Identification and Characterization of Antigenic Proteins of Excretory/Secretory and Somatic Products of *Fasciola gigantica* Collected from Ruminants

BY

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Identification and Characterization of Antigenic Proteins of Excretory/Secretory and Somatic Products of *Fasciola gigantica* Collected from Ruminants

A dissertation submitted in the partial fulfillment of the requirements for the degree of Master of Philosophy

In

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By

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بسم الله الرحمن الرحيم

Dedicated
To
My beloved parents

CERTIFICATE

This dissertation "Identification and characterization of antigenic proteins of excretory/secretory and somatic products of *Fasciola gigantica* collected from ruminants" is submitted by Mr. Muhammad Sajid is accepted in its present form by the department of Animal sciences, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Parasitology.

Supervisor _____

Dr. Kiran Afshan

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External Examiner_____

DECLARATION

I hereby declare that I have worked on my thesis "Identification and characterization of antigenic proteins of excretory/secretory and somatic products of *Fasciola gigantica* collected from ruminants. independently and the work presented here is original. This thesis has not been submitted in the current or a similar form to any other university.

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Muhammad Sajid
February 2018

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All the praises for Almighty Allah, Who created us as a Muslim and blessed us with knowledge to differentiate between the right and wrong. Many thanks to Him as He blessed us with the Holy Prophet Hazrat Muhammad, peace be upon him, for whom the whole universe is created and who enabled us to worship only one God. He (PBUH) brought us out of darkness and enlightened the way of Heaven.

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List of Abbreviations

Abbreviations	Full form
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
ELISA	Enzyme Linked Immunosorbent Assay
ES	Excretory Secretory
GDP	Gross Domestic Product
GIS	Geographic Information System
GOP	Government Of Pakistan
IgG	Immunoglobin G
KPK	Khyber Pakhtunkhwa
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PMSF	Phynylmethane sulfonyl fluoride
SDS	Sodium Dodecyl Sulfate
SPSS	Statistical Package for Social sciences
TBST	Tris Buffer Saline Tween
TMB	Tetra methyl benzidine
WHO	World Health Organization

ABSTRACT

Objectives

Fascioliasis is foodborne trematodes disease of ruminants caused by *Fasciola gigantica* and *F. hepatica* causing huge economic losses in term of reduced growth, fertility, meat and milk yield. The early diagnostic approaches and anthelmintic resistance are major obstacles in the control of fascioliasis. To improve the diagnosis and find alternative therapeutic measures, the current study was designed for the identification and characterization of somatic and ES products of adult worms collected from bile ducts of cattle and buffaloes. The immunogenicity of identified polypeptides in adult worm extracts were tested by Western Blot and indirect ELISA method.

Materials and Methods

Adult *F. gigantica* were collected from the bile ducts slaughtered of cattle and buffaloes. These flukes were processed to obtain somatic and ES products. Somatic proteins were obtained by homogenizing and centrifugation and ES products were obtained by incubation in PBS in which flukes were cultured. Protein concentration was quantified by Bradford method. The somatic proteins and ES products were then separated on SDS PAGE and electro-transferred to a nitrocellulose membrane to detect their immunogenicity. By Western blotting and Indirect ELISA immunogencity of different antigens and their reactivity against sera of *F. gigantica* infected animals. Chi-sqaure analysis was performed to find association of different risk factors with infection.

Results

The overall prevalence of fasciolosis was 4.7% in cattle and buffaloes. The disease was found to be associated with host locality ($\chi2$=42.247p=0.000), age of host ($\chi2$=22.828 p=0.000) and grazing management of the host ($\chi2$ =22.828 p=0.000). Somatic proteins separated by SDS were ranged of 15 to 146 kDa. Most frequent protein bands were ranged 16,27,44,45,78,80,90,110, and 130 kDa. Molecular weights of ES products were in range of 93,100,130,145,147, and 148 kDa. Western Blot of somatic products detected a polypeptide in these extracts is a range of size 130 to 250 kDa. Conclusion The immunogenic proteins identified were 38 to 72 kDa and 95 to 250 kDa and a band range of 130 to 250 kDa by Western blotting and indirect ELISA which can be used for early diagnosis. Immunogenic proteins in the somatic extracts must be tested in future for the

development of vaccine. The antigenic proteins in the ES products should also be identified.

Conclusion

The immunogenic proteins identified were 38 to 72 kDa and 95 to 250 kDa and a band range of 130 to 250 kDa by Western blotting and indirect ELISA which can be used for early diagnosis. Immunogenic proteins in the somatic extracts must be tested in future for the development of vaccine. The antigenic proteins in the ES products should also be identified.

INTRODUCTION

Livestock is the backbone of agriculture and plays a crucial role in finance of agricultural countries (Luqman *et al.,* 2013). Its role is very vital as for as human nutrition and socioeconomic development is concerned. Milk, meat and eggs are valuable products from livestock and they are indispensable source of protein, energy, minerals and micronutrients, and it produces 28% of protein and 13% of calories worldwide (FAO 2011). As in other developing countries, half of the population of Pakistan lives in rural areas and almost eight million families are directly depending on the livestock and dairy business for their income (Kregg-Byers and Schlenk, 2010; Luqman, 2017). During 2012-13, 11.9% of Pakistan's GDP was contributed by livestock which is 55.4% of total agriculture contribution to the national economy (Ashfaq *et al.,* 2014). For the last forty years, in developing countries due to increase in number of residents, per capita deployment of animal protein, with worldwide meat consumption anticipated to rise nearly by 73% in 2050 (FAO 2011). So, the production of meat and other products from animals will be required to be enhanced to overcome the burden of growing world population (Thornton, 2010). A vital part to attain this goal should come from better animal health care, which has key impact on farming efficiency of animals (Charlier *et al.,* 2014).

Parasitic infections are key in lowering livestock productivity worldwide (Kasib *et al.,* 2009; Vercruysse and Claerebout, 2001). These infections pose serious health threats limiting the output of livestock due to morbidity coupled with mortality (Nwosu *et al.,* 2007; Pradesh *et al.,* 2016). Helminth infections of ruminants limit efficient livestock production and we can discern several clinical features and symptoms of helminth infections from the primitive texts of Hippocrates, and the Holy Bible (Cox, 2002; Hotez *et al.,* 2006; Hotez *et al.,* 2008). Most grazing ruminants are infected by diverse helminths which incur huge economic losses due to the negative impact on nourishment, development, weight gain, carcass composition, wool growth, fecundity, and productivity of the host (Fitzpatrick, 2013). Platyhelminthes include trematode parasites cause serious economic losses and estimated prevalence of food borne trematodiasis exceeds 40 million in developing countries of East Asia (Hotez *et al.,* 2008).

1.1 Fascioliasis

Fascioliasis is a cosmopolitan food borne helminth infection, caused by digenetic trematodes species, *F. hepatica* and *Fasciola gigantica*. The disease is one of the most important and destructive liver damaging diseases of ruminants (Mas-Coma *et al.*, 2009). Its importance is increased because of its broad spectrum of final hosts (Rondelaud *et al.*, 2006) in which these fasciolids cause acute and chronic infections (Silva *et al.*, 1996). It is anticipated that more than 700 million ruminants and above 180 million people are in danger of fascioliasis (Rehman *et al.*, 2016). A wide range of herbivores, such as, cattle, pigs, horses, rodents, as well as human beings, are affected by it (Olivera *et al.*, 2012). It is a re-current infection in different parts of world, chiefly in Africa (Mas-Coma, 2004), most frequently described by fever, eosinophilia, and abdominal pain, although 50% cases may not be showing symptoms of the disease (Torres *et al.*, 2004). Blood loss may be (between 0.2 ml and 0.5 ml of blood per fluke per day), accompanied by low intake of diet, nutrients storage, and weaken energy metabolism due to liver damage, reduction in weight gain and milk production in animals (Dargie *et al.*, 1979).

1.2 Taxonomy

The taxonomic position of the flukes is presented as follow (WHO, 1995; Soulsby, 1968).

Phylum: Platyhelminths,

Class: Trematoda,

Sub- class: Digenea,

Super Family: Fasciolidea,

Genus: *Fasciola,*

Species: *F. gigantica*; *F. hepatica*

1.3 Morphology and Anatomy

F. gigantica and *F. hepatica* are commonly known as flukes. They are often termed as liver flukes since they infect livers of vertebrate hosts such as cattle buffalo sheep goat and even human. The structure of their bodies is leaf like which is flattened dorsoventrally tapered at back while flattened at the front. They have two suckers. the oral sucker is relatively small but strong and positioned at the end of a cone-shape projection at the

anterior end. The other sucker is known as acetabulum which is relatively larger compared to the oral sucker and is situated at the anterior end. They are largest flukes reaching a length of 30 mm *(F.hepatica)* and 75 mm *(F.gigantica*) and a width of 13 mm (Loos-Frank and Grencis, 2016). Both male and female reproductive organs are located within the same adult flukes. They are hermaphrodites. The variations more often highlighted between the species that of *F. gigantica* are comparatively longer with different general appearance of the body (Kendall, 1965).

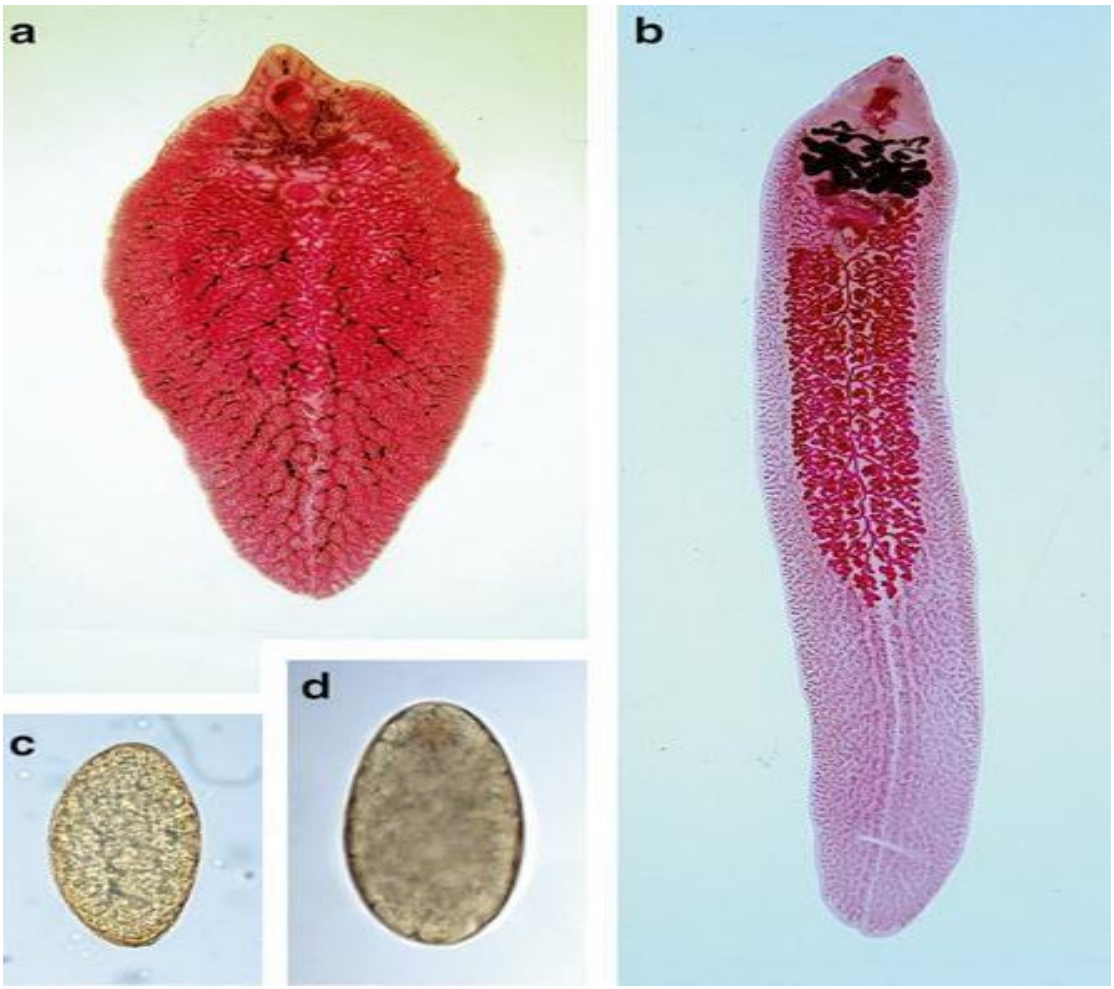


Figure 1 A) *F. hepatica* B) *F. gigantica* C) Egg of *F. gigantica* D) Egg of *F. hepatica*

1.4 Life Cycle

Fasciolids *(F.gigantica* and *F.hepatica)* are digenetic trematodes having a composite life cycle which requires *lymnaeid* snails as an intermediate host while the

definitive host is always a vertebrate (Usip *et al.,* 2014). Sexually mature flukes inhabit bile ducts of host and produce eggs which are released in the feces. Miracidium larvae emerge from eggs as these find moist conditions and temperature of the environment is above 5ºC. These miracidia require moisture to move into the tissue of suitable host snails within 24-30 hours. This larva completes many cycles of asexual reproduction and departs the intermediate host as cercariae. These cercariae then attach to watercress or other vegetation and transforms into metacercaria with a resistant cyst wall. If the definitive host ingests these metacercaria and is transformed into immature fluke which crosses the intestinal wall and enter the blood stream of host. The immature flukes reside in the liver parenchyma for about a month, increasing in size up to 10 mm. Once entered in the bile ducts they grow in size before they start laying egg about 10-12 weeks post invasion. Within the bile ducts each mature hermaphroditic fluke (both sexes in single worm) produces up to 50000 eggs per day. These eggs are passed with the bile liquids into the intestine and are carried onto the pastures in the feces (Whitfield, 1979; Andrews, 1999), (Figure 2).

1.5 Morbidity

Enormity of domestic animals and losses in food yield that results from fascioliasis has been reviewed by Burridge (Burridge, 1982). Liver flukes are important tissue parasites, which engender terrible pathology in sheep and cattle (Usip *et al.,* 2014). Because burrowing of parasites through hepatic tissue and inflammatory damage to host immune responses against secreted immunogenic proteins left by the parasite, causes physical damage to the host liver (Campillo *et al.,* 2017). Infection by *F. gigantica* or *F. hepatica* in very low numbers results in reduction of growth in farm animals (Sewell, 1966; Cawdrey *et al.,* 1977; Oakley *et al.,* 1979; Chick *et al.,* 1980; Malone *et al.,* 1982). Reduction in milk yield varies between 90 and 300kg/lactation which has been linked with *F. hepatica* infections (Both *et al.,* 1970, Randeli and Bradley, 1980; Ross, 1970). The establishment of pregnancy and conception is also affected by infections by flukes (Parr *et al.,* 1982).

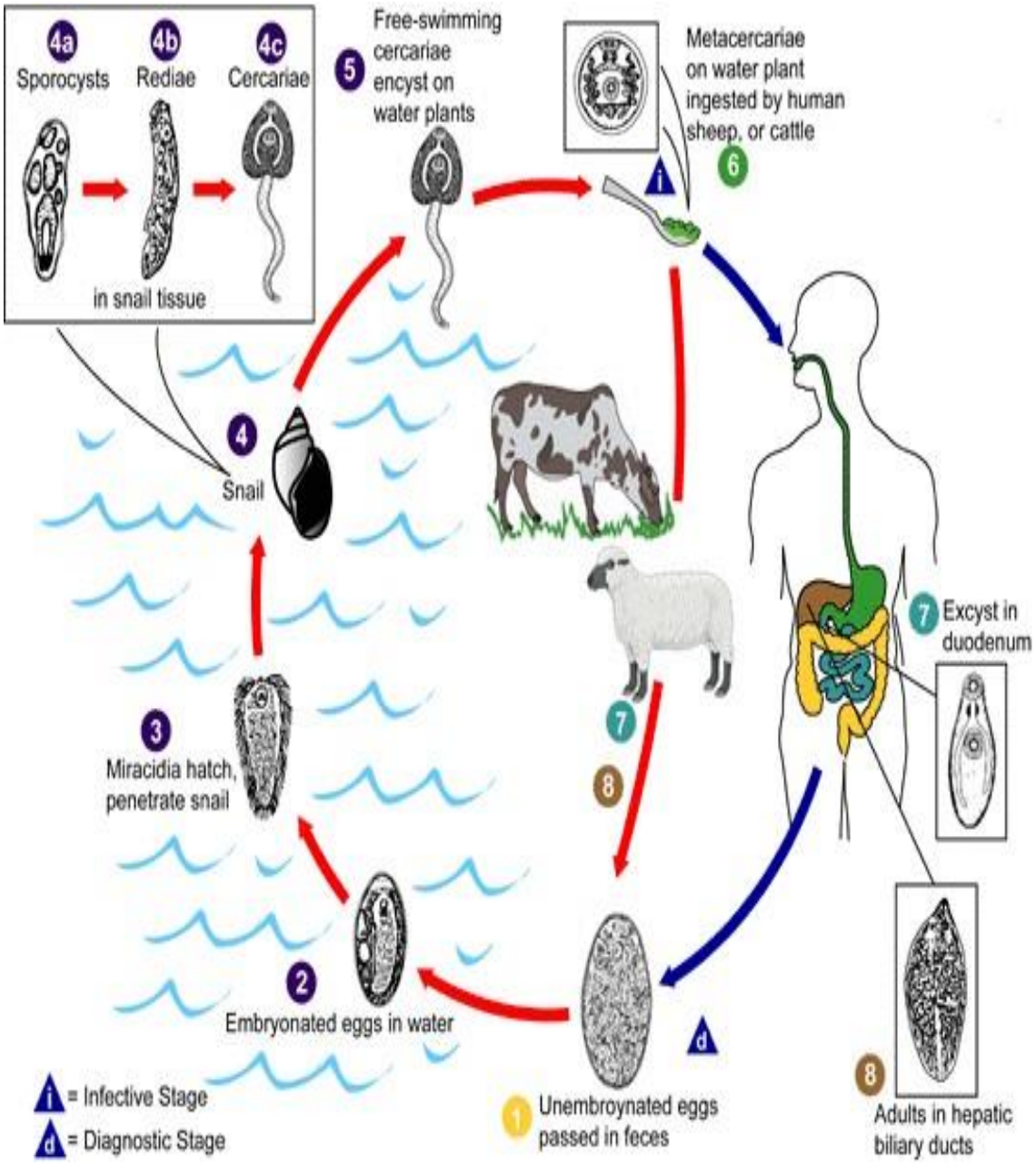


Figure 2: Life cycle of *Fasciola gigantica* (Source CDC website).

1.6 Clinical signs and symptoms

The clinical symptoms of fascioliasis range from the asymptomatic stage to severe episodes of bleeding within the abdomen requiring acute surgical abdomen (Torres *et al.*, 2004). Damage of liver and other metabolic changes are due migration and hematophagic activities of *Fasciola* sp. (Mas-Coma *et al.,* 2014). Infection of as low as 54 flukes per animal leads to10 % reduction in weight of the host animal (Cawdery *et al.,* 1977).

1.7 Economic Impact

Economic losses caused by fascioliasis can be estimated by factors like reduction in the body weight, reduction in milk production and the cost of treatment. Economic losses due to fascioliasis is estimated at US$ 3.2 billion worldwide (Khan *et al.,* 2017). Losses due to fascioliasis in the United Kingdom and Ireland alone are more than £70.67 million a year (Bekele *et al.,* 2010; Morgan *et al.,* 2013). Economic losses attributed to the sub-clinical infection of bovine fasciolosis were estimated to be £37.2 million in Switzerland (Morgan *et al.,* 2013). Furthermore, fascioliasis is recognized as emerging disease in humans. World Health Organization has anticipated that 180 million people are in danger to get infection and more than one million people are infected with fascioliasis (Mulatu and Addis, 2011).

1.8 Disease Transmission

Prevalence of fascioliasis is forecasted to increase because of climate change, more rainfalls and production of suitable conditions for snails (Hotez *et al.,* 2008). Climatic changes round the globe and a poor knowledge of livestock farmers about the animal diseases adds in development and growth of parasites (Raza *et al.,* 2017). Rainy seasons in addition to humidity are favorable for the growth and development of *Fasciola* species (Spithill, 1999). Studies have demonstrated that climatic factors and transmission of disease are associated with each other (Lari *et al.,* 2006; *Khan et al.,* 2009). The animals get infected by ingesting metacercaria when they graze in flooded meadows during dry season, with little or no infection between June and August. The production of eggs is at peak between August and September, 10-15 weeks post infection (Claxton *et al.,* 1997).

Studies showed relationship of infection between age, gender of the host (Sukhapesna *et al.,* 1990; Asanji and Williams, 1984). Different breeds of animals may have different susceptibility for a particular disease. Indian cattle **(***Bos indicus*) is more resistant to fascioliasis than European cattle (*B. taurus*). Besides, it is noticeable that variation in the prevalence of fascioliasis between the varieties of *B. indicus* may exist. Cattle are generally resistant to the fascioliasis. Although, there is inadequate evidence to propose that complete acquired immunity to *F. gigantica* occurs in cattle rather the evidence is consistent with a partial acquired resistance (Castelino and Preston, 1979).

1.9 Global prevalence

Prevalence of fascioliasis in an area depends upon a multifactorial system which comprises hosts, parasite, socio-economic status of the farmer and other environmental effects (Maqbool *et al.,* 2002). It is wide spread in many countries of Asia and Middle East including Iraq, Iran, Russia, Thailand, Turkey, Iraq, Iran, China, Vietnam, Nepal, Japan, Korea, Philippines, Pakistan, Bangladesh Saudi Arabia and Cambodia (Usip *et al.,* 2014). Apart from climate and ecological factors, other aspects of animals such as age, strain and herd level (stock rate and type of farming system) are also associated with the episodes of the disease (Kuerpick *et al.,* 2013; Petros *et al.,* 2013). A study conducted recently, on the prevalence of fascioliasis in the Nile Delta (Egypt) showed an overall infection of 9.77% infection (El-tahawy *et al.,*2017). Another study conducted in Vietnam showed an overall prevalence of 23.4% (Nguyen *et al.,* 2017). Similar studies conducted in India (Tarai, hills and plains in northern area) recorded infection rate 10.79% in cattle, 13.90% in buffaloes, 2.78% in sheep and 2.35% in goats (Dutta *et al.,* 1990; Garg *et al.,* 2009). A study conducted in Gorakhpur district India showed 94% of the buffaloes infected with *F. gigantica* (Singh and Agarwal, 1981).

1.10 Prevalence of fascioliasis in Pakistan

F. gigantica is a parasite indigenous to Africa and Asia not only infects ruminants, but also humans (Cotruvo *et al.,*2004). Pakistan is one of the Asian countries where both fasciolid species (*F. hepatica, F. gigantica*) overlap and are highly prevalent in livestock, including, cattle, buffaloes, sheep and goats (Afshan *et al.,* 2017; Khan *et al.,* 2010) and the average prevalence of fascioliasis is 6.5-29.99% in sheep, 0.66-28.75% in goat, 19.30-

20.42% in cattle and 14.71-30.50% in buffaloes. The reasons of increasing infection rate may be due to socio-economic status of the farmers (Jabbar *et al.*, 2006). Fascioliasis is of particular concern in bovines in the province of Punjab. It has been found to be widespread in different districts, with infection rate of 10.48-40.31%.

1.11 Diagnosis

The most conventional and broadly used way to detect fascioliasis is the microscopic observation of flukes eggs in the feces of host (Kato-Katz test) (Peters *et al.*, 1980). Due to complexity in different stages of the liver fluke, it is challenging to detect infection at initial stage because recently excysted juveniles enter through the wall of and appear in the peritoneum. These parasites then move towards liver, penetrate the liver tissue reach the bile ducts, where they gain maturity in about 20 weeks post infection (Mercedes, 2018) where they produce eggs. Therefore, fascioliasis cannot be detected by examination of eggs during this period. The juveniles cause maximum pathogenicity during such phase and they are more virulent compared to adult flukes. Although the method of egg detection is simple and confirms the infection but this cannot be applied during the pre-patent period and in case of low intensity of disease. The hurdles in the initial diagnosis of disease have commanded to the establishment of other more innovative methods for the detection of parasitic infections (Adedokun *et al.*, 2008).

Diagnosis of the fascioliasis by serological methods has been used as a substitute method for the examination of eggs and these methods can analyze large number of samples at the same time much earlier than the detection of eggs. Serological methods of detection use different immunogenic proteins of excretory/secretory products (ES products) in the blood of infected animal (Rodríguez-Pérez and Hillyer, 1995). Enzyme linked immunosorbent assay (ELISA) and Western blots are the major techniques which have been widely used in serodiagnosis of fasciolosis in animals and can detect serum antibodies to specific *F. gigantica* antigens from adult fluke extract or ES products (Adedokun *et al.*, 2008). For the diagnosis of fasciolosis, the serological test is very useful but as the antibodies persist for a long period even after treatment with drugs, the animals with drug resistant flukes' infection are unable to be identified and hence escape the further treatment.

1.12 Treatment

A number of preventive measures are available that can be used independently or in combined way to treat ruminant fasciolosis (Auxiliar *et al.,* 2012). The regulator strategy of parasites relies mainly on the use of drugs as they are safe, cheap and effective against a broad spectrum of parasites (Roberts, 2005). Both mature and juvenile flukes of this species can be effectively treated by the flukicides, such as triclabendazole (TCBZ), but there is an emerging tendency of anthelmintic resistance in liver flukes (Kelley *et al.,* 2016). Moreover, issues of residues in the food chain and environment have arisen, which threaten their sustained use (Vercruysse *et al.,* 2007).

Another way of attacking fascioliasis is the destruction of intermediate hosts (snails) in order to break the life cycle of *Fasciola*. This could be done by the application of various synthetic or botanical molluscicides in snail habitats. The most important synthetic molluscicides are niclosamide, sodium pentachlorophenate, N-tritylmorpholine, thiodicarb, and pyrethroids. Among these, niclosamide has the lowest toxicity for mammals, but highest for snails and their eggs. However, pest control using these synthetic molluscicides has created the problem of neurotoxicity to man and other non-targeted animals (Shafer, 2005).

1.13 Drug resistance

Effective vaccines are not available for fascioliasis (Toet *et al.,* 2014), chemotherapy is the sole option to combat the disease (Kelley *et al.,* 2016). Due to extensive use of drugs, there have been a number of reports on anthelmintic resistance in cattle (Anziani and Fiel, 2015). At present, control of fascioliasis is hindered by availability of anthelmintics. Most anthelmintic have a low efficacy against the immature stages of *Fasciola,* and there is evidence for the development of drug resistance in Pakistan (Rehman *et al.,* 2016).

1.14 Current Investigation

Many serological assays are rapidly developing, such as, ELISA and Western Blot to detect antibodies against specific antigens of *Fasciola sp*. requiring adult fluke somatic extracts or ES products. These methods can detect a number of sera at a time with the possibility to screen infection as early as two weeks post infection (Gonzales Santana *et al*., 2013; Mezo *et al*. 2004;

Ruiz-Navarrete *et al*., 1993). Excretory and secretory products are produced by the parasite and are commonly used to detect fasciolosis (Bautista *et al*., 1989). Specific antigens in the somatic extract and excretory-secretory (ES) product of *F. gigantica* adults were identified and characterized by SDS-PAGE and immunoblotting analysis (Almeida *et al*., 2007). Sobhon *et al*. (1996) characterized the polypeptides from whole body extract of *F. gigantica* and demonstrated that there were approximately 21 detectable bands, ranging in molecular weight from 17 to 110 kDa. Among these eleven polypeptides (97, 86, 66, 64, 58, 54, 47, 38, 35, 19 and 17 kDa), present in the tegument antigen which were extracted from the adult flukes. Meshgi *et al*. (2008) in Iran, separated 11 different bands of somatic extracts and 8 different size bands of ES products of *F. gigantica* using SDS PAGE. The molecular weights of these peptides were 18,22,24,33,36,42,46,57,60,62, and 68 kDa for somatic and 15,16,20,24,33, and 42 kDa for ES products respectively. Similarly, Latchumikanthan *et al*. (2012) separated 7 polypeptides of *F. gigantica* at 23, 25, 28, 43, 47, 52 and 66 kDa by SDS-PAGE. In another study, proteins with molecular weights of 97, 66, 54, 47, 38, 29, 27, 17, and 15kDa were separated (Krailas *et al*.,2002).

It is now recognized that many parasite proteases have the potential for chemotherapeutic or vaccine targets (McKerrow et al., 1999; McKerrow 1999; Selzer *et al*., 1999). Hannan Khan *et al*. (2017) in India, separated 12 immunogenic proteins using SDS PAGE from the ES products of *F. gigantica*. Fagbemi *et al*. (1995) demonstrated that 28 kDa cysteine protease of *F. gigantica* adult fluke was sensitive and enhanced the specificity of immunodiagnostic for fascioliasis in ruminants but this polypeptide showed cross reactivity. The use of adult fluke ES products in serodiagnosis indicated that these molecules evoke strong immune response during infection. And it was also demonstrated that during natural infection of *F. gigantica,* in cattle dynamic cellular and humoral responses were present (Phiri *et al*., 2006). Progress in the development of effective vaccine for *F. hepatica* and *F. gigantica* control in ruminants has been relatively slow. Despite intensive research very few circulating antigens have been reported in infected animals and these antigens have variable efficiencies (Anuracpreeda *et al*., 2013; Anuracpreeda *et al*., 2009; Fagbemi *et al*., 1995; Langley & Hillyer, 1989; Velusamy *et al*., 2004; Viyanant et al., 1997).

Therefore, the current study was an attempt to identify more antigenic polypeptides in the somatic and ES products of *F. gigantica* collected from cattle and

buffaloes. However, there are very limited studies on somatic and ES antigens of *Fasciola* stains from Pakistan. So, the current study was design to fill this knowledge gap. Furthermore, the information obtained from the study could be helpful in the development of diagnostic kits to detect fascioliasis in initial stages. In addition, these polypeptides can be tested in immunotherapeutic or vaccine development to combat disease.

Aims and Objectives

The aims and objectives of the current study are:

- To determine prevalence of *Fasciola gigantica* infection in large ruminants and their association with animal breed, age, gender, location, grazing habit and contact with water bodies.
- Isolation, identification and characterization of somatic and excretory/secretory product of *Fasciola gigantica* collected from large ruminants by using SDSPAGE.
- To detect immunogenic proteins from adult worm extracts of *Fasciola gigantica* by using Western blotting and Indirect ELISA.

MATERIALS AND METHODS

2.1 Study Area

Two sampling locations i.e. Sihala slaughter house (Rawalpindi, north Punjab) and slaughter house in Peshawar (headquarter of Khyber Pakhtunkhwa) were selected for present study. The sampling from slaughter houses was carried out for about six months. The animals are brought to the Sihala slaughter house from adjoining areas of Rawalpindi, while in case of Peshawar these are brought from the nearby regions Charsada and Mardan districts (Figure 2.1).

KPK is province of Pakistan, located along the border of Afghanistan, in the northwestern part of the country. It is third largest province by population and economy. Climate of KPK is very diverse because of its size. Monsoon season lasts between June and end in mid-September.

Punjab is the second largest province of Pakistan beside Baluchistan province. Nevertheless, it is largest province population wise with an estimated population in excess of 10 crores according to the census of 2017. Temperature of the Punjab varies between -2 to 45°C but it may reach its extremes (-10 to 50°C) during winters and summers respectively. Average annual rainfall in the Punjab ranges between 96cm to 46cm normally the rainy season is from July to September.

2.2 Sample collection

The adult flukes *(F. gigantica*) were collected from the bile ducts of the infected buffaloes and cattle, shortly after these animals were slaughtered. After removal from the host tissue and the flukes washed many times with 0.01M PBS and put into the labeled Eppendorf and falcon tubes and cryopreserved at -20°C.

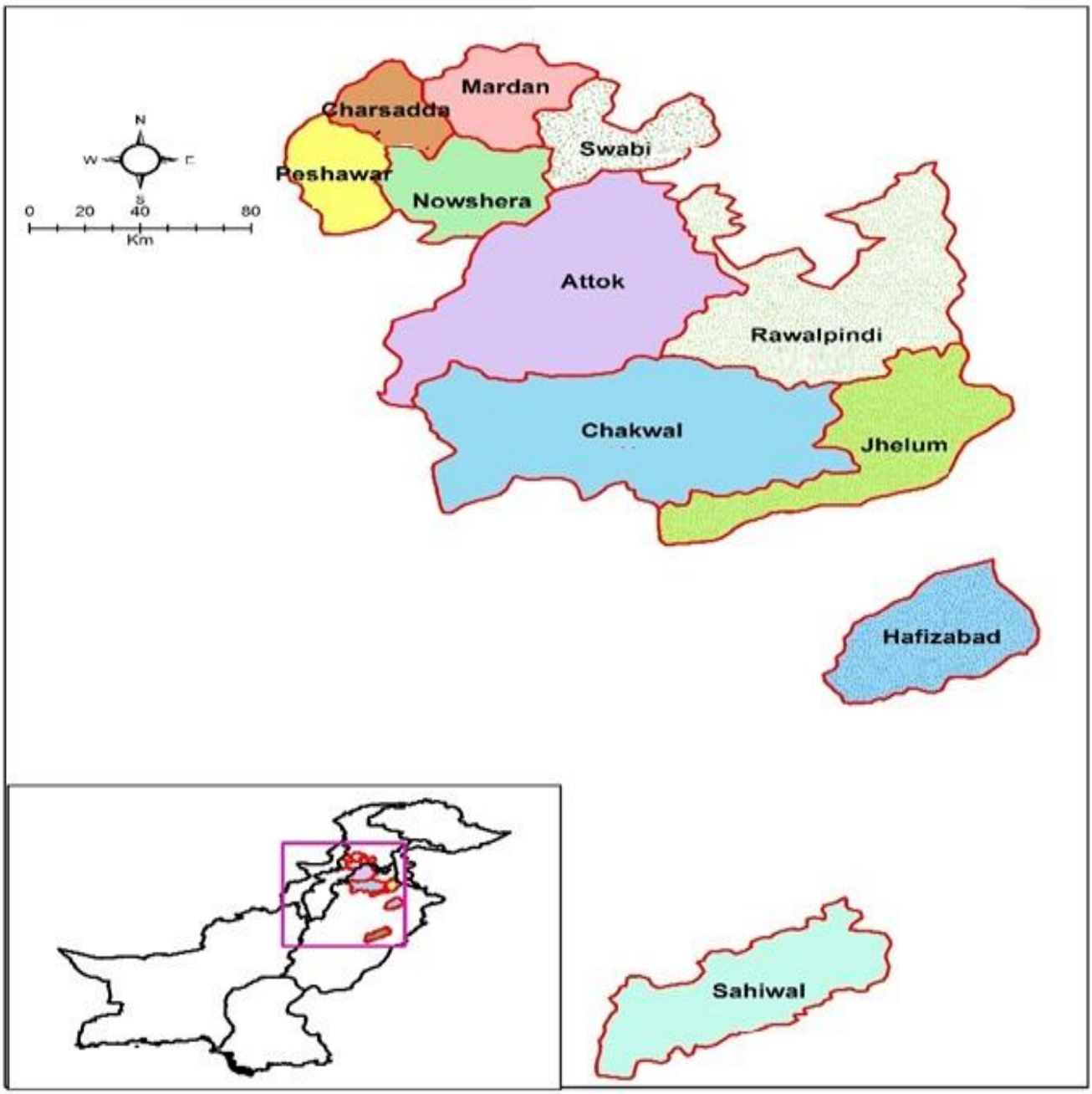


Figure 2.1: Map of Pakistan showing sampling sites.

2.3 Extraction of Somatic Antigens

In order to collect the somatic extracts, worms were washed many times with chilled PBS 0.01M (pH 7.1) to remove debris. After this, flukes were placed in the labelled tubes and stored at -20°C for subsequent use. Each fluke was placed in a separate Eppendorf labeled with animal identity given at the time of slaughtering. Flukes collected from different animals were weighed and stored separately. The tubes were labeled according to the animal identity given at the time of slaughtering. Each sample was homogenized by adding 10 ul of lysis buffer (Annexure I) for each mg weight of flukes. Besides, proteinase inhibitor (PMSF) cocktail was also added. After adding the lysis buffer, fluke tissue was grounded using tissue grinder. The whole process was carried out on ice to avoid the denaturation. This homogenized mixture was centrifuged at 1000rpm for 10 minutes at 4°C.The supernatant was then transferred to other separate tubes and stored at -20°C.

2.4 Extraction of ES Products

In order to get excretory/secretory (ES) products of the flukes, worms were placed in 0.01 M, 2.5 ml of PBS per worm at room temperature. After overnight incubation worms were removed and PBS containing fluke ES products was stored at -20°C for subsequent use.

2.5 Blood collection

Blood of the infected animal was collected at the time of slaughtering. The blood was allowed to clot and serum were separated and stored at 4 °C until used for further analysis.

2.6 Quantification of proteins

Protein concentration of crude and ES products was estimated following the process described by Bradford (1976) with slight modifications.

2.6.1 Assay procedure

Bradford reagent was prepared by mixing coomassie brilliant blue (0.05g), methanol (25ml), phosphoric acid (50ml) and water (100ml). Stock solution was kept in dark at 4°C covering the container with aluminum foil. Stock solution was prepared by mixing 25 ug of bovine serum albumin (BSA) in 1 ul of distilled water. Different serial (0.625 to 22.5 ug/ul) dilutions were prepared from this stock solution. Working solution was prepared by mixing Bradford reagent and distilled water in ratio of 1:4. Each sample was prepared by dissolving 100 ul of standards and 2900 ul of working solution. (Annexure 1)

2.6.2 Spectrophotometry

After running blank, absorbance of each sample (in triplicates) was measured at 595 nm for standard and each sample respectively. Means of absorbance of standards dilutions were plotted against their concentrations to obtain straight line equation. The regression equation was used to determine concentration of proteins in unknown samples.

2.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyze different types of proteins by separating them on the basis of their molecular weights as described by Laemmli (Laemmli, 1970). The samples were mixed with loading dye in the equal proportion. 7.5µl of protein sample and 7.5µl loading dye were added and a total volume of 15 ul was made to be loaded on gel. Samples were kept at 95°C for 5 minutes using water bath.

After loading the separating gel, stacking gel was loaded. After the complete polymerization combs were removed and samples loaded and Protein ladder was also loaded alongside samples. Gel were run at 45V and 90A current till sample reach at the bottom of the gel. Gel was placed in the fixation solution to fix protein into the gel for about 45 minutes. After fixation the gel was stained with coomassie blue for overnight on shaker. After staining the gel was put into destaining solution. So that the proteins bands and the space between them became clear. After destaining, a clear picture was taken by smart

mobile and the gel was analyzed using ImageJ Software. The molecular weight of proteins bands was calculated on comparison with the standard marker.

2.8 Western blot method

Somatic and ES products of *F. gigantica* separated by 10% SDS-PAGE gel, were electro transferred to nitrocellulose membrane according to the method described by Towbin *et al.*, (1979) After blotting has completed, the membrane was removed and washed with tris buffer saline tween (TBST). Membrane was incubated in blocking buffer and placed on metabolic shaker for about 30 minutes at room temperature. After that membrane was removed from the blocking buffer. The membrane was incubated in primary antibody (infected bovine serum) with dilution of 1:25 in blocking buffer overnight at 4 C. Next day antibody solution was discarded and membrane was washed with TBST (5x5 minutes), after that secondary antibody (anti-Rabbit Santa Cruz) was added with dilution 1:9999 in blocking buffer. The tray containing membrane was covered and again placed at shaker for 2 hours. That was followed by washing PVDF with TBST to remove unbound secondary antibody. 1 ml of substrate solution (NBT/BCIP Sigma) was added to the membrane and kept at room temperature until the color production. In the end the membrane was photographed to analyze the blots.

2.9 Indirect Enzyme Linked Immuno-Sorbent Assay

Plates were coated with eluted polypeptides of somatic products (200 ul) of *F. gigantica* using coating buffer (carbonate-bicarbonate buffer, pH9.6) followed by overnight incubation at 4 ºC. The indirect ELISA was performed as described by (Yadav *et al*., 2005). After overnight incubation plates were washed (5x5 minutes) by phosphate buffer Saline-Tween 20 (PBS/Tween 20). Microtiter plate wells were blocked with skimmed milk in PBS/Tween20. Again, washed and sera collected from infected cattle and buffaloes were diluted (1:25) incubated in well for 90 minutes at 37ºC temperature. After this, the plates were washed again and 100 ul of secondary antibody (anti-human IgG conjugated with horseradish peroxidase) was dispensed in all wells and incubated at room temperature for 30 minutes. Washed again and incubated with 100 ul of substrate solution (tetramethylbenzidine TMB) at room temperature for 15 minutes and kept in dark.

Enzymatic reaction was stopped by adding 100 ul of stop solution. Results were read at optical density (450) with microtiter plate reader.

2.10 Data Analyses

Statistical analyses were carried out by Statistical Package for Social Science (SPSS) using Chi-square test to find association of *F.gigantica* with different risk factors. Bradford method was used to find the concentration of proteins in the somatic ES extrcts. The molecular weights of the polypeptides on the gel were analyzed by Imagej software (Version 1.114).

RESULTS

3.1 Prevalence

The flukes were present in the bile ducts of the condemned livers, with hepatic tissues were severely affected. Liver fluke infection was recorded 4.7% of the animals examined 10 out 178 buffaloes while 2 out 78 cattle were infected. The prevalence of *Fasciola gigantica* infection was higher (5.6%) in buffaloes compared with cattle (2.6%) (Table 3.1).

3.1.1. Host type

Buffaloes and cattle were analyzed to determine whether disease is associated with host type or not. Investigation of data demonstrated that buffaloes were more affected compared to the cattle, while the results were not significant ($\chi 2=1.132$; $p=0.287^{NS}$) with respect to host type and infection (Table 3.1). Prevalence of disease in buffaloes was 5.6 % while 2.6% of cattle were (Fig.3.1).

Table 3.1: prevalence of *F.gigantica* infection in different host types

Host type	Total (%)	Non-Infected (%)	Infected (%)
Buffalo	178 (100)	168 (94.4)	10 (5.6)
Cattle	78 (100)	76 (97.4)	2 (2.6)
Total	256 (100)	244 (95.3)	12 (4.7)

Chi-Square Tests = 1.132 p=0.287^{NS}

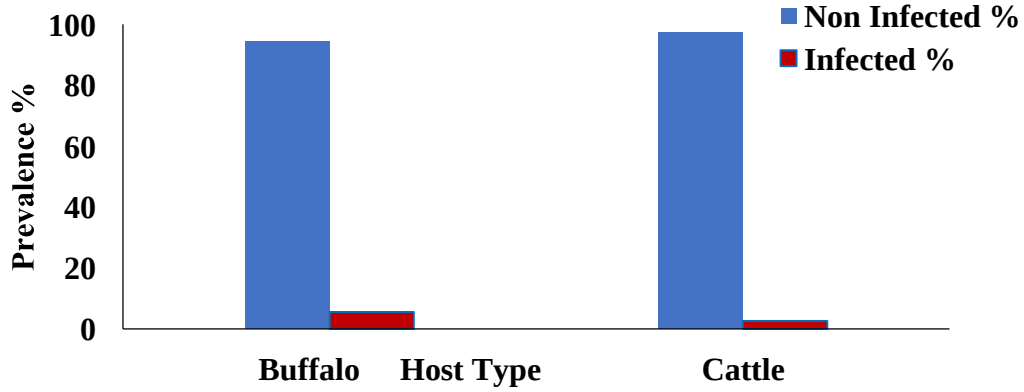


Figure 3.1: Prevalence (%) of *F. gigantica* infection with respect to host type

3.1.2 Animal breed

The highest incidence of *F. gigantica* infection was found 11.1 % followed by Nili Ravi 5.6 % and 2.8 % in Kundhi. The results were non- significant (x^2 =6.204; P=0.624) and showed that animal breed has not much effect on the disease prevalence (Table 3.2). On the other hand, in cattle highest prevalence was in Sahiwal (5.6%), and no infection was recorded in all other cattle breeds Fig. 3.2.

Table 3.2 Relationship of *F. gigantica* infection with breeds of cattle and buffaloes.

Animal breed	Total (%)	Non-infected (%)	Infected (%)
Azi kheli	36 (100)	32 (88.9)	4
Nili Ravi Kundhi	70 (100)	66 (94.3)	4
Kundhi	72 (100)	70 (97.2)	2
Bhagnari	8 (100)	8 (100)	0
Dhani	16 (100)	16 (100)	0
Lohani	2 (100)	2 (100)	0
Mix breed	14 (100)	14 (100)	0
Sahiwal	36 (100)	34 (100)	2
Total	256 (100)	244 (95.3)	12 (4.7)

Chi-square test=6.204 P =0.624^{NS}

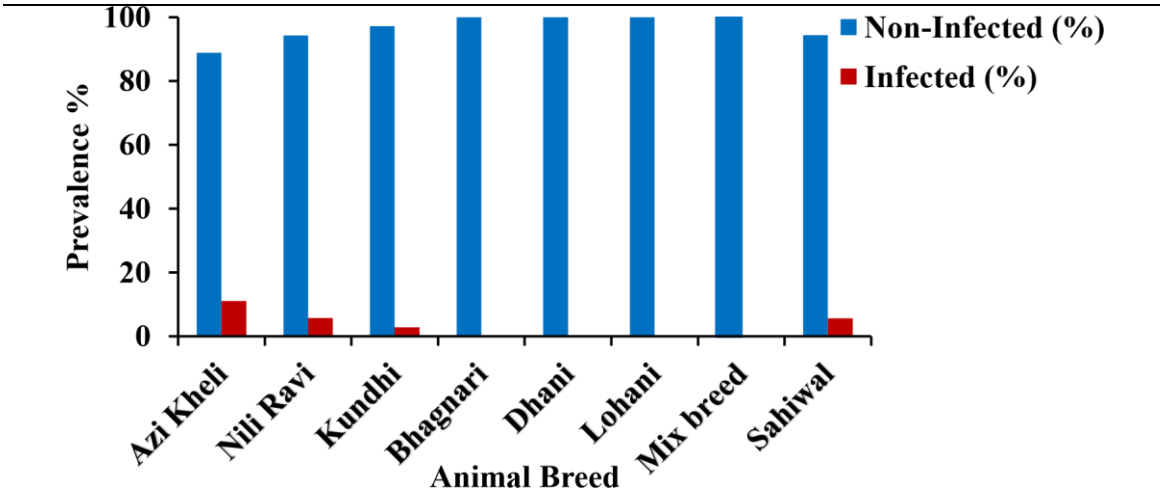


Figure. 3.2: Prevalence (%) of *F. gigantica* infection among breeds of cattle and buffaloes.

3.1.3. Host locality

The investigation of the data in Punjab province demonstrated that disease was most prevalent in district Chakwal (40 %). The other districts (Attock, Jhelum, Hafiz Abad, Rawalpindi and Sahiwal) included in the study did not show any case of disease (Table 3.3). In case of province KPK, the results revealed that the highest prevalence was in the district Charsada (12.5%) followed by Peshawar (5%). The animals from other districts (Mardan, Swabi, and Nowshera) were found to be non-infected. These results were highly significant ($\chi 2$ =42.247; P=0.000**) and showed that prevalence of disease and the locality of the host are allied to each other. Map of studied areas showing prevalence (%) is given in Fig. 3.3.

Table 3.3 Association of *F. gigantica* infection with respect to geographical areas of host.

Host locality	**Total (%)**	**Non-Infected (%)**	**Infected (%)**
Attock	32(100)	32 (100)	0(0)
Charsada	48(100)	42 (87.5)	6(12.5)
Hafiz Abad	2(100)	2 (100)	0(0)
Jhelum	14(100)	14 (100)	0(0)
Mardan	12(100)	12 (100)	0(0)
Nowshera	8(100)	8(100)	0(0)
Peshawar	40(100)	38 (95)	2(5)
Sahiwal	2(100)	2(100)	0(0)
Swabi	12(100)	12(100)	0(0)
Chakwal	10(100)	10(100)	4(40)
Rawalpindi	76(10)	76 (100)	0(0)
Total	256 (100)	244 (95.3)	12 (4.7)

Figure 3.3 Map of Pakistan indicating prevalence (%) of *F. gigantica* infection among study areas.

3.1.4 Host sex

The results did not show association between host sex and *F. gigantica* infection ($\chi 2 = 3.958$; $p=0.47^{NS}$) (Table 3.4). However, higher prevalence of infection was recorded in females (7%) as compared to males (1.2%) Fig.3.4.

Table 3.4: Relationship between *F. gigantica* infection and gender of host

Host sex	Total (%)	Non-Infected (%)	Infected (%)
Female	142(100)	132 (93.0)	10(7)
Male	114(100)	112 (98.2)	2(1.2)
Total	256(100)	244 (97.3)	12(4.7)

Chi-square test=3.958 P= 0.47^{NS}

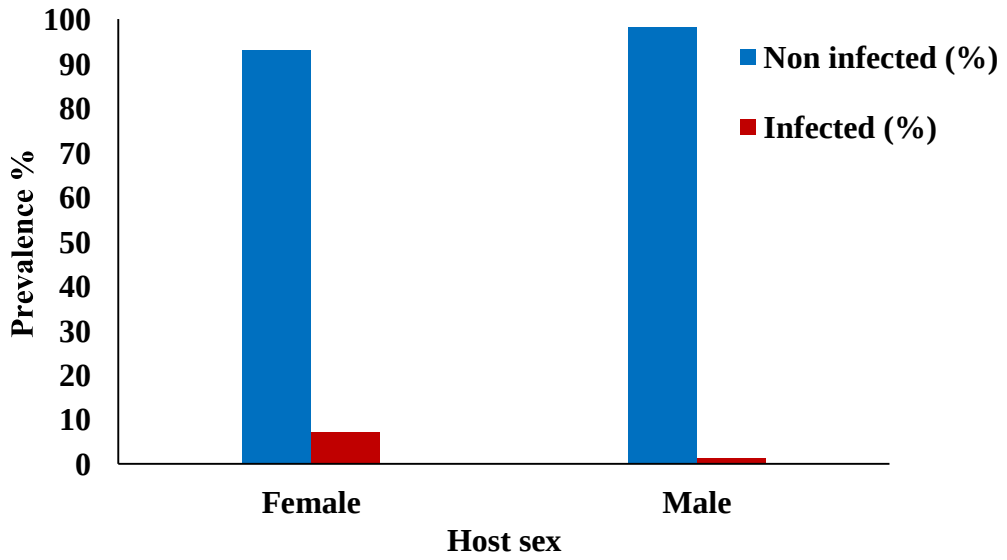


Figure 3.4 Prevalence (%) *F. gigantica* infection among gender of host (s)

3.1.5 Age of the animal

To determine whether age of animal and occurrence of disease were associated or not, data was analyzed by arranging examined animals in different age groups (Table 3.4). The results showed that disease was more prevalent in old age animals as compared to the age groups of younger animals. Highest prevalence was shown by animals of age group 8 to 10 years (14.3%) followed by age group 11 to 12 years (7.7%). Age groups 2-4 years and 5-7 years of the animals were found to be not infected Fig. 3.5. These results were highly significant ($\chi 2$ = 22.828; P=0.000**) and showed that animal age and disease prevalence are related.

Table 3.5 Association of *F. gigantica* infection among different age groups of hosts.

Animal Age(years)	Total (%)	Non-Infected (%)	Infected (%)
2 to 4	100(100)	100 (100)	0(0)
5 to 7	60(100)	60 (100)	0(0)
8 to 10	70(100)	60 (85.7)	10(14.3)
11 to 12	26(100)	24 (92.3)	2(7.7)
Total	256(100)	244 (95.3)	12(4.7)
Chi-square test= 22.828 P=0.000**			

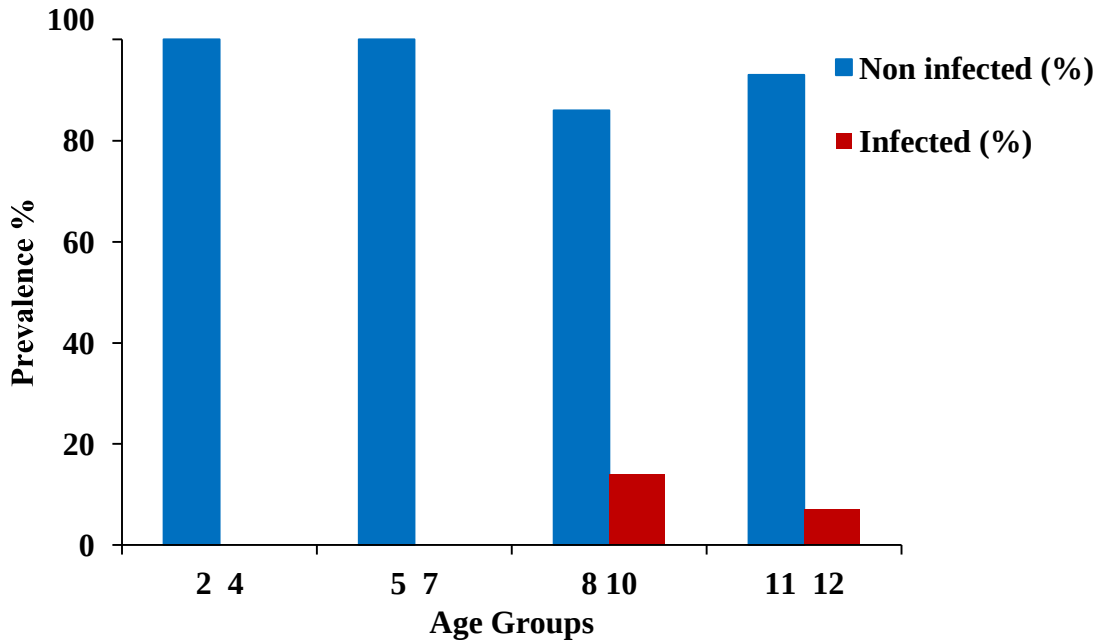


Figure 3.5 Represents prevalence % in association with types of animal age groups

3.1.6 Grazing Management

To find the relationship between grazing management of host by farmers and disease prevalence, infected and non-infected animals were grouped into three categories. These included intensive, extensive and semi-extensive grazing managements. The results were highly significant ($\chi 2$= 22.828; p=0.000**) and demonstrated that grazing management and disease occurrence are linked (Table3.6). This investigation showed that

disease prevalence was highest in animals with intensive grazing (10%) followed by the animals with extensive grazing (6.4%). Animals with semi extensive grazing management were shown to be least affected (2.8%) Fig.3.6.

Table 3.6 Association of *F. gigantica* infection and grazing managements of animals

Grazing	Total (%)	Non-Infected (%)	Infected (%)
Extensive	94(100)	88 (93.6)	6(6.4)
Intensive	20(100)	18 (90)	2(10)
Semi-Extensive	142(100)	138 (97.2)	4(2.8)
Total	256(100)	244 (95.3)	12(4.7)
Chi-square test=	**22.828**	**P=0.000****	

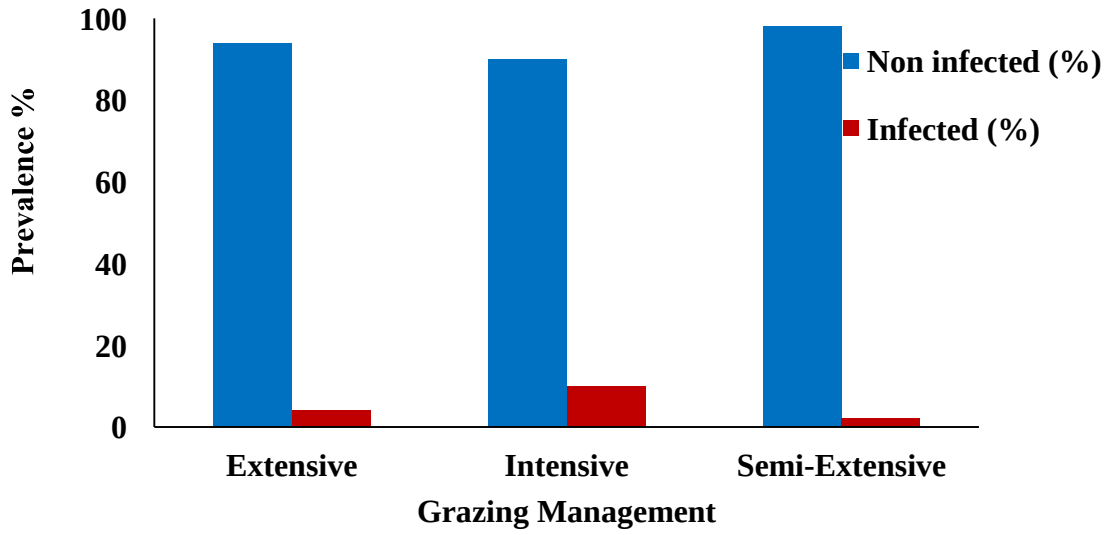


Figure 3.6 Prevalence (%) of *F. gigantica* among grazing habits of animals

3.1.7 Water bodies

The link between water bodies and disease of the host, are shown in Table3.7. The results demonstrated that disease was most prevalent in animals coming from the regions with water reservoirs (8.2%). Animals from the regions having all types of water bodies were affected 7.7 %. Lowest prevalence was shown by animals coming from areas having canal (2%) water bodies (Fig. 3.7). The results did not show significant ($\chi 2$= 6.239:

p=0.101^{NS}) difference for prevalence of infection with respect to types of water bodies near host.

Table 3.7 Relationship of F. gigantica infection with respect to the types of water bodies

Water bodies	Total (%)	Non-Infected (%)	Infected (%)
All types	52(100)	48 (92.3)	4(7.7)
Canal	99(100)	97 (98)	2(2)
Ponds	32(100)	32 (100)	0(0)
Reservoir	73(100)	67 (91.8)	6(8.2)
Total	256(100)	244 (95.3)	12(4.7)
Chi-square test=6.239 P=0.101[a]			

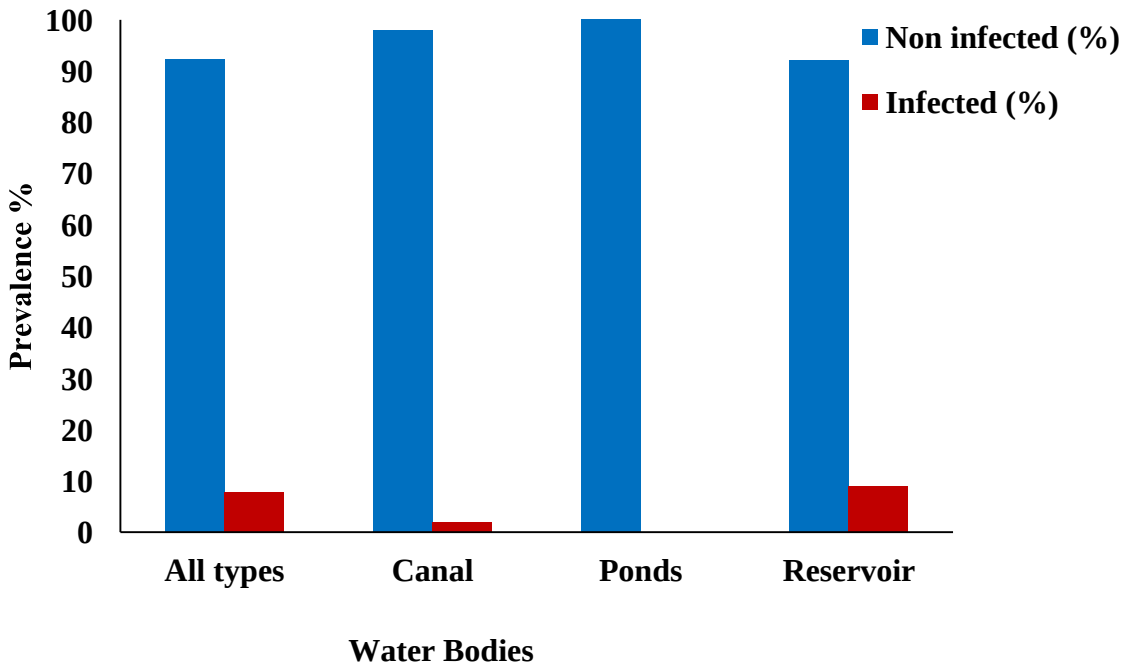


Figure 3.7 prevalence (%) of F. gigantica infection among different water bodies

3.2 Protein Quantification

Estimated through spectrophotometry, mean absorbances (595 nm) of standards of BSA dilutions (0.625 ug/ul to 22.5 ug/ul), ranged between 1.6 ug/ul to 2.8 ug/ul. These values of concentrations were plotted against standards to obtain standard curve, (Fig. 3.8a and 3.8b).

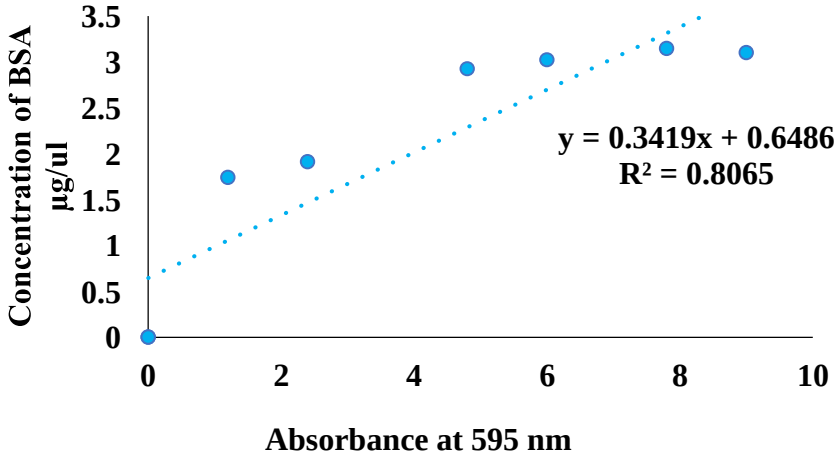


Figure 3.8 a: Standard curve obtained for BSA to determine the unknown protein concentration of adult worm extracts

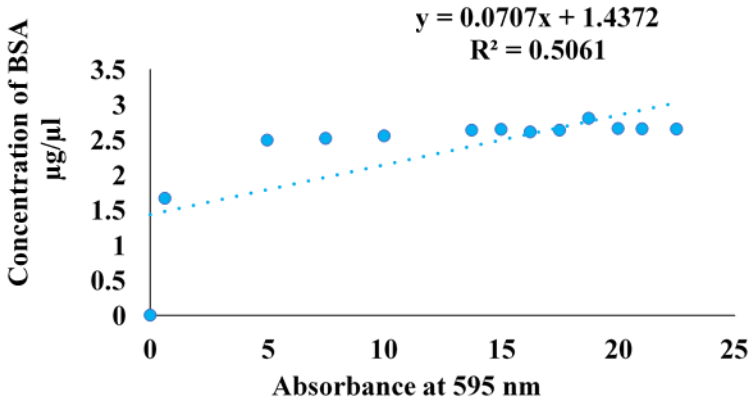


Figure 3.8 b: Standard curve obtained for BSA to determine the unknown protein concentration of adult worm extracts

Photo spectrometric analysis of parasites extracts gave values of mean absorbance (595 nm) that ranged between 1.8 ug/ul to 2.8 ug/ul. The proteins quantified in these extracts were between 6.5 ug/ul to 20.1 ug/ul (Table 3.8).

Table 3.8: Spectrophotometric analysis of adult worm of *Fasciola gigantica* excretory/secretory and somatic extracts.

Samples	Mean absorbance at 595 nm	Concentration of protein (ug/ul)
1	1.9232	6.942
2	1.9964	7.628
3	2.0324	8.228
4	1.8232	6.642
5	2.8405	20.042
6	2.6741	17.671
7	2.7111	18.2
8	2.6987	18.14
9	2.7241	18.38
10	2.6644	17.471

3.3 SDS-PAGE Analysis

` The somatic and ES products were fractionized by SDS-PAGE. Their molecular weights were determined by calculating their migration distance with respect to the protein ladder run as marker (Fig. 3.9).

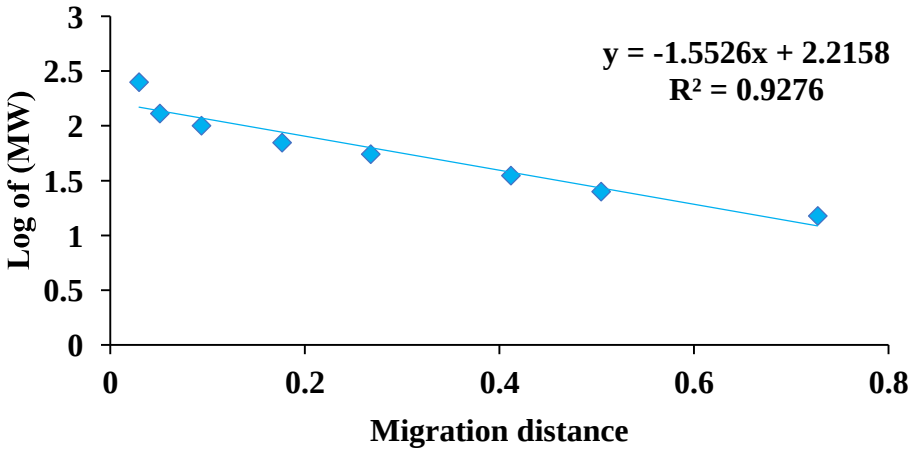


Figure 3.9: Migration trend of protein ladder to calculate the unknown molecular weight of somatic and excretory/secretory protein bands of *Fasciola gigantica* adult worms separated on SDS-PAGE gels.

3.3.1 Identified polypeptides of somatic extracts

The result of somatic extracts revealed different molecular weight polypeptide bands with reference to marker and equation calculated for ladder (Table 3.9). Lowest weight (kDa) peptide bands in the somatic extracts were found at 15 kDa while the highest molecular weight peptide band was at 146 kDa. Other polypeptides separated in somatic extracts were 15, 16, 27, 44,45,78, 80,90,110,130, and 146 kDa. The intensity of 130 kDa polypeptide was highest among all other somatic extracts (Fig. 3.10 a).

3.3.2 Identified polypeptides of E/S products

The analysis of these products showed a range of polypeptide bands having molecular weights in between 93 kDa and 148 kDa (Table 3.9). The other proteins of E/S products were as follow, 100 kDa, 130 kDa, 145 kDa and 147 kDa. The SDS PAGE polypeptide bands for ES, somatic extracts, and control groups are shown in Fig. 3.10a – 3.10b.

Table 3.9: Identified protein bands of different molecular weight by SDS-PAGE analysis of somatic and excretory/secretory antigens of *Fasciola gigantica* collected from large ruminants.

Number of worms extracted	**Major Band observed in excretory/secretory product of *Fasciola gigantica***	**Major Band observed in Somatic product of *Fasciola gigantica***
25	93	15-20
25	100	42-48
25	130	87-105
25	145	109-114
25	147	126-130
25	148	137-146

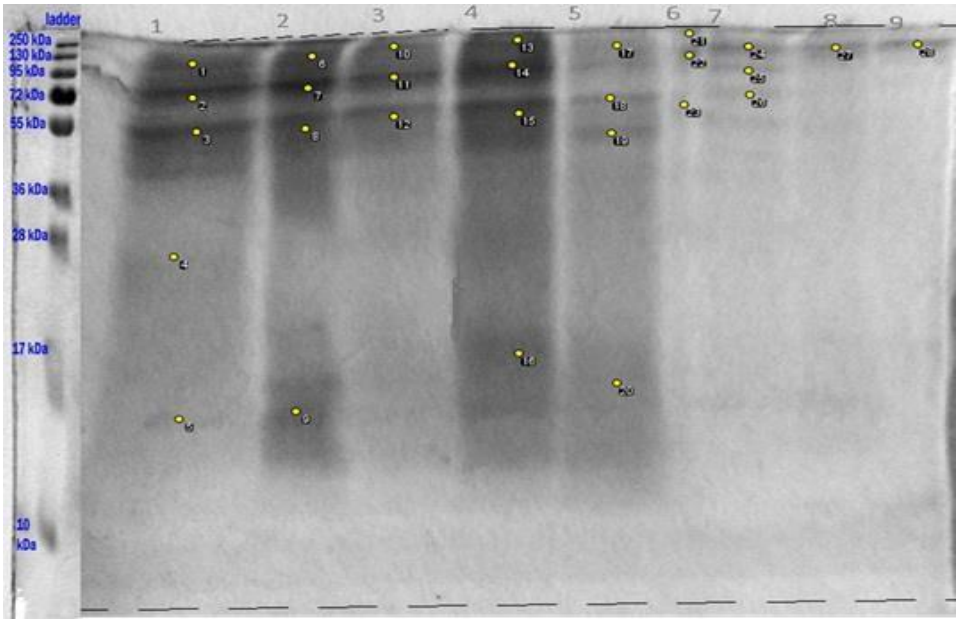


Figure 3.10 a: SDS-PAGE analysis of somatic and excretory/secretory extracts of adult worms of Fasciola gigantica collected from large ruminants.

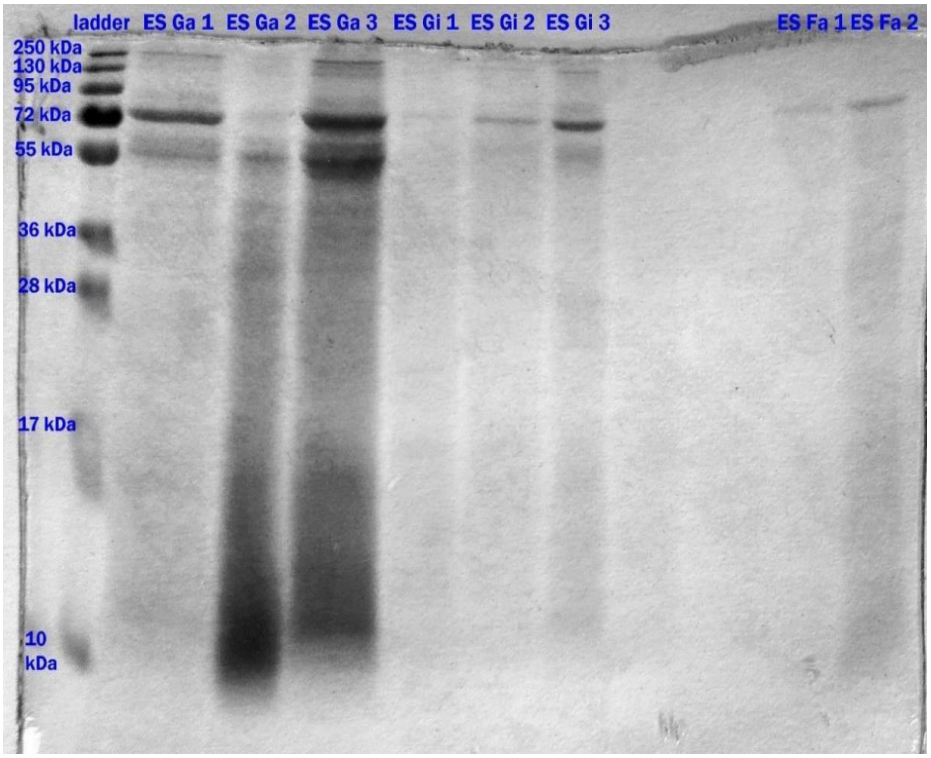


Figure 3.10 b: SDS-PAGE analysis of excretory/secretory extracts of adult worms of Fasciola gigantica and controls collected from large ruminants.

3.4 Western Blotting

Somatic extracts were blotted with antibody of *F. gigantica* (anti-FgS) to detect antigenic components in the worms. This analysis demonstrated that among many somatic polypeptide band separated by SDS PAGE, only a single polypeptide band of molecular weight between 130-250 kDa was immunogenic. The rest of the band did not show reactivity with the antibody (anti-FgS) (Fig.3.11).

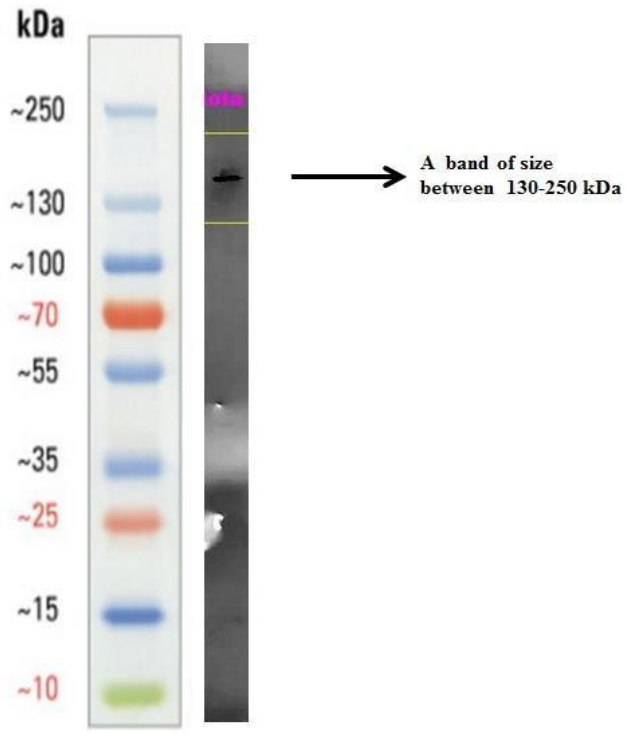


Fig. 3.11: Western blot analysis of somatic extracts of *F. gigantica* against sera of infected animals

3.5 ELISA

Indirect enzyme linked Immuno-sorbent assay (ELISA) was performed to confirm antigenic components in the crude extracts of *F. gigantica.* Proteins of different molecular weight separated by SDS PAGE were eluted and coated in ELISA plates to test their immunogenicity. Results of the assay revealed that different polypeptides in the crude extracts of *F. gigantica* did not show any immunogenic response among them with 15

infected sera of *F. gigantica* or any of the control sera. These bands included various polypeptides that had molecular weight range from 4 kDa to 37 kD. The most prominent immunogenic proteins of all bands were present in the range of 38-72 kDa. Out of 15 sera of *F. gigantica* 14 (93.3%) among them showed reactivity with the crude antigens of these molecular weight bands. The other most potent immunogenic proteins were between 95 to 250 kDa, 11 (73.3 %) out of 15 infected sera showed reactivity with *F. gigantica* infected sera. Polypeptides in range between 73-94 kDa showed 6.7 % reactivity against *F. gigantica* infected sera and showed cross-reactivity with control serum *Gigantocotyle explanatum.* (Table 3.10) The OD values at 450 nm for each tested sera against different molecular weight polypeptides is given in Fig. 3.12.

Table 3.10 Sera showing immunogenic proteins against somatic antigens of different polypeptides of *Fasciola gigantica* collected from cattle and buffaloes.

Molecular weight (KDa) of major immunogenic proteins	***Fasciola gigantica* infected sera**			**Normal control sera**
		***Gastrothylax* control sera**	***Gigantocotyle* control sera**	
4-20	15 (0)	9 (0)	10 (0)	10 (0)
21-37	15(0)	9 (0)	10 (0)	10 (0)
38-72	14(93.3)	9 (0)	10 (0)	10 (0)
73-94	1 (6.7)	9 (0)	1 (10)	10 (0)
95-250	11(73.3)	9 (0)	10 (0)	10 (0)
Total number of sera tested	15	9	10	10

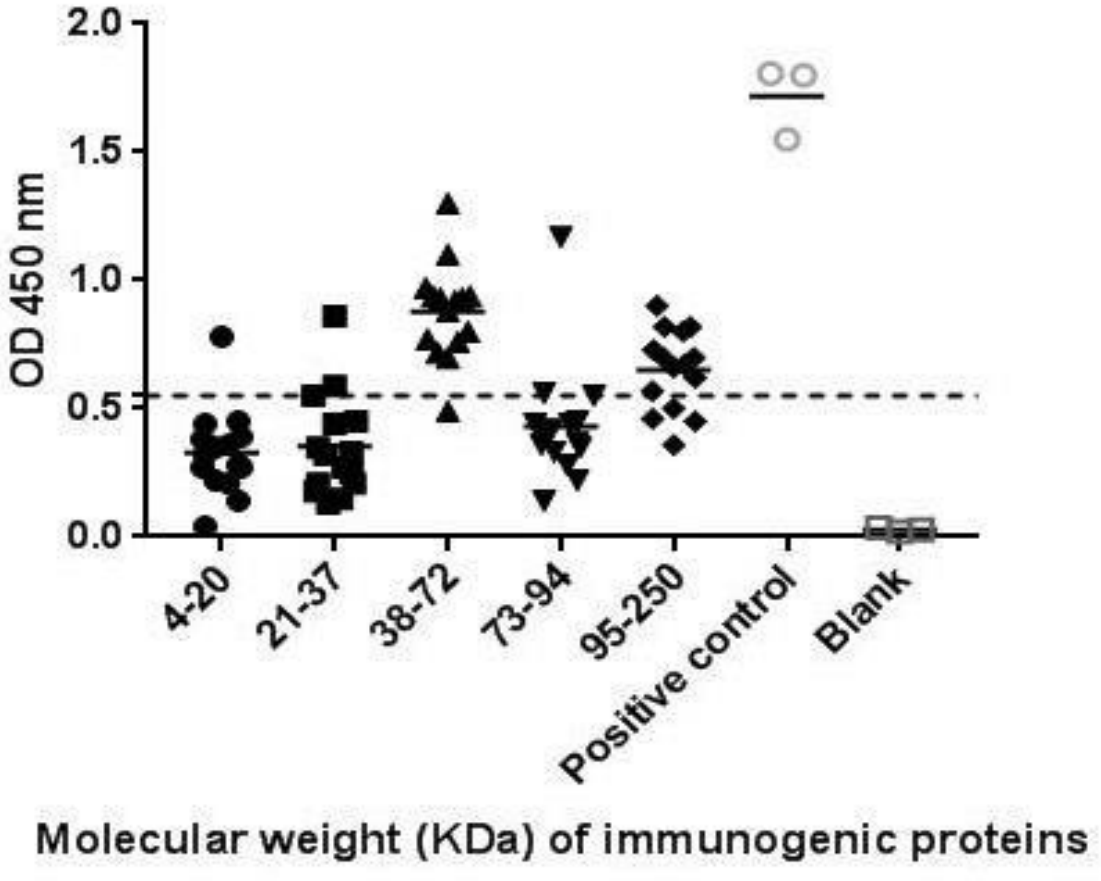


Figure 3.12: OD values at 450 nm of various molecular weights (kDa) proteins of somatic extract of *Fasciola gigantica* against infected cattle sera. The cut-off value was set at 0.6.

DISCUSSION

An early diagnosis and control of fascioliasis is very important as a livestock health concern, since disease is a global health threat to livestock. It is not only prevalent in Pakistan but also across the world and is associated with great economic losses due morbidity coupled with mortality. Pre-patent period is the time from the excystment of juveniles to the development of infection and maximum pathogenicity is associated with this time. The time flukes start producing eggs maximum loss has already occurred (Sajid and McKerrow 2002). So, an early diagnosis is the only answer to overcome this problem in a way that anthelmintic treatment effective against both the juveniles and the adult worms could be started since chemotherapy is the only choice in absence of an effective vaccine.

In present study, statistical analysis on disease incidence and host type indicated that buffaloes are more affected than cattle. These results are consistent with Kasib *et al*. (2009) who found similar distribution of disease. Main aspect of high predisposition of buffaloes to fascioliasis may include its habit to live in moist areas. Furthermore, intermediate hosts of *F. gigantica* are more aquatic than those of *F. hepatica* Spithill *et al*. (1999) that could be a reason of higher abundance of *F. gigantica* in buffaloes. However, these results do not show agreement with Rahman *et al*. (2017) who reported a higher incidence in cattle.

Breed wise relationship between prevalence of *F. gigantica* showed that all breeds of buffaloes examined were infected while one breed of all breeds of cattle were infected. Prevalence of infection in all breeds can be attributed to their habit to reside in swampy areas and by the fact that they are water loving. However, in case of cattle results showed agreement with Kato *et al*. (2005) who demonstrated high prevalence in particular breed of cattle in their study. But these results are not consistent with the investigations of Sánchez-Andrade *et al*. (2002) who demonstrated that risks of disease for cattle, in all breeds were alike.

The distribution of *Fasciola* species is associated with different climatic condition. Current study investigation on the locality of host and disease prevalence showed highest

prevalence in district Chakwal (Punjab) and Charsada (KPK). These results are highly significant ($p<0.00$) and show that host locality and occurrence of fascioliasis are linked to each other. Both the areas are at lower elevation with higher prevalence as compared to other areas in the study. These results are consistent with the finding of (Ashrafi *et al*., 2015) who demonstrated that *F. gigantica* dominate the infection of cattle and buffalo in lowlands. The incidence of trematode infestations naturally depends on the presence and distribution of freshwater snail which are intermediate hosts (Brown 1994; Van Veen *et al*., 1980) and thetheir distribution differs as climatic conditions get changed (Mungube *et al*. 2006). *Fasciola hepatica* is not found in lowlands because different studies have demonstrated that that *Lymnaea truncatula* is the only intermediate host of *F. hepatica* and is present in highlands (Oviedo *et al*., 1995; Bargues and Mas-Coma 1997, Samadi *et al*., 1999). However, the data is inadequate for various areas and their lower prevalence cannot be explained.

The gender distribution of fascioliasis was not significantly higher in females as compared to males, which indicates similar acquisition of the infection in both genders. The results are consistent with previous reports from the studies of (Asanji and Williams 1984) but contrary to the findings of (Aal *et al.,* 1999; Maqbool *et al*., 2002; Opara 2005) recorded no association between sex of the animal and incidence of disease. This may be attributed to the small sample size of the study. However higher infection in females than males can be explained by assuming hormonal influence and other physiological aspects such as milk production, and stress during pregnancy and parturition (Spithill,1999).

Evaluation of fascioliasis prevalence by age, demonstrated higher distribution in older animals compared to the younger counterparts. These results are consistent with the findings of Yildirim *et al*., (2007) who found a similar disease pattern. The higher prevalence in older animals might be attributed to grazing more often from contaminated vegetation increasing the risk of infection with *F. gigantica* metacercariae. The results of grazing managements showed that intensive grazers are more prone to fascioliasis than those with extensive and semi-extensive grazers. The results are concordant with previous finding of Khan *et al*. (2017). Significantly higher prevalence of fascioliasis in old cattle in Bangladesh has also been recorded by other authors (Affroze *et al*. 2013). Studies on water

bodies association with fascioliasis revealed that disease is more prevalent where water reservoirs are present in area of the host. The current result show agreement with the findings of (M. N. Khan *et al*. 2010).

ELISA and Western blot are the diagnostic methods that have revolutionized immunodiagnostic approaches. These methods lower the cross reaction to a large extent. Dutta *et al*. (1990) Somatic extracts of *F. gigantica* separated by SDS PAGE revealed various bands of molecular weights ranged from15 kDa to 146 kDa. These results are consistent with the findings of Yokananth *et al*. (2005) who reported a range of 14 kDa to 156 kDa. Simlarly, Morales and Espino (2012) separated 40 polypeptides bands from the *F. hepatica* tegument antigens with molecular weights in range of 10 to 150 kDa. In another study Allam *et al*. (2012) found different bands of 12 kDa to 117 kDa. These differences may be due to the presence of different strains of flukes from different host species or because of geographic variations (Gupta *et al*., 2003). Velusamy *et al*. (2004) separated different bands of somatic extracts of *F. gigantica* in range 8 and 54 kDa. Meshgi *et a*l. (2008) found separated 11 bands in somatic extracts of *F. gigantica* using SDS PAGE. These polypeptide bands ranged in 18 to 68 kDA. In the same study he resolved 8 different polypeptide bnads of molecular weight in range 18 to 62 kDa from the somatic extracts of *F. hepatica*. The results of electrophoresis of somatic extracts *F. gigantica* by Kumar *et al*., (2008) showed different bands in range of molecular weights 14 and 76 kDa. By using SDS PAGE Awad *et al*. (2009) fractionized 17 different polypeptides from the somatic extracts with molecular weights 13 and 262.3 kDa.

ES products of *F. gigantica* separated by SDS PAGE contained six bands of different molecular weights ranged between 93 kDa to 148 kDA. Hannan Khan *et al*. (2017) observed 24 different bands in ES products of *F. gigantica* the molecular weights of these bands ranged from 10 kDa to 170 kDa. Also Latchumikanthan *et al*. (2012) found seven different bands range in molecular weights of 23, 25, 28, 43, 47, 52 and 66 kDa. Intapan. (1998) separated six bands in the ES products of *F. gigantica* in range of 14.4 and 65 kDa and Demerdash *et al*. (2011) separated various polypeptides in range 14 and 100 kDa. Meshgi *et a*l. (2008) showed that ES products of *F. gigantica* and *F. hepatica* electrophoresed by SDS PAGE gave six common polypeptides whose molecular range was

in between 18 and 68 kDa . Awad et al. (2009) also resolved seven bands from ES products with molecular weight range of 15 and 101.7 kDa. Estuningsih *et al*. (2009) separated different polypeptides by SDS PAGE in the somatic extracts of fasciola gigantica that range in molecular weight in range 5 and 70 kDa. among them a polypeptide of 28 kDa and the other 60 kDa polypeptide immunized mice and showed that antigens were present in low molecular weight range. They demonstrated that ELISA was more sensitive (91%) as compared to fecal egg counts (87 %).

Immunogenic detection of *F. gigantica* somatic extract by western blotting method showed a single polypeptide with molecular weight in range between 130-250 kDa. These results are concordant with report from Yokananth *et al.* (2005) who found five bands in their analysis, to be immunogenic. One of these five bands was of molecular weight of 156 kDa. Similarly, a 37 kDa polypeptide was identified by Anuracpreeda *et al*. (2013) using Western blotting method. Intapan. (1998) identified a 27 kDa polypeptide from the ES products of *F. gigantica* that was immunogenic. Morales and Espino (2012) recorded that 12 to 14, 24 to 26, 38 and 52 kDa were immunogenic among 5,10,12,14,17,20,24,26,30,38,45,52,76 and 102 kDa polypeptides. Preyavichyapugdee *et al*., (2008) detected a 26 kDa polypeptide from the whole worm extracts of *F. gigantica* by western blot method. Almeida *et al*. (2007) detected 16 bands using infected sera of *F. hepatica* by Western blotting with molecular weights ranging from 8 kDa to 110 kDa.

In current study results of indirect ELISA showed that highly immunogenic proteins were present in molecular weight that range between 38 to 72 kD and they showed 93.3% sensitivity with infected sera. Our results are concordant with the findings of Awad *et al*. (2009) who showed that sensitivity of indirect ELISA was 93.3 % using *F. gigantica* somatic antigens, while the sensitivity of ELSA was between 93.3 % to 94.6% for ES products. These results are in accordance with (Rodríguez-Pérez and Hillyer 1995) who demonstrated that excretory/secretory antigens were more specific than the others somatic extracts. The results are consistent with the investigation of Meshgi *et al*. (2008) who found that 11 bands of somatic extracts that ranged between 18 to 68 kDa and showed sensitivity with infected sera. In another study, Guobadia and Fagbemi (1997) detected immunogenic polypeptides of molecular weights of 17,21,53, and 57 kDa using indirect ELISA method.

Demerdash *et al*. (2011) showed that immunogenic polypeptides in the ES products were 26,45,64, and 83 kDa. Velusamy *et al*. (2004) detected a 54 kDa immunogenic polypeptide from the somatic extracts of *F. gigantica.* The results of indirect ELISA in the study *by* Kumar *et al*., (2008) indicated that a 27 kDa polypeptide was 100 % sensitive to detect infection by *F. gigantica.*

Conclusion

The study recorded overall prevalence of 4.7 % of *F. gigantica* in cattle and buffaloes in different geographical areas. Results of risk factors showed that there was a substantial association between incidence of fascioliasis and host locality, sex, age of the animal, and breed. Areas with man-made irrigation system, low lands, and high precipitation areas have high risk of fascioliasis and should be prioritized for treatment, research, extension work and other control measures.

It is concluded that somatic extracts and ES products of the *F. gigantica* contain protein bands of different molecular weights, sizes and varied origin. These protein bands have prime importance as for as early diagnosis is concerned. Immuno assays such as indirect ELISA and Western blot are highly sensitive and specific and can detect different sera at a time in addition these methods can detect infection of very small number of worms.

The somatic polypeptides fractionized by SDS PAGE in range of molecular weight 38 and 72 kDa showed highly specific and reliable results. Similarly, a band of in range of molecular weight 130 and 250 was also detected by Western blotting. These polypeptides need further identification and characterization. The proteins bands that showed highest reactivity could further be used in immunodiagnosis. These immunogenic proteins of somatic extracts can be used for development of immuno assays Further description of these immunodominant proteins from the somatic and ES products of *F. gigantica* could be exploited as effective vaccine candidates.

Recommendations

- The study should be expanded to test antigenic proteins of somatic extracts for their immunogenic properties. This can be helpful in vaccine development against fascioliasis in future.
- The studies must be expanded to find immunogenic proteins in ES products of *F. gigantica*
- The isolated proteins must be tested for an early diagnosis of experimentally infected animals.

REFERENCES

Abdel Aal, A. A., Abou-Eisha, A. M., & El-Sheary, M. N. (1999). Prevalence of Fascioliasis among man and animals in Ismailia Province. *Assiut Vetrinary Medical Journal, 41*, 141–152.

Adedokun, O. A., Ayinmode, A. B., & Fagbemi, B. O. (2008). A comparative study of three methods for detecting Fasciola infections in Nigerian cattle. *Veterinarski Arhiv*, *78*(5), 411–416.

Affroze, S., Begum, N., Islam, M., Rony, S., Islam, M., Mondal, M., & Mondal, M. (2013). Risk Factors and Gross Pathology of Bovine Liver Fluke Infection at Netrokona District, Bangladesh. *Journal of Animal Science Advances*, *3*(2), 83.

Afshan, K., Fortes-Lima, C. A., Artigas, P., Valero, A. M., Qayyum, M., & Mas-Coma, S. (2014). Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan. *Geospatial Health*, *8*(2), 317–334.

Allam, G., Bauomy, I. R., Hemyeda, Z. M., & Sakran, T. F. (2012). Evaluation of a 14.5 kDa-fasciola gigantica fatty acid binding protein as a diagnostic antigen for human fascioliasis. *Parasitology Research*, *110*(5), 1863–1871.

Neelum, A., & Khan, M. (2017). Economics of milk production in District Mardan, Khyber Pakhtunkhwa (KP), Pakistan. *Sarhad Journal of Agriculture*, *33*(1), 42-46.

Anuracpreeda, P., Chawengkirtikul, R., Tinikul, Y., Poljaroen, J., Chotwiwatthanakun, C., & Sobhon, P. (2013). Diagnosis of Fasciola gigantica infection using a monoclonal antibody-based sandwich ELISA for detection of circulating cathepsin B3 protease. *Acta Tropica*, *127*(1), 38–45.

Anuracpreeda, P., Wanichanon, C., Chawengkirtikul, R., Chaithirayanon, K., & Sobhon, P. (2009). Fasciola gigantica: Immunodiagnosis of fasciolosis by detection of circulating 28.5 kDa tegumental antigen. *Experimental Parasitology*, *123*(4), 334–340.

Asanji, M. F., & Williams, M. O. (1984). The effect of sex on seasonal variation in single and double infection of cattle in sierra leone by Dicrocoelium hospes and faciola gigantica.

Veterinary Parasitology, *15*(3–4), 247–255.

Ashfaq, M., Muhammad, G., Ul-Haq, S., & Razzaq, A. (2014). Effects of livestock diseases on dairy production and in-comes in district Faisalabad, Punjab, Pakistan, (November 2014), 31.

Ashrafi, K., Valero, M. A., Peixoto, R. V., Artigas, P., Panova, M., & Mas-Coma, S. (2015). Distribution of Fasciola hepatica and F. gigantica in the endemic area of Guilan, Iran: Relationships between zonal overlap and phenotypic traits. *Infection, Genetics and Evolution*, *31*, 95–109.

Auxiliar, P., Lisboa, U. N. De, & Auxiliar, I. (2012). Genetic characterization of Portuguese Fasciola hepatica isolates, (December 2012).

Baldock, F. C., Arthur, R. J., & Lawrence, A. R. (1985). A meatworks survey of bovine hydatidosis in southern Queensland. *Australian Veterinary Journal*, *62*(7), 238–243.

Bautista-Garfias, C. R., López-Arellano, M. E., & Sánchez-Albarrán, A. (1989). A new method for serodiagnosis of sheep fascioliasis using helminth excretory-secretory products. *Parasitology Research*, *76*(2), 135–137.

Bekele, M., Tesfay, H., & Getachew, Y. (2010). Bovine Fasciolosis : Prevalence and its economic loss due to liver condemnation at Adwa Municipal Abattoir, North Ethiopia. *Ejast*, *1*(1), 39–47.

Blancas Torres, G., Terashima Iwashita, A., Maguiña Vargas, C., Vera Luján, L., Alvarez Bianchi, H., & Tello Casanova, R. (2004). Fasciolosis humana y compromiso gastrointestinal: Estudio de 277 pacientes en el Hospital Nacional Cayetano Heredia. 1970-2002. *Rev. Gastroenterol. Perú*, *24*, 143–157.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Analytical Biochemistry*, *72*, 248–254.

Brown, D. (1994). Freshwater snails of Africa and their medical importance (Taylor, Francis, Eds,). *London, UK*.

Castelino, J. B., & Preston, J. M. (1979). The influence of breed and age on the prevalence of bovine fascioliasis in Kenya. *British Veterinary Journal*, *135*(2), 198–203.

Charlier, J., Voort, M. Van Der, Kenyon, F., Skuce, P., & Vercruysse, J. (2014). Chasing helminths and their economic impact on farmed ruminants. *Trends in Parasitology*, *30*(7), 361–367.

Control of foodborne trematode infections. Report of a WHO Study Group. (1995). *World Health Organization - Technical Report Series*, *849*, 1–157.

Cotruvo, J. A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D. O., … Gannon, V. P. J. (2004). Waterborne Zoonoses: Identification, Causes, and Control. *Waterborne Zoonoses Identification, Causes, and Control*, 485 p.

Cox, F. E. G. (2002). History of human parasitology. *Clinical Microbiology Reviews*.

Dargie, J. D., Berry, C. I., & Parkins, J. J. (1979). The pathophysiology of ovine fascioliasis: studies on the feed intake and digestibility, body weight and nitrogen balance of sheep given rations of hay or hay plus a pelleted supplement. *Research in Veterinary Science*, *26*(3), 289–295.

De Almeida, M. A., Ferreira, M. B., Planchart, S., Terashima, A., Maco, V., Marcos, L., … Incani, R. N. (2007). Preliminary antigenic characterisation of an adult worm vomit preparation of Fasciola hepatica by infected human sera. *Revista Do Instituto de Medicina Tropical de Sao Paulo*, *49*(1), 31–35.

Dutta, J., Rathore, B. S., Mullick, S. G., Singh, R., & Sharma, G. C. (1990). Epidemiological studies on occurrence of haemorrhagic septicaemia in India. *Indian Veterinary Journal*, *67*(10), 893–899.

El-tahawy, A. S., Bazh, E. K., & Khalafalla, R. E. (2017). Epidemiology of bovine fascioliasis in the Nile Delta region of Egypt : Its prevalence , evaluation of risk factors , and its economic significance, *10*, 1241–1249.

Fagbemi, B. O., & Guobadia, E. E. (1995). Immunodiagnosis of fasciolosis in ruminants using a 28-kDa cysteine protease of Fasciola gigantica adult worms. *Veterinary Parasitology*, *57*(4), 309–318.

Fagbemi, B. O., Obarisiagbon, I. O., & Mbuh, J. V. (1995). Detection of circulating antigen in sera of Fasciola gigantica-infected cattle with antibodies reactive with a Fasciola-specific 88-kDa antigen. *Veterinary Parasitology*, *58*(3), 235–246.

Fitzpatrick, J. L. (2013). Ac ce pt us t. *Veterinary Parasitology*.

Ganesan, G. (2013). An extremely rare and a very intteresting report of endoscopic images of y-shaped mating of male and female hookworms. *International Journal of Current Research and Review*, *5*(20), 47.

Garg, R., Yadav, C. L., Kumar, R. R., Banerjee, P. S., Vatsya, S., & Godara, R. (2009). The epidemiology of fasciolosis in ruminants in different geo-climatic regions of north India. *Tropical Animal Health and Production*, *41*(8), 1695–1700.

Gonzales Santana, B., Dalton, J. P., Vasquez Camargo, F., Parkinson, M., & Ndao, M. (2013). The Diagnosis of Human Fascioliasis by Enzyme-Linked Immunosorbent Assay (ELISA) Using Recombinant Cathepsin L Protease. *PLoS Neglected Tropical Diseases*, *7*(9).

Guobadia, E. E., & Fagbemi, B. O. (1997). The isolation of Fasciola gigantica-specific antigens and their use in the serodiagnosis of fasciolosis in sheep by the detection of circulating antigens, *68*, 269–282.

Gupta, S. C., Ghosh, S., Joseph, D., & Singh, B. P. (2003). Diagnosis of experimental Fasciola gigantica infection in cattle by affinity purified antigen. *Indian Journal of Animal Sciences*, *73*(9), 963–966.

Haas, W., & Haberl, B. (1997). Host recognition by trematode miracidia and cercariae. In *Advances in trematode biology* (pp. 197–227).

Hannan Khan, M. A., Ullah, R., Rehman, A., Rehman, L., Ahammed Shareef, P. A., & Abidi, S. M. A. (2017). Immunolocalization and immunodetection of the excretory/secretory (ES) antigens of Fasciola gigantica. *PLoS ONE*, *12*(10), 1–15

Hossain, M. M., Paul, S., Rahman, M. M., Hossain, F. M. A., Hossain, M. T., & Islam, M. R. (2011). Prevalence and economic significance of caprine fascioliasis at sylhet district of Bangladesh. *Pakistan Veterinary Journal*, *31*(2), 113–116.

Hotez, P. J., Brindley, P. J., Bethony, J. M., King, C. H., Pearce, E. J., & Jacobson, J. (2008). Helminth infections: the great neglected tropical diseases. *The Journal of Clinical Investigation*, *118*(4), 1311–1321.

Hotez, P., Ottesen, E., Fenwick, A., & Molyneux, D. (2006). The neglected tropical diseases: The ancient afflictions of stigma and poverty and the prospects for their control and elimination. *Advances in Experimental Medicine and Biology*.

Jabbar, A., Iqbal, Z., Kerboeuf, D., Muhammad, G., Khan, M. N., & Afaq, M. (2006). Anthelmintic resistance: The state of play revisited. *Life Sciences*.

Kasib, M., Sohail, M., Nisar, M., Iqbal, Z., & Umair, M. (2009). Research in Veterinary Science Bovine fasciolosis : Prevalence , effects of treatment on productivity and cost benefit analysis in five districts of Punjab , Pakistan. *Research in Veterinary Science*, *87*(1), 70–75.

Kato, M., Murakami, Y., Shimizu, M., Yamamoto, H., Yonemoto, Y., Ishii, K.-I., & Kira, S. (2005). Survey of cattle fascioliasis in Tsuyama Abattoir. *Environmental Health and Preventive Medicine*, *10*(3), 162–165.

Kelley, J. M., Elliott, T. P., Beddoe, T., Anderson, G., Skuce, P., & Spithill, T. W. (2016). Current Threat of Triclabendazole Resistance in Fasciola hepatica. *Trends in Parasitology*.

Kendall, S. B. (1965). Relationships between the species of Fasciola and their molluscan hosts. In *Advances in parasitology* (Vol. 3, pp. 59–98).

Khan, M. K., Sajid, M. S., Khan, M. N., Iqbal, Z., & Arshad, M. (2017). Point prevalence of bovine fascioliasis and the influence of chemotherapy on the milk yield in a lactating bovine population from the district of Toba Tek Singh , Pakistan, (2011), 334–338.

Khan, M. K., Sajid, M. S., Khan, M. N., Iqbal, Z., & Iqbal, M. U. (2009). Bovine fasciolosis: Prevalence, effects of treatment on productivity and cost benefit analysis in five districts of Punjab, Pakistan. *Research in Veterinary Science*, *87*(1), 70–75.

Khan, M. N., Sajid, M. S., Khan, M. K., Iqbal, Z., & Hussain, A. (2010). Gastrointestinal helminthiasis: Prevalence and associated determinants in domestic ruminants of district Toba Tek Singh, Punjab, Pakistan. *Parasitology Research*, *107*(4), 787–794.

Kotpal, R. L. (2012). *Modern Text Book of Zoology: Invertebrates*. Rastogi Publications.

Krailas, D., Panomsuk, S., Janecharat, T., & Ukong, S. (2002). Production of monoclonal antibodies against partially purified surface tegument antigens of Fasciola gigantica. *The*

Southeast Asian Journal of Tropical Medicine and Public Health, *33 Suppl 3*(June), 92–96.

Kregg-Byers, C. M., & Schlenk, E. A. (2010). Implications of food insecurity on global health policy and nursing practice. *Journal of Nursing Scholarship*, *42*(3), 278–285.

Kuerpick, B., Conraths, F. J., Staubach, C., Fröhlich, A., Schnieder, T., & Strube, C. (2013). Seroprevalence and GIS-supported risk factor analysis of Fasciola hepatica infections in dairy herds in Germany. *Parasitology*, *140*(8), 1051–1060.

Kumar, N., Ghosh, S., & Gupta, S. C. (2008). Detection of Fasciola gigantica infection in buffaloes by enzyme-linked immunosorbent assay, 155–161

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685.

Langley, R. J., & Hillyer, G. V. (1989). Detection of circulating parasite antigen in murine fascioliasis by two-site enzyme-linked immunosorbent assays. *American Journal of Tropical Medicine and Hygiene*, *41*(4), 472–478.

Latchumikanthan, A., Soundararajan, C., Dhinakarraj, G., & Basith, S. A. (2012). Purification and characterization of excretory/secretory antigens of Fasciola gigantica. *Tamilnadu J Vet Anim Sci*, *8*, 14–18.

Loos-Frank, B., & Grencis, R. K. (2016). Parasitic Worms. *The Biology of Parasites*, 182.

Luqman, M., Shahbaz, B., Khan, I. A., & Safdar, U. (2013). Training need assesment of rural women in livestock management-case of southern Punjab, Pakistan *51*(*1).*

Maqbool, A., Hayat, C. S., Akhtar, T., & Hashmi, H. A. (2002). Epidemiology of fasciolosis in buffaloes under different managemental conditions. *Veterinarski Arhiv*, *72*(4), 221–228.

Mas-Coma, S. (2004). Human Fascoliasis: Epidemiological patterns in human endemic areas of South America, Africa and Asia. In *Southeast Asian Journal of Tropical Medicine and Public Health* (Vol. 35, pp. 1–11).

Mas-Coma, S., Adela Valero, M., & Dolores Bargues, M. (2014). Fascioliasis. *Advances in Experimental Medicine and Biology*, *766*, 77–114.

Mas-Coma, S., Bargues, M. D., & Valero, M. A. (2005). Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology*, *35*(11–12), 1255–1278.

Mas-Coma, S., Valero, M. A., & Bargues, M. D. (2009). Chapter 2 Fasciola, Lymnaeids and Human Fascioliasis, with a Global Overview on Disease Transmission, Epidemiology, Evolutionary Genetics, Molecular Epidemiology and Control. *Advances in Parasitology*. McKerrow, J. H. (1999). Development of cysteine protease inhibitors as chemotherapy for parasitic diseases: Insights on safety, target validation, and mechanism of action. In *International Journal for Parasitology* (Vol. 29, pp. 833–837).

McKerrow, J. H., Engel, J. C., & Caffrey, C. R. (1999). Cysteine protease inhibitors as chemotherapy for parasitic infections. *Bioorganic & Medicinal Chemistry*, *7*(4), 639–644.

Mercedes, M. (2018). The Use of MM3 Monoclonal Antibodies for the Early Immunodiagnosis of Ovine Fascioliasis Author (s): Mercedes Mezo , Marta González-Warleta and Florencio M . Ubeira Published by : Allen Press on behalf of The American Society of Parasitologists Stable U, *93*(1), 65–72.

Mezo, M., Gonzalez-Warleta, M., Carro, C., & Ubeira, F. M. (2004). ultrasensitive capture ELISA for detection of Fasciola hepatica coproantigens in sheep and cattle using a new monoclonal antibody (MM3). *Journal of Parasitology*.

Morales, A., & Espino, A. M. (2012). Evaluation and Characterization of Fasciola hepatica Tegument Protein Extract for Serodiagnosis of Human Fascioliasis, *19*(11), 1870–1878.

Morgan, E., Charlier, J., Hendrickx, G., Biggeri, A., Catalan, D., von Samson-Himmelstjerna, G., … Vercruysse, J. (2013). Global Change and Helminth Infections in Grazing Ruminants in Europe: Impacts, Trends and Sustainable Solutions. *Agriculture*, *3*(3), 484–502.

Mufti, S., Afshan, K., Khan, I. A., Zafar, Y., Rizvi, S. S. R., Nazir, F., & Qayyum, M. (2014). Genetic characterization of Fasciola samples from bovine hosts in Pakistan by sequences of ribosomal internal transcribed spacer regions. *Pakistan Veterinary Journal*, *34*(3), 361–366.

Mulatu, H., & Addis, M. (2011). Study on the prevalence and risk factors of fasciolosis in small ruminants in and around Hirna town, Ethiopia. *Global Veterinaria*, *7*(5), 497–501.

Mungube, E. O., Bauni, S. M., Tenhagen, B. A., Wamae, L. W., Nginyi, J. M., & Mugambi, J. M.

(2006). The prevalence and economic significance of Fasciola gigantica and Stilesia hepatica in slaughtered animals in the semi-arid coastal Kenya. *Tropical Animal Health and Production*, *38*(6), 475–483.

Nguyen, N. T., Le, T. C., Vo, M. D. C., Van Cao, H., Nguyen, L. T., Ho, K. T., … Matsumot0, Y. (2017). High prevalence of cattle fascioliasis in coastal areas of Thua Thien Hue province, Vietnam. *Journal of Veterinary Medical Science*, *79*(6), 1035–1042.

Nwosu, C. O., Madu, P. P., & Richards, W. S. (2007). Prevalence and seasonal changes in the population of gastrointestinal nematodes of small ruminants in the semi-arid zone of north-eastern Nigeria, *144*, 118–124.

Opara, K. N. (2005). Population dynamics of Fasciola gigantica in cattle slaughtered in Uyo, Nigeria. *Tropical Animal Health and Production*, *37*(5), 363–368.

80). Quick Kato smear for field quantification of Schistosoma mansoni eggs. *American Journal of Tropical Medicine and Hygiene*, *29*(2), 217–219.

Petros, A., Kebede, A., & Wolde, A. (2013). Prevalence and economic significance of bovine fasciolosis in Nekemte Municipal abattoir, *5*(8), 202–205. Phiri, A. M., Phiri, I. K., Sikasunge, C. S., & Monrad, J. (2005). Prevalence of fasciolosis in Zambian cattle observed at selected abattoirs with emphasis on age, sex and origin. *Journal of Veterinary Medicine Series B: Infectious Diseases and Veterinary Public Health*, *52*(9), 414–416.

Phiri, I. K., Phiri, A. M., & Harrison, L. J. S. (2006). Serum antibody isotype responses of Fasciola -infected sheep and cattle to excretory and secretory products of Fasciola species, *141*, 234–242.

PILE, E., SANTOS, J. A. A. dos, PASTORELLO, T., & VASCONCELLOS, M. (2001). Fasciola hepatica em búfalos (Bubalus bubalis) no município de Maricá, Rio de Janeiro, Brasil. *Brazilian Journal of Veterinary Research and Animal Science*, *38*(1), 42–43. Preyavichyapugdee, N., Sahaphong, S., Riengrojpitak, S., & Grams, R. (2008). Fasciola gigantica and Schistosoma mansoni : Vaccine potential of recombinant glutathione S - transferase (rFgGST26) against infections in mice, *119*, 229–237.

Puranik, P., & Bhate, A. (2007). *Animal Forms And Functions: Invertebrata*. Sarup & Sons.

Rahman, A. K. M. A., Islam, S. K. S., Talukder, H., Hassan, K., Dhand, N. K., & Ward, M. P. (2017). Fascioliasis risk factors and space-time clusters in domestic ruminants in Bangladesh, 29–31.

Raza, M. A., Iqbal, Z., Jabbar, A., & Yaseen, M. (2017). Point prevalence of gastrointestinal helminthiasis in ruminants in southern, (2007), 323–328.

Rehman, T., Khan, M. N., Abbas, R. Z., Babar, W., Sikandar, A., & Zaman, M. A. (2016). Serological and coprological analyses for the diagnosis of Fasciola gigantica infections in bovine hosts from Sargodha, Pakistan. *Journal of Helminthology*, *90*(04), 494–502.

Roberts, M. T. M. (2005). Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. *British Medical Bulletin*.

Rodríguez-Pérez, J., & Hillyer, G. V. (1995). Detection of excretory-secretory circulating antigens in sheep infected with Fasciola hepatica and with Schistosoma mansoni and F. hepatica. *Veterinary Parasitology*, *56*(1–3), 57–66.

Rondelaud, D., Vignoles, P., Dreyfuss, G., & Mage, C. (2006). The control of Galba truncatula (Gastropoda: Lymnaeidae) by the terrestrial snail Zonitoides nitidus on acid soils. *Biological Control*, *39*(3), 290–299.

Ruiz-Campillo, M. T., Hernandez, V. M., Escamilla, A., Stevenson, M., Perez, J., Martinez-Moreno, A., … Cwiklinski, K. (2017). Immune signatures of pathogenesis in the peritoneal compartment during early infection of sheep with Fasciola hepatica. *Scientific Reports*, *7*(1), 2782.

Ruiz-Navarrete, M. A., Arriaga, C., Bautista, C. R., & Morilla, A. (1993). Fasciola hepatica: characterization of somatic and excretory-secretory antigens of adult flukes recognized by infected sheep. *Revista Latinoamericana de Microbiologia*, *35*(3), 301–307.

Saiful Islam, K. B. M., & Taimur, M. J. F. A. (2008). Helminthic and protozoan internal parasitic infections in free ranging small ruminants of Bangladesh. *Slovenian Veterinary Research*, *45*(2), 67–72.

Sajid, M., & McKerrow, J. H. (2002). Cysteine proteases of parasitic organisms. *Molecular and Biochemical Parasitology*.

Sánchez-Andrade, R., Paz-Silva, A., Suárez, J. L., Panadero, R., Pedreira, J., López, C., … Morrondo, P. (2002). Influence of age and breed on natural bovine fasciolosis in an endemic area (Galicia, NW Spain). *Veterinary Research Communications*, *26*(5), 361–370.

Sarmiento Olivera, M., Gómez Olivera, I., Ordaz González, A. M., García Diaz, C. D., & Casanova Moreno, M. de la C. (2012). *Revista de ciencias médicas de Pinar del Río. Revista de Ciencias Médicas de Pinar del Río* (Vol. 16).

Selzer, P. M., Pingel, S., Hsieh, I., Ugele, B., Chan, V. J., Engel, J. C., … McKerrow, J. H. (1999). Cysteine protease inhibitors as chemotherapy: Lessons from a parasite target. *Proceedings of the National Academy of Sciences*, *96*(20), 11015–11022.

Silva, M. L. S., Da Costa, J. M. C., Da Costa, A. M. V., Pires, M. A., Lopes, S. A., Castro, A. M., & Monjour, L. (1996). Antigenic components of excretory-secretory products of adult Fasciola hepatica recognized in human infections. *The American Journal of Tropical Medicine and Hygiene*, *54*(2), 146–148.

Singh, D. J., & Singh, D. K. (2016). Anthelmintic activity of chlorophyllin against different larval stages of fasciola gigantica. *Revista Do Instituto de Medicina Tropical de Sao Paulo*, *58*(M).

Singh, O., & Agarwal, R. A. (1981). Toxicity of certain pesticides to two economic species of snails in northern India. *Journal of Economic Entomology*, *74*(5), 568–571.

Solomon, W. M., & Abebe, W. (2007). Prevalence study of ruminant Fasciolosis in areas adjoining Upper Blue Nile Basin, North western Ethiopia. *J Ethiopian Vet Assoc2007*, *11*, 67–81.

Soulsby, E. J. L. (1968). Helminths, arthropods and protozoa of domesticated animals. Seventh edition. In *Sixth edition of Monnig's veterinary helminthology and entomology* (pp. 176–325).

Spithill, T. W. (1999). Pathology, pathophysiology and clinical aspects. *Fasciolosis*, 185–224.

Sukhapesna, V., Sarataphan, N., Tuntasuvan, D., & Sa-ngiamluksana, S. (1990). A study on epidemiology of liver fluke infection in buffaloes. *Thai Journal of Veterinary Medicine*, *20*(4), 527–534.

Talukder, S., Bhuiyan, M., Hossain, M., Uddin, M., Paul, S., & Howlader, M. (2011). Pathological Investigation of Liver Fluke Infection of Slaughtered Black Bengal Goat in a Selected Area

of Bangladesh. *Bangladesh Journal of Veterinary Medicine*, *8*(1), 35–40.

Thornton, P. K. (2010). Livestock production: recent trends, future prospects. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *365*, 2853–2867.

Toet, H., Piedrafita, D. M., & Spithill, T. W. (2014). Liver fluke vaccines in ruminants: Strategies, progress and future opportunities. *International Journal for Parasitology*.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences*, *76*(9), 4350–4354.

Usip, L. P. E., Ibanga, E. S., Edoho, H. J., Amadi, E. C., & Utah, E. (2014). Prevalence of Fascioliasis and the economic loss of condemned liver due to Fasciola infection in Cattle slaughtered at three abattoirs in Eket Urban, Akwa Ibom State of Nigeria. *Global Advanced Research Journal of Food Science and Technology*, *3*(2), 54–75.

Van Veen, T. W. S., Folaranmi, D. O. B., Usman, S., & Ishaya, T. (1980). Incidence of liver fluke infections (Fasciola gigantica andDicrocoelium hospes) in ruminants in northern Nigeria. *Tropical Animal Health and Production*, *12*(2), 97–104.

Velusamy, R., Singh, B. P., Sharma, R. L., & Chandra, D. (2004). Detection of circulating 54 kDa antigen in sera of bovine calves experimentally infected with F. gigantica. *Veterinary Parasitology*, *119*(2–3), 187–195.

Vercruysse, J., & Claerebout, E. (2001). Treatment vs non-treatment of helminth infections in cattle: Defining the threshold. *Veterinary Parasitology*.

Vercruysse, J., Schetters, T. P. M., Knox, D. P., & Willadsen, P. (2007). Control of parasitic disease using vaccines : an answer to drug resistance ? Resistance against antiparasitic drugs, *26*(1), 105–115.

Viyanant, V., Krailas, D., Sobhon, P., Upatham, E. S., Kusamran, T., Chompoochan, T., … Prasittirat, P. (1997). Diagnosis of cattle fasciolosis by the detection of a circulating antigen using a monoclonal antibody. *Asian Pacific Journal of Allergy and Immunology*, *15*(3), 153–159.

Worku, T., Herago, T., & Amajo, M. (2017). Prevalence and Financial Losses Associated with Bovine Fasciolosis at Asella Municipal Abattoir, South Eastern Ethiopia. *Advances in Biological Research*, *11*(5), 258–264.

Yadav, S. C., Saini, M., Raina, O. K., Nambi, P. A., Jadav, K., & Sriveny, D. (2005). Fasciola gigantica cathepsin-L cysteine proteinase in the detection of early experimental fasciolosis in ruminants. *Parasitology Research*, *97*(6), 527–534.

Yildirim, A., Ica, A., Duzlu, O., & Inci, A. (2007). Prevalence and risk factors associated with Fasciola hepatica in cattle from Kayseri province, Turkey. *Revue de Médecine Vétérinaire*, *158*(12), 613–617.

Yokananth, S., Ghosh, S., Gupta, S. C., Suresh, M. G., & Saravanan, D. (2005). Characterization of specific and cross-reacting antigens of Fasciola gigantica by immunoblotting. *Parasitology Research*, *97*(1), 41–48.

Annexures

Annexure 1

Recipe of Lysis Buffer/ Extraction Buffer.			
.No.	**Reagents**	**Amount/%**	**Preparation**
1	M HEPES	50mM	(1M=238.3g) 50mM = 2383 x 50 / 1000 =1.1915g/100ml
2	NaCl	150mM	1M=(58.5g) 150mM = 58.5 x150 / 1000ml = 0.8775g/100ml
3	Sodium Azide	0.02%	0.02g in 100 ml
4	SDS	0.10%	0.1 g in 100 ml
5	Triton X – 100	1%	1 ml in 100 ml
6	PMSF	0.1 mg /ml	

Annexure 2

Recipe for Resolving Gel	
Reagents	**Amount**
Distilled water	3150 µl
30% Stock Acryl amide solution	2500 µl
4xResolving Tris Solution	1875 µl
10% Ammonium Persulphate	75 µl
Recipe for Stacking Gel.	
Distilled water	2100 µl
30% Stock Acrylamide Solution	325 µl
4x Stacking Tris Solution	800 µl
!0% Ammonium Persulphate	33.5 µl
TEMED	6 µl
Reagents used in Bradford method.	
Methanol	25ml
Phosphoric acid	0.05g
Coomassie brilliant blue	50 ml
Distilled water	100 ml
Phosphate-free, azide-free blocking solution, 1000 ml (adjust pH with 12 N HCl about 3.35 ml)	
150 mM NaCl	8.766 g
50 mM Tris-HCl (pH 7.5)	6.057 g
nonfat dried milk	5% (w/v)

Annexure 3

Formation of 0.1M PB	
Na2HPO4	1.09g
NaH2PO4	0.32g
NaCl	9g
Make 1000 ml adding water, this is we have 0.1M PBS.	

Stock solution for SDS PAGE	
Stock solutions	**Recopies and procedure**
Stock 30% Acrylamide Gel Solution	30.0g acrylamide, 0.8 g methylene bisacrylamide and dH_2O 100ml
Stock 4 x Resolving Gel Tris (1.5M Tris HCL pH8.8, 0.4% SDS)	To 110ml DH_2O add 36.4 g of Tris base Add 8 ml of 10% SDS, Adjust pH to 8.8 with 1N HCL Adjust the final volume to 200ml with distilled water
Stock 4 x Stacking Tris (0.5 M Tris. HCL pH6.8, 0.4% SDS)	To 110 ml DH_2O add 12.12 g of Tris base, Add 8ml of 10% SDS, Adjust pH to 6.8 with 1N HCL. Add Distilled water to a final volume of 200ml
Stock 4 x Tris-glycine tank buffer-SDS	For 300ml, Tris = 3.6g, Glycin = 17.28g, DH_2O = 300 ml Now to this 300ml of 4 xTris-Glycine buffer add 12ml of 10% SDS solution, Make volume up to 1200ml wit DH_2O.
10% AP solution	0.1g ammonium persulphate, 1m Distilled water

Stock 4 x Sampe Buffer	4ml glycerol, 2ml 2-mercaptoethanole, 1.2 g SDS, 5ml 4 x, Stacking Tris 0.003 g Bromophenole blue, Aliquot into 1.5ml micro centrifuge tubes, store at -20°C
10% SDS	5g SDS in 50ml distilled water
Fixing Solution	For 100 ml, 50 ml methanol, 10ml glacial acetic acid Make up to 100ml with distilled water
Gel Stain Solution	For 100ml, 50ml methanol, 10ml glacial acetic acid, 40 ml DH_2O, 0.1g coomassie blue. Make up to 100ml wit distilled water
Destain solution	For 100 ml, 40ml methanol, 10 ml glacial acetic acid Make up to 100 ml with distilled water
Storage solution	5ml glacial acetic acid, 95ml distilled water

Annexure 4

Western blotting transfer buffer (1000 ml).		
Tris Glycine SDS Methanol SDS: 3.7 ml of 10% SDS	48 mM 39 mM 0.037% 20%	5.814 g 2.928 g 0.37 g 200 ml
Blocking solution, 100 ml, in PBS		
Nonfat dried milk Antifoam A Sodium azide Sodium azide: 1 ml of 2% solution	5% 0.01% 0.02%	
Blocking solution, 10 ml, in PBS (pH 7.4)		
Nonfat dried milk Antifoam A Sodium azide	5% 0.01% 0.02%	

Annexure 5

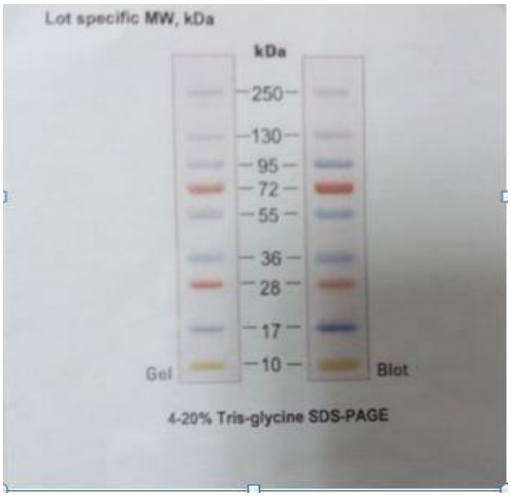
Preparation Serial dilution of BSA Standards.

Tubes	Blank	1	2	3	4	5	6	7	8	9	10	11	12
Volume of BSA (µl)	0	2 .5	20	30	40	55	60	65	70	75	80	84.1	90
Volume of water (µl)	100	97.5	80	70	60	45	40	35	30	25	20	15.9	10
BSA conc.(µg/100µl)	0	62.5	500	750	1000	1375	1500	1625	1750	1875	2000	2103	2250
Total volume	100	100	100	100	100	100	100	100	100	100	100	100	100
BSA conc.(µg/100µl)	0	0.625	p	7.5	10	13.8	15	16.3	17.5	18.8	20	21.03	22.5

Annexure 6

Protein Ladder

Protein ladder used as standard for protein identification was composed of proteins in rang 250-10 KDa molecular weight



Showing the migration distance and the molecular weights of different molecular weights proteins**.**