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Diagnosis of human trypanosomiasis, due to *Trypanosoma brucei gambiense* in central Africa, by the polymerase chain reaction

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Abstract

During a mass screening of sleeping sickness conducted in 1998 and 1999, and involving 27 932 persons in Cameroon and the Central African Republic, we tested the polymerase chain reaction (PCR) on whole blood for the diagnosis of human African trypanosomiasis due to *Trypanosoma brucei gambiense*. The 1858 samples obtained were from 4 groups: 155 infected patients, 1432 serological suspects detected by the card agglutination test for trypanosomiasis (CATT), 222 negative controls living in the prospected area (negative with the CATT and parasitological methods), and 49 negative controls (CATT and parasitological methods) and unexposed to the disease (Europeans). The technique of DNA extraction used made it possible to preserve the blood samples in the field. The primers used were specific for *T. brucei s.l.* Only 1 patient was PCR negative, and 3 of the negative controls, exposed to the disease, were PCR positive. Among the 1432 serological suspects, only 50 were PCR positive. During the 6-month follow-up after the surveys, the 3 negative controls, who were initially positive by PCR, were found to be negative. These initial positive PCR results are unlikely to have been due to a cross-reaction with *T. brucei brucei*, which is non-pathogenic for man, but are more likely to have resulted from a mislabelling of sample tubes. All control individuals, exposed or not to the disease, were negative by PCR. The PCR-negative patient was possibly a registration error. Among 50 PCR positive serological suspects, 39 of them were re-examined. Five were found to be positive by the kit for in-vitro isolation of trypanosomes, representing an increase in patients of almost 13%. At the end of the study, 160 patients were diagnosed, and the PCR was positive for 159 of them (99·4%). Moreover, the PCR made it possible to reduce the number of suspects to be re-examined (50 instead of 1432; a reduction of 96·5%).

Keywords: human African trypanosomiasis, sleeping sickness, *Trypanosoma brucei gambiense*, diagnosis, polymerase chain reaction, Cameroon, Central African Republic

Introduction

A mass survey within the framework of the fight against the sleeping sickness is usually done in 2 steps. First, a serological screening by the card agglutination test for trypanosomiasis (CATT) is done (MAGNUS *et al.*, 1978). Secondly, parasites are sought in lymph nodes, if adenopathies were noted, and in blood for CATT positive subjects. For this last purpose, one can use haematocrit centrifugation (WOO, 1971), the miniature anion-exchange centrifugation technique (mAECT; LUMSDEN, 1979) or quantitative buffy coat (QBC; BAILEY & SMITH, 1992). If the trypanosome is detected, a lumbar puncture is done to define the stage of the disease.

It has been proven that in many cases the parasite is not visualized under microscopy even if the subject is serologically and/or clinically suspect. The detection of trypanosomes in the blood is often made difficult because of the relatively poor sensitivity of the parasitological techniques and parasitaemia that is often low and fluctuating. However, direct microscopy detection of the parasites is essential to decide the best treatment for the patients. Because of the toxicity of melarsoprol, and in accordance with the recommendations of World Health Organization (WHO), the drug is administered only if the parasites are observed. Because of the limits of the techniques used, a certain number of patients are not detected. These false-negative cases can lead to the maintenance of a residual human reservoir in the prospected foci. At present, the most powerful diagnostic techniques include:

- mAECT, with a threshold of detection of 3–4 trypanosomes/mL blood (WHO, 1998) and,
- QBC, with a threshold of detection of 16 trypanosomes/mL blood (*loc. cit.*).

Polymerase chain reaction (PCR) makes it possible to preserve the samples in field conditions and treat them later in the laboratory. Its detection limit is 25 (KANMOGNE *et al.*, 1996) or 40 trypanosomes/mL (KABIRI *et al.*, 1999). With the DNA extraction technique described by PENCHENIER *et al.* (1996), the one used in our study, PCR detects, under experimental conditions, 1 trypanosome/mL blood. Therefore it is an excellent tool for the diagnosis of trypanosomiases.

The aims of this study were to test this PCR technique during mass screenings, determine its sensitivity and specificity and evaluate the number of false-negative patients using classical parasitological diagnostic techniques. For this purpose, we included and compared in our study a group of African subjects exposed to the disease and unexposed Europeans. Finally, we evaluated the proportion of serological suspects (positive with serological tests but negative with the parasitological diagnosis) who were positive by PCR in comparison with the serological suspects because these individuals represent the population at risk and thus need to be followed.

Material and Methods

Prospected foci

The surveys were conducted in 1998 and 1999 at 4 historical foci of sleeping sickness: 3 foci with a low prevalence in Cameroon (Campo, along the coast, at the frontier between Cameroon and Equatorial Guinea; Bipindi, in the forest belt of central Cameroon; and Fontem, in the west, down the Bamboutos mountains) and a focus with a higher prevalence, Batangafo, in Central African Republic (in the north of Bangui, close to the Chadian border). These surveys were the most exhaustive conducted up to date.

Controls

The blood samples of Europeans used as negative controls (all volunteers without contact with known foci of the disease), and blood samples of Africans living in sleeping sickness areas and used as further negative controls, were all negative by both serological and parasitological examinations.

Techniques

Each survey proceeded according to the standard protocol described previously (PENCHENIER *et al.*, 1999), including a serological screening by CATT, a search for adenopathies with lymph node aspiration, and QBC. Blood samples from all strongly positive serolo-

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gical suspects that were negative according to the other techniques used were cultured with the kit for in-vitro isolation of trypanosomes in the field (KIVI; AERTS *et al.*, 1992).

The blood samples obtained in vacutainers containing EDTA were stored at about 4°C and used as templates for PCR after DNA extraction with the kit Ready AMP™ genomic purification system (Promega, Madison, WI, USA) at the Molecular Biology Laboratory of OCEAC. This kit allows DNA extraction from more than 100 samples in a single day. The technique initially described by PENCHENIER *et al.* (1999) was modified by increasing the duration of the incubation period at 56°C to 30 min (instead of 20 min) and of incubation at 100°C to 15 min (instead of 10 min). The PCR technique was performed by using TBR1 and TBR2 *T. brucei s.l.* specific primers (MOSER *et al.*, 1989).

Study groups

The survey included 27 932 persons: 13 092 from Fontem, 5255 from Campo, 6696 from Bipindi, and 2889 from Batangafo. With the 49 Europeans used as negative controls, a total of 27 981 subjects were enrolled in this study.

We carried out PCR on 1858 blood samples corresponding as shown to the 4 following groups.

- Patients: 155 CATT positive and positive with the QBC and/or lymph-node aspiration.
- Serological suspects: 1432 CATT positive, but parasitologically negative.
- Negative controls exposed to the disease: 222 people living in foci, who were CATT negative and parasitologically negative.
- Non-exposed negative controls: 49 European volunteers without any contact with the disease who were CATT negative and parasitologically negative.

Reproducibility control

These control tests were carried out 1–3 months after screening on 22 patients who were PCR positive, and during a 6-month follow-up of 33 exposed PCR negative control individuals.

Survey of serological suspects and negative control individuals

All the serological suspects who were positive by PCR, except those of the Central African Republic, were followed-up by QBC and KIVI. We also examined under microscopy blood samples from a group of 111 persons strongly serologically suspect but PCR negative. Finally we followed-up the exposed negative control individuals who were PCR positive during the first sampling.

Results

First survey

The results obtained from mass screening are shown in the Table.

Of the 155 patients, only 1 was PCR negative. These patients was detected by CATT and diagnosed by lymph-node puncture. Among the serological suspects, 50 were PCR positive and 1382 PCR negative, which yields a positivity rate of 3·5%. Among the exposed controls, 3 were PCR positive. In the group of non-exposed controls, none was positive.

Reproducibility of the results

Of the 22 PCR positive patients re-examined 1–3 months after diagnosis, and before treatment, all remained positive by PCR; 33 PCR negative persons followed during 6 months remained negative.

To study the influence of blood conservation, we carried out PCR on samples stored during 1 month at room temperature in the laboratory and others stored for >3 months at 4°C. The results were identical to those obtained on samples treated immediately after sampling.

Survey of serological suspects and negative control individuals

The results obtained for control individuals are shown in the Table.

The PCR negative patient was tested 4 times; first, 1 month after diagnosis, then monthly. All the PCRs remained negative. At the end of the survey, there were 1432 serological suspects, including 50 PCR positive cases. Among these suspects, 7 were from the Batangafo focus and were not followed-up. Of the 43 remaining suspects, 39 (90·7%) were re-examined. Five were found to be positive by KIVI; the sixth suspect was negative by PCR.

Among the 111 PCR negative serological suspects examined, no parasitological test (QBC and KIVI) was able to find the trypanosome after 3 months, and PCR remained negative.

The 3 'negative exposed' control individuals who were PCR positive were followed-up after 3 months. They all became negative. There was thus no longer any PCR positive person among this negative control group.

At the end of the study, PCR was positive for 99·4% (159/160) of the patients and negative in all 222 exposed

Table. PCR results with *T. brucei*-specific primers, at the beginning of the study (1st survey) and on follow-up (2nd survey) in Cameroon and the Central African Republic

						Negative controls			
		Patients[a]		Serological suspects[b]		African exposed		European non-exposed	
	n	PCR+	PCR−	PCR+	PCR−	PCR+	PCR−	PCR+	PCR−
1st survey									
Fontem	13092	6	0	33	760	0	15	–	–
Campo	5255	17	0	4	369	3	146	–	–
Bipindi	6696	41	1	6	162	0	58	–	–
Batangafo	2889	90	0	7	91	–	–	–	–
Yaoundé	49							0	49
Total	27981	154 (99·4%)	1 (0·6%)	50 (3·5%)	1382 (96·5%)	3 (1·4%)	219 (98·6%)	0 (0·0%)	49 (100%)
2nd survey									
Total	27981	159 (99·4%)	1 (0·6%)	44 (3·1%)	1383 (96·9%)	0 (0%)	222 (100%)	0 (0·0%)	49 (100%)

[a]Positive by both the card agglutination test for trypanosomiasis (CATT) and parasitological examination.
[b]CATT positive but negative in parasitological tests (lymph-node aspiration and quantitative buffy coat).

control or 49 unexposed control individuals. PCR was negative for 96·9% (1383/1427) of the serological suspects. The increase in detected patients, among the re-examined suspects, was 12·8% (5/39).

Discussion and Conclusion

Our results show that PCR used according to our technique, compared to those used by KANMOGNE *et al.* (1996) or KABIRI *et al.* (1999), is more sensitive.

The infection in 1 PCR negative patient was detected by lymph-node puncture and confirmed by KIVI, meaning that trypanosomes were present in the blood and PCR should have been positive. Repeated PCR amplifications were carried out on this patient's blood samples from 1 to 4 months after treatment, but the results were all negative. However, a registration error cannot be excluded. The discordance noted between the PCR and parasitological methods is possibly due to poor or erroneous labelling of the sample tube during the survey. It is thus possible that this patient was PCR positive.

Despite this single exception, PCR remains a very sensitive technique since, after controls, 99·4% (159/160) of the patients were positive. Our PCR protocol is also very specific. On the one hand, the 111 PCR negative serological suspects were found to be uninfected during follow-up. On the other hand, all the non-exposed negative controls and 98·6% (219/222) of the exposed negative controls were PCR negative. For the 3 exposed negative control individuals (from the group of 222 examined) who were PCR positive, the most likely explanation could also be a mislabelling of the sample tubes, an easy mistake under field conditions during a mass survey. It is unlikely that the positive PCR results in these suspects could be explained by the (non-specific) amplification of DNA fragments of the non-pathogenic for humans species *T. brucei*. The fact that the other set of 49 non-exposed control samples from Yaoundé, collected under much less difficult conditions, were PCR negative as expected re-inforces this hypothesis.

PCR is an excellent diagnostic technique. One of its major advantages compared to the other techniques is its high sensitivity and specificity: it allowed the detection of a higher number of patients (5/39): an increase of nearly 13%.

Taking into account these results, does a PCR remaining positive after control make it possible to affirm that the person is still infected and, conversely, a negative PCR means that the person is not infected?

This question is fundamental for the follow-up of serological suspects. In practice, during a mass survey, after treatment of the diagnosis-confirmed patients, there still remains a high number of serological suspects to monitor. During our surveys, this population varied from 7·1% (Campo) to 2·5% (Bipindi), that is to say, for the whole of the 4 foci, a total of 1432 persons. With the PCR as a diagnostic test, the number of serological suspects to follow-up was decreased to 50, i.e., a reduction of 96·5% (1382/1432).

The good conservation under field conditions of the DNA in blood sampled in EDTA tubes allows their shipping to research laboratories for rapid PCR diagnosis. The laboratories can rapidly send back the results of the PCR to the medical teams in the field for treatment of these cases. This system is more flexible and cheaper than sending a medical team to find all the serological suspects. There remains the problem of the PCR positive serological suspects lost to follow-up, the proportion of which was, in this study, around 9% (4/43). But how many patients would we have lost if we had had to seek 1432 serological suspects?

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References

Aerts, D., Truc, P., Penchenier, L., Claes, Y. & Le Ray, D. (1992). A kit for *in vitro* isolation of trypanosomes in the field: first trial with sleeping sickness patients in the Congo Republic. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 394–395.

Bailey, J. W. & Smith, D. H. (1992). The use of acridine orange QBC technique in the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 630.

Kabiri, M., Franco, J. R., Simarro, P. P., Ruiz, J. A., Sarsa, M. & Steverding, D. (1999). Detection of *Trypanosoma brucei gambiense* in sleeping-sickness suspects by PCR amplification of expression-site-associated genes 6 and 7. *Tropical Medicine and International Health*, **4**, 658–661.

Kanmogne, G. D., Asonganyi, T. & Gibson, W. C. (1996). Detection of *Trypanosoma brucei gambiense*, in serologically positive but aparasitaemic sleeping-sickness suspects in Cameroon, by PCR. *Annals of Tropical Medicine and Parasitology*, **90**, 475–483.

Lumsden, W. H. R., Kimber, C. D., Evans, D. A. & Doig, S. J. (1979). *Trypanosoma brucei*: miniature anion-exchange centrifugation technique for detection of low parasitaemias: adaptation for field use. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **73**, 312–317.

Magnus, E., Vervoort, T. & van Meirvenne, N. (1978). A card-agglutination test with stained trypanosomes (C.A.T.T.) for serological diagnosis of *Trypanosoma b. gambiense* trypanosomiasis. *Annales de la Société belge de Médecine Tropicale*, **58**, 169–176.

Moser, D. R., Cook, G. A., Ochs, D. E., Bailey, C. P., McKane, M. R. & Donelson, J. E. (1989). Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology*, **99**, 57–66.

Penchenier, L., Dumas, V., Grébaut, P., Reifenberg, J. M. & Cuny, G. (1996). Improvement of blood and fly gut processing for PCR diagnosis of trypanosomosis. *Parasite*, **4**, 387–389.

Penchenier, L., Grébaut, P., Ebo'o Eyenga, V., Bodo, J. M., Njiokou, F., Binzouli, J. J., Simarro, P., Soula, G., Herder, S. & Laveissière, C. (1999). Le foyer de trypanosomose humaine de Campo (Cameroun). Historique et situation de l'endémie en 1998. *Bulletin de la Société de Pathologie Exotique*, **92**, 3, 185–190.

WHO (1998). *La Trypanosomiase Africaine: Lutte et Surveillance*. Geneva: World Health Organization, Technical Report Series, no. 881.

Woo, P. T. K. (1971). Evaluation of the hæmatocrit centrifuge and other techniques for the field diagnosis of human trypanosomiasis and filariasis. *Acta Tropica*, **28**, 298–303.

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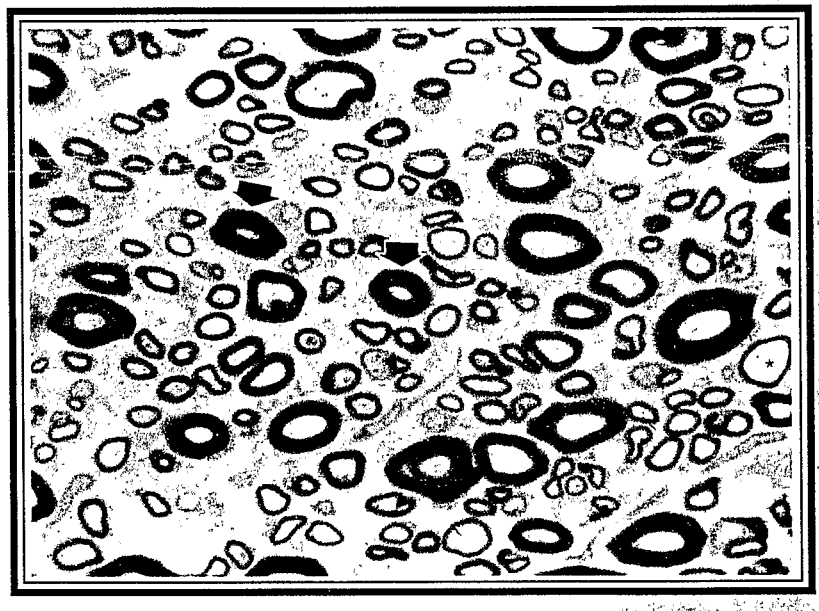

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