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Molecular and parasitological tools for the study of *Ascaridia galli* population dynamics in chickens.

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[Running title: Tools for population dynamics of *A. galli*]

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Abstract

Experiments were first conducted to compare and evaluate different methods of *Ascaridia galli* larval recovery from the chicken intestine. The number of larvae recovered from the intestinal wall of chickens infected with 1000 embryonated *A. galli* eggs and killed 15 days post infection (p.i.) by three methods (EDTA, pepsin digestion and scraping) were compared. The EDTA and pepsin digestion were found to be the most efficient methods with no significant difference ($P > 0.05$) in number of recovered larvae between the two. Subsequently, three different *A. galli* cohorts were established using the polymerase chain reaction linked restriction fragment length polymorphism (PCR-RFLP) technique. A 533 bp long region of the cytochrome c oxidase subunit 1 (*cox1*) gene of the mitochondrial DNA (mtDNA) was targeted and 22 *A. galli* females were allocated in to three different haplotypes. The four females with the highest embryonation rate from each haplotype group (total 12 females) were selected and used to inoculate each of 12 chickens with a dose of 1000 embryonated eggs. The chickens were killed 15 days p.i. and *A. galli* larvae were recovered from the small intestinal wall by the EDTA method and by sieving the lumen content on a 90 μm sieve. DNA of 40 larvae from each of the three different haplotypes was extracted using a worm lysis buffer (WLB) and PCR-RFLP analysis of these larvae revealed same haplotype as that of their maternal parent. The identification of distinguishable cohorts may be a powerful tool in population studies of parasite turnover within the animal host.

Introduction

Ascaridia galli (Schrank, 1788) is a nematode parasite that infects domestic as well as wild birds (Soulsby, 1982). Studies have suggested that *A. galli* is the most common nematode in all types of production systems and has a worldwide distribution. (Permin *et al.*, 1997; Ashenafi & Eshetu, 2004; Martin-Pacho *et al.*, 2005; Rabbi *et al.*, 2006; Abdelqader *et al.*, 2008). The adult stage inhabits the lumen of the small intestine of the host, feeding mostly on ingesta (Ackert *et al.*, 1940; Soulsby, 1982). *A. galli* infections have been associated with reductions in egg production in laying hens and in overall growth in chickens (Soulsby, 1982; Ramadan and Znada, 1991). *A. galli* may also play a role in transmission of *Salmonella* infections (Chadfield *et al.*, 2001; Eigaard *et al.*, 2006) and avian reoviruses (Calnek, 1997) resulting in disease and economic losses.

The life cycle of *A. galli* is direct and the infective stage is eggs harbouring third stage larvae. After ingestion, eggs reach the duodenum and hatch within 24 hours (Ackert, 1923), and larvae are released into the lumen of the intestine. The larvae then enter the mucosa of the small intestine and most larvae initiate a histotrophic phase between day 8 to 17 post infection (p.i.) (Tugwell and Ackert 1952, Herd and McNaught, 1975). Both onset and length of this phase depend on infection dose (Herd and McNaught, 1975).

New regulations in the European Union, setting standards for protection and welfare of laying hens (Anon, 1999), are implemented from 1st January 2012 and prescribe substitution of traditional cage systems with floor systems and enriched cage systems (Willer and Yussefi, 2004). In floor and free-range systems, the risk of *A. galli* infection is known to be very high (Permin *et al.*, 1997). In addition, the increased demand for more natural or ethical responsible products in recent years is making organic farming systems more common. As a consequence, the overall

prevalence of *A. galli* is expected to increase in coming years, and alternative control strategies are warranted in order to avoid increasing dependence on repeated use of anthelmintics.

Understanding the host-parasite relationships is a necessary prerequisite to develop such control strategies. For this purpose tools for recovery of the larval stages and identification of subpopulations will be very useful. The present study aimed at developing appropriate research tools for understanding the population dynamics of *A. galli*. A standard larval recovery technique for post mortem examination and a method to establish genetically different *A. galli* cohorts were therefore developed and evaluated.

Materials and Methods

Preparation of egg batches. 30 Hy-line breed chickens collected at an organic farm (no use of anthelmintics for 10 years) were killed by cervical disarticulation, eviscerated and a total of 30 adult *A. galli* female worms were collected from 20 hosts. *A. galli* eggs were isolated from the uterus of these worms separately and incubated for 6 weeks in 0.1N sulphuric acid (Permin et al, 1997). Twenty two egg batches were selected based on the highest embryonation rates (above 50%).

Experiment 1: Larval recovery. Infection of chickens. Twenty eight four-week old Lohmann Silver chickens were obtained from a conventional indoor pullet raising farm, supposedly uninfected premises, and randomly allocated to three groups of 10, 9 and 9 chickens and housed in separate sheds. After 2 days of acclimatization, 7 chickens in each shed were orally infected with a dose of 1000 embryonated eggs and the rest kept as uninfected controls. The egg batch was made as a mixture of eggs originating from the 22 females mentioned above in equal proportions.

Processing the intestinal contents. 15 days p.i. 27 chickens including 6 controls were killed by cervical disarticulation and randomly allocated to 3 larval recovery methods (see below) after stratification for shed. Pepsin digestion method was applied for controls. The gastrointestinal tract was removed from gizzard to cloaca. The intestines were cut open longitudinally and washed gently. All the luminal contents including the washings were collected on a 90 µm sieve and washed thoroughly with tap water. The retained material was transferred to a 50 ml tube and stored in 70 % ethanol at 5°C. The intestinal wall was subsequently subjected to either of three different methods: 1) EDTA, 2) pepsin digestion, 3) scraping. Furthermore, after initial processing by EDTA or scraping, intestines were pepsin digested.

EDTA method. 10mM EDTA in 0.9% saline solution was prepared (Kringel *et al.* 2002). The small intestine was cut into 4 pieces longitudinally and hung in 10 mM EDTA in 0.9% NaCl for 6 hours in a 500 ml conical glass. After removing the intestinal wall the contents were allowed to settle for 30 minutes. The supernatant was discarded and the sediment was collected and stored in 70% ethanol at 5°C.

Pepsin digestion method. The digestion fluid of 1L was prepared freshly (Kapel and Gamble, 2000). The small intestine was cut in to 2-3 cm pieces and digested in a beaker with 100 ml digestion fluid under moderate stirring (125 rpm) at 38°C for 45 min. The undigested intestinal pieces were rinsed on a metal sieve of 2-3 mm size with water and digested material was transferred to a conical glass, filled with water and left for 20 min. The supernatant was poured off and again filled with water and allowed to sediment for 20 minutes. This procedure was repeated for 5-6 times. The sediment was collected and stored in 70% ethanol at 5°C.

Scraping method. Using a glass slide the mucosal and sub-mucosal layers were carefully scraped off the small intestine and collected on a 90 µm sieve and washed thoroughly with water. The retained material was collected and stored in 70% ethanol at 5°C.

Measurement of length of A. galli larvae. With the help of tweezers, the length of each larva was measured manually using a measuring scale.

Comparison of quality of DNA of larvae following recovery. DNA from two times 20 larvae recovered by the EDTA and pepsin digestion methods, respectively, was extracted as described in the following section, PCR was conducted and bands were visualised after gel electrophoresis.

Experiment 2: Cohort study. *Extraction of DNA.* DNA was extracted from the 22 selected adult worms using the MasterPure™ DNA purification kit (Epicentre Biotechnologies) according to the manufacturer's protocol. DNA from single larvae was extracted using a worm lysis buffer (Maafi *et al.* 2003), and carried out according to Nejsun *et al.* (2008).

Selection of the target region (cox1 gene) in mtDNA. Sequence information of the *cox1* gene of the mtDNA was obtained from *Ascaris suum*, *Caenorhabditis elegans* and *Necator americanus* using BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Conserved regions were subsequently identified by aligning the sequences using Toffee (<http://igs-server.cnrs.mrs.fr/Toffee/toffee.cgi/index.cgi>) and used as target regions for primers. The forward (GCox18F-5': TTGTGGTCTGGTATGGTTGG) and reverse (GCox18R-5:

TGATGAGCTCAAACAACACAAC) primers which were designed using Primer3 (<http://frodo.wi.mit.edu/>) and flanked a region of ~800 bp.

PCR and sequencing. The PCR was performed in a TECHNE® TC-512 thermal cycler. The thermal profile for the PCR reaction was: 15 minutes at 95°C for initial activation of Taq polymerase followed by 35 cycles consisting of: 30 seconds at 95°C (denaturation), 40 seconds at 55°C (annealing), 1 minute at 72°C (extension) and a final elongation of 10 minutes at 72°C. Negative H₂O controls were included in all runs. The amplified fragment was purified by Qiaquick PCR-purification kit (Qiagen, Germany) and sequenced in both directions using a BigDye terminator sequencing system according to the manufacturer's instructions (Applied Biosystems, USA). After ethanol precipitation the sequencing products were run on an ABI3130XL (Applied Biosystems, USA).

Design of new primers. The sequence information was used to design new primers (GCox1F4F: 5'-ATTATTACTGCTCATGCTATTTTGATG and GCox14R: 5'-CAAACAAATGTTGATAAATCAA GG) in conserved regions of the *A. galli* sequence giving an amplicon with a length of 533 bp.

Selection of restriction enzymes. After obtaining the sequence information, single nucleotide polymorphisms (SNP) were identified manually and NEB cutter V2.0 (<http://tools.neb.com/NEBcutter2/index.php>) was used to identify two restriction enzymes (*Nla*III and *Sfc*I) that could allocate the 22 samples into three haplotypic groups and also had the property of sharing similar buffers and reaction conditions. The PCR products of all 22 *A. galli* samples were digested overnight at 37°C using 3 units of both *Nla*III and *Sfc*I (New England, Biolabs, USA) and

the digested products were subjected to gel electrophoresis on a 1.5% agarose gel (100 V, 90 minutes).

Infection of chicken. Twenty-five nine-week old chickens of Lohmann Sandy hybrid were obtained from another farm than in the former experiment. Ten chickens were immediately killed and all found to be *A. galli* negative. The remaining 15 chickens were randomly allocated into three groups of 5 and housed in three different sheds. Four chickens in each group were inoculated with 1000 embryonated *A. galli* eggs of the three different haplotypes and one chicken in each shed was kept as uninfected control. 15 days p.i. all chickens were killed and larvae recovered from the intestines by the EDTA method.

PCR-RFLP on larvae. The DNA of both the selected adult worms (positive controls) and larvae was subjected to PCR-RFLP as described above.

Data analysis. The recoveries of larvae from intestinal lumen and wall by different methods were compared by a generalized linear model (Proc Genmod, SAS v 9.1) by specifying a negative binomial distribution of larval counts. The difference in the length of the larvae recovered by different methods from the intestinal wall was analysed in a mixed effect variance model with the method as the fixed effect and the chicken as a random effect. The difference in the length of larvae between intestinal content and intestinal wall was analysed in a mixed effect variance model with location (lumen or wall) as the fixed effect and the chicken as the random effect.

One-way analysis of variance (ANOVA) was used to compare the recovery of larvae in the intestinal lumen, wall and total respectively, of three different haplotypes in the cohort experiment.

Animal ethics

The study was carried out in accordance with the requirements of The Danish Animal Ethics Committee (permit # 2005/561-1060)

Results

Experiment 1: Larval recovery. The percentage of total larvae recovered by different methods ranged from 0.83-2.56% of the infection dose (Table 1). The recoveries from intestinal wall were overall significantly different ($P = 0.018$) and subsequent pair wise analysis showed significant higher recovery by the EDTA method compared with scraping ($P = 0.006$) and higher recovery by pepsin digestion than scraping ($P = 0.003$). Pepsin digestion of the intestines after EDTA incubation yielded no more larvae while digestion after scraping yielded a single larva.

The recovered larvae had an overall mean length of 3.1mm. There was no statistical difference in the mean length of larvae recovered from the intestinal wall by either EDTA method or pepsin digestion method ($P = 0.66$). Similarly there was no significant difference in mean length of larvae recovered from the lumen and those recovered from the intestinal wall ($P = 0.90$).

Three chickens harboured nine adult worms indicating previous exposure to *A. galli*.

For all larvae ($n = 40$) recovered by EDTA and pepsin digestion, it was possible to amplify the *cox1* gene by PCR.

Experiment 2: Cohort study. In the three groups infected with eggs of haplotype I, II and III respectively, a mean percentage of 2.2, 2.6 and 1.2 of the infection dose with a mean number of

21.5 (individual numbers 1, 28, 43 and 14), 25.7 (individual numbers 3, 7, 85 and 8) and 12.0 (individual numbers 8, 4, 21 and 15) larvae were recovered. There was no significant difference in larval recovery between different haplotype groups. Forty larvae randomly selected from all 4 chickens of each of three maternal-haplotype groups (total 120) recovered from the chickens were PCR- RFLP typed and all were found to hold the same band pattern as their female of origin. The band pattern of some of the larvae is shown in Figure 1. The nucleotide sequences for the three haplotypes have been submitted to the GenBank under the following accession numbers GU138668- GU138670.

Discussion

Previous studies reported that the histotrophic phase is a normal part of *A. galli* life cycle (Herd and McNaught, 1975) and that most histotrophic larvae are in the mucosal layer and only rarely penetrate deeper into the intestinal wall (Ackert, 1923). A commonly used method for isolation of histotrophic larvae has been pepsin digestion e.g. as used by Herd and Mc Naught (1975) who incubated intestinal wall for 4 - 12 h at 37°C. However, Irvine and Dallas (2002) reported that digestion for prolonged period (more than 2h) may be associated with reduced PCR efficiency. We therefore compared a pepsin digestion technique developed for the release of *Trichinella* spp. larvae (Kapel and Gamble 2000) but with reduced digestion period (45 minutes) with the EDTA method which has been developed for the recovery of small immature *Trichuris suis* larvae from large intestine of pigs (Kringel *et al.* 2002). These methods were compared with manual scraping. Our study showed no difference in larval recovery by the EDTA and pepsin digestion methods and that both were superior compared to scraping. Following scraping it was very difficult to find the larvae

embedded in the clumps of mucosa. EDTA presumably loosens the connections between mucosal cells whereby embedded larvae are released whereas digestion with acid pepsin dissolves the mucosa and releases the larvae. The fact that *A. galli* larvae rarely penetrate deep in to intestinal tissue (Ackert, 1923) might be the reason for not finding any additional larvae when the intestines were pepsin digested after EDTA incubation. Based on this, and the fact that these samples were the easiest samples to read, the EDTA method is recommended for quantitative recovery.

We found no difference with respect to length neither between lumen and wall nor between EDTA and pepsin digestion method. It might have been suspected that a given larval recovery method would be better to recover larvae of a certain size, but we did not find any indication for such a selection in our study although the larvae had a relatively large variation in size.

Applying PCR-RFLP on the mitochondrial DNA it was possible to establish three different types of cohorts. We haplotyped 120 larvae and all showed a similar band pattern on the gel as that of their maternal parent. This supports the underlying hypothesis that mtDNA is maternally inherited in *A. galli* as is the case for e.g. *Ascaris suum* (Nejsum *et al.* 2008) and mammals in general (Hutchison *et al.* 1974).

The possibility to identify different cohorts makes it possible to follow different groups of *A. galli*, their location in the host and their relative establishment rates. Dobson *et al.* (1990) made a cohort study using levamisole resistant and susceptible strains of *Trichostrongylus colubriformis* whereas Roepstorff *et al.* (1996) used pyrantel resistant and susceptible strains of *Oesophagostomum dentatum* in his study. But this approach to cohort studies results only in two different cohorts and depends on availability of solidly resistant strains. The latter is not the case for a range of nematodes. In contrast Sørensen *et al.* (1999) used PCR-RFLP technique to distinguish between two isolates of *Schistosoma japonicum* whereas Nejsum *et al.* (2008) used the present

PCR-RFLP method on the mtDNA and were able to establish four different cohorts of *A. suum*. The utilization of the natural genetic variation in the mtDNA to identify different cohorts of parasites therefore not only makes it possible to identify several different cohorts but also an ability to work with naturally occurring worms in contrast to isolates or specific strains. However, similar establishment rates need to be confirmed before embarking on comparisons.

The development of reliable quantitative recovery methods in combination with genetically marked cohorts provides powerful tools for the study of population dynamics of nematodes in poultry and other livestock.

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Legend to Figure

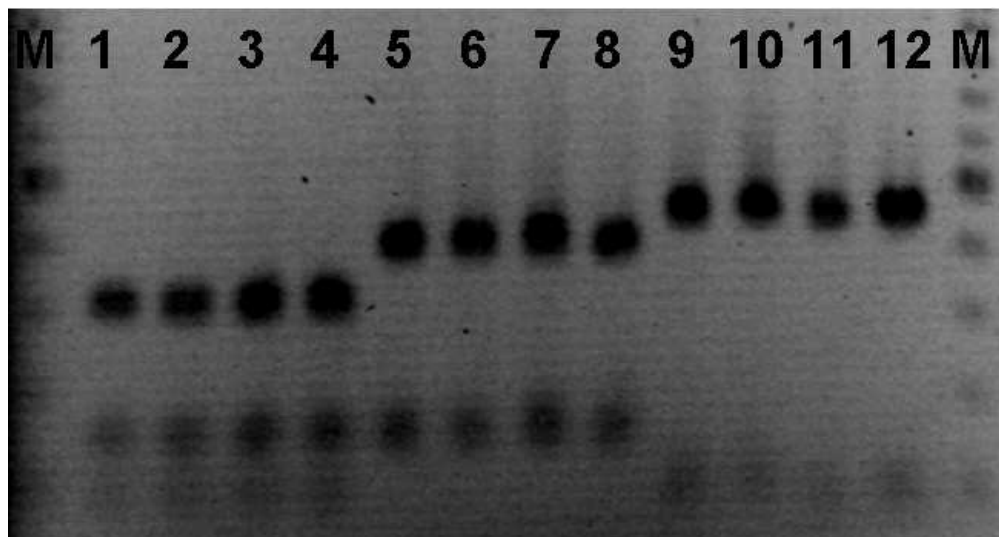
Figure 1. *PCR-RFLP on mtDNA. M: 100 bp size marker, lane 1 and 2-4: band patterns of adult female and larvae of haplotype I, lane 5 and 6-8: band patterns of adult female and larvae of haplotype II and lane 9 and 10-12: band patterns of adult female and larvae of haplotype III respectively.*

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Table 1.

The number of A. galli larvae recovered from intestinal wall and lumen contents of chicken experimentally infected with 1000 embryonated eggs and euthanized 15 days p.i. Intestinal wall recovery by EDTA, pepsin digestion or scraping methods.

Method	Intestinal wall		Lumen contents		Total larvae
	Number of larvae	Mean length (mm)	Number of larvae	Mean length (mm)	
EDTA method					
Mean (\pm s.d.)	5.4(\pm 8.2)	2.5(\pm 3.2)	21.3(\pm 20.8)	4.4(\pm 3)	26.7(\pm 23.6)
Range	0-23	1-9.5	3-54	1.5-7	5-62
Mean recovery (%)	0.5%		2.1%		2.7%
Pepsin digestion method					
Mean (\pm s.d.)	6.7(\pm 10.5)	2.5 \pm 1.6	20.1(\pm 16.4)	3 \pm 1.4	26.7 (\pm 20.9)
Range	0-30	1.5-4	2-47	1-8.5	3-57
Mean recovery (%)	0.7 %		2.0%		2.7 %
Scraping method					
Mean(\pm s.d.)	0.6(\pm 0.53)	2.6 \pm 0.2	7.6(\pm 9.84)	3.2 \pm 1.2	8.9(\pm 10.3)
Range	0-1	2.5-3	0-25	1.5-10	0-26
Mean recovery(%)	0.06%		0.8%		0.9%



172x92mm (96 x 96 DPI)

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