Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 156 (2008) 241–247
veterinary parasitology
www.elsevier.com/locate/vetpar

Haplotypic variability within the mitochondrial gene encoding for the cytochrome *c* oxidase 1 (*cox1*) of *Cylicocyclus nassatus* (Nematoda, Strongylida): Evidence for an affiliation between parasitic populations and domestic and wild equid hosts☆

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Received 4 January 2008; received in revised form 29 May 2008; accepted 30 May 2008

Abstract

This study investigated the genetic variability within *Cylicocyclus nassatus* (Nematoda, Strongylida, Cyathostominae) collected from different domestic and wild hosts (i.e. horse, donkey, Przewalskii horse, tarpan and Turkmen kulan) and localities in Europe and/or USA. The ribosomal Internal Transcribed Spacer 2 (ITS2) and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene were PCR-amplified and sequences characterized from seventy individual parasitic specimens. While ITS2 displayed 0–0.6% variation rate among all individual adult specimens of *C. nassatus* examined, 22 different sequence variants (haplotypes) of *cox1* were detected. Nucleotide variation was detected at 75 of the total 689 positions (overall 10.8% rate of intraspecific nucletidic difference) in the *cox1*, with the absence of invariable positions among specimens collected from each equid species or country. Conversely, two haplotypes were detected in horses from USA and in donkeys of Italy and Ukraine, respectively. The absence of haplotypes shared by the equid species suggests an affiliation of *C. nassatus* populations to their specific host. The results of the present study demonstrated that the characterization of mitochondrial regions may have important implications for studying the genetic structure and biology of equine cyathostomes, and to exploit taxonomic issues and practical implications related to the spread of anthelmintic resistance.

Keywords: *Cylicocyclus nassatus*; Horse; Donkey; Przewalskii horse; Tarpan; Turkmen kulan; Internal Transcribed Spacer 2; Cytochrome *c* oxidase subunit 1; Haplotypes

1. Introduction

Cyathostomes, cyathostomins or ‘‘small strongyles’’ (Nematoda, Strongylida), are presently considered the most important helminth parasites of equids. Adult and larval stages have been reported to be responsible for colic, weight loss, peripheral oedema, disorexia and

☆ Nucleotide sequence data reported in this paper are available in the GenBank™ under accession numbers from EU753193 to EU753214.

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doi:10.1016/j.vetpar.2008.05.031

lethargy (Uhlinger, 1991; Matthews and Morris, 1995; Murphy and Love, 1997). Most dramatically, larval stages encysted in the colon and caecum wall may synchronously emerge from the mucosa, thus causing the life-threatening larval cyathostominosis, characterized by severe colitis, protein-losing enteropathy, chronic diarrhoea, weight loss, and oedema (Love and McKeand, 1997; Love et al., 1999). Cyathostomes have a worldwide distribution and virtually 100% of horses and other equids are infected by them (Lyons et al., 1999). The emergence of drug-resistant populations has become a major concern in the control of cyathostomes in recent years and a constraint for equine health management programs (reviewed in Lyons et al., 1999; Kaplan, 2002, 2004). Of the approximately fifty cyathostome species described (Lichtenfels et al., 1998), 13 account for 98–99% total parasitic burden of infected animals (Lyons et al., 1999; Chapman et al., 2002; Kaplan, 2004). Within this group, *Cylicocyclus nassatus* is one of the most common, infects different wild and domestic hosts, has been shown to be resistant to common anthelmintics, and has been used as a model for molecular studies to evaluate the variation of genetic polymorphism within populations exposed to macrocyclic lactones (Tandon et al., 2005) and to investigate the genetic mechanism of drug resistance (Clark et al., 2005; Blackhall et al., 2006; Tandon et al., 2006). Hence, this species was chosen also for the purposes of the current study.

While non-encoding nuclear regions, i.e. Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) and InterGenic Spacers (IGS) of ribosomal DNA (rDNA) have been used for taxonomical delineation and identification of bursate nematodes (Chilton, 2004), including cyathostomes (Hung et al., 1999; Traversa et al., 2007), mitochondrial DNA (mtDNA) protein-coding genes are particularly useful to study genetic structures of parasitic populations for their maternal inheritance and higher mutation evolutionary rates (Blouin, 2002). In particular, the mtDNA gene encoding for the cytochrome *c* oxidase subunit 1 (*cox1*) has proven to be suitable as a molecular marker for population studies and haplotypic diversity evaluation in parasitic nematodes (e.g. Zhu et al., 2000; Hu et al., 2002; Otranto et al., 2005).

Indeed, the estimation of genetic variation within parasites is pivotal for studying their epidemiology and biology at population level, but information on the genetic variation within and among population of parasitic nematodes are still limited to few *taxa* (Hu and Gasser, 2006) and there are neither data on within-species nucleotidic heterogeneity or haplotypic diversity for equid cyathostomes nor information on their *cox1*, with the exception of a work for phylogenetic purposes for the strongylid group affecting equids (McDonnell et al., 2000). More specifically, there are no reports on the population genetics and sequence heterogeneity within cyathostome populations from different hosts and geographic areas. Thus, the aim of the present work was to provide information on the haplotypic diversity in a *cox1* region within different populations of *C. nassatus* (selected as a model cyathostome) collected from domestic and wild equid species in different geographic regions.

2. Materials and methods

2.1. Parasite source

Adult specimens of *C. nassatus* were collected after deworming or at necropsy of naturally infected equids, both domestic (i.e. horse: *Equus caballus*; donkey: *Equus asinus*) and wild (i.e. Przewalskii horse: *Equus przewalskii*; tarpan: *Equus gmelini*; Turkmen kulan: *Equus hemionus*) coming from different countries (Table 1). After morphological identification to species, the nematodes were washed extensively in physiological saline and fixed in absolute ethanol pending molecular analysis.

2.2. PCR protocols

Individual nematodes were exposed to liquid nitrogen and then subjected to the genomic DNA extraction using a commercial kit (DNEasy Tissue kit, QIAgen, Gmbh, Germany). All single DNA extracts (n. 70) were subjected to PCRs specific for the ribosomal ITS2 and for the *cox1* gene.

The ITS2 and rRNA flanking regions were amplified from each genomic DNA extract using the primer set D (5′-GAGTCGATGAAGAACGCAG-3′) and B1 (5′-GAATCCTGGTTAGTTTCTTTTCCT-3′) (Bachellerie and Qu, 1993) in 50 μl reaction containing 2.5 μl of template, 25 picomoles of each D and B1, 12.5 μl of Ready Mix REDTaq (Sigma, St. Louis, MO) and rinsed with distilled water. PCRs were performed in a thermal cycler (2700, Applied Biosystems, Foster City, CA) using the following cycling protocol: 94 °C for 12 min, and 30 cycles at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s, followed by a final extension for 7 min at 72 °C.

An informative 689 bp long region of the cox1 gene was PCR-amplified using the set of primers NTF (5′-TGATTGGTGGTTTTGGTAA-3′) and NTR (5′-ATAAGTACGAGTATCAATATC-3′) (Casiraghi et al.,