DETECTION OF PCR PRODUCTS VIA OLIGOCHROMATOGRAPHY (DIPSTICKS)

[1]FILIP CLAES, [2] CORIS BIOCONCEPT

[1] Institute of Tropical Medicine
Parasitology Department
Antwerpen

[2] Science Park Crealys,
Gembloux

Belgium

Abstract

A concept from CORIS was developed under IAEA contract number 12851/RBF. Establishment of the dipstick test in the laboratory (proof of principle) and comparison of sensitivity and specificity with classic agarose detection was made.

1. BACKGROUND

The detection of PCR products is usually made using horizontal electrophoresis in agarose gels. This detection method is rather time consuming (+/- 1 h), a U.V. detection system is needed, and there are health and environmental risks because of the need for ethidium bromide to detect DNA in agarose (carcinogenic, disposal of EtBr). Moreover, this system is costly and not suitable for individual sample testing. To overcome these problems, a biotech company involved in molecular diagnostics, Coris Bioconcept, developed a single test format detection system, the Oligochromatography. This system has been first developed for *Toxoplasma gondii*, but could be adapted for the detection of Trypanosomal DNA. Coris Bioconcept, who has the IPR of the system, expressed their interest in collaborating to develop dipstick tests for human African Trypanosomiasis.

Oligochromatography as explained in Fig. 1 is a simple and rapid dipstick test for detection of amplified PCR products.

A complex made of PCR products, with incorporated haptens, hybridized with a specific gold conjugate probe binds to an anti-hapten antibody immobilized on the stick. This binding reaction will give rise to a collared signal.

Advantages are:

(i) hybridisation takes place at constant temperature (55°C or lower), (ii) different anti-hapten antibodies can be immobilised on one dipstick and thus allow one-step detection of different gene amplification products, (iii) the test takes only 5 min and doesn't need any specific material nor skill to be performed.

18S ribosomal RNA gene (rDNA) was chosen as the new target gene for PCR since (i) it is a multi copy gene (200 copies) and (ii) it contains both conserved and polymorphic sequences.

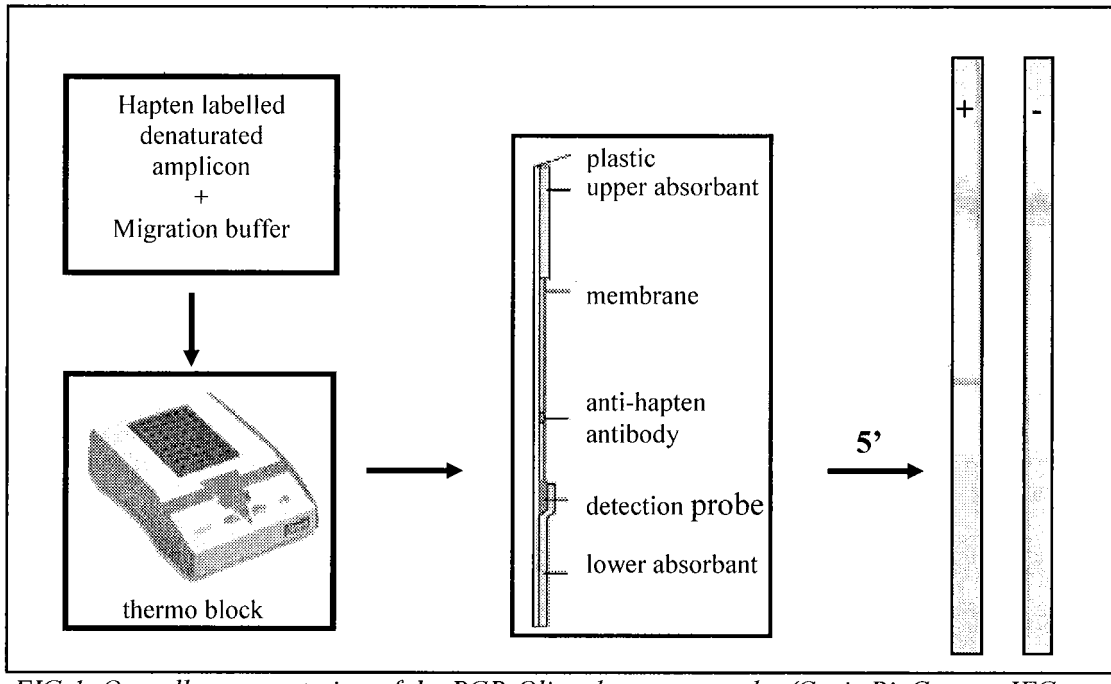


FIG.1. Overall representation of the PCR-Oligochromatography (Coris BioConceptIFG

2. OBJECTIVES OF DEVELOPMENTS THROUGH TECHNICAL CONTRACT

2.1. Development

Standard PCR protocols were adapted for use in the system: primers replaced by labelled primers and amplification conditions optimized. In first instance PCR-ISG and PCR-ITS, were used, since they seem to have the highest sensitivity. Later, more specific PCRs (SRA, TgsGP and RoTat) may be modified.

2.2. Evaluation

Use of PCR-dipstick detection test on the available DNA bank, including *T.b.brucei* (n=7), *T.b.gambiense* (n=9), T.b.rhodesiense (n=9), *T.evansi* (n=15), *T.equiperdum* (n=12), *T.congolense* (n=5) and *T.vivax* (n=4) (month 7-8). Use of PCR-dipstick detection test on available DNA from experimentally infected rabbits (n=50) and field samples (n=200).

3. RESULTS

- A Trypanosomatidae specific PCR on the 18S rRNA gene (rDNA) was developed and optimized in which the primers are situated in Trypanosomatidae conserved regions and the amplified region is polymorphic.
- A Trypanozoon specific Oligochromatography dipstick through specific probes was developed and optimized.

3.1. In silico selection of a target region in the 18S rRNA gene

In silico design of universal primers to amplify a short Trypanosomatidae specific DNA sequence by PCR. The reverse primer was biotin labelled.

Development of the PCR to amplify the Trypanosomatidae specific sequence.

Development of the PCR to amplify the specific sequences for all Trypanosomatidae.

- $MgCl_2$ concentration was optimized.
- Primers concentration was optimized.
- Annealing T was optimized
- Annealing and elongation time was optimized.
- Polymerase concentration was optimized.
- Cycle number was optimized.

Note. During optimisation of the PCR, there were problems with contamination. After sequencing this contamination was identified as the specific amplification of Bodo sp. rDNA. Bodo sp. is a free living flagellate which lives in water. This Bodo DNA was proven to be present in the QiaAMP extraction kit buffers. This problem is contained now.

3.2. Optimization results

The following PCR primers and protocol give the best results.

3.3. Primers

18S-F:	5'-CGCCAAGCTAATACATGAACCAA-3'	Tm: 66.6°C
18S-R:	5'-Biotin-TAATTTCATTCATTCGCTGGACG-3'	Tm: 66.6°C

3.4. PCR protocol

PCR mix:		**50 µL**
	Water	
	Buffer	1 X
	$MgCl_2$	2.5 m*M*
	dNTP	200 µ*M* each
	F-primer	0.2 µ*M*
	R-primer	0.8 µ*M*
	Hot Start Taq	0.5U
	DNA	

3.5. PCR programme

94°C	15 min	
94°C	30 s	45X
58°C	30 s	
72°C	30 s	
72°C	1 min	

3.6. In silico design of a Trypanozoon specific probe

Probe:

T. brucei: 18S-PR-B TTGTGTTTACGCACTTG Tm: 49.1°C 17bp

3.7. Development of the PCR-Oligochromatography detection system with avidin immobilized on the dipstick in collaboration with Coris Bioconcept

The avidin concentrates the amplicon and only if the *Trypanozoon* amplicon is present the dipstick will show a visible band.

(1) Primer concentration in PCR was modified for optimal performance of the Oligochromatography.

(2) Primer ratio in PCR was modified for optimal performance of the Oligochromatography.

(3) Avidin concentration on dipstick was optimized.

(4) Gold conjugation on probe was optimized.

(5) Migration buffer was optimized.

3.8. Detection Limits

The detection limit of the PCR assay on human blood spiked with decreasing numbers of *T.brucei.* Comparison of detection by agarose gel and detection by oligochromatography.

(1) 10^4 parasites / 180 µL blood

(2) 10^3 parasites / 180 µL blood

(3) 10^2 parasites / 180 µL blood

(4) 10 parasites / 180 µL blood

(5) 1 parasite / 180 µL blood

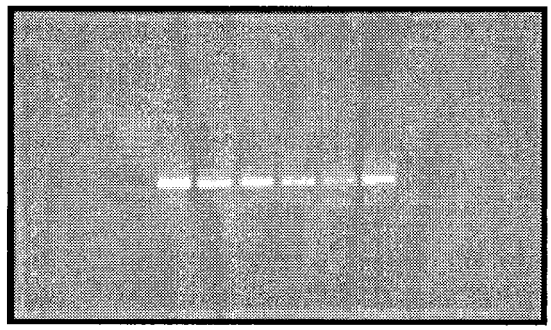
(6) Pos. control PCR *(T.b.* DNA)

(7) Neg. control extraction

(8) Neg. control PCR (H_2O)

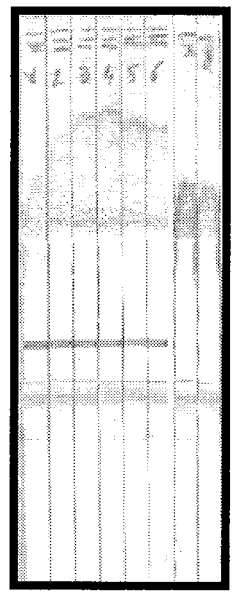
DNA extraction from the blood samples was performed using the QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Detection with 2% agarose gel



Detection limit 1 parasite / 180 µL blood

Detection with dipstick



Detection limit 1 parasite / 180 µL blood

3.9. Check on the specificity of the PCR assay on non-target pathogen DNA

(1) The PCR amplifies all *Trypanozoon* (*in silico*, not yet tested experimentally).

(2) The PCR does not amplify other pathogens present in the endemic regions (*Plasmodium falciparum*, *microfilaria* and *Mycobacterium* DNA, checked experimentally).

(3) The PCR amplifies *Leishmania* which is endemic in East-Africa because this genus also belong to the *Trypanosomatidae* group [detected by electrophoresis on agarose gel].

3.9. Check of the specificity of the Oligochromatography dipstick on human blood spiked with *Trypanozoon* and non target PCR product of *Leishmania.*

The Trypanozoon Oligochromatography test detects *T.b. gambiense, T.b.rhodesiense* and in silico all the other subspecies within the *Trypanozoon* group (not tested experimentally yet) but does not detect the Leishmania PCR products.

4. CONCLUSIONS

A first prototype PCR-Oligochromatography assay for molecular diagnosis of *Trypanozoon* infections was successfully developed. The assay, so far, is specific for *Trypanozoon* and has a detection limit of 1 parasite in 180 µL of blood.

5. FUTURE WORK

To test experimentally the specificity of the 18S rDNA PCR on all the subspecies within the Trypanozoon group.

To test experimentally the specificity of the oligochromatography dipstick on all the subspecies within the Trypanozoon group.

5.1. Development of the internal controls on the rear side of the dipstick

Control for PCR

A synthetic oligonucleotide will be added to the PCR mix which will be amplified by the same primers as the target sequence. This control oligonucleotide will always be amplified if the PCR works well. On the backside of the dipstick a gold conjugated probe specific for the internal control sequence will be placed in the probe conjugate pad. The anti-hapten antibodies will be immobilized on a specified place on the dipstick. During Oligochromatography the internal control PCR product will be detected on the backside of the dipstick through hybridisation with the internal control specific probe (see Fig. 2). In comparison with the gel detection of PCR products this inclusion of the internal control for PCR is a great improvement. The internal control may also be included in a gel detection format. However the Oligochromatography detection format has the advantage that an internal control with exactly the same length as the target amplicon can be used which is obviously not the case with the gel detection format.

Control for migration

Next to the internal control for PCR an internal control for migration will also be included on the backside of the dipstick. An oligonucleotide complementary to the internal

control probe for PCR will be immobilized (see Fig. 2) on a specified place on the backside of the dipstick. During migration the gold labelled internal control probe will hybridize on this oligonucleotide which will give rise to a coloured signal. If the dipstick works properly this control line has always to be positive.

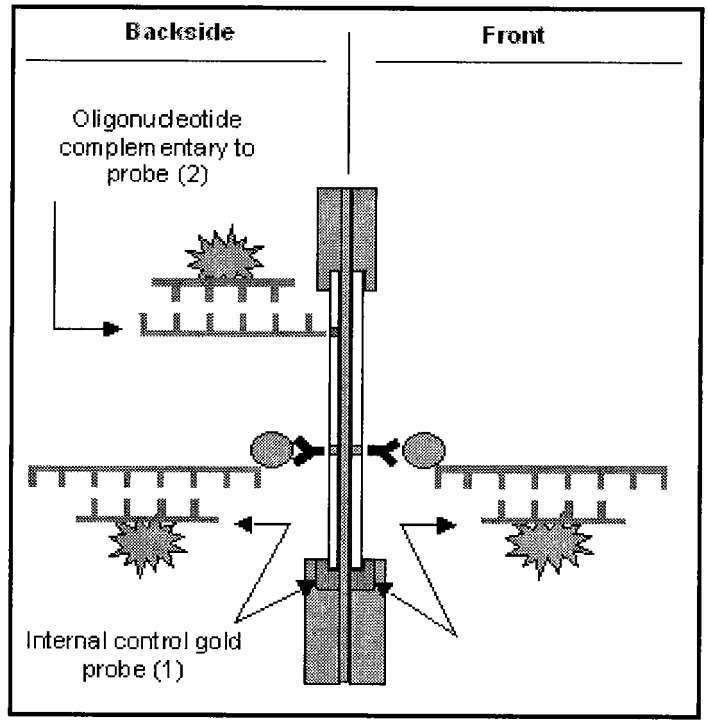


FIG. 2. Representation of the detection of the target PCR product at the front of the dipstick and internal controls for PCR (1) and for migration (2) on the rear of the dipstick.

Short laboratory (Phase I) evaluation of the prototype PCR-Oligochromatography for Trypanozoon detection

Production of sufficient prototype tests for the evaluation

Testing the diagnostic sensitivity and specificity of the prototype PCR-Oligochromatography test on available DNA from experimentally infected rabbits (n=50) and on a collection of blood samples from confirmed sleeping sickness patients (n=100) and the negative endemic controls (n=100) from R.D. Congo.

6. PERSPECTIVES

When this project is finished and a proof-of-principle evaluated prototype of the PCR-Oligochromatography for *Trypanozoon* detection is available, a Phase II evaluation can be started and managed by the IAEA in collaboration with Coris Bioconcept. If successfully evaluated a Phase III large scale evaluation can be started. Now the technique is available, the same strategy could be followed to diagnose animal Trypanosomiasis. *In silico* work showed us that the same PCR could be used to amplify *T.vivax, T.evansi, T.equiperdum, T. brucei, T.vivax* and *T.congolense* and that it will be possible to design specific probes to discriminate between the *T.congolense* group, the *T.vivax* group and the *Trypanozoon* group.

7. RING TESTING OF TRYPSTICK UNDER THE CRP

At the final RCM in Vietnam, 2005 it was decided to make a ring test to examine the performance of the Trypstick.

The reagents were supervised by F. Claes in Antwerp who also organised the transportation of the Trypsticks from the company.

Five contract or agreement holder laboratories were involved as shown in Table below as well as the laboratory in Antwerp.

	Name	Address	Info	RC number
1.	P.Solano	P. Solano Institut Pierre Richet s/c IRD, rue Fleming, 04BP 293, Abidjan 04 Côte d'Ivoire	Tel: (225) 21 35 43 70/ 21 35 70 67; Fax: (225) 21 35 40 15 E-mail: solano@ird.ci	IVC 11413
2.	J. Kangethe Kinyua	J. Kangethe Kinyua Kenya Agricultural Research Institute-Trypanosomiasis Research Center P.O. BOX 362 KIKUYU Kenya	Tel: 254 66 32960 Fax: E-mail: jkkinyuafr2001@yahoo.fr ; ketri@africaonline.co.ke	KEN 11414
3.	Viet Khong Nguyen	Viet Khong Nguyen National Institute of Veterinary Research 86 Truong Chinh, Dong Da, Hanoi, Vietnam	Tel: 0084 4 868 7642 Mobile 0913082035 Fax: 0084 4 8694082 nguykhon@fpt.vn	VIE 11420
4	J. Enyaru	J. Enyaru Livestock Health Research Institute (LIRI), P.O.Box 96, Tororo, Uganda	Tel: 226 20 97 20 53 Fax: 226 20 97 23 20 E-mail: jenyaru@hotmail.com	UGA 11418
5	P. Henning Clausen /	Peter Henning Clausen Institute of Parasitology and International Animal Health Freie Universitaet Berlin, Königsweg 67, D-14163 Berlin, Germany	Tel: +49 30 8386 2514 (office) Cell: + 49 179 862 42 00 (Cell Phone) E-mail: tropvetm@komma.zedat.fu-berlin.de	GFR 11412

Each laboratory received the following items.

- 4 boxes containing the PCR-Oligo Trypsticks (25 tests/kit, 4 kits, total: 100 tests)
- 1 box containing 23 control DNA samples for the Trypstick ring trial evaluation
- 4 boxes containing PCR-mix and polymerase, frozen
- The laboratories were told to fill in a receipt from and return it when they received the items.

A form was included to summarize data obtained in the study as shown below.

7.1. Checklist for Ring Test

For our own interpretation of the results of the *Trypanozoon*-PCR-OC ring trial, we kindly ask you to complete following checklist about the material you used during performing the tests.

(1) The PCR assay was performed in following thermocycler (please specify brand and type):

(2) The PCR assay was performed in following tubes (please specify brand and catalogue number):

3. The PCR assay was performed with ☐ heating lid

☐ mineral oil

4. The Oligochromatography assay was performed in a

☐ heating block with 13 mm tubes

☐ heating block with 1.5 mL tubes

☐ water bath with 13 mm tubes

5. The PCR mix at -20°C was only thawed one time?

☐ Yes

☐ No

If no please specify

6. I followed the protocol as described:

☐ Yes

☐ No

If no please specify

7. I encountered problems during performing the tests:

☐ Yes

☐ No

If yes please specify

8. Further remarks:

7.2. Test results 1

Please indicate + for positive result

- For negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control				
Neg. control				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

7.3. Test results 2

Please indicate + for positive result

- for negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control				
Neg. control				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

7.4. Test results 3

Please indicate + for positive result

- for negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control				
Neg. control				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

7.5. Actual results

CHECKLIST

For our own interpretation of the results of the *Trypanozoon*-PCR-OC ring trial, we kindly ask you to complete following checklist about the material you used during performing the tests.

(1) The PCR assay was performed in following thermocycler (please specify brand and type):

GeneAmP PCR Systems 9700 from Applied Biosystems Part no N8050200

(2) The PCR assay was performed in following tubes (please specify brand and catalogue number):

GeneAmP autoclaved reaction tubes with caps,part no N801-0612

(3) The PCR assay was performed with √ heating lid

☐ mineral oil

(4) 4. The Oligochromatography assay was performed in a

☐ heating block with 13 mm tubes

☐ heating block with 1.5 mL tubes

√ water bath with 13 mm tubes

(5) The PCR mix at -20°C was only thawed one time?

√ Yes

☐ No

If no please specify

(6) I followed the protocol as described:

√ Yes

☐ No

If no please specify

(7) I encountered problems during performing the tests:

☐ Yes

√ No

If yes please specify

(8) Further remarks:

The protocol was simple and straight forward to follow, with few manipulations to carry out.

TEST RESULTS 1

Laboratory: Livestock health Research Institute (LIRI), Tororo, Uganda

Executor: Dr. John Enyaru

Date: 09-02-2006

Please indicate + for positive result

- for negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control	+	+	-	+
Neg. control	-	+	+	-
1	+	+	-	+
2	-	+	+	-
3	-	+	+	-
4	-	+	+	-
5	-	+	+	-
6	-	+	+	-
7	+	+	-	+
8	+	+	-	+
9	-	+	+	-
10	-	+	+	-
11	+	+	-	+
12	-	+	+	-
13	+	+	-	+
14	+	+	-	+
15	-	+	+	-
16	-	+	+	-
17	+	+	-	+
18	-	+	+	-
19	+	+	-	+
20	-	+	+	-
21	+	+	-	+

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

TEST RESULTS 2

Laboratory: Livestock health Research Institute (LIRI), Tororo, Uganda

Executor: Dr. John Enyaru

Date: 09-02-2006

Please indicate + for positive result

- for negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control	+	+	-	+
Neg. control	-	+	+	-
1	+	+	-	+
2	-	+	+	-
3	-	+	+	-
4	-	+	+	-
5	-	+	+	-
6	-	+	+	-
7	+	+	-	+
8	+	+	-	+
9	-	+	+	-
10	-	+	+	-
11	+	+	-	+
12	-	+	+	-
13	+	+	-	+
14	+	+	-	+
15	-	+	+	-
16	-	+	+	-
17	+	+	-	+
18	-	+	+	-
19	+	+	-	+
20	-	+	+	-
21	+	+	-	+

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

TEST RESULTS 3

Laboratory: Livestock health Research Institute (LIRI), Tororo, Uganda

Executor: Dr. John Enyaru

Date: 09-02-2006

Please indicate + for positive result

- for negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control	+	+	-	+
Neg. control	-	+	+	-
1	+	+	-	+
2	-	+	+	-
3	-	+	+	-
4	-	+	+	-
5	-	+	+	-
6	-	+	+	-
7	+	+	-	+
8	+	+	-	+
9	-	+	+	-
10	-	+	+	-
11	+	+	-	+
12	-	+	+	-
13	+	+	-	+
14	+	+	-	+
15	-	+	+	-
16	-	+	+	-
17	+	+	-	+
18	-	+	+	-
19	+	+	-	+
20	-	+	+	-
21	+	+	-	+

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

8. OVERALL LABORATORY RESULTS

A sample set of 23 samples consisting of a positive (*T.b.brucei* DNA) and negative (human DNA) control and 21 coded "blind" identical DNA samples was sent to all participating laboratories together with the necessary standardized test reagents, a test report sheet, and a standard operating procedure (SOP), so the only sources of variability between the laboratories are the manipulator and the PCR thermocycler. Each laboratory received sufficient materials to perform PCR-Oligochromatography in triplicate for each sample.

The blind samples included a two-fold serial dilution series of *T.b.brucei* control DNA (7 samples) to evaluate the analytical sensitivity of the assay, the five different (sub)-species of *Trypanozoon* (*T.b.brucei*, *T.b.gambiense*, *T.b.rhodesiense*, *T.evansi*, and *T.equiperdum*), and nine non-*Trypanozoon* DNA samples to assess the analytical specificity of the diagnostic test (Table I).

DNA was extracted using the QIAamp DNA mini kit (Qiagen, Germany) according to manufacturer's manual and was quantified with a Nanodrop (Isogen, Belgium). 200 µL of each sample was sent on dry ice to each participant by express courier. The PCR-Oligochromatography protocol was performed as described by Deborggraeve et al. (2006).

The results of this multicenter trial were analysed using to the formulae described by Vandervoet et al. (2001). The advantage of these formulae is that they can be used to evaluate qualitative rather than quantitative data.

Two main parameters were analysed *in casu* the accordance (ACC) or intra-laboratory repeatability which is defined as the percentage chance of finding the same result for two identical DNA samples analysed in the same laboratory under standard operating conditions (independent from whether the result is correct or not), and the concordance (CON) or inter-laboratory reproducibility which is defined as the percentage chance of finding the same result for two identical samples analysed in different laboratories under standardized conditions. Confidence intervals were calculated via Monte-Carlo / Markov model with 5.000 iterations (ref).

Statistical evaluation from the data set, with 95% confidence intervals (CI) gave following results: an accordance of 88.7% (CI 84.4-92.5%), and a concordance of 88.1% (CI 84.3 – 92.3%). These data and their distribution are presented in Figs 1A and 1B respectively. Note that the results from lab 5 were excluded from this calculation due to multiple positive results in the negative sample population, possibly due to cross-contamination or errors during test performance. Thus, the final analysis was performed on the data from the 5 remaining laboratories.

The analytical sensitivity of the PCR-Oligochromatography assay was 2.5 fg of DNA per PCR reaction in 4 out of 5 laboratories (excluding the results from laboratory 5) and 20 fg DNA in laboratory 3. This means that in all laboratories evaluated, the assay can detect up to 1 parasite per reaction, since we assume that the genome of one Trypanosome is 0.2 pg. The target sequence of the assay is a multicopy 18S sequence which explains the higher analytical sensitivity on extracted DNA samples.

The assay was developed in such way to be *Trypanozoon* specific. Results from this trial show that this is indeed the case, except for some occasional false positive results (Table

8.1. Results for accuracy and concordance are satisfactory

This study shows that it is feasible to organize a trial in different continents (and including developing countries). To our knowledge, this is the first multicenter collaborative trial to be performed for diagnostic tests for human or animal Trypanosomiasis.

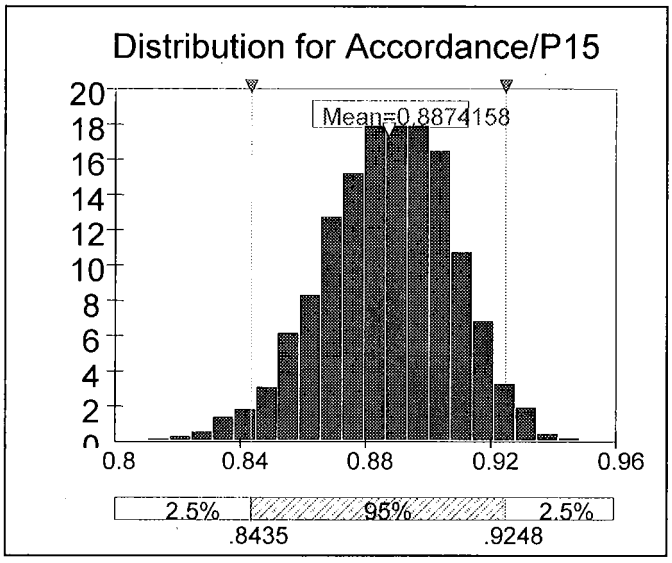
The results show that (i) PCR-oligochromatography may serve as diagnostic test for HAT, after a further phase III evaluation, (ii) this statistical approach may be used in the future to analyze other newly developed tests for HAT or animal Trypanosomiasis.

TABLE I. LABORATORY RESULTS IN THE COLLABORATIVE TRIAL OF THE PCR-OLIGOCHROMATOGRAPY ASSAY (3 REPETITIONS PER LABORATORY)

Sample	Expected	Lab 1			Lab 2			Lab 3			Lab 4			Lab 5			Lab 6		
	Result	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
T.b.brucei AnTat 2.2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Human DNA	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
T.b.brucei 2.5 fg	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1
T.b.brucei 5 fg	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1
T.b.brucei 10 fg	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1
T.b.brucei 20 fg	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
T.b.brucei 80 fg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.brucei 320 fg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.brucei 1280 fg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.gambiense LiTat 1.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.rhodesiense AnTat 25.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.evansi RoTat 1.2	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1
T.equiperdum OVI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.gambiense AnTat 9.1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1
T.congolense TRT 17	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
T.vivax ILRAD 700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Theileria parva	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Leishmania	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Plasmodium	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0
Schistosoma	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Bovine DNA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Trypanosoma cruzi	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0
Trypanosoma rangeli	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0

8.2. The accordance

Intra-laboratory repeatability was 88.7% (CI 84.4-92.5%)



8.3. The concordance

Inter-laboratory "reproducibility was 88.1% (CI 84.3-92.3%)