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Parasitology International

Fasciola hepatica infection in children actively detected in a survey in rural areas of Mardan district, Khyber Pakhtunkhawa province, northern Pakistan

Asma W. Qureshi[a,*], Aurang Zeb[b], Abu Mansoor[a], Azam Hayat[b], Santiago Mas-Coma[c,*]

[a] *Department of Zoology, GC Women University, Sialkot, Pakistan*
[b] *Department of Zoology, University of Hazara, Mansehra, Pakistan*
[c] *Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain*

ARTICLE INFO

ABSTRACT

Human fascioliasis is a freshwater snail borne, zoonotic disease caused by *Fasciola* liver flukes which are widely spread throughout Pakistan and has recently proved to be endemic in humans of the Punjab province. To verify whether fasciolids are also affecting humans outside this province, studies were conducted in four communities comprising rural and urban areas of Mardan district, Khyber Pakhtunkha province, northern Pakistan. Activities comprised two surveys, a coprological one to look for *Fasciola* infection and another to get information on potential human infection sources and risk factors by means of a questionnaire. Out of 540 subjects of all ages surveyed, only 4 children (0.74%) were found positive for *Fasciola* eggs: two 9- and 13-year old boys and two 7- and 16-year old girls. Burden per child ranged 73–146 eggs per gram of faeces (epg), with an average of 106 ± 15.4 epg. Based on the egg size, the infection was diagnosed to be caused by *F. hepatica*. Serum analyses showed eosinophilia and elevated enzyme values in liver function tests. The Mardan area fits well to all transmission and epidemiological requirements of *F. hepatica*, namely the presence of infected livestock reservoirs throughout, the appropriate climatic factors (mainly monthly temperature variation along the year), and the existence of adequate freshwater collections. The existence of the specific lymnaeid snail vector species for *F. hepatica* transmission was reported in the neighbouring Swat valley time ago. Results show that human fascioliasis may be widespread in Pakistan and that both *F. hepatica* and *F. gigantica* may be involved.

1. Introduction

Until 1990, human infection by *Fasciola* was considered of only secondary importance [1]. In the following decade, human fascioliasis was not only given importance in developed countries [2], but it began to show an increase of human infection reports and subsequently a progressive description of many human endemic in Europe, the Americas, Africa and Asia [3,4]. This is a waterborne parasitic disease caused by the two liver fluke species *F. hepatica* and *F. gigantica* transmitted by freshwater snail vectors of the family Lymnaeidae [5]. The World Health Organization includes fascioliasis in the list of the Neglected Tropical Diseases (NTDs) among the group of food-borne trematodiases [6].

The life cycle of these fasciolids is very susceptible to the environmental characteristics [7,8]. This explains why fascioliasis may be influenced by both climate and global changes, similarly as other trematodiases are recently also evincing [9,10]. Regarding climate change, fascioliasis reunites the most important characteristics to be a disease with high response capacity to environmental alterations, such as being zoonotic with low reservoir specificity [11,12] and a high number of lymnaeid snail species with transmission capacity [13]. Concerning global change, fascioliasis has shown to be influenced by both human and animal movements as well as by anthropogenic modifications of the external environment [14].

The high impact of this disease has been emphasized by studies on pathogenicity and immune suppression, which demonstrated the clinical problematics not only during the acute, migratory or invasive phase, as previously believed, but also during the long chronic, biliary or obstructive phase [15,16] in which almost all of the inhabitants of human endemic areas are diagnosed [4]. The public health importance of this disease highlights when considering extreme pathogenicity situations even including fatal cases [4] and the global estimations of around 17 million people infected worldwide. Anyway, this global picture is considered to be an underestimation owing to the wide regions of Asia and Africa where studies on human infection by *Fasciola* are lacking [3].

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* Corresponding authors.
E-mail addresses: asmawqureshi@yahoo.com (A.W. Qureshi), S.Mas.Coma@uv.es (S. Mas-Coma).

The south central region of Asia is a wide zone where knowledge on human fascioliasis is very scarce and even completely lacking in several regions and countries. No special importance was given to this wide zone until the detection of the large public health problem originated at the beginning of the XXI century in Vietnam [17,18], which moreover appears to spread to Laos [19] and southern China [20]. When considering the human fascioliasis situation in the Near East [21,22], the in between south central region of Asia poses a question mark. Human infection by *Fasciola* has already been reported from Bangladesh [23] and more recently highlighted from India [24] and also Nepal [25].

A worrying scenario has been moreover suggested by the recent discovery of a human endemic situation after a large survey of 7200 subjects in Pakistan, with an overall prevalence of 1.18% and a range between 0.67% and 1.75% according to localities, in the highly populated province of the Punjab, inhabited by almost 94 million people [26]. Such a situation in the Pakistani Punjab province increases the concern when projecting the conclusions of another study in which human fascioliasis was statistically demonstrated to have been influenced by climate change and the disease transmission to be modified from monoseasonal to biseasonal due to a global change impact [27].

The present study deals on an intervention to assess whether human fascioliasis poses public health problems in other provinces of Pakistan. Activities comprised two surveys, a coprological one to look for *Fasciola* infection and another to get information on infection risk by means of a questionnaire. The surveys were carried out in four localities covering urban and rural side areas of the Mardan district, in the province of Khyber Pakhtunkhawa, in northern Pakistan. Subjects found infected are clinically characterized, specifically diagnosed and epidemiologically discussed.

2. Materials and methods

2.1. Study area

The study was conducted in four communities of the district of Mardan, located in the province of Khyber Pakhtunkhwa, in the northern part of Pakistan. These communities are located in the same south western fertile plain near to the eastern hilly area and distributed northward of the district headquarters city of Mardan: Takht Bhai (34°16′45″ N, 71°56′10″ E, 353 m), Katlang (34°21′27″ N, 72°04′22″ E, 390 m), Babuzai (34°24′59″ N, 72°09′23″ E, 482 m), and Rustam (34°19′59″ N, 72°17′00″ E, 391 m) (Fig. 1A-C). The term "urban" is used for Takht Bhai because this village is a little bit larger than the others and houses of subjects surveyed are arranged around streets, whereas the term "rural" is applied to smaller villages where dwellings are in communities dispersed in the field.

The district of Mardan is located about 118 km westward from Islamabad, capital city of Pakistan, in the way to the frontier of Afghanistan and its capital Kabul. It is situated in a zone between two nearby divisions of Peshawar and Malakand. The total area of Mardan district is 1632 km^2 and its population is 3,000,545 inhabitants, mainly concentrated in Mardan city.

The average temperature is 34 °C, with an extremely hot summer season reaching up to 41.5 °C and a very cold winter with temperatures decreasing up to 2.09 °C (Fig. 2A).

2.2. Coprological survey

Faecal individual stool samples were collected randomly from a total of 540 subjects. The total study was designed in a way to assure that the sample size would be representative of the urban and rural areas of the zone selected. Both males and females as well as subjects of different ages from children to adults entered in the surveys. The nature

of the study was explained to the participants, who then were asked to try to pass a small volume of their own faeces into a clean numerated container. Despite the rarity of liver consumption in the study area, participants in the survey were asked at the beginning whether they had consumed animal liver in the previous days of the same week and/ or previous week. The only three subjects who answered positively were excluded from the study to avoid possible false positives due to the detection of eggs in transit. Anyway, no subject was found to shed eggs showing the typical deterioration after the transit through the human digestive system, as previously described [4].

One stool sample per subject was collected from all participating individuals and personal data (name, sex, and age) as well as date and locality of collection were noted on delivery of the container. Stool samples from a total of 60 subjects were obtained each month during the 9-month period from September to May.

After collection, samples were transported to the laboratory and whenever possible immediately examined by microscopic examination. Two different diagnostic techniques were applied to each stool sample. The Kato-Katz cellophane faecal thick-smear technique was made following WHO recommendations, using a template applied to a microscopic slide allowing for the analysis of only 1/24 g (= 41.7 mg) of faeces per slide [28]. Slides were initially examined within one hour of preparation to avoid over-clarification of some helminth eggs. The Kato-Katz slides were analyzed for the egg counts. Intensity of infection, measured as eggs per gram of faeces (epg) was used as an indicator of *F. hepatica* burden in the infected subjects to assess whether special precautions should be taken in the treatment of heavily infected children, according to WHO instructions [29,30].

A part of the remaining amount of faecal sample, which was considered sufficient for a significant analysis, was mixed with saline solution (0.9%) in a Petri dish. Coprological diagnosis was afterwards made by the direct wet-mount method, by placing the samples onto a glass slide with a coverslip. This simple direct smear method allows for detection and classification of helminth eggs without the problem of clarification posed by the Kato-Katz technique [4]. Faecal samples were finally kept in 10% formaline for verification purposes.

Helminth egg measurements were obtained with a calibrated microscope applied to the wet-mount slides. Egg perimeter was calculated by applying the formula $2 \times \pi \times \sqrt{(a2 + b2)/2}$ [31]. Burden was given in number of epg.

2.3. Questionnaire survey

In order to assess *Fasciola* infection risk and the potential liver fluke metacercarial sources for human infection, an appropriate questionnaire was developed including information on key aspects such as age, gender, symptoms, hygienic conditions, eating habits, drinking water sources, education, occupation, nearness to livestock, and awareness of fascioliasis. Question design was made following previous similar studies [32,33], but appropriately modified to adapt to the characteristics of the inhabitants and the environment of the Mardan area. Questionnaires were filled out on the base of personal interview.

2.4. Clinical assessment

Symptoms were noted individually for each patient, by focusing on those characterizing *Fasciola* infection [4], mainly fever, abdominal pain, epigastric pain, diarrhea, jaundice, and vomiting. Patient's routine peripheral blood examination furnished information on eosinophil counts and haemoglobin contents in the haemogram. The liver function enzymes are well known serum biomarkers for human fascioliasis [34]. Three liver damage biomarkers were analyzed: alanine aminotransferase (ALT or GPT, in international units per litre IU/L), aspartate
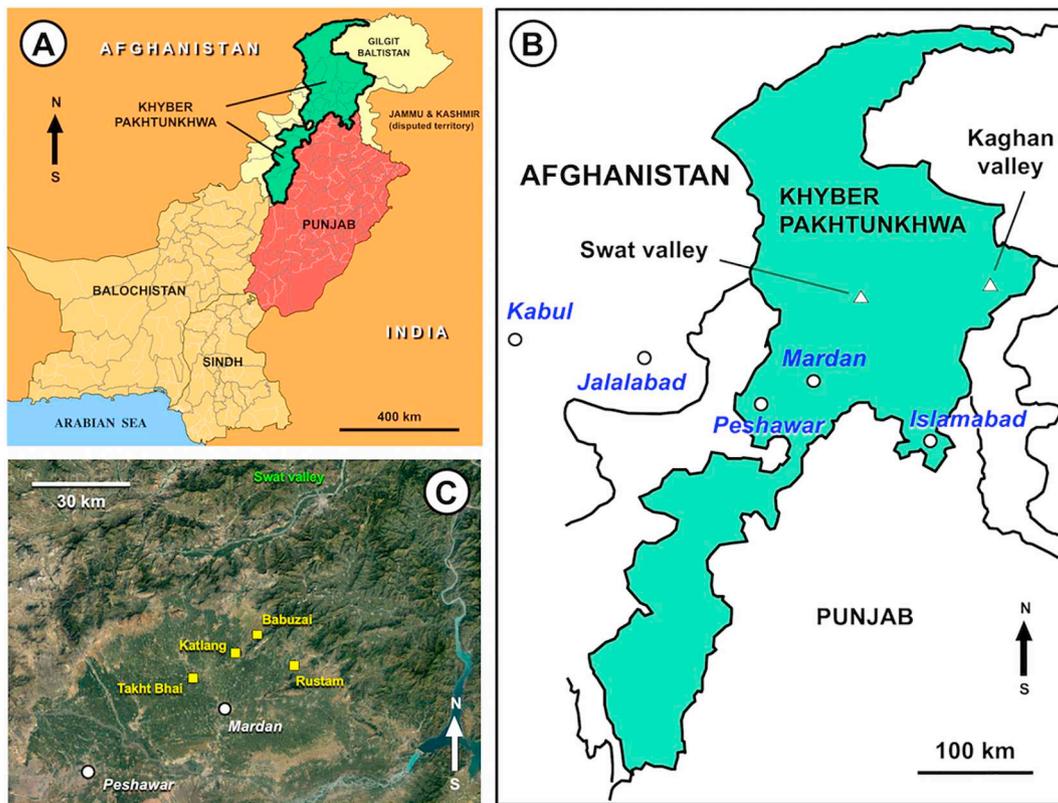
Fig. 1. A) Map showing the geographical location of the Khyber Pakhtunkhwa province in the northern part of Pakistan. B) Map of Khyber Pakhtunkhwa province showing the location of Mardan city in between Islamabad, capital city of Pakistan, and Peshawar, in the way to the frontier of Afghanistan, the city of Jalalabad and its capital Kabul. C) Map showing the location of the four communities surveyed of Takht Bhai, Katlang, Babuzai, and Rustam in the Mardan plain.

aminotransferase (AST or GOT, IU/L), and alkaline phosphatase (AP, IU/L). Values of these enzymes were analyzed before and after treatments by means of commercial kits (Randox Laboratories Ltd.).

2.5. Ethical procedures

The study was carried out under supervision and following the ethical rules approved by the Board of Studies (BoS) of Zoology and the Advanced Studies and Research Board (AS&RB) of the University of Hazara, Mansehra. The surveys were carried out after informed consent was obtained from the subjects to be surveyed, as well as from children's parents, the director and teachers of each school when dealing with children, following the principles expressed in the Declaration of Helsinki.

2.6. Statistical analyses

Statistical analyses were done using the computer software SPSS 10.0 (SPSS Institute, Chicago, IL) for Windows. A P value < .05 was considered significant when applying the Chi-Square (χ^2) test.
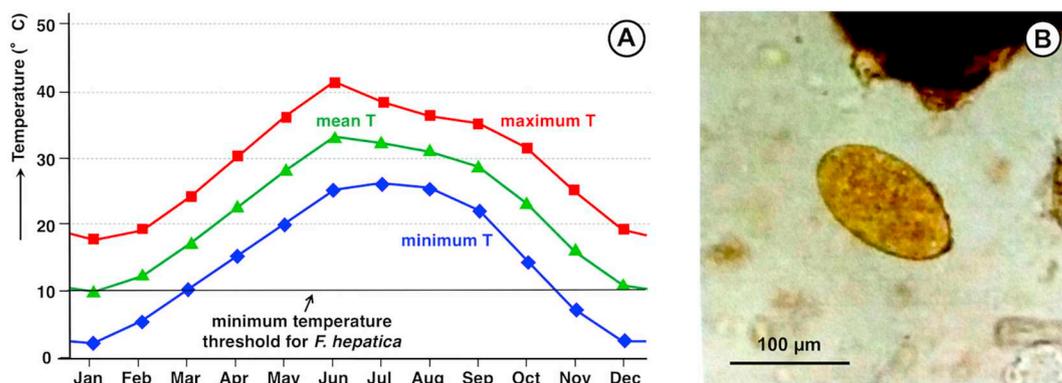

Fig. 2. A) Yearly evolution of mean monthly values of temperature in the Mardan plain, province of Khyber Pakhtunkhwa, northern Pakistan (data in ˚C): red squares and line = mean maximum temperature (MMT); green triangles and line = mean environmental temperature (MET); blue rhombuses and line = mean minimum temperature (MmT). B) Egg of *Fasciola hepatica* found in the children from the Mardan plain, province of Khyber Pakhtunkhwa, northern Pakistan; note typical roughened or irregular, more intense brownish dark protuberance at the abopercular end of the shell surface, at right. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Shedding and characteristics of *Fasciola* egg and nematode coinfections in the children found infected in the Mardan district, Pakistan.

Patients				
Gender	Female	Male	Male	Female
Age (years)	7 y	9 y	13 y	16 y
Locality	Katlang	Katlang	Takht Bhai	Rustam
Urban or rural	rural	rural	urban	rural
Month of detection	September	September	January	May
Fasciola egg measurements				
Burden (epg)	73	146	94	109
EL (µm)[a]	121.2	132.7	118.4	135.5
EW (µm)[a]	69.3	72.1	67.5	76.4
Egg perimeter[a]	310.0	335.32	302.6	345.38
Coinfections				
Ascaris lumbricoides	X	X	X	X
Ancylostoma duodenale	X	X	–	–

epg = No. of eggs per gram of faeces; EL = egg length; EW = egg width; EP = egg perimeter.

[a] means from six eggs measured in each patient.

3. Results

3.1. Coprological survey results

Out of 540 subjects surveyed, only 4 were found positive for trematode eggs, which means a total prevalence of 0.74%. Among them, one positive sample was within the 205 samples from the urban areas (0.48%) and three were within the 335 samples obtained in rural areas (0.89%).

These four positive samples corresponded to 2 males out of a total of 300 males surveyed (0.66%) and 2 were females out of a total of 240 females analyzed (0.83%). The 2 boys were 9 and 13-year old children, whereas the 2 females were 7 and 16 year old girls. No infection was detected in the age group above 20 years.

The two youngest 7-year female and 9-year male came from the locality of Katlang Babuzo, whereas the 13-year old male was from Takht Bhai and the 16-year female from Rustam. Two of them were detected in the September stool collection, another in January and the remaining one in May.

The low number of infected subjects explain the lack of significance ($P > .05$) regarding locality, gender, age and month of detection.

The aforementioned two youngest were coinfected by *Ascaris lumbricoides* and *Ancylostoma duodenale*, whereas the other two were additionally infected by only *A. lumbricoides* (Table 1).

3.2. Trematode egg characterization

The trematode eggs found in the four positive children were identified as belonging to *Fasciola* on the basis of their typical morphology in human infections, including operculum, brownish colour, non-embryonated, large-sized eggs, and showing the typical contents of fasciolid eggs (Fig. 2B).

Burden per child ranged 73–146 epg, with an average of 106 ± 15.4 epg (mean $\pm$ SD). The length of these *Fasciola* eggs varied between 118.4 and 135.5 µm (127.0 ± 4.21 µm) and their maximum width between 67.5 and 76.6 µm (71.33 ± 1.94 µm). The egg perimeter ranged 302.6–345.38 µm, with an average of 323.3 ± 10.15 µm (Table 1).

3.3. Clinical pictures and treatments

Only the two youngest positive children were showing evident symptoms (Table 2), although it should not be forgotten that they were coinfected by *A. lumbricoides* and *A. duodenale*. All the four *Fasciola* infected presented with eosinophilia, as well as with altered liver function enzymes (Table 2).

In these four children, *Fasciola* eggs disappeared from stools and liver function enzymes returned to normal values (Table 2) after local treatment with traditional herbal medicine known as "Hakeem" in the Pakistani language. Decreases of the values of ALT and AST after treatment proved to be statistically significant ($P < .05$).

This medicine is powdered seeds of *Nigella sativa* or "black cumin", an annual flowering plant (family Ranunculaceae) native to south and southwest Asia which has been used for centuries to kill worms. Treatment course was ¼th teaspoon daily of *Nigella sativa* seed powder for 2 weeks (dose of approximately 500 mg per day).

3.4. Questionnaire survey results

Results of the questionnaire survey and including the four positive children infected by *Fasciola* are noted in Table 3. All the results appear to be logical for a rural area in a developing country, including the very low awareness of the inhabitants about the disease. Among the different risk factors analyzed, only the awareness of the disease proved to be statistically significant ($P < .05$).

The usual consumption of raw vegetables and of natural, non-treated water from environmental sources such as streams and fountains, the presence of livestock, and unsufficient general hygienic measures, are high risk factors regarding *Fasciola* infection. The four *Fasciola* infected children fit perfectly to these high risk factors (Table 3).

4. Discussion

The length/width characteristics of the *Fasciola* eggs found in the four infected children fit well inside the species *F. hepatica*. Indeed, the egg size measurements obtained of 118.4–135.5/67.5–76.6 µm correspond to the lower band of the variability range of *F. hepatica* in humans which is of 100.6–162.2/65.9–104.6 µm in areas where *F. gigantica* is absent and of 106.5–171.5/63.9–95 in areas where both fasciolid species are present [4,35].

Confusion with similar eggs of other trematode species which are more or less frequent infecting humans in Asia, such as *Fasciolopsis buski* and *Gastrodiscoides hominis*, was ruled out by considering that (i) Pakistan is far away from the well-known geographical distribution of these two species in that continent, (ii) the only animal reservoir species of these two trematodes is the pig which has no presence in muslim communities such as those in Pakistan, and (iii) the detailed characteristics of shape, shell, colour, operculum and aboperculum end which allow for their differentiation [36–39].

The size of the eggs found in the children from Mardan district overlap in size with morphologically similar eggs of echinostomatid species having been reported in humans, such as *Echinostoma hortense* [40,41] or *Isthmiophora melis* [42,43]. Eggs of *E. hortense* are 111.2–124.3/74.1–79.0 µm and those of *I. melis* are 115–140 × 70–95 µm. The differentiation regarding eggs of these two echinostomatid species was based on three aspects: (i) the length/width ratio is higher in *Fasciola*, i.e. echinostomatid eggs are wider; (ii) in *Fasciola* eggs, at the aboperculat end of the shell surface, there is a typical roughened or irregular, more intense brownish dark

Table 2
Symptom characteristics and clinical parameters in the children found infected by *Fasciola* in the Mardan district, Pakistan.

Patients								
Gender	Female		Male		Male		Female	
Age (years)	7 y		9 y		13 y		16 y	
Symptoms								
Fever	X		–		–		–	
Abdomminal pain	X		X		–		–	
EpIgrastric pain	X		–		–		–	
Diarrhea	X		X		–		–	
Jaundice	–		–		–		–	
Vomiting	–		–		–		–	
Blood measurements								
Haemoglobin	11 (g/dL)		9 (g/dL)		13 (g/dL)		12 (g/dL)	
Eosinophils	1300		1600		800		700	
Liver function enzymes								
	Pretreat-ment	Posttreat-ment	Pretreat-ment	Posttreat-ment	Pretreat-ment	Posttreat-ment	Pretreat-ment	Posttreat-ment
ALT (U/dL)	74	38	62	27	54	23	71	31
AST (U/dL)	46	20	47	19	39	18	52	21
ALP (U/dL)	182	138	188	156	177	115	167	95

ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase.

Table 3
Results about potential human infection sources and risk factors obtained with the questionnaire survey from 540 subjects interviewed and the children found infected in the Mardan district, Pakistan.

	Subjects interviewed	Percentage (%)	Infected subjects
Education			
Uneducated	170	31.5	2
Secondary school	269	49.8	2
Degree and above	101	18.7	0
Occupation			
Servant (job in office)	108	20.0	0
Farmer	177	32.8	2
Other professions	255	47.2	2
Raw vegetable eating habits			
Consumption 1–3 times per week	184	34.1	0
Consumption 4–6 times per week	246	45.5	2
Consumption 7–9 times per week	110	20.4	2
Water drinking habits			
Stream water	39	7.2	1
Well water	416	77.0	3
Other sources	85	15.7	0
Water use and origin			
Well located near the animal farm	84	15.5	1
Well located far from the animal farm	456	84.4	3
Does wash hands regularly before eating	429	79.4	2
Does not wash hands regularly before eating	111	20.5	2
Does wash dishes regularly	463	85.7	3
Does not wash dishes regularly	77	14.2	1
Awareness of fascioliasis			
Knowledge about the disease	31	5.7	0
No knowledge about the disease	509	94.3	4

protuberance (Fig. 2B) which may sometimes appear laterally displaced [35]; (iii) human infection by these echinostomatids appears to be highly sporadic excepting *E. hortense* in eastern countries such as Korea [44].

The Mardan area does moreover fit well to all transmission and epidemiological requirements of *F. hepatica*, namely (i) the presence of infected livestock reservoirs throughout, (ii) the appropriate climatic factors, (iii) the existence of adequate freshwater collections, and (iv) the existence of the specific snail vector species.

At the neighbouring Peshawar, observations on cattle and buffalo brought for slaughter suggested a very high incidence of fascioliasis of between 70% and 80% already time ago [45]. The low specificity of fasciolid flukes regarding the definitive host species allow for liver fluke isolates from animal reservoirs to also infect humans [11,12,46].

The Mardan fertile plain runs down initially with a steep slope from the foot hills and includes both permanent and temporary water bodies. Streams generally flow from north to the south, carrying the rain water to the lower levels and finally to the Kabul river. Rainfall mainly occurs in the months of July, August, December and January, with August as the rainiest month, and there is a quite high relative humidity throughout the year. Present temperature variations in the Mardan plain throughout the year (Fig. 2A) are similar to those of southern Europe countries where *F. hepatica* typically follows a biseasonal transmission pattern including low transmission in spring and very high transmission in autumn, with highest definitive host incidences from August–September expanding even up to December–February [47–49].

The two fasciolid species show a different specificity regarding their lymnaeid snail vector species [5]. *Fasciola hepatica* uses small-sized, markedly amphibious species of the *Galba/Fossaria* group [50,51], including the high transmitting species *Galba truncatula* [52] which appears usually in temporary freshwaters, prefers cool temperatures such as those of altitudinal areas, and is present in Pakistan [45]. *Fasciola gigantica* is mainly transmitted by large-sized lymnaeids of the *Radix* group [13], such as *R. auricularia*, which prefers deep permanent freshwaters in warm lowlands, and is also present in Pakistan [45]. In this country, *G. truncatula* has been already described at 1675 m asl in the northern Swat district neighbouring the Mardan plain, and it was emphasized that this snail was never found at altitudes lower than 1200 m in that country [45].

The aforementioned lower altitudinal limit of *G. truncatula* in Pakistan appears to be the only aspect which does not completely fit and needs therefore further analysis. There are two ways to explain this apparent lack of correspondence.

The first one concerns the possibility that the presence of *G. truncatula* in the Mardan plain was overlooked because of its activity restricted to a short month window along the year. It cannot neither be disregarded that the present situation may have changed from the one assessed in the half of last century [45], *G. truncatula* having potentially colonized the Mardan plain posteriorly thanks to passive transport by livestock conducted along the Swat valley from altitude to the lowlands in transhumance movements. The passive transport of small, amphibious *Galba/Fossaria* lymnaeids attached to livestock hooves is well known [51]. Evidences indeed suggest that *G. truncatula* specimens may remain in mud rests attached to the feet of the ruminants, enter in hibernation or estivation in the drought attached mud during the movement and be able to reactivate once in the new location after contacting water [3]. Moreover, the possibility of *G. truncatula* arrival into the Mardan plain after dragging by stream waters downwards from the Swat valley cannot be ruled out, mainly when considering the increase of monsoon rains within the climate change impact of recent years [27]. The successful establishment of *G. truncatula* in the Mardan plain would have been subsequently facilitated by the appropriate temperatures during the cool month window of the year (Fig. 2A).

The second possibility would be to accept that the four children became infected in altitude areas instead of in the Mardan plain. The four infected children told to have occasionally visited mountainous areas where they have relatives. Altitude areas visited by the children were Swat valley and Kaghan valley where they mentioned to have eaten raw salads (mixture of raw, usually unwashed vegetables) and to have drunk natural water. These two valleys are breautiful areas frequently visited by local population and tourists during summer holidays. The altitude of the Swat Valley goes from 975 m upwards to 2900 m, whereas the altitude of the Kaghan valley reaches up to 2500 m (Fig. 1B). These altitudinal ranges fit well to the lower altitudinal limit of the distribution of *G. truncatula* in Pakistan as reported in the past [45].

The four positive children were shedding eggs, which means that they were already in the chronic, biliary or obstructive phase of fascioliasis. This disease phase has recently been demonstrated to also be potentially pathogenic [34,53–55]. The burdens of 73–146 epg detected in the Mardan children are low, but not as low as the few epg usually found in sporadic human infections in animal endemic areas, as it happens in European countries. Moreover, the coinfections with *A. lumbricoides* and *A. duodenale* diagnosed in the children remember the usual coinfection situations of the *Fasciola* infected children in human hyperendemic areas such as in Bolivia [56], Peru [57,58] and Egypt [59]. But the most important is that this indicates that these children were not diagnosed during the acute phase, which uses to be pronouncedly more symptomatic than the chronic phase [15]. Indeed, percentages of asymptomatic patients reported are only 12.5% in developed countries [2] and 15% in human hyperendemic areas of developig countries [60]. All in all, this suggests that human infection by *Fasciola* may be overlooked or misdiagnosed in children and consequently that human fascioliasis may be underestimated in many areas and provinces of Pakistan. This remembers the situation of other countries where higher infection rates in humans were to be expected but concrete epidemiological data were lacking due to the absence of appropriate surveys, as it was the case of Argentina [61].

The efficacy of the traditional herbal medicine based on a powder extracted from seeds of the Asian plant named black cumin, *Nigella sativa*, for fascioliasis treatment merits some comments. This plant has been widely analyzed [62] and its general anthelminthic properties have been the object of several studies [63–65]. Its action against trematodes has been experimentally studied in schistosomiasis [66,67] and its efficacy against *Fasciola* has been reported since long ago both in vivo in buffaloes [68,69] and also in vitro [70]. A two dose of 30, 40 and 50 mg/kg body mass treatment in buffaloes was noted to reduce *Fasciola* epg by 79.16%, 80.85% and 81.25%, respectively [69]. The present work in Mardan appears to be the first published report of the use of this herbal medicine for the treatment of human fascioliasis. The interest lies on the scarce alternative drugs available in front of the situation of the increasing triclabendazole resistance reports [33].

The questionnaire survey results obtained describe a scenario appropriate for the human infection by *Fasciola*. Daily raw vegetable consumption, natural water drinking, numerous livestock, and lack of hygienic habits are high risk factors for liver fluke transmission [71,72].

5. Concluding remarks

The detection of human infection by *Fasciola* indicates that, in Pakistan, human fascioliasis is not restricted to the wide Punjab province [26]. In the warm climate of the Punjab lowlands, environmental characteristics are appropriate for the transmission of *F. gigantica*, and also intermediate forms [73], excepting its northern cooler foothilly areas close to altitude mountains of the Hymalayan chain where livestock transhumance is followed in several places [27]. Results of the Mardan survey demonstrate that both *Fasciola* species may be involved in human infection in Pakistan.

The finding of children infected by *F. hepatica* in the Mardan plain suggests a worrying scenario, as children are the subjects typically infected in human endemic areas, as observed in Bolivia [56], Peru [57,58] or Egypt [59]. Child behaviour typically includes the habit of putting all kind of sylvatic herbs into the mouth for eating, sucking, chewing or stripping with the teeth, and also drinking from natural water collections of different types (streams, man-made canals, ponds, etc.), along their way to and from school in rural areas, in field trips, when playing outside, etc. [71]. Many such histories have been documented in reports on child infections everywhere. Vegetables directly taken from the nature by the children may not necessarily make part of the usual human diet in the area in question and therefore explain why, in a given area, children may show *Fasciola* infection whereas adult subjects do not [71].

Untreated children due to lack of diagnosis of *Fasciola* infection in Pakistan, as usually observed in rural human hyperendemic areas of developing countries, becomes a public health problem in the long term because of the long life span of up to 13.5 years of the liver fluke in the human host [36] and the sequelae of the infection even after effective treatment [74].

Additional surveys in other provinces of Pakistan are needed to assess the extent of the human fascioliasis public health problem in Pakistan.

Acknowledgements

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