Diagnosis, transmission and immunology of human Oesophagostomum bifurcum and hookworm infections in Togo

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Chapter 5

Prevalence of *Oesophagostomum bifurcum* and *Necator americanus* infections using specific PCR amplification of DNA from fecal samples

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Submitted for publication in the Transactions of Tropicale Medicine and Hygiene

SUMMARY

Until recently infection of humans with *Oesophagostomum bifurcum* has been regarded as a rare zoonosis. However, in northern Togo and Ghana, the prevalence of infection is as high as 80% in certain villages. Diagnosis of the infection is hampered by the fact that the eggs of *O. bifurcum* are morphologically identical to those of Hookworm (*Necator americanus*). Stools have to be cultured for 7 days to allow eggs to hatch to the characteristic third-stage larvae. In this study, the applicability was evaluated of specific PCR's to amplify DNA from fecal samples as an alternative method for differential diagnosis of the two infections. The PCR's did not show non-specific amplification with a range of control DNA samples. The *O. bifurcum* PCR amplified specific *O. bifurcum* products of ≈ 220 bp from 57/61 fecal samples known to contain *O. bifurcum* third-stage larvae, and the *N. americanus* PCR amplified specific *N. americanus* products of ≈ 250 bp from 137/145 fecal samples known to contain *N. americanus* third-stage larvae after coproculture. Moreover, PCR detected 26 additional *O. bifurcum* cases in 72 samples from *O. bifurcum* endemic villages in which no *O. bifurcum* larvae were found and 46 *N. americanus* cases in 79 samples in which no *N. americanus* larvae were found after coproculture. No *O. bifurcum* DNA was detected in 91 stool samples from individuals from two non-endemic villages. Therefore, PCR is a powerful tool to reflect the presence of *O. bifurcum* and *N. americanus* infections in a population.

INTRODUCTION

Until recently human infection with the parasitic nodular worm *Oesophagostomum bifurcum* (Strongyloida: *Oesophagostominae*) was regarded as a rare zoonosis. However, in northern Togo and Ghana, prevalences of infection have been described to as high as 50 percent or more in some villages (Polderman *et al.*, 1991, Pit *et al.*, 1999). The larval stages of *O.bifurcum* cause the formation caseous nodules in the bowel wall of the large intestines. The nodules give rise to clinical symptoms described as a "Dapaong Tumour" or "multinodular disease" (Haaf and van Soest, 1964; Gigase *et al*, 1987, Storey *et al.*, 2000).

Diagnosis of intestinal parasites is generally based on the detection of eggs in faeces, but eggs of *O. bifurcum* are morphologically indistinguishable from those of the hookworm *Necator americanus* which also occurs at high prevalence in northern Togo and Ghana (Polderman & Blotkamp, 1995). Therefore,

coprocultures are needed to allow eggs to hatch and develop to the characteristic third-stage (L3) larvae for the differential diagnosis (Polderman *et al.*, 1991).

In northern Ghana and Togo, several epidemiological studies on *O. bifurcum* and *N. americanus* infections that have been carried out during the last ten years (Krepel, Polderman, Pit) were all based on the use of coprocultures. The large-scale use of such cultures is hampered by the fact that the stool specimen mixed with vermiculite or charcoal are kept for one week at tropical temperatures in closed petri-dishes, under these circumstances development of maggots and overgrowth of fungi readily occurs. Furthermore, it is known from studies of several strongylid nematodes that there is a large variability in coproculture results (Dobson *et al.*, 1992; Pit *et al.*,1999). Therefore a new alternative diagnostic method for differential diagnosis would be useful for epidemiological studies. Especially in new study areas of unknown endemicity where facilities for coproculture are not available it is more convenient to transport samples to a central laboratory.

Experience with IgG_4 and IgE-specific serology indicated that acceptable levels of sensitivity and specificity can be reached (Polderman *et al.* 1993, Pit *et al.*,). However, such approach proved unsatisfactory because drawing blood is little accepted in the area of study and aspecific reactivity is seen in some non-endemic areas. Moreover, present and past infections can generally not be distinguished with serology. Polymerase chain reaction (PCR) methods (Saiki *et al.*, 1988) can provide a useful alternative method to demonstrate the presence of parasite-DNA. PCR has been used for the specific detection of minute amounts of DNA in faecal samples using species specific target sequences (Gasser, 1999). Genetic markers for *O. bifurcum* and *N. americanus* in the second internal transcribed spacer (ITS-2) of ribosomal DNA were described (Romstad *et al.* 1997) and specific PCR primers (OB and NA respectively) were designed to a species-specific region within ITS-2 together with a conserved primer (NC2) in the 28S rRNA gene for amplification. The efficiency of the method was determined for amplification of small amounts of DNA derived from adult worms of *O. bifurcum* and *N. americanus*, and tested its specificity using a range of control samples (Romstad *et al.*, 1997). Recently we established a two-step semi nested PCR method for the specific amplification of *O. bifurcum*

DNA from human faeces (Verweij *et al.*, 2000). In the present study, the previous work is extended for the detection of *N. americanus* in human faeces in order to apply both methods to determine the prevalence of *O. bifurcum* and *N. americanus* infections in an endemic region, and to compare these prevalences with those determined by coproculture.

MATERIALS AND METHODS

Samples

Human fecal samples were obtained from Tami and Ogaro in northern Togo , both villages known to be endemic for human infections with *Oesophagostomum bifurcum* and *Necator americanus.* Furthermore samples were obtained from Sagbadai in central Togo and Fiema in Ghana, both villages where human infections with *O. bifurcum* are not known. Copro-cultures were carried out as described previously (Polderman *et al.*, 1991). For DNA isolation 200 µl of feces suspension (≈ 0.5 gram/ml) was added to 200 µl of 4 percent polyvinylpolypyrrolidone (PVPP) (Sigma) suspension and heated for 10 minutes at 100 °C (Morgan *et al*, 1998). After sodium-dodecyl sulphate-proteinase K treatment (2 hours at 55 °C), DNA was isolated with the QIAamp Tissue Kit spin columns (QIAGEN). Other control samples were also included in the study (Table 1). All samples were stored at -20°C.

Enzymatic amplification and agarose gel electrophoresis

Regions of rDNA were amplified by PCR (Saiki *et al.*, 1988), and every precaution was taken to prevent contamination at every step of the PCR procedure (Kwok *et al.*, 1991). PCR conditions were optimized by titration of MgCl2 and dNTP concentrations, as well as varying PCR annealing temperatures, cycle numbers and times. PCR reactions (40 µl) were performed in 10 mM Tris-HCl, pH 8.4; 50 mM KCl; 3.0 mM MgCl2; 250 µM each of dATP, dCTP, dGTP and dTTP; 50 pmol of each primer with 1 U *Taq* polymerase (Promega). The following primers were used in separate reaction mixes: NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3'); OB (5'-TATATTGCAACAGGTATTTTGGTAC-3') and NC2; NA (5'-ATGTGCACGTTATTCACT-3') and NC2 (Romstad *et al*, 1997).

Table 1. Samples used in this study

Faecal samples	number
From humans from *Oesophagostomum bifurcum* and *Necator americanus* endemic area in Togo	
Tami, Northern Togo	**81**
Ogaro, Northern Togo	**52**
Sagbadai, Central Togo	**50**
Fiema, Central Togo	**41**
From humans with (mixed) infections	18
Necator americanus	**3**
Strongyloides stercorialis	**1**
Ascaris lumbricoides	**7**
Trichuris trichiura	**5**
Hymenolepis nana	**2**
Schistosoma	**1**
Taenia sp.	**1**
Giardia intestinalis	**2**
Entamoeba histolytica	**1**
Entamoeba dispar	**9**
Entamoeba coli	**4**
Entamoeba hartmanni	**4**
Endolimax nana	**4**
From healthy human with no known history of parasitism	20
Control DNA samples	10
Oesophagostomum dentatum	**1**
Oesophagostomum quadrispinulatum	**1**
Necator americanus	**1**
Strongyloides stercorialis	**1**
Ascaris lumbricoides	**1**
Trichuris trichiura	**1**
Hymenolepis nana	**1**
Schistosoma	**1**
Giardia intestinalis	**1**
Entamoeba histolytica	**1**

Cycling was performed in a thermocycler (Perkin Elmer Cetus) using the following parameters: initial denaturation at 94°C for 5 min, followed by 25 cycles (35 cycles in the second PCR) of 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing) and 72°C for 30 sec (extension), followed by a final extension at 72°C for 5 minutes.

A range of control DNA samples and DNA isolated from well-defined fe-

cal samples (table 1) were used to test the specificity of the two PCR procedures. The sensitivity was determined using DNA isolated from fecal samples which were known to produce L3 *O. bifurcum* or *N. americanus larvae* respectively after coproculture. Two µl (approx. 0.2 ng) of genomic DNA from adult *O. bifurcum* or *N. americanus* respectively was used as the 'positive' control in PCR, or as the heterologous 'negative' control. Samples without genomic DNA (no-DNA controls) were included in each amplification run. PCR products were run on 2% agarose-TBE (65 mM Tris-HCl, 27 mM boric acid, 1 mM EDTA, pH 9) gels and visualized with ethidium bromide.

Samples which did not produce an amplicon in either one of the two PCR's (i.e., were 'negative') were tested for inhibition in PCR. This was done by spiking individual negative samples with 2 µl (approx. 0.2 ng) of O. *bifurcum* adult worm DNA and subsequent PCR amplification with the NC1-NC2 reaction mix followed by PCR amplification with the OB-NC2 reaction mix

The lowest amount of DNA that could be isolated and amplified in both PCR's was determined in a spiking experiment adding different amounts of adult worm DNA in a fecal suspension.

RESULTS

The specificity of both two-step PCR's was evaluated using a range of controls: 9 DNA samples from the parasites, *O. dentatum, O. quadrispinulatum, Trichuris trichiura, Ascaris lumbricoides, Strongyloides stercorialis* (Nematoda), *Hymenolepis nana, Schistosoma mansoni* (Trematoda), *Giardia intestinalis* and *Entamoeba histolytica* (Protozoa), 15 DNA samples derived from stools from patients with *Ascaris lumbricoides*, *T. trichiura*, *S. stercorialis, Taenia spc.* (Cestoda), *H. nana, S. mansoni*, *E. histolytica, E. dispar, E. coli, E. hartmanni, Iodamoeba butschlii, G. intestinalis,* and 20 DNA samples derived from feces from individuals with no known history of parasitic infections. None of these DNA control samples produced a specific product upon secondary amplification in neither of the two PCR's.

DNA from adult *Oesophagostomum bifurcum* gave the specific 220 bp product in the *O. bifurcum* PCR and no specific product in the *N. americanus* PCR. DNA from adult *Necator americanus* gave no specific product in the *O. bifurcum* PCR and

Table 2 Coproculture and PCR results of human fecal samples from Tami, Ogaro and Sagbadai

	O. bifurcum PCR		*N. americanus PCR*	
	positive	negative	positive	negative
Tami **(N=81)**				
Larvae found by coproculture	35	2	62	2
No larvae found by coproculture	9	35	1	16
Ogaro **(N=52)**				
Larvae found by coproculture	22	2	41	2
No larvae found by coproculture	17	11	9	0
Sagbadai (**N=50**)				
Larvae found by coproculture	0	0	20	4
No larvae found by coproculture	0	50	18	8
Fiema (**N=41**)				
Larvae found by coproculture	0	0	14	1
No larvae found by coproculture	0	41	17	9

gave a specific 250 bp product in the *N. americanus* PCR.

The lowest amount of *O. bifurcum* DNA and *N. americanus* DNA added to a fecal sample (200 µl suspension) that could be isolated and subsequently amplified by *O. bifurcum* PCR and *N. americanus* PCR was estimated at 10 pg. As we used only $1/100^{th}$ of the isolated DNA in the reaction, the lower detection limit in both PCR's using DNA isolated from faecal samples was as little as 100 fg. Using DNA isolated from stool samples of individuals from Tami, Ogaro, Sagbadai, and Fiema no distinct ITS-2 amplicons were detected on ethidium-stained agarose gels after primary amplification (NC1-NC2) from samples known to contain *O. bifurcum* and/or *N. americanus* L3 larvae after coproculture. However, upon amplification in the second round of PCR, the primer set OB-NC2 amplified PCR products of ≈ 220 bp from 57/61 fecal samples known to contain *O. bifurcum* third-stage larvae and the primer set NA-NC2 amplified PCR products of ≈ 250 bp from 137/145 fecal samples known to contain *N. americanus* third-stage larvae after copro culture (table 2). Thus, an assay sensitivity of 93.4% and 94.5% for the *O. bifurcum* PCR and the *N. americanus* PCR respectively was achieved. Furthermore, *O. bifurcum* specific PCR products were amplified in 26/163 DNA samples in which no *O. bifurcum* L3 larvae were found after coproculture. Also *N. americanus* specific PCR products were amplified in 46/79 DNA samples from stools in which no *N. americanus* L3

larvae were found in after coproculture (table 2). Selected secundary amplicons produced from faecal samples were analysed by single-strand conformation polymorphism (SSCP) (Gasser, 1997; Gasser *et al.*, 1998) and sequencing, and proved to represent the correct DNA sequence (not shown).

All of the samples which did not produce an amplicon in either one of the two PCR's (i.e., were 'negative') were tested for inhibition in PCR. After spiking with adult *O. bifurcum* DNA all samples produced the specific 220 bp product after amplification using the *O. bifurcum* PCR, so there was no evidence of inhibition in any of the negative samples.

Additional bands of differing sizes were amplified from some of the samples, which appeared to be due to non-specific amplification. The fragments of 310 bp or 450 bp detected after the second PCR were NC1-NC2 amplicons of O. *bifurcum* or *N. americanus* produced as a consequence of primer carry-over.

Prevalence for *O. bifurcum* and *N. americanus* infections in Tami, Ogaro, Sagbadai and Fiema were calculated and compared with the outcome of the copro-culture and the results of the two specific PCR's. (Table 3). Prevalence of *N. americanus* infections were slightly higher in Tami and considerable higher in Ogaro, Sagbadai and Fiema when calculated with the results of the PCR's. The calculated prevalences of *O. bifurcum* infections based on the PCR results in Tami and Ogaro were also higher than when calculated with the outcome of coproculture.

DISCUSSION

In the present study, using well-defined DNA and stool samples as controls, specificity was 100 percent for both the *O. bifurcum* and *N. americanus* PCR. Comparison of the stoolcultures and PCR outcome in

Table 3 Prevalance of O. bifurcum and N. americanus infections based on coproculture and PCR results of human fecal samples from Tami, Ogaro, Sagbadai, and Fiema.

	O. bifurcum		*N. americanus*	
	coproculture	PCR	coproculture	PCR
Tami	45.7%	54.3%	77.8%	79.0%
Ogaro	46.2%	75.0%	82.7%	96.2%
Sagbadai	0%	0%	48.0%	76.0%
Fiema	0%	0%	36.6%	75.6%

the study villages revealed a sensitivity of 93-94 % for both PCR's. These findings are similar as found in an earlier but smaller evaluation of the *O. bifurcum* PCR on fecal samples (Verweij *et al.* 2000).

The false negative test results were not caused by inhibition of the amplification reaction. Possibly it is the result of the size of the sample from which the DNA was isolated, being 30 times less then the amount of stool used for coproculture. In combination with the non-random distribution of eggs in the feces this could account for negative PCR results. In cases of *N. americanus* PCR false negatives the possibility of misidentification of *Ancylostoma duodenale* third-stage larvae as being *N. americanus* larvae when examining the larvae found after stool-culture, can not be excluded.

In DNA isolated from stool samples from individuals of all four villages in which no *N. americanus* larvae were found in coproculture, we could amplify *N. americanus* DNA in a total of 46 out of 79 cases. Similarly in DNA samples from subjects in whom no *O. bifurcum* larvae were found after copro-culture, we could amplify *O. bifurcum* DNA in 26 out of 72 cases in Tami and Ogaro, both villages known to be endemic for human *O. bifurcum* infection. Knowing the variability in coproculture outcome to be very high, especially in individuals with low worm burdens (Pit *et al.*, 1999), this is not a surprising finding. It is also possible that these *O. bifurcum* PCR positive subjects only harbored tissue-dwelling larval stages causing disease but not resulting in positive stool cultures. Indeed, negative stool cultures are frequently seen in individual patients with Dapaong tumors (Storey *et al*, in press). No false positives were found in samples with a variety of other parasitic DNA. So, we can conclude that in infections where no or only few larvae can be found after copro-culture the PCR is a more sensitive method in diagnosis of hookworm- and *Oesophagostomum* infections than coproculture.

In Sagbadai (Central Togo) and Fiema (Central Ghana) where human infections with *O. bifurcum* are unknown, no *O. bifurcum* larvae were found after stool cultures and specific PCR was negative in all 91 cases which is a further indication of the specificity of the *O. bifurcum* PCR method as described.

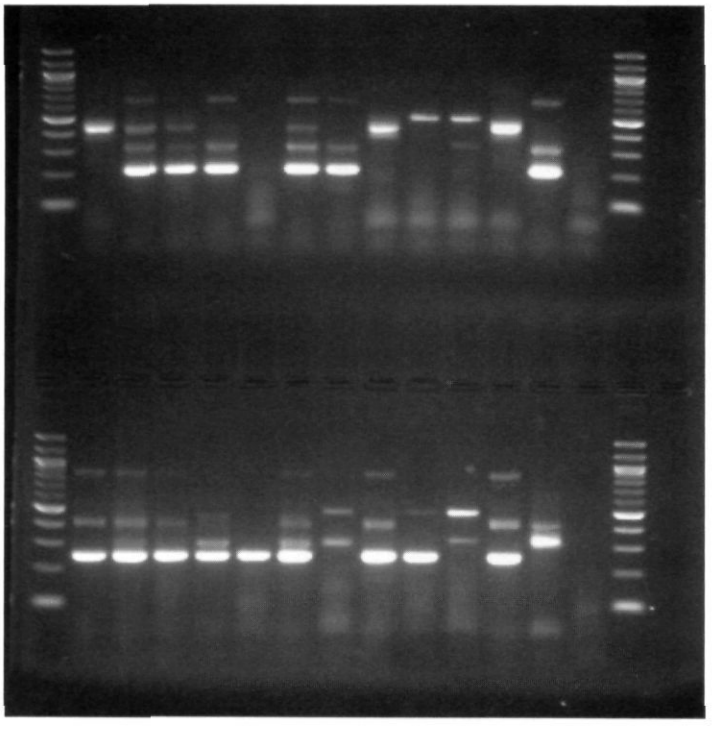
The findings of the present study indicate that PCR is a powerful tool to study the presence of parasites in a population. It may be particularly useful in situations, where on-the-spot-diagnosis based on cultures is

not feasible. The day to day variations of PCR results in comparison to those of stool-culture (Pit *et al.*, 1999), and the applicability to follow changes of infections over the seasons and after treatment need further study

Acknowledgement

The research was supported by the Ministry of Health and Education in Lomé, Togo. The assistance of Dr. P.T. Soboslay and A. Lamboni in the collection of the samples in Togo is greatfully acknowledged. This research was partly funded by the Netherlands Foundation for the Ad-

Fig. 1 *Representative example of an agarose gel showing Upper: Oesophagostomum bifurcum-specific products (220 bp) in lanes 2, 3, 4, 6, and 7 amplified from fecal samples by two-step semi-nested PCR. PCR using NC1-NC2 primer set followed by PCR using OB-NC2 primer set.; Lower Necator americanus- specific products (250 bp) in lanes 1, 2, 3, 4, 5, 6, 8, and 9 amplified from fecal samples by two-step semi-nested PCR. PCR using NC1-NC2 primer set followed by PCR using NA-NC2 primer set. lane 11 N. americanus adult worm control DNA; lane 12 O. bifurcum adult worm control DNA, water negative control, M = 100 bp ladder.*



vancement of Tropical Research (WOTRO). Support from the Parasitic Diseases Research Centre, Ministry of Health, Tamale, Ghana and the Australian Research Council, the Department of Industry, Science and Tourism and the Collaborative Research Program (The University of Melbourne) is acknowledged .

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