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Comparison of PCR with Parasitology and Serology in the Diagnosis of a Low Virulent Strain of *Trypanosoma brucei gambiense* in mice

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ABSTRACT

BALB/c mice infected with a low virulent strain of *Trypanosoma brucei gambiense* IL3253 were treated intraperitoneally (ip) with either Melarsoprol (Mel-B, obtained from WHO, Geneva) or phosphate-buffered saline (pH 7.8) containing 1% glucose (PSG) as controls. The mice were subsequently monitored regularly for parasites by direct microscopic examination of their tail blood or buffy coat and by polymerase chain reaction (PCR). Suratex® (AccuPharma, NY), an assay that detects circulating trypanosome parasite antigen, was also used. Mel-B was found to be an effective drug for treatment against *T.b. gambiense* and at the end of the first treatment schedule of 3 series of 3 injections at 7 days post-infection (DPI), all treated mice were negative for parasites even by PCR, while all the control animals were positive. Suratex® (AccuPharma, NY) was found to be inappropriate in the serodiagnosis of trypanosomosis due to *T.b. gambiense* in mice as it gave high levels of false positives. At 34 DPI, all the BALB/c mice were sacrificed and 0.5 ml of their blood injected ip into individual SCID mice. The SCID mice were likewise monitored for parasites or for evidence of infection by microscopic examination and PCR for up to 115 DPI. None of the SCID mice

injected with blood from BALB/c previously treated with Mel-B showed any parasite either in their tail blood or buffy coat over the entire sampling period, though some of these mice were shown to harbor an infection by PCR at some time over the sampling interval. This experiment affirms the importance of the various techniques used in the diagnosis of trypanosomosis. A repeated negative PCR test in combination with clinical and conventional microscopical examination may be a useful diagnostic regimen in the diagnosis of trypanosomosis.

INTRODUCTION

Human African trypanosomosis (sleeping sickness) constitutes one of the major problems of public health in many African countries. There are two forms of the disease: the chronic variety encountered in West and Central Africa, which is caused by *Trypanosoma brucei gambiense*, and the more acute form, which occurs in East Africa, and is caused by *T. b. rhodesiense*. The number of sleeping sickness cases is currently estimated at more than one million; conservative estimates put the number of new cases diagnosed at 25,000 per annum, with 50 million people at risk in the affected countries in tropical Africa (Komba et al. 1992).

Diagnosis of trypanosomosis is based on demonstrating, by direct microscopy, *in vitro* culture or inoculation of patients blood into mice to recover parasites, the presence of trypanosomes in peripheral blood, cerebrospinal fluid, bone marrow, lymph fluid or in chancre aspirates (Baker 1970; Lumsden et al. 1980). This, however, is often difficult to achieve. Firstly, in the chronic stage of the disease, there is scarcity and periodicity of trypanosomes in peripheral blood which often precludes the detection of parasites by direct microscopy. Secondly, examination of bone marrow or cerebrospinal, lymph and chancre fluids requires surgical manipulations that are difficult to carry out on a large number of clinical suspects or in epidemiological surveys. Despite the marked improvement in sensitivity afforded by trypanosome concentration methods such as the hematocrit centrifugation technique (Woo 1970), or its variant, the buffy coat technique (Murray et al. 1977) many infected animals still go undetected, particularly those with chronic infections (Masake and Nantulya 1991).

In an attempt to develop alternative diagnostic approaches, several indirect antibody based methods have emerged. These include the capillary-tube agglutination test (Ross 1971), immunoprecipitin tests (Taylor and Smith 1983), indirect immuno-fluorescence antibody tests (Wery et al.1970), indirect hemagglutination (Bone and Charlier 1975), card agglutination (Magnus et al. 1978) and enzyme immuno-assays (Nantulya 1989; Voller 1977). Antigen-based immunodiagnostic techniques such as the latex agglutination test have been

developed and used to a high level of specificity and sensitivity (Nantulya 1989; Nantulya et al. 1992; Nantulya 1994). Specific identification of *T. b. gambiense* by polymerase chain reaction (PCR) amplification of variant surface glycoprotein genes has been reported (Bromidge et al. 1993).

MATERIALS AND METHODS

Experimental design

Ten 5-week old male BALB/c mice were each challenged with 5×10^3 blood stream forms of *T. b. gambiense* IL3253 intraperitoneally (ip) in an inoculum of 0.5 ml of phosphate-buffered saline (pH 7.8) containing 1% glucose (PSG). This parasite is a low virulent strain as described earlier by Inoue et al. (1998). The mice were separated into two groups of 5 mice each and labeled as experimental and control groups respectively. At 4 days post-infection (DPI), mice from the experimental group were started on ip treatment with Melarsoprol (Mel-B, obtained from WHO, Geneva) at a dose of 10 mg/kg/day (Poltera et al. 1981). The treatment was by 3 series of 3 injections (1 injection per day during 3 consecutive days) separated by 7 days of rest. In the control group, PSG was administered using a similar protocol. One week after the last treatment, all the Mel-B and PSG-treated BALB/c mice were sacrificed and 0.5 ml of blood from each mouse was inoculated ip into a separate 5-week old male SCID mouse. All SCID mice were monitored regularly for presence of parasites.

Parasitological diagnosis

Parasitological diagnosis by wet blood film and buffy coat, measurement of packed cell volume (PCV), latex agglutination test and PCR were done at various DPI. The first series of diagnoses was done at 4 DPI (just before the start of the first schedule of treatment), 7 DPI (one day after the first schedule of treatment), 14 DPI (just before the second schedule of treatment), 17 DPI (a day after the second schedule of treatment), 24 DPI (just before the third and last schedule of treatment), 27 DPI (a day after the last schedule of treatment), and 34 DPI (one week after the last dose of the final treatment schedule).

Latex agglutination test

The latex agglutination test has been developed to detect circulating trypanosome antigens. In performing this test, briefly, 25 μl of plasma were transferred to a test circle on a special test slide. An equal volume of the Suratex® (AccuPharma, NY) reagent was pipetted next to the plasma. The two drops were mixed with a stirring rod and spread out onto the surface of this circle. The test slide was then tilted and rotated manually for 2 min and the slide examined in

clear light for evidence of agglutination. If the result was negative (no agglutination), the slide was rotated for another 3 min, to allow weak reactions to develop, and then it was examined again in clear light.

DNA extraction from small blood samples

About 10 μl of blood from the tail of a mouse was pipetted into 100 μl of lysis buffer containing a final concentration of 10 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 mM NaCl, 0.5% sodium dodecyl sulfate and 300 μg/ml of proteinase K. After incubation for two hrs at 55°C (Eyla, Dry Thermobath MG-1100, Tokyo), 100 μl of phenol:chloroform (1:1) was added and the mixture was mixed. The samples were then centrifuged at 4,000×g for 5 min at room temperature and the supernatant transferred to a new tube. This phenol:chloroform extraction procedure was repeated twice. To the supernatant was added 10 μl of 3 M sodium acetate (pH 5.2) and 250 μl of 100% ethanol, and store at -80°C for 30 min. After centrifugation at 4,000×g for 15 min at 4°C, the supernatant was removed and 500 μl of 70% ethanol was added to the DNA pellet. The samples were again centrifuged at 4,000×g for 5 min at 4°C and the supernatant removed. The micro-tubes with DNA samples were then air-dried (Eyela, Centrifugal Vaporizer, Tokyo). Each DNA sample was dissolved in 10 μl of Tris-EDTA buffer and kept frozen at -30°C till used.

PCR of variable surface glycoprotein (VSG) 117 genes

DNA preparations were amplified in a final volume of 30 μl in a Perkin Elmer PCR equipment (Gene Amp 2400, Perkin Elmer, CA). In preparation of the master mix, the following volumes were used; 20.4 μl DDW; 3 μl of 10× PCR buffer (Perkin Elmer, CA); 3 μl of 2 mM dNTPs (Perkin Elmer, CA), 0.3 μl of VSG 117 primer (50 pmol/μl each of sense and antisense), 0.3 μl *Taq* (*Thermus aquaticus*) DNA polymerase (Perkin Elmer, CA), and 3 μl of DNA sample was used as template per reaction program. In the PCR reaction program, samples were incubated at 94°C for 10 min in an initial denaturation step, followed by 40 cycles at 94°C (30 sec), 51°C (1 min) and 72°C (2 min). The PCR reaction products were analyzed by agarose gel electrophoresis and detected visually by UV transillumination (ATTO, Tokyo) of the ethidium bromide-stained gel.

RESULTS

A summary of results from various diagnostic tests used to detect *T. b. gambiense* IL3253 in BALB/c mice that were either treated with Mel-B or PSG is shown in Table 1. The animals were observed for evidence of harboring parasites for up to a period of 34 DPI. There was an excellent correlation between the tail

blood and buffy coat techniques over the entire sampling period for both groups of experimental animals whereby no evidence of infection was demonstrated in Mel-B treated animals, while parasites were observed from a good proportion of the control animals.

Table 1. Results of various diagnostic tests used to detect *Trypanosoma brucei gambiense* IL3253 in BALB/c treated with either Mel-B or PSG.

Test	Group	Days post-infection						
		4	7	14	17	24	27	34
Tail blood	PSG	0/5	1/5	0/5	0/5	0/5	2/5	0/5
	Mel-B	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Buffy coat	PSG	1/5	0/5	0/5	1/5	1/5	4/5	0/5
	Mel-B	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Latex	PSG	3/5	4/5	4/5	1/5	ND	ND	ND
	Mel-B	5/5	5/5	3/5	2/5	ND	ND	ND
PCR	PSG	2/5	5/5	2/5	1/5	1/5	4/5	1/5
	Mel-B	1/5	0/5	0/5	0/5	0/5	0/5	1/5

Results are shown as a proportion of the

$$\frac{\text{(Number of mice in which the presence of parasites was detected)}}{\text{(Number of mice tested)}}$$

ND: Not done. Tail blood, Buffy coat: Parasitological observation of parasites by direct microscopy and buffy coat respectively. Latex: Latex agglutination test (Suratex®). PCR: Polymerase chain reaction

SCID mice inoculated with blood from previously infected but Mel-B or PSG-treated BALB/c mice were observed for up to 115 DPI for evidence of parasites (Table 2). No parasites were observed from either the tail blood or buffy coat of all the SCID mice inoculated with blood from Mel-B treated BALB/c mice, while PCR at 24 DPI was negative. However, only 3 of the 5 SCID mice inoculated with blood from PSG-treated BALB/c developed parasitologically detectable trypanosome infection by wet blood film or buffy coat by day 100 post blood inoculation (PBI). One of the two apparently negative SCID mice was PCR positive for trypanosome infection on day 18 PBI, and was parasitologically and PCR negative on subsequent analyses up to day 100 PBI. However, on day 115 PBI, parasites were diagnosed from the tail blood of this mouse. Three out of five SCID mice previously inoculated with blood from Mel-B treated BALB/c mice showed evidence of infection by PCR at day 65 PBI (Table 2) but were negative by the other diagnostic techniques. However, at day 100 PBI, these SCID mice were all PCR negative.

Table 2. Results of various diagnostic tests used to detect *Trypanosoma brucei gambiense* IL3253 in SCID mice inoculated with blood from previously infected BALB/c treated with either Mel-B or PSG

Test	Group	Days post blood inoculation										
		2	8	11	16	18	24	65	80	86	100	115
Tail blood	PSG	0/5	1/5	3/5	3/5	2/5	3/5	2/3	2/3	2/3	2/3	3/3
	Mel-B	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Buffy coat	PSG	0/5	1/5	3/5	3/5	3/5	3/5	2/3	2/3	2/3	2/3	3/3
	Mel-B	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
PCR	PSG	ND	ND	ND	ND	1/2	0/2	0/1	0/1	1/1	0/1	ND
	Mel-B	ND	ND	ND	ND	0/5	0/5	3/5	1/5	0/5	0/5	ND

Results are shown as a proportion of the

$$\frac{\text{(Number of mice in which the presence of parasites was detected)}}{\text{(Number of mice tested)}}$$

ND: Not done. Tail blood, Buffy coat: Parasitological observation of parasites by direct microscopy and buffy coat respectively. Diagnosis by PCR for the control group was only performed on two mice that were parasitologically negative for trypanosomes. All the Mel-B treated mice were however screened by PCR.

DISCUSSION

Trypanosoma b. gambiense IL3253 is a low virulent parasite (Inoue et al. 1998), and therefore even in PSG-treated mice, parasitological diagnosis by either wet blood film or buffy coat was usually negative. The superior sensitivity of PCR was demonstrated at the end of the first treatment schedule (7 DPI) when all Mel-B treated mice were PCR negative, while all PSG-treated mice were PCR positive for trypanosome infection (Table 1). All the mice at this stage were negative for parasites when conventional parasitological diagnostic techniques by direct observation for parasites in tail blood or the more sensitive buffy coat method were used. Over an observation period of up to 27 DPI, all the Mel-B treated mice were negative for trypanosomes by all the diagnostic tests used. However, at 34 DPI, one mouse in this group was diagnosed as positive by PCR.

A total of 3 out of 5 mice were observed to have parasites from either their tail blood or buffy coat after blood from *T. b. rhodesiense*-infected PSG-treated BALB/c was inoculated into SCID, while 4 out of 5 were positive by PCR. On the other hand, no parasites were observed using the same techniques from SCID mice inoculated with blood from Mel-B treated BALB/c, though at day 65 PBI, some of them were shown to harbor an infection by PCR (Table 2). The single time that

SCID mice inoculated with blood from Mel-B treated BALB/c were PCR positive may indicate that a very small proportion of parasites do remain in some tissues in the animal even after treatment and the animals have been assumed to be cured. Because these mice were subsequently negative for PCR, it is conceivable that the parasite material was negligible, and having been rendered near non-virulent by drug treatment, that these parasites were ultimately cleared by the host immune response. These results show the difficulty associated with the treatment of sleeping sickness, and the extreme care and caution required in the diagnosis and treatment follow up of this disease.

The latex test repeatedly gave inconsistent results in the diagnosis of BALB/c or SCID mice experimentally infected with *T. b. gambiense*. In certain cases, mice that were naive to trypanosomal infection gave false positive results with this test (data not shown). This of course is in total contrast to the known efficacy of this test to diagnose trypanosomal infections in domestic ungulates and in humans (Komba et. al. 1992; Nantulya et al. 1992). We suspect that probably BALB/c and SCID mice inherently react positively with Suratex® (latex reagent) in a non specific fashion.

The criteria for cure is that in patients treated in hemolymph stage of the disease, there should be no clinical sign of the disease, or abnormality of blood or cerebrospinal fluid, after several examinations during two years. In patients treated in the meningoencephalitic stage there should be no clinical signs (except perhaps of irreversible damage to the nervous system done before treatment); the cell count of the cerebrospinal fluid should fall steadily to below 5 mm^3 and the protein content to below 0.3 mg/ml. Surveillance should continue for two to three years and for even longer in *T. b. gambiense* infections. There is no doubt that each of the techniques used for the diagnosis of trypanosomosis in this study (inoculation of blood into an animal model, parasitology, PCR) is a useful diagnostic tool. Further, these results show evidence of the efficacy of Mel-B, and further demonstrate the sensitivity of PCR in the diagnosis of trypanosome infections. A repeated negative PCR test in combination with conventional microscopical examination and clinical evaluation may be a useful diagnostic regimen in the diagnosis of trypanosomosis.

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