2009 International Nuclear Atlantic Conference - INAC 2009
Rio de Janeiro, RJ, Brazil, September 27 to October 2, 2009
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: 978-85-99141-03-8

COMPARISON OF KDNA PCR-HYBRIDIZATION ASSAY WITH THREE PCR METHODS FOR CANINE VISCERAL LEISHMANIASIS DIAGNOSIS

Marcia M. Pilatti[1], Sidney A. Ferreira[2] and Antero S. R. Andrade[1]

[1] Centro de Desenvolvimento da Tecnologia Nuclear (CDTN-MG)
Rua Professor Mário Werneck S/N°, Cidade Universitária-Campus da UFMG
31120-970 Belo Horizonte, MG
marciapilatti@yahoo.com.br
antero@cdtn.br

[2] Departamento de Parasitologia – Instituto de Ciências Biológicas
Universidade Federal de Minas Gerais
Av. Antônio Carlos 6627
31270-901 Belo Horizonte, MG
saninoalmeida@gmail.com

ABSTRACT

The sensitivity of the kDNA PCR-Hybridization assay, which uses radioactive DNA probes (labeled with ^{32}P), was compared with three conventional PCR methods used for canine visceral leishmaniasis diagnosis. All PCR methods had two steps: a first amplification followed by hybridization or by a new amplification (nested or semi nested). Two methods (kDNA PCR-Hybridization and kDNA snPCR) used primers addressed to kinetoplast minicircles and the other two methods to the coding (LnPCR) and intergenic noncoding regions (ITS-1 nPCR) of the ribosomal rRNA genes. The comparison was accomplished in two groups of 23 infected dogs using samples collected by the conjunctival swab procedure. In the Group 1 the DNA was extracted from cotton swabs by phenol-chloroform and in Group 2 by boiling. The most efficient PCR methods in the Group 1 were those based on kDNA targets. The kDNA PCR–Hybridization was able to detect parasites in 22/23 dogs (95.6%) and in 40/46 samples (86.9%). The kDNA snPCR was positive for 21/23 dogs (91.3%) and for 40/46 samples (86.9%). The positivities of the kDNA based methods were significantly higher than the positivities verified for the methods based on ribosomal rRNA genes ($p<0.05$). In the Group 2 the kDNA PCR–Hybridization showed a better performance detecting parasites in 18/23 dogs (78.3%) and in 31/46 samples (67.4%), significantly higher than the other three methods ($p<0.05$). The higher sensitivity of the minicircle kDNA based assays reported by others was confirmed in this study and kDNA PCR-Hybridization showed the best sensitivity among the assays evaluated.

1. INTRODUCTION

Visceral leishmaniasis (VL) is an endemic zoonotic disease caused by parasites of the *Leishmania donovani* complex. VL in the New World is caused by *Leishmania (Leishmania) chagasi* (= *L. (L.) infantum*) [1] which is transmitted mainly by the vector *Lutzomyia longipalpis* [2]. Dogs are considered the main domestic reservoir of the parasite, constituting part of the epidemiological cycle of human transmission with Brazil accounting for more than 90% of cases described in the New World [3]. The VL epidemiological control in Brazil involves the elimination of seropositive dogs, insecticide treatment (within domestic and peridomestic habitations) and systematic treatment of human cases [4]. The percentage of infected dogs in the endemic areas of the country ranges from 1% to 67%, however, the prevalence of infection is probably higher than reported from serological studies [5]. The classic methodology for leishmaniasis diagnosis includes direct microscopic identification of

the parasites on stained preparations, parasite isolation in culture medium and parasite inoculation in experimental animals. Serological tests are used for diagnosis in endemic areas and also for epidemiological studies. The most employed serological testes are complement fixation, indirect immunofluorescence (IFAT) [6] and ELISA [7]. Recently, immunohistochemistry and polymerase chain reaction (PCR) have been used with high sensitivity. PCR is a sensitive and specific method for detection of *Leishmania spp*. DNA in a variety of clinical samples and more sensitive than the classical procedures for canine VL diagnosis [8], suggesting that this methodology might become the gold standard for detecting *Leishmania*. In addition, PCR is an attractive alternative method to follow-up vaccinated dogs (an antileishmanial vaccine for dogs was licensed in Brazil) since these animals test positive in the conventional serological tests. Several kinds of biological samples can be used for the PCR, such as: spleen, lymph nodes, skin, conjunctival biopsy, blood and bone marrow. Blood and bone marrow are usually the most common tissues used [9]. Non invasive samplings are desirable since they are simpler, painless and more easily allowed by the dog owners. An interesting approach in this context is the conjunctival swab procedure, in which a sterile swab is used for sampling the dog conjunctivas. This method has showed high sensitivity [8] [10]. A major concern in the development and implementation o PCR for *Leishmania* diagnosis is the lack of standardization. Hundreds of works on PCR diagnosis of leishmaniasis have been published, but very few studies have compared different protocols [11]. In this study, we compared the sensitivity of the kDNA PCR-Hybridization assay, which uses radioactive DNA probes (labeled with ^{32}P), with the sensitivities of three conventional PCR methods used for canine visceral leishmaniasis diagnosis. The comparison was performed using DNA isolated from clinical samples obtained by the conjunctival swab procedure. All methods presented two steps: a first amplification followed by hybridization or a new amplification (nested or semi nested). Two of the methods (kDNA PCR-Hybridization and kDNA snPCR) used primers addressed to minicircles of kinetoplast (mitochondrial DNA) and the other two methods used primers to the coding (LnPCR) and intergenic noncoding regions (ITS-1 nPCR) of the ribosomal rRNA genes.

2. MATERIAL AND METHODS

2.1. Dogs and samples

Forty six symptomatic mongrel dogs, designated to compulsory euthanasia, were supplied by the Municipal Zoonotic Diseases Control Department of Belo Horizonte, Minas Gerais State, Brazil. The dogs were simultaneously positive for ELISA, IFAT and the complement fixation reaction. Exfoliative epithelial cells were collected from the right (RC) and left (LC) conjunctivas of each animal using sterile cotton swabs manufactured for bacteriological isolation. Each sample was analyzed separately to provide a replicate diagnosis for each animal. The cotton tips were broken and only the cotton parts were transferred to sterile tubes and stored at -20°C until being used. The 46 seropositive dogs were at random divided in two groups of 23 animals. For group 1, the DNA from cotton swabs was extracted by the phenol-chloroform method and for group 2 by boiling. This work was approved by the Animal Experimentation Ethics Committee of the Federal University of Minas Gerais (CETEA/UFMG) protocol number 198/2006.

2.2. DNA extraction

The DNA purification from cotton swabs of group 1 was carried out by the phenol-chloroform-isoamyl alcohol method as described by Strauss-Ayali (2004) [8]. The cottons from group 2 received 300 µl of double deionized water and were heated at 100°C for 15 min. The solution was eluted from the cotton and centrifuged at 15,000 x g for 10 min. The supernatant was collected and maintained at -20°C until being used.

2.4. PCR

Positive controls with *L. (L.) chagasi* (strain MHOM/1973/BH46) genomic DNA were used at 1.0 ng/µl. A negative control without DNA was included in all tests. The DNA sample volume of 1.0 µl was used for all methods. Four PCR protocols were tested.

2.4.1. kDNA PCR – hybridization

The PCR reaction mixture contained 0.2 mM of each dNTP, 0.2 nmol of each primer [5'–(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACAACCCC-3' and 5'GGGGAGGGGCGTTCTGCGAA-3'], 5.0 U of Taq DNA polymerase (Ludwig Biotec), 5.0 µl of 10X buffer (Tris-HCl 50 mM, [pH 8.3], KCl 50 mM), 2.0 mM $MgCl_2$ in a final volume of 50 µl. The amplifying conditions were: initial denaturation at 95°C for 15 min, 30 cycles including 94°C at 30 s, 50°C at 30 s, 72°C at 30 s, and a final extension at 72°C for 10 min. All amplification products were analyzed on 2% agarose gel and were stained with ethidium bromide. The target for amplification was a 120 base pairs (bp) sequence of Leishmania kDNA minicircle conserved region [12]. For the hybridization step 10 µl of each amplification product was mixed to 110 µl of NaOH 0.4 M, EDTA 25 mM [pH 8.0] solution and spotted on nylon membrane (Hybond –XL Amersham) using a Bio Dot apparatus (Hybri-dot manifold-BRL). After that, the membrane was rinsed with 2 x SSC (0.3 M NaCl, 0.3 mM sodium citrate) and dried. The DNA was fixed to the filter by UV light (0.12 J/cm^2). Cloned kDNA minicircles from *L. (L.) chagasi* were used as probes. The probe was labeled with ^{32}P[α]dCTP using the Random Primer DNA Labeling System (Invitrogen). Hybridization conditions were as previously described [13]. Briefly, the filters were pre-soaked at 58°C for 30 min in 0.5% non-fat milk, 1% sodium dodecyl sulphate (SDS) and 2 x SSC solution. The kDNA probe was added to the solution after being heated for 3 min in a boiling water bath. The filters were incubated for 14 h at 58°C, with shaking, then placed in 2 x SSC at room temperature for 20 min and washed in 0.5 x SSC, 0.5 x SDS, at 65°C for 30 min. Finally, it was dried and exposed in the cassette (BAS 2325 Fujifilm) for 2 h. The image was obtained using a Bio-Imaging Analyzer (Fujifilm).

2.4.2. kDNA semi nested PCR (kDNA snPCR)

The semi nested reaction was performed as previously described [14] with minor modifications. In brief, for the first amplification 1.0 µl of DNA solution was added to 49 µl of PCR mix containing 15 pmol specific *Leishmania* primers DRJ, 5´-CGA TTT TTG AAC GGG ATT TCT GCA C-3´, and KLK2, 5´-CTC CGG GGC GGG AAA CTG G-3´; 0.2 mM dNTP; 2 mM $MgCl_2$; 5.0 µl of 10X buffer (Tris-HCl 50 mM, [pH 8.3], KCl 50 mM) and 1,4 U of Taq DNA polymerase (Ludwig Biotec). The following cycling conditions were used: 94°C for 5 min, 30 cycles at 94°C for 30 s and 67°C for 30 s, with an extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Amplification products (800 bp) related

to the variable region of kDNA minicircles were visualized on a 2% agarose gel stained with ethidium bromide. For the reaction of reamplification 10 µl of a 1:40 dilution of the first PCR product was added to 15 µl of PCR mix under the same conditions as the first amplification, with 7.5 pmol DRJ primer and 7.5 pmol of a new *Leishmania* specific primer AJS31, 5´-GGG GTT GGT GTA AAA TAG GGC CGG-3´, 0.7 U of Taq DNA polymerase (Ludwig Biotec). Positive samples yielded a PCR product of 780 bp.

2.4.3. Internal Transcribed Spacer 1 nested PCR (ITS-1 nPCR)

This method was adapted from Schönian *et al*. (2003) [15]. Were used primers addressed to internal transcribed spacer 1 (ITS 1) between the genes coding for SSU rRNA and 5.8S rRNA. For the first amplification 1.0 µl of DNA solution was added to 49 µl of PCR mix containing 15 pmol of the primers 5´- CTGGATCATTTTCCGATG-3´ and 5´-TGATACCACTTATCGCACTT-3´ and 0.2 mM deoxynucleoside triphosphates, 2mM $MgCl_2$, 5.0 µl of 10X buffer (Tris-HCl 50 mM, [pH 8.3], KCl 50 mM), and 1.4 U of Taq DNA polymerase (Ludwig Biotec). The cicling conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Amplification products were visualized on 2% agarose gel and stained with ethidium bromide. The PCR product size stays between 300 and 350 bp. For the second amplification 10 µl of a 1:40 dilution of the first PCR product was added to 15 µl of PCR mix under the same conditions as the first amplification but with the following primers (15 pmol each): 5´-CATTTTCCGATGATTACACC-3´ and 5´-CGTTCTTCAACGAAATAGG-3´. Positive samples yielded a PCR product between 280 and 330 bp.

2.4.4. *Leishmania* nested PCR (LnPCR)

This method use primers addressed to the variable region of SSU rRNA (small subunit ribosomal ribonucleic acid) gene and was performed according Cruz *et al*. (2002) [16] with minor modifications. For the first amplification 1.0 µl of DNA solution was added to 49 µl of PCR mix containing 15 pmol of the Kinetoplastida-specific primes 5´-GGTTCCTTTCCTGATTTACG-3´ and 5´-GGCCGGTAAAGGCCGAATAG-3´, 0.2 mM deoxynucleoside triphosphates, 2mM $MgCl_2$, 5.0 µl of 10X buffer (Tris-HCl 50 mM, [pH 8.3], KCl 50 mM), and 1.4 U of Taq DNA polymerase (Ludwig Biotec). The cicling conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Amplification products were visualized on 2% agarose gel and stained with ethidium bromide. Samples revealing a 603 bp product were scored as positive. For the second amplification 10 µl of a 1:40 dilution of the first PCR product was added to 15 µl of PCR mix under the same conditions as the first amplification but with the following *Leishmania*-specific primers (15 pmol each): 5´-TCCCATCGCAACCTCGGTT-3´ and R333 5´-AAAGCGGGCGCGGTGCTG-3´and 0.7 U of Taq DNA polymerase (Ludwig Biotec) using an annealing temperature of 65°C in the cycles. Amplification products were visualized as before and positive samples yielded a PCR product of 353 bp.

2.5. Statistical analysis

The results were analyzed using the Pearson chi square test. The difference between the results was considered significant with P value < 0.05.

3. RESULTS

In the table 1 were showed the results from Group 1 dogs (DNA was extracted from cotton swab by the phenol-chloroform). The most efficient assays were those based on kDNA targets. The kDNA PCR – Hybridization was able detect parasites in 22/23 dogs (95.6%) and in 40/46 samples (86.9%), considering the right and the left conjunctivas. The kDNA snPCR was positive for 21/23 dogs (91.3%) and for 40/46 samples (86.9%). There was no statistically significant difference between the sensitivities of these two methods. The positivities of the kDNA based methods were significantly higher ($p<0.05$) than the positivities obtained by the two methods based on ribosomal rRNA genes. The ITS 1 nPCR and LnPCR were both able to detect the parasites in 17/23 dogs (73.9%) and respectively 29/46 (63%) and 30/46 (65.2%) of the samples. The results obtained from Group 2 were showed in the table 2 (DNA was extracted by boiling). The positivities for all methods were smaller in relation to Group 1. Again the kDNA PCR – Hybridization showed a better performance detecting parasites in 18/23 dogs (78.3%) and in 31/46 samples (67.4%), significantly higher ($p<0.05$) than the other three methods. The positivities of two methods, kDNA snPCR, and LnPCR, were below than the expected (based on the results obtained from Group 1) and these assays seem to be undergone some kind of inhibition. The kDNA snPCR and the LnPCR methods detected *Leishmania* respectively in 10/23 (39.1%) and 2/23 (8.7%) of the dogs and 14/46 (30.4%) and 3/46 (6.5%) of the samples. The three methods based on two amplifications steps (kDNA snPCR, ITS 1 nPCR and LnPCR) were able to detect parasites only after the second amplification.

Table 1- Comparison of PCR assays sensitivities using conjunctival swabs samples of dogs Group 1 (DNA extracted from cotton swabs by Phenol-chloroform)

METHOD	STEP	DOGS																																						Positive dogs/ Total dogs	Positive samples/ Total samples*
		1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19		20		21		22		23			
		L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R		
kDNA PCR - Hybrid.	1	+	-	+	+	-	-	+	-	-	-	+	+	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	16/ 23	27/ 46
	2	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	22/ 23	40/ 46
kDNA snPCR	1	-	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	11/ 23	19/ 46
	3	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	21/ 23	40/ 46
ITS 1 nPCR	1	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	7 / 23	10 /46
	3	+	-	+	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	17/ 23	29/ 46
LnPCR	1	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	7/ 23	13 /46
	3	+	-	+	+	-	-	+	+	-	+	+	+	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	17 / 23	30 /46

1 – First amplification; 2 – Hybridization; 3 – Second amplification; + Positive; - Negative; L – Left conjunctiva; R – Right conjunctiva; * Considering right and left conjunctivas.

Table 2- Comparison of PCR assays sensitivities using conjunctival swabs samples of dogs Group 2 (DNA extracted from cotton swabs by boiling)

METHOD	STEP	DOGS																																						Positive dogs/ Total dogs	Positive samples/ Total samples*
		1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19		20		21		22		23			
		L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R		
kDNA PCR - Hybrid.	1	-	-	-	-	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	11/ 23	18/ 46
	2	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	+	-	-	-	+	+	+	+	-	-	+	+	-	+	18/ 23	31/ 46
kDNA snPCR	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/ 23	0/ 46
	3	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-	10/ 23	14/ 46
ITS 1 nPCR	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/ 23	0/ 46
	3	+	-	-	-	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	8/ 23	11/ 46
LnPCR	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/ 23	0/ 46
	3	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/ 23	3/ 46

1 – First amplification; 2 – Hybridization; 3 – Second amplification; + Positive; - Negative; L – Left conjunctiva; R – Right conjunctiva; * Considering right and left conjunctivas.

4. CONCLUSION

The kDNA PCR - Hybridization seems to be the best choice among the assays evaluated since it was most sensitive and is able to differentiate *L. (L.) chagasi* and *L. (Viannia) braziliensis.* The hybridization using radiolabeled DNA probes increases the sensitivity and guarantees the specificity avoiding those spurious bands can be incorrectly scored. This assay also avoids procedures with two successive amplifications (nested or semi nested) that increase the likelihood of contamination. According to the results of this work the DNA extraction by boiling from cotton swabs is less efficient than the phenol-chloroform method and can not replace it. We have to consider that the sensitivities of all PCR assays reported here can be improved, as only 1.0 µl DNA sample was used in this study and this volume can be enlarged at least to 10.0 µl. Our results support the applicability of the conjunctival swab procedure for the canine leishmaniasis diagnosis. This procedure, associated to the DNA extraction by phenol-chloroform and the most sensitive PCR assays showed sensitivities above 90%. The conjunctival swab can be a valuable tool, mainly in circumstances where the serological tests are not adequate, how for example in the diagnosis and follow up of vaccine dogs, that after vaccination test positive in the conventional serological tests.

ACKNOWLEDGMENTS

This research was supported by International Atomic Energy Agency (IAEA), "Centro de Desenvolvimento da Tecnologia Nuclear / Comissão Nacional de Energia Nuclear" (CDTN/CNEN) and Minas Gerais State FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais). We gratefully thank to the Zoonosis Control Center of Belo Horizonte for supplying the dogs.

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