ENZYME-LINKED IMMUNOSORBENT ASSAY FOR T
DETECTION OF CIRCULATING *FASCIOLA GIGANTICA* A
IN SERA OF EXPERIMENTALLY INFECTED SHEE

BY

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APRIL 1997

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THIS IS MY ORIGINAL WORK AND HAS NOT BEEN SUBMITTED FOR A DEGREE IN ANY OTHER UNIVERSITY.

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DEDICATION

Dedicated to my dear PARENTS, Mr. JOSHUA and Mrs. RESPA OMEGA, and to my loving WIFE, MARGARET and SONS, REUBEN and JUDE.

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LMW	Low Molecular Weight
KCl	Potassium chloride
kDa	Kilo Daltons
M	Molar
MAFF	Ministry of Agriculture, Fisheries and Food
mg	Milligrams
mins	Minutes
ml	Millilitre
mm	Millimetres
mM	Millimoles
MW	Molecular Weight
N	Normal
$NaIO_4$	Sodium metaperiodate
$(NH_4)_2SO_4$	Ammonium sulphate
nm	Nanometres
N°	Number
OD	Optical Density
ODBG	Optical Density of Background
ODCNEG	Optical Density of Negative Control
ODS	Optical Density of Sample Test Serum
PAA	Polyacrylamide
PBS	Phosphate Buffered Saline

LIST OF ABBREVIATIONS

ABTS	Azino-bis-(3 ethylbenz-Thiazoline-6-Sulphonic acid)
APS	Ammonium persulphate
BSA	Bovine Serum Albumin
cm	Centimetre
DAB	3,3'5,5'-diaminobenzidine
DEAE	Diethylaminoethyl
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ES	Excretory/Secretory
FEST	Faecal Egg Sedimentation Technique
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
Fig	Figure
g	gravity
HCl	Hydrochloric acid
HMW	High Molecular Weight
H_2O_2	Hydrogen peroxide
HRPO	Horseradish peroxidase
IgG	Immunoglobulin G
I.P.R.	International Primate Research
K.A.R.I.	Kenya Agricultural Research Institute

PEG	Polyethylene glycol
PI	Post-infection
PLNUM	Plate Number
PM	Post-mortem
pp	Percent positivity
SDS-PAGE	Sodium dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'Tetraethylmethylethylenediamine
u	Microns
ul	Microliters
UV	Ultra Violet
wk	Week
°C	Degrees Celsius

ABSTRACT

Eighteen Dorper sheep aged between six and twelve months were obtained from an area known to be free of fasciolosis. The animals were divided into four groups (I-IV). Each of the first three groups comprised of five sheep while the fourth group had three sheep. Each of the five sheep in group I was orally infected with a low dose (250) of metacercariae of *Fasciola gigantica* whereas each of those in group II got a high dose (400) of the metacercariae. The five sheep of the third group were infected with varying doses of between 210 and 490 metacercariae per animal. The group III animals were slaughtered at weeks 7,11,13,14 and 15 post-infection and flukes recovered. These flukes were processed and their antigens compared with somatic antigens of *Paramphistomum* spp., *Schistosoma mansoni*, *Stilesia hepatica*, hydatid cyst fluid and *F. gigantica* (together with its excretory/secretory products) in a SDS-PAGE gel. An antigen, AgF.g28, was found to be both immunogenic and specific for *F. gigantica* by Western blotting.It was eluted from the SDS-PAGE gels and used to immunise the three sheep in the fourth group. Immunoglobulin G from the sera of the hyperimmunised sheep was isolated and conjugated with horseradish peroxidase enzyme.

Sera and faecal samples were collected weekly from the sheep in groups I and II from three weeks before infection to 21 weeks post-infection, after which they were slaughtered and the adult liver flukes in the bile ducts, together with the *Fasciola* eggs in the gall bladders, recovered and counted. The faecal samples were subjected to the FEST and the number of *Fasciola* eggs in 3 grams ascertained, while the sera were assayed for antigen and antibody by the antigen- and antibody-ELISA tests respectively.

The whole-worm SDS-PAGE protein profiles of the various ages of juvenile flukes and the

adult flukes recovered from sheep appeared identical. However, a protein band of a MW of about 42-kDa was present in the flukes recovered from sheep but was lacking in the adult fluke recovered from bovine, whereas a protein band of about 70-kDa was not as prominent in the fluke of bovine origin as it was in those from sheep. The protein bands in the ES products were fewer than those in the whole-worm extracts. Apart from a band of about 60 kDa, all the protein bands in the ES products were also present in the whole-worm extracts. Each of the other parasites had its own unique protein profile. Nevertheless, common protein bands existed between *F. gigantica* and *S. mansoni* (e.g bands of 40 and 94 kDa), *F. gigantica* and *S. hepatica* (e.g bands of 25 and 80-kDa) and also between *F. gigantica* and the hydatid cyst fluid (38-kDa). The 43-kDa band was present in all but the whole-worm protein profile of *Paramphistomum* spp.

A protein band of 28-kDa was identified and found to be specific for *F. gigantica*. This antigen, code named AgF.g28, was present in all ages of the *F. gigantica* whole-worm PBS extracts and also in the ES products. An antigen-ELISA developed for the AgF.g28 had a sensitivity of 75% and a specificity of 70% for the 21 weeks of infection. An antibody-ELISA for the same antigen had a sensitivity of 81% and a specificity of 20% for the same period. Eight out of ten sheep in both the low and high dose infection groups tested positive for fasciolosis at the first week post-infection by the antigen-ELISA, as compared to 2 out of 10 in the same period by the antibody-ELISA. The Faecal egg sedimentation technique first detected fasciolosis at the 13th week post-infection.

Student's T-tests conducted showed that none of the three diagnostic tests was able to distinguish between low and high levels of fasciolosis. Also, there was no significant difference between the number of adult flukes recovered from the bile ducts, the number of eggs in the

faeces and in the gall bladder between the animals in the low and high dose infection groups. A direct relationship was found to exist between the number of eggs in the faeces and those in the gall bladder, and also between the number of *Fasciola* eggs in the gall bladder and the number of adult flukes recovered. However, no relationship existed between the number of adult flukes recovered and the number of eggs in the faeces. A regression analysis at the 5% level revealed that the dose (i.e number of metacercariae ingested) did not influence the dependent variables such as number of eggs in the faeces and gall bladder, the percentage fluke take (i.e the proportion of metacercariae ingested that developed to adulthood and were recovered), and the optical density readings of the antigen- and antibody-ELISA. The statistical results effectively imply that quantitative diagnosis of fasciolosis cannot be ascertained by any of the three diagnostic tests used in the present study.

The fact that the AgF.g28 was found in both the whole-worm extracts and the ES products means that it was most probably an ES antigen. This was also evident from the SDS-PAGE protein profiles where the antigen in the ES products was more prominent and distinct than in the whole-worm extract. The antigen was not highly immunogenic, as shown by the Western blot and this probably explains why the sensitivities of both the Antigen and Antibody-ELISAs were low.

Under the conditions of the present study, it was found that the Antigen-ELISA was superior to both the Antibody-ELISA and the FEST. Its sensitivity and specificity were together higher than those of the other tests, and it was also able to detect fasciolosis in infected sheep relatively earlier. The success of a good diagnostic test is determined by its ability to detect disease in infected animals as early as possible, and also to have a high sensitivity and

specificity. If the antigen, AgF.g28, was produced in a purer form, and monoclonal antibodies used instead of polyclonal, then the Antigen-ELISA developed to detect AgF.g28 in serum would possibly be a good diagnostic test for fasciolosis.

1. INTRODUCTION

Fasciolosis is a disease caused by the parasites of the genus *Fasciola* of which *F. gigantica* and *Fasciola hepatica* are the most pathogenic. These parasites belong to the Phylum, Platyhelminthes; Class, Trematoda; Order, Digenea and Family, Fasciolidae (Froyd, 1969). Fasciolosis is found in domestic animals such as cattle, sheep, goats, camels, water buffalo and equinae, and wild animals such as the African buffalo, warthog, impala, kudu, Indian rhinoceros and hartebeest (Van Rensburg, Taljard and Van Wyk, 1991). The disease has also been found in man and in the monkey, *Macaca mulata* (Dawes and Hughes, 1964).

Fasciolosis is found almost anywhere in the world where both the intermediate hosts (snails) and the definitive hosts are present. However, *F. gigantica* is found mostly in Africa, the Indian sub-continent, central and south east Asia and other tropical and sub-tropical areas of the world (Hansen and Perry, 1994). Mixed infections with the two species of flukes may occur, especially in the highlands and the cooler regions (Dunn, 1978). *F. hepatica* occurs commonly in the temperate regions and to a lesser extent in other climatic areas (Pantelouris, 1965).

Among the domestic animals, sheep and cattle are the most severely affected by fasciolosis. The severity of the disease ranges from a devastating, highly fatal form in sheep to an asymptomatic infection in cattle. The severity, however, depends on the host species affected, the parasitic species, the number of parasites ingested (and whether they were ingested within a short or long period of time) and the immune status or response of the host (Kimberling, 1988).

When the disease is acute, it can cause severe anaemia and sudden death in sheep.

Deaths can occur in all seasons, and the clinical signs may include anaemia, reduced weight gain, decreased milk/wool/meat production, unthriftiness and submandibular oedema (Hansen and Perry, 1994). Death in sheep due to acute fasciolosis is a consequence of traumatic hepatitis caused by many juvenile flukes penetrating through the liver parenchyma at the same time. Dow, Ross and Todd (1968), found that by the eighth week after infection, 60% of the flukes were found migrating in the liver tissue. Post mortem done at this stage showed traumatic hepatitis, blood-stained fluid in the abdominal cavity, and enlarged, friable and haemorrhagic liver.

Fasciolosis is perhaps among the most important parasitic diseases hampering the development of the livestock industry in Kenya. The high mortality rate that occurs in outbreaks of the acute form of the disease, together with the losses incurred due to the less dramatic but insidious, long term and deleterious effects of the chronic, wasting condition (manifesting mainly as decreased meat and wool production, reduced growth rate, condemnation of livers at abattoirs and reduction of host fecundity) results in substantial economic loss (Karib, 1962; Froyd,1975; Haroun and Hillyer, 1986). It is estimated that upto 50% of livers may be condemned when animals from endemic areas are slaughtered (Ellwood,1973).

Fasciola hepatica and *F. gigantica* infections are estimated to result in losses of over US$3 billion per annum worldwide (Spithill, 1993). Froyd, (1975), estimated that liver condemnations and poor lamb growth-rates resulting from fasciolosis cost the British animal industry 1.5 million Sterling pounds annually. In Kenya, Preston and Castellino (1977), reported that about KSh 2,400,000 were lost annually from liver condemnation due to fasciolosis for the period 1972-1974. Losses due to condemnation of livers of sheep and goats alone due

to fasciolosis between 1974 and 1978 were found to be in excess of KSh 332,000 on average annually (Cheruiyot, 1980).

Diagnosis of fasciolosis in the field is done mostly by the identification of fluke eggs in the faeces, or by visualization of the parasites in the bile ducts and/or liver parenchyma at necropsy (Malek,1980). These methods have the advantages of being easy to perform, but have serious limitations. Although there are methods of quantitative assessment of the fluke eggs in the faeces,this does not necessarily correlate with the level of infection (Sewell,1966). The eggs are carried through the bile to the gall bladder which may serve as a reservoir of eggs for a considerable length of time. Eggs are expelled from the gall bladder when the latter contracts during digestion, and large numbers are released during the contractions. This means that the number of liver fluke eggs in a faecal sample is not an accurate indication of the number of parasites in the liver, nor of the amount of damage being done to the host (Hansen and Perry, 1994). Furthermore, the eggs are found in the faeces only after they have been laid by the adult flukes in the bile ducts, which means that this method is not useful in the diagnosis of prepatent infections when the immature flukes cause most pathological effects as they migrate through the liver parenchyma.

Visual identification of flukes in the liver and/or bile ducts is a sure method of diagnosis, but it is expensive in that the animals have to be slaughtered or allowed to die before the diagnosis can be made. This can be time-consuming and very wasteful as some of the animals may not be having the infection (Soulsby, 1965).

The limitations inherent in the above diagnostic methods for fasciolosis necessitate the development of other methods that are easy to run, sensitive, specific and which will also be

able to detect infection in its early stages. Several alternative methods for diagnosis of fasciolosis have been developed, most of which are serological. The advantages with serodiagnostic techniques are that apart from being very sensitive and specific, many animals can be sampled at once and in a short time. Also, they are capable of detecting pre-patent infections. For instance, Indirect Hemagglutination tests were able to detect infections as early as 10 to 14 days post-infection (Levieux, Levieux and Vinien, 1992). Other immunodiagnostic tests that have been developed for use in the diagnosis of *Fasciola* include Double immunodiffusion (Sewell, 1966), Fluorescent antibody tests (Schillhorn van Veen and Buys, 1979), Radio-Immunoassay (Dessaint and Capron, 1982) and Thin layer immunoassay (De Morilla, Paniagua, Ruiz-Navarrete, Bautista and Morilla, 1989). Other serological tests that can be used include immunoprecipitation and counter immunoelectrophoresis. Most of these tests have been tried in *F. hepatica* infections but their applicability in *F. gigantica* infections is largely untested. Bitakaramire,Movsesijan and Castelino (1971), developed a radioimmunoassay for detecting *F. gigantica* infections in cattle. Fluorescent antibody tests using single and multiple fluke antigens have been used in detecting chronic infections (Schillhorn van Veen and Buys, 1979). Intradermal tests with crude antigens have been used in developing diagnostic procedures, but their reliability has been questionable (Losos, 1986). Most of the serological methods that have been used in the diagnosis of fasciolosis do have limitations that make them impractical for use on a large scale or in the field situation. For instance, immunofluorescence is somewhat tedious and requires experienced and skilled personnel for the assessment of results. Radio immunoassay requires expensive counting equipment, the reagents have a short shelf-life and special safety measures for handling the reagents is necessary. Because of these difficulties,

the use of enzyme markers conjugated to antigen or antibody has increased. This relatively new technique is called Enzyme-linked Immunosorbent Assay (ELISA). Immunodiagnosis of fasciolosis using the ELISA technique has been reviewed by Hillyer (1988), and Santiago and Hillyer (1992). The reviews include work done by Burden and Hammett (1978), and Zimmerman, Nelson and Clark (1985). Enzyme labelled agents are relatively in-expensive, stable and sensitive. The Enzyme immunoassay produces assays which approach the sensitivity of radio immunoassay, and yet only requires the use of simple equipment.

ELISA can be used in the detection of either antigens or antibodies. Serological tests for antibody detection are of limited diagnostic value because antibody titres persist after the patients have been cured, and this gives false-positive results. In addition, it is possible that patients with recent infection have a negative test, a phenomenon demonstrated in experimental animals during the first one to four weeks of infection (Espino, Marcet and Finlay,1990). Immunological diagnosis of *Fasciola* by the direct detection of parasite products in serum samples has been reported (Yamasaki, Aoki and Oya, 1989; Espino *et al*,1990; Espino and Finlay, 1994; Fagbemi and Guobadia, 1995). These tests have an advantage over the antibody detection tests in that antigenaemia implies active infection.

The present study was carried out after considering all the above mentioned developments and limitations that have been encountered in the noble search for an effective diagnostic tool for fasciolosis in animals and man.

1.1 OBJECTIVES OF THE STUDY

The main objectives of the study were:-

1. To identify diagnostic (immunogenic) antigen(s) specific for *F. gigantica*.

2. To develop an ELISA for the detection of such antigen(s) in the sera of experimentally infected sheep for purposes of diagnosis of fasciolosis.

3. To compare the sensitivity and specificity of this Antigen-ELISA with the standard faecal egg sedimentation technique and the Antibody-ELISA.

4 To investigate whether the Antigen-ELISA so developed can be used to assess the level of infection by *F. gigantica* in the live sheep.

2. LITERATURE REVIEW

2.1 ECONOMIC IMPACT OF FASCIOLOSIS

Fasciolosis causes economic losses due to mortalities, abortions,retardation of growth, drop in meat/milk/wool production,condemnation of infected livers and sometimes condemnation of carcasses emaciated due to liver fluke infection. Animals with severe acute fasciolosis die either directly from the infection, or indirectly through predisposition to other diseases (Hughes, 1978).

In one farm in mainland Tanzania, 66% (79 out of 119) of the cattle, 92% (55 out of 60) of the sheep and 60% (3 out of 5) of the goats died of the disease (Hammond, 1956). In Kenya, an outbreak of acute fasciolosis in sheep was encountered in one of the regions endemic for the disease (Maingi and Mathenge, 1995). Sewell (1966), showed that in cattle infected with *F. gigantica*, each fluke reduced the annual live weight gain by about 198 grams. Oakley, Owen and Knapp (1979), demonstrated that *F. hepatica* in growing dairy heifers had a limiting effect on growth and impaired efficiency of feed conversion since infected animals consumed more feed to achieve the same or lower performance than non-infected calves. Hawkins and Morris (1978), observed that wool growth and body weight gain were reduced in young sheep infected with *F. hepatica* in comparison with control animals, when fed *ad libitum* in pens. The difference was statistically significant for a group with as few as 45 flukes and increased with increasing fluke burden.

Losses caused by both *F. gigantica* and *F. hepatica* globally are estimated to be over US$ 3 billion annually (Spithill, 1993). In the United Kingdom, Froyd and McWilliam (1975)

estimated that out of the 52 million pounds lost to the livestock industry annually, 2 million pounds was attributable to fluke damaged livers. In the Netherlands, Pekelder (1975) estimated that *F. hepatica* infection causes losses amounting to a minimum of from 125 to 150 million Guilders per annum. In Kenya, Bitakaramire (1967) recorded that the Kenya Meat Commission lost about KSh.800,000 annually between 1954 and 1966 from liver condemnation due to fasciolosis. Preston and Castellino (1977) put the figure at KSh.2,400,000 for the period from 1972 to 1974. In the 5 year period of 1974 to 1978, Cheruiyot (1980), estimated that losses due to fasciolosis in sheep and goats was averaging at KSh.332,800 annually. A later survey by the Agricultural Research Foundation (1986) showed the annual losses due to liver fluke infection in cattle, sheep and goats to be about KSh.326 million. It is not easy to assess all the economic losses caused by fasciolosis as other confounding factors present in a herd or flock make this task difficult. However, there are mathematical models available (Ogunrinade and Ogunrinade, 1980) by which estimates of such losses can be made.

2.2 AETIOLOGY

The two most important parasites that cause fasciolosis are *Fasciola hepatica* and *F. gigantica*. Even though it is generally agreed that *F. hepatica* and *F. gigantica* cause similar signs and damage to the hosts, there are glaring differences among them, apart from just the morphological, epidemiological and host preference. For instance, *F. gigantica* is more pathogenic in sheep than in cattle and the converse is also true (Pantelouris, 1965). *Fasciola gigantica* is found in the tropics and sub-tropics, especially in the whole of Africa, the Indian sub-continent, central and south east Asia whereas *F. hepatica* is more cosmopolitan in

distribution. It is found mostly in temperate regions and only in the highland and cooler areas in the tropics (Dunn, 1978). In Kenya, the disease is caused by both species of the parasite, although it is generally accepted that *F. gigantica* is the more important one (Froyd, 1959; Ogambo-Ongoma, 1969; Preston and Castellino, 1977).

2.2.1 Morphology

Fasciola gigantica and *F. hepatica* resemble one another in most of their morphological features, but the former is easily distinguished by its characteristic shape and larger size. The two parasites are similar in that they are both leaf-shaped and broader anteriorly. They have an anterior cone-shaped process which carries the oral sucker, and posterior to this is a pair of broad shoulders. There is a ventral sucker at the level of the shoulders and intestinal caeca with numerous branches extending to the posterior extremity (Soulsby, 1968).

The liver flukes are hermaphroditic. The testes are markedly branched and are present in the mid-field, about one-third of the body length from the anterior end. The ovary is also branched and is located in the midline, anterior to the testis. The uterus is anterior to the testis and is highly convoluted, appearing brownish when it is laden with oval eggs (Dunn, 1978).

The adult *F. gigantica* measures 27-75 mm long by 5-12 mm wide. It has an anterior cone smaller than that of *F. hepatica* and its shoulders are not as prominent. *Fasciola hepatica*, on the other hand, measures 20-30 mm long by 8-13 mm wide. It is leaf-shaped, broader anteriorly and brownish when living. The eggs of *F. gigantica* are larger, measuring 156-197 μ by 90-104 μ whereas those of *F. hepatica* are 130-145 μ by 70-90 μ (Soulsby, 1965).

Apart from the morphological differences, the two parasites can also be distinguished

epidemiologically and serologically by their specific intermediate host species and even biochemically. For instance, *F. gigantica* was found to produce toxins which affect the heart of the definitive host, leading to swelling of capillaries and haematomas under the pericardium. The spleen may be enlarged and there is a marked enlargement of the pulp during the acute stage of the disease. This was not seen with *F. hepatica* infection (Sogoyan, 1956).

2.3 LIFE CYCLE OF *FASCIOLA GIGANTICA*

The life cycle of *F. gigantica* was exhaustively described by Dinnik and Dinnik (1956); Sewell (1966); Bitakaramire (1969); and Hansen & Perry (1994).

The adult flukes inhabit the bile ducts of the definitive host. Eggs are released by the adult, hermaphroditic parasites. The eggs enter the duodenum with the bile and are expelled together with the host's faeces. In a moist habitat and a temperature of about 26°C, the eggs hatch into miracidia in about 17 days. Temperatures of between 5.5-19.5°C will inhibit egg hatching whereas temperatures less than 4°C will inhibit embryo development.

The ciliated miracidia are motile and actively seek and penetrate the suitable intermediate host-the snail- and lose the cilia in the process. The miracidia are specific for the intermediate hosts which they must penetrate within 24 hours or else they die. The intermediate hosts of *F. gigantica* are *Lymnaea auricularis* in the Indian sub-continent and *Lymnaea natalensis* in Africa. *Lymnaea truncatula* is the most important intermediate host of *F. hepatica* although *Lymnaea bulimoides* and *Lymnaea tomentosa* may also serve the purpose (Asanji, 1988). In the snail host, each miracidium subsequently develops into a sporocyst. Each sporocyst will develop into 5 to 8 first-generation rediae. A radium has a cluster of germinal cells and embryo balls from which

cercariae develop.

The infected snail host begins to shed cercariae about 36 days post-penetration. Shortly after emerging from the snail, the tadpole-like cercariae swim freely in the water and within a few minutes to 2 hours, they have made contact with herbage or any other objects just below the water surface. The cercariae cast off the tail and secrete a covering from their cystogenous glands, forming cysts of about 0.2 mm in diameter. These are metacercariae and are the infective forms of the parasite. They can remain infective for upto 6 months at 22-24°C in water.

Infection of the definitive host occurs by ingestion of herbage contaminated by the encysted metacercariae or by grazing on low-lying marshy infected pastures. In the abomasum and duodenum, the excystation process is accomplished by the enzymes pepsin and trypsin respectively. The excysted forms migrate through the gut wall, cross the peritoneum and peritoneal cavity, and penetrate the liver capsule. A few young flukes may also reach the liver through the blood-stream but this is not the usual route. Following the penetration of the liver capsule, the immature flukes migrate through the liver parenchyma for about 6 to 8 weeks before they enter the bile ducts, where they mature and commence egg production. Eggs are first seen in faeces 13 to 16 weeks post-infection. Adults can survive in the bile ducts for upto 11 years.

2.4 PATHOGENESIS, CLINICAL SIGNS AND PATHOLOGY OF *F. GIGANTICA* INFECTION

Liver fluke disease is a sequence of acute parenchymal hepatitis and chronic cholangitis, characterized by unthriftiness, weight loss, anaemia, oedema, eosinophilia and fibrosis, all

brought about by the trematode parasites. The degree of affection however depends on the host species affected, the number of parasites ingested and the duration through which the parasites are ingested by the host. In general, the liver fluke disease can be divided into the acute, sub-acute and the chronic forms. The acute form is caused by a traumatic invasion of liver parenchyma by numerous immature flukes, whereas the chronic form is a biliary fibrosis resulting from prolonged residence of adult flukes in the bile ducts of the host's liver (Kimberling, 1988).

While the liver is the tissue sought for by the young liver flukes, there are some instances when the parasite is known to establish itself and grow in other locations of the mammalian body. Such cases can not all be detected by the demonstration of eggs in the faeces. The most usual place is the lung, and the presence of flukes there has been recorded as the cause of acute verminous pneumonia and peribronchial inflammation in sheep (Muchilis,1959) and cattle (Cattelani, 1952). The parasites have also been found in lymph nodules (Dzekonske, 1947) and even in the uteri of cattle, causing sterility and endometritis (Thom,1956). It is thought that in this way, the young flukes may even come and settle in the foetus *in utero* (Bugge, 1935).

2.4.1 Acute Fasciolosis

The pathology of acute fasciolosis is that of a traumatic hepatitis. This is caused by many immature flukes penetrating through the liver parenchyma at the same time. Dow *et al*, (1968) reported that by the eighth week after infection, 60% are to be found migrating in the liver tissue. When post mortem is done to an animal with this kind of traumatic hepatitis, blood-stained fluid is found in the abdominal cavity, the liver is enlarged, haemorrhagic and friable.

Fibrinous clots are seen on the surface of the liver and there is a general fibrinous peritonitis. The liver is studded with migratory tracts, and blood clots which give the organ a hob-nailed appearance. The migratory immature flukes may be detected in the anterior ends of the tracts. A zone of haemorrhage may be seen in the middle part of the tract whereas the posterior part may have a zone of reddish-grey material containing mostly infiltrated leucocytes, especially eosinophils. If the cut surface is squeezed into water, a varying number of immature flukes are obtained with their sizes depending on the age of infection (Soulsby, 1965; Henderson, 1990).

Acute fasciolosis is commonly found in sheep. The tunnelling of the liver leads to inflammation, characterized by an abdominal pain with reluctance to move. In severe cases, there may be rapture of the liver capsule, with haemorrhage into the peritoneal cavity. Such animals die rapidly after a short period of showing clinical signs (Georgi and Georgi, 1990).

2.4.2 Sub-acute Fasciolosis.

The sub-acute form of fasciolosis occurs in animals, especially sheep, that have survived from an acute out-break or have been treated once before for the disease, and have also been exposed to the parasites for a long time. Grossly, the liver is uneven, dark brown in colour and the capsule is thickened. The marked hepatitis, characteristic of the acute form, is absent (Soulsby, 1965) but migratory tracts are still evident.The infiltration by white cells is more marked. There may be fibrosis in the distal parts of the tract and young but mature forms of the parasite are present in the bile ducts. Biliary cirrhosis has commenced, but is not as extensive as in the chronic form. Frequently the sub-acute form may be superimposed on an existing chronic form. A more marked cellular reaction may occur in cases of re-infection,

suggesting the initiation of an immune mechanism. However, under natural conditions, immunity does not appear to be playing any significant role in limiting the number of parasites acquired. The sub-acute disease is characterized by wasting, weakness and anaemia. Oedema, if present, may appear in the form of bottle-jaw and/or ascites (Soulsby,1965; Pantelouris, 1965).

2.4.3 Chronic Fasciolosis.

This is the most common form of fasciolosis in cattle. In sheep and other animals, the chronic form occurs when there is a cumulative low-level infection which does not produce a marked effect until the flukes have become adults in the bile ducts (Blood, Radostits and Henderson, 1984). Grossly, there is extensive biliary cirrhosis. In chronic infections, the liver lobes may be distorted and reduced in size (Urquhart,1956). The liver substance appears paler than normal and the lobular pattern is exerggerated. The walls of the extrahepatic bile duct are thickened. Their lumina may be several times larger than normal, especially when they are full of parasites. On incision, the liver is fibrotic, and the intrahepatic bile ducts are thickened. Many fibrotic areas in the liver parenchyma represent advanced stages of repair of the migratory tracts and infarcted areas. The fibrotic portal tract is frequently joined to the healed migratory tracts by a network of connective tissue, producing a coarse fibrosis (Soulsby,1965).

2.5 IMMUNOLOGY

The immune response of vertebrate hosts towards fasciolosis has been studied extensively under both experimental and natural conditions. Rats have been the animals of choice in

experimental studies but it is still a debatable issue whether to assume that their response to *Fasciola* spp. infection is an accurate representation of what happens in the natural hosts, and also under the natural field conditions (Hughes, 1987).

Animals infected with *Fasciola* spp. develop both natural and acquired immunity, but the acquired response is most readily studied under experimental conditions. Cattle show a higher degree of natural resistance to infection with *Fasciola* spp. than sheep (Haroun and Hillyer, 1986). The underlying reason for this difference in host susceptibility is not entirely clear. However, it has been suggested that natural immunity is expressed both during the juvenile migratory parenchymal stage and the adult bile duct stage of infection (Blood *et al.*, 1984).The former is considered a manifestation of differences in liver histology. Sheep liver contains only small amounts of connective tissue, hence, it offers less resistance than cattle which have a relatively large amount of fibroblastic tissue which helps to trap young migrating flukes (Ross, 1967; Boray, 1967). Immunity at the bile duct level has been shown in cattle only, where there is elimination of a large proportion of adult flukes between 5 and 10 months post-infection, followed by diminished faecal egg output by the flukes. It could be that these effects are caused by bile duct calcification which helps trap the adult flukes. Such trapped flukes probably die of starvation as they are immobilized and denied access to food supplies.

The presence of invading juvenile stages of *Fasciola* species provokes a significant immunological response in the host. The titre of specific antibody in the blood stream of sheep and cattle reaches a maximum during the first 6 weeks of infection, but declines once the flukes are established in the bile ducts (Movsesijan and Jovanovic, 1975; Hanna and Jura, 1977). It appears that the production of antibodies has little protective significance to the host, especially

during a primary infection. The flukes survive peak antibody titres while in the liver parenchyma and can exist for extended periods of time in the bile ducts of the natural host. Sheep appear unable to resist secondary and subsequent infections with *F. hepatica* (Sinclair, 1962; Boray, 1967; Haroun and Hillyer, 1986), but it is clear that infected cattle (Ross, 1967), rats (Hayes, Bailer and Mitrovic, 1972) and mice (Lang, 1967) can at least partially destroy a challenge infection.

During a primary infection, *Fasciola* species can evade the host response. Absorption of the host antigens onto their surface may mask them from immunological recognition (Smithers *et al.*,1969). It is also possible that the heptalaminate surface may be capable of rapid renewal when under immune attack (Perez and Terry, 1973; Wilson and Barnes, 1977). It is suggested that the young fluke burrowing in the liver is constantly moving out of range of the cellular effector mechanisms (Dawes and Hughes, 1964), while the adult is shielded from immune attack once it enters the bile duct (Lang, 1967). The tegumental surface of invading flukes is continually exposed to the host, so it is likely that an immunological attack is directed against the components of this surface. High titres of antibody specific for the surface of *F. gigantica* metacercariae have been detected in experimentally infected calves (Hanna and Jura, 1977). Thus, it might be expected that a mechanism of evading the host's immunological responses would be centred in the tegument of *Fasciola* (Hanna, 1980a).

Among the antibodies produced by sheep against fluke gut secretions, the predominant immunoglobulin class is IgG-1 and only small amounts of IgG-2 (Movsesijan and Jovanovic, 1975). Numerous IgA-producing cells appear in the liver after a single infection in calves with *F. hepatica*, but after a second infection, IgG-1 cells predominate both in the liver and in the

during a primary infection. The flukes survive peak antibody titres while in the liver parenchyma and can exist for extended periods of time in the bile ducts of the natural host. Sheep appear unable to resist secondary and subsequent infections with *F. hepatica* (Sinclair, 1962; Boray, 1967; Haroun and Hillyer, 1986), but it is clear that infected cattle (Ross, 1967), rats (Hayes, Bailer and Mitrovic, 1972) and mice (Lang, 1967) can at least partially destroy a challenge infection.

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hepatic lymph nodes (Flagstad and Eriksen, 1974). Significant amounts of haemocytotropic antibodies (showing affinities with human IgE) also develop in infected cattle (Doyle, 1973). The common assumption that the lumen of the bile ducts offers an immunologically secluded habitat which allows the juvenile flukes to mature to adulthood was confirmed by Hughes, Hanna and Symonds (1981). They found that the overall concentration of immunoglobulin in bile was much lower than in serum, meaning that the flukes derived significant protection from the host's immunological defenses by migrating into the bile ducts. The change in the nature of *F. hepatica* tegument antigenicity from a t1-dominated to a t2-dominated condition which accompanies maturation (Hanna, 1980a) may provide further protection, since apparently no antibodies against t2-antigens appear in the bile at any stage of infection.

It appears that there is no evidence to indicate that primary sensitization of sheep with *F. hepatica* stimulates any resistance to challenge in terms of reduction in number of worms recovered from challenge infection (Haroun and Hillyer, 1986). However, other manifestations such as retarded worm growth, decrease in worm size, reduced egg production by adult worms, delay of onset of anaemia and elevated antibody titres can be seen as effects of a primary sensitization. Sinclair (1962) infected sheep with 150 metacercariae of *F. hepatica* on four occasions before challenge, but could not find any resistance to challenge. However, he observed delayed and reduced egg production by the adult flukes. Boray (1967) infected sheep with large doses (1000 metacercariae) of normal *F. hepatica*, terminated the infections with an anthelmintic and then challenged these sheep and controls with 4000 metacercariae each. No appreciable difference in the number of flukes was found between the challenge infections and the controls. However, the previously infected sheep lived longer and developed anaemia later

than the controls. He attributed this response to the hepatic fibrosis that came about as a result of the repeated infection of the sheep. The fibrosis may have acted as a mechanical barrier which did not reduce the number and size, but probably reduced the mobility of the migrating flukes of the challenge infection. Results from studies by Sinclair (1971) led him to conclude that lymphocytes, which are the antigen-sensitive cells directly involved in antibody production and the carriage of immunological memory, are probably involved in the retarded development of the flukes in sheep that have been challenged with *F. hepatica*. Differences between the degree of eosinophils in the blood and within the liver tissues of the challenged and control sheep were also reported and it was suggested that this eosinophil presence is correlated with the retardation of the flukes. These results were confirmed later by other workers (Sinclair, 1973;Rushton, 1977; Knight, 1980).

It has been established that cattle acquire resistance to challenge with *F. hepatica* and *F. gigantica* when they are sensitized by primary homologous patent or drug-abbreviated infections (Haroun and Hillyer, 1986). The resistance is usually manifested by a decrease in the size and number of flukes recovered from challenged animals. Kendall, Sinclair, Everett and Parfitt (1978) showed that the resistance stimulated by primary infection with *F. hepatica* may persist for a long period after the removal of the sensitizing infection by anthelmintic treatment. Doy and Hughes (1984) found 56% resistance following a primary sensitization of 18 weeks duration. When they extended the period to 26 weeks, they obtained a higher level of resistance (94%) and thus suggested that the extra few weeks of exposure were necessary for the full development of resistance.

The immune response of rabbits towards fasciolosis has not been clearly described and

workers have come up with conflicting results. For instance, Ross (1966) reported a reduction in the size and number of worms recovered from a challenge following primary sensitization, but there was considerable individual variation and the reduction was not statistically significant. Kendall, Herbert, Parfitt and Piece (1967) attributed this reduction in the number of worms to an inhibition of growth due to overcrowding, rendering the worms difficult to find rather than to real resistance. Fortmeyer (1973) found significant resistance to oral challenge in repeatedly infected rabbits but not to intraperitoneal challenge. From this, he concluded that two kinds of immune mechanisms operate, one in the intestinal wall and the other during the migratory phase.

2.6 DIAGNOSIS

Diagnosis of fasciolosis can be achieved either by the demonstration of eggs in the faeces of infected animals, recovery of juvenile and/or adult parasites at necropsy, or by serological methods that can detect either specific antibodies or antigens in infected animals (Dunn, 1978). Of these methods, the serological methods have been found to be the most sensitive and specific (Hillyer, 1993), and more attention is being directed towards attaining a serological technique that can detect fasciolosis in its earliest stages before much damage is done.

Together with all these methods of diagnosis of fasciolosis, clinical signs and the pathology are useful in giving a tentative diagnosis. Sudden death (especially in sheep) or dullness, weakness, pallor of mucosae, dyspnoea and ascites are strong indicators of acute fasciolosis (Reid, 1973). On the other hand, chronic fasciolosis is characterized by weight loss, pallor of mucous membranes, submandibular oedema and/or ascites (Okao, 1975). The clinico-pathological picture obtained in animals suffering from fasciolosis includes anaemia, eosinophilia

and hypoalbuminemia (Reid, 1975). A direct correlation has been found to exist between liver enzyme levels in serum and worm burdens (Hillyer, Haroun, Hernandez and Soler De Galanes, 1987; Hillyer 1988).

2.6.1 Parasitological diagnosis of Fasciolosis.

This is done mostly by the identification of fluke eggs in the faeces or by visualization of the parasites in the bile ducts and/or liver parenchyma at necropsy (Malek,1980). In faeces of herbivorous animals, the presence of *Fasciola* eggs is a definitive diagnosis of the disease. However, identification of the eggs in the faeces of man, omnivorous or carnivorous animals is not confirmatory as they may have ingested uncooked liver or digestive organs of animals infested with *Fasciola* (Hillyer, 1988). Absence of *Fasciola* eggs is also not conclusive as the animal may be having the disease in its prepatent stages. Clinical manifestation of the disease may be seen from a few days to 2-3 months, yet the flukes require a period of at least 3-4 months to attain sexual maturity and begin laying eggs (Chen and Mott, 1990).

The methods used in processing faeces for microscopic examination include sedimentation (Boray and Pearson, 1960), floatation (MAFF, 1986) and a technique using acetic acid or hydrochloric acid together with centrifugal sedimentation (Coles, 1986).

Visualization of the parasites in the liver at necropsy gives a definitive diagnosis. However, this method of diagnosis loses meaning when the aim is to treat infected animals. It is a very expensive diagnostic technique and also wasteful, if animals have to be sacrificed or allowed to succumb to the disease in order for the diagnosis to be made.

and hypoalbuminemia (Reid, 1975). A direct correlation has been found to exist between liver enzyme levels in serum and worm burdens (Hillyer, Haroun, Hernandez and Soler De Galanes, 1987; Hillyer 1988).

2.6.1 Parasitological diagnosis of Fasciolosis.

This is done mostly by the identification of fluke eggs in the faeces or by visualization of the parasites in the bile ducts and/or liver parenchyma at necropsy (Malek,1980). In faeces of herbivorous animals, the presence of *Fasciola* eggs is a definitive diagnosis of the disease. However, identification of the eggs in the faeces of man, omnivorous or carnivorous animals is not confirmatory as they may have ingested uncooked liver or digestive organs of animals infested with *Fasciola* (Hillyer, 1988). Absence of *Fasciola* eggs is also not conclusive as the animal may be having the disease in its prepatent stages. Clinical manifestation of the disease may be seen from a few days to 2-3 months, yet the flukes require a period of at least 3-4 months to attain sexual maturity and begin laying eggs (Chen and Mott, 1990).

The methods used in processing faeces for microscopic examination include sedimentation (Boray and Pearson, 1960), floatation (MAFF, 1986) and a technique using acetic acid or hydrochloric acid together with centrifugal sedimentation (Coles, 1986).

Visualization of the parasites in the liver at necropsy gives a definitive diagnosis. However, this method of diagnosis loses meaning when the aim is to treat infected animals. It is a very expensive diagnostic technique and also wasteful, if animals have to be sacrificed or allowed to succumb to the disease in order for the diagnosis to be made.

2.6.2 Serological Diagnosis of Fasciolosis

Several serological techniques have been developed and used for the diagnosis of fasciolosis. The techniques can be divided into those that detect antibodies in the host produced against the parasites, and those that detect *Fasciola* antigens either in circulation or in faeces.

2.6.2.1 Detection of Antibodies.

Immunodiagnosis of fasciolosis by detection of antibodies in infected animals has been done extensively and many techniques have been employed to this end. Among the methods used in detection of antibodies against *Fasciola* infections are double immunodiffusion (Sewell, 1966), Fluorescent antibody tests (Schillhorn van Veen and Buys, 1979), Radio-immunoassay (Dessaint and Capron,1982) and Thin layer immunoassay (De Morilla *et al*, 1989).

Hillyer and Santiago de Weil (1981) reportedly developed a counter immunoelectrophoresis test that was able to detect 100% of infections with fasciolosis in mice, rats and rabbits. The technique could detect antibodies by 4-5 weeks post infection and in most rats as early as 2 weeks post-infection. Levieux *et al*, (1992) developed an Indirect haemagglutination test that could detect antibodies against *Fasciola* as early as 10 to 14 days post-infection.

Although antibody detection techniques are excellent for the early determination of infection with *Fasciola* in cattle and sheep, persistence of antibody for long periods after cure makes it unsuitable for the prediction of success of chemotherapy (Rodrigues-Perez and Hillyer, 1995). Added to this, the efficiency of the antibody detection technique depends on the

availability of defined, specific antigens (Hillyer, 1993). These are not commercially available and it is therefore difficult to have commercially prepared test systems.

2.6.2.2. Detection of antigens.

A sensitive and immunologically specific antigen detection test for *Fasciola* is an important goal since it should imply recent, active infection (Hillyer, 1993). Also, measurement of antigens as a parameter of active infection may be of potential use in the management of clinical disease . One of the main characteristics that a diagnostic test should possess is early detection of infection. This is one of the main advantages of antigen immunodiagnostic detection tests over antibody tests (Rodriguez-Perez and Hillyer, 1995). In parasitic infections, particularly those involving tissue parasites, early therapeutic treatments are usually most useful, as once parasites become established in target organs or viscera, it is much harder to eradicate them. Several studies have examined this approach and the search for appropriate antigens has been intensive.

Immature flukes have been shown to share antigens with other developmental stages of *F. hepatica*. Thorpe (1965) first showed immunofluorescent labelling of both immature and mature flukes with sera from infected rats. Moore and Halton (1976) found that carboxyl-esterase, acetylcholinesterase and alkaline phosphatase were similar in both immature and mature flukes. Antiserum raised in rabbits against a soluble extract of adult flukes was seen to react with all but the earliest forms of the immature flukes. The antisera reacted most strongly with migrating flukes (Bennett, 1978).

The most promising antigens that appear to qualify for antigen detection techniques have

been from the excretory/secretory products of *Fasciola* species. Although *in vitro* excretory/secretory products from 28 day old immature flukes produced significant resistance to challenge in rats but not in mice, those from adult flukes did not protect both rats and mice against challenge (Rajasekariah, Mitchell, Chapman and Montague, 1979). This meant that the excretory/secretory products could at least provoke an immune response which could even be strong enough to protect animals against challenge infections. Following this lead, various workers have identified and isolated different components of excretory/secretory products that could be used in immunodiagnosis. For instance, Hillyer and Soler De Galanes, (1988) identified two antigenic polypeptides of 17 and 63 kiloDaltons (kDa) from excretory/secretory products of *F. hepatica*. Espino, *et al.* (1990) used monoclonal antibodies to detect excretory/secretory antigens in circulation of humans with fasciolosis. Monoclonal antibodies against some excretory/secretory products of *F. hepatica* have been produced by Solano, Ridley and Minocha (1991). Similar work done by Rodriguez-Perez and Hillyer (1995) produced a test that could detect excretory/secretory antigens in circulation as early as 8 weeks post infection in sheep.

Apart from excretory/secretory antigens, Fagbemi and Goubadia (1995) have recently identified a 28-kDa cysteine protease from adult *F. gigantica* worms which seems to be good for immunodiagnostic purposes. Indeed, Fagbemi (1995) has gone further and developed a monoclonal antibody that is able to capture the 28-kDa protease. Earlier, Yamasaki *et al.* (1989) had identified a 27-kDa proteinase in an uncertain *Fasciola* species.

Cross-reactivity between *Fasciola* spp. and other parasites has been the single most unfortunate impediment in the search for the ideal immunodiagnostic antigen(s). Despite the high

sensitivity of the serological techniques that have been developed for the diagnosis of fasciolosis, their specificity is seriously hampered because of the possession of common antigen epitopes by various helminths. Pelley and Hillyer (1978) showed that *F. hepatica* and *S. mansoni* shared at least one common antigen epitope. Fagbemi and Obarisiagbon (1991) on the other hand, demonstrated common antigen epitopes between *F. gigantica, Dicrocoelium hospes* and *Schistosoma bovis*. Work done by Rodriguez-Perez and Hillyer (1995) revealed that there was cross-resistance between *F. hepatica* and *S. mansoni* in sheep and that sheep infected with *S. mansoni* and challenged with *F. hepatica* induced an anamnestic response to common epitopes shared between the two genera of trematodes. This cross-reactivity makes obtaining a specific immunodiagnosis more difficult, especially when using antibody detection techniques. In this work by Rodriguez-Perez and Hillyer (1995), *F. hepatica* detection appeared to be specific since the antigen detection results were not affected by primary *Schistosoma* infection. Until species-specific antigenic components of helminth parasites are obtained, most of the serodiagnostic methods in routine use may be more relevant to the study of experimental mono-infections (Hillyer *et al*, 1987) than to the diagnosis of natural infections in areas where poly-parasitism occur (Fagbemi and Obarisiagbon, 1991).

2.6.3 The Enzyme-Linked Immunosorbent Assay (ELISA).

Enzyme Immunoassays (EIA) were first described by Engvall and Perlman (1971) and Von Weemen and Schuurs (1971), independently. The principle was based on the original concept of Miles and Hales (1968) who indicated that enzymes or co-enzymes could replace the radioactive labels in non-competitive radioactive assays. Earlier, Nakane and Pierce (1967) had

shown that antibodies could be labelled with enzymes for use in histochemical staining of tissues.

Before the EIA was developed, labelling of antigens or antibodies was done using fluorescent dyes (immunofluorescence) or radio-isotopes (radio-immunoassay). Immunofluorescence is not easy to quantify for antibody assays, since it depends on subjective visual assessment of fluorescence and the results are usually expressed as the serial dilution of the serum that gives the least fluorescence (Voller, Bidwell and Bartlett, 1976). It therefore requires experience and skilled personnel for the assessment of results. Radioimmunoassay, on the other hand, is highly sensitive, but requires expensive and complex counting equipment, the reagents have a short shelf-life and because of the medical hazards, they must be handled only by highly trained personnel (Voller *et al*, 1976).

Because of these difficulties, the use of enzyme markers conjugated to antigen or antibody is increasing. Enzyme immunoassays overcome many of the disadvantages of the other methods and offers significant advantages. Enzyme labelled agents are relatively inexpensive, stable and sensitive. The EIA produces assays which approach the sensitivity of radioimmunoassay but requires only the use of simple equipment. The reagents present no hazards, they are stable and have long shelf lives (Voller *et al*, 1976). Moreover, the estimation of results can be visual, or be made with a simple spectrophotometer or by use of modern ELISA reader, which can be easily automated.

An important principle used in Enzyme immunoassays is that antibody or antigen can be bound to an inert carrier (e.g microtitre plate) without losing activity. Also, antigen or antibody can be linked to an enzyme with minimal loss of either immunological or enzymatic activity

(Anonymous, 1978).

Enzyme immunoassays can be classified into two groups:- the homogenous and the heterogenous EIAs.

2.6.3.1 Homogenous Enzyme Immunoassays

These assays do not require separation of free or bound labels because the assays depend on inhibition or inactivation of the enzyme label, through the binding antigen or antibody. The change in enzyme activity is measured in the presence of free and bound antigen and antibody (Voller, Bidwell and Bartlett, 1978).

2.6.3.2. Heterogenous Enzyme Immunoassays.

Heterogenous EIAs are those that involve at least one separation step in which the enzyme-labelled antigen or antibody is separated from the antigen/antibody complex, before measurement of enzyme activity in either fraction (Voller *et al* 1978, 1979). The heterogenous EIA that is best known is the Enzyme-linked immunosorbent Assay (ELISA) that was developed by Engvall and Perlman (1971). Various modifications of heterogenous EIAs have been described e.g Direct and Indirect-ELISA, competitive and non-competitive ELISA, Inhibition-ELISA, etc (Voller, *et al*, 1979).

The general principle of ELISA is as follows:-(Anonymous, 1978)

1) Antigen or Antibody is adsorbed to the carrier surface

2) Specific antibody or antigen from the test solution attaches to the prepared surface.

3) Enzyme labelled antiglobulin attaches to the antigen-antibody complex.

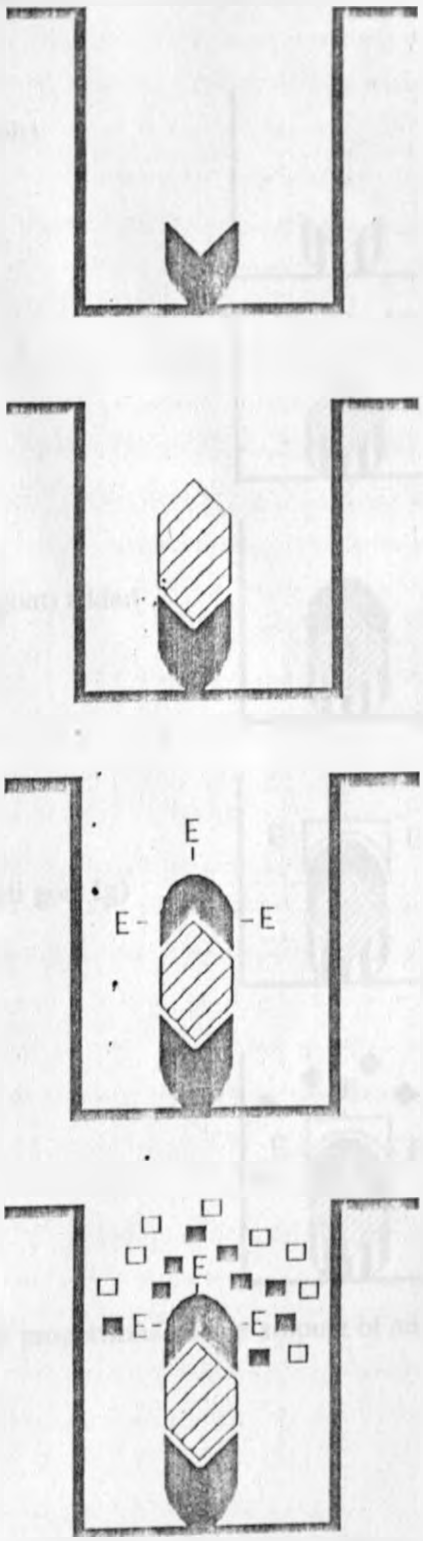
4) Enzyme label changes the colour of an added substrate so that the antigen-antibody complex can be detected.

5) Amount of antigen-antibody complex is directly proportional to the optical density of the final colour.

2.6.3.3. The Antigen-ELISA

When an antigen under investigation is adsorbed to the surface of specialized ELISA plates, the conformation of the antigen may change, thereby reducing the reactivity with the specific antibody (Al Moudallal, Altschuh, Briand and Van Regen Mortel, 1984; McCullough, Crowther and Butcher, 1985a). This happens in the Indirect ELISA. If the indirect assay is being used to detect antigen in test material, or if the antigen is heavily contaminated with other proteins (e.g. those derived from host tissue), interference may occur and could even cause total blocking of the interaction between the antigen and the ELISA plate surface (McCullough, 1993). The Antigen-ELISA circumvents the problems encountered with the indirect ELISA (McCullough and Spier, 1990, Porstmann and Kiessig, 1992). In the Antigen-ELISA, antibody which is specific for the antigen is absorbed onto the ELISA plate and the antigen is subsequently trapped by this "capture antibody". This capture can be detected by a second antibody reaction, that of the "detection antibody" (of a different species to the capture antibody). Conversely, the capture antibody can be labelled with the enzyme (Voller *et al*, 1976). The two types of Antigen-ELISA are presented diagrammatically in Figs. 1 and 2.

Figure 1. The Direct Double Antibody Sandwich ELISA for measuring antigen.



1 Antibody absorbed to plate

wash

2 Test solution containing antigen added

wash

3 Add enzyme labelled specific antibody

wash

4 Add enzyme substrate

The amount of hydrolysis of the substrate is directly proportional to the amount of antigen present (Voller.et. al,1976)

Figure 2: The modified double sandwich ELISA for measuring antigen

1 Plate coated with specific antibody A (e.g. rabbit)

Plate washed

2 Tested sample containing antigen reacted

Plate washed

3 Specific antibody B (of different species e.g. goat) added

Plate washed

4 Enzyme labelled anti B globulin added (eg anti goat Ig)

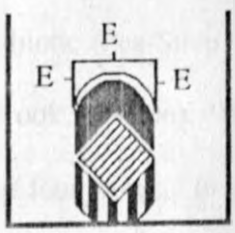


Plate washed

5 Enzyme substrate added

The amount of hydrolysis of the substrate is directly proportional to the amount of antigen present (Voller, et al, 1976).

3. MATERIALS AND METHODS

3.1 THE ANIMALS.

Dorper sheep were bought from the Maasai Rural Training Centre, Isinya in Kajiado District. This institution had a well managed flock of sheep and the area in which they were reared was known to be free of fasciolosis. Eighteen healthy sheep aged between 6 and 12 months were selected from the flock and transported to the pre-cleaned, well ventilated, concrete floor animal house at the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Upper Kabete Campus of the University of Nairobi. The animals were ear-tagged to facilitate identification and then washed thoroughly with acaricide (Almatix (R), Almandine Corporation, SA, Switzerland).

Faecal samples were collected from all the animals and examined. The animals were drenched with an albendazole (Valbazen (R),Kenya Swiss) and coccidiostats (Furaprol(R), ABIC LTD, Tel Aviv). They were also administered with a broad spectrum antibiotic (Pen-Strep (R), Norbrook, London) intramuscularly for 3 days and Multivitamins(R) (Norbrook, London). The animals were allowed to acclimatize to the new surroundings for a period of four weeks. In the third week of acclimatization, a blood sample (20 ml) was collected from each of the animals from the jugular vein by aseptic technique. The blood was allowed 8 hours to clot before it was centrifuged to separate the clot from the serum. The serum was collected using a pipette and stored at -20°C until use. This serum was considered as pre-infection and/or pre-immune serum.

The sheep were given enough hay and maize jam, and clean water was provided *ad libitum*.

3.2 EXPERIMENTAL DESIGN

The sheep were divided into 4 groups (I-IV). Each of the first three groups comprised of five sheep while the fourth group had three. Each group of animals was housed separately. Animals in group I were each given a low dose of about 250 metacercariae of *F. gigantica* orally. Animals in group II were each given a high dose of about 400 metacercariae and those in group III were each given doses of metacercariae ranging from 210 to 490 in number. The animals in group IV were immunised with the antigen (AgF.g28) so as to yield polyclonal antibodies (Table 1).

Table 1: Experimental design for the sheep.

Infection group	Dosage	Sheep Number	Number of metacercariae ingested	Time of Postmortem (weeks Post-infection)
I	Low	4502	292	21
		4509	256	21
		4544	204	21
		4546	225	21
		4549	199	21
II	High	4517	490	21
		4518	355	21
		4543	383	21
		4545	328	21
		4548	345	21
III	Mixed	4501	490	15
		4503	243	13
		4507	445	11
		4508	401	14
		4516	489	7
IV	Immunized with Antigen	4504	-	-
		4505	-	-
		4547	-	-

3.3 INFECTION OF SHEEP WITH METACERCARIAE.

3.3.1 *In vitro* culturing of *Fasciola* eggs to miracidia.

Livers from sheep slaughtered at the local abattoirs were examined and those found to have adult *F. gigantica* in the hepatic bile ducts had their gall bladders excised. The contents of the gall bladders were emptied into a clean glass bottle with a lid and transported to the laboratory. In the laboratory, the sedimentation technique described by Hansen and Perry (1994) was employed . Briefly, the gall bladder contents were thoroughly mixed with water before being filtered through a tea strainer and allowed 15 minutes for the eggs to sediment at the bottom of the beaker. The bile mixture was carefully decanted, leaving a small volume at the bottom containing the eggs. This procedure of addition of water and decantation was repeated twice. The final sediment volume was noted. This sediment volume was stirred so as to ensure uniform distribution of the eggs in the fluid. One millilitre was immediately pipetted out and placed on a slide. The eggs in the fluid on the slide were examined. The *Fasciola* eggs were identified by their characteristic colour, shape and size before they were counted. The number of eggs counted was multiplied by the volume containing all the eggs and recorded.

The total number of *Fasciola* eggs in the sediment was estimated by multiplying the number of eggs counted in 1 ml of the sediment by the total volume of the sediment.

A known number of *Fasciola* eggs was placed in universal bottles and kept in a warm (26°C) humid place for 14 days. Fully embryonated eggs were induced to hatch to miracidia by exposing the eggs to sunlight for 10 minutes (Bitakaramire, 1969).

3.3.2 Identification and rearing of the Snails

The intermediate host snails, *Lymnaea natalensis* were donated by the Kenya Agricultural Research Institute (KARI), Muguga. They had been identified on the basis of their shell structure and snail body colour as described by Mendal-Barth (1954). The shells were dextral and oblong with 4 increasing whorls, the ultimate whorl forming almost the entire shell. The columellar margin of the aperture was twisted and the outer lip sharp. The shells were either colourless, yellowish-brown or dark. The body was yellowish-brown or greenish in colour.

The snails were reared in a snail room that had much light and humidity and was maintained at 26°C. The snails were fed on boiled lettuce which was placed in four basins containing approximately 15 litres of pond water each. The pond water also had Guppy fish that feed on the worms *Chaetogaster lymnaeai*. These worms are prevalent in pond water and feed on *Fasciola* miracidia. Pond weeds, smooth stones and sand were also added to the aquaria to simulate the natural environment of the snails. The basins were left open to ensure maximum aeration of the water. The aeration was supplemented by using electrically powered aerators (Hy Flo(R), Medcalf Bros Ltd, England).

3.3.3 Infection of the snails with miracidia

Two hundred and sixty snails were placed in a plate with wells of about 5 ml capacity each. To each of the wells with a snail, about 10 miracidia were transferred and left overnight for the snails to be infected by the miracidia. It was ensured that the snails were completely submerged in the water containing the miracidia. Complete infection was ascertained by taking a few millimetres of the water in the well and examining it under a microscope for any motile

miracidia (Bitakaramire, 1969). The infected snails were transferred back to the aquaria.

3.3.4 Capturing of the metacercariae

Thirty days after infection, all the infected snails were transferred to aquaria which did not have the floating water weeds. Square pieces of transparent polythene paper (about 6 x 6cm) were placed on the surface of the water in the aquaria. The first metacercariae were seen encysted on the polythene paper on the 48th day post-infection. The metacercariae were seen against the light, but their presence was confirmed by examining the pieces of paper under a stereo microscope (Bitakaramire 1969).

The polythene papers dotted with numerous metacercariae were stored in pond water at 4°C and fresh ones placed in the aquaria. This procedure was repeated until a sufficient number of metacercariae was obtained.

3.3.5 Excystment of the metacercariae

The polythene papers studded with metacercariae were immersed in a beaker half full of concentrated sodium hypochlorite solution and stirred for about two minutes to allow complete detachment and excystation. The now metacercariae-free polythene papers were removed from the hypochlorite solution using hand forceps and discarded. The beaker was filled with water and allowed 15 minutes for the metacercariae to settle at the bottom. The water was decanted and the beaker refilled with water. This was repeated three times so as to dilute the hypochlorite solution and also to remove the unwanted debris (Waweru, 1995).

3.3.6 Counting the metacercariae

A few millilitres of the sediment were placed on a watch glass and examined under a dissecting microscope and the metacercariae counted. The live metacercariae were identified as transparent circles with butterfly-shaped internal organs in the centre. Dead metacercariae were either translucent, opaque or lacking a proper butterfly-formation of the internal organs (Fig. 3). Only the live metacercariae were counted. Metacercariae were put in gelatin capsules (at most 200 per capsule) using the pasteur pipette. The capsules were subsequently covered in wheat flour dough to make boluses and the appropriate number given orally to the sheep of groups I,II and III (Table 1).

3.3.7 Examination of the infected animals

All the infected animals were examined on a daily basis from the first day after infection. Rectal temperatures were taken daily and a thorough clinical examination conducted. Animals that showed clinical signs other than those related to fasciolosis were treated promptly. Faecal sample collection and examination was done weekly and those animals showing presence of nematode eggs were treated with levamisole (Nilverm$^{(R)}$, Coopers Kenya LTD) boluses. Levamisole has no effect on *Fasciola* species (Blood, Radostits and Henderson, 1984).

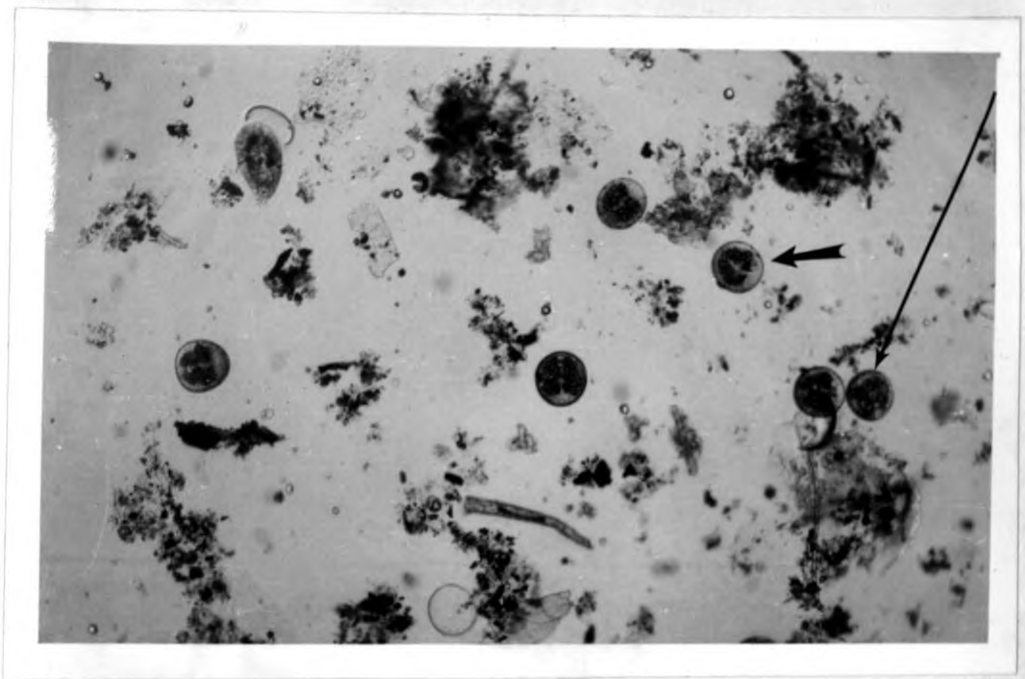


Figure 3: Normal and abnormal excysted metacercariae (x40 magnification). The normal metacercariae have a good butterfly formation of the internal organs, are transparent and circular (short, thick arrow), but the abnormal (or dead) metacercariae do not have a clear butterfly formation and/or are not transparent and/or are not circular (long, thin arrow)

3.3.8 Collection and processing of samples.

3.3.8.1 Collection and processing of blood samples.

About 20 ml of blood was collected from each of the infected sheep weekly. The blood was left at room temperature overnight for it to clot and the serum to separate. The clot was carefully removed and the serum centrifuged at 2000Xg for 10 minutes. Sodium azide was added to makea 0.1% solution. The bottles were properly labelled and stored at -20°C until use.

3.3.8.2 Faecal sample collection and examination.

Faecal samples were collected weekly from all the sheep of groups I and II from 3 weeks before infection upto 21 weeks post-infection. Faecal samples were obtained directly from the recti and examined soon after collection. Both the floatation (McMaster) technique (MAFF, 1986) and the Faecal egg sedimentation technique (Hansen and Perry, 1994) were employed.

3.3.8.3 Postmortem of infected animals

A complete postmortem was carried out on all the animals when either they died prematurely, or were slaughtered. The pathology caused by the liver flukes was noted, with special attention to the damage caused to the liver. The *Fasciola* eggs in the gall bladder and flukes in the liver parenchyma and/or bile ducts were recovered, counted and stored as described in 3.3.1, 3.4.1.1 and 3.4.1.2.

3.4 COLLECTION OF THE PARASITES.

3.4.1 *Fasciola gigantica*

3.4.1.1 Juvenile *Fasciola gigantica*

Juvenile *F. gigantica* parasites were obtained from slaughtered sheep of group III (Table 1) or those of groups I or II that died prematurely before the parasites had matured. They were recovered from the liver parenchyma by chopping the liver into small pieces of about 5 mm thick. These were then suspended in water while being pressed and squeezed manually to macerate and liquify the parenchyma. The suspension was washed several times with tap water and the flukes picked with hand forceps and counted. The numbers collected were recorded for individual animals. The juvenile parasites were washed 3 times for 15 minutes each in phosphate buffered saline (PBS) of pH 7.2 and stored at -20°C until required for use.

3.4.1.2 Adult *Fasciola gigantica*

Adult *F. gigantica* were found in the bile-ducts and gall bladders of animals of groups I and II (Table 1). They were removed carefully from the bile ducts by cutting open the large ducts first and following them up to the smallest ducts. The adult parasites were picked from the bile ducts. Those in the gall bladder were recovered by sieving the bile using a tea strainer. The livers were subjected to the same treatment as for the recovery of juvenile parasites. All the parasites were counted and the number recorded for individual animals. The parasites were washed 3 times for 15 mins each in PBS, pH 7.2 and stored at -20°C until required for use.

3.4.2 *Paramphistomum* species

The stomach flukes found attached among the ruminal papillae of infected sheep at the local abattoir were identified by their pinkish, cone-shape appearance (Soulsby, 1968) and carefully scrapped off after which they were washed 3 times for 15 minutes each in PBS, pH 7.2. They were then stored at -20°C until required for use.

3.4.3 **Hydatid Cyst fluid**

The hydatid cysts were obtained by dissecting them out from livers and lungs of infected sheep at the local abattoirs. The cysts were washed in distilled water several times and then dried on blotting paper. Fifty millilters of the hydatid cysts' fluid was harvested and centrifuged at 4,000Xg for 30 mins before being examined for hydatid sand. Only fertile cysts were used since it has been shown that non-fertile cysts are deficient in some antigens. The fertile cysts were identified by the fact that the hydatid sand in a fertile cyst sediments when the fluid is allowed to settle, whereas in non-fertile cysts there is no sedimentation even after centrifugation (Kagan and Agosin, 1968). The sediment and supernatant were mixed by stirring with a sterile rod. The fluid was stored at -20°C until required for use.

3.4.4 *Schistosoma mansoni*

About 120 adult *S. mansoni* were acquired from the Institute of Primate Research (IPR). They were washed 3 times for 15 minutes each in PBS, pH 7.2 and stored at -20°C until required for use.

3.4.5 *Stilesia hepatica*

Stilesia hepatica were recovered from bile ducts of livers from infected sheep at the local abattoirs. About 13 of them were washed 3 times for 15 minutes each in PBS, pH 7.2 and stored at -20°C until required for use.

3.5 PREPARATION OF CRUDE PBS EXTRACTS FROM VARIOUS PARASITES

3.5.1 Preparation of whole-worm antigens from Juvenile *F. gigantica*, Adult *F. gigantica*, *Paramphistomum* spp., *Schistosoma mansoni*, and *Stilesia hepatica*.

Varying numbers of *F. gigantica* (juveniles and adults), *Paramphistomum* spp., *S. mansoni* and *S. hepatica* were separately crushed in a frozen, sterile pestle and mortar with the addition of sterile sand. To every 20 parasites, 100 ml of PBS, pH 7.2 was added. Further crushing was done in a frozen Tin broeck tissue grinder (Kontes Glass Company, New Jersey) until a homogenous fluid was obtained. The fluid was centrifuged at 4,000Xg for 30 minutes and the sediment discarded. To each of the sample supernatants, a 0.2% sodium azide solution was added and stored in aliquots at -20°C until required for use.

The various crude extracts were given descriptive code names and numbers for ease of labelling. Hence, the juvenile *F. gigantica* extracts were code named JF.g( ) with a number in the brackets representing the age of the parasite in weeks. The adult liver flukes were code named AF.g, with those recovered from bovine livers having the code name AF.gb. *Paramphistomum* spp., *S. mansoni* and *S. hepatica* were code named P, S.m and St.h respectively.

3.5.2 Preparation of excretory/secretory antigens of *F. gigantica*.

Twenty live adult *F. gigantica* parasites were removed from the bile ducts of a liver from a sheep in group II and washed three times for 15 minutes each in 0.01M PBS, pH 7.2. They were then transferred to 100 ml of 0.15M PBS, pH 7.2 and incubated for 3 hours at 37°C. Finally, they were removed from the fluid, to which sodium azide was added to give a 0.2% solution (Fagbemi, Obarisiagbon and Mbuh, 1995). This crude excretory/secretory sample was stored at -20°C until use. It was given the code name ES.F.g.

3.5.3 Preparation of Hydatid cyst fluid extract

The hydatid fluid (collected as described in 3.4.3) was mixed with sterile sand and thoroughly crushed using both the pestle and mortar and the Tinbroeck tissue grinder. It was then centrifuged at 4,000Xg for 30 minutes. The supernatant was saved and sodium azide added to make a 0.2% solution. This sample was given the code name HyCF.

3.6 PROTEIN DETERMINATION OF THE VARIOUS PARASITE EXTRACTS

Protein determination was done for all the crude antigen extracts (prepared in 3.5) using the Folin-Ciocalteau method (Williams and Chase, 1968) which is a modification of the method described by Lowry, Rosebrough, Farr and Randal (1951). Briefly, 100-300ul of each of the crude antigen extracts was mixed with 2.5 ml of the Folin reagent and allowed 15 minutes for reaction to occur between the two. Dilute (1 Normal) phenol reagent was then added and allowed another 30 minutes at room temperature for the colour to develop. Bovine Serum Albumin (BSA), which was used as the protein standard was diluted serially. All samples were

run in duplicates. The OD values were read on the Beckman Spectrophotometer (Model 25. Scientific Instruments Division, California, U.S.A.) at the 570 wavelength. A graph of the OD values of the various dilutions of the standard against their known concentrations was plotted. The protein concentrations of the various parasite antigen samples were thereafter determined from the graph.

3.7 SEPARATION OF PROTEIN ANTIGENS AND MOLECULAR WEIGHT DETERMINATION.

3.7.1 Standardization of the sample concentrations.

Each of the crude antigen samples (prepared in 3.5 above) was standardized to a protein concentration of 3 mg/ml by diluting the more concentrated samples with PBS, pH 7.2 or by concentrating the more dilute samples. The crude sample from *S. hepatica* was concentrated by using polyethylene glycol 8000 (BDH chemicals, England). The sample was placed first in a dialysis tubing of 0.45 μm pore size (Medicell International Limited, London) before being covered by the polyethylene glycol (PEG).

The crude sample from *S. mansoni* was concentrated by lyophilization using Edwards(R) freeze drier (Model EF03, Britain) since the protein concentration was very low. It was then reconstituted to the required concentration using 0.15M PBS, pH 7.2.

3.7.2 Separation of proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was run according to the method described by Laemmli (1970). Briefly, the Mini Protean II electrophoresis cell (Biorad, USA) was set up to run two gels simultaneously. The SDS-PAGE gels were prepared and a comb with eight spikes inserted so as to make eight wells. The antigen samples of the various parasites were prepared for electrophoresis by mixing them with sample buffer at the ratio of 2:1 v/v to give a total volume of 30ul each of which was then placed in a well on the gel. Both low and high molecular weight markers (Sigma, USA) were run alongside the parasite samples. Bromophenol blue was used as tracking dye. A direct current was supplied by the LKB Biochrom (Model 2103, England) DC converter, set at 20mA for the two gels. At this current, the electrophoresis would run for about one and a half hours.

The initial electrophoretic runs were for the pore-gradient polyacrylamide separating gels of 7.5-15%, followed by that of 12.5% - 15%. The stacking gel for both of these runs was 5% polyacrylamide (PAA). Subsequently, runs were made with uniform PAA concentrations of 12.5% and 15% respectively. Molecular weight markers (Sigma, USA) were run alongside the samples.

In order to visualize the protein profiles, the gels that had gone through the electrophoresis process were stained with 0.5% Coomasie Brilliant Blue (R250, Merck, Germany) for 30 minutes and later destained 3 times for one hour each with a destaining buffer of 5% acetic acid and 16.5% methanol. The gels were then photographed and comparisons made between the protein profiles of *F. gigantica* and other parasites. Another comparison was made among the juvenile, adult excretory/secretory (ES) protein profiles of *F. gigantica*.

3.7.3 Molecular weight determination of the proteins.

Using proteins of known molecular weights, the molecular weights of the various proteins separated by the Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis were determined by first calculating the R_f values before plotting a graph of known molecular weights against their R_f values (Chung, 1987). R_f is calculated by the formula:-

$$R_f = \frac{\text{distance of migration of protein from origin}}{\text{distance of migration of tracking dye from origin}}$$

3.8 WESTERN BLOT

Western blot runs were carried out according to the method described by Towbin, Staehelin and Gordon (1979). Briefly, the SDS-PAGE was run (as described in 3.7.2) for a 15% PAA gel with samples of *F. gigantica*, other parasites and the excretory/secretory proteins of *F. gigantica*. The gels were not stained, instead the proteins were electrophoretically transferred onto nitrocellulose membrane (Immobilon-P, Millipore, U.S.A.) of pore size 0.45 μm using the Western Blot transfer cell (Model TE 50 Hoefer Scientific Instruments. San Francisco, USA). The Western blot transfers were done in duplicates. One of the nitrocellulose membranes was stained with Coomasie Blue (to show that the transfer was successful) whereas the other was processed for the immuno blot. The gels were also stained with Coomasie blue to show that the transfer was complete. No protein bands were to be observed when the transfer was complete. The unstained nitrocellulose membranes were incubated in serum (diluted 1/5 in distilled water) obtained from sheep that had been experimentally infected with *F. gigantica*. The incubation was carried out at room temperature for one hour with gentle agitation.

Unbound antibody was removed with washing buffer, afterwhich a secondary antibody, rabbit anti-sheep IgG conjugated to HRPO (Sigma,USA) and diluted to 1/3125 in conjugate diluent buffer, was added. This too was incubated for one hour at room temperature with gentle agitation. The excess conjugate was washed away and the substrate (prepared by adding 10 μl of 30% hydrogen peroxide to 0.05 gm of DAB in 100 ml of PBS), added. The colour was allowed to develop for 30 minutes before washing the membranes five times with distilled water. The nitrocellulose membrane was photographed and the results recorded.

3.9 IMMUNIZATION OF SHEEP WITH THE ANTIGEN

An antigen was found to be immunogenic and specific for *F. gigantica* by the Western blot. Its molecular weight was determined (as described in 3.7.3) and found to be 28 kiloDaltons (kDa). It was therefore code named AgF.g28.

3.9.1 Preparation and protein estimation of the antigen

A large amount of the AgF.g28 was obtained by performing several SDS-PAGE runs of *F. gigantica* whole-worm crude PBS, extracts. The antigen was cut out from the gels after staining them with ice-cold 0.25M potassium chloride solution (Hager and Burgess, 1980; Hames, 1990). The amount of antigen per gel strip was determined after eluting the antigen from the polyacrylamide gel (Hager and Burgess, 1980). Briefly, elution was done by adding 1 ml of elution buffer to each gel strip and crushed with 3 to 4 strokes of a pestle. The protein in the gel was allowed to elute for 1 hour at 25°C with occassional agitation. The mixture was then centrifuged for 1 minute in a clinical centrifuge at maximum speed to pellet the crumbled

gel.The supernatant was thereafter transferred into a siliconized 15 ml Corex tube. The protein determination was carried out using the method described by Williams and Chase (1968).

3.9.2 **Preparation of antigen and immunization of the animals.**

Several gel strips containing 3 mg of antigen were crushed in a tissue grinder to form a paste. Each of the 3 sheep in group IV (i.e the Specific-antigen-immunization group) were immunized subcutaneously with 0.4 mg of the paste antigen homogenized in 2 ml of Freund's Complete Adjuvant (FCA) (Sigma, USA) and 1 ml of PBS. Each of the immunized animals was given a booster after 28 days. The booster was composed of half the initial quantity (i.e 0.2 mg) of antigen homogenised in Freund's Incomplete Adjuvant (FIA) and PBS (Jurd and Bog-Hansen, 1990). Twenty five milliliters of blood was collected from each of the immunized animals 7 days after the booster and serum prepared (as described in 3.3.8.1) the serum was stored at -20°C in aliquots until use.

3.9.3 **Immunodiffusion**

Serum from each of the immunized animals was subjected to the simple immunodiffusion test (Hudson and Hay, 1991) against the antigen in order to ascertain the level of response (i.e the titre) of the animal towards the immunogen. The immunodiffusion gel used was a 1% preparation of purified Agar (Oxoid, England) in PBS, pH 7.4. The animals that responded poorly were given further boosters every two weeks, serum being collected just before the boosting. All sera from each animal that had titres of 1/8 or more were pooled together. The pooled serum for each animal was then subjected to the simple immunodiffusion test against the

antigen and also against the other related parasites' crude, whole-worm PBS extracts in order to check for cross reactions.

3.10 ISOLATION OF IMMUNOGLOBULIN G (IgG) FROM IMMUNE SERA.

3.10.1 **Separation of serum** proteins by precipitation and dialysis.

To each of the pooled sera (obtained in 3.9.4), immunoglobulins were precipitated with a 50% ammonium sulphate solution by adding an equal volume of saturated ammonium sulphate dropwise while stirring the mixture with a magnetic stirrer. The mixture was then centrifuged at 4,000Xg for 15 minutes and the supernatant discarded. The sediment was dissolved in 5 ml of 0.005M phosphate buffer, pH 8.0 and dialysed against a 0.005M phosphate buffer, pH 8.0 at 4°C. The buffer was changed frequently so as to maximize the rate of dialysis. The buffer was changed to one of 0.02M the day before running the ion-exchange chromatography.

3.10.2 **Setting up the ion-exchange chromatography column.**

One hundred and sixty grams of pre-swollen DEAE DE 52 (Whatman, New Jersey) for every 80 ml of serum was weighed and allowed to swell in 2 litres of 0.2M phosphate buffer, pH 8.0. It was then allowed to settle down before the buffer was siphoned out and 1 litre of 0.02M phosphate buffer, pH 8.0 added. The pH was adjusted using 1N HCl. The DEAE in 0.02M phosphate buffer was carefully introduced into the ion exchange column of 200 ml capacity. The DEAE was allowed to pack in the column and a reservoir of 0.02M phosphate buffer connected to ensure that the column did not dry up.

The dialysed serum proteins were centrifuged at 4,000Xg for 15 minutes and the supernatant introduced onto the DEAE column. The column was connected to the 7000 Ultrorac fraction collector (LKB Bromma, Sweden) which was set to collect 100 drops (approximately 8 ml) per tube. Two IgG fractions were collected. The amount of IgG in each of the pooled fractions collected was estimated by the use of ultraviolet light spectrophotometry to get optical density (OD) values, followed by calculation using the formula given by Hudson & Hay (1991) viz:

$$\text{Protein Concentration (Mg/ml)} = \frac{\text{OD x dilution factor x 10}}{\text{Molar Extinction coefficient}}$$

NB: The molar extinction coefficient used in this case was 14.5 as is reccomended for IgG of sheep (Hudson and Hay, 1991).

The two pooled IgG fractions were concentrated further by ultrafiltration using a PM 30 membrane (Amicon, USA) with a molecular weight cut-off of 30,000 daltons. The protein content of the resultant concentrated fractions was determined.

3.11 CONJUGATION OF SHEEP ANTI-AgF.g28 WITH HORSERADISH PEROXIDASE ENZYME

The conjugation of a fraction of the sheep anti-AgF.g28 with horseradish peroxidase enzyme was done following the method described by Wilson and Nakane (1978) and modified by Lindqvist, Gathuma and Kaburia (1982). Briefly, the ratio of protein (IgG) to Enzyme (HRPO) was calculated to 3:1. The protein concentration in this case was 19 mg/ml in 5 ml giving a total of 95 mg. Hence enzyme used was 31.6 mg.

The HRPO (31.6 mg) was dissolved in 9 ml of distilled water and oxidized by addition

of 1 ml of 0.15M sodium periodate ($NaIO_4$) to give a final volume of 10 ml. They were allowed to mix for 30 minutes with gentle magnetic stirring in a beaker covered with aluminium foil (to protect from light). The oxidized HRPO was dialysed against 500 ml of 1mM acetic acid, pH 4.4 containing 0.2 ml of ethylene glycol to get rid of the periodate and shorten the dialysis time. The acetate buffer was changed and 0.2 ml ethylene glycol added as before. Two more such changes were done, each being allowed 30 minutes to dialyse, this time without addition of ethylene glycol. The dialysed, oxidized HRPO was centrifuged at 2,000Xg for 10 minutes and the supernatant transferred into a clean sterile beaker. The pH was adjusted to 8.0 using 0.2M carbonate buffer, pH 9.0.

Five millilitres of sheep anti-AgF.g28 were added and pH adjusted to 9.0 using crystalline sodium carbonate while stirring gently. Conjugation was allowed to continue for 2hrs. The pH was thereafter adjusted to 7.5-7.8 with 1M HCl dropwise. Conjugation was left to go on overnight at 4°C without stirring. Twenty milligrams of glycine were added while stirring at room-temperature. The mixture was stirred for 1 hour before the pH was re-adjusted to 7.5 using 1M HCl. The mixture was thereafter centrifuged at 2,000Xg for 10 minutes and the supernatant transferred to a clean, sterile beaker. This supernatant was filtered through a 0.45 μm filter membrane into a sterile beaker and the volume found to be 9.8 ml. A 2% centrifuged, filtered, normal rabbit serum was added to the supernate and the new total volume found to be 10 ml. Finally, an equal volume (10 ml) of glycerol was added to the conjugate, mixed and stored at -20°c until required for use.

A chequerboard titration was done on a polyvinyl Nunc-immunoplate (Maxisorp, InterMed) in order to ascertain that the conjugation was successful.

3:12 THE DOUBLE-ANTIBODY (SANDWICH) ELISA FOR ANTIGEN DETECTION

3:12:1 Standardization of the Antigen-ELISA.

Chequerboard titrations were run in such a way as to find the optimum dilutions of the capture (coated) antibody (i.e sheep anti-AgF.g28) and the conjugate (i.e sheep anti-AgF.g28-HRPO) that would give minimal non-specific binding without compromising the detection ability of the test for the antigen.

3.12.2 Assaying the Negative Serum Samples.

The protocol used in these assays was as described by Voller *et al.*(1979) and Tijseen (1987). Briefly, polyvinyl Nunc-immunoplate (Maxisorp, USA) were coated with 100 μl of sheep anti-AgF.g28 antibody diluted 1/40 in coating buffer and incubated overnight at room temperature in a humid chamber. Wells A12, B12 and C12 were not coated as they were used for blanks and conjugate controls respectively. Excess coating antibody was washed off from the immunoplates 3 times for 5 mins each with washing buffer. One hundred microliters of test serum (i.e the negative serum samples) was added to all the wells except wells A12, B12, C12, D12 and E12. The test sera had been diluted serially with serum diluent buffer to give dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128. Incubation was allowed for one hour at 37°C before the plates being rinsed 3 times with washing buffer to clear off the excess or unbound antigen in the test sera. Each of the wells except A12 was given 100 μl of conjugated antibody (i.e sheep anti-AgF.g28-HRPO) diluted to 1/8000 in conjugate diluent buffer. This was incubated at 37°C for 1 hour and the excess conjugate washed off 3 times. Finally, 100 μl of

the substrate working solution containing the substrate (H_2O_2) and chromogen (ABTS) was added to all the wells and the plates incubated for 30 mins at room temperature. The absorbance (OD), was thereafter read on the ELISA reader (Titertek Multiskan MCC 340, Lab Systems, Finland) set at 405 nm. Seventy negative serum samples were assayed, each dilution of each serum sample being run in duplicate.

3.12.3 Assaying the serum samples from the low dose and the high dose infected sheep.

Pre-infection and weekly post-infection sera from each of the 5 sheep in low dose and high dose groups were assayed and the OD values noted. Upto 21 weekly post-infection sera were assayed. All sera assayed were serially double diluted from 1/2 to 1/128.

3:13 THE ANTIBODY-DETECTION ELISA

3.13.1 Standardization of the Antibody-ELISA

Chequerboard titrations were carried out for each of the reactants so as to ascertain the optimum dilutions required for them, i.e the coating antigen (AgF.g28), the test sera (sera of sheep infected with *F. gigantica*) and the conjugate (rabbit anti-sheep IgG-HRPO). All the buffers and ELISA plates used were of the same stock as those used in the antigen-ELISA (Appendix I I).

3.13.2 Assaying the Negative Samples

Fifty eight serum samples from animals known to be free from *Fasciola* were assayed

according to the protocol described by Voller *et al.*, (1976). Briefly, each of the wells on the ELISA plates, except wells A12, B12 and C12 were loaded with 100 μl of the partially purified antigen (i.e AgF.g28 eluted from the SDS-PAGE gel) diluted in coating buffer at the ratio of 1/2000. The plates were incubated at room temperature overnight in a humid chamber. Excess antigen was washed off 3 times using washing buffer before adding 100 μl of the test sera. The test sera had been diluted serially in serum diluent buffer to dilutions of 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600 and 1/3200. Each of the wells received test sera except wells A12, B12, C12, D12 and E12. The plates were incubated at 37°C for 1 hour before the excess antibody was washed off. All the wells except A12 were thereafter loaded with 100 μl of conjugated antibody (i.e rabbit anti-sheep IgG-HRPO) diluted to 1/2000 in conjugate diluent buffer. The plates were incubated for 1 hour at 37°C after which the excess conjugate was washed off. 100 μl of the substrate working solution (composed of 120 μl of 2% H_2O_2 ,25 ml of citric acid, pH 4.0 and 100 μl of 0.2 gm ABTS dissolved in 12.5 ml distilled water. This quantity was sufficient for every two ELISA plates) was ,finally, added to each of the wells on the plates. They were incubated for 30 mins at room temperature before the OD values at 405 nm were read on the ELISA reader.

3.13.3 Assaying the serum samples from the low dose and high dose infected sheep.

Pre-infection and post-infection sera from each of the 5 sheep in the low and high dose groups were assayed for antibody as was done for antigen assay in 3:12:3. However, the test sera were diluted serially to give the dilutions indicated in 3.13.2 above. The OD values were recorded.

3:14 STATISTICAL ANALYSIS

3:14:1 Evaluation of Sensitivity and Specificity.

Sensitivity is defined as the degree (percentage) to which a specified disease under study is correctly detected by a diagnostic test. Specificity, on the other hand, is the ability of a test to correctly detect healthy animals and/or those not having the specific disease under study (Sackett, Haynes and Tugwell, 1985).

In order to determine the sensitivity and specificity of a test, the health status of the animal must be established by other methods which are independent of the test under investigation (Thorner and Remein, 1961). In this study, the diagnosis of fasciolosis was established by post-mortem inspection of the slaughtered sheep.

The four-fold classification that was used in calculating the sensitivity and specificity of each of the 3 diagnostic tests in this study (i.e Faecal Egg Sedimentation Technique, Antigen detection ELISA and Antibody detection ELISA) is shown in Table 2:

Table 2: Classification of sheep with respect to post-mortem diagnosis and the 3 diagnostic test results under study.

	Post-mortem Diagnosis		
Test Results	Positive	Negative	Total
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	a+b+c+d=N

where a,b,c,d and N stand for the following:-

a-Sheep with fasciolosis and detected by the diagnostic test under study (true positives)

b-Sheep positive by the diagnostic test but negative at post-mortem (false positives)

c-Sheep negative by the diagnostic test but positive at post-mortem (false negatives)

d-Sheep negative for fasciolosis by both the diagnostic test and the post-mortem inspection (true negatives).

N-Total number of sheep being tested.

Sensitivity was therefore calculated as $= \frac{a}{a+c} \times 100$

Specificity was calculated as $= \frac{d}{b+d} \times 100$

3:14:2 **Determination of the optimum dilution of test serum for the ELISA tests.**

The optimum dilutions of serum samples for both the antigen- and the antibody-ELISA were determined by ascertaining the dilutions that gave the highest sensitivity and specificity.

3:14:3 **Determination of the cut-off points for the ELISA tests.**

The cut-off point is the measurable level above which a diagnostic test is regarded to have detected the presence of a specified disease and below which the test is regarded as not to have detected the disease (Sackett *et al.*,1985). In this study, the cut-off point was defined as the ratio between the OD of the test serum (ODS) and the OD of the negative control (ODCNEG)i.e ODS/ODCNEG that gave the highest sensitivity and specificity. The ratio between the OD of a sample and the negative sample is also known as percent

positivity,abbreviated, pp (Sackett *et al.*,1985)

3:14:4 Comparison of the three diagnostic tests' abilty to detect disease.

The earliest detection of fasciolosis by the FLST was taken to be the week post-infection in which *Fasciola* eggs were first seen in the faeces. For the Antigen- and Antibody-ELISA, the earliest detection was taken as the week post-infection in which the pp of the test sample was first seen to be equal to or greater than the cut-off point.

3:14:5 T-tests, Pearson Correlation and Regression Analysis.

All these tests were conducted as described by Steel and Torrie (1980). Student's T-tests were done to determine if there was a statistically significant difference between the low and high dose groups in the following parameters:-

(a) number of *Fasciola* eggs in 3 gm of faeces,

(b) number of *Fasciola* eggs in gall bladder,

(c) number of adult *F. gigantica* parasites recovered at post mortem,

(d) pp of the antigen-detection ELISA and

(e) pp of the antibody-detection ELISA.

The Pearson Correlation tests were done to ascertain the degree of relatedness between:-

(a) the number of adult flukes recovered from bile ducts and the number of *Fasciola* eggs in 3 gm of faeces at post mortem from the sheep in groups I and II.

(b) the number of *Fasciola* eggs seen in 3 gm of faeces and the number of *Fasciola* eggs seen in the gall bladder at post mortem and

(c) the number of adult flukes recovered and the number of eggs in the gall bladder at post mortem.

The Regression Analysis was done to find out whether the dose (i.e the number of metacercariae ingested) influenced the dependent variables (i.e the number of *Fasciola* eggs in gall bladder and faeces, the number of adult flukes and the OD values in both the antigen- and antibody-ELISA).

4. RESULTS

4:1 THE ANIMALS

4.1.1 Clinical Signs.

From about the 9th week post-infection, most of the sheep developed clinical signs typical of fasciolosis. These included sub-mandibular oedema, reduced weight, weakness, pale mucous membranes and reduced appetite among others. A total of 3 out of the 10 sheep of groups I and II succumbed to acute fasciolosis (see 4.1.2 below), but the other 7 survived and gradually had improved appetite and weight gain. Most of the clinical signs subsided with time, with the most persistent sign being parlour of the mucous membranes. All the sheep (both infected and non-infected) were drenched with a levamisole (Nilverm(R), Coopers) at week 7 post-infection when nematode eggs began appearing in the faeces. Levamisoles have no effect on *Fasciola* parasites (Blood *et al.*, 1984). Before they died, two sheep (4502 and 4545) were recumbent. The respiratory and heart rates were high (36 and 144 per min respectively). The third sheep (4544) died suddenly. Sheep 4545 that had verminous pneumonia died despite being treated with antibiotics for 3 days.

4:1:2 Postmortem findings in sheep infected with *F. gigantica*.

Sheep 4502 and 4544 of the low dose group, and sheep 4545 of the high dose group all died prematurely at around the 15th week post-infection. The liver of sheep 4545 was jaundiced, friable, enlarged and had numerous haemorrhagic tracts. Thirty four juvenile and 27 adult flukes were recovered from the liver parenchyma and bile ducts respectively of this particular

sheep. The gall bladder, which was distended with clotted blood and bile, had 3200 *Fasciola* eggs recovered from it. The sheep had bottle jaw, severe ascites and oedema of the lungs and abomasal mucosa. There was also anaemia, dehydration and the faeces were fairly dry. Fourteen *Fasciola* eggs were counted in 3 gm of faeces obtained from the rectum. The cause of death was anaemia and severe liver damage caused by the flukes.

Sheep 4544 died suddenly at the 15th week post-infection. Postmortem revealed a greatly enlarged, jaundiced liver with numerous haemorrhagic tracts. Twenty eight juveniles and 18 adult *F. gigantica* parasites were recovered from the liver parenchyma and bile ducts respectively. The gall bladder was greatly distended with clotted blood, greenish bile and 462 *Fasciola* eggs (Figs. 4 and 5). Fibrinous pneumonia and dehydration were also present. The cause of death was pneumonia and severe liver damage caused by the flukes.

Sheep 4545 died at the 14th week post-infection after a long period (one week) of weakness and recumbency, hence the emaciation and dehydration. The liver was thickly covered with fibrous adhesions and reduced in size. The hepatic parenchyma was firm and with an unusually high amount of connective tissue. Thirty six juvenile and 35 adult *Fasciola* were recovered from the liver tissue and bile ducts respectively. About 15 litres of straw-coloured ascitic fluid was drained from the abdomen. The lungs had areas of red hepatization and haemorrhage, but the most spectacular findings were three abscesses on the lung tissue adjacent to the heart (Fig. 6). Two of the abscesses contained two dead *Fasciola* parasites each but the third abscess was firm and the smallest in size of the three. The cause of death of this sheep was severe liver damage caused by the flukes and verminous pneumonia.

The postmortem findings in all the sheep except one (sheep 4546) that were slaughtered

at 21 weeks post-infection were similar. The livers were generally smaller than normal, firm and with a higher density of connective tissue. The tracts formed earlier in the migratory phases had healed and replaced with fibrous tissue. There were depressions on the liver capsule at the areas through which the flukes had gained entrance into the liver. The bile ducts were distended and on incision, revealed varying numbers of adult flukes. The gall bladders had clear bile and were only slightly distended. The animals' body condition was good, with no signs of ascites and haemorrhage as was frequently found in the animals that died prematurely. Sheep 4546 of group I was slaughtered at 21 weeks post-infection. The postmortem picture was primarily that of a healthy animal. However, the liver was shrunken in size, but devoid of the healed tracts present in the livers of the other sheep that were slaughtered at the same stage and time. The liver parenchyma was firm with increased connective tissue and thickened (but not distended) bile ducts. Three dead *Fasciola* parasites were recovered from peripheral bile ducts. They were small in size, white and shrunken. Only two liver flukes were found in the major bile ducts close to the gall bladder. They were alive, but smaller than their age mates from other sheep.

Each of the sheep in group III was slaughtered at the predetermined times. The sheep that were slaughtered at weeks 11, 13, 14 and 15 post-infection all had postmortem findings similar to the sheep 4502 and 4545 that died prematurely. However, none of the sheep of group III had adult flukes recovered from the bile ducts nor *Fasciola* eggs in the gall bladders, except sheep 4501 (slaughtered at week 15 post-infection). No parasites were recovered from sheep 4516 which was slaughtered at week 7 post-infection. The most common postmortem findings in sheep of this group (except sheep 4516 which was normal) were ascites, haemorrhagic tracts in the liver, distended gall bladders and juvenile flukes in the liver parenchyma. Table 3 below

is a summary of the data garnered from the sheep that were infected with metacercariae.

Table 3: Summary of data obtained from sheep infected with metacercariae of F. gigantica

Infection Group	Tag N°	Time eggs 1st seen in faeces (weeks PI)	N°. of eggs 1st seen in 3 gm of faeces	Time of death(weeks PI)	Time eggs last seen in faeces (weeks PI)	N°. of eggs last seen in 3 gm of faeces	N°. of eggs recovered from gall bladder	N°. of juveniles recovered from liver	N°. of adults recovered from bile ducts	Total N°. of flukes recovered
I	4502	14	3	15*	15	14	3,200	34	27	61
	4544	14	10	15*	15	1	462	28	18	46
	4546	20	1	21	21	4	9	0	5	5
	4549	13	1	21	21	257	43,175	0	29	29
	4509	16	15	21	21	153	545,700	0	33	33
II	4545	14	4	14*	15	22	9,400	36	35	71
	4543	16	8	21	21	71	5,200	0	29	29
	4548	14	2	21	21	204	1,328,040	0	53	53
	4517	14	3	21	21	15	4,900	0	28	28
	4518	15	4	21	21	25	1,640	0	9	9
III	4507	-	-	11	-	-	-	65	0	65
	4503	-	-	13	-	-	-	65	0	65
	4508	-	-	14	-	-	-	107	0	107
	4516	-	-	7	0	0	0	0	0	0
	4501	15	3	15	15	3	1800	20	15	35

NB:- * = Animal died prematurely

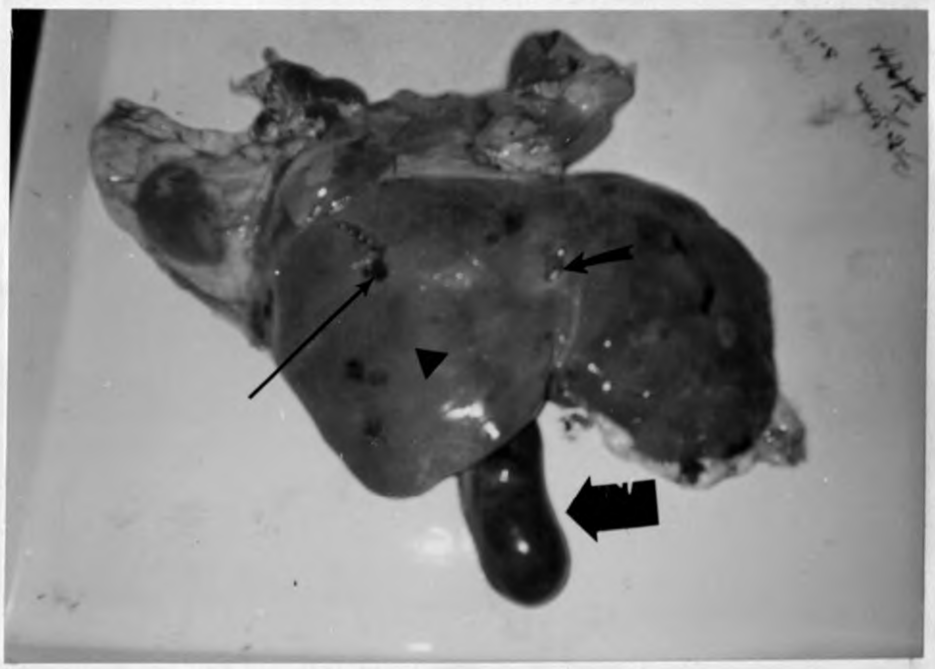


Figure 4: Dorsal view of the liver of sheep 4544 which died prematurely at the 15th week post-infection. Note the enlarged, jaundiced liver (arrow head), the haemorrhagic spots and tracts beneath the liver capsule (long arrow), distended gall bladder (thick arrow) and juvenile *Fasciola* penetrating through the liver capsule (small arrow).

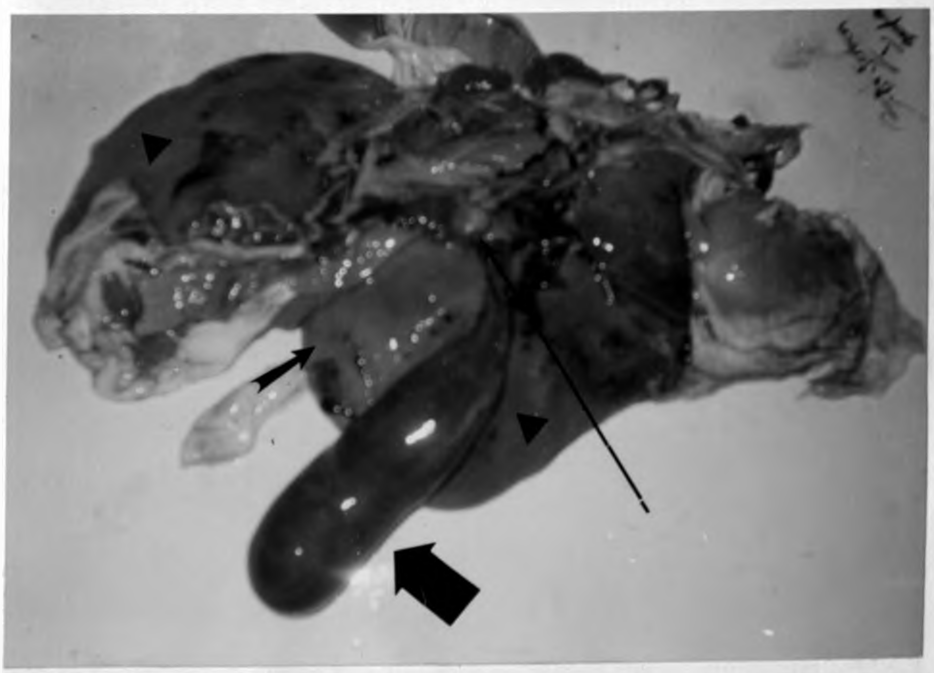


Figure 5: Ventral view of the liver of sheep 4544. Note the darkening of liver tissue due to haemorrhage (arrow head), jaundice (rocket arrow), the distended bile ducts (long arrow) and gall bladder (thick arrow).

Figure 6: Dorsal view of the lungs of sheep 4545 which died prematurely at the 14th week post-infection. Note the abscess on the lung (long thin arrow), the red hepatization and haemorrhage (thick, short arrow), and the adhesions on the lung surface, between the heart and the lungs, and around the abscess (arrow head).

4:2 PROTEIN CONCENTRATIONS OF THE VARIOUS PARASITES' WHOLE-WORM CRUDE PBS EXTRACTS.

The protein concentration of the crude PBS extracts of *Paramphistomum* ssp was the highest at 10.741 mg/ml whereas that of *S. mansoni* was the lowest at 0.798 mg/ml. The protein concentrations of all the juvenile flukes were about the same, but were lower than those of the adult liver flukes from both sheep and bovine. Table 4 below shows the protein contents of the crude extracts from the various parasites.

4:3 SDS-PAGE FOR COMPARISON OF PROTEIN PROFILES OF THE CRUDE PBS EXTRACTS OF VARIOUS PARASITES.

The protein profiles of the various ages of *F. gigantica* and excretory/secretory products on SDS-PAGE revealed that most of the proteins of *F. gigantica* were concentrated in the molecular weight range of 25 to 100-kDa (Fig. 7). All the protein profiles of the various ages of juvenile flukes (5, 6, and 7) appeared identical. However, there were some differences between the protein profiles of the juvenile flukes and the adults (9 and 10). The protein bands from the ES products (11) were fewer in number than those from the whole-worm extracts. All the bands in the ES protein profile were present in the profiles of the whole-worm extracts except the band of MW 50-kDa which was unique to the ES products.

Each of the protein profiles from the other parasite species was apparently unique and easily distinguishable from the rest. Despite this, there were some protein bands that appeared common in two or more parasites. The proteins of *Paramphistomum* spp. were mostly below 30-kDa whereas those of hydatid cyst fluid and *S. mansoni* were concentrated between 30 and 94-kDa. A few protein bands of *S. hepatica* had MW of more than 100-kDa (Fig. 8).

Table 4: Protein concentrations (mg/ml) of the crude PBS extracts of various ages of *F. gigantica*, Excretory/secretory antigens of *F. gigantica* and related parasites.

Code N°	Full Name of Crude PBS Extract	Code Name	Protein content (mg/ml)
1	*Paramphistomum* spp.	P	10.741
2	Hydatid cyst fluid	HyCF	7.231
3	*Schistosoma mansoni*	S.m	0.798
4	*Stilesia hepatica*	St.h	1.151
5	Juvenile *F. gigantica* (11 weeks)	JF.g (11)	3.458
6	Juvenile *F. gigantica* (13 weeks)	JF.g (13)	3.577
7	Juvenile *F. gigantica* (14 weeks)	JF.g (14)	3.836
8	Juvenile *F. gigantica* (15 weeks)	JF.g (15)	3.824
9	Adult *F. gigantica*	AF.g	4.828
10	Adult *F. gigantica* (from bovine)	AF.g b	4.364
11	Excretory/secretory products	ES.F.g	3.400

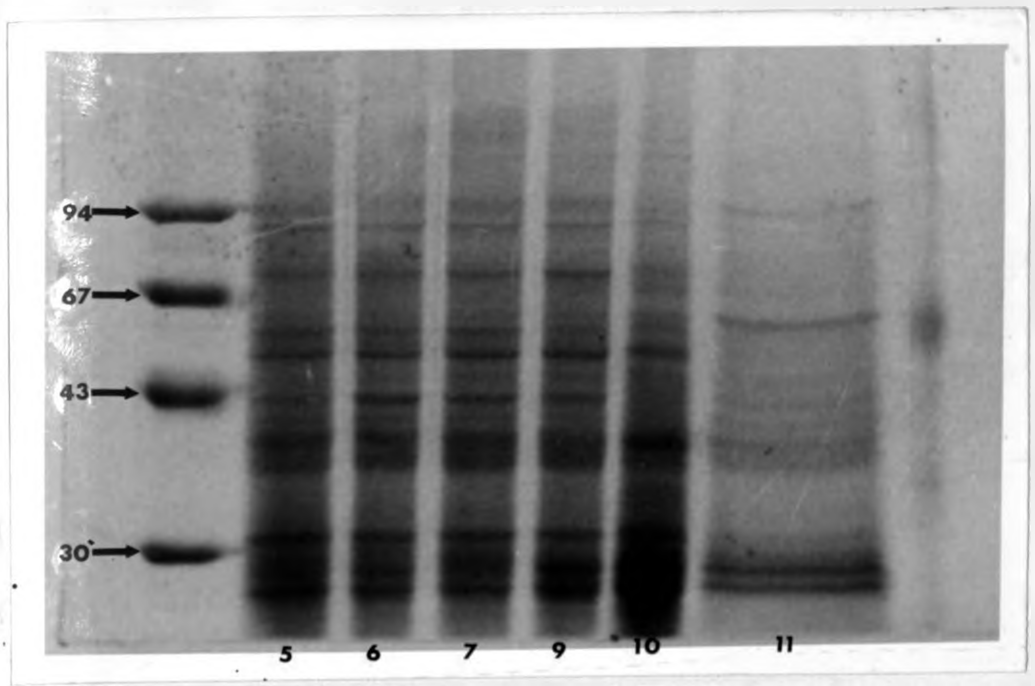


Figure 7: A 15% Poly acrylamide (PAA) gel showing protein profiles of the different ages of whole-worm crude PBS extracts and excretory/secretory products of *F. gigantica*: 5-juvenile, 11 weeks; 6-juvenile, 13 weeks; 7-juvenile, 14 weeks; 9-adult from ovine;10-adult from bovine and 11- Excretory/secretory products. The bands on the left are of proteins whose molecular weights are shown (in kiloDaltons).

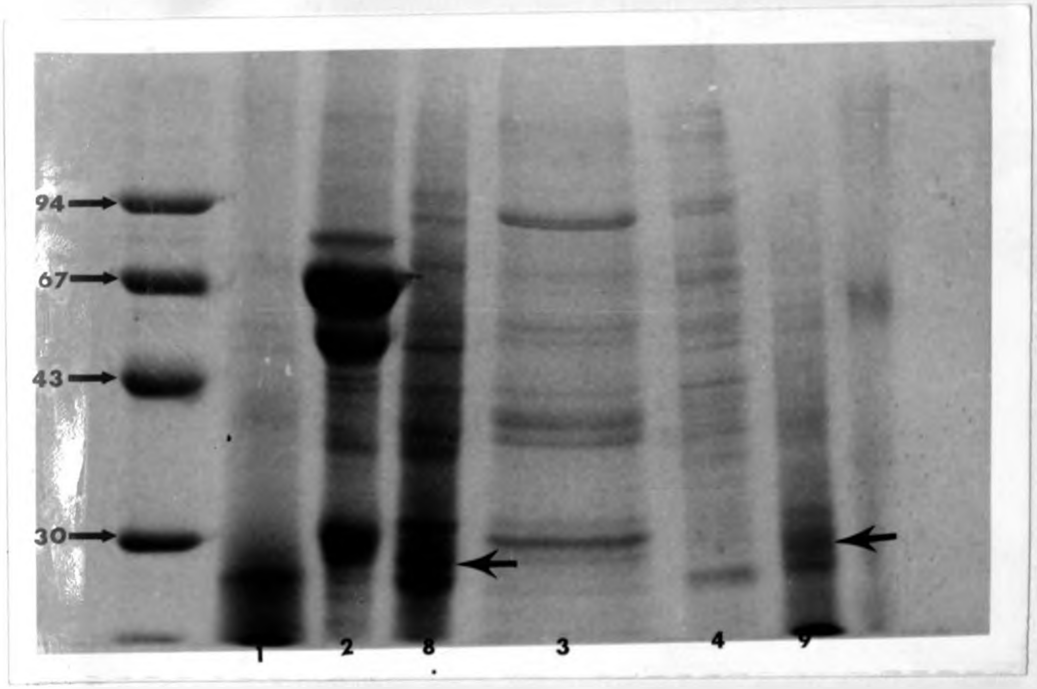


Figure 8: A 15% PAA gel showing proteins profiles of crude whole-worm PBS extracts of *Paramphistomum* spp. (1), hydatid cyst fluid (2), *Schistosoma mansoni* (3), *Stilesia hepatica* (4), juvenile *Fasciola gigantica* (8) and adult *Fasciola gigantica* (9). The bands on the left are of proteins whose molecular weights are shown (in kiloDaltons). The arrows directed at protein bands in profiles 8 and 9 point at the location of the antigen, AgF.g28.

Some of the blots were marred by vertical stripping, especially the protein profiles of the *Paramphistomum* spp. The protein bands on the nitrocellulose, enhanced by staining with Coomasie blue, showed that there was a band that appeared on the protein profiles of both the whole-worm (6) and the ES products (11) at the 28-kDa level (Fig. 9 and 10). This band was recognized by the antibodies distinctly and was not detected in any of the other parasites.

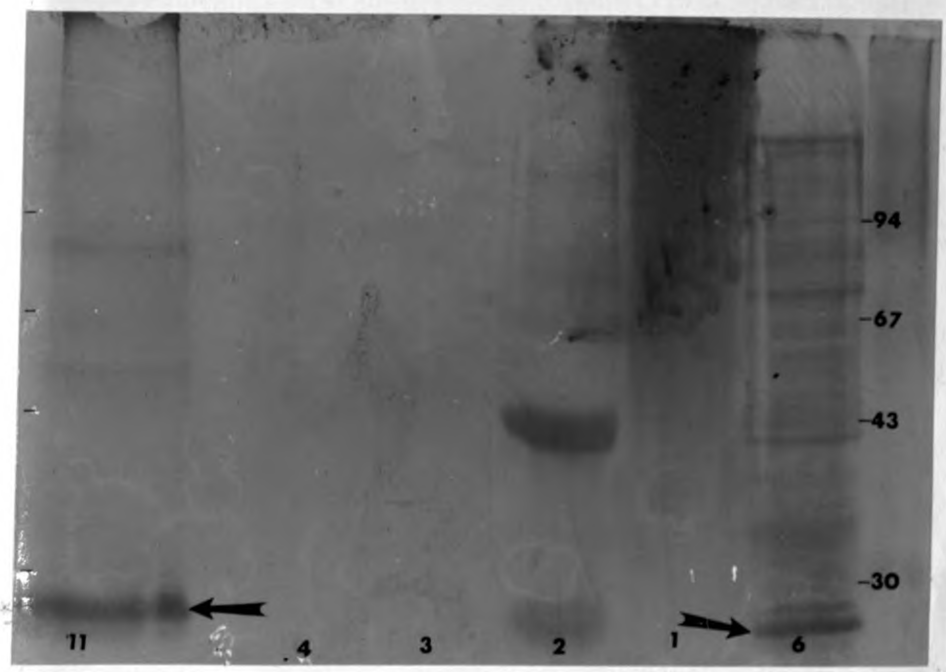


Figure 9: Western blot for protein profiles of *Fasciola gigantica* and related parasites. The AgF.g28 is shown by a rocket arrow on the protein profiles of both whole-worm (6) and excretory/secretory products (11) of *F. gigantica*. The molecular weights in kiloDaltons are shown on the right.

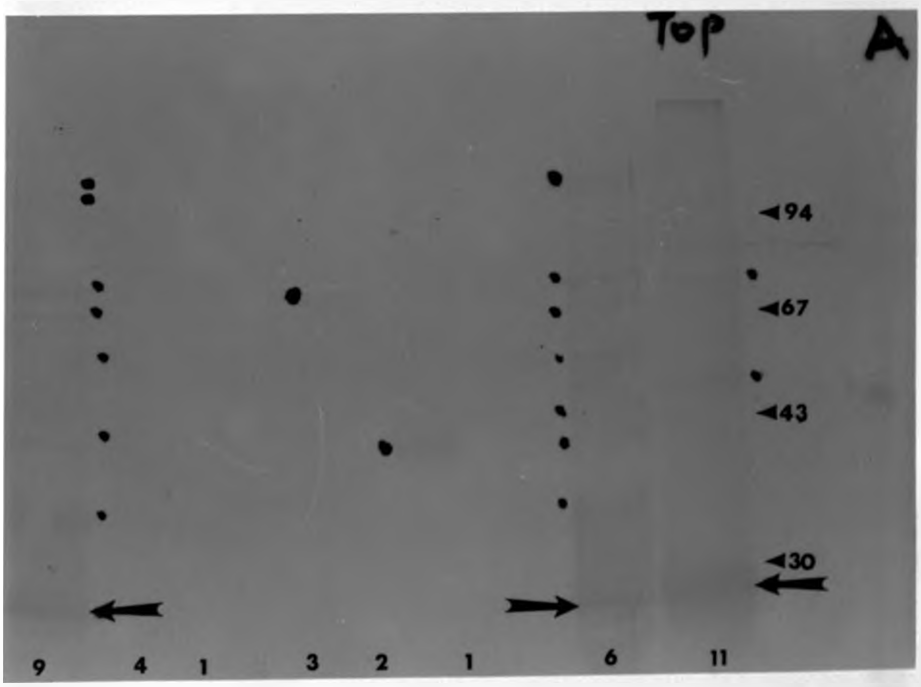
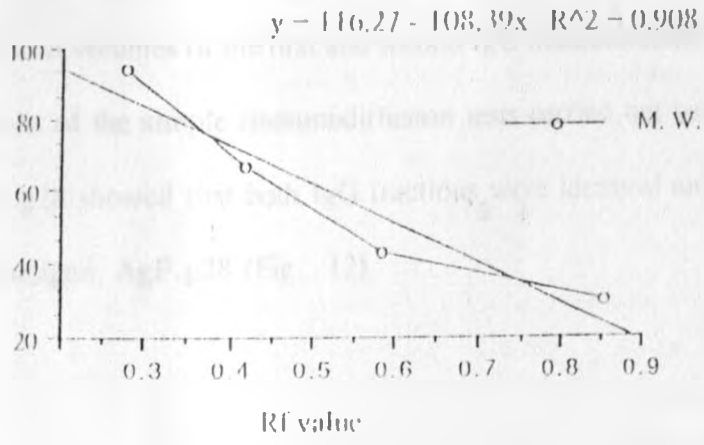


Figure 10: Western Blot for protein profiles of *Fasciola gigantica* (6 and 9) and related parasites; *Paramphistomum* spp (1), Hydatid cyst fluid (2), *Schistosoma mansoni* (3) and *Stilesia hepatica* (4). The nitrocellulose membrane was not stained but the faint protein bands that had been recognized by the antibody were represented by dots (inserted). Note that the antigen, AgF.g28, (shown by the rocket arrows) appeared only in the protein profiles of *F.gigantica* (6 and 9) and its excretory/secretory products (11). The molecular weights are in kiloDaltons.

Figure 11: Graph of R_f against known molecular weights. The antigen, AgF.g28, was found to have a molecular weight of 27.644-kDa, which was rounded up to 28-kDa.



4.5 PROTEIN ESTIMATION OF THE ANTIGEN, AgF.g28, IN THE SDS-PAGE GEL STRIP.

The protein concentration of the antigen, AgF.g28, was found to be 0.052 mg/ml per strip of gel after elution. The AgF.g28 used in coating the plates for the Antibody-ELISA was harvested from 15 gel strips found to have a protein content of 0.7813 mg/ml after elution.

4.6 PROTEIN ESTIMATION OF THE IgG FRACTIONS.

The protein contents of the two IgG fractions were found to be 15.17 and 19.0 μg/ml. The total volumes of the first and second IgG fractions collected were 40 and 50 ml respectively. Results of the simple immunodiffusion tests carried out between the two IgG fraction and the AgF.g28 showed that both IgG fractions were identical and also that both of them recognised the antigen, AgF.g28 (Fig. 12).

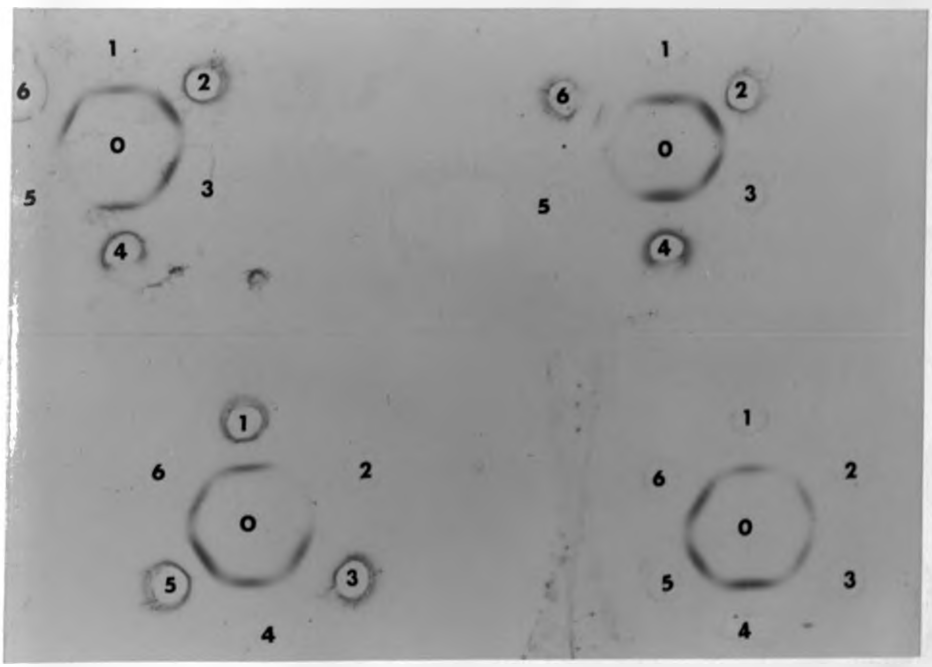


Figure 12: Simple Immunodiffusion test between the two IgG fractions and AgF.g28. The centre well (0) contains the AgF.g28 whereas the peripheral wells (1-6) contain the two IgG fractions alternately.

4:7 THE FAECAL EGG SEDIMENTATION TECHNIQUE (FEST) RESULTS.

4:7:1 Detection of *Fasciola gigantica* eggs in the faeces.

Fasciola gigantica eggs were first seen in the faeces on the 13th week post-infection. This was seen in sheep 4549 of the low dose group and only one egg was seen in 3 gm of faeces. By week 14 post-infection, 6 out of the 10 sheep of groups I and II were showing eggs in the faeces. Sheep 4509 of the low dose group had the highest number (15) of eggs first seen in the faeces but these first appeared at week 16 post-infection. Sheep 4546 of low dose group was the last to show eggs in the faeces. It first showed eggs in the faeces at week 20 post-infection and it was only one egg in 3 gm of faeces. Sheep 4549 of low dose group showed the highest number of eggs in the faeces (257 eggs in 3 gm of faeces) at week 21 post-infection. This is the same sheep which had eggs appearing in faeces earliest at week 13 post-infection. Two animals (4502 and 4544) of the low dose group died prematurely at the 15th week post-infection and one (4545) of the high dose group died at the 14th week post-infection. They all had began to show eggs in the faeces.

In general, the animals showed a gradual increase in the number of eggs in faeces with time except sheep 4544 whose eggs numbers declined at death. In this particular sheep, the gall bladder was greatly distended at death with clotted blood which literally blocked the neck of the bladder. Sheep 4548 of the high dose group showed fluctuations in the number of eggs in faeces with time.

Table 5 below gives a summary of the weekly faecal egg count (in 3 gm of faeces) for *F. gigantica* in the experimentally infected sheep.

Table 5: Weekly egg count (in 3gms of faeces) of *Fasciola gigantica* in experimentally infected sheep.

Infection group	Tag N°	Week 12	Week 13	Week 14	Week 15	Week 16	Week 17	Week 18	Week 19	Week 20	Week 21
Low Dose	4502	0	0	3	14	-	-	-	-	-	-
	4509	0	0	0	0	15	44	42	97	123	153
	4544	0	0	10	1	-	-	-	-	-	-
	4546	0	0	0	0	0	0	0	0	1	4
	4549	0	1	2	4	6	90	130	188	237	257
High Dose	4517	0	0	3	2	10	10	12	14	13	15
	4518	0	0	0	4	6	9	13	15	23	25
	4543	0	0	0	0	8	15	53	59	20	71
	4545	0	0	4	22	-	-	-	-	-	-
	4548	0	0	2	5	21	138	43	76	75	204

NB:- Low Dose = Infection with about 250 metacercariae

- High Dose = Infection with about 400 metacercariae

4.7.2 Percentage fluke take

This was defined as the proportion (%) of flukes that were recovered in the host sheep after infection with a known number of metacercariae of *F. gigantica*. The results showed that the highest number (53) of adult flukes recovered was from a sheep (4548) in the high dose group while the lowest (5) was from an animal (4546) in the low dose group. The highest fluke take (22.6%), however, was from an animal (4544) in the low dose group. It was evident that the animals that had the highest fluke takes were those which had died prematurely (i.e sheep 4502, 4544 and 4545) and therefore also had juvenile flukes. Among the animals that were slaughtered at 21 weeks post-infection, a fluke take of 15.4% was the highest (from sheep 4548 of the high dose group), whereas the lowest was 2.2% from a sheep (4546) in the low dose group. On average, the animals in the low dose group had a higher fluke take (14.68%) than those in the high dose group (10.56%). However, a Student's T-test showed that this difference was not statistically significant ($p > 0.05$). Table 6 shows the fluke recovery rates from the animals in the low and high dose infection groups.

Table 6: Fluke recovery rates in sheep experimentally infected with metacercariae of *Fasciola gigantica*.

Infection group	Tag N°	Dose	Time of death (Weeks PI)	N°. of adult flukes recovered	No. of juveniles recovered	Total No. of flukes recovered.	Percentage fluke take.
Low dose	4546	225	21	5	0	5	2.2
	4509	256	21	33	0	33	13.2
	4549	199	21	29	0	29	14.6
	4502	292	15	27	34	61	20.9
	4544	204	15	18	28	46	22.6
	Mean	**234.2**	-	**22.4**	**12.4**	**34.8**	**14.68**
High dose	4518	355	21	9	0	9	2.5
	4517	490	21	29	0	29	5.7
	4543	383	21	28	0	28	7.6
	4548	345	21	53	0	53	15.4
	4545	328	15	36	35	71	21.6
	Mean	**380.2**	-	**31.0**	**7.0**	**38.0**	**10.56**

4:7:3 Number of *Fasciola* eggs in gall bladder at postmortem

The results show that the highest number (1,328,040) of *Fasciola* eggs recovered from the gall bladder was from a sheep (4548) in the high dose group whereas the lowest number (9) was from a low dose group animal (4546). On average, the sheep in the high dose group had more (269,836) *Fasciola* eggs in their gall bladders than those (118,509.2) in the low dose group, although this difference was not statistically significant ($p > 0.05$). Another T-test showed that even though the number of adult flukes recovered (31) from the sheep in the high dose group were, on average, more than those recovered from animals in the low dose group (22.4), this was not statistically significant ($p > 0.05$). There was a direct relationship between the number of adult flukes recovered and the number of *Fasciola* eggs in the gall bladder, and also between the number of *Fasciola* eggs in 3 gm of faeces and the number of *Fasciola* eggs in the gall bladder. However, there was no relationship between the number of adult flukes recovered and the number of *Fasciola* eggs in the faeces. Table 7 shows the number of *Fasciola* eggs recovered from the gall bladders of the sheep of the low and high dose groups.

Table 7: Number of adult flukes recovered and eggs in gall bladders in sheep experimentally infected with metacercariae of *Fasciola gigantica*.

Infection Group	Tag N°	Time of death (weeks PI)	N°. of eggs in 3 gm of faeces at PM	N°. of adult flukes recovered at PM	N°. of juveniles recovered at PM	Total N°. of flukes recovered at PM	N°. of eggs in gall bladder at PM
Low Dose	4544	15	1	18	28	46	462
	4502	15	14	27	34	61	3,200
	4546	21	4	5	0	5	9
	4549	21	257	29	0	29	43,175
	4509	21	153	33	0	33	545,700
	Average	-	**85.8**	**22.4**	**12.4**	**34.8**	**118,509.2**
High Dose	4545	15	22	36	35	71	9,400
	4518	21	25	9	0	9	1,640
	4517	21	15	28	0	28	4,900
	4543	21	71	29	0	29	5,200
	4548	21	204	53	0	53	1,328,040
	Average	-	**67**	**31**	**7**	**38**	**269,836**

Range of eggs in gall bladder: 9-1,328,040
Range of eggs in faeces at postmortem: 1-257
Range of adult flukes in bile ducts at postmortem: 5-53
Range of flukes in the liver: 1-71
NB:- Low Dose = Infected with about 250 metacercariae.
- High Dose = Infected with about 400 metacercariae.

4.7.4 Comparison between low dose and high dose groups

4.7.4.1 Faecal egg counts

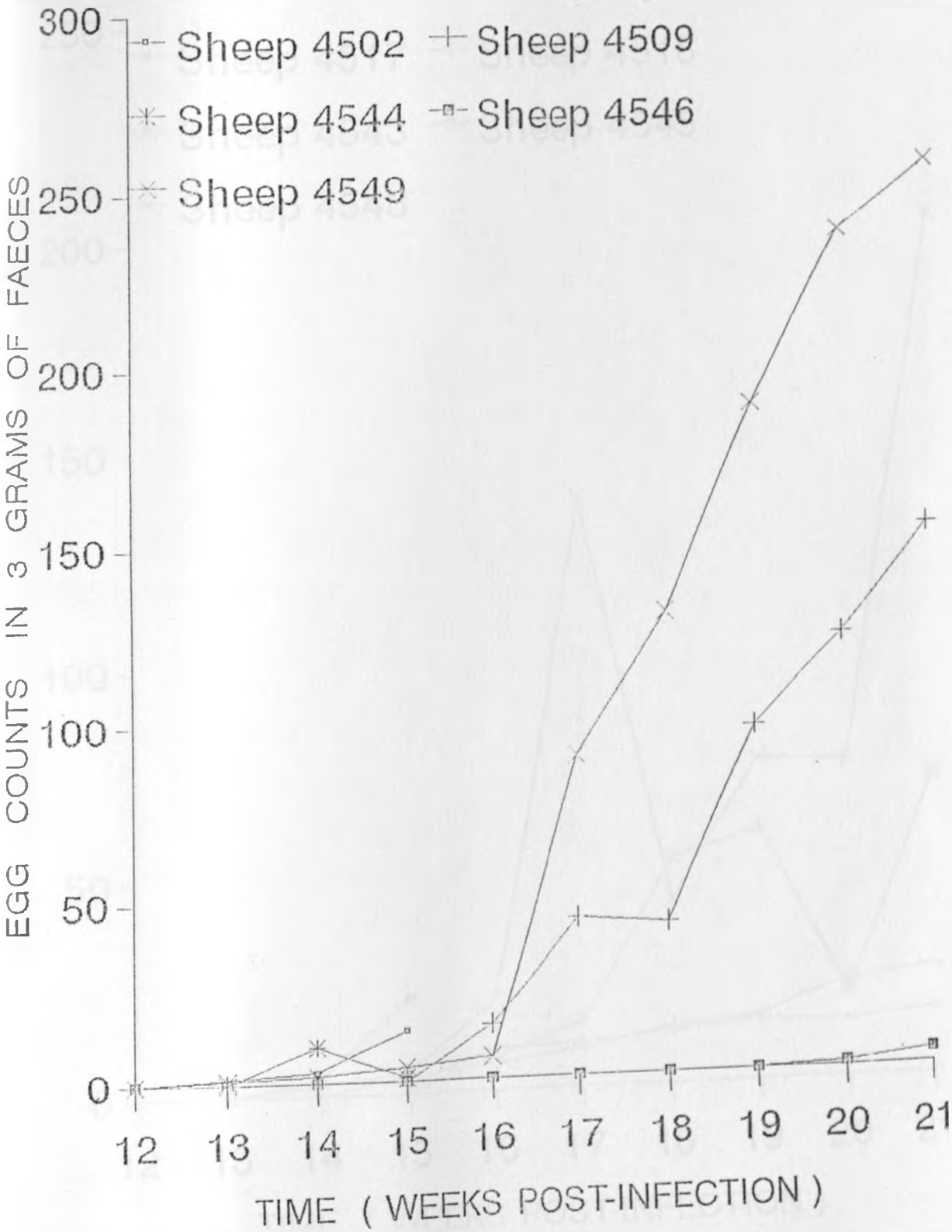
Figs. 13 and 14 show graphs of egg counts (per 3 grams of faeces) for individual animals in the low dose and high dose infection groups respectively, whereas Fig. 15 shows the mean faecal egg counts of the same two infection groups. There was a general increase in the number of eggs in the faeces with time for both infection groups from week 13 post-infection. However, there was a greater increase from week 16 post-infection for most of the animals in both groups.

One sheep (4549) from the low dose group had the highest number of eggs in the faeces at 21 weeks post-infection. The lowest number of eggs in the faeces in the same period was also from a sheep (4546) in the low dose infection group.

Animals 4517, 4518 (both of high dose group) and 4546 (low dose) maintained a low egg count throughout the experimental period. The same animals had the least number of adult *Fasciola* parasites when slaughtered (Table 3) at 21 weeks post-infection.

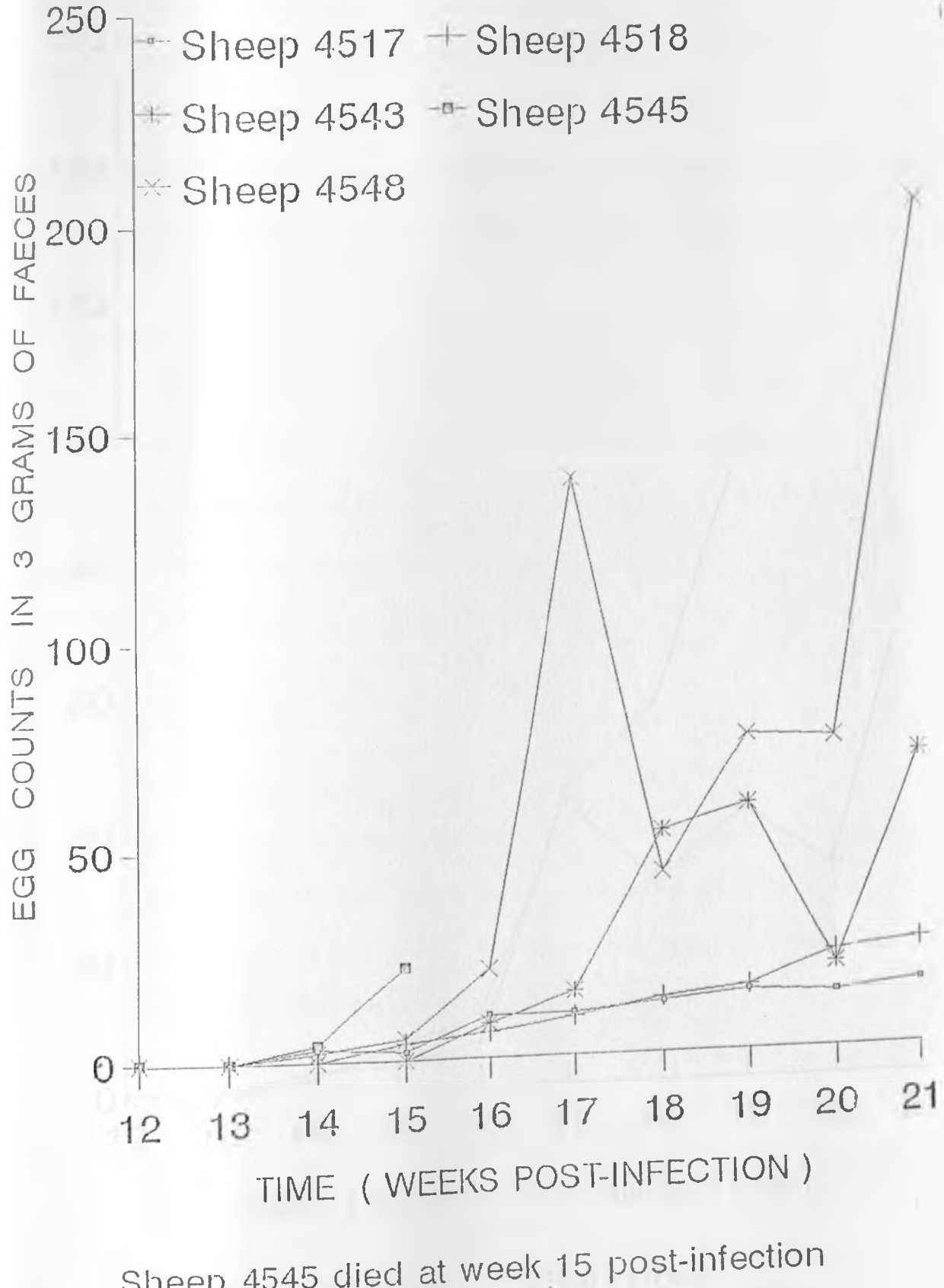
A Student's T-test done for the average number of eggs in 3 gm of faeces per infection group showed no significant difference ($p>0.05$) between the low dose and the high dose groups.

Figure 13: Time series plots for the number of *Fasciola gigantica* eggs in 3 grams of faeces from sheep experimentally infected with a low dose (250) of metacercariae.



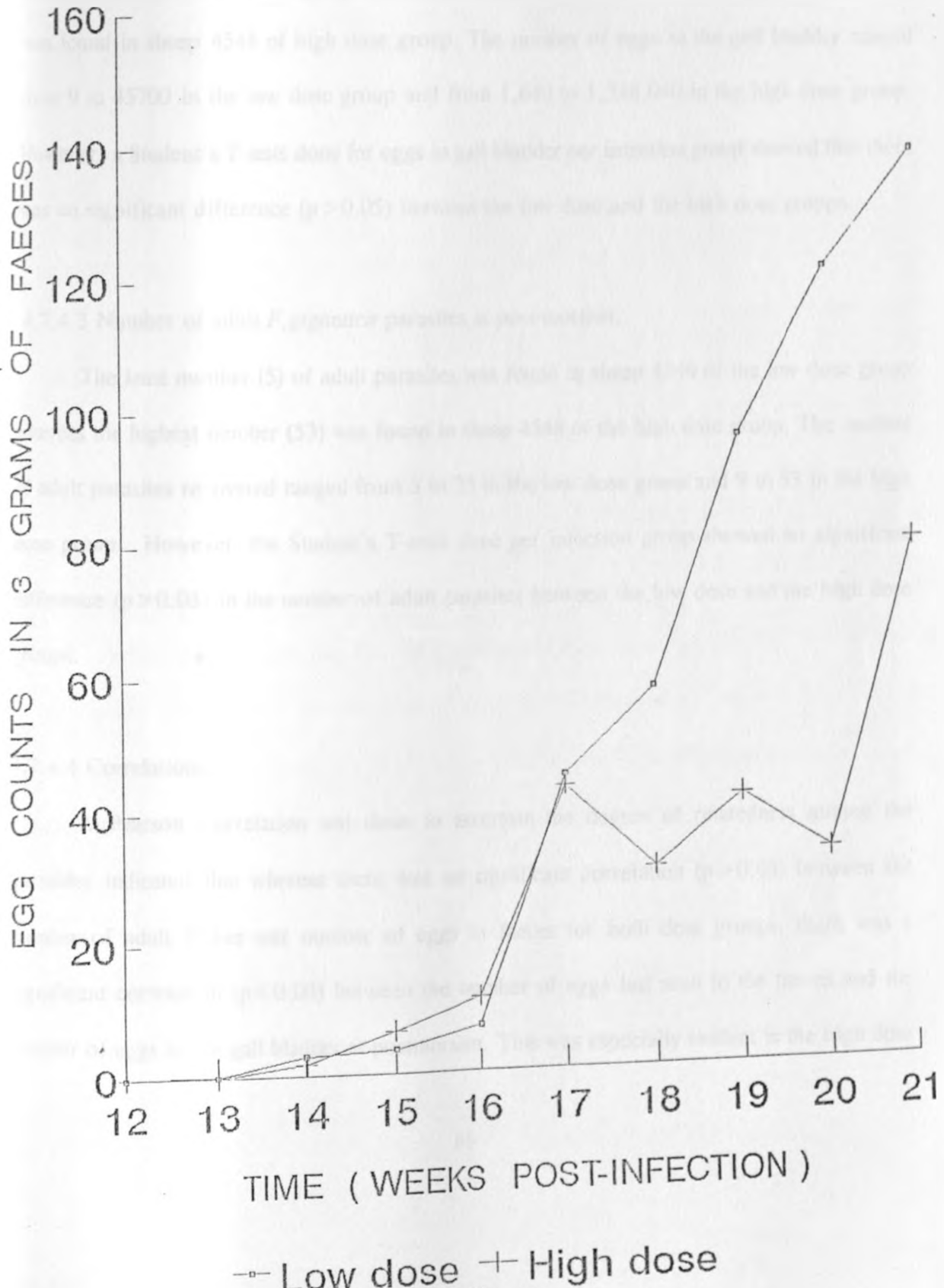
Sheep 4502 and 4544 died at week 15 post-infection

Figure 14: Time series plots for the number of *Fasciola gigantica* eggs in 3 grams of faeces from sheep experimentally infected with a high dose (400) of metacercariae.



Sheep 4545 died at week 15 post-infection

Figure 15: Mean weekly faecal egg counts of two groups of sheep infected with low (250) and high (400) doses of metacercariae of *Fasciola gigantica*.



4.7.4.2. Number of *Fasciola* eggs in the gall bladder

The lowest number (9) of eggs in the gall bladder at 21 weeks post-infection was found in sheep 4546 of the low dose group whereas the highest number (1,328,040) in a similar period was found in sheep 4548 of high dose group. The number of eggs in the gall bladder ranged from 9 to 45700 in the low dose group and from 1,640 to 1,328,040 in the high dose group. However, a Student's T-tests done for eggs in gall bladder per infection group showed that there was no significant difference ($p > 0.05$) between the low dose and the high dose groups.

4.7.4.3 Number of adult *F.gigantica* parasites at post-mortem.

The least number (5) of adult parasites was found in sheep 4546 of the low dose group whereas the highest number (53) was found in sheep 4548 of the high dose group. The number of adult parasites recovered ranged from 5 to 33 in the low dose group and 9 to 53 in the high dose group. However, the Student's T-tests done per infection group showed no significant difference ($p > 0.05$) in the number of adult parasites between the low dose and the high dose groups.

4.7.4.4 Correlations.

A Pearson Correlation test done to ascertain the degree of relatedness among the variables indicated that whereas there was no significant correlation ($p > 0.05$) between the number of adult flukes and number of eggs in faeces for both dose groups, there was a significant correlation ($p < 0.05$) between the number of eggs last seen in the faeces and the number of eggs in the gall bladder at postmortem. This was especially evident in the high dose

group.

There was also a direct relationship ($p<0.05$) between the number of adult flukes recovered from the liver and the number of eggs in the gall bladder at postmortem. This correlation was more significant in the low dose group.

4.7.4.5. Regression Analysis

The results of a regression analysis done for purposes of finding out whether the dose of metacercariae influenced the dependent variables showed that there was no influence of the dose over the dependent variables for the low dose, high dose and both groups combined at the 5% level.

4.8 THE DOUBLE-ANTIBODY (SANDWICH) ELISA

4.8.1 Optimum concentrations of Antigen and Antibody.

After running seven tests on a chequerboard, the optimum concentration for the coating (or capture) antibody (i.e the sheep anti AgF.g28) was found to be 1/40 whereas that of the conjugate (i.e sheep anti-AgF.g28-HRPO) was found to be 1/800. When controls were run with these concentrations it became apparent that there was a persistent background colour or" noise" that could not be removed without interfering with the sensitivity of the test. This background noise was created between the coating antibody and the conjugate and on analysis, it was found to be directly proportional to the OD values of the negative control serum (Fig. 16).
The negative control serum was obtained from naive sheep known not to have any infection.

Despite the presence of the background noise and interplate variations (Fig. 17), the post-infection OD values were consistently higher than the pre-infection readings. This was more evident in the high dose group in which none of the post-infection OD values of the 5 sheep went below that of the pre-infection readings (Fig. s 18 and 19). In both the low and high dose groups, there was an initial upsurge of OD values between the first and second weeks post-infection before declining gradually up to about the 10th week post-infection. The highest OD values were found between the 14th and 18th week post-infection. Towards week 21 post-infection, the OD values assumed a decreasing trend in almost all the sheep. The mean OD values of the low dose group gave a smooth graph when plotted against time, but those of the high dose group showed fluctuations. When the two graphs were plotted together (Fig. 20), they ran almost parallel to each other. The OD values in the low dose group were generally lower than those of the high dose group in the first 13 weeks post-infection, after which the pattern was reversed. The post-infection mean OD values for both infection groups never went below the pre-infection OD values except for the first week post-infection in the low dose group.

Figure 16: Scatterplot showing the direct relationship between the optical density values of the negative control sera and the optical density values of the background in the antigen-ELISA.

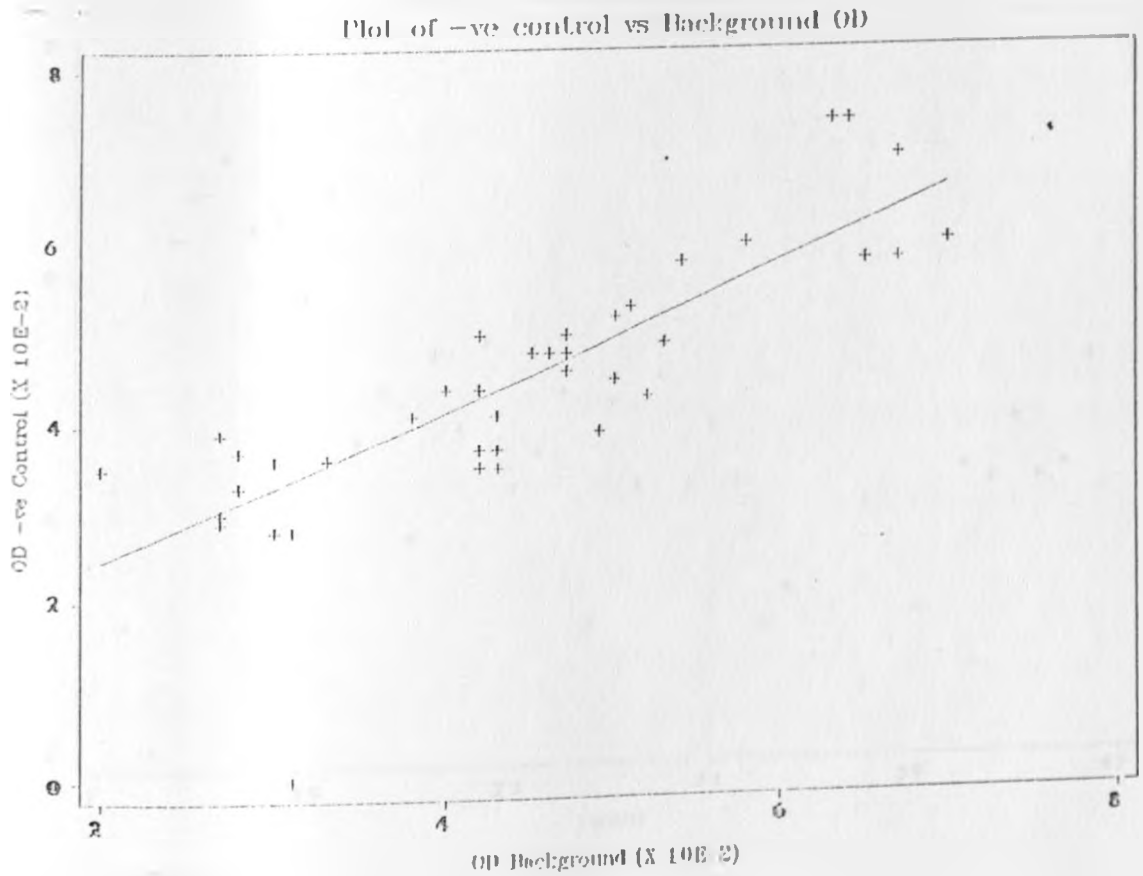


Figure 17: Scatterplot showing the inter-plate (PLNUM) variation of the optical density values of the background (ODBG) in the antigen-ELISA.

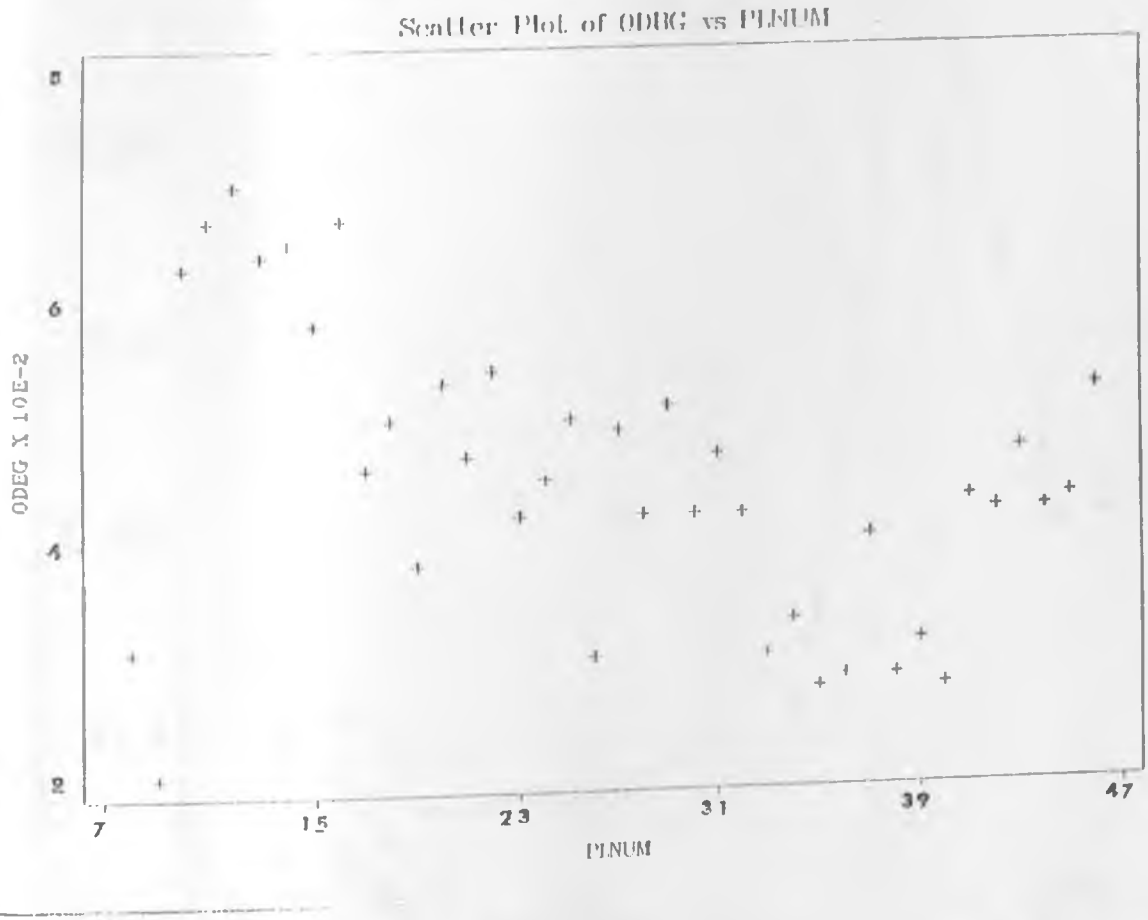
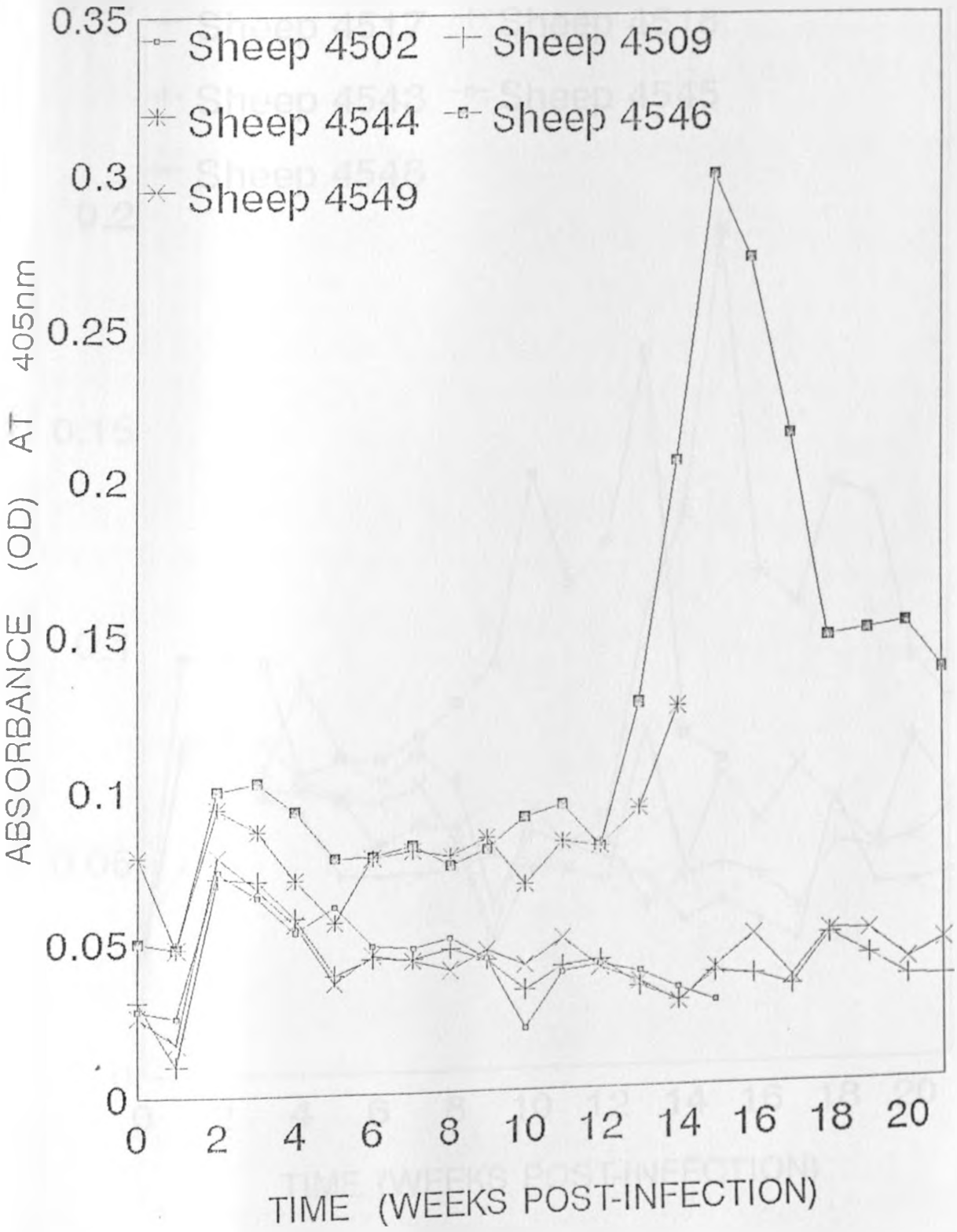
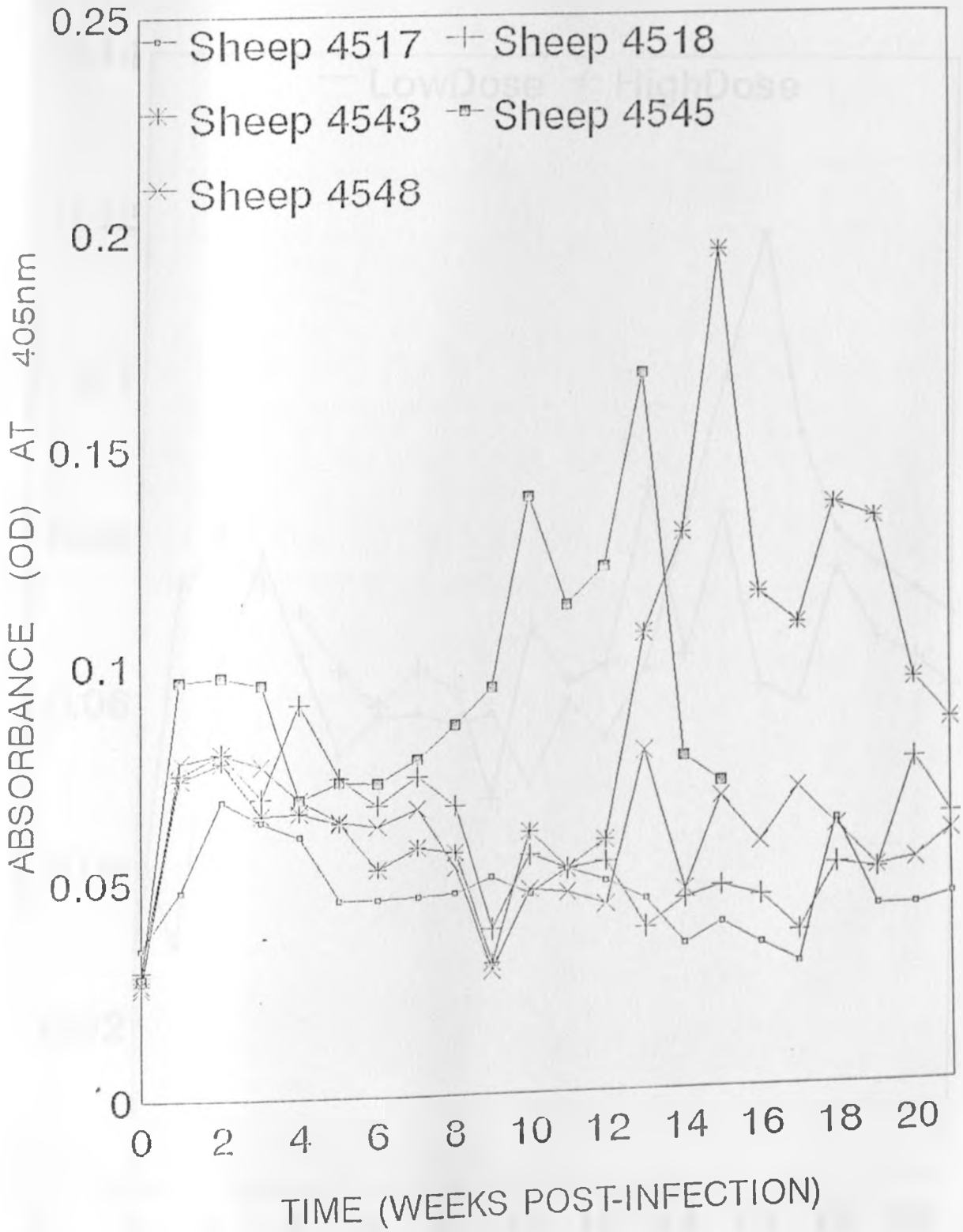


Figure 18: Optical Density (OD) values by the antigen-ELISA for the low (250) dose infection group against time.



Sheep 4502 and 4544 died at Week 15 Post-infection

Figure 19: Optical Density (OD) values by the antigen-ELISA for the high (400) dose infection group against time.



Sheep 4545 died at Week 15 Post-infection

Figure 20: Mean Optical Density (OD) values by the antigen-ELISA for the low (250) and high (400) dose infection groups with time.

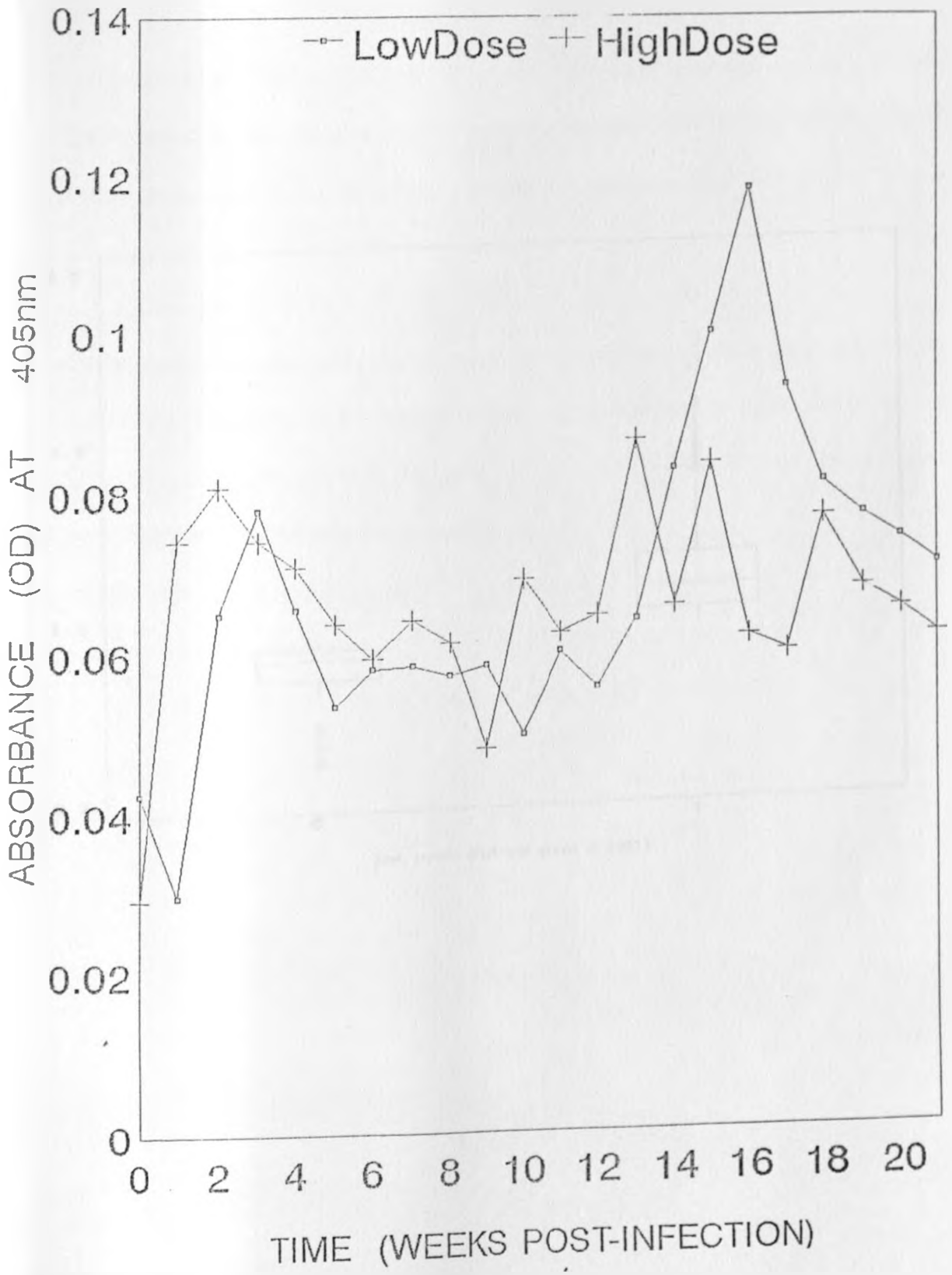
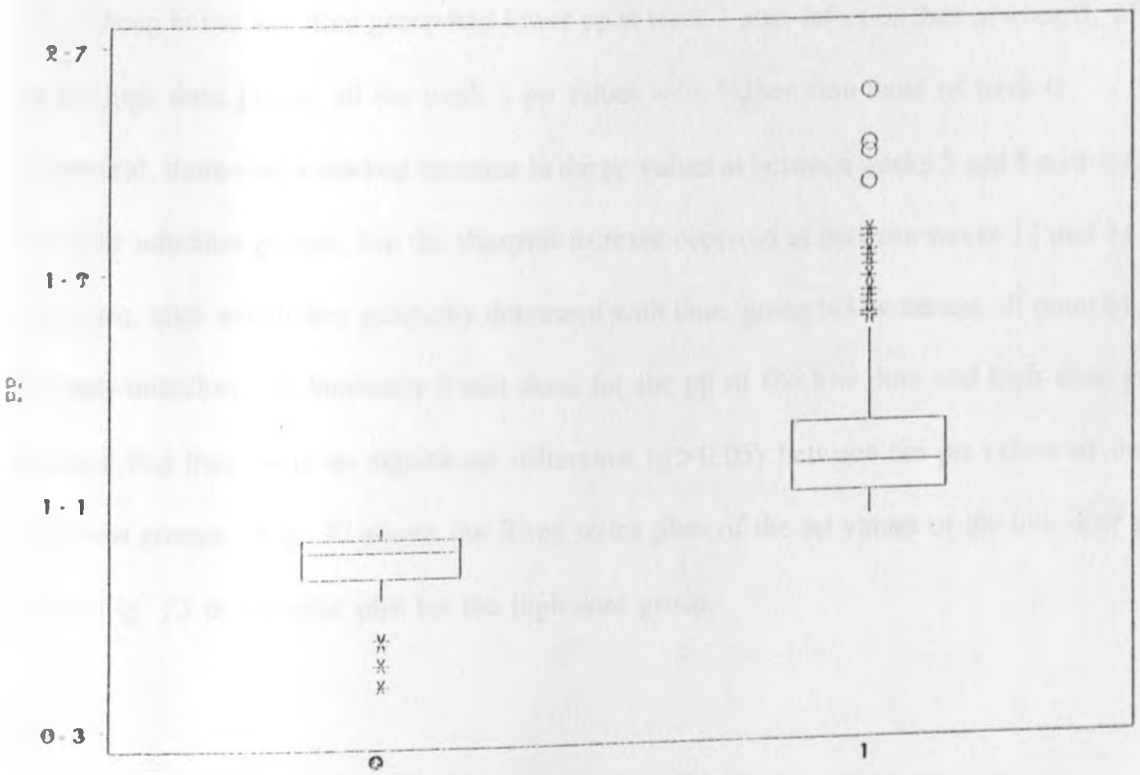


Figure 21: Box and Whisker plot showing the ability of the antigen-ELISA to differentiate between the negative (0) and the Positive (1) test sera.



4:8:2 Sensitivity and specificity

At the optimum cut-off point of 1.01, the sensitivity of the antigen-ELISA was found to be 75%, whereas the specificity was 70%.

4:8:3 Comparison of antigen-ELISA between Low dose and High dose infection groups.

Three out of the 5 sheep in the low dose group had their pre-infection sera (i.e at week 0) with pp greater than the cut-off point, as compared to none in the high dose group. 3 out of the 5 sheep in the low dose group had lower pp at week 1 post-infection than at week 0, whereas in the high dose group, all the week 1 pp values were higher than those of week 0.

In general, there was a marked increase in the pp values at between weeks 5 and 8 post-infection for both infection groups, but the sharpest increase occurred at between weeks 12 and 14 post-infection, after which they gradually decreased with time, going below the cut-off point by week 21 post-infection. A Student's T-test done for the pp of the low dose and high dose groups showed that there was no significant difference ($p > 0.05$) between the pp values of the two infection groups. Fig. 22 shows the Time series plots of the pp values of the low dose group while Fig. 23 is a similar plot for the high dose group.

Figure 22: Time series plots for the percent positivity values (pp) of the test sera of sheep from the low (250) dose infection group as detected by the antigen-ELISA. The cut-off point (1.01) is represented by a line (shown by the arrow).

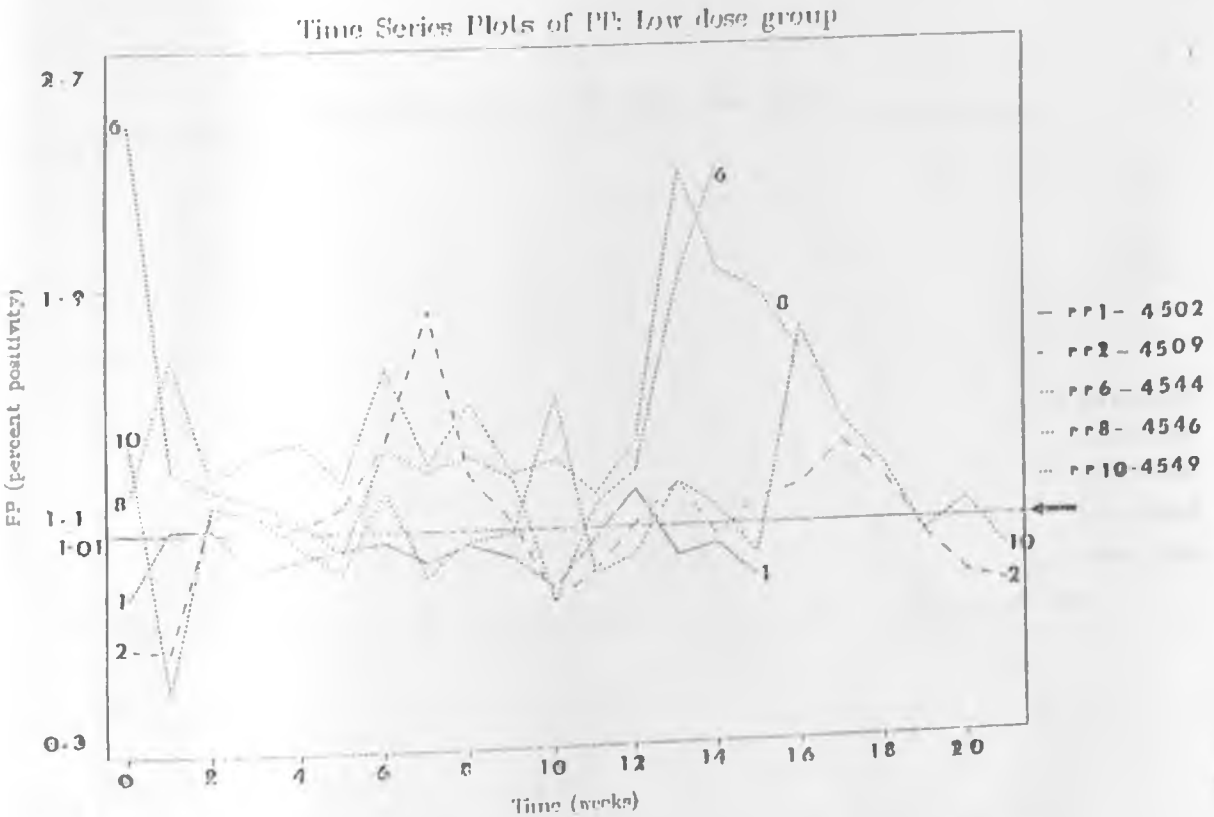
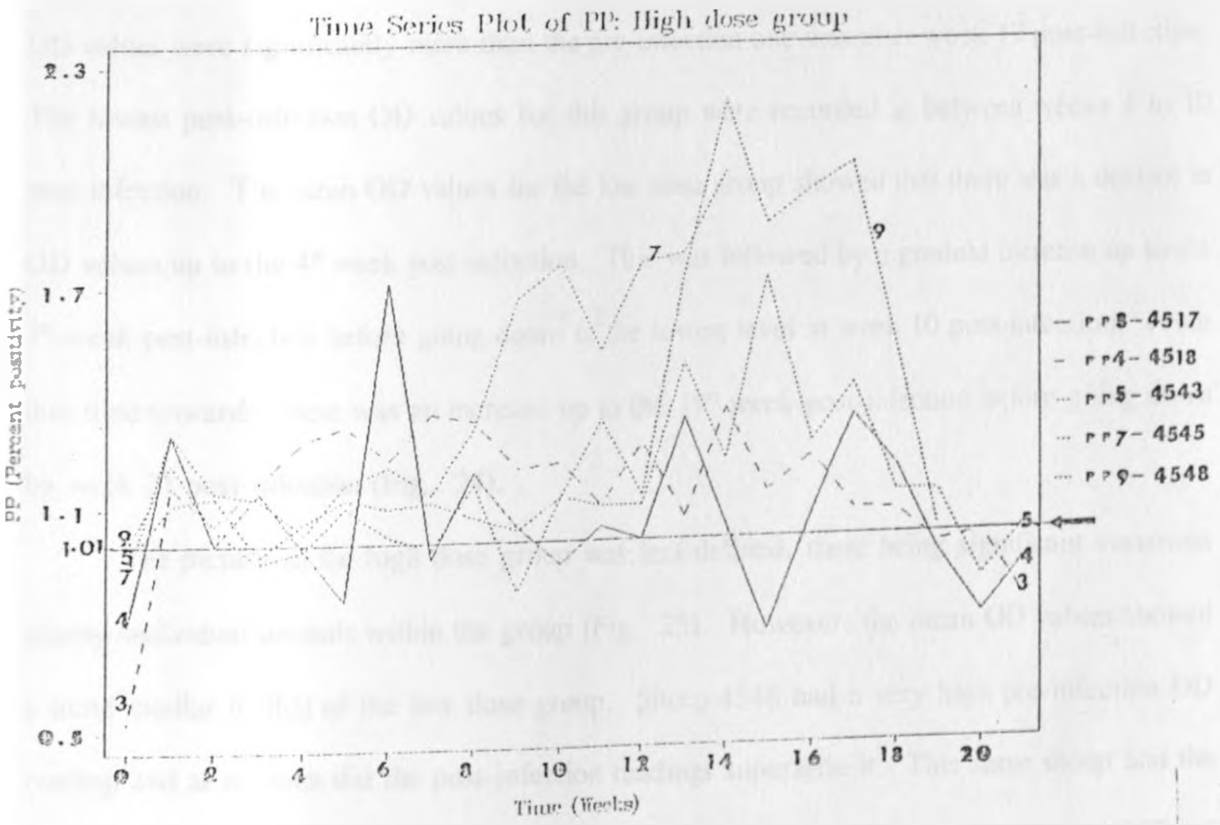


Figure 23: Time series plots for the percent positivity values (pp) of the test sera from sheep in the high (400) dose infection group as detected by the antigen-ELISA. The cut-off point (1.01) is represented by a line (shown by an arrow).



4:9 THE ANTIBODY DETECTION ELISA

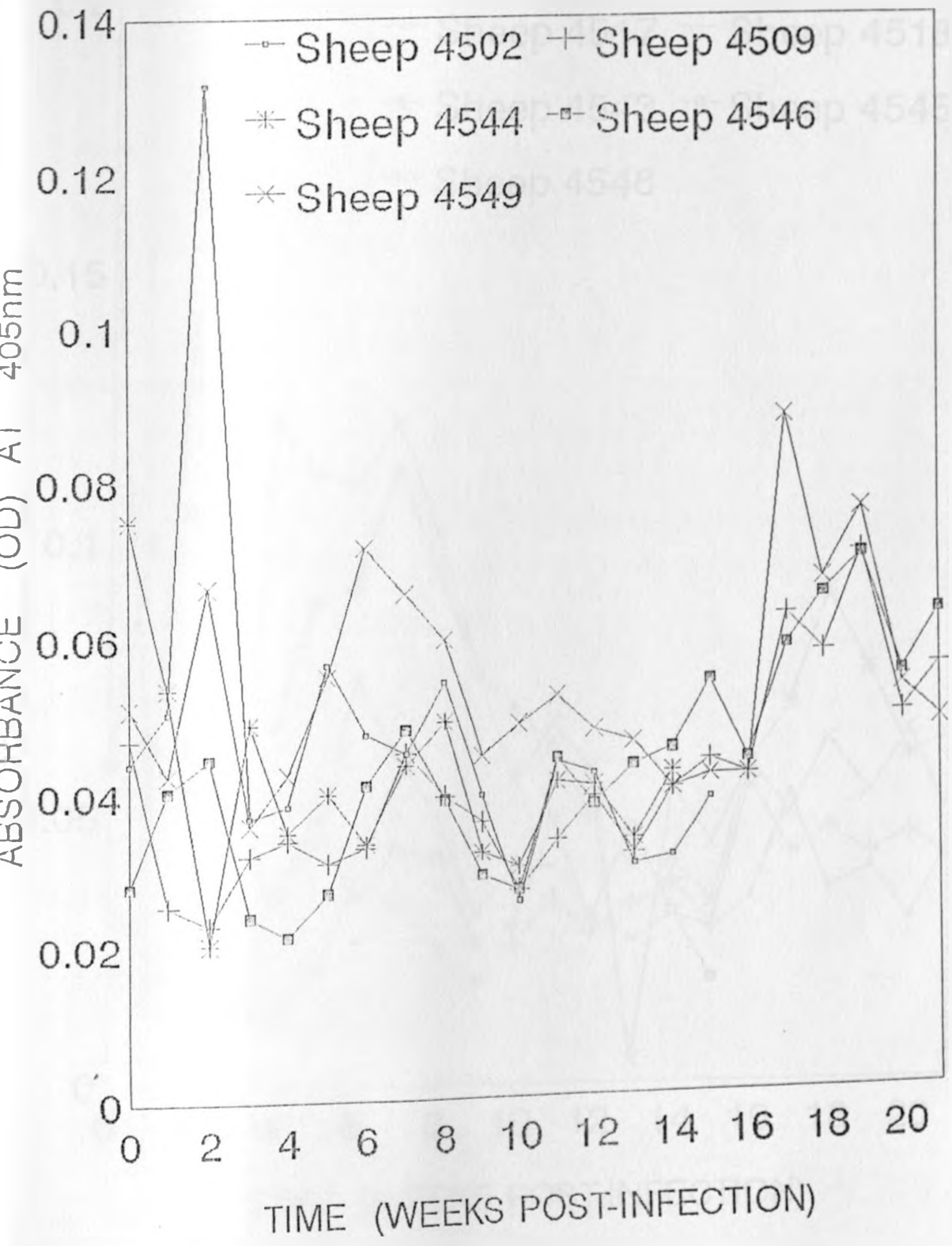
4:9:1 Optimum concentrations of antigen and antibody.

The optimum concentration for the partially purified AgF.g28 eluted from the SDS-PAGE was found to be 1/2000, the same as that of the conjugate (i.e Rabbit anti sheep IgG-HRPO). The background noise in this test was negligible. The OD values obtained from the antibody ELISA for both the low and high dose infection groups revealed that although they increased very gradually with time, the post-infection readings were most of the time less than the pre-infection one. Among the sheep in the low dose group, the only period when the post-infection OD values were significantly more than the pre-infection one was after week 17 post-infection. The lowest post-infection OD values for this group were recorded at between weeks 4 to 10 post-infection. The mean OD values for the low dose group showed that there was a decline in OD values up to the 4th week post-infection. This was followed by a gradual increase up to the 7th week post-infection before going down to the lowest level at week 10 post-infection. From that time onwards, there was an increase up to the 19th week post-infection before going down by week 21 post-infection (Fig. 24).

The picture in the high dose group was less defined, there being significant variations among individual animals within the group (Fig. 25). However, the mean OD values showed a trend similar to that of the low dose group. Sheep 4548 had a very high pre-infection OD reading and at no time did the post-infection readings supersede it. This same sheep had the lowest post-infection OD reading at the 13th week post-infection. OD values for sheep 4517 and 4518 remained low throughout the study, rising only slightly at week 16 post-infection.

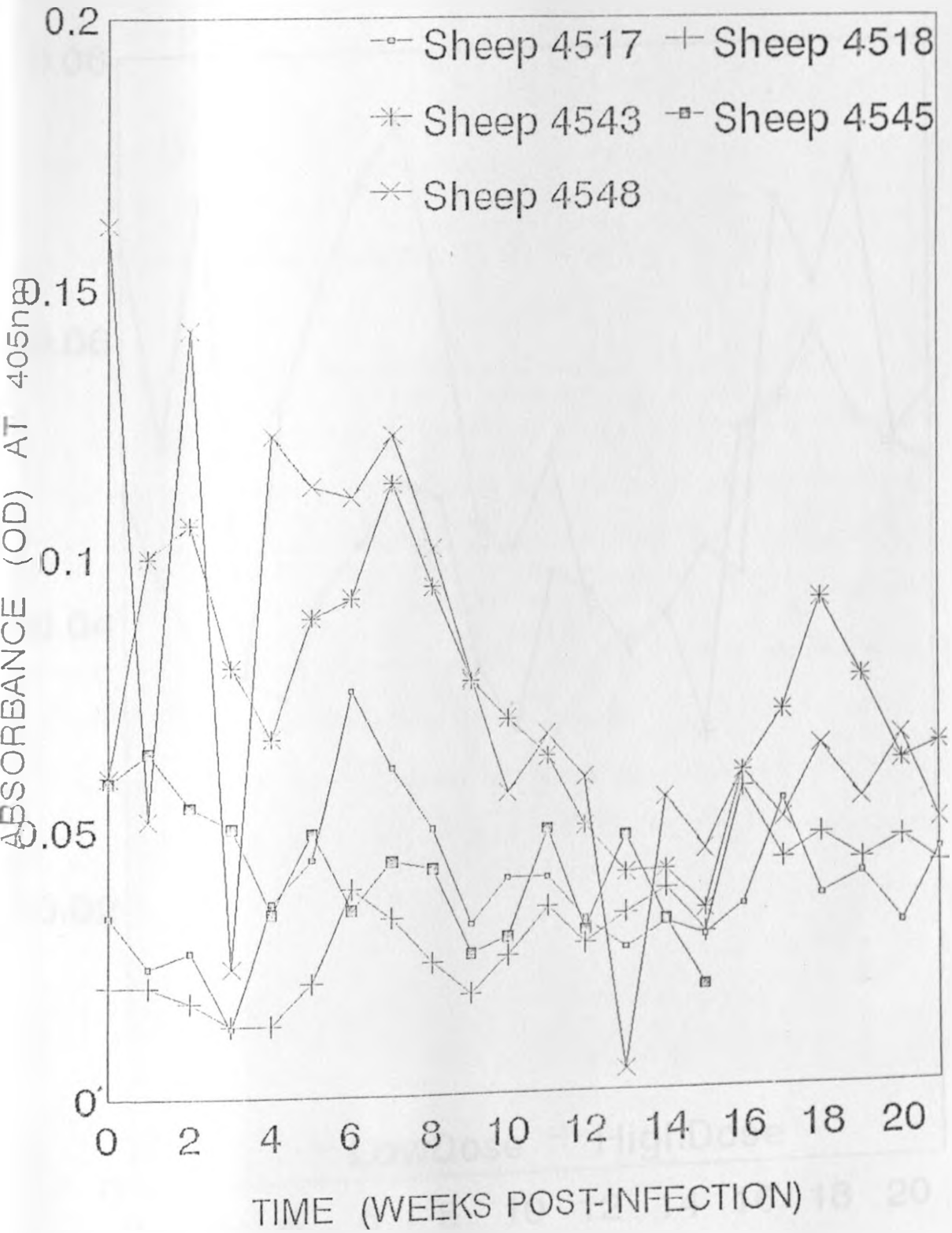
A comparison between the mean OD readings of the low and high dose groups with time showed that the two groups exhibited a similar trend, with the high dose group giving higher readings in the first 10 weeks post-infection (Fig. 26). The pre-infection OD readings for both groups were relatively higher than the post-infection readings for most of the time.

Figure 24: Optical Density (OD) values by the antibody-ELISA for the low (250) dose infection group with time.



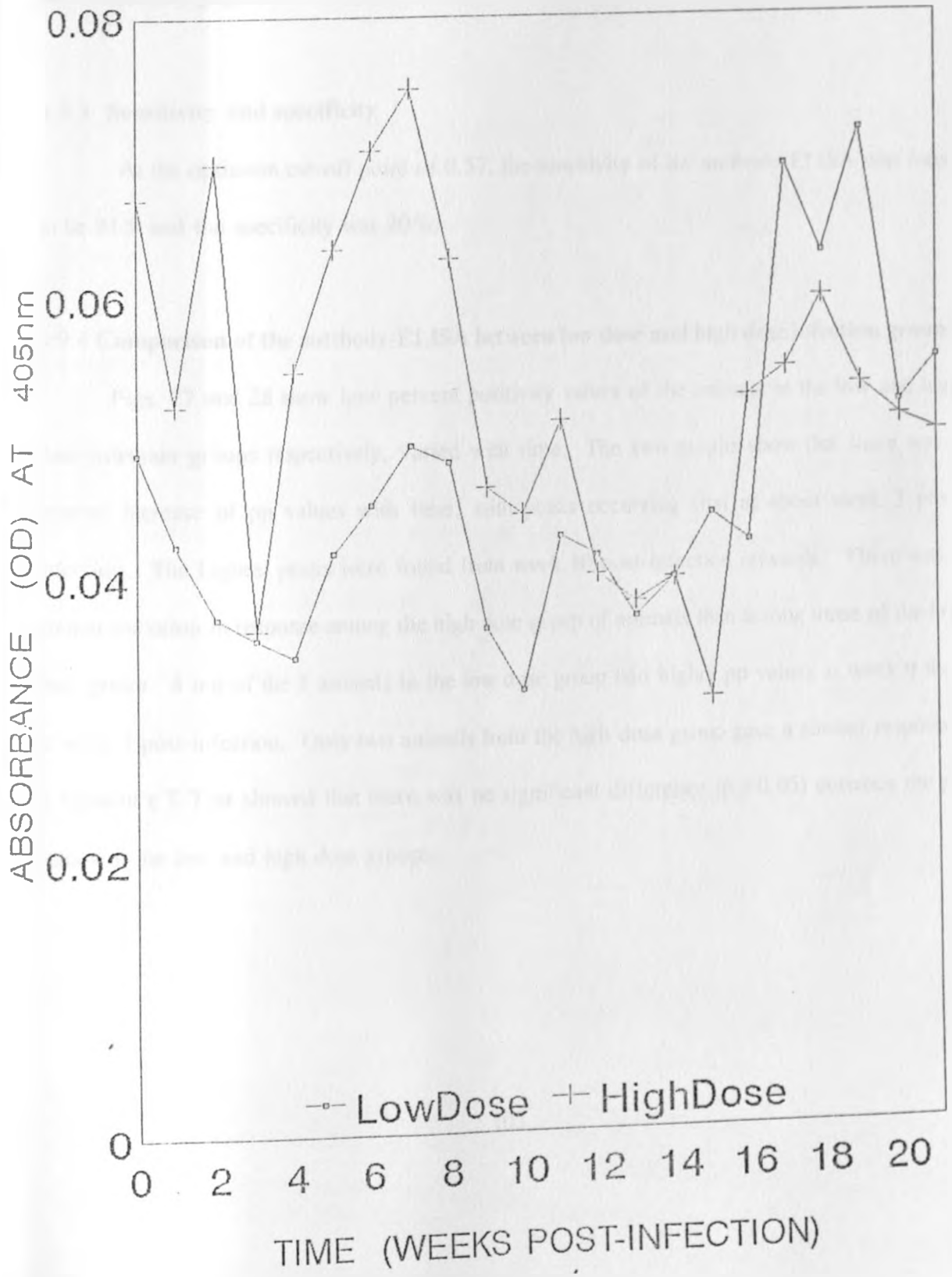
Sheep 4502 and 4544 died at Week 15 Post-infection

Figure 25: Optical Density (OD) values by the antibody-ELISA for the high (400) dose infection group with time.



Sheep 4545 died at Week 15 Post-infection

Figure 26: Mean Optical Density (OD) values by the antibody-ELISA for the low (250) and high (400) dose infection groups with time.



4:9:2 Determination of cut-off point

The cut-off point for this test was determined as described in 3.14.3 and found to be 0.57, meaning that any test sample whose pp value was found to be equal to or greater than 0.57 was considered positive.

4:9:3 Sensitivity and specificity

At the optimum cut-off point of 0.57, the sensitivity of the antibody-ELISA was found to be 81% and the specificity was 20%.

4:9:4 Comparison of the antibody-ELISA between low dose and high dose infection groups.

Figs. 27 and 28 show how percent positivity values of the animals in the low and high dose infection groups respectively, varied with time. The two graphs show that there was a general increase of pp values with time, with peaks occurring first at about week 3 post-infection. The highest peaks were found from week 10 post-infection onwards. There was a greater variation in response among the high dose group of animals than among those of the low dose group. 4 out of the 5 animals in the low dose group had higher pp values at week 0 than at week 1 post-infection. Only two animals from the high dose group gave a similar response. A Student's T-Test showed that there was no significant difference ($p > 0.05$) between the pp values in the low and high dose groups.

Figure 27: Time series plots for the percent positivity values (pp) of the test sera from sheep in the low dose infection group as detected by the antibody ELISA. The cut-off point (0.57) is represented by a line (shown by the arrow).

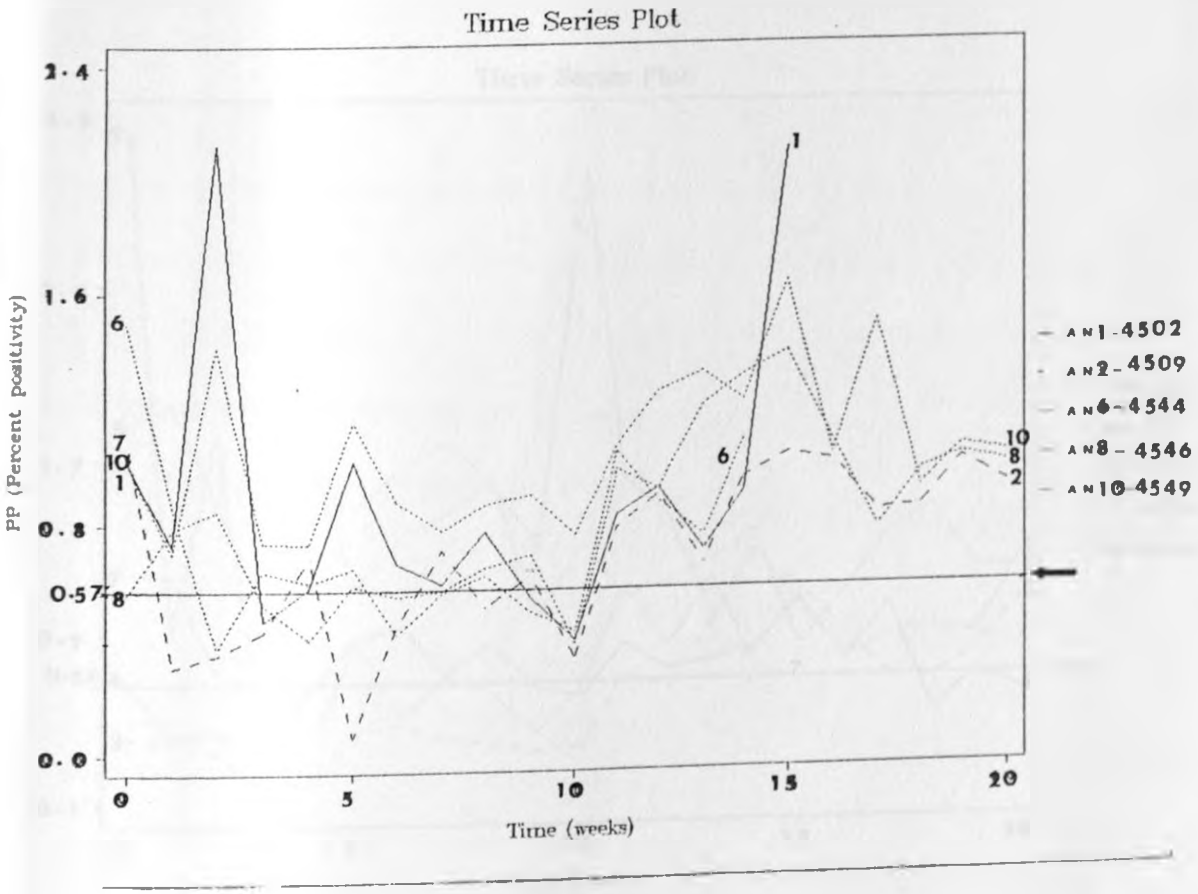
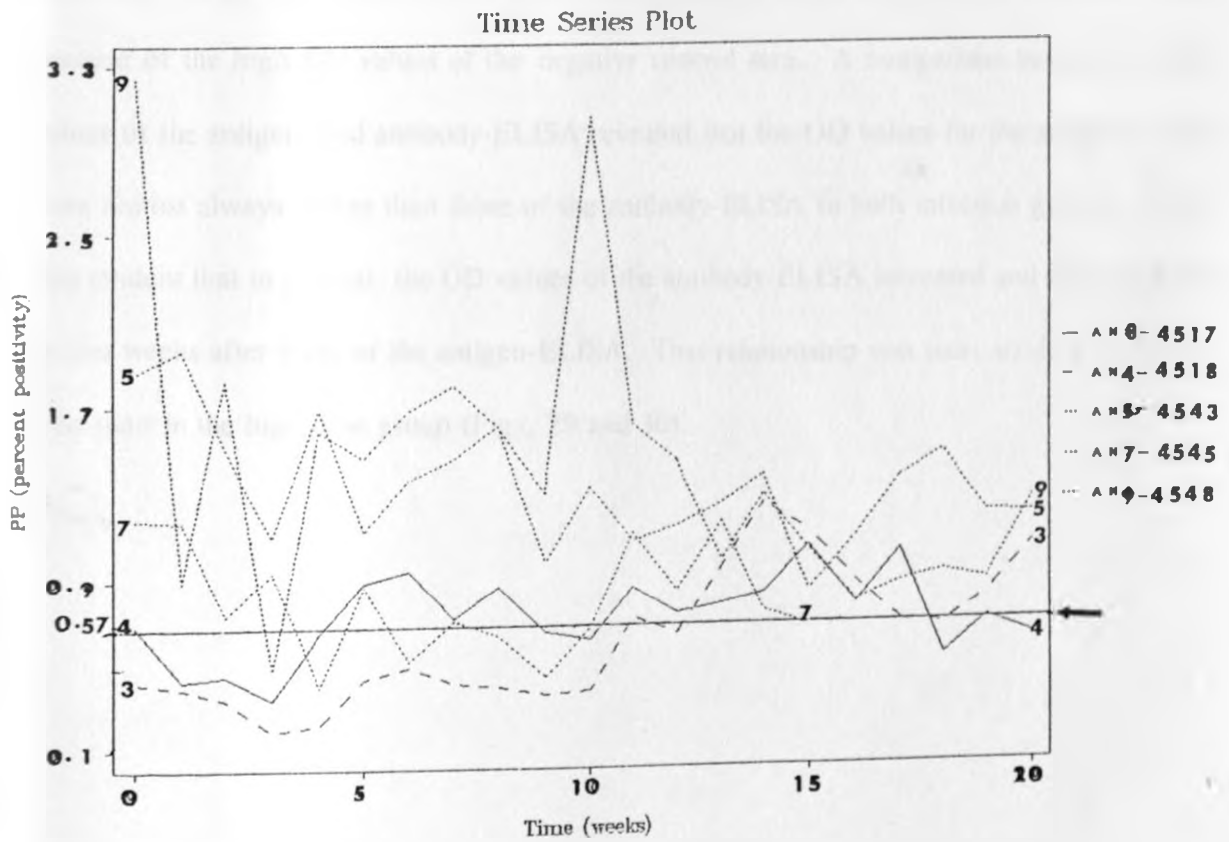


Figure 28: Time series plots for the percent positivity values (pp) of the test sera from sheep in the high dose infection group. The cut-off point (0.57) is represented by a line (shown by the arrow).



4:10 COMPARISON BETWEEN THE Antigen-ELISA AND OTHER DIAGNOSTIC TESTS

4:10:1 Earliest detection of fasciolosis

Eight out of the 10 animals of both the low and the high dose infection groups tested positive for fasciolosis at week 1 post-infection with the antigen-ELISA, as compared to 5 out of 10 with the antibody-ELISA. The FEST first tested positive at week 13 post infection. However, the pp values and cut-off point were generally much lower in the antibody-ELISA because of the high OD values of the negative control sera. A comparison between the OD values of the antigen- and antibody-ELISA revealed that the OD values for the antigen-ELISA were almost always higher than those of the antibody-ELISA in both infection groups. It was also evident that in general, the OD values of the antibody-ELISA increased and decreased one to two weeks after those of the antigen-ELISA. This relationship was more evident in the low dose than in the high dose group (Figs. 29 and 30).

Figure 29: Mean Optical Density (OD) readings of the antigen- and antibody-ELISA for the Low dose infection group against Time.

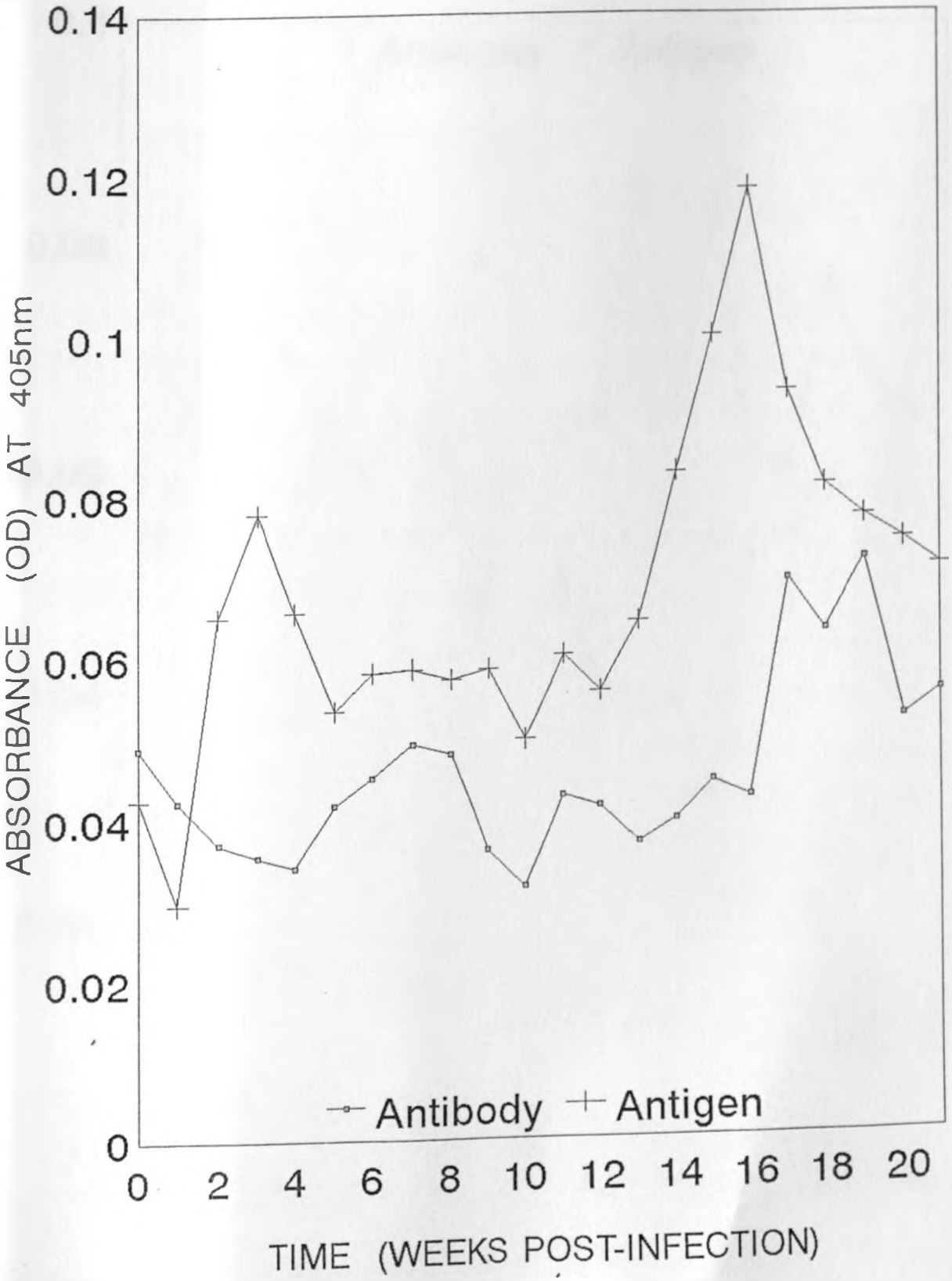
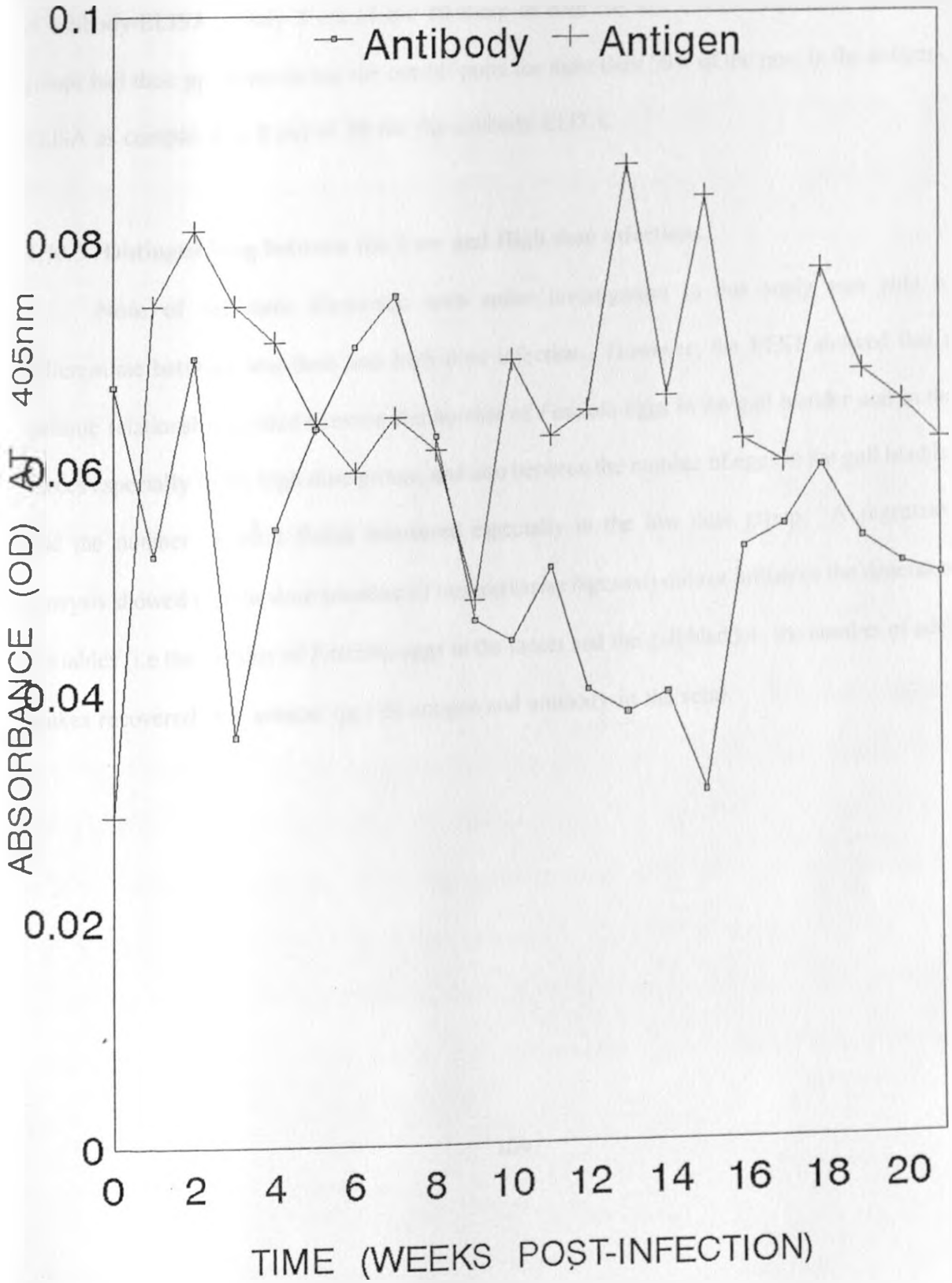


Figure 30: Mean Optical Density (OD) readings of the antigen- and antibody-ELISA for the High dose infection group against Time.



4:10:2 Sensitivity and Specificity

The sensitivity of the antigen-ELISA was 75% as compared to 81% of the antibody-ELISA. On the other hand, the specificity of the antigen-ELISA was 70%, as compared to 20% of antibody-ELISA. Only 2 out of the 10 sheep of both the low and the high dose infection groups had their pp values below the cut-off point for more than 50% of the time in the antigen-ELISA as compared to 8 out of 10 for the antibody-ELISA.

4.10.3 Distinguishing between the Low and High dose infections.

None of the three diagnostic tests under investigation in this study was able to differentiate between low dose and high dose infection. However, the FEST showed that a definite relationship existed between the number of *Fasciola* eggs in the gall bladder and in the faeces especially in the high dose group, and also between the number of eggs in the gall bladder and the number of adult flukes recovered especially in the low dose group. A regression analysis showed that the dose (number of metacercariae ingested) did not influence the dependent variables (i.e the number of *Fasciola* eggs in the faeces and the gall bladder, the number of adult flukes recovered, the amount (pp) of antigen and antibody in the sera).

5. DISCUSSION

The search for a specific antigen of *Fasciola* species that can be used in highly sensitive and specific serodiagnostic techniques has been intense, with more work being done in *F. hepatica* (Moore and Halton, 1976; Hillyer and Soler De Galanes, 1988; Yamasaki *et al*, 1989) than in *F. gigantica* (Fagbemi and Goubadia, 1995; Fagbemi, 1995). The most frequently discussed antigens that meet these qualifications are those whose molecular weights are about 28-kDa. Yamasaki *et al.* (1989) used monoclonal antibodies to identify a 27-kDa proteinase. Fagbemi (1995) also used monoclonal antibodies but found that some of them recognised a 27/28-kDa doublet and others recognised a single 28-kDa protein which he found to be a cysteine protease. The protein found in the present work is most likely the 28-kDa protease since it appeared as a single band on the Western blots. The protease found by Fagbemi was apparently obtained from adult whole-worm but the results in the present work show that the AgF.g28 was present in both the whole-worm and the ES products. Infact, it seems to be more prominent in the ES products than in the whole-worm extracts. The AgF.g28 was present in the whole-worm extracts of all ages of *F. gigantica* from 11 weeks to 21 weeks and in the ES products of adult flukes (ES products from juveniles were not tested in the present work). This agrees with the work done by Thorpe (1965) and Moore and Halton (1976) who found that some antigens are found in immature as well as in mature stages of *Fasciola*. Excretory/secretory antigens have been found to be good candidates for serodiagnosis (Rajasekariah *et al*, 1979; Hillyer and Soler de Galanes, 1988; Espino *et al*, 1990; Solano *et al*, 1991; Rodriguez and Hillyer, 1995). Somatic antigens are released periodically when the parasites shed their outer

glycocalyx garment, or when they die and disintegrate (Reddington, Wesleid and Wescott, 1984).

The Western blot results showed that whereas there were some antigens that were common in some or all of the parasites compared with *F. gigantica* in this study, it was clear that the AgF.g28 appeared only in *F. gigantica* whole-worm extracts and ES products. The first antibody used in the Western blot was from one of the sheep experimentally infected with *F. gigantica* and the fact that the anti-*Fasciola* serum could recognise antigens in other parasites is in line with what has been reported elsewhere (Pelley and Hillyer, 1978; Fagbemi and Obarisiagbon, 1991; Rodriguez-Perez and Hillyer, 1995). The Western blots were faint even after increasing the concentrations of the first antibody several times. This was probably due to the fact that the animals infected with living parasites did not have high titres of antibodies due to the adaptive mechanisms that *Fasciola* have perfected of evading immunological recognition by the host (Lang, 1967; Smithers *et al*, 1969; Perez and Terry, 1973; Wilson and Barnes, 1977). It has been reported that sera from sheep, horses and swine with chronic infections tend to react poorly even to crude adult-worm homogenates, thus exhibiting low sensitivity (Hillyer, 1993). There is also a possibility that the antigen, AgF.g28, was either not highly immunogenic or was available only in small quantities that could provoke a substantial antibody production. This is deduced from the observation that the OD values for the antibody-ELISA were relatively low (at most 0.08), whereas those of the antigen-ELISA went as high as 0.3. This could also explain why the Western blots were very faint.

Western blots have been used to identify immunogenic and specific antigens of *Fasciola* among others. Santiago and Hillyer (1986), using adult worm homogenates, found some clear

differences in the antigen profiles of sheep and cattle with fascioliosis. Hillyer and Soler de Galanes (1988) found by Western blot that the sera from humans, rabbits, cattle and sheep with fasciolosis all recognised two antigenic polypeptides of 17 and 63-kDa in the form of sharp bands. A 12-kDa band was identified and confirmed by Western Blot in *F. hepatica* found in humans (Hillyer, 1993). The Western Blot in the present study showed several distinct bands, many of which were also found in other parasites. Goubadia and Fagbemi (1995) used Western blotting to identify bands of 17-kDa, 57-kDa, 69-kDa and 87-kDa which were present in *F. gigantica* and absent in *Paramphistomum* and *Dicrocoelium* species.

The fact that the post-infection OD values of the antigen-ELISA were most of the time higher than those of the pre-infection readings is an indication that the test was actually able to detect the presence of the antigen in sera of infected sheep. The trend showed by the OD values of the antigen-ELISA (Fig. 20) is consistent with the pathogenesis of the liver-fluke disease (Sewell, 1966). The initial upsurge in antigen levels in serum at weeks 1 and 2 post-infection correspond to the time when the ingested metacercariae have penetrated the stomach wall and most of them are within the peritoneal cavity, searching for the liver. Many metacercariae die as they attempt to penetrate the stomach wall and are subsequently absorbed up by the host animal. The decrease in the antigen levels at between the 4th and 10th week post-infection corresponds to the span of time between when the juvenile parasites have penetrated through the gut wall and penetration of the liver capsule. During this same period, the antibody titer is high (Fig. 26). Most of the antigens in the blood are complexed with the antibodies and are therefore not free to be picked by the antigen-ELISA (Hughes, 1987). It is probable that at this point in time, most of the parasites are in the peritoneal cavity and therefore their antigens may not be

easily detected in circulation. Instead, they may be captured by the peritoneal macrophages. The fact that a sheep that was slaughtered at week 7 post-infection had no flukes recovered from the liver lends credence to this possibility. The gradual increase of antigen levels in serum between weeks 10 and 16 post-infection could have been as a result of increased release of secretory/excretory products by the rapidly growing liver-flukes which at this time were damaging the hepatic parenchyma as also they fed on liver tissue and blood. It is likely that some of the parasites die at this stage and their disintegrating body parts are assimilated into the bloodstream via the highly vascularised hepatic parenchyma. The decrease in antigen levels after the 16th week post-infection is probably explained by the fact that it is this period in the pathogenesis of fasciolosis that the parasites have attained adulthood and have reached the predilection sites - the bile ducts. The parasites are highly protected from the host's immune system (Hanna, 1980a). From the bile ducts, any products from the living or dead flukes are more likely to be excreted by the host animal through faeces than be absorbed into the blood.

The Double-antibody Sandwich ELISA had a sensitivity of 75% and a specificity of 70%. These were determined after eliminating the effects of the high background OD values. The cut-off point was determined by finding the percent positivity of each OD reading. The negative controls were a pool of about 20 sheep known not to have fasciolosis (Voller *et al.* 1976) and since the background readings were directly proportional to the negative control readings, and there was a significant interplate variation of the background readings, the effect of this background was eliminated by obtaining the ratio between the OD of the test sera and that of the negative control per plate. High background readings are a common phenomenon in enzyme immunoassays (Langley and Hillyer, 1989) and the determination of the cut-off point

by getting the percent positivity is preferred to cut-off points of OD values alone (Voller *et al*, 1976; Hillyer, 1993). The sensitivity and specificity of the antigen-ELISA in this study was fairly lower than most ELISAs whose sensitivities and specificities are normally 90% and above. The reason for this could probably be due to the fact that antigen-antibody complexes are highly specific interactions and any procedure that changes the molecular structure of the antigen or distorts the epitopic conformation is likely to reduce the sensitivity and specificity of the test (McCullough *et al.*, 1985a). The antigens used in this test were obtained directly from the SDS-PAGE gels without eluting them from the gel. Although Hames (1990) is of the contention that removal of antigens from the SDS-PAGE gel is not necessary (as it is immunologically inert) before immunization, Jurd and Borg-Hansen (1990) assert that antigens used as immunogens should be as pure as possible and in their native state. Indeed, eluted antigens were used in the Antibody-ELISA which had a sensitivity of 81% and a disappointing specificity of 20%.

The antigen-ELISA detected *F. gigantica* antigens in 8 out of 10 infected sheep by week 1 post-infection. Goubadia and Fagbemi (1995) found that *Fasciola* antigens could be detected at 2 weeks post-infection. Fagbemi, Obarisiagbon and Mbuh (1995) developed a antigen-ELISA that could detect an 88-kDa antigen in sera of cattle by the second week after infection.

There was a general increase of percent positivity values with time in the antigen-ELISA test results. The highest pp values were found between the 12th and the 16th week post-infection. This happens to be the same period in the pathogenesis of fasciolosis when the juvenile flukes are penetrating the bile ducts as they develop into adults (Sewell, 1966). It also coincided with the time when *Fasciola* eggs were first seen in the faeces. This agrees well with the findings of Bennett (1978) and Hanna (1980b) who found that antisera reacted most strongly to *F.*

hepatica infections at about the same time.

The antigen-ELISA was not able to significantly distinguish between low and high levels of infections ($p > 0.05$). This is despite the fact that two out of five sheep in the low dose group had their pp values lower than the cut-off point for more than 50% of the time, as compared to none in the high dose group. The inability to distinguish between low and high levels of infection by the antigen-ELISA probably lies in the fact that the percentage fluke take between the two infection groups was also not significantly different ($p > 0.05$). Infact, it would seem like liver flukes die in greater numbers when metacercariae are ingested in larger quantities. It is possible that most of the metacercariae that never make it to adulthood die soon after ingestion, this being explained by the fact that the pp values in most of the sheep increased in the first week after infection and then decreased up to around the 6th week post-infection before picking up again after the 12th week. The decrease in pp values after the 16th week of infection corresponds to the time when most of the flukes have entered the bile ducts and most antigens are released into the gut rather than through the blood (Lang, 1967; Hanna, 1980a).

The true results of the antibody-ELISA were masked by the high negative control OD values which had the effect of lowering the pp values and seriously reducing the specificity of the test. It is not that the OD values of the negative control sera were high in the antibody-ELISA assay, rather it was the OD values of the sera from the infected animals that were very low. The significantly, but unexpectedly, low OD values obtained by the antibody-ELISA for the sera from the infected animals may have come about as a result of formation of antigen-antibody complexes which had the effect of availing only a small quantity for detection. However, the general trend of antibody OD values are consistent with the pathogenesis of

fasciolosis (Sewell, 1966) and also with what Hanna and Jurra (1977) found in their studies of antibody response of calves to infection by *F. gigantica*. The high antibody levels at between the 4th and 8th week post-infection may be a response to the high antigenaemia experienced between the 1st and 3rd week post-infection. This agrees with observations by other workers (Movsesijan and Jovanovic, 1975; Hanna and Jurra, 1977) The reduction of antibody levels between the 10th and 16th week post-infection may be as a result of antibody/antigen complexes formation and also due to the heavy internal haemorrhage that occurs at this time as the juvenile flukes criss-cross the liver tissue. Indeed, all the three sheep that died prematurely did so during this period and they all had evidence of internal haemorrhage. The build-up of antibody titres after the 16th week post-infection is probably a response to the increased antigenaemia from the 14th week post-infection. The OD values of the antibody-ELISA were low throughout the experiment, perhaps due to the fact that the eluted antigen did not coat sufficiently onto the ELISA plates. Also, it is possible that the process of elution of the antigen from the SDS-PAGE gels may have altered the epitopic conformation of the antigens, thereby making it difficult for the antibodies to recognize them. It may also be that the antigen, AgF.g28, is not available to the host's immune system in sufficient amounts that can provoke production of high titres of antibody. Due to their migratory behaviour and subsequent disappearance into the bile ducts, the liver flukes do not give the host's immune system enough time to form high levels of antibody against them (Dawes and Hughes, 1964; Lang,1967).

Sheep 4548 of the high dose group had the highest pre-infection and post-infection OD values for both the antigen and antibody. This is the same animal that had the highest number of adult flukes recovered at post-mortem, the highest number of eggs in the gall bladder and also

in the faeces. Sheep 4546 of the low dose group had interesting results. Despite having been infected with 225 metacercariae, this sheep had only 5 flukes recovered from its liver at 21 weeks post-infection, this representing a fluke take of 2.2%. Of the five flukes, only two were in the major bile ducts. The other three had apparently been trapped in the smaller bile ducts. They were white and lifeless. The liver of this particular sheep was smaller than normal, firm and the liver parenchyma had a dense network of connective tissue. The smaller bile ducts were thickened and the lumina greatly reduced. The antigen levels in serum for this animal were moderate. It is difficult to know whether the liver had developed the fibrous tissue prior to or after infection with the metacercariae. Either way, the *Fasciola* were almost completely prevented from growing to adulthood or comfortably living within the bile ducts. Indeed, the first time the eggs were seen in the faeces was at the 20th week post-infection, an indication that the egg laying ability had been adversely affected. At post-mortem a week later, only two adults were recovered alive in the bile ducts. The gall bladder had only 9 *Fasciola* eggs and three grams of faeces had 4 eggs. The low levels of antigen and antibody in the pre-infection serum means that there was no prior exposure of this animal to *Fasciola*. It has been suggested that sheep liver cannot offer much physical resistance to migrating *Fasciola* parasites (Ross, 1967; Boray, 1967) because it has very little connective tissue, but this case study certainly disproves this line of thought. However, it is also probable that the physical resistance offered by this sheep was an exception rather than the rule.

The mean percentage fluke take was higher in the low dose group (14.68%) than in the high dose group (10.56%). This difference was not statistically significant ($p > 0.05$), however. The higher fluke take in the animals of the low dose group was probably as a result of more

animals in this group dying prematurely at a time when both juvenile and adult flukes were present in the liver. Since the percentage fluke take is the proportion of metacercariae that successfully developed and are recovered as flukes, the animals that died prematurely definitely had higher fluke takes. The fluke take of 10.56% is close to that of 10.44% obtained by Waweru (1995) in Dorper sheep, but it was much less than the 34.6% obtained by Guralp (1969) in an unspecified breed of sheep. It is also possible that animals infected with fewer numbers of metacercariae could have a higher percentage fluke take since the parasites would have less competition and more liver space to thrive in. Indeed, Guralp (1969) infected sheep with 200 metacercariae of *F. gigantica* and recovered 105 live adults at necropsy. This represented a percentage fluke take of 52.5%!

There was a significant ($p < 0.05$) and direct relationship between faecal egg counts and egg counts from the gall bladder. This was more so in the high dose group of infected sheep. Also, there was a direct and significant ($p < 0.05$) relationship between the number of adult flukes recovered and the number of eggs in the gall bladder, especially in the low dose group. However, no significant relationship existed between the number of adult flukes recovered and the number of eggs in the faeces at postmortem. These findings are consistent with those found by Ikeme and Obioha (1973) who examined cattle infected with *F. gigantica* at an abattoir. A loose relationship existed between the number of eggs in the faeces and the OD values of both the antigen and antibody-ELISA. There was a general increase in all the three parameters with time. However, eggs were first seen in faeces at the 13th week post-infection and slowly increased in number up to the 16th week before rising sharply up to the end of the experiment at 21 weeks post-infection. The OD values of the antigen-ELISA began rising from week 1

post-infection gradually up to week 16 before declining to low levels by week 21 post-infection. The initial, gradual increase of antigen tititres may have been due to the fact that the parasites at this time were frowing in weight and size and were therefore secreting and excreting more antigenic products. The decline in antigen titres after the 16th week post-infection may have come about as a result of he parasites having penetrated into the bile ducts where they were effectively shielded from circulation. The antibody-ELISA had high titres in the first 3 weeks after infection which thereafter decreased to their lowest level by week 10 and then gradually rose up to the 21st week post-infection. The decrease between the 3rd and 10th week post-infection may be attributed to the accelerated formation of antigen-antibody complexes. Towards the 21st week post-infection, there may have been fewer antigens available and hence, higher titres of free antibodies could be found in circulation.

The dose (number of metacercariae ingested) does not influence the dependent variables such as the number of eggs in the faeces and the gall bladder,the number of adult flukes recovered and the OD values for both the antigen and antibody-ELISA. This is probably because each animal responds differently to any infection. Furthermore, metacercariae have to encounter and overcome numerous obstacles, both physical and humoral, from the host animal on their way to developing into adult flukes. There is also the likelihood of the parasites missing the liver and landing into a tissue that is not favourable to their development. One of the sheep that died prematurely had *F. gigantica* in the lungs where they had died and precipitated abscess formation. Guralp (1969) found living flukes free in the peritoneal sac, in haematomas in the lungs, diaphragm and even in the wall of the portal vein.

The fact that none of the three diagnostic tests could distinguish between low and high

level infection is a serious setback because this means that even the highly sensitive and specific diagnostic tests cannot be used to determine the level of infection of fasciolosis in infected animals. It also means that fasciolosis will remain to be diagnosed only qualitatively and not quantitatively. Quantitative diagnosis of fasciolosis would indicate the worm burden, and this knowledge would be useful in ascertaining the state of health of the animal. This would, in turn help say, the clinician in deciding the best course of treatment and dosage. Two infective doses were used in this study to represent a low and a high worm burden. Investigation was done to check which of the three diagnostic tests could distinguish between the two levels of infection. Unfortunately, none of them could. However, there is the possibility that if the difference between the low and high doses is set further apart, the antigen-ELISA may be able to show the difference. Antigen-ELISA may also be improved by using a purer form of the antigen, and by employing the use of monoclonal rather than polyclonal antibodies. This may not only reduce the background noise in the immunoassays, but also increase the sensitivity and specificity of the assay.

CONCLUSIONS

1. The antigen, AgF.g28, is an immunogenic and specific protein of *F. gigantica* that has a great potential of being used in immunodiagnosis of *F. gigantica* infections.
2. The antigen-ELISA using the AgF.g28 has a high sensitivity and specificity and is also able to detect fasciolosis in the very early stages of the infection.
3. The antigen-ELISA is superior to both the antibody-ELISA and the faecal egg sedimentation

technique.

4. None of the three diagnostic tests in the present study were able to distinguish between low and high levels of infection with *F. gigantica* in sheep.
5. There is no influence of the dose of metacercariae on the dependent variables such as the number of eggs in faeces and gall bladder, fluke take and antigen/antibody levels in blood.
6. Further studies need to be done on the antigen AgF.g28 using monoclonal antibodies in order to improve the sensitivity and specificity of the test. The antigen should also be produced in a purer form.
7. A larger group of animals is required in order to have more representative statistical data. The death of two out of five animals in the low dose group greatly interfered with the analysis and interpretation of the results.
8. Further investigations are required to test the antigen-ELISA developed from the AgF.g28 antigen in animals that are naturally infected and also to test its specificity in animals infected with the other related helminth parasites.

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APPENDIX

REAGENTS AND BUFFERS

(A) GENERAL

(i)0.1% Sodium azide (NaN_3).

Used as an antimicrobial agent in stored serum samples. Prepared by dissolving NaN_3 in a known volume of serum so as to make a 0.1% w/v solution.

(ii)0.2% NaN_3.

Used as an antimicrobial agent in stored crude antigen samples. Prepared by dissolving NaN_3 in known a volume of crude antigen sample so as to make a 0.2% w/v solution.

(iii) Saline.

Added as an isotonic solution to antibodies after separation by ion-exchange chromatography and ultrafiltration. Prepared by adding analytical sodium chloride (NaCl) to a known volume of the antibodies so as to make a 0.9% w/v solution. Physiological saline can also be prepared by dissolving 8.5 gms NaCl in 1000 mls of distilled water.

(iv)Phosphate Buffered Saline (PBS).

Prepared by adding 1 volume of 0.15M phosphate buffer to 9 volumes of normal saline. 0.15M phosphate buffer was prepared by dissolving 21.294gms of disodium hydrogen phosphate (Na_2HPO_4) and 23.40gms of Sodium Dihydrogen phosphate

(NaH_2PO_4) in 1000mls of distilled water. pH adjusted using 4M NaOH or 1MHCl.

(v) 0.5M Phosphate buffer (stock) pH8

Na_2HPO_4 - 70.83gms,

NaH_2PO_4 - 70.00gms,

2000mls DDW

(B) Faecal Egg Examination Technique

(i)0.1% Methylene blue solution.

Used for background staining of faecal samples containing *Fasciola* eggs. Prepared by dissolving 1gm of Methylene blue powder in 1000mls of distilled water and stirred thoroughly.

(C) Immunodiffusion

(i) 1% Agar.

Used in simple immunodiffusion. Prepared by adding 1gm of purified Agarose (Oxoid,England) to 100mls of distilled water.The solution turns clear when completely dissolved. (Distilled water may be used instead of PBS). Add Sodium azide (1%) and store at 4_oC in universal bottles until use.

(ii)0.5% Coomasie blue stain.

Used for irreversible staining of proteins.

Glacial Acetic acid - 100mls

120 propyl Alcohol - 300mls

Distilled water - 600mls

Total volume 1000mls

5gms of Coomasie Brilliant blue powder added to the 1000mls, stirred thoroughly and sieved through filter paper.

(iii)Destaining buffer for Coomasie Brilliant blue stain

Glacial acetic acid 50mls

Methanol 165mls

Distilled water- 785mls

Total volume 1000mls

The destaining buffer may be re-used several times by physically removing the Coomasie blue stain using activated charcoal. The charcoal-destainer mixture is stirred thoroughly and allowed to separate. The liquid is filtered off as a clear destainer, ready for use.

(D) SDS - PAGE

(i) 10% Sodium Dodecyl sulphate (SDS)

Prepared by dissolving 5gms in distilled water to a final volume of 50mls. Stored at room temperature.

(ii) 10% Ammonium persulphate (APS)

Prepared by dissolving 0.1gms APS to 0.9mls of distilled water. Prepared fresh just before use.

(iii) 30% Acrylamide solution

Prepared by dissolving 29.2gms Acrylamide and 0.8gms N,N-methylene bis-acrylamide in distilled water to a final volume of 100mls. The mixture is filtered and stored at 4°C for 30-60 days.

(iv) 0.5 M Tris HCL.

Prepared by dissolving 6.05 gms Tris in distilled water to a final volume of 100mls.

(v) 1M Tris HCl PH 6.8

Prepared by dissolving 12.1gms Tris in distilled water (about 80mls). pH is adjusted before making up to 100mls with distilled stored at 4°C for not more than 60 days.

(vi) 1.5M Tris HCL pH 8.8

Prepared by dissolving 18.15 gms Tris in distilled water (about 80mls). pH adjusted and made up to 100mls with distilled water

(vii) TEMED (N,N,N'N'Tetra ethyl methyl ethylene Diamine)

Stored at 4°C and used directly with no dilution

(viii) 0.05% Bromophenol blue

Prepared by dissolving 0.5gms of the powder in 1000mls.

(ix) Sample buffer

Distilled water	4.0mls	0.7mls
0.5m Tris HCL	1.0mls	3.0mls
Glycerol	0.8mls	3.0mls
10% SDS	1.6mls	3.0mls
2 Mercaptoethanol	0.4 mls	0.3mls
0.05% Bromophenol blue	0.2mls	Trace

Total volume	8.0mls	10.00mls

(x)Separating gel (for SDS-PAGE)10mls	7.5%	12.5%	15%
30% Acrylamide (mls)	1.5	2.5	3.0
1M Tris HCL pH8.8 (mls)	3.75	3.75	3.75
Distilled water (mls)	4.4	3.425	2.925
----------Degas and add----------			
10% SDS (ul)	100	100	100
10% APS (ul)	33.75	33.75	33.75
TEMED (ul)	6.25	6.25	6.25

(xi) 5% stacking gel 4.4mls

Acrylamide (mls)	0.4
1m Tris HCL pH6.8 (mls)	0.5
Distilled water (mls)	2.86
10% SDS (ul)	40
10% APS (ul)	30
TEMED (ul)	6.25

(xii) Running buffer pH 8.3

Tris	3.0gms
Glycine	14.4gms
SDS	1.0gms

Dissolved in distilled water (about 600mls). pH adjusted before making upto a final

volume of 1000mls with distilled water. Prepared just before use.

(xiii) 0.25M Potassium chloride (KCl) solution.

Used as a reversible stain for proteins on SDS-PAGE gels. Prepared by dissolving 9gms of KCl in 500mls of distilled water before cooling the mixture. Used when it is ice cold. Destaining is performed by placing the stained gel in distilled water for 1 hour at room temperature.

(xiv) Elution buffer

Used for eluting proteins from SDS-PAGE gels. Prepared by mixing up the following reagents.

- 0.1% SDS i.e 0.1gms 100mls distilled water.
- 0.05m Tris HCl pH 7.9 i.e 0.605gms in 100mls distilled water
- 0.1mM EDTA i.e 0.004gms in 100mls distilled water
- 0.20M NaCl i.e 1.168gms in 100mls distilled water
- 0.1mg/ml Bovine serum Albumin (BSA) i.e 10mgs in 100 mls of distilled water.

(E) WESTERN BLOT

(i) Transfer buffer

25mM Tris	15.15gms
192 mM Glycine	72.07gms

100% Methanol	500mls
0.1% SDS	5gms

These reagents were dissolved in distilled water to make up a final volume of 5000mls.

(ii) Washing buffer pH7.2

NaCl	17.4gms
NaH_2PO_4	2.4gms
Na_2HPO_4	2.8gms

These reagents were dissolved in distilled water (about 1000mls). pH was adjusted to 7.2 before making a final volume of 2000mls with distilled water. 6mls of Tween 20 were thereafter added and stirred thoroughly. 0.5% skim milk solution was made by adding 10gms skimmed milk to the PBS/Tween buffer.

(iii) Substrate buffer

Prepared by adding 0.05gms of Diamino benzidine (DAB) to 100mls of PBS and 10 ul of 30% hydrogen peroxide (H_2O_2) just before use.

(F) Ammonium Cut

(i) Saturated Ammonium sulphate solution

Used in precipitating proteins, especially immunoglobulins. Prepared by adding ammonium sulphate ($(NH_4)_2 SO_4$) to distilled water and stirred thoroughly until when no more

salt dissolves. pH was adjusted to 7.5 by using either Ammonia solution or sulphuric acid.

(G) DEAE Ion Exchange Chromatography

(i) 0.2M Phosphate buffer pH 8.

Used for swelling the preswollen DEAE. Prepared by diluting the 0.5M stock solution 2 1/2 times.

(ii) 0.005M Phosphate buffer pH 8

Used in dialysis of immunoglobulins. Prepared by diluting the 0.5M stock solution 100 times.

(iii) 0.02M Phosphate buffer pH 8

Used in running the DEAE column. Prepared by diluting the 0.5M stock solution 25 times.

(H) Conjugation of Immunoglobulins

(i) 1mM Acetate buffer pH 4.4.

Prepared by dissolving 0.057mls of concentrated Acetic acid in 1000mls of distilled water. pH was adjusted using 0.1m NaOH.

(ii) 0.2M Carbonate buffer pH 9.0

0.2M $NaHCO_3$ (i.e 16.8gms in 1000mls distilled water was titrated against 0.2M Na_2CO_3 (i.e 21.1gms in 1000mls distilled water) until the pH 9.0 was attained.

(iii) 0.15M Sodium Metaperiodate ($NaIO_4$)

Prepared by dissolving 32.1mg of $NaIO_4$ in distilled water to make a final volume of 1ml.

(I) Enzyme-linked Immunosorbent Assay (ELISA)

(i) Coating (carbonate) buffer

Na_2CO_3 -31.59gms

$NaHCO_3$ -2.93gms

NaN_3 - 0.20gms

Dissolve in distilled water to make a final volume of 1000mls.

(ii) Washing buffer

It is basically 0.15M PBS pH7.2 containing 0.5% Tween 80

Prepared by dissolving the following in distilled water to make a final volume of 10,000mls.

KCl - 75gms

EDTA - 1gm

Benzoic acid - 2.5 gms

1ml of Tween 80 is added and pH adjusted to 7.5 with 4M NaOH.

(iii) Substrate diluent buffer pH 5

It is a preparation of 0.05M citrate buffer pH 5 in 0.1% benzoic acid i.e.

Citric acid - 10.5gms

Glacial acetic acid - 3.0gms

Benzoic acid - 1.0gms

pH was adjusted using Concentrated Ammonia and made up to 1000mls with distilled water.

(iv) Coniugate diluent buffer pH 7.5

Prepared by mixing the following reagents:-

0.05M phosphate buffer - 1000mls

KCl - 75gms

EDTA - 1gm

Benzoic acid - 2.5gms

5mls of Tween 80 were thereafter added and mixed thoroughly. pH was adjusted to 7.5 using 4M NaOH. NB - A blocking agent, 1% BSA was added when appropriate.

(V) Serum diluent buffer pH 7.5

Prepared by mixing the following reagents.

0.05M phosphate buffer - 1000Mls.

KCL - 75gms

EDTA - 1gm

Benzoic acid - 2.5gms

1ml of Tween 80 was added and pH adjusted to 7.5 with 4M NaoH.

(vi) Substrate (2% H_2O_2)

Prepared by diluting the stock solution (30% H_2O_2) 15 times.

(vii) Substrate working solution for ABTS (Azino-bis(3 ethylbenz-Thiazoline-6 sulphonic acid))

Prepared as follows.

Citric acid pH 4.0 (i.e 9.6g/l) - 25mls

2% H_2O_2 - 120ul.

ABTS (0.2gms in 12.5 mls distilled water) - 100ul.

This makes enough substrate working solution for two ELISA plates.