



## Epidemiological studies on some trematode parasites of ruminants in the snail intermediate hosts in three districts of Uttar Pradesh, Jabalpur and Ranchi

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### ABSTRACT

Seasonal prevalence of 5 trematode parasites in the 4 snail species, viz. *Lymnaea auricularia*, *L. luteola*, *Gyraulus convexiusculus* and *Indoplanorbis exustus* for the years 2012–2014 was studied in 3 districts of Uttar Pradesh and in Jabalpur and Ranchi districts of Madhya Pradesh and Jharkhand, respectively. Intramolluscan larval stages of *Fasciola gigantica*, *Explanatum explanatum*, *Paramphistomum epiclitum*, *Fischoederius elongatus* and *Schistosoma spindale* were identified using ITS-2, 28S rDNA, 12S mitochondrial (mt) DNA and Cox I markers. *F. gigantica* infection in *L. auricularia* had a significant ( $P < 0.05$ ) occurrence in the winter season followed by rains. Seasonality of *P. epiclitum* transmission in *I. exustus* was observed with significant occurrence of its infection in the rainy season followed by a sharp decline in other seasons. Prevalence of *S. spindale* infection in *I. exustus* was insignificant in 3 districts of Uttar Pradesh but highly prevalent in other 2 districts. Infection with *F. elongatus* in *L. luteola* was recorded in different seasons. *G. convexiusculus* were screened for *E. explanatum* and *Gastrothylax crumenifer* infection and a significant rate of infection with *E. explanatum* was observed in the rainy season. Climatic factors including temperature and rainfall influence the distribution of snail populations and transmission of trematode infections by these snail intermediate hosts.

**Key words:** Parasite, PCR, Prevalence, Season, Snail, Trematode

The most important trematode parasites affecting livestock in India are *Fasciola gigantica* and several species of amphistomes and schistosomes. Fasciolosis caused by *F. hepatica* and *F. gigantica* is an economically important helminthic disease of livestock (Spithill *et al.* 1999). The disease is recognized as an emerging food-borne zoonosis, with an estimated 17 million human beings infected and 180 million people are at risk of such infection worldwide (Mas-Coma 2005, Parkinson *et al.* 2007). In India, fasciolosis in domestic ruminants is caused by *F. gigantica*. Amphistomes are parasitic in the digestive tracts of many vertebrates including mammals causing a serious pathogenic condition ‘immature amphistomosis’ in domestic ruminants (Phiri *et al.* 2007). More than 70 species of amphistomes were reported from animals of food value from different parts of the globe (Roy and Tandon 1995). In India, several species of gastrothylacid amphistomes, viz. *Fischoederius cobboldi*, *F. elongatus*, *Gastrothylax crumenifer*, *Carmyerius spatiosus* and *Velasquezotrema tripurensis* along with 2 common amphistomes *Paramphistomum epiclitum* and *Explanatum explanatum* were recorded in

domestic ruminants (Prasad *et al.* 1994, Ghatani *et al.* 2012). *Schistosoma* are among the most abundant infectious agents of mankind; infecting about 200 million people and causing a chronic debilitating disease ‘schistosomosis’ (Chitsulo *et al.* 2000). Additionally, animals including cattle bear a heavy burden of infection with different species of *Schistosoma*. India has several endemic regions of schistosomosis with prevalence of *Schistosoma indicum*, *S. spindale*, *S. incognitum* (Chandler 1926), *S. nasale* (Rao 1933), *S. nairi* (Mudaliar and Ramanujachari 1945), *Orientobilharzia dattai* and *O. turkestanicum* (Dutt and Srivastava 1955, 1964) reported from different parts of the country.

Snails play an important role in the transmission of these parasites and monitoring the infection rate of snails with these trematodes is an important component of the epidemiological study on these parasites. In India, lymnaeid snails mainly *Lymnaea auricularia* and *L. luteola* act as intermediate hosts of *F. gigantica*, *S. incognitum*, *F. elongatus* and *O. dattai*. Other snail species like *I. exustus* is an intermediate host of *S. indicum*, *S. spindale*, *S. nasale* and *P. epiclitum*, whereas *G. convexiusculus* plays an important role in the transmission of *E. explanatum* and *Gastrothylax crumenifer*. Molecular approaches are the most effective and accurate means for identification of these parasites in the snail intermediate hosts. The systematic use

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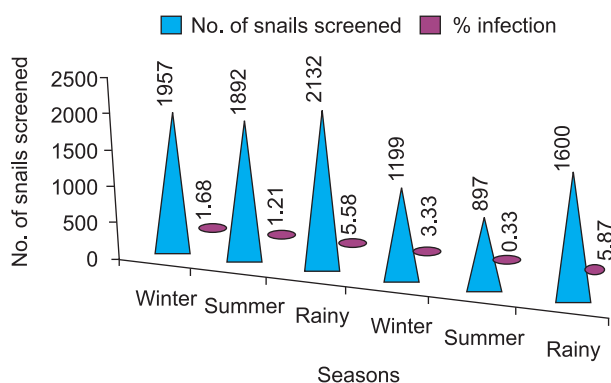


Fig. 1. Season-wise prevalence of different snail species positive for mammalian trematode infections in 3 districts of U.P during the period 2012–2014.

of the molecular approaches as a complement to the conventional approaches has enabled the identification of species and screening of genetic variants among populations (Velusamy *et al.* 2004, Cucher *et al.* 2006, Caron *et al.* 2007, Magalhaes *et al.* 2008). In India, studies on the epidemiology, dynamics of the snail transmission of these pathogens and forecasting systems for outbreaks of the snail-borne trematode diseases have been scanty. The present study was carried out to identify the intramolluscan stages of the commonly prevalent trematodes of domestic ruminants in India to species level using molecular approaches and to study their seasonal prevalence in various snail intermediate hosts in 3 districts of Uttar Pradesh and in Ranchi (Jharkhand) and Jabalpur (M.P) districts. Role of climatic factors like rainfall and seasons on the distribution of snail vectors and on their transmission of various trematode parasites was also studied.

## MATERIALS AND METHODS

### Collection of snails from different water bodies:

Snails belonging to 4 genera, viz. *L. auricularia*, *L. luteola*, *I. exustus* and *G. convexiusculus* were collected from different waterbodies of 3 districts of Uttar Pradesh viz. Meerut, Bareilly and Jhansi and Jabalpur (Madhya Pradesh) and Ranchi (Jharkhand) districts, with an average high temperature in the range of 21.6°–39.9°C and an average low temperature from 8.3°C–27.0°C. These snails were screened for prevalence of the mammalian trematodes for 2 years from 2012–2014 covering different seasons of the year. Snails were collected manually from a given area every month for 2-year period to study the prevalence of different trematode infections in them. Meteorological data on the average rainfall, relative humidity and temperature for 2012–2014 were collected from Meteorological Department, New Delhi, India. The results obtained on the seasonal prevalence of trematode infections in different snails were compared by Chi-square test (Snedecor and Cochran 2004).

### Molecular identification of the intramolluscan stages of trematodes transmitted by the above snails

**Isolation of genomic DNA of snail intermediate hosts:** Genomic DNA was isolated from *L. auricularia*, *L. luteola*, *I. exustus* and *G. convexiusculus* using commercial DNA isolation kit and following protocols provided by the manufacturer. Briefly, live snails of different sizes collected randomly from the water bodies were killed by gentle crushing in sterile petridishes. Shells were manually removed from these snails and whole snail tissue was processed for isolation of the genomic DNA with proteinase

Table 1. List of primers used in PCR amplification of different trematode parasites

Primer name	Sequence 5'–3'	Length (mer)	Amplicon size
<i>Schistosoma</i> (28S)	Forward TGAATTTAAGCATATCACTAAG Reverse GAAAGTGCAGTACAAGCAGAC	2222	600 bp
<i>Schistosoma Spindale</i> (12S)	Forward GTTTGGCGGTAAATTAGATTCG Reverse CTTAAGAGAATTTTCATCTACTT	2222	227 bp
<i>Schistosoma indicum</i> (12S)	Forward GTTTGGCGGTAAATTAGATTCG Reverse ATTCATAAGTGAATTTTCATCCC	2222	227 bp
<i>Explanatum explanatum</i> (ITS-2)	Forward TGTGTCGATGAAGAGCGCAG Reverse TGGTTAGTTTCTTTTCCTCCGC	20 22	492 bp
<i>Explanatum explanatum</i> (28S)	Forward AAAGCCCAGCACCGAAGCCTG Reverse CTCCTTGGTCCGTGTTTCAAG	2120	800 bp
<i>Paramphistomum epiclitum</i> (ITS-2)	Forward CTTATAAACTATCACGACGCCA Reverse TAGAACACCACAGTAGGGTATCGTC	2125	300 bp
<i>Paramphistomum epiclitum</i> (28S)	Forward AGGCACTTGTCAAGTGTGCG Reverse AGAACCGAGCTATTACCAGGC	2121	350 bp
<i>Gastrothylax crumenifer</i> (ITS-2)	Forward AGGCACTTGTCAAGTGTGCG Reverse TAACTGAACCCACGGTGGGT	2125	290 bp
<i>Fasciola gigantica</i> (ITS-2)	Forward ATAAACTATCACGACGCCA Reverse GGAAGACAGACCACGAAGG	2019	360 bp
<i>Fasciola gigantica</i> (28S)	Forward ACGTGATTACCCGCTGAAGT Reverse CTGAGAAAGGTGCACTGGCAAG	2022	618 bp
<i>Fischoederius elongatus</i> Cox-I	Forward -CTATACCAGTTACACCACCCA Reverse TTTTTTGGGCATCCTGAGGTT	2121	364 bp

K @ 20 µg / mg tissue digestion at 37°C over night. Subsequent steps of DNA isolation were carried out as per the manufacturer's instructions.

**PCR amplification of ITS-2 and 28S ribosomal DNA of *F. gigantica*:** Ribosomal (r) DNA sequences viz internal transcribed spacer-2 (ITS-2) and 28S rDNA were used as molecular markers for identification of *F. gigantica* in the snail tissue. Genomic DNA isolated from *L. auricularia* was subjected to ITS-2 and 28S rDNA amplification by PCR. ITS-2 (360 bp) and 28S rDNA gene (618 bp N-terminal partial sequences) were amplified using *Fasciola* specific primers (Table 1). Specific amplicons were sequenced and data were analyzed for species identification.

**PCR amplification of *P. epiclitum*, *E. explanatum* and *F. elongatus* ITS-2, 28S rDNA and Cox I markers in snail vectors:** Genomic DNA isolated from *I. exustus* was PCR amplified for *P. epiclitum* ITS-2 (290 bp) with species specific primers (Table 1). Likewise, genomic DNA isolated from *G. convexiusculus* was amplified for 442 bp fragment of ITS-2 of *E. explanatum* and *L. luteola* genomic DNA amplified for detection of mitochondrial Cox I DNA of *F. elongatus* using primers specific to *E. explanatum* and *F. elongatus*, respectively (Table 1). The sequences of the above three markers amplified by PCR were analyzed for identification of the respective species.

**Identification of mammalian schistosomes in *I. exustus*:** Genomic DNA was isolated from *I. exustus* and PCR amplification of mitochondrial 12S region of *Schistosoma* spp. with genus specific forward primer and reverse primers specific to *S. spindale* and *S. indicum*, respectively (Table 1) was carried out. The PCR amplicons were sequenced and the parasite species identified.

## RESULTS AND DISCUSSION

PCR amplification of different markers used in this study and their sequence results confirmed the intramolluscan stages as *F. gigantica*, *P. epiclitum*, *F. elongatus*, *E. explanatum* and *S. spindale*, respectively and indicated that these are the common trematodes prevalent in different aquatic snails in the study area.

### Identification and prevalence of *F. gigantica* infection in *L. auricularia*

Genomic DNA was isolated from randomly selected 173 *L. auricularia*. These snails were screened for *F. gigantica* infection by PCR amplification of ITS-2 and 28S rDNA regions. Sequence analysis of 360 bp ITS-2 region of 25 DNA samples confirmed the parasite larval stages as *F. gigantica* and it differed from *F. hepatica* ITS-2 at typical 6 nucleotide positions (207, 231, 270, 276, 327 and 334) and with one distinguishing deletion of T nucleotide at 327<sup>th</sup> position in *F. gigantica*. The 28S rDNA was also PCR amplified from the genomic DNA isolated from 28 randomly selected *L. auricularia* and sequence analysis of 618 bp of 12 samples confirmed the parasites as *F. gigantica*. None of the sequences matched to *F. hepatica* ITS-2 and 28S rDNA sequences. Therefore, using these two molecular

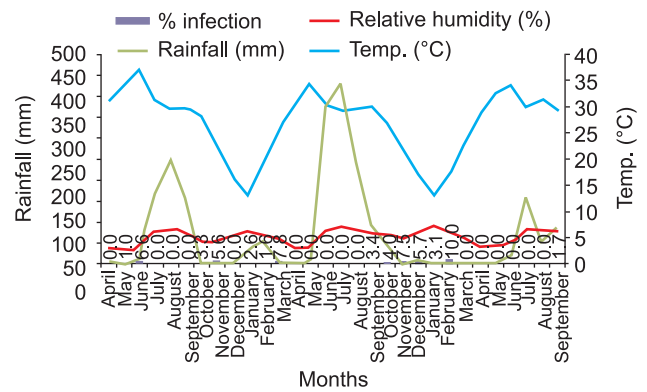


Fig. 2. Bio-climatograph of *L. auricularia* positive for *F. gigantica* infection (April 2012– September 2014).

markers it was confirmed that *F. gigantica* was the species prevalent in these snails. The overall prevalence of *F. gigantica* was studied in 3928 snails by observing the cercarial shedding from these snails for the period from 2012–2014. The snails were screened in 3 seasons of each year, viz. summer (March-June), rainy (July-Oct) and winter (Nov-Feb). *F. gigantica* infection in *L. auricularia* had a significant ( $P < 0.05$ ) occurrence in winter for both the years with 2.9% and 3.7% snails, respectively infected with the parasite, followed by the rainy season with prevalence of infection of 1.9% and 0.9%, respectively. There was a fluctuation of infection in summer between these 2 years, as a prevalence of 1.9% and 0.0% was recorded in 2012 and 2014, respectively (Fig. 2). Overall results indicated that *F. gigantica* infection in *L. auricularia* was prevalent throughout the year (Fig. 2). There was a significant ( $P < 0.05$ ) correlation of the seasons with the density of the snail population in the water bodies, as in hot summers a heavy mortality of the snails occurred while snail density/square meter was higher after rains.

### Identification and prevalence of *E. explanatum* infection in *G. convexiusculus* snails using ITS-2 and 28S rDNA markers

Genomic DNA was isolated from 173 *G. convexiusculus* for their screening for infection with *E. explanatum* by PCR using species specific ITS-2 and 28S rDNA primers (Table 1). PCR amplified 492 bp ITS-2 product and 800 bp region of 28S rDNA of *E. explanatum* in 21 snails. Sequence analysis of these PCR amplicons showed no variation in the ITS-2 and 28S rDNA vis-a-vis other isolates of *E. explanatum* described from several definitive hosts, thus confirming the species status of the parasite transmitted by these snails. Likewise, genomic DNA isolated from the above snails was screened for infection with *G. crumenifer* by PCR amplification of ITS-2 and 28S rDNA markers using species specific primers (Table 1). The PCR did not amplify the above marker sequences for *G. crumenifer*, indicating that none of these snails were harbouring the infective stages of *G. crumenifer*.

*G. convexiusculus* (n=1440) were also screened for

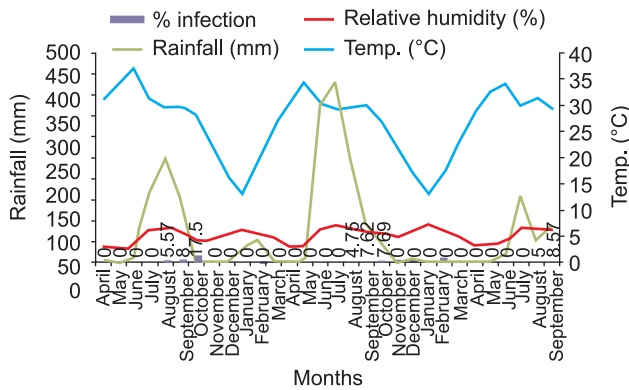


Fig. 3. Bio-climatograph of *I. exustus* positive for *P. epiclitum* infection (April 2012– Sep 2014).

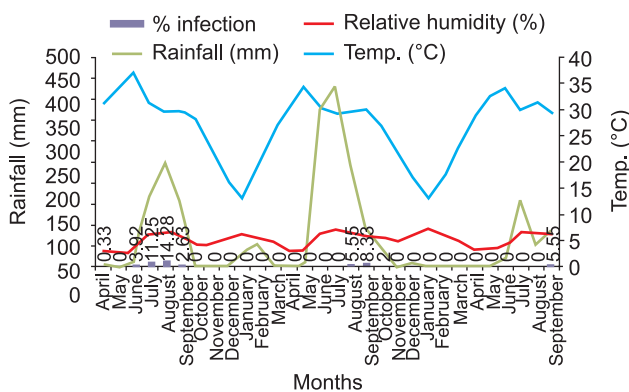


Fig. 4. Bio-climatograph of *G. convexiusculus* positive for *E. explanatum* infection (April 2012– Sep 2014).

cercarial shedding in different seasons in two years and an overall prevalence of 2.1% of *E. explanatum* was recorded, with each snail shedding very low number of cercariae. Rainy season recorded a significant ( $P < 0.05$ ) rate of infection with 6.5% and 4.0% prevalence for 2012–2013 and 2013–2014, respectively. No significant ( $P > 0.05$ ) rate of infection was observed in these snails in summer and winter seasons (Fig. 4). *E. explanatum* is a very common trematode infection of buffaloes and cattle in India (Prasad and Varma 1999, Hassan *et al.* 2005) and the parasite is transmitted by *G. convexiusculus*. The overall prevalence of infection in these snails was higher during rainy season and lowest in other seasons.

#### Identification and prevalence of *P. epiclitum* infection in *I. exustus* snails using ITS-2 and 28S rDNA markers

*I. exustus* ( $n=100$ ) were screened for infection with *P. epiclitum*. PCR was carried out on 100 snails for amplification of 290 bp ITS-2 and 28S rDNA (350 bp) of *P. epiclitum* with species specific primers (Table 1). Sequence analysis of 10 PCR products for these 2 markers showed a 100% match with *P. epiclitum* isolates reported from the definitive hosts.

*P. epiclitum* prevalence was significantly ( $P < 0.05$ ) affected with a change in temperature and rainfall. Higher

prevalence of *I. exustus* snails was recorded from July–Oct (rainy season), with a fall in their population in April, May and June. *I. exustus* is the intermediate host of *P. epiclitum* and *Schistosoma* spp. in India (Prasad *et al.* 1994, Agrawal *et al.* 2000). The overall prevalence of *P. epiclitum* in *I. exustus* for these 2 years was 6.9% (225/3249). Seasons had a direct effect on the infectivity of these snails, with cercarial shedding of *P. epiclitum* being higher in the months of July–Oct (rainy season) (Fig.3). This seasonality of the cercarial shedding is of great epizootiological significance and was reported earlier by Dutt and Bali (1980), Kohli and Agrawal (1995), Mishra and Agrawal (1998), Agrawal *et al.* (2000), Bedarkar *et al.* (2000), Khajuria and Kapoor (2003) and Mishra *et al.* (2012) for *Schistosoma* infection in *I. exustus* from different parts of India and from Bangladesh (Islam *et al.* 2011). Snail population density was dependent on combination of factors, of which seasonal rainfall had the highest influence. A lower prevalence in summer could be attributed to harsh dry conditions and lesser chances of infection due to non-availability of snail intermediate hosts as the water sources are scarce in this season.

#### Identification and prevalence of *F. elongatus* with Cox I marker

Genomic DNA from *L. luteola* ( $n=100$ ) was isolated for their random screening for *F. elongatus* infection and mitochondrial Cox I gene (364 bp) was PCR amplified using primers specific to *F. elongatus* (Table 1). Sequence analysis of 20 PCR products from as many number of snails confirmed the amphistome species as *F. elongatus* and the snail isolate showed 96% nucleotide identity with Cox I gene of the other isolates of this parasite of domestic ruminants. *L. luteola* ( $n=2361$ ) were also screened for infection with *F. elongatus* by cercarial shedding and only 17 (0.7%) were detected positive. These infected snails were shedding cercariae in all the 3 seasons with no significant ( $P > 0.05$ ) difference in rainy season, winter and summer (Fig.5). Lower infection rate in snails correlates with a low prevalence of *F. elongatus* infection in the definitive hosts in India (Ghatani *et al.* 2014).

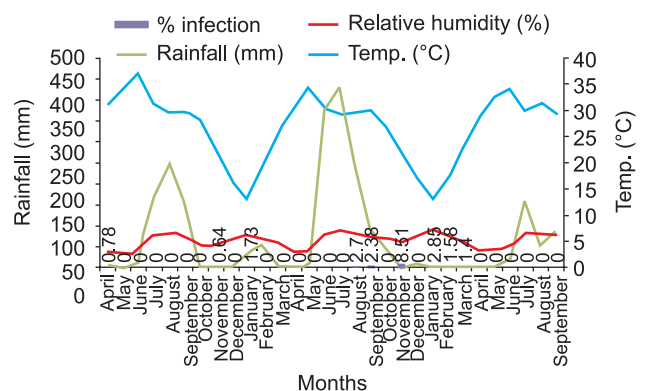


Fig. 5. Bio-climatograph of *L. luteola* positive for *F. elongatus* infection (April 2012–Sep 2014).

*Identification and prevalence of S. spindale in I. exustus*

*I. exustus* snails (3,249) were screened for *Schistosoma* infection in different seasons. None of these snails screened in the districts of Bareilly, Jhansi and Meerut were positive for *Schistosoma* infection. *I. exustus* (n=600) were screened for cercarial shedding for different *Schistosoma* spp. in Ranchi (Jharkhand) and Jabalpur (MP) districts but only 10 (1.7%) snails were positive for *S. spindale* infection. *I. exustus* has been reported as the intermediate host of *S. spindale*, *S. indicum* and *S. nasale* in India (Dutt and Srivastava 1968, Agrawal *et al.* 1991) but the snails positive for *Schistosoma* infection were harbouring *S. spindale* only and not *S. indicum* and *S. nasale* in the present study. The furcocercus cercariae shed by these snails were morphologically typical of mammalian schistosomes *i.e.* longicaudum and brevifurcus and were thus identified as *S. spindale*. Further confirmation of these *S. spindale* cercariae was done by amplification of 227 bp 12S mt DNA and 600 bp 28S rDNA. *I. exustus* (122) were also screened for infection with the prepatent stages of different species of mammalian schistosomes by PCR amplification of 12S mt DNA of *Schistosoma* spp. with genus specific forward primer and reverse primers specific to *S. spindale* and *S. indicum*, respectively. None of the snails screened with primers specific for *S. indicum* amplified a product and were negative for this infection.

Trematodes show distinct and direct relationship with the water temperature in their transmission process, with cercarial shedding being directly influenced by the water temperature (Poulin, 2005). Seasonality that is mirrored by changes in environmental variables can intervene in snail's ecology and influence the larval development of a trematode inside its host snail. Therefore, there is a significant correlation between infection in snails, climatic factors and snail population. The present preliminary study also indicated that climatic factors including rainfall and temperature influenced the snail populations in different seasons and also affected their rate of infection with the above trematodes (Fig.1).

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