# *SCHISTOSOMA JAPONICUM* IN THE PIG: A NEW TECHNIQUE FOR ESTIMATION OF INTESTINAL TISSUE EGG COUNTS

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**Abstract.** This study introduced a new method for estimating intestinal tissue *Schistosoma japonicum* egg counts, based on scraping of the mucosal layer of different sections of the intestines. Twenty-eight Danish Landrace/Yorkshire/Duroc crossbred pigs were divided into 3 groups of 15, 5 and 8 pigs, respectively. Pigs were fed either a high- or low- protein diet and were infected by an intra-muscular or per-oral route of infection with doses of either 1,000, 1,500 or 3,000 *S. japonicum* cercariae. The pigs were killed 9-11 weeks post infection. For all 28 pigs the intestines were divided into 3 sections: cecum, colon and rectum and the entire mucosa was scraped off the serosa of each section and homogenized. Subsequently, samples corresponding to 5 g homogenised mucosal tissue were digested and egg counts were determined and correlated to liver egg counts. In order to compare the relative distribution of eggs in the mucosa and the serosa, small intestinal wall subsamples formerly taken from each section from a subgroup of 5 pigs were homogenized and egg counts determined for both the mucosa and serosa. The number of eggs were significantly higher in the mucosa than in the serosa. Egg counts estimated from digestion of mucosa subsamples either over- or underestimated egg counts based on scrapings of the entire mucosa when compared, reflecting the very pathcy distribution of *S. japonicum* eggs in the intestinal wall. Correlating liver egg counts with the number of eggs based on scrapings from the entire mucosa from cecum, colon and rectum, respectively, significant correlations were found for 2 out of 3 groups of pigs. The present study revealed that estimating intestinal tissue egg counts based on scrapings of the entire mucosa is a reliable and convenient approach, nicely supporting the liver tissue digestion approach. In addition, a reduction of the processing time of intestinal tissue in general was achieved due to the very simple scraping technique.

## INTRODUCTION

*Schistosoma japonicum* infections in pigs have recently been introduced as a valuable model for studying host/parasite relationships in schistosome infections in the mammalian definitive host (Willingham *et al*, 1994). Particular attention has in this respect been paid to the host regulatory response to infection and the associated clinico-pathological and pathological consequences (Johansen *et al*, 1998; Willingham *et al*, 1998).

Schistosome egg deposition in the host tissue is a very central parameter in assessing host/parasite relationships because of reasonable correlations between tissue egg counts and disease burden (Cheever *et al*, 1994). This highlights the need for convenient and effective methods for estimating tissue egg counts. This need is particularly evident when dealing with hosts as large as pigs where total digestion of entire organs or intestinal sections is most difficult. As far as the pig/*S. japonicum* model is concerned, previous studies have shown that the very patchy distribution of eggs in the intestines makes subsampling extremely problematic (Bøgh *et al*, 1996). In contrast, Bøgh *et al* (1996) showed, as far as the liver is concerned, that egg counts in the left lateral lobe provides a reasonable estimate of total liver egg counts. However, from the point of view of studying the dynamics of the infection and the disease process, the possibility for proper enumeration of intestinal tissue egg counts is extremely crucial.

The present study introduces a new and convenient method for estimating intestinal tissue egg counts based on scrapings of the entire mucosa layer of different sections of the intestines. The relative efficiency of this new method is compared with the available standard technique, *ie* digestion of subsamples of intestinal sections and liver tissue.

## MATERIALS AND METHODS

The intestines from 28 Danish Landrace/Yorkshire/Duroc crossbred male and female pigs, bred under specific pathogen-free conditions, were used in the present study. All pigs had previously entered 3 different studies (Giver *et al*, 1999, 2000; Johansen *et al*, 1997).

Fifteen pigs were due to similarities in infection regimens considered as one group (A) in the present study. All 15 pigs were fed a protein-supplemented diet with access to water *ad libitum*. The protein content in the fodder was approximately 1/4 of total amount of fodder (dry matter). All individuals were at an age of 10-15 weeks infected with *S. japonicum* cercariae of a Chinese strain maintained at the Danish Bilharziasis Laboratory in *Oncomelania hupensis* snails. The infection doses were of either 1,000, 1,500 or 3,000 cercariae, and infections were given as intra-muscular injections of medium-suspended cercariae according to the method described by Willingham *et al* (1996). Nine to eleven weeks post infection (pi) the pigs were killed by an overdose of pentobarbital and perfused, as described by Bøgh *et al* (1997).

Initially was the mucosa layer from each of the 15 pigs in group A investigated. The intestine from each individual was divided into 3 sections consisting of cecum, colon and rectum, respectively and all sections were emptied for intestinal content. Before further investigation a small quadratic subsample at approximately 15 g was taken from the center of each section from a subgroup of 5 pigs each given 1,000 cercariae. The sample included both the mucosa and serosa and was saved for later examination. From each of the 15 pigs in group A the entire mucosa was hereafter scraped off the remaining intestinal wall sections according to the method introduced by Murell *et al* (1997) on determination of *Ascaris suum* larvae migration through the pig intestine. The mucosa was then homogenized in a blender for approximately 1 minute and from each section a sample corresponding to 5 g mucosal tissue was digested in 3% KOH at 37°C for 18 hours according to the method described by Bjørneboe and Frandsen (1979). Egg counts were determined as described by Bøgh *et al* (1996).

The remaining 13 pigs were divided into group B and C. Group B included intestinal mucosa from 5 pigs each given 1,000 cercariae by a per-oral infection route as described by Giver *et al* (1999). The pigs in this group were as pigs in group A fed a protein supplemented diet and water *ad libitum*. Group C included intestinal mucosa from 8 pigs fed a low-protein diet. The protein content in the fodder was approximately 1/7 of total amount of fodder (dry matter). Pigs in group C had been given concurrent infections with *S. japonicum* (1,500 cercariae) and *Trichuris suis* (4,000 larvae) (Johansen *et al*, 1997) and were as pigs in group A infected by the intra-muscular infection route. For both group B and C, conditions regarding study period, cercarial material used for infection as well as techniques used for obtaining information about intestinal egg counts, were equal to conditions described for group A. In order to compare the intestinal egg counts with liver egg counts, egg counts from the left lateral liver lobe from all 28 pigs were determined as described by Bøgh *et al* (1996). Female worm burdens earlier assessed by perfusion were also used in the present study.

In order to compare the relative distribution of eggs in the mucosa and the serosa, the small quadratic subsamples taken from the subgroup of 5 pigs from group A were used. The mucosa and serosa layer from each section was separated using the scraping technique just described. Homogenized 5 g samples from each tissue layer were digested and the egg number was determined as described by Bøgh *et al* (1996). Experimental design for pigs in groups A, B and C is presented in Table 1.

Data from the subgroup of 5 pigs taken from group A was log (x+1) transformed before Student's *t*-test was used for comparing the egg density between serosa and mucosa. Correlations between egg

Table 1
Experimental design.

| Group | No. of pigs | Infection method[d] | No. of cercariae | Protein content in fodder | Age at infection (weeks) | Duration of infection (weeks) |
|---|---|---|---|---|---|---|
| A | 5[a] | IM | 3000 | High | 10-12 | 9 |
| | 6[b] | IM | 1000 | High | 10-12 | 10 |
| | 4[c] | IM | 1500 | High | 15 | 11 |
| B | 5[b] | PO | 1000 | High | 10-12 | 10 |
| C | 8[c] | IM | 1500 | Low | 15 | 11 |

Experimental design is further described by [a]Giver *et al* (2000); [b]Giver *et al* (1999); [c]Johansen *et al* (1997).
[d] IM = intramuscular infection, PO = peroral infection.

counts from the mucosa and serosa seperately and the sum of egg counts from the mucosa and serosa were calculated using Pearson's correlation coefficient. The Wilcoxon signed-rank test was used on untransformed data to determine if mean egg counts based on quadratic mucosal subsamples and mean egg counts based on scrapings of the entire mucosa of cecum, colon and rectum taken from the same 5 pigs, was significantly different from zero.

Female worm burdens and tissue egg counts were log (x+1) transformed, before correlations between tissue egg counts and female worm burdens as well as correlations between egg counts in different tissue sections were calculated using Pearson's correlation test. Tissue egg counts were furthermore exposed to *a posteriore* pairwise comparison using Scheffe's range test.

## RESULTS

The geometric mean egg counts estimated from the quadratic subsamples of serosa and mucosa taken from 3 different intestinal sections (cecum, colon and rectum) of 5 pigs are presented in Table 2. For 4 out of the 5 pigs, egg counts from mucosa were significantly higher than egg counts from serosa. Significant correlations ($r = 0.96 - 1.00$, $p < 0.05$) were found for all pigs between the mucosa and total added value of mucosa and serosa, whereas significance was only found for one pig when considering correlation between serosa and added values. Large variation was seen between sampling methods when comparing mean mucosal egg counts based on quadratic subsamples and mean egg counts estimated from the scrapings of the entire mucosa (Fig 1). The mucosal subsamples from the cecum significantly underestimated egg counts from the entire cecum ($p < 0.05$), whereas subsamples from the colon and rectum overestimated egg counts based on the the entire mucosa of these sections, although the mean differences between counts from small samples and counts from the entire mucosa of colon and rectum were not significantly different from zero ($p = 0.5$).

Table 2

Geometric mean egg counts from intestinal wall subsamples of serosa and mucosa taken from 3 different intestinal sections (cecum, colon and rectum) of 5 pigs. Values from the different intestinal sections are for each pig pooled and presented as one mean value for either the mucosa or serosa. Confidence interval (ci) in parenthesis.

| Pig number | Eggs per gram tissue (Geometric means and ci) | | Student's *t*-test |
|---|---|---|---|
| | Mucosa | Serosa | |
| 1 | 93.2 (29.8-287.1) | 16.9 (2.7-85.8) | n. s. |
| 2 | 43.7 (15.9-116.4) | 0 (0-0) | $p < 0.001$ |
| 3 | 1,025.6 (427.9-2,456.4) | 35.6 (22.6-55.6) | $p < 0.001$ |
| 4 | 132.4 (59.1-295.5) | 7.4 (0.2-56.3) | $p < 0.005$ |
| 5 | 4,585.7 (3,289.2-6,393.0) | 174.5 (59.7-506.3) | $p < 0.001$ |

For each of the 3 groups of pigs, A, B and C, the geometric mean distribution of eggs per gram tissue from the 3 intestinal sections and the liver are presented in Fig 2. The very high egg number found in the cecum in group C is mainly due to the influence from 2 individuals in this group with extremely high egg counts. In general, egg counts from all tissue sections in group B seemed to be lower than counts from group A and C but no significant differences were seen neither between egg counts of different sections nor between each section of the different groups.

Correlating liver egg counts for all 3 groups of pigs with egg counts from the 3 different sections, revealed some differences between the groups (Table 3). For groups A and C, a significant correlation was found between egg counts of all 3 sections and liver egg counts. For group B, significance was found only for the correlation between liver and rectal egg counts. The number of female worms correlated for group A and C with tissue egg counts from all tissues including the liver, whereas no significant correlation was found between number of female worms and egg counts of any of the tissues in group B.

## DISCUSSION

The present study revealed that estimating intestinal tissue egg counts based on scrapings of the entire mucosa is a reliable and convenient approach, nicely supporting the liver tissue digestion approach.

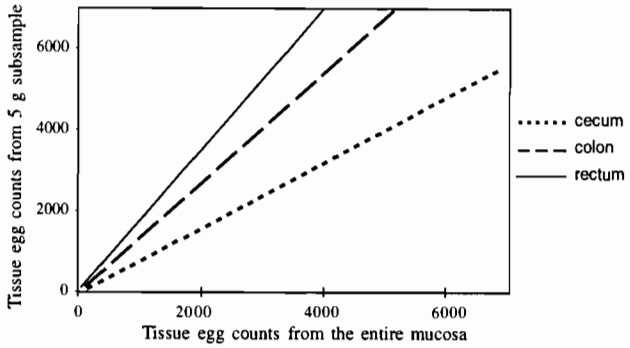


Fig 1–*Schistosoma japonicum* tissue egg counts estimated from mucosal subsamples taken from cecum, colon and rectum against egg counts from the same 3 sections based on scrapings of the entire mucosa.

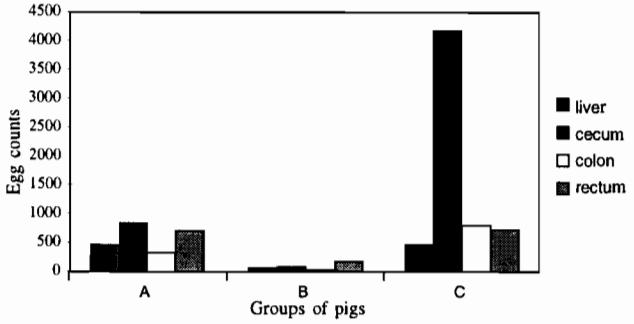


Fig 2–Distribution of eggs estimated from scrapings of the entire mucosa and based on geometric mean egg counts per gram tissue in 3 different groups of pigs (for further details see the text).

The differences seen in the present study between egg counts in mucosa and serosa indicate that eggs are mainly deposited in the mucosa. The correlation found between mucosa egg counts and added egg counts from mucosa and serosa support this. Willingham *et al* (1998) found that petechial hemorrhages and hyperemic foci in *S. japonicum* infected pigs were present in the mucosa throughout the large intestine after 11-24 weeks of infection, whereas pathological changes from the serosa were not reported. This is supported by histological investigations by Cheever *et al* (1980) in rabbits infected with a Japanese strain of *S. japonicum*. Thus, concentrating on the mucosa layer seems to be very appropriate when estimating egg counts in the intestinal wall as such.

The large variation seen between mean mucosal egg counts from the quadratic subsamples and mean counts estimated from scraping of the entire mucosa is a reflection of the patchy distribution of eggs in the intestinal wall reported by Cheever (1985), and is in line with results from a previous study by Willingham *et al* (1996). From that study it was concluded that, in consequence of a very large variation between counts from subsamples and total intestinal egg counts, subsampling in order to estimate intestinal egg counts is inappropriate. However the introduction of scraping technique circumvent these problems. When using the entire mucosa for estimating intestinal tissue egg counts, a reduction of the processing time of intestinal tissue in

Table 3
Correlation between egg counts from the liver and from different intestinal sections based on scrapings from the entire mucosa as well as between tissue egg counts and number of female worm.

| | | Correlation coefficients | | | |
|---|---|---|---|---|---|
| | | Liver | | Female worms | |
| | | r | p | r | p |
| Group A | Cecum | 0.8 | 0.05 | 0.6 | 0.05 |
| | Colon | 0.8 | 0.05 | 0.6 | 0.05 |
| | Rectum | 0.7 | 0.05 | 0.5 | 0.05 |
| | Liver | | | 0.6 | 0.05 |
| Group B | Cecum | - | - | - | - |
| | Colon | - | - | - | - |
| | Rectum | 0.9 | 0.05 | - | - |
| | Liver | | | - | - |
| Group C | Cecum | 0.8 | 0.05 | 0.9 | 0.01 |
| | Colon | 0.8 | 0.05 | 0.9 | 0.01 |
| | Rectum | 0.7 | 0.05 | 0.9 | 0.01 |
| | Liver | | | 0.9 | 0.01 |

general is seen due to the very simple scraping technique and the fact that digestion of a 5 g sample taken from the entire homogenized mucosa will be sufficient to give a reliable estimate of egg counts.

The comparison made between patterns of distribution of eggs in different intestinal sections and the liver in perorally infected pigs and in pigs fed a low-protein diet with the patterns in intramuscularly infected pigs fed an standard diet, did not reveal any statistically significant differences. Thus, eggs appear to be fairly equally distributed, when egg counts are expressed as eggs per gram tissue, between cecum, colon and rectum. This contrasts somewhat earlier observations by Willingham *et al* (1994) showing that rectal tissue counts markedly exceeded those in colon and cecum. Similarly, the markedly higher egg counts in the rectum than in the liver also demonstrated by Willingham *et al* (1994) could neither be confirmed in the present study. However, Willingham *et al* (1994) used digestion of small intestinal wall subsamples and this may partly explain the differences. On the other hand, the host/parasite interaction is of a dynamic nature, and differences between different experiments with different designs can be anticipated. The overall very low tissue egg counts in the perorally infected group is due to very low worm burdens (Giver *et al*, 1999) but again, all tissues appear equally affected. The very high egg counts in cecum of pigs fed a low-protein diet may more reflect large intrapig variations in worm establishment and tissue egg counts than a changed distribution induced by the low protein content of the fodder. In fact, the differences did not reach statistical significance.

The correlation observed in the present study between liver egg counts and intestinal egg counts and between female worm burdens and counts from different tissues, is very much in line with results from a previous study (Willingham *et al*, 1996). That study included 16 pigs each infected with 500 *S. japonicum* cercariae and a correlation was found between egg counts from the left lateral liver lobe and all intestinal sections, and also between female worm number and cecum, colon and liver egg counts. The lack of correlation in the present study in the per-orally infected group between liver and tissue egg counts as well as between female worms and tissue egg counts can probably be seen as a consequence of very low tissue egg densities (Giver *et al*, 1999).

In previous studies (Willingham *et al*, 1996; Johansen *et al*, 1997; Willingham *et al*, 1998) estimations of overall tissue egg counts in *S. japonicum* infected pigs have been based primarily on liver tissue egg counts as described by Bøgh *et al* (1996). This has partly been due to unclarity regarding patterns of distribution and patchyness of egg distribution in the intestines. The present study confirms the validity of that approach by showing correlations between liver egg counts and counts of the different intestinal sections. However, the scraping technique introduced makes it possible also to include reliable intestinal egg count estimates into the analysis of host/parasite relationships in continued efforts to understand aspects of regulatory processes and pathology pictures.

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*It is with deep regret that the authors note the untimely and sudden death of Professor Peter Nansen, who died in Copenhagen on 26 October 1999.