Sudan University of Science and Technology (SUST)

College of Graduate Studies

Protein Profile and Seroprevalence of Fasciola gigantica in Ruminants in the Sudan

شاكلة البروتين والإنتشار المصلي للدودة الكبدية في المجترات في السودان

By

Inas Ahmed Eltayeb Mahmoud

M.V.Sc. University of Khartoum (2003)

B.V.Sc. University of Khartoum (1994)

A Thesis Submitted in Fulfillment of the Requirements of the Degree of Doctor of Philosophy (PhD) In Veterinary Medicine (Parasitology)

Supervised By

Professor: *Ali Abd Almajid*-Main Supervisor

Professor: *Ahmed Ali Ismail*-1st Co-Supervisor

Professor: *Ibtisam Amin Goreish*-2nd Co-Supervisor

May 2017

Sudan University of Science & Technology

College of Graduate Studies

بسم الله الرحمن الرحيم

كلية الدراسات العليا

جامعة السودان للعلوم والتكنولوجيا

كلية الدراسات العليا

Approval Page

Name of Candidate: Inas Ahmed Eltayeb Mohamed

Thesis title: Protein profile of Fasciola gigantica and their role in the diagnosis of the disease

Approved by:

1. External Examiner

Name: Abbas Mohmed Ahmed

Signature: ..... Date: 9/5/2017

2. Internal Examiner

Name: Prof. Galal Eldin Elazhari Mohammed

Signature: ..... Date: 9/5/2017

3. Supervisor

Name: Prof. Ali Mohamed Abdel Majid

Signature: ..... Date: 9/5/2017

DEDICATION

To the soul of my parents,

My tuitors

Professor Ahmed Ali Ismail

Professor Mohamed Musa

and my family I dedicate this work with love

ACKNOWLEDGEMENT

All praises to Almighty Allah alone, the compassionate and merciful, who has always blessed me and guided me on the path of righteousness.

My deepest appreciation to my first co-supervisor the late ***Professor Ahmed Ali Ismaeil*** who left our world but, still his memory remains in our minds and hearts through his work, help, researches, guidness and love that will never forgotten.

My tremendous thanks to my supervisor ***Professor Ali M.A/Majid*** for his kind guidance and encouragement. I am very grateful to my second co-supervisor ***professor Ibtisam Amin Goreish*** for her deep concern, unconditional support and sincere encouragement to help me pursue my research in the veterinary Research Institute (VRI), Department of Parasitology.

My sincere appreciation goes to thank the staff of the Institute of Endemic Diseases for their co-operation and assistance especially ***Professor Moawia M. Mukhtar*** the director of the institute for his helpful devices and research expertise.

Words fail to express my special thanks to ***professor Sayyid Elsidig Elowni*** for his constructive comment and his critical reading of the thesis, revision and correction.

I would also like to acknowledge in admiration the cooperative altitude shown by the all members of Department of Parasitology (VRI) whenever, I approached them for their endless service and advice, especially worthy of mention are the services of ***Dr Osman Mukhtar***. The cooperation and help extended by ***Miss Randa*** was extraordinary.

My special thanks also extended to all staff of the Tsetse and Trypanosomiasis control Department, VRI for their tolerant attitude and priceless friendship throughout my research work at VRI.

I would like to express my gratefulness to the staff of Rabak Regional Laboratory mainly ***Dr. Abdelgadir and Dr. Tarig*** who were constantly providing me with technical support during the field work.

My deepest thanks go to my friend ***miss Zawahir*** for her company in the analysis of data.

Last but not least, I would like to thank my family for their constant encouragement and my ingenuous husband who has been very patient and tried to complete confidence in me when I doubted myself.

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ABSTRACT

The current study was designed to study the protein profile (antigenic structure) of adult *Fasciola gigantica* excecretory-secretory and somatic products from sheep and goats of the same locality (White Nile province) and from three cattle types (White Nile, Mangisto and Niyala types) from different localities in Sudan, using Sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This antigenic analysis was applied for detection of immunoreative protein that could be used for early diagnosis and establishment of protective vaccine. Furthermore, indirect ELISA (enzyme-linked immunosorbent assay) was evaluated for its effectiveness in immunodiagnosis of Fasciolosis in sheep and cattle in the area of Rabbak, White Nile Province, Sudan. It was also applied to compare ES Paramphistomum *spp.* extract versus ES and SO *F. gigantica* products in both sheep and cattle, to determine the possibility of cross reaction between the two parasite antigens.

Generally SDS-PAGE analysis showed the presence of several bands with lower molecular weights ranging from 12-95 KDa in the Fasciola ES products comparing with 14-123KDa in the somatic one. Dominant common bands for both ES and SO extracts were clustered between 27-30 KDa. Major common bands were identified in SO extracts of *F.gigantica* in sheep, goats and cattle at 45 and 66. Moreover, three common major bands with approximate molecular weight of 27-30, 45 and 66 KDa were found in somatic preparation of the parasite from the three different cattle types. >17, 57, 95 and 110 KDa were only seen in Niyala cattle type, while Mangisto type showed five polypeptide bands with specific bands above 95 and 123 KDa. Only one specific band (<80) was seen in White Nile type. *F.gigantica* somatic antigens in both sheep

and goats revealed 4 common dominant bands 14, 28, 45 and 66 KDa and three goat specific bands 19, 38, 50 KDa.

The dominant of *F.gigantica* ES extracts from sheep, goats and cattle were clustered between 17-24, 27-33 and 40 KDa. The electrophoretic migration showed some similarities and some differences between ES antigen extracts of *F.gigantica* existing in sheep, goats, and cattle. Twelve bands with molecular weight of 14, 17, 19, 21, 24, 28, 31, 33, 40, 55, 62 and 72 KDa were identified in sheep, whereas eleven bands with molecular weights of >17, 17, 20, 23, 27, 33, 40, 45, 80, 87, and 95 KDa were shown in cattle. On the other hand, nine bands with molecular weights of 12, 14, 17, 19, 21, 24, 40, 55 and 72 were found in goats. Considering this result, we noticed that the highest molecular weights 80, 87 and 95 of *F. gigantica* ES antigen were recorded only in cattle.

One hundred fifty six cattle faecal samples were examined, 30 were found positive with *Fasciola gigantica* with an overall prevalence of 19.2%. The results of indirect ELISA revealed that higher prevalence was detected by ES Ag (30%) comparing with SO Ag (18.7%). High specificity (81.6%) was recorded using SO antigen in cattle compared with 69% using ES antigen. Meanwhile, low sensitivities of 27.7% and 20% were recorded in both ES and SO antigens, respectively. Twenty one out of ninety two sheep faecal samples examined were found positive with *Fasciola gigantica* with overall prevalence rate of 22.8%. Moreover, the overall seroprevalence of Fasciola was 22.8% (21/92) and 20.6% (19/92) as assessed by *Fasciola gigantica* ES and SO antigens, respectively. Higher specificity in sheep was recorded using SO Ag (83%) (59/71) compared with ES Ag (78.9%). However, low sensitivity of 33% and 28.6% was determined when SO and ES antigens were used, respectively. From these results, we concluded that Indirect ELISA using

both ES and SO Fasciola antigens in sheep and cattle was not sensitive, although it showed slightly high specificity.

A remarkable observation occured in the current study is that the number of sheep and cattle negative to Fasciola ES and SO indirect ELISA and positive to coprological examination was high. In sheep 14 and 15 sera out of 21 positive faecal samples, while in cattle 22 and 24 sera out of 30 positive faecal samples resulted negative, respectively.

In cattle by using ES *Fasciola gigantica* antigen versus Paramphistomum faecal analysis, we observed that 17 sera out of 46 positive faecal Paramphistomum had positive values by Fasciola indirect ELISA. Similarily, in sheep considering both ES and SO Fasciola antigens, 2 sera out of 13 positive Paramphistomum faecal samples were positive to *Fasciola gigantica*. In addition, indirect ELISA tests using both paramphistomum and Fasciola ES antigens showed that among the 61 Paramphistomum positive sera, 26 sera were found positive for Fasciola. Similarly, the numbers of serologically positive Fasciola samples were 47. Among them 26 were found positive for Paramphistomum. Meanwhile, in sheep the results detected by indirect ELISA showed that among the 20 sera positive for Fasciola using ES, 4 sera were found to be positive for Paramphistomum. Similarly, among the 17 sera positive for SO Fasciola, 8 were found positive for Paramphistomum. So from these results we concluded that cross reaction between *Fasciola gigantica* and Paramphistomum *spp.* may exist.

ES extract for Paramphistomum was used to evaluate the diagnostic sensitivity and specificity of indirect ELISA for the diagnosis of Paramphistomiasis as well as its prevalence in cattle and sheep. The prevalence of Paramphistomiasis in cattle based on indirect ELISA was

higher than that detected by faecal analysis. However, We noted that the prevalence of Paramphistomiasis in sheep was higher by coprological examination (13.8%) than that detected by Indirect ELISA (11.5%).The sensitivity of indirect ELISA in both cattle and sheep was found to be 43.4% and 8.3%, whereas, the specificity was 62.7% and 88%, respectively. This result indicating that indirect ELISA for detection of Paramphistomum *spp.* is more specific but not sensitive.

ملخص الاطروحة

صممت الدراسة الحالية لتحليل مستضدات الدودة الكبديه البالغة *F.gigantica* المستخرجة من جسم الطفيل SO وكذلك التي تم إفرازها أو إخراجها بواسطة الطفيل ES من الأغنام والماعز المصابة في ولاية النيل الأبيض وأيضاً من ثلاثة سلالات مختلفة من الأبقار (سلالات النيل الأبيض والمنقستو ونيالا) من مناطق مختلفة في السودان ، باستخدام تقنية تعرف بـ(SDS polyacrylamide Gel Electrophoresis). أجري تحليل هذه المستضدات للتعرف علي البروتينات الممنعة التي يمكن استخدامها في التشخيص المبكر والتمهيد لإنتاج لقاح واقي في المستقبل .

كذلك استخدمت تقنية الاليزا ELISA (Immunosorbent assay) لتقييم فعاليتها في تشخيص مرض الـ Fasciolosis في الضأن والأبقار في منطقة ربك بولاية النيل الأبيض بالسودان . وطبقت أيضاً لمقارنة المستضدات التي تم إفرازها أو إخراجها بواسطة دودة البرمفستومم مقابل مستضد الفاشيولا المستخلص من جسم الطفيل والذي تم افرازة أو إخراجه بواسطة الطفيلES في الضأن والأبقار لتحديد احتمالية وجود أي cross reaction فيما بينهم.

بشكل عام عند إجراء تحليل SDS Polyacrylamide Gel Electrophoresis وضح وجود عدة أحزمة بأوزان جزيئية تتراوح من 12-95 كيلو دالتون في المستضد الذي تم افرازة أو اخراجه بواسطة دودة الفاشيلا ES مقارنة بـ14- 123كيلودالتون في المستضد المستخرج من جسم الطفيل SO .

الأحزمة المشتركة والمهيمنة علي كل من مستضدات دودة الفاشيلا المستخرجة من جسم الطفيل SO وكذلك التي تم افرازها بواسطة الطفيل ES وجدت متجمعة بين 27-30 كيلودالتون. أما الأحزمة الرئيسية المشتركة التي تم التعرف عليها فى مستضدات دودة الفاشيلا المستخرجة من جسم الطفيل SO من كل من الضأن والماعز والأبقار هي 45 و 66 كيلودالتون .

علاوة علي ذلك 3 احزمة رئيسية مشتركة بأوزان تقريبية هي 27-30 ، 45 ، 66 كيلودالتون وجدت فى مستضدات دودة الفاشيلا المستخرجة من جسم الطفيل SO في سلالات الأبقار الثلاثة المختلفة . أقل من 17، 17، 57 ، 95 ، 110 كيلو دالتون وجدت فقط من سلالات أبقار نيالا بينما سلالة المنقستو أظهرت وجود حزمتين معينة من 95 و 123 كيلو دالتون . لوحظ وجود حزام بروتيني واحد أعلي من 80 كيلودالتون يخص سلالة أبقار النيل الأبيض .

أربعة أحرمة بروتينية مشتركة ومهيمنة هي 14 ، 28 ، 45 ، 66 كيلو دالتون تم اكتشافها فى مستضدات دودة الفاشولا المستخرجة من جسم الطفيل فى كل من الضأن والماعز مع وجود 3 أحزمة محددة ومخصصة فى الأغنام ممثلة في 19 ، 38 ، 50 كيلو دالتون .

أما بالنسبة لمستضدات دودة الفاشيولا التي تم إفرازها أو إخراجها بواسطة الطفيل ES ، فقد وجد أن الأحزمة المهيمنة فى كل من الضأن والماعز والأبقار قد جمعت بين 17 – 24، 27 – 33 و 40 كيلو دالتون .

كذلك أظهرت تقنية SDS polyacrylamide Gel Electrophoresis بعض التشابه والاختلاف فى مستضدات دودة الفاشيولا التي تم افرازها أو اخراجها بواسطة الطفيل الموجود في كل من الضأن والماعز والأبقار .

12 أحزمة باوزان جزيئية 14،17 ، 19، 21،24 ، 28،31 ، 33، 40،55 ،62 ،72كيلودالتون وجدت من الضأن . بينما 11 أحزمة ذات أوزان جزيئية أقل من 17

17، 20 ، 23 ، 27 ، 33 ، 40 ، 45 ، 80 ، 87 ، 95 كيلو دالتون تم التعرف عليها من الأبقار . من ناحية أخري 9 أحزمة بأوزان جزئية 12 ، 14 ، 17 ، 19 ، 21 ، 24 ، 40 ، 55 ، 62 وجدت في الأغنام. بأخذ هذه النتيجة في الاعتبار نلاحظ أن أعلي وزن جزئي 80 ، 87 ، 95 لمستضدات دودة الفاشيولا التي تم إفرازها أو إخراجها بواسطة الطفيل قد تم تسجيلها فقط في الأبقار .

تم فحص 156 عينة براز من الأبقار ، وجد أن 30 عينة منها كانت موجبة لدودة الفاشيولا بمعدل إصابة 19,2% . أما بالنسبة لنتائج الفحص المناعي الممثل في الاليزا للفحص عن الأجسام المضادة لدودة الفاشيولا أثبت وجود أعلي معدل إصابة 30% باستخدام مستضد ES مقارنة بمستضد SO والذي أعطي نسبة إصابة بلغت 18,7% . أعلي نسبة خصوصية بلغت 81.6% سجلت باستعمال مستضد SO فى الأبقار مقارنة بـ69% عند استخدام مستضد الـ ES . بينما أقل نسبة حساسية للاختبار هي 27.7% و 20% عند استخدام كل من مستضد ES ومستضد SO ، علي التوالي .

21 عينة من أصل 92 عينة براز تم فحصها من الضأن وجدت موجبة بمعدل إصابة 22.8% . بينما وجد أن معدل الإصابة لفحص السيرم هي 22.8% و 20.6% باستخدام كل من مستضد الفاشيولا ES وSO علي التوالي .

أعلي خصوصية للاختبار من الضأن سجلت 83% باستخدام مستضد SO مقارنة بمستضد ES والذي سجل خصوصية بلغت 78.9% . لكن أقل حساسية حددت هي 33% و 28.6% باستخدام كل من مستضد SO ومستضد ES علي التوالي. من هذه النتائج نستخلص أن اختبار الاليزا الغير مباشر باستخدام كل من مستضد الفاشيولا ES و SO فى الضأن والأبقار ليس حساس بالرغم من أنه أظهر خصوصية مرتفعة بعض الشئ من الملاحظات الواضحة التي أظهرتها الدراسة الحالية أن أعداد الضأن والأبقار التي وجدت غي مصابة في اختبار الاليزا باستخدام كل من مستضد الفشيولا ES و SO ووجدت مصابة عن طريق فحص الترسيب للبراز كانت مرتفعة . فمثلاً فى الضأن 14 و 15 عينة سيرم من أصل عينة براز موجبة و 22 و 24 عينة سيرم من أصل 30 عينة براز موجبة وجدت سالبة علي التوالي .

باستخدام مستضد الفشيولا ES من الأبقار ، لوحظ أن 17 عينة سيرم من أصل 46 عينة كانت موجبة لدودة برامفستومم لديها قيمه موجبة لدودة الفاشيولا باستخدام فحص الاليزا . بالمثل في الضأن آخذين في الاعتبار كل من مستضد ES ومستضد SO ، 2 عينة سيرم من أصل 13 عينة براز موجبة لدودة برامفستومم كانت موجبة أيضاً لدودة الفاشيولا . بالاضافة إلي ذلك ، باستخدام كل من مستضد الـ برامفستومم ومستضد الفاشيولا ES أظهرت نتائج اختبار الاليزا أن من بين 61 عينة سيرم موجبة لدودة الـ برامفستومم أن 26 عينة سيرم وجدت موجبة لدودة الفاشيولا . بالمثل عدد العينات التي وجدت موجبة لدودة الفاشيولا كانت 47 عينة . من بينهم 26 عينة وجدت موجبة لدودة برامفستومم . بينما فى الضأن أوضحت النتائج التي تم الكشف عنها باستخدام فحص الاليزا أن 20 عينة سيرم كانت موجبة للفاشيولا باستخدام المستضد ES ، 4 عينات سيرم وجدت موجبة لدودة برامفستومم كذلك من بين17 عينة سيرم كانت موجبة لدودة الفاشيولا باستخدام المستضد SO ، 8 عينات وجدت موجبة لـ برامفستومم .

من هذه النتائج توصلنا إلي أن Cross reaction بين دودة الفاشيولا ودودة البرامفستومم ربما يكون موجود.

تم استخدام مستخلص ES لدودة الـ البرامفستومم لتقييم حساسية وخصوصية فحص الاليزا لتشخيص مرض ال البرامفستومم وفى الوقت نفسه تحديد معدل الاصابة في كل من الضأن والابقار . معدل الإصابة بمرض ال البرامفستوميسيس في الأبقار اعتماداً علي فحص الاليزا كان أعلي من الذي تم التحقق منه بواسطة فحص البراز . بالرغم من ذلك لوحظ أن معدل الإصابة بدودة البرامفستومم في الضأن كان أعلي باستخدام فحص البراز من الذي تم التحقق منه بواسطة

فحص الاليزا . حساسية فحص الاليزا من كل من الأبقار والضأن وجدت 43.4% و 8.3% بينما الخصوصية بلغت 62.7% و 88% علي التوالي . هذه النتيجة تشبر إلي أن فحص الاليزا للكشف عن دودة ال البرامفستومم أكثر خصوصية ولكنها أقل حساسية .

CHAPTER ONE

CHAPTER ONE

1. Introduction

Sudan is a vast country and is characterized by a wide spectrum of climatic conditions. Because of its enormous pastoral and agricultural potentialities, the country is regarded as one of the major food reservoirs in the world. 90% of the animal population is owned by nomads who are moving with their herds searching for water and pasture. Due to this system of animal husbandry, chances of contracting diseases are great in such a country where a wide range of helminthic, protozoal and external parasites thrive in abundance, **(*Eisa, 1963*)**. Parasitic diseases undoubtly have a significant role affecting the health and productivity of livestock, and, consequently, the national economy. Among major helminthic diseases of livestock in the Sudan, Fasciolosis is regarded as one of the gravest and economically the most important ***(Karib, 1962)***.

Fasciolosis is an economically important disease of domestic life stock; in particular cattle, sheep and occasionally man. The disease is caused by digenean trematodes of the genus *Fasciola*, commonly referred to as liver rot. The two species most commonly implicated as the aetiological agents of Fasciolosis are *F. hepatica* and *F. gigantica*. The former is found worldwide and is prevalent in almost all temperate regions where sheep and other ruminants are raised. It originated in the European continent and gradually migrated to other continents **(*Boray, 1969)***. *F. gigantica*, however, is restricted mainly to tropical areas such as Africa, South America, Southeast Asia, Southern Europe and Hawaii and also in the former USSR **(*McCarthy and Moore, 2000*)**. The two species of flukes show a wide distribution in African and Asian continents and have common characteristics.

Considering the worldwide spread, occurrence and zoonotic nature, Fasciolosis has emerged as a major global and regional concern affecting all domestic animals and infection is most prevalent in regions with intensive sheep and cattle production **(*WHO, 2007; 2008*).**

Tropical Fasciolosis caused by infection with *Fasciola gigantic*a is regarded as one of the most important single helminth infections of ruminants in Asia and Africa **(*Boray, 1985; Fabiyi, 1987; Murrell, 1994*).** Estimate of the prevalence of this parasite in ruminants range up to 80-100% in some countries **(*Schillhorn van Veen, 1980; Fabiyi, 1987*)**. Together with major nematodes infections, fasciolosis is a significant constraint to the productivity of domestic ruminants throughout Asia, South-East Asia and America and is thus a significant impediment to global food production **(*Dargie, 1987; Fabiyi, 1987; Murrell, 1994*).**

In Africa the prevalence rates range between 30-90% and up to 66% prevalence has been reported in Sudan **(*El-Azazy and Schillhorn Van Veen, 1983*)**.The disease is endemic in certain parts of the country, particularly all along the areas irrigated by the White Nile **(*Karib, 1962; Haroun and Hussein, 1975*)**. However, Fasciolosis was also reported in the western part of the country where prominent swamps and shallow-water valleys exist **(*Haroun, 1975*)**.

The reason for the high incidence of Fasciolosis in the areas of the White Nile and its tributaries appears to be due to the following:

a. The presence of *Lymnaea natalensis* snail, the intermediate host of *F.gigantica,* in large numbers in the flooded plains of the White Nile province and the river' s tributaries. The optimum environmental conditions such as the slow flowing water and the presence of abundant aquatic plant especially the water hyacinth facilitate the growth and survival of the snails.

b. The horizontal expansion in cultivated areas of both irrigation schemes and shifting agriculture has reduced the amount of natural pasture for both nomadic and settled herds which resulted in deriving the livestock to graze the swampy areas of the flood plain which harbour the infected snails during the dry season.

In such situations animals are often exposed to large doses of infection, sometimes taking the form of outbreaks of an acute disease with high mortalities, particularly in sheep **(*Karib, 1962; Haroun, 1975*)**. However, of even greater overall economic importance are the less dramatic but insidious, long term and deleterious effects of the chronic disease wasting condition. The sequel of such types of chronic disease may include inappetence, poor food utilization, loss in body weight, reduced milk and wool production and stillbirth. The highest risk of transmission occurs between December and May with peak egg excretion between August and September, a period coinciding with the rainy season. These wet conditions provide a suitable environment for the development of Fasciola eggs and hence infections of the intermediate host *Lymnaea natalensis*.

Human fasciolosis has been reported in many countries worldwide. In the last 25 years, infections were reported in 7071 persons in 51countries across several continents according to ***Esteban et al.,* (*1998*).** Serious underreporting of this zoonotic disease is suspected, caused by limitations of accurate diagnosis, and because human fasciolosis is not a notifiable disease **(*Mas-Coma et al., 2005*).**

Since tropical fasciolosis is a significant factor in limiting livestock production, the development of sustainable strategies for controlling *F.gigantica* is a priority. Control of fasciolosis has been shown to be very difficult. Despite all control efforts the parasite persists in most endemic areas and husbandry systems, including highly controlled conditions in

industrialized countries. Most control measures have been based on i) reducing infections in animals and humans by the use of anthelmintics; ii) combating the intermediate snail hosts; iii) reducing parasite transmission by management on the farm and by public health measures. Novel approaches for control include vaccination **(*Golden et al., 2010*),** breeding *Fasciola* resistant animals **(*Wiedosari and Copeman 1990*)** or using competition in snail intermediate hosts between *Fasciola* and other trematode species **(*Suhardono et al., 2006*).**

Triclabendazole has been the drug of choice for treatment of liver fluke infections in livestock for more than 20 years and it is also the only drug used for treatment of human fasciolosis **(*Fairweather, 2005*).** The drug has a high efficacy (approximately 99%) against both the mature adult worms in the bile duct and the migratory immature flukes **(*Fairweather and Boray, 1999*).** However, Triclabendazole resistant fluke populations have been reported in Ireland, the UK, Netherlands, Spain and Australia **(*Brennan et al., 2007*).** Although some flukicidal compounds such as Closantel **(*Coles et al., 2000*)** and Clorsulon **(*Coles and Stafford, 2001*)** retain their efficacy against Triclabendazole-resistant fluke population, novel fasciolicidal compounds or anti- *Fasciola* vaccines should be developed in the future. So the ultimate goal is a protective vaccine based on parasite antigens that stimulate the immune system and thus conferring an adequate level of protection.

The diagnosis of fasciolosis is generally carried out via coprological examination, and immunological technique like ELISA but such tests have many disadvantages. Pseudofasciolosis is the potential for misdiagnosis in such tests. Moreover, coprological diagnosis of fasciolosis is possible from 8-12 week post-infection (WPI) .Although ELISA can detect *F. hepatica* specific-antibodies as early as 2-4 week post-infection, thus providing the tool for early detection of the infection,

there is the possibility of cross-reactivity with other trematodes antibodies, leading to false positive results **(*Ievieux et al 1992; Hillyer, 1985; Hilyer and De Galanes, 1988*)**. In recent years, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot procedures have created a new era in immunodiagnosis which greatly reduced cross-reactions. These techniques were used as a verifying test in the field of parasitology **(*Sarimehmetoúlu, 2002*)**. Electrophoresis is a new methodological approach offers a new way not only to discover drugs and vaccines but also to study host–parasite interactions. Over the last two decades various studies to identify and characterized proteins of immunological significance have been carried out, especially the candidates for immunodiagnosis or vaccinations in parasitoses **(*Moxon et al., 2010 and Toledo et al., 2011*).**

Objectives of the Study:

Overall objective:

To study the protein profile and seroprevalence of *Fasciola gigantica* in ruminants in the Sudan.

Specific objectives:

1. To study the protein profile (antigenic structure) of *Fasciola gigantica* somatic and execretory–secretory products from sheep, goats and cattle.
2. To study the prevalence of Fasciolosis and Paramphistomosis in ovine and bovines in Rabbak (White Nile Province).
3. To assess the validity of the indirect ELISA for serological diagnosis of sheep and cattle Fasciolosis.
4. To test the cross reactivity of *F.gigantica* ES/SO and Paramphistomum ES antigens using indirect ELISA

CHAPTER TWO

CHAPTER TWO

2. Literature Review

2.1 Taxonomy and Classification:

Taxonomically *Fasciola* belongs to invertebrates and classification is presented as follows:

Kingdom	:	Animalia
Phylum	:	Platyhelminthes
Class	:	Trematoda
Order	:	Digenea
Family	:	Fasciolidea
Genus	:	*Fasciola*
Species	:	*Fasciola hepatica;* ***Linnaeus, 1758*** and *Fasciola gigantica;* ***Cobbold, 1856***

The adult mature and gravid fluke is flat with its body shaped like a leaf. The size range is 25 to 30 mm and 8 to 15 mm in length and width respectively, depending upon the species. The adult parasitizes the liver/or gallbladder of the final hosts **(*Despommier and Karapelou, 1987; Andrews and Dalton, 1999*).** The fluke has an elongated anterior end known as a cephalic cone in which has an oral and ventral sucker. The intestines are highly branched and present throughout the body. The male and female reproductive organs are present near the sucker in the centre of the body. The female reproductive tract is a dense ovary and is located just above the testes and is linked to a short convoluted uterus that opens into a genital pore above the ventral sucker. The vitellaria are highly dispersed and divided in the lateral and posterior region of the body. *F. gigantica* and *F. hepatica* parasites are very similar to each other, varying in length and width. In addition, the cephalic cone of *F. hepatica* is

shorter than *F. gigantica*. The shape of the eggs of the two flukes is also very similar **(*Soulsby, 1984*)** with the measurements of *F. hepatica* and *F. gigantica* being approximately 150µm x 90µm and 200µm x 100 µm, respectively **(*Dunn, 1978*).**

2.2: Epidemiology:

Fasciolosis is one of the most serious helminth diseases of the livestock in the world. It results in economic losses in livestock appearing in the form of mortalities from the acute disease in sheep and calves and decreased production and reproductive performance in the chronic form of the disease **(*Karib, 1962; Roseby, 1970; Haroun, 1975*).**

The disease is caused by two major species of Fasciola the most important of which are the *Fasciola hepatica* and *F. gigantica*. *F.hepatica* occurs in Europe, the Caribbean Islands, parts of North and South America , New Zeland, Australlia and the high lands of Africa and Asia **(*Over, 1982*).** *F.gigantica* is the most common cause of fasciolosis in domestic ruminants in tropical Africa, Asia and Middle East **(*Nwosu and Srivastarn, 1993; Diaw et al., 1994; Ndao et al., 1995*).**

F. hepatica is not a common liver fluke in Africa and it is only sporadically reported in Ethiopia, high lands of Kenya, Zaire, Egypt and South Africa. *F.gigantica* is the indigenous African liver fluke which is of wide occurrence throughout the continent. It occurs in both domestic and wild ruminants at both high and low altitudes from the north to the south of Africa. This large liver fluke is responsible for fasciolosis in cattle, sheep and goats in Africa and is the cause of considerable losses to livestock sector **(*Dinnik and Dinnik, 1959*).**

2.3 Life Cycle:

The life cycle of *Fasciola* spp (Figure 1) consists of a wide range of final host, consisting of both, domestic and wild mammals **(*Boray, 1969*).** The final host is mainly a vertebrate, including a diverse group of

herbivorous mammals, including cattle, buffalo, goat, sheep, horse, zebra, donkey and rabbit. Sometimes humans can also serve as an accidental host. Furthermore, infection has also been reported in birds **(*Vaughan, et al., 1997*)**.The life cycle of *F.hepatica* and *F.gigantica* is indirect involving an intermediate snail host belonging to the Family Lymnaeidae (Gastropods: Basommatophora) inhabiting water bodies **(*Mas-Coma et al., 2005*).** Eggs passed in the faeces of the definite host, develop and hatch releasing motile ciliated miracidia. The free swimming miracidia comes into contact with the intermediate snail host. It achieves entry into the snail by suctorial adhesion followed by cytolysis of the snail teguments **(*Dawes, 1959*).** After penetration into the snail, the miracidium shed its ciliated coat and develop asexually to sporocyst, rediae and finally cercarial stages. Cercariae swim freely in the water and become encysted on vegetation then develop into metacercariae, the infective stage. Herbivorous animals and human are usually infected by ingestion of aquatic plants (watercress, mint, spring water) that are contaminated with metacercariae **(*Markell and Voge, 1999; Rondelaud, et al., 2000*).** Several species of aquatic plants in Europe have been considered as an infection source for human fasciolosis **(*Mas-Coma et al., 1999*).** Furthermore, cercariae of *Fasciola* can float on water and encyst thus humans can become infected via metacercariae consumed with contaminated water **(*Chen and Mott, 1990*).** An experimental study by ***Taira et al.,* (*1997*)** suggested that humans consuming raw liver or semi-cooked liver dishes infected with juvenile flukes could easily be prone to infection. Research in Indonesia has established that infection with *F.gigantica* reaches a high prevalence in cattle and buffaloes in irrigated rice-producing areas where the snail intermediate host thrives **(*Copeman 1999*).** The common practice by farmers of using faeces from buffaloes and cattle as fertilizer in rice field, feeding the rice straw after harvest and

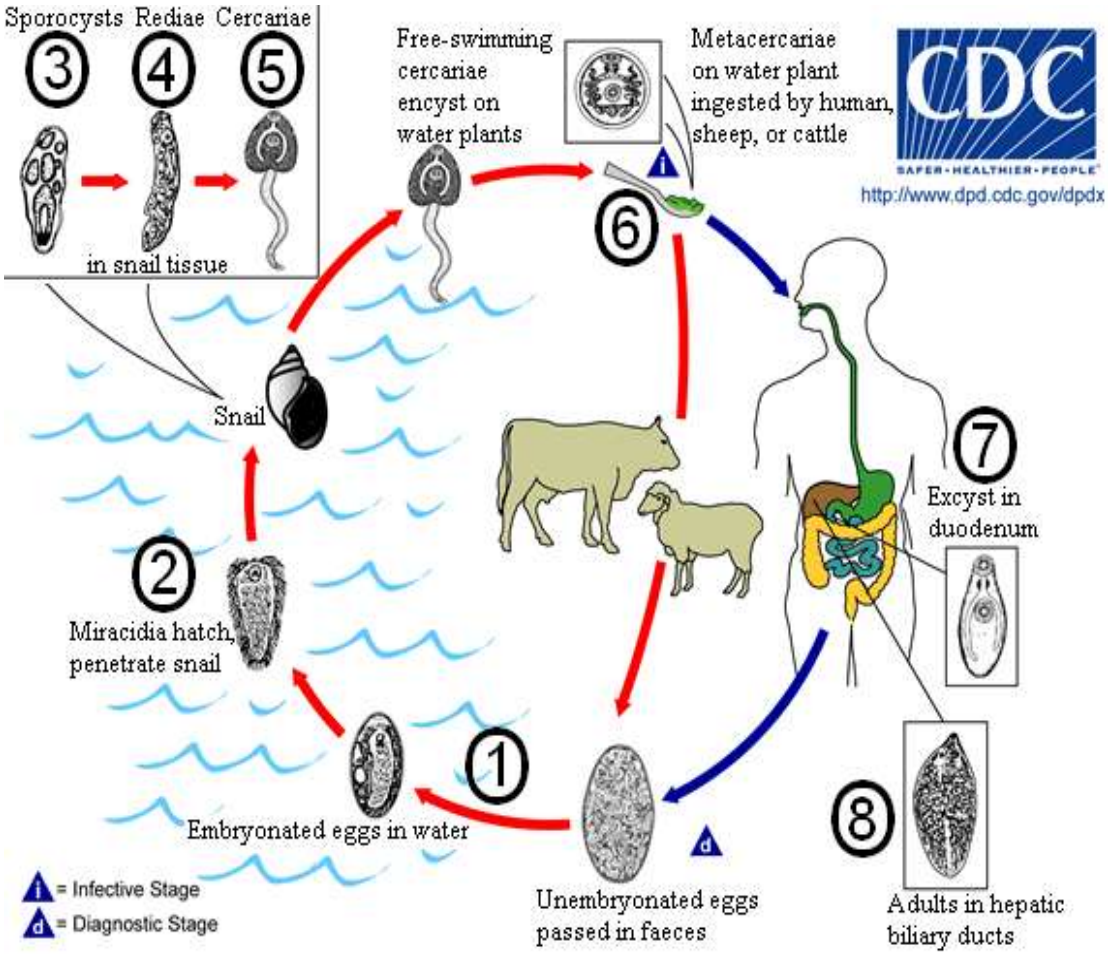

Figure 1.1: Lifecycle of *Fasciola*.

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allowing these animals access to newly harvested fields to graze promotes infection and facilitates the continuation of the life-cycle of *F.gigantica* **(*Suhardono et al., 2006* personal communication).**

2.3.1 The Definitive Hosts:

Throughout most of its range *F. gigantica* is of greatest importance as a parasite of cattle and buffalo although, regionally, infection may assume importance in goats, sheep and donkeys. ***Hammond and Sewell* (*1974*)** proposed that *F.gigantica* is better adapted to cattle than sheep in that it is more infective and lives longer. Wild herbivores are also susceptible; ***Hammond* (*1972*)** reported infection in 16 species in Africa. Laboratory animals are not readily infected with *F.gigantica* and there are conflicting reports regarding their susceptibility. Such conflicting reports may be the consequence of differences in susceptibility of the various strains of laboratory animals used and there may also be regional differences in infectivity of strains of *F. gigantica*.

***Hammond* (*1974*)** suggested that, due to inadequate diagnosis, human infections may be more common than indicated by the occasional case reports. A more recent review of human fasciolosis suggests that human disease results mainly from infection with *F.hepatica* with 2.4 million people infected and the further 180 million at risk **(*Anon., 1995*).** *F.gigantica* infections have been reported in Africa **(*Kyronseppa and Goldsmid, 1978*)**, Egypt **(*Ali et al., 1984*)**, the USSR **(*Sadykov, 1988*),** Germany **(*Schwacha et al., 1996*)** and Thailand **(*Kachintorn et al., 1988; Tesana et al., 1989*)**.

2.3.2 Snails as Intermediate Hosts:

The geographical distribution of *F. hepatica* and *F. gigantica* differ due to the different distributions of their intermediate snail hosts. *F. hepatica* is mainly found in temperate regions of Europe, America and Australia, where the lymnaeid amphibian snail *Galba truncatula* is the

most important intermediate host, being found mainly in mild and cold climatic zones **(*Mas-Coma and Bargues, 1997*)**. *F. gigantica* is present in tropical and sub-tropical regions of Africa and Asia, where *Lymnaea* spp. such as, *L. natalensis* and *L. rubiginosa* are among the main intermediate host species. However, some *Lymnaea* species (*Radix peragra*, *L. natalensis*, *Galba truncatuna*…) are suitable intermediate hosts for both *F. gigantica* and *F. hepatica.* As a result the distributions of these species overlap in a number of African and Asian countries **(*Graczyk and Fried, 1999; Mas-Coma et al., 2005*)**. ***Kendal* (*1954, 1965*)** regarded the race of the main intermediate hosts for *F.gigntica* in South, west and east Africa as *lymnaea natalensis* and in India, Bangladesh and Pakistan as *L. rufescens*. The two races merged, according to his accounts in Oman and lower Mesopotamia. He proposed the snail host in South East Asia as *L.rubiginosa*. A similar snail, *L. allula* is host to *F.gigantica* in Japan **(*Ueno et al., 1975*)** and Hawaii **(*Alicata, 1938*).**

The major role of *L.auricularia* as intermediate host for *F.gigantica*, proposed by ***Kendal* (*1954*)**, has not been challenged by subsequent authors. However, a number of other species of snail have also been shown to support the development of *F. gigantica* **(*Boray, 1965; Shahlapour., 1994*)**. Other Lymnaeids are *L. euphratic* in Iraq and *L. auricularia* in Oman. *L. cailliaudi* has been found responsible for transmission of both *F. hepatica* and *F. gigantica* **(*Farag et al., 1998*)**. In Australia *L. tomentosa* (host of *F. hepatica*) was shown to be receptive to miracidia of *F. gigantica* from East Africa, Malaysia and Indonesia.

***Dar et al.,* (*2002; 2003; 2004*)** reported that several snail species may contribute to the transmission of fasciolosis in Egypt among these are *L. truncatula* and *L. cailliaudi*, the main intermediate hosts in water bodies of the Nile Delta. However, *Radix natalensis* is the essential intermediate host for *F. gigantica* based on field and experimental studies, while

Galba truncatula was found naturally infected with *F. gigantica*. The detection of *F. gigantica*-like larval stages was detected in planorbids as *Biomphalaria alexandrina* in Egypt **(*Farag et al., 1993; Farag and El-Sayad, 1995; Farag et al., 1998; El-Shazly et al., 2002*).** Results based on the broad information acquired over many decades through studies indicate a clear preference of *F. hepatica* for Galba and of *F. gigantica* for Radix **(*Bargues et al., 2001; Bargues and Mas-Coma, 2005a*).**

2.3.3 Developmental Stages:

2.3.3.1 Development of the Eggs:

Eggs are shed by the adult mature flukes in the liver and bile ducts and pass into the intestine to be excreted in the faeces. The eggs consist of a fertilized ovum surrounded by a large number of yolk granules. They are yellowish brown in colour, oval in shape 130-150 um in length by 63-90um in width **(*Markell and Voge 1981; Soulsby, 1984*).** The eggs which are passed out in the faces onto pasture are undeveloped and undergo embryonation outside the host **(*Rowcliffe and Ollerenshaw, 1960*).** Several physic-chemical factors, especially temperature, humidity and oxygen tension, are known to influence embryonation. The development of *F.gigantica* eggs was found to be more variable than that reported for *F. hepatica.* It takes from 52-109 days at temperature between 10˚c and 22˚c **(*Dinnik and Dinnik 1959*).**

2.3.3.1.1. The Miracidium:

The miracidium is about 130 um in length, broad anteriorly and tapering posteriorly to a blunt end. The cuticle is ciliated, and there is an anterior papilliform protrusion and a pair of darkly staining eye spots visible near the anterior end of the body.

The time required for development of miracidia in eggs of *Fasciola gigantica* varies with temperature. It was 10-11 days at 37-38˚c, 21-24 days at 25˚c and 33 days at 17 -22˚c **(*Guralp et al., 1964*). *Grigoryan***

(*1958*) considered 24-26˚c and pH 6.5-7 optimal and found that, under such conditions, 70-80% of eggs would develop. He found that eggs did not survive at temperature higher than 43-44˚c and that desiccation also was rapidly fatal. ***Ollerenshaw and Smith*** **(*1969*)** considered that hatching of eggs doesn't occur if the temperature is below 10˚c. Eggs of *F.gigantica* don't all developed at the same rate so that, from the same batch, miracidia may hatch over a period up to 14 weeks, thus enhancing their opportunity to infect a snail **(*Guralp et al., 1964*).*Guralp et al.,* (*1964*)** also found that eggs were stimulated to hatch by exposure to sunlight or bright light. Once hatched from the egg the miracidium becomes active, immediately starting to swim at great speed(on average, 1mm s−1;***Wilson and Denison, 1970***) and survive in water for 18-26h **(*Asanji, 1988*).**

The miracidia respond to light, which bring them near the water's surface increasing their chances of encountering a potential intermediate host **(*Kalbe et al., 1997; Saladin, 1979*).** High temperatures dramatically reduce the miracidium life-span to as little as 6 hours at 25 °C **(*Smith and Grenfell, 1984*)**. However, a miracidium will only successful infect a snail if it locates and penetrates the snail within 3 hours of hatching **(*Kalbe et al., 1997*)**. After 7 hours at 18-24˚c, miracidial activity decreases and if the miracidium fails to penetrate a suitable snail host, it generally dies within 24 hours.

The ciliated free swimming miracidium comes into contact with the intermediate snail host. It achieves entry into the snail firstly through attachment by means of a saucer-shaped structure located at the anterior end of the miracidium, developed by an envagination of the epithelium followed by cytolysis of the snail tegument **(*Kendall,1965*).**The time the miracidium takes to penetrate the intermediate host is approximately 30 minutes. Once inside the snail, the miracidium shed its ciliated coat and

transform into a sporocyst containing up to six germinal balls **(*Dinnik and Dinnik, 1956 and 1959*)**.This then migrates to the snail's digestive gland and grows further.

2.3.3.2 Development in the Intermediate Host:

2.3.3.2.1. Sporocyst, Rediae and Cerariae:

The sporocyst is 70-100 um in length. Within the sporocyst, the germinal balls replicate to give rise to mother rediae, which are freed when the sporocyst ruptures. The mother rediae begin to feed in the snail tissues and about third week from the entrance of the miracidia, daughter rediae develop inside the mother rediae. The daughter rediae penetrate the digestive glands of the snail and give rise to a third generation of rediae between the 22^{nd} and 33^{rd} days from snail penetration. The third generation rediae begin to shed cercariae via the birth bore. The infected snails begin to shed cercariae through their mouth 36-40 days after infection **(*Dinnik and Dinnik, 1956*)** or 45 days after infection **(*Bitakaramire, 1968*).** It has been determined that thousands of cercariae can be produced from a single miracidium **(*Lapage, 1968*).** Cercarial shedding is initiated by the presence of fresh water **(*Kendall, 1965*)**.

Cercariae are tadpole-like with a discoidal body, long tail, oral sucker and ventral sucker in the centre of the body, similar to that seen in the adult fluke. Following shedding the cercariae swim very actively, they then cast off the tails and become encysted on vegetation to form the metacercariae, the infective stage. Here they remain until they are ingested by the final host. About two thirds cercariae attached to objects within 6.4 cm of the surface of water **(*Ueno and Yoshihara, 1974*).** The remainders don't attach but become" floating cysts" **(*Dreyfuss and Rondelaud, 1994*)**. The proportion of floating cysts is higher for *F.gigantica* than *F.hepatica* **(*Dreyfuss and Rondelaud, 1997*),** suggesting that such metacercariae may be more important as source of infection

with *F.gigantica* than they are for *F.hepatica*, when stock drink from habitats where cercariae are being released. Furthermore, the floating cysts may move with flow of water to be a source of infection at sites where the habitat is unsuitable for snails and therefore presumed safe from infection. This possibility was demonstrated by ***Suhardono et al.,* (*1999*)**, who successfully infected sheep with *F.gigantica* with the sediment of water flowing from rice field containing *L.rubiginosa* infected with *F.gigantica*. Cercariae can also be found floating freely in water **(*Esclaire et al., 1989*)** and thus the final host may be infected by drinking contaminated water **(*Bargues et al., 1996*).**

About 80% of cercariae are shed at night **(*Guralp et al., 1964; Da Costa et al., 1994*).** The total number of cercariae produced per snail is usually a few hundred but this varies from fewer than 100 to some thousands. ***Bitakaramine,* (*1968a*)** recovered a mean of 653 metacercariae of *F.gigantica* per snail from laboratory infection of *L.natalensis* but ***Grigoryan,* (*1958*)** reported that up to 2700 cercariae per snail may be produced and ***Guralp et al.,* (*1964*)** counted 7179 cercariae released from a snail over a period of 75 days.

Morphologically, the metacercaria is a round cyst, approximately 0.2 mm in diameter **(*Chen and Molt, 1990*)**. The structure of the cyst wall is complex. Essentially, it consists of an outer cyst and inner cyst. The outer cyst is composed of external layer of tanned protein and an underlying fibrous layer of mucoprotein. The inner cyst has a complex mucopolysaccharide layer subdivided into three, and an additional layer of laminated or keratinized protein. A region of keratinized layer is specialized to form the ventral plug, which is the point through which the fluke will hatch **(*Bennett and Threadgold, 1973*).** The outer cyst wall probably acts as a barrier against bacterial and fungal infections, and is also important for attachment to the substrate, normally grass **(*Dixon,***

***1965*)**. Strong adhesion to grass for long periods is important for the survival of metacerariae and the infection of the final host. The process of encysment takes approximately 24 hours, after which the metacercariae become infective **(*Chen and Mott, 1990*).**

The duration of survival of metacercariae is inversely related to temperature of storage and directly related to the degree of hydration.

2.3.3.3 Development in the Definitive Host:

The metacercariae are not fully infective just after encystations. At room temperature, they require at least 2-3days to become fully infective to definite host. In definite host the newly encysted metacercariae required at least 24 hrs to become infective **(*Boray, 1969*).** Once ingested by the definite host, metacercariae begin to exyst in the small intestine within approximately one hour after ingestion. Many factors concerned with exystation have been studied by ***Dawes and Huges* (*1964*).** They found that exystation induced by partial digestion of the cyst wall with acid pepsin, followed by the action of trypsin and bile salts. Following this they penetrate through the intestinal mucosa into the peritoneal cavity where they browse on the available tissue at about two hours after ingestion. It is thought that the process of penetration of the intestinal wall is aided by the secretions produced in the fluke′s gut **(*Bennet and Threadgold 1973*).** Within 24 hrs of infection the majority of the immature flukes occur in the abdominal cavity and by four to six days after infection the majority have penetrated the liver capsule and are found migrating in the liver parenchyma. **(*Soulsby, 1984; Andrews, 1999*)**.

After penetrating the liver capsule, the immature fluke burrows through the liver tissue, feeding on available tissue and causing extensive haemorrhage and fibrosis before eventually reaching the bile ducts. This period of migration through the liver parenchyma takes five to six weeks

in cattle **(*Dixon, 1965*)** and most of them reach the bile ducts by eight weeks. They then complete development to sexually mature adults, begin to produce eggs and the cycle repeats.

Reports of the duration of the prepatent period vary, usually from about 12 to 16 weeks **(*Grigoryan, 1958; Guralp et al., 1964; Sewell, 1966; Prasitirat et al., 1996*)**. Reasons for this variation may include the sensitivity of the method used to detect eggs in faeces, the number of flukes in the infecting dose, breed of host and strain of *F.gigantica*. The output of eggs rises for the first 4 to 12 weeks after eggs appear in the faeces **(*Sewell, 1966; Prasitirat et al., 1996*)** then fall to low levels, reducing the value of faecal egg counts as an indicator of level of infection **(*Sewell, 1966*).** With the same infecting dose faecal egg counts are up to 80% lower in buffalo than in cattle **(*Prasitirat et al., 1996; Wiedosari and Copeman, 1990*)**. Counts also vary between breed of cattle, in Bali cattle about 45% less than those in Ongoles with the same exposure to infection **(*Wiedosari and Copeman, 1990*)** but some survive at least 3 to 4 years **(*Alicata and Swanson, 1941; Hammond and Sewell, 1975*)**.

2.4 Identification of Fasciola spp.:

F. hepatica and *F. gigantica* are the main species belonging to the genus *Fasciola* (Platyhelminths: Digenea: Fasciolidae), causing fasciolosis in livestock and humans. These parasites occur in a wide range of definitive hosts and have a widespread geographical. Because of their public health and economic significance, an accurate identification of *Fasciola* species is necessary for clinical management of infection, and for epidemiological surveys **(*Ai, 2011*).** In order to understand the taxonomic relationships and the history of evolution (phylogeny) of *Fasciola* spp., this is an overview of the studies on identification of

Fasciola spp., based on morphological features, and emphasizes on the characterization of genetic variation.

2.4.1. Morphological and Merphometrical Identification:

The traditional identification methods are based on discrimination of morphology between species: *F. hepatica* and *F. gigantica*, described by ***Dunn* (*1978*) and *Soulsby* (*1984*).** Due to species overlap it is difficult to identify the prevalent species accountable for infection in these areas, as the distribution patterns of both *Fasciola spp* overlap **(*Lotfy and Hillyer, 2003*)**. This may be a reason that infections diagnosed due to *F. gigantica* are less compared to *F. hepatica* **(*Marcilla et al., 2002*).** The three species of *Fasciola (F. hepatica, F. gigantica,* with intermediate forms) are morphometrically very similar to each **(*Srimuzipo et al., 2000; Terasaki et al., 2000*)**. Earlier morphometric studies on adult flukes or their eggs were used to identify the different species. These were isolated from various domesticated animal hosts **(*Bergeon and Laurent, 1970*).** A more recent technical approach includes a computer image analysis system (CIAS) that is based on the standardized measurements of distances between organs of flukes. ***Valero et al.*, (*2005*)** and ***Periago et al.*, (2006)** used the computer image analysis system (CIAS) technique to measure the variations in morphometry between different populations of *Fasciola*. Previously this technique was performed on morphometry of both pure species of *Fasciola* adults in cattle from different regions **(*Valero et al., 2001a, b; Valero et al., 2002*)**. In Egypt (African region), ***Periago et al.*, (*2006*)** used the CIAS technique to investigate inter and intra specific morphological diversity by analyzing specific morphological characteristics. ***Ashrafi et al.*, (*2006*)** concluded that among various morphometrical parameters like VS-P, BL/BW, pharynx and oral sucker size can serve as a valuable taxonomic feature for species differentiation

by using CIAS technique. Moreover, ***Lotfy et al.,* (*2002*)** reported that fluke length, testes length and length of the area behind the testes were the most striking dimensions to discriminate the two species. Based on the morphometric characteristics obtained by CIAS and on the comparison with standard populations of *F. hepatica* and *F. gigantica* from other geographical areas, flukes with an "intermediate form" were identified **(*Ashrafi et al., 2006; Periago et al., 2006; Soliman, 2008*)**. A wide range of morphological types of *Fasciola* spp. has also been detected in many countries of the Far East such as Japan, Taiwan, Korea, China and Vietnam **(*Ashrafi et al., 2006; Le et al., 2008; Periago et al., 2008*)**.

Morphologic, morphoanatomic and morphometric information can certainly differentiate between species; however these approaches are inapplicable when the intermediate form is involved. Therefore, in such cases the soluble protein isoelectric focusing (IEF) method has been considered as a useful tool for differentiating two species **(*Lee and Zimmerman, 1992; Lotfy et al., 2002*)**. In endemic areas, where there was overlapping of the two *Fasciola* species, the molecular markers provided a better tool for species differentiation **(*Le et al., 2008*).** Specific differentiation of various species can only be made by a morphological study of adult flukes and by using molecular tools **(*Periago, 2008*).** In general fasciolids can be differentiated by morphometric techniques **(*Ashrafi et al., 2006*)**, but flukes with intermediate characters can cause misunderstanding **(*Itagaki et al., 2005a, b*)**. Especially in local areas, the existence of two species may result in hybrids through crossbreeding **(*Lotfy and Hillyer, 2003*)**. This phenomenon has lead to necessitate the use of molecular methods to recognize the existing species **(*Semyenova et al., 2003, 2005, 2006; Lin et al., 2007; Le et al., 2008*).**

2.4.2. Molecular Identification:

PCR technology and DNA sequencing techniques facilitate identification of species, clarification of strains and genetic populations. The selected gene or sequence must be common, highly conserved within, and sufficiently divergent between taxa. Ideally, the variable regions should have adjacent conserved regions so that "universal" oligonucleotide primers may be chosen **(*Kocher et al., 1989*).** The targeted sequence or gene must be long enough to provide sufficient numbers of informative characters for phylogenetic analysis. To date, thousands of complete or partially sequenced metazoan mitochondrial (mt) genomes and about 90,000 internal transcribed spacer-2 (ITS-2) sequences are available in GenBank (NCBI). Data from mitochondrial genomes and ITS genes have been extensively used in studies on animal evolution, phylogeny, biogeography, systematics, population genetics and related fields **(*Le et al., 2000*).**Several molecular methods such as, PCR, DNA sequencing, restriction fragment length polymorphism (RFLP) analysis and single-strand conformation polymorphism (PCR-SSCP) have been applied for the differentiation of the two *Fasciola* species **(*Le et al., 2002*).**

The PCR-RFLP assay, a rapid and simple test, has been used for identification of *Fasciola* spp. by ***Marcilla et al.,* (*2002*).** By using the common restriction enzymes *Ava*II and *Dra*II, the sequences of 618-bp in the 28S rRNA gene from liver fluke populations of South America, Europe and Africa could be distinguished; no intraspecific variations were found with this technique because there were a few nucleotide differences between *Fasciola* species. In Iran, RFLP patterns of *Fasciola hepatica* and *Fasciola gigantica* from Far provinces based on 263 and 356 bp fragments of 18s rDNA using DraI and BfrI restriction enzymes showed that BfrI restriction enzyme was obtained similar bands profile of

F.hepatica and *F.gigantica* whereas, restriction enzyme DraI can be created to differentiate between two species of *Fasciola* **(*Karimi, 2008*).**

With the results of sequencing of *nad1* and *ITS2* marker genes, the "Japanese form" of *Fasciola* was found: i) There was an 8.3% nucleotides difference in *nad1* sequences between a triploid (3n) Japanese *Fasciola* isolate and a *F. hepatica* isolate from Uruguay. Based on these *nad1* sequences the Japanese triploid worms could be categorized as *F. gigantica;* ii) however, there was homology of nucleotides at 8 positions in *ITS2* sequences between these isolates. Consequently, it appeared to be difficult to simply categorize this Japanese *Fasciola* spp. as *F. gigantica* **(*Itagaki and Tsutsumi 1998 b*).** These authors suggested that the maternal ancestor of the Japanese worms is *F. gigantica*, with mitochondrial DNA inherited maternally. On the other hand, judging from the *ITS2* sequence of nuclear DNA, the worms were also genetically similar to *F. hepatica*. It was concluded that the Japanese triploid worms might be a hybrid between *F. hepatica* and *F. gigantica* **(*Itagaki and Tsutsumi 1998b; Agatsuma et al., 2000*)**. Similar findings were reported in other Asian and non-Asian countries: Korea **(*Itagaki et al, 2005*);** China **(*Huang et al., 2003; Lin et al., 2007*);** Vietnam **(*Le et al., 2008; Itagaki et al., 2009); Russia (Morozova et al., 2003*)** and Niger **(*Ali et al., 2008*).** From these studies it is suggested that the intermediate forms may have originated in interspecific cross-hybridization between paternal *F. hepatica* and maternal *F. gigantica*, resulting in an "intermediate *Fasciola*".

Several studies have provided genetic evidence for the existence of "intermediate *Fasciola*" when characterizing *ITS1* and *ITS2* of rDNA in *Fasciola* isolates collected in various hosts (cattle, sheep, goat, including human), and geographical locations (i.e., Japan, Korea, China, Vietnam) **(*Huang et al., 2003; Lin et al., 2007; Le et al., 2008; Itagaki et al.,***

***2009; Peng et al., 2009*).** These findings strongly suggest that the "intermediate *Fasciola*" in Japan, Korea, China and Vietnam originated from the same ancestors and has recently spread throughout those countries because of movement of infected animals without geographical barriers in this area **(*Itagaki et al., 2005; Le et al., 2008; Peng et al., 2009*)**. Also Phylogeny links the origin of the *Fasciola* in Potohar (Pakistan) with *Fasciola* in China, Vietnam, Iran and Egypt **(*Mufti thesis 2011*)**. The *Fasciola* in these countries are closely related and serve as a point of origin. Moreover, it is not only in the Asian region but also in different host species and geographical localities in West Africa that the "intermediate *Fasciola*" was found together with *F. hepatica* and *F. gigantica*, as shown by ITS1 and ITS2 sequences of rDNA **(*Ali et al., 2008*)**. Due to the observed limited variation of nucleotides in the marker genes from mtDNA and rDNA **(*Itagaki et al., 2005; Peng et al., 2009*)** between samples collected in different regions of the world, it is suggested that a very recent geographical diffusion of *F. hepatica* from Europe into the other continents could be inferred **(*Mas-Coma et al., 2003, 2009; Lotfy et al., 2008*)**.

***Lotfy et al.*, (*2008*)** carried out a study on molecular phylogenetic of fasciolids based on their origin, diversity and geographical location by using the 28S, region of nuclear rDNA, and mitochondrial nicotinamide dehydrogenase subunit 1 (*nad*1) sequences of different fasciolid species. The study concluded that liver flukes migrated from Africa to Eurasia and then back to Africa, switching over their intermediate host from a planorbid to a lymnaeid. There was also an addition of another definitive host (human) and a change in habitat, intestinal to hepatic within mammals.

Finally they concluded that a wide range of morphological types of *Fasciola* spp. **(*Ashrafi et al., 2006; Periago et al., 2006*)** has been

observed. At the extremes of this morphological range, some resemble *F. hepatica*, whereas others resemble *F. gigantica*, with intermediate forms in between. Several reports indicated that there are few variations in nucleotides in some marker genes of the mtDNA and more variations in the rDNA of the Asian fluke population **(*Itagaki et al., 2005; Le et al., 2008; Peng et al., 2009*)**. These observations suggest that in the intermediate forms the mitochondrial genome may be inherited from *F. gigantica* and the nuclear genome from *F. hepatica*. However, the classification of new forms of the "intermediate *Fasciola*", and the production of interspecific hybridization between *F. gigantica* and *F. hepatica*, need to be further studied by using more genetic markers, more samples from different countries as well as from more different hosts.

2.4.2.1. SSR Marker:

Microsatellite markers, also known as simple sequence repeats (SSR's), these markers are most useful and commonly used amongst the different classes of molecular markers. These are DNA sequences repeatedly present in the genome of Eukaryotes **(*Philips et al., 2001*)** as well as Prokaryotes **(*Varshney, 2004*)** and in bacterial genomes **(*Gur-Arie et al., 2000*)**. According to ***Bruford et al.,* (*1996*)** and ***Turnpenny and Ellard* (*2005*)** SSR's are short repetitive DNA sequences of 1-6 base pairs that are distributed through the whole genome and are highly polymorphic and flanked by highly conserved sequences that varies in species and chromosomes, both in non-coding DNA and protein-encoding DNA **(*Toth et al.,2000*). *Metzgar et al.,* (*2000*)** suggest that SSR's are present in large numbers in non-coding regions in eukaryotic organisms. ***Gupta and Varshney* (*2000*)** suggest that frequency of microsatellites is higher in transcribed regions, especially in untranslated regions (UTR's) Yeast and vertebrate genomes possess a large number of these sequences **(*Hamada et al., 1982; Tautz and Renz, 1984*).**

***Chistiakov et al.,* (*2006*)** hybridized and reported different microsatellite sequences from a variety of organisms. Earlier repetitive DNA was termed as "junk" DNA due to its function being unknown.

SSR-based markers have been commonly used to investigate diversity in a species and hence make it an important research tool **(*Varshney, 2002; Rudd, 2003*)**. These markers are used as tools for techniques like genotyping, genetic mapping, medical genetics, forensics, positional cloning of genes, phylogeny, evolutionary biology and in oncology research. Utility of microsatellites as molecular markers in genome characterization and mapping is due to their small size 92-6 base pairs and they can be generated by amplifying and can be analyzed by PCR, whereas electrophoresis can be used for easy assessment. Developing SSR's is actually a laborious and time consuming process and steps in development limits their use in diversity evaluation. There are two general strategies for establishing SSR markers which include a search in the available data bases or their construction from the genome and establishing genomic or other libraries. Microsatellites applications are most practical in those species which possess a low level of genetic variation among interbreeding populations **(*Temnykh et al., 2000; 2001*).** ***Meuneir et al.,* (*2001; 2004*)** suggested that an important aspect of fasciolosis epidemiology is to consider the colonization of existing snail species and genetic variability in the presence of high environmental parasitism pressure. Their study was to compare the genetic diversity in snail samples from Bolivian Altiplano with samples from France, Morocco, Spain and Portugal. Isolation and identification of markers was done by ***Trouvé et al.,* (*2000*),** in which several loci of snails were studied with some variation present. ***Hurtrez-Bousses et al.,* (*2001; 2004*)** isolated six microsatellite markers from 52 sample of *F. hepatica* from the Bolivian Altiplano, an area with a high fasciolosis rate. The study

concluded that five microsatellite loci were polymorphic supporting the Hardy-Weinberg law of random mating. The considerable variability present proposes an interbreeding which is favorable for diversity. Their results showed no genetic differences existed in host species (sheep, cattle and pig). It was also suggested that species diversity in the liver fluke should be considered while developing treatments and vaccines.

2.4.3. Cytogenetic Identification:

The number and morphology of metaphase chromosomes have long been used to characterize species. Phylogenetic studies have mainly used information regarding chromosome numbers and morphology rather than fine details of chromosome organization, and hence have been applicable only for high-level comparisons **(*Session, 1990*).** The difference in size of metacentrics and subtelocentrics, as well as the number of chromosomes were firstly described in Japanese flukes by ***Sakaguchi* (*1980*).** Further study on karyotyping demonstrated differences in chromosome numbers of diploid (2n), triploid (3n) and mixoploid (2n/3n) specimens in populations of *F. gigantica* and *F. hepatica* from Japan, Korea **(*Terasaki et al., 1998*),** Vietnam **(*Itagaki et al., 2009*),** China **(*Yin and Ye 1990*),** Britain and Ireland **(*Fletcher et al., 2004*).** In these studies abnormal spermatogenesis was shown in triploid forms where the number of spermatozoa was smaller when compared to diploid forms. Different morphological forms of flukes appear to be related to the presence of triploids and diploids; triploids tend to be larger. Aspermic diploid and triploid forms have univalent chromosomes in primary oocytes, suggesting the potential of parthenogenesis **(*Terasaki et al., 2000*).** Diploid (2n), triploid (3n) and mixoploid (2n/3n) were found in both *F. hepatica* and *F. gigantica* **(*Fletcher et al., 2004; Itagaki et al., 2009*).**

These forms could be related to selective advantages in their reproduction and may not be of importance for the identification of *Fasciola* species.

2.4.4. Electrophoric Identification:

The availability of complete genome sequences for a large number of parasitic organisms has opened the door for large-scale proteomic studies to dissect both protein expression/regulation and function. Electrophoresis is one of the innovative tools to exploit proteome - the genome operating system by which the cells of an organism react to environmental signals **(*Anderson and Anderson, 1996*).** The techniques include the development of activity-based probes and activity-based protein profiling methods to screen for pharmacological tools to perturb basic biological processes. The standard method for quantitative proteome analysis combines protein separation by high resolution (isoelectric focusing, SDS-PAGE) two-dimensional gel electrophoresis (2DE) with mass spectrometric (MS) or tandem MS (MSyMS) identification of selected protein spots. Important technical advances related to 2DE and protein MS have increased sensitivity, reproducibility, and throughput of proteome analysis while creating an integrated technology. By using 2DE with extended pH range and high-sensitivity protein identification by electrospray ionization and MSyMS, **(*Steven, 2000*)** evaluated the potential of the 2DE-MS strategy to serve as the technology base for comprehensive and quantitative proteome analysis. Two dimensional Electrophoresis (2DE) form the first generation proteome tool as host proteome responses such as post-translational modifications of host proteins (phosphorylation, glycolysylation, acetylation and methylation) in reaction to parasite invasion can be detected and identified **(*Patton, 2000*).**

The variation in electrophoretic patterns between two economically important species of *Fasciola* is important tools for identification of these species and also for vaccine designing. Disease biomarker discovery is generally carried out using two dimensional polyacrylamide gel Electrophoresis (2D-PAGE) to compare and identify differences in the protein expression patterns of two parasites. After 2D-PAGE fractionation and staining, the protein(s) of interest are removed, proteolytically or chemically digested and identified by mass spectrometry (MS). Although 2DPAGE separation provides excellent resolution, the need for protein staining and the subsequent sample handling limits the sensitivity of the overall approach. Protein profiling is expected to discover unexpected targets for drug design by determining the function of thousands of unidentified proteins still likely to be found in the genome of *Fasciola hepatica* and *Fasciola gigantica*. Electrophoretic Protein profiling is expected to multiply the number of known drug targets 100-fold. This will encourage the pharmaceutical industry to develop new drugs against fascioliasis. It will be also an indispensable tool for designing the species specific vaccine and for determination of molecular taxonomy of two parasites on more scientific grounds.

2.4.4.1. Electrophoretic Pattern as a Tool for Refining the Taxonomic Status of Fasciola spps:

To discriminate the two principle species of fasciola and their isolates and varients of different areas is important for number of areas of research, particularly in defining taxonomy and monitoring transmission in epidemiological investigation. Much of the current knowledge of

Fasciola spp. taxonomy has stemmed from numerous observational and morphological studies. However, conventional methods of detection and differentiation of *Fasciola* do not accurately reflect the full diversity of *Fasciola* spp. Moreover, the identification of fasciola spp. by morphological distinction is quite unreliable **(*Ashrafi et al., 2006*).** A recent study investigated the extent of genetic variability among *Fasiola* collected from different host spp. and geographical localities **(*Lin et al, 2011*).**

Comprehensive protein characterization by electrophoresis using more variable markers along with proteomic analysis of *Fasciola* species can be used to refine the taxonomic status of the "intermediate*Fasciola*" and to assess its potential as a zoonotic agent. Nevertheless, molecular genetics studies over the past two decades have added significantly to our understanding of *Fasciola* taxonomy, genetics, and contributed to the development of advanced approaches for the accurate identification and differentiation of *Fasciola* spp. Importantly, these molecular methods have facilitated the identification of the hybrid "intermediate *Fasciola*". However, presently there is no molecular diagnostic method. Somatic and E\S antigens of two *spps*. have been contrasted using SDS PAGE, which provides the baseline to distinguish between two on electrophoretic basis. Differences between *F. hepatica* and *F. gigantic* somatic proteins have been noticed. *F. gigantica* has 11 major protein bands with molecular weights of 18, 22, 24, 33, 36, 42, 46, 57, 60, 62 and 68 kDa, whereas *F. hepatica* has proteins characterized by 8 distinct bands with molecular weights of 18, 22, 24, 33, 36, 42, 46 and 62 kDa. **(*Meshgi et al., 2008*).**

2.4.4.2. Electrophoresis as a Tool for Reconstruction of Phylogeny of *Fasciola* spps:

The origins, patterns of diversification, and biogeography of fasciolids

are all poorly known. Molecular phylogenetic studies will help us to better understand the origins, radiation, evolution, and patterns of host use of these important trematodes **(*Lotfy et al., 2008*).** Phylogenetic tree of Fasciola enzymes have been constructed by various researchers. The phylogenetic data (using cathepsin L and GST) also suggest that the *F. gigantica* and *F. hepatica* species separated approximately 19 million years ago, around the time that the ancestors of modern-day pecoran lineages diverged. These observations are consistent with both co-adaptation and co-speciation of the parasitic genes with the parasite's host. **(*Irving et al., 2003*).** Recent phylogenetic, biochemical and structural studies indicate that trematode cathepsins exhibit overlapping but distinct substrate specificities due to divergence within the protease active site. The developmentally regulated expression of these proteases correlates with the passage of parasites through host tissues and their encounters with different host macromolecules **(*Stack et al., 2011*).**

The analysis and characterizing the profile of cathepsin L proteases secreted by adult *F. hepatica* by two-dimensional gel electrophoresis (2-DE) and MS is used to determine the relative importance of the various cathepsin L groups to parasite virulence and adaptation. A phylogenetic analysis of 24 *F. hepatica* and eight *F.gigantica* full-length sequences revealed that these separated into five well supported clades that arose by a series of gene duplications The two initial gene duplications separated the cathepsins isolated from the infective newly excysted juvenile parasites (Clade 3, FhCL3 and Clade 4, FhCL4) from three clades expressed in the adult worm stage (clades FhCL1, -2, and -5). Following this, gene duplicationled to the separation of the adult clades FhCL1 andFhCL5 from clade FhCL2. The phylogenetic tree also showed that the *Fasciola* clade FhCL1 has undergone the greatest expansion and is

represented by three distinct subclades: FhCL1A, FhCL1B, and FhCL1C. It is noteworthy that all clades contain sequences from both *F. hepatica* and *F. gigantica*. However, subclades FhCL1A and FhCL1B are composed exclusively of *F. hepatica* sequences, whereas clade FhCL1C contains only one *F. hepatica* cathepsin L. This phylogenetic analysis, therefore, indicates that the early duplication events in the cathepsin L gene family occurred before the speciation of the *F.hepatica* and *F. gigantica* fasciolids and that expansion of subclades FhCL1A/FhCL1B and FhCL1C occurred after these gregation of these two species. ***Irving et al., (2003)*** made a similar observation and suggested that divergence of the FhCL1 clade reflected adaptation of the "temperate" *F.hepatica* and the "tropical" *F. gigantic*a to different host species **(*Robinson et al., 2008b*)**. The sample preparation of cathepsin L was done by 2D Electrophoresis and thus depicts its indispensable role for determining phylogeny of fasciolosis.

2.5 Antigenicity of Fasciola spp.:

2.5.1 Excretory/Secretory Material (ES):

The ES of many digenetic trematodes play vital roles in host-parasite interactions including digestion, invasion and immune evasion. Fasciola ES is composed of products secreted into the gut and expelled via the mouth, material excreted through the median pore, and components of the tegument which are actively shed. The composition and role of Fasciola ES is varied, with different life stages secreting distinct sets of proteins **(*Carmona et al., 1992; McGinty et al., 1993; Tkalcevic et al., 1995*).** ES is also shown to vary among flukes developing in different hosts **(*Lee et al., 1992a*).** At least 60 prominent ES proteins have been found, with a high number and abundance of protective enzymes, pointing to the central role of ES protecting Fasciola from host immune responses **(*Jefferies et al., 2001*).** Such proteins include oxidant scavenger enzymes, glutathione

S-transferase (GST) and fatty acid binding proteins (FABPs) **(*Piedrafita et al., 2000; Jefferies et al., 2001; Salazar- Calderon et al., 2003*).** Immune evasion and modulation is also assisted by ES **(*Jefferies et al., 2001*).** ES has been shown to play a role in modulating host immunity towards a Th2 response through induction of IL-4 and IL-10 cytokine production **(*Brady et al., 1999; Cervi et al., 2001; O'Neill et al., 2001; reviewed in Mulcahy et al., 1999; Piedrafita et al., 2004*).** This can facilitate the chronic disease state. It is also capable of digesting host immunoglobulin, aiding evasion of antibody-dependant cell cytotoxicity, as well as causing downstream release of cytokines, modulating the immune response **(*Berasain et al., 2000*).** It has been shown to kill lymphocytes in vitro **(*Goose, 1978*),** decreases nitrite production and the phagocytic ability of rat peritoneal cells **(*Cervi et al., 1996; Masih et al., 1996; Cervi et al., 1998*)**, and suppresses delayed-type hypersensitivity **(*Cervi et al., 1996*).** ES can also modulate lymphocyte proliferation **(*Cervi et al., 1998; Prowse et al., 2002*),** reduce CD4+ surface expression **(*Prowse et al., 2002*),** prevent peritoneal inflammatory cells killing NEJ in vitro **(*Goose, 1978*)** and may prevent antibody-mediated eosinophil attachment to juvenile flukes **(*Carmona et al., 1992; Carmona et al., 1993*).** Secreted material from the tegument may also modulate the immune response towards Th2 by activating mast cells **(*Trudgett et al., 2000; Trudgett et al., 2003*)**. Further, a Kunitz-type molecule isolated from *F. hepatica* has been proposed to play a role in immune evasion **(*Bozas et al., 1995*),** similar to that performed by protease inhibitors in other parasite species **(*Leid et al., 1987; Ghendler et al., 1994*).**

Adult Fasciola residing in the bile duct are primarily blood-feeders, though they also ingest hyper-plastic bile duct epithelium **(*Fairweather et al., 1999*).** Fasciola absorb nutrients through the tegument and intestinal epithelium of the gut **(*Tielens, 1999*).** Digestion is mainly an extracellular

process that is completed intracellular. A number of potential digestive enzymes have been recognised in ES that can degrade a large range of host substrates such as collagen, haemoglobin, albumin and immunoglobulin **(*Acosta et al., 1998; Piacenza et al., 1999; Berasain et al., 2000*)**.

Enzymes recovered from regurgitant are expected to play a role in feeding and nutrition, and the variety of enzymes present may reflect the range of food available or alternatively, different suites of enzymes may be expressed in different hosts. Many of the proteolytic properties associated with ES have been attributed to cysteine proteases, specifically cathepsin L-like and B-like proteases, with cathepsin L-like proteases accounting for a large proportion of the ES from adult fluke **(*Jefferies et al., 2001; Morphew et al., 2007; Robinson et al., 2008*).** Work on other helminth parasites suggest that proteases in ES from these parasites are involved in migration **(*Na et al., 2006; Williamson et al., 2006*),** and it has been proposed that several Fasciola ES enzymes, including cysteine proteases are involved in tissue migration **(*Law et al., 2003; Alcala-Canto et al., 2005*)**. Many ES products are immunogenic and can be used in diagnosis. A monoclonal antibody directed against ES and used for diagnosis in human fasciolosis **(*Espino et al., 1990; Espino and Finlay, 1994*)** also confers passive protection to mice against *F. hepatica* **(*Marcet et al., 2002*),** while several ES products have been characterised for use as vaccines or proposed drug targets **(*Wijffels et al., 1994; Dalton et al., 1996; Hawthorne et al., 2000; Dalton and Mulcahy, 2001*).**

SDS-PAGE analysis (12 % resolving gel) of total E/S antigen of *F.gigantica* revealed 7 polypeptide bands at 23, 25, 28, 43, 47, 52 and 66 kDa molecular weights**(*Latchumikanthan et al., 2012*).** On the contrary, four proteins bands of size 17, 21, 57 and 69 kDa were observed for E/S antigen of *F. gigantica* in 12.5 % SDS-PAGE by **(*Goubadia and***

***Fagbemi (1997*)**. ***El Ridi et al., (2007*)** also observed prominent bands at 62-60, 40, 30 and 28 kDa for excretory/ secretory products from *F. gigantica* by 12 % SDS-PAGE.

E/S antigen from *F. gigantica* by 75 % alcoholic fractionation revealed 3 protein bands at 28, 43 and 47 kDa. A single band at 28 kDa size was detected in anion exchange chromatography purified fraction (peak fractions 14, 15 and 16) ***Latchumikanthan et al., (2012),*** which is similar to the findings of ***Fagbemi and Hillyer* (*1992*)** who observed molecular weight between 20 to 100 kDa in SDS-PAGE with peak proteolytic fraction obtained from adult *F. gigantica* revealed 28.3 kDa protein under reducing and non-reducing SDS-PAGE. ***Sriveny et al., (2006*)** observed doublet 27- 29 kDa cathepsin L cysteine proteinase antigen from *F. gigantica* in 15 % SDS-PAGE on purification with anion exchange chromatography and ***Dixit et al., (2003*)** characterized 28 kDa cysteine proteinase from bubalian liver flukes using 15 % SDS-PAGE. Purification of *F. gigantica* functional antigens by chromatography method is expected to remove host components, which if present are liable to cross react with the conjugate and elicit false results in ELISA and Dot-ELISA. ***Fagbemi et al., (1997*)** reported that chromatography methods could be used as very effective tools for isolation of candidate diagnostic molecule from the parasites.

In recent years, Western blotting has greatly decreased the risk of cross reactions in studies carried out in humans and animals with fasciolosis **(*Rivera-Marrero et al., 1988; Ruiz-Navrrete et al., 1993*)**. ***Ruiz- Navarrete et al., (1993*)** studied the immune response of sheep to somatic components and E/S products of adult *F. hepatica*, and they determined that the bands of 20-23 kDa obtained from somatic antigens and 23-27 kDa obtained from E/S antigens could be used for diagnosis. ***Hillyer and De Galanes* (1991)** obtained sera from human patients,

calves, sheep and rabbits infected with *F. hepatica* and tested the enzyme-linked immunoelectrotransfer blot (Western blot) techniques with *F. hepatica* E/S antigens in order to evaluate their immunodiagnostic potential. They reported that serum samples from humans, rabbits, cattle, and sheep with fascioliosis recognized 2 antigenic polypeptides of 17 and 63 kDa in the form of sharp bands. ***Rivera-Marrero et al.,* (*1988*)** used Western blotting and reported that the bands of 25-30 kDa in E/S antigens were specific for acute and chronic fasciolosis in rabbits, cows and sheep. ***Fredes et al.,* (*1997*)** analyzed the antigenic components of E/S products of adult *F. hepatica* by SDS-PAGE followed by Western blotting. SDS-PAGE and Western blotting results using serum samples from infected sheep, pigs and horses showed that the 400, 150, 29 and <29 kDa fractions contained polypeptides, specifically recognized only by infected animals. When these fractions were evaluated with serum samples from sheep, horses and pigs by means of ELISA the most efficient fraction was the 29 kDa, exhibiting a mean sensitivity and specificity of 94.5% and 93.5%, respectively. They concluded that the 29 kDa fraction has the potential for the development of a specific ELISA test for the mass screening of fasciolosis. ***Sampaio-Silva et al.,* (*1996*)** reported that 25-27 kDa components were recognized by all 20 fasciolosis sera from humans using E/S products of adult worms of *F. hepatica*. ***Guobadia and Fagbemi* (*1995*)** found that 17, 21, 57 and 69 kDa protein bands were specific for *F. gigantica* infection in sheep. ES and crude antigens obtained from *F.hepatica* in sheep were separated by SDS-PAGE, seven protein bands and twenty three protein bands were detected between 6.5 and 205kDa, repectively **(*Gönen et al.,2004*).** The specific protein bands for *F. hepatica* infection were 33, 39.5 and 42 kDa in E/S antigens and 24, 33 and 66 kDa in crude antigen. The 33 kDa band is the common immune reactive band, and it is the most appropriate for use with this

antigen. Since this polypeptide was recognized by all infected animals using both E/S and crude antigens, this could play an important role in the diagnosis of sheep fasciolosis. According to **(*Sarimehmetoğlu, 2002*)** results, the specific protein bands determined in ES antigen obtained from *F.hepatica* in cattle were 36, 26 and 17kDa.

The immunological techniques offer an interesting option for the diagnosis of human fascioliasis **(*Rahimi et al., 2011; Almazan et al., 2001*)**. Among these tests, ELISA has been developed using ES antigens from adult flukes, purified antigens, or recombinant antigens **(*De Almeida et al., 2007; Dixit et al., 2002; Maleewong et al., 1996*)**. An ELISA using the 27 kDa native protein isolated from ES products of *Fasciola* spp. has shown high sensitivity and specificity in the diagnosis of human fascioliasis **(*Dalimi et al., 2004*).** Several groups have reported the purification of the immunodominant antigens from the ES products of the liver fluke **(*Silva et al., 2004; Yamasaki et al., 1989*).** Of these, only one group described the use of a purified 27 kDa cysteine proteinase from Japanese *Fasciola* spp. and its evaluation as an antigen in the diagnosis of human disease **(*Yamasaki et al., 1989*).** This proteinase demonstrated a high sensitivity and specificity in ELISA for human fascioliasis, showing only 1 case of cross-reactivity with sera from *schistosomiasis japonicum* patients. The purified fractions Fas 1 and Fas 2, which contain a cysteine proteinase of *F. hepatica* with an approximate molecular mass of 25-26kDa, are specific and sensitive antigens **(*Cordova et al., 1997*). *Kamel et al.,* (*2013*)** studied the dot-blot assay using *F. gigantica* adult worm regurge antigens (antigen I) vs. the partially purified fractions of the antigens with an approximate molecular mass of 27 kDa (antigen II) to evaluate their potential role in the diagnosis of human fascioliasis. The sensitivity, specificity, positive and negative predictive values using the adult worm regurge (antigen I) were 80%, 90%, 94.1%, and 69.2%,

respectively, while those using 27 kDa (antigen II) were 100%, which confirmed the diagnostic potential of this antigen.

Two fractions were isolated from coproantigen by ion exchange chromatography in which DEAE cellulose was utilized. Both fractions and crude antigen were characterized by SDS polyacrylamide gel electrophoresis which revealed 13 bands of molecular weight ranged from 205-31 in crude coproantigen. While fraction I resolved into six bands of molecular weight 198, 178, 148, 111, 101 & 45. Fraction II showed seven bands of 191KDa, 178 KDa, 166 KDa, 1I8 KDa, 98.5 KDa, 72 KDa & 32 KDa. Fraction II was higher immunoreactivity than fraction by ELISA. Three immunoreactive bands of 191 KDa, I18 KDa & 98.5 KDa were identified in fraction 11 using immunoblot assay. Five bands of 178 KDa, 148 KDa, 1I I KDa, 101 KDa & 45KDa were detected in fractionI **(*Abdel-Rahman and Abdel-Megged, 2000*).** Reports on the diagnosis of fascioliasis by detection of coproantigen in feces of different hosts included use of polyclonal sera and monoclonal antibodies developed against a 26-28KDa coproantigen **(*Espino et al., 2000; Paz-silva et al., 2002*). *Abdel-Rahman et al.,* (*1999*)** proved that this antigen is monomeric, highly glycosylated glycoprotein composed of 8 KDa protein cores which still contained the epitope recognized by the MoAb. The antigen did not posses protease activity but could be cleaved by trypsin without altering the reactive epitope. Indirect immunofluoresence of tissue sections of adult fluke indicated that 26-28KDa coproantigen was present in gut cells and tegument **(*Abdel-Rahman et al., 1999*).**

2.5.2. Surface and Somatic antigens:

Fasciola parasites in the bile ducts are able to survive for many years despite being exposed to the immune system. *Fasciola* worms present a large interface with the host, which means that they must possess an

evasion mechanism(s) to overcome antiworm immune strategies. One major interface between the parasite and the host is the tegument. The tegument of bile-dwelling *F.gigantica* is the interfacing layer that helps the parasite to maintain homeostasis, and evade the hostile environment, including the host's immune attack. The tegument is highly folded and invaginated into numerous ridges, pits and spines, which help to increase the surface area of the tegument for the absorption and exchanging of molecules, as well as for attachment. The outer membrane covering the tegument is a trilaminate sheet about 12 nm thick, and coated with a carbohydrate-rich glycocalyx layer that also bears high negative charges. Some host molecules may also be absorbed onto this layer. This unique characteristic enables the parasite to evade the antibody dependent cell-mediated cytotoxicity (ADCC) reaction excreted by the host. So the tegument carries out several functions, including osmoregulation, secretion, uptake of nutrients, and evasion from host immune responses **(*Dalton et al., 2004*).** Tegumental proteins of trematodes have been shown to be targets of several anthelmintic drugs **(*Meaney et al., 2004; Meaney et al., 2003*)** and vaccine candidates **(*Loukas et al., 2007; Mulvenna et al., 2010; Spithill and Dalton, 2010*)**. Migrating flukes make alterations to the outer tegument, secrete granules and are constantly shedding the tegument, only slowing down these processes once in the relative safety of the bile duct **(*Reddington et al., 1984; Sobhon et al., 1998*).** The constant sloughing of the tegument may help protect against specific antibodies and eosinophil attachment, or act as a decoy **(*Hanna, 1980; Keegan and Trudgett, 1992*).** Secreted granules have been proposed to promote production of irrelevant antibody **(*Piedrafita et al., 2004*).** The fluke's antigens that can elicit strong immunological responses in animal hosts are synthetized and released mainly from tegument and the caecum.

The electrophoretic pattern under reducing conditions of 12% SDS-PAGE showed some similarities and differences between crude somatic protein extraxt of *Fasciola gigantica* which revealed presence of 11 bands and 14 bands in case of *Fasciola hepatica* coexisting in bovines **(*Gul et al., 2013*).** The 11 bands in soluble protein fraction of *Fasciola gigantica* (bovines) reported in their study were in agreement with the study carried by **(*Meshgi et al., 2008*).** However the 14 bands found in *Fasciola hepatica* (bovines) was in close association to the results of (***El-Rahimy et al., 2012***) who noticed 13 bands. Dominant bands for both *Fasciola gigantica* and *Fasciola hepatica* in bovines clustered between 46 and 58 Kda; and also between 17 and 25 Kda. The identified clustered proteins during the current investigation were in accordance to ***Goreish et al.,* (*2008*)** and ***Espino et al.,* (*1993*)** respectively. In addition ~24 Kda and ~57 Kda being common protein band between the two species protein extract corresponds to Cathepsin L cystein proteases **(*Robinson et al., 2008*)** and leucyl aminopeptidase which are considered to be the relevant candidate for vaccine development against ruminant fascioliasis. **(*McManus and Dalton 2006; and Acosta, et al., 1998*).** The electrophoretic scanning also revealed the presence of ~ 110 Kda proteins in *Fasciola gigantica* which was also revealed by ***Maghraby et al.,* (*2007*). *Sobhon et al.,* (*1996*)** analyzed the proteins from the homogenized whole body of *F. gigantica:* it was found that there were approximately 21detectable bands, ranging in molecular weight from 17 to 110kDa. Eleven of the bands at 97, 86, 66, 64, 58, 54, 47, 38, 35, 19, and 17 kDa, were present in the tegument antigen which was extracted from the parasites' bodies by Triton X-100. The majority of antigens derived from the surface membrane and the tegument are MW 97, 66,58,54,47 and 14 KDa, while those released from the caecum are cysteine proteases of MW 27, 26 KDa **(*Sobhon et al., 1998*).** In

comparison, there has been considerable study of *F. hepatica*. ***Itagaki et al., (1995)*** found that the major antigens of adult *Fasciola spp*. were at 64-52, 38-28, 17, 15, 13, and 12kDa; it was also reported that the antigens at 66, 58, and 54kDa were more species-specific, they might be possible candidates for serodiagnosis of fascioliasis in cattle. Several protein bands between 21 to 110 kDa were found in somatic products of Sudanese *F.gigantica* **(*Goreish 2002*).**She reported that the immunoreactive polypeptides between 27 to 30 KDa were identified in both ES and SO preparation in naturally infected animals and experimentally infected animals after 4 weeks of infection. ***Ortiz et al., (2000)*** used E/S, somatic (SO) and surface (SU) antigens of adult *F. hepatica* for antibody response determination in dairy cattle naturally infected with *F. hepatica*. They reported that antibody responses were developed against 60-66 kDa in E/S and SU antigens and 17kDa in SO antigen. Monoclonal antibodies have been raised against some of these antigens, and have been embloyed in immunodiagnosis of infection. ***Viyanant et al., (1997)*** studied a monoclonal antibody specific to a 66kDa antigen of *F. gigantica* for the detection of circulating antigens in experimentally and naturally infected cattle, they reported that circulating antigens could be detected as early as the second and third weeks after infection; these antigens was associated with the crude surface tegument of the parasite. From the protection conferred to animal models and the vitro killing assays of young parasite by specific antibodies, candidate vaccines could be selected from these antigens such as antioxidant enzyme, glutathione-S-transferase, the digestive enzyme cysteine proteases, the surface–tegument proteins, such as fatty acid binding protein (14KDa), membrane protein (at 66 KDa), as well as muscle protein paramyosin, and hemoprotein. In an earlier study, ***Youssef and Mansour (1991)*** purified adult *F. gigantica* by Sephadex G-200. They

found 4 distinct protein peaks and used peaks II and III for further antigen study. Electrophoretic analysis of fraction 10 of the two peaks showed a protein band of between 10 and 66kDa. This indicates that the purification of the surface tegument antigen of *F. gigantica* is a sound method of antigen production. ***Krailas et al.,* (*2002*)** purified surface tegument antigens of *F. gigantica* by gel-filtration chromatography. The fractions were characterized and studied; proteins with molecular weights ranging from27 to 97kDa were present in the fractions of purified antigens. They differentiated the MAbs produced in this study by EITB. The first group recognized epitopes present in 60 and 38kDa; the second group had protein of 66, 60, and 38kDa. These MAbs were tested for their cross-reactivity with other trematodes commonly found to infect cattle and man. All of these MAbs showed some degree of cross-reactivity with other trematode species. Comparative studies on the sensitivity and specificity of various antigenic components for immunodiagnosis of human fascioliasis were reported previously. The antigenic components of *Fasciola gigantica* somatic extract were revealed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting technique using sera from patients with *F. gigantica* infection, patients with clinically diagnosed fascioliasis, patients with other infections/illness and healthy adults. By SDS PAGE, it was found that the somatic product comprised more than 22 polypeptides. Immunoblotting analysis revealed at least 13 components which were strongly recognized by sera of patients with fascioliasis. These antigenic components had molecular weights ranging from less than 14.4 to more than 94 kDa. One antigenic component, *i.e.* 38 kDa was found to give a consistent reaction with sera of patients with fascioliasis (100% sensitivity and 96.7% specificity) **(*Maleewong et al., 1996*).** The finding suggests that the 38 kDa components may be a potential diagnostic

antigen for fascioliasis. Similar results reported that sera from sheep and cows experimentally infected with *F hepatica* recognized prominent antigens located between 30 to 38 kDa of *F. hepatica* somatic antigen **(*Santiago and Hillyer, 1988*). *Shaker et al.,* (*1994*)** reported that the sera of patients with fascioliasis recognized the *Fasciola* somatic antigenic fractions at the 33 and 54 kDa.***Yamasaki et al.,* (*1989*)** demonstrated that the *Fasciola* proteinase, with an approximate molecular weight of 27 kDa, was valuable as a sensitive ELISA antigen for immunodiagnosis of human fascioliasis. Similar investigation evaluated the performance of a 27 kDa subunit of *F. hepatica* somatic antigen in two systems, immunoblotting and ELISA, and showed that this subunit has satisfactory validity in diagnosis of human fascioliasis **(*Shafiei, et al., 2015*).** This finding is also in agreement with ***Intapan et al.,* (*2003*),** which showed that a 27 kDa antigen of *F. gigantica* adult worm has a valuable performance in an ELISA system for the serodiagnosis of human fascioliasis. ***Rokni and Ghravi* (*2002*)** in comparison of adult somatic and cysteine proteinase antigens of *F. gigantica* for serodiagnosis of human fascioliasis, showed a higher specificity of cysteine proteinase than somatic antigen.

To date, three groups of *Fasciola* antigens (whole-worm extract, excretory-secretory, and recombinant proteins) have been used in different immunodiagnostic procedures for diagnosis of fascioliasis **(*Arias et al., 2006; Carnevate et al., 2001; ElRidi et al., 2007; Hillyer, 1999*).** Recently, the tegument of *F. hepatica* has been targeted by a number of proteomic approaches focused to investigate the distribution and abundance of surface protein composition of adult *F. hepatica* **(*Hacariz et al., 2011; Wilson et al., 2011*). *Morales and Espino* (*2012*)** demonstrated the value of FhTA as an antigen for serodiagnosis of human fascioliasis, they proceeded to identify the major components of this

extract that react specifically with infection sera. SDS-PAGE separation of FhTA revealed that this extract contains more than 40 components, which is consistent with the large number of proteins identified within the tegument of *F. hepatica* following proteomic approaches **(*Hacariz et al., 2012; Wilson et al., 2011*).** In the optimized Western blot conditions of their laboratory they found that the 12-to-14-, 24-to-26-, 38-, and 52-kDa bands were the components most frequently recognized (more than any other band) with fascioliasis sera irrespective of the antibody titer in the FhTA-ELISA. These bands were not detected in the sera of other parasitic infections; thus, these bands are considered to be highly specific for serodiagnosis of human fascioliasis. A proteomic approach identified enolase, aldolase, glutathione *S*-transferase, and fatty acid binding protein as the major immunoreactive components of the FhTA. Recently these proteins were found to be highly abundant either in the surface or the internal protein fraction of *F. hepatica* **(*Hacariz et al., 2011, 2012*).** FABP of *F. hepatica* has been recently categorized as an antioxidant molecule of the parasite **(*Robinson et al., 2008*).** Their abundance on the surface of *F. hepatica* indicates that these proteins play a role in the uptake of fatty acids from the environment, important for the parasite's lipid metabolism. GST is an enzyme found in all animals and plays a role in the detoxification and removal of harmful molecules. The 26-kDa GST from *F. hepatica* is an antigenic protein transiently expressed on tegumental surfaces excreted by the parasites **(*Abath and Werkhauser, 1996*).** Previous independent studies have shown that vaccines containing FABP or GST induce partial protection in experimentally infected mice and sheep **(*Lopez et al., 2007, 2008; Paykari et al., 2002; Preyavichyapugdee et al., 2008*).** Enolase was also recently identified as an abundant protein of the surface of *F. hepatica* **(*Gaudier et al., 2012*)** and as an important egg-associated protein **(*Moxon et al., 2010*).** It is a

multifunctional glycolytic enzyme that is present on the surface of several pathogens and has been characterized in detail as a plasminogen receptor **(*Bernal et al., 2004*).** In *Schistosoma bovis*, eight isoforms of enolase have been identified, and four of these have been shown to be immunogenic in sheep **(*Perez- Sanchez et al., 2006*).** Aldolase is a central glycolytic enzyme in carbohydrate metabolism; therefore, it is vital for energy production that is important for parasite activities and survival. Recombinant forms of aldolase from *S. mansoni* have been shown to induce high levels of IgG1 in mice, which were associated with 57% higher protection against a challenge infection **(*Marques et al., 2008*).**

Proteins of 50 kDa and 25 to 40 kDa have been identified in the tegumental syncytium of various *F. hepatica* stages using monoclonal antibodies **(*Abdel-Rahman et al., 1999; Hanna and Trudgett 1983*),** but these proteins have not yet been purified and characterized. Immunohistochemistry demonstrated that FhTP16.5 localizes to the surface of the tegument of various developmental stages and in parenchymal tissues of the adult fluke. Such specific localization makes FhTP16.5 an attractive target for immunoprophylaxis or chemotherapy. Antibodies to FhTP16.5 were detected in the sera of rabbits at 3 to 12 weeks of *F. hepatica* infection as well as in the sera of humans with chronic fascioliasis; these findings suggest that FhTP16.5 could be a good antigen for serodiagnosis of fascioliasis **(*Jose et al., 2012*).**

Of late, efforts are being focused on the use of larval antigens for serodiagnosis of human **(*Mousa et al., 1996*)** and ovine fasciolosis (***Mousa, 2001*)** with high level of specificity. SDS-PAGE and immunoblot analysis was carried out to evaluate the metacercarial antigen for serodiagnosis of fasciolosis in cattle **(*Velusamy et al., 2006*).** SDS profile of metacercarial antigen and *F.gigantica* somatic antigen was compared; in metacercarial antigen twenty four polypeptides ranging

from 96-12 kDa were resolved. Almost similar polypeptide profile was observed in *F.gigantica* somatic antigen except 21 kDa which was unique to metacercarial antigen. In immuoblot studies, the immunoreactive protein recognized against metacercarial antigen were 43, 32, 25, and 21 kDa. It was interested that the polypeptide which was unique to MAg (21kDa) reacted strongly as early as 10 days post infection and continued till the end of experiment, while reaction with 25 kDa polypeptide started on 2 weeks post infection and persisted till the end of the experiment. Also the comparative immunoblot studies with anti *Gigantocotyle explanatum* and anti *paramphistomum epiclitum* sera revealed that 21 and 25 kDa polypeptides of metacercarial antigens did not react with any of these sera and appear to be unique to *F.gigantica* and having the desirable qualities of early and specific immunodiagnosis. In contrast to this study **(*Mousa, 2007*)** observed a band of 32.5 kDa in cercarial antigen as early as 2 weeks post infection using sera from sheep infected with *F.gigantica*.

2.5.2.1. Comparison between ES and SO Antigens:

Following SDS-PAGE, E/S proteins of *F. hepatica* and *F. gigantica* were characterized by the presence of 6 common major peptide bands with molecular weights of 15, 16, 20, 24, 33 and 42 kDa. Differences between *F. hepatica* and *F. gigantica* somatic proteins were noticed. *F. gigantic*a had 11 major protein bands with molecular weights of 18, 22, 24, 33, 36, 42, 46, 57, 60, 62 and 68 kDa, whereas *F. hepatica* had proteins characterized by 8 distinct bands with molecular weights of 18, 22, 24, 33, 36, 42, 46 and 62 kDa ***Meshgi et al.,* (*2008*).**They also showed the presence of three common bands between E/S and somatic peptides of *F. hepatica* and *F. gigantica* (24, 33 and 42 kDa). ***Allam et al.,* (*2002*)** showed the presence of eight and five protein bands in whole worm antigens with lower molecular weights ranging from 25.5–48 in *F.*

hepatica and 27–57.6 kDa in *F. gigantica*. Finding on SDS-PAGE analysis of *F.gigantica* ***Upadhyay and Kumar* (*2002*)** reported eight ES bands with molecular weight 13-62 kDa and seven somatic protein bands. They also reported three common bands (24, 33 and 42 kDa) between ES and somatic antigen of *F.gigantica*. In ***Gupta et al.,* (*2003*)** report, somatic antigen of *F. gigantica* resolved by SDS-PAGE contained six proteins of 27.7–37.5 kDa. ***Rokni and Ghravi (2002*)** in comparison of adult somatic and cysteine proteinase antigens of *F. gigantica* for serodiagnosis of human fascioliasis, showed a higher specificity of cysteine proteinase than somatic antigen. In human, in addition to faecal examination, enzyme-linked immunosorbent assay (ELISA) is highly sensitive and specific for E/S antigen of *F. hepatica* **(*Khalili et al., 2001; Rokni et al., 2001*).** The results of ***Moazeni et al.,* (*2005*)** study showed that ES and somatic antigens of both species of *Fasciola* have cross reaction with each other and both antigens can be used for the detection of antibodies in the serum of immunized rabbit. These mean that some antigenic materials are common between ES and somatic antigens**.** ***Santiago et al.,* (*1986*)** reported that crude *F***.** *hepalica* ES products, when tested by ELISA, had a high reactivity with the sera from rabbit in acute fascioliasis. ELISA test using crude adult somatic antigen is advantageous for diagnosis of naturally occurring *Fasciola* infection in cattle **(*Itagaki et al., 1989*). *Cornelissen et al.,* (*1992*)** used ELlSA with somatic and ES antigens of *F. hepatica* for serodiagnosis of fascioliasis in naturally or experimentally infected sheep and reported the specificity of 98% and 95% with somatic and ES antigens, respectively. *Fasciola hepatica* somatic antigen, its partially purified fractions and excretion-secretion products were investigated as to serological, electrophoretic and biological properties **(*Cervi et al., 1992*).** In a Sephadex G-100 column (SG-100), *Fasciola hepatica* total antigen (FhTA) gave 5 fractions, and

SDS-PAGE analysis showed they were glycoproteins ranging from 14 to 94 kDa molecular weight (MW). When these fractions were analyzed by enzyme-linked immunotransfer blot (EITB) and immunodiffusion in gel (ID) with serum from immunized rats with FhTA, the presence of different antigenic components was revealed. In the SDS-PAGE of excretor-secretor antigen (ESA), it was possible to observe peptides from 12 to 22 kDa, which were also present in FhTA. When the FhTA, its fractions and the ESA were analyzed by EITB with the immune rat serum (IRS), it was observed that only some fractions of the SG-100 shared antigens with the FhTA and ESA. ***Lehner and Sewell* (*1980*)** reported no difference when comparing a crude ES with somatic antigen in the serodiagnosis of fascioliasis in sheep by ELISA**. *Maleewong et al.,* (*1996*)** compared somatic and ES antigens of *F. gigantica* for serodiagnosis of human fascioliasis and reported that the absorbance values in ELISA using somatic antigen are not significantly different from the values using ES antigen, therefore both somatic and ES antigens are effective for use in the diagnosis of human fascioliasis due to *F. gigantica.* ***Moazeni and Gaur* (*2003*)** found no difference between the antigenicity of ES and somatic antigens of *Fasciola spp* in gel diffusion test. The protein compositions of E/S and SO products of *F. gigantic*a isolates were investigated by SDS-PAGE **(*Goreish et al., 2008*).** They found that the E/S product of the adult parasite contains proteins bands fewer than those of SO product as it excluded the parasite somatic proteins. Electrophoresis patterns showed some similarities and some differences between the two-parasite preparations. A complex range of bands was identified in SO products between 40 and 90 KDa. A group of proteins clustered between 27 and 30 KDa, was common to E/S and SO preparation. Proteins within these molecular weight ranges were also identified in both parasite preparations by ***Ajanusi et al.,* (*1993*). *Yamchi***

***et al.*, (*2014*)** used Dot ELISA with ES antigen and crude antigens for sero-diagnosis of *F.gigantica* infection in cattle; they concluded that ES Ag was the best coating antigen in Dot-ELISA due to its high sensitivity, specificity, and precision rates. Also recently, native cathepsin-L cysteine proteinase was purified from the excretory secretory products of *Fasciola* and applied for sero-diagnosis of *Fasciola* infection in buffaloes using Dot-ELISA. The results demonstrated that cathepsin-L cysteine proteinase based Dot-ELISA achieved 90.00% sensitivity and 100% specificity **(*Varghese et al., 2002*).**

2.6. Diagnosis of Fasciolosis:

Diagnostic tools build the basis for successful prevention and control of infection diseases including fascioliasis. First and foremost, an accurate diagnostic method is obviously needed to identify infected individuals in a population and thus to enable proper intervention strategies, prevalence records, and ultimately accurate estimates of the global disease burden. In addition, a sensitive and specific diagnostic method is essential for evaluating treatment outcomes and community effectiveness of interventions, as well as verifying local disease eliminations and early detection of reappearances **(*Bergquist et al., 2009*).**

2.6.1. History and Clinical Diagnosis and Imagining Techniques:

Diagnosis of fascciolosis based on clinical signs is difficult to achieve especially if the disease is accompanied by other parasitic infections **(*Reid et al., 1967*).** In addition, several other diseases can also produce liver dysfunctions **(*Mullen, 1976*),** which may distort the clinical presentation of fasciolosis and complicate its diagnosis. Also in animals, the clinical features of fasciolosis present in different forms, depending on the animal species, the level of infection and the plane of nutrition of

the animals. Symptoms may also vary between animals within a group. Acute fasciolosis may cause sudden death and is mostly seen in sheep and goats, as a result of intake of large numbers of larvae over a short period. A history of grazing fluke-prone areas is usually found **(*Behm and Sangter, 1999*).** Animals presenting with the chronic form of *Fasciola* spp. infection often show no specific clinical signs, which makes diagnosis difficult. However, weight loss, pale mucous membranes, ventral oedema and diarrhea can be observed in this stage in both sheep and cattle. The most important feature of chronic fasciolosis is loss of production, mainly through reduction in weight gain **(*Hope-Cawdery et al., 1977*),** milk yield and fertility **(*Charlier et al., 2007*).** So confirmation of a presumptive case of fasciolosis by means of specific reliable method is important. The most commonly used diagnostic techniques for Fasciola infection is the parasitological examination of faecal samples and immunological tests for detection of specific antibodies or parasitic antigens in serum samples.

The infection in humans usually presents with very serious symptoms of pain in the right upper quadrant of the abdomen, mild fever, upper stomachache, loss of weight, indigestion, diarrhea and urticaria **(*Mas-Coma et al., 1999; De et al. 2005*)**. Imaging techniques, such as, ultrasound, computer tomography (CT) or magnetic resonant cholangiopancreatography (MRCP) are very useful to demonstrate the lesions caused by fasciolosis and to confirm clinical diagnosis **(*Adachi et al., 2005*).** Nevertheless, the clinical and imaging observations cannot always be differentiated from other hepatic diseases. The disease history of patients should also be considered for diagnosis, by using a short questionnaire for obtaining personal information: the history of hepatic disease and the habit of eating fresh aquatic vegetables. However, for

differential diagnosis of fasciolosis from other hepatic diseases, parasitological and immunological techniques may be required.

2.6.2. Parasitological Methods:

In animals, parasitological diagnosis of fasciolosis is based on examination for presence of immature or adult flukes in the liver, bile ducts and gall bladder, at slaughter or autopsy, and on the demonstration of fluke eggs by coprological examination. The specificity of liver examination is very high (93.4% to 100%) but the technique lacks sensitivity (47.4% to 63.2%) **(*Adedokun et al., 2008; Rapsch et al., 2006*).** Detection of liver fluke eggs in stool samples is a very simple method including flotation and sedimentation techniques, with high specificities of 93% - 100% **(*Anderson et al., 1999; Conceiçao et al., 2002; Charlier et al., 2008*).** The fluke eggs are heavy; therefore, the use of flotation solutions with high density is needed. (E.g. zinc chloride and sodium chloride (d = 1.6) or potassium iodomercurate solutions). Flotation methods are qualitative and the chemicals used are harmful for the environment. Sedimentation techniques appear to be more accurate and sensitive than flotation techniques **(*Boray, 1969*).** Sedimentation using tap water is the simplest and cheapest method, but it is more time consuming compared to flotation techniques. The visibility of the eggs in the sediment can be increased by adding a few drops of methylene blue solution. The sensitivity of this method is only 33.3% when the faecal material contains less than 1.5 eggs per gram faeces, but increases up to 100% for higher egg concentrations **(*Conceiçao et al., 2002*).** The sensitivity of this method can be increased by: using the shaking sieve method (66.7%) **(*Anderson et al., 1999*);** or increasing the weight of the faecal sample (from 43% for 4 g to 64% for 10 g) **(*Charlier et al., 2008*);** or repeating the examination on the same sample (from 69% for the first

examination to 91.9% if the method is repeated three times) **(*Rapsch et al., 2006*).**

The Kato-Katz method is a simple and widely used method on human stool samples and it has also been applied for detection of eggs of flukes **(*Katz et al., 1972; Esteban et al., 2002; Rokni et al., 2002*).** Both sedimentation and Kato-Katz methods can be used for the qualitative and quantitative assessment of the presence of eggs of *Fasciola* spp. in human stools **(*Anderson et al., 1999; Esteban et al., 2002*).**

While the parasitological methods used for detection of *Fasciola* flukes in the liver or their eggs in the faeces of definitive hosts have a very high specificity, their lack of sensitivity can be related with the following reasons: 1) during the migration phase, immature worms pass through the parenchyma but do not yet lay eggs; 2) in humans flukes often do not develop to the egg laying adult stage, or can migrate to ectopic locations; 3) in the case of low parasitic burdens, the egg output is low and egg laying may be irregular and pass undetected **(*Hillyer, 1999*).** So the parasitological diagnosis of fasciolosis is often unreliable because the parasite eggs are not found during the prepatent period, during which the maximum damage to the liver occurs. **(*Noureldin et al., 2004*).** Even when the worms have matured, the diagnosis may still be difficult since eggs are only intermittently released. Fluctuation occurs in egg execration within the same day and over a period of several days **(*Duwel and Reisenleiter, 1990*).** Moreover, in many infections, the fluke eggs are often not found in the faeces, even after multiple faecal examinations. If they are found, the number of eggs per gram (EPG) of faeces is almost never proportionate to the number of adult worms present and provides no estimate of infection severity **(*Anderson et al., 1999*).** Furthermore, fasciola and paramphistomum eggs have very similar morphologies which make them difficult to differentiate.

Eggs can be detected in cattle as from eight weeks after infection **(*De Leon and Quinons, 1981*)**. Cattle show diurnal variation in egg production **(*Boray, 1969; Dorsman 1962*),** but this cycle has not been demonstrated in sheep which can produce up to 2.5 million eggs per day depending on parasite burden **(*Happich and Boray, 1969*).** Despite the large amount of eggs produced, no coprological technique has been 100% sensitive **(*Doren et al., 1958*).** An alternative and effective method for detection of fasciolosis is greatly needed.

In conclusion coprological methods have the advantage of being cost-effective and relatively easy to perform, which render them widely applicable in resource-constrained settings **(*WHO, 2006*).** However, only chronic fasciolosis can be diagnosed, since eggs are excreted only from mature worms and hence ectopic fascioliasis and acute infections (first 3 months) remain undiscovered **(*WHO 2006*).** Coprodiagnostic methods are sufficiently sensitive in detecting moderate and high *Fasciola* spp. infection intensities, but the methods lack sensitivity for detecting low intensity infections. Still, repeated sampling can improve sensitivity significantly **(*WHO, 2006; Rapsch et al., 2006*).**

2.6.3. Hepatic Enzymes:

Before the development of immunological diagnostic techniques many researchers studied extensively damage to hepatic tissue and to bile duct epithelium and the effects of this on the hepatic enzymes. Enzymes from hepatocytes are: glutamate deshydrogenase (GLDH) and glutamateoxaloacetate aminotransferase. They indicate damage to hepatic cells, related to parasite migration. Their levels are increased during the migration of *F.hepatica*. Gamma-glutamyl transferase is present in the bile duct epithelium: its blood concentration increases after penetration of liver flukes into bile ducts, during the period from eight to twelve weeks after infection. This is followed by a decrease in this enzyme, frequently

interrupted by brief increases, between twelve to twenty weeks after infection **(*Wensvoort and Over, 1982*).** Several papers deal with the kinetics of these enzymes during fasciolosis illustrating the ongoing changes during infection **(*Salem et al., 2007*).** Unfortunately when parasitic burdens are very low the dosage of this enzyme is not useful **(*Gaasenbeek et al., 2001*).**

2.6.4. Immunodiagnosis:

Early diagnosis of Fascioliasis is necessary for institution of prompt treatment before irreparable damage of the liver occurs **(*Rokni et al., 2004*).** For these reasons, serology is the most dependable diagnostic method. It is an important procedure for the confirmation of fasciolosis that involves the analysis of antibody responses to fluke antigens as well as the detection of circulating antigens using defined sera and monoclonal antibodies **(*Spithill et al., 1999*).** Advances in immuno-diagnosis have focused on detection of parasite antigens in host body fluid; these tests have an advantage over antibody detection because antigenemia implies recent and active infection **(*Cornelissen et al., 1999*).** The somatic and ES antigen of fasciola spp. or their partially purified component are the commonest source of antigens used in protection trials and serodiagnosis **(*Cӧnen et al., 2004*).** Immunodiagnosis of parasitic disease is mainly based on antibody detection **(*Fagbemi et al., 1999*)** and revealed both recent and current infection with early diagnosis. To obtain reliable diagnostic method or to identify crude antigen, many authors prepared antigens from whole worm **(*Hillyer et al., 1987*)** or from tegument **(*Charmey et al., 1979*)** also coproantign **(*Allen et al., 1996*),** egg antigen **(*Khalil et al., 1989 and Abdel-Rahman and Abdel-Mageed, 2000*)** and execretory secretory products **(*Espino et al., 1994*).** Currently, haemoaglutination (HA), indirect fluorescence antibody test (IEAT), immunoperoxydase (IP), counter- electrophoresis (CEP)and enzyme

linked immunosorbent assay (ELISA) are used in the early diagnosis of this disease, but they have some disadvantages such as cross reactions with other parasites leading to false positive results. Therefore, the reliability of these tests is not high. In recent years, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting procedures have initiated a new era in immunodiagnosis which greatly reduced cross reaction. Evaluation of sandwich ELISA and Dot ELISA as an immunological assay is used for detecting fasciola coproantigen and serum antigen in infected sheep, thus presenting experimental trials that could be of value in providing a tool that may help in immunodiagnosis of fasciolosis.

2.6.4.1. Antigen Detection:

Active infection by *Fasciola* spp. can be demonstrated by the detection of metabolic products of flukes in the circulation. Such a test can also be used to confirm the efficacy of chemotherapy. Several assays have been developed to detect *Fasciola* spp. antigen in serum and faeces using monoclonal antibodies **(*Espino et al., 1990, 1994; Fagbemi et al., 1997; Mezo et al., 2004*)** (Table 1.2).

The antigen in blood can be detected by ELISA from one week post-infection onwards. However, Ag-ELISA has not been further developed because antigenaemia only develops when immature flukes are actively migrating through the liver parenchyma during 1-3 weeks post-infection and circulating antigens cannot be detected anymore once the flukes are established and mature to adult worms **(*Langley and Hillyer, 1989; Espino et al., 1997*).**

Copro Ag-ELISA has been applied to detect ES productions of *Fasciola* in stool samples by using monoclonal antibodies. The antigen can be detected as early as 3-4 weeks post-infection when the flukes reside in the host liver **(*Fagbenmi et al., 1997*).** In addition, a strong

correlation between copro-antigen levels and the numbers of flukes was seen **(*Abdel-Rahman et al., 1998*).** Circulating antigens were detected in 100% of sheep with 1 fluke and in 100% of cattle with 2 flukes, from five weeks post-infection (wpi) onwards **(*Mezo et al., 2004*).** Some false negatives were probably not detected because the flukes were immature and so there were no ES products in the bile ducts **(*Salem et al., 2007*).** It appears that the copro-antigen concentration correlated positively with parasitic burden and negatively with the time after infection at which copro-antigen was first detected. Even in animals with low fluke burdens (1-36 parasites) the first detection of *F. hepatica*-specific coproantigens by the MM3 capture ELISA preceded the first detection in egg count by 1-5 weeks **(*Dorchies, 2007*).** The copro-antigen became undetectable from 1 - 3 weeks after treatment with a flukicide in sheep and cattle **(*Mezo et al., 2004*)** and from 2 months post-treatment in 78.6% of patients **(*Espino et al., 1994*).** The copro Ag-ELISA was demonstrated to have a sensitivity and specificity close to 100%.

2.6.4.1.1. Sandwich ELISA:

Detection of circulating Fasciola antigen in both serum and stool was found to be more sensitive and specific **(*Hillyer, 1999*).** The majority of methods based on antigen detection are applied to *F. hepatica* infection, but only few are applied to *F. gigantica* infection **(*Estuningsih et al., 2004; Mezo et al., 2007*)**. A MoAb-based sandwich ELISA was employed for detection of circulating *F. gigantica* ES Ags in both serum and stool samples of *F. gigantica* infected patients. Identification of the target antigens recognized by anti-Fasciola MoAbs (12B/11D/3F and 10A/9D/10D) showed that they were glycoproteins and their MW lay in the 83, 64, 45 and 26 kDa regions **(*Demerdash et al., 2011*).** Close to those findings, ***Arafa et al.*, (*1999*)** produced mono specific antibodies against ES Ags of *F. gigantica* whose target antigens were recognized at

27.5, 32.5 and 55 kDa regions. They reported that cross reactivity with *Schistosoma mansoni* occurs at higher MW (110-120 kDa). ***Demerdash et al., (2011)*** showed that the sensitivity and specificity of MoAb-based ELISA in serum was 94% and 95.6%, while in stool samples it was 96% and 98.2%, respectively. A few antigen detection assays have been developed for diagnosis of *F. gigantica* in human fluids with varied ranges of sensitivities and specificities **(*Espino and Finlay, 1994; Espino et al., 1998; Arafa et al., 1999*)**. They also demonstrated that the diagnostic efficacy of MoAb-based sandwich ELISA in stool (coproantigens) was superior to serum samples (97.1% vs 94.3%); this could be due to the fact that coproantigens offered several advantages, e.g., the levels of coproantigens are less affected by immune complex formation than circulating Fasciola antigens, coproantigens are detectable during prepatent and patent phases of infection, non-invasive and finally the nature of these antigens of being glycoprotein making the coproantigen stable under several different storage conditions which ensured its diagnostic value **(*Espino and Finlay, 1994; Espino et al., 1998; Anuracpreeda et al., 2009; Velusamy et al., 2004; Valero et al., 2009*).**

A positive correlation was found between ova count/ gm stool of Fasciola infected patients and the OD readings of ELISA in both stool and serum samples **(*Demerdash et al., 2011*).** Other studies have demonstrated that coproantigens are correlated with Fasciola egg counts **(*Espino and Finlay, 1994*)** and the parasite burden **(*Anuracpreeda et al., 2009; Dumenigo et al., 1996*)**. On the other hand, ***Ubeira et al., (2009)*** reported that there was no correlation between number of ova/gm stool and coproantigens levels measured by ELISA.

1.6.4.2. Antibody Detection:

ELISA-based techniques or variant like Dot-ELISA are considered as rapid and reliable immunoassay with easy procedure for detection of antibody or antigen **(*Hillyer and De Galanes 1988*).** For these reasons, the vast majority of researchers nowadays have applied an ELISA-based assay or variant in diagnosis of different parasitological diseases such as fasciolosis, toxoplasmosis, schistosomiasis, hydatidosis and cysticercosis **(*Hassan et al., 2004*).**

Infection with *Fasciola* spp. results in a specific antibody response. These antibodies can be detected in either serum or milk **(*Charlier et al., 2007*).** Several techniques have been described for the detection of antibodies against *Fasciola* spp. infection in animals and man, such as the indirect hemagglutination test (IHA) **(*Levieux et al., 1992a, b*)**, indirect immunofluorescence assay (IFA) **(*Hanna and Jura, 1977*),** enzyme-linked immunosorbent assay (ELISA) and the Western immunoblot **(*Hillyer and De Galanes, 1988, 1991*),** Dot- ELISA **(*Shaheen et al., 1989*)** and Micro-ELISA **(*Carnevale et al., 2001*).**

1.6.4.2.1 The Enzyme Linked Immunosorbent Assay (ELISA):

Antibodies to *Fasciola* spp. in infected hosts can be detected by ELISA (Ab ELISA) as early as one to two weeks post-infection **(*Reichel, 2002; Kumar et al., 2008*).** They rise rapidly reaching a plateau by 3-6 weeks of infection. In one study with experimently infected calves, levels remained high throughout the 13 weeks of infection **(*Vignali et al., 1996*).** On the other hand, eggs of flukes are found in faeces only after 12 - 14 weeks of infection **(*Bürger, 1992*),** then drop sharply thereafter and become egg negative by one year **(*De León et al., 1981*).** This decrease in eggs is presumably due to the death of adult flukes, and is also followed by a decrease in ELISA absorbance values **(*Hillyer et al., 1985*).** Variable results were obtained in cattle. A significant increase in specific antibody titers was observed two weeks **(*Wyckoff and Bradley, 1986;***

***Santiago and Hillyer, 1988*)** and six to eight weeks after infection in cattle **(*Hillyer et al., 1985*)**. In sheep, a significant increase was observed at four weeks after infection **(*Santiago and Hillyer, 1988*).** Similar results were also reported in *Fasciola gigantica* infected goats **(*Goreish et al., 1988*).** The Ab ELISAs have sensitivities and specificities of 87-100% and 86-100%, respectively (Table 1.1). However, cross reactions were seen with serum samples obtained from patients with hydatidosis and toxocariasis **(*Rokni et al., 2003*)** when using crude excretory secretory products (ES) of adult worms as the antigen. Two *F. gigantica* antigens (Cr and ES) were applied to evaluate the diagnostic sensitivity, specificity, precision, positive predictive value and negative predictive value of indirect ELISA and Dot-ELISA for the diagnosis of *F. gigantica* infection in cattle **(*Yamchi et al., 2015*)**. Results of indirect ELISA showed that Higher sensitivity for the diagnosis of bovine fasciolosis was detected by Cr Ag (90 %) compared with ES Ag (88.33 %). High specificity (80 %) was recorded when ES Ag was used in indirect ELISA for diagnosis of *F. gigantica* infection in cattle compared with 75 % using Cr Ag. Higher precision (84.16 %) was obtained when using indirect ELISA with ES Ag for diagnosis of bovine fasciolosis compared with using Cr Ag for diagnosis (82.5 %). Using ES Ag in indirect ELISA gives high-accuracy rates. Their data also indicated that when ES antigens of *F. gigantica* used for indirect ELISA or Dot-ELISA designing, cross-reaction was not detected. Nevertheless, three positive bovine blood samples of dicrocoeliasis and three positive bovine blood samples of hydatidosis may be detected when indirect ELISA or Dot-ELISA was performed by crude antigens of *F. gigantica.* To improve the sensitivity and specificity of Ab ELISA, antigens purified from crude ES of flukes, recombinant antigens, or synthetic protein antigens should be used **(*Cornelissen, et al., 2001; Silva, et al., 2004; Yokanath et al., 2005*)**

(Table 1.1). Serodiagnosis of fascioliasis in human and animal species has been successfully carried out employing several antigenic fractions of *Fasciola* **(*Mezo et al., 2003; Sánchez-Andrade et al., 2008; Demerdash et al., 2011*),** purified antigens **(*O'Neill et al., 1998; Rokni et al. 2002*),** and recombinant antigens **(*O'Neill et al., 1999; Carnevale et al., 2001*).** Cathepsins L are the most frequently used target antigens for detecting anti-*Fasciola* antibodies **(*Carnevale et al., 2001; Rokni et al., 2002; Mezo et al., 2004, 2007, 2010; Intapan et al., 2005; Wongkham et al., 2005; Valero et al., 2009; Muiño et al., 2011*),** as circulating antibodies to these molecules remain at high levels for long periods **(*Valero et al., 2009*).** On pooled sera, herd prevalence of infection as low as 5 per cent, f2-antigen ELISA can be detected **(*Reichel, 2002*)**. Nevertheless, positive results do not determine whether or not live flukes are present: detectable antibodies may persist long after treatment or after the natural death of liver flukes. A positive result indicates that the animal is, or has been, infected by the parasite, antibodies detected in natural infection may or may not be related to an active infection. Under field conditions, diagnostic Se and Sp of, respectively 67–69% and 100% are reported for coproscopy and 86–92% and 83–94% for *F. hepatica* antibody-detection ELISAs **(*Anderson et al., 1999; Rapsch et al., 2006*)**. In contrast, in studies using two distinct populations, the reported Se and Sp are, respectively 97–100% and 96–100% for antibody detection ELISAs **(*Ibarra et al., 1998; Reichel, 2002; Molloy et al., 2005; Salimi-Bejestani et al., 2005*)** and 94% and 100% for a copro-antigen ELISA **(*Mezo et al., 2004*).** This confirms the value of evaluating tests in the area and situation where they are actually applied. Possible reasons for the lower Se and Sp in field situations are the greater proportions of animals with low levels of infection, the sampling of animals that have recovered from infection (i.e. with no flukes present) but in which *F. hepatica*-specific antibodies are

still circulating and the imperfect gold standard. By incision and inspection of the opened major bile duct, only 71% of all infected livers were detected and the remaining 29% were only detected after slicing and soaking of the livers. ***Rapsch et al., (2006)*** estimated the Se of detecting infection with *F. hepatica* at meat inspection at the abattoir as 69%. Therefore, worm counts at liver necropsy can only be considered as a gold standard if slicing and soaking of the liver is performed.

Isotype determination is also important. For example, IgM antibodies peak early by week 3 in infected sheep and drop sharply by week 6 post infection; IgG antibodies peak by 4-5 weeks of infection but remain high thereafter **(*Chauvin et al., 1995*).** In contrast, ***Clery et al., (1996)*** found IgG1 to be the dominant isotype over IgM, IgG2 and IgA in both chronically infected and acutely infected, previously naïve calves. Analysis of the isotypic responses of sera of fascioliasis patients to liver flukes tegumental extract **(*Morales and Espino 2012*)** revealed that the predominant antibodies elicited are IgG1 and IgG4, which are the antibody serotypes that also predominate against execretory-secretory products, crude-whole worm extract and cathepsin – L1 **(*O´Neill et al., 1998; Sabry et al., 2011 and Wongkham et al., 2005*). *O´Neill et al.,* (*1998*)** developed a diagnostic test for human fasciolosis based on the detection of antibodies to ES antigen or purified cathepsin L protease. The authors found that the assays were much improved by the use of conjugated second antibodies that detect IgG4 rather than total antibodies. Importantly, sera obtained from patients infected with Schistosomiasis mansoni, cysticercosis, hydatidosis and chagas' disease were negative in these tests. ***Morales and Espino* (*2012*),** in their study found that similar levels of both IgG antibodies, with mean level of IgG1 levels slightly higher than those of IgG4. Differences regarding the relative amount of both antibody isotypes could be attributed to the genetic background of

the infected individuals and the intrinsic properties of the antigen itself. Moreover, the cytokines elicited by different antigens also play a role in determing the main subclass of the antibody response **(*Garraud et al., 2003*).** But generally, the predominance of both antibody isotypes IgG1 and IgG4 are especially predominant in infections caused by n **(*Wongkhan et al., 2005*)** when antigenic exposure is chronic **(*Garraud et al., 2003*).** On the other hand, ***Osman et al., (1995*)** used an IgM ELISA to detect antibodies to ES antigens. Although all 38 patients with acute fasciolosis were positive by this test, only 77% of 14 chronic cases were positive. Therefore, an IgM ELISA may be a good indicator of early infection only.

The bulk-tank milk ELISA has been used to identify *F. hepatica* infected cattle herds and infection was linked to a decrease in productivity **(*Reichel et al., 2002; 2005; Charlier et al., 2007*).** However, the detection of infected herds from bulk milk samples appears to be more difficult than by the use of ELISA on blood samples. Bulk milk ELISA results are consistently lower than the corresponding bulk serum ELISA results **(*Salimi-Abejestani et al., 2005*).** Using a commercial kit with f2-antigen, it has been demonstrated that only bulk milks from herds with infection prevalence of at least 60 per cent were Identified **(*Reichel et al., 2005*).** With another test it was possible to identify herds in which more than 25 per cent of the cows were infected.

2.6.4.2.2. Dot ELISA:

More rapid, economic, direct and visually read, improved ELISA technique for the diagnosis of parasitic diseases as microenzyme-linked immunosorbent assay (dot-ELISA) was described by ***Rokni et al., (2006*).** This technique for diagnosis of bovine fascioliasis was used by ***Latchumikanthan et al., (2012*).** They cleared that nano-gram quantity of parasite antigen dotted onto a very small piece of nitrocellulose

membranes were considered enough to obtain a marked direct and accurate diagnosis for the parasite directly. ***Zimmerman et al.,* (*1985*)** used E/S antigens of *Fasciola hepatica* for diagnosis of ovine fascioliasis by Dot-ELISA. In that study, antibodies against the antigens were consistently detected by 4 weeks after the sheep were inoculated. ***Morilla et al.,* (*1989*)** evaluated Dot-ELISA in naturally and experimentally infected sheep using E/S antigen. The infected sheep gave very high titers, from 1:25600 to 1:204800, which was 1000 to 2000 times higher than with PHT (passive haemagglutination test) or with TIA (thin layer immunoassay). ***Shaheen et al.,* (*1989*)** applied partially purified antigens from a species of Fasciola at 180 ng protein/dot (2 μL) and serum samples at 1:20 dilution (1 μL) for diagnosis of human fascioliasis. The sensitivity of the assay was 100% and its specificity was 97.8%. The major cause of low specificity was human schistosomiasis, which showed cross reactivity with Fasciola infection.

***Dalimi et al.,* (*2004*)** evaluated *F.gigantica* partially purified fraction antigen (PPF) isolated from sheep liver flukes for the diagnosis of human fasciolosis. They found that the best sensitivity and specificity (94.23% and 99.36%) was obtained at the 1:800 sera dilution as the cut-off titer.

ES Ag was the best coating antigen in Dot-ELISA for the sero-diagnosis of fasciolosis in cattle due to its high sensitivity, specificity, and precision rates **(*Yamchi, 2014*).** Recently, native cathepsin-L cysteine proteinase was purified from the excretory secretory products of *Fasciola* and applied for sero-diagnosis of *Fasciola* infection in buffaloes using Dot-ELISA. The results demonstrated that cathepsin-L cysteine proteinase based Dot-ELISA achieved 90.00% sensitivity and 100% specificity **(*Varghese et al., 2012; Swarup et al., 1987*).**

Table 2.1 Immunodiagnosis: Antibody detection

	Antigen catalogue	Techniques	Agents	Sensitivity%	Specificity%	References
1	Somatic antigen	ELISA	Sheep serum		95	Cornelissen *et al.*, 1992
		IHA	Sheep serum		86	
		ELISA	Human serum	100	98	Maleewong *et al*., 1996
2	Crude execretory secretory products (ES)	ELISA	Sheep serum		95	Cornelissen *et al.*, 1992
		IHA	Sheep serum		86	
		ELISA	Cattle serum	98	96	Salimi-Bejestani *et. al.*, 2005a
		ELISA	Human serum	100	99.3	Maleewong *et al*., 1996
		ELISA	Cattle serum	87	90	Charlier *et al.*, 2008
3	27 KDa antigen (purified from ES)	ELISA	Human serum	100	100	Maleewong *et al.*, 1999 Tantrawatpan *et al*., 2003
		Dot-ELISA	Human serum	100	98.2	Intapan *et al.*,2003
4	Cathepsin L1 (purified from ES)	ELISA	Sheep serum	100	100	Mezo *et al.*, 2003
		Dot - ELISA	Sheep serum	to detect *F. hepatica* antibody from 3 weeks post-infection		
		ELISA	Human serum	Marker antigen for diagnosis And epidemiological surveys		O'Neil *et al.*, 1998; Strauss *et al.*, 1999; Rokni *et al.*, 2002
5	Fas 2 antigen (purified from ES)	ELISA	Human serum	95.5	86.6	Espinoza *et al*., 2005
			Bovine milk	95.	98.2	Reichel *et al*., 2002
6	28 KDa antigen (purified from ES)	ELISA	Sheep serum	100		Dixit *et al.*, 2002
		Western bot	Sheep serum	100		
			Dipstick-ELISA	100		
7	rCTL1 (recombinant cysteine proteinase) *F.hepatica*	ELISA	Human serum	100	100	O'Neil *et al.*, 1999
8	rCLT1 (recombinant cysteine proteinase) *F.gigantica*	ELISA	Human serum	100	98.92	Tantrawatpan *et al.*, 2005
9	Synthesized Antigen. Peptides L and V from *F.gigantica*	ELISA	Human serum	100	97.3	Carnevale, *et al.*, 2001; Intapan *et al*., 2005

Table 2.2 Immunodiagnosis: Antigen detection:

NO	**Monoclonal Antibody catalogues**	**Techniques**	**Agents**	**Sensitivity**	**Specificity**	**Detection time**	**References**
1	MAb ES78	Sandwich ELISA	Human Serum	detects 10 ng/ml antigen	100%	78.6% (11/14) becomes negative 2 months after treatment	Espino *et al.*, 1990;1994
			Human stool Sample	92.8% (detects>15ng/ml antigen)	100%		
2	Monoclonal antibody to whole worm extract of *F. gigantica*	Sandwich ELISA	Cattle serum sample (experimental infection)			Positive from 3 weeks post-infection and negative 2 weeks after chemotherapy	Fagbemi *et al*., 1997
3	Monoclonal antibody (MoAb) 1C12 to 66 kDa surface tegumental (ST) antigenof *F.gigantica*	Sandwich ELISA	Cattle serum sample (experimental and natural infections)	86.6% (cut-off point of 32 uninfected cattle) 100% (cut-off based on commercial fetal calf and trematode-free baby calves sera)		Positive from first week post-infection	Viyanant *et al*., 1997
4	mAb M2D5/D5F10	Copro ELISA	Cattle stool Sample	300 pg of 26-28 kDa glycoprotein of *F. hepatica* (>10 flukes)		Positive from 6 weeks post-infection onwards	Abled-Rahman *et al*., 1998
5	mAb MM3	Copro ELISA	Sheep stool Sample	0.3 ng/ml of *F. hepatica* ES antigen (100% with 1 fluke)	100%	5 weeks postinfection onwards and undetectable from 1-3 weeks posttreatment	Mezo *et al*., 2004; 2007 Valero *et al*., 2009
			Cattle stool Sample	0.6 ng/ml of *F. hepatica* ES antigen (100% with 2 fluke)			
			Human Stool Sample	100%	100%		Ubeire *et al*., 2009
6	mAb Cathepsin L	Copro ELISA	Cattle stool Sample	95%	91%		Estuningsih *et al.*, 2009

Zimmerman et al., (1985) used E/S antigen of *F. hepatica* in dot ELISA format and antiparasite antibodies were detected by 2weeks PI in animals experimentally infected with 500 mc and 4weeks PI in sheep infected with 250 mc; further, in rabbits the antiparasite antibodies were detected even at initial dose of infection with 50 mc **(*Yadav and Gupta 1993*).** A previous document showed that sandwich-Dot- ELISA had better sensitivity and specificity than S-ELISA for both stool and serum, and may be used as a rapid screening test in field **(*El Amir et al., 2008*).**

2.6.4.2.3 Haemagglutination Test:

Haeagglutination (HA) test has also been used to diagnose and evaluate chemotherapy success in *F.hepatica* infected sheep and cattle. A specificity of 85% with this technique was obtained when a group of *F.hepatica* infected sheep was tested compared with 95% specificity obtained in an ELISA with E/S products of the parasite **(*Cornelissen et al., 1992*).** In other work haemagglutination titers as high as 1:204.800 were obtained in infected sheep while negative controls gave 1:800 **(*Arriage de Morilla, 1989*).**

The HA test using fractionated antigen **(*Levieux et al., 1992a*)** detected specific antibodies at 2-4 weeks after infection in cattle. These antigens were detected for up to 28 weeks **(*Levieux et al., 1992b*),** while in goats antibodies were detected 2-3 weeks per infection **(*Levieux and levieux, 1994*).** The authors suggest that the HA test may be useful for detecting infection in goats during the prepatent period.

2.6.5. Electrophoretic pattern as tool for diagnosis of fasciolosis:

The diagnosis of fasciolosis is generally carried out via coprological examination, and immunological technique like ELISA but such tests have many disadvantages. Pseudofasciolosis is the potential for misdiagnosis in such tests. Moreover, coprological diagnosis of

fasciolosis is possible from 8-12 week post-infection (WPI) .Though ELISA can recognize *F. hepatica* specific-antibodies since 2-4 week post-infection, thus providing early detection of the infection but there is some possibility of cross-reactivity with the schistosomiasis antibodies. Moreover, in many human infections, the fluke eggs are often not found in the faeces, even after multiple faecal examinations. Furthermore, eggs of *F. hepatica*, *F. gigantica* and *Fasciolopsis buski* are morphologically indistinguishable. In recent years, sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting procedures have initiated a new era inimmunodiagnosis which greatly reduced cross-reaction. These techniques were used as a verifying test in the diagnosis of viral and bacterial infections at first, but lately these techniques have been used in the field of parasitology **(*Sarimehmetoúlu, 2002*).**

Thus electrophoresis ensures sensitive potential tool for serodiagonosis of fasciolosis. Electrophoretic bands of 21 and 25KDA derived from metacercarial antigens (MAg) of *Fasciola gigantica* enhance the sensitivity and specificity of test for early diagnosis of fasciolosis. It can be used as promising alternative to conventional method of faecal egg detection .This will encourage early chemotherapy to save animal prior to damage in the form of traumatic hepatitis **(*Velusemy et al, 2006*).**The 8 kDa protein of *F.hepatica* obtained by gel electrophoresisisis suggested as one of the diagnostic antigens in human fascioliasis without cross-reaction with other human trematodiasis **(*kwangsig et al, 2003*).** A 28kDa cysteine proteolytic enzyme extracted from *F. gigantic*a by electrophoresis has a potential use for the serodiagnosis of ruminant fasciolosis as a supplement to the usual coprological methods. **(*Benjamin, 1995*)**.The 17kDa *F. hepatica* excretory secretory antigen is an excellent candidate for the immunodiagnosis of acute and chronic

fascioliasis. Purification of this antigen by electrophoresis and its application to quantitative serologic tests will permit further analysis of its predictive value to evaluate cure **(*Hillyer et al., 1988*).**

2.6.6 Molecular Diagnosis:

Several molecular tests to detect parasites have been developed in the last decade. Their specificity and sensitivity have gradually increased, and parasites that were previously difficult to diagnose using conventional techniques began to be identified by molecular techniques. As a result, currently these parasites can be easily treated before causing major harm to the infected population. Before the availability of PCR-based approaches, DNA probes were the alternative choice for the genotypic detection of *Fasciola* spp. **(*Heussler et al., 1993*).** However, DNA probe-based assays usually require the use of radioactive isotopes and can have bio-safety concerns. Over the last two decades, several PCR-based approaches including PCR-linked restriction fragment length polymorphism (PCR-RFLP), PCR-linked single-strand conformation polymorphism (PCR-SSCP) and specific PCR assays, have been developed for the accurate identification of *Fasciola* spp. **(*Ichikawa and Itagaki , 2010; Lin et al., 2007; Huang et al., 2004; Marcilla et al., 2002; Alasaad et al., 2011*)**. Recently, several specific PCR assays have been developed to differentiate *F. hepatica* from *F. gigantica* and detect *Fasciola* infections in the intermediate host snail and definitive hosts (such as buffalo), utilizing various genetic markers, such as *cox*1, ITS, non-coding repetitive DNA fragment as well as RAPD-derived sequences **(*Ai et al., 2010; Velusamy et al.,2004; McGarry et al., 2007*).**

For detecting *F. gigantica* or *F. hepatica* infection in snails, conventionally the snails are screened for cercarial shedding or teased for the microscopic examination of the developmental stages of the parasite. This process is tedious, expensive and lacks specificity, as earlier

intramolluscan developmental stages of the parasite cannot be specifically identified **(*Caron et al., 2007*).** Therefore, there is a role of PCR for the specific and early detection of infection in snails. A PCR assay was used to detect *F. gigantica* infection in the snail vector host, *Lymnaea auricularia* **(*Velusamy et al., 2004*).** The specific primers amplified a *F. gigantica* specific 124-bp non-coding repetitive DNA fragment from infected *L. auricularia* snails. ***Kaplan et al.,* (*1995*)** also identified a 124-bp repetitive DNA sequence which was used as a specific probe for detection of *F. hepatica* infections in intermediate host snails (*Fossaria cubensis* and *Pseudosuccinea columella*) **(*Kaplan et al., 1995*). *Baran et al.,* (*2012*)** determined prevalence of *Fasciola gigantica* infection in field-collected snails of *Radix gedrosiana* in northwestern Iran. A fragment of 618 bp of 28s rRNA gene was amplified by polymerase chain reaction (PCR). The PCR products were subjected to restriction fragment length polymorphism (RFLP) using DraII and AvaII enzymes. PCR-RFLP patterns revealed that 3.12% of the snails were infected with *F. gigantica*. It was also found that the infected snails had a limited distribution over the water bodies located in the central part of the region. It was concluded that PCR-RFLP was a reliable approach to detect Fasciola infection in pond snails and may be useful to establish control measures for livestock and humans' fasciolosis in the region. A multiplex PCR assay was able to detect *F. hepatica* DNA in *L. viatrix* snails, which were even formalin-fixed and paraffin-embedded **(*Magalhaes et al., 2008*).** TaqMan chemistry was adopted by ***Schweizer et al.,* (*2007*)** to establish a real-time PCR assay. The combined use of primers and probe targeting an 86-bp target of a repetitive 449-bp genomic DNA fragment facilitated the detection of *L. truncatula* naturally infected with *F. hepatica*. These PCR assays are highly specific and sensitive, providing useful and practical tools for the epidemiological investigation of *Fasciola* in the snail hosts

(*Schweizer et al., 2007*). Loop-mediated isothermal amplification (LAMP) allows amplification of target nucleic acids under isothermal conditions with high sensitivity, specificity, rapidity and precision, which has found broad applications for the detection of pathogens **(*Notomi et al., 2000; Nagamine et al., 2002*)**. ***Ai et al.,* (*2010*)** developed a LAMP assay for the sensitive and rapid detection and discrimination of *F. hepatica* and *F. gigantica*. The assay can be done in 45 min under isothermal conditions at 61°C or 62°C by employing a set of 4 species-specific primer mixtures and the results can be checked visually. This LAMP assay was approximately 10^5 times more sensitive than the conventional specific PCR assays, and may find applicability in the field settings or in poorly-equipped laboratories in endemic countries **(*Ai et al., 2010*)**.

A molecular technique (PCR) was applied to diagnose fasciolosis infection in sheep and goats, not only in the fecal samples but also bile samples were used to confirm the *Fasciola* spp. in Pakistan **(*Shahzad et al., 2012*)**. Forty three (10.75%) bile and fecal samples were found positive for *F. hepatica* through PCR and out of these 43 samples, 27 (13.5%) were positive from bile samples where as 16 (8%) were positive from fecal samples. All samples positive by microscopic method were also positive by PCR, whereas, out of the 43 samples positive by PCR test, 11 were negative by microscopy. In conclusion, PCR for the detection of *F. hepatica* is specific and sensitive. The test is suitable for tracing infected animals and provides a quantitative validated measure that is useful in epidemiological surveys and follow up for drug treatment in cattle, buffalo, sheep and goats. In addition, it would be useful for designing fasciolosis control programs in endemic areas.

2.6.7. Immunodiagnosis of *Fasciola gigantica* in White Nile province in Sudan:

***Osman,* (*2010*)** evaluated the potentially of some immunological techniques in the diagnosis of Fasciolosis in cattle in the area of Rabak, White Nile province, Sudan. Firstly the samples were subjected to feacal examination using sedimentation method. The result revealed that 41% of the slaughtered animals, 31.6% at livestock farms and household cattle and 30.9% at veterinary hospital were infected with *F.gigantica*. Overall infection was 34.2%. Secondly a total of 224 were examined at postmortem, one hundred and thirty of these cattle were found infected with *F.gigantica* and 94 were negative. For the validation of indirect ELISA assay, the positive references sera considered of these 130 positive samples at postmortem and the negative reference sera were the 94 negative samples. The first indirect ELISA was based on Saposine like protein recombinant antigen (SAP). The result indicated that 111 out of 130 references positive were positive with the (SAP) giving a sensitivity of 85% , while the number of negative samples at postmortem that react negative in the ELISA test were 81 with a specificity of 86%. The second ELISA was based on execretory and secretory antigen. The results indicated that 104 out of 130 references positive were positive with the ES antigen giving a sensitivity of 80% while, the number of negative samples at postmortem that react negative in the ELISA test were 50 with a specificity of 53%.

2.7. Resistance to Fasciolosis:

Resistance to fasciolosis is mediated by various immune and nonimmune mechanisms.While many mammalian species can be infected with fasciola; there is a wide variation in their degree of suscepility to infection, and in their ability to acquire resistance to re-infection. Natural resistance to fasciolsis differs according to animals' species. For example sheep exhibits a lower degree of natural resistance while cattle and goats develop significant levels to resistance **(*Haroun and Hillyer, 1986*).** In

contrast to sheep cattle are capable of eliminating an initial infection and are also found to resist re-infection **(*Boray, 1969; Goreish et al., 1988*).** So cattle rarely die from liver fluke due to the development of self-cure phenomenon between 9 and 26 months after infection. This self-cure is most likely related to the calcification and thickening of the bile duct walls that is observed in chronically infected cattle and not observed in sheep. Fibrosis of the liver capsules prevents fluke penetration into liver parenchyma **(*Anderson et al., 1978*)** and the resistance to re-infection in cattle is mediated by this physical barriers.

Sheep often die from acute fasciolsis, while some infections can last as long as 11 years **(*pantelouris, 1965*).** However, there have been some reports of differing levels of susceptibility of sheep with different genetic backgrounds to liver fluke infection **(*Boyce et al., 1987*).** Most notably, Javanese thin-tailed sheep have been found to be highly resistant to infection with *Fasciola gigantica* **(*Wiedosari and Copeman, 1990; Roberts et al., 1997*).**

2.8. Immunology:

2.8.1 The Host Immune System:

The host immune system protects it from invading pathogens using layers of defense mechanisms of increasing specificity. Initially, physical barriers, such as the epithelium, prevent the infectious agents from entering the host. If these are breached the hosts' innate, followed by the adaptive, immune system act to try to expel the pathogen. The innate immune system is fast acting and uses non-specific defense mechanisms to expel invading bodies. The specific acquired immune system is slower acting but highly adaptable **(*Mulcahy et al., 1999*).** CD4+ T helper cells (Th), B lymphocytes (B cells) and T regulatory (Treg) lymphocytes have critical roles in host defence and immunoregulation **(*Hirahara et al., 2011*).** T cells are a major source of cytokines and bear receptors (TCR)

which recognize antigen peptides (T cell epitopes) in association with major histocompatibility complex (MHC) presenting cells **(*Bhattacharya and Sinha, 2006*)**.Th cells can be divided into subsets which each differ in phenotype and function, e.g. Th1, Th2 and Th17 cells **(*Hirahara et al., 2011*)**.

The Th1 cells produce interferon-γ (INF- γ), interleukin-2 (IL-2) and interleukin-3 (IL-3) which promote a type 1 immune response **(*O'Neill et al., 2000*)**. These cytokines promote the production of activated macrophages, antibodies; mediate delayed type hypersensitivity reactions and inflammatory responses **(*O'Neill et al., 2000*).** This response is often elicited against invading intracellular organisms (viruses, bacteria and protozoa) but can also be effective against extracellular organisms. For example, induced immunity to schistosomiasis in mice is mediated by activated macrophages, which can kill larval *Schistosoma mansoni* in vitro **(*James et al., 1982*).**

Th2 cells produce a number of cytokines including interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and interleukin-10 (IL-10) which promote a type 2 immune response. This has been shown to be important in the control of helminth infections **(*Urban et al., 1995; Estes et al., 1994; Mulcahy et al., 1999*)**. The cytokines promote B cell proliferation, the secretion of immunoglobulins (IgA, IgG1 and IgE) and mediate production / activation of mast cells and eosinophils. Eosinophils bind to antibodies on the surface of extracellular organisms, such as *F. hepatica* and release compounds, such as nitric oxide which are toxic to the invading pathogen **(*Anthony et al., 2007; Mulcahy, 1999*).**

The cytokines released by the different T cell subsets regulate the type of immune response generated. For instance, the type 1 cytokine INF- γ suppresses type 2 responses where as IL-4, IL-10 and IL-13 inhibit the

effects of INF- γ and thus the development of type 1 responses **(*Mulcahy, 1999*)**.

2.8.2 Parasite Evasion of Immunogenic Attack:

Flukes have evolved a number of mechanisms to evade the immune system and thus survive for long periods within their host **(*Fairweather and Boray, 1999*)**. However, the ways in which the parasite evades or modulates the immune system are not fully understood. The final residence in the bile ducts is a relatively immunologically 'safe' environment from immune attack **(*Hanna, 1982*)** for the parasite but, to get there, it must evade the immune system as it migrates through the intestinal wall and liver tissue **(*Haroun and Hillyer, 1986*)**. Only 5-10% and 20-25% of the inocula in cattle or sheep, respectively, reach maturity in experimental infections, indicating that a large proportion of the NEJ either fail to enter the gut or are killed during the migrating phase **(*Haroun and Hillyer, 1986; Piacenza et al., 1999*).**

The tegument of *F. hepatica* differs from that of related species such as the schistosomes. Instead of the schistosomes' two-lipid bilayer (thin layer composed of two layers of hydrophobic molecules) liver flukes have a single surface membrane covered with a tough glycocalyx (carbohydrate-based structure) **(*Threadgold, 1976*).** This tough tegument protects the liver fluke from the immune system in a number of ways. Using surface radio-labelling techniques, ***Dalton and Joyce* (*1987*)** showed that the glycoproteins on the surface of the NEJ, immature (liver) and the adult (bile duct) stage differed. So, as the parasite develops from a NEJ through to the adult stage, the surface composition alters, presenting the immune system with a changing target, therefore protecting the parasite from immune recognition by specific antibodies **(*Tkalcevic et al., 1995*).** The glycocalyx on the surface is also shed and replaced

approximately every 3 hours as the parasite migrates to the bile ducts **(*Hanna, 1980*).** This prevents the definitive host's immune defense mechanisms, such as the eosinophils, making sufficient contact with the fluke to cause damage to their surface **(*Hanna, 1980*).** In addition to this, the glycocalyx shed from the parasite binds any circulating antibodies, which then prevents further up regulation of the host immune response **(*Duffus and Franks, 1981*).**

The tracts made by flukes in the liver tissue are filled with immune effecter cells such as T and B lymphocytes, macrophages and granulocytes (eosinophils and neutrophils) but these are not attached to any of the parasites present **(*Meeusen and Brandon, 1995*).** Flukes from a secondary infection (where a host which has previously been infected with *F. hepatica* but the infection was cleared and the host re-infected) are never found in cavities generated by flukes in the primary infection which indicates flukes may avoid areas where there are high levels of immune response mechanisms **(*Meeusen and Brandon, 1995*)**. Parasites may also modulate short range immune responses; this may explain the presence of undamaged flukes in tissues filled with immune effector cells **(*Meeusen and Brandon, 1995*).** The way in which the parasites do this is not fully understood, but they may secrete enzymes such as glutathionine *S*-transferase to deactivate the effector cells **(*Brophy et al., 1990; Creaney et al., 1995*)**. ***Carmona et al.,* (*1993*)** identified a cathepsin L protease in NEJ excretory secretory (E/S) material and showed that it can prevent antibody-mediated attachment of eosinophils to this stage, thus evading immune detection.

Furthermore, parasites release immunosuppressive factors to modulate the definitive host's immune system **(*Sandeman and Howell, 1981; Zimmermann et al., 1983*).** As an infection proceeds in an ovine host the proliferative capacity of the peripheral blood lymphocytes reduces after

just 4 weeks of infection, indicating that modulatory effects occur as the parasite migrates to the liver tissue **(*Zimmermann et al., 1983; Mulcahy et al., 1999*)**. In addition, the fluke's E/S products can block the differentiation and maturation of eosinophils by bone marrow cells in mice **(*Milboume & Howell, 1990; Milboume & Howell, 1993*).** A 28kDa protein isolated from the E/S fluid can mimic the action of IL-5 **(*Milboume & Howell, 1993*)** and, thus, direct the definitive host's immune system to a less effective Type 2 response, allowing the host to tolerate the parasite leading to chronic infection **(*Clery et al., 1996*).** Furthermore, the *F. hepatica* E/S proteases, cathepsin L1 and cathepsin L2, can also degrade all subclasses of human IgG, which assist the parasite in evading immune attack **(*Berasain et al., 2000*).**

2.9 Cross Reaction as a Common Phenomenon among Tissue Parasites:

Infection of animals with parasites continues to cause worldwide great economic losses. Accurate serological diagnosis of parasitic infections is a good initial approach to improve its control. However, the low specificity of current serological techniques constitutes a problem due to cross-reactions among animals with different infections. Cross-reactivity is a widely spread trait among phylogenetically related and unrelated parasites. It emerges from the wide existence of common antigens, which suggest that antigenic continuity is the rule rather than the exception. One explanation of this phenomenon is that all parasites emerged from common components at the beginning of creation. With the evolution and under stress of host immune responses, some of these parasites acquired exclusive molecules to defend themselves against these responses. Therefore the existence of common structure is the rule. Analysis of cross reactivity among parasite is of significance for understanding the

evolutionary conservation of antigens. Although cross reaction has a negative impact on accurate diagnosis, the utilization of monoclonal antibodies as well as pure fractions of the parasitic extracts in the serological discrimination between different infections could participate in minimizing this drawback **(*Mezo et al., 2003 and Dalimi et al., 2004*)**. Moreover, adoption of more sensitive techniques rather than the conventional ones in the diagnosis or detection of parasite antigens or its DNA, also share in eliminating cross reactivities in diagnosis assays. Examples for cross-reactivity between some tissue parasites infesting farm animals were documented such as *Taenia* and *Echinococcus*; *Taenia saginata, Taenia solium* and *Echinococcus granulosus, Trichinella spiralis* and *Toxoplasma gondii*. Cross-reaction is not only restricted to species belong to the same phylum, but also extended to helminthes of different phyla such as the high degree of cross-reactivity between sera of animals infected with *T. solium, Hymenolepis nana* and *E. granulosus.* Also, a cross-reaction between three important zoonotic helminthes *Fasciola gigantica, T. spiralis* and *E. granulosus* was recorded **(*El-Moghazy and Abdel-Rahman 2012*).** Despite their drawbacks in accurate immunodiagnosis, extensive similarities in the antigenic composition among parasites often permit the use of a diagnostic antigen from one species potentially to protect from another. This approach solves the problem of antigen scarcity. Evidence for protective immune cross-reactivity between *Schistosoma mansoni* and *Fasciola hepatica* is well documented. The low molecular weight cross-reactive component of 14 KDa isolated from *S. mansoni* (Sm14) could form the basis of a single effective cross- protective vaccine against both parasites based on its proved prophylactic potentials **(*El-Moghazy and Abdel-Rahman 2012*).** Consequently, optimism of developing multi-purpose vaccine candidates

against parasitic infections, in the near future, is a reality rather than imagination.

2.10. Paramphistomosis:

Paramphistomes or stomach flukes are conically shaped digenetic trematode parasites belonging to the superfamily, Paramphistomatidae **(*Hafeez 2003*).** Like other digenetic trematodes, paramphistomes require a snail to complete the life cycle. The immature flukes of this parasite live in the small intestine and abomasums from where they migrate to reticulum and rumen and mature into adults there.

They are fleshy, pear-shaped, measuring 5-12mm (length) x 2-4mm (width) and are pink or light red in colour, Juvenile flukes are however smaller (1-2mm long).

Paramphistomosis is distributed all around the world, but its highest prevalence has been reported in tropical and subtropical regions particularly in Africa, Asia, Australlia, Eastern Europe and Russia **(*Boray, 1959, 1969; Horak, 1971; Gupta et al., 1978*).**

Paramphistomosis has been a neglected trematode infectious disease in as an important cause of productivity loss, that cause high morbidity and mortality and by affecting health, production and reproduction of ruminants particularly in young stock. **(*Jehangir et al., 2016*).** Older animals can however develop resistance but may still harbor numerous adult flukes in the rumen and reticulum without showing overt symptoms, however in case of heavy infection, damage to the rumen has been recorded in the form of unthrifitness, emaciation, lower feed conversion rate, decrease in milk yield and reduction of fertility **(*Meshgi et al., 2009*; *Kamaraj et al., 2010*; *Sanchis et al., 2012*)**. In case of acute infection, large number of immature flukes while migrating through the intestinal tract causes acute parasitic gastroenteritis especially in young ruminant

(*Ilha et al., 2005; Khan et al., 2008*). Also the young flukes in the duodenum and upper ileum are plug feeders and they embedded in the mucosa causing necrosis and haemorrhage and may reach the muscular coat. Thus haemorrhagic duodenitis may occur in heavy infection **(*Soulsby, 1984*).**

A transitory diagnosis of paramphistomosis is based on the history and clinical signs of the disease. Further confirmation can be obtained by examining the faecal samples for the presence of parasite eggs. However, this method is unreliable because the parasite eggs are not found during prepatent period and hence often results in misdiagnosis. Moreover, this method lacks sensitivity, especially in light infections or during subclinical disease **(*Horak, 1971; Bida and Schillhorn van Veen, 1977*).** Early diagnosis of paramphistomosis is necessary for rapid treatment before irreparable damage to the rumen and bile duct occurs **(*Wang et al., 2006*)**. Different immunodiagnostic tests such as ELISA, immunofluorescence assay have been used in the early immune diagnosis of paramphistomosis, but they have some disadvantages, such as cross reactions with other trematodes, leading to false positive results **(*Hillyer, 1985*)**. In recent years, SDS-PAGE and western blot procedures have created a new era in immunodiagnosis and greatly reduced cross reaction. ***Sanchis et al.,* (*2012*)** recorded that ELISA sensitivity and specificity using secretory excretory antigen of Paramphistomum were 82 % and 79 % respectively. Also ***Hussan et al.,* (*2005*)** recorded 82 % sensitivity of *Paramphistomum somatic* antigen but they recorded higher specificity of 90%. On the other hand, ***Shivjot et al.,* (*2009*)** in their study showed higher sensitivity 85.71% and a lower specificity 23.65% using somatic antigen of *Paramphistomum* and ***Tariq et al.,* (*2011*)** who reported sensitivity of dot ELISA as 100 % for detection of *Paramphistomum cervi* antibodies in hyper immune rabbit using crude antigen of

Paramphistomum. ***Salib et al., (2015)*** concluded that ELISA is more specific and accurate but less sensitive than western blotting for the diagnosis of Amphistomes infection in cattle and buffaloes. The sensitivity, specificity and accuracy for ELISA and western blotting were (74% and 100%), (82.4% and 33.3%) and (79.76% and 77.78%), respectively.

CHAPTER THREE

CHAPTER THREE

3. Materials and Methods

3.1 Collection of Parasites:

Infected livers with Fasciola were obtained from sheep and goats in White Nile Province and from three cattle types of three different localities of Sudan, e.g. Niyala type (Western Sudan), White Nile type (Southern Sudan) and Mangisto type (Eastern Sudan). Adult Fasciola worms were collected from the bile ducts of infected livers at slaughterhouses. They were washed six times with 0.01 M phosphate buffer saline solution (PBS, pH 7.2) at room temperature. Then the flukes were used to prepare the parasitic products.

3.1.1 Preparation of the Parasite Products (Antigens):

3.1.1.1 Execretory /Secretory Products (E/S):

Isolated adult parasites of both *F.gigantica* and Paramphistomum *spp.* were washed six times with 0.01M PBS, pH 7.2 and further six times with RPMI-1640 medium (Rose Marry memorial Institute Media). The worms were then incubated overnight in culture flasks in RPMI-1640 medium with 2% glucose, 30mM Hepes and 25mg/ml gentamycin per milliliter of medium (one worm per 2.5 ml) at 37˚C. Following the incubation period, the culture medium was harvested, centrifuged at 14900 Xg for 30 minutes and the supernatant was estimated for protein concentration by using the Thermo Scientific™ Micro BCA Protein Assay Kit. The antigen was then aliquoted and stored at -20˚C till use.

3.1.1.2 Somatic Products:

After collection of Fasciola E/S antigens, the worms were separated and ground in dry ice using a pestle and morter. Approximately four volumes of PBS were added and the ground material was left to stand for

30 minutes with occasional shaking on ice. The soluble somatic extract was collected by centrifuging the homogenate at 5000g at 4˚C. After dialysis, the homogenate was centrifuged again for 500g at 4˚C for 15 minutes. The protein concentration of antigens was measured by using the Thermo Scientific™ Micro BCA Protein Assay Kit. The prepared antigens were then aliquoted and stored at -20□c until use.

3.1.2 Determination of Antigen Protein Concentration:

The Thermo Scientific™ Micro BCA Protein Assay Kit (Product No. 23225), was used for the quantification of the total protein concentration in the E/S and somatic antigens as follow:

3.1.2.1 Preparation of the Micro BCA Working Reagent (WR):

The WR was prepared by mixing 25 parts of Micro BCA Reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1, Reagent MA:MB:MC). 150µL was pippated of each standard or unknown sample replicate into a microplate well (Product No. 15041). 150µL of the WR were added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered using sealing tape for 96-Well Plates (Product No. 15036) and incubated at 37°C for 2 hours. Then the plate was cooled to room temperature and the absorbance was read at or near 562nm on a plate reader.

The calculation of standard curve was made by plotting the average reading for each BSA standard vs. its concentration in µg/mL. The standard curve was used to determine the protein concentration of the samples. The curve was made by Microsoft Excel programme.

3.1.3. Sodium, Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Somatic and E/S antigens were run by 10% acrylamide gel slabs containing SDS as described by (Laemmli, 1970). Details of standard

protocol regarding the preparation of polyacrylamide gel were illustrated in (Tables 3 and 4). The resolving gel (Table 3) was poured till 3/4 of the plate's length approximately; small layer of isopropanol was added on the top of it directly prior to polymerization. The purpose of isopropanol was to produce a smooth, completely level surface on the top of the resolving gel, so that bands were straight and uniform.

Before adding the stacking gel (Table 4), the isopropanol was poured off the resolving gel into a sink and excess isopropanol removed from the surfaces by rinsing the top layer of the gel with water and dry off as much of the water as possible by using filter paper. Then the stacking gel was poured to the top and the comb was inserted between the plates, taking care not to catch bubbles under the teeth.

For sample preparations, the two antigen extracts were diluted with equal volume of samples buffer (2% SDS and 0.1 M dithiotreitol, sigma chemical Co.) in tris buffer (pH 6.8) and heated for 3 minutes at 100˚C.

After 20 -30 minutes the comb was removed and the wells were washed with water. Twenty micrograms of protein of each E/S and SO products were loaded slowly in each well of separate gel. 5ul of molecular weight standard markers (Roti®-mark prestained, Roth) were run in one of the outside lanes of the gel. Samples were electrophoresed at 100 V in SDS running buffer until the lowest molecular weight standard band had migrated to approximately 1cm from the bottom of the gel; about 60 minutes. The gels were then stained with Coomasie blue (Sigma chemical Co.) at room temperature overnight to visualize the different bands. The gels were then destained with destaining solution several times to wash the excessive stain and to clarify the bands.

Preparation of Polyacrylamide Gel:

Table 3.1: Resolving Gel: (10ml)

Component	**Quantity**
Acrylamide	4.2ml
dH2O	3.2ml
Resolving buffer	2.5ml
SDS 10%	100 µl
Ammonium persulfate (APS) 10%	100µl (10ul/ml) 0.1gm/ml
TEMED	14µl

Table 3.2: Stacking Gel: (8ml)

Component	**Quantity**
Acrylamide	1.3ml
dH2O	4ml
Stacking buffer	2.5ml
SDS 10%	100µl
Ammonium persulfate (APS) 10%	100µl (10µl/ml)
TEMED	10µl

3.2. Collection of Samples:

One hundred fifty six and ninety two rectal faecal and blood samples were collected from both sheep and cattle, respectively at Rabbak and Kosti (White Nile State, Sudan) an area known to be endemic with fasciolosis. Faecal samples were transported to the laboratory and stored at 4˚C until the test was performed within 48 hours. The sera were separated from the blood samples and stored at -20˚C for further studies.

3.2.1 Coprological Examination:

Detection of *F.gigantica* and paramphistomum eggs in faeces was carried out by sedimentation method using (Brumpt' s technique) 3 gm of faeces were weighed and placed in a container. 40-45 ml of tap water was added, mixed and filtered through a tea strainer. The filtered material was placed in test tube for 5 minutes. Then the supernatant was removed carefully. The sediment was stained with drop of methelene blue, transferred to slide with a coverslip and examined under the microscope for the presence of *F.gigantica* and *Paramphistomum* eggs.

3.2.2 Antibody detection by Indirect ELISA:

Each well of a polystyrene microtiter plates (Greneir, Germany) was filled with 100 µl of 5mg/ml of either E/S or SO products of adult *F.gigantica* and Paramphistomum ES antigen in 0.1 M carbonate/bicarbonate buffer (pH 9.6). The plates were incubated overnight at 4˚C (Harlow and Lane 1988).The wells were washed three times with PBST (PBS pH 7.2, 0.05% Tween-20) to get rid of excess unbound antigen and the remaining free binding sites were blocked with blocking buffer (3% skimmed milk in PBS), 200ul/well for 1 hour at 37˚C. The plates were again washed three times with PBST. Sera samples collected from different sources were diluted (1:100) in 1% skimmed milk prepared in PBS (pH 7.2) and 100µl of each diluted serum was dispensed as duplicate in each well and incubated at 37°C for 1h. Wells

were washed again three times with PBST and 100ul/ml of the conjugate (1:10000 dilutions in blocking buffer, 7.4) were added to all wells and incubated for 1 hour at 37˚C. Finally after three washes with PBST, 100ul of the substrate TMB were added to all wells. The plates were incubated 10 minutes at 37˚C. The reaction was stopped after 2 minutes using 50µl of 2M sulphuric acid in each well. The absorbance readings were taken at 450 nm using an ELISA reader. The data were expressed as the mean of the optical density. The limit for discriminating negative from positive results was determined by the mean value of the negative controls plus 2 standard deviations. The sensitivity and the specificity of ES/SO indirect ELISA was calculated as follows:

Sensitivity%= TP/TP+FPx100

Specificity%=TN/TN+FNx100

Table 3.3: Summary of the ELISA protocol:

Assay Steps	**Incubation Period**	**Incubation Temperature**	**Wash Steps**
Coat ES antigen	Overnight	4 ^{o}C	3X
Blocking	1 hour	37 ^{o}C	3X
Add Test sera	1 hour	37^{o}C	3X
Add conjugate	45 min	37^{o}C	3X
Add substrate	10 min	Room temp	-
Add Stopper	None	Room temp	-
Read reaction	At 450 nm		

CHAPTER FOUR

CHAPTER FOUR

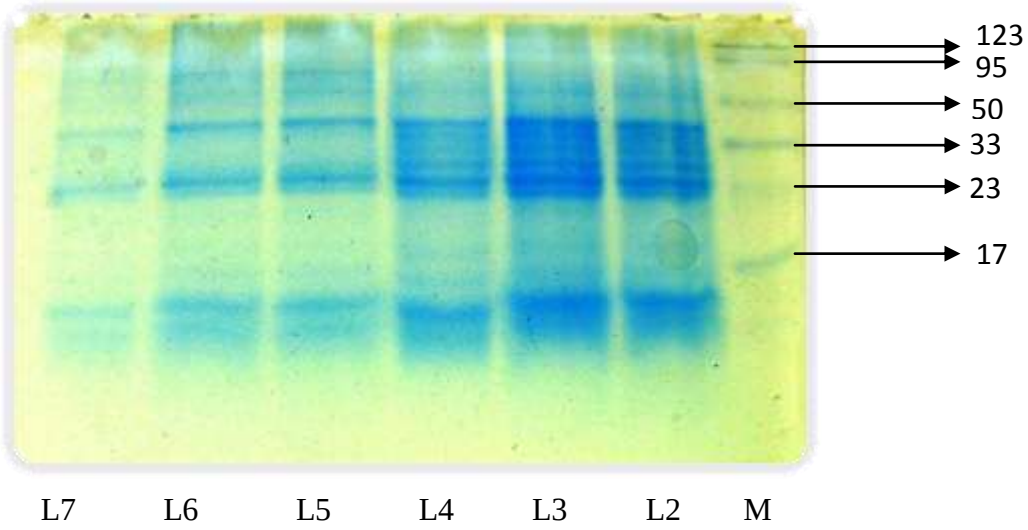
4. Results

4.1 Electrophoresis of *F.gigantica* ES/SO Antigens in Sheep and Goats Origin:

The electrophoretic profile of *F.gigantica* execretory secretory products (E/S) and somatic extracts (SO) from different isolates of sheep and goats under reducing conditions in 10% SDS-PAGE were illustrated in Fig (1, 2, 3, 4). SDS-PAGE analysis of E/S antigens of *F.gigantica* existing in both sheep and goats revealed different patterns: In sheep E/S protein had 12 major peptide bands with molecular weight of 14, 17, 19, 21, 24, 28, 31, 33, 40, 55, 62 and 72 KDa whereas, in goat E/S protein had nine bands with molecular weights of 12, 14, 17, 19, 21, 24, 40, 55 and 72KDa (Fig 1&2). Comparison between sheep and goat *F.gigantica* E/S antigen revealed eight common dominant bands (14, 17, 19, 21, 24, 40, 55 & 72KDa).

In contrast, *F.gigantica* somatic antigens in both sheep and goat revealed four common dominant bands (14, 28, 45 & 66 KDa) and three goat-specific bands (19, 38 & 50 KDa) (Fig 3&4).

Moreover, between E/S and SO in sheep, there were two common major bands with molecular weight of 14 & 28KDa. Only two distinct bands (14, 19) were shared by goat somatic and E/S *F.gigantica* antigens.



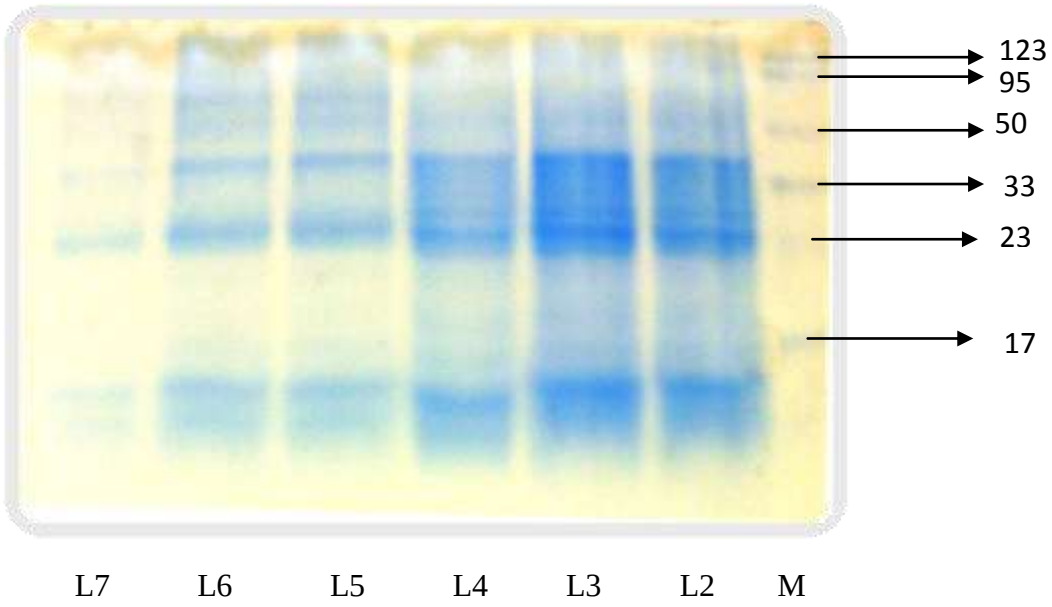


Fig 1&2: Protein profile of *F.gigantica* secretory/execretory extract from different liver of both sheep (lane2, 3, 4) and goat (lane 5, 6, 7), molecular weight marker (lane1)

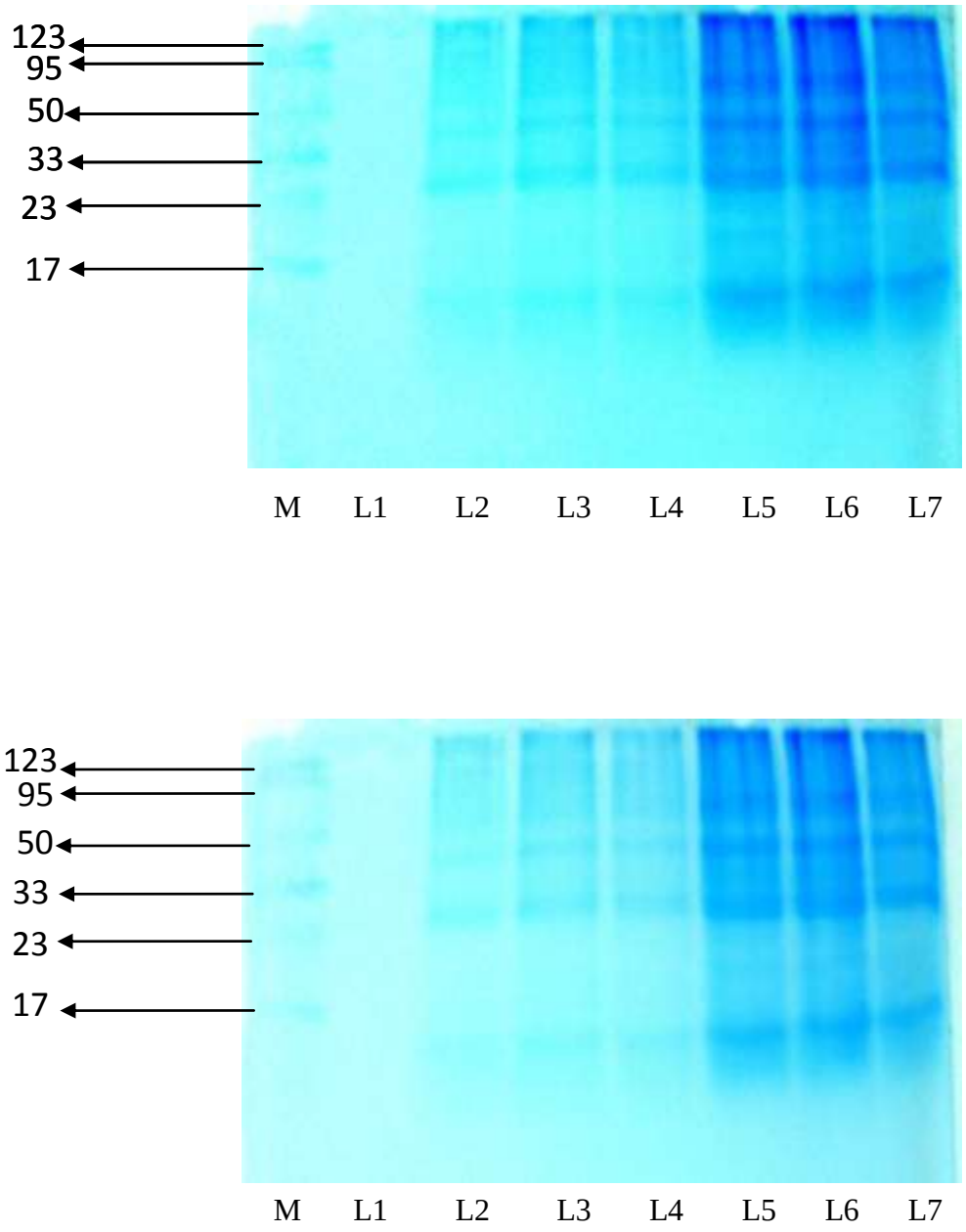


Fig 3&4: Protein profile of *F.gigantica* somatic extract from different liver of both sheep (lane 2, 3, 4) and goat (lane5, 6, 7), molecular weight marker M

4.2 Electrophoresis of *F.gigantica* ES/SO antigens in three different cattle ecotype:

Three common major bands with approximate molecular weight of 27-30, 45 and 66kDa were identified in somatic preparation of the parasite from three different bovine type (White Nile, Mangisto and Niyala) (Fig 5).

Bands of protein less than 17, 57, 95 and 110 kDa were only seen in the somatic extract of fasciola from Western cattle type, while Mangisto type showed 5 polypeptide bands with specific bands above 123 and 95kDa. Only one specific band (above 80) was seen in White Nile type.

Approximately similar electrophoretic pattern for E/S products of *F.gigantica* in the three cattle type, which included 7 common major protein bands with molecular weight of 17, 20, 23,27,33,40 and 80kDa. Nevertheless, the electrophoretic separation of the E/S antigen in both Mangisto and Niyala ecotype showed two different bands with molecular weight of less than 17, 45 and 87, 95 kDa, respectively.

4.3. Electrophoretic pattern of *F.gigantica* ES/SO antigens in cattle, sheep and goats:

The general conclusion of the electrophoretic migration pattern of *F.gigantica* E/S and SO extract from sheep, goats and cattle were characterized by several bands with molecular weights ranging from 12-95 and 14-123KDa, respectively. Dominant common bands for both ES and SO products were clustered between 27-30 KDa.

Major common bands were observed in SO extracts of *F.gigantica* in sheep, goats and cattle at 45 and 66, there were at least five bands 47, 80, 95, 110 and 123 KDa identified only in cattle, two bands 38 and 50 KDa

were goats-specific and only one band 14 KDa in sheep. The electrophoretic pattern also showed some similarities and some differences between ES protein extracts of *F.gigantica* existing in sheep, goat and cattle. Eleven bands with molecular weight of 14, 17, 19, 21, 24, 28, 31, 33, 40, 55, 62 and 77 were identified in sheep, where as ten bands with molecular weight of 17, 20, 23, 27, 33, 40, 45, 80, 87 and 95 KDa were showed in cattle. However nine bands with molecular weights of 12, 14, 17, 19, 21, 24, 40, 55 and 72 were found in goats. Dominant bands of *F.gigantica* ES extracts in sheep, goats and cattle were clustered between 17-24, 27-33 and 40 KDa. The differences between sheep, goats and cattle *F.gigantica* ES antigen revealed two common dominants bands 55 and 72KDa in both sheep and goats and four cattle-specific bands with molecular weights of 45, 80, 87 and 95 KDa.

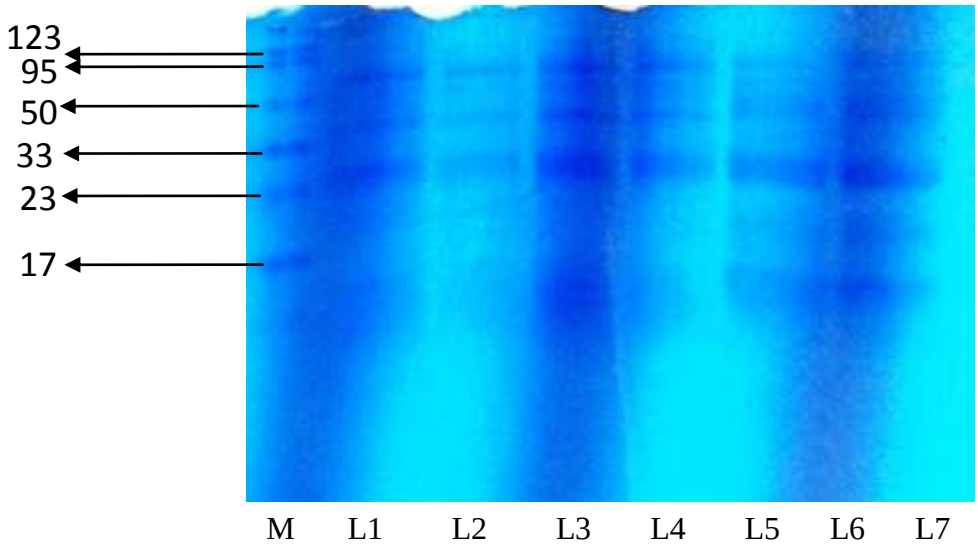


Fig 5: Protein profile of *F.gigantica* somatic extract from different livers of White Nile cattle type (lane1, 2), Mangisto type (lane3, 4) and Niyala type (lane5, 6)

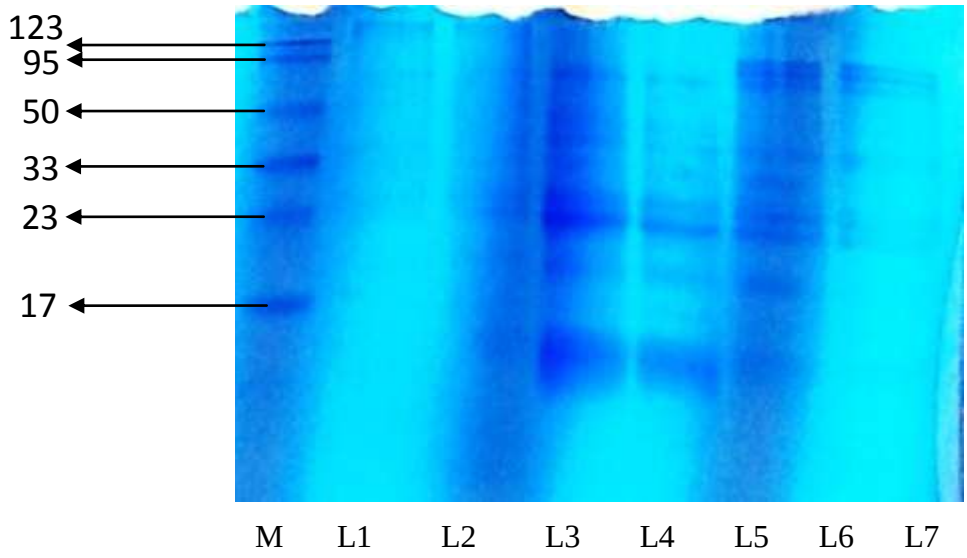


Fig 6: Protein profile *F.gigantica* secretory / extract of different livers of White Nile cattle type (lane1, 2), Mangisto type (lane 3,4) and Niyala type (lane 5,6)

4.4. Results of indirect ELISA and sedimentation tests in cattle:

4.4.1. Results of *Fasciola gigantica*:

Out of 156 cattle faecal samples examined, 30 were found positive with *Fasciola gigantica* indicating prevalence of 19.2%. Using ES Fasciola antigen in indirect ELISA test 47 out of 156 blood samples were positive indicating seroprevalence of 30%. Using SO Fasciola antigen in indirect ELISA test 29 out of 155 blood samples were positive indicating seroprevalence of 18.7%. (Tables 4.1&4.2)

The diagnostic performance of the indirect ELISA using ES *Fasciola gigantica* antigen to detect Fasciola infection was illustrated in (Table 6). When using ES Fasciola antigen 8 sera out of 30 positive for Fasciola by faecal examination resulted positive at indirect ELISA and faecal analysis indicating a sensitivity of 27%. Similarly, 87 sera out 126 negative for Fasciola by faecal examination resulted negative at both tests indicating a specificity of 69%. Furthermore, 39 sera samples out of 126 negative for Fasciola by faecal examination resulted positive at ELISA, while 22 sera samples out of 30 positive for Fasciola by faecal examination resulted negatively. (Table 4.1)

Assessing the validity of an indirect ELISA test for serological diagnosis of cattle fasciolosis somatic antigen was tested comparing with Fasciola faecal analysis (Table 4.2). We found that a total number of 102 sera out of 125 negative for Fasciola by faecal examination resulted negative at indirect ELISA test and faecal analysis indicating a specificity of 81.6%. Moreover, 6 sera out of 30 positive for Fasciola by faecal examination were positive at both tests indicating a sensitivity of 20%. We also observed that 23 sera out of 125 negative for Fasciola at faecal examination had positive values of indirect ELISA and negative by faecal

analysis, whereas, 24 sera samples resulted positive to faecal analysis and negative to ELISA test.

Table 4.1: Sensitivity and Specificity of indirect ELISA Test using ES Fasciola Antigen from Cattle

Count				
		ES fasciola		
		Negative ES ELISA Fasciola	Positive ES ELISA Fasciola	Total
Sed* Fasciola	Negative faecal Fasciola	87	39	126
	Positive faeca Fasciola	22	8	30
Total		109	47	156

Sed* Sedimentation

ES Execretory/Secretory

Sensitivity = 27% (8/30) Specificity = 69% (87/126)

Table 4.2: Sensitivity and Specificity of indirect ELISA Test using Somatic Fasciola Antigen from Cattle

Count				
		SO Fasciola		
		Negative SO ELISA Fasciola	Positive SO ELISA Fasciola	Total
Sed* Fasciola	Negative faecal Fasciola	102	23	125
	Positive faecal Fasciola	24	6	30
Total		126	29	155

SO Somatic

Sensitivity = 20 %(6/30) Specificity =81.6 %(102/125)

When the indirect ELISA data obtained from sera of cattle were analyzed by using ES and SO fasciola antigens (Table 4.3), it was observed that among 47 positive fasciola using fasciola ES antigen 22 sera were found positive for fasciola somatic antigen. While, 25 sera samples resulted negative for fasciola somatic antigen and positive for fasciola ES antigen. Similarly, among the 107 sera samples of negatively ES antigen 6 sera were found positive for fasciola SO antigen and 101 resulted negative for both.

By using ES *Fasciola gigantica* antigen versus paramphistomum faecal analysis (Table 4.4), it was found that 17 sera out of 46 positive for Paramphistomum by faecal examination were positive for both indirect ELISA test and faecal examination. Meanwhile, the number of negative sera samples that react negative in both faecal analysis and indirect ELISA were 80 out of 110 negatively Paramphistomum faecal samples. Moreover, 30 sera samples were positive by indirect ELISA and negative by faecal examination, while, 29 samples were negative by indirect ELISA and positive by faecal examination.

For performance of indirect ELISA using both ES Paramphistomun antigen and ES Fasciola antigen were given in (Table 4.5 & 4.6).The results detected by serological indirect ELISA test showed that among the

61 sera of positively Paramphistomum 26 sera were found positive for Fasciola, while 35 sera samples resulted negative for Fasciola and positive for Paramphistomum. Similarly, among the 95 sera samples of negatively Paramphistomum 21 sera were found positive for Fasciola and 74 sera resulted negative for both. Furthermore, the numbers of serologically positive Fasciola samples were 47. Among these positive

samples 26 were found positive for paramphistomum, while, 21 sera samples resulted negative for paramphistomum and positive for Fasciola.

Table 4.3: Cross-tabulation Result of indirect ELISA comparing ES and SO Fasciola Antigens from Cattle.

Count				
		SO Fasciola		
		Negative SO ELISA Fasciola	Positive SO ELISA Fasciola	Total
ES ELISA Fasciola	Negative ES ELISA Fasciola	101	6	107
	Positive ES ELISA Fasciola	25	22	47
Total		126	28	154

ES Execretory/secretory

SO Somatic

Table 4.4: Cross-tabulation Result of Cattle Faecal Examination for Paramphisomum and indirect ELISA using Somatic Fasciola Antigen.

Count				
		SO Fasciola		
		Negative SO ELISA Fasciola	Positive SO ELISA Fasciola]	Total
Sed* Param**	Negative Faecal Param**	80	30	110
	Positive Faecal Param**	29	17	46
Total		109	47	156

Sed* Sedimentation

Param** Paramphistomum

Table 4.5: Cross-tabulation Result of indirect ELISA using ES Paramphistomum Antigen versus ES Fasciola Antigen from Cattle.

Count				
		ES Fasciola		
		Negative ES ELISA Fasciola	Positive ES ELISA Fasciola	Total
ES Param**	Negative ELISA param**	74	21	95
	Positive ELISA param**	35	26	61
Total		109	47	156

ES Execretory/Secretory

Param** Paramphistomum

Table 4.6: Cross-tabulation Result of indirect ELISA using ES Fasciola Antigen versus Paramphistomum ES Antigen from Cattle

Count				
		ES Param**		
		Negative ES ELISA Param**	Positive ES ELISA Param**	Total
ES Fasciola	Negative ES ELISA Fasciola	74	35	109
	Positive ES ELISA Fasciola	21	26	47
Total		95	61	156

ES Execretory/secretory

Param** Paramphistomum

Among the 109 sera samples which were serologically negative for Fasciola 35 sera samples were found positive for Paramphistomum and 74 samples resulted negative for both antigens.

4.4.2. Results of Paramphistomum:

Out of 156 cattle faecal samples examined 46 were positive for Paramphistomum *spp.* indicating prevalence of 29.5%. However, by using ES Paramphistomum antigen in indirect ELISA test 61 out of 156 blood samples were positive indicating seroprevalence of 39 %.

After comparing data from ELISA using ES Paramphistomum antigen and faecal analysis, it was found that 20 sera out of 46 positive for Paramphistomum at faecal examination were positive for both tests giving a sensitivity of 43.4%. Meanwhile, the numbers of negative faecal samples that react negative in ELISA test were 69 with specificity of 62.7 %.(Table 4.7)

On the other hand, 41 samples of the sera analyzed had positive values of indirect ELISA and negative by faecal examination. Also 26 sera were negative by indirect ELISA and positive by faecal examination. Considering the ES Paramphistomum antigen versus *Fasciola gigantica* faecal samples (Table 4.8), a total number of 77 sera out of 126 negative for Fasciola by faecal examination resulted negative in both indirect ELISA and faecal examination. While, 12 sera samples out of 30 positive for Fasciola by faecal examination were found positive to indirect ELISA. Moreover, 49 of the sera samples analyzed had positive values of indirect ELISA and negative by faecal analysis, while 18 sera resulted negative at ELISA test and positive at faecal examination.

Table 4.7: Sensitivity and Specificity of indirect ELISA Test using ES Paramphistomum Antigen from cattle.

Count				
		ES Param**		
		Negative ES ELISA Param	Positive ES ELISA Param	Total
Sed Param**	Negative faecal Param	69	41	110
	Positive faecal Param	26	20	46
Total		95	61	156

Param** Paramphistomum

ES Secretory/Execretory

Sensitivity = 43.4% (20/46)

Specificity = 62.7 %.(41/110)

Table 4.8: Cross-tabulation Result of Cattle Faecal Examination for Fasciola and indirect ELISA Test using Paramphistomum ES Antigen

Count				
		ES Param**		
		Negative ELISA Param	Positive ELISA Param**	Total
Sed* Fasciola	Negative faecal Fasciola	77	49	126
	Positive faecal Fasciola	18	12	30
Total		95	61	156

Sed* Sedimentation

Param** Paramphistomum

ES Secretory/Execretory

4.5. Results of indirect ELISA and sedimentation tests in sheep:

4.5.1. Results of *Fasciola gigantica*:

Out of 92 sheep faecal samples examined 21 were positive for *F.gigantica* indicating prevalence of 22.8%. By using ES Fasciola antigen in indirect ELISA test 21 out of 92 blood samples were positive indicating seroprevalence of 22.8%. Moreover, by using SO Fasciola antigen in indirect ELISA test 19 out of 92 blood samples were positive indicating seroprevalence of 20.6%.

After comparing data from indirect ELISA using both ES and SO antigens versus faecal analysis (Table 4.9 & 4.10), considering ES antigen; it was observed that 6 sera out of 21 positive for Fasciola at faecal examination were positive for both tests indicating a sensitivity of 28.6%. Meanwhile, the number of negative samples that react negative in both faecal analysis and indirect ELISA were 56 with specificity of 78.9%. On the other hand, 15 samples of the sera analyzed had positive values of indirect ELISA and negative by faecal examination. While 15 sera out of 71 negative for Fasciola by faecal examination resulted positive. (Table 4.9)

When using SO *Fasciola gigantica* antigen 7 sera samples out of 21 positive at faecal examination resulted positive to both indirect ELISA test and Faecal examination indicating a sensitivity of 33%, while 59 sera samples were negative to indirect ELISA and faecal analysis indicating a specificity of 83%(59/71). Furthermore 12 sera samples out of 71 negative by faecal examination resulted positive at ELISA, while 14 sera samples out of 21 positive by faecal examination resulted negative. (Table 4.10)

Table 4.9: Sensitivity and Specificity of indirect ELISA Test using ES Fasciola Antigen from Sheep

Count				
		Fasciola ES		
		Negative ES ELISA Fasciola	Positive ES ELISA Fasciola	Total
Sed* Fasciola	Negative faecal Fasciola	56	15	71
	positive faecal Fasciola	15	6	21
Total		71	21	92

Sed* Sedimentation

ES Secretory/Execretory

Sensitivity 28.6% (6/21). Specificity 78.9% (56/71)

Table 4.10 Sensitivity and Specificity of indirect ELISA Test using Somatic Fasciola Antigen from Sheep

Count				
		Fasciola SO		
		Negative SO ELISA Fasciola	Positive SO ELISA Fasciola	Total
Sed* Fasciola	Negative faecal Fasciola	59	12	71
	Positive faecal Fasciola	14	7	21
Total		73	19	92

Sed* Sedimentation

SO Somatic

Sensitivity 33% (7/21) Specificity 83% (59/71)

When the indirect ELISA data obtained from sera of sheep were analyzed by using ES and SO Fasciola antigens (Table 4.11, it was observed that among 21 positive fasciola using ES fasciola antigen 4 sera were found positive for fasciola somatic antigen. While, 17 sera samples resulted negative for fasciola somatic antigen and positive for fasciola ES antigen. Similarly, among the 71 sera samples of negatively ES antigen 15 sera were found positive for fasciola SO antigen and 56 resulted negative for both.

Assessing the validity of an indirect ELISA test for serological diagnosis of sheep fasciolosis, both ES and SO fasciola antigens were tested comparing with Paramphistomum faecal result Table (4.12& 4.13). Based on ES antigen, it was shown that a total number of 60 sera out of 79 negative for Paramphistomum at faecal examination resulted negative in both indirect ELISA and faecal analysis. Meanwhile, 2 sera out of 13 positive for Paramphistomum at faecal examination were positive to both tests. We also observed that 19 sera out of 79 negative for Paramphistomum at faecal examination had positive values of indirect ELISA, whereas, 11 resulted positive to faecal analysis and negative to ELISA. Considering the SO *Fasciola gigantica* antigen, We also observed that 17sera out of 79 negative for Paramphistomum at faecal examination had positive values of indirect ELISA, whereas, 11 samples resulted positive to faecal analysis and negative to indirect ELISA. A total number of 62 sera out of 79 negative for Paramphistomum at faecal examination resulted negative in both indirect ELISA and faecal sedimentation test. While, 2 sera out of 13 positive for Paramphistomum at faecal examination resulted positive by indirect ELISA.

Table 4.11: Cross-tabulation Result of indirect ELISA Test comparing Fasciola ES antigen versus Fasciola Somatic Antigen from Sheep

Count				
		Fasciola SO		
		Negative SO ELISA	Positive SO ELISA	Total
Fasciola ES	Negative ES ELISA Fasciola	56	15	71
	Positive ES ELISA Fasciola	17	4	21
Total		73	19	92

ES Execretory/Secretory

SO Somatic

Table 4.12: Cross-tabulation Result of Sheep Faecal Examination for Paramphistomum and indirect ELISA test using Fasciola ES Antigen

Count				
		Fasciola ES		
		Negative ES ELISA Fasciola	Positive ES ELISA Fasciola	Total
Sed* Param**	Negative faecal Param**	60	19	79
	Positive faecal Param**	11	2	13
Total		71	21	92

Sed* Sedimentation

Param** Paramphistomum

ES Secretory/Execretory

Table4.13:Cross-tabulation Result of Sheep Faecal Examination for Paramphistoum and indirect ELISA Test using Fasciola Somatic Antigen

Count				
		Fasciola SO		
		Negative SO ELISA Fasciola	Positive SO ELISA Fasciola	Total
Sed* Param**	Negative faecal Param**	62	17	79
	Positive faecal Param**	11	2	13
Total		73	19	92

Sed* Sedimentation

Param** Paramphistomum

SO Somatic

In sheep, (Tables 4.14 &4.15) after comparing data from indirect ELISA using ES Paramphistomum and ES/SO *Fasciola gigantica* antigens we concluded that among the 20 sera of positively ES Fasciola 4 sera were found positive for Paramphistomum, while 16 sera samples resulted negative for Paramphistomum and positive for ES Fasciola antigen. Similarly, among the 67 sera samples of negatively Fasciola 6 sera were found positive for Paramphistomum and 61 sera resulted negative for both. Furthermore, the numbers of serologically positive SO Fasciola samples were 17. Among these positive samples 8 were found positive for Paramphistomum, while, 9 sera samples resulted negative for Paramphistomum and positive for SO Fasciola antigen. Among the 70 sera samples which were serologically negative for SO Fasciola antigen 2 sera samples were found positive for Paramphistomum and 68 samples resulted negative for both antigens.

4.5.2. Results of Paramphistomum spp.:

Out of 87 sheep faecal samples examined 12 were positive for Paramphistomum sp. indicating prevalence of 13.8%. Using ES Paramphistomum antigen in indirect ELISA test 10 out of 87 blood samples were positive indicating seroprevalence of 11.5%.(Table 4.16,4.17) The diagnostic performance of the indirect ELISA using ES Paramphistomum antigen to detect Paramphistomum infection is illustrated in table (21). The result revealed that 1 serum sample out of 12 positive at faecal examination resulted positive to both indirect ELISA test and faecal examination indicating a sensitivity of 8.3% (1/12), while 66 sera samples were negative to indirect ELISA and faecal analysis indicating a specificity of 88% (66/75). It was also found that 9 sera out of 75 negative by faecal examination had positive values of indirect ELISA; whereas, 11sera samples resulted positive to faecal analysis and

negative to ELISA. On the other hand, the indirect serological ELISA was assessed to detect Paramphistomum ES antigen versus Fasciola faecal samples result (Table 4.17). A total number of 60 sera out of 66 negative for Fasciola by faecal examination resulted negative in both indirect ELISA and faecal analysis. While, 4 sera samples out of 21 positive for Fasciola by faecal examination were found positive to indirect ELISA. Moreover, 6 of the sera samples analyzed had positive values of indirect ELISA and negative by faecal analysis, while 17 sera resulted negative at indirect ELISA test and positive at faecal examination.

Table4.14: Cross-tabulation Result of indirect ELISA Test comparing Fasciola ES Antigen versus Paramphistomum ES Antigen from sheep

Count				
		ELISA param**		
		Negative ELISA Param**	Positive ELISA Param**	Total
Fasciola ES	Negative ES ELISA Fasiola	61	6	67
	Positive ES ELISA Fasciola	16	4	20
Total		77	10	87

Param** Paramphistomum

ES Secretory/Execretory

Table4.15: Cross-Tabulation Result of indirect ELISA Test comparing Fasciola Somatic Antigen versus Paramphistomum ES Antigen from sheep.

Count				
		Param** ES		
		Negative ES ELISA Param**	Positive ES ELISA Param**	Total
Fasciola SO	Negative SO ELISA Fasciola	68	2	70
	Positive SO ELISA fasciola	9	8	17
Total		77	10	87

Param** Paramphistomum

SO Somatic

Table4.16: Sensitivity and Specificity of indirect ELISA Test using Paramphistomum ES Antigen from Sheep

Count				
		Param** ES		
		Negative ES ELISA Param**	Positive ES ELISA Param**	Total
Sed* Param**	Negative faecal Param**	66	9	75
	Positive faecal Param**	11	1	12
Total		77	10	87

Sed* Sedimentation

Param** Paramphistomum

Sensitivity = 8.3% (1/12)

Specificity = 88% (66/75)

Table 4.17 Cross tabulation Result of sheep faecal examination for Fasciola and indirect ELISA using Paramphistomum Antigen

Count				
		Param** ES		
		Negative ES ELISA Param**	Positive ES ELISA Param**	Total
Sed* Fasciola	Negative faecal Fasciola	60	6	66
	Positive faecal Fasciola	17	4	21
Total		77	10	87

Sed* Sedimentation

Param** Paramphistomum

ES Secretory/Execretory

CHAPTER FIVE

CHAPTER FIVE

5. Discussion

The parasitic helminth antigens are commonly defined in the term of molecular weight and their chemical nature. The antigenic epitope that may stimulate the immune system and the profile of immunoglobulin classes elicited by these antigens play a crucial role in the host-parasite interplay. The antigenic components of *F.gigantica* ES and somatic products were contrasted using SDS-PAGE which provide the base line to distinguish between the two antigens on electrophoresis basis. To compare the electrophoretic pattern of somatic and ES antigens of *F.gigantica* by SDS-PAGE, the adult flukes were collected from infected livers of cattle, sheep and goats. ES and somatic isolates were prepared by incubation and homogenization of adult flukes, respectively.

The general conclusion of SDS-PAGE analysis showed the presence of several bands with lower molecular weights ranging from 12-95 KDa in the ES products comparing with 14-123KDa in the somatic one.

Dominant common bands for both ES and SO extracts were clustered between 27-30 KDa. The identified clustered proteins during the current investigation are in accordance to **(*Goreish et al., 2008, Espino et al., 1993*)** and partially agree with the previous finding of Upadhyay and ***Kumar* (*2002*).** They reported that three common bands (24, 33, and 42 KDa) were occurred between the ES and somatic antigen of *F.gigantica*.The result of ***Moazeni et al.,* (*2005*)** study showed that ES and SO antigens of both species of Fasciola have cross reaction with each other. This means that some antigenic materials are common between ES and SO antigen. Indirect immunoflouorescence of tissue sections of adult fluke indicated that 26-28 KDa coproantigen was present in gut cell and

tegument **(*Abdel-Rahman et al., 1999*). *Sabhon et al.*, (*1998*)** stated that 26 and 27 KDa were most likely derived from the deeply localized tissue, such as caecum which also continuously released its content to the exterior. The two proteins have been purified and characterized for their amino acid composition and sequence and are believed to be cysteine proteases **(*Kiatpathomchai et al., 1995*). *Chaithirayanon et al.*, (*2002*)** also revealed that 28.5 KDa antigen present in the tegument, ES material of the adult, whole – body extracts of newly excysted juveniles, 5-week-old juvenile and adult fasciola. It did not cross react with antigen from other trematode parasites, including S. mansoni.

A single band at 28 KDa size was detected in anion exchange chromatography purified fraction from *F.gigantica* ES antigen **(Latchumikanthan *et al., 2012*)**. This result is similar to the findings of ***Fagbemi and Hillyer* (*1992*)** who revealed 28.3 KDa protein from adult *F. gigantica* under reducing and non reducing SDS-PAGE. ***Sriveny et al.*, (*2006*)** observed doublet 27-29 KDa Cathepsin L cystein proteinase antigen from *F.gigantica* in 15% SDS-PAGE on purification with anion exchange chromatography. Dixit *et al.*, (2003) also characterized 28 KDa cysteine proteinase from bubalian liver flukes using 15% SDS-PAGE. ***Rivero-Marrero et al.*, (*1988*)** used western blotting and reported that the bands of 25-30 KDa in the ES antigen were specific for acute and chronic fasciolosis in rabbits, cows and sheep. ***Sampaio-Silva et al.*, (*1996*)** reported that 25-27 KDa components recognized by all 20 fasciolosis sera from human using ES product of adult *Fasciola hepatica*.

Protein of MW of 27 and 26 Kda were also purified from the caecum of *F.gigantica* by ***Sabhon et al.*, (*1998*),** who analyzed the protein from homogenized whole body of the worm. Similar findings were identified by ***Gupta et al.*, (*2003*)** and ***Allam et al.*, (*2002*).** They reported the

presence of 27 KDa protein in the whole worm antigen on SDS-PAGE analysis of *F. gigantica*. Other investigations evaluated the performance of a 27 KDa subunit of *F.hepatica* somatic antigen in two systems, immunoblotting and ELISA, and showed that this subunit has satisfactory validity in diagnosis of human fasciolosis **(*Shafiei et al., 2015; Intapan et al., 2003 and Abdel M Farghaly et al., 2009*).** Cysteine proteinases are intensely immunogenic in cattle and sheep **(*Kiatpathomchai et al., 1995 and Wijffels et al., 1994*).** *F.hepatica* cysteine proteinase has been tested as possible vaccine candidate in sheep and cattle **(*Dalton et al. 1996*).**

The 26 KDa GST from *F.hepatica* is an antigenic protein transiently expressed on the tegument surface execreted by the parasites **(*Abath and Werkhauser 1996*)**. Vaccines containing FABP or GST induce partial protection in experimently infected mice and sheep **(*Lopez et al., 2007, 2008; Paykari et al., 2002; Preyavichyapugdee et al., 2008; Sexton et al., 1990*).**

Major common bands were identified in SO extracts of *F.gigantica* in sheep, goats and cattle at 45 and 66. This result is in close association to the result of ***Viyanant et al.,* (*1997a, b*),** who identified 66 and 47. Similarly ***Gul et al.,* (*2013*)** noticed that dominant bands for both *F.gigantica* and *F.hepatica* in bovines clustered between 46 -58 KDa. ***Sabhon et al.,* (*1998*)** discovered major antigens from the surface of tegument of adult *F.gigantica* at 66, 58, 54 KDa **(*Sabhon et al., 1996 and Viyanant et al., 1997b*)**.***Viyanant et al.,* (*1997*)** studied a monoclonal antibody specific to 66 KDa antigen of *F.gigantica* for the detection of circulating antigens in experimently and naturally infected cattle. They demonstrated that circulating antigen could be detected as early as the second and third week after infection. The 66 KDa has been specifically located and found to be concentrated in the surface membrane. Its

potential as vaccine candidate should be explored, since hosts immunological attacks elicited by this protein may result in the disruption of the membrane and tegument and hence, which in turn could injure and kill the parasites.

Three common major bands with approximate molecular weight of 27-30, 45 and 66 KDa were found in the somatic preparation of the parasite from three different cattle type. >17, 57, 95 and 110 KDa were only seen in western cattle type, while Mangisto showed 5 polypeptide bands with specific bands above 95 and 123 KDa. Only one specific band (<80) was seen in White Nile breed. The electrophoretic scanning revealed the presence of 110 KDa protein in *F.gigantica* **(*Maghraby et al., 2007*). *Sabhon et al.,* (*1996*)** analysed the proteins from homogenized whole body of *F.gigantica,* it was found that there were approximately 21 bands, ranging in molecular weight from 17-110KDa. Closely similar protein bands between 21-110 KDa were detected in *F.gigantica* of Sudanese cattle **(*Goreish et al., 2008*). *Maleewong et al.,* (*1997*)** revealed at least 13 components by immunoblotting analysis of somatic extracts of *F.gigantica*. These antigenic components had molecular weights ranging from less than 14.4 to more than 94 KDa.

***Krailas et al.,* (*2002*)** purified surface tegument antigens of *F.gigantica* by filteration chromatography, protein with molecular weights ranging from 27-97 were present in the fraction of purified antigens. Immunohistochemistry demonstrated that FhTP 16.5 localized to the surface of the tegument of various developmental stages and parenchymal tissues of the adult fluke. FhTP 16.5 could be a good antigen for serodiagnosis of fasciolosis. **(*Joe et al., 2012*).**

The differences in the reported molecular weights of *F.gigantica* in the three different cattle type may be due to the existence of different isolates from different cattle species or geographical variation. ***Chauke et al.,* (*2014*)** detected polymorphic genetic variation between the isolates of *F.gigantica* isolated from cattle in different geographical locations using RAPD-PCR. Specific RAPD assays have been developed for differentiation of fasciolid species in UK, Peru, Ghana and Sudan **(*MCGarry and Ortiz 2007*)** and the technique enabled differentiation of *F.hepatica* and *F.gigantica* from cattle and sheep hosts from countries mentioned above.

F.gigantica somatic antigens in both sheep and goats revealed 4 common dominant bands 14, 28, 45 and 66 KDa and three goat specific bands 19, 38, 50 KDa. These results are in concept with ***Guobadia and Fagbemi* (*1995*)** who demonstrated that 17, 21, 57 and 69 KDa protein bands were specific for *F.gigantica* infection in sheep. However, ES and crude antigens obtained from *F.hepatica* in sheep were 33, 39.5 and 42 KDa in ES antigen and 24, 33 and 66 KDa in crude antigen.

The dominant of *F.gigantica* ES extracts from sheep, goats and cattle were clustered between 17-24, 27-33 and 40 KDa. Nearly similar results were obtained by ***Meshgi et al.,* (*2008*).** They stated that ES protein of *F.hepatica* and *F.gigantica* were characterized by the presence of 6 common major peptide bands with molecular weights of 15, 16, 20, 24, 33 and 42 KDa.

The electrophoretic migration showed some similarities and some differences between ES antigen extracts of *F.gigantica* existing in sheep, goats, and cattle. Twelve bands with molecular weight of 14, 17, 19, 21, 24, 28, 31, 33, 40, 55, 62 and 72 KDa were identified in sheep, whereas

eleven bands with molecular weights of >17, 17, 20, 23, 27, 33, 40, 45, 80, 87, and 95 KDa were shown in cattle. On the other hand, nine bands with molecular weights of 12, 14, 17, 19, 21, 24, 40, 55 and 72 were found in goats. Considering this result, we noticed that the highest molecular weights 80, 87 and 95 of *F. gigantica* ES antigen were recorded only in cattle. Meanwhile, the numbers of ES protein bands appeared in the three different animals' species are nearly the same. Our findings were not consistent with those reported by ***Upadhyay and Kumar* (*2002*)** on the number of ES bands (eight bands). In addition ***Meshgi et al.*, (*2008*)** recorded 6 common ES peptide bands with molecular weights of 15-42 KDa for both *F.gigantica* and *F.hepatica*. However, ***Silva et al.*, (*2004*)** showed 11 polypeptides in the adult *F.hepatica* of which five were detected in sera of 20 patients infested with this parasite.

SDS-PAGE analysis (12% resolving gel) of total ES antigen revealed 7 polypeptides at 23, 25, 28, 43, 47, 52, 66 KDa molecular weight **(*Latchumikanthan et al., 2012*).** On the contrary, 4 protein bands of size 17, 21, 57 and 69 KDa were observed for ES antigen of *F.gigantica* in 12.5% by ***Goubadia and Fagbemi* (*1997*). *El Ridi et al.*, (*2007*)** also observed prominent bands at 62-60, 40, 30 and 28 KDa for ES products from *F.gigantica* by 12% SDS-PAGE. In the present study, 10% SDS-PAGE analysis showed that 12, 10 and 9 polypeptide bands for total *F.gigantica* ES antigen from different animal species, however the previous investigators recorded 4 to 7 bands with more or less similar molecular weights. This difference may be due to the dilution of resolving gel SDS-PAGE or may be to the variation of strains of *F.gigantica* in different parts of the world. Meanwhile, similar results obtained by ***Sampaio-Silva et al.*, (*1996*).**

It is well known fact that helminth parasites during their development undergo antigenic polymorphism which induces drastic alteration in immune response. So use of these different developmental stage antigens in the immunodiagnosis is very important. Development of simple and specific immunological tests for the diagnosis of helminth infections has been a major goal for immunological research. Several immunodiagnostic methods have been used recently for the diagnosis of trematode infections in ruminants and also to assess the immune response elicited by these parasites in the experimental animals. Immunodiagnostic methods for the detection of these parasitic infections however, usually suffer from problems of low sensitivity.

Fasciolosis is a parasitic disease caused significant economic losses in domestic livestock, particularly cattle and sheep. The pathogenicity of Fasciolosis is based on expansion of lesions in the liver of the host by migrating and feeding immature flukes as well as host immune responses to parasite secreted molecules and tissue damage alarm signals **(*Molina-Hermández et al., 2015*).** Unfortunately, conventional detection methods can only reveal the infestation between 10 and 14 weeks after infection when eggs can found in faeces of hosts **(*Conboy and Stromberg 1991*).** Therefore, designing a more accurate diagnostic assay may be valuable. It seems that serological methods can be evaluated as an alternative assay for the diagnosis of Fasciolosis because of the relative simplicity and early sero-conversion (usually 1-2 weeks) **(*Sanchez-Andrade et al., 2000*).**

In the current study, the coproscopy by sedimentation and antibody indirect ELISA techniques were utilized to attain the prevalence of bovine and ovine fascioliasis comparing with bovine and ovine

Paramphistomiasis in Rabbak, White Nile Province, considering the associated ES *Paramphistomum sp.* and ES / SO *Fasciola gigantica* antigens as well as their sensitivity and specificity.

Forty six out of 156 cattle faecal samples examined were found positive with *Paramphistomum sp.* with overall prevalence rate of 29.5%. However, the seroprevalence of *Paramphistomum sp.* using ES Paramphistomum antigen was 39% (61/156). On the other hand, out of 156 cattle faecal samples examined, 30 were found positive with *Fasciola gigantica* with an overall prevalence of 19.2%. The seroprevalence of *Fasciola gigantica* when assessed by indirect ELISA using ES Fasciola antigen was 30% (47/156). In the present study, we noted that the prevalence reports based on antibody ELISA test using ES Fasciola and Paramphistomum antigens were consistently higher than that detected by faecal analysis. This could be related to the development of antibodies much earlier than presence of eggs in faeces during the course of infection. ELISA is more clearly more sensitive than faecal examination because many animals with mature infections don't shed detectable numbers of eggs. The ability to diagnose and treat early is a big advantage of the ELISA because, it minimizes tissue damage in the infected animals caused by the immature flukes as they migrate through the liver. So early diagnosis of bovine Fasciolosis using antibody detection tests, especially during the prepatant periods makes ELISA for curbing the negative impact of infection on productivity and hence to avert huge economic losses **(*Sanchez - Andrade et al., 2000*).** However, our trends are not in agreement with ***Aliyu et al., (2014)*** in Nigeria. The reason for the differences in results is that they detected *F.gigantica* infection with antigen and monoclonal antibody of *F. hepatica*. So much

lower infection level was recorded with ELISA, as the two species might have dissimilarities in their antigenic epitopes.

The low sensitivity of coprological examination could be due to the presence of a large number of animals with low levels of infection, in the sampled group, and sampling animals that have recovered from infection(i.e. with no fluke present), but in which anti-fasciola antibodies are still circulating. However, sensitivity could be improved by increasing sample size. ***Charlier et al.,* (*2008*)** found that increasing the amount of faeces analyzed from 4g to 10 g led to substantial improvement of sensitivity. However, when 10 g was used, the specificity was no longer 100%. Previously, it was shown that the Se of coproscopy improved from 69% to 90% when three serial samples (of 10g) of the same sample were used rather than a single sample **(*Raspsch et al., 2006*).**The apparent reduction in Sp is probably due to a combination of detecting light infections that were not picked up by the gold standard and the increase amount of debris under the coverslip, hampering the microscopial reading of the sample. So detection of Fasciolosis is hampered due to low sensitivity of methods, which cannot detect small numbers of ova in large volumes of ruminant faeces.

In Sudan, epidemiological studies on Fasciolosis are mostly based on faecal examination or inspection at slaughter house and few researchers have employed immune-serological methods. The prevalence of *Fasciola gigantica* infection as determined by coprological examination and meat inspection at abattoir was firstly reported by ***Karib,* (*1962*),** he reported 26% prevalence in sheep and cattle at Kosti region (White Nile Province). ***Eisa* (*1963*)** reported 37% prevalence in the upper Nile region (now Republic of Southern Sudan). In Western Sudan, ***Sayda* (*2012*)** reported 5.66% in cattle, 0.57% in goats and no infection was observed in sheep.

The highest infection rate was observed in cattle in the eastern part of Southern Darfur State (14.3%). Furthermore, ***Osman* (*2011*)** reported 34.2% prevalence in the White Nile Province. The prevalence of 19.2% obtained in this study in Rabbak (White Nile Province) is much lower than 34.2% recorded by ***Osman* (*2011*).** This could be attributed to the small samples size; the current survey was limited to small area and did not cover all the seasons of the year.

In the current study, two *Fasciola gigantica* antigens (ES/SO) were applied to evaluate the diagnostic sensitivity and specificity of indirect ELISA for the diagnosis of *F.gigantica* infection as well as its prevalence rate in cattle and sheep. The results of indirect ELISA revealed that higher prevalence rate for diagnosis of bovine Fasciolosis was detected by ES Ag (30%) comparing with SO Ag (18.7%). High specificity (81.6%) was recorded when SO antigen was chosen in indirect ELISA for diagnosis of *F.gigantica* infection in cattle compared with 69% using ES antigen. Meanwhile, low sensitivities of 27.7% and 20% were shown in both ES and SO Fasciola antigens, respectively.

Twenty one out of ninety two sheep faecal samples examined were found positive with *Fasciola gigantica* with overall prevalence rate of 22.8%. Moreover, the overall seroprevalence of Fasciola was 22.8% (21/92) and 20.6% (19/92) as assessed by *Fasciola gigantica* ES and SO antigens, respectively. In this study, we noted that the seroprevalence of Fasciola in sheep was found to be nearly the same as the coprological analysis. This result may indicate that the sheep' s immune system did not respond adequately to the antigenic stimulus from migrating or immature flukes. ***Person and Allonby* (*1979*)** reported breed differences for *F.hepatica* infection. They reported that it may be due to general resistance of some breeds to parasitic infection which is genetically

controlled. Moreover, certain hosts do possess some immunity to infection /reinfection indicated that the parasite isn't altogether invincible. While many mammalian species can be infected with Fasciola, there is a wide variation in their ability to acquire resistance to reinfection. Sheep often die from acute Fasciolosis, while some infections can last as long as 11 years **(*Pantelouris, 1965*).** However, there have been some reports of different levels of susceptibility of sheep with different genetic back grounds to liver fluke infection **(*Boyce et al., 1987*).** Most notably, Javanese thin-tailed sheep have been found to be highly resistant to infection with *Fasciola gigantica* **(*Wiedosari and Copeman, 1990; Roberts et al., 1997*)**.

After contrasting data from indirect ELISA using both ES and SO Fasciola antigens versus faecal analysis, higher specificity for the diagnosis of *F.gigantica* in sheep was recorded using SO Ag (83%) (59/71) compared with ES Ag (78.9%). However, low sensitivity of 33% and 28.6% was determined when SO and ES antigens were used, respectively. So from these data results, we concluded that Indirect ELISA using both ES and SO Fasciola antigens in sheep and cattle was not sensitive, although it showed slightly high specificity.

To the contrary, some studies have reported high sensitivities and specificities of 87-100% and 86-100% of indirect ELISA, respectively as previously mentioned in literature review (Table 2.2).

The low sensitivity occurred in this study, because the presence of antibodies does not always correlate with the existence of active infection, it may just show exposure to parasite **(*Espino et al., 1998; Sanchez-Andrade et al., 2000*).** Furthermore, using polyclonal secondary antibody instead of monoclonal antibody increases the test sensitivity.

Also possible reasons for lower sensitivity and specificity in field situatations are the greater proportions of animal with low level of infection, the sampling of animals that have recovered from infection (i.e. with no fluke present) but in which *F.gigantica* specific antibody are still circulating and the imperfect gold standard. In Sudan ***Osman* (*2011*),** in his study when using ES indirect ELISA the sensitivity was 80% and the specificity was 53% using postmortem examination as gold standard. On the other hand, he reported that 98 of 130 positive at postmortem were positive in sedimentation giving a sensitivity of 75%. By incision and inspection of opened major bile ducts, only 71% of all infected livers were detected and the remaining 29% were only detected after slicing and soaking of the livers **(*Charlier et al., 2008*). *Raspech et al.*, (*2006*)** estimated the sensitivity of detecting infection with *F. hepatica* at meat inspection at the abattoir as 69%. Therefore, worm count at liver necropsy can only be considered as a gold standard if slicing and soaking of the liver is performed. However, we must admit that even very light or prepatent infections could still be missed, affecting the calculated Sensitivity and Specificity of the evaluated tests. On the other hand, crude Fasciola antigen in ELISA studies **(*Khalil et al., 1989 and Khalil et al., 1990*).** The investigators gave a sensitivity of 66% and specificity of 84%. Later ***Hassen et al.*, (*2004*)** reported an IgG ELISA of 61.11% sensitivity and 70% specificity. ***Vongpakorn et al.*, (*2001*)** stated that ELISA was highly sensitive but gave low specificity. They showed that this method, using ES antigen, might not be suitable for the diagnosis of Fasciolosis as they showed cross reaction with many parasitic infections. The sensitivity, specificity, positive predictive value and negative predictive value were found to be 100%, 23.4%, 23.4% and 100%, respectively. ***Anderson et al.*, (*1999*)** reported that positive and negative predictive value for ES antigen of *F.gigantica* with 72 sera samples of stool positive

and 20 sera of stool negative cattle were 91.2% and 58.3%. ***Fagbemi and Guobadia* (*1995*)** reported that the negative predictive value was 45.5%. They concluded that there was no correlation between the faecal egg and the adsorbance values in ELISA. ***Yagi et al.,* (*1986*)** also found that primary infection with *F.gigantica* could cause cattle resistance with *S.bovis*. They showed that cross-reaction occurred among different species trematodes.

Another reason that may lead to low sensitivity in our test is that we use a total IgG. Isotype determination is also important. For example IgM antibodies peak early by week 3 in infected sheep and drop sharply by week 6 post infection; IgG antibodies peak by 4-5 weeks of infection but remain high thereafter **(*Chauvin et al., 1995*).** In contrast, ***Clery et al.,* (*1996*)** found IgG1 to be the dominant isotype over IgM, IgG2 and IgA in both chronically infected and acutely infected, previously näive calves. Analysis of the isotypic responses of sera of Fascioliasis patients to liver fluke tegumental extract **(*Morales and Espino 2012*)** revealed that the predominant antibodies elicited are IgG1 and IgG4, which are the antibody serotypes that also predominate against execretory- secretory products, crude-whole worm extracts, and Cathepsin – L1 **(*Maher et al., 1999; O'Neill et al., 1998; Sabry et al., 2011 and Wongkham et al., 2005*). *O'Neill et al.,* (*1998*)** developed a diagnostic test for human Fasciolosis based on the detection of antibodies to ES antigen or purified cathepsin L protease. The authors found that the assays were much improved by the used of conjugated second antibodies that detect IgG4 rather than total antibodies. ***Morales and Espino* (*2012*)** in their study found that similar levels of both IgG antibodies, with mean level of IgG1 levels slightly higher than those of IgG4. Differences regarding the relative amount of both antibody isotypes could be attributed to the

genetic background of the infected individuals and the intrinsic properties of the antigen itself. Moreover, the cytokines elicited by different antigens also play a role in determing the main subclass of the antibody response **(*Garraud et al., 2003*).** But generally the predominance of both antibody isotypes IgG1 and IgG4 are especially predominant in infections caused by *F.gigantica* **(*Wongkham et al., 2005*)** when antigenic exposure is chronic **(*Garraud et al., 2003*).**

The false negative results obtained from this study in both sheep and cattle may be due to modulation of the host immune response by liver flukes as reported by ***Anderson et al., (1999*).** Also for sheep and cattle that were negative on coprological examination and positive in indirect ELISA suggest that juvenile flukes may have been migrating through the liver parenchyma, but had not reached the bile duct so eggs could not be found in faeces **(*Rǿdriguez-Perez and Hillyer, 1995*).** Another explanation was previous exposture to infection **(*Espino et al., 1997; Sanchez-Andrade et al., 2000*)** and the animals were not infected at the time of sampling **(*Espino et al., 1998*).**

A remarkable observation occured in the current study is that the number of sheep and cattle found negative to Fasciola ES/SO indirect ELISA and positive to coprological examination was high. The results revealed that in sheep using both ES and SO Fasciola antigens 14 and 15 sera out of 21 positive faecal samples, while in cattle 22 and 24 sera out of 30 positive faecal samples resulted negative, respectively. So failure to detect antibodies in sera from coprologically positive animals could be due to persistence of very low antibodies against *F.gigantica* at the time of sampling. ***Thammasart et al., (1995)*** found 47.6% false positive in the ELISA result for *F. gigantica* from 233 cattle sera using crude somatic antigen. Furthermore, ***Welch et al., (1987)*** recovered that 26% of animals

shedding fasciola ova were ELISA negative, and ***Hillyer et al., (1996)*** found seven animals as ELISA negative, which were positive in faecal examination. Antibodies to *Fasciola sp.* in infected hosts can be detected by Ab ELISA as early as one to two weeks post infection ***(Hillyer and De Galanes, 1991; Chauvin et al., 1995; Reichel, 2002; Kumar et al., 2008)***.They rise rapidly reaching a blateau by 3-6 weeks of infection. On the other hand, egg excretion rates in infected calves' peak at 14-20 weeks then drop sharply thereafter and become egg negative by one year ***(De Léon et al., 1981)***. This decrease in eggs is presumably due to the death of adult flukes, and is also followed by a decrease in ELISA absorbance value ***(Hillyer et al., 1985).*** Variable results were also obtained in cattle. A significant increase in specific antibody titers was observed two weeks ***(Wyckoff and Bradley, 1986; Santiago and Hillyer, 1988)*** and six to eight weeks after infection in cattle ***(Hillyer et al., 1985).***

Furthermore, although indirect ELISA providing early detection of infection, but there is some possibilities of cross reactivity with other trematodes antibodies leading to false-positive results ***(Levieux et al., 1992; Hillyer et al., 1985; Hillyer and Soler, 1998).*** Another explanation of very low antibody titers against *F.gigantica* is that antigens and eggs liberated by adult flukes are carried out in the intestines with the bile juices which limit the immune stimulation and immunopathology induced by these. Consequently, antibody titers, measured in mice, rats, sheep and cattle were observed to decline, albeit slowly, after the parasites had enter the bile ducts ***(Hanna, 1980a; Meussen and Brandon, 1994; Clery et al., 1996).***

In cattle by using ES *Fasciola gigantica* antigen versus Paramphistomum faecal analysis, we observed that 17 sera out of 46 positive faecal paramphistomum had positive values of Fasciola indirect ELISA. Similarily, in sheep considering both ES and SO Fasciola

antigens, 2 sera out of 13 positive paramphistomum faecal samples were positive to *Fasciola gigantica*. In addition, the serological indirect ELISA tests using both paramphistomum and Fasciola ES antigens showed that among the 61 Paramphistomum positive sera 26 sera were found positive for Fasciola. Similarly, the numbers of serologically positive Fasciola samples were 47. Among these positive samples 26 were found positive for Paramphistomum. Meanwhile, in sheep the results detected by serological indirect ELISA showed that among the 20 sera of positively ES Fasciola 4 sera were found positive for Paramphistomum. Similarly, among the 17 sera of positively SO Fasciola sera 8 were found positive for Paramphistomum. So from these results we concluded that cross reaction between *Fasciola gigantica* and Paramphistomum may occur. Many investigators have shown that immunological cross-reactivity exists between antigens of Fasciola, Schistosoma, Paragoniums and some other *Paramphistomum spp.* The immunological cross-reactivity between Fasciola and other parasite species may reduce the accuracy of immunodiagnostic assay for Fasciolosis. However, the utilization of monoclonal antibodies and pure fractions of parasitic extracts could participate in minimizing this drawback. Moreover, adoption of more sensitive techniques rather than conventional ones in diagnosis or detection of parasite antigen or its DNA, also share in eliminating cross-reactivity in diagnosis assay.

In the present study, ES paramphistomum antigen was applied to evaluate the diagnostic sensitivity and specificity of indirect ELISA for the diagnosis of paramphistomiasis as well as its prevalence rate in cattle and sheep. As mentioned above, the prevalence reports of cattle paramphistomiasis based on indirect ELISA was higher than that detected by faecal analysis. However, twelve out of 87 sheep faecal samples

examined were found positive with Paramphistomum sp. with overall prevalence rate of 13.8% and overall seroprevalence of 11.5%. We noted that the seroprevalence of ovine Paramphistomiasis was not higher than the coprological examination. This may be due to general resistance of some type to parasitic infection which is genetically controlled as discussed above in ovine Fasciolosis.

The sensitivity of indirect ELISA in both cattle and sheep was found to be 43.4% and 8.3%, whereas, the specificity was 62.7% and 88%, respectively. This result indicating that also indirect ELISA for detection of Paramphistomum spp. is more specific but not sensitive. ***Sanchis et al., (2012)*** recorded that ELISA sensitivity and specificity using secretory excretory antigen of Paramphistomum were 82 % and 79 % respectively. Also ***Hassan et al., (2005)*** recorded 82 % sensitivity of *Paramphistomum somatic* antigen but they recorded higher specificity of 90%. On the other hand, ***Shivjot et al., (2009)*** in their study showed higher sensitivity 85.71% and a lower specificity 23.65% using somatic antigen of *Paramphistomum* and ***Tariq et al., (2011)*** who reported sensitivity of dot ELISA as 100 % for detection of *Paramphistomum cervi* antibodies in hyper immune rabbit using crude antigen of *Paramphistomum*. ***Salib et al., (2015)*** concluded that ELISA is more specific and accurate but less sensitive than western blotting for the diagnosis of Amphistomes infection in cattle and buffaloes. The sensitivity, specificity and accuracy for ELISA and western blotting were (74% and 100%), (82.4% and 33.3%) and (79.76% and 77.78%), respectively. False positive results may be attributed to migration of early immature larvae through the wall of small intestine, cross reactivity with antibodies from other infections especially trematodes as *Fasciola gigantic*a and also may be due to treated cases ***Sanchis et al., (2012).*** On the contrary, false negative results may be attributed to low levels of specific IgG, variant Ig antibody expression and/or formation of circulating immune complexes.

Conclusion

In summary the general conclusion of SDS—PAGE analysis of execretory/seretory and somatic products of adult *Fasciola gigantica* from sheep, goat and cattle showed the presence of several bands with lower molecular weight in the execretory/secretory product comparing with the somatic one.

The differences in the reported molecular weights of *F.gigantica* in the three different cattle type from different localities; sheep and goats from the same locality may be due to the existence of different isolates from different animals' species or geographical variation.

Indirect ELISA was carried out to evaluate its effectiveness in the imunodiagnosis of fasciolosis and Paramphistomiasis in sheep and cattle in area of Rabbak White Nile Province. It was concluded that indirect ELISA using both Fasciola ES/SO and Paramphistomum ES antigens in sheep and cattle was not sensitive, although it showed slightly high specificity.

The study showed that cross-reaction between Fasciola and Paramphistomum may occur which, may reduce the accuracy of immunodiagnosis of both worms.

Recommendation

Suggestions for further work:

1. The application of molecular technique for identification of DNA polymorphism of Sudanese liver flukes and further work is required to study The DNA sequence of the parasite.
2. The immunogenic components of the ES and SO extracts identified in this study require further extensive research on isolation and purification e.g. purified antigen, recombinant antigen or synthetic antigen in order to improve the sensitivity and specificity of anti-bodies indirect ELISA.
3. To evaluate the antigen, further investigation should be performed by the detection subclass immunoglobulin.
4. Immunoblotting analysis of *F.gigantica* ES/SO antigens and Paramphistomum ES antigens, to determine the bands of Fasciola which are cross reacted with Paramphistomum and the band which is specific for Fasciola diagnosis.

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Appendix 1

Preparation materials.

1. **Resolving buffer MW(OH) = 121.14**
   Tris – OH (base) 18.15
   D. W 100ml
   Then adjust pH to 8.8 and complete to 100ml.
2. **Stacking buffer:**
   Tris – Hcl 3gm
   D.W 40 ml
   Adjust pH to 6.8 and complete to 50 ml
3. **SDS Running buffer:**
   Tris-OH 3.02 gm
   Glycine 14.4 gm
   SDS 1gm or 10ml 10% SDS
4. **Staining buffer:**
   Glacial acetic acid (GAA) 5.75ml
   Coomassie Blue dye 0.157 – 0.2 gm
   Methanol 28.5 ml
   dH2O 62.5 ml
5. **De-staining buffer:**
   Glacial acetic acid (GAA) 10 ml
   Methanol 50 ml
   dH2O 100 ml
6. **SDS sample loading buffer:**
   0.5 Tris-OH (pH 6.8) 100 ml
   dH2O 4 ml
   Glycerol 0.8 ml
   SDS 10% 1.6 ml
   2-ß mercaptoethanol 400 ul
   0.05% Bromophenol blue 200 ul

Appendix2

Composition of ELISA reagent and buffer:

1- Coating buffer:-

Dissolve 1 tablet of carbonate-bicarbonate in 100ml D.W

2- Phosphate Buffered Saline (PBS):

Dissolve 10 tablets of (PBS) in 1000 ml D.W

3- Washing Buffer:

1000 ml (PBS) + 500 µl Tween twenty 0.05%

4- Blocking Buffer:

Washing buffer + Skimmed milk (5%)

25ml (PBST) + 1.25g Skimmed milk

5- Diluent Buffer (Blocking Buffer):

50ml (PBST) + 2.5g Skimmed milk

6- Conjugate:

Anti-Bovine IgG (whole molecule)-Peroxidase, antibody produced in rabbit (SIGMA- Germany) IgG fraction of antiserum.

7- Substrate:

TMP Peroxidase Substrate – Peroxidase Substrate Solution B

8- Stopping Solution: M sulphuric acid.