spp.) which were sensitive

to cefoxitin in the antibiogram (*screening*) were submitted to the test by disc approximation (confirmatory), in which a cefoxitin disc (30 µg) was placed in the center of the plate, 20 mm (center to center) away from a ceftriaxone disc (30 µg) and from a ceftazidime disc (30 µg). The plate was incubated at 35 ºC for 18-24 h. Cefoxitin works as an inducer of the AmpC enzyme and the reading was considered positive, when the flatness of the halo around the ceftriaxone and/or ceftazidime disc was observed[22].

ESBL: The screening of the ESBL phenotype was done with the antibiogram for the isolates identified as *Escherichia coli, Klebsiella pneumonie* and *Klebsiella oxytoca*, through five substrates: aztreonam 30 µg, cefotaxime 30 µg, cefpodoxime 10 µg, ceftazidime 30 µg and ceftriaxone 30 µg. The isolates that showed resistance to at least one of the antimicrobials used in the screening were submitted to the confirmatory test by disc approximation or double disc synergism. In this test, one amoxicillin/clavulanic acid disc (20 µg/10 µg) was placed in the center of the plate and 20 mm (center to center) away from an aztreonam disc (30 µg) and from a ceftazidime disc (30 µg). The plate was incubated at 35 ºC for 18-24 h. The test was considered positive when there was an increase or distortion of the inhibition zone (ghost zone) between any antimicrobial marker and the amoxicillin/clavulanic acid disc[22].

KPC*: Enterobacteriaceae*, mainly *Klebsiella pneumoniae*, with resistance to some third generation cephalosporins (ceftazidime, ceftriaxone or cefotaxime) and to some carbapenem (imipenem or meropenem) in the antibiogram, were subjected to the modified Hodge test (confirmatory). To carry out the modified Hodge test, an *E. coli* ATCC® 25922 inoculum corresponding to a 0.5 McFarland standard was prepared and sowed over the surface of a Mueller-Hinton agar plate. A 10 µg meropenem disc was placed in the center of the plate. With the aid of an inoculation loop, three to five freshly-grown colonies (24 h) from the test sample were sowed from the center of the meropenem disc to the periphery of the Petri plate, in order to trace out an imaginary line of 20 to 25 mm. After incubation at 35 ºC for 16-20 h, the test was considered positive when there was growth of the *E. coli* ATCC® 25922 strain in the meropenem inhibition zone (distortion of the inhibition zone)[5].

Processing and analysis of results: The data collected from the workers were codified and organized in the IBM software program *Statistical Package for Social Sciences* (SPSS) for Windows (version 18.0), then analyzed through descriptive analysis.

RESULTS

Colonized workers: During the study period (18 months), the oral cavities of 55 (18.7%) subjects were colonized by *Enterobacteriaceae;* of these workers, 36 were healthcare providers, and 19 (13.1%) were support staff. Among the colonized individuals, 49.1% (27/55) carried enterobacteria with a profile of multiresistance to antimicrobial agents, 90.9% (50/55) carried only one species of enterobacteria, and 9.1% (5/55) carried two to three species simultaneously, that is to say, they were colonized by multiple species of *Enterobacteriaceae.*

Bacterial isolates: 64 enterobacteria of different genera and species were isolated (Table 1). The most common genera were *Enterobacter* (46.9%), *Klebsiella* (18.8%) and *Citrobacter* (17.2%), whereas the most prevalent species was *Enterobacter gergoviae* (17.2%). Potentially pathogenic bacteria were also isolated, including *Klebsiella pneumoniae* (12.5%), *Klebsiella oxytoca* (6.2%), *Escherichia coli* (6.2%) and *Serratia marcescens* (3.1%).

Table 1
Species of *Enterobacteriaceae* (n = 64) isolated from the oral cavity of workers in an oncology hospital. Goiânia, Goiás, 2009-2010

Microorganism	Isolates (f)	Total (%)
Enterobacter gergoviae	11	17.2
Enterobacter sakasaki	08	12.5
Enterobacter aerogenes	08	12.5
Klebsiella pneumoniae	08	12.5
Citrobacter koseri	07	10.9
Pantoea agglomerans	05	7.8
Klebsiella oxytoca	04	6.2
Escherichia coli	04	6.2
Enterobacter cloacae	03	4.7
Citrobacter amalonaticus	02	3.1
Citrobacter freundii	02	3.1
Serratia marcescens	02	3.1
Total	64	100

Profile of antimicrobial susceptibility: Table 2 shows the profile of susceptibility of the enterobacteria to the 16 antimicrobial agents: 57.8% (37/64) of the isolates were resistant to amoxicillin/clavulanic acid, 45.3% (29/64) to cefoxitin, 15.6% (10/64) to tetracycline and 10.9% (7/64) to cefpodoxime. In addition, all (100.0%) of the enterobacteria were sensitive to cefepime, ciprofloxacin, gentamicin, imipenem, meropenem and levofloxacin.

Forty-two (65.6%) isolates presented some type of resistance, and 31 (48.4%) were resistant to two or more classes of antimicrobials, characterizing a profile of multiresistance. Of these, 6.4% (2/31) were simultaneously resistant to three, and 6.4% (2/31) to four different classes.

Phenotypic production of β-lactamases: In this study, the phenotypic production of Inducible Chromosomal AmpC β-lactamase was researched among the 43 (67.2%) CESP group isolates identified: (*Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., *Providencia* spp.). All were positive for AmpC production. However, these microorganisms showed different mechanisms. Twenty-two (51.2%) isolates were sensitive to cefoxitin (tracer) and positive for the confirmation test (disc approximation) of production of the enzyme, characterizing an induction mechanism. Nevertheless, 21 (48.8%) of the isolates were resistant to cefoxitin, indicating the presence of mutant strains that hyper-produce the AmpC enzyme (Table 3).

The Extended Spectrum β-lactamase (ESBL) was studied for the 16 (25.0%) isolates identified as *E. coli* and *Klebsiella* spp.; however, none was a producer of the enzyme. All of the enterobacteria (100.0%) were negative for the production of KPC-type carbapenemase (Table 3).

Table 2
Profile of antimicrobial susceptibility of *Enterobacteriaceae* (n = 64) isolates from the oral cavity of workers in an oncology hospital. Goiânia, Goiás, 2009-2010

Antimicrobial Agent	S	I	R	Total R (%)
	Isolates (f)			
Amoxicillin-clavulanic acid	18	9	37	57.8
Cefoxitin	35	0	29	45.3
Tetracycline	53	1	10	15.6
Cefpodoxime	49	8	7	10.9
Trimetoprim-sulfmetoxazol	61	1	2	3.1
Ceftazidime	63	0	1	1.6
Ceftriaxone	63	0	1	1.6
Piperacillin tazobactam	63	0	1	1.6
Cefotaxime	63	1	0	0
Aztreonam	63	1	0	0
Cefepime	64	0	0	0
Ciprofloxacin	64	0	0	0
Gentamicin	64	0	0	0
Imipenem	64	0	0	0
Levofloxacin	64	0	0	0
Meropenem	64	0	0	0

Table 3
β-lactamases produced by *Enterobacteriaceae* (n = 64) isolated from the oral cavity of workers in an oncology hospital. Goiânia, Goiás, 2009-2010

Enzymes	Isolates evaluated	Positive isolates	Total
	(f)	**(f)**	**(%)**
KPC	64	0	0
AmpC/induction	43	22	51.2
AmpC/hyperproduction	43	21	48.8
ESBL	16	0	0

KPC: *Klebsiella pneumoniae* Carbapenemase; ESBL: Extended Spectrum β-lactamase.

DISCUSSION

A large number of HAIs have an endogenous origin and are difficult to prevent. However, the number of preventable infections is significant, especially those resulting from the cross-transmission of microorganisms[9]. Thus, the problem of colonization of healthcare workers by pathogenic and multiresistant bacteria is clear[4, 17].

In health services, the multidisciplinary team deserves special attention because it is the base level of care provided to patients. The support team maintains the infrastructure necessary for the hospital's operation[34]. These workers can spread microorganisms, and their identification as possible reservoirs and sources of infectious agents is an important measure to prevent and control HAIs[6].

Nevertheless, studies on the oral cavity and gram-negative bacteria of clinical and epidemiological importance are rare in literature. Despite the limits, these studies are very important for the control of microbial dissemination, and consequently the control of infection rates. The oral cavity can serve as a potential reservoir of *Enterobacteriaceae*, which are spread to the environment and to susceptible individuals through saliva. This fact becomes more important when considering the hospital environment, as most *Enterobacteriaceae* infections take place in this setting[13, 35].

The condition of being a carrier is also damaging to the health of the worker. In a situation of defense mechanism imbalance, endogenous microorganisms can unleash severe infections[9,35]. Hosting enterobacteria in the oral cavity is a predisposing and aggravating factor for many oral and systemic diseases[11,26].

In this study, the prevalence of colonization in the oral cavity by *Enterobacteriaceae* was 18.7%; however, individual analysis of the groups of workers showed that 24.2% of the healthcare workers and 13.1% of the support workers were carriers.

A similar study performed with 278 members of a healthcare team at a university hospital found a 69.6% colonization rate by gram-negative bacteria (GNB) (enterobacteria and/or non-fermenters)[20]. In studies with immunocompromised individuals, the presence of *Enterobacteriaceae* in the oral cavity varied from 32.5% to 60.7%[10, 21]. In another study, the colonization rate of the oral cavity by enterobacteria and/or *Pseudomonas* spp. was 51.0%[24].

The prevalence of colonization observed in this study was lower than those found in other studies involving the oral cavity. Nevertheless, this data can be considered relevant, because *Enterobacteriaceae* are enteric microorganisms that do not normally inhabit the oral cavity[13]. The natural habitat of this family of coliforms is the intestinal tract of humans and animals, being among the primary agents of HAIs, and responsible for a variety of clinically important illnesses, such as infections of the urinary tract, respiratory tract, wounds, central nervous system and bloodstream[35].

Among the colonized study subjects, a greater prevalence was observed for the healthcare team (24.2%) in comparison to the support team. This may be explained by the fact that these workers are responsible for healthcare, and are frequently exposed to direct and indirect contact with patients and biological agents. Additionally, many of these professionals work in other healthcare institutions, a factor that favors colonization by diverse agents, including multiresistant bacteria[32].

Colonization is a dynamic process dependent upon various factors[35]. Conditions such as hospitalization, compromised immunological response, inadequate hygiene habits, salivation reduction and natural chewing movements favor the colonization and proliferation of *Enterobacteriaceae* in the oral cavity[1]. Furthermore, there is a hypothesis that the incidence of these microorganisms in the mouth is also related to the presence of coliforms in water and foods[27].

Another concerning factor is that 9.1% of the carriers were colonized by different species of enterobacteria. In one study performed with the healthcare team of a teaching hospital, 49.2% of the workers were multicolonized by GNB[20]. The large microbial diversity of the oral cavity reflects, among other factors, the presence of the dental biofilm, which enables special conditions for survival and growth[14]. Additionally, anatomical and physiochemical properties of the oral cavity make it an ecosystem that is highly complex, heterogeneous and distinct from all others[13, 21].

In regard to the phenotypic characterization of the isolated microorganisms, the most common bacteria were *Enterobacter*, *Klebsiella* and *Citrobacter*, and the most prevalent species was *Enterobacter gergoviae* (17.2%). In one study with health workers, the genera *Enterobacter* and *Klebsiella* were also described as the most frequent in the oral cavity[20]. Similar data were reported in another study, in which *Enterobacter cloacae* (31.0%) was the most isolated enterobacteria among individuals in dental treatment, followed by *Klebsiella pneumoniae* (18.3%)[24].

The species of enterobacteria isolated in this study are opportunistic hospital pathogens, which eventually may be found in the oral cavity and in subgingival samples of healthy individuals[24,26,35]. Bacteria known for their virulence and capacity to cause severe infections (*Klebsiella*, *Escherichia, Serratia*) were isolated[30].

The species *E. gergoviae* can be isolated from environmental sources, as well as the respiratory and urinary tracts and the blood of human beings. This microorganism is the most common cause of nosocomial bacteremia. Its presence in the oral cavity constitutes a risk factor for infections such as severe adult periodontitis and pneumonias[24,26,35].

The highest resistance rates were observed for the group of β-lactams: amoxicillin/clavulanic acid (57.8%), cefoxitin (45.3%) and cefpodoxime (10.9%) (Table 2). The β-lactams constitute the most traditional antimicrobial agents employed in the treatment of infections. The increase of resistance in gram-negative bacteria is due to the production of β-lactamase enzymes[28,31], as verified by this study.

High rates of resistance to quinolones, aminoglycosides and β-lactams have been reported at various institutions[23,30]. Yet, in this study, all of the isolates were sensitive to quinolones (ciprofloxacin and levofloxacin), gentamicin, cefepime and carbapenems (imipenem and meropenem). One study that evaluated the antimicrobial susceptibility of enterobacteria isolated from the oral cavity also found 100.0% sensitivity to quinolones[25].

Sensitivity to quinolones and aminoglycosides is an important finding. These pharmaceuticals are the drug of choice for the treatment of a variety of infections by gram-negative rods, including respiratory infections caused by isolated producers of AmpC β-lactamase[23].

Cefepime is also active against producing strains of AmpC β-lactamase, and the first choice therapy against this type of microorganism[23]. Carbapenems are a broad spectrum antimicrobial group, especially used in situations of severe infection by multiresistant enterobacteria[3].

Of the workers studied, 49.1% were colonized by multiresistant enterobacteria. Isolates with a multiresistant profile correspond to 48.4% of the total enterobacteria, with some (6.4%) being resistant to four distinct classes of antimicrobials. This result represents a concern, since these colonization agents were isolated from healthy carriers. As a consequence of this profile, various antimicrobials become less active, reducing therapeutic options and increasing the clinical impact of infectious diseases[7].

In the last few years, *Enterobacteriacae* have been shown to be resistant to a variety of antimicrobial agents. This increase in resistance is primarily related to the frequent use of antimicrobials and to how easy it is for these microorganisms to build up resistance[22,31]. This profile has been particularly observed in the hospital environment, where outbreaks of infections of β-lactamases-producing enterobacteria are described[8,33].

In regard to β-lactamases of clinical importance, the phenotypic production of ESBL and KPC was not observed. Yet, among the CESP group, all of the isolates were producers of AmpC β-lactamases. This increased production was different between the isolates due to a mechanism of induction and mutation.

In the group of AmpC producing bacteria, 51.2% of the isolates were sensitive to cefoxitin (tracer) in the antibiogram, and subsequently were confirmed to produce AmpC β-lactamases. This means that these isolates only express resistance when exposed to an antimicrobial inducer (induction), that is, when the therapy is started. In these cases, therapeutic failure can occur during treatment[23].

On the other hand, 48.8% of the CESP isolates were resistant to cefoxitin in the antibiogram, and therefore considered mutant strains. As the result of a mutation, this type of isolate permanently hyper-produces AmpC β-lactamases[31]. This phenomenon results in the constant production of high levels of AmpC β-lactamase regardless of exposure to an induction agent. *Enterobacteriaceae* mutants may be selected from populations of inducible strains during therapy by using weak inducing antimicrobial agents[23].

The AmpC β-lactamase enzyme belongs to the C molecular class and functional group 1, does not suffer the action of β-lactamase inhibitors and has inducible expression, being produced in low quantities (intrinsic mechanism) by the CESP group. AmpC-producing strains are intrinsically resistant to penicillin, cephalosporin and monobactams[31].

In healthcare institutions, the prevalence of AmpC-producing strains is variable, and rates of up to 22.7% may be found[16]. Many intensive care units have reported outbreaks of *Enterobacteriaceae* that produce inducible AmpC, which stands out for its difficult treatment due to the profile of multiresistance of the isolates[2].

In this study, the prevalence of carriers of *Enterobacteriaceae* among the workers at the institution investigated was considered to be significant and the phenotypic profile of the isolates was rather concerning, as it features multiresistant colonization agents and AmpC β-lactamase producers.

It is believed that the detection of multiresistant *Enterobacteriaceae* in the oral cavity of the workers at this institution will permit the tracing and identification of carriers, as well as knowledge of the profile of the colonizing microorganisms, in order to monitor the emergence of

resistance and new pathogens. Additionally, it is hoped that the results may contribute to supporting the identification of contamination routes, and consequently, losses caused by these agents. Such information is also useful to improve healthcare practices, keeping in mind the quality of life of the worker, healthcare service users and the community in general, in consonance with the principles of safety.

RESUMO

Enterobacteriaceae isoladas da cavidade bucal de trabalhadores de hospital oncológico do Centro-Oeste brasileiro

A investigação de trabalhadores dos serviços de saúde como reservatório e disseminadores de bactérias patogênicas tem sido referida como estratégia de prevenção e controle das infecções relacionadas à assistência à saúde. Este estudo buscou avaliar a presença de *Enterobacteriaceae* na cavidade bucal de trabalhadores de hospital oncológico do Centro-Oeste brasileiro, bem como caracterizar o perfil fenotípico dos isolados. Foi coletada amostra de saliva de 294 trabalhadores pertencentes às equipes de saúde e de apoio. Procedimentos microbiológicos foram realizados segundo técnicas referendadas. Dentre os participantes, 55 (18,7%) estavam colonizados por *Enterobacteriaceae* na cavidade bucal. Foram isoladas 64 bactérias, incluindo espécies potencialmente patogênicas. A espécie mais prevalente foi *Enterobacter gergoviae* (17,2%). As maiores taxas de resistências foram observadas para os β-lactâmicos e 48,4% dos isolados foram considerados multirresistentes. Para as enterobactérias pesquisadas, a produção de ESBL e KPC foi negativa. Porém, dentre os 43 isolados do grupo CESP, 51,2% foram considerados produtores de β-lactamase AmpC por indução e 48,8% mutantes hiperprodutores. Considera-se a prevalência de portadores de *Enterobacteriaceae* significativa e o perfil fenotípico dos isolados preocupante, especialmente pela multirresistência e produção de β-lactamases AmpC.

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Rickettsia typhi IN RODENTS AND *R. felis* IN FLEAS IN YUCATÁN AS A POSSIBLE CAUSAL AGENT OF UNDEFINED FEBRILE CASES

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SUMMARY

Rickettsia typhi is the causal agent of murine typhus; a worldwide zoonotic and vector-borne infectious disease, commonly associated with the presence of domestic and wild rodents. Human cases of murine typhus in the state of Yucatán are frequent. However, there is no evidence of the presence of *Rickettsia typhi* in mammals or vectors in Yucatán. The presence of *Rickettsia* in rodents and their ectoparasites was evaluated in a small municipality of Yucatán using the conventional polymerase chain reaction technique and sequencing. The study only identified the presence of *Rickettsia typhi* in blood samples obtained from *Rattus rattus* and it reported, for the first time, the presence of *R. felis* in the flea *Polygenis odiosus* collected from *Ototylomys phyllotis* rodent. Additionally, *Rickettsia felis* was detected in the ectoparasite *Ctenocephalides felis* fleas parasitizing the wild rodent *Peromyscus yucatanicus*. This study's results contributed to a better knowledge of *Rickettsia* epidemiology in Yucatán.

KEYWORDS: *Rickettsia typhi;* Murine typhus; Rodents.

INTRODUCTION

Rickettsia typhi is the causal agent of murine typhus; a worldwide zoonotic infectious disease. The clinical manifestations in humans are commonly nonspecific and include fever, headache, chills and rashes[7].

The classical biological cycle of *Rickettsia typhi* involves rodent species like *Rattus rattus* and *Rattus norvegicus* as hosts and the oriental rat flea *Xenopsylla cheopsis* as a vector. However, *Rickettsia typhi* has been reported by molecular methods in other species like *Apodemus agrarius*, opossums and fleas (*Ctenocephalides felis, Leptopsylla segnis, Ctenophthalmus congeneroides* and *Rhadinopsylla insolita*)[3,5,12]. Rodent diversity in Yucatán, Mexico includes species belonging to the Muridae family: *Mus musculus, Peromyscus yucatanicus, Ototylomys phyllotis*, *Reithrodontomys gracilis*, *Sigmodon hispidus*, *Rattus rattus* and *Oligoryzomys fulvescens*, and the Heteromyidae family: *Heteromys gaumeri*. *Peromyscus yucatanicus* and *Heteromys gaumeri* are considered endemic rodent species in the Yucatán Peninsula[6,11].

Human cases of murine typhus in the state of Yucatán have been reported since 2009, with single infections and family clusters in places that have been associated with rodent presence[8,21]. The people in the municipalities of Yucatán mostly work in agriculture and livestock with close proximity to vegetation, domestic, peridomestic and wild animals around their houses, which are conditions that could favor a vector-borne disease transmission, such as rickettsiosis, ehrlichiosis or leptospirosis.

In the absence of any evidence for the presence of *Rickettsia typhi* in any host or vector in Yucatán, aside from the increment of human cases of murine typhus in the past five years, this study evaluates the presence of *Rickettsia typhi* in rodents and their ectoparasites in a small municipality of Yucatán, where nonspecific febrile illnesses are often reported, and the presence of rodents is associated with most of the cases.

MATERIALS AND METHODS

Study area: This study was conducted in the municipality of Oxkutzcab, in the state of Yucatán, Mexico (20° 18' 10" N, 89° 25' 6" W). There are no previous reports of rickettsiosis infection or detection of *Rickettsia* in hosts and/or vectors in this municipality.

Rodent and ectoparasite collection: From February to July 2012, 16 patients living in Oxkutzcab, Yucatán, were visited. These patients were previously diagnosed in 2011 with nonspecific febrile illnesses and with a negative dengue test, but the presence of rodents inside and/or outside their house was noted in their clinical history. The houses of each patient were visited and additional visits were made to houses in the surrounding areas. In this study, 3 x 3.5 x 9" Large Folding Aluminum traps (H.B. Sherman Traps, Inc.) were used to collect rodents. Traps were placed inside and outside the house in kitchens, basements, bedrooms and terraces, as well as in places where people reported the presence of rodents. Traps were placed for between three and five consecutive

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days. Trapped rodents were inspected to collect ectoparasites and blood samples. Rodents that survived the blood sample collection after inspection were released on the outskirts of the town. Ectoparasites collected were stored in 1.5 mL tubes and maintained at -70 °C until needed. Collected rodents and ectoparasites were identified by one of the authors using identification keys[1,10,18].

Samples were analyzed individually using whole blood or the entire ectoparasite. DNA was purified using DNA blood and tissue kit (QIAGEN, Valencia, CA). PCR amplicons were purified using Qiagen Gel Extraction Kit (QIAGEN, Valencia, CA); according to the manufacturer's instructions with a final elution volume of 100 µL.

PCR and sequencing: Conventional Polymerase Chain Reaction (PCR) technique was selected to amplify DNA fragments from two different rickettsial genes: 17 kDa lipoprotein and rickettsial outer membrane protein B (*ompB*). A 434 bp fragment from the rickettsial gene 17 kDa was obtained using primers Fw1: 5´-GCTCTTGCAACTTCTATGTT-3´ and Rv2: 5´-CATTGTTCGTCAGGTTGGCG-3´) [22] and a 990 - 999 bp fragment of the rickettsial gene *ompB* using primers ompB330(1) fw (5'-ATGGCTTCAAAAACCAAATTTTCTAA-3') and ompB330(1) Rv (5'- AGCTCTACCTGCTCCATTATCTGTACC-3')[15]. PCR reaction was performed using Platinum® *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and using a Multigene Thermalcycler (Labnet International, Inc). Ten microliters of each PCR product underwent electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and were examined in a UV transilluminator.

PCR products were sequenced by the PRISM Big Dye Terminator Cycle 3.1 Sequencing (Applied Biosystems, Foster City, CA) method. The sequenced products were purified with an ABI XTerminator kit and separated on a 3130*xl* genetic analyzer from Applied Biosystems. Sequence similarities were determined using the BLAST search engine from the National Center for Biotechnology Information (NCBI) website.

RESULTS

Rodent and ectoparasite collection: Forty-two rodents (n = 42) from three different species belonging to the Muridae family were collected: *Rattus rattus* (n = 32), *Peromyscus yucatanicus* (considered endemic in the state of Yucatán[20]) (n = 6), and *Ototylomys phyllotis* (n = 4).

Six *Polygenis odiosus* (*P. odiosus*) were obtained from fleas of the Rhopalopsyllidae family in two *O. phyllotis* of the four collected, and four *Ctenocephalides felis* fleas were obtained from one *P. yucatanicus* out of the six rodents collected (Table 1). No ectoparasites were obtained from any *R. rattus* collected.

PCR and sequencing: PCR amplification was achieved in 11 samples: eight from *R. rattus* blood samples, one from *a C. felis* flea collected from *P. yucatanicus*, and two from a *P. odiosus* flea collected from *Ototylomys phyllotis*. All amplicons were sequenced.

The 17 kDa gene fragment sequence, obtained from whole blood from *R. rattus* (GenBank accession no. KF241855.1), was 100% identical to *Rickettsia typhi* isolated from human blood in Yucatán (GenBank accession no. JX198507.1), *Rickettsia typhi* str. B9991CWPP (GenBank accession no. CP003398.1), *Rickettsia typhi* identified from *Ctenophthalmus congeneroides* in rodents in Korea (GenBank accession no. EU532435.1), and *Rickettsia typhi* str. in Wilmington (GenBank accession no. AE017197.1).

Table 1
Positive rodents and fleas tested for *Rickettsia* spp.

Rodent species	Rodents tested	Fleas tested	
	Rodent Positive (total tested)	*C. felis* Positive (total tested)	*P. odiousus* Positive (total tested)
R. rattus	8 (32)	--	--
O. phyllotis	0 (4)	--	2 (6)
P. yucatanicus	0 (6)	1 (4)	--

Sequences obtained from an *ompB* gene fragment obtained from blood sample of *R. rattus* (GenBank accession no. KF241858.1) were 99% identical to *Rickettsia typhi* str. B9991CWPP (GenBank accession no. CP003398.1), *Rickettsia typhi* str. TH1527 (GenBank accession no. CP003397.1), *Rickettsia typhi* str. in Wilmington (AE017197.1).

The 17 kDa gene fragment sequences obtained from *C. felis* (GenBank accession no. KF241853.1) and *P. odiosus* (GenBank accession no. KF241854.1), collected from *P. yucatanicus* and *O. phyllotis* rodents respectively, were 100% identical to *Rickettsia felis* identified in *Carios capensis* ticks in the United States (GenBank accession no. DQ102709), *Rickettsia felis* URRWXCal2 (GenBank accession no. CP000053), *Rickettsia* sp. Hf187 identified in ticks in Japan (GenBank accession no. AB114813); 99% identity to *Rickettsia felis* identified in *Pulex echidnophagoides* from opossums in Yucatán, Mexico (GenBank accession no. GU447234.1) and *Rickettsia rickettsii* identified in *Rhipicephalus sanguineus* ticks in Yucatán (GenBank accession no. KC713872.1).

Sequences obtained from an *ompB* gene fragment from *C. felis* (GenBank accession no. KF241856.1) and *P. odiosus* (GenBank accession no. KF241857.1) collected from *P. yucatanicus* and *O. phyllotis* rodents respectively, were 99% identical to *Rickettsia felis* URRWXCal2 (GenBank accession no. CP000053.1), *Rickettsia felis* identified in the insect pest *Liposcelis bostrychophila* (GenBank accession no. GQ385243.1), and 94% identical to Candidatus *Rickettsia hoogstraalii* (GenBank accession no. EF629536.1).

DISCUSSION

Small mammals are key components in the process of succession and regeneration of tropical forests and rainforests because they play an important role in predation and the dispersion of seeds[17]. The presence of rodent diversity in Yucatán, Mexico in domestic areas is, in most cases, due to the constant expansion of residential areas which encroaches upon wildlife ecosystems, thereby favoring the presence of vector-borne diseases, such as leishmaniasis[4,19], hantavirus[16] or rickettsiosis[12].

In this study, the presence of *Rickettsia felis* was identified in *Polygenis odiosus* fleas as a potential new vector of *R. felis*. This flea species is widely distributed and considered endemic in the Yucatán peninsula, with high preference to *O. phyllotis* which could possibly be a host of *R. felis*[9]. The presence of *R. felis* in *C. felis* collected from *P. yucatanicus* suggests the possible role of this rodent as a new reservoir of *R. felis*. This hypothesis is supported by documented evidence that demonstrates that other species of *Peromyscus* are involved in the ecology of Rickettsiae[13,14]. There is a need for further studies to focus on the identification of the presence of *Rickettsia* spp. in *P. yucatanicus*, in order to confirm it. The high presence of *R. typhi* in blood samples from *R. rattus* confirms its presence in the state of Yucatán and supports the importance of rodent control, as rodents are a disease host.

One of the considerations of these results is the need for comprehensive work with government authorities and the community to develop rodent control strategies in small communities, where living conditions favor the presence of rodent species, due mainly to poverty, agriculture and food habits (corn is the main food source), in order to prevent human cases of this, in the eyes of the authors, neglected febrile disease.

RESUMEN

Rickettsia typhi y *R. felis* en roedores y sus pulgas en Yucatán como posible agente causal de casos febriles indefinidos

Rickettsia typhi es el agente causal del tifo murino; una enfermedad zoonótica transmitida por vector mundialmente distribuida, comúnmente asociada con la presencia de roedores domésticos y silvestres. Los casos humanos de tifo murino en el Estado de Yucatán son frecuentes. Sin embargo, no existe evidencia de la presencia de *Rickettsia typhi* en mamíferos o vectores en Yucatán. En la búsqueda de vectores y reservorios de *Rickettsia typhi*, evaluamos la presencia de bacterias del género *Rickettsia* en roedores y sus ectoparásitos de un pequeño municipio del estado de Yucatán por medio de técnicas de PCR convencional y secuenciación de ADN. Se identificó la presencia de *Rickettsia typhi* en muestras de sangre obtenidas de *Rattus rattus* y reportamos por primera vez la presencia de *Rickettsia felis* en la pulga *Polygenis odiosus* colectado de *Ototylomys phyllotis*. Complementariamente, *Rickettsia felis* fue detectado en la pulga *Ctenocephalides felis* parasitando al roedor *Peromyscus yucatanicus.* No se identificó especie de *Rickettsia* en las muestras de sangre de *O. phyllotis* y *P. yucatanicus* analizados. Nuestros resultados contribuyen también en el conocimiento de ciclo de vida biológico del género *Rickettsia.*

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SPATIOTEMPORAL TRENDS OF CASES OF PANDEMIC INFLUENZA A(H1N1)PDM09 IN ARGENTINA, 2009-2012

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SUMMARY

The aim of this paper was to analyze the spatiotemporal variations of cases of influenza A(H1N1)pdm09 in Argentina. A space-time permutation scan statistic was performed to test the non-randomness in the interaction between space and time in reported influenza A(H1N1)pdm09 cases. In 2009, two clusters were recorded in the east of Buenos Aires Province (May and June) and in the central and northern part of Argentina (July and August). Between 2011 and 2012, clusters near areas bordering other countries were registered. Within the clusters, in 2009, the high notification rates were first observed in the school-age population and then extended to the older population (15-59 years). From 2011 onwards, higher rates of reported cases of influenza A(H1N1)pdm09 occurred in children under five years in center of the country. Two stages of transmission of influenza A(H1N1)pdm09 can be characterized. The first stage had high rates of notification and a possible interaction with individuals from other countries in the major cities of Argentina (pattern of hierarchy), and the second stage had an increased interaction in some border areas without a clear pattern of hierarchy. These results suggest the need for greater coordination in the Southern Cone countries, in order to implement joint prevention and vaccination policies.

KEYWORDS: Influenza A(H1N1)pdm09; Cluster analysis; Spatiotemporal analysis; Argentina.

INTRODUCTION

In April 2009, the first cases of influenza A(H1N1)pdm09 were discovered in Mexico and California (USA). At the end of that year, the new virus had caused, at least, 18,000 deaths[14]. Unlike what happened in these countries, the arrival of the influenza A(H1N1)pdm09 in the Southern Hemisphere coincided with the period of circulation of seasonal influenza. In Argentina, the first cases of influenza A(H1N1) pdm09 were detected in early May, registering a peak circulation of the virus in late June[18]. Compared with other countries in the Southern Hemisphere, Argentina recorded the highest rates of mortality from influenza A(H1N1)pdm09[18].

There are very few studies that analyze the spatiotemporal interactions of cases of influenza A(H1N1)pdm09. Furthermore, these studies were conducted primarily in developed countries and have focused only on the dynamics of 2009, the first year of the appearance of the virus[6,10,13,19,20]. After the first year of the onset of influenza A(H1N1)pdm09, there have been new outbreaks in some countries (New Zealand[2], the United Kingdom[5] and Mexico[3]). However, since the emergence of the virus, no studies have thus far been conducted to analyze the spatial-temporal trends in the medium term.

A more detailed study on patterns of the intranational spatial spread of the virus would raise new hypotheses about the factors that influence its rapid circulation. Moreover, this would allow more effective design policies for prevention and control measures in the context of a significant increase in demand on health systems.

The aim of this paper is to analyze the spatiotemporal variations of cases of influenza A(H1N1)pdm09 in Argentina, between 2009 and 2012.

MATERIAL AND METHODS

Data collected by the National Surveillance System based on Laboratories (SIVILA) were used. Health establishments present in all provinces of Argentina reported to this system. More detailed information on the monitoring system can be found in VAN KERKHOVE *et al.*[18]

All laboratory confirmed cases of pandemic influenza A(H1N1) pdm09 between 2009 and 2012 that were confirmed with registered information about a department of residence were evaluated.

The spatial units were composed of the 509 departments that make up the Argentine territory, and the autonomous city of Buenos Aires. Figure 1 shows the 24 jurisdictions that comprise the Argentine territory.

In addition, age and sex were recorded when available. In 2009,

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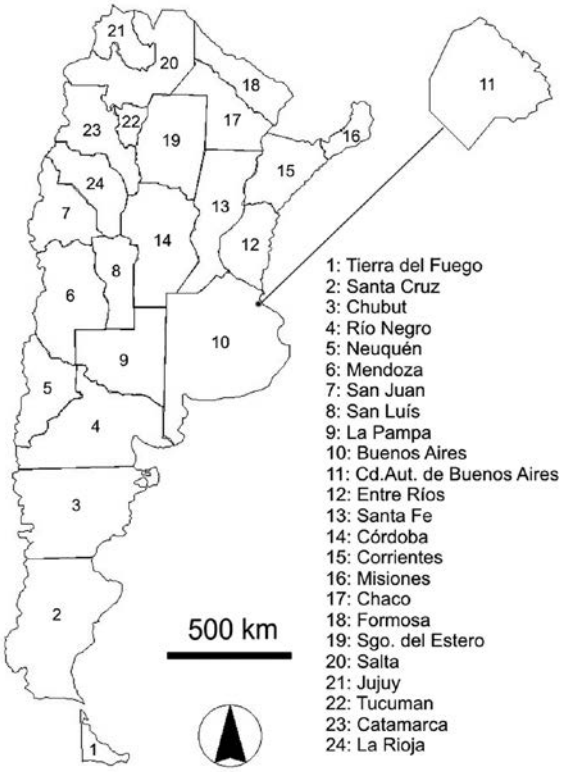

Fig . 1 - The Autonomous City of Buenos Aires and Provinces of Argentina.

66.2% and 70.2% of the reported cases had data on age and sex, respectively. In 2011, this percentage was 97.2% for both variables, while in 2012, 85.9% and 73.1% were the respective totals for this information. Notified cases were divided into six age groups (0-4, 5-14, 15-29, 30-44, 45-59 and 60 + years) and two groups according to sex. For each age and sex group, population data from the 2010 Census[7] were used as the denominator for notification rates. Because there are no population data by age group and sex for the years 2009, 2011 and 2012, data from the 2010 census were used as the denominator of the rates over the years.

Cluster definition: the general concept of cluster means a more heterogeneous 'clumped' distribution of disease cases than would be expected from the variation in population density and chance fluctuations[1]. A space-time permutations scan statistic was conducted to test the non-randomness in the interaction between the space and time of the recorded cases of influenza A(H1N1)pdm09[8,9]. This analysis consists of thousands of cylinders that move through the different points (formed by the geographic center of each department). Each cylinder has a base, which represents the geographical area, and a height, which is the time (in this case, the unit of time is the month). The base of each cylinder comprised a maximum of 50% of the population, while the height was a maximum period of six months. The cylinder with more observed cases than expected, with respect to the cases reported outside the cylinder, is called 'cluster 1' (or 'most likely cluster'). The expected number of cases was estimated assuming complete spatial-temporal randomness, which is synonymous with assuming a constant risk (Poisson) distribution. Since the space-time permutation scan statistic requires only case data, for each department and month the expected cases were calculated as the proportion of all cases that occurred in a department ('x') times the total number of cases during month ('y'). The expected number of cases in each cylinder is the summation of every department's expected cases that comprised that cylinder[8]. The space-time permutation scan statistic also calculates secondary clusters. To test the hypothesis of the non-random location of these clusters, 9999 Monte Carlo permutations were performed. If the Poisson generalized likelihood ratio of the 'cluster 1' (or other secondary clusters) exceeds 5% of the maximum ratios calculated by permutations, the cluster is significant at $p < 0.05$. The space-time scan statistic was performed separately for each year.

The SaTScan version 9.1.1 software developed by Martin Kulldorff, Harvard Medical School (Boston, USA) and the Information Management Services Inc (Maryland, USA) was used.

RESULTS

The year 2009 had the highest viral circulation, with 9007 reported cases of influenza A(H1N1)pdm09 in Argentina. During this year, there were two space-time clusters: the main cluster was located in the eastern part of the province of Buenos Aires, during the months of May and June, while the secondary cluster was located in central-northern Argentina, without occupying the provinces of Entre Rios, Corrientes and Misiones, during the months of July and August (Table 1, Fig. 2). In 2010, the lowest number of cases (n = 14) was reported and no space-time clusters were detected. In 2011, the number of cases increased (n = 160) and there were two clusters: the first, between the months of May and July, was located in central-western Argentina, mainly occupying the provinces of Mendoza, San Juan and San Luis, while the second cluster was located in the north of the country during the months of September and November (Table 1, Fig. 3). Finally, 2012 showed the highest number of cases (n = 341) recorded after 2009, the year of the onset of the virus in Argentina. There were three clusters: the first was recorded in northern Argentina, between February and July, the second cluster was located in the center-west, between the months of September and October, and the third cluster was located in the northeast of the country, mainly in the province of Corrientes, during the month of August (Table 1, Fig. 4).

Tables 2 and 3 show the rates of notifications of influenza H1N1 in each spatial-temporal cluster. In 2009, within cluster 1 there were higher rates of notifications of influenza A(H1N1)pdm09 in the young population (0-14 years) and lower rates in the older population (60+) compared to the other age groups. Cluster 2 showed lower rates in the age groups: 5-14, 60 and older, compared to the other groups. When comparing the two clusters, lower rates were observed in children under 14 years and a higher rate in the group aged 15 to 29 in cluster 2 (Table 2). No differences between sexes (Table 3) were observed.

In 2011, there was a significantly higher rate of notifications in individuals under five years compared to other age groups in cluster 1 (Table 2). There was also a higher rate in men as compared to women in cluster 1 but not in cluster 2. In cluster 2 there were no large variations between notification rates by age and gender groups.

In 2012, only cluster 2 showed a higher rate of notifications in the age group under five years compared to all other age groups, meanwhile cluster 1 showed higher rates for the 30-44, 45-59 and 60 + age groups (Table 2). In cluster 3, there were also higher rates of notification in

Table 1
Characteristics of clusters of cases of influenza A(H1N1)pdm09 in Argentina, 2009-2012

	Period (months)	Observed cases	Expected cases	Test statistic	*p*
Year 2009					
Cluster 1	May-June	2093	1442.77	157.22	< 0.001
Cluster 2	July-August	1779	1207.01	139.60	< 0.001
Year 2011					
Cluster 1	May-July	64	28.48	21.61	< 0.001
Cluster 2	September-November	72	37.03	18.43	< 0.001
Year 2012					
Cluster 1	February-July	49	11.64	35.28	< 0.001
Cluster 2	September-October	75	35.64	19.09	< 0.001
Cluster 3	August	102	63.64	12.54	< 0.001

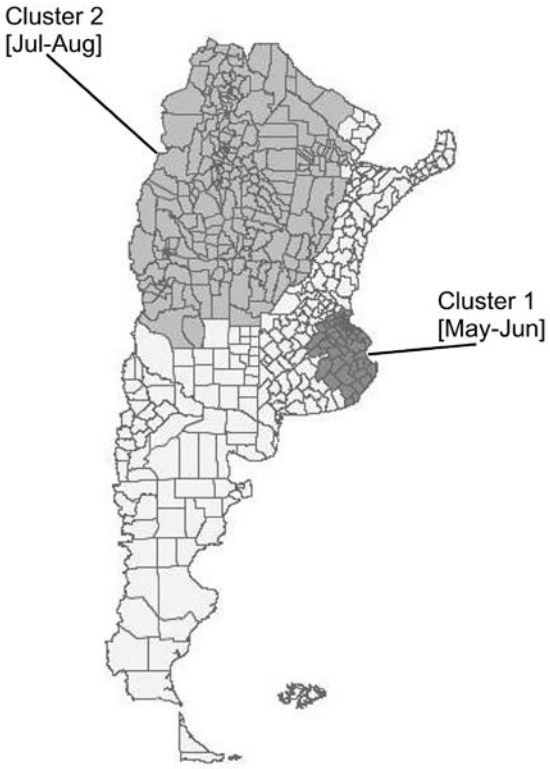

Fig. 2 - Spatiotemporal clusters of cases of Influenza A H1N1 in Argentina, 2009. Months within brackets.

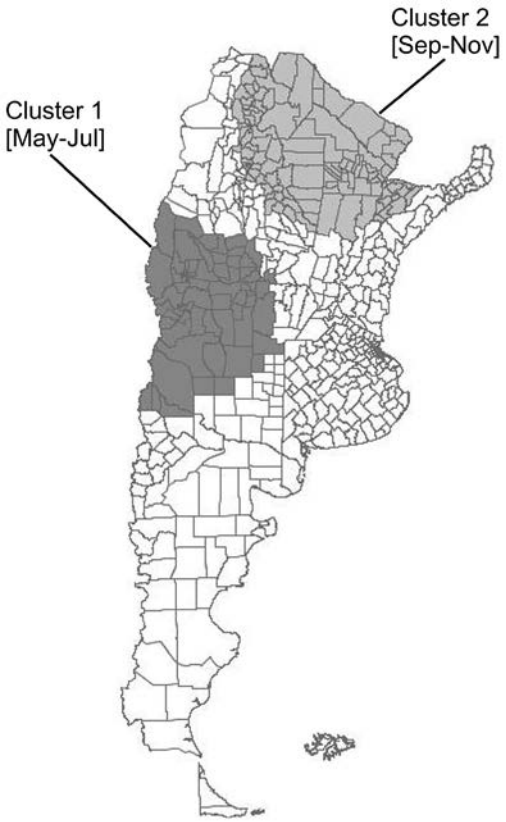

Fig. 3 - Spatiotemporal clusters of cases of Influenza A H1N1 in Argentina, 2011. Months within brackets.

individuals under five years, compared to the 5-14, 45-59 and 60 + age groups (Table 2). In the three clusters, there was no difference between the rates by sex. In general, notification rates by age group and sex were higher in cluster 3 than in clusters 1 and 2.

DISCUSSION

Spatiotemporal variations were observed in cases of influenza A(H1N1)pdm09 in Argentina between 2009 and 2012, and the spatiotemporal clusters showed differences in the composition of age groups.

There was a difference between the spatiotemporal pattern recorded in 2009, the year of the emergence of influenza A(H1N1)pdm09, and the patterns found between 2011 and 2012. In May and June of 2009, cluster 1 was located around agglomerate Buenos Aires, the main entrance of individuals from countries in the Northern Hemisphere to

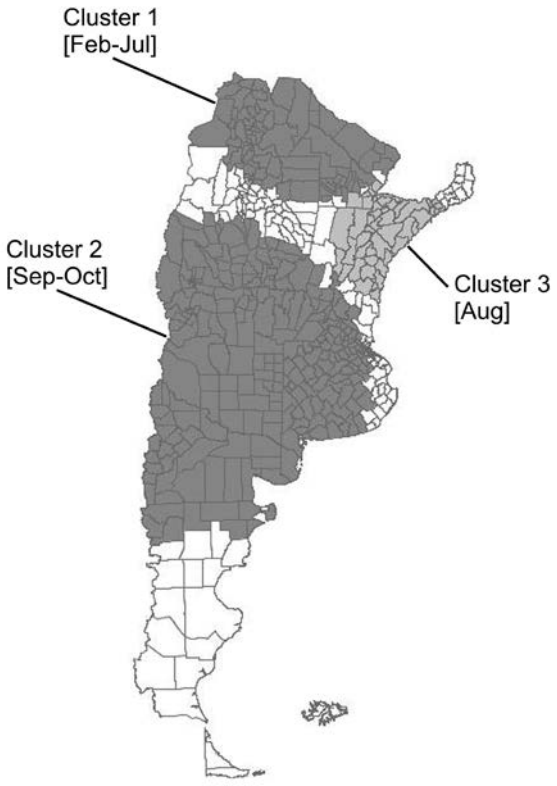

Fig. 4 - Spatiotemporal clusters of cases of Influenza A H1N1 in Argentina, 2012. Months within brackets.

South America, through Ezeiza International Airport, and south-east of the province of Buenos Aires. Between the months of July and August, there was another cluster in north-central Argentina, apart from the Mesopotamian provinces located to the east of the country (Entre Rios, Corrientes and Misiones). This cluster 2 comprised six of the 10 largest cities of Argentina, according to the 2001 Census. Apparently, the spatiotemporal distribution of cases of influenza A(H1N1)pdm09 followed a hierarchical pattern along the settlement system in 2009. After a year with very few cases, there was a change in the location of spatiotemporal clusters between 2011 and 2012, now being distributed near the border areas with neighboring countries. In 2011, the location of cluster 1, which comprises the provinces of Mendoza and San Juan, could be related to an abrupt increase in cases of influenza A(H1N1) pdm09 in Chile since epidemiological week (EW) 27 (n = 48)[16]. The location of cluster 2, in northern Argentina, during the months of October and November, coincided with a peak of confirmed cases (n = 343) in southern Bolivia (data from Santa Cruz) for EW 39 (end of September) and the emergence of cases of influenza A(H1N1)pdm09 in Paraguay between epidemiological weeks 42 and 46 (October and November)[16].

In 2012, there was also a relationship between increased cases registered in Santa Cruz de la Sierra (southern Bolivia) during February and June, and a peak of cases in Paraguay during the last week of June and the spatial-temporal cluster located in northern Argentina, in February and July[16]. The duration of cluster 1 was unusually long when compared to that of the other clusters. The relationship with the two outbreaks in the two bordering countries can be related to the unusual length of cluster 1. The outbreak observed in Paraguay could also be related to the spatiotemporal cluster mainly located in Corrientes, during the month of August. In the case of cluster 2, located in the center of the country and bordering Chile, there appears to be no interaction because there were very few cases in this country. It is likely that there could be a transmission of cases from cluster 3, recorded in August, to cluster 2, with cases observed between September and October.

These spatiotemporal changes between 2009 and 2011-2012 correspond to a transition of transmission of influenza A(H1N1)pdm09 in two stages. The first stage is characterized by an interaction with individuals from Northern Hemisphere countries through major cities of Argentina (hierarchical transmission). The second stage is characterized by greater interaction in some border areas without a clear pattern of hierarchical transmission. Related to this, in the coastal area of Peru, the first outbreak occurred in 2009, which included the major metropolitan area of the country

Table 2
Reported cases of influenza A(H1N1)pdm09 per 100000 inhabitants (2010 Census) by age group, Argentina 2009-2012

	0-4 yr		5-14 yr		15-29 yr		30-44 yr		45-59 yr		60+ yr	
	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI	Rate	95% CI
2009												
Cluster 1	23.80	21.25-26.57	17.23	15.67-18.90	10.94	09.94-12.00	11.82	10.70-13.01	12.40	11.09-13.82	6.06	5.16-7.07
Cluster 2	12.29	10.40-14.44	8.13	7.06-9.31	13.43	12.24-14.71	13.85	12.47-15.34	12.60	11.09-14.27	5.21	4.22-6.38
2011												
Cluster 1	6.64	4.22-9.97	2.38	1.38-3.84	1.46	0.81-2.43	1.42	0.72-2.54	0.56	0.14-1.53	0.20	0.01-0.98
Cluster 2	2.79	1.65-4.44	0.62	0.29-1.18	1.09	0.66-1.69	1.44	0.87-2.26	1.40	0.76-2.38	0.30	0.05-0.99
2012												
Cluster 1	3.19	1.73-5.42	1.44	0.78-2.45	1.23	0.69-2.06	0.79	0.32-1.65	0.56	0.14-1.52	0.48	0.08-1.59
Cluster 2	1.60	1.02-2.41	0.26	0.12-0.52	0.20	0.09-0.39	0.38	0.21-0.64	0.58	0.33-0.95	0.47	0.25-0.82
Cluster 3	9.30	5.98-13.86	2.02	1.06-3.52	4.78	3.36-6.60	4.14	2.63-6.21	3.05	1.65-5.19	0.98	0.25-2.66

Table 3
Reported cases of influenza A(H1N1)pdm09 per 100000 inhabitants (2010 Census) by sex group, Argentina 2009-2012

	Female		Male	
	Rate	95% CI	Rate	95% CI
2009				
Cluster 1	12.77	12.02-13.55	12.70	11.93-13.51
Cluster 2	13.48	12.62-14.37	12.19	11.36-13.06
2011				
Cluster 1	1.10	0.69-1.67	2.39	1.75-3.20
Cluster 2	1.04	0.73-1.45	1.40	1.03-1.87
2012				
Cluster 1	0.94	0.59-1.45	1.38	0.93-1.98
Cluster 2	0.42	0.29-0.58	0.52	0.37-0.69
Cluster 3	3.50	2.61-4.60	4.16	3.16-5.37

(Lima) in June[4]. This outbreak mainly affected children of school age. In July, the outbreak was dispersed to the rest of Peru, affecting all age groups[4]. The southernmost provinces of Argentina have not shown space-time clusters. However, it has been found that colder and drier temperatures, as recorded in these regions, increase the transmission of influenza virus[6,11]. There may be other spatial and sociodemographic factors affecting the circulation of influenza A(H1N1)pdm09. The increase in public transport use or a younger population structure has been associated with higher incidences and hospitalizations due to influenza A(H1N1)pdm09[12,20]. These Patagonian provinces are characterized by low population densities and relatively large buffers between the main urban centers. These spatial and socio-demographic factors could play a more important role in the transmission of influenza A(H1N1)pdm09. Furthermore, the results analyzed in this study seem to suggest that the seasonality of influenza A(H1N1)pdm09 appears to be similar to other types and subtypes of flu virus in Argentina[16].

In 2009, while in cluster 1 (May-June) the highest rates of notifications were recorded in individuals under 15, in cluster 2 (July to August) rates were similar in most age groups, but with lower reporting rates in the 5-14 and 60+ age groups. This significant decrease in the first age group could reflect the role that school closures could have had on the level of transmission in the school population. This strategy has had a positive effect on the reduction of cases of influenza A(H1N1) pdm09 pandemic in Tierra del Fuego, at the southern end of Argentina[15].

Except in 2010, the clusters located in the center of the country reported higher rates of case notification of influenza A(H1N1)pdm09 in children under five years compared to other age groups, while during 2011 and 2012, there were no differences in notification rates between age groups and sexes between clusters located in northern Argentina.

The highest notification rates in children under five years are consistent with data analyzed by BANDARANAYAKE *et al.*[2], who found higher rates of hospitalization for influenza A(H1N1)pdm09 in children under five years in New Zealand, during the two outbreaks of 2009 and 2010. This skewed distribution towards younger individuals agrees with the hypothesis of an extended immunization of older adults, born before 1969, exposed to any of the three pandemics during the first half of the twentieth century[3,17]. On the other hand, in Mexico, the proportion of hospitalizations for influenza A(H1N1)pdm09 was higher in older individuals in an outbreak in 2011-2012 compared to 2009[3].

This study has some limitations, mainly due to the quality of the data used and the strategy for obtaining the samples used by the health system. Regarding the quality, in areas of lower population density and less access to clinical virological laboratories the number of notifications could have been underestimated. However, SIVILA (Sistema Nacional de Vigilancia por Laboratorios) already had sentinel units in all provinces of Argentina in 2009. Concerning sampling strategies, this was changed in mid-June 2009, when the sampling of severe cases was prioritized. This change may also have affected the spatiotemporal patterns recorded in 2009. In addition, other studies are needed to describe the spread of the virus through genetic and antigenic data.

In conclusion, this study's results suggest important spatial and temporal variations in notifications of influenza A(H1N1)pdm09 in Argentina, that pose the need for greater coordination in the Southern Cone countries at the time of carrying out the policies of prevention and vaccination.

RESUMEN

Tendencias espacio-temporales en los casos de gripe A(H1N1)pdm09 en Argentina, 2009-2012

El objetivo de este trabajo es analizar las variaciones espacio-temporales de los casos de gripe A(H1N1)pdm09 en Argentina. Se realizó un escaneo estadístico espacio-temporal por permutaciones para poner a prueba la no aleatoriedad en la interacción entre espacio y tiempo de los casos registrados de gripe A(H1N1)pdm09. Durante 2009 se identificaron dos conglomerados espacio-temporales, en el este de la provincia de Buenos Aires (mayo y junio) y en la mayor parte del centro-norte de la Argentina (julio y agosto). Durante 2011 y 2012 se registraron conglomerados próximos a zonas limítrofes con otros países. Al interior de los conglomerados, primero se observaron mayores tasas de notificación en población de edad escolar para luego extenderse a población mayor (15-59 años). A partir de 2011, las mayores tasas se observaron en menores de 5 años residentes en el centro del país. Se pudieron caracterizar dos etapas de transmisión espacio-temporal de la gripe A(H1N1)pdm09. La primera etapa se caracterizó por altas tasas de notificación y una posible interacción con individuos provenientes de otros países llegados a las grandes ciudades de la Argentina (patrón de jerarquía). La segunda etapa mostró una mayor interacción en algunas zonas fronterizas y sin un patrón claro de jerarquía. Estos resultados plantean la necesidad de generar una mayor coordinación en países del Cono Sur, con el objetivo de implementar políticas más efectivas de prevención y vacunación.

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PREVALENCE OF INTESTINAL PARASITES AMONG FOOD HANDLERS OF SARI, NORTHERN IRAN

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SUMMARY

Parasitic infection is highly prevalent throughout the developing countries of the world. Food handlers are a potential source of infection for many intestinal parasites and other enteropathogenic infections as well. The aim of this study was to determine the prevalence of intestinal parasite carriers among food handlers attending the public health center laboratory in Sari, Northern Iran for annual check-up. The study was performed from August 2011 through February 2012. Stool samples were collected from 1041 male and female food handlers of different jobs aged between 18 to 63 years and were examined following standard procedures. Sociodemographic, environmental and behavioral data analysis of the food handlers were recorded in a separate questionnaire. Intestinal parasites were found in 161 (15.5%) of the studied samples. Seven species of protozoan or helminth infections were detected. Most of the participants were infected with *Giardia lamblia* (53.9%) followed by *Blastocystis hominis* (18%), *Entamoeba coli* (15.5%), *Entamoeba histolytica/dispar* (5.5%), *Cryptosporidium* sp. (3.1%), *Iodamoeba butschlii* (3.1%) and *Hymenolepis nana* (1.9%) as the only helminth infection. The findings emphasized that food handlers with different pathogenic organisms may predispose consumers to significant health risks. Routine screening and treatment of food handlers is a proper tool in preventing food-borne infections.

KEYWORDS: Prevalence; Intestinal parasites; Food handlers; Iran.

INTRODUCTION

Intestinal parasitic infections cause significant problems in individuals and public health, particularly in developing countries, with a prevalence rate of 30-60%[27]. Transmission of intestinal parasites that occurs directly or indirectly through food, water or hands indicates the importance of fecal-oral human-to-human transmission[37]. Foodborne diseases are large problems in developed and developing countries. The spread of disease by food handlers is a common and persistent problem[5,38] worldwide. Food handlers with poor personal hygiene working in the food service settings can be infected by different enteropathogens[35], possibly causing fecal contamination of foods by their hands during food preparation, and finally, may be implicated in the transmission of many infections to the public in the local community[19]. Therefore, a proper screening procedure for food handlers is helpful in the prevention of probable morbidity and the protection of consumer health.

According to National Food Safety Standards in Iran, all food handlers must undergo parasitological stool examination prior to receiving their health certificate. Therefore, all food handlers were referred to a health center medical diagnosis laboratory in order to check for intestinal parasitic infections. The objective of this study was to assess the prevalence of intestinal parasites among food handlers working in the food service setting who were attending the Health Center Diagnostic Laboratory in Sari, Northern Iran, for annual check-ups.

MATERIALS AND METHODS

A cross-sectional study was carried out on 1041 food handlers (620 male and 421 female; age range 18-63) attending the Health Center Diagnostic Laboratory in Sari, Northern Iran from August 2011 to February 2012.

Sari lies at the center of the Mazandaran province in Northern Iran (lat. 35°58–36°50N, long. 52°56–53°59E). Sari has a population of approximately 261,293 individuals. The mean yearly relative humidity is 85.83%, with rainfall occurring in all seasons of the year, and the average temperature is 17 °C.

The sample size was originally calculated to be 896 participants, based on the prevalence of 30% in Northern Iran[11,30]. The sample size was extended to 1041, to take into account possible technical difficulties in the study. The study was carried out on food handlers of different jobs, from different geographical areas in the city of Sari, during a five-month period, with a total of 421 (40.5%) females and 620 (59.5%) males and an age range of 18-63 years (mean age 33 ± 15 yrs). The participants were categorized according to the nature of their occupational duties. The

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subjects under study were, 112 (10.7%) bakers, 137 (13.2%) butchers, 59 (5.7%) cooks and kitchen helpers, 36 (3.4%) confectioners, 67 (6.4%) chicken store workers, 185 (17.8%) fast-food workers, 56 (5.4%) fruit/vegetable sellers, 198 (19%) restaurant workers, 18 (1.8%) school cafeteria workers and 173 (16.6%) supermarket staff. Written consent was obtained from both the health center administration and participants.

A structured questionnaire was used to collect data on age, sex, educational level, income and the hygienic status of each study subject.

Fresh fecal specimens were collected in a clean stool cup. Each container was labeled and immediately transferred to the parasitology laboratory at Sari Medical School. The diagnosis was made on direct wet mount, formalin-ether concentration, and with confirmation of positive stool specimens on Ziehl-Neelsen and Trichrome stained slides.

A Chi-square test was used to assess associations among the variables. The significance level was 5%. SPSS 16 software was used for statistical analysis. The study was approved by the research committee of Mazandaran University of Medical Sciences, Sari, Iran.

RESULTS

Overall, positive stool results for intestinal parasites were 161 (15.5%). As shown in Table 1, the positive rates among males and females were 19% (118/620) and 10.2% (43/421). There was an equal distribution of parasitic infection among all age groups of food handlers, ranging from 11.2-16.8% with a higher prevalence in the 30-39 years group and a lower one in the below 19 years group.

Table 1
Prevalence of intestinal parasitic infections, as determined by analysis of stool specimens, according to age group and sex of 1041 food handlers in Sari, Northern Iran, 2011-2012

Variable	Total n (%)	Positive n (%)	OR	*p*-value
Sex				
Male	620 (59.5)	118 (19)	0.48 [0.33,0.70]	0.0001
Female	421 (40.5)	43 (10.2)		
Age group				
<19	197 (18.9)	22 (11.2)	0.62 [0.37,1.02]	0.06
20-29	170 (16.3)	28 (16.8)		
30-39	499 (47.9)	84 (16.8)		
40-49	155 (15)	24 (15.5)		
>50	20 (1.9)	3 (15)		

The prevalence of the intestinal parasites detected in the study is shown in Table 2. Of the study subjects, 15.2 % (n = 158) were infected with a single parasite and 0.3% (n = 3) had a double infection. The prevalence of *G. lamblia* as a predominant species was 82 (50.9%), followed by *B. hominis* 29 (18%), *E. coli* 25 (15.5%), *E. histolytica* 9 (5.6%), *Cryptosporidium sp.* 5 (3.1%), *I. butschlii* 5 (3.1%), and *H. nana* 3 (1.9%).

Table 2
Prevalence of intestinal parasitic infections among 1041 food handlers, aged 18-64 years in Sari, northern Iran, 2011-2012

Intestinal parasite	n	Positive (%) n = 162	Positive (%) n = 1041
Single infection			
G. lamblia	**82**	**50.9**	7.9
B. hominis	**29**	**18**	2.8
E. coli	**25**	**15.5**	2.4
E. histolytica	**9**	**5.6**	0.8
Cryptosporidium spp.	**5**	**3.1**	0.5
I. butschlii	**5**	**3.1**	0.5
H. nana	**3**	**1.9**	0.3
Double infection			
G. lamblia + I. butschlii	**2**	**1.3**	0.2
H. nana + E. coli	**1**	**0.6**	0.1
Total infected	161	100	15.5

The occurrence of parasites was associated with occupational category ($p < 0.0001$). Fruit/Vegetable sellers 23/56 (41.1%), school cafeteria workers 5/18 (27.8%), chicken store workers 16/67 (23.9%), cooks and kitchen helpers 13/59 (22%), restaurant workers 38/198 (19.2%), fast-food workers 33/185 (17.8%), confectioners 3/36 (8.3%), bakers 9/112 (8%), butchers 11/137 (8%), and supermarket staff 10/173 (5.8%) (Table 3).

Table 3
Prevalence of intestinal parasitic infections occurrence of enteroparasites in six occupational categories of 1041 food handlers, aged 18-64 years in Sari, Northern Iran, 2011-2012

Occupation	Total examined n (%)	Positive n (%)
Fruits/vegetables sellers	56 (5.4)	23 (41.1)
School cafeterias	18 (1.8)	5 (27.8)
Chicken store workers	67 (6.4)	16 (23.9)
Cooks and kitchen helpers	59 (5.7)	13 (22)
Restaurant workers	198 (19)	38 (19.2)
Fast-food workers	185 (17.8)	33 (17.8)
Confectioners	36 (3.4)	3 (8.3)
Bakers	112 (10.7)	9 (8)
Butchers	137 (13.2)	11 (8)
Supermarket staff	173 (16.6)	10 (5.8)
Total	1041 (100)	161 (15.5)

As shown in Table 4, factors related to intestinal parasitism included educational level, hand washing prior to eating and after using the toilet,

Table 4
Relationship between prevalence of intestinal parasitic infections and certain assumed risk factors among 1041 food handlers aged 18-64 years in Sari, northern Iran, 2011-2012

Risk factors	Total examined n (%)	Positive n (%)	OR	*p*-value
Place of living				
Rural	350 (33.6)	60 (17.1)	1.2 [0.85,1.71]	0.31
Urban	691 (66.4)	101 (14.6)		
Educational level				
Illiterate	186 (17.9)	56 (30.1)	2.12 [1.39,3.24]	<0.005
Primary school	332 (31.9)	58 (17.5)		
Secondary school	475 (45.6)	43 (9)		
College studies	48 (4.6)	4 (8.3)		
Economic status				
≤200 US $	816 (78.4)	131 (16)	1.24 [0.81,1.9]	0.34
>200 US $	225 (21.6)	30 (13.3)		
Hand washing after toilet				
Yes	938 (90.1)	136 (14.5)	0.52 [0.32,1.9]	0.01
No	103 (9.9)	25 (24.3)		
Eating with unwashed hands				
Yes	348 (33.4)	74 (21.3)	1.88 [1.33,2.64]	0.0004
No	693 (66.6)	87 (12.5)		
Eating unwashed vegetables				
Yes	796 (76.5)	123 (15.4)	0.99 [0.67,1.47]	1.00
No	245 (23.5)	38 (15.5)		
Contact with soil				
Yes	38 (3.7)	29 (76.3)	21.26 [9.48,45.93]	<0.0001
No	1003 (96.3)	132 (13.1)		
Contact with cat or dog				
Yes	95 (9.1)	21 (22.1)	1.63 [0.97,2.74]	0.07
No	946 (90.9)	140 (14.8)		
Number living in home				
2-4	615 (59.1)	107 (17.4)	1.45[1.01,2.06]	0.04
≥5	426 (40.9)	54 (12.7)		

contact with soil, and number of people sharing the same home. Parasitic infections were negatively associated with household income level, contact with dogs and cats, place of living, and eating raw vegetables that were not washed with detergent ($p > 0.05$).

DISCUSSION

Several authors across the world have emphasized the significance of food handlers with poor personal hygiene as a risk in the transmission of parasitic and bacterial diseases[35]. In this study, stool microscopic examination of 1041 food handlers investigated the presence of intestinal parasites. The overall prevalence of protozoan infections was 15.1% and that of helminthic infections was 0.4%. Mixed intestinal parasite infections were detected in 1.9% of the study samples. Higher prevalence rates have been reported from food handlers in Hamadan, Western Iran (74%)[13], Khuzestan, Southern Iran (34/25%)[28], while lower ones have been reported in Golestan, Northern Iran (6%)[20]. Recent reports in Iran showed the prevalence of intestinal parasites as between 18.4 to 33.3% in different studies[11,16,23,24,29]. When the data of the present study were compared with those of the reports of recent years, which were carried out on different groups of people in Iran, the lowest prevalence of parasitic infections was found among food handlers in Sari, Northern Iran. Possible explanations include geographic difference, the time of the study, and that the participants in this study had taken medical examination and might have been treated for intestinal parasites. Other studies on food handlers of countries neighboring Iran showed prevalence rates of 23%[2], 8.8% and 52.2%[15,31], 33.9%[1] and 6.7 to 52.2% in other countries of the world[10,14,34]. The differences in reported prevalence in various studies may be due to socioeconomic status, climatic conditions, poverty, personal and community hygiene, different study population and the year in which these surveys were conducted. According to recent reports on different groups of people in Iran, the prevalence of the intestinal helminthic

infections was 0-3.6%[17,18,29,30]. The prevalence of helminthic infections in this survey was 2.5%, which is comparable to some earlier studies of intestinal parasites from Iran and inhabitants of other countries 2.7-13.6%[1,7,18,37].

It should be mentioned that, in recent years, the prevalence of intestinal parasites, particularly of helminth infections, has decreased significantly in Iran, which could be explained by the following reasons: (a) installation and operation of a reliable sewage system, (b) Substitution of untreated human excrement with chemical fertilizer, (c) education on disinfecting vegetables before use, (e) routine drug therapy with mebendazole in health centers and (d) implementing health educational programs. The most predominant species identified were *G. lamblia* (50.9%), *B. hominis* (18.1%) and *E. coli* (15.5%). Previous similar studies conducted in Iran and other countries revealed that the leading parasites were *G. lamblia* and *B. hominis* among food handlers, as well as general population[2,6,10,11,34]. Among protozoa, *G. lamblia* is the common cause of diarrhea worldwide. The majority of individuals infected with *G. lamblia* and *B. hominis* are asymptomatic and excrete large numbers of cysts that remain viable for long periods of time in the environment, contaminating water and food. *G. lamblia* can be directly transmitted to consumers if ingested via food or water that has been contaminated by infected food handlers.

In the current study, the prevalence was higher in males (19%) than in females (10.2%), with a statistically significant difference ($p < 0.0001$). Several studies have reported a higher prevalence of infection in males than in females[8,12,22], whereas other data have indicated the contrary[26,30]. No significant difference was found in the distribution of parasitic infection among all age groups of food handlers, ranging from 11.2-16.8% prevalence, with a higher prevalence in the 20-39 years group and lower in the below 19 years group. This shows that there is equal exposure to the infection and suggests an effect of environmental conditions on infection. However, it is in accordance with a study performed in Khartoum, where the prevalence of infection with no significant difference was 29.9 to 31.7%[7], albeit countered by other studies conducted in Northern Iran that have revealed a significant association[18]. In this survey, the prevalence of intestinal parasites was found to be higher among individuals living in rural areas (14.6 to 17.1%) with no significant difference ($p = 0.31$). Again, the inadequate infrastructure in these areas and the lack of sanitation and hygienic conditions were probably the main reasons for this relationship[3,9,32]. In the current study, more intestinal parasites were detected in people with a lower income ($p = 0.34$); however, it is estimated that the better the living conditions are, the lower the prevalence rates are**.** A significant association was observed between the educational level of food handlers and parasitic infection ($p < 0.0001$), assuming that they were highly aware of the importance of personal hygiene. Similar results were obtained in a study on Jakarta sidewalk food vendors[33], a study among food handlers in a tertiary care hospital in Mecca, Saudi Arabia[37] and another survey in Santa Catarina, Brazil among 238 workers of a fast-food company[25]. The prevalence of intestinal parasites was higher in crowded families ($p = 0.04$). This result was similar to a study conducted on 1200 individuals in Qaemshahr, Iran[18]. Person-to-person transmission seems to be the initial mechanism of parasite infection in crowded families. Several environmental and behavioral variables were significantly associated with intestinal parasite infection. In the current study, reduced hand washing with soap prior to eating, after using the toilet, or in both situations, and contact with soil, significantly increased the risk of infection ($p = 0.0004$, 0.01, 0.0001), which is in agreement with the data found in Mecca, Saudi Arabia[37]. Improper hand washing before handling food is one obvious route for dissemination of infections. Parasite eggs in the soil can be transmitted to vegetables, then on to hands and hence directly into the mouth[21], or ingested by eating raw vegetables[36]. The observations of the present study suggest a lack of a significant relationship between parasitic infection and eating raw vegetables ($p = 1.00$). The present study showed that the occurrence of parasites was associated with occupational category ($p < 0.0001$). Fruit/Vegetable sellers (41.1%) and supermarket staff (5.8%) contributed most to this association. The result of a study conducted among 238 workers of a fast food company in Santa Catarina, Brazil revealed a statistically significant difference between the prevalence of the infection and occupational category[25]. The fruits and vegetables may be contaminated by the hands of the sellers who don't wash their hand after defecation.

In conclusion, the intestinal parasitic infection rate of food handlers of Sari city was relatively high. The findings emphasize that food handlers with different pathogenic microorganisms may predispose consumers to significant health risks. Therefore, constant epidemiological surveillance through biannual routine parasitological tests and treatment of the infected cases along with the improvement of environmental sanitation are recommended to control the parasitic infection in food handlers.

RESUMO

Prevalência de parasitas intestinais entre manipuladores de alimentos de Sari, Norte do Iran

Infecção parasitária é altamente prevalente entre os países em desenvolvimento do mundo. Manipuladores de alimentos são fonte potencial de infecção de muitos parasitas intestinais assim como de outras infecções entero-patogênicas. O propósito deste estudo foi determinar a prevalência de portadores de parasitas intestinais entre pessoas que manipulam alimentos e atendem o centro de saúde pública em Sari, norte do Iran para possível check-up. O estudo foi conduzido de agosto de 2011 até fevereiro de 2012. Amostras de fezes foram coletadas de 1041 manipuladores de ambos os sexos e de diferentes funções entre a idade de 18 e 63 anos e foram examinados de acordo com procedimentos standard. Em questionário separado foram colocadas as análises sócio-demográficas, ambientais e de comportamento dos manipuladores de alimentos. Parasitas intestinais foram encontrados em 161 (15,5%) das amostras estudadas. Sete espécies de protozoários ou infecções por helmintos foram observadas. A maior parte dos participantes estava infectada com *Giardia lamblia* (53,9%) seguida por *Blastocystis hominis* (18%), *Entamoeba coli* (15,5%), *Entamoeba histolytica/dispar* (5,5%), *Cryptosporidium* sp. (3,1%), *Iodamoeba butschlii* (3,1%) e *Hymenolepis nana* (1,9%) como a única infecção por helminto. Os achados enfatizaram que os manipuladores de alimentos, com diferentes organismos patogênicos podem se constituir risco significante para os consumidores. Exames rotineiros e tratamento dos manipuladores de alimentos são as ferramentas apropriadas para prevenir as infecções originadas de alimentos.

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CONFLICT OF INTEREST

The authors have no conflicts of interest regarding the content of this article.

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CLINICAL AND EPIDEMIOLOGICAL PROFILE OF ELDERLY PATIENTS WITH CHAGAS DISEASE FOLLOWED BETWEEN 2005-2013 BY PHARMACEUTICAL CARE SERVICE IN CEARÁ STATE, NORTHEASTERN BRAZIL

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SUMMARY

By controlling the transmission of Chagas disease, the challenge of providing assistance to millions of infected patients that reach old age arises. In this study, the socioeconomic, demographic and comorbidity records of all elderly chagasic patients followed at the Pharmaceutical Care Service of the Chagas Disease Research Laboratory were assessed. The information related to the clinical form of the disease was obtained from medical records provided by the Walter Cantídio University Hospital. The profile of the studied population was: women (50.5%); mean age of 67 years; retired (54.6%); married (51.6 %); high illiteracy rate (40.2%); and family income equal to the minimum wage (51.5%). The predominant clinical forms of Chagas disease were cardiac (65.3%) and indeterminate (14.7%). The main electrocardiographic changes were the right bundle branch block (41.0%), associated or not with the anterosuperior left bundle branch block (27.4%). The average number of comorbidities per patient was 2.23 ± 1.54, with systemic arterial hypertension being the main one found (67.0%). It was found that the elderly comprise a vulnerable group of patients that associate aging with cardiac and/or digestive disorders resulting from the evolution of Chagas disease and other comorbidities, which requires special attention from health services to ensure more appropriate medical and social care.

KEYWORDS: Chagas disease; Elderly; Cardiac form; Comorbidities.

INTRODUCTION

Chagas disease (CD) is a chronic infection caused by the protozoan *Trypanosoma cruzi* and transmitted to humans by blood-sucking insects (kissing bugs) of the family *Reduviidae* and subfamily *Triatominae*[30,47]. As a result of the success achieved in the efforts to control vectorial and transfusional transmissions of CD during the last decades, the challenge of providing assistance to millions of infected in its chronic phase arises[59]. It is estimated that about 18 million people are infected with the disease, particularly in Latin America, with about 21,000 deaths reported each year[63].

Several studies have demonstrated a progressive reduction of seropositivity for CD in young age groups and the consequent increase of the prevalence of infection among older individuals[3,6,34]. It is believed that a large proportion of individuals with CD are either already in old age, or will become elderly in the near future[21].

It is essential that importance be given to the association between CD and the aging process of its carriers. Along with the decline that occurs with advancing age[21], added to cardiac and/or digestive disorders that result from the progression of CD, this group of patients is susceptible to other chronic diseases of advanced age, such as ischemic heart disease, diabetes mellitus, hypertension and arthrosis[41]. This increases the morbidity and worsens the quality of life of the individuals that age in this unfavorable condition[3,31,38,64].

These associations lead to a significant demand for health services and drugs, predisposing this vulnerable population to many risks such as polypharmacy[21,43,60]. The scarcity of research on CD among the elderly contrasts with the importance of this disease in this age group[34].

Therefore, the aim of this study was to better understand the clinical and epidemiological profile of elderly chagasic patients from a Pharmaceutical Care Service reference in the State of Ceará, since these aspects of this population in particular should receive attention, given that medical and social care is one of the main challenges of this neglected disease in Brazil[3,15].

MATERIAL AND METHODS

This was a descriptive cross-sectional survey conducted in the Pharmaceutical Care Service on the chagasic patients of the Laboratory of Research in CD at the Federal University of Ceará (LPDC/UFC). This

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service is linked to the Cardiology Outpatient Unit of the Walter Cantídio University Hospital (HUWC), responsible for the clinical diagnosis of patients with CD. Once diagnosed, the patient is referred to this Service to perform the drug treatment for CD.

The records of all patients followed in the Service during the period of July 2005 (date of commencement of this Service) to June 2013 were analyzed, totaling eight years. The study included elderly patients of both sexes selected according to the criteria of the Brazilian Institute of Geography and Statistics (IBGE), which sets the age as 60 years or over[29]. Patients whose medical records were not available for consultation at HUWC or presented insufficient data for the proposed objectives and analyses were excluded from the clinical evaluation of the study.

The following variables: gender, age, occupation, marital status, education, family income, place of origin, comorbidities, medication use and symptoms related to CD, were obtained from records filed in the LPDC obtained by interview during the first contact with the patient on the Service. The clinical information related to CD, the records of radiological studies of the esophagus, colon and heart, and electrocardiographic results were obtained through medical records filed in the HUWC.

To characterize the clinical form of the disease, the following criteria were used in accordance with the literature[6,11]:

I - Cardiac form: Symptomatic or asymptomatic individuals with electrocardiographic (ECG) changes suggestive of cardiac involvement and/or registration of cardiomegaly (detected by chest X-ray) in the medical record;

II - Digestive form: Individuals with test results in their medical records (esophagogram or endoscopy and/or barium enema or colonoscopy) compatible with megaesophagus and/or megacolon, and/or history of surgery for megaesophagus and/or megacolon.

III - Indeterminate form: Asymptomatic individuals presenting normal electrocardiogram (ECG), without registration of cardiomegaly in the medical records, and no changes in X-ray compatible with megaesophagus and/or megacolon;

IV - Mixed Form: Individuals presenting association between cardiac and digestive form.

This study was approved by the Research Ethics Committee of the HUWC in June 2012 under protocol number 031.05.12.

For statistical analysis, the GraphPad Prism Program (version 6.0) was used. For values with normal distribution, the "t" Student test was used, whereas the Mann Whitney test was used for values that did not show normal distribution. To investigate possible associations between variables, the Fisher's exact test was used. A significance level of $p < 0.05$ was adopted.

RESULTS

From the total of 411 patients followed at this Service between July 2005 and June 2013, 97 (23.6%) were individuals aged over 60 years.

A progressive increase in the percentage of elderly patients relative to the total number of patients enrolled in the Service has been observed during its eight years of activity. Between July 2005 and June 2007, only two elderly patients were registered among 59 patients (3.4%). In the period from July 2007 to June 2009, there were 16 elderly patients from a total of 80 patients (20.0%). In the following period, July 2009 to June 2011, 105 patients were enrolled, 22 of whom were elderly (21.0%). And finally between July 2011 and June 2013, 167 patients were registered and 57 were elderly (34.1%) (Fig. 1). Comparing the percentage of elderly patients corresponding to the first four years of activity of the service (12.9%) with the subsequent four years (29.0%), there was a significant increase in this percentage ($p = 0.003$).

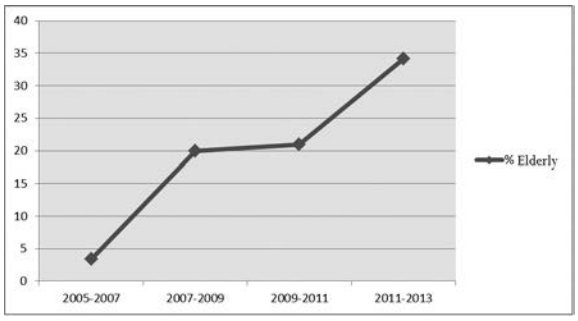

Fig. 1 - Biannual increase of the percentage of elderly patients with Chagas disease enrolled in the Pharmaceutical Care Service in Ceará State during its eight years of activity (n = 97).

Regarding the sociodemographic characteristics, it was found that most patients were women (50.5%); 70.1% were aged between 60 and 69 years; the mean age was 66.9 ± 6.5 years; 54.6 % were retired, and among those still working, the main occupation registered was farming (55.0 %); 49.5% had not finished elementary school and a high percentage of illiteracy was found (40.2 %); 51.5% had a monthly income equal to the minimum wage; and most (51.6 %) were married .

With respect to the place of origin, 81 patients (83.5%) were reported to reside in the countryside of the state of Ceará and 16 (16.5%) were from the capital, Fortaleza. The main inland towns cited by the patients as places of origin were: Quixeré (18;18.5%), Jaguaruana (16;16.5%), Russas (14;14.4%) and Limoeiro do Norte (13;13.4%), municipalities located in the Low Jaguaribe Microregion, in Jaguaribe Valley. The other cities mentioned by patients as places of residence are shown in Figure 2.

When asked how they were diagnosed with CD, the majority of patients (30.9%) reported that the presentation of symptoms of the cardiac and/or digestive form led them to seek medical care; 27.8% were diagnosed by routine serological tests; 19.6% by electrocardiographic changes characteristic of CD; and 6.2% through blood donation.

In terms of symptoms related to CD, 18.5% of patients reported being asymptomatic, while 81.5% reported at least one symptom attributable to CD. The most frequently reported cardiac symptoms were chest pain (42.3%), dyspnea (36.1%), palpitations (23.7%), fatigue (17.5%), dizziness (7.2%) and syncope (6.2%). The most cited digestive symptoms were constipation (36.1%) and dysphagia (23.7%).

Two patients were excluded from the evaluation of the clinical form of CD due to insufficient information in their medical records (loss of

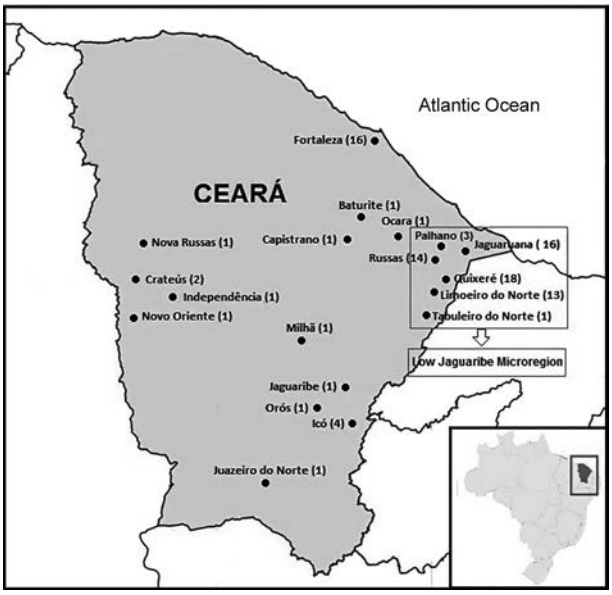

Fig. 2 - Place of origin of the elderly patients with Chagas disease in the state of Ceará (n = 97).

2.1%). Thus, the 95 analyzed patients had the following distribution according to the clinical form: predominance of the cardiac form (65.3%), followed by indeterminate form (14.7%), mixed (13.7%) and digestive (6.3%) (Fig. 3).

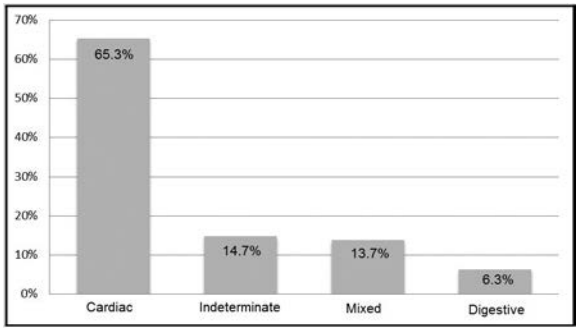

Fig. 3 - Clinical forms of the Chagas disease of elderly patients followed at the Pharmaceutical Care Service in Ceará State (n = 95).

Among patients with the mixed form, the association between cardiac form and chagasic megacolon (61.5%) was dominant, followed by cardiac form in association with megaesophagus (30.8%). One patient (7.7%) presented the cardiac form associated with both megacolon and megaesophagus. As for those ranked in the digestive form, four patients (66.7%) suffered from megacolon while two (33.3%) had megaesophagus.

Among these 95 patients, 75 (77.3%) had at least one change in ECG characteristics of CD. The major electrocardiographic abnormalities found among elderly patients with the isolated cardiac form of CD or associated with the digestive form (mixed form) are described in Table 1.

With regard to the existence of other diseases associated with CD, only 13.4% of patients reported no comorbidities, while 86.6% reported having at least one concomitant disease to CD. The average number of

Table 1
Electrocardiographic changes found in elderly patients with Chagas disease followed at the Pharmaceutical Care Service in Ceará State (n = 95)

Electrocardiographic changes	*n*	%
Right bundle branch block (RBBB)	39	41.0
Anterosuperior left bundle branch block (ASDB)	26	27.4
Changes in ventricular repolarization (CVR)	16	16.8
Ventricular extrasystole (VES)	13	13.9
Low voltage of the limb leads (LVLL)	06	6.3
First-degree atrioventricular block (AVB)	05	5.3
Sinus bradycardia	05	5.3
Electrically inactive area (EIA)	04	4.2
Left bundle branch block (LBBB)	03	3.2
Atrial fibrillation (AF)	03	3.2
Left ventricular hypertrophy (LVH)	03	3.2

comorbidities per elderly patient in this study was 2.23 ± 1.54 (CI: 1.92-2.54) and the main ones reported are described in Table 2. In relation to changes possibly resulting from the evolution of CD, 12.4% of the followed patients had arrhythmia and 8.2% congestive heart failure (CHF).

Table 2
Comorbidities associated with Chagas disease in elderly patients followed in the Pharmaceutical Care Service in Ceará State (n = 97)

Comorbidities	*n*	%
Systemic Arterial Hypertension (SAH)	65	67.0
Dyslipidemia	31	31.9
Dyspepsia	16	16.5
Diabetes Mellitus	14	14.4
Osteoporosis	11	11.3
Depression	06	6.2
Arthritis	06	6.2
Ischemic Heart Disease	04	4.1
Asthma	04	4.1
Rheumatism	04	4.1
Hypothyroidism	03	3.1
Cerebrovascular Accident (CVA)	03	3.1
Chronic Renal Failure (CRF)	03	3.1

With respect to medication use, 84.5% of the subjects reported continuous use of at least one drug. The average number of medications per patient was 3.12 ± 2.44 (CI: 2.63-3.61). The therapeutic agents most frequently used were diuretics (45.4%), angiotensin-converting enzyme inhibitors (ACEI) (36.1%), α and β-blockers (21.6%), anticoagulants

(21.6%), statins (17.5%), antacids/anti-ulcer (16.5%), anxiolytic/sedative (15.5%), antagonists of the angiotensin receptor 1 (14.4%), antidiabetic agents (13.4%), antiarrhythmic drugs (12.4%), calcium channel blockers (10.3%), laxatives (8.2%), drugs to treat osteoporosis (8.2%) and positive inotropic agents (7.2%).

DISCUSSION

The present study demonstrated a significant increase in the number of elderly patients enrolled in the Pharmaceutical Care Service throughout its eight years of activity. This finding corroborates the study of GUARIENTO *et al.*[22], which showed a progressive increase in the percentage of elderly patients enrolled in the Outpatient Unit of the Group for Studies into Chagas Disease (GEDoCh) at the Clinical Hospital of Campinas State University (UNICAMP) over 25 years (1980-2005).

The increased prevalence of CD among individuals with older age can be attributed to: the reduction in the incidence of this disease in Brazil, resulting from the success of the vector and transfusion transmission control campaigns; the improving social status of the population with housing improvement in endemic regions; and greater efficiency in diagnostic and therapeutic approaches[16,21,62]. Furthermore, the Brazilian population in general has experienced major changes in its demographic profile with substantial aging of the population as a result of the fall in the rate of mortality associated with the rapid and marked decline of the fecundity rate[28].

The rise of an elderly population with CD deserves special attention, since this association may have particularities that need further investigation[9], making the studies regarding the clinical aspects of CD in this geriatric age group a priority[15]. Due to the fact that the elderly population has specific and peculiar characteristics, the healthcare for this group of patients requires a more careful evaluation from health professionals[51], so that a clinical diagnosis of CD in the elderly is not confused with other diseases most prevalent in these individuals, such as dilated cardiomyopathy, ischemic heart disease, heart disease by arterial hypertension and cancer of the esophagus[9].

This study allowed the identification of a sociodemographic standard for chagasic elderly patients followed in the Pharmaceutical Care Service. The following groups were predominant in the population: women, patients between 60 and 69 years old, retirees, married individuals, and those with a poor education and low income. A very similar profile was observed by ALVES *et al.*[4] when 90 elderly chagasic patients from the Outpatient Clinic GEDoCh of the Hospital of UNICAMP were evaluated. It is known that the epidemiological profile of a patient with CD is that of an adult, from a rural region and with a low level of schooling, demonstrating the close relationship of the disease with underdevelopment and poverty[14].

It is important to emphasize the high percentage (40.2%) of illiterate patients that were detected in this study. ALVES *et al.*[4] reported in their study that this reality is a reflection of the socioeconomic conditions of chagasic patients who have few social opportunities, low wages and limited education.

Despite the small difference, the percentage of elderly chagasic women (50.5%) was higher than the percentage of men (49.5%) in the present study. In other studies, also involving elderly patients, the female sex was predominant compared to the male sex[3,4,22]. GUARIENTO *et al.*[22] point out that this aspect may be related to worse prognosis of CD associated with the male sex. This fact is well evidenced in the literature, where the male sex acts as a risk factor for worse outcomes among chronic carriers of this disease so that men tend to die younger, not reaching old age[49,52]. Furthermore, a study showing the situation of the elderly in Brazil documented a higher mortality of men in relation to women[35]. This is what the literature on gerontology refers to as the feminization of aging, a result of increased life expectancy among women at 60, 70 and 80 years old[32,48].

Regarding the place of origin, it was observed that most of the elderly patients (67.0%) were from the Low Jaguaribe Microregion. The Jaguaribe Valley is a region in Ceará that has always aroused interest among researchers as to the epidemiological importance of CD. The pioneering studies were performed by ALENCAR[2] and demonstrated high rates of human infection and capture of triatomines in this region. More recent studies have focused on the detection of seroprevalence in municipalities of the Lower Juaguaribe Microregion such as Limoeiro do Norte[18], Jaguaruana[6] and Russas[13]. According to BORGES-PEREIRA *et al.*[6], from the literature about CD in Ceará, the municipality of Jaguaruana and neighboring regions have always been in the group of areas with the highest prevalence of *T. cruzi* infection. These same authors found that 52.9% of the seropositive patients for CD were over 50 years old and there were no individuals younger than 16 years old. This reduction of seropositivity in younger age groups and the consequent increase in the prevalence of the disease among older individuals is a result of the success of measures to control vectorial transmission. Thus, the active search in this region has led to the diagnosis of patients with CD mainly in the elderly age group, which is possibly a result of vectorial infection acquired in the past. Once diagnosed, these patients are referred to reference services in Fortaleza, including the Pharmaceutical Care Service of patients with CD of the LPDC, a fact that may explain the high prevalence of elderly patients from this region.

The predominant clinical form of CD among patients included in this study was the cardiac, followed by the indeterminate, mixed and digestive forms. For mixed and digestive forms, the incidence of megacolon was higher than that of megaesophagus. These findings are consistent with other studies in the literature, where the cardiac form is prevalent among the elderly chagasic[3,4,22].

The high percentage of cardiac form found in this study (65.3%) can be explained because it was performed with patients aged over 60 years. Such an interpretation is associated with the fact that an older age is related to a more severe clinical form of CD with impairment of cardiac function[21]. This phenomenon has been documented in the literature, showing that the evolutionary character of CD reflects the progressive aging of its carriers, as the disease transmission has been interrupted[16].

SILVA *et al.*[58] have shown that the age variable acts as a risk factor associated with the development of Chagas heart disease. They observed that patients over 60 years old were three times more likely to have heart disease when compared to those aged 50-59 (OR = 2.89, 95% CI = 1.09-7.61). This finding seems plausible, since the rate of conversion of chronic indeterminate to cardiac form, although small, is time dependent.

According to PIANCASTELI[50], the true prevalence of the digestive form is not well known or may be underestimated due to the greater propaedeutic difficulty and, in the case of colonopathy, to the lack of consensus on the interpretation of results. Moreover, it is important to mention that patients included in this study come from the Cardiology Clinic of the HUWC and are not always followed by a gastroenterologist, which may lead to an underestimation of the actual percentage of patients with the digestive form of the disease.

The patients classified as indeterminate (14.7%) constitute a group characterized by a long asymptomatic period (which may last a lifetime), with no clinical, electrocardiographic and radiological manifestations, and their diagnosis is based on positive serology and/or parasitology[11,12]. In most cases, chagasic individuals with this clinical form are unaware of the presence of infection and show good prognosis in the medium and long terms. However, they must still be regularly monitored, since about 2-5% of these patients annually advance to the cardiac and/or gastrointestinal forms[39].

The pathogenesis of Chagas heart disease involves mechanisms such as direct injury caused by *T. cruzi*; autoimmunity induced by *T. cruzi* antigens, with consequent destruction of myocytes, sympathetic and parasympathetic ganglia; microvascular disease and neurogenic mechanisms with sympathetic and parasympathetic dysfunction, inducing the inflammatory process and fibrosis[26]. In severe cases the disease evolves to CHF, cardiomegaly, arrhythmias, conduction block of the electrical stimulus, thromboembolic events, ischemic stroke and sudden death[11,54]. Among individuals who develop this clinical form of the disease, a significant group becomes candidate for the use of artificial heart devices, such as defibrillators and pacemakers, with CD being the major cause of the implantation of such devices[53].

The presence of electrocardiographic changes is a fundamental element in the characterization of significant cardiac involvement in CD[11]. The electrocardiographic findings in this study are in agreement with the literature. ALMEIDA *et al.*[3], when evaluating 61 elderly chagasic patients, found ECG abnormalities in 85.2%, the most prevalent of which were: ASDB (41.0%), RBBB (32.8%), VES (22.9 %) and CVR (11.5%). The most prevalent conduction disorder in the sample of the present study was the RBBB (41.0%), associated or not to ASDB, data also convergent with the literature. NETTO et al.[44] also studied elderly patients and confirmed the RBBB as the most frequent alteration in the chagasic group. According to the Brazilian Consensus on CD[11], electrocardiographic changes characteristic of CD include: RBBB with or without ASDB, VES, sinus bradycardia with a cardiac frequency lower than 40 bpm, AVB of 2nd degree or total, CVR, presence of EIA, sinus node dysfunction, AF and LBBB.

With respect to the existence of chronic diseases concomitant with CD, the high percentage (86.6%) of patients that reported at least one comorbidity associated with *T. cruzi* infection in this study was expected, since, according to the literature, the elderly have higher morbidity rates than other groups of patients[61,64].

A similar result was found by ALVES *et al.*[4], where the average number of comorbidities associated with CD by elderly patients was 2.8 ± 1.8, the most prevalent of which were: SAH (56.7%), osteoporosis (23.3%), osteoarthritis (21.2%), dyslipidemia (20%), ischemic heart disease, diabetes mellitus and dyspepsia in equal proportions (10%), as well as CHF and hypothyroidism, which had an incidence of 7.78% each.

Statistics show that the main cause of morbidity and mortality among elderly Brazilians is cardiovascular diseases and the main ones include: CVA, CHF, coronary heart disease, hypertrophic cardiomyopathy, valvular disease (aortic stenosis and mitral valve disease), arrhythmia and SAH[36,37,55,64]. In a survey based on the analyses of data from the Ministry of Health, it was found that in Maringá - PR, between 1970 and 1990, there was a significant increase in the number of deaths associated with SAH (119%) and an increased risk of death associated with cardiovascular disease in both sexes as age advances[42]. In the present population, the most frequent cardiovascular diseases were SAH (67%), CHF (8.2%), ischemic heart disease (4.1%) and CVA (3.1%). It is important to note that hypertension and dyslipidemia, major comorbidities found in this study with prevalence rates of 67% and 31.9% among elderly patients with CD, are important factors for the development of ischemic heart disease and cerebrovascular disease[4, 37].

Previous publications have shown that SAH is the most common cardiovascular disease in chagasic populations[3,27,46,57] and that it has a higher prevalence in patients aged over 50 years, showing a cumulative effect between the two pathologies[5,24]. Greater myocardial damage was observed by GUARIENTO *et al.*[20] in patients with this combination of diseases. They determined that more severe forms of heart disease occurred in chagasic patients suffering from SAH than in those who did not have SAH. Also, high levels of heart damage from SAH in elderly chagasic patients who died and underwent necropsy have been observed[45].

There is still scarce data in the literature about concomitant hypertension and CD. Research related to this association is relevant due to the known presence of parasympathetic nervous system involvement in CD, which determines a higher sympathetic activity, leading to a probable influence on the genesis of hypertension in these patients[25,26]. Some authors[23,24] point out trypanosomiasis pathophysiology as a contributing factor to high blood pressure, also indicating that 50% of individuals with this combination of pathologies are over 45 years old and already have CHF to some degree.

GURGEL *et al.*[25], when correlating the frequency of SAH in chronic CD carriers with age, observed that the 225 chagasic and hypertensive patients had a median age distribution of 55 years with significant differences of hypertensive degree between age groups for both sexes ($p < 0.001$ for females and $p < 0.05$ for males). Higher levels of blood pressure were found in elderly chagasic patients, particularly females, showing the association of both diseases in advanced age groups.

Thus, it is plausible to suppose that there is a higher risk of death among aging chronic carriers of CD, and that this fact is associated with a higher incidence of cardiovascular disease (particularly the higher incidence of SAH)[4]. Moreover, it cannot be forgotten that the physiological changes resulting from the cardiovascular aging process contribute to the deterioration of cardiac function, leading to a functional decline[33,40].

The cardiac complications resulting from the process of evolution of Chagas heart disease are important causes of hospitalization and increase mortality associated with clinical decompensation[10]. BOZELLI *et al.*[7]

studied the medical records of 95 patients diagnosed with CD treated at the University Hospital of Maringa (HUM). They observed that the mean age of patients treated in the inpatient service (61.2 years ± 12.8) was significantly higher than that of the outpatient unit (50.1 years ± 12.5). In most cases, hospitalization was assigned to the symptoms resulting from CHF. According to FRANÇA & ABREU[17], the presence of these complications increases therapeutic costs, highlighting the importance of University Hospitals in providing a differentiated care to this group of patients.

The most commonly used therapeutic agents were consistent with the most prevalent clinical form of CD in the elderly population studied (cardiac form). The main drugs used were found to act in the cardiovascular function, as follows: diuretics (45.4%), ACEI (36.1%), α and β-blockers (21.6%) and anticoagulants (21.6%). The average of three medications per patient was expected, since the elderly, due to accumulation of chronic diseases, comprise the most medicalized group in society[19].

Polypharmacy is an important issue among the geriatric population, given that the consequence of the wide use of drugs affects their clinical and economic context, impacting on patient safety[56]. Risks related to polypharmacy are higher in the older age group due to the physiological changes resulting from aging (eg.: reduced metabolic capacity of the liver and renal involvement), which lead to an increased incidence of adverse reactions and more serious repercussions which may increase morbidity and mortality[1,8,56].

It can be concluded, therefore, that the chagasic elderly comprise a vulnerable group of patients that have an association between the risk of morbimortality for Chagas heart disease and the other comorbidities often present in this age group, as well as the risk of drug interactions due to polypharmacy. Since the growth of the chagasic population above 60 years is a reality, this finding should draw the attention of health authorities to improve access to health services as well as the training of qualified professionals in the treatment and recognition of clinical differences inherent to this group of patients.

RESUMO

Perfil clínico e epidemiológico de pacientes idosos com doença de Chagas atendidos entre 2005-2013 por um serviço de atenção farmacêutica no estado do Ceará, nordeste do Brasil

Controlando-se a transmissão da doença de Chagas, surge o desafio de prestar assistência a milhões de pacientes infectados que chegam à velhice. Neste estudo, foram avaliados os registros socioeconômicos, demográficos e de comorbidades de todos os pacientes chagásicos idosos acompanhados no Serviço de Atenção Farmacêutica do Laboratório de Pesquisa em Doença de Chagas. As informações relacionadas à forma clínica da doença foram obtidas a partir de registros médicos disponibilizados pelo Hospital Universitário Walter Cantídio. O perfil da população estudada foi de: mulheres (50,5%); idade média de 67 anos; aposentados (54,6%); casados (51,6%); alta taxa de analfabetismo (40,2%); e renda familiar de um salário mínimo (51,5%). As formas clínicas predominantes da doença de Chagas foram a cardíaca (65,3%) e a indeterminada (14,7%). As principais alterações eletrocardiográficas foram o bloqueio de ramo direito (41,0%), associado ou não ao bloqueio ântero superior esquerdo (27,4%). O número médio de comorbidades por paciente foi de 2,23 ± 1,54, sendo a hipertensão arterial sistêmica a principal encontrada (67,0%). Verificou-se que os idosos constituem grupo vulnerável de pacientes que associam o envelhecimento com as alterações cardíacas e/ou digestivas resultantes da evolução da doença de Chagas e outras comorbidades, o que exige atenção especial dos serviços de saúde para um atendimento médico e social mais adequado.

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PREDICTIVE FACTORS FOR THE PROGRESSION OF CHRONIC CHAGAS CARDIOMYOPATHY IN PATIENTS WITHOUT LEFT VENTRICULAR DYSFUNCTION

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SUMMARY

The identification of predictors for the progression of chronic Chagas cardiomyopathy (CCC) is essential to ensure adequate patient management. This study looked into a non-concurrent cohort of 165 CCC patients between 1985 and 2010 for independent predictors for CCC progression. The outcomes were worsening of the CCC scores and the onset of left ventricular dysfunction assessed by means of echo-Doppler cardiography. Patients were analyzed for social, demographic, epidemiologic, clinical and workup-related variables. A descriptive analysis was conducted, followed by survival curves based on univariate (Kaplan-Meier and Cox's univariate model) and multivariate (Cox regression model) analysis. Patients were followed from two to 20 years (mean: 8.2). Their mean age was 44.8 years (20-77). Comparing both iterations of the study, in the second there was a statistically significant increase in the PR interval and in the QRS duration, despite a reduction in heart rates (Wilcoxon < 0.01). The predictors for CCC progression in the final regression model were male gender (*HR* = 2.81), Holter monitoring showing pauses equal to or greater than two seconds (*HR* = 3.02) increased cardiothoracic ratio (*HR* = 7.87) and time of use of digitalis (*HR* = 1.41). Patients with multiple predictive factors require stricter follow-up and treatment.

KEYWORDS: Chagas cardiomyopathy; Clinical progression; Prognosis; Cohort studies.

INTRODUCTION

Although it was first described over a century ago, Chagas disease (CD) still remains a relevant endemic ailment in Latin America as it threatens some 16 million people in the continent. As the transmission via vectors has been controlled in several countries, the clinical follow-up of the millions still infected remains an important challenge. Chronic Chagas cardiomyopathy (CCC) is the main morbidity resulting from CD[10]. Left ventricular dysfunction is the strongest predictive factor for morbidity and mortality in CCC[8,9]. The identification of markers for disease progression before the occurrence of ventricular dysfunction may allow for earlier treatment and better prognosis. Although this disease has been extensively studied, the natural history of CCC and its independent predictive factors in outpatients – examined through the most sophisticated non-invasive cardiovascular methods such as echo-Doppler cardiography (ECHO), Holter monitoring, and exercise testing (ET) – are not completely understood. Most previous studies resorted to simpler risk stratification methods, such as electrocardiography (ECG) and chest radiography, and were conducted based on small selected heterogeneous groups including Chagas patients with varied prognoses, some of them for a short period of time[23]. Some authors[26,31] have looked at earlier stage CCC patients, with differing findings on the ECG, no left ventricular dysfunction, and unnoticeable symptoms. Very few studies have measured prognostic factors of Chagas cardiomyopathy among asymptomatic *Trypanosoma cruzi*-infected persons[26].

This study aimed to identify the predictive factors for the progression of CCC in patients with no left ventricular dysfunction and to check if this progression is different in patients who have an ECG with abnormalities indicative of CCC when compared to one who have unspecific ECG abnormalities.

MATERIALS AND METHODS

Patients: This non-concurrent cohort study covered adult CCC patients with no left ventricular dysfunction living in the metropolitan area of Belo Horizonte seen at the Referral Center for Training on Infectious and Parasitic Diseases of the *Hospital das Clínicas* of the Federal University of Minas Gerais (HC-UFMG) for their first and return visits, between 1985 and 2010. Patients were not selected for gender, age range, ethnicity or social status. *Ethics:* this study was approved by the UFMG Research Ethics Committee (COEP-UFMG), as per process ETIC 347/09, and found to be in compliance with the 1975 Declaration of Helsinki. All the workup was carried out with the consent of the patients and the database was protected by user passwords granted only to the group of researchers.

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Enrollment criteria: proven diagnosis for CD by two positive conventional serologic test results for *T. cruzi* infection[32] having completed the initial assessment protocol (iteration 1) comprised of an interview, physical examination, chest radiography, ECG, and ECHO; being diagnosed with CCC (abnormal ECG); having undergone clinical assessment, ECG, and ECHO at least two years since the initial assessment (iteration 2); having the tests at iteration 1 done within ≤ 12 months.

Exclusion criteria: having other heart conditions (ischemic, hypertensive, congenital or valvular heart disease, alcoholic cardiomyopathy) referred or investigated through clinical and/or complementary tests, found at any stage of the follow-up; having left ventricular systolic or diastolic dysfunction on the ECHO on iteration 1; using a pacemaker (PM) or having ventricular tachycardia (VT) - defined as three or more consecutive premature ventricular complexes with a heart rate of more than 100 beats per minute - on the ECG at the initial assessment; patients with time intervals between tests > 12 months.

Cardiac non-invasive studies: They were all carried out as per the standard routines at HC-UFMG, from the preparation of the patients up to the interpretation of their results. All of them were conducted by personnel with previous experience of CCC and blind interpreted in relation to the clinical form of the disease. The date on which the first tests were done on the patients was considered as the patient's date of entry into the study.

Conventional resting 12-lead ECG: interpreted by two examiners using the diagnostic criteria for CCC3 accepted by the World Health Organization.

ECHO: Abnormal test results were characterized by LV (left ventricular) dysfunction and/or anomalous segmental contractility, in addition to the presence of apical aneurysm. Systolic dysfunction was considered when EF < 54% and classified as mild to moderate (≥ 40%) and severe (< 40%), whereas diastolic disorder was characterized for dysfunction stages ≥ II (patients were classified according to diastolic function patterns: normal, impaired relaxation known as stage I, pseudonormal pattern known as stage II and restrictive pattern known as stage III). Segmental disorder was defined as the presence of akinesia, hypokinesia or dyskinesia in a defined area.

Chest radiography: with images taken from two views: posteroanterior and lateral – using the cardiothoracic ratio (CTR) as reference and those patients with a CTR ≥ 0.50 were deemed as abnormal. *Exercise Testing* (ET): It was completed by patients for whom the test was not contraindicated. They were considered abnormal when any of the following were observed: ventricular arrhythmia, blood pressure alterations, chronotropic response, myocardial ischemia criteria - namely J-point depression (the point at which the QRS complex meets the ST segment) ≥ 1 mm, with a horizontal or downsloping ST segment with duration ≥ 0.80 seconds (sec); Y-point depression 80 milliseconds (msec) after point J ≥ 1.5 mm with an upsloping ST segment; J-point elevation ≥ 1 mm. *24-hour Holter monitoring*: They were considered abnormal when any of the following were found: arrhythmias with complexity ≥ Lown 2[18], intra or atrioventricular conduction disorders, pauses of ≥ 2.0 seconds and changes in the ST segment matching myocardial ischemia criteria.

Categorization: After cardiac studies results had been analyzed, patients were divided into two groups at iteration 1: group 1 (G1): ECG with at least two unspecific abnormalities; and group 2 (G2): ECG with abnormalities indicative of CCC. Further on, considering ECG and ECHO, patients were independently categorized for CCC[8], taking the most abnormal test result into account, thus describing four stages: 1- ECG: at least two unspecific abnormalities (G1) – sinus bradycardia (HR > 40 bpm), low voltage, incomplete right bundle branch block (RBBB), left anterior hemiblock (LAHB), first-degree atrioventricular block (AV block), unspecific ST-T alterations. ECHO: normal. 2- ECG: abnormalities indicative of CCC (G2) – complete RBBB in association or not with LAHB, isolated monomorphic ventricular extrasystoles (VES), sinus bradycardia (HR ≤ 40 bpm), second-degree AV block, T primary alterations. ECHO: abnormal, but no ventricular dysfunction. 3- ECG: abnormalities indicative of CCC (G2) – polymorphic or sustained VES, electrically inactive area, sinus node dysfunction. ECHO: diastolic ventricular dysfunction or abnormal EF, however ≥ 40%. 4- ECG: abnormalities indicative of CCC (G2) – atrial fibrillation (AF), complete AV block, left bundle branch block (LBBB), non-sustained ventricular tachycardia (NSVT) and PM at time 2. ECHO: diastolic ventricular dysfunction or EF < 40%.

Outcomes or response variables: worsening of the CCC scores and onset of left ventricular dysfunction.

Analyzed independent explanatory variables:

Social, demographic and epidemiologic variables: age; gender; ethnicity; intensity and duration of physical effort in previous and current occupations2; time spent in rural (RA) and CD endemic areas (EA); location and time spent at current residence; family history (FH) for CD, heart disease and sudden death – the last two occurring in family members aged 40 or less; drinking – weekly alcohol intake (in grams[29]) and period of abuse (in years); smoking (pack-year). C*linical variables:* symptoms, thromboembolism, comorbidities, systemic hypertension, body mass index (BMI), specific complete etiologic treatment with benznidazole, CCC score, regular and continuous use (in years) of cardiovascular drugs for at least two years including: loop diuretics, hydrochlorothiazide (HCTZ), beta blockers, spironolactone, amiodarone, angiotensin converting enzyme inhibitors (ACE inhibitors) or angiotensin II receptor blockers (ARBs). *Cardiac non-invasive studies variables:* abnormalities on ECG, chest radiography, ECHO, 24-hour Holter monitoring and on exercise testing.

Data collection tools and analysis: A structured questionnaire was completed during first and return visits and all codified responses were entered into Microsoft Access. Statistical packages *MINITAB for Windows 14.10*, *nQuery Advisor 4.0, SPSS 15.0* and *EXCEL* were used for data analysis purposes.

Descriptive analysis: Frequency distribution tables were used for nominal categorical variables, whereas central tendency (means, medians) and variability measures such as minimum, maximum, and standard deviation (SD) were used for numerical variables. McNemar's test was used to compare the variables resulting from initial and final ECG and ECHO tests, while the Wilcoxon test was adopted to compare continuous variables in paired data groups. *Survival analysis:* The outcomes considered for survival analysis were the worsening of CCC score over time and the onset of left ventricular dysfunction. CCC scores collected

at the beginning and end of the study were considered when calculating the worsening of the CCC score. CCC score worsening was defined as having a greater score at the end of the study than the one collected at the beginning. Patients categorized as stage 4 were excluded from the aforementioned analysis because it was impossible for them to receive a worse score at the end of the study. *Univariate analysis* the Kaplan-Meier estimator was used to build survival curves, alongside Cox's univariate model and the differences in survival between groups were assessed by the log-rank test. *Multivariate analysis*: The Cox regression model was used and a *p*-value of 0.20 was used to enter predictive variables into the Cox model and a 5% significance level was adopted as a cutoff threshold for the variables to be considered in the model. A new final model using only variables obtained in univariate analysis with a *p*- value of 0.5 was performed in order to avoid "overfitting phenomena". A final model was considered adequate to be interpreted when cox proportional risk was tested by using a logarithm of cumulative risk function against time (in months) for each covariate.

Observations: A hazard ratio (HR) with a CI of 95% was calculated. A 5% significance level was adopted in all analyses. Some data sets were stratified in accordance with the literature in order to explore them better.

Analysis of the sample stratified into ECG groups: In order to assess whether the intensity of the initial abnormality of the ECG could predict CCC progression, groups G1 and G2 were analyzed solely in relation to the onset of left ventricular dysfunction – as the ECG accounted for part of the CCC score, thus the outcome "worsening of the CCC scores" should not be considered. The detection power of the sample was calculated using a 95% CI[27].

RESULTS

Patients were considered as lost when there was a failure to establish communications with patients after at least three phone calls on different days and at different times, sending a letter, and attempting to contact the patient's neighbors (Fig. 1). The lost group was similar to the excluded and studied ones, there was no statistically significant difference between these groups.

Descriptive analysis:

Social, demographic and epidemiologic profile of the patients: the mean age of patients was 44.8 years (20-77 years) (SD = 10.6); mainly born in RA (97.0%) and residing in CD EA (88.5%) for a mean of 16 years (SD = 8.7). Most patients have lived away from RA and EA for a mean of 23.4 years (SD = 11.1). At the start of the study, patients had been involved in intense (49%) occupational physical effort for a mean of 14.6 years. Mean alcohol intake was 194.9 grams/week for 21.5 years (SD = 474.1 g/week), median 60 grams/week for 20 years; smoking history was quantified at a mean of 18.4 pack-years (SD = 16.3).

Clinical profile: Most patients were asymptomatic (63.6%). Systemic hypertension was the most prevalent comorbidity (21.8%) in iteration 1; incidence increasing to 28.5% in iteration 2, an increase which was also seen in the use of cardiovascular drugs, going from 30.3 in iteration 1 to 43.6% in iteration 2. The increase was statistically significant in both cases (McNemar's: $p < 0.01$). Prevalence rates of the stages of CCC reduced from 49.7% (iteration 1) to 39.4% (iteration 2) in stage 1; from 42.4% to 40.6% in stage 2; while an increase from 6.7% to 13.3% was noticed in stage 3 and from 1.2% to 6.7% in stage 4.

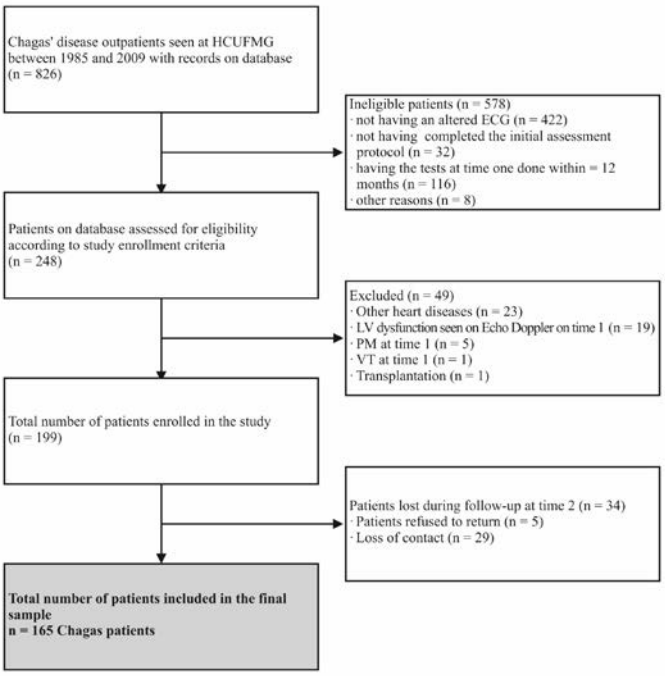

Fig. 1 - Workflow from the study 'Predictive factors for chronic Chagas cardiomyopathy patients without left ventricular dysfunction', CD Outpatient ward/HC-UFMG.

Cardiac non-invasive studies: ECG (Table 1) and ECHO (Table 2) explanatory variables were described and compared in both iterations of the study. They show a statistically significant (*p*-value Wilcoxon Test < 0.01) increase in both PR interval (56.4% of the patients) and QRS duration (40.8%), and in the reduction of heart rate (57.6%) all in iteration 2 of the study.

One-hundred patients underwent ET (61%), and 142 (86%) the 24-hour Holter monitoring.

Follow-up*:* More than 91% of the patients survived until the end of the study, while seven deaths were observed (4.5% lethality rate), two of which (28.6%) were due to sudden death, two (28.6%) due to decompensated CCC and three (42.8%) due to causes unrelated to CD. Eight (4.8%) patients were lost for over a year after completing the protocol in iterations 1 and 2 of the study. The minimum follow-up time in the study was two and the maximum was 20 years (mean = 8.2; DP = 3.2). The worsening of the CCC score was observed in 37 (22.7%) patients, while five (3.1%) improved their scores and 121 (74.2%) remained stable.

Analysis of the outcome "worsening of the CCC scores": In univariate analysis, all studied and statistically significant explanatory variables are shown in the tables and figures below (Table 3, Table 4 and Table 5 and Fig. 2, 3, 4, 5). In multivariate analysis, three adequate models were obtained, the first using the variables that had $p < 0.20$ and the others using variables that had $p < 0.5$ in the univariate analysis. The best final model was one of the $p < 0.5$ variables. It presented HR estimates with a more stable and shorter CI of 95% and showed the following remaining variables to be significant: male gender, pauses ≥ 2 seconds on Holter,

Table 1
Descriptive comparative analysis of ECG variables. CD patients. HC-UFMG

Variable		Time 1		Time 2	
		Occurrences (n)	Percentage (%)	Occurrences (n)	Percentage (%)
1st degree AV block*	No	150	90.9	136	82.4
	< 230 msec	14	8.5	17	10.3
	≥ 230 msec	1	0.6	12	7.3
Sinus bradycardia*	< 60 bpm	62	37.6	69	41.8
Complete RBBB	No	113	68.5	108	65.5
	yes	52	31.5	57	34.5
Inomplete RBBB	No	153	92.7	152	92.1
	yes	12	7.3	13	7.9
Isolated VES	No	150	90.9	154	93.3
	yes	15	9.1	11	6.7
LAHB	No	117	70.9	117	70.9
	yes	48	29.1	48	29.1
Unspecific ST alterations	No	142	86.1	137	83.0
	yes	23	13.9	28	17.0
Rhythm	sinus	164	99.4	158	95.8
	multifocal atrial	1	0.6	-	-
	AF			2	1.2
	PM			5	3.0
2nd degree AV block - grade I	No	163	98.8	164	99.4
	yes	2	1.2	1	0.6
LBBB	No	163	98.8	162	98.2
	yes	2	1.2	3	1.8
Low voltage	yes	3	1.8	3	1.8
NSVT	yes			1	.6

* *p*-value McNemar's test < 0.05.

Table 2
Descriptive comparative analysis of ECHO variables. CD Outpatient ward/HC-UFMG

Variable		Time 1		Time 2	
		Occurrences (n)	Percentage (%)	Occurrences (n)	Percentage (%)
LV final diastol.[a] diameter	normal	151	91.5	137	83.0
	altered	14	8.5	28	17.0
LV*[c] final systol.[b] diamater	normal	155	93.9	134	81.2
	altered	10	6.1	31	18.8
Ejection fraction	normal	165	100.0	144	87.3
	53 to 40%			17	10.3
	<40%			4	2.4
Shortening fraction	normal	144	87.3	136	82.4
	altered	21	12.7	29	17.6
Contractility alterations	absent	148	89.7	131	79.4
	global dysf.[d]	1	0.6	9	5.5
	segmental dysf.[d]	16	9.7	25	15.2
Diastolic function alteration	absent	133	80.6	75	45.7
	grade I dysf.[d].	32	19.4	85	51.8
	grade II dysf.[d]	-	-	4	2.4
Degenerative* valve disorders	no	158	95.8	140	84.8
	yes	7	4.2	25	15.2

a: diastol: diastolic; b: systol: systolic; *c: *p*-value McNemar's test < 0.05; d: dysf: dysfunction.

Table 3
Univariate analysis for outcome 'worsening of CCC scores'. CD Outpatient ward/HC-UFMG

Variable	Absolute # (n) / relative # (%) or stratification	Worsening incidence (%)	*p*-value[a]	HR	95% CI for HR	
					Lower limit	Upper limit
Female gender	yes (103/62.4)	13.7		1.00		
	no (62/37.6)	37.7	**0.010**	2.59	1.25	5.36
Age	< 50 years (112/67.8)	21.9		1.00		
	≥ 50 years (53/32.2)	24.5	**0.024**	2.18	1.11	4.26
Caucasian ethnicity	no (112/67.9)	21.8		1.00		
	yes (53/32.1)	24.5	0.615	1.19	0.60	2.36
Permanence at RA	<10 years (41/24.8)	31.1		1.00		
	≥ 10 years (124/75.2)	19.5	0.079	0.55	0.28	1.07
Permanence at EA	< 10 years (34/20.6)	24.4		1.00		
	≥ 10 years (131/79.4)	15.6	0.363	0.64	0.25	1.66
Currently resides at RA	no (160/97)	22.2		1.00		
	yes (05/3)	40.0	**0.012**	6.54	1.50	28.50
Currently resides at EA	no (140 /84.8)	20.3		1.00		
	yes (25/15.2)	36.0	**0.002**	3.39	1.56	7.40
Remains currently at RA or EA	< 10 years (163/98.2)	10.7		1.00		
	≥ 10 years (2/1.2)	25.2	0.071	2.99	0.91	9.77
FH of Chagas' disease	yes (127/77)	21.6		1.00		
	no (38/23)	23.0	0.912	1.05	0.47	2.32
FH of heart disease	yes (74/44.8)	22.2		1.00		
	no (91/55.2)	23.3	0.656	0.86	0.45	1.66
FH of sudden death	yes (59/35.8)	22.1		1.00		
	no (106/64.2)	24.1	0.839	1.05	0.63	1.77
Physical effort at current job	mild (36/21.8) + moderate(48/29.1)	25.3		1.00		
	intense (67/40.6)+ VI[b] (14/8.5)	20.0	0.183	0.64	0.33	1.24
Time at current job	< 10 years (42/25.5)	19.1		1.00		
	≥ 10 years (123/74.5)	25.3	0.318	1.42	0.72	2.80
Physical effort previous job	mild (11/8.9) /moderate (33/26.8)	18.2		1.00		
	intense (49/39.8) / VI[b](30/24.4)	22.1	0.362	1.48	0.64	3.45
Time at previous job	< 10 years (112/67.9)	19.5		1.00		
	≥ 10 years (53/32.1)	24.2	0.359	1.48	0.64	3.44
Drinking present/past	yes (75/45.5)	18.0		1.00		
	no (90/54.5)	28.4	0.438	1.30	0.67	2.51
Smoking present/past	yes (58/35.2)	23.4		1.00		
	no (107/64.8)	21.4	0.639	0.84	0.41	1.72
Thrombo-embolics	no (159/96.4)	22.8				
	yes (6/3.6)	20.0	0.564	0.556	0.08	4.08
Comorbidities	no (117/70.9)	25.2				
	yes (48/29.1)	16.7	0.941	0.97	0.44	2.14
Systemic hypertension	no (129/78.2)	23.6				
	yes (36/21.8)	19.4	0.617	1.24	0.54	2.84
BMI	↓ weight (9/5.5)	12.5	0.714			
	normal (92/55.8)	22.8	0.845	1.22	0.16	9.20
	↑ weight (47/28.5)	21.7	0.784	1.33	0.17	10.51
	obese (17/10.3)	29.4	0.485	2.15	0.25	18.51

Table 3
Univariate analysis for outcome 'worsening of CCC scores'. CD Outpatient ward/HC-UFMG (cont.)

Variable	Absolute # (n) / relative # (%) or stratification	Worsening incidence (%)	*p*-value[a]	HR	95% CI for HR	
					Lower limit	Upper limit
CTR	normal (144/87.3)	16.8				
	altered (21/12.7)	65.0	**0.000**	6.419	3.213	12.823
Ventricular arrhythmia grade on ET	Lown 0 (63/63)	17.5				
	Lown 1- 4 (37/37)	36.1	**0.041**	2.36	1.04	5.39
Top HR on ET	normal (81/81)	26.3				
	altered (19/19)	15.8	0.418	0.61	0.18	0.418
Blood pressure on ET	normal (26/26)	24.7				
	altered (74/74)	23.1	0.587	0.77	0.30	1.98
AHA functional class on ET	I (27/27)	25.9	0.651			
	II (44/44)	25.6	0.513	0.68	0.21	2.17
	III+IV (29/29)	20.7	0.871	1.09	0.40	2.96
cond.[c] dis.[d] AV/ IV Holter	no (79/55.6)	26.6				
	yes (63/44.4)	18.0	0.111	0.55	0.26	1.15
pauses ≥ 2 sec. on Holter	no (133/93.7)	21.4				
	yes (9/6.3)	44.4	**0.043**	2.99	1.04	8.64
SV[e] arrhythmia on Holter	no (107/75.4)	22.9				
	yes (35/24.6)	22.9	0.384	1.46	0.62	3.40
Ventricular arrhythmia complexity on Holter	0 and 1 (74/52.1)	16.4				
	2 to 4 (68/47.9)	29.9	0.056	2.05	0.98	4.28
Sustained VA[f] (pairs) on Holter	no (100/70.4)	16.2				
	yes (42/29.6)	39.0	**0.004**	3.09	1.42	6.72
NSVT on Holter	no (124/87.3)	18.7				
	yes (18/12.7)	52.9	**0.003**	1.47	1.14	1.90

a *: Cox's univariate model; b: VI: very intense; c: cond.: conduction; d: dis.: disorders; e: SV: supraventricular; f: VA: ventricular arrhythmia.

Table 4
Univariate analysis for outcome 'worsening of CCC scores'. CD Outpatient ward/HC-UFMG. Drinking (weekly alcohol intake and time of abuse) and smoking (pack-year)

Worsening of CCC scores	Estimation	Weekly alcohol intake (in grams)	Time of drinking abuse (in years)	Smoking (pack-year)
No	Quartile 1	22.0	12.0	458.5
	Median	58.0	21.0	1160.0
	Quartile 3	157.5	27.5	4042.5
Yes	Quartile 1	20.0	13.5	603.0
	Median	66.0	20.0	1020.0
	Quartile 3	197.0	27.0	4028.0
	p - value*	0.979	0.959	0.598
	HR	1.00	1.00	1.01
	95% CI to HR	[0.99; 1.01]	[0.96; 1.04]	[0.97; 1.05]

(*): Cox's univariate model.

Table 5
Univariate analysis of time (in years) patients have taken cardiovascular drugs for outcome 'worsening of CCC scores'. CD Outpatient ward/HC- UFMG

Variable	*p*-value*[a]	HR	95% CI to HR	
			Lower limit	Upper limit
Loop diuretics	**0.036**	1.17	1.01	1.36
HCTZ[b]	0.276	0.92	0.79	1.07
Digitalis	**0.000**	1.47	1.23	1.77
B-blockers	0.449	0.45	0.06	3.52
Spironolactone	**0.004**	1.42	1.12	1.80
ACEi/ARBs	**0.030**	1.13	1.01	1.26
Amiodarone	0.320	1.08	0.93	1.25

*[a]: Cox's univariate model; b: HCTZ: hydrochlorothiazide.

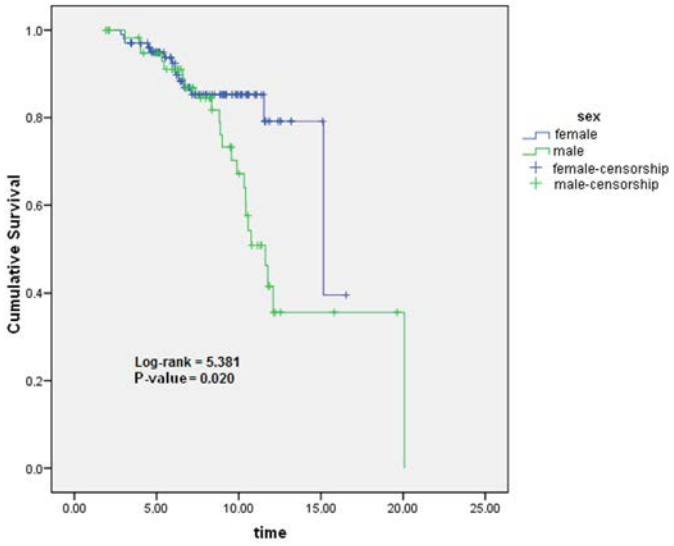

Fig. 2 - Kaplan-Meier estimator for time (in years) until the worsening of CCC scores by gender, CD Outpatient ward/HC-UFMG.

increased CTR, and time of use of digitalis (Table 6). The Median survival time to CCC score worsening was 15.15 (8.91-21.39) years (standard error = 3.18; 95% CI).

Analysis of the outcome "onset of left ventricular dysfunction": Even after including the variables that had $p < 0.20$ in the univariate analysis (Table 7), an adequate final regression model was not attained for multivariate analysis.

Analysis of sample stratified into ECG groups: 88 (53%) patients were categorized into group 1 (G1) and 77 (47%) into group 2 (G2). Left ventricular dysfunction was seen in 11 (12.5%) patients in G1 and in 13 (16.9%) in G2. Univariate analysis did not reveal a statistically significant difference between both groups (*HR* = 0.89) for the onset of left ventricular dysfunction ($p = 0.788$; 95% CI: 0.39-2.02). Relative risk (RR) was 1.4 and calculated sample detection power found to be 12.23%.

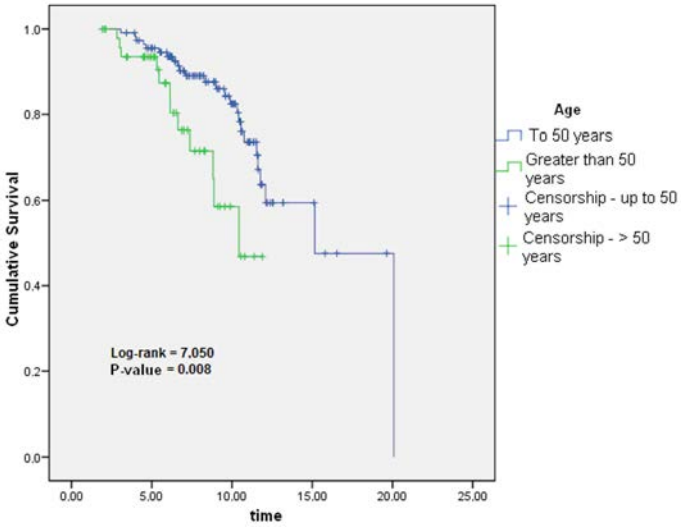

Fig. 3 - Kaplan-Meier estimator for time (in years) until the worsening of CCC scores by age range, CD Outpatient ward/HC-UFMG.

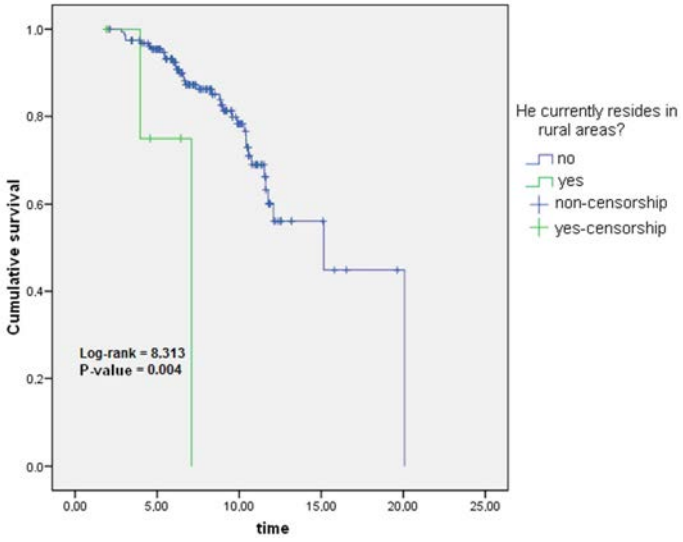

Fig. 4 - Kaplan-Meier estimator for time (in years) until the worsening of CCC scores for those currently residing in rural areas, CD Outpatient ward/HC-UFMG.

DISCUSSION

Chagas disease is a complex heterogeneous illness with wide variation in clinical course and prognosis. The advantages of the present study include the homogeneous sample of patients at earlier stages of CCC with no left ventricular dysfunction, having minimal abnormalities on ECG, examined through noninvasive risk markers that can be routinely measured, the duration of follow-up (mean 8.2 years), and the use of multivariate methods of statistical analysis. This study demonstrated that the variables finally found to be predictive factors for CCC progression for both outcomes - worsening of the CCC scores and the onset of left ventricular dysfunction – were male gender, living in rural areas, time of use of digitalis and increased cardiothoracic ratio.

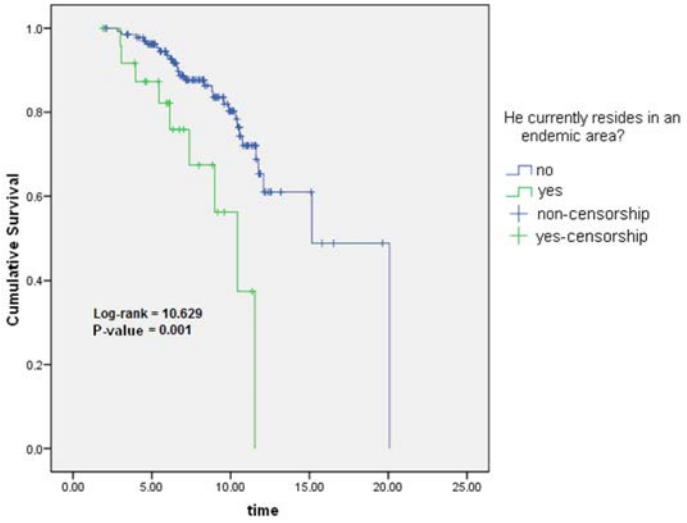

Fig. 5 - Kaplan-Meier estimator for time (in years) until the worsening of CCC scores for those currently residing in endemic areas, CD Outpatient ward/HC-UFMG.

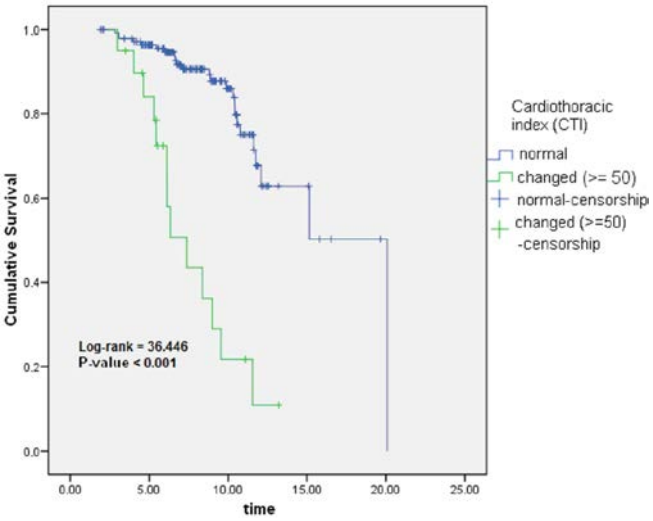

Fig. 6 - Kaplan-Meier estimator for time (in years) until the worsening of CCC scores based on CTR. CD Outpatient ward/HC-UFMG.

Table 6
Multivariate analysis of time until the worsening of CCC scores. CD Outpatient ward/HC-UFMG

Variable	Ratio	Standard error	Wald's test	*p*-value	HR	95% CI to HR	
						Lower limit	Upper limit
Male gender	1.03	0.39	6.99	0.008	2.81	1.31	6.03
Pauses ≥ 2 seconds on Holter	1.11	0.55	4.07	0.044	3.02	1.03	8.83
CTR ≥ 0.50	2.06	0.42	24.51	<0.001	7.87	3.48	17.82
Time taking digitalis (years)	0.35	0.11	10.15	0.001	1.41	1.14	1.75

Resorting to univariate analysis, this paper – as did other important longitudinal studies – has revealed the following as statistically significant variables found to be predictive factors for CCC worsening as an outcome: male gender[12,14], age > 50 years[20,25,28]; CTR ≥ 0.50[13,14,22,25]; time of use of digitalis[30], identification of ventricular arrhythmias in the ET[24] categorized from Lown 1 to 4; Holter monitoring showing pauses equal to or greater than two seconds, and ventricular arrhythmia – sustained and non-sustained VT[22,24]. A statistically significant finding – time taking cardiovascular medication – may be related to more severe cases of CCC (patients who needs treatment) as well as the variable "time of use of digitalis". However, this study was not designed to discuss such findings.

Upon looking into patients' current residential addresses in RA and CD EA two interesting issues surface: (1) the impact of exposure to reinfection in rural and endemic areas upon the deterioration of the patient's clinical status. The progressive decrease in morbidity and mortality seen in CD in all controlled endemic areas was clearly predicted by DIAS[11], who observed this in Bambuí, Minas Gerais, Brazil, where a pioneering effort was made to systematically eradicate the vector insect. The author attributed the decrease to the cessation of exogenous reinfections, probably by the same strain, responsible for the more severe cases of CD. And (2): access to healthcare services is more precarious in rural areas. It is clear that patients followed up at CD specialized care centers or at cardiology outpatient wards have greater access to therapy, enhanced compliance to treatment, better quality of life, and live longer, as the therapeutic arsenal used to treat heart failure from Chagas disease is highly effective and beneficial for the patient's survival and quality of life, in addition to reducing hospitalization rates.

It was observed that patients with abnormal CTR have almost a 13-fold risk of this outcome happening, a very high risk, confirming the important predictive value of chest radiography. When evaluating a patient with abnormal CTR, attention should be turned to their adequate treatment as soon as possible. Less than one fourth of the patients with CCC had abnormal CTR, stressing the diagnostic value of ECG, due to its significant sensitivity in detecting abnormalities in early stage CCC[8,28]. Although ECG unspecific abnormalities can commonly occur in the elderly as a result of atherosclerosis, hypertension, ischemia etc., the presence of electrocardiographic repolarization abnormalities increase sensitivity for the diagnosis of acute Chagas disease[1]. Those abnormalities seem to persist. Some longitudinal studies have found unspecific abnormalities in the ECG to be independent predictive factors for worse prognoses and the progression of CCC[15,22,25], which reinforces the point

Table 7
Univariate comparison of ECG and CTR variables in both iterations of the study for outcome 'onset of ventricular dysfunction on ECHO'. CD Outpatient ward/HC-UFMG

Variable			Incidence of ventricular dysfunction on ECHO (%)	*p*-value*[a]	HR	95% CI to HR	
						Lower limit	Upper limit
TIME 1	PR interval* (msec)	≤ 200	14.02				
		> 200 & < 230	21.4	0.772	1.20	0.36	4.02
	HRate* (bpm)	< 60	17.5				
		≥ 60 & < 100	9.7	0.321	0.62	0.25	1.58
	QRS duration* (msec)	< 120	14.4				
		≥ 120	14.8	0.324	0.64	0.26	1.55
	RBBB + CRBBB	no	13.9				
		yes	15.6	0.352	0.67	0.28	1.57
	CRBBB	no	15.0				
		yes	13.5	0.212	0.56	0.22	1.40
TIME 2	PR interval* (msec)	≤ 200	11.8	0.169			
		> 200 & < 230	17.6	0.871	1.11	0.32	3.82
		≥ 230	41.7	0.061	2.62	0.96	7.19
	HRate* (bpm)	< 60	14.7				
		≥ 60 & < 100	14.5	0.612	0.81	0.36	1.83
	QRS duration* (msec)	< 120	11.3				
		≥ 120	19.1	0.852	1.08	0.47	2.49
	RBBB + CRBBB	no	13.7				
		yes	15.7	0.586	0.79	0.34	1.83
	CRBBB	no	14.8				
		yes	14.0	0.225	0.57	0.23	1.41
	CTR	normal	8.3				
		altered	57.1	**0.000**	12.73	5.34	30.33

a*: Cox's univariate model.

that ECG unspecific abnormalities are prevalent from acute phases of the disease. In the present study, a statistically significant difference between both groups (G1 and G2) for the onset of left ventricular dysfunction was not found, which can be explained by two ideas: the possibility that both groups were not really particularly different, thereby not allowing the authors to lessen the risk of patients with more than one unspecific abnormalities in the ECG progress CCC, and the low calculated sample detection power found to be 12.23% in the present study.

In the present study, the most prevalent abnormalities found in ECG are in accordance with the literature[19] as well as the presence of ECG alterations being an independent predictive factor for poor prognosis of CCC[14,20,22,25]: QRS width was associated with mortality, as seen in the papers by RIBEIRO *et al*.[24]. A PR interval ≥ 0.16 seconds was shown to be an independent predictive factor[15]. Some papers have indicated that increased – and not reduced – heart rates are a predictive factor for mortality, but such a finding, albeit not correlated to the worst prognosis in several longitudinal studies, has been frequently reported in clinical settings in association with heart disease progression and severe sinus bradycardia (HR under 40 bpm).

Concerning ECHO, stage I diastolic dysfunction was the most prevalent abnormality found in both iterations of the study, further supporting the finding seen in the literature that it may occur even in Chagas patients without heart disease and precede systolic dysfunction in CCC4. Some authors[13] have described increased LV diastolic diameter as a predictive factor for poor prognosis of CCC, while PETTI *et al*.[20] correlated increased final LV diastolic diameter to death.

The authors' data show that occupational reorientation is needed as the disease progresses to cardiac involvement, but many factors act as barriers to successfully finding a new career: the limits imposed upon the patient by the disease's clinical manifestations and complications[8,16], the lack of specialized training seen among CD patients due to adverse socioeconomic circumstances, the migration from rural areas to urban regions – where the types of jobs available differ tremendously, the stigma that haunts those diagnosed with CD – even when the diagnosis is not accompanied by occupational disability.

In the present study, a significant share of the patients were overweight (BMI > 25) or obese (BMI > 30) due to their lifestyle and a third of them had comorbidities, with systemic hypertension ranking highest. However, the prevalence rate of systemic hypertension in the studied population, made up exclusively of CD patients, was lower than

that found for the Brazilian population in general within the same age range[7]. In spite of some controversy, there are publications to further support this finding[17]. This study, however, was not designed to this aim. The statistically significant increase in the incidence of systemic hypertension and in the use of cardiovascular drugs between iterations 1 and 2 of the study supports the verification of its increased prevalence rates as patient age increases while CCC progresses, giving both diseases a summational character. Some patients took inadequately prescribed digitalis, despite the authors' efforts to convince patients and doctors of other services to stop it once it was not indicated. Increased CTR can result in a lack of opportunity of ready access to ACE inhibitor (this drug was first prescribed about ten years after the beginning of the study).

Low lethality rates in this paper relate to the overall status of the patients in the study. Mean follow-up was long enough to ensure that time was plenty for events to unfold, with regard to the onset of left ventricular dysfunction and CCC progression. Four patients died due to Chagas disease during the follow-up in this study, two due to sudden death and two due to heart failure requiring internation. Nowadays, there is a noticeable displacement of deaths to older patients, mainly among those from 50 to 79 years old[21]. More than 50% of the internations due to Chagas disease in Brazil occurred among this population. Heart failure is the main cause of hospitalization (approximately 50%) of the South American population and it presents high mortality especially in patients with Chagas etiology[6].

The limitations of the present study: selection bias might have occurred because patients with missing data were excluded and the multivariate analysis was restricted to the 165 patients who had complete data on all the variables. Although the authors believe that the patients in this cohort were representative of outpatients with Chagas heart disease in other areas of Brazil, further investigations involving this population are desirable. Patients with minimal abnormalities in their ECG were studied, thus it was necessary to define as G1 (group 1) and stage 1 those patients with two or more unspecific abnormalities, despite the low calculated sample detection power hindering the authors' ability to draw certain conclusions. The authors could not assess the effect of therapy because treatment was not controlled in the study. The loss of follow-ups was more than 5%, although these patients and those excluded were statistically similar.

Conclusion: The identification of factors associated with the progression of CCC is essential to adequate patient management. In the present study the most important predictors were: male gender, Holter monitoring showing pauses equal to or greater than two seconds, time of use of digitalis and increased cardiothoracic ratio. Several other potential prognostic factors were pointed out in the univariate analysis, which will also contribute to the improvement in CCC patients care.

AUTHORS CONTRIBUTIONS

Silvana de Araújo Silva, Eliane Dias Gontijo and Carlos Faria Santos Amaral: Study design; collection, analysis, and interpretation of data; writing of the paper; and decision to submit it for publication. João Carlos Pinto Dias: interpretation of data; writing of the paper; and decision to submit it for publication. Camila Gomes de Souza Andrade: collection, writing of the paper.

RESUMO

Preditores da evolução da cardiopatia chagásica crônica em pacientes sem disfunção ventricular esquerda

A identificação de preditores da progressão da cardiopatia chagásica crônica (CCC) é essencial ao manejo adequado do paciente. Estudo coorte não concorrente de 165 pacientes portadores de CCC entre 1985-2010 quanto a preditores independentes da evolução da CCC. Os desfechos foram piora da classificação da CCC e surgimento de disfunção ventricular esquerda ao ecoDopplercardiograma. Variáveis sócio-demográficas, epidemiológicas, clínicas e propedêuticas foram estudadas e realizadas análise descritiva, análise de sobrevida com análise univariada (Kaplan-Meier e modelo univariado de Cox) e multivariada (modelo de regressão de Cox). O seguimento foi de dois a 20 anos, com média de 8,2 anos. A média de idade dos pacientes foi de 44,8 anos (20-77 anos). Comparando ambos os tempos do estudo, no tempo 2 houve significância estatística do aumento do intervalo PR e da duração do QRS, além da redução da frequência cardíaca (Wilcoxon < 0,01). Os preditores da evolução da CCC no modelo final de regressão foram sexo masculino (*HR* = 2,81), pausas iguais ou maiores que dois segundos ao Holter (*HR* = 3,02), aumento do índice cardiotorácico (*HR* = 7,87) e tempo de uso de digital (*HR* = 1,41), destacando-se necessidade de seguimento e tratamento mais rigoroso para os chagásicos que cumulam estes fatores.

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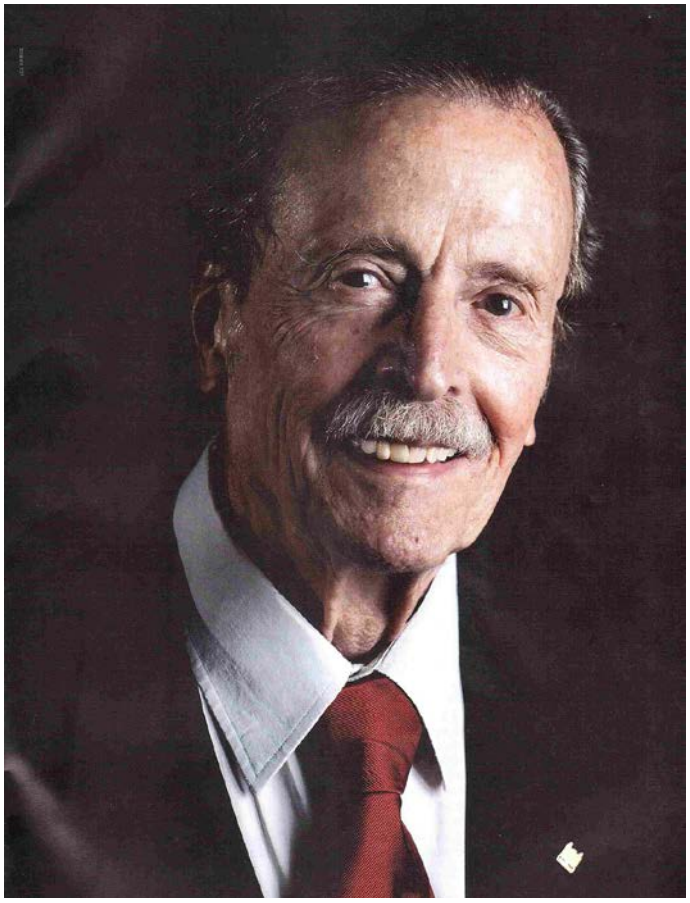
TRIBUTE

LUIZ HILDEBRANDO PEREIRA DA SILVA (1928-2014)



Luiz Hildebrando Pereira da Silva (1928-2014) graduated from the University of São Paulo Medical School in 1953 and shortly thereafter joined the Department of Parasitology, then chaired by Professor Samuel Pessoa, a leading Brazilian parasitologist.

Samuel Pessoa and Luiz Hildebrando both shared a deep commitment to social problems. They considered it their duty to direct their scientific research towards Brazilian people to lessen the burden of parasitic diseases. Therefore, they actively participated as scientists and policy makers in the fight against prevailing parasitic diseases, such as ancylostomiasis, schistosomiasis, Chagas disease and malaria.

Following the military coup of 1964, Luiz Hildebrando and many other scientists were suspended from the University of São Paulo due to undefined "subversive activities". Hildebrando returned to Europe, where he had previously worked for two years in René Thomas' laboratory in Belgium. This time, he went to André Lwoff's laboratory at the Pasteur Institute in Paris, where he met and worked with two other future Nobel Prize winners, François Jacob and Jacques Monot.

After some time, Hildebrando secured his own laboratory at Pasteur Institute and resumed experimental work on malaria, mainly that of African populations, caused by *Plasmodium falciparum.*

When democracy was restored in Brazil in the 1980's, Hildebrando began the long process of returning home, to collaborate on a project studying malaria in Rondônia with colleagues of the University of São Paulo. Finally, in 1997, after his retirement from the Pasteur Institute, Hildebrando formally returned as a professor to the Department of Parasitology that he had left 30 years ago. This time, he became fully involved with Amazonian malaria and, in 1998, he moved to Porto Velho to live and work amidst the disease.

Hildebrando's permanent fight to solve the practical, day-to-day problems caused by malaria in Rondônia includes over 200 publications, which underpin his scientific contribution to basic and applied research on parasitic diseases. Particularly important was his participation in unveiling the widespread prevalence of asymptomatic malaria in Amazonian native populations and the urgency for its control. Among his latest achievements is the creation of an Institute (IPEPATRO) for the study of tropical pathologies, alongside his permanent involvement with decision making, at both a state and federal level, for improving the control of parasitic diseases.

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BRIEF COMMUNICATION

ANTIFUNGAL ACTIVITY OF SILVER NANOPARTICLES OBTAINED BY GREEN SYNTHESIS

Eduardo José J. MALLMANN(1), Francisco Afrânio CUNHA(1,2), Bruno N.M.F. CASTRO(2), Auberson Martins MACIEL(1,2), Everardo Albuquerque MENEZES(2) & Pierre Basílio Almeida FECHINE(1)

SUMMARY

Silver nanoparticles (AgNPs) are metal structures at the nanoscale. AgNPs have exhibited antimicrobial activities against fungi and bacteria; however synthesis of AgNPs can generate toxic waste during the reaction process. Accordingly, new routes using non-toxic compounds have been researched. The proposal of the present study was to synthesize AgNPs using ribose as a reducing agent and sodium dodecyl sulfate (SDS) as a stabilizer. The antifungal activity of these particles against *C. albicans* and *C. tropicalis* was also evaluated. Stable nanoparticles 12.5 ± 4.9 nm (mean ± SD) in size were obtained, which showed high activity against *Candida* spp. and could represent an alternative for fungal infection treatment.

KEYWORDS: Silver nanoparticles; Antifungal activity; *Candida* spp.

Candida albicans and *Candida tropicalis* yeasts are responsible for a number of major diseases as well as recent cases of resistance to the main antifungals. Therefore, new substances should be researched as an alternative to combat such resistance[4,11]. *C. albicans* and *C. tropicalis* are the main yeasts isolated from samples of patients admitted to hospitals in the state of Ceará, Brazil[12].

Nanotechnology is responsible for the production and study of metal nanoparticles. These structures present several applications that highlight antimicrobial activity, and this property is an important tool in combating microorganisms resistant to conventional drugs[18].

Silver nanoparticles (AgNPs) are a new kind of material with several applications, such as sensors, catalysts, anticancer agents and antimicrobial agents. AgNPs have exhibited activity against bacteria, fungi and viruses[8]. However, synthesis of AgNPs produces toxic waste, such as ammonia[14], which can affect human health and the environment[18]. The green synthesis of AgNPs has used various routes: plants, microorganisms and non-toxic substances[6,15,19].

The aim of this study was to synthesize AgNPs using ribose sugars as reducing agents and sodium dodecyl sulfate (SDS) as the capping agent. The antifungal activity of these nanoparticles was evaluated against strains of *C. albicans* and *C. tropicalis*.

The synthesis of AgNPs was performed using ribose (Sigma-USA). First, 500 mL of a 5 mM AgNO3 (Merck-Brazil) solution was added to 1.0 g of ribose and 0.5 g of SDS (Sigma-USA) was used as the stabilizer. This solution was stirred and the temperature was raised to 50 °C. SDS had the function of preventing agglomeration and subsequent precipitation of the AgNPs. The reaction was considered complete when the solution acquired a pale yellow color, characteristic of AgNPs (Fig. 1a)[2,14].

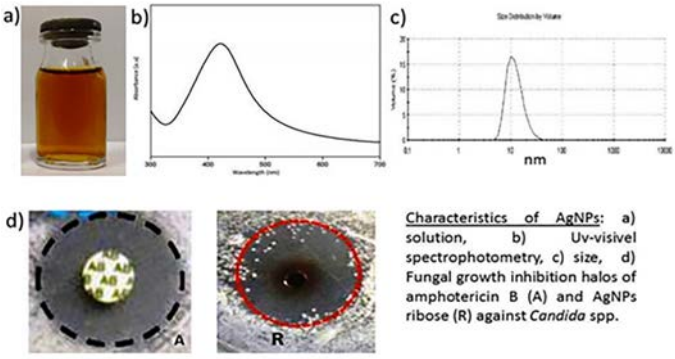

Fig. 1 - Characterization of the AgNPs and their antifungal effects.

The purification was carried out by centrifugation at 10,000g/10min. Characterization of the synthesized AgNPs was carried out using a UV-Visible spectrophotometer (Thermo Scientific GENESYS™ 10s), by scanning of the absorbance spectra in a 300-700 nm range of wavelength.

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Table 1
Effect of AgNPs produced by green synthesis and Amphotericin B against *C. albicans* and *C. tropicalis*

Strains (*n*)	**AgNPs**		**Amphotericin B**		*p*
	Range (mm)	Halo (mm) (Mean ± SD)	Range (mm)	Halo (mm) (Mean ± SD)	
C. albicans (14)	17-30	23 ± 4	15-25	20 ± 3	
C. tropicalis (16)	12-30	21 ± 4	15-25	20 ± 3	0.02

The size of the AgNPs was analyzed on Zetasizer, NanoZS Malvern® by dynamic light scattering (DLS)[5].

In this study, 30 strains of *Candida* spp. were selected (14 *C. albicans* and 16 *C. tropicalis*) and isolated from blood samples of patients hospitalized in the state of Ceará, Brazil. *C. albicans* was purified and identified in a chromogenic medium, with production of chlamydospores in rice extract agar containing Tween-80, and germ tube formation. This identification was carried out by molecular biology with the primer *hwp*1 (cr-f-5'-GCT ACC ACT TCA GAA TCA TCA TC-3'; cr-r-5' GCA CCT TCA GTC GTA GAG ACG-3') and the PCR conditions were 95 °C for five min, followed by 30 cycles of 94 °C for 45 s, 58 °C for 40 s, and 72 °C for 55 s; extension was performed at 72 °C for 10 min. The DNA fragment size that was produced had 945 bp. The molecular identification of *C. tropicalis* was performed using the *trf*4 gene. The following primers were used (trf4 5'-ATT GGC TGA AAC AGA GGT-3 '; trf4-5' CAA CCC TGC TAA GTC ATT AC-3') and the PCR conditions were 95 °C for five min, followed by 30 cycles of 94 °C for one min, 50 °C for one min, and 72 °C for 90 s; extension was performed at 72 °C for 10 min. The DNA fragment size that was produced had 324 bp[7,16].

The sensitivity of *Candida* spp. was evaluated by the well diffusion method on a Mueller-Hinton medium supplemented with 2% glucose and 0.05% methylene blue. In mediums containing *Candida* spp, wells were made and filled with 80 µg of AgNPs. Discs of amphotericin B 10 µg were used as control. The plates were incubated at 35 °C for 24h, and after this period fungal growth inhibition halos were measured (mm). Each test was conducted three times, according to the protocol of CLSI M44-A2[3,20].

Production of AgNPs using ribose as a reducing agent and SDS as a capping agent was simple and easy to perform. The entire process was completed in 30 min. The chemical reaction proposed can be represented by the following equation:

$$C_5H_{10}O_5 + 2Ag^+ \rightarrow C_5H_8O_5 + 2H^+ + 2Ag^0\,(AgNPs)^{(1)}$$

These AgNPs showed strong spectrophotometric absorbance, around 420 nm, as shown in Figure 1b. This is typical behavior of these structures. Use of SDS as the capping agent provided prolonged stability for up to four months when stored at room temperature and exposed to ambient light. Some sugars have reducing properties and are used in the production of nanoparticles. The process does not harm the environment because it does not produce toxic waste and requires no accelerator[13].

AgNPs produced in this study had a size of 12.5 ± 4.9 nm (mean ± SD), with a narrow particle size distribution, as shown in Figure 1c. This feature gives a high surface area, better for antimicrobial activity and good order. In previous studies using glucose as the reducing agent, the size of AgNPs was around 15 nm[10].

The AgNPs exhibited high antimicrobial activity, and this property can be very useful, especially against microorganisms resistant to conventional antimicrobials[17]. *C. albicans* and *C. tropicalis* showed high sensitivity to AgNPs (Fig 1d). The activity of 80 µg of AgNPs can be compared with the activity of amphotericin B, a powerful antifungal (Table 1). Studies highlight this same result with activity of AgNPs against *Candida* spp[9,14]. The statistical analysis of the results, carried out by Student's t-test, showed that *C. albicans* was more sensitive than *C. tropicalis* ($p = 0.02$).

In conclusion, AgNPs were easily prepared by green synthesis using ribose as a reducing agent and SDS as a stabilizer. Additionally, they showed high activity against *C. albicans* and *C. tropicalis*, a similar activity observed by the antifungal amphotericin B, and may represent an alternative for treating fungal infections.

RESUMO

Atividade antifúngica de nanopartículas de prata obtidas por síntese verde

Nanopartículas de Prata (AgNPs) são estruturas metálicas em escala nanométrica. AgNPs apresentam atividades antimicrobianas contra fungos e bactérias; no entanto, a síntese de AgNPs pode gerar resíduos tóxicos e devido a isso novas rotas utilizando compostos atóxicos têm sido buscadas. O objetivo desse estudo foi sintetizar AgNPs utilizando a ribose como agente redutor e dodecil sulfato de sódio (SDS) como estabilizante e avaliar a atividade antifúngica dessas partículas contra *C. albicans* e *C. tropicalis*. Foram sintetizadas nanopartículas estáveis com 12,5 ± 0,2 nm (média ± DP) que apresentaram elevada atividade contra *Candida* spp. e podem representar boa alternativa no tratamento de infecções fúngicas.

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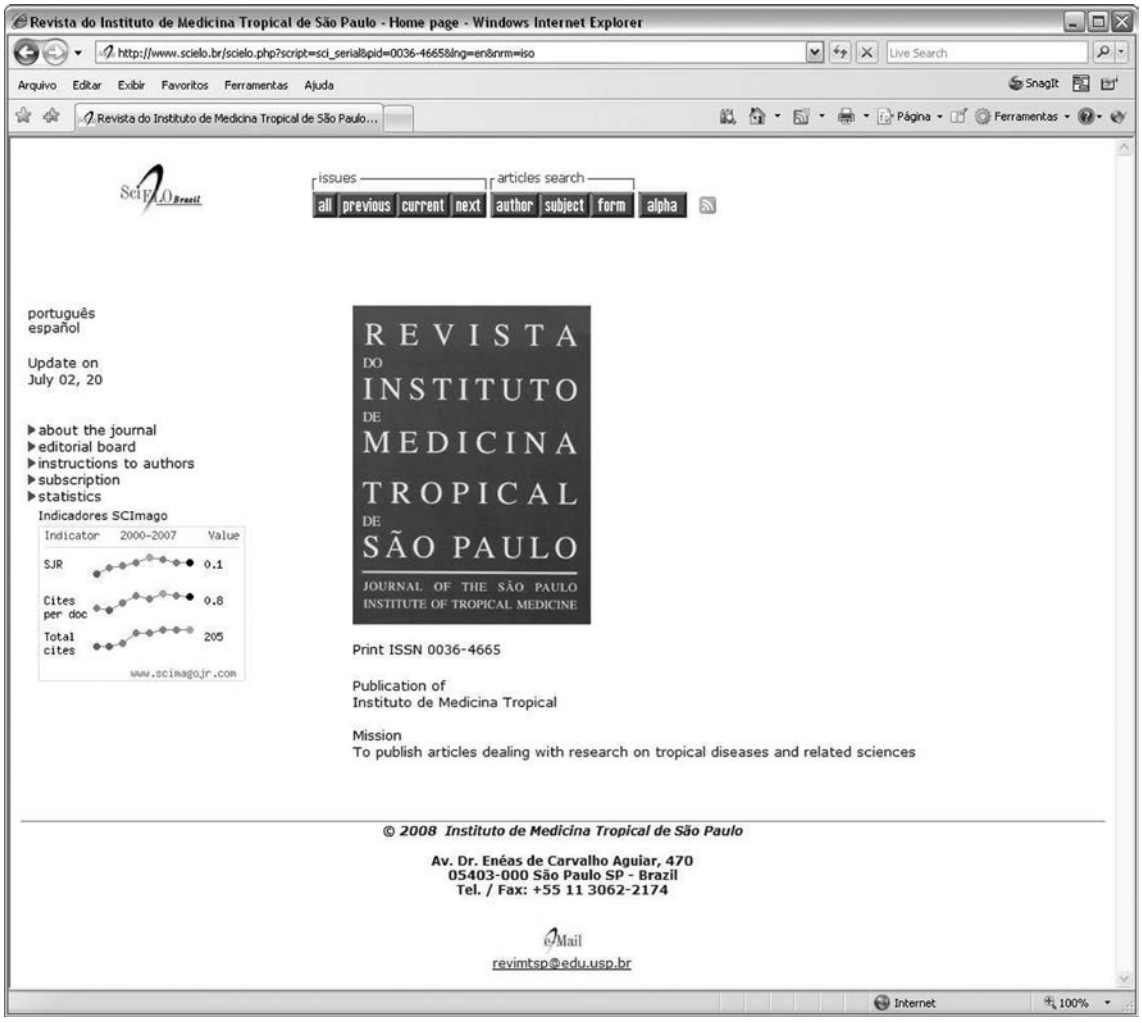

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BRIEF COMMUNICATION

EFFECT OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY ON VAGINAL *Candida* spp. ISOLATION IN HIV-INFECTED COMPARED TO HIV-UNINFECTED WOMEN

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SUMMARY

Vulvovaginal candidiasis (VVC) in HIV-infected women contributed to the impairment of their quality of life. The aim of this study was to evaluate the effect of highly active antiretroviral therapy (HAART) use on the vaginal *Candida* spp. isolation in HIV-infected compared to HIV-uninfected women. This cross-sectional study included 178 HIV-infected (HIV group) and 200 HIV-uninfected women (control) that were studied at the Specialized Assistance Service (SAE) for sexually transmitted diseases (STD)/ AIDS of the city of Maringá, Brazil, from April 1 to October 30, 2011. The yeasts were isolated and identified by phenotypic and molecular methods. The *in vitro* antifungal susceptibility to fluconazole, itraconazole, nystatin and amphotericin B was tested by the reference microdilution method. Higher frequencies of total vaginal *Candida* spp. isolation were found in the HIV-infected group than in the control group. However, both groups showed a similar frequency of colonization and VVC. Although *C. albicans* was the most frequent and sensitive to azolics and polyenes in both HIV-infected and uninfected women, the emerging resistance of *C. glabrata* to amphotericin B in the HIV-infected women was observed. Although higher frequency of vaginal *Candida* spp. isolation had been observed in the HIV-infected than in HIV-uninfected women, colonization and VVC showed similar frequency in both groups, indicating that HAART appears to protect against vaginal colonization and VVC.

KEYWORDS: HIV; Vulvovaginal candidiasis; *Candida* spp.; Antiretroviral therapy.

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a disease caused by the abnormal growth of yeast-like fungi in the mucosa of the female genital tract by members of the genus *Candida*[21]. These yeasts, in particular *C. albicans*, are well adapted to the human body, and are capable of colonizing it without producing signs of disease in conditions of physiological equilibrium[28]. However, under conditions that disrupt the delicate balance between the host and this commensal fungus, a parasitic relationship may occur, resulting in the development of infections termed candidiasis, including VVC[2]. For development of VVC, predisposing factors related to the host are very important, mainly being immunosuppressive diseases, such as HIV infection[13,15].

Because it strikes millions of women annually, causing great discomfort, interfering with sexual and affective relations and impairing work performance, VVC has been considered an important worldwide public-health problem[5,24]. In HIV-infected women, the impact of VVC on top of other medical complications that stem from the viral infection and its treatment certainly contributes to the impairment of their quality of life. It must still be considered that out of HIV-infected individuals worldwide (around 40 million), nearly half are women[16], raising concerns about VVC. In Brazil, HIV infection occurs in 0.5% of the population, with a trend toward expansion of the epidemic among women, from a ratio of 18.5 men:1 woman in the 1980s to 1.5:1 in 2004[2].

Use of the highly active antiretroviral therapy (HAART) has extended the life span of HIV-infected persons. However, some studies have reported that even for users of this continuous therapy opportunistic infections remain a serious problem[3,18]. Nevertheless, to the authors' knowledge, few studies have related vaginal *Candida* colonization and VVC in HIV-infected women to HAART use in different populations, have enrolled relatively few HIV-infected women[15], only treated specific clinical conditions as *Candida* colonization[14], lacked appropriate matched controls[9,15], or have been restricted to specialized subpopulations such as women with symptoms of vulvovaginitis[23]. There are some available

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studies that were performed before the broad use of HAART or in populations that do not regularly use this therapy[1,7,9,23,27].

The aim of this study was to evaluate the effect of HAART use on the vaginal *Candida* spp. isolation in two clinical conditions: colonization and VVC, in HIV-infected compared to HIV-uninfected women. In addition, the antifungal susceptibility of *Candida* species to the most commonly used antifungal drugs was evaluated.

MATERIAL AND METHODS

This was a cross-sectional study that included a group of 178 HIV-infected and 200 HIV-uninfected women, who were studied at the Specialized Assistance Service (SAE) for sexually transmitted diseases (STD)/AIDS in the city of Maringá, Brazil, from April 1 to October 30, 2011. Inclusion criteria were women having diagnoses of HIV/AIDS confirmed by two different methods (HIV group), or confirmed diagnosis as HIV-uninfected (control group). Exclusion criteria were: hysterectomized women, pregnant or postpartum women, women of less than 18 years of age, women with no history of sexual activity, women that had some degree of difficulty in understanding the study, women suffering vaginal bleeding, or women that had undergone sexual intercourse/vaginal douching within the 48 hours preceding collection of the vaginal sample.

The women signed the consent form to participate in the study, and completed a standardized questionnaire with information regarding symptoms of VVC. The HIV group also responded to a questionnaire regarding socio-demographic characteristics, obstetrical and gynecological history, and sexual behavior. Data regarding HIV infection, including the period of infection (years), $CD4^+$ T lymphocyte count, HIV viral load values, and HAART use were obtained from the medical records of each woman. A single health professional was responsible for contacting the women, administering the questionnaire, and collecting the vaginal sample. This research was approved by the Committee for Ethics in Research Involving Humans at the State University of Maringá, Paraná, Brazil (reports No. 185/2007 and No. 085/2011).

Vaginal samples were collected with sterile swabs and a disposable vaginal speculum, inoculated in sterile saline, and immediately seeded onto plates containing Sabouraud dextrose agar (SDA) (Difco, USA), with the addition of 100 mg/mL chloramphenicol, and incubated at 25 °C for up to five days. A pool of the colonies grown on each plate was subcultured on CHROMágar *Candida*® (Probac, France) to assure the purity of the isolates and to identify mixed cultures. Beginning with the pure culture, the yeasts were identified by classical phenotypic methods[11]. Additionally, the identification of yeasts was confirmed using matrix-assisted laser-desorption/ionization time-of-flight mass spectroscopy assay (MALDI TOF-MS). For MALDI TOF-MS identification, yeasts were prepared[26] and the measurements were performed[19] with Microflex LT mass spectrometer (Bruker Daltonics, Germany) using FlexControl software (version 3.0, Bruker Daltonics, Germany). The yeasts were stored in Sabouraud dextrose broth (SDB) (Difco, USA) with 10% glycerol at -20 °C.

Women were evaluated for the presence of clinical signs and symptoms of VVC by SAE doctors. *Candida* vaginal colonization was defined as culture positive for yeasts from women without signs and symptoms of VVC. Women with a positive culture were considered to have VVC if they reported at least two symptoms of this pathology (discharge, burning, vaginal itching, dysuria or dyspareunia), and signs of VVC reported by doctors[12].

The *in vitro* antifungal activity assay was performed for fluconazole (FLU, Pfizer Inc., NY, USA), itraconazole (ITRA, Janssen Pharmaceutical, NJ, USA), nystatin (NYST, Sigma Pharma, MO, USA) and Amphotericin B (AMB, Squibb Pharmaceutical, NJ, USA). All yeasts isolated were tested by means of the Clinical Laboratory Standards Institute reference broth microdilution method for fluconazol and itraconazol, with modifications for other drugs[4,5]. The minimum inhibitory concentration (MIC) for azoles was defined as the first well with a significant growth reduction (approximately 50%) compared to that of the positive control. In the case of NYST and AMB, the MIC was defined as the lowest concentration capable of inhibiting 90% of the growth[17]. The endpoints for antifungal agents: isolates with MIC between 16 and 32 µg/mL for FLU, 0.25 to 0.5 µg/mL for ITRA, and 8 to 32 µg/mL for NYST were considered as dose-dependent susceptibility (DDS). Isolates with an MIC ≤ 8 µg/mL for FLU, ≤ 0.125 µg/mL for ITRA, ≤ 4 µg/mL for NYST, and ≤ 1 µg/mL for AMB were susceptible (S). Those with an MIC ≥ 64 µg/mL for FLU, ≥ 1 µg/mL for ITRA, ≥ 64 µg/mL for NYST and ≥ 2 µg/mL for AMB were resistant (R).

The statistical analysis was performed using the STATA for Statistics and Data Analysis 9.1 software. All variables were expressed as absolute and relative frequencies. The frequencies of *Candida* spp. isolation from the vaginal mucosa, and also colonization and VVC, were calculated by the crude odds ratio (OR) with a 95% confidence interval (CI), and were evaluated between groups by the Chi-square test (χ^2) with Yates correction. A value of $p < 0.05$ was considered significant.

RESULTS

Figure 1 is an overview of the study and results.

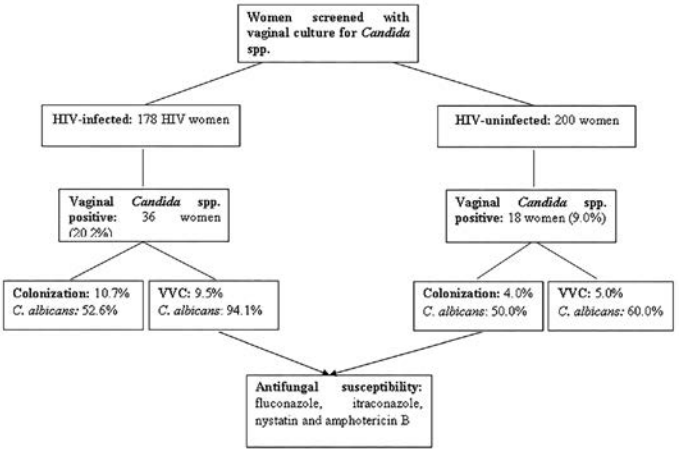

Table 1 describes the socio-demographic and clinical characteristics of the two groups. The median age of the HIV group was 41.24 ± 10.31 years, and for the control group it was 42.22 ± 14.14 years ($p > 0.05$). Considering all women studied, *Candida* spp. isolation from the vaginal

Table 1

Relationship between total *Candida* vaginal isolation, colonization and VCC, as well as the socio-demographic characteristics, obstetrical and gynecological history, sexual behavior and data regarding HIV of 178 HIV-infected women

Characteristics of *Candida* spp. in HIV-infected women	HIV-infected *n = 178*		Positive *Candida* isolation *n = 36*		*p*-value	Colonization *n = 19*		*p*-value	VVC *n = 17*		*p*-value
	n	(%)	*n*	(%)		*n*	(%)		*n*	(%)	
Age (years)											
15 to 30	28	15.7	10	27.8	0.4206	4	21.1	0.7867	6	35.3	0.2861
31 to 40	60	33.7	17	47.2	0.0286*	7	36.8	0.2285	10	58.8	0.0131*
≥ 41	90	50.6	9	25.0	0.1400	8	42.1	0.646	1	5.9	0.2103
Marital status											
married/cohabiting	93	52.2	19	52.8	0.9494	7	36.8	0.4337	12	70.6	0.2264
unmarried/non-cohabiting	85	47.8	17	47.2	0.9521	12	63.2	0.3204	5	29.4	0.4202
Education											
< 8 years	85	47.8	12	33.3	0.3414	5	26.3	0.3515	7	41.2	0.7375
≥ 8 years	93	52.2	24	66.7	0.1994	14	73.7	0.8782	10	58.8	0.6832
Family income											
< $ 240/month	25	14.0	4	11.1	0.8763	2	10.5	0.8911	2	11.8	0.9316
≥ $ 240/month	153	86.0	32	88.9	0.6519	17	89.5	0.6906	15	88.2	0.8139
Skin color											
white	109	61.2	18	50.0	0.3803	11	57.9	0.8311	7	41.2	0.3025
brown	50	28.1	12	33.3	0.7177	6	31.6	0.8582	6	35.3	0.7105
black	15	8.4	6	16.7	0.5635	2	10.5	0.9222	4	23.5	0.3957
yellow	4	2.2	0	0.0	-	0	0.0	-	0	0	-
Employ out side the home											
yes	98	55.1	23	63.9	0.4397	13	68.4	0.3652	10	58.9	0.8136
No	80	44.9	13	36.1	0.5500	6	31.6	0.5254	7	41.1	0.8467
Age of first sexual intercourse											
< 18	112	62.9	24	66.7	0.7329	15	78.9	0.2254	9	53.0	0.5527
≥ 18	66	37.1	12	33.3	0.8071	4	21.1	0.5201	8	47.0	0.584
Number of lifetime sexual partners											
< 10	129	72.5	25	69.4	0.7923	14	73.7	0.953	11	64.8	0.6127
≥ 10	49	27.5	11	30.6	0.8635	5	26.3	0.9545	6	35.2	0.7146
Number of deliveries											
< 3	87	48.9	17	47.2	0.8922	8	42.1	0.7135	9	53.0	0.8198
≥ 3	91	51.1	19	52.8	-	11	57.9	0.6708	8	47.0	0.8287
Period of HIV infection (years)											
< 10	131	73.6	25	69.4	0.6346	15	78.9	0.6576	10	58.9	0.3021
≥ 10	47	26.4	11	30.6	0.7579	4	21.1	0.8175	7	41.1	0.4101
HAART use											
yes	141	79.2	22	61.1	0.0670	13	68.4	0.3676	9	53.0	0.0724
no	37	20.8	14	38.9	0.1990	6	31.6	0.5588	8	47.0	0.1340
Current CD4+ T lymphocyte count											
< 200 cells/mm^3	15	8.4	6	16.7	0.5635	2	10.5	0.9222	4	23.5	0.3957
200 and 350 cells/mm^3	32	18.0	8	22.2	0.7873	5	26.3	0.6634	3	17.6	0.9863
> 350 cells/mm^3	131	73.6	22	61.1	0.2136	12	63.2	0.4401	10	58.9	0.3021
Current viral load											
< minimum limit copies/ml	104	58.4	11	30.6	0.0848	6	31.6	0.2001	5	29.4	0.2039
Minimum limit - 100,000 copies/mL	69	38.8	22	61.1	0.0726	11	57.9	0.2360	11	64.7	0.1134
> 100,000 copies/mL	5	2.8	3	8.3	0.7502	2	10.5	0.6904	1	5.9	0.8637

VVC, vulvovaginal candidiasis; *$p < 0.05$ was considered significant.

mucosa and VVC was significantly associated with the 31 to 40 years of age group ($p = 0.0286$ and $p = 0.0131$, respectively). For the groups studied, only the control group showed association with age, since vaginal yeast colonization was significantly associated with the 31 to 40 years of age group ($p = 0.0185$). Data regarding HIV infection, including the period of HIV infection, $CD4^+$ T lymphocyte count, HIV viral load values and correct use of HAART were not significantly associated with *Candida* spp. total isolation, colonization or VVC.

The HIV infected group showed a higher frequency of colonization than VVC (52.8% and 47.2%, respectively, $p = 0.05$), and the control group showed more frequent VVC than colonization (55.5% and 44.4%, respectively; $p = 0.01$). However, comparing the two groups, *Candida* spp. total vaginal isolation was more frequent in the HIV group (n = 36/178, 20.2%) than in the control group (18/200, 9.0%; $p = 0.003$). For clinical conditions, the HIV group showed a similar frequency of vaginal colonization (19/178, 10.7%) (OR = 2.9; 95% CI 0.91-9.6; $p = 0.1072$) and VVC (17/178, 9.5%) (OR = 1.879; 95% CI 0.8657-1.994; $p = 0.4057$) to the control group (n = 8/200, 4.0%; n = 10/200, 5.0%, respectively) (Table 2).

With respect to yeast species in the present study, *C. albicans* was the most frequently isolated in both the HIV (n = 26, 72.2%; $p = 0.02$) and control groups (n = 10, 55.6%; $p = 0.05$). With respect to yeast species in different clinical conditions, the HIV group showed more *C. albicans* than the control in VVC ($p = 0.007$; OR 3.1; 95% CI 1.24-9.05) and in colonization ($p = 0.005$; OR 2.9; 95% CI 0.91-10.87). In the HIV group, the following non-*albicans* species were identified: *C. glabrata* (n = 7), *C. parapsilosis* (n = 2) and *C. rugosa* (n = 1). In the control group, the following were identified: *C. glabrata* (n = 5), *C. parapsilosis* (n = 2) and *C. tropicalis* (n = 1). Thus, in both groups, *C. glabrata* was the second most common yeast isolated (19.4% and 27.8%, for the HIV and control groups, respectively) (Table 2).

Table 3 shows the interpretation of the antifungal activity results for drugs as well as for susceptibility, dose-dependent susceptibility or resistance. In general, the *C. albicans* isolates showed no resistance to the antifungal agents tested for both the HIV and the control group. The results for amphotericin B were 100% sensitivity. For nystatin, the results showed elevated rates of vaginal isolates of *C. albicans* and non-*albicans* species with dose-dependent susceptibility. Of the non-*albicans* species identified in the HIV group, only *C. glabrata* showed resistance, one (4.8%) to FLU, two (9.5%) to ITRA, and another two (9.5%) to AMB. In the control group, two non-*albicans* yeasts (23.3%) showed resistance to FLU and one (6.7%) to ITRA; the two resistant species found in this group were *C. glabrata* and *C. tropicalis.*

DISCUSSION

In the present study, *Candida* spp. total vaginal isolation was significantly more frequent in the HIV group than in the control group. However, a similar frequency of colonization and VVC in the HIV and

Table 2
Frequencies of *Candida* spp. vaginal total isolation, colonization and vulvovaginal candidiasis (VVC) in HIV-infected and uninfected women in southern Brazil

Candida species	HIV-infected group n = 178						HIV-uninfected group n = 200					
	Total isolation		Colonization		VVC		Total isolation		Colonization		VVC	
	n	%	n	%	n	%	n	%	n	%	n	%
C. albicans	26	72.2	10	52.6	16	94.1	10	55.6	4	50.0	6	60.0
Non-*albicans* species	10	27.8	9	47.4	1	5.9	8	44.4	4	50.0	4	40.0
Total	36	100.0	19	52.7	17	47.3	18	100.0	8	44.4	10	55.5

Table 3
Interpretation of the results for minimal inhibitory concentration (MIC) for antifungal drugs in vaginal yeasts from HIV-infected (n = 36, *C. albicans* = 26) and HIV-uninfected groups (n = 18, *C. albicans* = 10)

Antifungals	HIV-infected group												HIV-uninfected group											
	C. albicans						non-*albicans* species						*C. albicans*						non-*albicans* species					
	S		DDS		R		S		DDS		R		S		DDS		R		S		DDS		R	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
FLU	24	92.3	2	7.7	-	-	4	71.4	5	23.8	1	4.8	10	100.0	-	-	-	-	1	33.3	5	43.4	2	23.3
ITRA	25	96.1	1	3.9	-	-	8	90.5	-	-	2	9.5	9	90.0	1	10.0	-	-	-	-	7	93.3	1	6.7
NYST	25	96.1	1	3.9	-	-	8	66.7	2	33.3	-	-	3	30.0	7	70.0	-	-	6	76.7	2	23.3	-	-
AMB	26	100.0	-	-	-	-	8	90.5	-	-	2	9.5	10	100.0	-	-	-	-	8	100.0	-	-	-	-

FLU = fluconazole; ITRA = itraconazole; NYST = nistatyn and AMB = amphotericn B; S (susceptible): isolates with MICs ≤ 8 µg/mL for FLU; ≤ 0.125 µg/mL for ITRA; ≤ 4 µg/mL for NYST; ≤ 1 µg/mL for AMB. DDS (dose-dependent susceptibility): isolates with MIC between 16 and 32 µg/mL for FLU; ≤ 0.25 to 0.5 µg/mL for ITRA; ≤ 8 to 32 µg/mL for NYST. R (resistant): Isolates with MIC ≥ 64 µg/mL for FLU; ≥ 1 µg/mL for ITRA; ≥ 64 µg/mL for NYST; ≥ 2 µg/mL for AMB.

control groups was shown, which is consistent with the results of the most HIV-infected women enrolled in this study that showed excellent control of the infection. Further, the data regarding HIV infection were not associated with *Candida* spp. isolation from the vaginal mucosa, colonization or VVC.

The results found in this study differ from those described in other populations that also had good control of HIV infection. In these studies, the frequency of VVC in the HIV group was similar to the control group, but the frequency of colonization was higher[7,15]. Also, for vaginal *Candida* colonization, the results differ from those in which the risk of colonization in HIV-infected women with CD4+ T-cell counts below 100-200 cells/mm^3 was three or four times higher, compared to immunocompetent HIV-infected or HIV-uninfected women[1,7].

The results are encouraging, since HAART seems to protect against vaginal colonization and VVC, and some investigators have reported a relationship between vaginal *Candida* colonization and VVC with heterosexual transmission of HIV. Recent research has shown that women with vaginal yeasts were more likely to acquire HIV, and the condition may contribute more to the HIV epidemic than previously thought[27]. It was shown that treatment of VVC in HIV-infected women can reduce genital shedding of HIV RNA and DNA by 3.2 and 3 times, respectively[17].

For the HIV and control groups, *C. glabrata* was the second most common yeast isolated, in agreement with other studies[1,15]. Older studies described higher rates of vaginal colonization with non-*albicans* species in HIV-infected than in uninfected women[7,17]. With respect to yeast species in different clinical conditions, the HIV group showed more *C. albicans* than the control group in VVC and in colonization, similar to other studies[1]. In some studies, *C. albicans* was related to symptoms in HIV-infected women[22], but in others no relationship between symptoms and the yeast species isolated[15] was found.

In relation to antifungal susceptibility, *C. albicans* showed no resistance to the antifungal agents tested for both the HIV and the control group and 100% of sensibility for amphotericin B. These results reinforce previous findings showing that amphotericin B is an excellent and highly efficacious therapeutic option for vaginal *C. albicans* including in HIV-infected women[8]. For nystatin, the results of this study are in accordance with others that have shown elevated rates of vaginal isolates with dose-dependent susceptibility, and also some resistance[5]. Nystatin has been used for several decades as one of the principal treatments for vaginal *Candida* spp. in Brazil[23]. This history may partly explain the elevated dose-dependent susceptibility rate observed in both groups studied. Large-scale use of fluconazole began more recently, which may also partly explain the better therapeutic activity of this drug against *C. albicans* found in the present study.

Non-*albicans* species showed resistance to fluconazole and itraconazole. In general, these results are not surprising since the management of women with non-*albicans* species, mainly *C. glabrata,* is difficult because of the lower sensitivity of non-*albicans* species to both azoles[6,25] and polyenes[10]. To the authors' knowledge, this is one of the first studies to show amphotericin B resistance in *C. glabrata* isolated from HIV-infected women. The importance of the results in relation to antifungal susceptibility is confirmed by the report which found no trials that addressed treatment of VVC in HIV-infected women[20]. However, there is a need to evaluate drugs and drug regimens for VVC treatment and prophylaxis in HIV-infected women.

In conclusion, this study found higher frequencies of total vaginal *Candida* spp. isolation in the HIV-infected women with prolonged HAART use, in relation to HIV-uninfected. However, a similar frequency of colonization and VVC in the HIV and control groups was shown. Thus, the results are encouraging, since HAART seems to protect against vaginal colonization and VVC. Although *C. albicans* was the most frequent and sensitive to azolics and polyenes in both HIV-infected and uninfected women, the emerging resistance of *C. glabrata* to amphotericin B in the HIV-infected women studied was observed. If this proves to be correct, implementing routine culture identifications of vaginal *Candida* spp. in HIV-infected women could help in guiding treatment, assisting in care, and improving the quality of life of these patients. Once the resistance of *C. glabrata* to amphotericin B is detected, and this yeast is intrinsically resistant to azoles, it is important to have knowledge of their involvement in VVC of HIV-positive women.

RESUMO

Efeito da terapia anti-retroviral altamente ativa no isolamento vaginal de *Candida* spp. em mulheres infectadas por HIV comparado às não infectadas

Candidíase vulvovaginal (CVV) em mulheres infectadas pelo HIV contribuiu substancialmente para a diminuição da sua qualidade de vida. O objetivo deste estudo foi avaliar o efeito do uso de terapia anti-retroviral altamente ativa (HAART) no isolamento de *Candida* spp. vaginais em mulheres HIV positivas comparado às não infectadas por HIV. Este estudo transversal incluiu 178 mulheres infectadas pelo HIV (grupo HIV) e 200 mulheres não infectadas (grupo controle) acompanhadas no Serviço de Assistência Especializada (SAE) para as doenças sexualmente transmissíveis (DST)/AIDS da cidade de Maringá/Brasil, de 1 abril a 30 de outubro de 2011. As leveduras foram isoladas e identificadas por métodos fenotípicos e moleculares. A susceptibilidade *in vitro* aos antifúngicos fluconazol, itraconazol, nistatina e anfotericina B foi avaliada pelo método de referência de microdiluição. Nós encontramos maior frequência de isolamento vaginal total de *Candida* spp. no grupo HIV do que no grupo controle. Entretanto, foi observada frequência similar de colonização e CVV entre os dois grupos. Apesar de *C. albicans* ser a mais frequente e sensível a azólicos e polienos em mulheres infectadas pelo HIV e não infectadas, foi detectada emergente resistência de *C. glabrata* a AMB nas mulheres infectadas pelo HIV. Embora tenha sido observada maior frequência de isolamento vaginal de *Candida* spp. nas mulheres infectadas pelo HIV do que nas não infectadas, colonização e CVV apresentaram frequência similar em ambos os grupos, o que indica que HAART parece proteger contra colonização vaginal e CVV.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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BRIEF COMMUNICATION

IDENTIFICATION OF *Leishmania infantum* IN PUERTO IGUAZÚ, MISIONES, ARGENTINA

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SUMMARY

The emergence of zoonotic visceral leishmaniasis (ZVL) in Latin America is a growing public health problem. The urbanization of ZVL has been observed in different countries around the world, and there are a growing number of reports drawing attention to the emergence of this infection in new locations, as well as its increase in previously established areas of endemicity. In the city of Posadas, Misiones province, Northeastern Argentina, the transmission of ZVL associated with canines and *Lutzomyia longipalpis* was first reported in 2006. In the city of Puerto Iguazú, also in Misiones province, the first human case of ZVL was reported in February 2014. From 209 surveyed dogs, 15 (7.17%) were identified as positive by serological and/or parasitological methods. Amplification was observed in 14 samples and in all cases the species implicated was *Leishmania infantum*. To the authors' knowledge, this is the first molecular characterization of *L. infantum* from dogs in this area.

KEYWORDS: *Leishmania infantum;* Molecular characterization; New focus; Puerto Iguazú, Argentina.

Visceral leishmaniasis (VL) is one of the most important parasitic diseases in the world. The domestic dog (*Canis familiaris*) plays a fundamental role as a reservoir of zoonotic visceral leishmaniasis (ZVL), favoring the urban cycle of the disease in the presence of the phlebotomine vector. In Latin America, canine leishmaniasis is widespread and it is among the most important canine zoonotic vector-borne diseases. The estimated number of infected dogs is in the millions and a high prevalence of canine leishmaniasis (CanL) is associated with the transmission of infection to humans[4].

In 2006, the first autochthonous human case of VL in Argentina was reported in Posadas, Misiones province (Northeastern Argentina), and was associated with canines and *Lutzomyia longipalpis*[9]. The presence of *Leishmania infantum* was further described in *Lu. longipalpis* sandflies and dogs using molecular methods[1,5]. To date, 104 human cases of visceral leishmaniasis have been reported in Misiones province (0 in Puerto Iguazú). The department of Puerto Iguazú (northwest of Misiones) has been considered an endemic area for *Leishmania braziliensis,* and until February 2014 was considered free of *L. infantum*.

The city of Puerto Iguazú is located in the northwest region of Misiones province, Northeastern Argentina, 300 km from Posadas. The city is bordered by Brazil (Iguazú falls), to the north, and by Paraguay, to the east (Ciudad del Este). The objective of this study was to determine if *L. infantum* is the etiologic agent of canine visceral leishmaniasis in domestic dogs from the city of Puerto Iguazú. To date, CanL by *L. infantum* has not been reported in this area.

In May 2013, a parasitological and serological pilot survey was carried out on 209 domestic dogs randomly sampled from a total of 21,466 properties included in the City Land Registry. The research protocol was reviewed and approved by the bioethics committee of the Ministry of Health in Misiones, Argentina (Comité de Bioética, División de Zoonosis de la Subsecretaría de Atención Primaria y Salud Ambiental Salud del Ministerio de Salud de Misiones; Resolución Ministerial Nº: 2640/2008). Written informed consent was obtained from each dog owner before clinical examination, whole blood (1 mL) and popliteal node (with ethanol 70%) extraction was performed.

The detection of anti-*Leishmania* antibodies was carried out by two serological tests: i) Kalazar Detect® immunochromatographic test (ICT) was performed according to the manufacturer's instructions (InBios International, Seattle, WA, USA). Briefly, 20 µL of plasma plus three drops of chase buffer were placed on the pad of the dipstick, and; ii) *In house* immunofluorescent antibody test (IFAT) was performed following a standard method using 10 µL of 2 x 10^7 *L. infantum* promastigotes/mL in 1x PBS per well as antigen (reference strain MHOM/FR/78/LEM-75) and serial two-fold dilutions of plasma for the analysis[3]. The

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IFAT threshold title for positivity was 1/160. From 209 surveyed dogs, 13 (6.22%) were seropositive by IFI (13/209) and/or by ICT (12/209).

Lymph node samples were washed with 500 µL of PBS and DNA was extracted by conventional phenol-chloroform extraction and ethanol precipitation[7], and further eluted in 100 µL sterile distilled water. Purified DNA was stored at -80 ºC until further use. Parasite DNA detection was done by means of PCR targeting the *Leishmania* intergenic transcript spacer (ITS-1)[10]. Amplicons were visualized in seven out of 209 dogs. A total of 15 (7.17%) dogs were positive by serological and/or parasitological methods.

Later, those 15 positive dogs were subjected to a nested-PCR with outer pair previously used[7] and inner pair SAC (5´-CATTTTCCGATGAT TACACC- 3´) and VAN2 (5´-GCGACACGTTATGTG AGCCG-3´) to amplify an internal region (280 to 330 bp) of the fragment as described by CRUZ *et al.* in 2013[6]. Direct sequencing of the PCR products was performed with forward and reverse primers; using the Big-Dye Terminator Cycle Sequencing Ready Reaction Kit V3.1 and the automated sequencer "3730 DNA analyzer" (Applied Biosystems, Foster City, CA). Sequences obtained were analyzed and edited using BioEdit v7.2.5.©1999-2013 software (Tom Hall, Ibis Biosciences, Carlsbad, CA). Amplification was observed in 14 of the 15 samples studied and in all cases the species implicated *was L. infantum*.

In February 2014, the first fatal case of VL in humans was reported in the city of Puerto Iguazú[8]. Although the infecting species in this patient is not known, 29 human-VL patients at species level have been identified in the following cities; Posadas, Oberá, San Ignacio, Candelaria and Apóstoles (Misiones Province), as well as in 52 dogs sampled in 2006[5] and 53 dogs in 2010, all from Posadas. *L. infantum* was the only *Leishmania* species identified infecting humans and dogs (unpublished data).

In conclusion, the changes in the urbanization of the vector, the existence of susceptible reservoir hosts (dogs), vectors (*Lu. Longipalpis*) and the proximity of the disease to Paraguay, Brazil[2] and other cities of Misiones, may have favored the establishment of ZVL by *L. infantum* in Puerto Iguazú. It is important to highlight the strategic position of the city of Puerto Iguazú as a contributing factor to the spread of the disease, because of its geographical location (a location at which the Argentine, Paraguayan and Brazilian borders meet) and the influx of tourists it receives throughout the year due to the nearby Iguazú National Park (Iguazú falls). CanL control policies should be established in order to prevent further human and canine cases.

RESUMEN

Identificación de *Leishmania infantum* en Puerto Iguazú, Misiones, Argentina

La emergencia de leishmaniosis visceral zoonótica (LVZ) en América Latina es problema de salud pública en aumento. La urbanización de la LVZ es un fenómeno observado en diferentes países alrededor del mundo y hay un número creciente tanto de denuncias respecto a la aparición de esta infección en nuevas ubicaciones, como su aumento en zonas endémicas previamente establecidas. En la ciudad de Posadas, provincia de Misiones, nordeste de Argentina, la transmisión de LVZ asociada a canes y *Lutzomyia longipalpis* fue descrita por primera vez en 2006. En la ciudad de Puerto Iguazú, provincia de Misiones, el primer caso humano de LVZ tuvo lugar en febrero de 2014. De 209 perros muestreados, 15 (7.17%) resultaron positivos mediante métodos serológicos y/o parasitológicos. Se observó amplificación en 14 muestras y en todos los casos la especie implicada fue *Leishmania infantum*. Según nuestro conocimiento, esta es la primera caracterización molecular de *L. infantum* en perros procedentes de este área.

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BRIEF COMMUNICATION

DETECTION OF *Leptospira* spp. AND *Brucella abortus* ANTIBODIES IN FREE-LIVING JAGUARS (*Panthera onca*) IN TWO PROTECTED AREAS OF NORTHERN PANTANAL, BRAZIL

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SUMMARY

This study aimed to assess the exposure of free-living jaguars (*Panthera onca*) to *Leptospira* spp. and *Brucella abortus* in two conservation units in the Pantanal of Mato Grosso, Brazil. The presence of antibodies in blood samples of eleven jaguars was investigated using autochthonous antigens isolated in Brazil added to reference antigen collection applied to diagnosis of leptospirosis by Microscopic Agglutination Test (MAT). The Rose Bengal test was applied for *B. abortus* antibodies. Two (18.2%) jaguars were seroreactive for the *Leptospira* spp. antigen and the serovar considered as most infective in both animals was a Brazilian isolate of serovar Canicola (L01). All jaguars were seronegative for *B. abortus*. These data indicate that the inclusion of autochthonous antigens in serological studies can significantly increase the number of reactive animals, as well as modify the epidemiological profile of *Leptospira* spp. infection.

KEYWORDS: Zoonotic diseases; *Leptospira* spp.; *B. abortus*; Serology; *Panthera onca.*

INTRODUCTION

The jaguar (*Panthera onca*) is the largest felid in the Americas, where retaliation from ranchers due to livestock predation, illegal hunting tourism activity, and habitat loss associated with agricultural expansion are threats to the species in the Pantanal biome, an important area for jaguar conservation in the long-term[4].

Leptospirosis is a zoonosis of worldwide distribution and global importance[1]. The incidence of human infection is higher in the tropics where conditions for its transmission are favorable, but the disease occurs in both industrialized and developing countries[2]. The genus *Leptospira* is divided into 20 species based on DNA hybridization studies[2], and these 20 species are classified into more than 280 serovars, according to their antigenic relatedness[5], which affect various vertebrate hosts and remain in the environment by a dynamic process through a variety of domestic and wild animals. Leptospires are shed in the urine of carrier animals and the transmission is strongly affected by environmental conditions[2]. In Brazil, serological surveys have shown exposure to *Leptospira* spp. in various captivity and free-living wild species, of which the serovars Castellonis, Hardjo[10], and Copenhageni[15] were the most likely to cause infection in captive jaguars. Pomona was the most prevalent serovar found in free-living sampled jaguars[7,21]. In addition to the death of a female puma in Rio de Janeiro's zoo which showed clinical signs of leptospirosis and titers ≥ 400 to serovar Pomona by MAT[15], the high titers found in free-living neotropical felids in the same biome studied[12] suggest that *Lepstospira* spp. exposure may affect the conservation of wild felids. Due to the fact that transmission occurs mainly in wet environments and that wild animals are relevant in leptospirosis epidemiology, studies are necessary in the Pantanal region to clarify the potential impact of *Leptospira* spp. exposure on wild populations.

The occurrence of brucellosis in humans is highly dependent on the occurrence of the disease in animals' reservoirs, including wildlife[8]. The main clinical signs of *Brucella abortus* in wild mammals are abortion, orchitis, epididymitis and infertility[24]. In Brazil, antibodies against *B. abortus* have been detected in free-living and captive white-lipped and collared peccaries[11,13,18,21], in free-living and captive maned wolves (*Chrysocyon brachyurus*)[16], in a free-living jaguar in the Atlantic Forest[21] and in another in the Emas National Park[12]. The present study aimed to detect antibodies to *Leptospira* spp. and *B. abortus* in jaguars from two conservation units in the Pantanal region, Brazil.

MATERIAL AND METHODS

The studied areas comprised two federal conservation units (Taiamã

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Ecological Station - 16° 50' 34.31" S, 57° 35' 03.70" W, and Pantanal Matogrossense National Park - 17° 50'47. 33" S, 57° 24'12.67" W) in the Pantanal of Mato Grosso, considered one of the largest floodplains in the world. Annual rainfall ranges from 1,200 to 1,300 mm across the region, defining dry and rainy seasons with seasonal fluctuations in water level, which have great influence on ecological processes[17]. The average annual temperature is 25 °C[3].

Eleven jaguars were captured between July 2010 and November 2012, under license granted by the Authorization System and Biodiversity Information - SISBIO, numbers: 30896-1 and 18699-1, immobilized with a combination of tiletamine and zolazepam (Zoletil 100®, Virbac SA, Carros-Cedex, France) and fitted with radio-collars. After clinical examination and collection of biological samples, all animals were released at the same site at which they were captured. Sera blood samples were frozen and stored at -20 °C until testing and analysis at the Biological Samples' Bank of National Research Center for the Conservation of Carnivorous Mammals of Brazil (CENAP/ ICMBio).

Serum samples were examined for different leptospiral antibodies by Microscopic Agglutination Test (MAT)[6] with the cut off 1:100 dilution against the following pathogenic serovars: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whiticombi, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Mini, Javanica, Panama, Pomona, Pyrogenes, Hardjo, Wolffi, Shermani, Tarassovi, Andamana, Patoc and Sentot, which were cultivated in modified EMJH medium. In addition to the reference strains, eleven Brazilian isolates of *Leptospira* spp. were used in this study: Brasiliense serovar isolated from *Didelphus marsupialis* (Strain 4B), Pomona serovar isolated from domestic *Sus scrofa* (Strain M7/87), Guaricura serovar isolated from *Bubalus bubalis* (Strain M4/98), Copenhageni serovar isolated from *Rattus norvegicus* (Strain M9/99), Canicola serovar isolated from *Canis familiars* (Strain L01), Canicola serovar isolated from domestic *Sus scrofa* (Strain L04), Canicola serovar isolated from *Bos Taurus* (Strain L014), Bananal serovar isolated from *Hydrochaeris hidrochaeris* (Strain 2A CAP), Bananal serovar isolated from *Hydrochaeris hidrochaeris* (Strain 21A CAP), Pomona serovar isolated from domestic *Sus Scrofa* (Strain Gr6) and M110/06 isolated from *Cerdocyon thous* (probably a new species). The positive sera were titrated by testing serial twofold serum dilutions and the reciprocal of the highest serum dilution that showed 50% agglutinated leptospira was defined as the serum titer[20].

For brucellosis, serum samples were examined by the Rose Bengal test (RBT) for screening and 2-mercaptoethanol test (2-ME) as a confirmatory test[14]. The antigen used was an inactive suspension of *B. abortus* 1119-3 produced by the Institute of Technology of Paraná, Brazil.

RESULTS

All animals were considered adults based on tooth wear and color, ranging from four to 10 years old. Only two (18.2%) jaguars tested were seroreactive for *Leptospira* spp. antigen by MAT, one from each conservation unit of this study. The serovar considered as most infective in both animals was a Brazilian isolated antigen, serovar Canicola (L01) with titers = 3200. One of the seropositive animals reacted only to serovars of Brazilian isolate antigens (Canicola L01, T = 3200. Canicola L04, T = 800; Canicola L014, T = 400), and the other animal showed a low titer for serovar Copenhageni (10A), the only reaction for antigen of the reference collection (Canicola L01, T = 3200; Canicola L04, T = 400; Canicola L014, T = 400; Copenhageni 10A, T = 200: Copenhageni M9/99, T = 200). Both animals were in good overall health at the time of capture and no clinical signs were correlated with the infections dealt with in the present paper. All eleven jaguar serum samples were negative for *B. abortus* antigen by RBT.

DISCUSSION

Despite the absence of clinical signs at the time of capture, high antibody titers to serovar Canicola (L01) isolated from *Canis familiaris* in the state of Paraná, southern Brazil, were detected in two jaguars highlighting the importance of using local antigens in serological surveys, usually carried out only with collection of reference antigens performed by MAT. This high titer (T = 3200) also suggests a recent or frequent contact with this or a closely related agent, which probably circulates in these two regions of the northern Pantanal, despite the low frequency of positive animals found in this study (18.2%). Although the specificity of MAT is good, there is significant serological cross-reactivity among serovars that may result in an equal or even higher antibody titer[19]. Therefore, serological tests may suggest, but not definitively identify, the infecting serovar, and isolation of the agent and molecular analysis should be required.

Serological surveys are commonly performed in studies of wildlife exposure to pathogens in South America to determine whether wild animals have been exposed to an antigen, due to the concern of disease transmission across the interface between wildlife and domestic animals. However, local Brazilian antigens have rarely been tested in studies involving free-living species in serological investigations for leptospiral infections. VIEIRA *et al.* (2013)[23] did not detect seropositivity to serovar Canicola (L01) using local strains in a study of exposure to *Leptospira* spp. in wild mammals from the southern Pantanal of Mato Grosso do Sul, and the only other antigen isolated in Brazil of serovar Canicola (L014) was detected in *Cerdocyon thous.* In the present study, the results showed high antibody titers for serovar Canicola, of which the main natural reservoir is the dog, suggesting an occasional contact between jaguars and domestic dogs. This is a concern for the conservation of wild carnivores and requires further investigations in the Pantanal region. Furthermore, the inclusion of autochthonous antigens in serological inquiries should be considered because they can significantly increase the number of reactive animals, as well as modify the epidemiological profile of infections, likewise observed in a study conducted on cattle by SARMENTO *et al.* (2012)[22].

The serological method used to detect *B. abortus* antibodies in the present study is recommended by the Brazilian Department of Livestock Health[14] and is also performed in serological surveys for brucellosis in wildlife[7,21]. FURTADO (2010)[7] reported the contact of a jaguar with *Brucella* sp. in the Pantanal biome, suggesting that predation of cattle infected with *B. abortus* may explain seroconversion. In the present work, although all samples were negative for antibodies against *B. abortus*, zoonotic diseases included in sanitary control programs need to be further investigated in wildlife to assist decision-makers in the development of effective action plans. The negativity of exposure to *B. abortus* in jaguars from these two conservation units suggests a low level of environmental anthropogenic alteration, commonly related to livestock

areas, with consequent predation of cattle. However, the validation of diagnostic techniques, such as serology, requires careful analysis of the isolation of *Brucella* agent, especially when it comes to wildlife[9], in order to determine the possible transmission chain of the disease and the role of certain species in the maintenance of the agent in the environment.

Despite the low number of reactive animals for *Leptospira* antigens and the absence of individuals positive for *B. abortus* antibodies in the present study, more extensive investigations are necessary to determine the likelihood of the impact of infection by these pathogens on the health and reproductive parameters of wild populations. These data are important for the development of management plans of protected areas, as well as for an evaluation of the role played by this species in the epidemiological cycle of this important zoonotic disease.

Preventive measures for *Leptospira* spp. infection, such as water treatment for consumption and chemoprophylaxis, can also be recommended for people who visit these areas for professional reasons or for recreational activities.

RESUMO

Detecção de anticorpos para *Leptospira* spp. e *Brucella abortus* em onças-pintadas (*Panthera onca*) de vida livre em duas áreas protegidas no Pantanal Norte, Brasil

Este estudo teve como objetivo avaliar a exposição de onças-pintadas de vida livre (*Panthera onca*) para *Leptospira* spp. e *Brucella abortus* em duas unidades de conservação no Pantanal de Mato Grosso, Brasil. A presença de anticorpos em amostras de sangue de onze onças foi investigada utilizando antígenos autóctones isoladas no Brasil adicionais a coleção de antígenos de referência aplicada usualmente ao diagnóstico da leptospirose pelo teste de soroaglutinação microscópica (MAT). Para os anticorpos de *B. abortus*, foi utilizado o teste de Rosa Bengala. Duas onças-pintadas (18,2%) foram reagentes para *Leptospira* spp. e o sorotipo considerado como o mais provável pela infecção em ambos os animais foi um isolado brasileiro do sorovar Canicola (L01). Todas as onças-pintadas foram soronegativas para *B. abortus*. Estes dados indicam que a inclusão de antígenos autóctones em estudos sorológicos pode aumentar significativamente o número de animais reativos, assim como modificar a caracterização do sorotipo mais prevalente.

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BRIEF COMMUNICATION

NEW WILDLIFE HOSTS OF *Leptospira interrogans* IN CAMPECHE, MEXICO

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SUMMARY

Leptospira interrogans has been identified to cause leptospirosis, a widespread zoonotic disease that has been identified in domestic and wild animals. This work analyzed kidneys from two species of wild rodents from the state of Campeche, Mexico. Analyses were made by PCR using specific primers for detection of *Leptospira interrogans* DNA. The rodent species that tested positive were *Heteromys gaumeri* and *Ototylomys phyllotis*, both of which are new hosts for the bacteria in Southeastern Mexico. These records provide new insights into the disease's transmission that should be studied carefully in order to identify other potential host species, including humans, which are at risk of becoming infected if they are in contact with infected wildlife.

KEYWORDS: Wildlife hosts; *Leptospira interrogans*; Campeche; Mexico.

Several species of the genus *Leptospira* cause leptospirosis, a zoonosis of urban distribution[3,6]. Wild and domestic mammals (160 species) have been identified as hosts for these bacteria worldwide[2,6]. *Leptospira interrogans* has mainly been identified in domestic mammals because they have direct contact with humans[4,5,6]. However, in Neotropical areas, such as Panama[21], the Peruvian Amazon[6,8] and the city of São Paulo[16], some wild mammals (bats, carnivores, marsupials and rodents) have been identified as hosts of *L. interrogans*.

In Mexico, records of wildlife hosts for *L. interrogans* are scarce and widely scattered across different states (e.g. *Didelphis virginianus* in Yucatán[25] [Southeastern Mexico], *Odocoileus virginianus* in Coahuila[9] [Northern Mexico] and *Zalophus californianus* in the Gulf of California[1,18]). A study carried out in Cozumel, Quintana Roo, identified a 21.5% seroprevalence of *Oryzomys couesi cozumelae*[24]. In Tamaulipas, Northeastern Mexico, five species of wild rodents (*Baiomys musculus*, *Liomys irroratus*, *Oryzomys alfaroi*, *Peromyscus leucopus* and *Sigmodon hispidus*) tested positive for different serovars of *L. interrogans* by Microscopic Agglutination Technique (MAT)[22]. However, there are no records of wildlife hosts reported in Campeche, and in the Yucatan Peninsula only one species of rodent has been previously reported[24]. For this reason, the aim of this paper is to report two new species of wild rodents that are hosts of *L. interrogans* in Calakmul, Campeche, Mexico.

Ten rodents were collected (collection permit FAUT-0170) on August 17th, 2013 from the Yaax'che camp, Calakmul, Campeche, Mexico (located 43 km SSE from the archeological zone of Calakmul, 18° 29' 14'' N, 89° 53' 57'' W). These specimens were killed in compliance with the guidelines of the American Society of Mammalogy for the Use of Wildlife Mammals in Research[17]. All specimens were identified and deposited at the Museo de Zoología "Alfonso L. Herrera" in the Facultad de Ciencias (MZFC) of the Universidad Nacional Autónoma de México.

For the identification of *Leptospira* DNA in these rodents, one kidney was aseptically collected and deposited in 70% ethanol. A portion of 25 mg of kidney tissue was processed for DNA extraction using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's specifications (using the Purification of Total DNA from Animal Tissues Protocol). After extractions were done, a multiplex PCR was performed using primer sets G1/G2 (specific for the detection of pathogenic leptospires) and B64I/B64II (specific for *Leptospira kirschneri*) with expected products of 285 bp and 563 bp, respectively[19]. Additionally, the positive samples were analyzed using specific primers for the identification of pathogenic leptospira species[23]. The reaction mixture consisted of 12.5 µL of GoTaq® Green Master Mix, 2X of Promega Corporation (Madison, WI, USA), using a pair of primers, Intergroup A fwd and Intergroup A rev (100 ng each), 6.5 µL nuclease-free water and 200 ng DNA in a final volume of 25 µL.

In order to minimize cross-contamination and to avoid false positive results, a negative control (i.e. reaction mix without DNA) and a positive

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control (i.e. reaction mix and *L. interrogans* serovar Pomona DNA) were both included. Each PCR reaction was performed in triplicate.

The PCR products were analyzed by electrophoresis on 1.5% agarose gels, using a 100 bp molecular weight marker (Nucleic Acid Markers, LMW DNA Ladder of BioLabs) in 1X TAE buffer. Gels were stained with SYTO® 60 nucleic acid stain (Invitrogen by Life Technologies CA, USA) and visualized using an ODYSSEY CLx Imaging System (LICOR Biosciences).

Two rodents collected at Yaax'che camp, Calakmul, Campeche, Mexico that tested positive using the G1/G2 primers were identified as *Heteromys gaumeri* (temporary catalog RAVGA014) and *Ototylomys phyllotis* (temporary catalog RAVGA013).

These tests were confirmed positive with primers of Intergroup A designed by REITSTETTER[23], which specifically amplify a segment of 396 bp of *L. interrogans* DNA. *Leptospira kischneri* was not detected in any of the samples analyzed, and the DNA of *L. interrogans* was not found in any of the negative controls (Fig. 1).

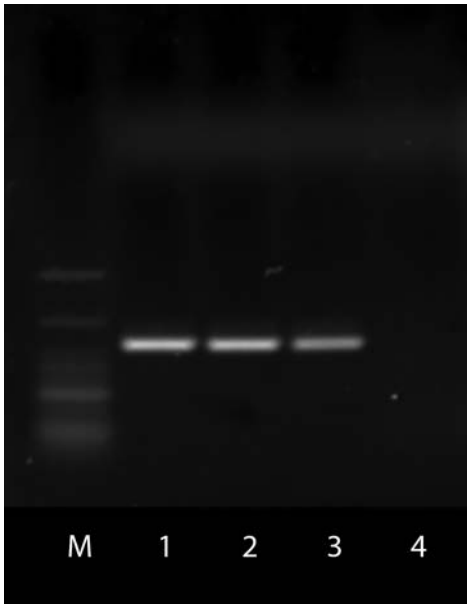

Fig. 1 - Agarose-gel electrophoresis of the single PCR products amplified with primers of Intergroup A designed by REITSTETTER[23]. M: 100 bp DNA marker ladder; Lane 1: *Heteromys gaumeri* (RAVGA014); Lane 2: *Ototylomys phyllotis* (RAVGA013); PCR Controls; Lane 3: Positive control (396 bp, *L. interrogans* serovar Pomona DNA); Lane 4: Negative control (without DNA).

Climate affects the timing and intensity of outbreaks of infectious diseases[14,15]. It has been stated by several authors[3,6,20,26] that adverse climatic events, such as hurricanes and floods, are related to the timing and intensity of *Leptospira* outbreaks. In the case of the present study, the presence of two tropical storms that occurred before and after the specimen's collection[12,13], allowed for speculation regarding the study's findings of *L. interrogans*.

This is the first work that identifies *Heteromys gaumeri* and *Ototylomys phyllotis* as new hosts for *L. interrogans*, by using the set of primers designed by REITSTETTER[23] to identify pathological samples. Moreover, the study area in which the specimens were collected corresponds to a new locality in Mexico, where the presence of the bacteria had not been previously reported. The presence of *L. interrogans* in wild rodents from the same locality should be studied carefully in order to identify the possibility of other species and particularly humans of this area being infected. The author's suggestion is based on previous studies made on domestic animals and humans. In the case of domestic animals (bovines, pigs and dogs) a study revealed a general positivity of 30.5%[10], while a more recent study showed a general positivity of 21.3% registered in dogs of Campeche city[7]. Particularly in the case of human leptospirosis, incidence varied from 0.7-2.2/100,000 inhabitants, with a general seroprevalence of 14.2%[11,25].

Since extreme weather events have been reported to promote the presence of *Leptospira* outbreaks[6], it is essential to further analyze potential reservoirs of several pathogenic species of *Leptospira* in order to identify the dynamics of the transmission between wild mammals and peri-urban human populations, in order to reduce the risks of a potential leptospirosis outbreak in vulnerable groups such as biologists, national and foreign campers and tourists that visit the study area.

RESUMEN

Nuevos huéspedes silvestres de *Leptospira interrogans* en Campeche, México

Lepstospira interrogans ha sido identificada como uno de los agentes causantes de la leptospirosis, una zoonosis ampliamente distribuida, la cual se ha identificado en numerosos animales domésticos y silvestres. En este trabajo se analizaron los riñones de dos especies de roedores silvestres procedentes del estado de Campeche, México mediante la técnica de PCR con iniciadores específicos para la detección de DNA de *Leptospira interrogans*. Las especies de roedores que resultaron positivas corresponden a *Heteromys gaumeri* y *Ototylomys phyllotis*, ambas representan nuevos registros de huéspedes para la bacteria en el sureste de México. Estos nuevos huéspedes deberán ser estudiados cuidadosamente con el fin de determinar la posibilidad de que otras especies de animales, y en particular los humanos, entren en contacto con el patógeno presente en animales silvestres.

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CORRESPONDENCE

FALSE-NEGATIVE DENGUE CASES

September 24, 2014

Dear Editor, we would like to discuss on the recent publication on false-negative dengue cases[1]. ACOSTA *et al.*[1] reported that "Health care providers should therefore be aware of samples tested negative by NS1 antigen assays, especially when clinical symptoms and other laboratory data results show evidence of dengue infection". In fact, the problem of false-negative in dengue diagnosis is an important issue for discussion. First, the problem of the NS1 assay in dengue diagnosis is noted worldwide[2]. Combining with other immunological test is suggested for improvement of diagnostic accuracy[2]. Also, it should be noted that the diagnosis of dengue in many tropical countries is usually a presumptive diagnosis without the laboratory confirmation[3]. This implies that there can be both false-positive and false-negative of dengue diagnosis. Sometimes, other arboviral infection such as Chikungunya virus infection can have the similar clinical feature to dengue and mis-diagnosed as dengue. In addition, there can be more than one arboviral infection in a case (such as co-infection between dengue and Mayaro virus infection)[4]. Nevertheless, the definitive diagnosis is more useful for epidemiological purpose than therapeutic purpose. The symptomatic and supportive treatment is usually the basic effective management for the patient with dengue and other similar arboviral infections.

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