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Fasciola hepatica: An assessment on the vectorial capacity of *Radix labiata* and *R. balthica* commonly found in Belgium

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Abstract

A previous study conducted in Belgium revealed that genetic material of *Fasciola* sp. was present in snail species belonging to the genus *Radix*. Here, these snails were collected and identified by DNA-based techniques as *Radix labiata* and *Radix balthica*. These two species and *Galba truncatula* (the major intermediate host in Europe) were experimentally infected with *Fasciola hepatica*. The resulting metacercariae were fed to rats and the infection was monitored using several techniques. Microscopy revealed the presence of larval stages in 78.3, 45, and 6.25% of *G. truncatula*, *R. labiata*, and *R. balthica* snails, respectively. These results were confirmed by a PCR that amplifies a *Fasciola* sp. specific sequence. Furthermore, this PCR was found to be more sensitive than microscopic examination. *R. labiata* shed fewer metacercariae than *G. truncatula* but these were as infective to rats as those shed by *G. truncatula*. This study demonstrates that *R. labiata* may act as an incidental intermediate host for *F. hepatica* in Belgium.

Keywords: *Fasciola hepatica*; Lymnaeidae; Polymerase chain reaction (PCR); Vectorial capacity; Microscopy; Second internal transcribed spacer (ITS-2)

1. Introduction

Fasciolosis is a food-borne trematode infection, which has a worldwide distribution. It is responsible for marked economic losses in livestock due to reduced weight gain, milk production, fertility, and condemnation of livers (Genicot et al., 1991). The parasite is prevalent in Europe and a recent study from Belgium reported that 17.3% of cattle were serologically positive (Lonneux et al., 2000). This is a vectorial disease transmitted by freshwater snails of the family Lymnaeidae (Mollusca: Gastropoda: Basommatophora) (Boray, 1982; Malek, 1984). In Europe, the principal intermediate host for *Fasciola hepatica* is *Galba truncatula* O.F. Müller, 1774.

While the morphological identification of *G. truncatula* is quite easy, other lymnaeids belonging to the genus *Radix* Montfort, 1810 are more difficult to identify morphologically (Mas-Coma, 2005; Pfenninger et al., 2006) except for *Radix auricularia auricularia* Linnaeus, 1758. The analysis of nuclear ribosomal DNA ITS-2 sequences has been used for the identification of the different species (Bargues et al., 2001; Mas-Coma et al., 2005). Several authors (Bank et al., 2002) have reported the presence of the following species of the genus *Radix* in Belgium: *R. auricularia auricularia*, *Radix labiata*, Rossmässler, 1835, and *Radix balthica* Linnaeus, 1758. During a previous research programme dedicated to the study of biodiversity in small water surfaces in Belgium, several *Radix* spp. were collected and examined at our

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laboratory. A specific *Fasciola* DNA sequence was amplified by polymerase chain reaction (PCR) according to Kaplan (Kaplan et al., 1995). The study revealed that specific DNA material was present in adult *Radix* spp. (Caron, 2004).

Microscopic examination is the most frequently used technique to detect *F. hepatica* in the intermediate host. Historically, three approaches have been used to diagnose trematode infections in snails: (1) observation of cercarial shedding, (2) dissection and (3) crushing, followed by microscopic examination (Kaplan et al., 1997). Although those techniques are simple, fast, and very cheap, they have low sensitivity and/or specificity. Indeed, prior to the release of rediae from the sporocyst, and their subsequent migration through the tissue of the snail (around day 21 post-infection), the detection of the parasite is difficult. Nevertheless, young rediae might be seen 9–10 days after infection. Before cercarial development, however, the intra-molluscan stages of the different trematode species are difficult to differentiate (Kaplan et al., 1995). For this reason, several DNA- or RNA-based techniques were developed (Shubkin et al., 1992; Rognlie et al., 1994; Kaplan et al., 1995; Kramer and Schnieder, 1998; Mostafa et al., 2003; Magalhaes et al., 2004; Cucher et al., 2006). These techniques are specific and sensitive (Kaplan et al., 1997) but are rarely used to detect naturally occurring infections, although this should be one of their main applications. They would be suitable, for example, for monitoring seasonal transmission during epidemiological studies (Rognlie et al., 1994; Kaplan et al., 1997).

The present study was aimed at the molecular identification of two populations of snails belonging to the genus *Radix* and commonly found in Belgium. Furthermore, the vectorial capacity of these species for *F. hepatica* was evaluated under experimental conditions. Infection of the snails was monitored using a microscopic examination and a PCR technique. Finally, the infectivity of the different populations of metacercariae was evaluated in an experimental model of infection in rats.

2. Materials and methods

2.1. Snails

G. truncatula was used as a positive control. The colony originated from a French population (46°40′34.81″N and 1°22′58.58″W), living in the commune of Migné, department of Indre. This population was used because past experimental infections proved that this population was highly susceptible to *F. hepatica* (Vignoles et al., 2001). *Radix* sp. 1 and *Radix* sp. 2 were collected from the field in 2 locations in Belgium. *Radix* sp. 1 was collected in the commune of Ernonheid (50°23′45.86″N and 5°40′43.79″E) and *Radix* sp. 2 in Sart Tilman (50°34′51.03″N and 5°35′43.17″E) respectively, Province of Liège. The coordinates were calculated with Google Earth (version 4, Mountain View, CA, USA).

2.1.1. Snail identification

The ITS-2 region sequence was analysed according to Bargues and Mas-Coma (2005). The snail DNA was extracted from half of the foot using a commercially available kit (High Pure PCR Template Preparation Kit, Roche) and DNA concentration was measured with a spectrophotometer (Nanodrop, Wilmington, DE, USA). The ITS-2 sequence was amplified by PCR using a commercial PCR kit (Core System 1, Promega Benelux, Leiden, The Netherlands). The primers used were News2 (sense) 5′-TGT-GTC-GAT-GAA-GAA-CGC-AG-3′ and Its2Rixo (antisense) 5′-TTC-TAT-GCT-TAA-ATT-CAG-GGG-3′ (Almeyda-Artigas et al., 2000; Bargues et al., 2001). Amplification was performed in a total volume of 100 μl. The PCR mixture was prepared according to the manufacturer's recommendations and 200 ng of template DNA was added. Amplification was performed in a Peltier Thermal Cycler (MJ Research, Biozym, Landgraaf, Belgium) with an initial denaturation step at 94 °C for 2 min, followed by 35 denaturation cycles at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 30 s followed by a final extension at 72 °C for 7 min. The amplification products were electrophoretically resolved in 1% agarose gels and stained with ethidium bromide. The DNA strips were cut and then purified with a kit (Geneclean, MP Biomedicals, Brussels, Belgium) and sequenced with a genetic analyser (ABI PRISM® 3100, Applied biosystem, Lennik, Belgium). The ITS-2 sequences obtained were compared with the BLASTn genomic database (Altschul et al., 1997) and those presenting the higher identity was consider as the most probable identification.

2.1.2. Snail breeding

The three snail species were maintained in the laboratory for breeding. Briefly, snails were kept on plastic dishes (*G. truncatula* and *Radix* sp. 2) or in aquaria (*Radix* sp. 1) to minimize their motality rate, in an air-conditioned room (PVG, Schoten, Belgium), under the following conditions: constant temperature of 20 °C, diurnal photophase of 12 h with a light intensity

of 3000–4000 lux. They were fed with leaves of pesticide free lettuce and left for 5 days in standing spring water (Romy Spring) before use (Van der Steen et al., 1969). Plastic dishes and aquaria were checked every day and rinsed every week. Dead snails were removed daily.

2.2. Rats

Eighteen 15-week-old female Wistar rats were used (University of Liège breeding unit). All animals were individually marked, housed in conventional cages with grid floors, and fed a commercial rodent diet ad libitum for the entire experiment (Prestige, Versele-Laga, Belgium).

2.3. Parasites

Eggs of *F. hepatica* were recovered at a local slaughterhouse in Liège from the bile of infected cattle. *F. hepatica* eggs were incubated for 20 days at 20 °C in complete darkness for miracidial development (Ollerenshaw, 1971).

2.4. Experimental infections

2.4.1. Snail infection

In order to compare the mortality in infected and uninfected snail populations, mortality rates in the breeding unit were calculated for each species. One hundred newly hatched uninfected snails were raised for 50 days. Dead snails and egg masses were regularly removed. At the end of the 50-day period, the remaining snails were counted.

One hundred juvenile (1–2 mm) snails of each species were used in each infection experiment as it was described in previous studies (Busson et al., 1982; Vignoles et al., 2002). Each snail was placed in one hole of a 24 well micro-plate filled with spring water. Each snail was exposed to two miracidia for 5 h. The choice of two, rather than one, miracidia per snail was prefered because the infection rate of *G. truncatula* was higher and in 85% of infected snails, only one of the two miracidia developed (Preveraud-Sindou and Rondelaud, 1995). Thereafter the exposed snails were maintained in the breeding unit for a maximum of 50 days as described above. Twenty snails of each species were collected on days 15 and 30 post-infection (PI). On day 50, thermal shock was provoked by applying ice to the surviving snails, in order to stimulate the shedding of cercariae. Young, uninfected snails were used as negative controls.

2.4.2. Rat infections

Rats were divided into three groups of six animals each. Groups 1 and 2 were infected with metacercariae from the cercariae shed by *G. truncatula* or *Radix* sp. 1 respectively. Group 3 acted as negative control. Twenty 2-week-old metacaercariae were suspended in an adraganth solution (VWR, Fontenay-sous-Bois, France) and administered orally to each rat. Group 3 was given an equivalent volume of the adraganth solution.

2.5. Monitoring of snail infection

The experimental infection of snails with *F. hepatica* was monitored using two techniques: microscopy and specific amplification of a parasite DNA sequence.

Twenty snails collected on days 15 or 30 PI were examined by squashing and dissection. Firm pressure was applied with forceps on the upper side of the last whorl near the suture to free the intra-molluscan larvae. Snail bodies were finely dissected under a stereoscopic microscope (×40). The larvae, if present, were examined and characterised (Augot et al., 1998).

The bodies of each batch of snails were then pooled and DNA was extracted with a commercial kit (High Pure PCR Template Preparation Kit, Roche). The DNA concentration was measured with a spectrophotometer (Nanodrop, Wilmington, DE, USA). *F. hepatica* DNA was amplified by Polymerase Chain Reaction (PCR) using a commercial kit (Core System 1, Promega Benelux, Leiden, The Netherlands). Primers were designed based on the DNA sequence of a 124 bp DNA *Fasciola* sp. specific probe (Kaplan et al., 1995): Fsh1 (sense) 5′-GAT-CAA-TTC-ACC-CAT-TTC-CGT-TAG-TCC-TAC-3′ and Fsh2 (antisense) 5′-AAA-CTG-GGC-TTA-AAC-GGC-GTC-CTA-CGG-GCA-3′. Amplification was performed in a total volume of 50 μl. The PCR mixture was prepared according to the manufacturer's recommendations and 200 ng of template DNA was added. Amplification was performed in a Peltier Thermal Cycler (MJ Research, Biozym, Landgraaf, Belgium) with an initial denaturation step at 95 °C for 5 min, followed by 40 denaturation cycles at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min. The amplification products were electrophoretically resolved in 1% agarose gels and stained with ethidium bromide.

2.6. Monitoring of rat infection

Faecal examination, copro-antigen detection and serology were all used to monitor *F. hepatica* infection in rats. Animals were weighed and blood was collected

from the caudal vein and centrifuged (1000 × *g* for 10 min) before infection and weekly thereafter up to 12 weeks PI. Individual fresh stools were collected twice a week. Sera and stools were stored at −20 °C or 4 °C, respectively, prior to examination.

A standard sedimentation and flotation technique was used to analyse faecal samples. Briefly, 2 g of fresh faeces were mixed with 56 ml of water and then filtered (150 μm, Retsch, Aartselar, Belgium). The supernatant was centrifuged up to 1000 × *g*. The pellet was re-suspended in a saturated solution of $ZnCl_2$–NaCl and centrifuged up to 100 × *g*. The tubes were filled with the $ZnCl_2$–NaCl solution in order to obtain an upper meniscus. A coverslip was deposited onto the meniscus. Five minutes later the coverslip was removed and examined at x40 for the presence of *F. hepatica* eggs. Copro-antigens were detected in 3 g of stools using a commercially available kit (*F. hepatica* Ag ELISA Kit, BIO K 201, Bio-X, Jemelle, Belgium) according to the manufacturer's instructions. This test detects excretory-secretory antigens of *F. hepatica* in the faeces of the definitive host. Specific antibody detection was based on an Enzyme-Linked-Immunosorbent-Assay (ELISA) that used somatic *F. hepatica* antigen (FhAg), as previously described (Bossaert et al., 2000). The parasites were extensively washed in phosphate buffered saline (PBS 0.05 M), homogenised in a Ten Broeck tissue grinder at 4 °C and sonicated. After centrifugation (24,000 × *g* for 30 min at 4 °C), the supernatant was collected, referred as FhAg and stored at −20 °C until further use. Protein concentration was determined according to the Bradford protein assay. Micro-plates (Maxisorb, Greiner, Wemmel, Belgium) were coated with 100 μl per well of FhAg (10 μg/ml) diluted in PBS, overnight at 4 °C. A positive and a negative controls were included each time. Optimal antigen and conjugate concentrations were determined by checkerboard titration. After three washes in PBS-Tween 0.05% (PBST), the plates were saturated with a 10% powder skimmed milk solution in PBST (PBSTM) for 1 h at 37 °C. The plates were then washed three times in PBST and 100 fold diluted rat serum samples in PBST were added. The plates were incubated 1 h at 37 °C and after three washes in PBST, a rabbit polyclonal anti-rat conjugated to horseradish peroxidase (Dako, Heverlee, Belgium) was added at a dilution of 1/4000 in PBSTM. The plates were incubated 1 h at 37 °C. After 3 washes in PBST, the 3,3′-5,5′-tetramethyl-benzidine (TMB, Fluka, Buchs, Switzerland) substrate in citrate buffer (pH 4.2) with 1.5 ml/l of H_2O_2 (30%), was added. After 10 min, the reaction was stopped by the addition of sulphuric acid (1M). Optical densities (OD) were read at 450 nm with a microplate autoreader (Multiskan RC, Thermolab-system, Rødovre, Denmark). The determination of the cut-off was based on the average of the OD of 70 negative reference sera plus 2 S.D.

On week 12 PI all rats were euthanasied. Post-mortem examination was carried out and all flukes were collected from the bile duct and measured.

2.7. Statistical analysis

The results of experimental infections in the different snail species and the mortality rates between infected and uninfected snails were analyzed by Chi-square (0.05) test. A two-way analysis of variance with interaction for paired data was used to analyse the body weigh and the specific antibody response. A t test was used (0.05) to compare the number and the size of the flukes collected from the rats at post-mortem. All analyses were carried out using the SAS software (version 6.12, Cary, NC, USA).

3. Results

3.1. Snails

The ITS-2 based identification resulted in the identity of *G. truncatula* (identities = 97%) as *G. truncatula* (Genbank accession nos. AJ243017), *Radix* sp. 1 (identities = 98%) as *R. labiata* (Genbank accession nos. AJ319637) and *Radix* sp. 2 (identities = 97%) as *R. balthica* (Genbank accession nos. AJ319634). Results were further confirmed by the Parasite and Vector Molecular Analysis Centre of the Department of Parasitology, University of Valencia, Spain (Bargues et al., 2001; Bargues et al., 2003; Bargues and Mas-Coma, 2005).

The mortality rates in uninfected snails were 7, 4, and 38%, for *G. truncatula*, *R. labiata*, and *R. balthica*, respectively.

G. truncatula and *R. labiata* were exposed to miracidia three times while *R. balthica* was exposed twice (Tables 1 and 2). On day 15 PI, 28.3 ± 17.5% of *G. truncatula* and 26.6 ± 12.5% of *R. labiata* individuals showed intra-molluscan stages of *F. hepatica* at microscopic examination. There was no statistical difference between these two species. On day 30 PI, the mean infection rates reached 78.3 ± 10.4 and 45 ± 13.2% for *G. truncatula* and for *R. labiata* respectively. These rates were significantly different ($p < 0.05$) (Tables 1 and 2). PCR performed on snails at days 15 and 30 PI confirmed the microscopic results for

Table 1
Microscopic and PCR results on days 15 and 30 PI in *Galba truncatula* and *Radix labiata* (*Radix* sp. 1) experimentally infected with *Fasciola hepatica*

	Galba truncatula	*Radix labiata*
D15		
Squashing and dissection	+	+
Infection rates	28.3 ± 17.5%	26.6 ± 12.5%
PCR	+	+
D30		
Squashing and dissection	+	+
Infection rates	78.3 ± 10.4%	45 ± 13.2%
PCR	+	+

The infection rates are the mean of 3 infestations ± S.D. for each species.

Table 2
Microscopic and PCR results on days 15 and 30 PI in *Radix balthica* (*Radix* sp. 2) experimentally infected with *Fasciola hepatica*

Infections	*Radix balthica*	
	1	2
D15		
Squashing and dissection	−	+
Infection rates	0%	5%
PCR	+	+
D30		
Squashing and dissection	−	+
Infection rates	0%	12.5%[a]
PCR	−	+

The infection rates are the result of two infestations. The S.D. is 3.5 for D15 and 8.8 for D30
[a] Calculated on 8 individuals only.

these two species (Fig. 1). All infections in *G. truncatula* led to the shedding of cercariae (∼100 metacercariae/snail) until day 55 PI. Only one infection out of three led to the shedding of cercariae in the case of *R. labiata* (∼4 metacercariae/snail) and no shedding was observed in the case of *R. balthica*. However, 2.5 ± 3.5% (day 15 PI) and 6.25 ± 8.8% (day 30 PI) of *R. balthica* snails contained *F. hepatica* larvae. PCR confirmed the results obtained by squashing and dissection. Furthermore, specific fluke DNA was amplified on day 15 PI following the first infection, whereas no intra-molluscan larvae were observed microscopically (Table 2).

The average mortality rates were calculated for each species of snail, reaching 31, 25, and 54% for *G. truncatula*, *R. labaita*, and *R. balthica*, respectively. These mortality rates were found to be statistically different to the mortality rates in uninfected snails ($p < 0.05$).

3.2. Rats

Body weights did not change during the course of the experimental infections (data not shown). Eggs of *F. hepatica* were observed on day 42 onwards in one rat infected with metacercariae originating from *G. truncatula*. On day 70 PI, all infected animals were passing *F. hepatica* eggs. The results of copro-antigen detection are illustrated in Fig. 2. Copro-antigen were already detectable on day 35 PI.

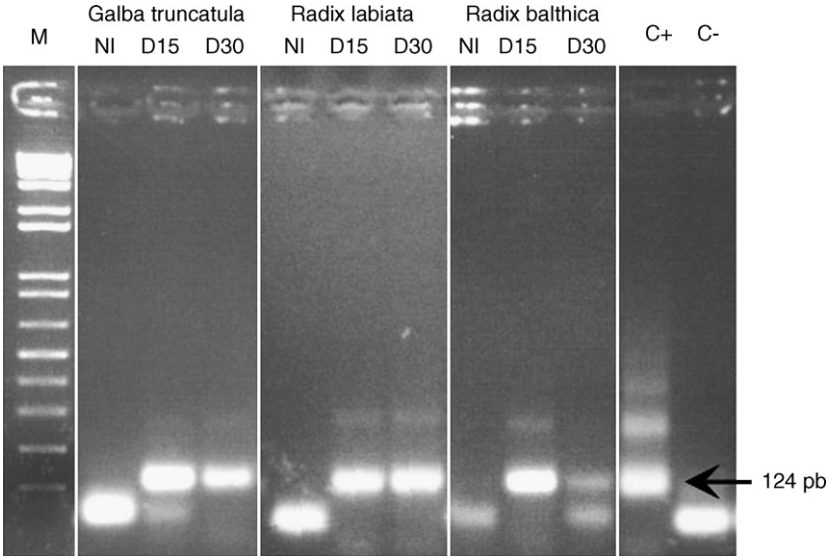

Fig. 1. Agarose gel electrophoresis following PCR performed on experimentally infected or control snails. (M) Molecular size marker DNA of 1000 bp; (NI) non-infected snails; (D15) day 15 PI; (D30) day 30 PI; (C+) positive control; (C−) negative control.

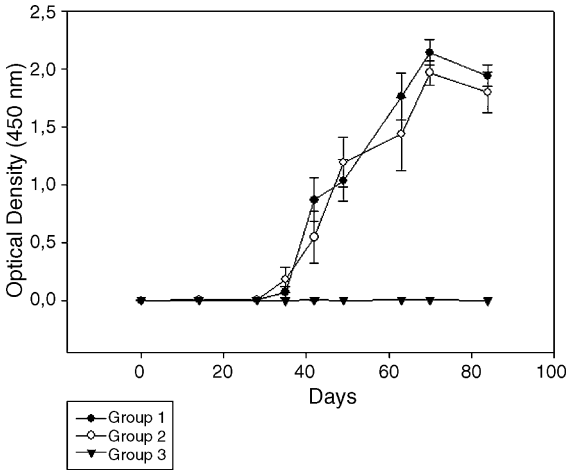

Fig. 2. Coproantigen detection in rats experimentally infected with 20 *F. hepatica* metacercariae. Group 1—rats infected with metacercariae shed by *G. truncatula*; Group 2—rats infected with metacercariae shed by *R. labiata (Radix* sp. 1*)*; Group 3—controls. Vertical lines represent standard errors.

There was no difference between the two groups of infected rats.

Serum antibodies to *F. hepatica* were detected by ELISA as little as 2 weeks after infection and titres remained elevated until the end of the experiment (data not shown). Again there were no statistically significant differences between the two infected groups.

At post-mortem examination, 21 and 23 flukes were collected from rats infected with metacercariae produced by *G. truncatula* (3.5 ± 2 /rat) and *R. labiata* (3.8 ± 2.7 /rat) respectively. The average size of the parasite was 2.2 ± 0.33 and 2.0 ± 0.33 cm in flukes originating from *G. truncatula* and *R. labiata* respectively. There were no statistical differences between the number and the size of the flukes collected from the two experimental groups.

4. Discussion

Morphological features are not always sufficient to differentiate sibling species due to inter- and intraspecific variations. This is particularly the case for the lymnaeid snails belonging to the genus *Radix* (Bargues and Mas-Coma, 2005; Pfenninger et al., 2006). Currently, their identification based on the analysis of ITS-2 DNA sequences seems to be the most reliable approach (Bargues et al., 1997, 2001, 2003; Bargues and Mas-Coma, 1997, 2005). However, one study (Anderson, 2001) pointed out that molecular data from a single genetic marker can lead to ambiguous conclusions, which emphasizes the importance of utilizing more than one marker in studies on parasite species (Carvalho et al., 2004). For instance another marker, the Molecularly defined Operational Taxonomic Units (MOTU), inferred from mitochondrial COI sequence variation, has been shown to be congruent with biological species in the genus *Radix* (Pfenninger et al., 2006).

Here, the molecular identification based on ITS-2 sequence analysis led to *R. labiata* (=*R. peregra* sensu Ehrmann, 1933) for *Radix* sp. 1 and for *R. balthica* (=*R. peregra*, O.F. Müller, 1774 = *R. ovata* Draparnaud, 1805) for *Radix* sp. 2 (Bargues et al., 2001; Mas-Coma et al., 2005). It is difficult to compare these results with those obtained in previous studies based solely on morphological criteria. However, several considerations can be made:

(1) Microscopic examination was not very sensitive in determining infection status of snails. *F. hepatica* DNA was amplified by PCR on day 15 PI in *R. balthica* although larvae were not observed. Nevertheless, squashing and dissection are easy and cheap to perform. Furthermore, PCR was not performed on each individual snail, so the different infection rates were calculated using microscopic techniques. Therefore, considering the lack of sensitivity of microscopic examination, we only used the data recorded on day 30 PI to calculate infection rates (Kaplan et al., 1997).
(2) Experimental infection of the three snail species showed significant variation in susceptibility towards *F. hepatica*. Exposed *R. labiata* snails were able to harbour the parasite and allow its multiplication, leading to the shedding of infective metacercariae. The average infection rates reached 78, 45, and 6.25%, for *G. truncatula*, *R. labiata*, and *R. balthica*, respectively. Infection rates of 67.8% have been reported for *G. truncatula* (Belfaiza et al., 2004). However, it must be pointed out that, in a single species, important variations exist in infection rates. For example, the infection rates of seven different populations of *G. truncatula* varied greatly, from 5 to 80% (Rondelaud et al., 2004). Furthermore, differences in susceptibility can also depend on the size of the snails (Dreyfuss et al., 1997, 2000). Only young *Radix* other than *R. auricularia* and its variety allow *F. hepatica* multiplication with cercariae excretion (Boray, 1969, 1978). This capacity increases with previous contacts between *G. truncatula* offspring and the parasite (Rondelaud, 1993).
(3) The PCR technique used in this study was found to be more sensitive than microscopic examination, especially in alternative host such as *R. balthica*.

According to Kaplan et al. (1995), 1 ng of *F. hepatica* DNA allows the amplification of the 124 bp probe. This sensitivity level allows the detection of one miracidium, the minimal biological unit that can be found in an infected snail. The DNA probe used in this study is not able to differentiate *F. hepatica* from *F. gigantica* but the latter species has a tropical distribution and has not been discuted in Northern Europe (Torgerson and Claxton, 1999). However, this technique is unable to assess whether full intra-molluscan development (leading to cercarial shedding) takes place or not in a potential host. In the present study specific DNA was detected in exposed *R. balthica,* but the absence of cercarial shedding indicates that the infection was abortive.

The mortality rate for uninfected *G. truncatula* (7%) was lower than the 38% reported in a previous study (Vignoles et al., 2004). Here, mortality rates for *R. labiata* and *R. balthica* reached 4 and 38%, respectively. The maintenance and breeding of *R. balthica* was particularly difficult and infectivity could not be verified due to the lack of metacercariae.

G. truncatula did not support easily the effects of aestivation (Rondelaud and Morel-Vareille, 1975; Rondelaud, 1994) and, under such conditions, other potential vectors could play an important role in parasite transmission. In Belgium where the three species are present the respective role of each of them remained to be determined although *G. truncatula* is the main intermediate host. However in other European countries where *G. truncatula* is rare or absent, other species belonging to the genus *Radix* could play a major role in the epidemiology of fasciolosis as already suggest (Boray, 1969; Caron, 2004).

Both *G. truncatula* and a population of *R. labiata* were able to produce *F. hepatica* metacercariae under experimental conditions, although at very different levels. In most previous studies on the vectorial capacity of different snail species, the viability and infectivity of metacercariae have not been considered. In the present work, experimental infection of rats with metacercariae produced by either *G. truncatula* or *R. labiata* was performed and no differences were observed between the two groups of rats. The antibody response as measured by ELISA was in agreement with a previous report of experimental infection in rats (Mulcahy et al., 1999). Copro-antigen detection was found to be particularly useful and reliable: faecal samples are easy to obtain and a positive signal is associated with the presence of viable flukes in the bile ducts (Mezo et al., 2004). Furthermore, copro-antigens can be detected before patency.

To summarize the results of the present study, *R. labiata* harboured the intra-molluscan development and allowed subsequent shedding of *F. hepatica* cercariae, although in fairly low numbers. These metacercariae were as infective in a rat as those produced in *G. truncatula* as judged by parasitological and serological data. Therefore, this population of *R. labiata* can be considered as an incidental intermediate host for *F. hepatica* in Belgium and elsewhere. This is probably not the case, however, for this population of *R. balthica*. These laboratory results should be confirmed in the field in naturally-infected potential vectors.

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