The zoonotic potential of Oesophagostomum bifurcum in Ghana. Epidemiological, morphological and genetic studies

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CHAPTER 7

PCR-BASED DIFFERENTIAL DIAGNOSIS OF *ANCYLOSTOMA DUODENALE* AND *NECATOR AMERICANUS* INFECTIONS IN HUMANS IN NORTHERN GHANA

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Abstract

In the present study, a two-step semi-nested PCR-based approach was evaluated for the specific detection of *A. duodenale* DNA in human faeces, which was used to determine as to what extent this species of hookworm is present in two regions (i.e., Bolgatanga and Garu) of northern Ghana. Initially, the sensitivity and specificity of the PCR were tested using a range of well-defined control samples. Subsequently, a total of 378 human faecal DNA samples from Bolgatanga and Garu were subjected to the PCR. The results were compared with those obtained using a previously-established PCR for the specific detection of *N. americanus* DNA in human faeces. Infection with *A. duodenale* or *N. americanus* was recorded in 74 (19.6%) or 278 (73.5%) samples, respectively, of which 64 (16.9%), represented co-infections with both species. While *A. duodenale* was predominantly detected in the samples from Bolgatanga, infections in Garu related almost exclusively to *N. americanus*. The results showed that the present PCR approach is a valuable complementary tool for the diagnosis of *A. duodenale* infection in humans in Ghana, having implications for epidemiological studies and for monitoring the success of control programs in regions in Africa.

Introduction

Hookworms are among the most common intestinal parasitic nematodes infecting an estimated 1.3 billion people worldwide.[2,171] Infection causes iron-deficiency anaemia, which may result in mental retardation, heart problems and growth deficit, particularly in children.[8,172,173] Two principal species of hookworm infecting humans are *Necator americanus* and *Ancylostoma duodenale*.[2] In northern Ghana, where presently extensive efforts are being undertaken to control hookworm disease and oesophagostomiasis in humans, *N. americanus* has been considered as the predominant species of hookworm, but it is unclear whether *A. duodenale* co-exist.

For monitoring the efficacy of mass treatment with anthelmintics (i.e., albendazole) in northern Ghana, the accurate identification and/or differentiation of the species involved is central. Currently, diagnosis of hookworm infection in humans relies on the detection of the eggs in the faeces. However, this is hampered by the fact that the eggs of *A. duodenale* are morphologically indistinguishable from those of other strongylid nematodes, including *N. americanus* and *Oesophagostomum bifurcum*. For this reason, coproculture is required to allow eggs to develop and hatch to release infective third-stage larvae (L3s) for subsequent identification or differentiation. Although there are some clear morphological characteristics to distinguish the L3s of *A. duodenale* from those of *N. americanus*,[127,174-176] reliable identification is time consuming and requires skilled personnel.

The use of DNA methods for the identification of parasites can overcome some of the limitations of traditional coproscopic methods. In particular, PCR based methods using appropriate DNA target regions have proven to be useful alternatives. For instance, the first and second internal transcribed spacer (ITS-1 and ITS-2, respectively) of ribosomal DNA (rDNA) provide genetic markers for the specific identification of a range of parasitic nematodes, including *A. duodenale* and *N. americanus*.[49,79,168,170,177] Previously, employing the ITS-2, a highly sensitive PCR was established for the specific amplification of *N. americanus* and *O. bifurcum* DNA from human faeces, and used to determine the prevalence of these two parasitic nematodes in northern Ghana.[46] Interestingly, the results of the latter study revealed a number of samples, which were known to contain hookworm eggs (i.e., larvae were identified morphologically after coproculture), but were test-negative using the PCR. One of the explanations given for this finding was that the larvae in the coproculture of these samples might have represented *A. duodenale*. In the present study, a PCR assay was validated for the detection of *A. duodenale* DNA from human faeces and used to determine to what extent *A. duodenale* is present in northern Ghana. The results were compared with those obtained using a PCR which specifically detects *N. americanus* infection.

Materials and methods

Samples

Faecal samples from humans were collected from two distinct regions (Bolgatanga and Garu) in northern Ghana. In the Bolgatanga region (10:78N; 0:85W), faecal samples were collected from 253 patients who visited the outpatient Department of the Regional Hospital (between August 1999 and November 2000), for which a request was made for an examination for intestinal parasites. Patients ranged in age from 1-65 years (median 24 years), and 60% lived within 15 km of the town of Bolgatanga. The majority of these individuals were pregnant women visiting the hospital for routine antenatal monitoring but with no clinical signs of parasitic diseases. Other patients complained of relatively mild abdominal discomfort. In the Garu region (10:85N, 0:18W), 125 faecal samples were collected from a number of localities in four neighbouring villages, as part of a cross sectional survey on intestinal nematodes.[30] Collection of these samples took place in September-December 2001. Participants were equally distributed by gender, and represented all age groups in the community (median 14 years). From each faecal sample collected, a small aliquot was either frozen directly or stored in 70% ethanol at room temperature. All aliquots were transported to the Department of Parasitology, Leiden University Medical Center (LUMC), The Netherlands, for DNA isolation. The ethical considerations and the methodology of sample collection are described in detail elsewhere.[30,40]

For establishing the PCR, 19 faecal DNA samples from humans shown by coproculture to be infected with *A. duodenale* and/or *N. americanus* were used. Also, genomic DNA samples representing individual adults of *A. duodenale* were included. Control samples to test the specificity of the PCR included genomic DNA samples from individual adults of *N. americanus*, *Ternidens deminutus*, *O. bifurcum*, *O. dentatum*, *Trichuris trichiura*, *Strongyloides stercoralis*, and faecal DNA samples derived from humans infected with *Entamoeba histolytica*, *Giardia intestinalis*, *Cyclospora cayetanensis*, *Cryptosporidium parvum*, as well as 12 faecal DNA samples from humans with no known history of parasitism (see Table 1).

Coproculture

Within 24 h of collection of the faecal samples, a duplicate coproculture of each sample was made to identify L3s.[44] In brief, 2 grams of faeces were mixed with an equal amount of vermiculite and placed on filter paper in a petridish under moist conditions for one week. Culture fluid was poured off and 100 µl of sediment was microscopically examined. L3s of *O. bifurcum*, *S. stercoralis* and hookworm were identified morphologically using published keys and description.[12,20,127,176,178] For all samples from Garu (n=125) and the 19 samples from Bolgatanga used for establishing the PCR, an experienced microscopist performed the morphological differentiation of the L3s of *A. duodenale* from those of *N. americanus*. Differentiation was based on the following criteria: measuring the total length of the sheath;

measuring the total length of the larva; examining the buccal cavity for the presence of a clearly visible spear; examining the shape of the larval tail and measuring the larval tail from the anus to the tip of the tail and establishing whether the cuticular striation was faint or strong.[127,176,178]

Isolation of DNA

For the isolation of DNA from faecal samples, 250 µl of an ethanol/faeces suspension was centrifuged (13000xg) for 30 sec. The ethanol layer was removed and the pellet was washed with 0.9 % NaCl. Subsequently, the pellet was resuspended into 200 µl of 2% polyvinylpolypyrrolidone (PVPP) and incubated at 100°C for 10 min. After sodium-dodecyl sulphate-proteinase K treatment (2 h at 55°C), DNA was isolated using spin columns (QIAamp Tissue Kit, QIAGEN, Germany)[179] and eluted into 250 µl of H_2O. Genomic DNA from individual worms was isolated by sodium dodecyl-sulphate/proteinase K digestion,[134] purified over spin columns (Wizard™ DNA Clean-Up; Promega, Madison, WI, USA) and then eluted into 50 µl of H_2O. Samples were stored at -20°C until required for PCR amplification.

Enzymatic amplification by PCR

PCR reactions were performed in 40 µl volumes using 2 µl of faecal DNA sample, 25 pmol of each primer, 250 µM of each dNTP, 3 mM $MgCl_2$, and 2 U *Taq* polymerase (Promega). Control samples without DNA (negative control) and with genomic DNA of adult *A. duodenale* (positive control) were included in each PCR run. For the first amplification round primers NC1 (forward: 5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3')[134] were used. Amplification consisted of 94°C for 5 min, 25 cycles at 94°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec, followed by a final step of 5 min at 72°C and was performed in an iCycler (Biorad, Hercules, CA, USA). Subsequently, 2 µl of the first round amplicon were subjected to a second amplification round using primers AD1 (forward: 5'-CGACTTTAGAACGTTTCGGC-3') and NC2. The cycling conditions for the second round amplification consisted of 94°C for 5 min, 35 cycles at 94°C, 1 min; 55°C, 1 min; 72°C, 1 min, then 72°C for 5 min. For comparison, all samples collected were also subjected to a previously-described PCR which detects specifically *N. americanus* DNA in human faeces.[46] A volume of 10 µl of each second-round amplicon was mixed with 3 µl loading buffer and examined on ethidium bromide-stained 2% agarose-TBE (65 mM Tris-HCl, 27 mM boric acid and 1 mM EDTA, pH 9.0; Bio-Rad) gels.

Results

Validation of the PCR approach

The PCR for the amplification of *A. duodenale* DNA from faeces was established using samples (n = 19) from humans from Bolgatanga known to contain *A. duodenale* and/or *N. americanus* eggs (Table 1). Based on detailed microscopic examinations of coproculture supernatants, L3s of *A. duodenale* were detected in 12 of these faecal samples, and L3s of *N. americanus* were detected in all 19 samples. The latter was confirmed by the specific detection of *N. americanus* DNA by PCR.

Initially, a direct PCR approach using the primer combination Ad-NC2 was tested, but this approach did not achieve effective amplification of a fragment of the expected size (~130 bp) from faecal DNA samples known to contain *A. duodenale* DNA. Subsequently, a two-step semi-nested PCR approach was evaluated. In the first PCR round, the primer set NC1-NC2 was used, which amplifies the ITS-2 region from different species of strongylid nematodes, including that of *A. duodenale* (~310 bp). Subsequently, a second PCR was performed using the primer combination Ad-NC2 to amplify an internal fragment within the ITS-2 region of *A. duodenale*. Using this approach, a fragment of ~130 bp could be amplified from faecal DNA samples known to contain *A. duodenale* (Fig. 1).

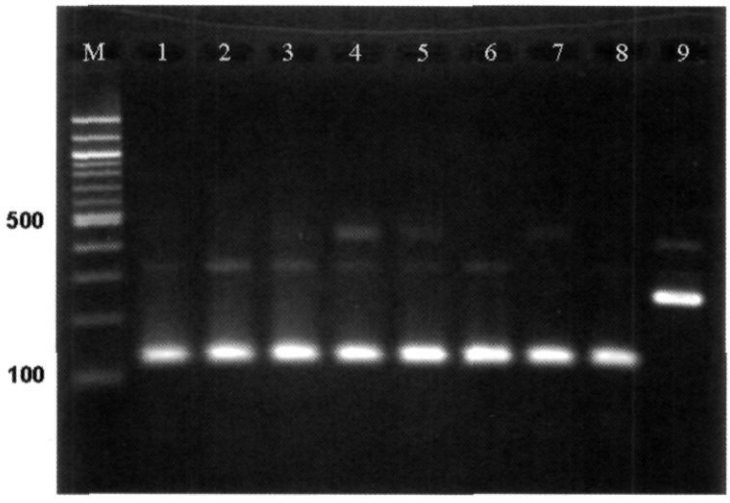

Figure 1 Example of an agarose gel showing the fragments of ~130 bp amplified from human faecal samples known to contain *Ancylostoma duodenale* DNA (lane 1-7). Lane 8 and 9 represent control samples with DNA from individual adult *A. duodenale* and *Necator americanus*, respectively. M represents a 100 bp ladder.

Table 1 Samples used in this study

Samples	Number
Faecal samples	
From humans from	
Bolgatanga	253
Garu	125
From humans with infection of	
Ancylostoma duodenale and *Necator americanus*	12
Necator americanus	7
Cryptosporidium parvum	1
Cyclospora cayetanensis	1
Entamoeba histolytica	1
Giardia intestinalis	1
From humans with no history of parasitism	12
Genomic DNA samples from adult	
Ancylostoma duodenale	1
Necator americanus	1
Oesophagostomum bifurcum	1
Oesophagostomum dentatum	1
Strongyloides stercoralis	1
Ternidens deminutus	1
Trichuris trichiura	1

Additional (fainter) fragments of ~310 and/or ~420 bp were amplified from some samples, and these were interpreted to represent NC1-NC2 amplicons produced due to primer 'carry over' into the second PCR round. Amplification from each of the 12 faecal DNA samples known to contain *A. duodenale* DNA produced a band of ~130 bp using the semi-nested PCR. Also, 2 samples, for which no L3s of *A. duodenale* were detected upon microscopic examination after coproculture, tested positive in the secondary PCR using primers Ad-NC2. The secondary amplicons of all 14 PCR-positive samples were subjected to sequencing, and their sequences were shown to be consistent with the ITS-2 of *A. duodenale*.[see 54] The specificity of the two-step semi-nested PCR was tested using a range of control samples (see Materials and methods

section). Although some of these control samples amplified some faint, non-specific bands, none of them produced a fragment of ~130 bp, demonstrating the specificity of the PCR primers and conditions for the amplification of *A. duodenale* DNA (data not shown).

Differential diagnosis by PCR

Based on the results from the validation, faecal DNA samples (n = 378) were subjected to PCR for the differential diagnosis of *A. duodenale* and *N. americanus* using the approach described herein and the one described previously for *N. americanus* (Table 2).[see 46] The partial ITS-2 amplicon (~130 bp) specific for *A. duodenale* was detected for 74 samples (19.6%), of which 64 (16.9%) also showed the *N. americanus*-specific product (~250 bp). With the exception of one, all of the *A. duodenale*-positive samples originated from Bolgantanga. For *N. americanus*, specific amplicons were detected for 276 (73.0%) of the 378 samples tested. Based on these results, the prevalence of infection with *A. duodenale* in the samples from Bolgatanga was 28.9%, whereas in the samples from Garu this was only 0.8%. The prevalence of *N. americanus* infection for the samples from Bolgatanga and Garu was 62.4% and 94.4%, respectively (Table 2). Samples from which no amplicons were produced using either PCR assay were tested for PCR inhibition by faecal constituents. This was verified by spiking them with *A. duodenale* DNA (~0.5 ng) and subjecting them to amplification using the semi-nested *A. duodenale*-PCR. Agarose gel electrophoresis of the resultant amplicons showed bands of ~130 bp for all spiked samples, and thus there was no evidence of inhibition in the PCR. For 307 of the 378 faecal DNA samples subjected to PCR, coproculture was carried out successfully, and hookworm L3s were detected in 197 (64.2%) of them. For samples from Bolgatanga, no species differentiation of L3s was performed. For samples from Garu, one hookworm-positive culture was found to contain both *A. duodenale* and *N. americanus* L3s.

Table 2 Number (percentage) of *Ancylostoma duodenale* and/or *Necator americanus* infection(s) detected in humans from two areas in northern Ghana as determined by PCR

	PCR negative	PCR positive		
		N. americanus only	*A. duodenale* only	*N. americanus* and *A.. duodenale*
Bolgatanga (n=253)	85 (33.6%)	95 (37.5%)	10 (4%)	63 (24.9%)
Garu (n=125)	7 (5.6%)	117 (93.6%)	0	1 (0.8%)

This sample was also test-positive using both the *A. duodenale*- and *N. americanus*-specific PCRs. Table 3 summarizes the results of coproculture versus those obtained using the PCR assays. The *A. duodenale*- and/or *N. americanus*-specific PCR were/was positive for 186 (94.4%) of the 197 coproculture-positive samples. Also, 53 samples in which no hookworm L3s were detected by microscopy were shown to be positive for *A. duodenale* and/or *N. americanus* when subjected to the specific PCR assays. Eleven samples for which hookworm L3s were detected after coproculture were test-negative in both PCR assays.

Discussion

In Africa, human infection with the hookworms *N. americanus* and *A. duodenale* is endemic in many countries.[180-183] Prior to this study, it had been assumed that the principal species of hookworm infecting humans in northern Ghana was *N. americanus*. However, this study demonstrated that *A. duodenale* is also present in this region. This finding may have important implications for public health and for the control of hookworm disease in northern Ghana. Transmission of infection with *N. americanus* and *A. duodenale* both occur percutaneously. However, L3s of *A. duodenale* can also be transmitted by the oral route.[178,184] Furthermore, in contrast to *N. americanus*, L3s of *A. duodenale* may stay in a state of 'arrested development' in tissues after invasion of the human host.[185] Although the repositories of the dormant larvae are somewhat unclear, they have been found in muscles tissue and/or mammary glands. In the latter case, this may result into transmammary transmission.[186] Also, infection in humans with *A. duodenale* can cause more severe pathological effects and produce symptoms with fewer worms compared with *N. americanus* infection.[187] It has been shown that infection with

Table 3 PCR compared with coproculture results for 307 human faecal samples from two areas in northern Ghana

	PCR negative	PCR positive		
		N. americanus only	*A. duodenale* only	*N. americanus* and *A. duodenale*
Coproculture				
negative (n=110)	57	41	2	10
positive (n=197)	11	144	6	36

A. duodenale causes an approximately five times greater blood loss, and therefore leads to a higher degree of iron-deficiency compared with *N. americanus*.[188] However, the number of epidemiological studies showing the impact of endemic *A. duodenale* infection on the iron status of populations is still limited.[189]

Interestingly, in contrast to *N. americanus*, these initial findings suggest that the geographical distribution of *A. duodenale* varies significantly between different areas in northern Ghana. While a large number of samples from Bolgatanga were found to contain *A. duodenale* DNA, only one case of *A. duodenale* infection was detected in the samples from Garu just 100 km away. Such variation in distribution of *A. duodenale* between different geographical areas has also been reported in other studies[190] and could relate to environmental (ecological) differences and/or differences in social or cultural habits of the people in the different geographical locations. A larger number of faecal samples from a wider range of geographical locations within Ghana should be collected to accurately establish the distribution and variation in prevalence of *A. duodenale* infection within this country.

Of the 197 samples in which hookworm L3s were detected by morphological examination after coproculture, 11 samples were found to be negative using both hookworm PCRs. The fact that *A. duodenale*-specific amplicons were detected for these samples after spiking them with adult *A. duodenale* DNA showed that there was no faecal inhibition in the PCR. A possible explanation for the 'false-negative' PCR results might be the amount of faecal sample used for DNA isolation, which was approximately 30 times less compared with that used for coproculture. The fact that most (n = 8) of the false-negative samples represented light infections, with less than 10 larvae detected microscopically after coproculture, supports this proposal. Furthermore, while coprocultures were performed on fresh faecal samples, PCR was carried out on samples which had been stored in ethanol or frozen for 3-4 years. Some DNA degradation might have occurred resulting in false negative PCR results.

As the ITS-2 region of rDNA of *A. duodenale* and *A. ceylanicum* (a hookworm of dogs and cats) have very similar ITS-2 regions, with only a few nucleotide differences between them, primer AD1 is likely to also anneal to *A. ceylanicum* DNA, and (in combination with primer NC2) amplify a similar size fragment as for *A. duodenale* DNA. Therefore care should be taken when using the present PCR approach in countries were human infection with *A. ceylanicum* has been reported, as is the case in some regions in the Asia-Pacific.[191-194] Although it cannot be totally excluded that *A. ceylanicum* infection in humans occurs in northern Togo and Ghana, it is highly unlikely as, to our knowledge, there are presently no published reports of human infection with this species of hookworm in Africa. Furthermore, sequencing of secondary amplicons of a number of PCR-positive DNA samples detected in the present study were all shown to represent *A. duodenale*.

In conclusion, the present study showed that specific PCR to detect human infection with *A. duodenale* is more effective compared with the traditional approach of identifying L3s after coproculture. It was found that this method is rapid and relatively easy to perform. Moreover, the detection of *A. duodenale* infection in Ghana using this approach can be carried out using either fresh or preserved faecal samples (i.e., frozen or stored in ethanol). Therefore, it represents a powerful tool for conducting epidemiological and ecological studies of *A. duodenale* in this country, and for monitoring the effectiveness of treatment and control strategies. While validated in Ghana in the present study, this PCR assay should have applicability in other countries where *A. duodenale* is known to be endemic.