Ascaris: Development of selected genotypes in mice

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Abstract

Using nucleotide variation in the first internal transcribed spacer of nuclear ribosomal DNA, five different genotypes (designated G1–G5) have been identified and the preponderance of genotype G1 in humans and of genotype G3 in pigs led to the proposal that parasites bearing the two genotypes have an affinity for a particular host species. A subsequent study using eggs of genotype G1 from humans and G3 from pigs to infect pigs and mice indicated that there is a significant difference in the ability to infect and establish as larvae in mice and as adults in pigs between the two genotypes. Extending previous investigations, the present study investigated whether there are differences in development as designated by egg hatching, larval migration and distribution in the mice between the Ascaris strains with known genotypes. Ascaris eggs of genotypes G1 (predominating in human-derived worms) and G3 (predominating in pig-derived worms) were used to infect C57BL/6 mice orally. Eggs/larvae were examined from the small and large intestines, thoracic and abdominal cavities, peripheral blood, livers and lungs at intervals of 2 h until 12 h post-infection, then periodically until 34 days of infection. Results showed distinct differences in egg hatching (the timing and location of hatching, and the numbers hatched), and in larval migration and distribution (the means and constituent ratios, the time of peak recovery, and larval re-pairing in intestines) between the two strains. The results can explain the findings of significantly higher larval recovery of genotype G1 than G3 in the mice, and may shed some enlightenment to understand the difference in host affiliation of Ascaris of different genotypes.

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1. Introduction

Although Ascaris lumbricoides Linnaeus, 1758 and Ascaris suum Goezee, 1782 are important and widespread nematode parasites of humans and pigs, respectively, their taxonomic status has been controversial (Stewart and Hale, 1988; Crompton, 1989; WHO, 1996; Peng et al., 1998a; O’Lorcain and Holland, 2000; Stephenson, 2002). Various studies using morphological (Sprent, 1952; Maung, 1973), karyotypic (He et al., 1986; Mutafova, 1983), immunological and/or biochemical (Kurimoto, 1974; Kennedy et al., 1987; Nadler, 1987; Christie et al., 1990; Abebe et al., 2002), experimental cross-host species transmission (Takata, 1951; Galvin, 1968) and experimental epidemiological criteria (Peng et al., 2001) have attempted to address this question. Because of the absence of any reliable morphological characters to discriminate the two presumed species, and the occasional reports of cross-infection between the two hosts (Anderson, 1995; Maruyama et al., 1996), molecular methods have been employed in recent years to investigate the genetic make-up of Ascaris populations. Results using molecular methods have suggested population sub-structuring within the genus and also contemporary cross-transmission between populations of human and pig Ascaris (Anderson, 1995; Anderson et al., 1993, 1995; Peng et al., 1998b, 2003, 2005; Zhu et al., 1999; Nejsum et al., 2005; Criscione et al., 2007; Leles et al., 2010).

Extending previous studies, a series of recent investigations has been conducted to gain a better insight into the genetics and epidemiology of Ascaris in China (cf. Peng et al., 2007). Nucleotide variation in part of the first internal transcribed spacer (pITS-1) of nuclear ribosomal DNA within and among a large number of Ascaris individuals (n = 815) from humans and pigs from six endemic regions in China was investigated. Five different genotypes (designated G1–G5) were identified in human Ascaris, of which three (genotypes G1–G3) were detected also in pig-derived worms. Of the five Ascaris genotypes identified, genotype G1 infected mainly humans (~63–74%), whereas genotype G3 infected predominantly pigs (~79–86%). There were significant differences in the frequency of genotypes between human- and pig-derived Ascaris (Peng et al., 2003). The preponderance of genotype G1 in humans and of genotype G3 in pigs led to the proposal that parasites
bearing the two genotypes have an affinity for a particular host species. A subsequent study employing markers in the cytochrome c oxidase subunit 1 and NADH dehydrogenase subunit 1 genes of mitochondrial DNA reinforced this idea (Peng et al., 2005).

Since host affiliation of parasites may relate to unconnected host, environmental and/or parasite factors (Wakelin and Bradley, 2002), it is not yet possible to be sure whether genotypic disparities in Ascaris truly reflect biological differences pertinent to host specificity. A subsequent study was therefore conducted to test the proposal that there might be a particular host affiliation for Ascaris of selected genotypes (Peng et al., 2006). By excluding environmental effects or variation relating to the exposure of hosts to eggs, and minimizing genetic variation between host individuals, experimental infections in pigs and mice were carried out using eggs of genotype G1 derived from humans and G3 derived from pigs. Initial findings indicate that there is a significant difference in the ability of Ascaris eggs of genotype G1 and G3 to infect and establish as larvae in mice and as adults in pigs. The disparities in recovery rates of adults from pigs and of larvae from mice infected with genotypes G1 and G3 strengthens the idea that genetic variation of Ascaris may contribute to host affiliation. Two main possibilities arise. First, there may be differences in development such as egg hatching, larvae migration and distribution in the initial phases of invasion by Ascaris of the different genotypes, which can cause different recovery in the hosts. Second, there might be differences in host species in susceptibility/resistance as well as immune responses to Ascaris of different genotypes. The present study therefore focused on the first of these by experimental infections of C57BL/6 mice with Ascaris eggs of genotype G1 and G3 to compare the dynamics of egg hatching and larval migration and distribution after infection.

2. Materials and methods

2.1. Ascaris eggs, genotyping and viability test

Female adults of Ascaris were collected from humans after treatment with pyrantel pamoate (Hangzhou Minsheng Pharmaceutical Group Co., Ltd., 10 mg/kg, one dosage) from Changyi Township, Xinjian County, Jiangxi Province, China, and from pigs from a local slaughter house. Eggs from the distal part of the uterus from individual adult females of Ascaris were isolated and incubated as reported previously (Jungersen et al., 1996) until 95% of the eggs developed into the infective stage, and were then stored at 4–10 °C for subsequent use.

The genotypes of Ascaris individuals and eggs had been previously identified using molecular approaches. Briefly, A ~300 bp ITS-1 DNA region (pITS-1) known to contain at least six variable nucleotide positions (see Zhu et al., 1999) was amplified by PCR from individual worms or eggs and then a single-strand conformation polymorphism (SSCP)-based approach (Gasser et al., 2004) was performed. For selected samples from different SSCP gels, the entire ITS-1 region was amplified and subjected to PCR-RFLP or/and automated sequencing (see Peng et al., 2003, 2006). The eggs used in the present study were batch E9 and E13 from a single adult female of Ascaris from human and pig host, and genotyped as G1 and G3. These eggs had been used in our previous study to infect 47 pigs (E9) and 27 pigs (E13), respectively, and after 60 days of the infection, only one immature worm was found from the 47 pigs and 305 adult worms recovered from the 27 pigs after chemotherapy (cf. Table 3, Peng et al., 2006). Prior to the present study, the viability/infectivity of each batch of eggs was tested by gastric inoculation of ten mice at the dosage of ~1000 eggs. If larvae were recovered from the lungs of these mice on day 7 after inoculation, the eggs were considered infective.

2.2. Mice infection and maintenance

Specific pathogen free (SPF) C57BL/6 mice (n = 160, males, 6–7 weeks old and 18–20 g body weight) were bought from Vital River Laboratory Animal Technology Co., Ltd. Beijing, China. The animal was provided ad libitum with sterilized feed and water during the entire 34 days of experimental period. After the viability/infectivity of eggs was proved (see below), the mice were randomly divided into the group G1 and group G3, and each mouse was orally inoculated with ~1000 eggs of G1 or G3 about after 12 h starving. All experimental protocols were approved by the Faculty of Experimental Animals of Nanchang University.

2.3. Recovery of eggs and larvae

After inoculation, 5 mice from each group infected with eggs of genotype G1 or G3 were euthanised at the intervals of every 2 h until 12 h or at the intervals of 2–10 days during the period of day 1 to 34, to recover eggs/larvae from following locations/organs.

1. The small and large intestines from each mouse were separated and the contents inside were squash out. Then 10 ml of normal solution was injected into the cavity. The contents and the washed solution were collected together and mixed with 10 ml of 10% formalin solution, labeled and kept in room temperature for later examination under microscope.

2. Thoracic and abdominal cavities were washed with 0.5 ml and 1 ml normal solution, respectively. The washed solutions were collected and mixed with the same volume of 10% formalin solution, labeled for later examination.

3. Peripheral blood of each mouse was collected from eyes, mixed with 4 times of volume of tape water for hemolysis. Precipitation was obtained by centrifugation at 1500 rpm for 5 m and then mixed with the same volume of 10% formalin solution for later examination.

4. Larvae in lungs, livers and the walls of small and large intestines of each mouse were recovered by using the modified Baermann funnel technique (Lewis et al., 2006).

2.4. Statistical analysis

Raw data were transformed using a common logarithm before statistical analysis. A repeated measures ANOVA was employed to test the significance of the recovered number at an organ or location of mice over time points between the two genotypes. In addition to compare absolute quantity (means of recovered larvae/eggs), relative quantity (e.g. infection rate of mice, and constituent ratio of larval from different sites) was also analyzed using Chi-square test with Yates’ correction.

3. Results

3.1. Infection success in mice

The infection of two groups of mice with Ascaris eggs of two selected genotypes was proved to be of similar success in the present study. This was first demonstrated by an initial test of 5 mice in each group. It showed 4 and 5 were infected, respectively, representing a similar level of infectivity ($\chi^2 = 0.0, P = 1.0$). Subsequently, at the end of the experiment, among the 80 mice in each group, 61 and 54 had worm(s) recovered, approximately indicating an infection percentage of 76.25% and 67.5%, respectively, which also indicated a similar infection percentage between the two groups ($\chi^2 = 1.113, P = 0.2914$).
3.2. Site and timing of egg hatching

In both groups, the unhatched eggs stayed in small intestines only for first 6 h, and the peak was at 2 h. At the 4 h there was still a relatively large part of eggs of G1 remained but for G3 eggs only small part remained (Table 1). Along with the time passed the unhatched eggs were moving to the large intestine (Table 2), which showed that most of G3 eggs were not hatched in contrast to G1 eggs. At 4 h, a maximum number of eggs recovered in both groups, followed by a gradual decline, although the decrease in group G1 was more marked.

3.3. Newly hatched larvae in the lumen of intestines

Newly hatched larvae were recovered from the lumen of small intestines at hours 2, 4, 6 and 12 in group G1 and only at hours 2 and 4 for group G3 (Table 3), but larvae were recovered from the lumen of large intestines at every time point (Table 4). Except for hour 2, more larvae were recovered from large intestines than from small intestines in both groups, and more larvae were recovered from group G1 than group G3 in both small and large intestines (Tables 3 and 4). From 24 h onwards, no larvae were found in intestines of both groups until 168. Although larvae were again detected in small intestine (at 216 h) and large intestines (at 168, 216 and 264 h), these larvae were not considered as newly hatched larvae and thus not listed and statistically processed. After 264 h, no larvae were recovered from intestines any more.

3.4. Larval migration into the wall of intestines, abdominal and thoracic cavities

The wall of small and large intestines were examined for larvae at only hour 4. Larvae were recovered from both locations from mice of G1 genotype, and no significant difference was detected for the means between small and large intestines. From mice of group G3, however, larvae were only found in the wall of large intestines. Significant differences between the two genotypes were found in both sites (Table 5).

Larvae were found in abdominal cavities only on days 5, 7 and 9 for group G1 and on days 7 and 9 for group G3. From thoracic cavities, larvae were only recovered on day 9 from group G1 and on day 5 from group G3. No larvae were detected in all peripheral blood samples.

3.5. Larvae migration and distribution in livers

The earliest time at which larvae were recovered from livers was at 6 h for both groups. Larval recovery from livers showed two peaks in group G1. The first appeared at 12 h and the other higher at 120 h. In group G3, only one peak appeared around 24 h, which was much less pronounced compared with group G1 (Fig. 1). Larvae were not recovered from livers since 168 h in group G3 but existed even on 360 h in a mouse of group G1 (Fig. 1). Statistical significance of the factor of genotype on the recovery numbers from lungs was detected (F = 82.225, df = 1, P < 0.001).

3.6. Larvae migration and distribution in lungs

The earliest time at which larvae appeared in lungs was at 4 h for both groups, two hours earlier than that in livers. There were two peaks of larvae recovered in both groups. The first peak appeared at 10 h for group G1, which was created by a maximum (131) of larval recovery from 1 mouse (for the other 4 mice larval recovery ranged 1–4), and 2 h later for group G3 when the number of larvae recovered from group G1 was much lower. The second peak in group G1 was on 216 h but in group G3, it appeared 2 days earlier than that in group G1 (Fig. 2). Statistical significance of the factor of genotype on the recovery numbers from lungs was detected (F = 85.39, df = 1, P < 0.001).

3.7. The constituent ratios of larvae distribution in main organs

The dynamic changes of constituent ratios of larvae distributed in internal organs were illustrated by Fig. 3. Because larvae were mainly recovered from intestines, livers and lungs, for succinctness, larvae recovered from body cavities were not included in this figure. At hour 2, all larvae were found both in small and large intestines (roughly half to half), then almost all of larvae were recovered from large intestines, and few from livers (4–6 h). At hour 8, obvious changes can be seen between the two groups because the larvae from livers in group G1 started to occupy a quite large part in comparison with group G3. At hour 10, about a half of larvae still recovered from large intestines in group G3, but about a half of larvae recovered from lungs in group G1. At hour 12, half larvae were found from lungs and still some part of larvae were from liver and large intestines in group G3, but about seven eighths of larvae were recovered from livers and others from lungs in group G1. Afterwards and before 7 days, most larvae were recovered from livers in both groups although the percentage of larvae

### Table 1

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<th>Genotype</th>
<th>Hours after inoculation</th>
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<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>G1</td>
<td></td>
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<tr>
<td>G3</td>
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*Statistical significance of the factor of genotype on the recovery numbers was detected (F = 6.541, df = 1, P = 0.034).

### Table 3

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<tr>
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<tr>
<td>G1</td>
<td></td>
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<tr>
<td>G3</td>
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*Statistical significance of the factor of genotype on the recovery numbers was detected (F = 121.062, df = 1, P < 0.001).

### Table 2

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<tr>
<td></td>
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<td>G1</td>
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<td>G3</td>
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*Statistical significance of the factor of genotype on the recovery numbers was detected (F = 121.062, df = 1, P < 0.001).
in livers between the 2 groups still showed some difference. At 7 days and afterwards, most larvae were found from lungs, large intestines and livers but in group G3 no larvae were found from livers (Fig. 3). Chi-square test showed significant difference in the ratios between the two genotypes at hour 6–12, and day 9 ($\chi^2 = 8.089–45.619$, $P = 0.044–0.000$).

### 4. Discussion

Mice have previously been used to study the migration of *Ascaris* larvae (cf. Slotved et al., 1998; Lewis et al., 2006), although eggs of *Ascaris* from pigs only used. Also, the migration of *Ascaris* (mainly *A. suum*) in mice has been reported by several authors (e.g. Jenkins, 1968; Bindsell, 1970; Douvres and Tromba, 1971; Slotved et al., 1997, 1998; Lewis et al., 2006). Mice of different strains, *Ascaris* from different pigs and locations, eggs of various dosages of infection and different recovery methods were used, all of which can cause difficulties in comparing results. For example, a recent study employing pig *Ascaris* eggs to infect similar mouse strain (Lewis et al., 2006) reported much higher recoveries from mice than our present and previous studies (Peng et al., 2006). We think that the disparities could be attributed to the source of eggs used. Eggs from different females can become a mixture of different genotypes, by which the diversity in biological characteristics and host response arising from different strains can be altered (Wakelin and Bradley, 2002). Thus the widespread use of mixtures of eggs from several worms, and/or from several donor hosts, may be influential to the reproducibility of, and comparability between, studies of this kind.

A potential confounding factor with comparative studies of this kind, in which parasites and their eggs have been obtained from different host species, may lie in the differences in viability and infection potential of eggs obtained from the different sources. In the present study, however, we are able to exclude this confounding factor and ensure approximately equivalent infection potential between batches of G1 and G3 eggs by testing each in both mice and pigs (see Peng et al., 2006). Thus, while G1 eggs produced few if any adult worms in pigs, they gave high recoveries of larvae in mice. Similarly, while G3 eggs produced poor larval recoveries in mice, they yielded high recoveries of adults from pigs.

In the present study we used two isolates of different genotypes and each from only a single donor worm of each host origin, which may be vulnerable to criticism in sample size and replication. Anyway, we would like to stress first, that the present study is a direct

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<tr>
<td>G1</td>
<td>26.40 ± 15.37</td>
</tr>
<tr>
<td>G3</td>
<td>2.60 ± 1.95</td>
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* Statistical significance of the factor of genotype on the recovery numbers was detected ($F = 10.718$, df = 1, $P = 0.014$).

Table 5

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<th>Mice</th>
<th>Small intestines</th>
<th>Large intestines</th>
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<tr>
<td>Group G1</td>
<td>20.00 ± 17.21</td>
<td>50.80 ± 43.73</td>
</tr>
<tr>
<td>Group G3</td>
<td>0</td>
<td>2.00 ± 1.00</td>
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<tr>
<td>$P (F)$ values</td>
<td>&lt;0.001 (64.00)</td>
<td>0.001 (22.839)</td>
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Fig. 1. Mean number of larvae recovered from livers at different hours after inoculation (±SEM). The broken line and solid line represent the group of mice infected with *Ascaris* eggs of genotypes G1 and G3, respectively.

Fig. 2. Mean number of larvae recovered from lungs at different hours after inoculation (±SEM). The broken line and solid line represent the group of mice infected with *Ascaris* eggs of genotypes G1 and G3, respectively.
Extending from our study series (see Peng et al., 2007) by which two important findings were obtained. One is the affiliation of predominated genotype G1 in humans (~63–74%) and G3 in pigs (~79–86%) from an epidemiological investigation based on a large collection of Ascaris samples (Peng et al., 2003). The other is the finding of difference in host affiliation between the selected genotypes from an experimental infection of either mice and pigs using eggs of genotypes G1 and G3 (Peng et al., 2006). We need to point out that the batches of eggs used in the present study are as the same genotype as previously used, but from different single females. In contrast with the results from previous study (cf. Table 1, Peng et al., 2006), the present study did reproduce a similar result in overall larval recovery showing again that Ascaris with genotype G1 has significantly higher larval recovery than that with G3 in the C57BL/6 mice. Second, as recently reported that offspring even from a single female worm can be from different paternal lines (Zhou et al., 2010), and that even considering a single egg as an individual, ITS1 intra-individual variation was demonstrated by the presence of 2–4 genotypes/haplotypes in Brazilian Ascaris (Leles et al., 2010). Thus use of more batches of eggs from multiple females may bring in more unknown factors which may serve as confounding actors concomitant with the treatment factor, and should be excluded as much as possible in any research designs. In consideration of scanty knowledge on this field, using eggs with identified genotype from a single worm might be prudent and justifiable and acceptable choice. In addition, the workload in this study was very time and labor consuming and an increase of one more sample in each genotype will lead the workload up to fourfold, thus making the use of more samples less feasible.

Collectively, results from our experiment and previous reports could indicate the following points about migration routes of Ascaris (mainly A. suum) in mice, but these may differ according to mouse strains. First, eggs may hatch either in small and large intestine and newly hatched larvae penetrate through wall of both small and large intestine, but the large intestine may be the main location of invasion. Second, because larvae were not found in the body cavity until 5 days post-infection in our study and by Slotved et al. (1998), they may enter the liver and lung via blood vessels at a very early stage (Jenkins, 1968; Bindseil, 1970). Third, larvae can enter the lungs by bypassing the liver since we recovered larvae from lungs at hour 4, but not until hour 6 from livers (both groups). Fourth, larvae can penetrate from body cavities to re-enter the intestine after visceral migration. Finally, the survival time of larvae in mice is more prolonged than is usually accepted, being up to 20 days.

In conclusion, based on previous findings of predominant genotype G1 in humans and G3 in pigs and the difference in host affiliation in mice and pigs between the two genotypes and extending foregoing investigations (Peng et al., 2003, 2006), the present study investigated whether there are differences in development as designated by egg hatching, larvae migration and distribution in the mice between the Ascaris strains with known genotypes. The study using eggs with the same genotypes but from different single females not only reproduced the results that G1 has significantly higher overall larval recovery than G3 in the C57BL/6 mice (cf. Table 1, Peng et al., 2006), but also revealed details of difference between the two genotypes in egg hatching (the timing and location of hatching, and the numbers hatched), and in larvae migration and distribution (the means and constituent ratios, the time of peak recovery, and larvae reappearing in intestines). These can explain the different recovery rate of genotype G1 and G3 in the mice, and may shed some enlightenment to understand the difference in host affiliation of Ascaris of different genotypes. However, it should be cautious that the genotypes we used can only be considered markers that fortuitously associate with the biological characteristics that determine host affiliations (pigs versus humans; Peng et al., 1998b, 2003, 2006) and may well segregate independently from those (multiple?) genetic loci that determine success in one or other host species. Further studies are needed to investigate the relationship between parasite strains representing different genotypes and host affiliation as well as genes controlling host affiliation.

Acknowledgments

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References


