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

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CHAPTER 4

SCREENING FOR HAPLOTYPIC VARIABILITY WITHIN *OESOPHAGOSTOMUM BIFURCUM* (NEMATODA) EMPLOYING A SINGLE-STRAND CONFORMATION POLYMORPHISM APPROACH

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Abstract

Genetic markers in the mitochondrial genome have proven useful for population genetic studies because of their maternal inheritance and relatively rapid evolutionary rates. In this study, we exploited the high resolution capacity of PCR-coupled single-strand conformation polymorphism (SSCP) to screen for sequence variation in part of the cytochrome *c* oxidase subunit 1 gene (p*cox*1) among individuals representing the parasitic nematode, *Oesophagostumum bifurcum* from human or Mona monkey hosts from Ghana. SSCP analysis revealed distinct profiles among some of the individuals, and subsequent sequence analysis of representative samples defined 10 different haplotypes. For comparative purposes, the p*cox*1 sequences for representatives of four other species of *Oesophagostomum* from livestock were included. While there were high levels (11.5-13.7%) of sequence difference among the latter species, there was no fixed nucleotide difference between *O. bifurcum* individuals from humans and those from monkeys. The data support the proposal that *O. bifurcum* from the two primate hosts represents a single species and that the haplotypic variability in p*cox*1 represents population variation. The results reinforce the usefulness of the SSCP-sequencing approach for studying genetic variation in nematode populations using mitochondrial markers.

Introduction

Infection of non-human primates and humans with the nodule worm, *Oesophagostomum bifurcum* (Nematoda: Strongylida) causes significant disease due to granulomata and caseous nodules in the wall of the large intestine, produced by encysted larvae. In spite of the human health impact of oesophagostomiasis in northern Togo and Ghana, little is known about its epidemiology.[24] There are suggestions that non-human primates may act as a reservoir host for human infection with *O. bifurcum*.[39] However, there is evidence of a significant difference in the geographical distribution of the parasite between monkeys and human hosts. For instance, while in Baobeng-Fiema (central Ghana) the Mona monkey (*Cercopithecus mona*) is known to harbour patent infection at relatively high prevalence (~90%), human oesophagostomiasis has not yet been detected in this region (chapter 2). Moreover, comparative morphological study of mature, adult *O. bifurcum* from both species of primate has indicated that significant variation in parasite morphology between them can occur (chapter 3). These observations have been suggestive of population variation within *O. bifurcum,* possibly relating to distinct genetic, biological and/or epidemiological characteristics.

We recently used a PCR-based mutation detection (single-strand conformation polymorphism, SSCP) approach to scan for nucleotide variability in the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA) among individuals representing *O. bifurcum* from human or Mona monkey hosts from Ghana.[21] Although some sequence microheterogeneity was detected among individuals, no unequivocal sequence difference existed in the ITS-2 between *O. bifurcum* from the two host species. Thus, the ITS-2 rDNA region did not display sufficient sequence variation to investigate the genetic make-up of *O. bifurcum* populations. More variable DNA sequences, such as those of the mitochondrial genome, are likely to be valuable for studying population structures because of their maternal inheritance and relatively rapid mutation rates.[132] For example, the cytochrome *c* oxidase subunit I (*cox*1) gene has been shown to be applicable to population-based studies of a range of parasitic helminths, including platyhelminths and enoplid nematodes.[62,131,133] However, currently there are neither sequence data for the *cox*1 of nodule worms nor information on within-species sequence heterogeneity. In this study, we employed SSCP in combination with DNA sequencing to analyse halpotypic variability in a portion of the *cox*1 gene (p*cox*1) within *O. bifurcum* from human and the Mona monkey hosts, and assessed the usefulness of *cox*1 sequences for studying the population genetics of nodule worms.

Materials and Methods

Parasites and isolation of genomic DNA

Adult worms of *O. bifurcum* were obtained from humans and Mona monkey (*Cercopithecus mona*) in Ghana (see Table 1). Worms from humans were obtained from the faeces of infected patients after treatment with pyrantel pamoate, as described previously,[44] whereas worms from Mona monkey were removed from the large intestine at necropsy. Also obtained for comparative purposes were representatives of other species of nodule worm from livestock hosts (Table 1). Adult *O. dentatum*, *O. quadrispinulatum* and *O. venulosum* were removed from the large intestine at necropsy, while third-stage larvae (L3) of *O. columbianum* were cultured from the faeces of mono-specifically infected sheep. Nematodes were washed extensively in physiological saline and frozen at -20°C until required for DNA isolation. With the exception of the larval sample, each adult specimen was identified to the species level using morphological criteria.[12,20,128] Genomic DNA was isolated by a method of sodium dodecyl-sulphate/proteinase K treatment,[134] purified over spin columns (Wizard™ DNA Clean-Up; Promega) and eluted into 50 µl H2O.

Enzymatic amplification and single-strand conformation polymorphism (SSCP) analysis

The p*cox*1 was enzymatically amplified with primers JB3 (forward: 5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (reverse: 5'-TAAAGAAAGAACATAATGAAAATG-3').[133] Primers were end-labelled with [γ-33P]ATP (NEN, DuPont) using T4 polynucleotide kinase (Promega). The PCR amplification was performed in 50 µl volumes using 25 pmol of each primer, 250 µM of each dNTP, 3 mM $MgCl_2$ and 2 U *Taq* polymerase (Promega) under the following conditions: after an initial denaturation at 94°C for 5 min, 30 cycles at 94°C, 30 sec (denaturation); 50°C, 30 sec (annealing); 72°C, 30 sec (extension), followed by a final extension of 5 min at 72°C in a thermocycler (Perkin Elmer Cetus, USA). Control samples without DNA were included in each PCR run, and human DNA was subjected to the same amplification procedure as for parasite DNA. In no case were products detected in these control samples. A volume of 7 µl of each PCR product was mixed with 3 µl loading buffer (aqueous solution of 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanole and 30% glycerol) 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 using ΦX-174-*Hae*III (Promega) as a size marker.

Table 1 Genomic DNA samples isolated from members of the genus *Oesophagostomum*. All samples represented single worms, except for Oec 16.9 which represented a pool of third-stage larvae (L3)

Species	Sample codes	Life cycle stage	Host	Location
O. bifurcum	HDAM1-HDAM3	Adult male	Human	Ghana
	HIAM1-HIAM3	Adult male	Human	Ghana
	HDAF1&HDAF2	Adult female	Human	Ghana
	HIAF1-HIAF3	Adult female	Human	Ghana
	HJM1-HJM3	Juvenile male	Human	Ghana
	HJF4-HJF9	Juvenile female	Human	Ghana
O. bifurcum	AAM1	Adult male	Mona monkey	Ghana
	AAF1-AAF5	Adult female	Mona monkey	Ghana
O. dentatum	Oed2	Adult male	Pig	Denmark
O. quadrispinulatum	Oeq3	Adult male	Pig	Denmark
O. venulosum	Oev3	Adult male	Ox	Australia
O. columbianum	Oec16-9	L3	Sheep	Australia

SSCP analysis was carried out as described recently.[97] In brief, 10 µl of PCR product were mixed with an equal volume of SSCP loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanole). After denaturation at 95°C for 5 min and snap-cooling on a freeze block (-20°C), 3 µl of individual samples were loaded into the wells of a 0.4 mm thick, non-denaturing gel (0.5 x mutation detection enhancement (MDE solution; FMC), and subjected to electrophoresis in a conventional sequencing rig (BaseRunner, IBI). The conditions for electrophoresis (7 W for 15 h at 24°C) were standardised for optimal resolution of bands, and the MDE concentration was as recommended by the manufacturer. Gels were dried on to blotting paper and subjected to autoradiography using RP1 film (Agfa).

DNA cycle sequencing and analyses

PCR products were purified over spin columns (Wizard™ PCR Preps, Promega) prior to sequencing. Cycle-sequencing was carried out using the *f*mol™ kit (Promega) according to a modification of the original protocol described by Gasser and co-workers.[134] The same primers as used for enzymatic amplification were employed (individually) to sequence in both orientations. The cycling conditions used were: 94°C, 5 min (initial time delay), then 94°C, 30 sec (denaturation); 45°C, 30 sec (annealing); 72°C, 30 sec (extension) for 30 cycles, followed

by final extension at 72°C for 5 min. Sequences were aligned manually, and pairwise comparisons were made of the level of sequence differences (D) using the formula D = 1-(M/L), where M is the number of alignment positions at which the two sequences aligned have a base in common, and L is the total number of alignment positions over which the two sequences are compared.[135] Amino acid sequences were deduced from nucleotide sequences using the program MacVector™ 4.1.4. Codon positions were determined by comparative alignment with the *cox*1 sequence of *Ascaris suum*.[59] Dendrograms were constructed from these data using the Unweighted Pair Group Method using Arithmetic averages (UPGMA).[136]

Results and discussion

SSCP was employed to screen for sequence variation within and among p*cox*1 amplicons representing *O. bifurcum* from human and Mona monkey hosts. Initial agarose gel electrophoretic analysis of the p*cox*1 amplicons (~450 bp) revealed no variation in size. Autoradiographic exposure of agarose gels indicated the specificity of the PCR products and conditions, in that each product appeared as a single band, with no evidence of non-specific background amplification.

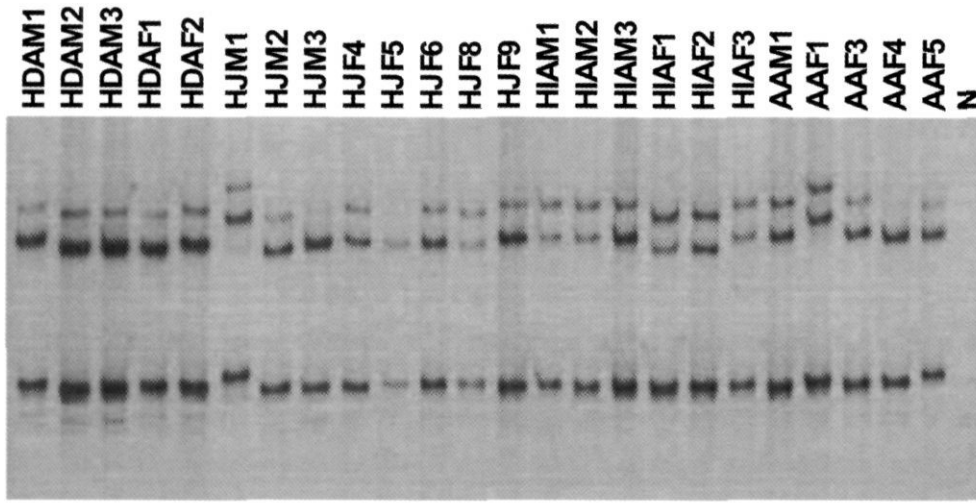

Figure 1 Single-strand conformation polymorphism analysis of sequence variation in a portion of the mitochondrial cytochrome *c* oxidase subunit 1 gene among specimens of *Oesophagostomum bifurcum* from human (first 19 lanes) and Mona monkey (following five lanes) (refer to Table 1 for sample codes). N represents a no-DNA control.

SSCP analysis of 24 p*cox*1 amplicons representing *O. bifurcum* from humans (codes HDAM1-3, HDAF1-2, HJM1-3, HJF4-6, HJF 8-9, HIAM1-3 and HIAF1-3) and Mona monkey (codes AAM1, AAF1 and AAF3-5) displayed 10 distinct profiles, each consisting of 2-3 strong bands, and 1-2 additional faint bands for the majority of samples (Fig. 1). Amplicons representing these 10 profiles were subjected to sequencing. Sequences of 393 bp in length were obtained, and their G+C content varied from 30.8-32.8%. Alignment of the p*cox*1 sequences revealed one polymorphic site for sample HDAF1 at alignment position 358 (alignment available from authors upon request). Pairwise comparison between the *O. bifurcum* samples showed sequence differences ranging from 0.3-8.4% (Table 2). Although sample AAF5 from the Mona monkey host showed the highest percentage of sequence differences (7.6-8.4%) with respect to the other nine *O. bifurcum* samples (0.3-2.5%), it was lower than that among any of the p*cox*1 sequences representing the five species of *Oesophagostomum* (Table 2).

Table 2 Pairwise comparison of sequence difference (%) in the pcox1 among samples representing *O. bifurcum* from humans and Mona monkey, and those representing *O. colombianum* (Oec), *O. dentatum* (Oed), *O. quadrispinulatum* (Oeq) and *O. venulosum* (Oev) from livestock hosts (also refer to Table 1)

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Oesophagostomum bifurcum														
1.HDAM1	-													
2.HDAF1	2.3	-												
3. HJM1	2.3	1.8	-											
4. HJM3	1.8	1.5	0.5	-										
5. HIAF1	2.5	1.5	1.8	1.3	-									
6. HIAF3	2.0	1.8	0.8	0.3	1.5	-								
7. AAM1	2.3	2.0	1.0	0.5	1.8	0.8	-							
8. AAF1	2.3	2.0	1.0	0.5	1.8	0.8	1.0	-						
9. AAF4	2.5	1.8	0.8	0.8	2.0	1.0	1.3	1.3	-					
10. AAF5	7.6	8.4	8.4	7.9	8.4	7.6	8.1	8.4	8.1	-				
Representatives of other species of *Oesophagostomum*														
11.Oec16.9	11.2	12.2	12.2	12.0	12.0	12.2	12.2	12.2	11.2	11.7	-			
12. Oed2	13.0	13.7	13.2	13.5	14.5	13.5	13.2	13.5	14.0	13.0	13.7	-		
13. Oeq3	11.5	12.5	11.5	10.9	11.7	10.9	10.7	11.5	11.7	12.7	13.0	12.0	-	
14. Oev3	12.5	14.0	13.2	13.0	13.7	12.7	13.0	13.0	13.0	13.5	11.5	12.7	12.7	-

Nucleotide differences were detected at 44 alignment positions in the p*cox*1 sequence. Of these, 42 (95.5%) were single base substitutions, including 34 (81%) transitions (A ‹–› G, n = 22; C ‹–› T, n = 12) and 8 (19%) transversions (A ‹–› C, n = 1; A ‹–›T, n = 7). Multiple substitution events were detected at 2 (4.5%) nucleotide positions. At sequence positions 111 and 216, a transition (A ‹–› G and T ‹–› C, respectively) and a transversion (A ‹–› T) were detected. Most of the sequence differences (n = 41; 93.2%) were at the third codon position, whereas the remainder of the sequence differences (n = 3; 6.8%) were at the first codon position. One amino acid difference (L ‹–› F) was detected at alignment position 2 in sample AAF5 (not shown). This amino acid difference was related to a transversion (A ‹–› C) at the third codon position at alignment position 6. In spite of the variation in the nucleotide and amino acid sequences within *O. bifurcum*, there was no unequivocal (i.e., fixed) nucleotide difference between *O. bifurcum* individuals from humans and those from Mona monkey.

The p*cox*1 sequences for representatives of four species of *Oesophagostomum* from different livestock species were compared with *O. bifurcum*. The p*cox*1 sequences (393 bp) determined for the five species of *Oesophagostomum* showed no variation in length, and their G+C content ranged from 29.5-32.3%. No nucleotide polymorphism was detected in the p*cox*1 sequence for any species of *Oesophagostomum* examined. The sequence differences (11.5-13.7%) in the p*cox*1 among these nodule worms, determined by pairwise comparison, are shown in Table 2. Among all sequences, interspecific nucleotide differences occurred at 91 (23.2%) of the 393 alignment positions, consisting of 40 (44%) transitions between purines (A ‹–› G, n = 30) or pyrimidines (C ‹–› T, n = 10), and 34 (37.4 %) transversions (G ‹–› T, n = 11 ; A ‹–› T, n = 23). Multiple substitution events were detected at 17 alignment positions. Of the 91 substitutions, most (n = 86) were found to be at third codon positions. The minority of substitutions was found at the first codon position (n = 2) and the second codon position (n = 3), which is typical for mitochondrial genes of nematodes.[137] Changes in the predicted amino acid sequences were detected at amino acid alignment positions 7 (F ‹–› S), 89 (V ‹–› I), 98 (S ‹–› L) and 120 (D ‹–› V), and related to substitutions at the first and second codon positions among the *O. bifurcum* samples at sequence alignment positions 20, 265, 293 and 359, respectively.

A dendrogram depicting the genetic differences among all haplotypes representing *O. bifurcum* from human and Mona monkey hosts and among species representing four heterologous species of *Oesophagostomum* (for comparative purposes) is shown in Fig. 2. In this dendrogram, the *O. bifurcum* haplotypes formed three groups to the exclusion of the other species of *Oesophagotomum*. There was no relationship between haplotype groupings and the specific primate host infected. Also, *O. bifurcum* and *O. quadrispinulatum* were found to be genetically most similar, followed by *O. columbianum*, *O. venulosum* and *O. dentatum*.

Compared with the magnitude of sequence differences in the p*cox*1 among representatives of the five species of *Oesophagostomum* examined, the absence of any

unequivocal (fixed) nucleotide difference between *O. bifurcum* from human and those from Mona monkey hosts provided support for the hypothesis that *O. bifurcum* from humans and Mona monkeys represents a single species, and that the haplotypic variability detected represents population variation. These findings are consistent with previous results for the ITS-2 nuclear rDNA region.[21]

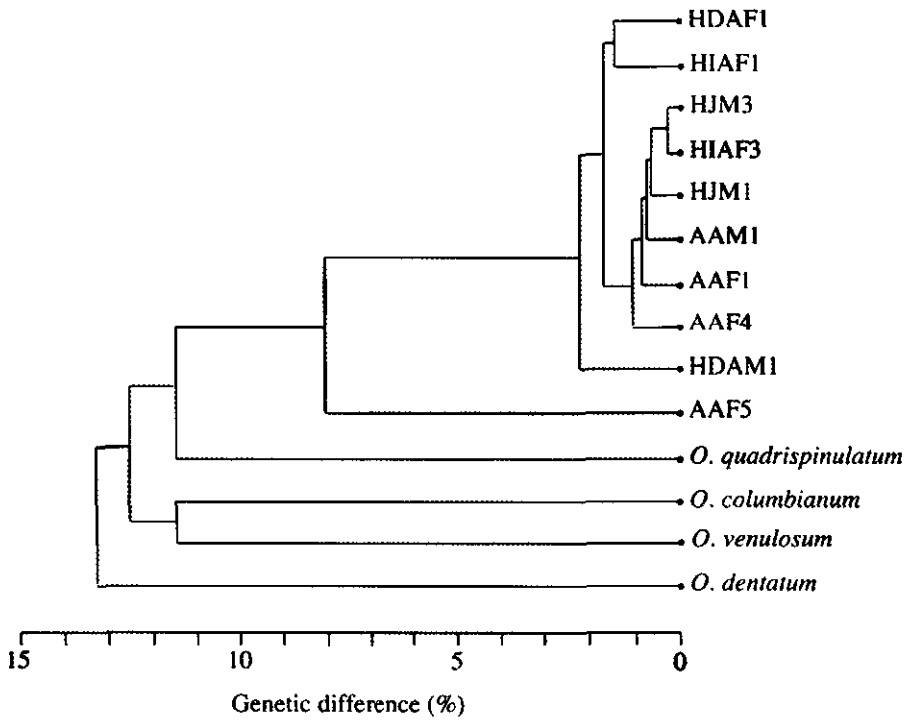

Figure 2 Dendrogram based on the sequences of part of the mitochondrial cytochrome *c* oxidase subunit 1 gene (393 bp) for *Oesophagostomum bifurcum* specimens from human and Mona monkey hosts, and other species of *Oesophagostomum* from livestock for comparative purposes (refer to Table 1 for sample codes).

Interestingly, the geographical distribution of human oesophagostomiasis appears to be localised to a well-defined area in northern Togo and Ghana, concentrated in several foci and with a decreasing prevalence toward the south of these countries.[22,23,37,40] In this particular area, the number of non-human primates has decreased significantly over the last decades (Polderman unpublished), which may suggest that humans have become a 'preferred' host for *O. bifurcum*. To the south, there are villages (e.g., Baobeng-Fiema) where *O. bifurcum* is commonly found in the Mona monkey, but not in humans, although both primate species live in close association. To date, there is no explanation for this fascinating observation, although recent experimental results may suggest that *O. bifurcum* is poorly adapted to lower primate hosts.[138] It is possible that differences in ecological factors, such as differences in host preference, feeding, social and cultural habits, climatic and environmental conditions and/or presence of transport hosts[24,139] are related to the difference in geographical distribution of *O. bifurcum* between humans and Mona monkeys. However, it remains possible that genetic substructuring does indeed exist between *O. bifurcum* from the two primate hosts, although this is not adequately reflected in the p*cox*1 data presented herein or in the ITS-2. Further work is required to examine large numbers of *O. bifurcum* obtained from a broad geographical and primate host range in Ghana and Togo. Future work will investigate the population genetic structures within *O. bifurcum* from human and non-human primates employing data obtained by AFLP™ or random amplification of polymorphic DNA (RAPD) analyses. This may provide insights into the epidemiology and ecology of this parasite, which causes serious health problems in northern Togo and Ghana.