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CIAS detection of *Fasciola hepatica*/*F. gigantica* intermediate forms in bovines from Bangladesh

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Abstract

Fascioliasis is an important food-borne parasitic zoonosis caused by two trematode species, *Fasciola hepatica* and *Fasciola gigantica*. The characterisation and differentiation of *Fasciola* populations is crucial to control the disease, given the different transmission, epidemiology and pathology characteristics of the two species. Lineal biometric features of adult liver flukes infecting livestock have been studied to characterise and discriminate fasciolids from Bangladesh. An accurate analysis was conducted to phenotypically discriminate between fasciolids from naturally infected bovines (cattle, buffaloes) throughout the country. Morphometric analyses were made with a computer image analysis system (CIAS) applied on the basis of standardised measurements and the logistic model of the body growth and development of fasciolids in the different host groups. Since it is the first ever comprehensive study of this kind undertaken in Bangladesh, the results are compared to pure fasciolid populations of *F. hepatica* from the European Mediterranean area and *F. gigantica* from Burkina Faso, geographical areas where both species do not co-exist. Principal component analysis showed that the biometric characteristics of fasciolids from Bangladesh are situated between *F. hepatica* and *F. gigantica* standard populations, indicating the presence of phenotypes of intermediate forms in Bangladesh. These results are analysed by considering the present emergence of animal fascioliasis, the local lymnaeid fauna, the impact of climate change, and the risk of human infection in the country.

Keywords

Fasciola hepatica, *Fasciola gigantica*, forms, multivariate analysis, CIAS, Bangladesh.

Introduction

Fascioliasis, a food-borne zoonosis caused by liver flukes of the genus *Fasciola* (Trematoda: Digenea), is a serious animal health problem worldwide and is considered the most important helminth infection in ruminants causing considerable socioeconomic problems in tropical countries (Mas-Coma *et al.* 2009a). This disease in ruminants causes substantial economic losses to rural agricultural communities and commercial animal producers due to the death of infected animals, condemnation of affected livers and production losses associated with reduced feed conversion efficiency worldwide (Torgerson and Claxton 1999; Spithill *et al*. 1999).

Additionally, studies performed in recent years have shown it to be an important public health problem as well. Human cases have been increasing in the five continents, including estimates of human infection of up to 17 million people, or even higher depending from the hitherto unknown situations in Asia and Africa (Mas-Coma *et al.* 2009a). This disease has recently proven to have a great morbidity impact throughout the biliary or chronic period of the disease (Valero *et al.* 2003, 2006a, 2008; Girones *et al.* 2007), and not only due to the pathogenicity of the acute phase as hitherto believed (Chen and Mott, 1990). Clinical studies have shown this disease to be pronouncedly complex, giving rise to progressive general deterioration of the patients, with sequelae sometimes

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leaving them handicapped and frail, even including fatal cases (Mas-Coma *et al.* 2014a). The diagnosis of the liver fluke infection in humans poses, moreover, many problems related to the different epidemiological scenarios and transmission patterns, as well as to the different situations in individual patients and due to the different periods this disease shows in humans (Valero *et al.* 2009a; Mas-Coma *et al.* 2014b). Additionally, recent epidemiological studies have proven it to be related to climate and global changes (Mas-Coma *et al.* 2008, 2009b; Afshan *et al.* 2014), as a consequence of the pronounced dependence of the fluke life cycle stages regarding the weather and other abiotic factors of the environment (Fuentes *et al.* 1999, 2001). The World Health Organization (WHO) has recognized fascioliasis as an emerging human disease, with human infection reported in more than 71 countries and 180 million people presently at risk of infection (WHO, 2013).

The two causal agents of this disease, *Fasciola hepatica* and *F. gigantica*, are two closely related species. The definitive host range of both fasciolid species is very large, including herbivorous mammals, mainly livestock species, while freshwater snails of the family Lymnaeidae act as intermediate hosts or vectors (Bargues and Mas-Coma, 2005). Whereas *F. gigantica* occurs mainly in tropical areas of Africa and Asia, *F. hepatica* prefers temperate and cooler areas throughout the five continents, and both species overlap in subtropical zones of Africa and Asia (Mas-Coma *et al.* 2009a). Intermediate forms appearing in overlap zones of southern Asia and Southeast Asia pose problems in the identification of the flukes involved in the infection of both humans and animals (Mas-Coma *et al.* 2009a; Ichikawa *et al.* 2011).

Despite the importance of distinguishing between infections by one or another fasciolid species, due to their different transmission, epidemiology, pathology and control characteristics, there is, unfortunately, neither a direct coproantigen test nor an indirect immunological test available for their differential diagnosis (Valero *et al.* 2009a, b, 2012a, b; Mas-Coma *et al.* 2014b). Diagnostic tools available at present are only useful to differentiate fascioliasis from other diseases. Hitherto, specific differentiation can only be made by either a phenotypic study of adult flukes by means of morphometric tools (Periago *et al.* 2006; Valero *et al.* 2009a, 2012c; Ashrafi *et al.* 2006, 2015; Asfhan *et al.* 2013) or genotypically by molecular tools (Mas-Coma *et al.* 2009a).

The overlapping distribution of *F. hepatica* and *F. gigantica* has also led to a long-lasting controversy concerning the taxonomic identity of the *Fasciola* species found in countries of the Far East, in which some resemble *F. hepatica*, whereas others resemble *F. gigantica*, with intermediate forms also being present and involving phenomena such as abnormal gametogenesis, diploidy, triploidy and mixoploidy, parthenogenesis and hybridisation events between different genotypes (Mas-Coma *et al.* 2009a). A comprehensive multidisciplinary study has, however, demonstrated that *F. hepatica* and *F. gigantica* should be considered valid species, despite their capacity to hybridise and give rise to intermediate forms in overlapping areas (Mas-Coma *et al.* 2009a).

Bangladesh is one of the Asian countries where fascioliasis is the most prevalent parasitic disease in cattle, buffaloes, goats and sheep (Nooruddin and Islam, 1996; Hossain *et al.* 2011). An earlier survey revealed that 71.55% of cattle, 14.6% of sheep and goats and 100% of buffaloes are affected by the liver-fluke (Bhuiyan, 1970). A more recent report (BAS-USDA, 2012) showed that 10.42% cattle, 9% goats and 36.61% of buffaloes are infected with *Fasciola* among snail borne trematode infections in Bangladesh. Recent post-mortem examinations revealed a *Fasciola* infection rate of 35.27% irrespective of geographical areas and host animal species (unpublished data, 2013).

Unfortunately, appropriate phenotypic analyses to assess the presence and phenotypic features of the fasciolids have never been carried out in Bangladesh. The present study represents the first attempt within that endeavour. Bovines, as the most widespread livestock species in the country, were been the hosts selected. An approach by means of a computer image analysis system (CIAS) (Valero *et al.* 2005, 2012c) was applied for the morphometric characterisation of the liver flukes.

Materials and Methods

Geographic location, collection and examination of flukes

Bangladesh is a low-lying, riverine country located in south Asia with a vast marshy jungle coastline of 710 km on the northern littoral of the Bay of Bengal threatened by floods and droughts. It has a tropical monsoon climate characterised by heavy seasonal rainfall, high temperatures, and high humidity. The country has an area of 147,570 square kilometers and is bordered on the west, north, and east by a 4,095-km land frontier with India and, in the southeast, by a short land and water frontier (193 km) with Burma (Myanmar). In the south, there is a highly irregular deltaic coastline of about 580 km, fissured by many rivers and streams flowing into the Bay of Bengal. Bangladesh comprises mostly irrigated fields which provide excellent breeding grounds for the development and survival of freshwater snails serving as potential intermediate hosts for a variety of digenetic trematode parasites.

Adult fasciolids were collected directly from the livers of naturally infected Nili-Ravi and Murrah type domestic water buffalo (*Bubalus bubalis*) (n = 51) and Indian zebu (*Bos indicus*) (n = 51), at necropsy from different locations in four different broad agro-ecological zones of Bangladesh between April and November 2012 (Table I and Fig. 1) with the help of rubber-coated forceps in order to avoid any structural damage to the flukes. Fasciolid specimens providing the largest worm variability in their size, maturity and gravid uteri were used for characterisation. Individual worms were washed extensively in physiological saline (0.85% NaCl) to remove blood and bile. The living flukes were washed with and incu-

Table I. Breeding sites of the bovine definitive hosts (with latitude, longitude and altitude) and traditional agro-ecological zones in Bangladesh where the liver-fluke samples were collected. Location on map (Fig. 1) indicated by letters

Localities (*Administrative Units)	Location on map	Latitude	Longitude	Altitude (feet)	Altitude (meter)	Traditional agro-ecological zones
Chittagong / Cox´s Bazar / Chakaria	A	21°45´41.36″N	92°04´33.45″E	37	11.21	Coastal
Barisal /Bhola/ Bhola Sadar	B	22°41´12.48″N	90°38´30.76″E	26	7.88	
Chittagong/Rangamati/Rangamati Sadar	C	22°39´28.51″N	92°10´24.54″E	150	45.45	Hilly
Sylhet/Sylhet/Sylhet Sadar	D	24°54´23.77″N	91°50´50.29″E	70	21.21	
Rajshahi/Naogaon/Patnitola	E	25°02´40.55″N	88°45´19.68″E	80	24.24	Barind (ricepads)
Rajshahi/Naogaon/Shapahar	F	25°07´30.31″N	88°35´24.50″E	148	44.85	
Rajshahi/Naogaon/Naogaon Sadar	G	24°48´39.24″N	88°56´37.04″E	68	20.61	
Rajshahi/Pabna/Ishwardi	H	24°07´12.43″N	89°04´03.56″E	61	18.48	Floodplains
Dhaka/Mymensingh/Ishwarganj	I	24°41´10.20″N	90°35´36.88″E	55	16.67	
Rangpur/Lalmonirhat/Lalmonirhat Sadar	J	25°58´26.15″N	89°17´02.26″E	124	37.58	
Dhaka/Mymensingh/Mymensingh Sadar	K	24°44´04.60″N	90°25´43.80″E	66	20.00	
Rajshahi/Bogra/Dhunat	L	24°41´29.12″N	89°32´10.33″E	54	16.36	
Khulna/Jhenaidah/Shailkupa	M	23°40´45.47″N	89°14´48.28″E	43	13.03	
Khulna/Jhenaidah/Jhenaidah Sadar	N	23°32´43.15″N	89°10´28.16″E	47	14.24	

*Administrative units of Division / District / Upazila

bated in PBS at 37 °C for a short while to allow them to expel the gut contents. The flukes were afterwards placed between two microscopic slides and adult worms were observed using a stereomicroscope to verify the presence of eggs in the uterus.

Fasciolid specimens were fixed in AFA solution (95% alcohol – 20 parts, formalin – 6 parts, glacial acetic acid - 1 part, distilled water – 40 parts) between two slides with little pressure, depending on the thickness of the flukes. The flukes were stained with Semichon's carmine solution (glacial acetic acid and distilled water by equal amount, saturation by dissolving carmine powder heated at 95–100 ^{0}C for 15 minutes and on filtration dilute with the same amount of 70% alcohol as stock solution) and subsequently differentiated, dehydrated and mounted with DPX.

Morphometrics

The morphometric study was conducted to phenotypically discriminate adult flukes from the naturally infected ruminants. Studies were performed using a computer image analysis system (CIAS) on the basis of standardised measurements known to be useful for the differentiation of fasciolid species (Valero *et al.* 2005, 2009a; Periago *et al.* 2006, 2008; Afshan *et al.* 2013). Standardised measurements were taken using a microscope and images captured by a digital camera (Nikon Coolpix), which were then analysed by image analysis software (ImagePro plus version 5.0 for Windows, Media Cybernetics, Silver Spring, Maryland, USA). Since it is the first time that such a study has been performed in Bangladesh, the results are compared to pure fasciolid populations also from boivines, namely: (i) *F. hepatica* from Valencia, Spain and Corsica, France; and (ii) *F. gigantica* from Burkina Faso. These pure fasciolid geographical origins are areas where both species do not co-exist (Periago *et al.* 2006). All the previously published data on *F. hepatica* from Spain (Europe) and *F. gigantica* from Burkina Faso (Africa) (Periago *et al.* 2006) are used as a standard references.

The following standardized measurements were taken (Fig. 2):

– Lineal biometric characters (mm): body length (BL), maximum body width (BW), body perimeter (BP), body roundness (BR), distance between the oral sucker and ventral sucker (OS–VS), distance between the ventral sucker and the union of the vitelline glands (VS–Vit), distance between the union of the vitelline glands and the posterior end of the body (Vit–P), distance between the ventral sucker and the posterior end of the body (VS–P), pharynx length (PL), pharynx width (PW).

– Areas (mm^2): body area (BA).

– Ratios: BL over BW (BL/BW), BL over the distance between the VS and P (BL/VS–P), PL over PW (PL/PW), BP over BL (BP/BL), BL over distance between VS and Vit

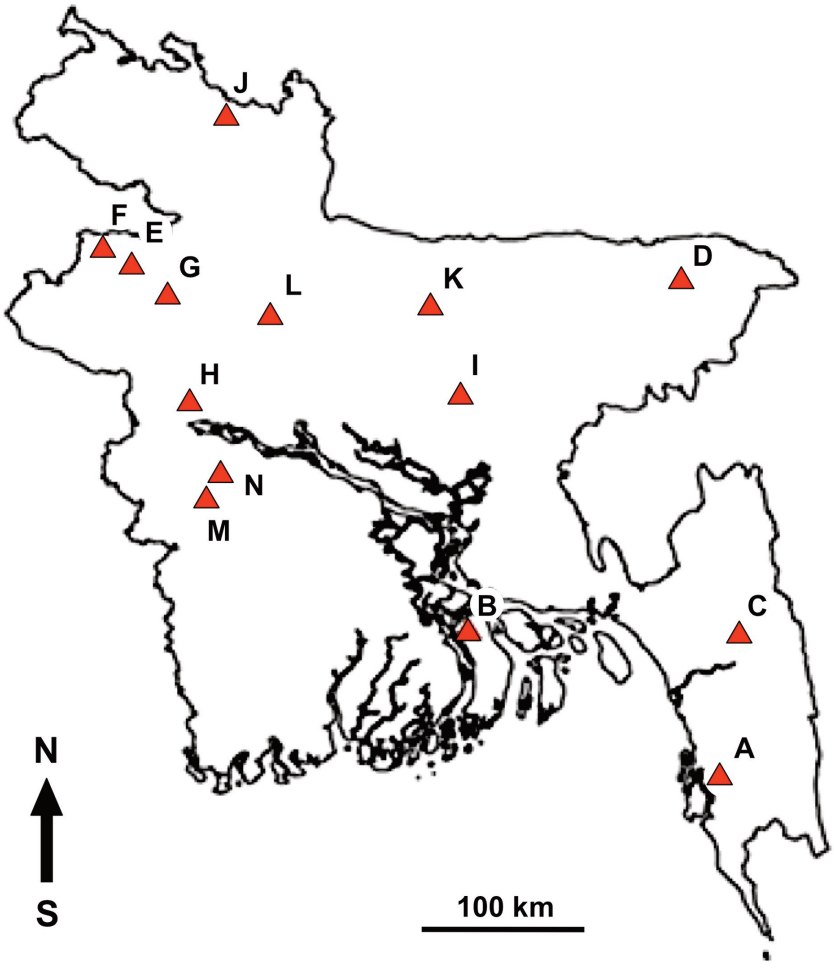

Fig. 1. Geographic map of Bangladesh, showing the sites where the hosts of the liver flukes analysed were bred. GIS data of each site are indicated in Table I. **A** – Chakaria; **B** – Bhola Sadar; **C** – Rangamati Sadar; **D** – Sylhet Sadar; **E** – Patnitola; **F** – Shapahar; **G** – Naogaon Sadar; **H** – Ishwardi; **I** – Ishwarganj; **J** – Lalmonirhat Sadar; **K** – Mymensingh Sadar; **L** – Dhunat; **M** – Shailkupa; **N** – Jhenaidah Sadar

(BL/VS-Vit) and BL over distance between Vit and P (BL/Vit-P); BA over BL (BA/BL), BW (BA/BW), BP (BA/BP), distance between VS and Vit (BA/VS-Vit), distance between Vit and P (BA/Vit-P) and distance between VS and P (BA/VS-P).

– Body roundness (BR = BP2/4πBA) was used to measure the body shape. A circular object will always have a roundness of 1.00, while more irregular objects will have larger values, indicating how circular an object is (Periago *et al.* 2006).
– Morphometric measurements used for fasciolid adults follow a logistic growth model with respect to time (Valero *et al.* 2005). This implies that the morphometric development of the fasciolid adult is not limited but 'damped' and does not exceed certain characteristic maxima (Valero *et al.* 2001a, b, 2006b, 2011). Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study (Table II).

Data analysis

The fasciolid adult stage undergoes a marked developmental process in the definitive host. The changes in different biometrical parameters of adult flukes at different times have been previously analysed (Valero *et al.* 1998, 2001a, b, 2005, 2011). The corresponding growth curves were all logistic, which implies that the morphometric development of the adult fluke is not unlimited but "damped" and cannot exceed certain characteristic maxima of ym (= maximum value attained by a biometric variable). This model describes the variation in adult fluke dimensions along time, from parasite migration to the adult location in the bile duct. Entry into the bile duct induces maturation and egg production. The logistic model which represents body growth and development is characterised by two phases (Valero *et al.* 1998, 2005). The "exponential" part of logistic growth corresponds to body development during migration in the abdominal cavity and liver parenchyma, as well as to development and sexual mat-

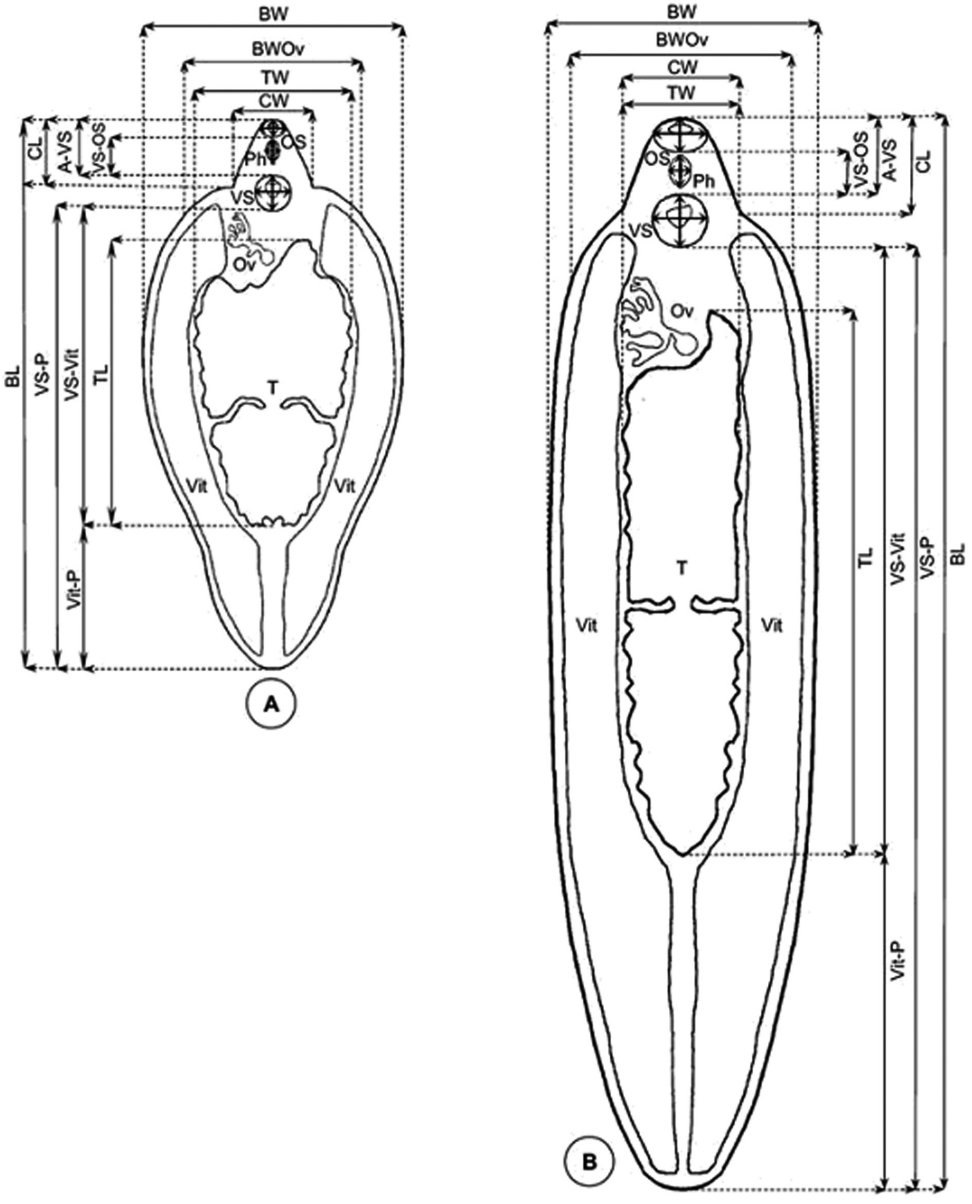

Fig.2. Standardised measurements applied for the morphometric characterisation of fasciolid adult specimens (see text for abbreviations). **A** – *Fasciola hepatica*-like forms; **B** – *Fasciola gigantica*-like forms

uration in the biliary duct system up to the onset of egg production.

Consequently, to avoid the influence of age and growth on the trematode comparison, morphological variation is quantified by geometrical morphometrics (Rohlf and Marcus, 1993), a technique offering an estimate of size by which different axes of growth are integrated into a single variable (Afshan *et al.* 2013). The estimate of size is contained in a single variable reflecting variation in many directions, as many as there are landmarks under study, and shape is defined as their relative positions after correction for size, position and orientation. With these informative data, and the corresponding software freely available to conduct complex analyses, significant biological and epidemiological features can be quantified more accurately (Dujardin, 2008).

Current statistical techniques in morphometrics make it possible to test the null hypothesis of conspecific populations being simply the allometric extension of each other, provided a common allometric trend is identifiable (Klingenberg, 1996; Dujardin and Le Pont, 2004). Multivariate analyses were applied to calculate the phenotypic variations among fasciolid adults, using size-free canonical discriminant analysis of the covariance of log-transformed measurements to assess phenotypic variations between the samples. These analyses are applied to exclude the effect of within-group ontogenetic variations by reducing the effect of each character on the first

Table II. Comparative morphometric data of adult liver flukes from bovines of Bangladesh with previously published data of standard populations from Spain, France and Burkina Faso described by Periago *et al.* (2006)

Adult measurements	Bangladesh N=102	Spain n=84	Corsica n=86	Burkina Faso n=81
Body length, BL	30.29 ± 0.77 (20.19–53.58)	11.64–22.93 17.41 ± 0.23	12.22–29.00 20.45 ± 0.37	28.82–52.30 39.72 ± 0.58
Body width, BW	10.32 ± 0.17 (6.98–15.39)	6.41–13.88 10.02 ± 0.17	4.88–14.07 10.71 ± 0.18	6.03–11.84 8.45 ± 0.14
BW at ovary level, BWOv	9.03 ± 0.19 (5.10–12.90)	4.53–11.67 7.90 ± 0.14	4.46–11.46 8.06 ± 0.15	5.33–11.55 7.59 ± 0.13
Body perimeter, BP	81.23 ± 1.71 (57.22–130.96)	28.58–54.40 43.05 ± 0.55	30.21–66.43 49.21 ± 0.81	63.19–113.71 85.25 ± 1.21
Body roundness, BR	1.69 ± 0.02 (1.39–2.25)	1.06–1.58 1.23 ± 0.01	1.10–1.55 1.25 ± 0.01	1.71–3.65 2.47 ± 0.05
Cone length, CL	2.46 ± 0.05 (1.90–3.30)	1.09–2.92 2.02 ± 0.04	1.58–3.04 2.21 ± 0.03	2.10–3.36 2.67 ± 0.04
Cone width, CW	3.24 ± 0.09 (2.00–5.00)	2.35–4.21 3.20 ± 0.04	2.30–4.01 3.08 ± 0.04	2.23–5.08 3.74 ± 0.06
OS maximum diameter, OSmax	0.72 ± 0.01 (0.50–1.00)	0.57–1.03 0.85 ± 0.01	0.60–0.99 0.84 ± 0.01	0.52–1.17 0.83 ± 0.02
OS minimum diameter, OSmin	0.47 ± 0.02 (0.20–0.90)	0.44–0.77 0.61 ± 0.01	0.40–0.83 0.65 ± 0.01	0.21–0.95 0.62 ± 0.02
VS maximum diameter, VSmax	1.12 ± 0.03 (0.85–1.85)	0.92–1.49 1.13 ± 0.01	0.69–1.38 1.10 ± 0.01	0.87–1.92 1.50 ± 0.02
VS minimum diameter, VSmin	0.98 ± 0.02 (0.79–1.80)	0.86–1.35 1.12 ± 0.01	0.83–1.26 1.08 ± 0.01	0.80–1.83 1.38 ± 0.02
Distance between anterior end of body and VS, A-VS	2.50 ± 0.03 (2.00–3.00)	1.12–2.92 2.05 ± 0.04	1.35–3.04 2.48 ± 0.04	1.46–3.01 2.36 ± 0.03
Distance between suckers, OS-VS	1.82 ± 0.08 (0.70–4.30)	0.57–2.41 1.42 ± 0.04	0.89–2.32 1.82 ± 0.03	1.15–2.22 1.71 ± 0.03
Distance between VS and union of vitelline glands, VS-Vit	18.36 ± 0.53 (9.35–36.04)	6.57–14.31 9.60 ± 0.17	5.97–18.22 11.64 ± 0.26	12.26–34.11 22.68 ± 0.45
Distance between Vit and posterior end of body, Vit-P	9.42 ± 0.32 (5.20–17.02)	2.63–7.57 4.73 ± 0.10	2.90–8.99 5.17 ± 0.15	8.97–21.43 13.45 ± 0.32
Distance between VS and P, VS-P	27.78 ± 0.77 (17.71–50.82)	9.51–19.94 14.40 ± 0.22	8.86–25.08 16.90 ± 0.36	26.28–50.09 36.39 ± 0.59
Pharynx length, PhL	0.74 ± 0.03 (0.27–1.57)	0.52–1.00 0.70 ± 0.01	0.10–0.97 0.76 ± 0.01	0.46–1.06 0.78 ± 0.02
Pharynx width, PhW	0.60 ± 0.02 (0.26–1.14)	0.26–0.83 0.44 ± 0.01	0.29–0.63 0.41 ± 0.01	0.23–0.68 0.42 ± 0.01
Testicular space length, TL	17.17 ± 0.38 (12.00–29.00)	5.45–11.68 7.91 ± 0.15	5.38–15.99 9.85 ± 0.23	12.76–29.38 18.76 ± 0.38
Testicular space width, TW	5.27 ± 0.14 (3.00–8.00)	4.18–8.93 6.61 ± 0.12	3.17–10.11 7.39 ± 0.14	3.24–8.66 5.51 ± 0.12
Testicular space perimeter, TP	44.88 ± 1.02 (30.00–74.80)	17.10–34.34 25.32 ± 0.42	15.75–40.29 29.85 ± 0.56	25.97–68.06 44.62 ± 0.87
Body area, BA	318.41 ± 11.20 (164.11–637.60)	54.90–197.40 123.83 ± 3.28	46.80–261.71 153.61 ± 4.70	162.58–482.91 249.38 ± 7.48
Oral sucker area, OSA	0.36 ± 0.02 (0.10–0.90)	0.25–0.56 0.41 ± 0.01	0.22–0.59 0.43 ± 0.01	0.10–1.11 0.46 ± 0.03
Ventral sucker area, VSA	1.16 ± 0.06 (0.68–3.33)	0.67–1.57 0.99 ± 0.02	0.45–1.30 0.93 ± 0.02	0.56–3.52 1.86 ± 0.08
Pharynx area, PhA	0.50 ± 0.03 (0.08–1.79)	0.16–0.57 0.31 ± 0.01	0.04–0.54 0.31 ± 0.01	0.12–0.64 0.33 ± 0.02
Testicular space area, TA	95.20 ± 4.65 (36.00–243.60)	17.85–70.62 40.45 ± 1.24	13.77–100.47 56.30 ± 1.96	41.05–187.50 80.75 ± 3.44
BL/BW ratio	2.96 ± 0.07 (1.83–4.87)	1.29–2.77 1.74 ± 0.03	1.33–2.80 1.91 ± 0.03	3.40–6.77 4.70 ± 0.08
BWOv/CW ratio	2.89 ± 0.06 (1.97–4.08)	1.52–3.46 2.47 ± 0.04	1.47–3.86 2.62 ± 0.05	1.34–3.63 2.03 ± 0.05
Sucker ratio, OSA/VSA	0.30 ± 0.01 (0.15–0.48)	0.24–0.60 0.41 ± 0.01	0.21–0.99 0.46 ± 0.01	0.08–0.40 0.25 ± 0.01
BL/VS-P ratio	1.10 ± 0.003 (1.05–1.15)	1.14–1.31 1.21 ± 0.004	0.95–1.38 1.21 ± 0.01	0.95–1.20 1.09 ± 0.004

Measurements are in mm; all values are shown as range (extreme values) with mean ± SE; SE = Standard error, n = sample size

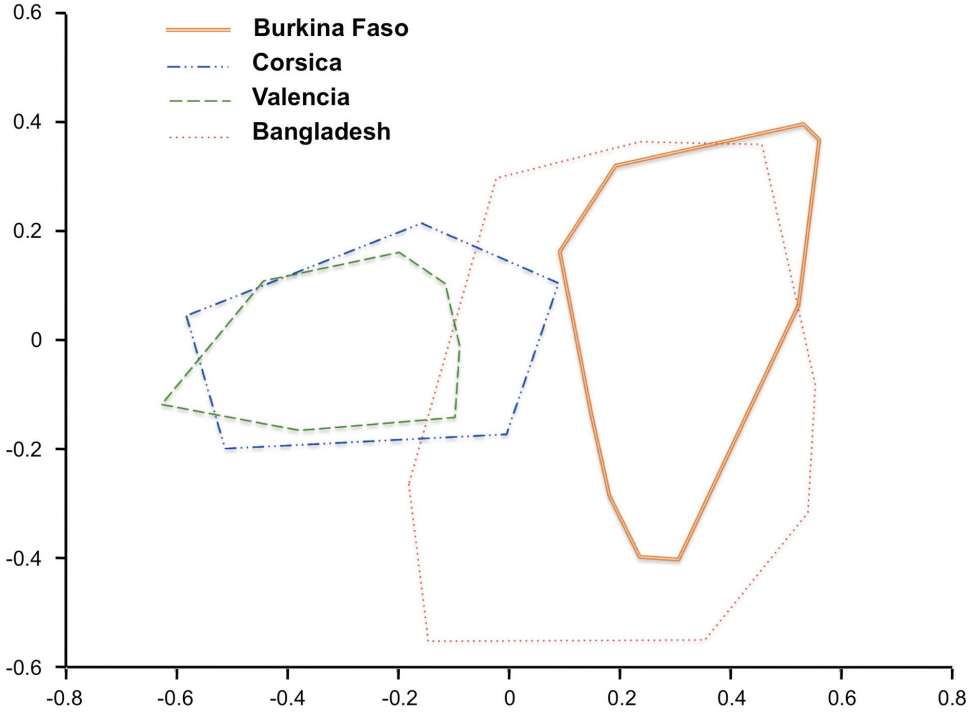

Fig. 3. Principal component analysis of adult fasciolids from natural infections in bovines of Bangladesh (dotted line) compared with *F. hepatica* from Valencia, Spain (dashed line) and Corsica, France (dashed-dotted line) and *F. gigantica* from Burkina Faso (solid line) (Periago *et al.* 2006). Samples are projected on to the first (PCI, 58%) and second (PCII, 29%) principal components

pooled within-group principal component (a multivariate size estimator) (Dos-Reis *et al.* 1990). The principal component analysis is used to summarize most of the variations in a multivariate dataset in a few dimensions (Dujardin and Le Pont, 2004).

Phenotypic analysis of fasciolid adults was conducted by using various modules of the CLIC package (by J.P. Dujardin, http://momedujardin.wordpress.com). The results were statistically significant when $P<0.05$. The following non-redundant measurements (one measurement is not included in another) used for fasciolid adults were BL, BW, BP, VS-Vit, Vit-P, PL and PW, where at least one dimension was measured among the most important morphological characters. The remaining variables (PCII, Y axis) were all significantly correlated with the first principal component (PCI, X axis), contributing 58% to overall variations. The PCI could therefore be accepted as a general indicator of size (Afshan *et al.* 2013), so that the resulting factor maps (Fig. 3) can clearly illustrate global size differences in the populations analysed.

Although metacercarial infectivity does not appear to differ in isolates from different livestock species (Valero and Mas-Coma, 2000), the definitive animal host species is known to pronouncedly influence the phenotype of both adult stage and eggs of the liver fluke, mainly due to the different size of the liver duct microhabitat (Valero *et al.* 2001a, 2002, 2009a). For this reason, only parasites obtained from bovines were used in the principal component analysis carried out in the present study.

Results

Fasciolid populations from cattle from Bangladesh were grouped according to the maximum and minimum values of given differentiating morphological measurements previously proposed for *F. hepatica*, *F. gigantica* or *Fasciola* sp. (= intermediate forms) (Periago *et al.* 2006): BR: 1.06–1.58 in *F. hepatica*, 1.71–3.65 in *F. gigantica*; BL/BW: 1.29–2.80 in *F. hepatica*, 3.40–6.78 in *F. gigantica*; and VS–P: 8.86–25.08 mm in *F. hepatica*, 26.28–50.09 mm in *F. gigantica*.

The specimens from Bangladesh were first grouped according to BR and BL/BW, and, secondarily, according to VS–P. Using these criteria, adult specimens from cattle were grouped into *F. hepatica*-like (11.76%), *F. gigantica*-like (47.06%) or *Fasciola* sp.-like (41.18%). Similarly, adult specimens from buffaloes were grouped into *F. hepatica*-like (5.88%), *F. gigantica*-like (39.22%) or *Fasciola* sp.-like (54.90%).

The resulting factor maps clearly illustrate global size differences in the cattle and buffalo populations analysed, each group being represented by its perimeter (Fig. 3). Two independent zones can be distinguished: one zone is made up of

samples from Spain and Corsica, while the other zone consists only of samples from Burkina Faso. These zones overlap with the samples from Bangladesh.

The multivariate analysis used to measure the changes in size of fasciolid adults from Bangladesh and compared with the above-mentioned standard populations for each pure fasciolid species showed that the size of most fasciolids from Bangladesh is situated between *F. hepatica* and *F. gigantica* standard populations. These results demonstrate that intermediate forms of fasciolids exist in Bangladesh. Nevertheless, it is worth mentioning that the samples from Bangladesh overlap with *F. hepatica* and *F. gigantica* standard populations, but in the absence of specimens of standard *F. hepatica* and *F. gigantica* from Bangladesh it is also possible that the forms identified as *F. hepatica*-like and *F. gigantica*-like could be the extreme values of the morphometric distribution of the *Fasciola* sp.-like specimens.

Discussion

The CIAS detection of the three phenotypic groups of *F. hepatica*-like forms (cattle: 11.76%; buffalo: 5.88%), *F. gigantica*-like forms (cattle: 47.06%; buffalo: 39.22%) or *Fasciola* sp.-like forms (cattle: 41.18%; buffalo: 54.90%) represent highly interesting results and pose several question marks.

The first aspect to be highlighted is the geographical and physiographical similarities between Bangladesh and the lowlands of Pakistan, where human fascioliasis has been reported recently (Qureshi *et al.* 2005; Qureshi and Tanveer, 2009) and where human infection by *Fasciola* has more recently proved to be related to climate and global changes (Afshan *et al.* 2014). Bangladesh presents abundant water bodies, irrigation fields and canals, making it a suitable habitat for lymnaeid snail vectors. Geographical locations, agro-ecological and agro-biological conditions, and climate change with the particular trend of increasing temperatures are enhancing the abundance, seasonality, reproduction and distribution of different species of snail borne trematodes throughout the country. Bangladesh is globally recognised as the most vulnerable country to climate change, e.g. in Bangladesh the average temperature has registered an upward trend of about 1°C in May and 0.5°C in November along the 14 year period from 1985 to 1998 (IPCC, 2007). According to general analyses, climate change is bound to have a severe impact on the transmission of this disease in Bangladesh (Mas-Coma *et al.* 2008, 2009b). Thus, similarity results suggest that a risk for human infection in Bangladesh should be considered. In that sense, the detection of *F. hepatica*-like forms becomes important, given the well-known capacity of *F. hepatica* to infect humans which is markedly higher than in *F. gigantica*. Present widespread fascioliasis with high pathogenicity in ruminants from Bangladesh is a good indicator for the disease likely to be diagnosed in humans if properly investigated. The higher proportion of *F. gigantica*-like forms in the country may suggest more severe pathogenic characteristics related to the larger size of *F. gigantica* if it infects humans (Girones *et al.* 2007; Valero *et al.* 2008).

The percentages of the three forms found in the bovines studied, with similar proportions in both cattle and buffaloes, and with a patent domination of *F. gigantica*-like forms and *Fasciola* sp.-like forms closer to *F. gigantica* than to *F. hepatica*, agree with the known evolutionary-historical scenario throughout southern Asia (Mas-Coma *et al.* 2009a). In relation to the distribution of fasciolids in Asia, historical, archeological, biogeographic, climatic and lymnaeid faunal data indicate that two different main routes from the Fertile Crescent and eastwards separated by the large Himalayan chain need to be considered (Mas-Coma *et al.* 2009a). One northward from the Himalaya includes the spread of mainly *F. hepatica* and another route southward from the Himalaya concerns the spread of mainly *F. gigantica* through the southern Asian region. This southward spread appears to have been linked to the higher temperatures and presence of the appropriate *Radix* vector species in the lowlands of Afghanistan, Pakistan, India and eastward up to South East Asia. This spread should have been facilitated by the extensive trade between the two primary centres of India and the Fertile Crescent during the 4000–1000 BC period and the later, very intense and long-distance commercial exchanges between those southern Asian countries and Near East countries, for instance, through the southern routes of the "Silk Road", which was active over 15 centuries, from around 138 years BC until the 15th century. Camels, taurine and zebu cattle were mainly used for the transportation of goods and merchandise, while dromedaries were later incorporated into the most southern routes of the Silk Road through Afganistan, Pakistan and India because of their better adaptation to warmer climates (Mas-Coma *et al.* 2009a).

The detection of *F. hepatica*-like forms poses the question mark about the lymnaeid vectors which may be needed for their transmission in Bangladesh. *Fasciola hepatica* is mainly transmitted by small size lymnaeid species of the *Galba*/*Fossaria* group (Bargues *et al.* 2007, 2011a), including *Galba truncatula* as the main vector and the only one in Europe, but also present in Asia, Africa and South America. *Fasciola gigantica* is transmitted by lymnaeids of larger size of the *Radix* group (Bargues and Mas-Coma, 2005). The presence of lymnaeid vectors defines not only the distribution of fascioliasis, but may also explain the distribution of human infection within a country, as has been recently observed in different countries (Artigas *et al.* 2011; Bargues *et al.* 2011b, c), and within an endemic area, as well as its seasonality or permanent transmission (Bargues *et al.* 2012). In southern Asia, *G. truncatula* is restricted to the highlands in countries such as Afghanistan and Pakistan (Kendall, 1954, 1965) and its absence as well as the lack of appropriate *Galba*/*Fossaria* lymnaeid species for the development of *F. hepatica* in India and eastward up to South-east Asia is well known (Mas-Coma *et al.* 2009a). In Bangladesh, *G. truncatula* has never been

found, nor does the country have temperatures below 15°C (except for about 10 days a year), which indicates a scenario not appropriate for the transmission of *F. hepatica*. Consequently, it may be perhaps better to consider that the forms identified as *F. hepatica*-like could indeed correspond to the extreme values of the morphometric distribution of the *Fasciola* sp.-like specimens.

Previous studies have shown the presence of morphologically intermediate forms in geographically sympatric areas of *F. hepatica* and *F. gigantica* in Africa and Asia. In Africa, the presence of intermediate forms has been morphometrically evidenced by means of the CIAS methodology in Egypt (Periago *et al.* 2008). In Asia, a varied spectrum of morphological forms of fasciolids has been described based on traditional microscopic measurements in several countries, such as India (Varma, 1953), Japan (Watanabe, 1962; Terasaki *et al.* 2000), Korea (Chu and Kim, 1967), the Philippines (Kimura *et al.* 1984) and Thailand (Srimuzipo *et al.* 2000), and more recently by CIAS also in Iran (Moghaddam *et al.* 2004; Ashrafi *et al.* 2006, 2015) and Pakistan (Asfhan *et al.* 2013).

The present phenotypic study by the CIAS method on fasciolids infecting bovines demonstrates, for the first time in Bangladesh, the presence of intermediate forms (here noted as *Fasciola* sp.-like forms) and *F. hepatica*-like forms, besides the *F. gigantica*-like forms always reported in the country as simply *F. gigantica*. Interestingly, and agreeing with these results, fascioliasis caused by a shorter strain of *F. gigantica* has been sporadically described in Bangladesh (Robertson, 1976; Nooruddin and Islam, 1996). An additional study of liver flukes from livestock by means of PCR-RFLP of nuclear rDNA ITS-1 and short fragment (535 bp) sequences of the mtDNA *nad*1 also detected a heterogeneity in the liver flukes from that country, when finding that (i) 29 out of 127 aspermic flukes presented a combination of ITS-1 sequences of *Fasciola gigantica* and *Fasciola hepatica*, and (ii) all flukes presented *nad*1 sequences identical to aspermic *Fasciola* sp. from other Asian countries (Mohanta *et al.* 2014)

The increasing recent prevalences found in livestock, the proved impact of climate change on Asian latitudes such as those of Bangladesh, and the risk for human infection due to the aforementioned climate change effects on the disease transmission as already reported in the similar situation in Pakistan, suggest a worrying scenario. Further studies on the transmission and epidemiology of the disease in humans as well as animals are already under way to obtain the baseline on which to establish appropriate control measures against fascioliasis in Bangladesh.

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