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Original Article

A preliminary serological study of *Trypanosoma evansi* and *Trypanosoma lewisi* in a Chinese human population

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ABSTRACT

Trypanosoma evansi, known as an animal trypanosome, is widely distributed in many countries of Africa, Asia and South America; it causes significant economic loss in these countries. A few cases have also occurred in some countries of Europe due to the importation of infected animals from endemic regions. Rare human *T. evansi* infections were attended by the health departments and international health organizations in these endemic countries. *Trypanosoma lewisi*, a cosmopolitan parasite of rats, sometimes found in humans, is currently considered as a zoonotic pathogen and has gained special attention from scientists and international health organizations such as the World Health Organization. The current study considered the serological screening of human infection by *T. evansi* and *T. lewisi* in a Chinese human population. None of the 622 samples was found positive for *T. evansi* infection using the card agglutination test for the trypanosome antigen Rotat 1.2, while, 2.41% of the examined serum samples exhibited some seropositivity to *T. lewisi* using enzyme-linked immunosorbent assay. No significant difference was found between the samples from areas in the South (Zhaoqing, Guangdong) and Central (Zhengzhou, Henan) China.

Introduction

Trypanosoma evansi, a *Trypanozoon* parasite derived from *T. brucei* lineage, is mainly found in Africa, Asia and South America though naturally, this parasite can infect almost all warm-blooded animals with the exception of humans (Hoare, 1972; Brun et al., 1998). Surra is a disease caused by *T. evansi* in animals, which originally developed in camels in North Africa, but has since spread through the Arabian Peninsula and Middle East toward China and Southeast Asia, and has also was imported into Latin America by the conquistadores (Desquesnes et al., 2013b). However, the first well documented *T. evansi* human infection case was observed in India in 2005; it was investigated by the World Health Organization under a request of the Health Department of Maharashtra State (Joshi et al., 2005; World Health Organization, 2005; Powar et al., 2006). In this case, the patient was proved to produce an inefficient apolipoprotein-L1 (ApoL1) because of two genetic mutations on each allele of the gene ApoL1 (Vanhollebeke et al., 2006). ApoL1 is considered as the key component of HDL in normal human serum (NHS) for lysing animal trypanosomes; also called the trypanolytic factor (Vanhamme et al., 2003). A number of similar cases were reported in Southeast Asia (Desquesnes et al., 2013a). However, in a recent case identified in Vietnam, a patient found infected by *T. evansi* exhibited no immunosuppression and a normal ApoL1 level, which suggests that *T. evansi* may be a pathogen in humans (Van Vinh Chau et al., 2016).

Trypanosoma lewisi is a worldwide *Herpetosoma* trypanosome found in *Rattus norvegicus*, *R. rattus* and other related species such as *R. tanezumi* (Milocco et al., 2012; Pumhom et al., 2014). Rats are infected with *T. lewisi* mainly by ingestion of infected fleas or by

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contact with the feces of infected fleas (Hoare, 1972; Desquesnes et al., 2002). *Trypanosoma lewisi* has long been considered as a rat-specific and non-pathogenic trypanosome to its natural hosts. However, an increasing number of human cases of infection by *T. lewisi*, with associated clinical signs, mainly fever, but also coughing, edema, anemia and anorexia, have been reported in some Asian countries such as India and Thailand (Johnson, 1933; Shrivastava et al., 1974; Howie et al., 2006; Kaur et al., 2007; Sarataphan et al., 2007; Banerjee et al., 2008; Verma et al., 2011). These reports suggested that *T. lewisi* might be a neglected zoonotic pathogen (Lun et al., 2009). Recent studies on the activity of human serum demonstrated that *T. lewisi* is resistant to NHS, which confirms its potential as a human pathogen (Lun et al., 2015). In addition, further studies have indicated that *T. lewisi* infection could induce an immunosuppressive effect on its hosts, resulting in increasing susceptibility to other pathogens, such as *Salmonella typhimurium, Cryptococcus neoformans* and *Toxoplasma gondii* (Nielsen et al., 1978; Catarinella et al., 1998; Gross et al., 2006; Carrera et al., 2009). Consequently, more attention should be paid to this cosmopolitan parasite.

Trypanosoma evansi is widely distributed in South China and thousands of animals (mainly water buffalos) are still infected by this parasite (Lun et al., 1993), while *T. lewisi* is also widely distributed in most provinces of China (Liu and Liu, 1990; Lun et al., 2015). In addition, a review of these atypical human cases was recently published (Truc et al., 2013), to make a point of these rare human infections.

Due to the wide distribution of these two animal trypanosomes, occasionally infecting humans, and exhibiting a pathogenic potential, it was necessary to conduct and evaluate serological screenings in an “exposed” human population, particularly those from the countryside in the endemic regions.

Materials and methods

Human data and samples

Blood samples were collected from 622 volunteers by medically trained staff from Zhengzhou, Henan (Central China) and by health examination for those seeking working permission in Zhaoqing, Guangdong province, South China. Wet smears of the blood were made to detect trypanosomes using microscopy. Serum samples were separated using centrifugation and kept in Eppendorf tubes at −80 °C until laboratory tests were performed. Based on the medical records, most of the persons grew up in villages around 25–250 km from towns or cities. Seventy-three percent of persons from Guangdong had an experience of close contact with water buffalo when they grew up in their hometown, while those from Henan had experienced close contact with cattle; however, Henan province is not an enzootic region of *T. evansi* (Lun et al., 1993). Most of the persons from the South and Center mentioned that they had experienced close contact with wild rats when they grew up in their hometowns.

The present protocol was properly reviewed and approved by the Ethical Committee of the School of Life Sciences, Sun Yat-Sen University, Guangzhou, China.

Rat serum samples

Three Sprague-Dawley (SD) rats were infected using intraperitoneal injection with 1×10^6 *T. lewisi* (COP02) after thawing the injectant from liquid nitrogen conservation. Two months after infection, serum samples were collected from the infected rats for the positive control. Serum samples of non-infected SD rats ($n = 3$) were used as the negative control. All serum samples were kept frozen at −80 °C until used in the enzyme-linked immunosorbent assay (ELISA).

Serological tests for detection of antibodies directed against T. evansi: *card agglutination test for* Trypanosomosis/Trypanosoma evansi

The card agglutination test for *Trypanosomosis/Trypanosoma evansi (*CATT/*T. evansi;* antigen lot No.12C3B2-2) was carried out on sera diluted 1:4 as described by the manufacturer (Prince Leopold Institute of Tropical Medicine; Belgium) and according to Bajyana Songa et al. (1987 & 1988). Interpretation of the results is based on visual appreciation of the agglutination, according to a reference picture included in the commercial protocol leaflet; it results in negative (−), doubtful (±), or positive results (+, ++ or +++). Negative and positive reference samples are provided with the kit, in order to validate the quality of reagents.

Serological tests for detection of antibodies directed against T. lewisi: *enzyme-linked immunosorbent assay* Trypanosoma lewisi

All serum samples were tested for *T. lewisi*-specific antibody using ELISA currently under development, according to a protocol described below.

A sample of *Trypanosoma lewisi* (Wery L307 24/9/68) was purified from the blood of the rats experimentally infected with *T. lewisi* (Dethoua et al., 2013) using anion exchange chromatography using DEAE cellulose. These live parasites were washed in medium (Ringer Lactate; glucose 0.6%, KCl 0.4%, $NaHCO_3$ 0.125%, polymixin B 5 mg/mL, L-glutamine 2 mM, MEM non-essential amino acids, pH 8.0) according to Holzmuller et al. (2008). Parasites were added to each well (4×10^5/well) of a microplate (NUNC-Immuno™ MicroWell™ 96 well solid plates; Sigma-Aldrich, France) as previously described (Williams et al., 1996). The plates were emptied and filled with phosphate-buffered saline (PBS) containing 0.05% Tween 20, 10% glycerol and 1% bovine serum albumin for 3 h at room temperature for blocking, washed twice with PBS 0.05% Tween and air dried. Plates were wrapped in aluminum paper for shipping from France to China. When received at the Sun Yat-Sen University, Guangzhou, China, the plates were kept at −20 °C less than 8 mth. Before use, plates were placed at room temperature for 1 h.

In the absence of human reference serum samples available against *T. lewisi*, rat serum samples were used from *T. lewisi*-infected and non-infected SD rats as positive and negative controls, respectively. The secondary antibodies used for these samples were rabbit anti-rat IgG (this conjugate was shown not to react with human IgG; Boster; China). For human samples, the secondary antibodies were rabbit anti-human IgG (this conjugate was shown not to react with the mouse or rat IgG; Boster; China). Sera collected from infected SD rats 2 mth after infection with *T. lewisi* were used as the positive control, while those from non-infected SD rats were used as the negative control.

The ELISA procedure was carried out by diluting 200 μL of serum sample at 1:100 in PBS (containing 0.05% Tween 20, 5% glycerol and 2% BSA) which was added to each well, and the plates were incubated for 2 h at room temperature. The plates were then washed, and 200 μL of HRP conjugated detection antibody solution (secondary antibodies diluted in PBS containing 0.05% Tween 20 and 2% BSA) were added. After incubation for 40 min at room temperature and washing, color was developed by the addition of a substrate solution containing tetramethylbenzidine chromogenic substrate and stopped by the addition of 50 μL of stop solution to each well. The optical densities (ODs) were measured at 450 nm in a microplate reader. Each serum sample was run in triplicate. Validation of

the procedure was based on the result obtained with SD-rat negative (NC = negative control) and positive reference samples (PC = positive control), expected to exhibit low and high optical densities, respectively, using anti-rat conjugate.

Interpretation of the results for human samples: this ELISA being under development, and in the absence of positive reference human samples, there was no cut off line available, so the interpretation of the test was made as follows. When the relative absorption ratio of a sample, described as $(OD_{Sample} - OD_{Blank})/(OD_{Negative\ control} - OD_{Blank})$, was greater than 2, it was arbitrarily determined that this serum sample was highly suspect. Intermediary values, between 1.6 and 2, were also explored as doubtful results.

Ethics statements

The present protocol was properly reviewed and approved by the Ethical Committee of the School of Life Sciences, Sun Yat-Sen University, Guangzhou, China.

Results

Serological tests for T. evansi *infection: card agglutination test for* trypanosomosis//Trypanosoma evansi

Following the kit instructions, negative and positive reference samples provided in the CATT/*T. evansi* kit gave expected results, as positive reference samples produced strong agglutination, while negative reference samples did not show any visible agglutination. Visible agglutination was not observed in any of the 622 human serum samples tested, and thus they were all considered negative using the CATT/*T. evansi*.

Serological test for T. lewisi infection: enzyme-linked immunosorbent assay of T. lewisi

The rat reference samples and the pool of 622 human serum samples were examined for the anti-*T. lewisi* antibody using ELISA. The positive and negative reference serum samples of the SD rats proved to react as expected, with optical densities of PC = 3.476, NC = 0.921. For human samples, the relative absorption ratio $(OD_{Sample} - OD_{Blank})/(OD_{Negative\ control} - OD_{Blank})$ of three samples was greater than 2.000, with a mean of 2.086. In samples from Guangdong, the mean relative absorption ratio was 2.018, while that of samples from Henan was 2.372.

From the total of 622 serum samples, as shown in Fig. 1, the three samples (0.48%) exhibiting a relative absorption ratio > 2.000 were considered as "strongly suspect" samples. Exploring lower rates, five samples (0.80%) exhibited a relative absorption ratio of 1.800–2.000, which were considered as "medium suspects" and seven samples (1.13%) exhibited a relative absorption ratio of 1.600–1.800, which were considered as "low suspects". In this study, seroprevalence could not be obtained since the ELISA has not yet been standardized; however three samples were strong suspects and another 12 were considered as "suspects", making a total of 2.4%. The frequency distribution of the optical densities is presented in Fig. 2; the mean response in these 622 samples was optical density 1.332 ± 0.367.

The serological response to *T. lewisi* antigens detected in Guangdong province was slightly higher than that found in Henan province (all samples with relative absorption ratio greater than 1.6 were included; 2.59% versus 2.27%, $p = 0.797$; Table 1). However, statistical analyses using a chi square test showed that these differences were not significant. In addition, the serological response to *T. lewisi* antigens in females was higher than in males (total, 3.23% versus 1.42%, $p = 0.145$; Henan, 3.02% versus 1.31%, $p = 0.287$ and Guangdong, 3.52% versus 1.56%, $p = 0.312$); however, statistical analyses using a chi square test showed that these differences were not significant.

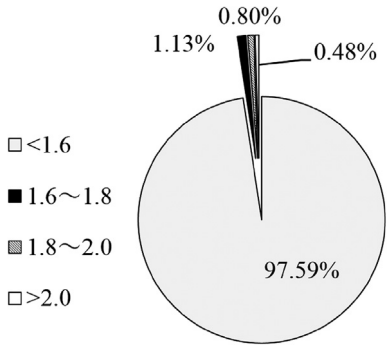

Fig. 1. Serological response to *Trypanosoma lewisi* antigens in humans from Henan and Guangdong, in Central and South China, respectively, where the ratio method was used to describe positive or negative serum samples based on optical density (OD) and the relative absorption ratio = (ODSample - ODBlank)/(ODNegative - ODBlank).

Discussion

Trypanosoma evansi and *T. lewisi* are worldwide-distributed animal heamoflagellates, so both of them can be found in the study area.

Trypanosoma evansi

Amongst mammals, *Trypanosoma evansi* has long been considered as pathogenic to animals only; humans were considered as resistant, due to the trypanolytic factor present in the serum, whereas animals may have sub-clinical expression, mild or strong clinical signs up to death but including abortion, anemia, loss of production (Hoare, 1972; Lun et al., 1993). Without appropriate treatment for horses and dogs, it usually causes death. However, the cases reported in India (Verma et al., 2011), as well as a recent

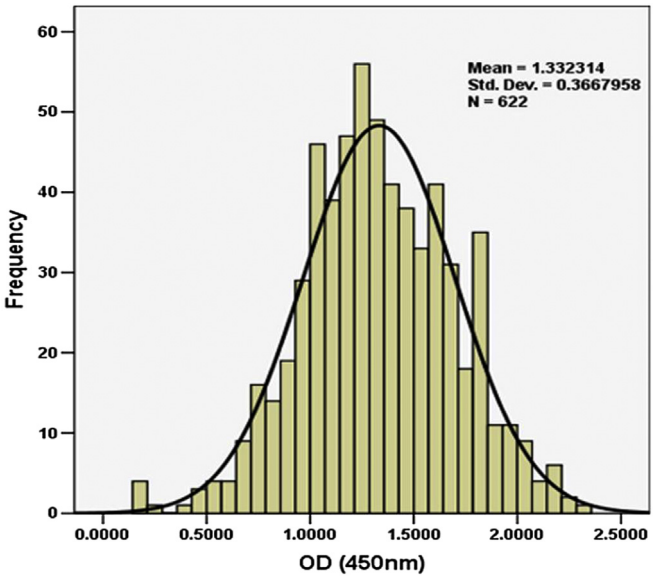

Fig. 2. Frequency distribution of optical density (OD) values of all human samples from Henan and Guangdong (using Statistical Package for Social Sciences (SPSS) version 13.0; SPSS Inc.; USA).

Table 1
Serological response to *Trypanosoma lewisi* antigens in humans from Henan and Guangdong, China.

Sampling site	Number of tested samples			Number of suspect samples				Seroprevalence (%)		
	All	Male	Female		All	Male	Female	All	Male	Female
Henan	352	153	199	>2.0	1	0	1	0.28%	0	0.50%
				1.8–2.0	3	1	2	1.96%	0.65%	1.00%
				1.6–1.8	4	1	3	1.14%	0.65%	1.51%
				>1.6	8	2	6	2.27%	1.31%	3.02%
Guangdong	270	128	142	>2.0	2	0	2	0.74%	0	1.41%
				1.8–2.0	2	1	1	0.74%	0.78%	0.70%
				1.6–1.8	3	1	2	1.11%	0.78%	1.41%
				>1.6	7	2	5	2.59%	1.56%	3.52%
Total	622	281	341	>1.6	15	4	11	2.41%	1.42%	3.23%

case in an adult woman detected in Hochimin city, Vietnam (Van Vinh Chau et al., 2016), confirm the ability of *T. evansi* to infect humans with significant clinical signs. The cases of *T. evansi* infection in humans caused a shock regarding public health since this species is largely distributed and thus a large human population may be exposed to infection. In terms of potential pathogenicity, *T. evansi* being a species close to pathogens of African sleeping sickness (World Health Organization, 2005), strong and possibly fatal effects may be expected. In the first fully documented case observed in India, the patient exhibited a lack of efficient ApoL-1 which explained the infection (Vanhollebeke et al., 2006). *T. evansi* in such a case, is not sensitive to the lysis by a non-efficient ApoL-1; however, in the recent case described in Vietnam, gene sequence and seric concentration of ApoL-1 were normal (Van Vinh Chau et al., 2016), suggesting a real potential as a human pathogen.

In 2005, a serological survey was conducted using CATT/*T. evansi* in the residential area of the Indian patient of 2004. Out of 1806 individuals tested with CATT/*T. evansi*, 81 were positive using plain serum. No trypanosome was detected in the blood of the 60 persons positive to the test at a significant serum dilution (minimum 1:4). These results may suggest a frequent human exposure to *T. evansi* in the study area and, maybe a frequent transmission of parasites to humans leading in a “normal” immune population to transient infections. The specificity of the CATT/*T. evansi* test has not been previously evaluated for human screening and was used in this study for the first time in Asia (Shegokar et al., 2006).

The absence of a CATT-positive result in the current study might have been because there was no chance to collect serum samples from the farmers taking care of buffaloes. Indeed, farmers are in very close contact with their animals and could be considered as an “exposed population”. On the other hand, the negative result from the samples collected from Henan province was expected since this province is not an endemic region of *T. evansi* (Lun et al., 1993). As a matter of fact, the serum samples collected from Henan province were originally planned for the detection of *T. lewisi* infection and were used for the negative control for *T. evansi* infection. It would be very useful to work on the human *T. evansi* infection by establishing collaboration with the local CDC in China in order to survey surely exposed populations.

Our results showed the absence of contact between the parasite and the human population investigated. However, CATT/*T. evansi* can mainly detect multivalent immunoglobulins such as IgM which have a pentameric structure; consequently, it may be positive only in case of recent circulation of the parasite in the blood. Thus, for nonrecent contact, such a test would remain negative. To increase the accuracy of such investigations, it might be advisable to use both CATT/*T. evansi* and IgG detection ELISA/*T. evansi* in the future. However, regarding the ELISA method, it is necessary to obtain reference positive samples from human patients who have been duly identified.

Trypanosoma lewisi

Trypanosoma lewisi is considered as a non-pathogenic species for its natural host of rats (Hoare, 1972). However, clinical signs were reported in humans, mainly fever. The increasing number of cases of *T. lewisi* infection in humans has drawn the attention of international organizations such as the World Health Organization (Truc et al., 2013). *Trypanosoma lewisi* can cause clinical signs in infants and young patients (see reviews by Lun et al., 2009; Truc et al., 2013), and its presence in the vicinity of human habitats was recently shown to be quite high in Southeast Asia (Pumhom et al., 2015). Importantly, unlike *T. evansi* which can be lysed by normal human serum, *T. lewisi* is naturally resistant to such lysis (Lun et al., 2015); thus it can naturally infect humans. Therefore, *T. lewisi* has been considered as a potential zoonotic trypanosome (Lun et al., 2015). Moreover, when evaluating the efficacy of trypanocides against *T. lewisi*, although initial work showed the efficacy of fexinidazole and pentamidine (Dethoua et al., 2013), other workers demonstrated that all human and animal trypanocides were inefficient against this parasite in laboratory rats, including fexinidazole and pentamidine (Desquesnes et al., 2016a, 2016b).

In the present study, out of the 622 human serum samples collected from South and Central parts of China, 0.48–2.41% of the samples reacted with *T. lewisi* antigens (using cut-off values of 2.0 OD and 1.6 OD, respectively). The three-highest seropositive samples were from females 27–29 years old, who had grown up in the countryside before they left home as adults to work in the city; more accurate epidemiological information was not available. If these samples are considered as true positives, the results from the present study indicate that although a low seroprevalence of *T. lewisi* antibodies was found, contact between humans and *T. lewisi* antigens or live *T. lewisi* can exist, leading to an immune response and probably to transient or undetected infections or both. Such results would support the conclusion of Liu and Liu (1990) who reported a seroprevalence of 0.96% (15/1564 based on ELISA) using hemagglutination and an agar diffusion test for *T. lewisi* infection in human serum samples from Jilin province (Northeast China).

More work, particularly more serum samples from populations in the countryside of different regions, will be greatly beneficial to understanding *T. lewisi* infection in the Chinese population. In addition, the environmental status of the residents should be carefully investigated in future epidemiological surveys.

Trypanosomes of the *lewisi* group express and secrete receptors for the immunoglobulins Fc region (Balber and Sturtevant, 1986; Vincendeau and Daëron, 1989). This nonspecific binding, independent of an antigen-antibody reaction, has to be taken into account in serological assays. This indicates the needs to optimize and develop reliable ELISA *T. lewisi* to improve the investigations on *T. lewisi* infections in humans. To standardize such an ELISA test, it is

necessary to get positive reference human serum samples from active infection cases.

Detection of active human infections by *T. evansi* or *T. lewisi* is necessary to generate and make available positive reference human samples. This requires surveillance, which would be supported by extensive distribution of the e-leaflet recently released by the Network on Atypical Human Infections by Animal Trypanosomes (NAHIAT) and that is available on the web.

More case reports will certainly help to develop diagnostic tools and to improve the surveillance of atypical human infections by animal trypanosomes.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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