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Short communication

ITS1 intra-individual variability of *Ascaris* isolates from Brazil

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ABSTRACT

The zoonotic potential of *Ascaris* infecting pigs has stimulated studies of molecular epidemiology with internal transcribed spacer 1 (ITS1) as the target. The aim of this study was to determine the value of *Ascaris* ITS1 as a molecular marker through assessing the intra-individual genetic diversity of *Ascaris* isolates from two geographical areas of Brazil. DNA was extracted from single isolated eggs, ITS1 PCR was performed, and the PCR products were cloned and sequenced. Clone analysis showed high ITS1 intra-individual variability revealed by 2–4 ITS1 genotypes/haplotypes per sample (egg). Two genotypes, G1 and G6, and 13 new haplotypes were detected and characterized. The most prevalent in humans, G1 and/or the Brazilian G6, were detected in all samples. Except for genotype G1, no relationship was observed between Brazilian ITS1 genotypes/haplotypes and those previously described in China, Bangladesh, Japan, United Kingdom, Australia, and Denmark, with respect to geographic origin or host affiliation. However, an association between the two geographically separated Brazilian ITS1 isolates was observed. The ITS1 intra-individual variability revealed in this study indicated that the use of this genetic region to discriminate human and pig *Ascaris* genotypes should be reconsidered.

1. Introduction

One of the major soil transmitted helminthiases (STHs) is the intestinal nematode *Ascaris lumbricoides* (L.), which is widely prevalent and has a cosmopolitan distribution [1,2]. Transmission is normally through the ingestion of infective *Ascaris* sp. eggs in contaminated soil and vegetables. A closely related species, *Ascaris suum* Goeze, infects pigs. Discrimination between *A. lumbricoides* and *A. suum* is difficult due to the absence of distinguishing morphological characteristics. Cases of cross-host infection have been reported in North America and Denmark [3,4]. Molecular epidemiology investigations have been proposed to determine if pigs are a potential reservoir of the *Ascaris* that infects humans [3–6]. Studies of genetic characterization and molecular diagnosis have used nuclear internal transcribed spacer 1 (ITS1), mitochondrial cytochrome c oxidase subunit 1 (*cox*1), NADH dehydrogenase subunit 1 (*nad*1) targets, and recently, microsatellite markers [5–8]. Specifically, through the PCR–RFLP approach it was possible to identify ITS1 restriction patterns corresponding to *Ascaris* from human and pig hosts [7,9]. Genotype cross-infection was detected in North America by the presence in humans of the ITS1 pattern of *Ascaris* from pigs [9]. Similar results were observed in Denmark using ITS1 PCR–RFLP and AFLP approaches [4]. Using the SSCP technique, five ITS1 genotypes (G1–G5) were identified in human *Ascaris* and 3 in pig *Ascaris* (G1–G3) [5]. In six endemic regions of China, the genotype G1 frequently infected humans, and the genotype G3 was predominant in pigs, while the other three genotypes were detected at lower frequencies in their respective hosts. These studies suggested that there is a particular host affiliation of ITS1 genotypes [5]. Recently, an ITS1 *Ascaris* analysis in Brazil detected G1 and also revealed the genotype G6 in the human population [10].

The aims of this research were to assess the ITS1 intra-individual variability in *Ascaris* isolates from Brazil and determine the value of this region as a molecular marker of human and pig *Ascaris*.

2. Material and methods

Human fecal samples positive for *A. lumbricoides* ($n=9$) from Rio de Janeiro ($n=7$) and Santa Isabel do Rio Negro ($n=2$), Amazon, in southeast and northern Brazil, respectively, were analyzed (Table 1).

Table 1
Brazilian *Ascaris* samples, geographic origin, and ITS1 genotypes and haplotypes.

Samples	Geographic origin	Genotypes	Haplotypes
DL01	Rio de Janeiro—RJ, SE	G1/G6	–
DL02	Rio de Janeiro—RJ, SE	G1/G6	H1
DL04	Rio de Janeiro—RJ, SE	G6	H2–4
DL13	Rio de Janeiro—RJ, SE	G1	H5–6
DL15	Rio de Janeiro—RJ, SE	G1	H7
DL16	Rio de Janeiro—RJ, SE	G1	H8–9
DL17	Rio de Janeiro—RJ, SE	G6	H10–11
041–1	Santa Isabel do Rio Negro—AM, NO	G6	H12
104–5	Santa Isabel do Rio Negro—AM, NO	G1/G6	H13

RJ: Rio de Janeiro State, AM: Amazonas State, SE: Brazil southeast region, NO: Brazil north region.

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Table 2
ITS1 polymorphic sites on genotypes/haplotypes of the *Ascaris* isolates from Brazil and worldwide.

				Nucleotide variation at alignment position									
Genbank	Genotypes/haplotypes	Country	Host	120	124	127	128	129	130	133	150	155	156
AJ554036	G1	Ba/Br/Ja/Ch	H/P	T	T	T	–	–	G	G	T	A	T
AJ554037	G2	Ch	H/P	.	.	.	T	.	.	S	.	.	.
AJ55403	G3	Ja/Ch	H/P	.	.	.	T	.	.	C	.	.	.
AJ554039	G4	Ch	H	.	.	.	.	.	.	.	.	.	.
AJ554040	G5	Ch	H	.	.	.	.	.	.	S	.	.	.
EF153621	G6	Br	H	–	.	.	.	.	.	.	.	.	.
AJ000895	Al	Au	H	.	.	.	.	.	.	.	.	.	.
AJ000896	As	UK/De	P	.	.	.	T	T	.	C	.	.	.
GQ339794	H1	Br	H	.	–	.	.	.	.	.	.	.	.
EU635686	H2	Br	H	.	.	–	.	.	.	.	.	.	C
EU635687	H3	Br	H	.	.	–	.	.	.	.	.	.	.
EU635688	H4	Br	H	.	.	–	.	.	.	.	.	.	C
GQ339795	H5	Br	H	–	.	.	.	.	.	.	.	.	.
GQ339796	H6	Br	H	.	.	.	.	.	.	.	C	.	.
GQ339797	H7	Br	H	–	.	.	.	.	.	.	.	.	.
GQ339798	H8	Br	H	C	.	.	.	.	.	.	.	.	.
GQ339799	H9	Br	H	.	–	.	.	.	.	.	.	.	.
EU635694	H10	Br	H	.	.	–	.	.	.	.	.	.	.
EU635695	H11	Br	H	.	.	–	.	.	T	.	.	G	C
GQ339800	H12	Br	H	–	.	.	.	.	.	.	.	.	.
GQ339801	H13	Br	H	.	C	.	.	.	.	.	.	.	.

Numbers correspond to nucleotide position on reference sequence AJ554036 [5]. H: human, P: pig, Al: *A. lumbricoides* (without nomenclature), As: *A. suum* (without nomenclature), Ba: Bangladesh, Br: Brazil, Ch: China, Au: Australia, UK: United Kingdom, De: Denmark, Ja: Japan. S: nucleotide G or C, R: nucleotide A or G, W: nucleotide A or T. –: nucleotide deletion, dots: similar to reference sequence G1. ▼: in this paper.

To verify ITS1 intra-individual variability of *Ascaris*, DNA was extracted from a single egg isolated from each sample, and PCR was performed following the methodology of Leles et al. [10], using the primers XZ5 forward 5′-TGATGTAATAGCAGTCGGCG-3′, XZ1 reverse 5′-GGAATGAACCCGATGGCGCAAT-3′ and NC13 reverse 5′-GGCTGCG TTCTTCATCAT-3′ [7]. To prove the intra-individual variability the PCR products were cloned into the pGEM-T Easy Vector System (Promega), and at least 3 clones for each sample were sequenced on both strands in a 3100 Automated DNA Sequencer (Applied Biosystems) as described by the suppliers. Chromas v 1.45 (School of Health Science, Griffith University, Queensland, Australia), Bio Edit v 5.0.9 (Department of Microbiology, North Carolina State University, USA), and DAMBE v 4.2.13 were used for editing and sequence analysis. The nucleotide sequences from this study were deposited in the GenBank database under the accession numbers: EF1536919–23, EU635683–95, and GQ339794–GQ339801.

3. Results

Twenty eight ITS1 clones of isolated *Ascaris* eggs were obtained. Sequence analysis revealed 15 ITS1 sequence clones corresponding to 2 genotypes previously characterized, G1 and G6 [5,10], and 13 new haplotypes (sequence variants of ITS), referred to as H1–H13 (Table 1). In each sample, 2–4 genotypes/haplotypes were found. None of the samples showed the polymorphisms characteristic of the genotypes G2, G3, G4, or G5 (Table 2). The genotypes G1 and/or G6 were found in all samples, and each new haplotype in a single sample. The presence of G1 and G6 genotypes in both Brazilian geographical areas revealed a relationship between them. Except for genotype G1, no relationship was observed between Brazilian ITS1 genotypes/haplotypes and those previously described in China, Bangladesh, Japan, United Kingdom, Australia, and Denmark (Table 2).

4. Discussion

Criscione et al. [8] analyzed microsatellite loci of 129 *Ascaris* from humans and pigs from China, Guatemala, and Nepal. They concluded that hybridization occurs in *Ascaris* infecting sympatric populations of humans and pigs, and, consequently, long-term control measures should be re-evaluated. The two geographically separated locations studied in Brazil have a high prevalence of *Ascaris*. Costa-Macedo et al. [11] recorded a prevalence of *Ascaris* infection of 25% in children from low-income families in Rio de Janeiro. The Santa Isabel do Rio Negro Amazonas region showed a prevalence of 40% in the general population [12]. In Brazil, diagnosis of parasite infection is generally by microscopy, which cannot discriminate between eggs of *Ascaris* from humans and pigs, and studies of the molecular epidemiology are rare.

In a previous study we identified two ITS1 *Ascaris* genotypes, G1 and G6, in the same fecal sample [10]. This suggested the possibility of intra-population diversity of ITS1 in *Ascaris*, due to co-infection with different *Ascaris* genotypes. However, it was not possible to assess ITS1 intra-individual variation. In the present study, considering a single *Ascaris* egg as an individual, ITS1 intra-individual variation was demonstrated by the presence of 2–4 genotypes/haplotypes in each sample. Considering that ribosomal spacers are non-coding regions and occur in multiple copies (~42 copies in *Ascaris* [13]), it is possible that differing ITS1 sequences occur in an individual egg. Some researchers have utilized two ITS1 in *Ascaris* based on 6-bp differences to discriminate the two species: *A. lumbricoides* and *A. suum* [5,7]. Peng et al. [5] proposed five *Ascaris* genotypes (G1–G5), of which genotypes G2, G4, and G5 contain ambiguous nucleotide positions. These ambiguous positions were interpreted by the authors as indicative of hybridization or interbreeding of individuals with different genotypes. We believe that each of these genotypes in fact represents two different genotypes characterized in an individual. Consequently, this suggests *Ascaris* ITS1 intra-individual diversity, which was not detected, because ITS1 cloning was not performed. Pecson et al. [13] developed a real-time PCR for quantifying viable *Ascaris* eggs based on ITS1 sequence. The authors used cloning and sequence analysis to verify the probe sequence. Interestingly, a human genotype clone was observed in eggs collected exclusively from pig intestine. We interpreted this finding as indirect evidence of ITS1 intra-individual variability (or intra-population variability, since more than one egg was used), which was observable only through *Ascaris* ITS1 cloning. ITS1 analysis without cloning assays revealed only one ITS1 copy, possibly the most frequent ITS1 copy, or a copy with ambiguous nucleotide positions, leading to genotype misclassification and/or data suppression of the genetic structure of the *Ascaris* population.