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Phylogenetic patterns of *Haemonchus contortus* and related trichostrongylid nematodes isolated from Egyptian sheep

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Abstract

Haemonchus contortus is a major parasite of small ruminants and its blood-feeding behaviour causes effects ranging from mild anaemia to death. Knowledge of the genetic variation within and among *H. contortus* populations can provide the foundation for understanding transmission patterns and aid in the control of haemonchosis. Adult male *H. contortus* were collected from three geographical regions in Egypt. The second internal transcribed spacer (ITS2) of nuclear ribosomal DNA was amplified using the polymerase chain reaction (PCR) and sequenced directly. The population genetic diversity and sequence variations were determined. Nucleotide sequence analyses revealed one genotype (ITS2) in all worms, without genetic differentiation. The similarity in population genetic diversity and genetic patterns observed among the three geographical regions could be attributed to possible movement between the sites. This is the first study of genetic variation in *H. contortus* in Egypt. The present results could have implications for the rapid characterization of *H. contortus* and other trichostrongyloid nematodes, and evaluation of the epidemiology of *H. contortus* in Egypt.

Introduction

Haemonchus contortus (order Strongylida) is a common parasitic nematode that infects small ruminants and causes significant economic losses worldwide. The order Strongylida is divided into four suborders (Durette-Desset & Chabaud, 1993) that are distinguished by morphological characteristics, such as the mouth and caudal bursa, as follows: the Ancylostomatina (hookworms), Strongylina (strongyles), Trichostrongylina (trichostrongyles) and Metastrongylina (lungworms).

An understanding of the genetic variation within and among *H. contortus* populations can facilitate deeper comprehension of transmission patterns and establishment of a control strategy (Yin *et al.*, 2013). The blood-feeding activity of adult worms causes anaemia, oedema, diarrhoea and even death (Gasser *et al.*, 2008). There is high variation among breeds in their resistance to haemonchosis, with a significant advantage to locally adapted breeds (Besier *et al.*, 2016). Molecular phylogenies of the phylum Nematoda, based on the internal transcribed spacer 2 (ITS2), have been proposed recently (Kampfer *et al.*, 1998). Attempts to develop phylogenetic classification of the group using cladistic methods have, until recently, been restricted to the family Trichostrongylidae (Hoberg & Lichtenfels, 1994), and the superfamily Trichostrongyloidea (Durette-Desset *et al.*, 1999). Molecular studies have been numerous among these groups (Gasser & Newton, 2000), but

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have used mostly the ITS1 and ITS2 regions of rDNA for diagnostic purposes (Audebert *et al.*, 2000; Dallas *et al.*, 2000), with some phylogenetic reconstructions (Hoste *et al.*, 1998; Chilton & Gasser, 1999) limited to the intragenic level. Genetic variability of *H. contortus* from sheep and goats, using the mitochondrial DNA cytochrome oxidase subunit I gene (mtDNA COI) sequences revealed high rates of gene flow among populations (Hussain *et al.*, 2014). A major high-level molecular phylogenetic study on the Strongylida, based on ITS2 sequences, was limited to the suborder Strongylina (Chilton *et al.*, 1997). Other studies focusing on domestic and wild animals reported high genetic variation and relatively low host specificity for *H. contortus* in Brazil and Italy (Cerutti *et al.*, 2010; Brasil *et al.*, 2012). Population genetic investigations of *H. contortus* have been conducted in a wide range of topographical locales worldwide, including Australia, Brazil, Europe, Malaysia and the USA (Blouin *et al.*, 1995; Troell *et al.*, 2006; Hunt *et al.*, 2008). However, to our knowledge, genetic variability of *H. contortus* in Egypt has not yet been considered. Thus, in the present study, we investigated genetic variation within and among *H. contortus* in Egypt, employing the ITS2 of nuclear ribosomal DNA and the mtDNA COI gene as markers.

Materials and methods

Collection of adult worms

Fifteen adult male *H. contortus* worms were collected from sheep in three geographical regions in Egypt (Cairo, Giza and Qalubia). Both the abomasum and its contents were examined carefully and individual adult male worms were collected and identified by microscopic examination of spicules, according to the procedures outlined by Whitlock (1960) and MAFF (1986). The worms were preserved in 70% ethanol and stored at −20°C, until DNA extraction was performed.

Molecular analysis

Adult worm specimens were cut into fine pieces, and then ground in a sterile mortar, in which liquid nitrogen was used to disrupt the cells. DNA extraction kits (Qiagen) were used for extraction of DNA from the worm pellets, according to the manufacturer's protocols. The *H. contortus* DNA extracts were stored at −20°C. The primer sets used in the polymerase chain reaction (PCR) assay to amplify partially the ITS2 and COI gene of the *H. contortus* genome were, respectively, ITSF: 5′-ACGTCTGGTTCAGGGTTGT-3′, ITSR: 5′-TTAGTTT CTTTTCCTCCGCT-3′ and COIF: 5′-CCTACTATAATT GGTGGGTTTGGTAA-3′, COIR: 5′-TAGCCGCAGTAAA ATAAGCACG-3′, according to Stevenson *et al.* (1999) and Kanzaki & Futai (2002).

PCR was performed in a total volume of 50 µl containing 1 × PCR buffer (20 mM Tris–HCl, pH 8.4, and 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mixture (dATP, dCTP, dGTP and dTTP), 100 pmol of each primer, 2.5 units (U) *Thermus aquaticus* (*Taq*) polymerase, 0.1 µg of extracted parasite genomic DNA and nuclease-free sterile double-distilled water up to 50.0 µl. The resulting mixture was then subjected to a precise thermal profile in a programmable thermocycler (Biometra) as follows: for ITS2 an initial denaturation was made at 94°C for 120 s; 35 cycles at 94°C for 40 s, 36°C for 40 s and 72°C for 60 s; then followed by a final extension at 72°C for 600 s. For the COI gene an initial denaturation was made at 95°C for 120 s; 35 cycles at 95°C for 50 s, 55°C for 45 s and 72°C for 60 s; then the final extension at 72°C for 600 s. The resulting PCR amplicons (10–15 µl) were analysed using 1.5% agarose gel electrophoresis, as described by Sambrook & Russell (2000). The DNA bands were visualized after gel staining with ethidium bromide (0.5 µg/ml) against GeneRuler 100 bp Plus ready-to-use DNA ladder (molecular weight marker) (Fermentas).The PCR amplicons of the proper predicted size were gel purified using a DNA gel purification kit (ABgene).

The PCR DNA amplicon products were directly sequenced with the same primers used to generate PCR amplicons, using the BigDye Terminator v.3.1 Cycle Sequencing Kit on an automatic sequencer (3500 Genetic Analyzer; Applied Biosystems) (Sanger *et al.*, 1977).

The resulting nucleotide sequence data of the selected regions of ITS2 and the COI gene of *H. contortus* from local Egyptian sheep were compiled and submitted to GenBank (accession numbers KF176320 and KT826575). These sequence data were compared to those of other related family isolates accessed via GenBank. The nucleotide sequences were aligned using the ClustalW (1.82) program of the European Bioinformatics Institute.

Phylogenetic analysis

Phylogenetic analysis of the partial 361 bp length of ITS2 and 709 bp length of the COI gene of *H. contortus* included multiple and pairwise sequence alignments constructed using the ClustalW algorithm. Furthermore, the phylogenetic tree was constructed using the neighbour-joining method of the MegAlign program from the Laser Gene Biocomputing Software Package (DNASTAR, Madison, Wisconsin, USA).

Results

Agarose gel electrophoretic analysis of the PCR amplicons indicated that amplified DNA fragments encoding the ITS2 and COI gene corresponded to the expected lengths of about 361 bp and 709 bp, respectively.

Sequence comparisons of the partial genomic sequences of *H. contortus* ITS2 and the COI gene were performed for accurate, robust genotyping, and sequence alignment was performed using the multiple-alignment algorithm in the MegAlign program with sequences of 14 reference genotypes retrieved from GenBank. The sequences of the species under investigation in the present study showed no variation with 15 other sequences of worms from Egyptian sheep, and little variation with published ITS2 sequences. Analysis of the nucleotide sequence to establish the phylogenetic relationship with the genomic groups of PCR amplicons from ITS2 revealed a single open reading frame (ORF). A homology search revealed sequence similarity between this ORF and other published sequences. Therefore, the sequenced fragment of the local Egyptian strain under investigation was

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identified as the ITS2, as the location of the ITS2 is conserved throughout the subfamily.

In order to understand further the population structure, a comparison was performed of the partial genomic sequences (235 bp) of the *Haemonchus* COI gene obtained from sheep of various geographical areas in Egypt with 22 reference genotype sequences from other countries, retrieved from GenBank. Sequences from worms of Egyptian sheep showed little variation among each other, based on published reports, ranging in difference from seven to ten substitutions. On the other hand, sequences originating from Egypt showed great variation from sequences originating from other countries.

The percentage nucleotide identity between the Egyptian *Haemonchus* isolates and those of other ITS2 relatives in GenBank ranged from 98.3 to 99.6%, with a divergence range from 0.4 to 1.3%, indicating how closely related the species were. Our results showed that ITS2 sequence identity was recorded with the reference *H. contortus* intergenic spacer, ITS, isolate 15 (98.7%). Comparison of the local Egyptian *H. contortus* sequence with that of other *Haemonchus* isolates showed single or triplet mismatches or substitutions. Divergence and identity between the isolate of Egyptian *Haemonchus* and those of known *Haemonchus* strains worldwide have been reported previously. The isolate of the present study showed typical identity (99.6%) with HQ844231, KC998714, JQ342246 and JN128898, and 0.4% divergence, whereas the percentage identity with EU084684 was lowest at 98.3% and 0.9% divergence. The isolates of the present study showed highest identity with those of *H. contortus* isolated in Pakistan, including KJ724402 (94.9% identity and 4% divergence).

Phylogenetic analysis of aligned ITS2 and COI gene sequences of these Egyptian *H. contortus* families showed distinct clusters that revealed close ancestral genetic relationships with those retrieved from GenBank (fig. 1A and B). The phylogenetic tree was constructed to calculate and examine the evolutionary relationships of the sequences, in which the length of the horizontal line was proportional to the estimated genetic distance between the sequences.

Discussion

Although parasitological examination of sheep faeces is considered the gold standard for detection of trichostrongylid eggs, the technique can sometimes lack sensitivity and cannot identify the worm species (Lichtenfels *et al.*, 1994). A considerably more efficient approach entails amplification of a specific genetic region, using a technique such as PCR, one of the most advanced tools in recognizing almost all pathogens of veterinary importance (Learmount *et al.*, 2009). To address the shortcomings of the conventional diagnostics of parasitic gastroenteritis, many PCR assays have been created for specific, sensitive and rapid recognition and characterization of *H. contortus*.

Haemonchus contortus samples subjected to PCR expressed a 361 bp fragment of the ITS2, which is a stable, conserved region among trichostrongyloid genomes. The PCR assay can reliably distinguish and characterize *H. contortus* infections from those induced by other trichostrongyloids (Gharamah *et al.*, 2012). The PCR products obtained in the present study were purified and sequenced for proper confirmation. Alignment results revealed 100% homology among the three groups of sequenced Egyptian isolates, which suggests that the adult worms isolated from all animals were identical. On the other hand, PCR used in the present study amplified a segment from a highly conserved *H. contortus* gene (ITS2), which was used for the sequence alignment. The level of variation in ITS2 of the *H. contortus* species between sites was low. This value was 0.43% between *H. placei* from Uzbekistan and *H. placei* of bovine animals from Australia (Stevenson *et al.*, 1999), whereas among *H. contortus* isolates from various locations, this value reached 0.86%. Comparative sequence analysis revealed 99.6% homology with the *H. contortus* 18S ribosomal RNA gene (accession no. HQ844231.1), 99.6% homology with the *H. contortus* field variant 2 5.8S ribosomal RNA gene (accession no. KC998714.1) and 99.2% homology with the *H. contortus* field variant 15 (accession no. KC998713.1). Previous studies have confirmed that the causative worm isolated from cases of parasitic gastroenteritis in sheep is *H. contortus*, which has been identified phylogenetically and is consistent with other reports of *H. contortus* worldwide. Over the past few years, methods of molecular taxonomy have been applied to the study of polymorphism. *Haemonchus contortus* and *H. placei* are more closely related to each other than to other species (Jacquiet *et al.*, 1995). Stevenson *et al.* (1999) conducted a comparative study of various sites of the ITS2 in *H. contortus* and *H. placei*, and revealed only three differences in nucleotide sequences of ITS2. To clarify the objectivity of these conclusions, we conducted a comparative study of DNA samples of *H. contortus* collected from hosts inhabiting different regions. This comparison, in our view, will facilitate examination of intraspecific variability of DNA segments and aid in improving the effectiveness of molecular applications that determine species taxonomy of parasitic nematodes. The ITS region is suitable for molecular diagnosis across a diverse range of trichostrongyloid nematodes (Gasser *et al.*, 1994).

Nematodes such as *Haemonchus* species possess high levels of prolificacy, in addition to a high rate of infection and direct life cycle without an intermediate host, thus leading to a large effective population size with wide genetic variability (Prichard, 2001). The mitochondrial DNA genome has a higher rate of substitution than that of nuclear DNA, making it possible to resolve differences between closely related individuals (Anderson *et al.*, 1998; Blouin, 1998, 2002).

The present results show genetic diversity among populations of *H. contortus*, including those from sheep in Egypt. The COI gene sequences exhibited a high frequency of major differences, including insertions in 60 positions, deletions in four positions and substitutions in 56 positions. These results are consistent with those of many investigators, including Brasil *et al.* (2012) in Brazil and Hussain *et al.* (2014) in Pakistan, who have reported variation in COI gene sequences among *Haemonchus* species from various hosts worldwide.

The high level of genetic diversity observed in Egyptian sequences is typical of trichostrongylids (Blouin *et al.*, 1995; Braisher *et al.*, 2004; Silvestre *et al.*, 2009), and is

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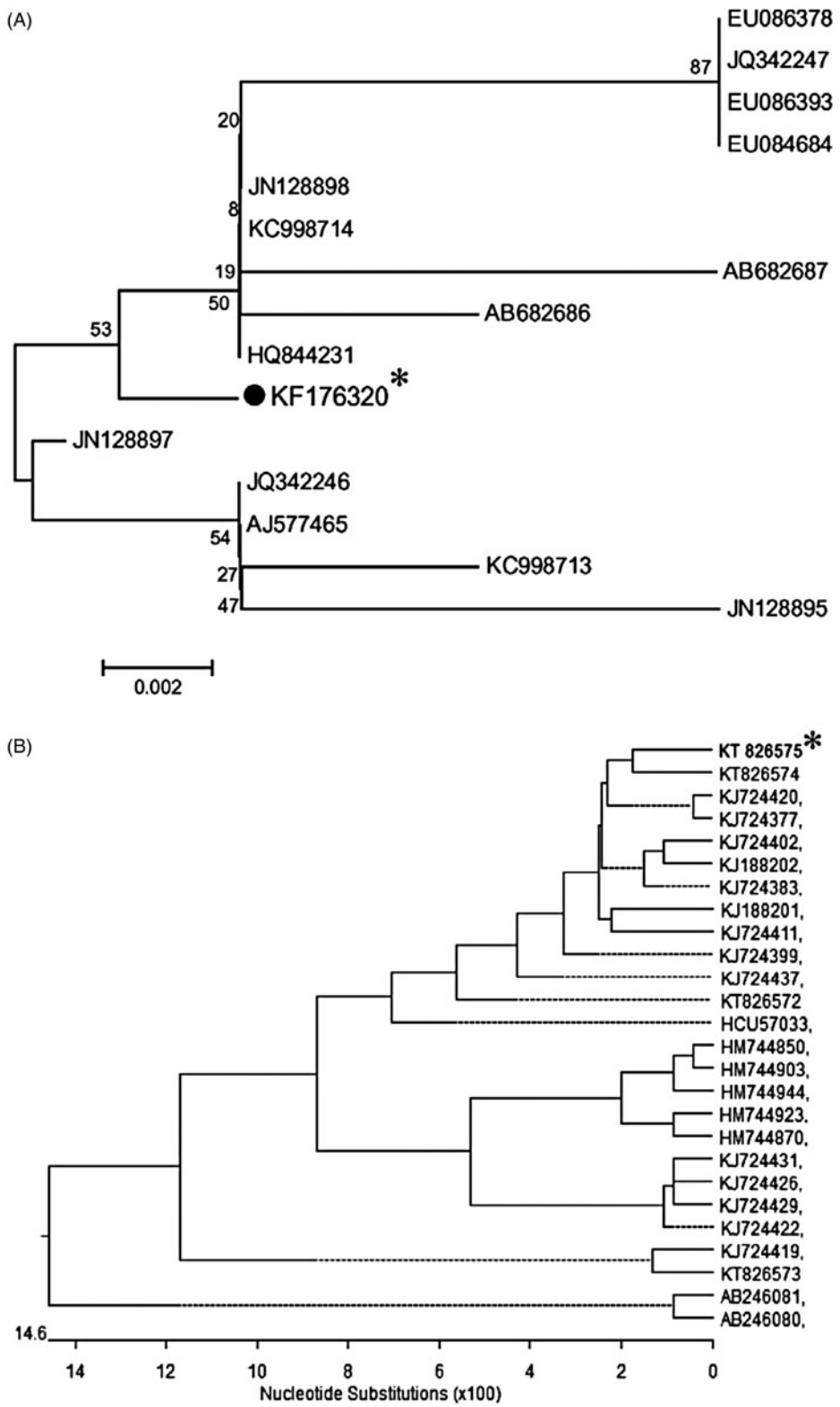

Fig. 1. Phylogenetic trees of *Haemonchus contortus* isolated from Egyptian sheep and other related isolates generated from (A) ITS2 and (B) the COI gene. Sequences were analysed using the ClustalW (1.82) program and neighbour-joining method of the MegAlign program. Genetic distances are indicated below the tree; * indicates the specific GenBank numbers of *H. contortus*.

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thought to be a consequence of both parasite-related and host-related factors. Parasite-related factors include high biotic potential, large population size, short life cycle and high infection rate, as well as high mutation rates observed in these highly polymorphic nematodes and persistence of the infective stage in the host environment that facilitates cross-species migration (Blouin *et al.*, 1995; Brasil *et al.*, 2012; Hussain *et al.*, 2014). These findings are also consistent with those of Riggs (2001), who attributed the differences in diversity parameters among *Haemonchus* species to variations in their prolificacy, prepatent period, host preponderance and evolutionary rate. Host-related factors include: (1) differences in ruminant species; (2) the presence of *H. contortus* among heterologous hosts, such as sheep, goats and cattle that share common pastures, thereby promoting transmission of infection from one host species to another; (3) absence of anthelmintic selection and subsequent potential infection in intensively managed flocks; and (4) intense gene flow across subpopulations resulting from the transfer of hosts (Akkari *et al.*, 2013).

In conclusion, for the first time, we obtained data on the DNA structure of *Haemonchus* nematodes collected from Egyptian sheep. Further investigations of *Haemonchus* nematodes in other ruminants in Egypt are required. The application of molecular techniques such as PCR and sequencing could lead to accurate methods for the detection of various genotypes. Sequence information facilitates a better understanding of the evolution and dissemination of *Haemonchus* in the field. Furthermore, knowledge of the genotypes present in Egypt will guide the application of efficient control strategies.

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Conflict of interest

None.

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