DURATION OF PERSISTENCE OF *ONCHOCERCA GIBSONI* DNA IN CATTLE BLOOD

LAMA KEBERADAAN DNA *ONCHOCERCA GIBSONI* DALAM DARAH SAPI

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ABSTRACT

The aim of the study was to investigate the usefulness of the polymerase chain reaction (PCR) and PCR-ELISA to detect the presence of *O. gibsoni* DNA in serum of cattle after chemotherapy as an indicator of successful treatment. As targets for PCR, three different DNA repeat sequences that are present in the *O. gibsoni* genome were used; namely O-87, O-123 and O-150 repeat families. The sensitivity of the PCR methods for each sequence was 100 pg DNA in 0.5 ml serum from Tasmanian cattle. These primers were used to amplify sequences extracted from serum of *O. gibsoni*–infected cattle, collected before and after treatment. No detectable band was observed in agarose gel even after reamplification of 1 µl of the first PCR product. To determine the duration of persistence of DNA in serum of cattle, 5 calves were injected with 200 µg of *O. gibsoni* DNA into the jugular vein, and blood samples were collected before injection, and 1,2, 3, 4, 5, 10 and 30 minutes after injection. Using O-150 primers, PCR amplified product could only be detected from sera up to 5 minutes after injection. The study demonstrated that DNA of *O.gibsoni* in the circulation is rapidly degraded by the host, thus supporting a conclusion that detection of *O. gibsoni* DNA in serum by PCR-ELISA as a tool to evaluate drugs is not an appropriate approach.

Key words: *O. gibsoni*, PCR, PCR-ELISA

ABSTRAK

Penelitian ini awalnya bertujuan untuk menggunakan metode PCR dan PCR-ELISA untuk DNA *O. gibsoni* dalam serum sapi setelah pengobatan sebagai salah satu indikator keberhasilan khemoterapi. Sebagai target DNA untuk PCR adalah tiga untai DNA yang berulang yang terdapat dalam genom *O. gibsoni* yaitu O-87, O-123 and O-150. Sensitifitas metoda PCR untuk masing-masing untai DNA adalah 100 pg dalam 0,5 ml serum sapi dari Tasmania. Ketiga metoda PCR ini digunakan untuk mendeteksi keberadaan DNA *O. gibsoni* dalam serum sapi sebelum dan sesudah khemoterapi. Tidak ada pita DNA yang nampak pada elektroforesis gel bahkan setelah hasil PCR pertama di PCR lagi. Penentuan lamanya waktu keberadaan DNA *O. gibsoni* dalam darah sapi, lima ekor sapi diinjeksikan 200 µg DNA O. *gibsoni* melalui vena jugularis dan sampel darah diambil sebelum dan 1, 2, 3, 4, 5, 10 dan 30 menit setelah diinjeksikan. PCR dilakukan dengan memakai primer dari untai DNA O-150. Hasil DNA yang diperbanyak dengan PCR hanya mampu mendeteksi keberadaan DNA *O. gibsoni* dalam darah sapi sampai 5 menit setelah penyuntikan. Penelitian ini membuktikan bahwa deteksi DNA *O. gibsoni* dalam serum darah sapi dengan PCR-ELISA untuk mengevaluasi obat bukan merupakan pendekatan yang sesuai.

Kata kunci: *O. gibsoni*, PCR, PCR-ELISA.

INTRODUCTION

During the past 10 years, the diagnosis of parasitic diseases has incorporated nucleic acid-based detection. The use of DNA probes and PCR in detecting parasites now plays a major role in the epidemiology, prevention, and treatment of diseases. High accuracy, sensitivity and specificity offered by these techniques are among the advantages they have over many more traditional methods (Weiss, 1995).

PCR has been successfully used for diagnosis of filarial infection caused by *B. malayi* (Poole and Williams, 1990; *L. loa* (Touré *et al.*, 1997); *W. bancrofti* (Ramzy, *et al.*, 1997) and *O. volvulus* (Meredith et al., 1991, and Zimmerman *et al.*, 1994). The usual targets for PCR are repeated sequences of nucleic acid bases in the target parasite. For example, PCR assays for *B. malayi* target the *Hha* I family; for *W. bancrofti* the *Ssp* I repeat, for *L. loa* 15r3; and for *O. volvulus*, the O-150 family are target sequences. The O-150 family is present across species of the genus *Onchocerca* with some degree of variation in each isolate or species (Meredith *et al.*, 1989). In addition to the O-150 sequence, two other repeated sequences (so far found only in *O. gibsoni* and *O. volvulus* genomes) are O-87 and O-123 families (Catmull *et al.*, 1992 and 1994). These two sequences have not previously been used as targets for PCR in a diagnostic test. However, they together with O-150 family, were used in PCR and PCR-ELISA in this study, to determine the duration of *O. gibsoni* DNA persist in bovine blood.

MATERIALS AND METHODS

Five calves about six months of age were used to study the duration that *O. gibsoni* DNA remains detectable in the circulation. All calves were injected with 200 µg of *O. gibsoni* DNA into the jugular vein. Five milliliter of blood was drawn from each animal immediately before injection of DNA and 1, 2, 3, 4, 5, 10 and 30 minutes after injection and held in tubes without anticoagulant for serum collection. All tubes were spun at 3000 *g* to clarify serum prior to storage as 0.5 mL aliquots at -70 ^{0}C.

Extraction was done according to Fahrimal *et al.* (1992). Serum was digested with a final concentration 200 µg proteinase-K/mL and 1 % SDS (sodium dodecyl sulfate) at 37^{0}C over night. The next day, a final concentration of 1 M NaCl was added and extracted twice with an equal volume of equilibrated phenol and once with chloroform isoamyl alcohol (24:1). The mixture was centrifuged for five minutes at 10,000 *g* at each extraction. The aqueous phase was precipitated with two volumes of cold absolute ethanol and stored at –70^{0}C for at least one hour before centrifugation at 10,000 *g* for 15 minutes. The pellet was washed once with 70 % ethanol and reformed by centrifugation for five minutes. The pellet was then air-dried and re-suspended with 50 µL TE buffer (10 mM Tris-HCL pH 8.0 and 1mM EDTA).

For the 87 base pairs repeat, the forward primer used was GAA GTT AAG ATG GAG CAA CC and the reverse primer CGC CTT TTT TCT CTT TTT ATC GT. For the O-123 bp, the forward primer was GTC GAG AAT TTG GAA AAAA TC and the reverse primer CTT TGG CAA GCG AAC TGA CG. The primers used in PCR amplification of O-150 were published by Meredith *et al.* (1991). Forward and reverse primers were GAT T(CT)T TCC G(AG)C GAA (AGCT)A(AG) and GC(AGCT) (AG)T(AG) TAA AT(AGCT) TG(AGCT) AAA TTC respectively. One fifth of the extracted suspension of DNA was used for the PCR process.

Final and optimum concentrations of each component for the O-87 sequence were primer 10 pM, 200 µM dNTPs, 2 µM $MgCl_2$, 1 x PCR buffers (670 mM Tris-HCl, pH 8.8; 166 mM $(NH4)_2SO_4$; 2 mg/mL gelatin; 4.5%Trixon X-100) and one unit Taq polymerase in a total volume of 25 µL. The PCR amplification was carried out for 30 cycles. Each cycle consisted of one minute at 94^{0}C (an additional five minutes at 95^{0}C for the first cycle), 30 seconds at 45^{0}C and 30 seconds at 72^{0}C with an additional five minutes at 72^{0}C after the last cycle. Ten µL of PCR product was run in ethidium bromide-stained 2% agarose gel. DNA in agarose gel was visualized in GELDOC apparatus from Bio Rad.

Optimum concentration of each component required for amplification of the O-123 sequence was primer 20 pM, 150 µM dNTPs, 2 µM $MgCl_2$, 1 x PCR buffers (670 mM Tris-HCl, pH 8.8; 166 mM $(NH_4)_2$ SO_4; 2 mg/mL gelatin; 4.5%Trixon X-100) and one unit Taq polymerase in a total volume of 25 µL. The PCR amplification was carried out for 30 cycles. Each cycle consisted of one minute at 94^{0}C (an additional five minutes at 95^{0}C for the first cycle), 30 seconds at 45^{0}C and 30 seconds at 72^{0}C with additional five minutes at 72^{0}C after the last cycle.

For the O-150 sequence the requirement was primer 40 pM, 200 µM dNTPs, 4 µM $MgCl_2$, 1 x PCR buffers (670 mM Tris-HCL, pH 8.8; 166 mM $(NH_4)_2$ SO_4; 2 mg/mL gelatin; 4.5%Trixon X-100) and one unit Taq polymerase in a total volume of 25 µL. The PCR amplification was carried out for 30 cycles. Each cycle consisted of one minute at 94^{0}C (an additional five minutes at 95^{0}C for the first cycle), 30 seconds at 45^{0}C and 30 seconds at 72^{0}C with additional five minutes at 72^{0}C after the last cycle.

The PCR products were run in two percent agarose gel (w/v) stained with a final concentration of 5 µg/mL ethidium bromide. A 0.5 kb DNA ladder (Bresatec, Ltd.) was used as a marker.

Detection of amplified products was also done using the PCR-ELISA kit from Boehringer Mannheim (Germany, 1997). Amplified target DNA was labeled by substituting dTTP with combination of dTTP and digoxigenin-11-dUTP (19:1 ratio) in the normal PCR process. Capture probes were selected based on the G-C rich content within the amplified target sequences. The probe sequences for each target sequence were TTGAAGAGATGAAGCC for O-87, CTG AGT TCA ACG TTA GAA TCA ACA G for O-123, and CGG GTA CGT ACC TTC AAA TTG AGT C for O-150. The probes were attached to biotin on their 5' prime ends.

Ten µL of PCR product was added to 40 µL of denaturation solution containing 0.5% sodium hydroxide in each sterile centrifuge tube, topped up to 500 µL with hybridization solution containing biotin-labeled capture probe and mix the mixtures thoroughly. Two hundred µL of the mixture from each tube was added to each well of a streptavidin-coated microtitre plate (MTP) supplied with the kit and incubated in a shaker at 37^{0}C for three hours. The solution was then discarded and wells were washed. Two hundred µL of anti-DIG-POD antibody was added to each well. The MTP was then incubated at 37^{0}C incubator on a shaker for 30 minutes. The plate was again washed three times and 200 µL of ABTS was added and incubated as before in the dark. The plate was photographed.

Two protocols were used to determine the sensitivity of the PCR and PCR-ELISA reactions using the three sets of primers for each sequence. First, serial dilutions of *O. gibsoni* DNA ranging from 1 pg to 10 ng were added to 0.5 mL aliqouts of serum from Tasmanian cattle (*O. gibsoni* is not present in Tasmania) and extracted with phenol:chloroform:isoamyl alcohol and PCR as described above. The amplicon was visualized in agarose gel after electrophoresis. Second, for the PCR-ELISA the same extracted DNA and procedures were applied but dig-dUTP was used and processed with the PCR-ELISA method.

RESULTS AND DISCUSION

The three sets of primers were able to assist amplification of *O. gibsoni* genomic DNA extracted from spiked Tasmanian bovine serum in amount of 10 pg for O-87 and O-123 sequenses and 1 pg for O-150 sequence. The sensitivity to detect the *O. gibsoni* DNA in serum was increased by using PCR-ELISA method to 10 fg and 1 fg for O-87 or O-123 sequences and the O-150 sequence respectively (Figure 1).

Initially, the PCR and PCR-ELISA methods developed in this study was intended to detect DNA released from fragmenting *O. gibsoni* as an indicator of successful adulticidal chemotherapy. *Onchocerca gibsoni* in cattle has been used for tertiary drug screening methods for *O. volvulus* because of great similarity between these two species. However, since the methods failed to detect the presence of *O. gibsoni* DNA in serum of treated cattle, the methods were used to study the persistence of *O. gibsoni* DNA in cattle blood.

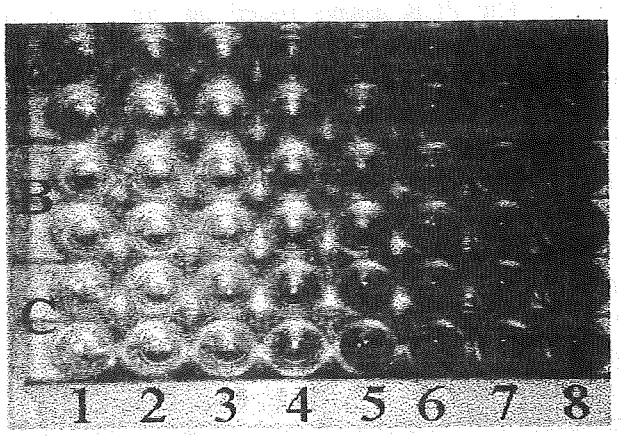

Figure 1. PCR-ELISA sensitivity of detection of *O. gibsoni* DNA in 0.5 mL of bovine serum from three sets of primers. A. duplicate of PCR-ELISA result using primers from O-87 sequence; B. using primers from O-123 sequence; C. using O-150 primers. Columns 1 to 7 refer to *O. gibsoni* DNA concentration in 0>5

mL of serum (10^0, 10^1, 10^2, 10^3, 10^4, 5 x 10^5 and 10^6 fg).

The polymerase chain reaction is commonly used to diagnose infectious microbial disease in humans and animals and its application to diagnosis of diseases caused by helminths is increasing, especially those caused by filarial worms (Singh 1997). The technique was considered worth investigating as an indirect mean of detecting an adulticidal effect against *O. gibsoni* because PCR has high sensitivity for detecting DNA in biological samples. Furthermore, it was reasoned that DNA may enter the circulation from disintegrating adult worm following their death and thus be detectable in serum or plasma.

The three target sequences used were chosen for amplification using PCR because they are highly repeated in the genome. It was reasoned that this would make it more likely that they would be detected, as more copies would be present than for unique or lowly repeated sequences, especially if the DNA was degraded into fragments. O-123 and O-150 are highly repetitive in the genome of both *O. volvulus* and *O. gibsoni* (Meredith *et al* 1991 and Catmull *et al.*, 1994). The O-87 repeat sequence has high copy numbers in *O. gibsoni* (Catmull *et al.*, 1992), but it is not known if it is also present in *O. volvulus*.

The PCR assays based on the 3 sets of primers chosen for this study were very sensitive (10 pg for O-87 and O-123 sequences and 1 pg for the O-150 sequence). Assuming the amount of DNA in a microfilaria is 250 pg (Meredith *et al.*, 1989), the assays were capable of detecting DNA in serum from a fragment of one microfilaria of *O. gibsoni*. It would seem unlikely that all three sets of primers used failed to assist amplification of the target sequences in *O. gibsoni* DNA if it was present in serum of infected cattle before or after chemotherapy. Nevertheless, after several unsuccessful attempts, it was apparent that these primers were not able to demonstrate the presence of *O. gibsoni* DNA in serum of cattle infected with *O. gibsoni* either before or after their treatment with filaricidal drugs.

To investigate the presence and duration of persistence of *O. gibsoni* DNA in the blood of cattle, 200 µg of *O. gibsoni* DNA was intravenously injected into five calves. The PCR products (only O-150 was used for this experiment) in PCR-ELISA were only detected from serum collected up to five minutes after DNA was injected (Figure 2). It was concluded that DNA of *O. gibsoni* in circulation of cattle is rapidly degraded since the PCR product signals declined progressively which each successive collection of blood over the first five minutes.

It was concluded that the negative result in PCR and PCR-ELISA of bovine serum before and after chemotherapy was likely to be due to the absence of DNA in the circulation. Adult *O. gibsoni* are confined inside nodules while microfilariae are in dermal interstitial tissues. It is thus unlikely that DNA released from fragmenting adults or microfilariae of *O. gibsoni* after successful chemotherapy will enter the circulation directly but will be taken up and degraded by the cells of the surrounding inflammatory response which dead worms induce.

Even if DNA from dead *O. gibsoni* reached the circulation, it is likely to be undetectable by PCR within minutes. DNA of *O. gibsoni* was only detectable (by agarose gel or PCR-ELISA) in the serum of a calf injected iv with 200 µg *O. gibsoni* DNA for five minutes after injection. Furthermore, even with *W. bancrofti* infection, where microfilariae are in the circulation, William *et al.* (1996) were unable to detect DNA of *W. bancrofti* following treatment of patients with diethylcarbamazine or Ivermectin, even though their microfilarial count had dropped sharply by two hours after treatment.

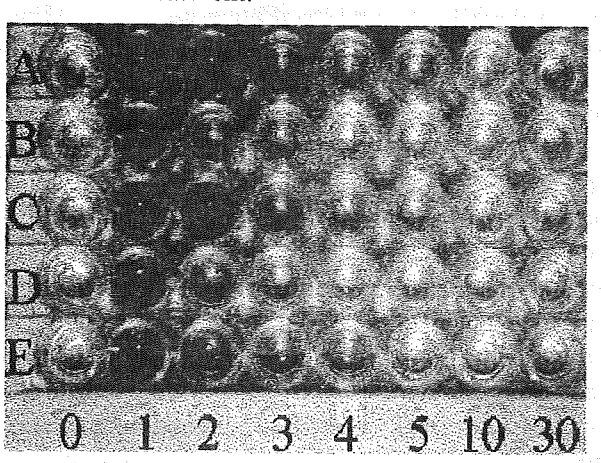

Figure 3. PCR-ELISA from a trial with *O. gibsoni* DNA injection into five cattle (A – E). Numbers on the horizontal axis are minutes after injection of DNA at which a sample of blood was taken. Note that for animal B there was no blood taken at three and five minutes after DNA injection.

The results obtained support a conclusion that the use of PCR to amplify DNA or fragments of DNA of *O. gibsoni* in serum is not a useful method of determining the success or failure of adulticidal (or microfilaricidal) chemotherapy. Furthermore, it is reasonable to assume that this conclusion also applies to *O. volvulus* because of the similarity between *O. gibsoni* and *O. volvulus* in their anatomy, genome, host-parasite relationship and response to drugs.

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