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Short communication

A novel technique for identification of *Ascaris suum* cohorts in pigs

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Abstract

The objective of the present study was to develop a fast, cheap and reliable technique for identifying different cohorts of the swine parasite, *Ascaris suum*. A polymerase chain reaction linked restriction fragment length polymorphism (PCR-RFLP) technique on mt-DNA was used to identify unique haplotypes of four gravid *A. suum* females on agarose gels after eggs were recovered from each of the worms. Each of four pigs was inoculated with 2000 embryonated eggs originating from one of the four identified *Ascaris* haplotypes, respectively. *Ascaris* larvae were isolated from the small intestine at day 14 post-infection using an agar technique. Single larvae from each pig were transferred to 96-well PCR plates and a simple DNA extraction using a worm lysis buffer was carried out and followed by the PCR-RFLP analysis. More than 100 larvae from each of the four pigs were analysed and all were found to have the same haplotype as the parental female. We conclude that unique haplotypes of female *A. suum* and offspring can be identified by means of PCR-RFLP on mt-DNA and suggest that this method can be used in future research on *Ascaris* population biology using cohorts with distinct mt-DNA profile.

Keywords: Ascaris; Cohorts; Challenge infection; Host–parasite interaction; Genotypes

1. Introduction

An ability to trace and follow the fate of different cohorts of endoparasites in a host will facilitate detailed studies of the host–parasite relationship under experimental conditions. It will be possible to investigate basic knowledge on population biology like, when and to what degree do parasites establish during continuous exposure and which role do parasite genetics play on the host–parasite interaction. Previously, cohorts of radiolabelled infective larvae have been used successfully in ruminants (e.g. Barger and Lejambre, 1988; Seaton et al., 1989) whereas similar studies in pigs have been unsuccessful (Roepstorff and Johansen, unpublished data). Later studies have applied cohorts of anthelmintic resistant and susceptible nematodes (e.g. Dobson et al., 1990; Roepstorff et al., 1996) but this approach has several limitations. Firstly, the availability of anthelmintic resistant strains will most often limit the number of cohorts to two and secondly, very solidly susceptible and resistant strains are needed in order to avoid any

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residual population after anthelmintic treatment and following inconclusive results. More recently Sørensen et al. (1999) used PCR-RFLP to distinguish between two laboratory maintained isolates of *Schistosoma japonicum* and these authors subsequently followed the isolates as separate cohorts in the same animals.

Mitochondrial DNA is in general considered to be maternally inherited (e.g. Lansman et al., 1983), what also seems to be the case for pig roundworm, *A. suum* (Anderson et al., 1995). mtDNA may thus serve as a useful target for a marker for cohort studies as the offspring from a given female will carry the same mtDNA haplotype, irrespectively of the male(s) which she has mated. Therefore, by using eggs extracted from different female haplotypes in experimental infections, one may expect that the offspring, i.e. the nematode population establishing in the host, will hold the same mtDNA profile (e.g. as determined by PCR-RFLP) as that of their parental female.

We here present a fast PCR-based method for identifying and tracing different cohorts of *A. suum* in experimentally infected pig.

2. Materials and methods

2.1. Production of single female egg pools

Adult pig roundworms, *A. suum*, were collected from the small intestine of pigs from a Danish abattoir originating from different farms. Immediately after arrival at the lab, the individual uteri were removed from 16 worms and eggs were extracted from the lowest part (3–4 cm) of the uteri into a Petri dish by squeezing the eggs out of the uteri with a scalpel. The worms were subsequently stored in 70% ethanol at 5 °C while the eggs from each female were transferred to 50 ml centrifuge tubes using 10 ml of water. Extreme care was taken not to transfer eggs from one female egg pool to another by using new Petri dishes and scalpels for each worm and by heat treatment of the tweezers. The eggs were decoated by adding 10 ml of 1.0% sodium hypochlorite (NaClO) and gentle inverting the tubes for 8 min. Then, 25 ml of water were added and the tubes were centrifuged for 5 min at 70 × *g* and the supernatant discharged. The eggs were washed with 45 ml water, centrifuged for 5 min at 70 × *g* and the water was discharged. This washing procedure was repeated 4 times in order to remove excess hypochlorite. Then 20 ml of 0.05 M H_2SO_4 was added and the eggs were transferred to 50 ml cultivation flasks. The eggs were counted and in order to avoid density-dependent inhibition of egg development (Eriksen, 1990) concentrations of less than 25 eggs/μl were ensured by adding additional acid. The eggs were stored in a dark place at room temperature and embryonation was followed twice a week for at total of 12 weeks. After embryonation the eggs were stored at 10 °C.

2.2. PCR-RFLP

The four *A. suum* females with the highest number of infective eggs were selected. DNA was extracted from 2 cm of muscle tissue from the anterior part of the worm using QIAGEN tissue kit (Qiagen, Germany) according to the manufacturer's instructions except that DNA was eluted in 100 μl elution buffer. DNA was stored at 5 °C. As described by Anderson et al. (1995) a 633 bp fragment of the mtDNA comprising the d-loop region was amplified. PCR cycling conditions: 95 °C for 15 min followed by 30 cycles consisting of 95 °C for 30 s, 63 °C for 40 s and 72 °C for 1 min, and a final extension cycle of 72 °C for 7 min.

The amplified fragment was purified by Qiaquick PCR-purification kit (Qiagen, Germany) and subsequently sequenced in both directions. The same PCR-primers and a BigDye terminator sequencing system were used according to the manufacturer's instructions (Applied Biosystems, USA). After ethanol precipitation the sequencing products were run on an ABI3130XL (Applied Biosystems, USA).

The sequence information and NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php) were used to identify restriction enzymes, which could be used to differentiate between the four *Ascaris* females. *Ssp*I, *Hpy*188III and *Dra*I were identified as potential restriction enzymes and also had the property that they could be performed in a single multiple digestion reaction.

In order to verify the restriction sites 5 μl of the PCR product from each of the 4 females were digested with 2U *Ssp*I, 2 U *Hpy*188III and 5U *Dra*I using NEBuffer 2 (New England Biolabs, USA) in a total volume of 10 μl for 2 h at 37 °C. After gel electrophoresis of 5 μl of the digested PCR product on a 1.5% agarose gel (120 V, 90 min) the 4 females could be identified by a unique fragment pattern.

2.3. Experimental infections

Each of four SPF-pigs (crossbreed Landrace, Yorkshire, Duroc; 10 weeks of age) were inoculated by stomach tube with 2000 embryonated eggs originating from one of the four identified *Ascaris* haplotypes (A, B, C and D), respectively. At day 14 post-infection the pigs were killed, eviscerated and immature *Ascaris* (4th stage larvae) were isolated from the entire small intestine using