

Title page

PCR-RFLP on the rDNA cannot unequivocally assign *Ascaris* to species level or identify hybrids

Authors:

Peter Nejsum, Department of Clinical Medicine, Faculty of Health, Aarhus University, Denmark

Martha Betson, School of Veterinary Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK

Russell Stothard, Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool, UK

Conflict of interest: None

Funding: N/A

Number of words: 708

Number of references: 6

Table: 1

Accepted Manuscript

To the Editor—We read with interest the report by Chelladurai et al. [1] where 100 adult *Ascaris* were obtained from pigs at local abattoirs in Iowa, USA and characterized by DNA sequencing of a part of the mitochondrial *cox1* gene and by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the nuclear ribosomal DNA (rDNA) complex. The debate on the epidemiological and evolutionary distinction between *A. lumbricoides* and *A. suum* continues, for the latter PCR-RFLP method, as used by Zhu et al. [2], cannot unequivocally designate worms into “human” (*A. lumbricoides*), “swine” (*A. suum*) and “hybrid” genotypes simply based on the number of restriction fragments, i.e. two, three and four bands, respectively.

In an alternative multi-locus fingerprinting analysis, amplified fragment length polymorphism (AFLP) was used by Nejsum et al. [3]; 151 polymorphic markers were used to genotype *Ascaris* obtained from humans and pigs in Denmark. Cluster analysis separated worms into two major groups – human and pig derived, except the human worms which were identified as cross-infections. Among the 55 pig worms, PCR-RFLP of the rDNA revealed three profiles (i.e. 2 bands, 3 bands and 4 bands) in the following proportions 6.3%, 60.0% and 32.7%, respectively. Similar proportions were observed among *Ascaris* (n=32) from humans from Denmark which all were identified as cross-infections. This suggests that all three rDNA PCR-RFLP genotypes could be found among *A. suum* from pigs in Denmark. We note that these frequencies amongst the Danish *A. suum* are also very close to the lowland percentages of 12.9%, 54.8% and 32.2%, respectively [1].

In a study by Betson et al. [4], 8 multi-locus microsatellite markers were used to genotype *Ascaris* from humans and pigs and we now have applied the PCR-RFLP analysis method on a random subset of these worms from Africa identified as *A. lumbricoides* and *A. suum* (see Table). Most human and pig worms showed either 2 and 3 band patterns, despite the fact that none of these worms were identified as hybrids, both *A. suum* and *A. lumbricoides* displayed the four-band pattern and, as noted by Nejsum et al. [3], to be most frequent for *A. suum*.

It is interesting to speculate why some worms have the four fragment PCR-RFLP type which resembles a heterozygote profile and a putative hybrid worm, but is at odds with the dynamics of heterozygosity at other loci. The dynamics of the rDNA array is more complex than a simple single locus; it is a multi-copy gene in tandem repeated orientation and subjected to several molecular drive mechanisms or DNA turnover processes such as slippage, gene conversion and unequal crossing over. For *Ascaris*, it has been estimated that there are about 40 copies per cell [5] and the *HaeIII* sites are located in the internal transcribed spacer region 1 (ITS 1) with presence and absence giving rise to two and three fragments, respectively [2]. Worms that have both types of ITS-1 copies will, therefore show a ‘mixed profile’, i.e., have four fragments.

The reason why all human and pig worms do not exhibit either two or three fragments, respectively, most likely relates to incomplete homogenization of the rDNA array. Heterogeneity within the rDNA array is known at an intra-individual level, having been described in several other parasitic nematodes, including *Trichuris trichiura* [6]. This is likely due to the very close evolutionary relationship between *A. lumbricoides* and *A. suum* and insufficient time for the arrays to homogenize fully with fixation of species-specific alleles.

Thus, we caution against firm interpretation that worms exhibiting four bands using the PCR-RFLP represent ‘recent’ hybrid worms, rather than simply the retention of an ancestral rDNA array prior to

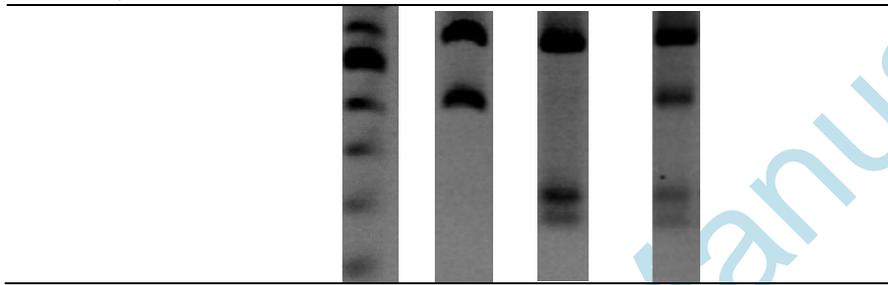
speciation which has failed to homogenize. Similarly, worms cannot be unequivocally assigned as *A. lumbricoides* based on the presence of two fragments; hence, we advocate that PCR-RFLP analysis be used in conjunction with other multi locus genotyping methods.

References

1. Jesudoss Chelladurai J, Murphy K, Snobl T, Bader C, West C, Thompson K, Brewer MT. Molecular epidemiology of *Ascaris* infection among pigs in Iowa. *J Infect Dis* **2017**; 215:131-138.
2. Zhu X, Chilton NB, Jacobs DE, Boes J, Gasser RB. Characterisation of *Ascaris* from human and pig hosts by nuclear ribosomal DNA sequences. *Int J Parasitol* **1999**; 29:469–78.
3. Nejsum P, Parker ED, Frydenberg J, et al. Ascariasis is a zoonosis in Denmark. *J Clin Microb* **2005**; 43:1142–8.
4. Betson M, Nejsum P, Bendall R, Deb RM, Stothard JR. Molecular epidemiology of ascariasis : A global perspective on the transmission dynamics of *Ascaris* in people and pigs. *J Infect Dis* **2014**; 210: 932–41.
5. Pecson BM, Barrios JA, Johnson DR, Nelson KL. A real-time PCR method for quantifying viable *Ascaris* eggs using the first internally transcribed spacer region of ribosomal DNA. *App Environ Micro* **2006**; 72: 7864–72.
6. Nissen S, Al-Jubury A, Hansen TVA, et al. Genetic analysis of *Trichuris suis* and *Trichuris trichiura* recovered from humans and pigs in a sympatric setting in Uganda. *Vet Parasitol* **2012**; 188:68–77.

Table. Number of fragments generated for *Ascaris* samples after PCR-RFLP analysis of the rDNA with *Hae*III. Worms were identified as *Ascaris lumbricoides* and *A. suum* using microsatellite markers [4]. The fragment pattern on the agarose gel is given below with the 100 bp ladder to the left.

	Number of fragments			Total
	2	3	4	
<i>A. lumbricoides</i> , Uganda	45	0	1	46
<i>A. suum</i> , Uganda	0	48	8	56
<i>A. suum</i> , Tanzania	0	10	2	12



Accepted Manuscript