FASCIOLOSIS IN PAKISTAN: ADULT AND EGG PHENOTYPING, RISK MAPPING AND SEROLOGICAL STUDIES IN SUB-TROPICAL PUNJAB

KIRAN AFSHAN
05-arid-343

Department of Zoology
Faculty of Sciences
Pir Mehr Ali Shah
Arid Agriculture University, Rawalpindi
Pakistan
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FASCIOLOSIS IN PAKISTAN: ADULT AND EGG PHENOTYPING, RISK MAPPING AND SEROLOGICAL STUDIES IN SUB-TROPICAL PUNJAB

by

KIRAN AFSHAN
(05-arid-343)

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Pakistan
2014
In the name of Allah, the Most Beneficent, the Most Merciful
CERTIFICATION

I hereby undertake that this research is an original one and no part of this thesis fall under plagiarism. If found otherwise, at any stage, I will be responsible for the consequences.

Name: Kiran Afshan          Signature: ______________________
Registration No: 05-arid-343    Date: ______________________

Certified that the contents and form of thesis entitled “Fasciolosis in Pakistan: Adult and Egg Phenotyping, Risk Mapping and Serological Studies in Sub-Tropical Punjab” submitted by Ms. Kiran Afshan have been found satisfactory for the requirement of degree.

Supervisor: _______________________  (Prof. Dr. Mazhar Qayyum)

Member: ________________________  (Prof. Dr. Mirza Azhar Beg)

Member: ________________________  (Prof. Dr. S. M. Saqlan Naqvi)

Chairman, Department of Zoology: ________________________________

Dean, Faculty of Sciences: ________________________________

Director, Advanced Studies: ________________________________
Dedicated

To My Beloved Father

Mr. Ghazanfar Ali
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<tbody>
<tr>
<td>A-VS</td>
<td>Distance between the anterior end of the body and ventral sucker</td>
</tr>
<tr>
<td>BA</td>
<td>Body area</td>
</tr>
<tr>
<td>BL</td>
<td>Body length</td>
</tr>
<tr>
<td>BL/BW</td>
<td>Body length over body width</td>
</tr>
<tr>
<td>BL/VS-P</td>
<td>Body length over the distance between the ventral sucker and the posterior end of the body</td>
</tr>
<tr>
<td>BP</td>
<td>Body perimeter</td>
</tr>
<tr>
<td>BR</td>
<td>Body roundness</td>
</tr>
<tr>
<td>BW</td>
<td>Maximum body width</td>
</tr>
<tr>
<td>BWOv</td>
<td>Body width at ovary level</td>
</tr>
<tr>
<td>BWOv/CW</td>
<td>Body width at ovary level over cone width</td>
</tr>
<tr>
<td>CF</td>
<td>Conical Factors</td>
</tr>
<tr>
<td>CFI</td>
<td>First Conical Factors</td>
</tr>
<tr>
<td>CFII</td>
<td>Second Conical Factors</td>
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<tr>
<td>CIAS</td>
<td>Computer image analysis system</td>
</tr>
<tr>
<td>CL</td>
<td>Cone length</td>
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<tr>
<td>CS</td>
<td>Centroid size</td>
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<tr>
<td>CW</td>
<td>Cone width</td>
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<tr>
<td>EA</td>
<td>Egg area</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EL</td>
<td>Egg length</td>
</tr>
<tr>
<td>EL/EW</td>
<td>Egg length over egg width</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>EP</td>
<td>Egg perimeter</td>
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<tr>
<td>ER</td>
<td>Egg roundness</td>
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<tr>
<td>ESAs</td>
<td>Excretory secretory antigens</td>
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EW  Egg width
GDP  Gross domestic product
Mab  Monoclonal antibodies
MET  Mean environmental temperature
MEv  Mean evaporation
MMT  Mean maximum temperature
MmT  Mean minimum temperature
MSH  Mean sunshine hours
MSR  Mean solar radiation.
NDVI  Normalized difference vegetation index
OD  Optical density
OS max  Maximum diameter of oral sucker
OS min  Minimum diameter of oral sucker
OSA  Oral sucker area
OSA/VSA  Oral sucker area over ventral sucker area
OS-VS  Distance between the oral sucker and ventral sucker
PC  Principal component
PCI  First principal component
PCII  Second principal component
PhA  Pharynx area
PhL  Pharynx length
PhW  Pharynx width
PI  Postinfection
RAIN  Precipitation
rDNA  ribosomal DNA
RH  Relative humidity
TA  Testicular space area
TL  Testicular space (taking both testes together) length
<table>
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<tr>
<td>TP</td>
<td>Testicular space perimeter</td>
</tr>
<tr>
<td>TW</td>
<td>Testicular space width</td>
</tr>
<tr>
<td>Vit-P</td>
<td>Distance between the union of the vitelline glands and the posterior end of the body</td>
</tr>
<tr>
<td>VS max</td>
<td>Maximum diameter of ventral sucker</td>
</tr>
<tr>
<td>VS min</td>
<td>Minimum diameter of ventral sucker</td>
</tr>
<tr>
<td>VSA</td>
<td>Ventral sucker area</td>
</tr>
<tr>
<td>VS-P</td>
<td>Distance between the ventral sucker and the posterior end of the body</td>
</tr>
<tr>
<td>VS-Vit</td>
<td>Distance between the oral sucker and the union of the vitelline glands</td>
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<td>Wb-bs</td>
<td>Water budget based system</td>
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Layyah; 15 = Kot Addu; 16 = Farooquia; 17 = Dunya Pur; 18 = Muzaffargarh; 19 = Lodhran; 20 = Jalalpur.

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To my beloved father
Memories of my father still linger in my mind.…
My father has always been a hero of mine………
He set good examples that I can follow………
I value them today and continue to value them tomorrow…..

KIRAN AFSHAN
ABSTRACT

Fasciolosis also known as fascioliasis is a highly pathogenic liver infection caused by *F. hepatica* and *F. gigantica*. The study was aimed to use morphological markers for identification of fasciolids, generation of risk maps and use of rapid and cost effective diagnostic tool to reduce the impact of fasciolosis on animal health. The phenotypic features of adults and eggs of fasciolid species infecting buffaloes from central Punjab area, Pakistan, have been studied to characterize the fasciolid populations involved. Morphometric analyses were made by applying standardized measurements with a computer image analysis system (CIAS). The current investigation is first time conducted in Pakistan to confirm the taxonomic status of fasciolids by comparing with other pure standard populations viz., *F. hepatica* of European Mediterranean origins and *F. gigantica* representing Burkina Faso (Africa). In these geographical areas there is no overlapping of both fasciolids species. Only parasites obtained from bovines were employed. The climatic factors influencing fascioliasis presence and potential spread were analyzed from five meteorological stations during 1990-2010. The fascioliasis forecast risk Mt and Wb-bs (Water-Budget-Based System) indices and NDVI (Normalized Difference Vegetation Index), known to be useful for fascioliasis assessment, were obtained and correlated with geographical distribution, seasonality patterns and two-decade evolution of fascioliasis in livestock throughout the province. These two climatic forecast indices and a remote sensing marker are used to characterize the climatic factors and the earth surface in order to ascertain the epidemiological complexity and time-lag dynamics of fascioliasis. The seroprevalence of fascioliasis was also determined in sub-tropical Punjab with the application of a very sensitive and
specific ELISA test by using monoclonal antibodies which are able to detect even very low intensity infection. The MM3 Sero-ELISA was applied to check the status of fascioliasis. The increase of disease transmission risk in the lowlands should be highlighted, given that the largest part of the Punjab province includes low altitude, highly irrigated plains. The importance of livestock in this province makes this phenomenon to be given forecast priority assessment henceforth in order to establish the adequate control measures. The use of cost effective diagnostic tools would be helpful to reduce the impact of fascioliasis on animal health by selecting the appropriate anthelmintic treatment. An annual treatment scheme to effectively control the disease is finally recommended to be applied throughout the whole Punjab province.
Chapter 1

INTRODUCTION

Livestock rearing is a common practice of human beings in early times, served to them by making their lives productive and secure. In Pakistan mostly farmers have small flock holdings and livestock rearing is a vital part of the rural economy, which in return contributes significantly to national gross domestic product (GDP). Domesticated ruminants are the major source of milk and meat productions. Livestock is the main source of animal protein for local human population in Pakistan. Furthermore it also generates income for resource poor farmers and thus play key role in poverty alleviation for rural masses (Hasnain and Usmani, 2006).

Pakistan is rich in livestock diversity based on small ruminants and large ruminant inhabits various agro-ecological zones of Pakistan. Buffaloes (*Bubalus bubalis*) of Pakistan are mainly kept for milk production with beef as an important by-product (Khan, 2003; Bilal *et al.*, 2006). The famous buffalo’s breeds are Nili-Ravi and Kundi that are confined in Punjab and Sindh provinces. There are three types of cattle breeds (*Bos indicus*) found in Pakistan i.e. dairy (Sahiwal, Red-Sindhi and Cholistani), dual purpose (Tharparkar and Kankrej) and draught type (Bhagnari, Dajal, Dhanni, Lohani and Rojhan). These cattle are generally kept for draught purposes with beef as a by-product (Shah, 1994; Aslam *et al.*, 2002). Livestock population of Pakistan contains 32.7 million buffaloes, 36.9 million cattle, and livestock contributed approximately 11.6 percent to national GDP (GOP, 2012).
There is the number of reasons that triggers low productivity in domesticated animals in Pakistan. Among these infectious diseases is of prime important contributing factor. Presently the livestock production system in the country is dominated by subsistence and small-holdings, the farmers give more importance to diseases than other factors which low productivity in livestock including nutrition, breeding and reproduction. In international market the increasing demand of livestock and its products makes the infectious diseases of extreme importance, as they affect the livestock production system, and can be transmitted through contact with air, feed, water and vectors.

Pakistan is semi-tropical country which provides most favorable environmental conditions for survival and propagation of wide variety of parasitic organisms. The parasitic diseases are of prime importance, causes low productivity in livestock, usually ignored by the livestock owners due to low mortality with parasitic infections. Among parasitic diseases fascioliasis is an important limiting factor in livestock of Pakistan, thus its treatment and control is of extreme important to increase the productivity of livestock.

1.1 FASCIOLIASIS

Fasciolosis also described as fascioliasis is a plant-borne health hazard resulted by two helminths namely *Fasciola hepatica* and *Fasciola gigantica*. *F. hepatica* is prevalent in all over the world mainly in temperate areas of Europe, Americas and Australia; whereas *F. gigantica* is restricted to tropical countries and the geographic distribution of these fasciolids species co-exist in several parts of
Africa and Asia (Mas-Coma et al., 2005). Both fasciolid species have similar life cycle with the involvement of two hosts. The final host spectrum is wide, which parasitizes many herbivores ranging from mammals to humans. The freshwater lymnaeids serve as an intermediate hosts, in which some of the developmental stages of parasites occurred (Torgerson and Claxton, 1999).

1.2 ETIOLOGY

The taxonomic status of *Fasciola* is following:

<table>
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The fasciolids have life like body and *F. gigantica* differentiated on the basis of general appearance having narrow and elongated body while *F. hepatica* are broad and short in length with curved body in shape of lancet (Kendall, 1965). The adult flukes parasitized the bile duct of the liver or gallbladder. In Japan mitochondrial analysis indicated the existence of diploid, triploid and chimeric flukes referred as *Fasciola* species and reproduced by parthenogenetically (Sakaguchi, 1980; Itagaki and Tsutsumi, 1998). The adult stage of both fasciolids,
of relative large size, with a body length/width of up to 29/14 mm in *F. hepatica*, and 52/12 mm in *F. gigantica*. Recently in phenotypic studies use of computer image analysis system (CIAS) is helpful to make differentiate between both fasciolid species, which is not possible with traditional microscopic methods. The criteria used most recently for differentiation into *F. hepatica* and *F. gigantica* is 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* (Periago, *et al.* 2006).

The egg of *Fasciola* are operculated, large and broadly ellipsoidal, size range found in the literature for domestic animal hosts: *F. hepatica* 130-148/60-90 µm, 130-145/70-90 µm or 130-150/63-90 µm and *F. gigantica* 150-196/90-100 µm (Boray, 1982; WHO, 1991; Mas-Coma and Bargues, 1997). The standard borderline measurement differentiating two fasciolids was found to be 150 µm in length and 90 µm in width. Therefore it is anticipated that, lower values indicating *F. hepatica* while higher values *F. gigantica* (Valero *et al.*, 2009).

### 1.3 Intermediate Snail Hosts

The transmission and spread of fasciolid infection is related to specificity of fasciolid species at the snail intermediate host level. The liver flukes larval stages are species specific for freshwater snails belong to the family Lymnaeidae. The lymnaeid snails of the genus *Radix* are mainly infected by *Fasciola gigantica*, while *Fasciola hepatica* infects the species of *Galba/Fossaria* group. The American lymnaeid species *Pseudosuccinea columella* have also documented to transmit fasciolid infection (Bargues *et al.*, 2001). In Egypt, snails belongs to
family Planorbidae of the species *Biomphalaria alexandrina* were found parasitized with *F. gigantica* but this finding still needs further confirmation (Farag and El Sayad, 1995). The *F. hepatica* has developed ability to co-exist with local lymnaeid species ensuring its spread in the Americas, Asia, Hawaii, Papua New Guinea, Philippines, Japan, Australia, and New Zealand (Mas-Coma and Bargues, 1997; Bargues *et al.*, 2001). On the other hand the *F. gigantica* have restricted geographical distribution due to limited specificity for their intermediate snails, the African *Radix natalensis* and the Eurasian *R. auricularia* (Mas-Coma *et al.*, 2005).

### 1.4 LIFE CYCLE

The fasciolid species require two hosts for completion of its life cycle (Figure 1) and presenting a wide spectrum to definitive mammalian host, mainly ruminants especially cattle, sheep and buffalo (Boray, 1982; Mas-Coma and Bargues, 1997). The intermediate host are freshwater snails in which developmental stages of parasites occurs.

Eggs are released in the faeces of definitive host, undergoes development and hatch out into a free living miracidia. Miracidia have a short life span and penetrates suitable intermediate snail host within 24 hours (Thomas, 1883; Hope Cawdery *et al.*, 1978). Inside the body of infected snail, parasite reproduces asexually and develops sporocysts, redial stages and cercaria. The cercariae emerge from the body of snail and attach themselves to aquatic plants and encyst as metacercariae. The metacercariae are ingested by the ruminant, or by humans
eating uncooked foods such as watercress and infected liver (Markell and Voge, 1999; Rondelaud, 2000). The excystment of metacercariae occur in the duodenum and, juvenile flukes travel through the parenchyma and finally reside in the bile duct.

1.5 CLIMATE AND ENVIRONMENT

The variation in altitude, rainfall, temperature, animal husbandry practices at farm level, suitability of the external environment may involve in the dissemination of the parasite along with its intermediate host. The distribution pattern of fascioliasis in the given area is directly proportion to the availability of specific freshwater snails (Urquhart et al., 1996). The development of *Fasciola* in the snail is dictated by external temperature. The optimum temperature required for both fasciolids (*Fasciola hepatica* and *F. gigantica*) is found to be 10 °C and 16°C, respectively (Malone et al., 1998). The optimum temperature 25-27°C is needed for the development of *Fasciola* within the snail host. At low temperature the parasite reproduction will be effected (Howell, 2011). The moisture also effect the transmission of disease and survival of the eggs and metacercariae is high in moist, cool conditions. The perfect moisture conditions are prerequisite for the reproduction and development of both parasite and its freshwater snails. It has been observed these ideal conditions are achieved when rainfall surpasses transpiration rate leading to availability of surface water. These climatic conditions are prerequisite for the development of eggs and free living larval stages (Urquhart et al., 1996). The altitude is also considered as a limiting factor for the distribution of fascioliasis. The *F. gigantica* in Ethiopia (Africa) prefer to live at low altitudes of
1800 m and *F. hepatica* requires 1200 to 2560 m altitude above sea level. Both fasciolid species co-existing at 1200-1800 m altitudes (Yilma and Malone, 1998).

### 1.6 GEOGRAPHIC DISTRIBUTION

Fascioliasis has worldwide distribution, infecting human and animals (Torgerson and Claxton, 1999) and propagates in regions where environments are encouraging for the survival of freshwater lymnaeids. The fascioliasis occurs in areas having high cattle and sheep production.

#### 1.6.1 Human Fascioliasis

Several studies have been conducted throughout the world on human fascioliasis (Chen and Mott, 1990). The human fascioliasis has been reported in 51 countries (Esteban *et al.*, 1998). Previously it was thought that human infection is only sporadic in areas endemic for animal fascioliasis but it has been reported that human fascioliasis is endemic to these areas (Mas-Coma *et al.*, 1999a). The high prevalence rates recorded for human fascioliasis in endemic areas are independent or do not related to high animal prevalences (Mas-Coma *et al.*, 1999c). The worldwide estimates of human fascioliasis increased from 2,000 cases (Chen and Mott, 1990) to 2.4 million (Rim *et al.*, 1994; WHO, 1995). Most recently it has been reported that 17 million individuals of the world are infected with fascioliasis (Hopkins, 1992) or might be more are at even high risk because of lack of knowledge about the situation in African and Asian countries (Mas-Coma, 2004a). In Asia the frequency of fascioliasis is high in (Gilan) Iran (Mas-Coma *et al.*, 1999).
Figure 1: Life cycle of *Fasciola* spp. showing different developmental stages with the courtesy of Bob Hanna, 2003.
1.6.2 Animal Fascioliasis

Fascioliasis is of an important veterinary problem throughout the world, inflicting countless economic losses to livestock industry, predominantly in ovine and bovine (Keyyu et al., 2005; Menkir et al., 2007). Most of the studies were carried out on the prevalence of Fasciola in cattle. In tropical developing countries prevalence rates reported to be (28.4-78.0%) through examining faecal and liver examinations (Anderson et al., 1999; Phiri et al., 2005, 2006; Keyyu et al., 2006).

In United Kingdom and Ireland economic losses, due to fascioliasis, are more than 18 £ million every year (Mulcahy and Dalton, 2001). Bovine fascioliasis in Switzerland caused the economic losses attributed to be €52 million per year (Schweitzer et al., 2005). In Kenya liver condemnations in slaughtered cattle are up to 0.26 million US$ per annum (Kithuka et al., 2002), whereas 100% liver condemnation rates are reported in Tanzania (Keyyu et al., 2006).

1.7 PATHOLOGY AND SYMPTOMATOLOGY

The metacercariae after ingestion in the body of final host passes through parenchymal and biliary phase (Dubinsky, 1993). In parenchymal phase excystment metacercariae take place in small intestine and juvenile penetrates the intestine or invade the abdominal cavity and finally reside the liver (Chen and Mott, 1990; Boray, 1969). During migration phase in the liver and associated tissues it causes damage and inflammation. In the biliary phase the parasites get entry into bile ducts, where the flukes mature, feed, reproduce and cause tissue damage and hypertrophy of the ducts.
The signs and symptoms observed in animals due to fascioliasis are related to intensity of metacercariae ingested. The sheep, cattle, buffalo and goat are categorized as the most common definitive hosts. The clinical manifestation is classified into four types (Dubinsky, 1993; Behm and Sangster, 1999). The Acute Type I Fascioliasis occur with ingestion of more than 5000 metacercariae. The sudden death of the host is observed when metacercariae infestation rate is high. The clinical signs commonly observed in hosts are lethargy, ascites and abdominal haemorrhage. In Acute Type II Fascioliasis the infestation of metacercariae is 1000-5000, most frequent signs are animal die showing pallor, loss of conditions and ascites. The Subacute Fascioliasis occurs when animal ingested 800-1000 metacercariae. The most dominant signs are weight loss, animal become anemic, lethargic and may die. The Chronic Fascioliasis is mostly asymptomatic and it may develop ascites and bottle jaw. The infestation rate is low with 200-800 metacercariae. The weight loss is also commonly observed. In all types of fascioliasis the most commonly observed signs in blood are anaemia, hypoalbuminemia, and eosinophilia (Behm and Sangster, 1999), the enzymatic activities of certain enzymes also increased in case of subacute and chronic fascioliasis (Sykes et al., 1980; Anderson et al., 1981). The damaged liver tissue sometime co-infected with bacteria, most commonly Clostridium novyi causes black disease, which is not curable and animals die quickly.

1.8 FASCIOLIASIS RESISTANCE

Several studies have been conducted on different animal species to investigate the mechanism of resistance against fascioliasis. It has been
documented that cattle acquired resistance against both fasciolid species when they are immunized (Haroun and Hillyer, 1986). In rats the resistance against fascioliasis is also well documented (Milligen et al., 1998). However, sheep and goat are not resistant to re-infection with fasciolids (Chauvin et al., 1995; Moreno et al., 1997). The reports about development of resistant against fascioliasis are still not available.

1.9 ECONOMIC SIGNIFICANCE

Fascioliasis caused huge economic losses in livestock resulted due to animal mortality, morbidity, reduced weight gain, condemnation of liver and predisposed animals to secondary infections (Malone et al. 1998). The economic losses recorded for cattle are contamination of livers which have high market value, and production losses especially reduction in weight gain (Phiri et al., 2006). The huge production losses in sheep occurred due to sudden animal deaths, poor growth rate and poor quality wool production (Sinclair, 1962; Roseby, 1970).

In acute fascioliasis (Chick et al., 1980; Spithill et al., 1997; Schweizer et al., 2005) animals become anaemic due to blood sucking and approximately 0.2-0.5 ml blood per day per animal (Dawes and Hughes, 1970). The heavy blood loss decrease the total proteins may lead to hypoalbuminaemia (Soulsby, 1987). The chronic fascioliasis causes decrease in growth rate, feed and wool production (Oakley et al., 1979). The reduction in quantity and quality of milk due to fascioliasis is well documented, and a 14 percent decrease in milk yields was recorded (Ross, 1970). However, fascioliasis in cattle causes negligible effects on
milk yield (Dargie, 1987; Whitehead, 1976; Castagnetti et al., 1982; Hope Cawdery, 1984). In general fascioliasis causes massive economic losses to the agriculture budget of 200 million US$ per annum (Ramajo et al., 2001). The estimated economic losses due to fascioliasis was over 3.2 billion US$ annually all over the world (Spithill et al., 1999).

1.10 DIAGNOSIS

1.10.1 Humans

The human fascioliasis is diagnosed by applying various techniques such as parasitological examination and immunodiagnostic techniques. The fecal examination is most important in epidemiological studies and for post treatment monitoring. Immunological testing is being applicable throughout the course of infection and concern with the detection of circulating antibodies, circulating antigens and immune complexes. The non-invasive diagnostic techniques namely x-rays, use of radioisotope scanning, ultrasound, computed tomography (CT) and magnetic resonance imaging (Esteban et al., 1998; Hillyer, 1999).

Recently purified excretory/secretory antigens or recombinant molecules are used to improve sensitivity and specificity of serological assays. The adult and immature fasciolids secreted cysteine proteinases (Law et al., 2003) which are extremely antigenic in small and large ruminants (Cornelissen et al., 2001; Neyra et al., 2002) and humans (Cordova et al., 1997). A new ultrasensitive capture ELISA seems promising for both immunological and coprological antigen detection (Mezo et al., 2003, 2004).
The discrimination between *F. hepatica* and *F. gigantica* cannot possibly be performed with coprological, pathological and immunological methods. Most recently PCR-RFLP was described to differentiate both fasciolid species (Marcilla *et al*., 2002; Huang *et al*., 2004).

### 1.10.2 Animals

In animals fascioliasis are usually diagnosed with coprological examination and by using immunological testing most commonly with ELISA. The faecal examination is most commonly used technique which detects fluke eggs in faeces 8-12 weeks of post-infection. However the technique is not useful for early detection of infection. The biochemical and haematological profiling is also taken into account (Torgerson and Claxton, 1999; Graczyk and Fried, 1999). In most of the countries the incidence of fascioliasis are recorded at abattoir by meat inspection (Khaitsa, 1994). The immunological techniques are applied for early diagnosis of infection after 2-4 week post-infestation (Zimmerman *et al*., 1982; Dumenigo *et al*., 2000).

### 1.11 TREATMENT

#### 1.11.1 Humans

Triclabendazole treatment is given at rate of 10-12 mg/kg body weight in case of human fascioliasis (Savioli *et al*., 1999). The drug is active against both adult and juvenile flukes. The adverse reactions following treatment are usually mild for short duration. The nitazoxanide and bithionol are also applied...
successfully against fascioliasis (Rossignol et al., 1998; Ramachandran, 2000). The newly used drug of choice registered in Egypt, myrrh (Mirazid®) (Massoud et al., 2001), need further investigations for future use. Most recently the artemisinin derivatives showed high fasciolicidal activity (Keiser, 2006).

1.11.2 Animals

Presently a verity of drugs has been applied for treatment of animal fascioliasis and triclabendazole is most commonly used drug of choice. The long term use of this drug in different countries has developed resistance in animals against the drug (Moll et al., 2000). The newly developed drug called 'Compound Alpha', chemical composition similar to triclabendazole was proofed effective against fascioliasis (Ibarra et al., 2004).

1.12 CONTROL

In human fascioliasis the preventive and control strategies are same as suggested for veterinary fascioliasis (Roberts and Suhardono, 1996; Spithill et al., 1999; Torgerson and Claxton, 1999). For effective control strategies recommendations are prepared for many tropical and sub-tropical regions on the basis of temperature and rainfall patterns (FAO 1994). These control measures includes strategic anthelmintic treatment to eliminate the number of fluke in the host at the most appropriate time for effective prevention of pasture contamination (Fabiyi, 1987), the intermediate host snail population reduction by drainage and other agricultural practices, efficient farm and grazing management to reduce the
chances of infection (Keyyu et al., 2005 and 2006).

1.13 STUDY RATIONALE

Fascioliasis has been documented in all parts of Pakistan by using traditional parasitological protocols but no comprehensive study based on standardized methods with advanced techniques have ever been applied. Preliminary studies carried out on phenotyping of fasciolids in Pakistan were performed with traditional microscopic methods and results were not compared with pure standard of fasciolids. The current study new approach, namely Computer Image Analysis System (CIAS) is used to measure the intraspecific variations in adult fasciolid and eggs by taking into account standardized measurements proposed by Valero et al. (2005) and Periago et al. (2006, 2008). The CIAS technique is helpful in phenotypic characterization of fasciolid adults and eggs. The CIAS is convenient for fasciolids, as many structures show complex morphology such as reproductive organs and ceca.

The climatic factors have very crucial role in the transmission of fascioliasis. The occurrence of disease has been related to many environmental factors such as temperature, precipitation and evapo-transpiration rate. Forecast indices based on climatic factors have been effectively used for the forecasting of animal and human fascioliasis in the world. Previously very few studies in Pakistan were conducted to find the correlation of infection with climate but no one worked on forecasting the risk of fascioliasis by applying mathematical models and remote sensing. The current study was design to devise climatic indices and remote
sensing for forecasting fascioliasis risk values in Punjab, Pakistan.

The presence of permanent water bodies and irrigation system in some areas of northern and central Punjab propagates the survival and dissemination of snail intermediate host throughout the year inspite of seasonality. Previously different parasitological techniques were used for screening of animal fascioliasis, which is only possible 8-12 weeks of post infection. Few studies were conducted on serology of animals but current study applied the monoclonal antibody (Mab) MM3 Sero-ELISA for screening fascioliasis, which is most sensitive and specific test. This technique was applied first time in northern and central Punjab, Pakistan for early diagnostic of bovine fascioliasis. As the climate condition of the area favors the transmission of infection there is a dare need to screen out animal fascioliasis during early stages to obtain the exact situation of disease in area.

The study was aimed to use morphological markers for identification of fasciolids, generation of risk maps and use of rapid and cost effective diagnostic tool. The application of standardized morphological markers for adult flukes and gravid eggs provided information about existence of fasciolids prevalent in Punjab, Pakistan. The forecasting was the first approximation of potential impacts of climate changes on fascioliasis risk in Punjab, Pakistan. The information generated would be helpful in targeting fascioliasis in order to mitigate the impact of fascioliasis on animal health. The present study also provided most reliable and state-of-the-art diagnostic technique for screening large number of animals at an affordable price in Pakistan.
Keeping in view the significance of fascioliasis the current study was designed with following hypothesis.

“Phenotypic variations and epidemiological pattern of fasciolids may be affected under prevailing climatic conditions of sub-tropical Punjab, Pakistan.”

The objectives of present study were:

- Phenotypic characterization of fasciolids adults/eggs from the buffalo of central Punjab Pakistan by applying computer image analysis system (CIAS).
- Development and analysis of risk mapping by using climatic data and forecast indices to mitigate the impact of fasciolosis in sub-tropical Punjab, Pakistan.
- Detection of anti-*Fasciola* IgG antibodies by using monoclonal antibodies (Mab) based MM3 Sero-ELISA in cattle and buffalo of sub-tropical Punjab, Pakistan.
Chapter 2

PHENOTYPIC CHARACTERIZATION OF FASCIOLIDS ADULTS/EGGS FROM THE BUFFALO OF CENTRAL PUNJAB PAKISTAN BY APPLYING COMPUTER IMAGE ANALYSIS SYSTEM (CIAS)

2.1 INTRODUCTION

Fascioliasis is food-borne helminth infection occurred due to trematode of the genus *Fasciola* (Trematoda: Fasciolidae) *F. hepatica* and *F. gigantica* (Mas-Coma, 2005; Ashrafi *et al*., 2006a). The geographical dissemination of both these fasciolids coexists in several countries of Africa and Asia (Mas-Coma *et al*., 2005, 2009). The discrimination among both liver fluke species is of great significance but unfortunately there is no direct coprological method or nor an indirect immunological technique which make differential diagnosis between them (Valero *et al*., 2009b, 2012a, b). Presently diagnostic methods applied are only used to discriminate fascioliasis from other parasitosis. Until now, the morphometrical characterization of fasciolid adults and eggs can only be applied for discrepancies among species or with molecular tools (Marcilla *et al*., 2002; Periago *et al*., 2006; Mass-Coma *et al*., 2009; Valero *et al*., 2009a, 2012c).

The overlapping distribution of *F. hepatica* and *F. gigantica* has generated debate on the taxonomic ranking of their intermediate form documented so far in Far East countries, where intermediate forms are present along with their resemblance either with *F. hepatica* and *F. gigantica* like. The phenomena of hybridization and abnormal gametogenesis between different genotypes are well
documented in fasciolids which supported the existence of different intermediate forms (Mas-Coma and Bargues, 1997; Mas-Coma et al., 2009). Numerous comprehensive studies on fasciolids validated that both *F. hepatica* and *F. gigantica* should be considered as distinct species, regardless of their ability to crossbreed and give rise to intermediate forms in overlapping areas (Mas-Coma et al., 2009).

The phenotypic description has been regularly applied in systematic studies on fasciolid species. The differences between both species are mostly highlighted as increased length, body narrow and elongated of *F. gigantica* as compared to *F. hepatica* having short, broad and curved body in the shape of lancet (Kendall, 1965). Some scientist reported that both these species are differentiated on the basis of cuticular scales, branching of intestine and reproductive structures (Jackson, 1921; Varma, 1953; Watanabe, 1962; Bergeon and Laurent, 1970).

In Asian countries, where both species coexist, it has become challenging task to differentiate both fasciolids based on their morphological features. Previously much of research were focused on geographical areas where liver flukes species overlap especially in Iran (Sahba et al., 1972), Philippines (Kimura et al., 1984), Thailand (Srimuzipo et al., 2000) and Egypt (Lotfy et al., 2002).

Pakistan is one of the Asian countries where both fasciolid species overlap, and fascioliasis is highly prevalent in livestock, including cattle, buffaloes, sheep and goats (Khan et al., 2010). The two fasciolid species are frequently reported (Maqbool et al., 1994, 2002; Siddiqi and Shah, 1984; Chaudhry and Niaz, 1984;
Masud and Majid, 1984; Khan et al., 2009). In Punjab province, Pakistan fascioliasis is of special concern in domesticated animals. It has been reported in several districts having 10.5 to 40.3 percent prevalence rate (Chaudhry and Niaz, 1984; Masud and Majid, 1984; Maqbool et al., 2002; Khan et al., 2009) and results indicate the presence of *F. gigantica* to be more widespread than *F. hepatica* in five Punjabi districts (Khan et al., 2009). Furthermore, human fascioliasis cases have also been recorded in rural areas of Lahore, central Punjab, Pakistan, posing greater public health problem (Qureshi et al., 2005).

Faced with the present situation, it is evident that further studies need to carry out on the transmission pattern of disease to find out the epidemiological status of fascioliasis in humans and animals. The present study aimed to provide phenotypic description of liver flukes infecting the buffalo of central Punjab, Pakistan. The studies carried out on standardized measurements of fasciolids are helpful in differentiating of both liver flukes (Periago et al., 2006). Therefore, a computer image analysis system (Valero et al., 2005) was used in present study to differentiate liver flukes of central Punjab. The definitive animal hosts is known to pronouncedly influence the phenotype of both adult worms and eggs because of microhabitat inside the duct (Valero et al., 2001a, 2002, 2009a). Therefore only fasciolids of bovine origin were used in this work. The current study was designed with following hypothesis.

“Phenotypic characterization could be used for species identification”

The objective of present study was:
2.2 REVIEW OF LITERATURE

Fascioliasis is parasitic disease of veterinary importance the discrimination between the etiological agents of disease is very complicated task in geographical areas where intermediate forms of these two species co-exist. Several studies have been conducted on phenotypic differentiation of fasciolid adults and eggs throughout the world. Lack of literature about standardized measurements of adults and eggs of fasciolid species in Asian countries, particularly in Pakistan has necessitated developing current morphological study on fasciolid adults and eggs measurements in Punjab province of Pakistan.

2.2.1 Geographical Distribution of Fasciolids

2.2.1.1 Fasciola hepatica

*F. hepatica* (Linnaeus, 1758) is common parasite of ruminants and other domestic animals, adults are mostly found in the bile duct and gall bladder of the animals. Geographical distribution comprises of temperate and subtropical zones of Europe; north, central and south America; northern and central Asia; Oceania; and northern, eastern and southern Africa; and islands of: New Zealand, Tasmania, the United Kingdom, Iceland, Cyprus, Corsica, Sardinia, Sicily, Japan, Papua New Guinea, the Philippines and several islands of the Caribbean (Boray, 1982; Mas-
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Coma and Bargues, 1997; Pantelouris, 1965).

2.2.1.2 *Fasciola gigantica*

*F. gigantica* (Cobbold, 1855) common parasite located in bile ducts and gall bladder of ruminants (Losos, 1986; Round, 1968). First time described from *Giraffa camelopardalis* in Sub-Saharan Africa (Cobbold, 1855) and later re-described from cattle in Senegal (Raillet, 1895). *F. indica* (Varma, 1953) was reported from the liver of *Bos indicus*, *Bubalus bubalus*, *Capra hircus* and *Sus cristatus* in India and later synonymized with *F. gigantica* (Kendall, 1965; Kendall and Parfitt, 1959; Sarwar, 1957). Geographically it is distributed in Africa, South Africa (Losos, 1986; Schillhorn Van Veen, 1980), and in tropical Asia, Southeast Asia, and the Pacific regions, including old Union of Soviet Socialist Republics (USSR; Tashkent, Uzbekistan, Turkmenia, Samarkand region), Iran, Iraq, China, Korea, Japan, India, Pakistan, Vietnam, Thailand, Laos and in the Pacific, Malaysia, Philippines and Hawaii. The *F. gigantica* infection is one of the most important menaces of the livestock populations of India, Pakistan, Indonesia, Indochina and the Philippines (Boray, 1982; Mas-Coma and Bargues, 1997).

2.2.2 Fasciolid Adults Phenotyping

The morphological phenotype of a living organism is an extremely complex and dynamic system. Previously morphological phenotyping of fasciolids was performed in different regions of world. Kimura *et al.* (1984) reported that morphoanatomic characteristics even in case of *Fasciola* species are difficult to analyze. The Mas-Coma *et al.* (1984) proposed standardized measurement criteria
for morphological characterization of adult brachylaimid trematodes. Later on all these standardized measurements were modified by Valero et al. (1996) for the Fasciolidae. Valero et al. (1999) conducted a morphological study on comparison of adult liver flukes and eggs from highland and lowland populations of Bolivian and Spanish sheep. The results of standardized measurements for morphological characterization of flukes and eggs indicated the existence of *F. hepatica* in Bolivian sheep. The measurements were made with computer linked to a stereomicroscope 3CCD colour video camera by using image analysis software. In the northern Bolivian Altiplano Valero et al. (2001) reported that the final host species decisively influences the size of the *F. hepatica* adults and eggs even within the same endemic area with the use of image analysis software. Moreover, findings indicated that in endemic areas in which *F. hepatica* and *F. gigantica* coexist, thorough studies on egg morphometry must be carried out.

The Valero et al. (2005) used computer image analysis system (CIAS) for the quantification of size and shape of *Fasciola hepatica* and *Fasciola gigantica* gravid adults and eggs for Europe and Africa. The computer image analysis system (CIAS) was also used by Periago et al. (2006) to quantify the different size and shape of *F. hepatica* and *F. gigantica* adults and eggs in allopatric populations from Europe and Africa. These findings may be useful for the identification of the species of *Fasciola* present in geographical areas where both species coexist.

In province of Gilan, Iran Ashrafi et al. (2006) used calibrated stereomicroscope and microscope for morphological characterization of fasciolids by using all standardized measurements and concluded that simple, traditional
microscopic measurements may be sufficient for phenotypic discrimination of fasciolids, even in areas where intermediate forms are present. The finding also indicated discriminating criteria (VS-P, BL/BW) in allopatric populations.

Periago et al. (2008) reported the presence of *F. hepatica*, *F. gigantica* and intermediate forms (*Fasciola* sp.) in Egypt and validate the usefulness of CIAS for the morphological characterization of fasciolid adults. The BR, BL/BW and VS-P proofs useful tool for finding inter- and intraspecific morphological variations in *Fasciola* adults. Valero et al. (2012) applied CIAS on the basis of standardized measurements to characterize *F. hepatica* infecting sheep present in human fascioliasis endemic areas of Cajamarca Valley and Mantaro Valley and the northern Bolivian Altiplano.

The fasciolid species are very similar to each other in their morphological forms and the discrimination between them is very difficult (Srimuzipo et al., 2000; Terasaki et al., 2000). Several studies have been conducted in countries where both fasciolid species overlap such as Iran, the Philippines, Thailand and Egypt for comparing both species (Sahba et al., 1972; Kimura et al., 1984; Srimuzipo et al., 2000; Lotfy et al., 2002). In Asian countries, especially Japan, Taiwan, Philippines, Korea and Pakistan a wide range of morphological types has been detected using traditional microscopic measurements (Watanabe, 1962; Oshima et al., 1968; Akahane et al., 1970; Kimura et al., 1984; Srimuzipo et al., 2000; Terasaki et al., 2000; Mufti et al., 2011). The specimens could not be discriminated clearly in all these studies because overlapping was obtained in the measurements. The overlapping in the measurements supported the phenomena of
abnormal gametogenesis, diploidy, triploidy and mixoploidy, parthenogenesis or facultative gynogenesis and hybridization events (Itagaki et al., 1998; Terasaki et al., 2000; Fletcher et al., 2004).

2.2.3 Fasciolid Eggs Phenotyping

Periago et al. (2006) reported the morphometric values of eggs somewhat overlap in areas of Europe and Africa but significant differences were recorded in egg length (EL), egg perimeter (EP) and egg area (EA) of the two species. The variations were observed in the size of *F. hepatica* eggs in livestock present in different geographical locations (Tinar, 1984), and in Asian countries where both liver fluke species exist, a large overlap of egg measurements have been recorded (Kimura et al., 1984; Sahba et al., 1972; Srimuzipo et al., 2000; Watanabe, 1962).

Valero et al. (2001) recorded the influence of host species on size of fasciolid eggs measurements and found significant differences between the different host species. The experimental studies also conducted and have shown that the size of *F. hepatica* eggs isolated from a given host changed when experimentally transferred to a different host species.

Itagaki et al. (2005) and Semyenova et al. (2006) confirmed the existence of morphologically intermediate forms between the two fasciolid species and genetic hybrids of both in overlapping areas, endemic for animals and humans in Africa (Periago et al., 2008) and Asia (Ashrafi et al., 2006; Le et al., 2008). The existence of these intermediate forms has been molecularly verified in these overlapping areas (Inoue et al., 2007). The question arises that whether egg
characteristics are suitable tool for the differentiation of fascioliasis caused by either species.

Valero et al. (2009) recorded that in endemic areas of Asia and Africa where both liver flukes species co-exist; human became simultaneously infected by both *F. hepatica* and *F. gigantica*. In such areas of fasciolids overlapping an appropriate coprological diagnosis is required for the measurements of large number of eggs, and shall not rely on only one or very few eggs. The measurement of large number of eggs allows assessing egg size variations and confirmation of existence of fasciolid species infecting the patient. Moreover, intermediate forms of eggs may produce in stools when co-infection with both fasciolid species was observed and they crossbred.

### 2.2.4 Present Study

The studies performed in various parts of the world on phenotyping of liver flukes adults and eggs are helpful in characterization of local strain of Pakistan. However, the liver flukes adults and eggs phenotypes are not clear in Pakistan especially in areas of central Punjab. In current study comprehensive phenotyping of liver flukes adult and eggs were made with the use of computer image analysis system (CIAS) which was not yet being applied before in Pakistan.

The study is unique in sense that no such study was conducted before in Pakistan based on CIAS for morphological characterization of liver fluke adults and eggs as previously traditional microscopic measurements were used which make impossible to calculate all standardized measurements proposed for fasciolid
adult and eggs. In current study the results were compared with standard populations of *F. hepatica* (Spain) and *F. gigantica* (Burkina Faso) from the areas where both species do not overlap published by Periago *et al.* (2006). Previously no such study was conducted in Pakistan which used the standard populations for comparison. As the literature showed that the type of host also influenced the size and shape of liver fluke adults and eggs, so the current study also used the same host to avoid such discrepancies. This study is an attempt to discriminate the fasciolid adults and eggs by using morphological markers.

### 2.3 MATERIALS AND METHODS

#### 2.3.1 Study Area

It comprises irrigated land with a well-established water canal system of the Indus river basin commonly categorized as central Punjab in Pakistan. The buffaloes originated from different districts of the central Punjab area, including the districts of Lahore, Faisalabad, Okara, Sahiwal and Jhang (Fig. 2). The rivers and canals of central Punjab provides suitable breeding grounds for freshwater snails serving as potential intermediate hosts for a variety of digenetic trematodes.

#### 2.3.2 Morphometry

An accurate morphometric study has been conducted to establish phenotypic differentiation of both *F. hepatica* and *F. gigantica*. The adult flukes parasitizing the buffalo were used to avoid both definitive host species and staining procedures biases. The eggs were analyzed without fixation and CIAS with
standardized methodologies for measurements of both eggs and adults were used (Valero et al., 1996, 2001, 2005).

### 2.3.2.1 Liver flukes collection

In September 2011, the post-mortem examinations of 19 buffaloes were conducted immediately after slaughtering to ascertain the presence of adult liver flukes. The adult worms found in bile ducts were collected with the help of rubber-coated forceps in order to avoid any structural damage. Only adult flukes having gravid uteri (n = 81) were included in the study. Fasciolid specimens providing the largest worm variability in their size, maturity and gravid uteri were used for characterization. Previously published data on *F. hepatica* (Spain) and *F. gigantica* (Burkina Faso) were used as standard or genetically pure populations (Periago et al., 2006; Mas-Coma et al., 2009).

### 2.3.2.2 Staining

Fasciolid specimens were fixed between slides with little pressure and placed in Bouin’s solution. The fasciolids were stained in Grenacher’s borax carmine (Appendix # 1) followed by dehydrated and mounted in Canada balsam.

### 2.3.2.3 Egg collection from adult liver fluke uteri

The eggs were collected from the distal part of the uteri of fully gravid flukes by putting slight pressure on acetabulum to squeeze out mature eggs via genital pore. More mature eggs were obtained by making small cut in front of
Figure 2: Map of Pakistan indicating the five districts of central Punjab from where the liver flukes infected buffaloes originated (darkly coloured).
acetabulum with the help of fine needles. In total, 345 eggs were included being fully mature, dark brownish and having well-formed shell walls. The comparison was made with data representing pure eggs isolated from two standard populations of Spain and Burkina Faso origin (Periago et al., 2006).

2.3.3 Morphometrical Measurements

The morphometric study was conducted to work out phenotypic differentiation of both *F. hepatica* and *F. gigantica*. The adult fasciolids prevalent in naturally infected buffaloes were used for the phenotypic differentiation. All measurements of adult worms and eggs were made according to a standardized methodology previously described (Valero et al., 2005, 2009a; Periago et al., 2006, 2008). After egg collection from the uteri of flukes, standardized measurements were taken using a microscope and images were captured with the help of digital camera (Nikon Coolpix) and further analyzed by image analysis software (ImagePro plus, Silver Spring, USA). All the measurements were presented in millimeters (mm) for adult worms, and in micrometers (µm) in the case of eggs.

2.3.3.1 Morphological characteristics of adult fasciolids

For adult fasciolids, the following standardized measurements were taken (Fig. 3):

A) **Lineal biometric characters:** body length (BL), maximum body width (BW), body width at ovary level (BWOv), body perimeter (BP), body roundness (BR), cone length (CL), cone width (CW), maximum diameter of
oral sucker (OS max), minimum diameter of oral sucker (OS min), maximum
diameter of ventral sucker (VS max), minimum diameter of ventral sucker
(VS min), distance between the anterior end of the body and ventral sucker
(A-VS), distance between the oral sucker and ventral sucker (OS-VS),
distance between the oral 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 (PhL), pharynx width (PhW), testicular
space (taking both testes together) length (TL), testicular space width (TW),
testicular space perimeter (TP);

**B) Areas:** body area (BA), oral sucker area (OSA), ventral sucker area (VSA),
pharynx area (PhA), testicular space area (TA);

**C) Ratios:** body length over body width (BL/BW), body width at ovary level
over cone width (BWOv/CW), oral sucker area over ventral sucker area
(OSA/VSA), and body length over the distance between the ventral sucker
and the posterior end of the body (BL/VS-P).

### 2.3.3.2 Eggs characteristics

For fasciolid eggs, the following standardized measurements were taken (Fig. 4):

**A) Lineal biometric characters:** egg length (EL), egg width (EW), egg
perimeter (EP), egg roundness (ER)

**B) Area:** egg area (EA)

**C) Ratios:** egg length over egg width (EL/EW).
2.3.3.3 Shape of adult liver flukes and eggs

Liver fluke body roundness (BR = BP²/4πBA) and egg roundness (ER = EP²/4πEA) were applied to measure the body and egg shapes, respectively. The criteria set for circular object is 1.00 for roundness, more than 1 for irregular objects (Anonymous, 2001).

2.3.3.4 Grouping criteria

The liver flukes collected from central Punjab, Pakistan were categorized on the basis of maximum and minimum values of morphological measurements of *F. hepatica*, *F. gigantica* or their intermediate forms described previously as a discriminating criteria (Periago *et al.*, 2006): BR: 1.06–1.58 for *F. hepatica*; 1.71–3.65 in case of *F. gigantica*; BL/BW: 1.29–2.80 in *F. hepatica*; 3.40–6.78 for *F. gigantica* and VS-P: 8.86–25.08 mm for *F. hepatica*; 26.28–50.09 mm in *F. gigantica*. The grouping was made with respect to BR and BL/ BW, and also on VS-P. Morphometric of adult *F. hepatica* follow a logistic growth model with reference to time (Valero *et al.*, 2001a, b, 2005). The morphometric maximum values are characteristic for each population (Table 1).

2.3.4 Statistical Techniques

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.
Multivariate analyses were applied to calculate the phenotypic variations among fasciolid adults, using size-free canonical discriminant analysis on 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 pooled within-group principal component (Dos Reis et al., 1990). The principal component analysis is used to summarize differences in a multivariate dataset in few dimensions (Dujardin and Le Pont, 2004). The log-transformation was done by base-10 logarithms and used to stabilize the data for obtaining linearity. The adult fasciolids morphometric analysis were conducted by using BAC v.2 software and the PAD (Permutaciones, Analisis Discriminante) which are module of the CLIC programme (http://www.npl.ird.fr/morphometrics) (Dujardin, 2002; Dujardin and Le Pont, 2004; Dujardin et al., 2010). The results were reflected 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, OS max, OS min, VS max, VS min, A-VS, VS-Vit, Vit-P, PhL, PhW and TL, where at least one dimension was measured among the most important morphological characters. The remaining variables were all significantly correlated with the first principal component (PC1), which contributed 72% to overall variations. PCI could therefore be accepted as a general indicator of size (Bookstein, 1989). The canonical discriminant analysis was also performed by using the software PAD V. 98 (Dujardin, 2010). Discriminant analyses excluding size were graphically represented along first two conical discriminant factors.
Figure 3: Standardized measurements of fasciolid adults described by Valero et al. (2005) and Periago et al. (2006, 2008): A) Fasciola hepatica; B) Fasciola gigantica.
Figure 4: Standardized measurements of fasciolid eggs.
2.4 RESULTS

The comparison of morphometric measurements of fasciolid adults and eggs from buffaloes from the central Punjab area, Pakistan, with standard populations of *F. hepatica* from Spain and *F. gigantica* from Burkina Faso previously measured (Periago *et al.*, 2006) is displayed in tables 1 and 2, respectively, showing their extreme values, mean and standard deviation.

Adult fasciolids from Pakistan were categorized based on BR, BL/BW and VS-P criteria into *F. hepatica*-like (3.7%), *F. gigantica*-like (22.2%) or *Fasciola* sp.-like (74.1%). The EL and EW of Pakistan *F. gigantica* and *Fasciola* spp are $158.46 \pm 7.92$, $92.31 \pm 5.22$ and $145.73 \pm 15.94$, $93.62 \pm 6.40$ respectively. These measurements are considered basic for eggs phenotyping and indicate the existence of intermediate forms of eggs in Pakistan. The application of these markers shows that the vast majority of specimens analyzed are intermediate forms (or *Fasciola* sp.-like). The morphometric measurements of adult fasciolids and eggs (Plate 1, 2 and 3) recorded from central Punjab, Pakistan are given in Table 3 and 4 with their extreme values, mean and standard deviation.

The multivariate analysis used to measure the changes in size of fasciolid adults from Pakistan and compared with the above-mentioned standard populations for each pure fasciolid species showed that the size of fasciolids from Pakistan lay between *F. hepatica* and *F. gigantica* standard populations. The samples were plotted against first (PCI) and second (PCII) principal components. The fasciolid adult variables from Pakistan, Spain and Burkina Faso were significantly (p<0.05)
correlated with PC1. The factor map of first two principal components (PC) is shown in Figure 5. The PCI and PCII contributed 72% and 14% size variations respectively. The size of Pakistan adult liver flukes population is located between the standard populations. The resulting scatter plot illustrates global size difference between fasciolids from Pakistan versus standard populations. These results confirm that intermediate forms of fasciolids exist in central Punjab.

The centroid size variation (Fig. 6) of fasciolid species were statistically significant (p<0.05) from smallest to largest among three groups of population: Spain < Pakistan < Burkina Fasso. Although it was observed that size of Pakistan fasciolids are intermediate, as it lay between the fasciolids of standard populations.

Size-free conical discriminant analyses of fasciolid adults from Pakistan were carried out along standard populations. The scatter plot of conical factors (CFI and II) showed that standard population from Spain and Burkina Faso are completely separated. Where, fasciolids population from Pakistan showed discriminant tendency from other two populations along CFI and CFII (Fig. 7). The small (close to 0) value of Wilks' lambda = 0.062, P<0.05 showed that the three populations are significantly discriminated although some overlapping of size was observed.

Similarly, the morphometric measurements of fasciolid eggs from central Punjab were between those of eggs from F. hepatica and from F. gigantica standard populations, also indicating the existence of intermediate forms of fasciolid eggs in Pakistan.
Plate 1: Mounted Fasciola spp. from central Punjab, Pakistan.

Plate 2: Mounted Oral Suckers (OS), Ventral Suckers (VS) and Pharynx (Ph) of Fasciola spp. collected from central Punjab, Pakistan.
Plate 3: Shape and size of *Fasciola* spp. eggs from uteri of adult liver flukes residing in the buffaloes of central Punjab, Pakistan.
Table 1: Comparative morphometric measurements data showing range, mean and standard deviation of adult fascioloid species from buffaloes of Pakistan with previously published data of pure populations from Spain and Burkina Faso described by Periago et al. (2006).

<table>
<thead>
<tr>
<th>Adult measurements (mm)</th>
<th>Pakistan Fasciola sp.</th>
<th>Burkina Faso Fasciola gigantica</th>
<th>Spain Fasciola hepatica</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>81</td>
<td>81</td>
<td>84</td>
</tr>
<tr>
<td>Body length (BL)</td>
<td>17.43 - 41.34, 29.09 ± 5.23</td>
<td>28.82-52.30, 39.72 ± 0.58</td>
<td>11.64-22.93, 17.41 ± 0.23</td>
</tr>
<tr>
<td>Body width (BW)</td>
<td>6.37 - 12.45, 9.64 ± 1.29</td>
<td>6.03-11.84, 8.45 ± 0.14</td>
<td>6.41-13.88, 10.02 ± 0.17</td>
</tr>
<tr>
<td>Body width ovary level (BWOv)</td>
<td>5.48 - 10.49, 7.83 ± 1.07</td>
<td>5.33-11.55, 7.59 ± 0.13</td>
<td>4.53-11.67, 7.90 ± 0.14</td>
</tr>
<tr>
<td>Body perimeter (BP)</td>
<td>43.42 - 96.87, 70.84 ± 11.40</td>
<td>63.19-113.71, 85.25 ± 1.21</td>
<td>28.58-54.40, 43.05 ± 0.55</td>
</tr>
<tr>
<td>Body roundness (BR)</td>
<td>1.53 - 2.65, 1.91 ± 0.21</td>
<td>1.71-3.65, 2.47 ± 0.05</td>
<td>1.06-1.58, 1.23 ± 0.01</td>
</tr>
<tr>
<td>Cone length (CL)</td>
<td>1.59 - 2.68, 2.16 ± 0.26</td>
<td>2.10-3.36, 2.67 ± 0.04</td>
<td>1.09-2.92, 2.02 ± 0.04</td>
</tr>
<tr>
<td>Cone width (CW)</td>
<td>2.59 - 4.37, 3.52 ± 0.37</td>
<td>2.23-5.08, 3.74 ± 0.06</td>
<td>2.35-4.21, 3.20 ± 0.04</td>
</tr>
<tr>
<td>Maximum diameter of oral sucker (OS max)</td>
<td>0.72 - 1.78, 1.00 ± 0.12</td>
<td>0.52-1.17, 0.83 ± 0.02</td>
<td>0.57-1.03, 0.85 ± 0.01</td>
</tr>
<tr>
<td>Minimum diameter of oral sucker (OS max)</td>
<td>0.32 - 1.62, 0.66 ± 0.16</td>
<td>0.21-0.95, 0.62 ± 0.02</td>
<td>0.44-0.77, 0.61 ± 0.01</td>
</tr>
<tr>
<td>Maximum diameter of ventral sucker (VS max)</td>
<td>1.08 - 1.88, 1.55 ± 0.13</td>
<td>0.87-1.92, 1.50 ± 0.02</td>
<td>0.92-1.49, 1.13 ± 0.01</td>
</tr>
<tr>
<td>Minimum diameter of ventral sucker (VS max)</td>
<td>0.61 - 1.66, 1.41 ± 0.14</td>
<td>0.80-1.83, 1.38 ± 0.02</td>
<td>0.86-1.35, 1.12 ± 0.01</td>
</tr>
<tr>
<td>Distance between anterior end of body and VS (A-VS)</td>
<td>0.97 - 2.48, 1.94 ± 0.29</td>
<td>1.46-3.01, 2.36 ± 0.03</td>
<td>1.12-2.92, 2.05 ± 0.04</td>
</tr>
<tr>
<td>Distance between suckers (OS-VS)</td>
<td>0.16 - 1.91, 1.29 ± 0.28</td>
<td>1.15-2.22, 1.71 ± 0.03</td>
<td>0.57-2.41, 1.42 ± 0.04</td>
</tr>
<tr>
<td>Distance between Vs and union of vitelline gland (VS-Vit)</td>
<td>10.71 - 26.60, 18.67 ± 3.59</td>
<td>12.26-34.11, 22.68 ± 0.45</td>
<td>6.57-14.31, 9.60 ± 0.17</td>
</tr>
<tr>
<td>Distance between Vit and posterior end of body (Vit-P)</td>
<td>1.38 - 15.32, 8.83 ± 2.26</td>
<td>8.97-21.43, 13.45 ± 0.32</td>
<td>2.63-7.57, 4.73 ± 0.10</td>
</tr>
<tr>
<td>Distance between VS and posterior end of body (VS-P)</td>
<td>15.88 - 39.57, 27.50 ± 5.30</td>
<td>26.28-50.09, 36.39 ± 0.59</td>
<td>9.51-19.94, 14.40 ± 0.22</td>
</tr>
<tr>
<td>Pharynx length (PL)</td>
<td>0.63 - 1.28, 0.93 ± 0.11</td>
<td>0.46-1.06, 0.78 ± 0.02</td>
<td>0.52-1.00, 0.70 ± 0.01</td>
</tr>
<tr>
<td>Pharynx width (PW)</td>
<td>0.34 - 0.75, 0.56 ± 0.09</td>
<td>0.23-0.68, 0.42 ± 0.01</td>
<td>0.26-0.83, 0.44 ± 0.01</td>
</tr>
<tr>
<td>Variable</td>
<td>Minimum - Maximum</td>
<td>Mean ± Standard Deviation</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-------------------</td>
<td>--------------------------</td>
<td></td>
</tr>
<tr>
<td>Testicular space length (TL)</td>
<td>7.80 - 22.26</td>
<td>13.79 ± 3.14</td>
<td></td>
</tr>
<tr>
<td>Testicular space width (TW)</td>
<td>3.71 - 7.64</td>
<td>5.68 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>Testicular space perimeter (TP)</td>
<td>22.48 - 59.63</td>
<td>37.29 ± 7.43</td>
<td></td>
</tr>
<tr>
<td>Body area (BA)</td>
<td>87.33 - 332.11</td>
<td>212.47 ± 54.59</td>
<td></td>
</tr>
<tr>
<td>Oral sucker area (OSA)</td>
<td>0.28 - 0.84</td>
<td>0.52 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Ventral sucker area (VSA)</td>
<td>1.03 - 2.46</td>
<td>1.75 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Pharynx area (PA)</td>
<td>0.21 - 0.64</td>
<td>0.40 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Testicular space area (TA)</td>
<td>22.41 - 123.46</td>
<td>62.10 ± 19.65</td>
<td></td>
</tr>
<tr>
<td>Ratio between BL and BW (BL/BW)</td>
<td>2.02 - 4.85</td>
<td>3.04 ± 0.53</td>
<td></td>
</tr>
<tr>
<td>Ratio between BWOv and CW (BWOv/CW)</td>
<td>1.66 - 2.88</td>
<td>2.24 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>Ratio between Suckers area OSA/VSA</td>
<td>0.17 - 0.44</td>
<td>0.30 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Ratio between BL ad VS-P (BL/VS-P)</td>
<td>1.01 - 1.12</td>
<td>1.06 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: The morphometric data with their ranges, mean and standard deviation values of mature eggs of fasciolids from Pakistan with data previously published by Periago et al. (2006) for pure populations from Spain and Burkina Faso.

<table>
<thead>
<tr>
<th>Egg measurements (µm)</th>
<th>Pakistan Fasciola species, n= 345</th>
<th>Spain F. hepatica, n=113</th>
<th>Burkina Faso F. gigantica, n=142</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg length, EL</td>
<td>115.07-186.68, 150.75±14.73</td>
<td>107.30-152.70, 129.80±0.83</td>
<td>129.61-204.51, 156.80±1.07</td>
</tr>
<tr>
<td>Egg width, EW</td>
<td>79.12-114.09, 93.1±5.99</td>
<td>52.44-89.11, 69.59±0.60</td>
<td>61.63-112.56, 89.45±0.75</td>
</tr>
<tr>
<td>Egg perimeter, EP</td>
<td>323.21-481.64, 411.16±30.46</td>
<td>270.45-360.07, 319.29±1.70</td>
<td>335.52-471.84, 390.14±2.26</td>
</tr>
<tr>
<td>Egg roundness, ER</td>
<td>1.00-1.57, 1.24±0.06</td>
<td>1.05-1.33, 1.17±0.01</td>
<td>1.00-1.34, 1.09±0.01</td>
</tr>
<tr>
<td>Egg area, EA</td>
<td>8223.94-14045.6, 10925.41±1332.61</td>
<td>5137.25-9183.46, 6983.80±75.9</td>
<td>7846.34-15890.70, 11144.09±124.34</td>
</tr>
<tr>
<td>EL/EW ratio</td>
<td>1.19-2.14, 1.63±0.19</td>
<td>1.46-2.54, 1.88±0.02</td>
<td>1.32-2.64, 1.77±0.02</td>
</tr>
</tbody>
</table>
Table 3: Morphometric data (ranges, mean and standard deviation) of adult liver flukes from buffaloes of central Punjab, Pakistan.

<table>
<thead>
<tr>
<th>Adult measurements (mm)</th>
<th>Fasciola gigantica</th>
<th>Fasciola sp.</th>
<th>Fasciola hepatica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>18</td>
<td>60</td>
</tr>
<tr>
<td>Body width ovary level (BWOv)</td>
<td>5.550 - 8.675, 7.211 ± 0.885</td>
<td>5.48 - 10.49, 7.98 ± 1.04</td>
<td>6.89 - 9.85, 8.50 ± 1.49</td>
</tr>
<tr>
<td>Body perimeter (BP)</td>
<td>66.546 - 96.872, 82.436 ± 7.256</td>
<td>43.42 - 90.45, 67.95 ± 10.17</td>
<td>55.58 - 64.80, 58.92 ± 5.11</td>
</tr>
<tr>
<td>Body roundness (BR)</td>
<td>1.946 - 2.645, 2.155 ± 0.182</td>
<td>1.61 - 2.13, 1.85 ± 0.14</td>
<td>1.53 - 1.59, 1.55 ± 0.03</td>
</tr>
<tr>
<td>Cone length (CL)</td>
<td>1.906 - 2.613, 2.299 ± 0.184</td>
<td>1.59 - 2.68, 2.12 ± 0.27</td>
<td>1.81 - 2.43, 2.02 ± 0.35</td>
</tr>
<tr>
<td>Cone width (CW)</td>
<td>2.843 - 4.169, 3.662 ± 0.319</td>
<td>2.59 - 4.37, 3.48 ± 0.37</td>
<td>2.72 - 3.80, 3.28 ± 0.54</td>
</tr>
<tr>
<td>Maximum diameter of oral sucker (OS max)</td>
<td>0.725 - 1.111, 0.975 ± 0.104</td>
<td>0.77 - 1.78, 1.01 ± 0.13</td>
<td>0.91 - 1.01, 0.95 ± 0.05</td>
</tr>
<tr>
<td>Minimum diameter of oral sucker (OS max)</td>
<td>0.388 - 0.859, 0.676 ± 0.122</td>
<td>0.32 - 1.62, 0.66 ± 0.17</td>
<td>0.47 - 0.80, 0.63 ± 0.17</td>
</tr>
<tr>
<td>Maximum diameter of ventral sucker (VS max)</td>
<td>1.328 - 1.825, 1.567 ± 0.132</td>
<td>1.08 - 1.88, 1.54 ± 0.13</td>
<td>1.48 - 1.60, 1.55 ± 0.06</td>
</tr>
<tr>
<td>Minimum diameter of ventral sucker (VS max)</td>
<td>1.243 - 1.619, 1.430 ± 0.113</td>
<td>0.61 - 1.66, 1.41 ± 0.15</td>
<td>1.39 - 1.53, 1.47 ± 0.07</td>
</tr>
<tr>
<td>Distance between anterior end of body and OS (A-OS)</td>
<td>0.371 - 0.952, 0.701 ± 0.140</td>
<td>0.33 - 1.07, 0.65 ± 0.14</td>
<td>0.47 - 0.87, 0.65 ± 0.20</td>
</tr>
<tr>
<td>Distance between anterior end of body and VS (A-VS)</td>
<td>1.115 - 2.413, 2.046 ± 0.308</td>
<td>0.97 - 2.48, 1.91 ± 0.29</td>
<td>1.72 - 2.22, 1.95 ± 0.25</td>
</tr>
<tr>
<td>Distance between suckers (OS-VS)</td>
<td>1.025 - 1.720, 1.400 ± 0.224</td>
<td>0.16 - 1.91, 1.25 ± 0.28</td>
<td>0.85 - 1.60, 1.30 ± 0.40</td>
</tr>
<tr>
<td>Distance between Vs and union of vitelline gland (VS-Vit)</td>
<td>17.351 - 26.603, 22.189 ± 2.695</td>
<td>10.71 - 25.00, 17.80 ± 3.16</td>
<td>12.61 - 16.62, 15.03 ± 2.13</td>
</tr>
<tr>
<td>Distance between Vit and posterior end of body (Vit-P)</td>
<td>7.955 - 15.323, 11.005 ± 1.854</td>
<td>1.38 - 12.87, 8.31 ± 1.97</td>
<td>5.99 - 6.38, 6.19 ± 0.20</td>
</tr>
<tr>
<td>Pharynx length (PL)</td>
<td>0.772 - 1.071, 0.937 ± 0.105</td>
<td>0.63 - 1.28, 0.93 ± 0.12</td>
<td>0.89 - 1.12, 0.97 ± 0.13</td>
</tr>
<tr>
<td>Pharynx width (PW)</td>
<td>0.397 - 0.710, 0.520 ± 0.086</td>
<td>0.34 - 0.75, 0.57 ± 0.08</td>
<td>0.46 - 0.71, 0.59 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>Minimum - Maximum, Mean ± Standard Deviation</td>
<td>Minimum - Maximum, Mean ± Standard Deviation</td>
<td>Minimum - Maximum, Mean ± Standard Deviation</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------------------------------</td>
<td>---------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Testicular space width (TW)</td>
<td>3.711 - 6.762, 5.407 ± 0.788</td>
<td>3.74 - 7.64, 5.74 ± 0.94</td>
<td>5.25 - 7.11, 5.90 ± 1.04</td>
</tr>
<tr>
<td>Testicular space perimeter (TP)</td>
<td>31.379 - 59.630, 43.198 ± 7.001</td>
<td>22.48 - 53.73, 35.87 ± 6.84</td>
<td>28.08 - 35.39, 32.29 ± 3.78</td>
</tr>
<tr>
<td>Testicular space roundness (TR)</td>
<td>1.711 - 2.516, 2.118 ± 0.266</td>
<td>1.45 - 2.27, 1.75 ± 0.20</td>
<td>1.48 - 1.84, 1.62 ± 0.19</td>
</tr>
<tr>
<td>Body area (BA)</td>
<td>133.231 - 332.113, 254.228 ± 44.712</td>
<td>87.33 - 308.64, 201.63 ± 52.24</td>
<td>159.59 - 210.75, 178.65 ± 27.96</td>
</tr>
<tr>
<td>Oral sucker area (OSA)</td>
<td>0.300 - 0.708, 0.527 ± 0.114</td>
<td>0.28 - 0.84, 0.52 ± 0.10</td>
<td>0.38 - 0.59, 0.50 ± 0.10</td>
</tr>
<tr>
<td>Ventral sucker area (VSA)</td>
<td>1.308 - 2.333, 1.771 ± 0.277</td>
<td>1.03 - 2.46, 1.75 ± 0.26</td>
<td>1.62 - 1.92, 1.79 ± 0.15</td>
</tr>
<tr>
<td>Pharynx area (PA)</td>
<td>0.239 - 0.584, 0.376 ± 0.085</td>
<td>0.21 - 0.62, 0.41 ± 0.08</td>
<td>0.31 - 0.64, 0.44 ± 0.17</td>
</tr>
<tr>
<td>Testicular space area (TA)</td>
<td>37.560 - 123.461, 71.836 ± 20.848</td>
<td>22.41 - 101.25, 59.84 ± 18.88</td>
<td>42.32 - 65.27, 51.98 ± 11.90</td>
</tr>
<tr>
<td>Ratio between BL and BW (BL/BW)</td>
<td>3.405 - 4.849, 3.784 ± 0.396</td>
<td>2.20 - 3.36, 2.85 ± 0.31</td>
<td>2.02 - 2.76, 2.27 ± 0.43</td>
</tr>
<tr>
<td>Ratio between BWOv and CW (BWOv/CW)</td>
<td>1.665 - 2.414, 1.974 ± 0.232</td>
<td>1.66 - 2.88, 2.30 ± 0.26</td>
<td>2.54 - 2.63, 2.59 ± 0.05</td>
</tr>
<tr>
<td>Ratio between Suckers area OSA/VSA</td>
<td>0.216 - 0.362, 0.297 ± 0.047</td>
<td>0.17 - 0.44, 0.30 ± 0.05</td>
<td>0.21 - 0.36, 0.28 ± 0.08</td>
</tr>
<tr>
<td>Ratio between BL and VS-P (BL/VS-P)</td>
<td>1.026 - 1.076, 1.054 ± 0.014</td>
<td>1.01 -1.12, 1.06 ± 0.03</td>
<td>1.11 - 1.07, 1.09 ± 0.02</td>
</tr>
</tbody>
</table>
**Table 4:** Morphometric data (ranges, mean and standard deviation) of gravid eggs in uteri of fasciolid adults from central Punjab, Pakistan.

<table>
<thead>
<tr>
<th>Egg measurements (µm)</th>
<th><em>F. gigantica</em> n= 136</th>
<th><em>Fasciola</em> species n= 209</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg length, EL</td>
<td>135.93 - 177.43</td>
<td>115.07 - 186.68</td>
</tr>
<tr>
<td></td>
<td>158.46 ± 7.92</td>
<td>145.73 ± 15.94</td>
</tr>
<tr>
<td>Egg width, EW</td>
<td>80.16 - 109.56</td>
<td>79.12 - 114.09</td>
</tr>
<tr>
<td></td>
<td>92.31 ± 5.22</td>
<td>93.62 ± 6.40</td>
</tr>
<tr>
<td>Egg perimeter, EP</td>
<td>375.74 - 481.09</td>
<td>323.21 - 481.64</td>
</tr>
<tr>
<td></td>
<td>425.58 ± 18.32</td>
<td>401.78 ± 33.05</td>
</tr>
<tr>
<td>Egg roundness, ER</td>
<td>1.10 - 1.57</td>
<td>1.00 - 1.46</td>
</tr>
<tr>
<td></td>
<td>1.26 ± 0.06</td>
<td>1.22 ± 0.06</td>
</tr>
<tr>
<td>Egg area, EA</td>
<td>9130.94 - 14045.60</td>
<td>8223.94 - 13871.62</td>
</tr>
<tr>
<td></td>
<td>11455.77 ± 846.08</td>
<td>10580.29 ± 1472.68</td>
</tr>
<tr>
<td>EL/EW ratio</td>
<td>1.45 -2.09</td>
<td>1.19 - 2.14</td>
</tr>
<tr>
<td></td>
<td>1.72 ± 0.13</td>
<td>1.56 ± 0.19</td>
</tr>
</tbody>
</table>
Figure 5: Factor map corresponding to adult fasciolid specimens from naturally infected buffaloes from Pakistan, compared to pure populations of *F. hepatica* from Spain and *F. gigantica* from Burkina Faso (Periago *et al.*, 2006). Samples are plotted onto the first (PCI, 72%) and second (PCII, 14%) principal components. Each group is indicated by its perimeter.
Figure 6: Centroid size variations among fascioid adult specimens from Pakistan with standard population from Spain and Burkina Faso (Periago et al., 2006) are presented as quantile plots. Vertical lines under the quantiles are number of organisms examined. Each box characterizes the median as a line across the middle and quartiles (10th and 90th percentiles) as its ends. Units are in millimetre (mm).
Figure 7: Discriminant analysis, showing conical factors (CFI and CFII) maps of adult fasciolid specimens from naturally infected bovines from Pakistan with standard population from Spain and Burkina Faso (Periago et al., 2006).
2.5 DISCUSSION

In current study morphologically both species are found different in shape and size *F. gigantica* (28.82-52.30 mm) having increased length, body narrow and elongated where *F. hepatica* (11.62-22.93) have short, broad and curved body (Kendall, 1965). Other parasitologists has documented that both these species are differentiated depending on the branching pattern of testes and ovaries (reproductive organs) and of intestine (Jackson, 1921; Watanabe, 1962; Bergeon and Laurent, 1970). Periago *et al.* (2006) reported that in morphological characterization much emphasis has given to size and shape of fasciolids. Most of the parasitologist believed that it is very difficult to classify both species precisely on external phenotypic features (Kimura *et al*., 1984).

The present morphometric phenotypic study on fasciolids infecting buffaloes from central Punjab demonstrates the presence of intermediate forms (*Fasciola* sp.) together with *F. hepatica* and *F. gigantica* in Pakistan (Table 3, Fig. 5). Previous studies showed 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 demonstrated 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 in several countries, such as India (Varma, 1953), Japan (Watanabe and Iwata, 1954; Watanabe, 1962; Oshima *et al*. 1968a, b; Terasaki *et al*., 2000), Korea (Chu and Kim, 1967), the Philippines (Kimura *et al*., 1984), Thailand (Srimuzipo *et al*.,
2000), Iran (Moghaddam et al., 2004), Vietnam (Itagaki et al., 2009) and China (Peng et al., 2009) based on traditional microscopic measurements. In Iran, however, the presence of intermediate forms was verified by the same methodology as the one applied in this study (Periago et al., 2008). The intermediate form of fasciolids has recently been reported in Pothwar region of Pakistan by using traditional microscopic measurements technique (Mufti et al., 2011).

In Pakistan, fascioliasis in humans from Lahore, central Punjab has been diagnosed in the chronic stage of the disease, i.e. through the coprological detection of fasciolid eggs (Qureshi et al., 2005). Liver fluke disease has recently proved to be highly pathogenic when chronic, and not only in the acute phase as previously thought. This aspect is of great importance in human prevalent areas of developing nations where the majority of infected subjects, mainly children, are detected with prolonged infection stages (Valero et al., 2003, 2006, 2008). Additionally, appropriate surveys in these human disease areas have shown that liver fluke infected patients are usually concomitantly infected by other parasites, which have shown to be related to an immune-suppression caused by Fasciola infection in chronic fascioliasis (Girones et al., 2007). This pathological scenario of great concern may be aggravated when considering that results of recent studies indicate that the occurrence of F. gigantica is more widespread than F. hepatica in five Punjabi districts (Khan et al., 2009), similarly to northern Iran (Ashrafi et al., 2004). The considerably larger size of F. gigantica adult worms when compared to F. hepatica and the consequent higher pathogenicity of F. gigantica may hence be an additional factor to be taken into account in Pakistan. Moreover, fascioliasis is a
trematode disease highly susceptible to climatic factors (Fuentes et al., 1999, 2001), which explains the influence of environmental variations on transmission of this disease (Mas-Coma et al., 2008). Pakistan’s latitudinal situation is prone to be affected by environmental variations, which has indeed already been detected, showing an increase in the mean temperature in coastlines, a 10-15% decrease of rainfall in the coastline, the presence of hyper arid plains, and rise in rainfall in the northern part of the country during summer and winter seasons (Farooq and Kahn, 2004; Cruz et al., 2007). When considering all the afore-mentioned facts, it becomes evident that assessing the present situation of liver fluke species is a priority for Pakistan.

Thirty morphological measurements were observed in present study for Pakistan F. hepatica, F. gigantica and intermediate forms, collected from bovine population. The studies previously conducted on fasciolid species made comparison between different host species. The fasciolids showed a wide spectrum for its definitive mammalian hosts from herbivores to omnivores (Mas-Coma and Bargues, 1997; Mas-Coma, 2004a). The variations in Fasciola adults sizes dependent on its definitive host (Valero et al., 2001). In current study comparison was made with bovine standard populations of both liver fluke species from the areas where both these species do not overlap. The F. hepatica like specimens from Pakistan has found to be higher maximum values in various measurements from standard population.

Because of existence of both fasciolid species in Pakistan the variation in
adult *Fasciola* morphology was observed. In the areas where both species overlap, existence of mixed morphological types was observed which was considered due to occurrence of abnormal spermatogenesis (Terasaki *et al.*, 1982) and reproduction by parthenogenesis in helminths (Agatsuma *et al.*, 1994). The aspermic triploid liver flukes have been reported in UK which indicated facultative gynogenesis (Fletcher *et al.*, 2004).

The phenomenon of hybridization is well documented in trematodes and the existence of fasciolid hybrids was confirmed when molecular characterization of Japanese fasciolids was made by rDNA ITS2 sequencing and mtDNA CO1 and ND1 sequencing (Itagaki and Tsutsumi, 1998; Itagaki *et al.*, 1998). The molecular classification of fasciolids presented small differences in nucleotide for both fluke species with rDNA markers, generally ITS-2, 28S (Barker *et al.*, 1993; Itagaki and Tsutsumi, 1998; Marcilla *et al.*, 2002), and mtDNA, CO1 and ND1 (Itagaki *et al.*, 1998).

In present study the morphology of adult fasciolid eggs showed overlapping of size and shape between *F. gigantica* and *Fasciola* sp. The most frequently used criteria for diagnosis when they are released in the faeces; eggs of fasciolids have specific length and width ranges and are large, operculated, yellowish brown, ellipsoidal and non-embryonated (Valero *et al.*, 2009). The overlapping of intraspecific size variabilities among fasciolid eggs were found in current study. The eggs of other trematodes e.g. Fasciolopsis, Gastrodiscoides and Echinostoma (Mas-Coma *et al.*, 2005; WHO, 1991) showed overlapping of intraspecific size variabilities with fasciolid species. This may leads to confusion in the identification
of fasciolid eggs from other trematode species, if other diagnostic criteria are not taken (Belisario et al., 2007). The abopercular end of Fasciola eggs are irregular rough which is not observed in Fasciolopsis eggs (Ash and Orihel, 1997).

The size measurements of eggs of fasciolid intermediate forms in the current study, of 115.07-186.68/79.12-114.09 µm (Table 2), show intermediate values when compared to the standard fasciolid populations of both species described previously (Periago et al., 2006) and which molecularly proved to be genetically pure F. hepatica and pure F. gigantica (Mas-Coma et al., 2009). Fasciolid egg size ranges from various livestock species and from different geographical areas were previously described by applying the CIAS methodology (Valero et al., 2009a). Egg length minimum values obtained in the present study approach minimum values found in F. hepatica from Bolivian cattle, F. hepatica from Egyptian cattle and those from Georgia. Egg length maximum values approach maxima obtained in F. gigantica from Egyptian cattle and F. gigantica from Vietnamese cattle, but are smaller than the maxima described in those from Burkina Faso. In Asia and Africa where both fasciolid species were found, the intermediate forms of eggs were observed in stool samples of human and animal populations may be due to crossbreeding of fasciolid species (Valero et al., 2009). The results obtained from fasciolids encountered in the Central Punjab area agree with results from previous studies carried out in other Asian countries, where a large egg measurement overlap was detected (Watanabe 1962; Akahane et al., 1970; Sahba et al., 1972; Kimura et al., 1984; Srimuzipo et al., 2000).
According to Mas-Coma et al. (2009), Iran and Pakistan are examples of Asian countries presenting a zonal overlap of the both liver fluke species. The zonal overlap refers to areas with different altitudes where the highlands offer the necessary cold-mild weather conditions for *Galba/Fossaria* lymnaeid species and *F. hepatica*, and where the lowlands offer the warm-hot climate necessary for *Radix* lymnaeid species and *F. gigantica*, definitive hosts becoming co-infected by both fasciolids when moving from lowlands to neighboring highlands and vice-versa. In this manner, animals and humans may become co-infected because of interzonal transhumance, nomadic migrations, transportation, trade and/or other reasons (Mas-Coma et al., 2009). Previous studies in Pakistan (Kendall, 1954; Kendall and Parfitt, 1959) indicated that *F. hepatica* is confined to the highlands due to the presence of its intermediate snail host *Galba truncatula*, while *F. gigantica* is found in lowland areas with *Radix auricularia*.

In the case of Iran, in Gilan province, *G. truncatula* is frequent in the Talesh Mountains, where it has been found to transmit *F. hepatica* in nature (Ashrafi et al., 2007), whereas *R. gedrosiana* is well distributed in lowlands and does not appear to have the capacity to colonize highland regions (Ashrafi et al., 2004). This explains why *F. gigantica* is the most prevalent species (91.1%) in the human endemic area around the cities of Rasht and Bandar-Anzali, whereas the scarcity of *F. hepatica* in animals in these lowlands (8.9%) might be the consequence of *F. hepatica*-infected animal transportation from highlands to lowlands or be hybrid-intermediate, long-time introgressed flukes showing a *F. hepatica*-like form (Ashrafi et al., 2006b). Although altitudinal livestock transhumance does not
appear to take place in Gilan nowadays, it might have been taken place in the past (Mas-Coma et al., 2009a).

### 2.5.1 Conclusion

The intermediate forms, as well as the *F. hepatica*-like and *F. gigantica*-like adult worms, found in buffaloes from the Central Punjab area in the present study may also be explained by zonal overlap (Mas-Coma et al., 2009a). In fact, nomads move their animals from the highlands to the lowlands in the winter season looking for grazing lands. Similarly, animals are moved from the lowlands to the cooler highlands when warmer conditions return. Further work on this very complex orographic and transhumance scenario is needed to comprehend the epidemiological status of disease in each locality, to assess how transmission of fascioliasis occurs throughout the Central Punjab area and thus establish the appropriate control measures for each endemic subzone in the future.
Chapter 4

DETECTION OF ANTI-

Fasciola IgG ANTIBODIES BY USING

MONOCLONAL ANTIBODIES (Mab) BASED MM3 SERO-

ELISA IN CATTLE AND BUFFALO OF SUB-TROPICAL

PUNJAB, PAKISTAN

4.1 INTRODUCTION

Fascioliasis an important veterinary problem in livestock reported all over
the world inflicts deleterious economic losses in ruminants due to reduction in
weight, milk yield, fertility rate and condemned livers (Schweizer et al., 2005;
Elitok et al., 2006; Charlier et al., 2007). Furthermore, fascioliasis has recently
been included as an important human disease among neglected diseases by WHO,
human cases having been reported from countries in Europe, America, Asia, Africa
and Oceania (Mas-Coma et al., 2009a). The control of this parasitic disease was
limited in the past due to lack of appropriate early diagnostic test.

Historically, Fasciola infection was diagnosed by traditional microscopic
fecal egg examination, which is still a widely used diagnostic technique. This
technique has several disadvantages such as it is not effective until 10-12 week
postinfection (PI), laborious and requires an appropriate amount of faeces for
examination (Anderson et al., 1999).

As the disease is asymptomatic and early diagnosis is required for its
control, numerous immunological tests were developed all over the world for early
detection of antibodies against *Fasciola* in blood (Molloy et al., 2005; Reichel et al., 2005). In present study monoclonal antibody (Mab) based MM3 Sero-ELISA was performed for early detection of fasciolids infection in cattle and buffalo of sub-tropical areas of Punjab, Pakistan. The MM3 Sero-ELISA was confirmed to be most specific and sensitive test for detection of anti-*Fasciola* antibodies in serum samples (Mezo et al., 2007; Valero et al., 2009b). The MM3 Sero-ELISA test was applied first time in Pakistan for early diagnosis of fasciolid infections.

From last two decades all over the world, investigations were made to search specific and sensitive methods for early diagnosis of fascioliasis in ruminants. Numerous ELISA tests were performed by using different antigens including whole *F. hepatica* excretory secretory antigens ESAs (Espino et al., 1987; Rivera Marrero et al., 1988; Itagaki et al., 1995; Ortiz et al., 2000; Salimi-Bejestani et al., 2005), purified recombinant cathepsins (O’Neill et al., 1999; Cornelissen et al., 2001; Neyra et al., 2002; Espinoza et al., 2005; Sriveny et al., 2006) and other recombinant antigens (Silva et al., 2004; Paz-Silva et al., 2005; Arias et al., 2006). But specificity and sensitivity of these ELISA tests is usually below 100%, not been tested for low-intensity infections and most of them are not available commercially. Mezo et al. (2004) reported that MM3-sero sandwich ELISA was 100% specific and have ability to test even low intensity infection.

Previously in Pakistan coprological examination of faecal samples based on slaughterhouse screening of large ruminants were performed for diagnosis of fasciolosis. However the use of Monoclonal antibody (Mab) based MM3 Sero-ELISA in naturally infected field animals has not yet been done in Pakistan. The
data about animal age, sex, breed and areas were sought out on prescribed questioners (see Appendix # 2). The current study was aimed to find out the sero-epidemiological pattern of fasciolids under prevailing climatic conditions of subtropical Punjab, Pakistan. The study was hypothesized as “MM3 Sero-ELISA could be sensitive and specific for early diagnosis of infection”.

The objective of the current investigation was:

- Detection of anti-\textit{Fasciola} IgG antibodies by using monoclonal antibodies (Mab) based MM3 Sero-ELISA in cattle and buffalo of sub-tropical Punjab, Pakistan.

4.2 REVIEW OF LITERATURE

Fascioliasis affects the livestock production potential all over the world, mainly in tropical countries where conditions are suitable for the development of infection. On seroprevalence of fascioliasis a lot of information has been generated throughout the world. However, little serological information is available on fascioliasis in Pakistan. The present study has necessitated diagnosing infection at early stages in order to devise effective control strategies against fasciolosis.

4.2.1 World Fascioliasis Occurrence

Torgerson \textit{et al.} (1999) and Tum \textit{et al.} (2007) reported prevalence rates of fascioliasis in cattle from endemic areas of the world. They had reported fascioliasis in Chile (94 per cent), the USA (California 52.7 per cent; Florida 68 per cent; Louisiana 25 per cent), Ireland (45 per cent), Spain (29.5 per cent), Turkey
(29.3 per cent), Peru (29 per cent), Germany (10.7 per cent), Morocco (10.4 per cent), Cambodia (10 per cent) and New Zealand (8.5 per cent). In Africa, Megrad (1978) worked out prevalence rates of 37 per cent in Sudan, 45 per cent in Cameroon, 30-90 per cent in Ethiopia and 62 per cent in Central Africa. The fascioliasis recorded in bovine was 19.8 percent in Brazil (Luz, et al., 1992), 29.19 percent in China (Wang BingYun, et al., 2001), 9.73 percent in Egypt (El-Shazly et al., 2002), 7 percent in Iraq (Al-Khafaji et al., 2003), 26 percent in Kenya (Mungube et al., 2006). Similarly in India the both, Fasciola gigantica and Fasciola hepatica were prevalent with 30 per cent and 57.3 per cent in cattle, respectively (Bhatia et al., 1989).

4.2.2 Fascioliasis in Pakistan

In Pakistan different prevalence records of fascioliasis in livestock were reported infected with fasciolid species, with F. gigantica being the most important (Maqbool et al., 1994, 2002; Siddiqi and Shah, 1984; Chaudhry and Niaz, 1984; Masud and Majid, 1984; Sahar, 1996). Fascioliasis has been reported in all parts of the country and some areas of Punjab, Sind and Khyber Pakhtunkhwa are badly affected (Kendall, 1954; Sarwar, 1956; Buriro, 1981). The accurate amount of economic losses due to fascioliasis in livestock is not yet available. The so far estimated prevalence rate of fascioliasis based on coprological examination were, 25.46 per cent in Faisalabad (Khan et al., 2009), Punjab 14.71 per cent (Maqbool et al., 2002), Bahawalpur 17.68 per cent (Chaudhry and Niaz, 1984), Multan 23.97 per cent (Masud and Majid, 1984), Lahore 10.48 per cent (Sahar, 1996) and 55 per cent in Peshawar (Siddiqi and Shah, 1984).
4.2.2.1 *Fasciola* species

The studies carried out on the prevalence of *Fasciola* species was reported in all parts of the Punjab. And most of these studies were based on coprological examinations. In Punjab, Pakistan, Suhail *et al.* (2003) recorded the prevalence of bovine fascioliasis as 6.8 percent. Khan *et al.* (2009) reported bovine fascioliasis in Sargodha 40.31 percent (387/960), Jhang 34.27 percent (329/960), Muzaffargarh 20.83 percent (200/960), Lodhran 20.10 percent (193/960), Layyah 11.77 percent (113/960). In Toba Tek Singh Khan *et al.* (2010) reported *Fasciola* species infection 24.65 percent (281/1140) in cattle, 32.36 percent (369/1140) in buffalo, 35 percent (294/840) in sheep and 35.19 percent (219/660) in goat. In Islamabad and Rawalpindi prevalence record was 4.44 percent (4/90) in sheep while in goat 0.64 percent (2/310). In Quetta Ahmed *et al.* (2005) conducted a histopathological study and find *Fasciola* species infection 19.92 percent (52/216) in sheep and 27.9 percent (12/43) in goat.

Qureshi and Tanveer (2009) in different areas of Punjab conducted serological study based of indirect haemagglutination (IHA) test in buffaloes and found fascioliasis in Sheikhupura 8 percent (4/50), Gujranwala 10 percent (5/50), Kasur 14 percent (7/50), Shahdara 20 percent (10/50), Kamoke 32 percent (16/50) and Muridke 26 percent (13/50).

4.2.2.2 *Fasciola hepatica*

The *Fasciola hepatica* prevalence rates were recorded separately in most parts of the country. Azam *et al.* (2002) in Dir (Khyber Pakhtunkhwa) reported
5.93 percent (7/118) *F. hepatica* infection in buffalo by fecal examination. Raza *et al.* (2007) reported infestation of *F. hepatica* in cattle (9 percent) and in buffaloes (4 percent) from various parts of the country. In Quetta histopathological examination were conducted by Kakar *et al.* (2008 and 2011) and recorded the infestation of *F. hepatica* in cattle (16.16 percent (64/396) and 15.20 percent (44/288) respectively) and buffalo (11.47 (39/340) and 14.40 (29/201) respectively). In the Province of Punjab coprology based studies were conducted to find out the *F. hepatica* infestation rate in most of the areas. Iqbal *et al.* (2007) reported bovine fascioliasis in Farooqia 70.62 percent (476/674), Kot Addu 16.29 percent (22/135), Dunya Pur 32.12 percent (53/165), Layyah 21.42 percent (12/56), Mor Mandi 40 percent (26/65), Shorkot 46.23 percent (43/93) and Jalalpur 24.78 (29/117). In Jhang and Sargodha districts Khan *et al.* (2009) recorded 7.36 percent (106/1440) infection rate. In Toba Tek Singh Khan *et al.* (2010) reported *F. hepatica* infestation 4.56 percent (52/1140) in cattle, 6.75 percent (77/1140) in buffalo, 7.02 percent (59/840) in sheep and 7.58 percent (50/660) in goat.

### 4.2.2.3 *Fasciola gigantica*

The separate *F. gigantica* infestation was recorded in different areas of Pakistan. In Jhang and Sargodha districts of Punjab Khan *et al.* (2009) recorded 19.86 percent (286/1440) *F. gigantica* infestation from fecal examination. Khan *et al.* (2010) in Toba Tek Singh reported 20.09 percent (229/1140) in cattle, 25.61 percent (292/1140) in buffalo, 27.98 percent (235/840) in sheep and 25.61 percent (169/660) in goat. In Quetta histopathological examination were conducted by Kakar *et al.* (2008 and 2011) and recorded the infestation of *F. gigantica* in cattle
(12.37 percent (49/396) and 3.4 percent (10/288) respectively) and buffalo (13.52 (46/340) and 3.9 (8/201) respectively). Bhutto et al. (2002) in Hyderabad (Sindh) reported 4 percent (8/200) *F. gigantica* infestation in buffalo calves from faecal examination. Shaikh et al. (2004) reported a histopathological study in Hyderabad with 14.8 percent (49/330) *F. gigantica* infestation in buffalo. Bhutto et al. (2012) in Sindh recorded the overall prevalence of fascioliasis due to *F. gigantica* infestation 42.06 percent (768/1800) in buffaloes, Larkana 41.83 percent, Hyderabad 53.5 percent and Badin 53.50 percent.

4.2.3 Serodiagnosis of Fascioliasis

Fascioliasis cause deleterious loses in livestock and its control is limited due to absence of accurate and practicable tests for early diagnosis. Historically the microscopic examination of parasite eggs in faeces was common practice, this traditional diagnostic technique is still widely used and not effective until at least 10–12 week postinfection (PI). The microscopic fecal examination has various drawbacks less sensitive, hard to perform, requires an appropriate amount of faeces, unable to diagnose infection at early stages, in chronic infection sporadic eggs release in faeces leads to misdiagnosis of infection (Anderson et al., 1999).

In all of over the world for control purpose, studies on fascioliasis concentrated on the significance of immunological testing to diagnose infection during the prepatent period. Numerous immunological tests applied for detection of anti-*Fasciola* antibodies in bovine serum are well studied (Bossaert et al., 2000; Cornelissen et al., 2001; Phiri et al., 2006).
Several investigations have been made to search specific and sensitive methods for the serodiagnosis of fascioliasis in ruminants from last two decades. Several antigenic fractions of *Fasciola* has been effectively applied in ELISA tests (Mezo *et al.*, 2003; Sanchez-Andrade *et al.*, 2008; Demerdash *et al.*, 2011), whole excretory secretory antigens ESAs of *F. hepatica* (Espino *et al.*, 1987; Rivera Marrero *et al.*, 1988; Itagaki *et al.*, 1995; Ortiz *et al.*, 2000; Salimi-Bejestani *et al.*, 2005), purified antigens (O’Neill *et al.*, 1998; Rokni *et al.*, 2002) and more recently, purified and recombinant cathepsins antigens (O’Neill *et al.*, 1999; Cornelissen *et al.*, 2001; Neyra *et al.*, 2002; Espinoza *et al.*, 2005; Sriveny *et al.*, 2006). The most frequently used target antigens for detecting anti-*Fasciola* antibodies are Cathepsins L (Carnevale *et al.*, 2001; Rokni *et al.*, 2002; Mezo *et al.*, 2004, 2007, 2010; Intapan *et al.*, 2005; Wongkham *et al.*, 2005; Valero *et al.*, 2009b; Muñó *et al.*, 2011), as circulating antibodies remain at high levels to these molecules for long periods (Valero *et al.*, 2009b). The other recombinant antigens are also used for detection of anti-*Fasciola* antibodies (Silva *et al.*, 2004; Paz-Silva *et al.*, 2005; Arias *et al.*, 2006).

Most of these immunological tests proved to be sensitive enough to detect fasciolids infection during the prepatent period in ruminants but not yet been tested for the diagnosis of low-intensity infections i.e. animal harboring with fewer number of flukes. The specificity and sensitivity of these tests is usually below 100 percent, and most of them are not commercially available (Mezo *et al.*, 2007).
Mezo et al. (2003) reported that an antigenic fraction obtained by FPLC fractionation of *F. hepatica* ESAs (peak IV) can be used in indirect ELISA for the serodiagnosis of ovine fascioliasis with 100 percent specificity, even in animals with low-intensity infections. However, several relevant antigens included in this purified fraction (range 7-40 kDa) are also recognized by monoclonal antibodies (mAb) MM3.

Several monoclonal antibodies have been produced against *F. hepatica* ESAs and somatic antigens (Hanna and Trudgett, 1983; Hanna et al., 1988; Solano et al., 1991), only two of them have been described to be appropriate for the detection of *Fasciola* coproantigens in humans and animals. The monoclonal antibody (mAb) named F10 (Abdel-Rahman et al., 1998, 1999) precisely identified the faeces from calves infested with 10 or more flukes, whereas mAb ES78 (Espino and Finlay, 1994; Dumenigo et al., 2000) identified faeces from calves infested with 5 or more flukes, however not tested in animals with less than 5 flukes. Mezo et al. (2004) reported mAb MM3 proved to be useful for detection of coproantigens by adult flukes. The MM3-Copro ELISA has several disadvantages as it can only detect active infection.

Mezo et al. (2007) used mAb MM3 for serodiagnosis and performed sandwich ELISA. The MM3-Sero ELISA applied was proved 100 percent sensitive and specific for the early detection of infection, and shows a number of advantages over the peak-IV ELISA. The MM3-Sero ELISA detected lambs harboring only 1–2 flukes by week 4 PI, and lambs with 3 or more flukes by week 3 PI. In contrast, peak-IV ELISA revealed the same infections more frequently by week 5–6 PI and
production of the purified antigens is time consuming and may show interbatch variations.

4.2.4 Present Study

The literature on serology of fascioliasis carried out all over the world is useful to develop such a study in Pakistan. Previous studies on fascioliasis were based on coprological examination of faecal samples based on slaughter house screening of large ruminants conducted in all parts of Pakistan. The early diagnosis is required for timely treatment of disease and to adapt control measures this could necessitate to develop current study based on serological assay.

The uniqueness of current study is the application of a very sensitive and specific ELISA test by using monoclonal antibodies which are able to detect even very low intensity infection. However, the Monoclonal antibody (Mab) based MM3-Sero ELISA has not only provides new protocol for diagnosis but also give us detail sero-epidemiological record of fasciolosis in cattle and buffalo grazing in northern and central Punjab, Pakistan.

4.3 MATERIALS AND METHODS

4.3.1 Study Area

The study area comprises of two regions of Punjab province viz; Pothwar region and some area of central Punjab (Fig. 28).

4.3.1.1 Pothwar region
The Pothwar region (32° 30” N to 34° latitude and 71° 45” E to 73° 45” E longitude) is situated in the northern part of the Punjab province of Pakistan. It covers an area of about 23,160 sq / km, located at an elevation of 472.2 to 609.6 meter. Pothwar region is bound by salt ranges in the South, River Jhelum in the East, in West River Indus and in North by Murree Hills, Foot Hills of Himalayas. It comprises of hilly and plane areas with five districts namely, Rawalpindi (33°36′0″N latitudes and 73°02′0″E longitudes), Islamabad (33° 43’ 0″ N latitudes and 73° 4’ 0″ E longitudes), Chakwal (32° 55′ 49″ N latitudes and 72° 51′ 20″ E longitudes), Attock (33° 54′ 26″ N latitudes and 72° 18′ 40″ E longitudes) and Jhelum (32°56′00″N latitudes and 73°44′00″E longitudes).

Climatically it is considered as semi-arid zone with hot summers season and cold winter season. The area is mainly considered as barani / rain-fed with agriculture mainly being dependent on rainfall as no irrigation system is found. The Pothwar region is comprises of pasture land and livestock rearing is main source of income for the farmers. The mean daily temperature is 38 °C in summer and 3–6 °C in dry cold winter season. The mean monthly rainfall in summer is 200 mm and 36–50 mm in winter.

4.3.1.1 Rawalpindi

The district Rawalpindi lies at elevation of 500 m with humid sub-tropical climate. The climate of Rawalpindi varies because of its location in northern part of the Punjab province. The main rivers are River Indus and River Jhelum. The
summer season is long and hot with maximum temperature 54 °C, monsoon season is short with more rainfall. The annual rainfall is 990 mm. The winter season is mild with minimum temperature of – 4 °C.

4.3.1.1.2 Islamabad

Islamabad capital of Pakistan lies between the elevations of 457-1604 m with humid subtropical climate. Islamabad's climate is controlled by three artificial reservoirs; Rawal, Simli, and Khanpur Dam. The May to July is hottest months of the years with average temperature of 38 °C. The heavy rainfalls occur from July through September in monsoon.

4.3.1.1.3 Chakwal

Chakwal is a semi-arid area with 498 m elevation. The 70% of the population involves in agriculture mainly dependent on rainfall due to scarcity of irrigation system.

4.3.1.1.4 Attock

It is located at the bank of the Indus River runs on the northern and western boundaries of the district. The climate of the area is hot in summers and cold in winters, average annual rainfall is 783 mm. The climate in the northern part is humid comparative to the southern part of the district. The area is mostly comprises of hills, plateaus and separated plains.

4.3.1.1.5 Jhelum
The Jhelum district lies at the bank of River Jhelum with elevation of 250 m in the north of the Punjab. The summer is hot and humid while winter is warm and dry. The maximum temperature in summer is 45.7 °C and minimum temperature 1.8 °C in winter, with annual rainfall of 850 mm.

4.3.1.2 Central Punjab

The central Punjab comprises of irrigated land having well established water channel system of Indus river basin. The areas of central Punjab includes in the study are Lahore (31°32′59″N 74°20′37″E), Sargodha (32°08′00″N latitudes and 72°67′00″E longitudes) and Faisalabad (31°21′52″N latitudes 72°59′40″E longitudes) districts. In irrigated areas of central Punjab every farmer allots a piece of land for planting fodder crops. The milking cows and buffalos are usually stall-fed with green fodder and concentrates. The animals used for power purpose are sustained on hays, maize and communal grazing lands.

4.3.1.2.1 Lahore

The district Lahore lies at elevation of 217 m having semi-arid climate and bounded by Ravi River from the northern side. The summer is long hot and rainy and dry and warm winter. The maximum temperature recorded is 40-48 °C and minimum temperature – 1 °C, with rainfall of 221 mm.

4.3.1.2.2 Sargodha
Sargodha district lies at elevation of 100-200 m bounded by River Jhelum on the western and northern sides while River Chenab on the eastern side. The maximum temperature recorded in the summer is 50 °C while in the winter the minimum temperature as low as freezing point. The area mainly covers flat, fertile plains with alluvial soil.

4.3.1.2.3 Faisalabad

Faisalabad district lies at elevation of 184 m bounded by Chenab River to the north-west while the River Ravi to the south-east. The irrigation water which meets 80% requirement of the cultivated crop lands derives from lower Chenab canal. The maximum temperature recorded is 50 °C while the minimum temperature is – 2 °C in the winter. The average annual rainfall recorded is 300 mm, with alluvial soil deposits making it very fertile.

4.3.2 Animals

The study population comprises of cattle and buffalo reared at different public and private farms located in sub-tropical Punjab. The simple random sampling without replacement was done to find out the prevalence of fasciolid infection. A total of 598 (414 from cattle and 184 from buffaloes) blood samples were collected. Only those herders were included in this study that had shown their willingness for cooperation in animal blood and data collection.

4.3.2.1 Management practices
In northern Punjab farmers usually follow semi-extensive farming production system. Cattle are taken to the communal land, roadsides for grazing. The flocks are taken out for grazing early in the morning and brought back to their farm. The animals are kept in kraal (confined wall area) having mud-plastered walls, but use of thorny bushes is also common to prevent animals from predators at night. In the kraal animals are supplemented with green fodder and concentrate feed made up of oilseed cakes are given on occasional basis when green fodder is scare in hot summer season (June-July) and winter season December-January. The concentrate supplement was given @4-5 Kg / day for adult and 0.5-1 Kg for calves. Straws of the cereals and other by-products are commonly used to overcome feed shortages. The animals were irregularly dewormed depending upon the availability anthelmintics; most common drug of choice is albendazole.

4.3.2.2 Animal age and grouping criteria

The age of cattle and buffalo was determined from animal records maintained at publically and privately owned farms of sub-tropical Punjab. The conventional methods were also applied to determine the age of animal by using their dental eruption formula as described by Hornsveld et al. (1996). Animal grouping were made by categorizing them into four different age groups i.e. 1-3, 4-6, 7-10 and ≥ 11.

4.3.2.3 Animal data statistics

The animal characteristics data such as age, sex, area and altitude were sought on the prescribed questionnaires. This data was used to investigate the
relationship of age, sex, area and altitude of area from which cattle and buffalo blood samples were obtained with the prevalence of fasciolid infection.

4.3.3 Serology

In present study MM3-Sero ELISA was performed for measuring the antibodies level against *Fasciola* antigen in serum samples of cattle and buffaloes from Pakistan where both fasciolid species co-exist. Effect of serum dilutions on the OD values were obtained for MM3-Sero ELISA and specific serum dilutions were selected for accurate diagnosis. Mezo *et al.* (2007) developed MM3 Sero-ELISA and proved it as highly sensitive and specific test for ruminants.

4.3.3.1 Collection and preparation of sample

The blood samples were collected in 5 ml of vacutainers without EDTA from cattle (n= 414) and buffaloes (n=184) located in different animal herds of subtropical Punjab. The blood was taken from jugular vein of the animals and allowed to clot for few hours, centrifuged at 3000 rpm for 20 min. Serum samples were obtained and stored in small 1 ml eppendorfs tubes at -20 °C until assayed.

4.3.3.2 Diagnostic techniques

4.3.3.2.1 MM3-Sero ELISA procedure

The level of antibodies against *Fasciola* antigen d in serum samples collected from cattle and a buffalos were determined using MM3- Sero ELISA. The assay was executed in 96 well microtiter plates, coated with monoclonal
antibody (Mab) MM3. The odd rows of plate were coated with antigen where the even rows were without antigen. The MM3 Sero-ELISA kits are provided by World Health Organization Collaborating Centre on Fascioliasis and Its Snail Vectors (Valencia, Spain). The MM3-Sero ELISA was performed according to method proposed by Mezo et al. (2007) with some modification (see Appendix #3).

The aliquots of serum samples in PBS-T/BSA (0.2% / 1%) were added to the MM3 antigen coated plates (100 µl / well) in duplicate and incubated at 37°C for 2 hours. The plates were then washed 3 times with PBS-T 200 µl/ well. The 100 µl / well anti-bovine biotin of Sigma (ref B9780-5ml) (1:120,000 dilution in PBS-T/BSA 0.2% / 1%) were added to each well and incubated for 1 hour. After incubation, the plates were washed again 3 times. The secondary antibody NeutrAvidin Horseradish Peroxidase conjugated of Pierce-Thermo (ref 31001), 100 µl / well added (1:4000 dilution in PBS-T/BSA) incubated for 1 hour at 37ºC. The plates were washed and substrate solution SigmaFast OPD, was added at 100ul / well and incubated for 20 minutes at room temperature. The reaction with yellowish coloration was stopped by adding 25 µl/ well of 3N H₂SO₄ and was read using ELISA-reader at 490 nm. The OD values were calculated by taking the mean of ODA₁ – ODB₁. The cut off value is 0.100.

4.3.4 Statistical Analysis

The ELISA test was repeated twice for each sample and results were taken as the mean OD 490 nm of each sample. The cut-off value was set with the mean of OD values of serum samples of *Fasciola*-negative bovine (n= 15) herds. The
Software GraphPad Prism V. 5 was used for graphical representation of OD values of each sample plotted against sex, age, species and area wise for both cattle and buffalo separately.

The chi-square analysis was applied to find out the relationship of age, sex, species and area with prevalence of fasciolid infection. And simple liner regression model was applied to find out the relationship between prevalence of infection and altitudes of areas samples were collected. The data analysis was conducted in statistical package SPSS V. 17. The results were considered statistical significant when $p < 0.05$.

4.4 RESULTS

The usefulness of MM3-Sero ELISA for serodiagnosis of bovine fascioliasis was determined and the effectiveness of different serum dilutions on OD values of the assay was investigated. The maximum OD values were obtained with sera at 1:100 dilutions; the assay was also able to detect anti-*Fasciola* IgG antibodies at higher serum dilutions of 1:10000. Titration curve for different serum dilutions was given in Figure 29.

The specificity and sensitivity of the assay was determined by evaluating the OD values of serum samples collected from *Fasciola*-negative and *Fasciola*
Figure 28: Map of Pakistan showing Pothwar region and central Punjab.
Plate 4: Animal bathing and drinking sites.
positive bovine herds. The OD values of serum samples from Fasciola-negative herds (-0.074 ± 0.125) were extremely lower when compared with serum samples of Fasciola-positive (1.42 ± 0.18) bovine herds (Table 12 and 13). The OD value ≥ 0.100 was calculated the optimal cut-off for the MM3-Sero ELISA. The 100% specificity and sensitivity of the assay was observed at this cut-off point.

The MM3-Sero ELISA was used to test the sera from cattle and buffalo naturally infected with Fasciola antigens. The results of MM3-Sero ELISA are summarized in Table 14. A total of 35 cattle samples (8.45%) of the sera examined had OD values ranges from 0.102 - 0.633 and high percentage of negative cattle samples 379 (91.54%) with OD values (ranges from -0.499 - 0.099) were recorded. The sera from buffalo (n= 184) were examined for Fasciola-antigen, 4(2.17%) were found positive to infection with OD values ranges from 0.133 - 0.414 and extremely high percentage of negative buffalo sera were obtained.

4.4.1 Level of Fasciola-antibodies in Cattle and Buffalo

Level of Fasciola-antibodies in serum samples of cattle and buffalo along with control group was recorded. The antibody level was above the cut-off value in all infected cattle and buffalo (OD ≥ 0.100). The high serum antibody level was recorded for cattle as compared to buffalo (Fig. 30).

The seroprevalence of fascioliasis in cattle and buffalo were recorded in Table 15. The prevalence of infection was found statistically significant ($\chi^2 = 8.241, p < 0.05$).
4.4.2 Level of *Fasciola*-antibodies in Age Groups of Cattle and Buffalo

Level of *Fasciola*-antibodies in serum samples of four age groups of cattle and buffalo along with control group was observed. Serum antibody level in all infected cattle was above the cut-off point (OD \( \geq 0.100 \)) between the age groups of 1-3, 4-6 and 7-10 years but in the age group \( \geq 11 \) years the serum antibody level was below the cut-off point (Fig. 31). The *Fasciola*-antibody level recorded in buffalo was above the cut-off value (OD \( \geq 0.100 \)) in the age groups of 7-10 and \( \geq 11 \) years. The age groups 1-3 and 4-6 years were found negative to the infection as the antibody level was below the cut-off point (Fig. 32).

The seroprevalence of fascioliasis in four age groups of cattle and buffalo were recorded. The prevalence of infection in different age groups of cattle were found statistically non-significant (\( \chi^2 = 4.889, p > 0.05 \)) (Table 16). And the highest seroprevalence 12.61% (14/111) was recorded for the age group of 7-10 years. While in buffalo the highest seroprevalence 4.35% (3/69) was recorded for the age group of \( \geq 11 \) years and statistically non-significant difference (\( \chi^2 = 2.746, p > 0.05 \)) was observed between the age groups (Table 17).

4.4.3 Level of *Fasciola*-antibodies in Studied Areas of Cattle and Buffalo

Level of *Fasciola*-antibodies in serum samples from studied areas of cattle and buffalo along with control group was observed. The antibody level in control sera was below the cut-off point indicating no infection. Serum antibody level in all infected cattle from Rawat, Attock, Rawalpindi, Gujar Khan, Kallar Syedan, Jhelum and central Punjab areas were found above the cut-off point (OD \( \geq 0.100 \))
but no infection was recorded in Chakwal and Dhudial areas as the serum antibody level was below the cut-off point (Fig. 33). The high antibody titer was obtained in Rawat and Attock.

Buffalo are mostly comprises in areas of central Punjab and less population is found in Northern of the Punjab. In buffalo serum antibody level was detected above the cut-off point (OD ≥ 0.100) in central Punjab, whereas Rawalpindi and Jhelum were found negative to the infection as the antibody level was below the cut-off point (Fig. 34). The infection in these areas is less because of sampling as very small sample size was taken from these areas.

The seroprevalence of fascioliasis in studied areas of cattle and buffalo were recorded. The prevalence of infection among different areas for cattle were found statistically highly significant ($\chi^2 = 48.90$, $p < 0.01$) (Table 18). And the highest seroprevalence 28.57% (14/49), 21.43% (6/28) and 17.14% (6/35) were recorded for Rawat, Attock and Rawalpindi respectively. The highest seroprevalence in buffalo 2.37% (4/169) was recorded for central Punjab and statistically non-significant difference ($\chi^2 = 0.363$, $p > 0.05$) was observed among studied areas (Table 19).

4.4.4 Level of Fasciola-antibodies according to Sex of Cattle and Buffalo

Level of Fasciola-antibodies in sera of male and female population of cattle and buffalo along with control group was observed. The antibody level in control sera was found below the cut-off point indicating no infection. In all infected males
and females cattle *Fasciola*-antibodies level were found above the cut-off point (OD ≥ 0.100). The high antibody titer was recorded in female as compare to male (Fig. 35).

In buffalo serum antibody level was observed above the cut-off point (OD ≥ 0.100) for females and males were found negative to the infection as the antibody level was below the cut-off point (Fig. 36).

The number of female animals were higher in farms as compare to male because male are slaughtered at very young age and female are more preferable because of their economic values. The seroprevalence of fascioliasis in cattle and buffalo according to sex was recorded in Table 20 and 21. The prevalence of infection with respect to sex for cattle were found statistically non-significant ($\chi^2 = 1.856, p>0.05$). And the highest seroprevalence 9.39% (31/330) was recorded for female cattle. In buffalo statistically non-significant difference ($\chi^2 = 0.499, p > 0.05$) was observed with respect to sex and no infection was recorded in male as they are less in number.

### 4.4.5 Relationship between Prevalence and Altitude

The relationship between prevalence of infection and altitude of the studied area was determined and regression lie was fitted (Fig. 37). The results indicated a positive trend between prevalence and altitude of area but weakly correlated ($R^2 = 0.24, P > 0.05$) with each other.
Figure 29: Effects of serum dilutions obtained in the MM3-Sero ELISA. The cut-off values indicated with horizontal line (OD ≥ 0.100) of assay.
Table 12: The MM3-Sero ELISA diagnostic performance for the detection of specificity of the test.

<table>
<thead>
<tr>
<th>Assay results</th>
<th>Fasciola free herds (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Serum OD Min-Max</td>
</tr>
<tr>
<td>Negative</td>
<td>15</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
</tr>
</tbody>
</table>

*assay results with OD value ≥ 0.100 were classified as positive.

Table 13: The MM3-Sero ELISA diagnostic performance to measure the sensitivity of the test.

<table>
<thead>
<tr>
<th>Assay results</th>
<th>Fasciola infected herds (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Serum OD Min-Max</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>18</td>
</tr>
</tbody>
</table>

*assay results with OD values ≥ 0.100 were categorized as positive.
Table 14: MM3-Sero ELISA test for comparison of serum samples of cattle (n= 414) and buffalo (n= 184).

<table>
<thead>
<tr>
<th>Animals</th>
<th>n (%)</th>
<th>Serum OD Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle Serum (+)</td>
<td>35 (8.45)</td>
<td>0.102 - 0.633</td>
</tr>
<tr>
<td>Cattle Serum (-)</td>
<td>379 (91.54)</td>
<td>-0.499 - 0.099</td>
</tr>
<tr>
<td>Buffalo Serum (+)</td>
<td>4 (2.17)</td>
<td>0.133 - 0.414</td>
</tr>
<tr>
<td>Buffalo Serum (-)</td>
<td>180 (97.82)</td>
<td>-0.361 - 0.097</td>
</tr>
</tbody>
</table>

* OD values ≥ 0.100 were considered as positive.
Figure 30: MM3-Sero ELISA for the detection of level of *Fasciola* antibodies in serum taken from cattle. The cut-off value of assay is showed with horizontal line. Dotted lines represent the mean OD Values.

Table 15: The prevalence of fascioliasis related with cattle and buffalo.

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>414</td>
<td>35</td>
<td>8.45</td>
</tr>
<tr>
<td>Buffalo</td>
<td>184</td>
<td>4</td>
<td>2.17</td>
</tr>
<tr>
<td>Total</td>
<td>598</td>
<td>39</td>
<td>6.52</td>
</tr>
</tbody>
</table>

Ch-Sq ($\chi^2$) = 8.241, p< 0.05
Figure 31: MM3-Sero ELISA performed to measure the level of *Fasciola* antibodies in serum from studied age groups from cattle. The cut-off value indicated with horizontal line, dots represent the mean OD values.

Table 16: Fascioliasis prevalence among age groups of cattle.

<table>
<thead>
<tr>
<th>Ages(years)</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>176</td>
<td>14</td>
<td>7.95</td>
</tr>
<tr>
<td>4-6</td>
<td>107</td>
<td>7</td>
<td>6.54</td>
</tr>
<tr>
<td>7-10</td>
<td>111</td>
<td>14</td>
<td>12.61</td>
</tr>
<tr>
<td>≥11</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>414</td>
<td>35</td>
<td>8.45</td>
</tr>
</tbody>
</table>

Chi-Sq ($\chi^2$) = 4.889, p > 0.05
Figure 32: MM3-Sero ELISA representing the *Fasciola* antibodies level in serum of different age groups of buffalo. The cut-off value of assay presented with horizontal line, dots show the mean OD Values.

Table 17: Seroprevalence of fascioliasis among age groups of buffalos.

<table>
<thead>
<tr>
<th>Ages(years)</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>24</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>4-6</td>
<td>24</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>7-10</td>
<td>67</td>
<td>1</td>
<td>1.49</td>
</tr>
<tr>
<td>≥11</td>
<td>69</td>
<td>3</td>
<td>4.35</td>
</tr>
<tr>
<td>Total</td>
<td>184</td>
<td>4</td>
<td>2.17</td>
</tr>
</tbody>
</table>

Ch-Sq ($\chi^2$) = 2.746, p > 0.05
Figure 33: MM3-Sero ELISA measuring the level of Fasciola antibodies in serum of cattle from different studied areas. The cut-off values represented with horizontal line, dotted lines indicate the mean OD values.

Table 18: The cattle presented the seroprevalence of fascioliasis in different areas.

<table>
<thead>
<tr>
<th>Areas</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rawat</td>
<td>49</td>
<td>14</td>
<td>28.57</td>
</tr>
<tr>
<td>Attock</td>
<td>28</td>
<td>6</td>
<td>21.43</td>
</tr>
<tr>
<td>Rawalpindi</td>
<td>35</td>
<td>6</td>
<td>17.14</td>
</tr>
<tr>
<td>Gujar Khan</td>
<td>68</td>
<td>2</td>
<td>2.94</td>
</tr>
<tr>
<td>Kallar Syedan</td>
<td>51</td>
<td>3</td>
<td>5.88</td>
</tr>
<tr>
<td>Jhelum</td>
<td>65</td>
<td>3</td>
<td>4.62</td>
</tr>
<tr>
<td>Chakwal</td>
<td>50</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Dhudial</td>
<td>50</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Central Punjab</td>
<td>18</td>
<td>1</td>
<td>5.56</td>
</tr>
<tr>
<td>Total</td>
<td><strong>414</strong></td>
<td><strong>35</strong></td>
<td><strong>8.45</strong></td>
</tr>
</tbody>
</table>

Ch-Sq ($\chi^2$) = 48.901, p< 0.05
Figure 34: MM3-Sero ELISA presenting level of *Fasciola* antibodies in serum samples of studied in different areas. The cut-off value indicated with horizontal line, dots show the mean OD values.

Table 19: The seroprevalence of fascioliasis in different areas of buffalo.

<table>
<thead>
<tr>
<th>Areas</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rawalpindi</td>
<td>11</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Jhelum</td>
<td>4</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>central Punjab</td>
<td>169</td>
<td>4</td>
<td>2.37</td>
</tr>
<tr>
<td>Total</td>
<td>184</td>
<td>4</td>
<td>2.17</td>
</tr>
</tbody>
</table>

$\text{Ch-Sq (χ}^2\) = 0.363, \ p > 0.05$
Figure 35: The MM3-Sero ELISA detected in serum samples of studied sex groups of cattle. The cut-off value of assay presented with horizontal line, dotted lines showed the mean OD Values.

Table 20: The seroprevalence of fascioliasis according to sex of cattle.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>330</td>
<td>31</td>
<td>9.39</td>
</tr>
<tr>
<td>Male</td>
<td>84</td>
<td>4</td>
<td>4.76</td>
</tr>
<tr>
<td>Total</td>
<td>414</td>
<td>35</td>
<td>8.45</td>
</tr>
</tbody>
</table>

Ch-Sq (χ²) = 1.856, p > 0.05
**Figure 36:** MM3-Sero ELISA detected level of *Fasciola* antibodies in serum samples of sex of buffalo. The cut-off value of assay presented with horizontal line, dots indicated the mean OD values.

**Table 21:** The seroprevalence of fascioliasis in sex groups of buffalo.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>164</td>
<td>4</td>
<td>2.44</td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Total</td>
<td>184</td>
<td>4</td>
<td>2.17</td>
</tr>
</tbody>
</table>

Ch-Sq ($\chi^2$) = 0.499, p > 0.05
Figure 37: Scatterplot of the prevalence achieved with serological assay against altitude of the areas from where serum samples were collected. A linear regression line was fitted to the data.
4.5 DISCUSSION

Fascioliasis is a parasitic disease most commonly infecting ruminants and causes detrimental economic losses in term of reduced weight, milk yield, fertility and contaminated liver (Hillyer, 1999; Schweizer et al., 2005; Suzuki et al., 2006; Elitok et al., 2006; Charlier et al., 2007). For control purpose early detection are needed which is not possible with traditional coprological examination as infection appeared 10-12 weeks of postinfection (PI). Various immunological testing had been done all over world but limited studies in Pakistan on immune-diagnosis of fasciolosis. Highly sensitive methods are needed for the diagnosis of fascioliasis during prepatent period in order to adopt control strategies and to diminish the negative economic effect.

The present study emphasized on monoclonal antibody (Mab) based MM3-Sero ELISA for the detection of *Fasciola*-antibodies in serum samples of naturally infected cattle and buffalo from sub-tropical Punjab. The MM3-Sero ELISA is a consistent and easy to use diagnostic tool which is useful for screening large number of animals (Mezo et al., 2003 and 2010).

The results of current study showed MM3-Sero ELISA was extremely sensitive and specific for early detection of *Fasciola*-antibodies in serum samples of cattle and buffalo. Previously it was reported that MM3-Sero ELISA as highly specific and sensitive diagnostic tool which is enable to detect infection even at very low intensity. Only 1-2 flukes can be detected through this ELISA 4 week of PI while more than 3 flukes can be detected 3 week of PI (Mezo et al., 2007).
detection of low intensity infections is essential as many subclinical infections occurred in ruminants because of availability of contaminated pasture which favors the dissemination of life cycle of parasite (Anderson et al., 1999; Mezo et al., 2004 and 2007). However, the indirect ELISA determined infection 1-2 week of PI with sensitivity of only 91% and specificity of 86% (Arias et al., 2006) and never used for low intensity infections.

In the current investigation sera diluted up to 1: 10000 and test was reliable to detect the positive serum even at very high dilution. The usefulness of MM3-Sero ELISA that assay was able to detect positive infection even at high serum dilution (Mezo et al., 2007).

The assay was performed for measuring the level of antibodies against Fasciola in serum of cattle and buffalo. Overall very low infection rate (6.52%) was recorded in present study for both cattle and buffalo. Previously high prevalence rates were recorded for both species in different areas of Pakistan namely in central Punjab 25.45% (Khan et al., 2009), 23.97% in Multan (Masud and Majid, 1984), 10.48% in Lahore (Sahar, 1996) and 55% in Peshawar (Siddiqi and Shah, 1984). In domestic animals prevalence of fascioliasis had also been reported from various parts of the country (Ahmed, 1983; Javed et al., 1993; Nasreen et al., 2000; Azam et al., 2000; Bhutto et al., 2002; Ahmad, 2002; Raza et al., 2007 and Kakar and Kakarsulemankhel, 2008). The reasons for low infection rate in present study might be due to the serum samples collected from farm animals and these farms are well managed and they have regularly deworming activities. Moreover the farm animals usually have
stall-feeding habits and they do not allow for free grazing. The farm animals having free grazing activities close to water bodies have more chance of infection as they are exposed to infective stages of parasite and its intermediate host (Lymnaeids). The type of sampling and season in which serum samples had been collected may also affect the prevalence of infection. The fascioliasis was reported as seasonal disease and mainly restricted to two seasons (Swarup and Pachauri, 1987; Chaudhri et al., 1993).

In present study highest infection rate was recorded in cattle (8.45%) as compared to the buffalo (2.17%). The present results were inconsistent with previously reported study in Quetta having high infection rate in buffalo (27.3%) as compared to cattle (24.6%) (Kakar and Kakarsulemankhel, 2011). Similarly in buffaloes incidence of fascioliasis was 30.50% and 20.42% in cattle (Khan et al., 2009). Previously high susceptibility of infection was recorded in buffalo as they live in moist swampy areas (Ligda, 1998). In current study high infection rate of fascioliasis observed in cattle might be due to free grazing activities of cattle as compare to the buffalo which are mostly lived in captive conditions. The other plausible explanation for low infection rate in buffalo might be due to development of resistance against parasitic stages (cercariae and metacercariae) and its intermediate hosts because of its habit to live in swampy areas from a long period of time.

The area wise infection rate of fascioliasis was recorded for both cattle and buffalo in current study. The highest infection rate in cattle was recorded in areas of northern Punjab including Rawat, Attock and Rawalpindi as compared to central
Punjab. The reason for high prevalence in northern Punjab is might be due to effect of climatic conditions, as the northern areas have more rainfall and humidity which are favorable conditions for development and transmission of intermediate host of fasciolids (Torgerson and Claxton, 1999).

For buffalo more infection rate was recorded in central Punjab as compare to north of the Punjab. This might be due to number of samples from central Punjab because buffalo population is mostly confined to these areas as compared to northern Punjab. The other possible explanation might be due to climatic conditions of the areas and canal irrigated croplands in central Punjab which facilitate the propagation of intermediate hosts life cycle (Maqbool et al., 2003; Narcis et al., 2004; Diaz et al., 2007).

In cattle statistical non-significant high infection rate was found in younger animals as compared to old ones. The results were found in agreement with work executed in various part of the world (Firreria et al., 1981; Shah-Fischer and Say, 1989; Kiyyu, 2003; Nganga et al., 2004; Kuchai et al., 2011). The young animals might have high susceptibility towards infection as compared to old age animals. The reason for low infection rate in old animals might be due to development of immune system against the infection because of more exposure to the parasite and have ability to expel the parasites before they cause infection (Dunn, 1978; Shah-Fischer and Say, 1989). In the case of buffalo high susceptibility to infection was recorded in old animals then younger animals as previously reported (Khan et al., 2009). The statistically higher significant difference was recorded in old buffalo (Shrestha et al., 1992; Ghir-mire and Karki, 1996; Maqbool et al., 2002;
Pfukenyi et al., 2005; Qureshi et al., 2005a). This might be because of lowering of resistance due to environmental factors (Maqbool et al., 2002). The other plausible explanation might be due to more exposure of old animals to the parasitic stages and its intermediate host and relaxation of immune system.

Females were found more susceptible to infection as compared to males for both cattle and buffalo although found statistically non-significant. The non-significant gender wise difference was also reported in buffalo (Aal et al., 1999; Maqbool et al., 2002; Qureshi et al., 2005a and Khan et al., 2009). And statistically significant results were reported in previous studies for high susceptibility of infection in female cattle (Asanji and Williams, 1984 and Phiri et al., 2005a, b). Similar nature of results had been reported in other animals (Dobson, 1966a; Dobson and Owen, 1978). The high infection rates in females were might be due to high level of stress during parturition period (Spithill et al., 1999). The females are usually weak and malnourished making them more susceptible to infection (Blood and Radostits, 2000). The other possible explanation for high infection rate in female might be due to high female to male ratio and might be because of maintenance of female for breeding and milk production (Phiri et al., 2005b). The low infection rate was recorded in male. This might be due to less number of male samples, as males are slaughtered at very young age and kept mostly for selective breeding. Another possible explanation for low infection rate is males are mostly used for draft purposes and not allowed for free grazing, having less exposure to moisture (Uriarte et al., 1985).
The weak trend was observed between altitude of the areas and prevalence of infection which indicated that there are some other factors which influence the infection inspite of altitude. The results of current investigation are not in agreement with previous studies in world. Previously it was reported in different parts of the world that high land areas have high rainfall and humidity providing suitable conditions for more prevalence of infection as compared to dry lowland areas (Needham, 1977; Davies, 1982; Cheruiyot, 1983; Chambers, 1987; Majok et al., 1993; Vassilev, 1999; Pfukenyi and Mukaratirwa, 2003; Pfukenyi et al., 2006). And the positive relationship between fascioliasis and rainfall was also observed (Ollerenshaw and Smith, 1969). The temperature might be another important limiting factor at highland areas for the development of parasitic stages as the temperature exceeds 10 °C is favorable for the development of parasitic stages (Ollerenshaw, 1958). For current investigation the possible explanation for no relationship between altitude and prevalence may be existence of permanent water bodies and irrigation system in sub-tropical Punjab propagating the survival and existence of intermediate snail host (Lymnaeids) which in turn favors the transmission and existence of fascioliasis throughout the year. Under these conditions it would be useful to set up control strategies against fascioliasis which causes detrimental economic losses in livestock industry.

4.5.1 Conclusion

The results of this comprehensive evaluation led us that MM3-Sero ELISA provided sero-epidemiological record of fasciolosis in bovine for timely diagnosis of infection in order to adapt control strategies to mitigate the infection.
Chapter 5

GENERAL DISCUSSION

In present study the intermediate morphological forms of fasciolids are recorded in central Punjab, Pakistan using computer image analysis system (CIAS). The morphological characterizations of fasciolids are very complicated due to differences in external phenotypic features (Kimura et al., 1984), and thus traditional microscopic measurements may not be adequate in morphological characterizations (Ashrafi et al., 2006). In many Asian countries with the application of traditional microscopic measurements varied morphological forms of fasciolids have been reported, most recently the intermediate forms in Iran and Egypt has been described by using CIAS (Periago et al., 2008). The intermediate fasciolid forms also recorded in Pothwar region of Pakistan by using traditional microscopic measurements (Mufti et al., 2011).

Previous studies (Kendall, 1954; Kendall and Parfitt, 1959) indicated that in Pakistan *F. hepatica* is confined to highlands due to presence of its intermediate snail hosts *Galba truncatula*, while *F. gigantica* is found in lowlands with *Radix auricularia*. The intermediate forms recorded in present study may be because of overlapping of these two species in lowland areas of Pakistan. It is to be pointed out that overlapping might be occurred due to nomadic movements from highlands to lowland during winter season in search of grazing grounds. There might be the possibility that grazing animals carried *Galba truncatula* along with mud in their hooves. Earlier studies have shown that the *Galba truncatula* is not completely aquatic and have ability to live in mud by undergoing aestivation. They resume
development when favorable agro-climatic conditions return and thus have become the source of intermediate form fasciolids. Furthermore the existence of *G. truncatula* to lowlands area might have sporadic and temporary adaptation during cold rainy seasons (Kendall, 1954; Kendall and Parfitt, 1959). Similarly animal moves from lowlands to highland in summer season may also transfer *Radix auricularia* to mountainous areas (Mas-coma et al., 2009). The phenomenon of regional overlap is well documented in Nile Delta region in Egypt (El-Azazy and Schillhorn Van Veen, 1983; Looss, 1896; Soliman, 1998), Iran (Ashrafi et al., 2004, 2006, 2007) and in East Africa (Mas-Coma et al., 2009).

Another plausible explanation for co-existence of both fasciolid species occurred due to translocation of *G. truncatula* from highland to lowlands areas attributed to the River Indus and migratory birds. The River Indus runs throughout the country as it originated from the Karakoram, Hindu Kush and Himalayan ranges of Tibet, Kashmir and Northern Areas of Pakistan and irrigate the lowland areas. So there might be a possibility that *G. truncatula* have settled in lowlands as well. It has also been observed that the migratory bird’s move from Siberia to Afghanistan, Karakorum Range, across river Indus in Pakistan and finally moves towards India in search of breeding grounds. There might be a possibility that these migratory birds introduced different intermediate hosts (Lymnaeids) while making stopovers at lakes and water bodies come in their way in Pakistan.

Previously in Pakistan no information is available for comparing standard population with local fasciolid of bovine origin. It has been confirmed that the
Fasciola spp size variations are definitive host dependent (Mas-Coma and Bargues, 1997; Mas-Coma, 2004a; Valero et al., 2001). In present study comparison was made first time with bovine pure populations of both liver flukes from the areas where these species do not overlap, and result clearly indicated the presence of intermediate fasciolid forms.

This study indicated the existence of both fasciolid species in central Punjab, Pakistan, when BR BL/BW and VS-P measurements were applied to study inter- and intraspecific variations among Fasciola adults. These findings are in agreement with Periago et al. (2008) in Iran and Egypt, where these fasciolids were overlapped. In overlapping areas the plausible explanation might be the existence of hybrid forms due to abnormal spermatogenesis (Terasaki et al., 1982) and reproduction by parthenogenesis in helminths (Agatsuma et al., 1994) and facultative gynogenesis (Fletcher et al., 2004). Another possibility for phenotypic variations may be due to occurrence of fasciolids originated from different geographical areas having distinctive environmental conditions (Mayr, 1969).

The present study is also focused on the morphological characterizations of gravid eggs collected from mature fasciolids uteri, which was done first time in Pakistan. The size and shape of eggs collected from adult fasciolids uteri are same as collected from bile duct or in the faeces of animals (Valero et al., 2009). The results indicated overlapping of size and shape between Fasciola spp like (intermediate form) eggs. The most frequently used criteria for fasciolid diagnosis is based on eggs morphology recovered from the faeces. This may leads to
incorrect identification of fasciolid eggs in hosts having mixed infection especially
in case of Fasciolopsis, Gastrodiscoides (Mas-Coma et al., 2005) and Echinostoma
(WHO, 1991). In Pakistan, gastrointestinal amphistomes are widely distributed in
different agro-ecological region (Khan et al., 1988b; Khan et al., 1989; Pal and
Qayyum, 1993; Malik et al., 1995; Azad et al., 1997) and might have lead to
incorrect identification with fasciolid eggs.

In current study the traditional microscopic measurements EL, EW, EL/EW
along with other measurements EP, ER and EA were also taken using CIAS as
differential criteria for both species. The intermediate forms of eggs were recorded;
results were in agreement with Asian and African countries where intermediate
forms of eggs reported along with both fasciolid species and its existence may be
associated with crossbreeding of fasciolid species (Valero et al., 2009a).

One of the most important aspects of current study was analyzing the
climatic factors influencing the presence and potential dissemination of fascioliasis
with reference to climatic and global changes in the Punjab province. Two climatic
forecast indices and a remote sensing marker are used to characterize the climatic
factors and the earth surface in order to ascertain the epidemiological complexity
and time-lag dynamics of fascioliasis in the aforementioned model area.

The broad term of ‘global change’ includes all kind of environmental
modifications (McMichael et al., 2002; Smith et al., 2002; Clavero et al., 2011),
although in recent times there is the trend to differentiate climate change from
global change, the latter becoming increasingly used as result of anthropogenic modifications of biotic and abiotic factors. Inappropriately, climate change relates with damaging human activities and environmental variations causing the outbreaks of parasitic diseases by their own. Thus, it is very challenging to determine that whether the causation of disease emergence is by climate change or by global change. The global change is caused by so many factors considered as the major drivers of these changes (Molyneux, 1997; Molyneux, 2003; Childs et al., 1998; Gubler, 1998; Fayer, 2000, Patz et al., 2000; Patz et al., 2004; Gajadhar and Allen, 2004; MacPherson, 2005; Chomel et al., 2007; Chomel, 2008). Most of these studies emphasize the impact of global change on parasitic diseases which are vector-borne and of zoonotic importance, as it is the case of fascioliasis.

There are many factors included in global change which may impact on fascioliasis. Among them, and just to mention a few ones related to the present study in Punjab, modifications of land use become crucial for that disease when they imply changes of water collections, such as the different structures constructed for irrigation. Influences of irrigation on fascioliasis at local level have already been highlighted in Peru (Esteban et al., 2002) and at larger scale in Cambodia (Tum et al., 2004 and Tum et al., 2007). The effects of import and export and also movements of livestock in the spread of fascioliasis have also been emphasized, such as the introduction of domestic animal species from Europe into the Americas since the beginning of the New World conquest by Columbus in 1492 (Mas-Coma et al., 2009). Differences in livestock management are also well known to influence Fasciola infection in the animals (Walker et al., 2011) and have even been
highlighted in livestock of the Punjab Plain in Pakistan (Maqbool et al., 2002).

Among parasitic diseases, trematodiases and nematodiases transmitted by snails are the helminthiases most affected by climate change (Mas-coma et al., 2008; 2009 and 2010). Climate change has been shown to affect trematode transmission at different levels of the parasite’s life cycle, including aspects as (i) cercarial output, (ii) cercarial production variability, (iii) degree of cercarial production, cercarial size and size of intermediate host (iv) cercarial quality, (v) period of cercarial production rise and host mortality, and (vi) latitude. The common scenario comprises an increase of a few temperature degrees leading to great increases in cercarial appearance from snail vectors and consequently in disease transmission (Mas-coma et al., 2009; Poulin, 2006).

Although the aforementioned important global-warming-induced impact affects all trematodiases, data available so far suggests that flukes that follow a two-host life cycle and whose epidemiological characteristics are strongly marked by their zoonotic origin are the trematodiases showing a higher, or at least faster detectable, climate change impact. Changes in prevalences, intensities and geographical distribution at definitive host level, including both human and other mammal host infection, are more directly influenced in these cases due to the absence of any intermediate host population buffering this effect in between snail vector and definitive host. Moreover, the existence of animal reservoir host species additional to the human host allow to enhance this transmission increase effect in a faster way. This explains why, within plant-borne trematodiases, the liver
Fascioliasis by *F. hepatica* and *F. gigantica* using a large spectrum of herbivorous and omnivorous mammal hosts are more affected than the intestinal fasciolopsiasis and gastrodiscoidiasis which only use the pig as animal reservoir host (Mas-coma et al., 2008; 2009 and 2010).

Fascioliasis fulfills all adequate requirements for an helminthic disease to be highly and relatively rapidly (in comparison to other helminthiases) affected by climate change: (i) two host life cycle without any intermediate host between snail vector and mammal host; (ii) very dependent on climatic variables in most of its life cycle; (iii) amphibious snail vectors very dependent on climatic conditions; (iv) high zoonotic component due to the very low specificity at mammal host level (Mas-coma et al., 2009). This is why fascioliasis has always been the model most used to illustrate helminth-climate interactions (Ollerenshaw and Smith, 1969) as well as to develop climate forecasting methods and evaluate their usefulness.

Science-based indicators of vulnerability to climate change and of adaptability furnish crucial information for prioritization (Füssel, 2009). Southern countries of Asia top climate change's most vulnerable list, owing to fluctuations in weather condition lead to succession of upsetting natural disasters. According to the Global Climate Risk Index (CRI) 2012, Pakistan ranked 8th in the world country list according to the Long-Term Climate Risk Index for the 1991-2010 period and first in the world country list concerning 2010. This was due to heavy floods affecting more than 20 million people (over 10% of the total population of the country) and killing more than 1,700 people (Harmeling, 2012). Pakistan also
appears in the developing country list of the climate change Economic Vulnerability Index (EVI) (Bruckner, 2012). Anyway, the climate change problem for Pakistan does not only concern extreme catastrophic weather events, but as a developing nation it is also highly vulnerable to modest climate change (Yohe et al., 2006).

Additionally, Pakistan appears within the 16 countries rated within the group of the highest risk category by the Climate Change Vulnerability Index (CCVI) (Maplecroft, 2010). CCVI enables to identify areas of risk by evaluating 42 social, economic and environmental factors to assess national vulnerabilities across three core areas: (i) contact to natural disasters and sea-level rise; (ii) human sensitivity, in terms of population patterns, development, natural resources, agricultural dependency and conflicts; and (iii) future vulnerability by considering the adaptive capacity of a country’s government and infrastructure to combat climate change. According to the CCVI 2011 map, Pakistan enters in the extreme risk category mainly due to the Punjab province, which shows the highest CCVI risk almost throughout its whole area.

In Pakistan, climate change influences already reported include a rise in mean temperature in coastal areas, a 10-15% decrease of precipitation in the coastal belt and the hyper arid plains, and an increase in summer and winter precipitation in the northern part of the country (Farooq, 2004). Climate change impact on future water availability in Pakistan refers to glacier melting and forecasts on less rains in summer and more rains in winter (IPCC, 2001). There are reports indicating that
climate change is going to affect the deglaciation of Western Himalaya glaciers. In
the early few decades of the 21st century, forecast studies indicate that there will be
(i) excessive melting of Karakoram glaciers, (ii) flows of river Indus at Besham
Qila will be increased by about 50%, and (iii) thereafter there will be terrifying
reduction in flows (reduced to about 40% of year 2000 value by the end of the
century) (Rees and Collins, 2004). Global warming will yield additional flows of
the order of 5.2 MAF (= million acre feet) annually for the second decade of the
century and afterwards there will be steady decline of 22 MAF in Besham Qila
flows in the subsequent 80 years (Basharat and Hashmi, 2010). Moreover, the
results of nine out of ten global circulation models show that there will be an
increase of the order of 8-24% in South Asian Monsoons. This additional water
will be available and intense in space and time and can be readily stored in fresh
ground water aquifer of Punjab, preferably in eastern and south eastern doabs
(IPCC, 2001).

Results obtained in long-term analyses of the two fascioliasis forecast
climatic indices Mt and Wb-bs fit well with the aforementioned general estimates
of climate change for Pakistan. The crucial aspect is the trend of increasing
fascioliasis risk that the two indices show for the meteorological stations of
Sargodha and Faisalabad of the Punjab Plain in months of both summer and winter.
In the case of the more accurate index of Wb-bs, regression line trends for both F.
hepatica and F. gigantica appear to be statistically significant. The latter results
should be highlighted, given that the fasciolid species in the warmer lowlands is F.
gigantica, due to the presence of its specific lymnaeid vector species Radix
auricularia in the water collections of the Punjab Plain (Kendall, 1954).

It should be taken into account here that Mt and Wb-bs are indices able to estimate the influence that rainfall increases will directly develop on fascioliasis risk throughout the Punjab Plain, but are unable to detect the potential effects that rainfall increases may have on fascioliasis through artificial irrigation. The results obtained in the present study have shown that irrigation plays a decisive role in the fascioliasis transmission risk in winter. Otherwise said, there would be no disease transmission in winter if there would not be the large irrigation system available in the Punjab Plain. Moreover, the transmission monthly window in summer would be (i) relatively short and (ii) only restricted to narrow riversides, if there would not be the irrigation system assuring water availability in subsequent weeks and months and throughout areas far away from the river banks. Consequently, the already verified increase in summer and winter precipitation in the northern part of the country (Farooq and Khan, 2004) and the pronounced increases of the monsoons (IPCC, 2001) together with the increasing flows related to the increasing rates of deglaciation of Western Himalaya glaciers due to global warming (Basharat and Hashmi, 2010; Rees and Collins, 2004) will give rise to pronounced increases of water flow through the five great rivers and subsequent canals of the very wide irrigation system throughout the irrigated areas of the Punjab Plain which show fascioliasis risk in the NDVI maps.

A recent multidisciplinary analysis has shown that fasciolids in Asia derive from the Eurasian Near East region (Mas-Coma et al., 2009). Wild herbivore domestication began around 10,000 years ago at the dawn of the Neolithic in the
region known as the Fertile Crescent, a formerly fertile area in the Near and Middle East which was an agricultural region. By adapting to early domesticated animals in the Fertile Crescent, *F. hepatica* and *F. gigantica* took a crucial step which would enable them to spread from that region, to colonize almost the whole world as seen today. Biogeographic, climatic and lymnaeid faunal data indicate that the eastward spread followed two different main routes from the Fertile Crescent, one northward and another southward separated by the large Himalayan chain. In the southern Asian region, the restriction of the specific lymnaeid vector species *Galba truncatula* to highlands became a barrier for the spread of *F. hepatica* through the warmer lowlands. However, those high temperatures were permissive for the presence of *Radix* species and consequently *F. gigantica* in the lowlands of Afghanistan, Pakistan, India and South East Asia. This southward spread should have been facilitated by the extensive trade between the two primary centers 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 (Mas-Coma et al., 2009). Within all this long historical scenario, Punjab was part of several old empires and civilizations (Grewal, 2004), in which livestock and other domestic animals such as equines and camelids played a major role for food and transport. The British empire finally provided the Punjab Plain with the largest irrigation system, made mainly during the first half of the 20th century to counteract the dryness of the very wide lowland areas of Pakistan by taking advantage of the waters running down through large rivers from the heavy monsoon rainfall as well as from the thaw of snow on the Himalayan mountains (Visser, 1996; Basharat and Hashmi, 2010).
Thus, human history is at the origin of the high complexity of fascioliasis in the Punjab Plain, despite its pronounced topographic uniformity and a priori climatic and land in appropriateness for the transmission of this trematode disease. This complexity concerns the overlap of several aspects which draw a complicated disease picture, including:

A) the coexistence of both *F. hepatica* and *F. gigantica* infecting livestock (Khan *et al.*, 2010), together with morphologically intermediate forms recently described in Pakistan (Afshan *et al.*, 2013), similarly as in close countries (Ashrafi *et al.*, 2006; Periago *et al.*, 2008);

B) the presence of populations of the lymnaeid species *Radix auricularia* allowing for the transmission of *F. gigantica* but a priori not that of *F. hepatica* (Kendall, 1954), although the *F. hepatica* specific vector *G. truncatula* has been quite recently mentioned not only to be present but also to be involved in fascioliasis transmission in different lowland districts such as Gujranwala, Lahore, Sheikhupura, Sargodha, Jhang and Faisalabad of Punjab province (Buriro and Chaudhry, 1981; Maqbool *et al.*, 2003);

C) the coexistence of mid-sized ruminants as sheep and goats and large ruminants as cattle and buffaloes showing different susceptibilities; these definitive host species are known to influence aspects as fluke development and egg production and shedding (Valero *et al.*, 2002; Valero *et al.*, 1998; Valero *et al.*, 2001) as well as disease prevalences and intensities (Mas-Coma *et al.*, 2009; Mas-Coma and Bargues, 1997; Molina *et al.*, 2005);
D) the detection of highly varying prevalences of up to 70% in livestock, depending on animal species and localities, areas or districts;

E) the finding of different monthly prevalence dynamics patterns, including from monoseasonality to biseasonality, also depending on localities, areas or districts as well as on definitive host species (Qureshi, 2008; Qureshi and Tanveer, 2009; Maqbool et al., 2002; Khan et al., 2010)

F) the reports of important differences in infection rates in the same animal host species (e.g., buffalo) according to different management conditions (e.g., slaughterhouses, livestock farms, veterinary hospitals, household animals) (Maqbool et al., 2002);

G) the involvement of humans, including liver fluke infection mainly affecting children and young subjects and prevalence data based on results from low sensitivity diagnostic techniques suggesting an underestimation of a probably larger public health problem (Qureshi et al., 2005; Qureshi, 2008; Qureshi and Tanveer, 2009), which could be solved by the application of more sensitive and specific modern techniques (Valero et al., 2012; Espinoza et al., 2007).

The combination of a concentrated fascioliasis transmission risk in winter due to irrigation and the wider window of transmission risk during summer and autumn due to overlap of rainfall and irrigation, together with local differences in irrigation management according to districts and local agricultural needs, may explain the heterogeneity of prevalence figures observed in different animal
surveys, whether in complete year surveys as well as in point prevalence studies in different localities, areas and districts.

Another Important aspect of current study was application of monoclonal antibody based (mAb) MM3 Sero-ELISA for early diagnosis of fascioliasis in subtropical Punjab, Pakistan. Early detection of infection are needed for control measures, which is not possible with currently used coprological methods which detect infection 10-12 weeks of postinfection (PI). Limited work on immunological testing has been reported in Pakistan.

Overall very low infection rate (6.52%) was recorded in present study for both cattle (8.45 %) and buffalo (2.17 %). Previously high prevalence rates with coprological examination were recorded for both species in different areas of Pakistan namely in central Punjab 25.45% (Khan et al., 2009), 14.71% in Punjab (Maqbool et al., 2002), 17.68% in Bahawalpur (Chaudhry and Niaz, 1984), 23.97% in Multan (Masud and Majid, 1984), 10.48% in Lahore (Sahar, 1996) and 55% in Peshawar (Siddiqi and Shah, 1984). The reasons for low infection rate in present study might be due to the serum samples collected from farm animals which are well managed with regular deworming. The feeding habitat of animals may be another plausible factor for low infection rate in current study, stall-feeding is common practice in farm animals and they do not allow for free grazing. The farm animals having free grazing activities close to water bodies have more chance of infection as they are exposed to infective stages of parasite and its intermediate host (Lymnaeids). The seasonality and type of
sampling may be another possible reason which affects the prevalence of infection.
The fascioliasis is documented as seasonal disease (Swarup and Pachauri, 1987; Chaudhri et al., 1993). The geographical area and agro-climatic condition may also reduce the infection level as it effects the development and transmission of snail intermediate hosts population.

The MM3-Sero ELISA is proved highly sensitive and specific for the detection of *Fasciola*-antibodies in serum samples even when infection is at very low intensity. This ELISA enable to detect 1-2 flukes at 4th week of PI while more than 3 flukes can be detected 3 week of PI. This assay is useful to detect positive infection even at high serum dilution (Mezo et al., 2007). The results of current study are in agreement that MM3-Sero ELISA is a reliable and easy to use diagnostic tool, which is useful for screening large number of animals (Mezo et al., 2003 and 2010).

5.1 RECOMENDATIONS

The intermediate forms, *F. hepatica*-like and *F. gigantica*-like adult worms and eggs, are prevalent in buffaloes of central Punjab, Pakistan. Fasciolid intermediate forms have attained an endemic status in central Punjab, Pakistan. Further work on this complex orographic and transhumance scenario is needed to be carried out on the epidemiology of fasciolosis in various agro-ecological zones of Pakistan and that would enable for establishing appropriate control measures.

The combined approach by Mt, Wb-bs and NDVI indices illustrates how
climate change and global change are influencing fascioliasis risks in Pakistan, both with regard to geographical distribution and seasonality in Punjab province. The economic importance of livestock in this the most densely populated province of the country makes this phenomenon to be given forecast priority assessment henceforth in order to establish the adequate control measures. Through the use of these three indices one could predict the geographical risk and the seasonality of fascioliasis in other ecological zones of Pakistan. This study also recommends the future requirement for standardization of data sampling and examination/analysis procedures, in the way for the development of computerized spatial databases which can be frequently updated with new material and merged into an advanced Geographic Information System (GIS) model.

The application of MM3 Sero-ELISA appeared to be effective tool for accurate diagnosis of fasciolid infections in cattle and buffaloes of sub-tropical Punjab, Pakistan. Furthermore sero-ELISA is a reliable and state-of-the-art diagnostic technique for screening large number of animals. Using MM3 based Sero-ELISA epidemiological studies could be suggested in different agro-ecological regions of Pakistan to determine the extent of fascioliasis in domesticated animals. It will also help to devise effective mitigation and adaptation strategies. For prevalence studies the sampling should be conducted in winter season as in summer animal picks infection and it appears in winter season. The infection pattern recorded in this way could be used for recommending appropriate dosage of flukicide treatments.
In Pakistan, animal fascioliasis control is mainly related to chemotherapy. Strategic chemotherapy needs appropriate knowledge about disease epidemiology at local level to enable treatment planning for maximum effectiveness with least possible drug dosification. According to the results obtained in the current investigation about monthly forecasts and fascioliasis transmission patterns in Punjab province and basing on international recommendations on strategic treatment of *Fasciola* (FAO, 1994), the following annual scheme to control the disease successfully caused by either fasciolid species may be recommended to be applied for all livestock species in the Punjab province:

- A first preventive-curative treatment should be applied in the months of February-March in the Punjab Plain and April-May in the highlands, to remove fluke adults from the liver.
- A second preventive-curative treatment should be applied in September-October in throughout the endemic areas of the Punjab province. This treatment is crucial.
- An intermediate prophylactic treatment should be applied at the end of the dry season (June) when growth of free-living stages and intramolluscan fluke phases are arrested and reproduction and activity of snails is minimum. If not all treatments could be afforded due to economic restrictions, this intermediate prophylactic treatment is the one to be avoided.

With regard to humans, the evident first priority is to obtain the needed knowledge baseline enabling for subsequent appropriate action. For this purpose,
surveys should be carried out on populations inhabiting the central and southern high risk endemic parts of Punjab Plain, and to repeat surveys in northern Punjab Plain by means of modern, highly sensitive and specific diagnostic techniques to assess the real epidemiological situation of human fascioliasis and its geographical spread throughout the Punjab province.
SUMMARY

In Pakistan, the province of Punjab, the most important by extension and population, presents livestock fascioliasis throughout, causing high economic losses, but also affecting humans inhabiting rural areas. Prevalences in livestock vary pronouncedly in space and time (1-70%), conforming a heterogeneous picture. Fascioliasis is a parasitic liver infection caused by trematode species of the genus *Fasciola* (Trematoda: Fasciolidae): *F. hepatica* and *F. gigantica*. The phenotypic features of adults and eggs of fasciolids infecting buffaloes inhabiting the central Punjab area, Pakistan, have been studied to characterize fasciolid populations involved. The multivariate analysis used showed that the characteristics of fasciolids from Pakistan are between standard populations of liver fluke species. Similarly, the morphometric measurements of fasciolid eggs from central Punjab are also between *F. hepatica* and *F. gigantica* standard populations. These results demonstrate the presence of fasciolid intermediate forms in the endemic area studied in Pakistan.

The climatic factors influencing fascioliasis presence and potential spread were analyzed and results showed the combined use of the three Mt index, Wb-bs index and NDVI index/prevalence correlation time-lags proves to be an appropriate way to approach the geographical distribution risk areas and the seasonality of disease transmission risk in the case of fascioliasis in Punjab province, Pakistan. The results of current investigation show how the combined use of these three indices allow for a complete analysis in such an heterogeneous epidemiological
situation of fascioliasis including (i) mid-sized and large-size livestock species presenting different immunological responses to fasciolids, (ii) the overlap of both *F. hepatica* and *F. gigantica*, (iii) overlapping of highlands and lowlands in the area studied, and (iv) disease transmission following biseasonality with one peak related to natural rainfall and the other peak related to man-made irrigation. Results suggest a human infection situation giving cause for concern and illustrate how climate and global changes are influencing both geographical distribution and seasonality of fascioliasis risks. Mainly in summer but also in winter, the increase of fascioliasis risk throughout the Punjab Plain and its decrease in the northern highland part of the province become evident during the 1990-2010 period. The increase of disease transmission risk in the lowlands should be highlighted, given that the largest part of the Punjab province includes low altitude, highly irrigated plains. The importance of livestock in this province makes this phenomenon to be given forecast priority assessment henceforth in order to establish the adequate control measures. An annual treatment scheme to effectively control the disease is finally recommended to be applied throughout the whole Punjab province.

In present study highest infection rate was recorded in cattle (8.45%) as compared to the buffalo (2.17%). The results of current study showed MM3-Sero ELISA was very sensitive and specific for the diagnosis of *Fasciola*-antibodies in serum samples of cattle and buffalo. Moreover, climate change is shown to be able to modify the characteristics of fascioliasis transmission and epidemiology affecting both humans and livestock in the Punjab Plain and whole Pakistan. These results suggest potential situations of concern in other south Asian countries in the
near future. The Punjab Plain, where liver flukes have shown to be a constant threat to livestock development giving rise to important economic losses, may thus conform a useful fascioliasis model to be extrapolated to other areas of country.
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APPENDICES

Appendix # 1

PREPARATION OF STAIN

Borax carmine stain was prepared by the following method.

Recipe for 400ml

Borax Crystal / Disodium tetraborate decahydrate  8g
Carmine 5g
70% Alcohol 200ml
Distilled water 200 ml or 200 cc

Procedure

8 g of borax was dissolved in 200 ml of distilled water in round bottle then add 5 g carmine (Sigma) and a piece of porcelain in the bottle connected with distillator. Set the heater at 150-200°C heat the mixture for 30 minutes. Then allowed to cool and add 200 ml of 70% alcohol and left for 24 hours. Then filter the solution and stored in dark.
1. The flukes were kept in borax-carmine overnight on a Mini-Rocker MR-I for balancing the flukes.

2. After overnight staining the flukes were put in 70 percent alcohol with few drops of 37 percent HCL. When internal organs of flukes become visible then passed through different grades of alcohol.

3. Flukes with little pressure by placing slide on it were passed through 96 percent, 100 percent and absolute butanol for 45 minutes.

4. Flukes were then transferred and passed through xylene for 45 minutes.

5. Finally the flukes were mounted in Canada balsam and slides were dried in hot oven over night and examined for morphometric measurements.
Appendix # 2

PROFORMA OF EACH ANIMAL

Date of sampling: __________

Field information

- Host type: __________
- Animal breed: __________
- Host locality: __________
- Host sex: __________
- Animal age: __________

(Record 1 counting no, of incisor teeth)

- Grazing management: intensive/ extensive/semi extensive
- Type of water bodies: canal/reservoir/pond/tube well
- Temperature: Max / Min
- Altitude of animal grazing area
Appendix # 3

SEROLOGICAL ANALYSIS

MM3-Sero ELISA (Enzyme Linked Immunosorbent Assay).

Preparation of Reagents:
96 well-flat bottom antigen coated micro-titer plates (Provided by WHO, World Fascioliasis Center).

a) 0.01 M phosphate-buffered saline solution, pH 7.4 (PBS).

Solution A
- NaH$_2$PO$_4$, pM 120.00
- Dist. Water to 1000 ml

Solution B
- Na$_2$HPO$_4$, pM 141.96
- Dist. Water to 1000 ml

b) PBS PREPARATION
Solution A + Solution B + Dist. Water = PBS 0.01 M
19 ml + 81 ml + 1900 ml = 2000 ml

c) Washing buffer, 0.01 M PBS, pH 7.4 containing 0.2% Tween-20 (PBS-T).
PBS 0.01 M 1000 ml
Tween-20 2 ml

d) Sample diluent, PBS-T containing 1% BSA (bovine serum albumin)
Sample 100 ul for each well
PBS-T 9600 ul
BSA 0.1 gram

e) Primary antibody (Monoclonal Anti-Bovine IgG Clone BG-18 Biotin Conjugate) (Sigma)
Primary antibody 1:120,000

f) Secondary antibody (NeutrAvidin™ Horseradish Peroxidase conjugated) (Pierce Biotechnology).
Secondary antibody 1:4000.

g) Substrate buffer, pH 5.0 SIGMAFAST™ OPD (o-Phenylenediamine dihydrochloride)

One OPD tablet and one urea hydrogen peroxide/buffer tablet, dissolved in 20 ml of water, provides 20 ml of ready-to-use substrate. When the tablet is dissolved in this manner, the final concentrations will be 0.4 mg/ml OPD, 0.4 mg/ml urea hydrogen peroxide, and 0.05 M phosphate-citrate, pH 5.0. Tablets are individually packaged in foil packets.
Strip of ELISA Plate

Samples run in duplicate as indicated in darkly colored wells.