

Development of a versatile *in vitro* method for understanding the migration of *Fasciola hepatica* newly excysted juveniles

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(Received 24 July 2015; revised 6 October 2015; accepted 8 October 2015; first published online 2 November 2015)

SUMMARY

Fasciola hepatica is a parasitic trematode that causes serious losses to livestock producers, and also zoonotic disease. The limitations of chemotherapy for the control of fasciolosis have led to significant interest in the development of vaccines to protect cattle and sheep from infection. However, relatively few studies have concentrated on the mechanisms of invasion of the gut by newly excysted juvenile liver flukes (NEJ) and the host response triggered by this event. The aim of this work was to develop an *in vitro* model to study invasion by NEJ, while also reducing the requirement for challenge infections of experimental animals. *Fasciola hepatica* metacercariae were excysted *in vitro* and placed into compartments containing rat distal jejunal sheets. Variations in incubation medium, chamber size and incubation temperature were used to identify optimal conditions for NEJ migration across the gut. Histological examination showed increased migration until 120 min post-incubation. The use of RPMI, without gassing at 39 °C, as the incubation medium was found to be optimal, with 40.5% of NEJ migrating after 150 min. This study describes a readily-reproducible method for studying the migration of *F. hepatica* NEJ within the definitive host. It will be useful for identifying potential drug and vaccine targets.

Key words: *Fasciola hepatica*, NEJ, gut, jejunum, method optimization, horizontal diffusion chamber.

INTRODUCTION

Fasciola hepatica is a trematode, distributed worldwide, with a high economic impact in the livestock industry. Cattle and sheep become infected by ingestion of metacercariae that excyst in the digestive tract, releasing the newly excysted juveniles (NEJ), which are capable of burrowing through the intestine and migrating to the liver through the peritoneal cavity. Relatively little work has been done to understand the mechanisms through which NEJ orchestrate invasion and the host response that they trigger at gut level. Most of the existing literature dates from 1970s to the 1990s (Hanna and Ballawy, 1975; Van Milligen *et al.* 1999) with only one publication in the last decade (McGonigle *et al.* 2008). Just after NEJ excystation, the NEJ are able to recognize the intestinal environment, changing their behaviour in the presence of duodenal extracts (Sukhdeo and Metrick, 1986). They also have the ability to detect the presence of bile salts, triggering migration through the bowel (Tielens *et al.* 1981). The NEJ excretory/secretory (ES) products comprise a cocktail of important molecules for tissue degradation, feeding and immunoregulation including those belonging to the cathepsin clade. The up-regulation and secretion of cathepsin Bs and L3, which have high collagenase activity, is particularly

important, in view of the necessity for the NEJ to migrate across the intestine (Robinson *et al.* 2009).

The earliest study reported on NEJ migration is that of Schumacher (1938) who observed the 24 h period following infection of guinea pigs. Since then, it has been shown *in vitro* that NEJ have the ability to penetrate rat mid-small intestine (Doy and Hughes, 1981), rat small and large intestine (Hanna and Ballawy, 1975; Kawano *et al.* 1991; McGonigle *et al.* 2008) and small and large intestine of other species like mice and rabbits (Kawano *et al.* 1991). The percentages of successful NEJ migration found in the different models vary from 37% (Kawano *et al.* 1991) to 77% (Van Milligen *et al.* 1998a, b). A similar NEJ migration rate was reported by Hanna and Ballawy (1975), with *Fasciola gigantica* NEJ for 5 h *in vitro*. However, when the same experiment was performed in chickens, minimum NEJ invasion was reported (Kawano *et al.* 1991). Most of the experiments employed for NEJ invasion assessment required the use of various *in vitro* methods based on large portions of intestine or intestinal loops where screening of the whole sample was difficult because of the large volume to examine. Therefore, the localization of the NEJ within the different mucosal compartments or gut layers remained unknown. An *ex vivo* model was developed by Van Milligen *et al.* (1998b), which was useful to clarify both the dynamics of *F. hepatica* migration and the mucosal immunity that naïve and primed infected rats developed after infection (Van Milligen *et al.* 1998a, b). This method had the

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advantage that the blood supply in the gut was guaranteed, providing conditions more akin to those *in vivo*. However, the use of *ex vivo* models is ethically more difficult to justify than *in vitro* alternatives.

The mechanisms that NEJ employ for interacting with the gut epithelium prior to penetration and the involvement of glycans and C-type lectin receptors are still unclear. The aim of this work was to develop a more ethical, robust and efficient *in vitro* model for studies on the early stages of *F. hepatica* invasion that minimize the requirement for live animals and that will be valuable in understanding these mechanisms.

MATERIAL AND METHODS

Reagents

All chemical and reagents were purchased from Sigma-Aldrich Company Ltd. (Ireland) unless otherwise stated.

Excystation of metacercariae in vitro

Fasciola hepatica metacercariae were obtained from Ridgeway Research Ltd., (St Briavels, UK) or Baldwin Aquatics, Inc. (Monmouth, Oregon) and stored at 4 °C until use. Excystation of metacercariae was performed as described by Carmona *et al.* (1993) with minor modifications. NEJ were collected by pipetting and maintained in 38 °C Roswell Park Memorial Institute medium (RPMI) 1640 medium (Gibco, Ireland) until infection.

Tissue donors

All procedures involving animal tissue were carried out following approval by the University College Dublin (UCD) Animal Research Ethics Committee (AREC-14-28). Twenty-three 6 weeks old male Wistar rats (250–300 g) were housed in the Biomedical Facility in UCD in controlled environmental conditions. The animals were euthanized by stunning, then cervical dislocation. Laparotomy was performed, the caecum exposed and the small intestine was identified. The portion of small intestine between 25 and 5 cm just before the cecum was dissected and kept in temperate incubation medium.

Chamber configuration

The method was based on modifications of a horizontal diffusion chamber developed for *ex vivo* bacterial studies (Soni *et al.* 2006). The chamber was composed of two sections: the basolateral and apical compartments. The basolateral compartment consisted of a half-filled 9 mL glass vacutainer tube. The apical compartment was either a 1.5 mL or 200 μ L eppendorf tube whose lid was drilled to create an aperture. NEJ were introduced into the chamber from the

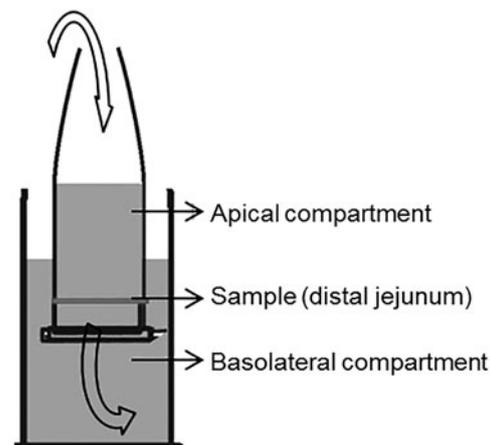


Fig. 1. Scheme of the modified horizontal diffusion chamber and the NEJ flow. The Horizontal diffusion chamber consisted of the assembly of an apical and a basolateral compartment. The apical compartment was made from an eppendorf tube, the size of which was selected depending on the conditions employed for each method. The basolateral compartment consisted of medium half-filled 9 mL vacutainer tubes mounted inside a temperature controlled system. An aperture was drilled into the tip of the apical chamber to facilitate introduction of NEJ. The lid of the chamber was also drilled to create an aperture in which the tissue sample was mounted. NEJ that completely traversed through the jejunum were collected at the bottom of the basolateral compartment. Abbreviation: NEJ, newly excysted juvenile.

drilled tip side whereas the lid region was the area where NEJ came in contact with gut tissue. The apical compartment fitted into the basolateral compartment, with the tip region facing to the upper side of the basolateral compartment (Fig. 1).

Tissue preparation, chamber assembly and NEJ challenge

Intestinal contents were removed by flushing with medium. The jejunum (3–4 cm) was opened along its mesenteric border to create a sheet which was pinned on a moistened dissection board with the serosal surface in contact with the board. The tissue was regularly irrigated with medium. Several adjacent sheets were obtained from each donor. The apical compartments of each chamber were opened and their lids were positioned between the corkboard and the serosal layer. The apical compartments were then closed making sure that the tissue covered the entire aperture. The chambers were then filled with medium. Tissue debris was removed and each chamber assembled. NEJ were added to the apical compartment using a 200 μ L pipette.

Experimental conditions and assessment of migration in chambers

Seven different protocols were evaluated, as described in Table 1. The conditions that varied

Table 1. Parameters for the protocols assessed during method optimization

Protocol	Donor specie	Sex	Age	Number animals	Chambers per time point	Chamber size (mL)	Medium	Temp (°C)	Heating system
1	Wistar Rat	Male	6 w.o.	3	3	1.5	5% CO ₂ Krebs-Henseleit	37	Water bath
2	Wistar Rat	Male	6 w.o.	4	4	1.5	5% CO ₂ Krebs-Henseleit	37	Water bath
3	Wistar Rat	Male	6 w.o.	2	2	0.2	5% CO ₂ RPMI 1640	37	Heating block
4	Wistar Rat	Male	6 w.o.	4	4	0.2	5% CO ₂ RPMI 1640	37	Heating block
5	Wistar Rat	Male	6 w.o.	4	4	0.2	RPMI 1640	37	Heating block
6	Wistar Rat	Male	6 w.o.	4	6	0.2	RPMI 1640	39	Heating block
7	Wistar Rat	Male	6 w.o.	3	18	0.2	RPMI 1640	39	Heating block

Protocol	Metacercariae supplier	Metacercariae storage time	NEJ inoculation dose	Accuracy of NEJ inoculation dose	NEJ infecting rate (NEJ mm ⁻²)	Time points (min)	Histology
1	Ridgeway research	Less than 6 months	37.38 ± 5.22	NO	0.48	60, 120, 180, 240	NO ^a
2	Norm Baldwin	Less than 6 months	40	NO	0.51	60, 120, 180, 240	NO ^a
3	Norm Baldwin	More than 6 months	36 ± 2.77	NO	1.83 ²	30, 60, 90, 120, 150	YES
4	Norm Baldwin	Less than 6 months	31 ± 8.82	NO	1.58	30, 60, 90, 120, 150	YES
5	Norm Baldwin	Less than 6 months	33 ± 4.95	NO	1.68	30, 60, 90, 120, 150	YES
6	Norm Baldwin	Less than 6 months	42 ± 3.75	NO	2.14	30, 60, 90, 120, 150	YES
7	Norm Baldwin	Less than 6 months	43.56 ± 1.6	YES	2.21	30, 60, 90, 120, 150	NO

NEJ, newly excysted juvenile; Temp, temperature; w.o., weeks old.

^a NEJs counted under stereoscope with no distinction of their localization.

were as follows: incubation medium, the supply of CO₂ gas, size of apical compartment, incubation time, temperature, heating system and number of NEJ per chamber. Fluid from the apical compartment was collected carefully at each time point and placed in a watch glass. NEJ found here were counted using a stereoscope. In parallel, fluid from the basolateral compartment was allowed to settle for 10 min so that all NEJ sedimented to the bottom of the chamber before being counted.

Assessment of NEJ migration in distal jejunum: tissue embedding and histology staining

In order to assess the NEJ pattern of migration in distal jejunum tissue samples were fixed separately in 10% buffered formalin (Fisher Chemical, UK) at each time point, using one of two approaches:

Methodology used for Protocols 1 and 2. After tissue fixation, specimens were placed on a microscope slide. Slides were viewed with an Olympus BX40FA camera (Olympus Optical Co, Ltd. Japan) using 4x and 20x Ach objectives (Olympus, Japan).

Methodology used for Protocols 3, 4, 5 and 6. Histological examination was performed following collection of semi-circular tissue samples. The tissue pieces were mounted into cassettes, processed and embedded in paraffin wax. Sections of 5 µm thick were cut from the paraffin wax block. One in every ten cuts was mounted in slides and incubated at 58 °C overnight. Slides were dewaxed and haematoxylin and eosin (H&E) staining was performed on all samples in a Shandon Gemini Varistain automatic slide stainer (Thermo Scientific). Slides were viewed as previously described. Localization (mucosa or serosa) and number of NEJ were recorded. NEJ located at the luminal surface were considered as NEJ located in the mucosa.

Accuracy of NEJ infective dose

NEJ were placed into the apical compartment by pipetting, and tips that were used for the NEJ challenge were kept and checked under a stereoscope. Numbers of NEJ that remained adhered in the tips were subtracted from the original infective dose to obtain an accurate NEJ inoculation dose.

Methodology refinement and validation

Once the conditions for NEJ migration had been optimized, the protocol was repeated to establish the kinetics of migration. Eighteen apical chambers were moved to fresh basolateral compartments every 30 min until 150 min of incubation. After 150 min, fluid from the apical chamber was collected and NEJ found was counted as previously described. The fluid from each basolateral compartment was

allowed to settle for 10 min and NEJ was counted as described.

Graphs and statistical analysis

Results were plotted using GraphPad Prism 5 software. Numbers of NEJ at each location and time point were expressed as means (±S.D.). Data were analysed using the Friedman test and Dunn's multiple comparison test was employed to compare differences in migration between time points.

RESULTS

Protocols 1, 2, 3 and 4

The results of all the protocols employed are given in Table 2. Using Protocol 1, although NEJ were detected in the apical compartment at all the time points none were found in the basolateral compartment at any time point. On microscopic examination 15% of NEJ were detected in the tissue 180 min post-infection. However, the layers where the NEJ were located could not be distinguished. Changing the strain of metacercariae (Protocol 2) did not produce any increase in NEJ migration, suggesting that the incubation medium, the size of the chamber or the heating system were critical factors to consider. Changing these three factors (Protocol 3) did not result in any increase in NEJ counts in the basolateral compartment. However, there was a slight increase in the NEJ counts found within the gut tissue, with percentages that varied from 4.13 (±2.02) to 26.49% (±18.36). No significant differences were detected in NEJ counts from mucosal or serosal layers. Considering that this was the first time we were able to identify NEJ in the distal jejunum we concluded that using small chambers immersed in RPMI 1640 medium worked better than large chambers immersed in Krebs–Henseleit solution.

The low infection rate led us to conclude that the viability of the metacercariae was too low as they had been stored for more than 6 months. In order to assure that metacercarial viability did not interfere with the assay, metacercariae less than 6 months old were used for the further optimization experiments. Using fresh metacercariae in the same conditions as Protocol 3 (Protocol 4) did increase NEJ counts in the basolateral region, with 5.88% (±5.61) of NEJ detected after 120 min incubation. However, the NEJ counts in histological sections were lower than in the previous method suggesting that further changes were necessary. We concluded that although standardizing metacercarial viability was an important variable to take into account, modification of other factors such as the incubation medium or the incubation temperature would be useful to investigate.

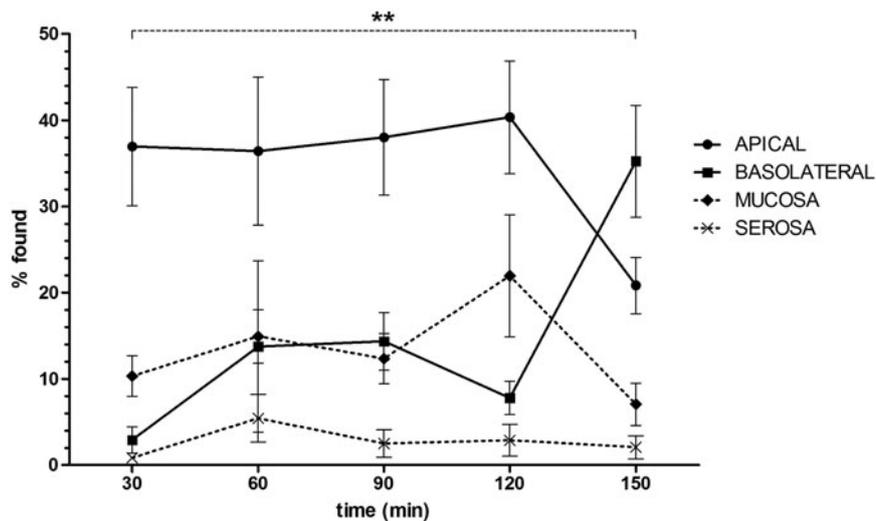


Fig. 2. Kinetics of NEJ invasion in rat distal jejunum using Protocol 6 NEJ were inoculated into the *in vitro* system as described and allowed to migrate through rat distal jejunum for 30–150 min. NEJ located in apical (continuous line circles) and basolateral (continuous line squares) were counted. Jejunum was fixed and serial histological examination was performed every 50 μm to assess the percentage of NEJ located in the mucosa (dashed line diamonds) and serosa (dashed line crosses) at the different time points. Total percentages were calculated according to the initial NEJ inoculation dose. Results were calculated based on six independent experiments. Vertical bars represent s.e. Asterisks represent significant differences in the percentage of NEJ found in the basolateral chamber between time points (** $P \leq 0.01$). Abbreviation: NEJ, newly excysted juvenile.

The influence of incubation medium and incubation temperature were the key factors that triggered NEJ invasion into the basolateral compartment

The following protocols were modified from the previous ones in two respects. For the first modification, which corresponded to Protocol 5, RPMI 1640 medium was no longer gassed with 5% CO_2 . This protocol showed for the first time a continuous migration of the NEJ from the apical to the basolateral chamber at all the time points, with values of 22.73% (± 3.49) and 16.37% (± 1.97) at 120 and 150 min, respectively. In addition, the NEJ counts in the distal jejunum were higher than the NEJ counts found in the previous protocol. We concluded that the lack of additional CO_2 supplied to the medium was responsible for these differences.

In spite of this increase in migration, NEJ migration at 150 min was lower than the NEJ migration detected by other *in vitro* models (Hanna and Ballawy, 1975; Van Milligen *et al.* 1998a, b; McGonigle *et al.* 2008). For that reason, we performed Protocol 6. This was based on Protocol 5 with the modification of increasing the temperature of the system from 37 to 39 $^{\circ}\text{C}$. As shown in Fig. 2, the percentage of NEJ found in the apical compartment was stable from time 30 to 120 min, this value varying between 36.42% (± 21.08) and 40.34% (± 16.01). After 120 min, a large decrease of NEJ was observed in the apical compartment, of the order of 20.84% (± 7.99). The number of NEJ observed in the basolateral compartment increased over time, with 150 min the time point with most NEJ found in this chamber (35.23

$\pm 15.84\%$). This was the first time where we observed a significant difference in NEJ migration between 30 and 150 min with a final NEJ migration rate comparable with previous studies. We concluded that an increase of 2 $^{\circ}\text{C}$ in the incubation temperature provided this improvement.

There were no significant differences in the NEJ counts in mucosa and serosa in Protocol 6

The percentage of NEJ found in the mucosa (Fig. 2) increased at all the time points, and peaked at 120 min post infection at 21.94% (± 17.29). After this peak, a drop in this percentage was observed at 150 min post infection which correlates with the increase of the NEJ detected previously in the basolateral compartment. There were no significant differences between the counts at 30 and 150 min. Fewer than 6% NEJ were detected in the serosa, with 5.41% (± 6.78) the highest value at 60 min post infection. Examples of NEJ located in the mucosa and serosa are shown in Fig. 3.

NEJ detection methods of all the previous protocols underestimated the true NEJ counts

Taking into account the NEJ counts in the apical and basolateral compartments and within the gut in all the protocols assessed, the highest level of NEJ detection, as a percentage of NEJ inoculated, was seen in Protocol 6 with an average of 65.37% (± 20.42) suggesting that significant numbers of

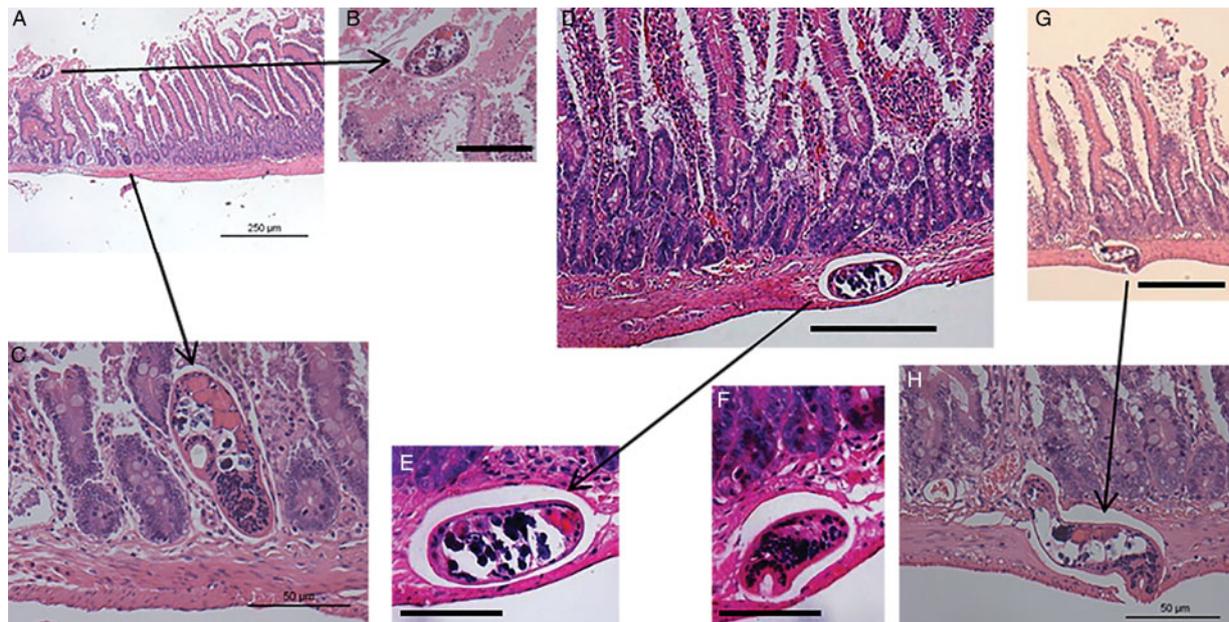


Fig. 3. Detection and localization of NEJ in rat distal jejunum by histology. NEJ were inoculated into the *in vitro* system as described and allowed to migrate through rat distal jejunum. Jejunum was fixed and the presence of NEJ in the mucosa (A, B, C) and in the serosa (D, E, F, G, H) was assessed every 50 μm of tissue after H&E staining at 60 min (A, B, C), 90 min (D, E), 120 min (F) and at 150 min (G, H) post NEJ inoculation. Scale bar = 250 μm (A, G). Scale bar = 50 μm (B, C, D, E, F, H). Abbreviations: H&E, haematoxylin and eosin; NEJ, newly excysted juvenile.

NEJ were missing and/or that the NEJ infecting dose could have been overestimated. In order to investigate this and to obtain a more accurate NEJ migration curve, Protocol 7 was designed.

Protocol 7 reproduced the kinetics seen in Protocol 6 and also increased the NEJ detection rate in the basolateral compartment by 40.53%

Protocol 7 was carried out taking into account the possibility of overestimating the initial NEJ dose inoculated into the chambers. We found that 7.3% (± 3.01) of the NEJ remained attached to the tips that were used for infecting the chambers, thus confirming the initial overestimation of the NEJ dose in previous protocols.

Once we calculated the exact NEJ dose we wanted to reproduce and obtain a more realistic cumulative NEJ invasion curve using a larger number of chambers. Our results confirmed the reproducibility of the method counting NEJ in the basolateral compartment at all the time points (Fig. 4). Although the highest percentage of NEJ in the basolateral compartment was detected at 150 min (40.53 ± 7.17) significant differences were already observed in NEJ numbers from 30 to 90 min post infection. In addition, NEJ counts in the apical chambers decreased from 20.84% in Protocol 6 to 15.93% (± 6.37) in the current protocol suggesting that more NEJ were invading the mucosa and serosa. Although histology was not performed and NEJ counts in the jejunum were not used for assessing this protocol the percentage of NEJ detection from

the tips, the apical and the basolateral compartment only (i.e. excluding NEJ within the tissue) was 61.51%.

DISCUSSION

We aimed to develop an optimized robust and reproducible *in vitro* model for assessing *F. hepatica* NEJ migration. Sheets of isolated intestine have been used successfully to examine pathogen entry (Baird *et al.* 2004) as well as ion transport responses of previously sensitized mucosal sheets to specific antigen (Baird and O'Malley, 1993). We modified the structure of the horizontal diffusion chamber used in previous microbiological studies (Soni *et al.* 2006) to our requirements for parasite studies. Optimization of the method was carried out by investigating seven different protocols to assess *in vitro* NEJ invasion under physiological conditions. Protocols 5, 6 and 7 showed active NEJ migration. We observed that incubation temperature, incubation medium and chamber size/rate of infection were the most important parameters in determining migration from apical to basolateral compartment. Temperature also correlated with the efficiency of the heating system. When a water bath was used as the heating system, variability due to vaporization, room temperature changes etc. could mitigate against the correct temperature being reached. However, the use of a heating block to surround the whole basolateral chamber eliminated these factors. Because steel has a higher thermal conductivity than water (52 W mK^{-1} for steel *vs* 0.6 W mK^{-1} for water at

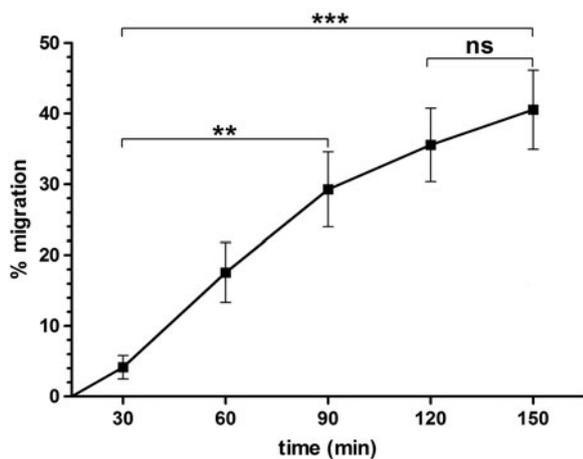


Fig. 4. Kinetics of NEJ invasion in rat distal jejunum using Protocol 7 NEJ were inoculated into the *in vitro* system as described and allowed to migrate through rat distal jejunum from 30–150 min. A total of 18 chambers were used in the confirmation of the method. NEJ located in basolateral compartments were counted and the total percentage was calculated after recalculation of the NEJ inoculation dose. Results were calculated based on three independent experiments. Vertical bars represent S.E. Asterisks represents significant differences in the percentage of NEJ found in the basolateral compartment between different time points (** $P \leq 0.01$, *** $P \leq 0.001$). Abbreviation: NEJ, newly excysted juvenile.

20 °C, (Young, 1992), heat is conducted more efficiently, reaching the desired temperature very quickly. Because of the higher percentage of NEJ detected in the basolateral compartment in Protocols 6 and 7, we concluded that 39 °C is the most reliable temperature to maximize NEJ migration in this system.

Chamber size and rate of infection are complementary factors. Because the number of NEJ used in each chamber was very similar (40 NEJ per chamber approximately), the size of the chamber determined the area of the sample, being larger in the 1.5 mL chambers and smaller in the 200 μ L chambers. Although other authors proposed that rate of infection does not interfere with the percentage of migration (Van Milligen *et al.* 1998a, b), these calculations were based on a gut loop model, by using a bigger portion of intestine. By using the gut loop model, homogeneity of NEJ distribution on the lumen surface is not guaranteed because it is possible that a considerable proportion of NEJ are crowded unevenly in some focal portions of the loop and the rest of the NEJ disperse in the rest of the sample. In our methods, because of the size of the chamber, a homogenous dispersion of NEJ is more likely, especially with the smaller compartment.

We observed that the incubation medium used had a considerable effect on NEJ migration. This is consistent with what has been described previously (Hanna and Ballawy, 1975). We found that RPMI 1640 without CO₂ was the best medium to use in the current method. Carbon dioxide is typically

used in mammalian tissue and cells for maintaining constantly a neutral-acid pH of the medium where cells are cultured for periods of days or weeks. It has been shown that the pH detected in the lumen of jejunum and ileum in rats tends to be quite high (pH = 7.9) (Ward and Coates, 1987). Therefore, it can be suggested that, under these experimental conditions, CO₂ gasification is not a crucial factor for NEJ and jejunum survival. In the present study, distal jejunum was cultured for less than 3 h. As the pH indicator did not vary from the beginning to the end of the experimental period, we conclude that this was not long enough to change the pH to non-physiological conditions. The use of bile as a supplement to enhance migration was considered as it has been described that it is a direct stimulus for NEJ to leave the intestine (Tielens *et al.* 1981). Nevertheless, bile is also detrimental to NEJ survival and changes the NEJ glycocalyx (Bennett and Threadgold, 1975), which it is desirable to keep intact for assessing host–parasite interaction.

Taking into account the NEJ numbers found at apical and basolateral compartments in Protocols 6 and 7 we can confidently conclude that active migration was observed. Particularly in Protocol 7, there were significant differences in the NEJ migration between 30 and 90 min post-infection. This could explain the requirement for NEJ to recognize receptors in the gut as a trigger for the upregulation of the expression and secretion of active molecules such as cathepsin L3 and cathepsin Bs (Cancela *et al.* 2008; Robinson *et al.* 2009) and start migration very quickly in order to avoid host digestion or expulsion from the host. The 40.53% migration rate observed after 150 min is comparable with migration observed in another *ex vivo* method where the same value was obtained in cumulative penetration at that time point (Van Milligen *et al.* 1998a, b), with another *in vitro* model created for *F. gigantica* invasion in distal ileum (Hanna and Ballawy, 1975) and with Japanese *F. hepatica* in rats (Kawano *et al.* 1991). Regarding the localization of the NEJ in the intestine at the different gut layers we found no significant differences between mucosa at serosa in Protocol 6 at any time point. For that reason histological examination was not performed for Protocol 7.

In the first attempt at optimization, 65.37% of total NEJ were detected between apical and basolateral compartments along with in the gut tissue. The fact that this value was lower than that reported for an *ex vivo* model (Van Milligen *et al.* 1998a, b) made us consider that further improvements were required in NEJ identification to achieve an even higher rate of detection. After verifying with Protocol 7 that the dose of infection used in each chamber was actually a bit lower than originally calculated, because some NEJ were attached in the tip of the pipette, we obtained very similar NEJ detection rates. Although histological examinations were not

performed in this last method we suggest that NEJ detection was improved compared with Protocol 6.

The method we used for the detection of NEJ within host tissue was based on previous work (Van Milligen *et al.* 1998a, b). Using our pattern of tissue screening, which is to examine every 50 μm , some NEJ located in tissue may have been hidden due to their orientation. As the images indicate, NEJ migrated actively in the tissue, with the oral sucker facing the gut tissue. When NEJs reached the serosal layer, they appeared to be surrounded by an empty cavity. As the serosa is a distinct barrier, we hypothesized that the cavity may be filled with ES products (Robinson *et al.* 2009; Cancela *et al.* 2010). However, since tissues were fixed by formalin, we cannot rule out the possibility that these were artefacts.

A limitation of this method is the progressive deterioration in the integrity of the mucosal tissue, as judged histologically, after 150 min, thus limiting the time post-infection that can be investigated. Nevertheless, considering that mucosa was only compromised after 150 min and that no significant differences were detected in the migration of the NEJ between 120 and 150 min we concluded that this limitation did not interfere with our experiments.

All the modifications carried out during this optimization have led to the development of a reliable *ex vivo*, method which is in accordance with the 3Rs (replacement, reduction and refinement) principle as no invasive procedures are needed and the number of animals required is minimized. The main advantage is that various experimental conditions can be applied to specimens from the same donor, which avoids the use of one animal for a single experimental condition, thus reducing intra animal variability. In addition, it is versatile as it can be applied to all the intestinal segments and to different animal species. Up to 12 chambers can be mounted from a single rat jejunum in the optimal time frame for assuring tissue integrity (data not shown). However, it is noteworthy to point out that the intestinal tract and the animal species chosen in the study are the key factors that determine the number of chambers that are possible to produce. In order to acquire statistically robust data, the number of chambers required for each experimental condition is dependent on the research question as well as other factors such as the power of the analysis and the confidence interval required, among others. Based on the data obtained in this study, we recommend that for similar work no less than three chambers per experimental condition and donor should be used.

In conclusion, we can say that the modified horizontal diffusion system we have developed and validated is a useful tool for assessment of gut penetration by *F. hepatica* NEJ *in vitro*. Multiple, matched tissues may be obtained from a single donor which permits good

experimental design. This will be particularly useful in determining the migration pattern of NEJ that have been exposed for example to vaccine induced antibodies, glycan ligands, host lectin receptor agonists and putative chemotherapeutic agents. This method in combination with other *in vitro* methods will allow us to improve understanding of the main factors that contribute to the earliest stages of fasciolosis in the definitive host, and ultimately to increase the range of control methods available for this important parasitic disease.

ACKNOWLEDGEMENTS

We are grateful to Patsy Kearns and Fiona McCartney for animal care and preparation before euthanasia. We also thank Joseph Brady and Margot Coady for their assistance during the histology process.

FINANCIAL SUPPORT

This work was supported by the European Community's Seventh Framework Programme (PARAVAC – Project Number 265862 to G.M.).

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