A NEW DIAGNOSTIC METHOD FOR THE RAPID DETECTION OF LEISHMANIASIS USING PCR.

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SUMMARY

A new method has been designed for the early detection of *Leishmania donovani* from blood using DNA technology. The designed PCR was based on the detection of the Lmet2 repeat sequence which is an imperfect 60-bp repeat sequence and is specific to *L. donovani* complex. Two PCR primers, Lmet2A and Lmet2B and a short capture probe, Lmet2P are used. To improve PCR specificity, a simplified post-hybridization step has been developed which includes the probe in the PCR and enables the hybridization to be performed automatically as part of the PCR programme. After PCR, the hybrids are detected by capture in microtiter wells and colorimetric visualization. Preliminary results have indicated that this method is diagnostic for *Leishmania donovani*.

INTRODUCTION

Human visceral leishmaniasis has been known in the Maltese Islands since the beginning of this century. Symptomatic visceral leishmaniasis is usually fatal if left untreated. There are many more cases of visceral leishmaniasis in dogs than there are in people in the Mediterranean basin. The parasite responsible for the condition in Malta in both humans and dogs is *Leishmania infantum* which forms part of the *Leishmania donovani* complex. Studies carried out by an MRC team assisted by WHO (Geneva) have established that local domestic and stray dogs are the only known reservoir of the disease. Also small scale screening surveys have suggested that there is a high prevalence of oligosymptomatic and asymptomatic infection in both the human population and the local canines.

There is, therefore, a need for a rapid diagnostic method for leishmaniasis. The usual method of parasitological diagnosis is by microscopy and/or culture. Serological testing may offer an alternative to microscopy for patient diagnosis but cross-reactions with other parasites may occur and the presence of antibodies may not indicate a current infection.

PCR is an alternative very sensitive technique which has been applied to the detection of many disease-causing

organisms. Qiao et al (1995) have reported the use of PCR in the diagnosis of leishmaniasis in infected laboratory animals. However, their method was not sensitive enough to detect the presence of parasites in blood from all infected animals.
Here we report the use of this PCR method with modifications which have increased the sensitivity of this diagnostic test.

MATERIALS AND METHODS

Blood was collected using EDTA as anti-coagulant. Red cells were lysed by the addition of 100 ul of 0.5% saponin, left to stand for 20 minutes, centrifuged and the supernatant was discarded. The pellet was washed twice in TBS, resuspended in 100 ul of water, mixed well and boiled for 5 minutes. After cooling, an equal volume of chloroform was added. At this stage the samples may be stored at -20 ^{O}C until required. The mixture was centrifuged at 12,000g for 3 minutes and 5 ul of the upper aqueous layer was used directly in the PCR reaction. The PCR reaction mix also contained 400 uM nucleotides, 3 mM $MgCl_2$, 10% DMSO, 0.5% DNA polymerase enzyme and reaction buffer.,,

Lmet2 is an imperfect 60 bp sequence which is specific to the *L. donovani*-complex (Howard et al., 1991). The two PCR primers Lmet2A and Lmet2B and the short probe, Lmet2P, are shown against the Lmet2 sequence in Fig.1. Lmet2B and Lmet2P were labelled at the 5' ends with digoxigenin and biotin respectively during their commercial synthesis. During PCR the Lmet2 sequence was amplified by the primers Lmet2A and Lmet2B. Lmet2P was present throughout the PCR but could not take part in the reaction because the annealing temperature used in the PCR reaction was too high for this oligonucleotide to bind to its target sequence. A tail of dTTPs at the 3' end of this sequence also prevented extension with the corresponding Lmet2 sequence. After PCR, any product was denatured and the temperature of the reaction was dropped to allow Lmet2P to anneal to its complementary product sequence. After hybridization, the biotin label on the Lmet2P enabled hybrids to be captured in avidin-coated microtiter plates. The captured hybrids were then detected with anti-digoxygenin alkaline phosphatase antibody conjugate by virtue of the digoxigenin labelled Lmet2B which was incorporated into the complementary strand of the PCR product.

The PCR and solution hybridization conditions programmed into the thermal cycler were:

93^{O}C for 3 min 1 cycle

93^{O}C for 0.5 min
68^{O}C for 1 min 35 cycles PCR analysis
72^{O}C for 1 min

72^{O}C for 10 min 1 cycle

99^{O}C for 20 min 1 cycle

55^{O}C for 90 min 1 cycle Solution hybridization

The PCR samples were immediately placed on ice, diluted with 0.25 ml of TBS 0.5% Tween 20 buffer and mixed well. 100 ul of each diluted sample was analysed in avidin-coated microtiter plate wells. The microtiter plate was coated with avidin 10 ug/ml in 50 mM carbonate buffer, pH 9.6 and left for 1 hour at 37^{o}C. After coating, the wells were washed twice with TBS 0.5%Tween 20 buffer and blocked with sonicated herring sperm DNA for 30 minutes at room temperature. The wells were washed twice with the TBS 0.5%Tween 20 buffer and then used for assaying the PCR products. The PCR products were captured for 30 minutes after which the wells were washed twice with TBS 0.5% Tween 20. The captured product was detected using 100 ul/well anti-digoxigenin alkaline phosphatase antibody enzyme conjugate (diluted 1/5000 TBS 0.5% Tween 20 plus 3% milk powder). After incubating for 30 minutes at room temperature, the wells were washed four times with TBS 0.5% Tween 20. 100 ul of substrate (Sigma 104) in 50 mM carbonate buffer pH 9.6, 1mM $MgCl_2$ was added per well. The absorbance at 405 nm after 1 hour was measured using a microplate reader.

RESULTS

Samples positive for leismaniasis gave a yellow colour when assayed using this method. However, there were a few samples that gave a false positive result. It would seem that non-specific binding of either the biotin-labelled probe to the avidin coated microplate wells or the digoxigenin-labelled DNA in the absence of any PCR product is occurring. To block this non-specific binding, the microplates were coated with herring sperm DNA, but this did not completely block the non-specific binding.

DISCUSSION

When looking for parasites in blood, a very sensitive PCR method, which employs many PCR cycles, highly active Taq enzyme and a high concentration of primers and probe, is required. At such high sensitivity a certain amount of non-specific colour would be expected. However, further work needs to be done to eliminate this non-specific binding completely and hence have this new diagnostic method validated.

The PCR product gives a smear when run on a gel, hence a colorimetric method of detection needs to be used. Automation of the PCR and hybridization followed by this colorimetric detection of product considerably simplifies the whole assay. Adaptations such as these to make PCR more user-friendly are essential in promoting the widespread application of the PCR method. This is particularly important if PCR is to be applied to diseases that are endemic to a particular region.

REFERENCES

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Qiao, Z., Miles, M.A. and Wilson, S.M. (1995) Detection of parasites of the Leishmania donovani- complex by a polymerase chain reaction-solution hybridization enzyme linked immuno assay (PCR-SHELA). *Parasitology* 110, 269-275.

Figure 1.

The partial sequence of Lmet2 showing one complete repeat subunit (within the dashed lines) and showing the relationship of the two primers, Lmet2A and Lmet2B, and the probe, Lmet2P.

Figure 2.

Diagrammatic representation of the probe, Lmet2P, hybridized with the digoxygenin-labelled complementary strand of the PCR product and colorimetric detection of the captured hybrid. A, avidin; D, digoxigenin; B, Biotin; Ab/AP, anti-digoxigenin/alkaline phosphatase conjugate; O, substrate; O, product.

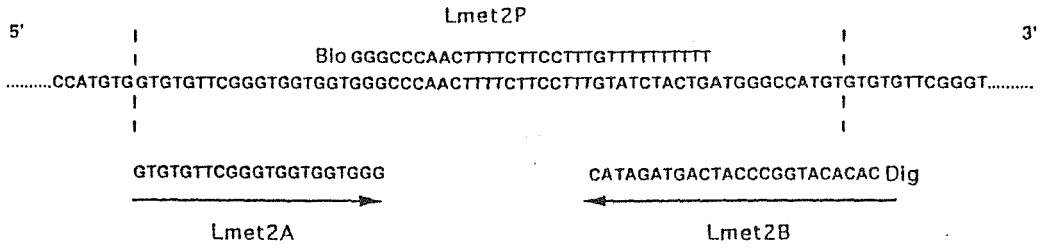
Lmet2P
5'
3'
Bio GGGCCCAACTTTTCTTCCTTTGTTTTTTTTT
.....CCATGTGGTGTGTTCGGGTGGTGGTGGGCCCAACTTTTCTTCCTTTGTATCTACTGATGGGCCATGTGTGTGTTCGGGT.....
GTGTGTTCGGGTGGTGGTGGG
CATAGATGACTACCCGGTACACAC Dig
Lmet2A
Lmet2B

Fig 1

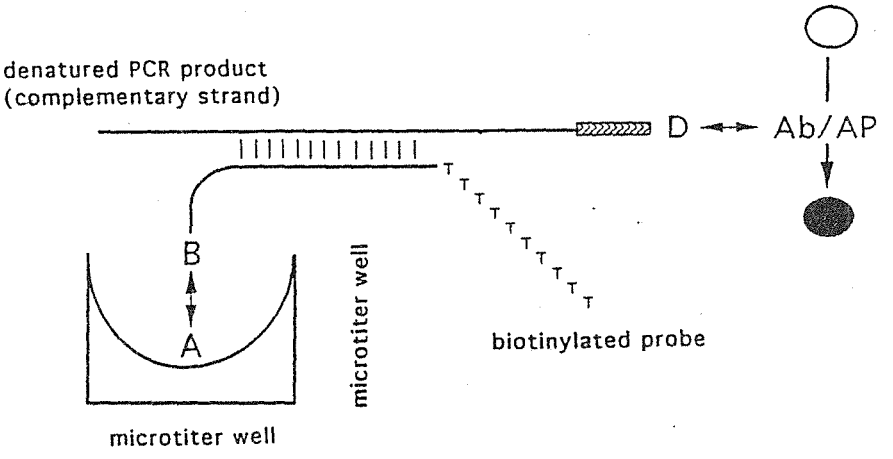
denatured PCR product
(complementary strand)
D
Ab/AP
B
A
microtiter well
biotinylated probe
microtiter well

Fig 2