Am. J. Trop. Med. Hyg., 77(1), 2007, pp. 102–106

Comparison of *Trypanosoma cruzi* Lineages and Levels of Parasitic DNA in Infected Mothers and Their Newborns

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Abstract. To better understand the factors involved in maternal–fetal transmission of *Trypanosoma cruzi*, we compared DNA levels—obtained by use of quantitative real-time PCR and parasitic genotypes determined by PCR amplification followed by hybridization—in Bolivian mothers and their congenitally infected newborns. Mothers and their neonates displayed markedly different parasitic DNA levels, as most maternal estimated parasitemias (> 90%) were < 10 parasites/mL, whereas those of 76% of their newborns were > 1,000 parasites/mL. Comparison of *T. cruzi TcII* sublineages infecting mothers and newborns showed identity, without evidence of mixed infection in mothers or neonates. Analysis of minor variants of *TcIId*-genotyped parasites using sequence class probes hybridizing with hypervariable domains of kDNA minicircles showed discrepancies in half of mother/newborn pairs.

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi,* agent of Chagas disease, infects 16–18 million people in Latin America. Parasites are mainly transmitted by blood-sucking vector bugs releasing excreta containing infectious agents, transfusion of infected blood, or congenitally from mothers to their fetuses.[1] In endemic countries where national programs of vectorial control and selection of blood donors have been developed, maternal–fetal transmission of parasites has evolved as the most important route of *T. cruzi* infection, making possible transmission from one generation to another.[2,3]

Such transmission occurs in 1–12% of infected mothers, and morbidity and mortality of congenital infection vary from asymptomatic to severe and mortal clinical forms of disease.[3,4] Host as well as parasitic factors might be involved in such variations. Indeed, recent studies have highlighted associations between a higher frequency of positive hemocultures in infected mothers and their lower capacity to develop a parasite-specific immune response and congenital infection.[5] We have also recently shown that neonates could be infected with *T. cruzi* of various nDNA genotypes, such as *TcIIb*, *TcIId*, and *TcIIe* sublineages, and with kDNA variants of the *TcIId* sublineage, independently of their parasitemia or clinical status, indicating that the DNA polymorphism of *T. cruzi*, at least of type *TcIId*, was not associated with the clinical diversity of congenital infection.[6]

To better understand the factors involved in the maternal–fetal transmission of *T. cruzi*, the present work aims to compare the sublineages of *T. cruzi*, as well as their DNA levels, using quantitative real-time PCR (qPCR), in blood of infected mothers and their infected neonates.

MATERIAL AND METHODS

Patients and blood samples. The present work considered 36 newborns congenitally infected with *T. cruzi* and 15 of their mothers (from whom samples were available), coming from the Bolivian regions of Cochabamba (Maternity German Urquidi, University Hospital Viedma, "Universidad Mayor de San Simon") and Tarija (Maternity Hospital "San Juan de Dios" and the district hospital of Yacuiba). Clinical data of such patients have been reported elsewhere.[4,6] Umbilical cord and maternal venous blood samples were immediately mixed with the same volume of guanidine-HCl 6 M, EDTA 0.1 M (pH 8), boiled for 15 min, and kept at 4°C until use. This study has been approved by the scientific/ethic committees of U.M.S.S. and Université Libre de Bruxelles (U.L.B.), and written informed consent was obtained from the mothers before blood collection.

Diagnosis of *T. cruzi* infection. Maternal infection was assessed using standard *T. cruzi*-specific serological tests (ELISA, hemagglutination, and immunofluorescence) performed as previously described.[4] Congenital infection with *T. cruzi* was assessed by direct microscopic detection of parasites in blood buffy coat collected in 4–6 centrifuged microhematocrit heparinized tubes ($n = 35$) or by hemoculture ($n = 1$) as previously described.[4] The detection limit of such parasitological detection was estimated to be 40 parasites/mL.[7] The number of positive microhematocrit tubes containing parasites, as well as the number of detected parasites per tube allowed estimation of parasitemia as either low (≥ 40 to < 150 p/mL), medium (≥ 150 to < 400 p/mL), or high (≥ 400 p/mL).

DNA extraction and standard PCR. DNA was isolated from 0.2 mL of blood/guanidine solution, using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. Purified DNA was dissolved in 200 μL of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and conserved at –20°C until use. Presence of *T. cruzi* DNA in extract was first tested by standard PCR using primers amplifying either nDNA (Tcz1/Tcz2, giving amplicons of 200 pb) or kinetoplast sequences (Tc121/Tc122, giving amplicons of 320 pb).[8]

***T. cruzi* typing.** Identification of (sub)lineages of *T. cruzi* and of variants of the sublineage *TcIId* was performed by hybridization of DNA amplicons resulting from the Tc121/Tc122 PCR amplification, with oligonucleotides or kDNA probes as previously described.[6] The *TcIId* variants were

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grouped according to their relative hybridization pattern with probes Oli-IId-1 and Oli-IId-2 as described by Virreira and others in 2006.[6] Although our *TcIIe* probe hybridizes also to some extent with the *TcIIb* lineage, samples hybridizing with the *TcIIe* probe but not with the *TcIIb* probe are unambiguously typed as the *TcIIe* sublineage.

Quantitative real-time PCR (qPCR). Assays of qPCR were performed using the SYBR Green system (Roche Applied Science, Mannheim, Germany) in a LightCycler 2.0 System (Roche), according to the manufacturer's instructions and using kDNA S35/S36 modified primers as previously described.[9] However, the hybridization temperature of the amplification program was increased from 57°C (as described by Cummings and Tarleton in 2003[9]) to 63 or 65°C, to delay the appearance of unspecific bands (background) originating from human DNA.[10] All samples were analyzed in duplicate, in at least two separate experiments. Under standard conditions, the reaction medium (10 μL final volume) was prepared in glass capillaries mixing 2 μL of LightCycler FastStart DNA Master SYBR Green I buffer containing *Taq* polymerase, 0.8 μL of 25 mM $MgCl_2$, 0.5 μL of each 10 μM S35/S36 modified primers, 5.2 μL of sterile water, and 1 μL of template DNA. Standard curves were performed using 10-fold serial dilutions of an initial suspension of 10^5 parasites (trypomastigote form) in 1 mL of a mixture containing 0.5 mL of human blood and 0.5 mL of 6 M guanidine-HCl, 0.1 M EDTA, pH 8, and boiled for 10 minutes. The reaction mix was centrifuged in a Roche LightCycler Carousel Centrifuge 2.0 before amplification with the following program: denaturation phase of 95°C for 15 minutes and 40 cycles of 95°C for 10 sec, 63°C for 10 sec, and 72°C for 5 sec.

RESULTS

Evaluation of qPCR in detecting *T. cruzi* DNA levels in blood samples. Reproducible agreement between the number of cycles of qPCR and the number of parasites per assay was obtained using the serial dilutions of parasites in human blood. Figure 1A shows values of a standard curve performed at a hybridization temperature of 63°C, allowing reliable detection down to 0.001–10 parasites DNA equivalent/assay, corresponding to 2–20,000 parasites/mL of blood. Unspecific background amplification, obtained with various negative blood samples, resulted in a crossing point of 27 cycles or more (dashed area in Figure 1A). The crossing point of the lowest concentration tested (0.001 parasite/assay) was ≈ 1 cycle less than the crossing point of negative controls. By increasing the hybridization temperature to 65°C, the crossing point of the cycle number corresponding to the background was increased to 30 cycles, and the lowest concentration tested resulted in a crossing point ≈ 2 cycles less than the crossing point of negative controls. Consequently, this last condition (hybridization temperature of 65°C) allowed better appreciation of very low parasitemia.

Fluorescence intensities of amplicons obtained after standard PCR (performed using both nDNA Tcz1-Tcz2 and kDNA Tc121/122 primers) and the qPCR in blood samples of infected individuals were compared. Figure 1, B and C, shows good agreement between fluorescence intensities of amplicons in standard PCR and qPCR.

Determination of *T. cruzi* DNA blood levels in congenitally infected newborns and their mothers. The 36 studied umbilical cord blood samples of congenital cases were positive in the standard Tcz1-Tcz2 PCR.[8] Determination of parasitic DNA levels by qPCR indicated parasitemias ranging between 6 and 55,600 parasites/mL in these samples. Figure 2 shows the distribution of such parasitic load, indicating that 76.3% of these blood samples contained > 1,000 parasites/mL. As shown in Figure 3, agreement was observed between the qPCR data and the semiquantitative estimation of parasitemia performed by microscopic analysis of capillary tubes. Three umbilical cord blood samples of these confirmed congenital cases harbored a qPCR-estimated parasitemia of < 40 parasites/mL: 2 were detected positive by direct microscopic examination, and the third was positive after hemoculture but negative by microscopic examination.

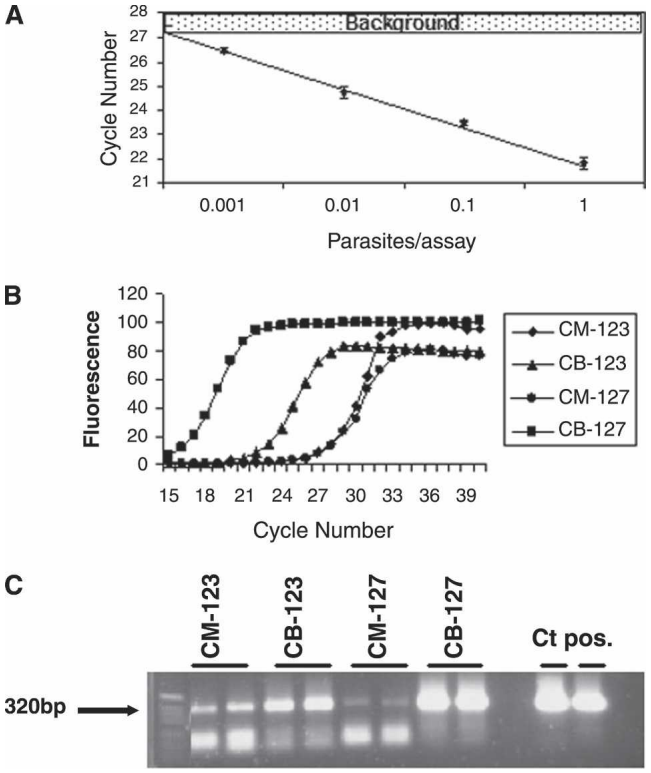

FIGURE 1. Amplification of *T. cruzi* DNA extracted from human blood. (**A**) Standard curve of qPCR using the SYBR Green system (Roche) in the LightCycler apparatus performed at a hybridization temperature of 63°C with blood artificially infected with known amounts of parasites. A background of unspecific amplification of various negative samples was observed at > 27 cycles (dashed area). (**B**) Amplification curves of DNA extracted from various blood samples. Fluorescence was recorded after each cycle. CM stands for "mothers"; CB stands for "baby." (**C**) Classic PCR of DNA extracted from the same samples as in (**B**), using the Go*Taq* polymerase kit (Promega, Madison, WI). Amplicons were separated on TAE 1.2% agarose gel in the presence of ethidium bromide (0.50 μg/mL).

The standard Tcz1-Tcz2 PCR was positive in all 15 of the available blood samples from mothers of congenitally infected newborns. Quantitative PCR performed in these samples showed parasitic DNA levels much lower than those of their infected newborns, corresponding to 2–12 parasites/mL. Fourteen of the 15 mothers (93.3%) displayed parasitemias < 10 parasites/mL. There was correlation neither between maternal and neonatal qPCR-estimated parasitemias nor between *T. cruzi*-specific antibody maternal levels and estimated maternal or neonatal parasitemias ($P > 0.05$).

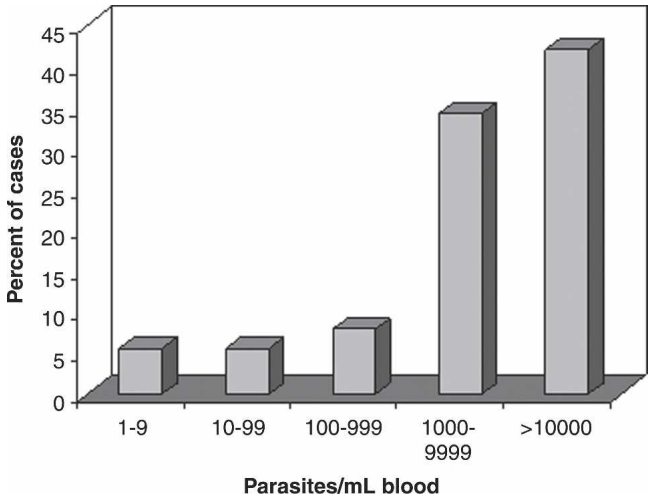

FIGURE 2. Distribution of parasitemias estimated from DNA levels in umbilical cord blood. DNA of 36 samples of umbilical blood of congenitally infected babies was extracted and amplified by qPCR (see Materials and Methods).

***T. cruzi* typing in infected mothers and newborns.** Data of *T. cruzi* typing of the 36 studied umbilical cord blood samples of congenital cases were reported previously.[6] Briefly, 34 samples were infected with the *TcIId* sublineage (94.4%), whereas 2 other samples harbored the sublineages *TcIIb* and *TcIIe*. Fifteen mothers of the above-mentioned neonates showed 14 (93.3%) and 1 (6.7%) infected with the *TcIId* and *TcIIe* sublineages, respectively. Mixed infections with different *T. cruzi* sublineages were detected neither in mothers nor in fetuses. Comparison of such *T. cruzi* typing in the 15 mother/neonate pairs showed the parasitic sublineages being identical in all mothers and their newborns (14 pairs infected with *TcIId* and 1 pair infected with *TcIIe*).

The complementary kDNA genotyping of the 14 mother/neonate pairs harboring the *TcIId* sublineage showed 7 mother/neonate pairs displaying an identical pattern of hybridization with both oligonucleotide Oli-*TcIId*-1 and Oli-*TcIId*-2 probes, indicating the presence of similar *TcIId* variants in both samples (Figure 4A). The 7 other mother/neonate pairs displayed markedly different relative patterns of hybridization with the Oli-*TcIId*-1 and Oli-*TcIId*-2 probes, indicating that these mothers and their neonates were infected with different *TcIId* variants (Figure 4B).

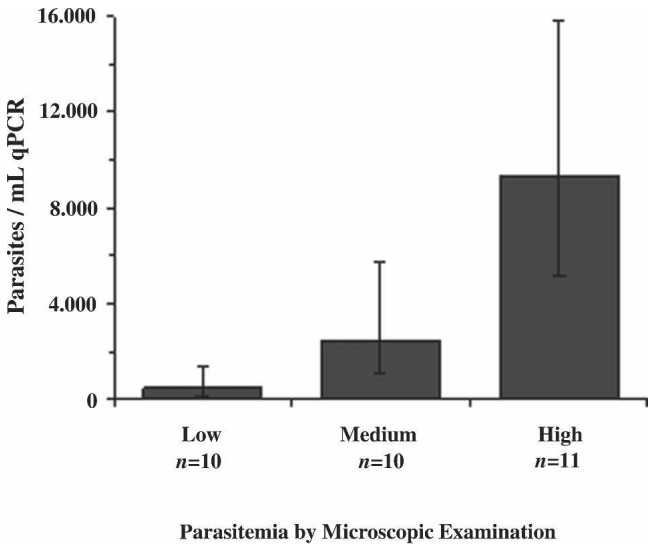

FIGURE 3. Agreement between parasitemias in umbilical cord of *T. cruzi* congenitally infected newborns as estimated by qPCR and microscopic examination of centrifuged capillary tubes. Results are expressed in geometric means ± SEM; n = number of samples.

DISCUSSION

Altogether, our results show that 1) mothers display low parasitic DNA levels, corresponding to parasitemia < 10 parasites/mL in 90% of them, whereas their newborns displayed high DNA levels corresponding to parasitemia levels > 1,000 parasites/mL in more than 76% of them; 2) comparison of *T. cruzi* sublineages infecting mothers and their newborns

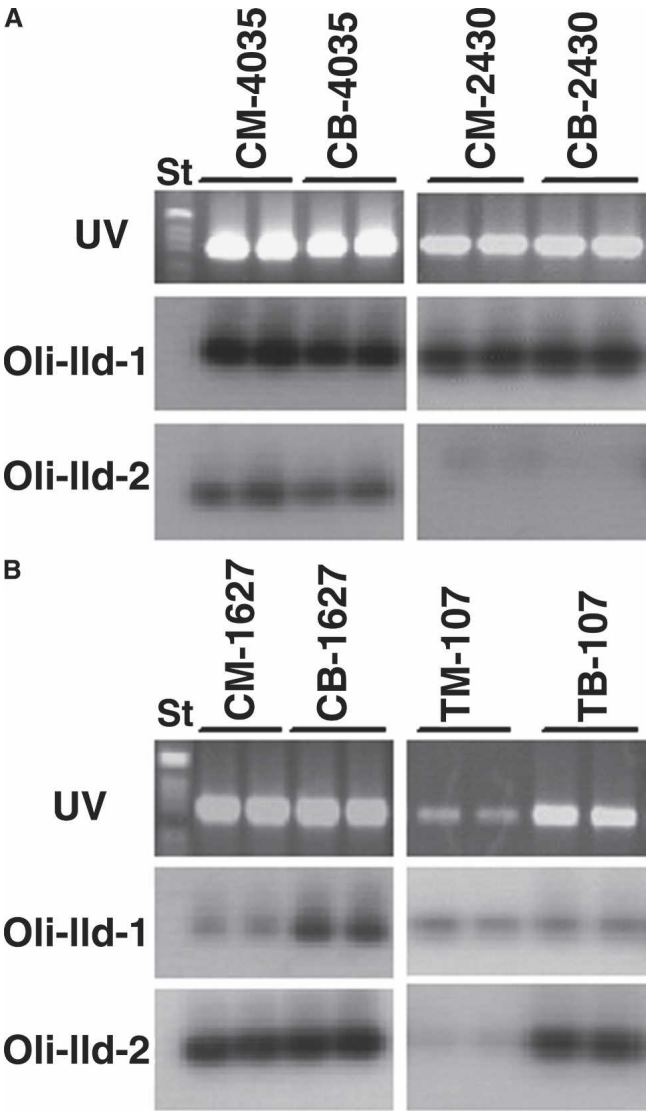

FIGURE 4. Relative hybridization with oligonucleotides probes of amplicons isolated from maternal and umbilical cord blood. (**A**) Samples presenting identical patterns of relative hybridization with oligonucleotides Oli-*IId*-1 and Oli-*IId*-2. (**B**) Samples presenting different patterns of relative hybridization with oligonucleotides Oli-*IId*-1 and Oli-*IId*-2.

(*TcIId* and *TcIIe*) showed identity without evidence of mixed infection in mothers or in neonates; 3) comparison of minor variants of *TcIId* showed discrepancies in half of mother/newborn pairs.

Our data emphasize the capacity of qPCR to estimate *T. cruzi* DNA levels directly from human blood, as was also the case in amniotic fluid, as previously shown.[10] In our experimental conditions, the limit of detection of qPCR could be estimated to 2 parasites/mL of blood using a hybridization temperature of 65°C (i.e., a detection limit similar to that of the standard Tcz1-Tcz2 PCR that we used for qualitative appreciation of the presence of parasitic DNA).[8] By contrast, the microscopic examination of parasites concentrated in capillary tubes allows to detect parasitemia $\geq$ 40 parasites/mL.[7]

Although the parasite detection threshold of PCR techniques is low, it is important to note that such procedures detect parasitic DNA without giving information on parasite viability, whereas the above-mentioned parasitological method indicates unambiguously the presence of live parasites. Indeed, PCR detection of low levels of parasitic DNA could correspond to debris of dead parasites not yet degraded by host blood DNase, without current infection. In addition, kDNA of *Trypanosomatidae* is organized in multiple circles, probably much more difficult to digest than linear nDNA. These considerations might complicate the diagnosis by PCR of congenital infection in newborns, as we may not exclude that parasitic DNA might be transferred from the mother to the fetus without the passage of live parasites (i.e., in the absence of infection), as is likely the case for other parasitic molecules.[11] This has to be kept in mind when interpreting qPCR results that show low levels of foreign DNA in blood, and the existence of infection must be confirmed by other data or other PCR analysis of blood.[12] However, qPCR remains a sophisticated and expensive technique useful as an investigation tool to improve our understanding of the maternal–fetal transmission of *T. cruzi* but is hardly applicable in the control strategies for congenital Chagas disease.[2,3]

Agreement between parasite levels obtained by qPCR and parasite counts in capillary tubes highlights the reliability of detecting parasitic DNA levels to estimate parasitemia. The high parasitemia observed in > 76% of congenitally infected neonates confirms the intense parasite multiplication occurring in fetal tissues in this particular form of acute chagasic infection. However, some neonates also displayed lower parasitemia. Such differences could relate to the timing of the first transplacental transmission of parasites during gestation, a weaker neonatal parasitism, or the closer to delivery such transmission might have occurred. Another non-exclusive possibility lies in the capacity of fetuses/neonates to control infection. Indeed, it has been previously shown that *T. cruzi*-infected fetuses/neonates were able to overcome their physiologic immunologic immaturity by developing a cytotoxic- and IFN-γ producing CD8 T-cell response toward parasites.[13] Both factors (timing of maternal–fetal parasite transmission and fetal capacity to develop its own immune response) likely contribute to explain the absence of a correlation between maternal and neonatal estimated parasitemia.

Parasitic DNA levels detected in infected mothers were close to the lower detection limit of qPCR. However, such low parasitic DNA levels can be considered as corresponding to living parasites (see above) because high levels of *T. cruzi*-specific antibodies were detected in such mothers: half of them displayed *T. cruzi*-positive hemocultures,[5] and they have transmitted parasites to their fetuses. These low maternal parasitemias, contrasting with the 100-fold higher parasite blood levels observed in most neonates, likely relates to the stronger *T. cruzi*-specific immune response developed by such chronically infected mothers than in their fetuses/neonates.[5,13]

The *T. cruzi* typing of blood samples of congenital cases and of some mothers confirmed previous data that we and others obtained, indicating the sublineage *TcIId* as largely distributed in the Bolivian population[6,14] and that various (sub)lineages of *T. cruzi* can be involved in congenital chagasic infection.[6] A remarkable result of the present study comes from the comparison of *T. cruzi* genotypes infecting the mothers and their neonates, which shows similar (sub)lineages (*TcIId*, *TcIIe*) in all mother/neonate pairs, whereas different minor kDNA variants of the *TcIId* sublineage were found in half of such pairs. The observation of parasite genotypes similar in both mothers and newborns is the expected result in case of maternal infection with only 1 parasite genotype directly transmitted to the fetus. Discrepancies between the *T. cruzi* genotypes observed in mothers and their newborns, as observed for minor *TcIId* kDNA variants, can be differently interpreted. One hypothesis relates to maternal infection with only 1 parasite genotype that is transmitted to the fetus. A much more intense parasite multiplication occurring in the infected fetuses in comparison to mothers would allow susceptible mutations to rapidly generate new variants detected in the neonates, although contradictory data have been reported about the mutation rates in the hypervariable regions in kDNA minicircles.[15,16] Another possible interpretation of such discrepancies relates to possible mixed maternal infections with various *TcIId* variants (which cannot be excluded using our protocol), followed by either a transplacental passage of only one of these variants to be transmitted to the fetus or by a transplacental transmission of various variants and a more rapid multiplication of one of these variants becoming dominant in fetus. Interestingly, previous histopathologic studies of placentas performed by our team indicated that parasite multiplication occurred in the chorionic plate and the membranal chorion (i.e., within regular cells for *T. cruzi* multiplication), without invasion of trophoblastic villi[3,17] (and unpublished data), suggesting that the capacity of placenta to select the parasite genotype during maternal–fetal transmission is probably limited.

In conclusion, comparison of parasitic features in infected mothers and their newborns highlights the contrasting levels of high parasitemia observed in neonates and the low parasitemia detected in their mothers. Mixed infections by various sublineages were detected neither in mothers nor in neonates, and these sublineages of *T. cruzi* were found to be identical in mothers and their neonates. However, further studies will be necessary to clarify the mechanism leading to the detection of different minor *T. cruzi* variants in mothers and their neonates using probes hybridizing with hypervariable domains of kDNA minicircles.

Received February 21, 2007. Accepted for publication April 18, 2007.

Acknowledgments: The authors thank Dolores Rangel and Nelly Aguado (Hospital Universitario San Juan de Dios and Laboratorio de Epidemiologia—SEDES, Tarija, Bolivia). M. Virreira is a recipient of a "Xénophilia" grant (ULB, Belgium). C. Alonso-Vega is fel-

low of the "Association pour la Promotion de l'Éducation et la Formation à l'Étranger" (APEFE, "Communauté Française de Belgique").

Financial support: This study was supported by the Conseil Interuniversitaire de la Communauté Française de Belgique (CIUF) and the Fonds National de la Recherche Scientifique Médicale (Belgium, conventions 3.4.595.99/3.4.615.05).

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