EPIDEMIOLOGY AND IMMUNODIAGNOSIS OF *FASCIOLA HEPATICA* INFECTION IN CATTLE

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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March 2004

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Abstract

Fasciola hepatica (the common liver fluke) is a trematode parasite particularly of ruminants, that is found throughout the world. Disease may be acute or chronic with consequent economic losses to farmers. Accurate and early diagnosis is necessary to implement control. Diagnosis is normally based on the observation of eggs of *F. hepatica* in faeces or serological tests for the detection of parasitic antigens or specific antibodies in serum samples. Diagnosis by faecal examination is not possible during the 8 - 12 week prepatent period of the infection, and this method of diagnosis has a low sensitivity following patency due to the relatively low number of eggs shed. To improve diagnosis during both early and chronic phases of infection several ELISA techniques have been described. Some of these tests rely on antibody detection using crude extracts or excretory/secretory products of *F. hepatica*. A particular advantage of these tests is that they can detect prepatent infections. There are some tests that have been fully validated using sera from cattle of known *F. hepatica* infection status, but so far there are no reports of tests using milk for detection of infection in cattle. This study was designed to develop and evaluate a milk antibody detection ELISA to diagnose *F. hepatica* infection in cattle that could be used to provide a convenient method of assessing the prevalence of infection within a herd. In addition a bulk tank milk test was developed to improve routine surveillance of the disease, and which would be more readily available for farmers to improve diagnosis and control of the parasite. For this purpose an ELISA was developed based on excreted/secreted antigens of *F. hepatica* and validated using 514 sera from cattle of known infection status. The diagnostic sensitivity of the test was 98% (95% confidence intervals, 96% - 100%) and the diagnostic specificity was 96% (95% confidence intervals, 93% - 98%) using a diagnostic cut-off value of 15 percent positivity (PP) and a 1:800 dilution of sera. The ELISA was evaluated against a commercially available test. The *Kappa* statistic for the two tests was 0.82. This indicates almost perfect agreement between the tests. This diagnostic ELISA was then adapted to detect antibodies to *F. hepatica* in milk. Using a total of 1565 individual sera and milk samples from 61 different farms. A diagnostic cut-off value of 20 PP was established, and at a 1:2 dilution, the diagnostic sensitivity of the milk ELISA was shown to be 92% (95% confidence intervals, 89% - 96%) and diagnostic specificity was 88% (95% confidence intervals, 85% - 91%). The correlation coefficient comparing the serum test and milk test was 96%. Sixty-one bulk tank samples were collected from herds of known seroprevalence. The results indicated that bulk tank milk samples tested using in-house ELISA gave an accurate estimate of herd seroprevalence. The correlation between the herd seroprevalence and the bulk tank ELISA value was 83%. A survey to establish prevalence of *F. hepatica* infection in dairy herds in England and Wales was undertaken. Two thousand two hundred bulk tank milk samples collected over a two year period suggested that the proportion of herds that demonstrated evidence of exposure to *F. hepatica* was higher in 2002 and 2003 than in 2001 and that there was an increase in the proportion of infected herds in the autumn and winter periods compared to the spring and summer. The proportion of infected dairy herds varied between regions with the highest reported prevalence in western areas of England and Wales, notably Carmarthen, Starcross, Aberystwyth and Preston.

Two hundred and ninety four blood samples were collected from infected cattle at post-mortem and were used to develop a means of estimating intensity of infection in cattle. The level of fluke-induced liver pathology in each animal was assessed using a scoring system. There was a highly significant difference ($P < 0.001$) in the PP values of sera from cattle categorised as having a low, medium or high intensity of infection.

Declaration

I declare that the work presented in this thesis is based entirely upon my own research.

Mohammad Reza Salimi Avval Bejestani


For my Family


For my Family


For my Family

Acknowledgements

I would like to thank the following people for their help and guidance during this research:

Special thanks go to Dr Diana Williams for being my supervisor, for her patience, kindness and constant support all the way through my study.

Thanks to all the members of Vet Parasitology Department, Professor Trees, Dr McGarry, Dr Hodgkinson, Dr Makepeace, Catherine Guy, Sarah Felstead, Debbie Kelleher and everybody else whom without their co-operation, this research would have not been possible.

Thanks to Dr Peter Cripps for his help with the statistical analysis of data and Dr Roger Daniel and his colleagues for sending samples from Carmarthen Veterinary Investigation Centre.

Thanks to the Ministry of Science, Research and Technology of Iran (my beloved country) and University of Semnan for their financial support. Also thanks to Dr Shams the Scientific Representative of the Islamic Republic of Iran and Director of Iranian students in the UK and Ireland for his support.

Special thanks to my beloved family, my dear wife Masoumeh Minou for her patience, encouragement and support. My lovely children; Sahar and Saeid for understanding that Dad is busy studying and playing quiet games to avoid disturbing me.

And at last all my friends for their encouragement and support.

Abbreviation

µg	microgram
µl	microlitre
°C	degree Celsius
APS	Ammonium persulphate
BTM	bulk tank milk
CI	confidence interval
DIG	Diffusion in gel
DIPA	dot immunoperoxidase
E/S	excreted and secreted (antigen)
ELISA	enzyme linked immunosorbent assay
epg	eggs per gram of faeces
et al.	et alia (and others)
FEC	faecal egg count
FABP	fatty-acid binding protein
g	gram
GIS	geographical information system
γGT	gamma-glutamyl transpeptidase
GLDH	glutamate dehydrogenase
GLM	general linear model
GST	glutathion-S-transferase
HA	haemagglutination
IFN-γ	interferon gamma

Ig	immunoglobulin
IL-	interleukin-
IU	international Unit
L	litre
LAP	leucine aminopeptidase
LSTM	Liverpool School of Tropical Medicine
mg	milligram
ml	millilitre
ng	nanogram
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PP	percent positivity
SDS	Sodium Dodecyle Sulphate
SPM	skimmed-milk powder
Th	T helper subset
TMB	3,3,5,5-tetramethyl benzidine
VIC	Veterinary Investigation Centre
VLA	Veterinary Laboratory Agency

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1.1. General introduction

Fasciolosis (liver fluke disease) caused by the digenetic trematode *Fasciola hepatica* is of major health, welfare and economic importance to cattle and sheep production in temperate regions of the world. Economic losses due to this infection in Great Britain and Ireland alone are greater than £80 million annually and world wide are approximately US$2000 million annually (Spithill et al., 1999; Mulcahy and Dalton, 2001). Economic losses fall into three main categories: acute loss due to animal mortality; loss at the abattoir because of liver condemnation and finally losses in production. Tropical fasciolosis caused by the related species *Fasciola gigantica*, is the most important infection of ruminants in Asia and Africa (Spithill et al., 1999). Furthermore fasciolosis has recently been recognised as a common and important pathogen of humans in countries such as Bolivia, Peru, Equador, Egypt and Iran (Esteban et al., 1997; O'Neil et al., 1998; Mas-Coma et al., 1999; Rokni et al., 2002). It is estimated that 2.4 million people are infected with liver fluke world-wide (Mas-Coma et al., 1999) and a further 17 million people are considered to be at risk (Mas-Coma et al., 1999; Rokni et al., 2002). Moreover sporadic cases occur in Western Europe, and it is likely that a significant number of cases go undiagnosed. The Word Health Organisation has now recognised that *F. hepatica* is a serious zoonosis in certain areas of the world (WHO, 1995).

Mammals acquire infection by ingestion of vegetation or water contaminated with encysted *F. hepatica* metacercariae. A juvenile fluke hatches from the cyst in the intestine and migrates through the intestinal wall and into the liver. In the liver

parasites feed actively and cause extensive haemorrhage and tissue damage. After approximately 8-12 weeks, the parasite matures, migrates to the bile ducts and starts to produce eggs. The eggs, together with bile, drain into the intestine and are passed out onto the pasture with the faeces. Intermediate snail hosts, of the genus *Lymnaea*, become infected with miracidiae that hatch from eggs. In the snail they develop and multiply and some time later they emerge as swimming cercariae which then encyst on vegetation.

Fasciolosis ranges from a peracute syndrome characterised by sudden death, to chronic disease (Boray, 1969; Behm and Sangster, 1999). Acute disease has been reported more commonly in sheep and goats, which seem to be more susceptible to the effects of infection (Behm and Sangster, 1999). Calves are also susceptible to disease but in excess of 1000 metacercariae and after infection with 10,000 metacercariae similar to sheep disease characterized by death (Boray, 1969). Flukes do not only blood feed, they also eat their way through the liver, feeding mainly on hepatic cells causing parenchymal damage with extensive haemorrhage. In the acute form, death often results from circulatory collapse and haemorrhagic anaemia. In chronic fasciolosis clinical signs include lethargy, ascites and subcutaneous oedema, low weight gain and wool break due to poor liver function, hypoalbuminaemia and anaemia. Blood eosinophilia appears soon after infection and increases rapidly during the parenchymal stage. Macrophages and fibroblasts gradually fill the tracks created by the migrating flukes resulting in fibrotic granulation. The presence of adult flukes in the bile ducts causes bile duct thickening and calcification.

The role of parasite intensity and frequency of infection on induction of protective immunity in cattle and sheep, is not fully understood. Cattle, unlike sheep are reported by some authors to develop resistance to re-infection. However it is not clear whether this resistance is due to an acquired immune response or is effected by means of a physical barrier due to fibrosis and calcification to fluke migration through the liver and into the bile ducts (Boray, 1969; Hoyle et al., 2003).

Diagnosis of fasciolosis is based on observation of eggs of *Fasciola* in faeces, immunodiagnostic tests for the detection of parasitic antigens in serum or specific antibodies in serum and milk samples together with clinical signs. Diagnosis based on clinical signs alone is difficult, because several other diseases can produce similar clinical signs (Mullen, 1976) and fasciolosis may be accompanied and exacerbated by other infections such as *Ostertagia* spp and *Clostridium novyii* (Reid et al, 1967).

As fasciolosis causes such significant economic losses and is a potential epidemic and endemic disease in humans, its control in domestic animals is extremely important. Chemotherapy, which eliminates the parasite from the ruminant host, is the main method used to control fasciolosis. Land management practices, like drainage, and restricting grazing to snail free areas, are also applied where adequate resources are available. However these methods are expensive, it is not always feasible to apply them everywhere and they require frequent attention for effective control to be maintained during the year. There are reports of resistance in certain *F. hepatica* populations to some chemotherapeutic drugs and there is also public concern regarding chemical residues in food and in the environment.

Therefore, interest in developing a vaccine for fasciolosis has increased in recent years.

Vaccine design should be based upon a good understanding of the natural immune processes occurring during the various phases of infection and should give a benefit that would be visible to farmers at a competitive price.

Several candidate fluke proteins have been isolated and used in vaccine trails but till now only a few vaccine candidates have been shown to produce a degree of protection against fasciolosis; these include glutathions-S-transferase (GST), fatty-acid binding protein (FABP), leucine aminopeptidase (LAP) and cathepsin L proteinases (Mulcahy and Dalton, 2001; Dalton et al., 2003a). The cathepsin L proteinases, which are secreted by liver flukes at all stages of their development in the mammalian host, are believed to play important roles in facilitating parasite migration (tissue degradation), feeding and immuno-evasion (Mulcahy and Dalton, 2001) and have shown excellent prospects for the development of first generation anti-fluke vaccines (Dalton et al., 2003a).

The work described in this thesis investigates immunodiagnosis of *F. hepatica* infection in cattle. There are several diagnostic tests reported in the literature review, but only one was commercially available at the start of this work. The aim of this project was to develop a fully validated diagnostic test with high sensitivity for detecting infection. No validation data were available for the commercial test. Therefore an ELISA test to diagnose *F. hepatica* infection in cattle by measuring antibody in milk has been developed. This is a rapid method for detecting the

infection, without causing stress to the cattle by collecting blood. To develop a milk antibody detection test, first an ELISA to detect serum antibodies was developed. Finally a bulk tank milk test was developed that can be used to assess the level of infection within a herd.

1.2. Literature Review

1.2.1. The life cycle of *F. hepatica*

The discovery of the life cycle of *F. hepatica* has been formally credited to two scientists, Thomas and Leuckart, working at the end of the nineteenth century (summarised by Reinhard, 1957). The parasite has an indirect life cycle involving a snail (*Lymnaea* spp) intermediate host, within which asexual reproduction (sporocysts, rediae, and cercariae) occurs and a mammalian (cow, sheep, human, rabbit, etc.) definitive host, the site of sexual reproduction (Figure 1.1).

Mammals are infected by ingesting metacercarial cysts via contaminated vegetation (grass, watercress, mint, etc). Within the mammalian intestine they excyst and penetrate the intestine wall and migrate across the peritoneal cavity to the liver. The juvenile flukes penetrate through the liver capsule and start to migrate and feed the parenchymal cells, until they ultimately enter the bile ducts where they develop to adults. The time from ingestion of metacercariae to becoming mature adult flukes in the bile ducts varies between different host species, but on average ranges from 8 – 12 weeks in sheep and cattle (Boray, 1969).

The fluke is hermaphrodite and self-mating may occur (Hurtrez-Bousses et al., 2001). Eggs are produced by each worm and laid unembryonated and are passed from the common bile ducts into the duodenum and then voided with the faeces onto pastureland. The eggs must be washed into fresh water to complete their development and hatch. Hatching usually takes place within 10 – 15 days, but temperature, moisture and oxygen tension levels all affect the embryonation process within the fluke egg.

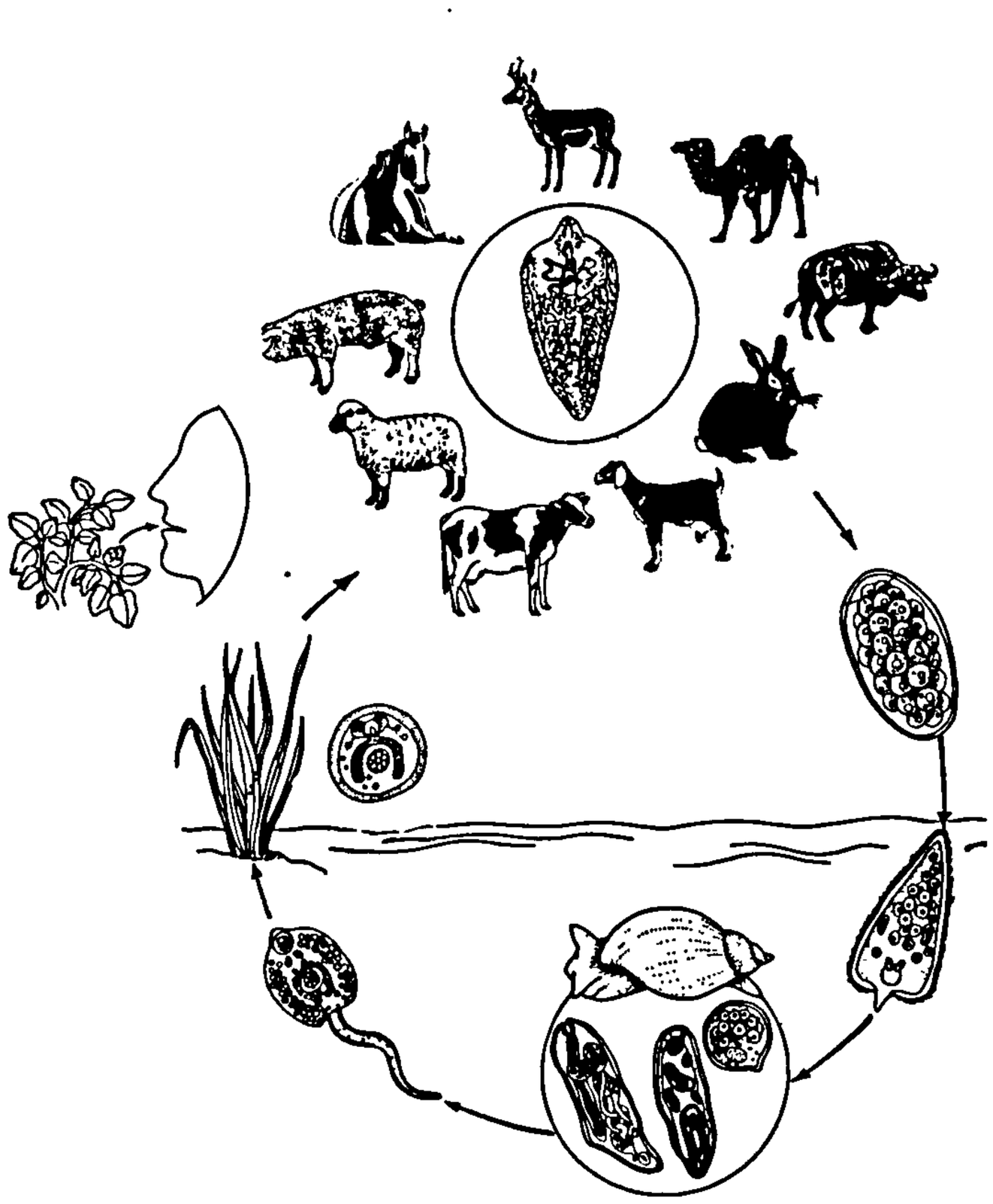

Figure 1.1. Life cycle of *Fasciola hepatica* (FAO, 1994)

Thus at low temperatures, development can be delayed for several months - a minimum temperature of 10°C is required for egg development. Immediately after hatching, the ciliated miracidium swims, actively seeking its appropriate snail

host. It penetrates the foot of the snail and develops into a sporocyst (Figure 1.2). Asexual multiplication occurs within the sporocyst, with germinal cells forming many rediae. When these are mature the sporocyst bursts, releasing the rediae. Rediae similarly develop and give birth to cercariae. The cercariae are subsequently shed from the snail into water (Figure 1.3) and after finding a suitable site usually on vegetation, they encyst, lose their tails and become metacercariae (Figure 1.4) which are resistant to mild changes in temperature and other environmental parameters.

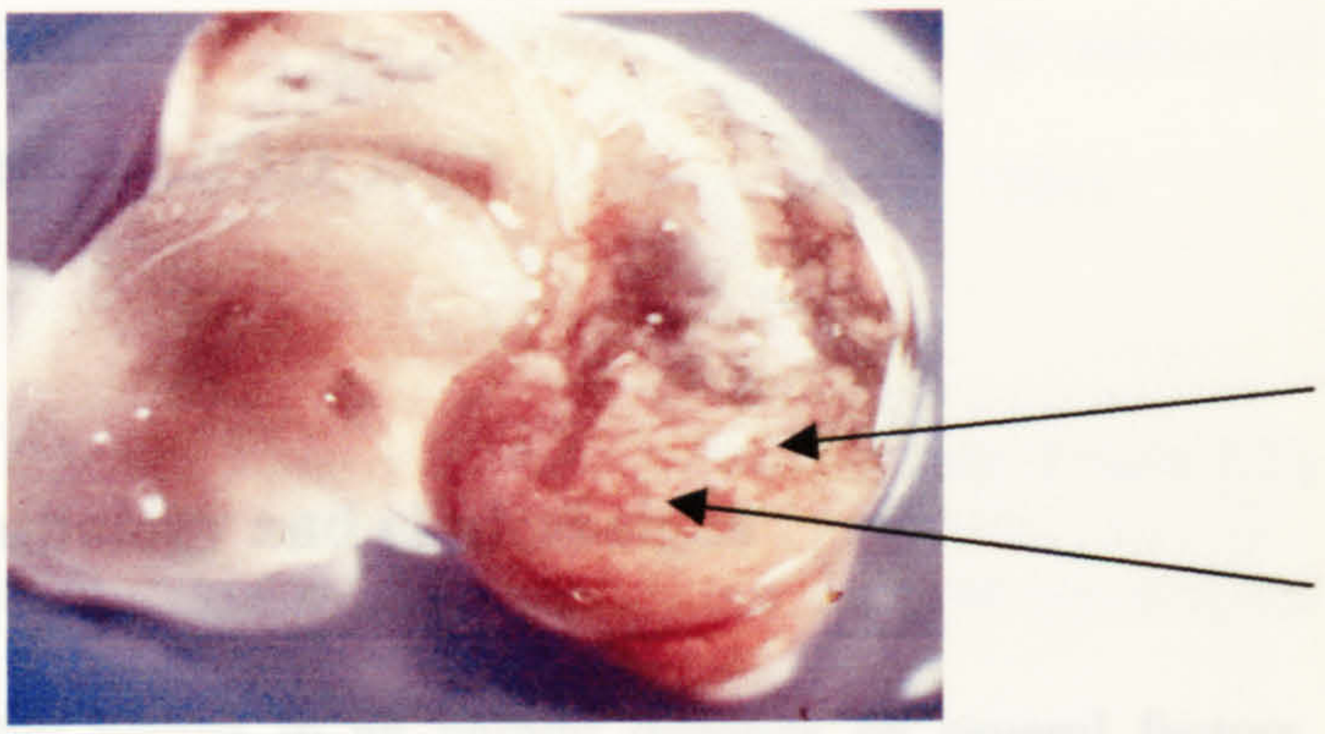

Figure 1.2. A *Lymnaea* sp snail infected with *Fasciola* cercariae (cercariae are visible in the body cavity of the snail) (FAO, 1994).

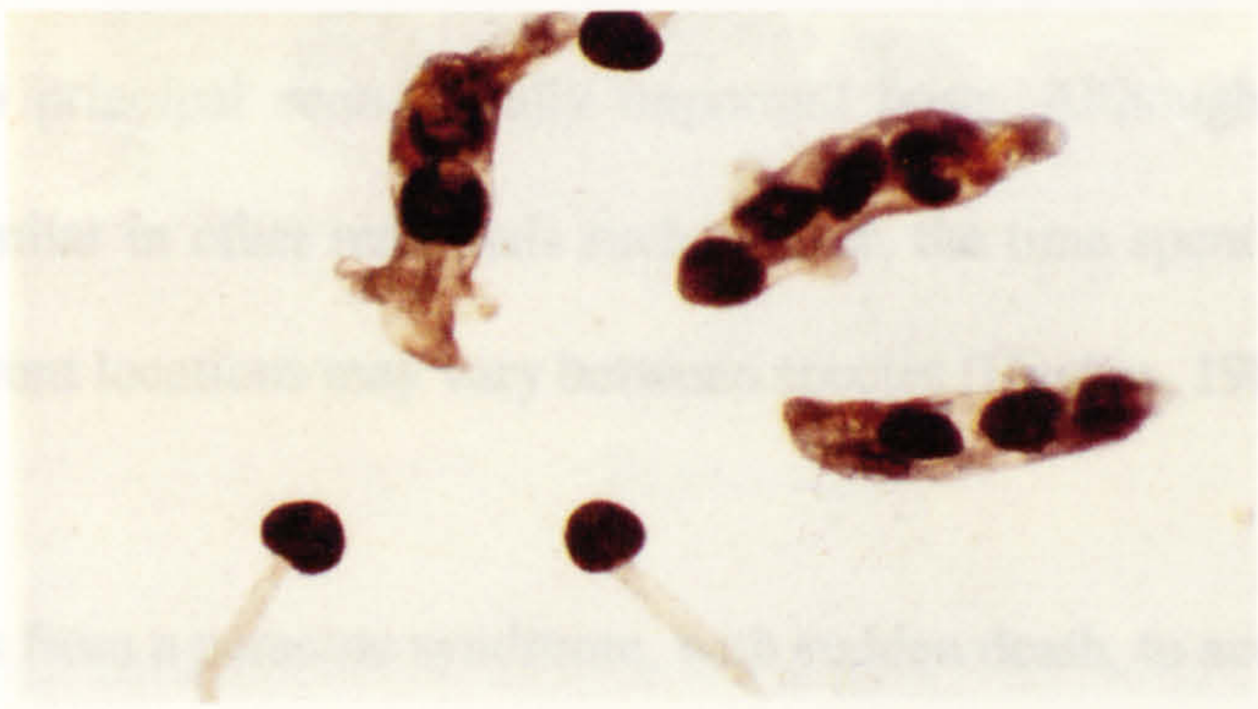

Figure 1.3. *Fasciola* larvae (rediae and cercariae) removed from snail (FAO, 1994)

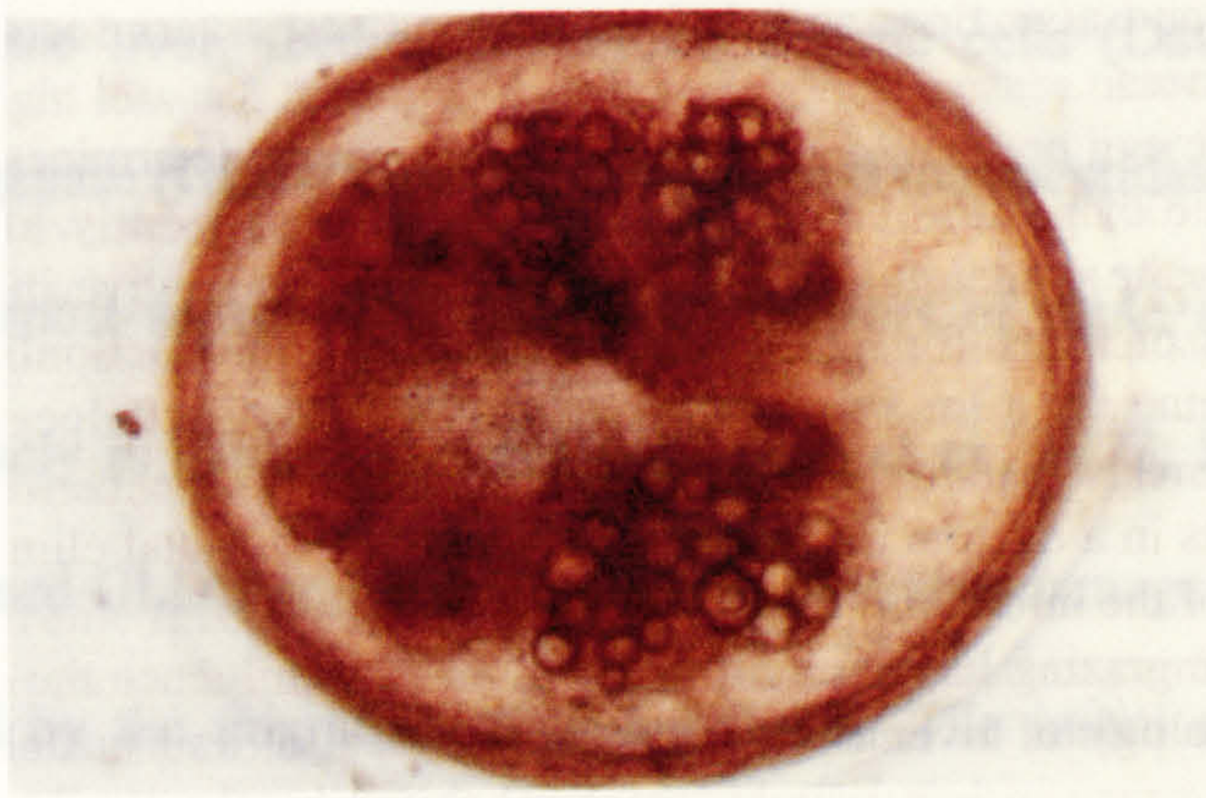

Figure 1.4. *Fasciola* metacercariae (FAO, 1994)

1.2.2. Pathogenesis, pathology and clinical signs

The form of fasciolosis that occurs in an animal depends on several factors, including the host species, the number of metacercariae ingested and the current health status of the animal (Boray, 1969). The majority of current knowledge concerning the nature of fluke infection in mammals has been obtained from examination of the clinical and pathological effects of infection in sheep and cattle, the two principal economically important hosts. Although the course of infection is similar in other mammals such as deer, the time spent by the various stages in different locations may vary between species (Hughes, 1987).

Disease ranges from a peracute syndrome, with sudden death, to acute and chronic disease. Acute disease has been reported more commonly in sheep and young calves, which seem to be more susceptible to the effects of infection (Boray, 1969; Behm and Sangster, 1999). In outbreaks of acute ovine fasciolosis, losses of

up to 25% of a flock are reported. Flukes do not only blood feed, they also eat their way through the liver, feeding mainly on hepatic cells (Dawes, 1963). Glutamate dehydrogenase (GLDH) and gamma-glutamyl transpeptidase (γGT) are reported to be the most sensitive biochemical indicators of acute and chronic fasciolosis respectively in cattle and sheep (Anderson et al., 1977; Ferre et al., 1994, 1996). Increased GLDH activity is indicative of severe damage to the liver parenchyma caused by the migration of young flukes. The initial rise in γGT reflects penetration of the bile ducts by migrating flukes and the associated damage to bile duct cells (Sykes et al., 1980; Bulgin and Anderson, 1984).

In the acute form, gross haemorrhage and acute liver failure are seen (Figure 1.5), due to the massive structural damage to blood vessels and the liver parenchyma by migrating juveniles.

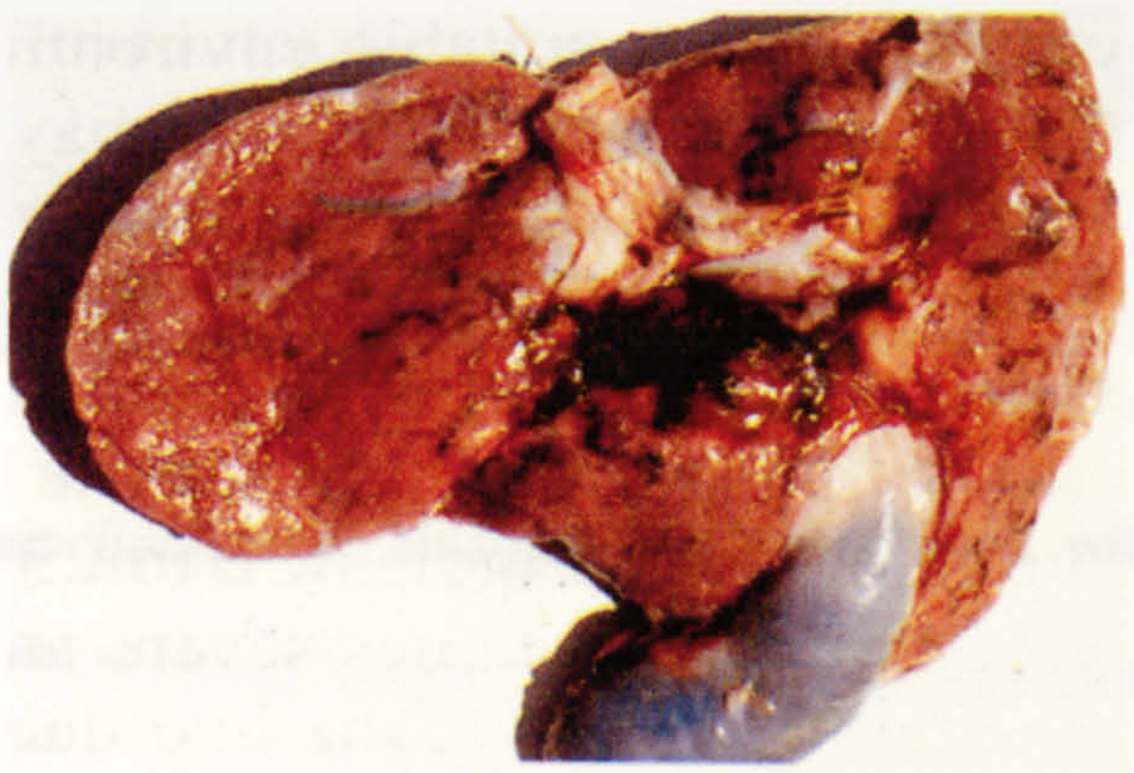

Figure 1.5. Acute fasciolosis with massive bleeding (FAO, 1994).

Without treatment, death often results from circulatory collapse and acute haemorrhagic anaemia. In comparison, lethargy, ascites and subcutaneous oedema, low weight gain and wool break are signs of chronic fasciolosis (Figure 1.6). The clinical signs of chronic disease are a result of poor liver function, hypoalbuminaemia and anaemia. The gross pathology seen in chronically infected sheep and cattle are different. Cirrhosis and fibrosis of the liver parenchyma and bile ducts occur (Figure 1.7) in both cattle and sheep, but in cattle, unlike sheep, bile duct calcification is also seen (Dow et al., 1967; Sinclair, 1971; Sinclair, 1973: Hughes et al., 1981).

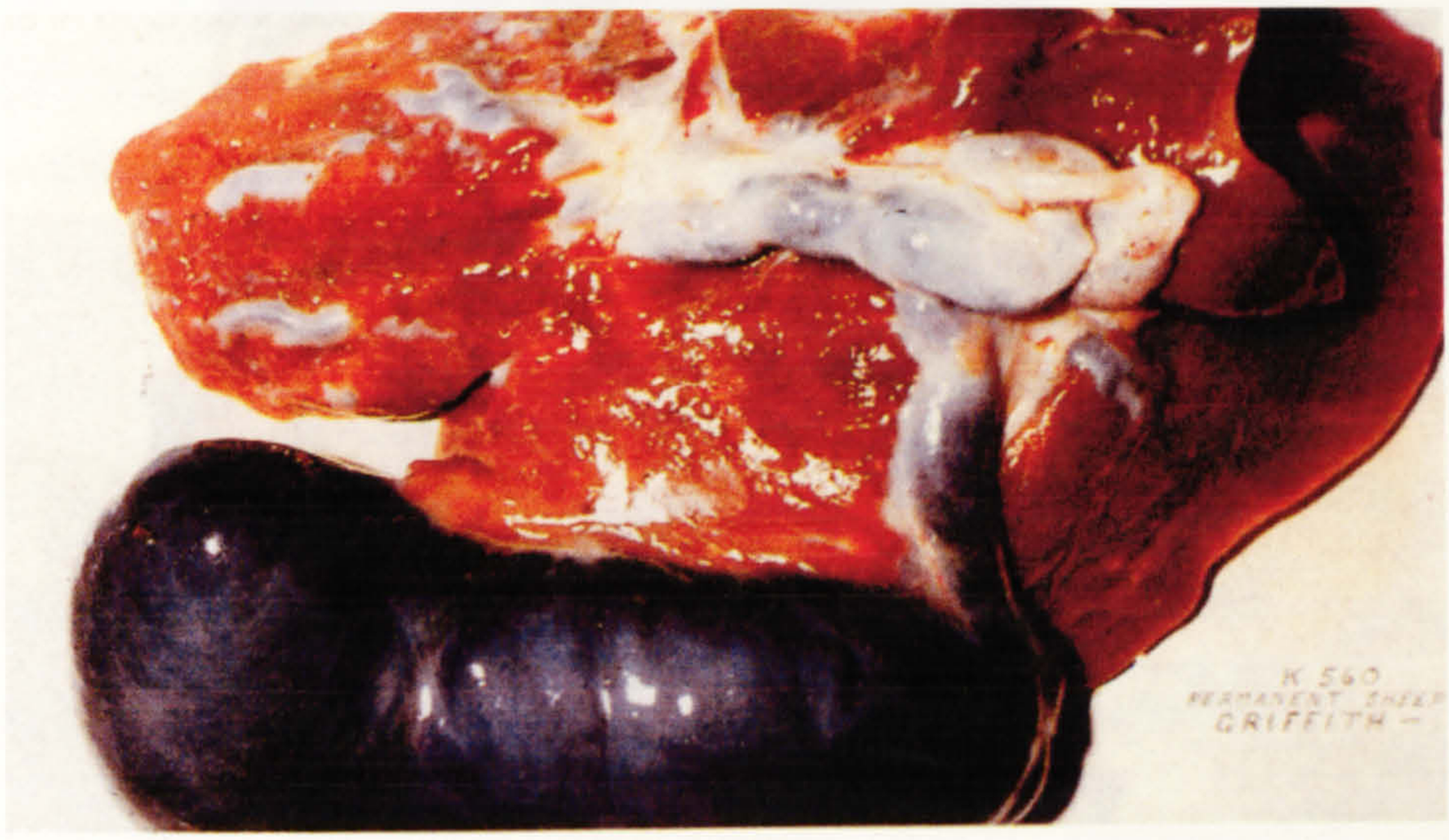

Figure 1.6. Chronic fasciolosis: enlarged bile ducts filled with flukes (FAO, 1994).

It has been suggested that this calcification and other fibrotic changes of the bile ducts is the reason for the partial resistance seen in cattle, perhaps because it may physically impair fluke feeding (Boray, 1969). However, it is not certain that

calcification or physical damage are the only factors involved in the partial resistance seen in cattle. In cattle, chronic disease is increasingly common and often manifests as production losses. Reductions of 13 kg per carcass for cattle have been reported (Marley et al., 1996). In the subclinical form of the disease, whilst no clear clinical signs are evident, reduced productivity is also reported (Behm and Sangster, 1999).

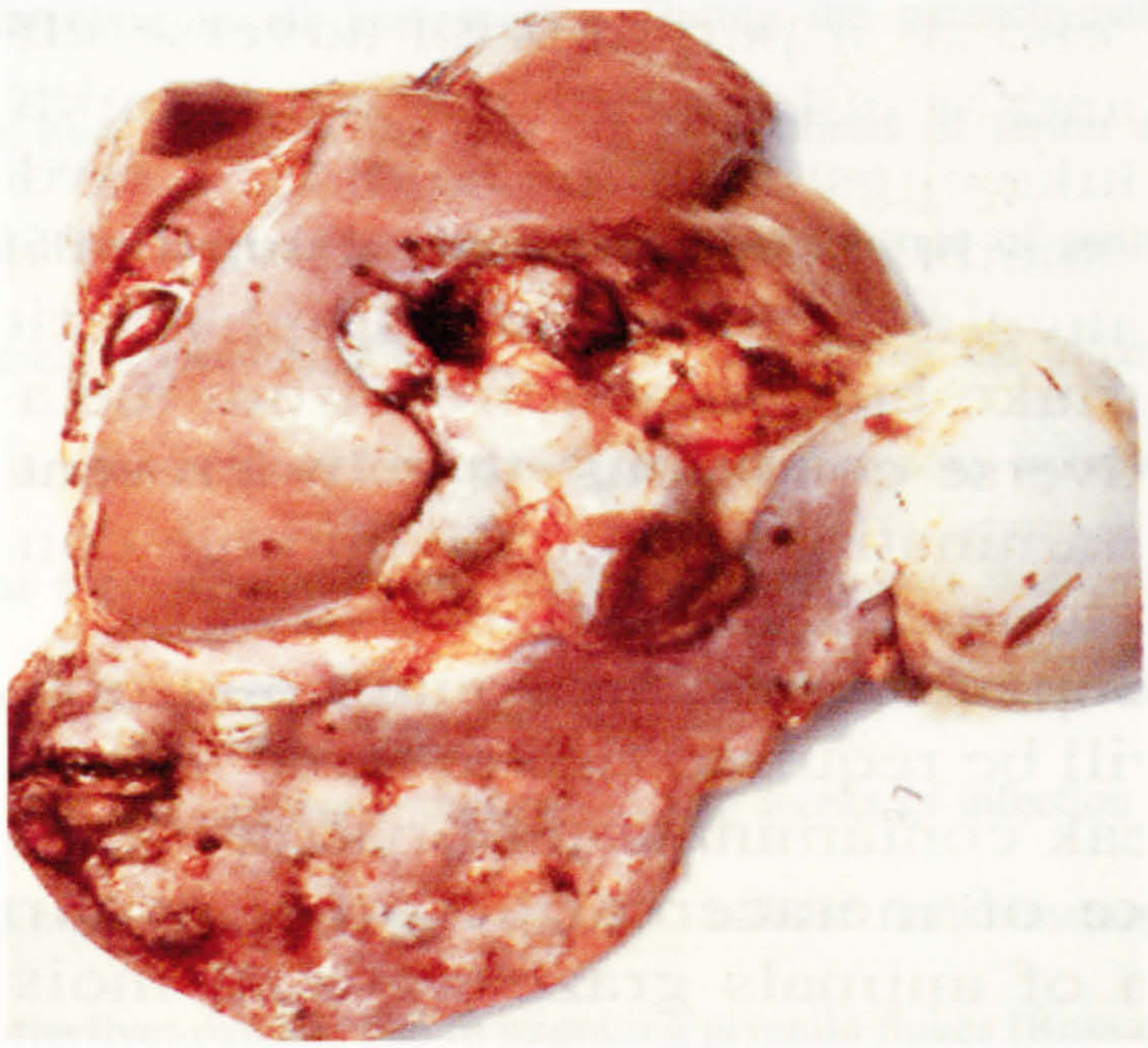

Figure 1.7. Chronic infection in young cattle: liver with severe fibrosis, enlarged bile ducts (FAO, 1994).

The pathology of fasciolosis occurs in two phases: the parenchymal phase associated with migration of flukes through the liver parenchyma and the biliary phase due to presence of adult flukes in the bile ducts. Infection rates and the severity of disease vary considerably between individual animals. Flukes are concentrated in the ventral portion of the liver and many of them become trapped

in the parenchyma where they face a hostile inflammatory reaction. The bile ducts become thickened due to epithelial hypertrophy, fibrosis and calcification of the duct walls occur from 16 to 20 weeks of infection (Behm and Sangster, 1999). Anaemia is probably one of the most important factors contributing to host morbidity and mortality in fluke infection and the rate of blood loss (Figure 1.8) is estimated to be 0.2 – 0.5 ml per day per fluke (Dawes and Hughes, 1964; Jennings, 1976). Hypoalbuminaemia and hyperglobulinaemia commonly occur in liver fluke infection in all host species. During the parenchymal phase of infection, the liver, which is the only site of synthesis of serum albumin, is damaged and liver function is compromised, thus the rate of serum albumin synthesis decreases (Anderson et al., 1977). Also during the biliary stage of infection the amount of blood lost exceeds the liver's capacity to replace the lost albumin. Thus a progressive loss of plasma albumin occurs in all infected host species (Behm and Sangster, 1999). Levels of immunoglobulins particularly IgM, IgG1, IgG2a and IgE increase within two to four weeks of infection (Hughes et al., 1981; Knight and Becker, 1987). A cellular inflammatory reaction, accompanies the liver damage due to migrating juvenile flukes (Ross et al., 1966; Dow et al., 1967). The tracks created by migrating flukes fill with erythrocytes, lymphocytes, neutrophils, eosinophils and macrophages (Behm and Sangster, 1999). Blood eosinophilia appears soon after infection and increases rapidly during the parenchymal stage and persists at a high level after the flukes enter the bile ducts (Ross et al., 1966; Sinclair, 1975; Poitou et al., 1992). Macrophages and fibroblasts gradually fill the older part of the tracks and create fibrotic granulation (Behm and Sangster, 1999).

Figure 1.8. *Fasciola* consume large amounts of blood. This figure shows regurgitated blood from a single fluke isolated from a cow. (FAO, 1994).

1.2.3. Immunology of fluke infection

1.2.3.1. Immune response to *F. hepatica* in cattle and sheep

Investigations have shown that, in contrast to sheep, cattle infected with a single infective dose of metacercariae followed by drug termination of the infection, develop some degree of resistance to re-infection (Ross, 1966; Doyle, 1971; Haroun and Hillyer, 1986). Other studies showed that the type of infection, either a single or a trickle administrated dose, had no effect on the development of protective immunity (Bossaert et al., 2000). Doyle (1973), Doy and Hughes (1984), and Brown et al. (1999) found that resistance to a challenge infection increased with the duration of the sensitising period. It has been suggested that the

rejection of the challenge infection may occur at the level of the gut or peritoneum rather than in the liver (Hayes and Mitrovic, 1977; Doy et al., 1978; van Milligen et al., 1998a). In contrast, cattle appeared to remain fully susceptible to a trickle challenge infection with metacercariae in the presence of an existing infection under natural grazing conditions (Clery et al., 1996).

Research has shown that the antibody response to *F. hepatica* in infected cattle, measured using either a whole-worm extract (Wyckoff III and Bradley, 1986; Santiago and Hillyer, 1988), a fractionated crude adult antigen (Oldham, 1983), somatic antigen (Bossaert et al., 2000), or ES products (Santiago and Hillyer, 1988; Bossaert et al., 2000) is predominantly an IgG1 response (Hoyle et al., 2003). Cornelissen et al., (1992) compared ELISA results obtained using unfractioned somatic and ES antigens and sera from infected sheep and found no difference between the two antigens. In both cattle and sheep, circulating antibodies to ES or somatic fluke preparations were detected within 2 weeks of infection (Santiago and Hillyer, 1988; Chauvin et al., 1995; Clery et al., 1996). Western blot analyses have been used to detect proteins present in crude fluke preparations that are recognised by immune sera. Santiago and Hillyer (1988), reported that antibodies in sheep and cattle sera recognised different somatic proteins. Cattle antibodies detected proteins of 56, 64 and 69 kDa but these were not recognised by antibodies in sheep. Both cattle and sheep antibodies recognised bands of 30-38 kDa, but antibody appeared slightly later than to the higher weight fractions recognised by cattle. Secondary challenge enhanced the response to the lower molecular weight antigens (Chauvin et al., 1995; Itagaki et al., 1995; Hoyle et al., 2003). The specificity of antibody responses in cattle naturally exposed to

F. hepatica in groups of calves, heifers and adult cows were investigated and results indicated that naturally infected cattle from all three groups had antibodies that detected surface and excretory-secretory proteins of 60 – 66 kDa as well as a somatic protein of 28 kDa. Heifers and adult cows also recognised somatic proteins of 17 kDa and only adult cows had antibodies that recognised a 28 kDa protein of E/S antigens (Ortiz et al., 2000).

Naive cattle experiencing a primary infection and chronically infected cattle produced specific antibodies predominantly of the IgG1 isotype, but it was suggested that this isotype was non-protective (Clery et al., 1996; Mulcahy et al., 1998). The IgG2 response was low or even absent (Duffus and Franks, 1981; Clery et al., 1996; Mulcahy et al., 1998). Similarly Hoyle et al., (2003) found a predominantly IgG1 response to different fluke antigens irrespective of how the cattle had been sensitized. In naturally infected cattle, Ortiz (1997) found that IgG1 and IgG2 responses were equal in animals that had presumably been exposed to repeated infection.

There are three phenotypes of T lymphocyte described in cattle: CD4+, CD8+ and gamma delta T cells (Howard and Morrison, 1994). CD4+ T cells, have been further sub-divided in to T helper 1 (Th1) and T helper 2 (Th2) subsets and these generate help for the immune response. CD8+ T cells have cytotoxic actions on infected host cells. Gamma delta T cells use a different class of T-cell antigen receptor and their function is not fully understood. The cytokines IFN-γ, IL2 and TNFβ are associated with the Th1 subset and IL4, IL5 and IL6 with a Th2 profile (Cherwinski et al., 1987). Th1 cells can induce a delayed type hypersensitivity

response (Cher and Mosmann, 1987) and are associated with cell mediated immunity, whilst Th2 cells provide help for antibody production by B cells. However, Th1 cells are also able to stimulate antibody production, mainly that of the IgG2a isotype (Coffman et al., 1988; Stevens et al., 1988). Each T helper subset responds to different antigen presenting cells, with macrophages stimulating Th1 cells and B cells Th2 cells (Gajewski et al., 1991). Antibody production in response to Th2 help is mainly of the IgG1 and IgE isotypes, which are seen normally in helminth infections. The bovine immunoglobulin isotypes include IgG1, IgG2 (IgG2a1, IgG2a2, IgG2b), IgA, IgM and IgE (Knight and Becker, 1987). IL4 is involved in stimulation of B cell activity and the production of IgG1 and IgE isotypes, whilst IFN-γ, is involved in the production of IgG2 (Estes et al., 1994; 1995; Estes, 1996).

It has been shown that Th cell clones specific for *F. hepatica* enhanced IgG1 synthesis through IL-4 expression (Brown et al., 1999). The capacity to increase IgG2 production is associated with the production of IFN-γ (Estes et al., 1994) which seemed to be co-expressed with IL-4 by the majority of *Fasciola* –specific CD4+ T cell clones (Brown et al., 1998). However low ratios of IFN-γ to IL-4 transcript levels were detected in those clones (Brown et al., 1998), confirming that a dominant Th2-like response is observed in animals chronically infected with *F. hepatica* (Clery et al., 1996; Brown et al., 1998). Recently, higher levels of IgG2 specific antibodies have been associated with protection in vaccinated cattle (Mulcahy et al., 1998).

The role of parasite intensity and frequency of infection in inducing protective immunity in cattle is unknown (Bossaert et al., 2000). The presence of adult flukes in the bile ducts appears to suppress the antibody response, but the host's resistance or susceptibility to infection appears to be unrelated to the antibody response (Bossaert et al., 2000). The substantial parenchymal fibrosis attributed to the primary infection, suggested that resistance was effected by means of a physical barrier to fluke migration through the liver and into the bile ducts rather than an acquired, protective immune response (Boray, 1969; Hoyle et al., 2003). Haroun and Hillyer (1986) and Keegan and Trudgett (1992) all reported that there was no correlation between antibody titres and fluke burden. In contrast, a positive correlation between specific IgG1 antibody titre and fluke burden was reported by Mulcahy et al. (1998) and Bossaert et al., (2000).

Waldvogel et al. (2004) reported that the polarisation of the immune response in infected cattle is apparently stronger in hepatic lymph nodes, which drain the site of infection, than in retropharyngeal lymph nodes. Brady et al. (1999) working with mice demonstrated that immune responses dominated by the Th1 cell subset, induced at one site in the body, could exert a bystander modulation of the reciprocal T cell subset at another site in the body. Furthermore, Brady et al., (1999) suggested that the Th2 cell may have a dominant effect in Th1-Th2 cross regulation in vivo. Furthermore these authors showed that *F. hepatica* infection induced Th2 responses that suppressed a Th1 response induced by *Bordetella pertussis* infection (Brady et al., 1999) and Th1 responses induced in *Schistosoma mansoni* infection (Sher et al., 1991). Excretory/Secretory (E/S) components of *F. hepatica* exert direct immune suppressive effects through the activity of

proteinases on immunoglobulin molecules (Carmona et al., 1993) and may also, by induction of IL-4 and perhaps IL-10, suppress IFN-γ production by Th1 cells and inhibit the activation of macrophages (Brady et al., 1999). Using *F. hepatica* infected mice, it was shown that spleen cells secreted high levels of IL-4 and IL-10 in response to liver fluke antigens in vitro (O'Neill et al., 2000). Prowse et al., (2002) reported that E/S products of *F. hepatica* could suppress sheep lymphocyte proliferation in vitro. In summary, Cathepsin L-like enzymes, which are the major component of E/S products of juvenile flukes, and are produced by all stages of the fluke, have been associated with cleavage of immunoglobulin (Chapman and Mitchell, 1982; Smith et al., 1993b), suppression of T cell responses (O'Neill et al., 2001; Prowse et al., 2002) and prevention of eosinophil migration (Carmona et al., 1993).

1.2.3.2. Immune response to *F. hepatica* in rat

The course of *F. hepatica* infection in rats appears to be similar to that in cattle, providing a useful model to analyse the humoral and cellular immune responses after infection (Armour and Dargie, 1974; Doy and Hughes,1982; Keegan and Trudgett, 1992; van Milligen et al., 1998b). Research has indicated that rats develop a high level of protection against a challenge infection with *F. hepatica* metacercariae. A challenge as low as one parasite resulted in 76% protection against a challenge infection (Hayes et al., 1973). Although a fluke infection in rats generated a strong resistance to subsequent infection, the immune responses did not protect against the primary infection and did not result in fluke expulsion (Mulcahy et al., 1999). Keegan and Trudgett (1992), found that during a primary infection, IgG antibodies to whole fluke antigen appeared early, within seven days

of infection and peaked within three weeks. No significant correlation was found between antibody titre and the number of flukes recovered at post-mortem during the primary infection, suggesting that the antibody response was non-protective and that rats did not generate an immune response quickly enough to eliminate the parasite in primary infection. Rats were resistant to secondary challenge and the antibody response to challenge was rapid, peaking within 5 days. A circulating eosinophilia was seen throughout infection (Cervi et al., 2001) and large numbers of neutrophils, macrophages, lymphocytes and eosinophils were found in liver sections surrounding flukes and fluke tracts (Milbourne and Howell, 1990; Keegan and Trudgett, 1992). Poitou et al. (1992) confirmed the early appearance of antibody to ES proteins. Poitou et al. (1993) detected IgG1, IgG2a, IgM and IgE antibody isotypes at different times throughout the infection period. Continual sequential release of new antigens throughout fluke development was shown by finding IgM antibody not only during the first few days but also throughout infection (Poitou et al., 1993). Peripheral eosinophil and neutrophil numbers increased during infection and in the spleen the proportion of B cells increased, whilst that of CD4+ and CD8+ T cells decreased (Poitou et al., 1993).

Local immune responses to fluke infection were examined in rats and it was shown that in a chronic primary infection, only the hepatic lymph nodes produce parasite specific antibodies, whereas during a secondary challenge mesenteric lymph nodes produce antibodies specific to newly exysted juvenile proteins (Meeusen and Brandon, 1994). Comparing responses after oral and intraperitoneal challenge it was shown that protection against secondary challenge occurred at both at the level of gut and peritoneal cavity (Meeusen and Brandon, 1994). These

authors showed during the normal migration of the parasite from intestine to bile ducts only the hepatic lymph nodes are stimulated and this stimulation occurs only after the parasites pass into the liver. Examination of the gut response to secondary challenge revealed that resistance to re-infection is significantly correlated with tissue levels of IgE, IgG1 and the presence of eosinophils (van Milligen et al., 1998b). Thus, the same sort of response that is seen after the primary infection, i.e. an IgG1, IgE, eosinophilic reaction, which is not able to prevent primary invasion, is also found during a secondary challenge, where it appears to be protective (van Milligen et al., 1999). Immature flukes appear to stimulate a more effective protective immune response than adult flukes (Tkalcevic et al., 1996).

1.2.4. The diagnosis of bovine fasciolosis

Diagnosis of *Fasciola* infection may be made using several different methods: the observation of eggs of *Fasciola* in faeces (Boray, 1985); serological tests for the detection of parasitic antigens or specific antibodies in serum samples; measurement of liver enzymes, GLDH and γGT, and clinical signs. Diagnosis based on clinical signs and liver enzyme profiles is difficult, because several other diseases can produce similar clinical signs (Mullen, 1976), the disease may be accompanied by other parasitic infections (Reid et al, 1967) and charges in liver enzymes are not specific to *F. hepatica* infection. Therefore parasite specific methods are required to confirm diagnosis.

1.2.4.1. Diagnosis by parasitological methods

1.2.4.1.1. Diagnosis of *F. hepatica* by identification of the eggs in faeces:

Definitive diagnosis of chronic infection is usually achieved by finding eggs in faeces (Boray, 1985; Hillyer et al., 1992). Adult worms begin to lay eggs from about eight weeks (De Leon et al., 1981) to 14 weeks after infection (Urquhart et al., 1997) depending on the host species. Thus parasitological diagnosis during the prepatent period, may be difficult (Anderson et al., 1999). Repeated stool examinations are usually required for the detection of eggs and in many cases, no eggs are recovered because the sensitivity of the technique is low (Hillyer et al., 1992). It is recommended that freshly collected rectal samples should be used for helminthological faecal examination (Ministry of Agriculture, Fisheries and Food, 1971; Thienpont et al., 1979). But Bonita and Taira (1996) reported that preservation with 5% formalin solution is an acceptable means of storing faecal samples for later examination of *Fasciola* eggs using the Beads technique (sieving technique using a glass bead layer for the detection of *F. hepatica* eggs from cattle faeces) and by other sedimentation methods. These authors reported that the egg recovery rates from 5% and 10% formalin preserved samples were 57.2% and 60.8% respectively, and these values were similar to the value of 58.8% for fresh *Fasciola* eggs in cattle faeces. Eggs of *Fasciola* are 130 to 150 µm length and 63 to 90 µm wide with an oval shape and a characteristic yellow colour (Figures 1.9 and 1.10). Abrous et al., (1998) reported that there is morphological variability of *F. hepatica* eggs in ruminants, rodents and lagomorphs and size of *F. hepatica* eggs was affected by the host species in rats and rabbits.

There are two methods described for the detection of *F. hepatica* eggs in faeces.

1.2.4.1.1.1. Flotation technique

The flotation method has poor sensitivity for the detection of *F. hepatica* eggs (Happich and Boray, 1969). In this method saturated Zinc sulphate solution is used, which has a higher specific gravity than *F. hepatica* eggs. Thus *F. hepatica* eggs float on the surface, but they frequently collapse due to the high concentration of Zinc sulphate (Sewell and Hammond, 1972).

1.2.4.1.1.2. Sedimentation technique

Sedimentation relies on the relatively high density of fluke eggs which means that they sink in water. Anderson et al., (1999) using a modification of a technique described by Young and Trajstman (1980), reported the sensitivity of this method was 66.7% and specificity 100%. Also Braun et al., (1995) reported the sensitivity of faecal egg counts as a method of detecting infected animals was 68%. Lumbreras et al., (1962) identified *F. hepatica* eggs in 98% of samples from infected animals using three rounds of 2 to 4 minute sedimentation in water. If the sedimentation period was too long, sedimenting faecal debris interfered with the test, although detergents can help to separate contaminating material from *Fasciola* eggs (Boray, 1969). Using this method, fluke eggs appeared yellowish brown in colour (Figures 1.9, 1.10) and could be distinguished easily from paramphistome eggs, which are colourless, but have the same size range (Figure 1.11).

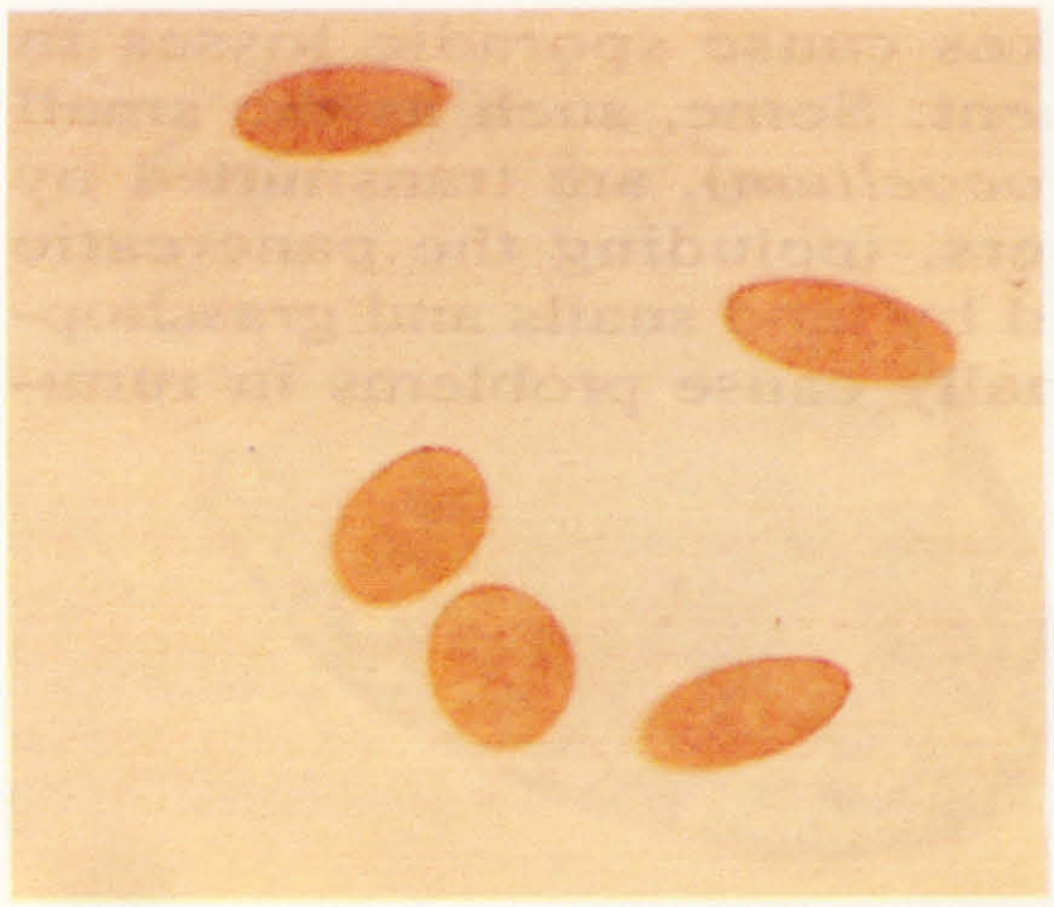

Figure 1.9. *Fasciola* eggs (FAO, 1994).

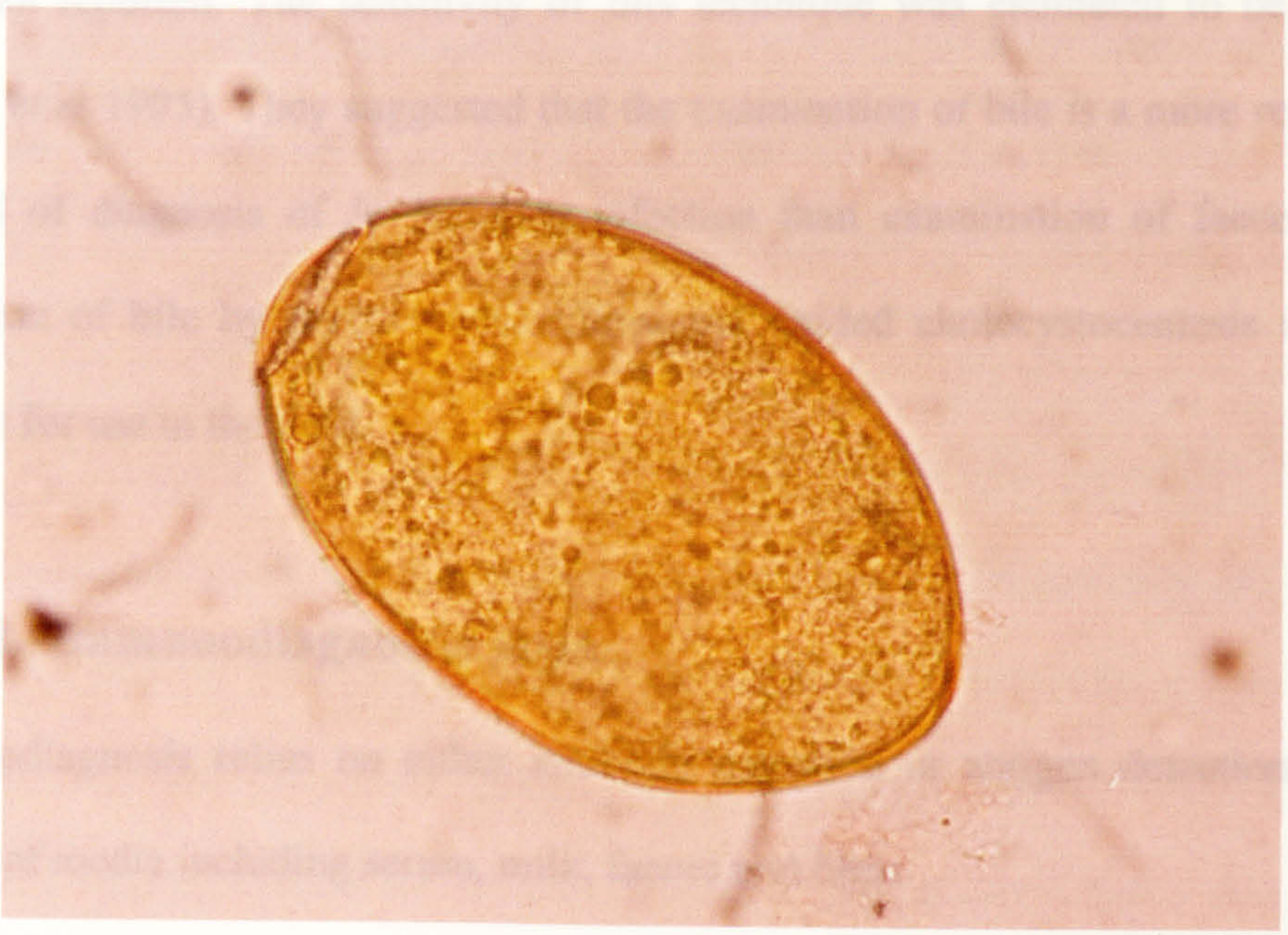

Figure 1.10. *Fasiola* egg

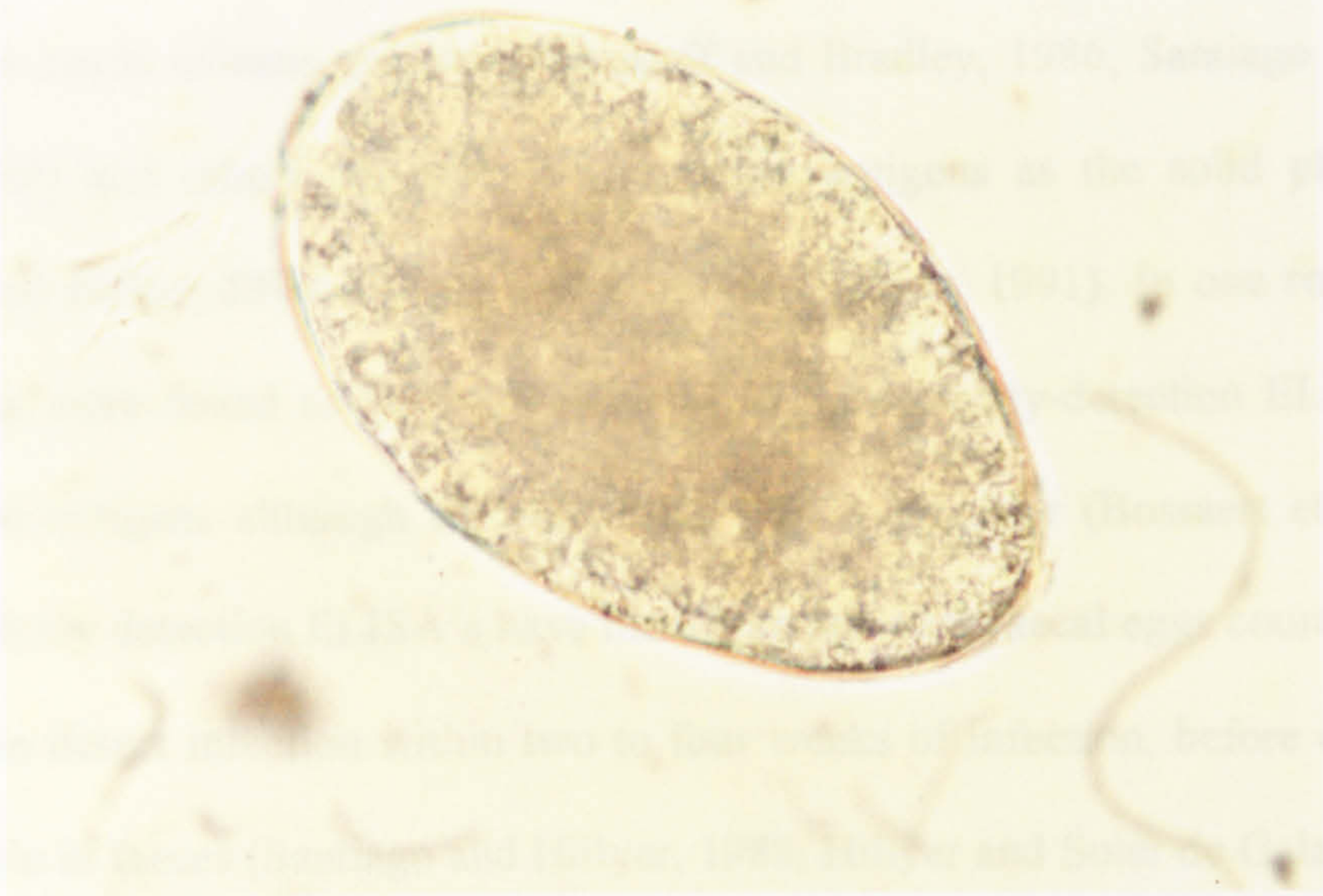

Figure 1.11. *Paramphistome* egg

1.2.4.1.1.3. Diagnosis of *F. hepatica* by identification of the eggs in bile.

Aspiration of bile which was then examined microscopically for *F. hepatica* eggs has been reported. The sensitivity of this technique was estimated to be 98% (Braun et al 1995). They suggested that the examination of bile is a more reliable method of diagnosis of *F. hepatica* infection than examination of faeces but collection of bile by percutaneous ultrasound guided cholecystocentesis is not feasible for use in the field.

1.2.4.2. Immunodiagnostic tests.

Immunodiagnosis relies on either antibody detection or antigen detection in a variety of media including serum, milk, faeces and bile.

1.2.4.2.1. Antibody – detection assays

A vide variety of serum antibody-detection ELISA's have been described. Some are based on crude somatic antigens (Wyckoff and Bradley, 1986; Santiago and Hillyer, 1988) and others use excretory/secretory antigens as the solid phase (Santiago and Hillyer,1988; Hillyer and Soler de Galanes, 1991). In one report E/S antigens were found to be more effective in an antibody-detection ELISA than somatic antigens although the reason for this is not clear (Bossaert et al., 2000). Antibody detection ELISA's have the advantage over faecal eggs counts in that they can detect infection within two to four weeks of infection, before eggs are detectable in faeces (Santiago and Hillyer, 1988; Hillyer and Soler de Galanes, 1991; Fagbemi and Guobadia, 1995; Bossaert et al., 2000). Antibody levels have been shown to reach a peak 8 – 10 weeks after infection (Santiago and Hillyer, 1988; Hillyer and Soler de Galanes, 1991; Fagbemi and Guobadia, 1995).

The diagnostic sensitivity and specificity have been evaluated for several antibody –detection tests. Anderson et al., (1999) using the method described by Wijffels et al., (1994) reported that the diagnostic sensitivity of an ELISA based on E/S antigens was 86.1% and the diagnostic specificity was 70%. Ibarra et al., (1998) carried out a comparison of three ELISAs. They reported that an indirect ELISA showed a sensitivity of 96.5% and specificity of 98.8%, a Diffusion in gel (DIG) ELISA 97.5% and 80.0% and a dot-ELISA, 93.1% and 95.4%, respectively. Maisonnave (1999) using a dot immunoperoxidase assay (DIPA) to detect specific antibodies against *F. hepatica* in cattle, reported that the DIPA had a diagnostic sensitivity of 82%, a specificity of 90%, good repeatability, and a significant association with the reference ELISA. Sanchez-Andrade et al. (2000) reported that

the diagnostic sensitivity and specificity values for an indirect ELISA and a sandwich ELISA were 92% and 92%, and 86% and 100% respectively in infected cattle.

In all the reports described so far, serum was used to detect antibodies against *F. hepatica.* However nowadays milk is widely used as a medium for diagnosis and surveillance of different diseases in dairy cattle and milk antibody testing plays a significant role in cattle disease control and eradication programmes in many countries (Pritchard et al., 2002). There are no reports of any ELISAs that have been evaluated for diagnosis of *F. hepatica* using milk. However many milk ELISA's developed to detect other infections have been reported. A milk ELISA test is used to detect bovine viral diarrhoea virus (BVDV) (Pritchard, 1998; Beaudeau et al., 2001; Pritchard et al., 2002), Foot-and-Mouth Disease (FMD) (Armstrong, 1997; Armstrong et al., 2000; Armstrong and Mathew, 2001), bovine herpesvirus-1 (BHV-1) (Hartman et al., 1997; Nylin et al., 2000; Stahl et al., 2002) and bovine respiratory syncytial virus (Elvander et al., 1995). A milk ELISA test is also used for surveillance of *Hypoderma* spp infection (Frangipane di Regalbono et al., 2003) and brucellosis (Vanzini et al., 1998, 2001).

As well as individual milk ELISAs, bulk tank milk ELISAs have also been developed to detect different diseases in cattle. Bulk tank tests are employed particularly for screening and monitoring of surveillance of different diseases in cattle. Bulk tank milk sampling is used in several countries for screening of dairy populations for BVDV (Niskanen et al., 1991; Niskanen, 1993; Radwan et al., 1995; Paton et al., 1998; Lindberg and Alenius, 1999; Melendez and Donovan,

2003). Bulk tank milk samples have also been used to screen for bovine herpesvirus-1 (Hartman et al., 1997; Nylin et al., 2000; Stahl et al., 2002), Foot-and-Mouth Disease (Armstrong and Mathew, 2001), bovine respiratory syncytial virus (Elvander et al., 1995) and brucellosis (Vanzini et al., 2001).

Detection of antibody as a means of diagnosing *F. hepatica* infection has limitations, because serum and possibly milk antibodies may be present if the animal has previously been infected (Leclipteux et al 1998). In other words, antibodies indicate past exposure to the parasite rather than the presence of an active infection (Dumenigo et al., 1996). Ortiz et al., (2000) reported that the total antibody response to ES antigens measured by ELISA, remained constantly high over a 2-year period in groups of cattle of different ages, which were naturally exposed to *F. hepatica* in an endemic area. Also ELISA methods that measure antibody levels are not always appropriate if animals are under chemotherapy (Leclipteux et al., 1998). Several studies have shown that the detection of parasite antigens is more reliable than the detection of antibody in the diagnosis of a current parasite infection (Kaliraj et al., 1981).

1.2.4.2.2. Antigen – detection assay

Antibody responses to different *F. hepatica* antigens are detected at different times during the course of infection (Bennett, 1975; Hanna, 1977; Rajasekariah and Howell, 1978; Hanna, 1980; Hughes et al., 1981; Bennett et al., 1982). As a result, several antigens have been identified for use in serological and faecal antigen detection tests (Dumenigo et al., 1996). Several antigens have been reported that are associated with migrating flukes (Ruiz-Navarette et al., 1993)

and the pattern of antigen recognition changes after the fluke reach the bile ducts (Tkalcevic et al., 1996).

Circulating antigens were detected in almost 90% of cattle sera at 6 and 8 weeks after infection, which generally coincided with increased host circulating antibody to *F. hepatica* E/S antigens (Langley and Hillyer, 1989). A monoclonal antibody has been produced which can detect *F. hepatica* antigen in serum (Espino et al., 1990) and faeces of infected humans and cattle (Espino and Finlay, 1994; Dumenigo et al., 1996). There was a direct correlation between antigen concentration in faeces and the number of adult flukes in cattle (Dumenigo et al., 1996). Similarly Espino and Finlay (1994) showed that there was a direct correlation between the amounts of antigen in human faeces and the faecal egg count, suggesting that antigen release might be correlated with worm burden. This ELISA was reported to detect antigen in 95 – 100% of infected cattle (Dumenigo et al., 1996). Abdel–Rahman et al. (1998) reported that, using a monoclonal antibody–based capture ELISA for detection of a 26 to 28 kDa coproantigen of *F. hepatica* in the faeces, infection could be detected at 6 weeks post infection. In contrast another monoclonal antibody used to detect E/S antigen in faeces was not able to detect infection during the liver migration phase (Leclipteux et al., 1998).

1.2.4.3. Other immunodiagnostic tests

Haemagglutination (HA) tests have been used to diagnose *F. hepatica* in cattle. The HA test using a fractionated antigen (Levieux et al., 1992b) detected specific antibodies from 2 – 4 weeks after infection up to 28 weeks after infection (Levieux et al., 1992a). These authors suggested that this test may be useful to

predict chemotherapeutic success in *Fasciola* infected cattle (Levieux et al., 1992b).

Bossaert et al., (2000) reported that Western blotting revealed a major antigenic fraction in *F. hepatica* E/S antigen of 26-30 kDa recognised specifically by sera from *F. hepatica* infected calves as early as 6-8 weeks post-primary infection. Similarly, using Western blotting, Ortiz (1997) reported that the antibodies were detected to 27 – 30 kDa proteins in the E/S and somatic antigens of adult fluke at 5 weeks post infection and recognition increased in intensity up to 11 weeks after infection, when the experiment was stopped. Ortiz (1997) also reported that the 27 – 28 kDa immunodominant antigen in fluke E/S antigens, the Cathepsins L1 and L2 were detectable in naturally and experimentally infected cattle at three weeks after infection.

Cathepsin L proteases isolated from adult *F. hepatica* flukes have been used to develop serological diagnostic tests. The *F. hepatica* Cathepsin L1 is secreted by all stages of the developing parasite; it is capable of cleaving host immunoglobulins and can prevent in vitro attachment of eosinophils to newly excysted juveniles (Carmona et al., 1993; Smith et al., 1993a, b). Furthermore, Cathepsin L1 can degrade extracellular matrix and basal membrane components thus assisting the migration of the juvenile parasites through the liver (Berasain et al., 1997). This protease has been shown to be highly immunogenic in infected animals, and is a potential vaccine candidate (Wijffels et al., 1994; Dalton et al., 1996; Spithill and Dalton, 1998). Cathepsin L1 purified from the E/S products of *F. hepatica* as single antigen and used in an ELISA provided a more accurate

diagnosis compared with whole E/S extract, discriminating more clearly between sero-positive and sero-negative populations (O'Neill et al., 1998). However, production of sufficient quantities of pure Cathepsin L1 is a complex, time-consuming and expensive process. To solve this problem, recombinant Cathepsin L1, produced in yeast (Roche et al., 1997) was used in an IgG4-ELISA as antigen and showed a similar performance to the native antigen (O'Neill et al., 1999). This test has been employed to detect infected humans using purified Cathepsin L1 or the recombinant protein, as antigen (O'Neill et al., 1998, 1999).

Cathepsin L proteases, as well as peptides defining selected epitopes of Cathepsin L1, have been used in order to develop reliable tests for the diagnosis of *F. hepatica* infections in sheep and cattle (Wijffels et al., 1994; Cornelissen et al., 1999, 2001). It seems that the recombinant protein procathepsin L1 from *F. hepatica* can be employed as a useful antigen to diagnose infection in cattle (Carnevale et al., 2001).

1.2.5. Epidemiology and Control of Fasciolosis

Epidemiology is the study of the frequency and distribution of disease in populations and the factors that affect this distribution for the purpose of disease prevention. The study of epidemiology of fasciolosis in ruminants includes factors that affect prevalence and intensity of infection. These factors are susceptibility of the host species, the presence of the intermediate host (snail) population, the factors which affect the snail population and the role of reservoir hosts. Investigation of each of these factors is necessary to improve the control the

disease. The following section reviews the epidemiology of fasciolosis and explores the different strategies that are currently applied to the control and eradicate of the disease to prevent consequent economic losses and improve health and welfare of domestic livestock.

1.2.5.1. Host species.

1.2.5.1.1. Definitive hosts. Cattle and sheep are the most important species of livestock animals that are affected by *Fasciola* spp. As *Fasciola* spp., particularly *F. hepatica* has a word-wide distribution, it can affect all populations of livestock animals around the world. Also buffalo, goats, horses, donkeys, deer and other species of herbivore can be infected by this parasite. Human fasciolosis is also reported from different areas of the world (Esteban et al., 1997; O'Neil et al., 1998; Mas-Coma et al., 1999; Rokni et al., 2002).

There are several drugs available that are effective at terminating fluke infections in cattle and sheep including closantel, clorsulon, rafoxanide, nitroxynil and triclabendazole (Fairweather and Boray, 1999). Some, such as nitroxynil and closantel, are only effective against late juvenile and mature fluke stages, whilst others such as albendazole, only kill the adult fluke (McKellar and Kinabo, 1991). However, one drug is effective against very early juvenile through to the adult stages of the parasite, triclabendazole (Boray et al., 1983; Stansfield et al., 1987; Fuhui et al., 1989; Richards et al., 1990; Thomas and Coles, 2000). Triclabendazole is the preferred drug where there is significant risk of acute disease, because it can kill the early migrating juvenile flukes, thus preventing

liver damage. It is also useful in the treatment of herd and flocks where individual animals may harbour differing ages of the parasite.

Drug treatment only removes the current infection and does not prevent re-infection, except by possibly reducing transmission levels. Where infective metacercariae are present on pasture for several months, frequent dosing throughout the grazing season may be required. Chemotherapy is therefore not the ideal means of control, being expensive, manpower intensive and requiring constant access to supplies of the drug. Furthermore, the development of *F. hepatica* resistance to triclabendazole has recently been reported in Great Britain (Anon, 1995; Mitchell et al., 1998; Thomas and Coles, 2000), Ireland (Anon, 1995), Australia (Overend and Bowen, 1995; Fairweather and Boray, 1999), and the Netherlands (Moll et al., 2000; Gaasenbeek et al., 2001). Although triclabendazole resistance is not yet a widespread phenomenon, it is a cause for concern and is further evidence for the need to develop alternative methods of fasciolosis control. Strategic control programmes incorporating grazing management and minimising anthelmintic treatment will slow the development of anthelmintic resistance (Barnes et al., 1995). Nevertheless, fasciolicides are likely to remain as the main form of fluke control for the foreseeable future (Fairweather and Boray, 1999).

1.2.5.1.2. Intermediate host. *Lymnaeid* snails are the intermediate host of *F. hepatica* and liver fluke transmission is absent in areas where conditions for development of snails are not suitable. Several species of the genus *Lymnaea* can act as intermediate hosts. In the UK the principal species is *Lymnaea trunculata*

(Boray, 1969; Amato et al., 1986). One of the clearest targets for control of fasciolosis is elimination of the snail intermediate host. Chemical treatment of land with molluscides has been tried over the years, but there are many problems associated with this method. Molluscides must be applied on a regular basis for any consistent benefit to be achieved, at least once annually, as snails can repopulate the cleared area rapidly. The chemicals used are toxic and so there are concerns over pollution and hazards to wildlife and humans. Another method of control is drainage since *Lymnaea* snails are amphibious and rely on water for their life cycle (Roberts and Suhardono, 1996). Thus, snail species involved in transmission live in wet, mild habitats and are not found in well drained, dry areas. However, drainage may not be beneficial for the local environment, and in some areas farm irrigation schemes may favour survival of the snails. On a smaller scale, antagonistic trematode schemes have been tried. The snail can play host not only to *F. hepatica*, but also to other trematodes and when infected with one species, it cannot host another (Lie et al., 1966). However, such biological methods of control have not been successful when tried on a larger scale (Boray, 1969). Kaplan et al. (1995 and 1997) reported that, using a DNA probe for detection of *F. hepatica* infected snails, prevalence of fasciolosis in the snail population could be measured and this provided a useful measure of the success of control programmes. However this method is expensive and has limitations. Populations of wild waterbirds, particularly ducks, may also control the snail and consequently, *Fasciola* populations (Gordon and Boray, 1970). If the snail population cannot be removed, reducing exposure of stock to snails should be attempted by restricting grazing to safe areas free of snails. Fencing off wet areas

and ditches is simple and cost-effective, but if grazing land is scarce and much of it is suitable habitat for the snail, then this is not possible.

1.2.5.2. The climate and environment

To complete the life cycle of *Fasciola* spp., suitable conditions of moisture and temperature are required for the development of the larval stages and intermediate host itself. Moisture is necessary for the transmission, proliferation and survival of both flukes and snails and the areas where there is seasonal rain fall, transmission will be limited to the wet season, unless land is irrigated or there are permanent water sources (Torgerson and Claxton, 1999). The minimum and maximum critical temperatures for development of *F. hepatica* eggs are about 9.5 °C and 30 °C respectively (Torgerson and Claxton, 1999). A minimum temperature of 10 °C is necessary for development of *Lymnaea trunculata* and maximum growth occurs at 18-27 °C (Kendall, 1953). The minimum and maximum critical temperatures for development of parasite in the snail are 10 °C and 20 °C respectively (Kendall, 1970) and the optimum temperature for the survival of 100% of metacercariae is 12-14 °C (Torgerson and Claxton, 1999). In areas which have a mean day and night temperature above 10 °C for approximately 6 months of the year, fasciolosis is endemic and a cycle of summer/winter snail infection has been reported (Torgerson and Claxton, 1999). In contrast, in areas where there are only 2 months of the year with temperatures greater than 10 °C, parasites will have to overwinter in the snail to complete their development.

Various predictive models based on climatic conditions, specifically rainfall have been developed to aid in the control of fasciolosis and sometimes together with temperature provides a measure of the risk of fasciolosis for the following autumn and winter (Ollerenshaw and Rowlands, 1959; reviewed by Torgerson and Claxton, 1999). More advanced models in addition to climate conditions contain data on liver condemnation rates from abattoirs, which have made them more accurate (Goodall et al., 1993). However, the ideal models, using geographical information system (GIS), can include data on soil type, hydrology, vegetation and climate (Malone et al., 1998). These models are of significant value because by forecasting the risk of fasciolosis, farmers could be assisted in making decisions regarding dosing and grazing strategies. Previously, only western countries could benefit from these models where centralised veterinary support and finances allowed their implementation. But, GIS models have been developed to predict the risk of fasciolosis and been applied in the design of chemotherapy strategies in Ethiopia (Malone et al., 1998). Such models could be used for the control of fasciolosis in other developing countries.

In summary, applying current methods of fasciolosis control have been proved to be expensive, time consuming and require considerable management. On the other hand immunological control in the form of a vaccine maybe a more efficient, simple and cost effective means of reducing disease.

1.2.5.3. Vaccine development strategies

The future of liver fluke control will almost certainly depend on the development of a protective vaccine. In order to design an effective vaccine, the type and nature

of the immune response in the host to infection must be considered. Knowledge of means by which the parasite evades the host's immune effector mechanisms, will help in the development of vaccines.

From the host's point of view, killing a small invading fluke would be easier than killing a much larger adult. Therefore, if juveniles migrating through the gut and peritoneal cavity were targeted, elimination without any damage to the host could be achieved. In other words it would seem more logical if flukes were targeted before they have caused significant structural damage to the liver. Thus a vaccine which only works by stimulating responses against the adult fluke stages would still result in the host being subjected to substantial pathology.

The liver fluke is composed of many thousands of proteins, some of them have structural roles, and others are involved with metabolism. Damage or death to the fluke can be caused by targeting an immune response against proteins, which are essential to the survival of the fluke, either for maintaining structural integrity or for general metabolic processes. Potential vaccine components among these proteins have been identified partly as a result of recent progress in molecular biology (Spithill et al., 1997). Research on a number of molecules including Cathepsin Ls, glutathione S-transferase (GST), leucine aminopeptidase (LAP) and fatty acid binding proteins (FABP), has demonstrated the feasibility of inducing protective responses (Morrison et al., 1996; Spithill and Dalton, 1998; Spithill et al., 1999). *F. hepatica* Cathepsin L proteases were the first of this class of enzyme to be described in a helminth (Smith et al., 1993a). Cathepsin L proteases of *F. hepatica* have given consistent positive results when used as vaccines against

fasciolosis in cattle and sheep and therefore have excellent prospects for the development of first generation anti-fluke vaccines (Dalton et al., 2003a). It has been suggested that vaccination with fluke Cathepsin L induces a Th1 response which unlike Th2 responses appear to be protective in cattle and sheep, and can produce levels of protection of up to 72% and 79% respectively (Mulcahy and Dalton, 2001). It has been shown that Cathepsin L alone or in combination with fluke haemoglobin could induce 42 – 69% and 52 – 72% protection respectively against *F. hepatica* infection in vaccinated cattle (Dalton et al., 1996). Also Cathepsin L as well as inducing protection, had an anti-fecundity effect by inhibiting the embryonation of eggs (Dalton et al., 1996; Mulcahy et al., 1998). Sm14, a *Schistosoma mansoni* derived antigen, the first member of the FABP family to be identified in parasites, has been shown to induce complete protection against a *F. hepatica* challenge infection in vaccinated mice (Almeida et al., 2003). In addition vaccination with fluke FABP induced 55% (Hillyer et al., 1987) and 69 – 78% (Hillyer, 1985) protection against *F. hepatica* challenge infection in vaccinated cattle and mice respectively. Using leucine aminopeptidase as a vaccine in sheep, induced the production of neutralising antibodies and evoked 89% protection against fasciolosis (Piacenza et al., 1999).

Although effective protective antigens have been isolated and characterised, none have yet reached the manufacturing and commercialisation stage (Dalton and Mulcahy, 2001). To develop a commercial vaccine several items should be considered such as fermentation process development, purification process development, formulation and stability development and finally manufacturing (Dalton et al., 2003b). In manufacturing, quality assurance and quality control

should be applied before marketing (Dalton et al., 2003b). However the future of control of fasciolosis will probably depends on the development of a protective vaccine which should give a benefit that would be visible to farmers at a competitive price (Dalton et al., 2003a; Cervi et al., 2004).

1.2.3. Objectives

Bovine fasciolosis has been reported to be increasing in prevalence in Great Britain in recent years (VLA report, 2002c), but the prevalence of disease in cattle, particularly in dairy cattle, is not known. Infections in many herds probably go undiagnosed or misdiagnosed but may lead to considerable economic loss. Therefore a test that can detect the disease easily and readily without being expensive in terms of time and money would assist in both the diagnosis and the surveillance of infection. Surveillance is vital to assess the significance and spread of fasciolosis in UK.

To achieve this goal, this study was designed to:

Develop and evaluate an in-house diagnostic ELISA test for *Fasciola hepatica* infection in cattle.

Adapt this diagnostic ELISA to detect antibodies against *F. hepatica* in milk and bulk tank milk samples.

Characterise the nature of the antibody responses to *F. hepatica* in serum and milk.

Determine if cattle naturally exposed to *F. hepatica* developed immunity to infection.

Determine if the in-house ELISA can be used to assess the intensity of infection in naturally infected cattle.

Assess prevalence of infection in dairy herds in the UK using bulk tank milk samples.

Chapter Two

Materials

and

Methods

2.1. Samples

2.1.1. Cattle

For developing an in-house ELISA test to detect the antibody response to *F. hepatica*, a total of 258 sera from naturally infected cattle, and 256 sera from non-infected cattle were used. Serum and faeces were collected from 258 cattle, kept on farms in the Cajamarca region of Northern Peru. Fasciolosis is endemic in Cajamarca, where it is estimated that 95% of cattle are infected with *F. hepatica* (Claxton et al., 1997). Cattle were classed as positive if *Fasciola* eggs were detected in their faeces by the sedimentation technique (Claxton, 1996). Serum from 256 cattle from two dairy farms located in Cheshire, UK, were used as the negative population. There were assumed to be fluke free because all the cattle were bred on the farms and were zero-grazed. No fasciolosis had ever been diagnosed in cattle on either farm. Six calves were infected with approximately 1000 viable *F. hepatica* metacercariae (Akca, 1999). Sera that were collected at weekly intervals were tested to determine the time point after infection calves were first shown to be positive in the in-house ELISA. To compare and evaluate the in-house ELISA test with the commercially available Bio-X test, a subset of 39 positive sera and 47 negative sera were randomly selected from the samples used.

For analysis of the antibody response in milk to *F. hepatica* by in-house ELISA, a total of 1565 individual sera and 765 individual milk from 61 different farms were used. In addition 61 bulk milk tank samples one from each farm were collected at the same time. Also from 27 of these farms, 715 faeces samples were collected

and were used. Twenty – 34 cattle were sampled on each farm. Details of age, parity and days into lactation were recorded.

To estimate the national prevalence of *F. hepatica* infection, a total of 2182 bulk tank milk samples from 13 Veterinary Investigation Centres in England and Wales, were collected between September 2001 and April 2003 and were tested using the LSTM ELISA. These samples were collected in collaboration with Sutton Bonington Veterinary Investigation Centre, and formed part of a national surveillance scheme for Bovine Viral Diarrhoea disease in dairy herds.

For analysis of intensity of infection by ELISA, a total of 294 individual serum samples from cattle with different levels of infection (Low, Medium, High, assessed from fluke burden and intensity of liver pathology) were collected among nine hundred and sixty nine inspected cattle from Shrewsbury Abattoir (Shropshire, England, UK) and were tested by LSTM ELISA.

The negative control serum used in the ELISA was from a cow, which had been maintained on a fluke – free farm (The Faculty of Veterinary Science Animal Husbandry Farm, Leahurst) and was negative by faecal egg count. The positive control was a pool of serum taken between 2 and 9 weeks after infection from a cow that had been infected orally with 1000 *F. hepatica* metacercariae (Akca, 1999).

2.1.2. Collection of samples

2.1.2.1. Blood samples

Blood samples were collected by jugular or tail venepuncture in vacutainer tubes with no added anticoagulant and were left at room temperature for 4 hours to clot. Then they were refrigerated for at least 15 minutes. In order to separate serum from the clot, they were centrifuged at 550g for 10 minutes. The serum samples were aliquoted and stored at –20°C until used.

2.1.2.2. Faecal samples

Faecal samples were collected from the rectum into 50 ml polyethylene screw topped containers or disposable plastic hand gloves.

2.1.2.3. Milk samples

Milk samples were collected directly from the teat or at milking time from the collecting jar, into universal tubes without preservative. In order to separate milk from fat or cream, each sample was centrifuged at 1000g for 20 minutes. The milk samples then were aliquoted and stored at –20°C until use.

2.1.2.4. Bulk tank milk samples

Bulk milk tank samples were collected from the main milking container on the same day of blood and milk sampling. They were kept in universal tubes with preservative Bronopol (2-bromo-2-nitropropane-1, 3-diol) (150 mmol / 25ml) and were centrifuged at 1000g for 20 minutes in order to separate milk from the fat. The bulk tank milk samples then were aliquoted and stored at –20°C until use.

2.2. Preparation of *F. hepatica* E/S antigen

Live adults flukes were collected from the bile ducts of either condemned bovine livers at Shrewsbury Abattoir or experimentally infected rats and were kept in sterile PBS. In the lab each fluke was washed 6 times with sterile PBS and then 6 times with RPMI 1640 tissue culture medium (Gibco Ltd, Paisley, Scotland) containing 100 U/ml Penicillin and 100 mg/ml Streptomycin, to remove all traces of blood, host tissue and bile. Flukes were then incubated in 1ml RPMI medium overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. The following day, the supernatant was collected and centrifuged at 10,000g for 30 minutes at 4°C to remove particulate material. The protein concentration was estimated according to Warburg and Christian (1941) and the samples aliquoted and stored at -20°C.

2.3. ELISA procedure

2.3.1. LSTM (in-house) ELISA

2.3.1.1. Serum ELISA

Immulon-2 ELISA plates (Dynatech Laboratories, Virginia,USA) were coated with 100 µl/well of 0.5 µg/ml of E/S antigens in 0.1 M carbonate buffer (pH 9.6) (Appendix 1). Following overnight incubation at 4°C, the plates were washed 6 times (4 short washes and 2 five min washes) with PBS (pH 7.2) containing 0.05% Tween–20 (PBS/Tween) and blocked with 200 µl per well of a solution containing 2% skimmed–milk powder (Marvel, Premier Beverages, Stafford, UK) in PBS/Tween (SMP/PBS/T) for 1 hour at 37°C. After blocking and washing, 100

µl of sera diluted 1:800 in SMP/PBS/T were added to the well and incubated for 1hour at 37°C. The plates were washed as before and incubated with 100 µl per well of a 1:6000 monoclonal anti–bovine IgG conjugated to horse radish peroxidase (MAST Diagnostics, Bootle, Merseyside) for 1 hour at 37°C. After washing, 100 µl of freshly prepared TMB substrate (hydrogen peroxide solution in acetate buffer pH 5.0 and tetramethylbenzidine in a methanol based solution, MAST Diagnostics, Merseyside, UK) were added to the well. The colour change was recorded after 20 minutes incubation in the dark, at a wavelength of 450 nm on an automatic ELISA reader (Dynex Technologies, Revelation 3.2). The results are given as the mean of the optical density (OD) obtained from duplicate samples expressed as a percentage of the positive control (C+), using the following formula:

$$\text{Percent Positive (PP)} = \frac{\text{Mean OD of test sample}}{\text{Mean OD of C+}} \times 100$$

In each ELISA test, negative and positive controls were included and tested in quadruplicate.

2.3.1.2. Milk ELISA

The same procedure was used for milk, except that milk samples, were tested at a 1:2 dilution in SMP/PBS/T.

2.3.1.3. Bulk tank milk ELISA

The same procedure was used for bulk milk samples except that each sample was tested undiluted.

2.3.2. Bio-X ELISA

2.3.2.1. Serum

Bio-X kit is an indirect ELISA for sera which is developed by Bio–X, S.P.R.L. Rue de Luxembourg, 34 B6900 Marche-en-Famenne, Belgium. The test was used according to the manufacturer's instructions given below:

The test uses 96-well microtitration plates sensitized with specific F. hepatica antigens. The odd columns on each plate (1, 3, 5, 7, 9, 11) contain these specific antigens, whereas even columns (2, 4, 6, 8,10, 12) are used to control the specificity of the test. This is a genuine negative control to differentiate specific anti-F. hepatica antibodies from non-specific ones.

The test blood sera were diluted 1:100 in the dilution buffer. The plate was incubated for one hour and washed, then 100µl of the conjugate, a peroxidase-labelled anti-bovine IgG1 monoclonal antibody added to the wells. The plate was then incubated for one hour at room temperature, washed again and 100 µl/well of the enzyme's substrate (hydrogen peroxide) and the chromogen added. If specific anti-F. hepatica immunoglobulins are present in the test sera the conjugate remains bound to the microwell that contains the antigen and the enzyme catalyses the transformation of the colorless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the amount of specific antibody in the sample. The signal read off the negative control microwell is subtracted from that of the positive microwell sensitised by the antigen. Comparison of the signals obtained for the unknown samples with those

obtained for a reference serum supplied with the kit make it possible to rank the serum's positivity on a five-point scale.

Value	*Positivity*
*0.2<val**	*0*
0.2< val <0.4	+
0.4< val <0.6	+ +
0.6< val <0.8	+ + +
0.8<val <1.00	+ + + +
1.00< val	+ + + +

**Val = Value*

2.3.2.2. Milk

The same procedure was used for milk, except that milk samples were tested at a 1:2 dilution in dilution buffer.

2.3.2.3. Bulk tank milk samples

The same procedure was used for bulk milk samples, except that bulk milk samples were tested at a 1:2 dilution in dilution buffer.

2.4. Sedimentation technique for faecal egg count

One gram of faeces was homogenised in water and passed through a 250 μm pore sieve. The retained material was washed thoroughly, the filtrate was transferred to a beaker that was allowed to stand for 10 minutes and the supernatant discarded. The remaining 12 – 15 mls was transferred to a 10-cm petri dish whose base had been scored into 1-cm squares with a marker pen. After a further 10 minutes

sedimentation, the supernatant was again drawn off and a few drops of iodine added. The petri dish was examined using a low power stereomicroscope microscope and the eggs counted. The number of eggs counted was used to calculate the number of eggs per gram of faeces.

2.5. Sodium Dodecyle Sulphate-Polyacrylamide Gel Electrophoresis (SDS- PAGE) and Western Blotting

2.5.1. SDS PAGE

SDS PAGE electrophoresis was performed according to Laemmli (1970), using 10% non-gradient polyacrylamide gels under non-reducing conditions. Mini-protean® 3 cell apparatus (Bio-Rad laboratories Ltd., UK) was cleaned with methanol. The apparatus was assembling according to the manufacture's instructions and a 10% resolving gel (Appendix 2) was poured between the glass plates to 2 cm below the top. Ultra pure water was immediately added to the top to level the surface. The gels were allowed to polymerise at room temperature for one hour. The ultra pure water was poured off and a 4% stacking gel (Appendix 2) was poured on the top of the polymerised separating gel. A separating comb with ten teeth was placed into the stacking gel and then the gel was allowed to polymerise for one hour as before. After one hour, the gels were placed in the tank and the comb was removed slowly.

The samples were mixed equal parts with 2X sample buffer and boiled for 90 seconds and were loaded into the wells; also 6µl of Biotinylated SDS-PAGE marker (Bio-Rad) was applied to each gel. Samples were overlaid with running

buffer so that gels were fully submerged. The lid was put on and connected to a power supply and the gels were run at constant current at 50mA (25mA per gel) for one hour until the dye (bromophenol blue) reached the end. The gels were gently removed and either stained with Coomassie blue or used for Western Blotting.

For staining, gels were put in a container and Coomassie Brilliant Blue R-250 (Appendix 2) was poured on and then the container was put on the top of a rocking table for one hour and then destained in destaining solution (Appendix 2) until the background was clear. The relative molecular weights of the bands were estimated by reference to a range of low molecular weight markers.

2.5.2. Western Blotting

Gels were removed and a corner cut corresponding to position of the markers, and then placed carefully in transfer buffer for at least 15 minutes to equilibrate. Meanwhile a piece (8.5 x 5.5 cm.) of Immobilon™-P (transfer membrane = Membranes, Immobilon-P Polyvinylidene difluoride, Sigma, Poole, Dorset, England, UK) was cut and was prepared by wetting it directly in 100% methanol for 15 seconds, transferring to ultra pure water for 2 minutes, finally placing it in transfer buffer for 5 minutes. The Mini trans-blot electrophoretic transfer cell (Bio-Rad) was prepared according to the instruction manual and the proteins transferred from the SDS-PAGE onto the membrane in transfer buffer (25mM Tris, 192mM Glycine, 20% v/v Methanol, pH 8.3). The excess binding sites on the membrane were blocked with 4% skimmed milk powder in PBS for 30

minutes on the bench then overnight at 4°C. The following day, the membrane was washed three times twice for 10 minutes and once for 30 minutes in PBS / 0.05%Tween and then it was cut in strips 5mm wide. Each strip was incubated with serum diluted 1:50 and 1:100 or milk diluted in 1:2 and 1:5 in incubation buffer (4% skimmed milk powder / PBS / 0.05%Tween) for one hour. After a further three washes as before, the strips were incubated with a monoclonal anti bovine IgG conjugated to horseradish peroxidase diluted 1:500 for one hour. After further washes the membrane strips were incubated in DAB (0.7 $mgml^{-1}$ 3,3-diaminobenzidine plus 0.17mg/ml Urea hydrogen peroxide dissolved in 15 ml ultra pure water and filtered through a 0.2 μm filter, Sigma) for 10 to 15 minutes, then washed in distilled water and air-dried on filter paper.

2.6. Statistical analysis

All the serum and milk samples in the ELISA assay were analysed in duplicate. All data were analysed by using Minitab for Windows, version 13.32 (Minitab Inc.) and SPSS for Windows, version 11 (SPSS Corporation Software). Descriptive statistics were applied to describe and characterize the data distribution and summarize the findings. Charts and graphs were drawn to show the sum of observations divided by the number of observations in the set of data. To measure the degree of linear association between two variables, to show the relationship between a dependent and independent variable and to compare proportional distribution of the data, Pearson's correlation coefficients, regression plots and chi-squared analyses were applied respectively. The mean differences were significant at the 0.05 level. The agreement between the two tests was

assessed by calculating the *Kappa* statistic according to Thrusfield (1995). Upper control limits (UCL) and lower control limits (LCL) were established using the 95th and 5th percentiles of the replicate OD values for each of the controls. The percentiles values were also obtained by using Minitab for Windows, version 13.32. The diagnostic sensitivity and specificity and 95% confidence intervals were calculated using WinEpiscope 2.0 (N. de Blas, C. Ortega, K. Frankena, J. Noordhuzen, M. Thrusfield: http://www.clive.ed.ac.uk/winepiscope/).

Chapter Three

Development and evaluation of an antibody-detection ELISA for *Fasciola hepatica* compared to a commercially available test

3.1. Introduction:

Fasciolosis is a parasitic disease that occurs world–wide and is caused by the digenetic trematodes, *Fasciola hepatica* and *F. gigantica.* The disease causes significant economic losses, combined estimates of the world-wide cost of ruminant infections with *F. hepatica, F. gigantica* (the tropical liver fluke) and stomach flukes (*Paramphistomum* and other genera) is approximately US $ 3 billion per year (FAO, 1994). Chronic fasciolosis can reduce growth rates, feed conversion (Oakley et al. 1979) and wool production (Hawkins and Morris, 1978). Diagnosis is normally based on observation of eggs of *Fasciola* in faeces (Boray, 1985). Diagnosis by faecal examination is not possible during the 8-10 week prepatent period of the infection and this method of diagnosis has a poor sensitivity during the patent period due to the relatively low number of eggs shed (Happich and Boray, 1969). To improve diagnosis during both early and chronic phases of infection several ELISA techniques have been described. Some of these tests rely on antibody detection using crude somatic extracts or excretory/secretory (E/S) products of *F. hepatica* (Hillyer and Santiago de Weil 1979; Zimmerman et al., 1982, 1985). A particular advantage of these tests is that they can detect prepatent infections (Wyckoff and Bradley 1986). The specificity of antibody responses to *F. hepatica* varies during the course of infection (Bennett, 1975; Hanna, 1977; Rajasekariah and Howell, 1978; Hanna, 1980; Hughes et al., 1981; Bennett et al., 1982), and as a result, several antigens have been identified for use in serological immunodiagnosis (Espino et al., 1991; Dumenigo et al., 1996; Hillyer,1993; Tuntasuvan, 1993). However few of these tests have been fully validated using sera from cattle of known *F. hepatica*

infection status and none is used routinely for the diagnosis of infection in individual cattle. Here we evaluate an ELISA based on E/S antigens of *F. hepatica* and compare it to the commercially available Bio-X ELISA kit.

3.2. Materials and Methods

3.2.1. Cattle:

For analysis of the antibody response to *F. hepatica* by ELISA, a total of 258 sera from naturally infected cattle, and 256 sera from non-infected cattle were used. In addition sera from six calves experimentally infected with *F. hepatica*, were included in the study. Serum and faeces were collected from 258 cattle (Holsteins and Brown-Swiss), kept on farms in the Cajamarca region of Northern Peru. Fasciolosis is endemic in Cajamarca, where it is estimated that 95% of cattle are infected with *F. hepatica* (Claxton et al., 1997). Cattle were classed as infected if *Fasciola* eggs were detected in their faeces by the sedimentation technique (Claxton, 1996). Two hundred and fifty six Holstein cattle from two dairy farms located in Cheshire, UK, were used as the negative population. All the cattle were bred on the farms and were zero-grazed as adult cows. Fasciolosis had not been diagnosed in cattle on either farm. Eight, three to four-month-old Belgium Blue X Jersey calves were purchased from a farm known to be free of fasciolosis. Faecal samples were examined and serum samples were tested by ELISA to establish that the calves were not infected with *F. hepatica*. Six calves were infected with approximately 1000 viable *F. hepatica* metacercariae (Akca, 1999). To compare the tests, a subset of 39 positive sera and 47 negative sera were randomly selected from the samples used to evaluate the in-house test.

The negative control serum was from a cow that had been kept indoors throughout its life on the husbandry farm, Leahurst, University of Liverpool. This farm is known to be free of *F. hepatica* and *Lymnaea trunculata* snails. The positive control was a pool of serum taken between 2 and 9 weeks after infection from a single cow that had been infected orally with 1000 *F. hepatica* metacercariae on three occasions (0, 14, and 21 weeks post infection) (Akca, 1999).

Three sera from calves experimentally infected with *Dictyocaulus viviparus* were the kind gift from Dr J Matthews, Department of Veterinary Clinical Science, Faculty of Veterinary Science, University of Liverpool. Sera from one calf with a mono-infection of *Nematodirus helvetianus* and two calves infected with *Ostertagia ostertagi* were the kind gift of Prof J Verycrusse, University of Ghent, Belgium.

3.2.2: Production of Excretory and Secretory (ES) Antigens of *F. hepatica*:

Wistar rats were infected with 100 metacercariae and killed 10 weeks after infection. Live adult flukes were collected from the bile ducts and were washed 6 times with sterile PBS and then 6 times with RPMI tissue culture medium (Gibco Ltd, Paisley, Scotland) containing 100 U/ml Penicillin and 100 mg/ml Streptomycin, to remove all traces of blood, host tissue and bile. Flukes were then incubated in 1ml RPMI medium overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. The following day, the supernatant was collected and centrifuged at 10,000g for 30 minutes at 4°C to remove particulate material. The protein

concentration was estimated according to Warburg and Christian (1941) and the samples aliquoted and stored at -20°C.

3.2.3. LSTM ELISA procedure:

Immulon-2 ELISA plates (Dynatech Laboratories, Virginia,USA) were coated with 100µl/well of 0.5µg/ml of E/S antigens in 0.1 M carbonate buffer (pH 9.6). Following overnight incubation, the plates were washed 6 times (4 short washes and 2 five min washes) with PBS (pH 7.2) containing 0.05% Tween–20 (PBS/Tween) and blocked with 200µl per well of a solution containing 2% skimmed–milk powder (SMP) (Marvel, Premier Beverages, Stafford, UK) in PBS / 0.05%Tween (SMP/PBS/T) for 1 h at 37°C. After blocking and washing, 100µl of sera diluted 1:800 in SMP/PBS/T were added to the well and incubated for 1 h at 37°C. The plates were washed as before and incubated with 100µl per well of a 1:6000 monoclonal anti–bovine IgG conjugated to horse radish peroxidase (MAST Diagnostics, Bootle, Merseyside) for 1 h at 37°C. After washing, 100µl of freshly prepared TMB substrate (hydrogen peroxide solution in acetate buffer pH5.0 and tetramethylbenzidine in a methanol based solution, MAST Diagnostics, Merseyside, UK) were added to the well. The colour change was recorded after 20 min incubation in the dark, at a wavelength of 450 nm on an automatic ELISA reader (Dynex Technologies, Revelation 3.2). The results are given as the mean of the optical density (OD) obtained from duplicate samples expressed as a percentage of the positive control (C+), using the following formula:

$$\text{Percent Positive (PP)} = \frac{\text{Mean OD of test sample}}{\text{Mean OD of C+}} \times 100$$

In each ELISA test, negative and positive controls were included and tested in quadruplicate.

3.2.4. Bio X Kit:

The Bio-X bovine *Fasciola hepatica* ELISA Kit (Bio-X Company S.P.R.L. Rue de Luxembourg, 34 B6900 Marche-en-Famenne, Belgium) was used according to the manufacturer's instructions on a subset of the samples used to validate the LSTM ELISA

3.2.5. Statistical analysis:

The diagnostic sensitivity was calculated as the proportion of the number of ELISA positive results to the number of known positive sera and the diagnostic specificity was calculated [using WinEpiscope 2.0 (N. de Blas, C. Ortega, K. Frankena, J. Noordhuzen, M. Thrusfield: http://www.clive.ed.ac.uk/winepiscope/)] as the ratio of the number of ELISA negative results to the number of known negative sera. Six control plates were used on different days to establish upper control limits (UCL) and lower control limits (LCL) by using the 95^{th} and 5^{th} percentiles of the replicate OD values for each of the controls used (Table 3.1). The percentile values were obtained by using Minitab for Windows, version 13. The correlation coefficient between the ELISA test and BioX ELISA kit was calculated by using Minitab for Windows, version 13 and the agreement between the two test was assessed by calculating the *Kappa* statistic according Thrusfield (1995).

Control	No. of replicate	Range OD	LCL (OD)	UCL (OD)
Blank	96	0.038-0.090	0.046	0.050
Conjugate	96	0.040-0.089	0.051	0.055
Negative	96	0.046-0.098	0.057	0.061
Positive	96	1.020-1.976	1.441	1.526

Table 3.1: Range of optical densities (OD) observed in replicates of four controls included on six ELISA plates run on different occasions. The controls were: the substrate (Blank), conjugate control, negative serum control and positive serum control. The lower control limit (LCL) is the 5^{th} percentile and the upper control limit (UCL) is the 95^{th} percentile.

3.3. Results:

3.3.1. ELISA test:

A total of 514 serum samples from cattle of known *F. hepatica* status were tested in the LSTM ELISA. The results are shown in Figure 3.1.

To calculate the optimum diagnostic cut-off value for the test, the diagnostic sensitivity and specificity were calculated for cut-off values ranging from 10PP to 25PP. At a cut-off value of 15PP, the diagnostic sensitivity was 98% and the diagnostic specificity was 96% (Table 3.2).

Sera from two calves experimentally infected with *D. viviparus*, one from a calf infected with *O. ostertagi* and two from calves infected with *N. helvetianus* were

tested in the LSTM ELISA. The PP values for all the sera tested were below 10PP.

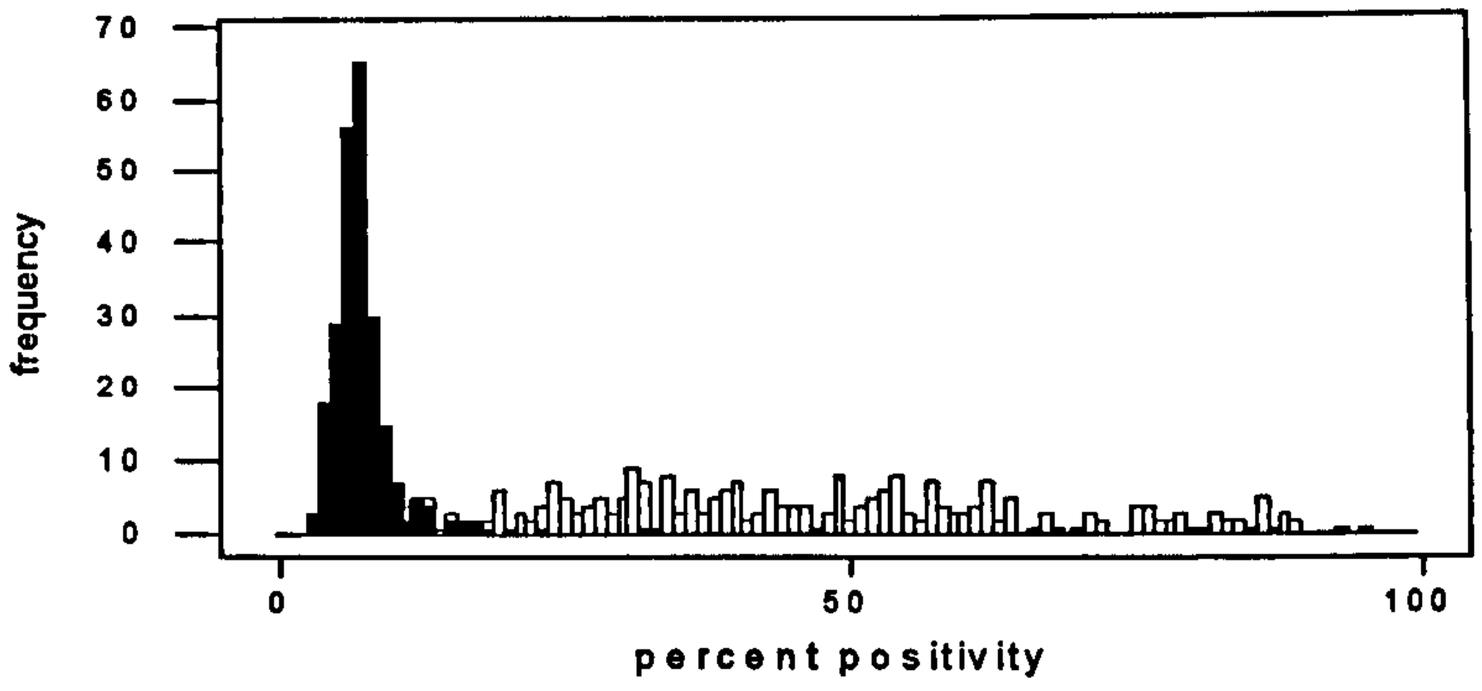

Figure 3.1 Graph showing the frequency distribution of ELISA values for serum samples from known positive and negative cattle. (■ = negative and □ = positive)

PP Value	Sensitivity (95% CI)	Specificity (95% CI)
10	100% (100 - 100)	87% (83 - 91)
15	98% (96 - 100)	96% (93 - 98)
20	95% (93 - 98)	99% (98 - 100)
25	87% (85 - 93)	100% (99 - 100)

Table 3.2: Calculation of diagnostic sensitivity and specificity (with 95 % confidence intervals) at different cut-off values.

3.3.1.2. Detection of antibodies in experimentally infected calves.

Sera from six calves, experimentally infected with *F. hepatica*, collected at weekly intervals, were tested to determine the time point after infection calves

were first shown to be positive in the LSTM ELISA. The results are shown in Figure 3. 2. One calf became positive at week two, three calves became positive at week three and two calves became positive at week four after infection.

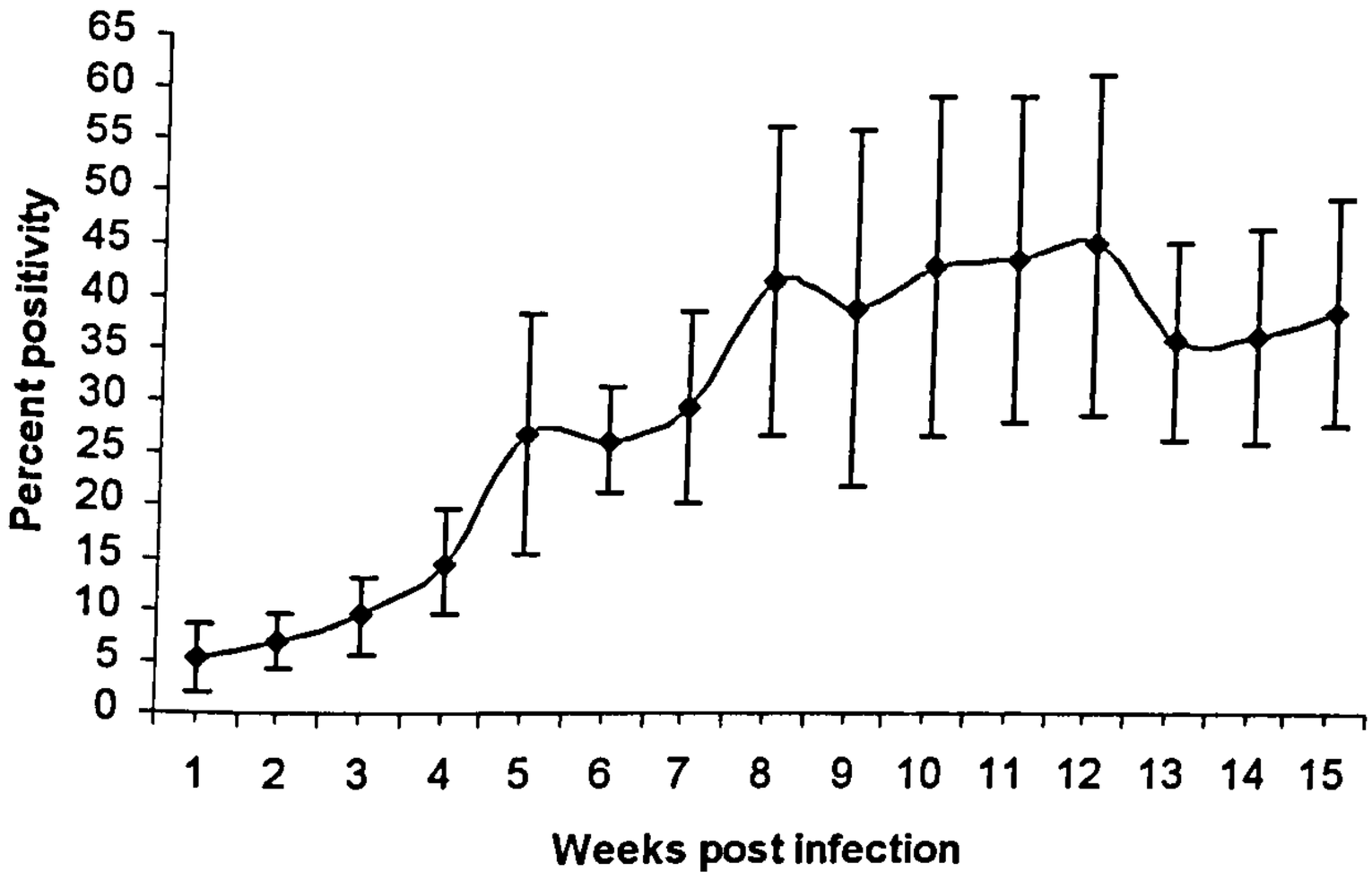

Figure 3.2. Graph showing antibody response in six calves after infection with 1000 metacercariae of *F. hepatica*, tested using LSTM ELISA. Each point represents the mean percent positivity plus/minus one standard deviation.

3.3.2. Comparison between the LSTM ELISA and the Bio - X kit:

Thirty-nine positive and forty-seven negative sera were tested in both the LSTM ELISA and the Bio-X ELISA. The correlation coefficient (Figure 3.3) between the LSTM ELISA test and Bio-X ELISA was 93.9 percent and the *Kappa* value was 0.82. A *Kappa* value of more than 0.81 indicates almost perfect agreement between two tests (Thrusfield, 1995). Hence there was almost perfect agreement between the LSTM ELISA and the Bio-X ELISA.

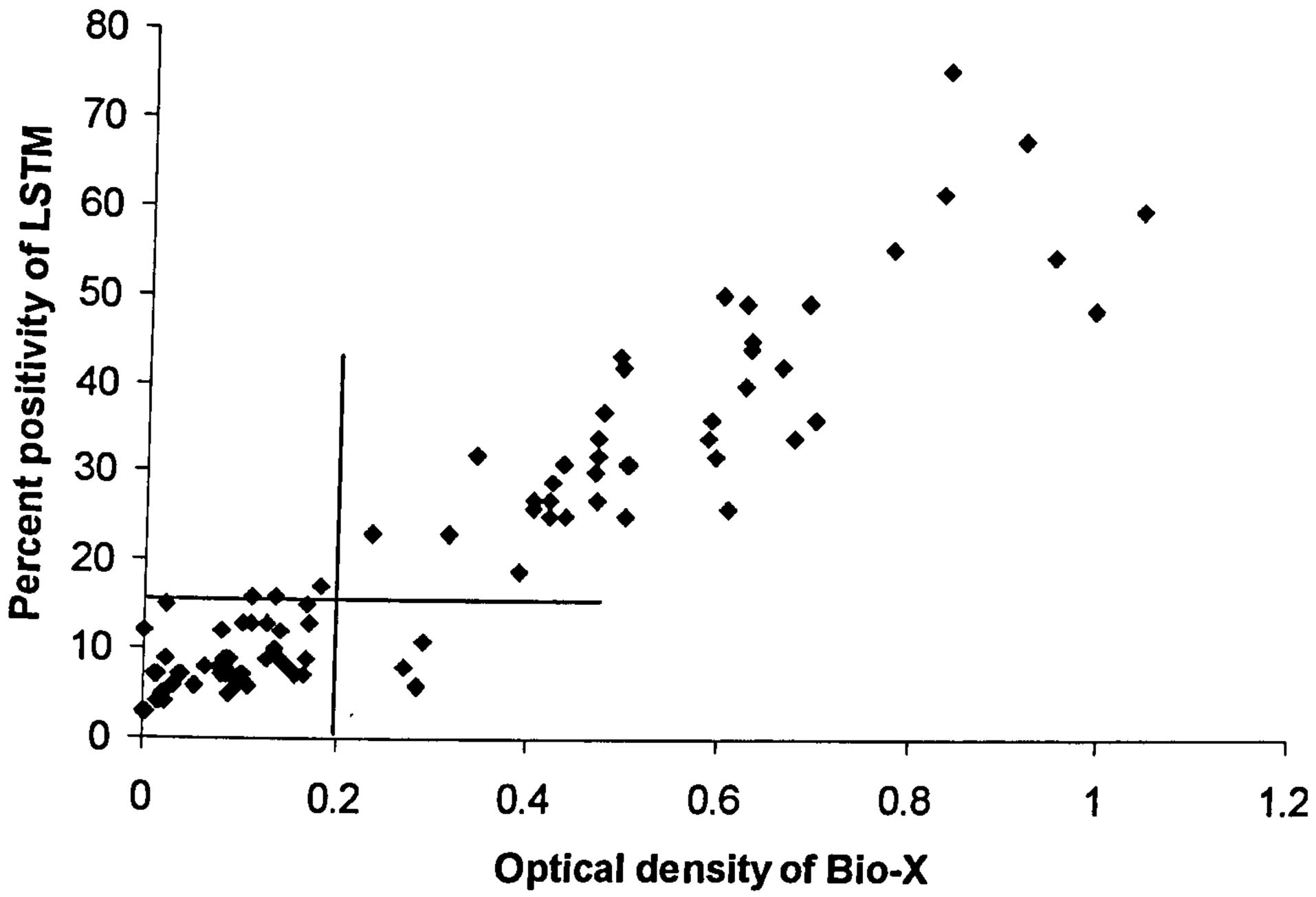

Figure 3.3. A Comparison between the ELISA and the Bio – X kit.

Thirty nine sera from faecal egg count positive cattle and 47 sera from negative cattle that had never been exposed to *F. hepatica* were tested in the LSTM ELISA and the Bio – X kit. Results are expressed as the ratio between the percent positivity value (LSTM ELISA) and the optical Density value (Bio-X kit).

3.4. Discussion:

We have developed and evaluated a diagnostic antibody-detection ELISA for *F. hepatica* infection in cattle. The test is based on E/S antigens of *F. hepatica* that contain predominantly fluke cysteine proteases, which are known to be immunodominant in cattle, naturally exposed to *F. hepatica* infection (Ortiz et al, 2000).

A new diagnostic test should ideally be evaluated against a gold standard. To this end, we used two populations of cows of known infection status. The positive population was from Northern Peru, where the prevalence of infection is considered to be high, up to 95% (Claxton et al, 1997). All the cows in the positive population had positive faecal egg counts indicating that each animal was currently infected. The negative population consisted of cattle that were not grazed and so had not been exposed to the parasite. Using these populations, the diagnostic sensitivity and specificity of the ELISA at a cut off value of 15PP, were 98% and 96% respectively. At this diagnostic cut off value, there was no cross-reaction using serum from cattle with mono-infections of *D. viviparus, N. helvetianus* and *O. ostertagi*, common nematode parasites of cattle in temperate areas.

An advantage of using a serodiagnostic test rather than faecal egg counts, for diagnosis, is that cattle with prepatent infections can be detected. In six experimentally infected calves, antibodies were detected between 2 and 4 weeks after infection. This is consistent with results reported by others, using a variety of sero-diagnostic tests (Farrell et al, 1981; Wyckoff and Bradley, 1986; Sinclair and Wassall, 1988; Hughes et al, 1981; Santiago and Hillyer, 1988; Abdel-Rahman et al, 1998; Fagbemi and Guobadia, 1995; Bossaert et al, 2000; Reichel, 2002). However it is possible that the size of the infectious dose of metacercariae will affect the development of a detectable antibody response (Cornelissen et al, 1999). Cattle exposed under natural conditions are likely to ingest lower doses of metacercariae over a prolonged period. Nevertheless serodiagnostic tests are likely to detect infection more rapidly than faecal egg counts.

Whilst antibody-detection tests can identify animals with prepatent infections, a disadvantage with these tests is that a positive result does not necessarily indicate a current infection, but rather a history of exposure. Antibodies persist in fluke-infected cattle after treatment with triclabendazole for up to 7 months (Levieux et al, 1992a; Castro et al, 2000). This means that any antibody-detection test used to diagnose infection in individual animals must be interpreted with care. The six experimentally infected calves used in our study, were treated with triclabendazole twenty weeks after infection. They remained positive by ELISA for at least 11 weeks after treatment, when sampling was stopped (results not shown).

The LSTM ELISA was compared to the Bio-X ELISA, a commercially available test for serodiagnosis of *F. hepatica* infection in cattle. Both tests use E/S antigen, as the solid phase component but the interpretation of the optical density results are different. For the LSTM ELISA, results are expressed as the percent positivity against a positive control, thus standardising day to day variability in the test. In contrast the Bio-X test results are optical densities that are compared to a table of OD's provided by the manufacturer and classed as either negative (0, representing an OD below 0.2) or positive, ranging from 1+ to 4+. This does not allow for daily variation or batch variation in test operation. Nevertheless calculation of the *kappa* statistic indicated that agreement between the two tests was almost perfect.

In conclusion we have developed a sensitive and specific diagnostic ELISA for *F. hepatica* infection in cattle that has been fully validated using sera from populations of cattle of known infection status.

Chapter Four

Modification and validation of the LSTM ELISA and the Bio-X ELISA to detect *Fasciola hepatica* specific antibody in individual milk samples

4.1. Introduction

The use of milk for diagnosis and surveillance of different diseases in cattle, has become routine in recent years and milk antibody testing now plays a significant role in cattle disease control and eradication programmes in many countries (Pritchard, 2001; Pritchard et al., 2002). It has been shown for many infections that there is generally a good correlation between milk and serum antibody titres (Boulard, 1985; Niskanen et al, 1989; Kloosterman et al, 1993; Pritchard, 2001; Pritchard et al., 2002), but that milk sampling is easier, cheaper and not invasive compared to blood sampling. The number of cases of fasciolosis diagnosed in cattle in England and Wales has increased significantly over the past 5 years particularly in dairy cattle and yet it is thought that the majority of chronic infections probably go undiagnosed or misdiagnosed (SAC, 2000). Hence a test that can detect antibodies in milk samples would assist in both the diagnosis and the surveillance of infection. This would enable farmers to treat their cattle appropriately and improve milk yield and production. Surveillance is vital to assess the significance and spread of fasciolosis in UK.

To this end the LSTM ELISA test, and Bio-X ELISA – a commercially available kit used routinely by the VLA of England and Wales were adapted for use with milk from dairy cattle. Both tests have been developed for sera diagnosis. To ensure that the population of antibodies in milk were representative of that in serum, milk and serum were compared by Western Blotting.

4.2. Materials and methods

4.2.1. Samples

For analysis of the antibody response in milk to *F. hepatica* by ELISA, 606 individual serum and milk samples from 21 farms were collected. Also 277 faecal samples were collected from 11 of these farms. Milk samples were tested at two different dilutions (1:2 and 1:5) to determine the optimum dilution. In addition the sensitivity and specificity at three cut off values were calculated to identify the optimum cut off value for LSTM Milk ELISA test. After this initial evaluation a further 1565 milk and serum samples from 61 different farms were collected. Also a total of 715 faecal samples from 27 of these farms were collected. Twenty – 34 cattle were sampled on each farm. The herd size ranged between 30 and 374 and the mean herd size was 107. Details of age, parity and days into lactation were also recorded. 24/61 these farms were located in south and mid Wales, Carmarthen, and were identified by the VLA because of presenting clinical problems associated with fasciolosis (abortion due *Salmonella dublin* or Black disease). The remaining 37 farms were located in Cheshire, England and were investigated as part of a study on *Neospora caninum* infection.

4.2.2. Sodium Dodecyle Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

4.2.2.1. SDS PAGE

SDS PAGE electrophoresis was performed according to Laemmli (1970), using 10% non-gradient polyacrylamide gels under non-reducing conditions. Mini-protean^{R} 3 cell apparatus (Bio-Rad laboratories Ltd., UK) was cleaned with

methanol. The apparatus was assembling according to the manufacture's instructions and a 10% resolving gel (Appendix 2) was poured between the glass plates to 2 cm below the top. Ultra pure water was immediately added to the top to level the surface. The gels were allowed to polymerise at room temperature for one hour. The ultra pure water was poured off and a 4% stacking gel (Appendix 2) was poured on the top of the polymerised separating gel. A separating comb with ten teeth was placed into the stacking gel and then the gel was allowed to polymerise for one hour as before. After one hour, the gels were placed in tank and the comb was removed slowly.

The samples were mixed in equal parts with 2X sample buffer and boiled for 90 seconds and were loaded into the wells; also 6µl of Biotinylated SDS-PAGE markers (Bio-Rad) was applied to one lane on each gel. Samples were overlaid with running buffer so that gels were fully submerged. The lid was put on and connected to a power supply and the gels were run at constant current at 50mA (25mA per gel) for one hour until the dye (bromophenol blue) reached the end. The gels were gently removed and either stained with Coomassie blue or used for Western Blotting.

For staining, gels were put in a container and Coomassie Brilliant Blue R-250 (Appendix 2) was poured on and then the container was put on the top of a rocking table for one hour and then destained in destaining solution (Appendix 2) until the background was clear. The relative molecular weights of the bands were estimated by reference to a range of low molecular weight markers.

4.2.2.2. Western Blotting

Gels were removed and a corner cut corresponding to position of the markers, and then placed carefully in transfer buffer for at least 15 minutes to equilibrate. Meanwhile a piece (8.5 x 5.5 cm.) of Immobilon™-P (transfer membrane = Membranes, Immobilon-P Polyvinylidene difluoride, Sigma, Poole, Dorset, England, UK) was cut and was prepared by wetting it directly in 100% methanol for 15 seconds, transferring to ultra pure water for 2 minutes, finally placing it in transfer buffer for 5 minutes. The Mini trans-blot electrophoretic transfer cell (Bio-Rad) was prepared according to the instruction manual and the proteins transferred from the SDS-PAGE onto the membrane in transfer buffer (25mM Tris, 192mM Glycine, 20% v/v Methanol, pH 8.3). The excess binding sites on the membrane were blocked with 4% skimmed milk powder in PBS for 30 minutes on the bench then overnight at 4°C. The following day, the membrane was washed three times twice for 10 minutes and once for 30 minutes in PBS / 0.05%Tween and then it was cut in strips 5mm wide. Each strip was incubated with serum diluted 1:50 and 1:100 or milk diluted in 1:2 and 1:5 in incubation buffer (4% skimmed milk powder / PBS / 0.05%Tween) for one hour. After a further three washes as before, the strips were incubated with a monoclonal anti bovine IgG conjugated to horseradish peroxidase diluted 1:500 for one hour. After further washes the membrane strips were incubated in DAB (0.7 $mgml^{-1}$ 3,3-diaminobenzidine plus 0.17mg/ml Urea hydrogen peroxide dissolved in 15 ml ultra pure water and filtered through a 0.2 μm filter, Sigma) for 10 to 15 minutes, then washed in distilled water and air-dried on filter paper.

4.2.3. Quality assurance

Five control plates were used on different days to establish upper control limits (UCL) and lower control limits (LCL) by using the 95th and 5th percentiles of the replicate OD values for each of the controls used. The percentiles values were obtained by using Minitab for Windows, version 13.32. Pearson correlation coefficient for the ELISA tests was calculated by using Minitab for Windows, version 13.32 (Table 4.1).

	No. of replicates	Range OD	LCL OD	UCL OD
Blank	80	0.032 – 0.059	0.041	0.044
Conjugate	80	0.032 – 0.091	0.048	0.053
Negative	80	0.034 – 0.104	0.048	0.053
Positive	80	0.356 – 0.655	0.495	0.527

Table 4.1. Range of optical densities (OD) observed in replicates of four controls included on five ELISA plates run on different occasions, the controls were: the substrate (Blank), conjugate control, negative serum control and positive serum control. The lower control limit (LCL) is the 5th percentile and the upper control limit (UCL) is the 95th percentile.

4.2.4. Statistic analysis

The Pearson correlation coefficients, Regression plots, and analysis of all data were calculated by using Minitab for Windows, version 13.32 (Minitab Inc.). The diagnostic sensitivity and specificity and confidence intervals were calculated

using WinEpiscope 2.0 (N. de Blas, C. Ortega, K. Frankena, J. Noordhuzen, M. Thrusfield: http://www.clive.ed.ac.uk/winepiscope/).

4.3. Results

4.3.1. Validation of the LSTM milk ELISA

4.3.1.1. Diagnostic sensitivity, diagnostic specificity and cut-off value

The optimum milk dilution and diagnostic cut-off values for the LSTM ELISA, together with the diagnostic sensitivity and specificity, were calculated using either sera or faecal egg count tests as the gold standard. The sensitivity and specificity were calculated for cut-off values ranging from 15PP to 25PP and at dilutions 1:2 and 1:5 (Tables 4.2 and 4.3).

Test	Gold standard	Dilution	Cut off value	Sensitivity (95%CI)	Specificity (95%CI)
Milk	Sera	1:2	15	94% (92 – 98)	67% (62 – 71)
Milk	FEC	1:2	15	99% (98 – 100)	30% (23 – 37)
Milk	Sera	1:2	20	92% (88 – 95)	86% (83 – 90)
Milk	FEC	1:2	20	98% (96 – 100)	43% (36 – 51)
Milk	Sera	1:2	25	87% (83 – 92)	93% (91 – 96)
Milk	FEC	1:2	25	95% (91 – 99)	53% (45 – 61)

Table 4.2. Diagnostic sensitivity and specificity for LSTM ELISA for milk tested at dilution of 1:2 at cut off values ranging from 15 to 25 PP, using, either the serum test or the faecal egg count test as gold standards.

Test	Gold standard	Dilution	Cut off value	Sensitivity (95%CI)	Specificity (95%CI)
Milk	Sera	1:5	15	91% (88 – 95)	83% (79 – 87)
Milk	FEC	1:5	15	97% (94 – 100)	43% (35 – 50)
Milk	Sera	1:5	20	89% (85 – 93)	96% (94 – 98)
Milk	FEC	1:5	20	97% (94 – 100)	58% (50 – 66)
Milk	Sera	1:5	25	85% (80 – 90)	98% (96 – 99)
Milk	FEC	1:5	25	95% (91 – 99)	64% (56 – 71)

Table 4.3. Diagnostic sensitivity and specificity for LSTM ELISA for milk tested at dilution of 1:5 at cut off values ranging from 15 to 25 PP, using, either the serum test or the faecal egg count test as gold standards.

A cut off value of 20 and dilution of 1:2 were chosen to give the optimum diagnostic sensitivity and specificity.

For a full evaluation of the milk LSTM ELISA, a total of 1565 individual sera and 765 individual milk samples from 61 different farms and also 715 faecal samples from 27 of these farms were tested. The diagnostic sensitivity and specificity for LSTM milk ELISA test, using either serum or faecal egg counts as the gold standard, is shown in table 4 – 4. The diagnostic sensitivity and specificity for milk were 92% and 88% respectively, when the serum test was considered as a gold standard. Using the FEC as the gold standard, the diagnostic sensitivity for

both sera and milk was high (96% and 99% respectively) but the diagnostic specificity was low (68% for both).

Test	Number tested	Gold standard	Sensitivity (95%CI)	Specificity (95%CI)
Milk	765	Sera	92 (89 – 96)	88 (85 – 91)
Sera	715	FEC	96 (93 – 98)	68 (63 – 72)
Milk	715	FEC	99 (98 – 100)	68 (63 – 74)

Table 4.4. Diagnostic sensitivity and specificity with 95% confidence intervals for milk using serum or faecal egg counts as the gold standards.

4.3.1.2. Herd prevalence measured by serum and milk ELISA, and faecal egg count

The prevalence of infection measured by serum antibody, milk antibody, and faecal egg count were calculated for each of the 61 farms tested. The results are shown in Figures 4.1, 2 and 3.

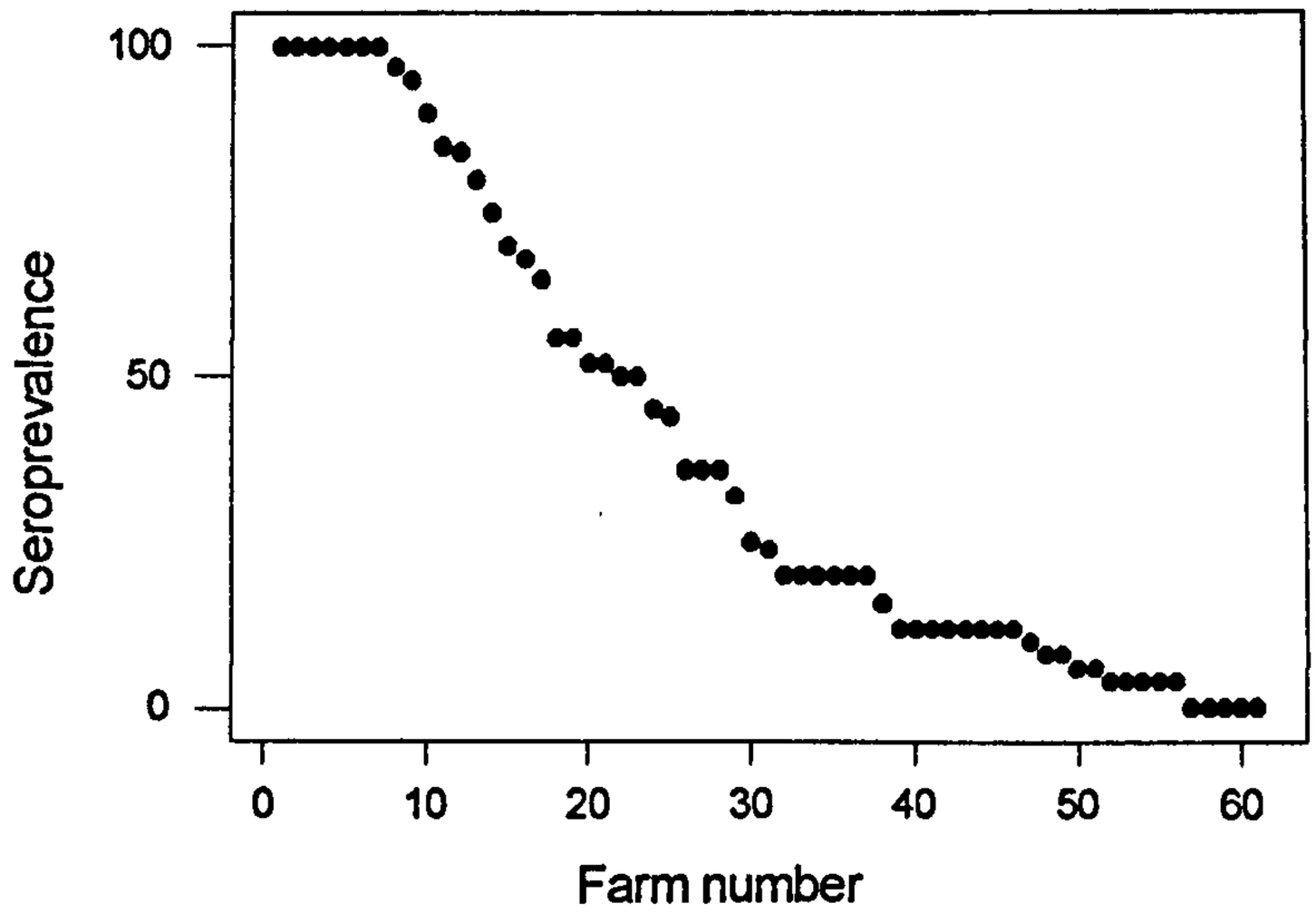

Figure 4.1. Herd prevalence measured by serum (N = 61)

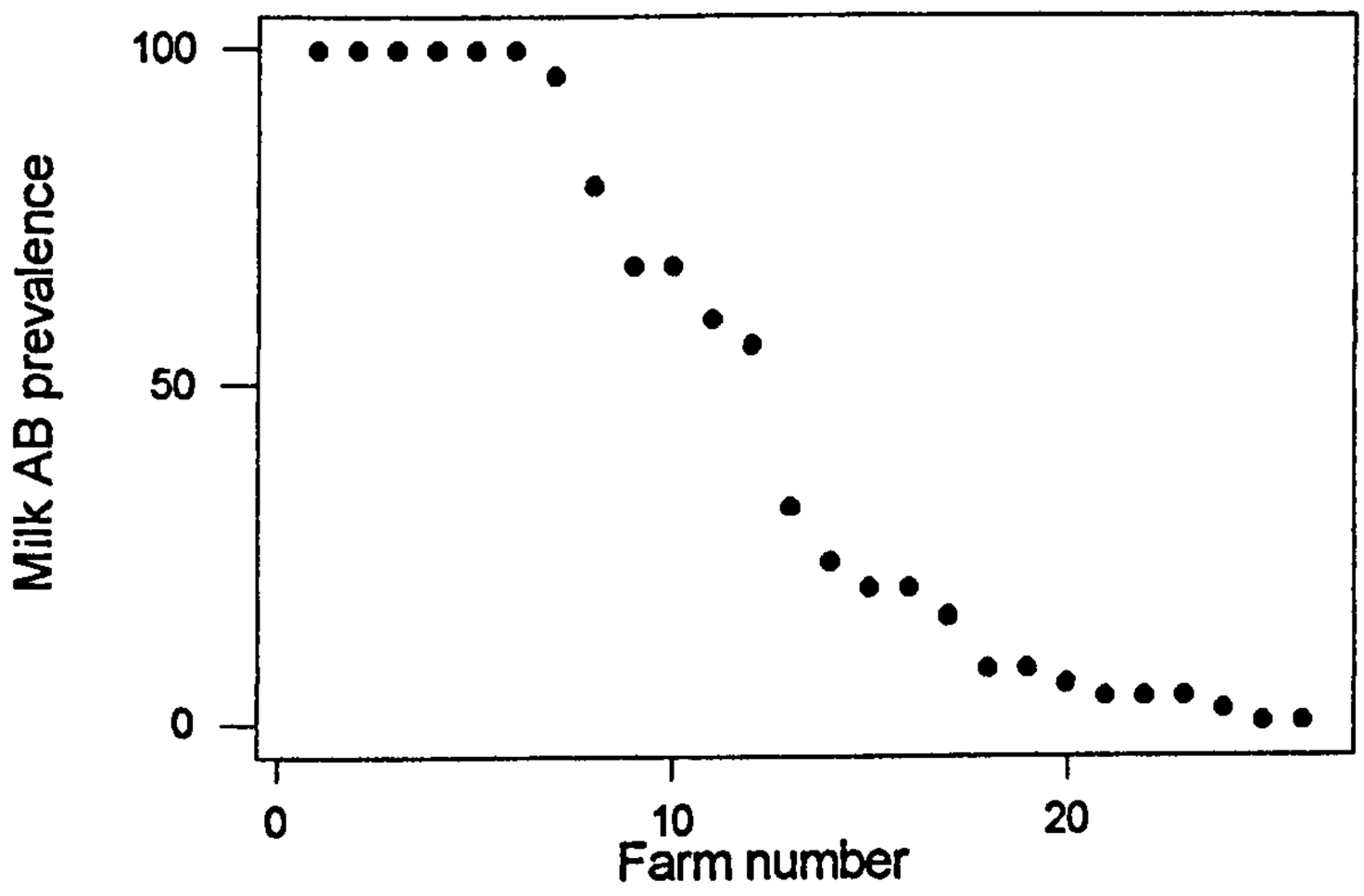

Figure 4.2. Herd prevalence measured by LSTM milk ELISA (N = 26)

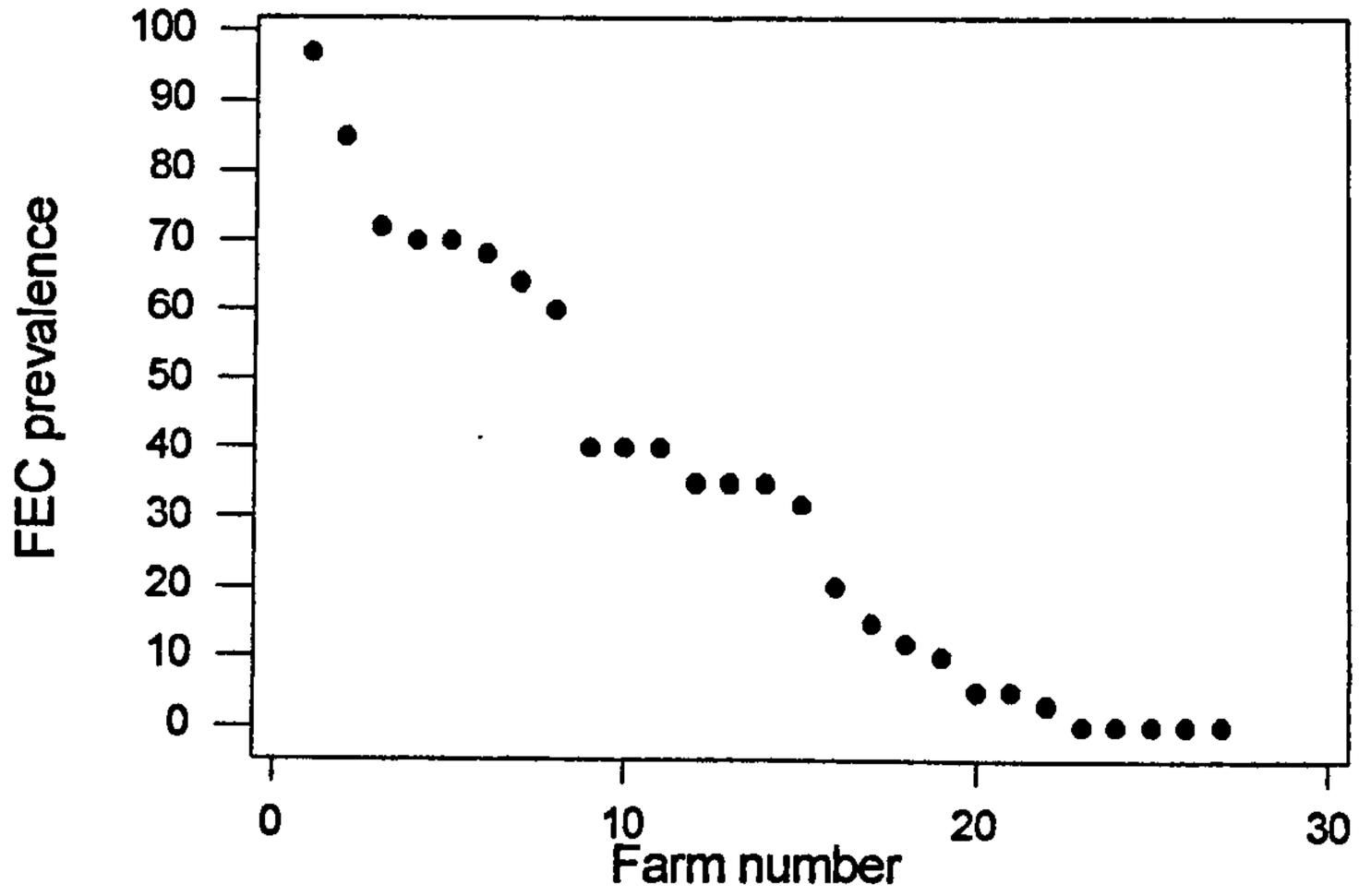

Figure 4.3. Herd prevalence measured by faecal egg count (N = 27)

The milk antibody prevalence was evaluated against the seroprevalence within the herd by calculating correlation coefficient and drawing regression graphs with 95 percent confidence intervals. The correlation coefficient between milk antibody prevalence and seroprevalence was 96% (Figure 4.4). The correlation coefficient between prevalence measured by faecal egg count and both seroprevalence and milk antibody prevalence within the herd were calculated and are shown in figures 4.5 and 4.6 The correlation coefficient was 87% for both.

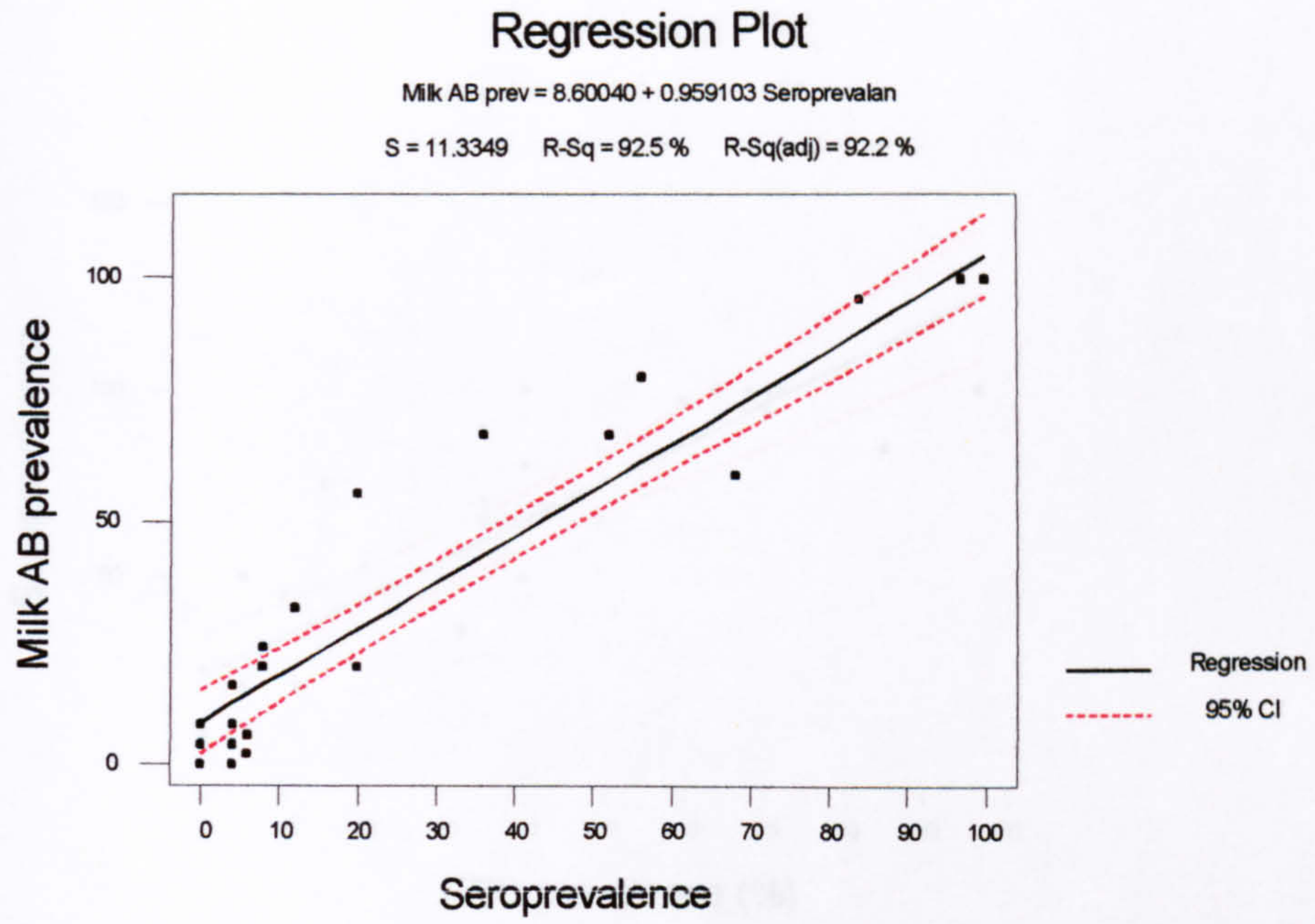

Figure 4.4. Regression plot between milk antibody prevalence and seroprevalence for 26 herds. The Pearson correlation coefficient was 96%.

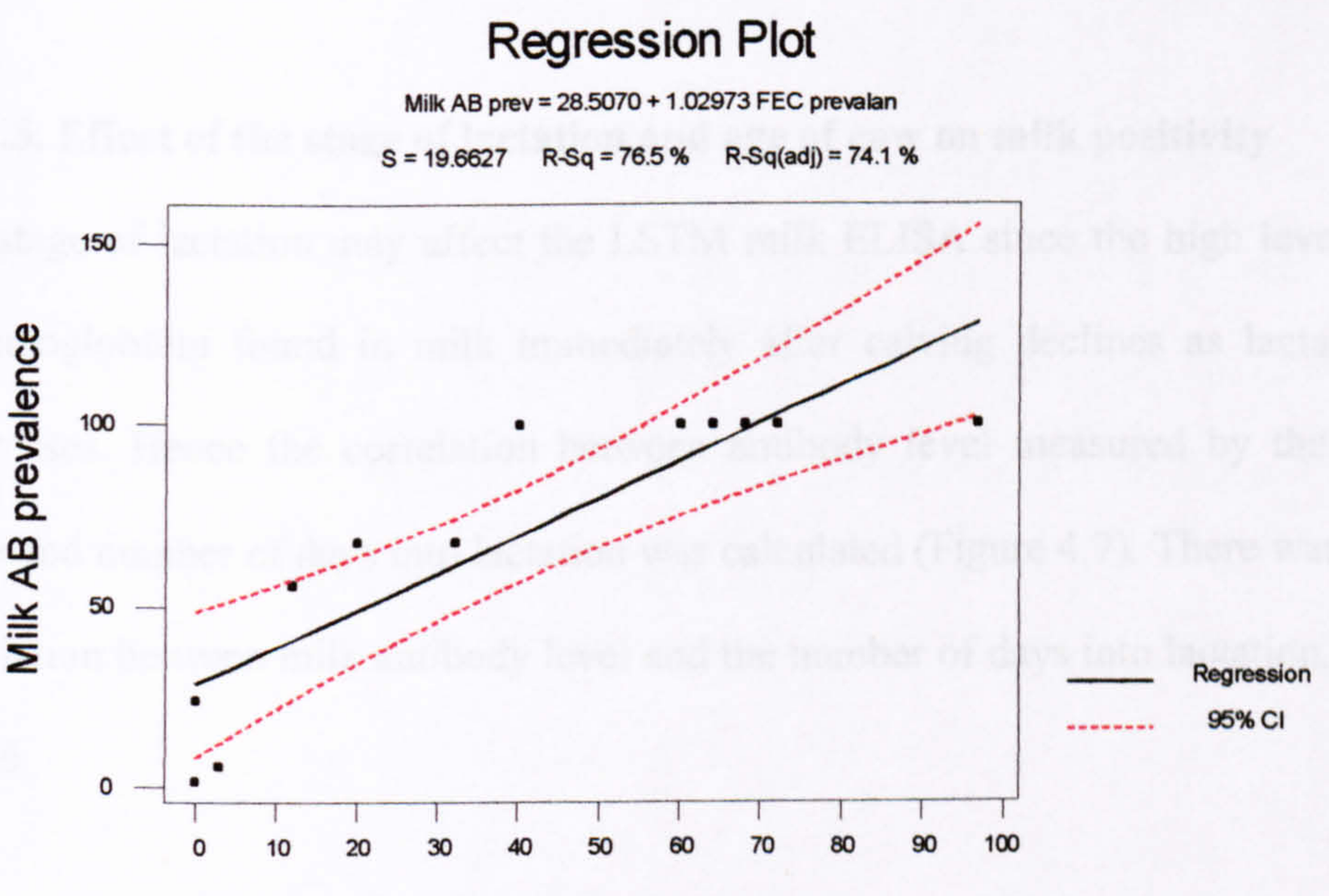

Figure 4.5.Regression plot between milk antibody prevalence and herd prevalence measured by faecal egg count. The Pearson correlation coefficient was 87%.

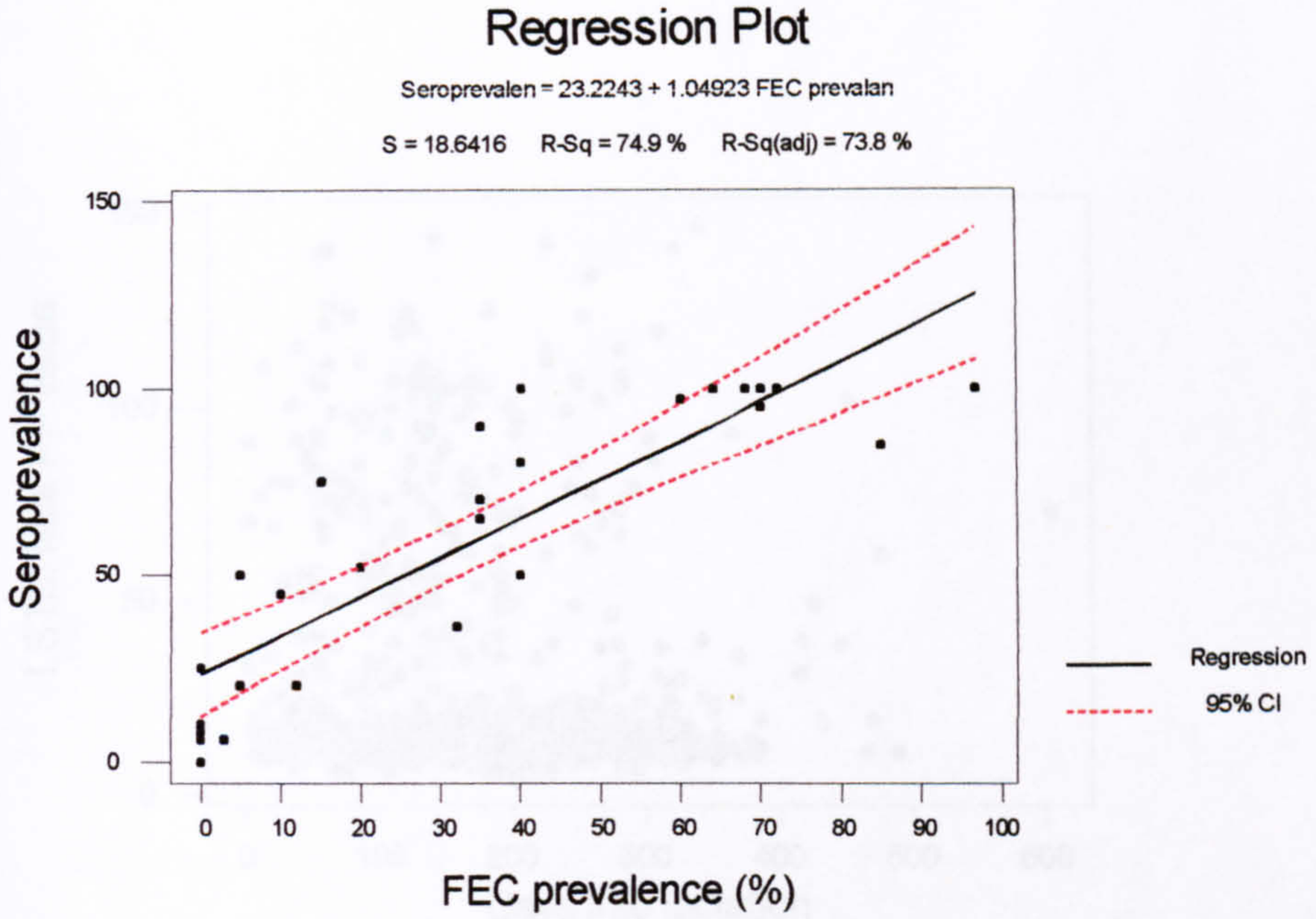

Figure 4.6. Regression plot between seroprevalence and herd prevalence measured by FEC. The Pearson correlation coefficient was 87%.

4.3.1.3. Effect of the stage of lactation and age of cow on milk positivity

The stage of lactation may affect the LSTM milk ELISA since the high level of immunoglobulin found in milk immediately after calving declines as lactation progresses. Hence the correlation between antibody level measured by the PP value and number of days into lactation was calculated (Figure 4.7). There was no correlation between milk antibody level and the number of days into lactation, r = -0.056.

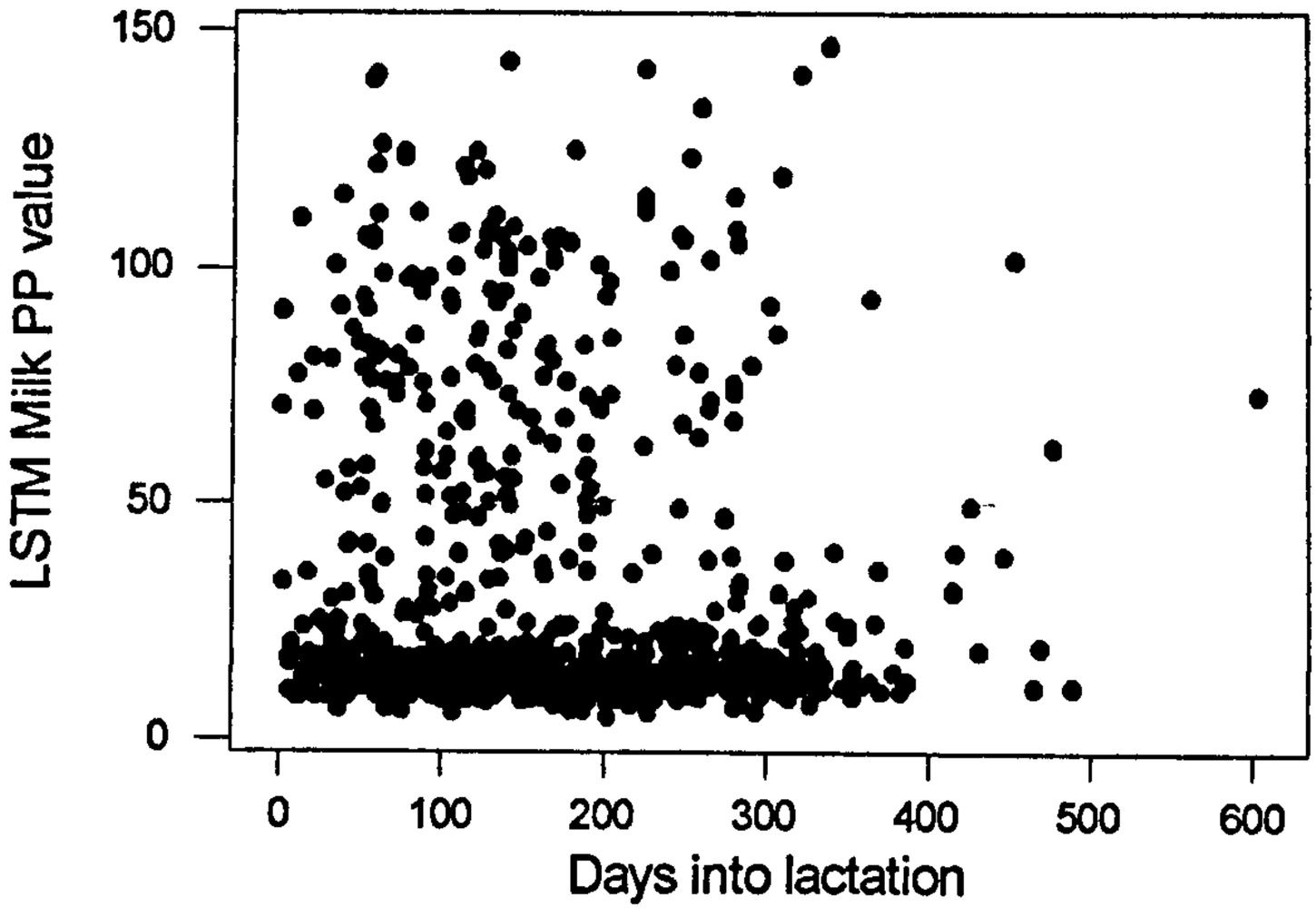

Figure 4.7. Milk antibody PP values in cattle at different stages of lactation.

We wanted to determine if there was any evidence that cattle acquired immunity to infection. Therefore the correlation between age and intensity of infection measured by faecal egg count was calculated. This assumes that eggs per gram of faeces is proportional to numbers of fluke. The results (Figure 4.8) suggest that the intensity of infection does not decrease with age.

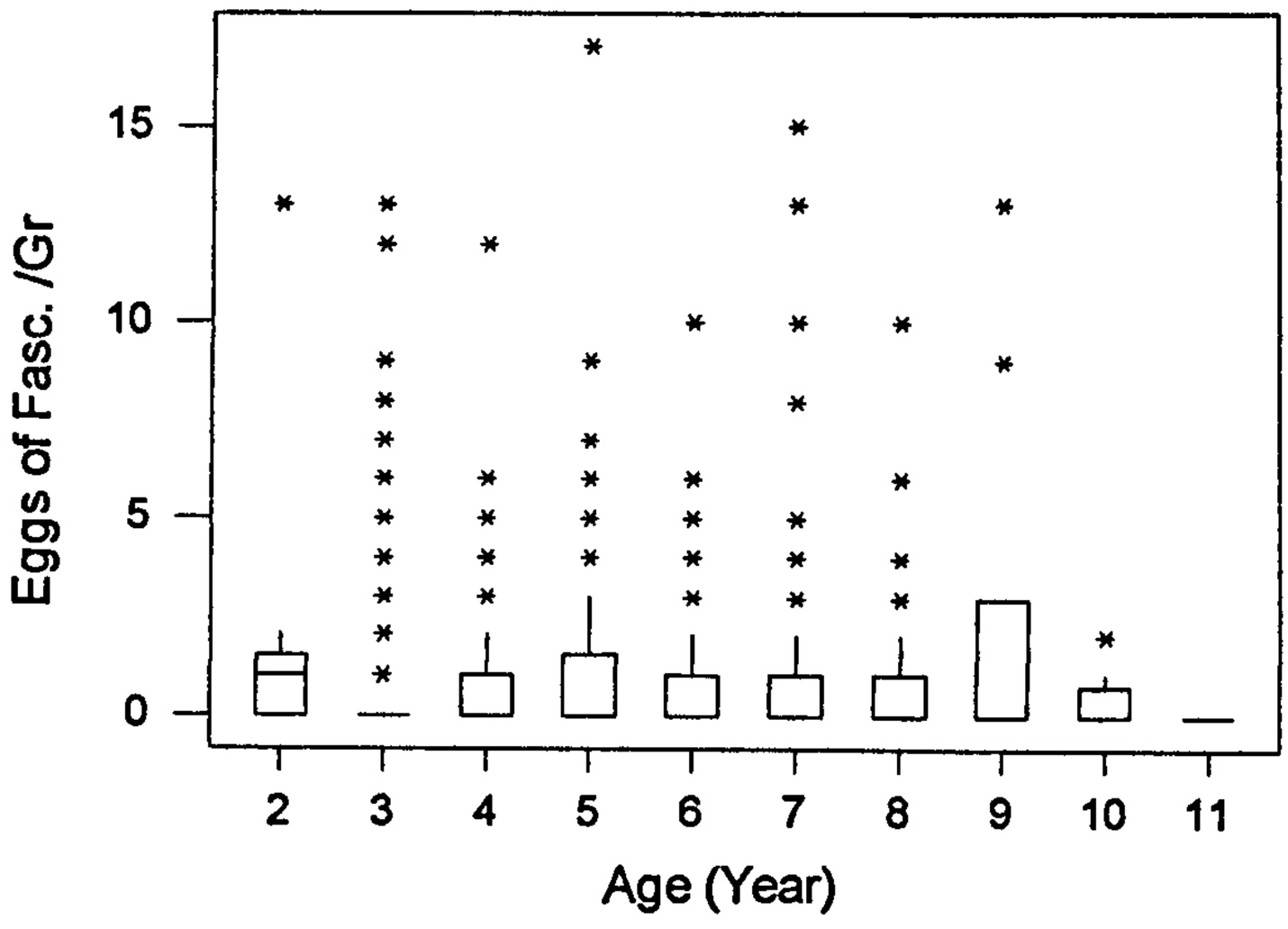

Figure 4.8. Intensity of infection for cattle of different ages (Correlation, r = 0.061).

4.3.2. Validation of the Bio-X ELISA

4.3.2.1. Diagnostic sensitivity and specificity

The Bio-X ELISA test, which is a commercial available test that detects specific antibody to *F. hepatica* in serum of infected cattle, was adapted for use with milk. A total of 228 serum, milk and faecal samples from 9 different farms were used to calculate the optimum dilution of milk for use in the test, the diagnostic sensitivity and specificity. The Bio – X test serum results and the faecal egg counts were used as gold standards, and were calculated for milk dilutions of 1:2 and 1:5 (Table 4.5).

Test	Gold standard	Sensitivity (95%CI)	Specificity (95%CI)
Bio-X Milk AB (1:2)	Bio-X Sera	92% (88 – 97)	84% (76 – 91)
Bio-X Milk AB (1:5)	Bio-X Sera	83% (77 – 90)	93% (87 – 98)
Bio-X Sera	FEC	97% (94 – 100)	55% (47 – 63)
Bio-X Milk AB (1:2)	FEC	97% (94 – 100)	53% (45 – 61)
Bio-X Milk AB (1:5)	FEC	93% (88 – 99)	65% (57 – 73)

Table 4.5. Diagnostic sensitivity and specificity calculated for the milk test using serum and faecal egg count as gold standards.

From these results, a milk dilution of 1:2 was selected and used to test 1300 individual milk and serum samples from 50 farms. Six hundred and fifty four faecal samples from 24 of these farms were also analysed.

The diagnostic sensitivity and specificity for the Bio-X milk ELISA test, using sera and faecal egg counts as the gold standards, is shown in table 4.6. The diagnostic sensitivity and specificity for Bio-X milk were 72 and 93% respectively when the Bio-X serum test was considered as a gold standard. Using the FEC, the diagnostic sensitivity for both Bio-X sera and Bio-X milk was high but the diagnostic specificity was low (Table 4.6).

Test	Gold standard	Sensitivity (95% CI)	Specificity (95% CI)
Bio-X Milk	Bio-X Sera	72% (67 – 76)	93% (90 – 95)
Bio-X Milk	FEC	97% (95 – 100)	72% (67 – 77)
Bio-X Sera	FEC	99% (97 – 100)	63% (59 – 68)

Table 4.6. Diagnostic sensitivity and specificity for Bio-X milk ELISA, using sera or FEC as the gold standards.

4.3.2.2. Herd prevalence measured by serum antibody, milk antibody and faecal egg count

The results of herd prevalence using serum, milk, and faecal egg count are shown in figures 4.9, 4.10 and 4.11.

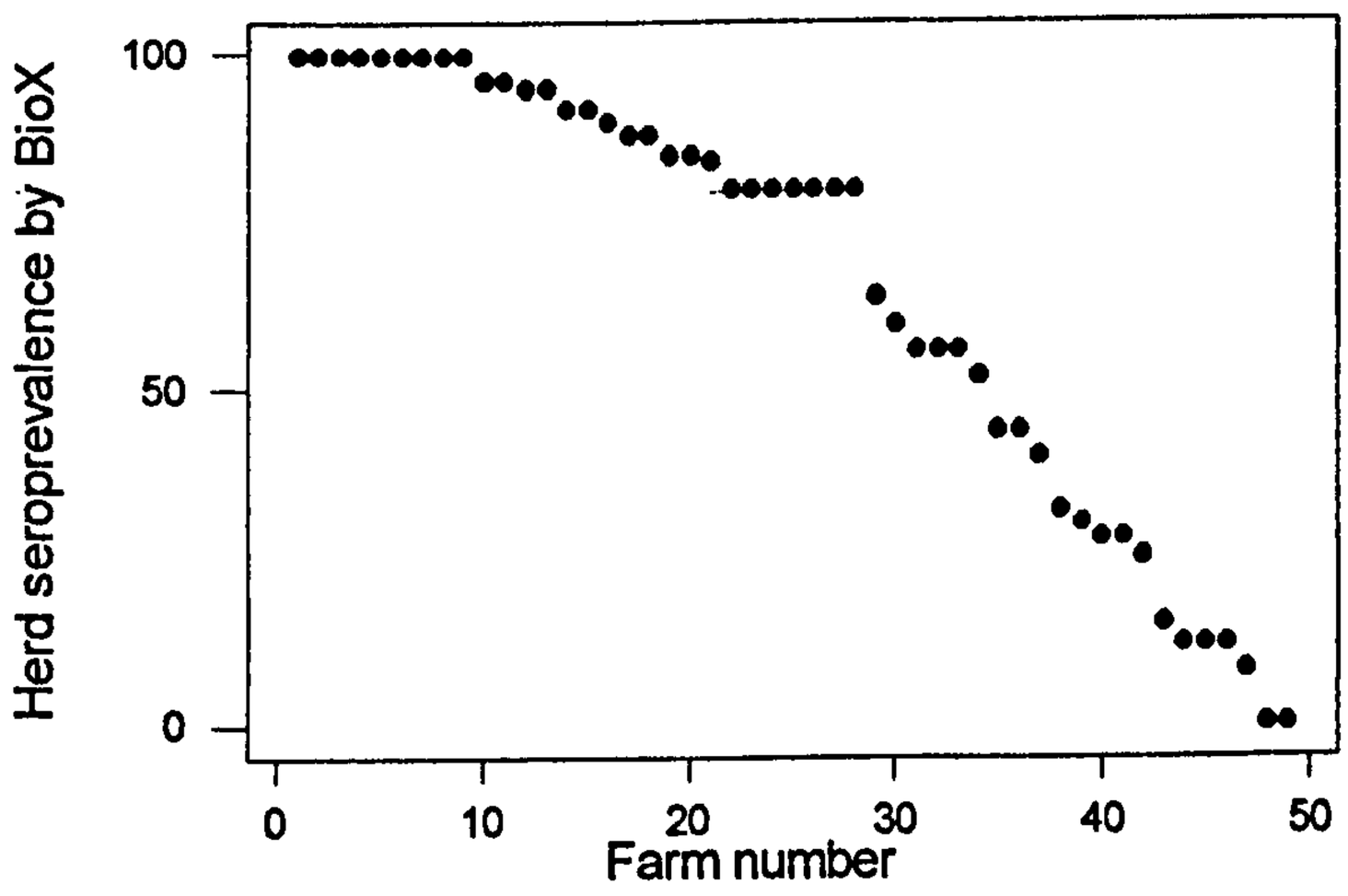

Figure 4.9. Herd prevalence measured by Bio-X serum ELISA (N = 50).

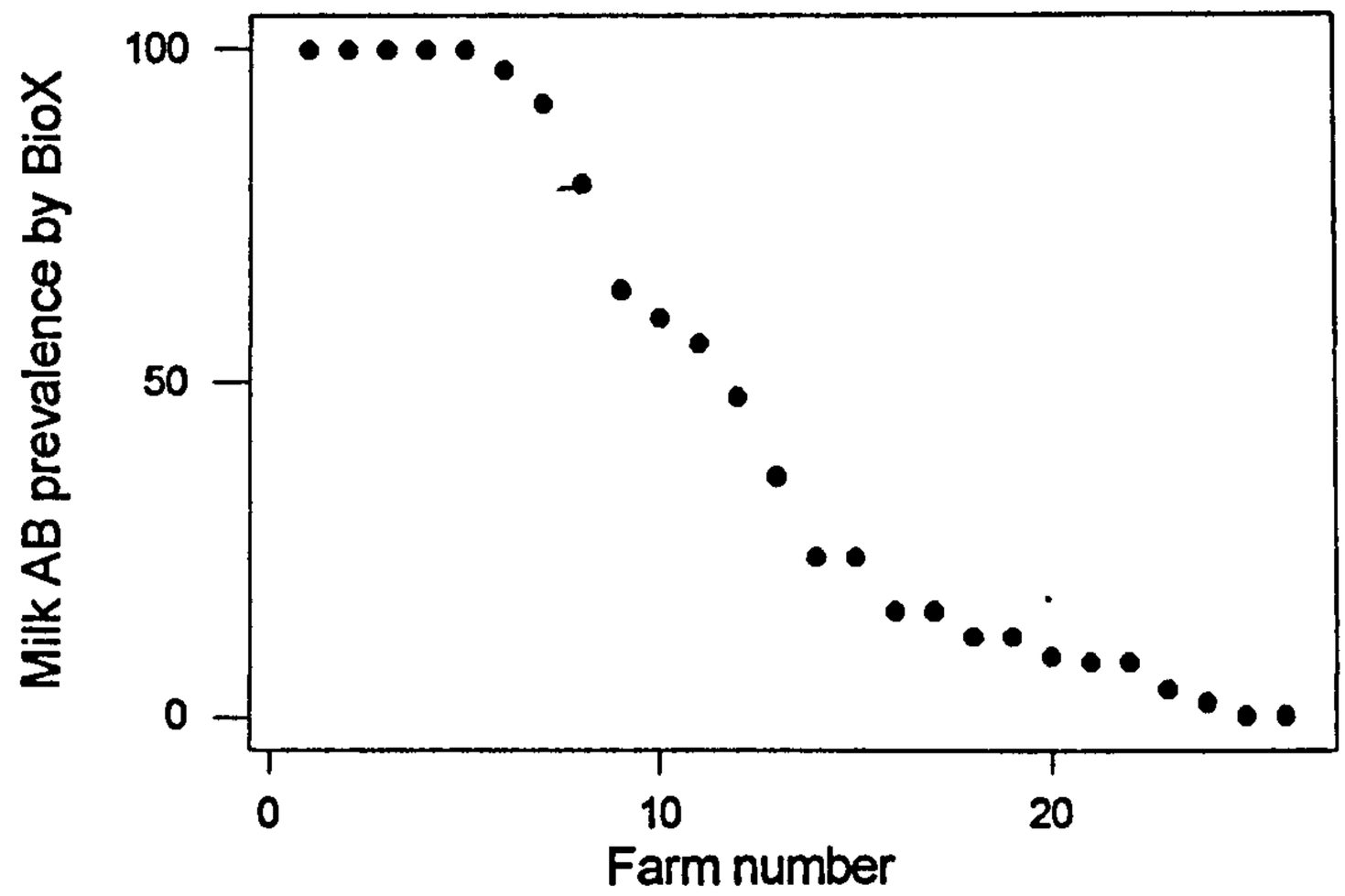

Figure 4.10. Herd prevalence measured by Bio-X milk ELISA (N = 26).

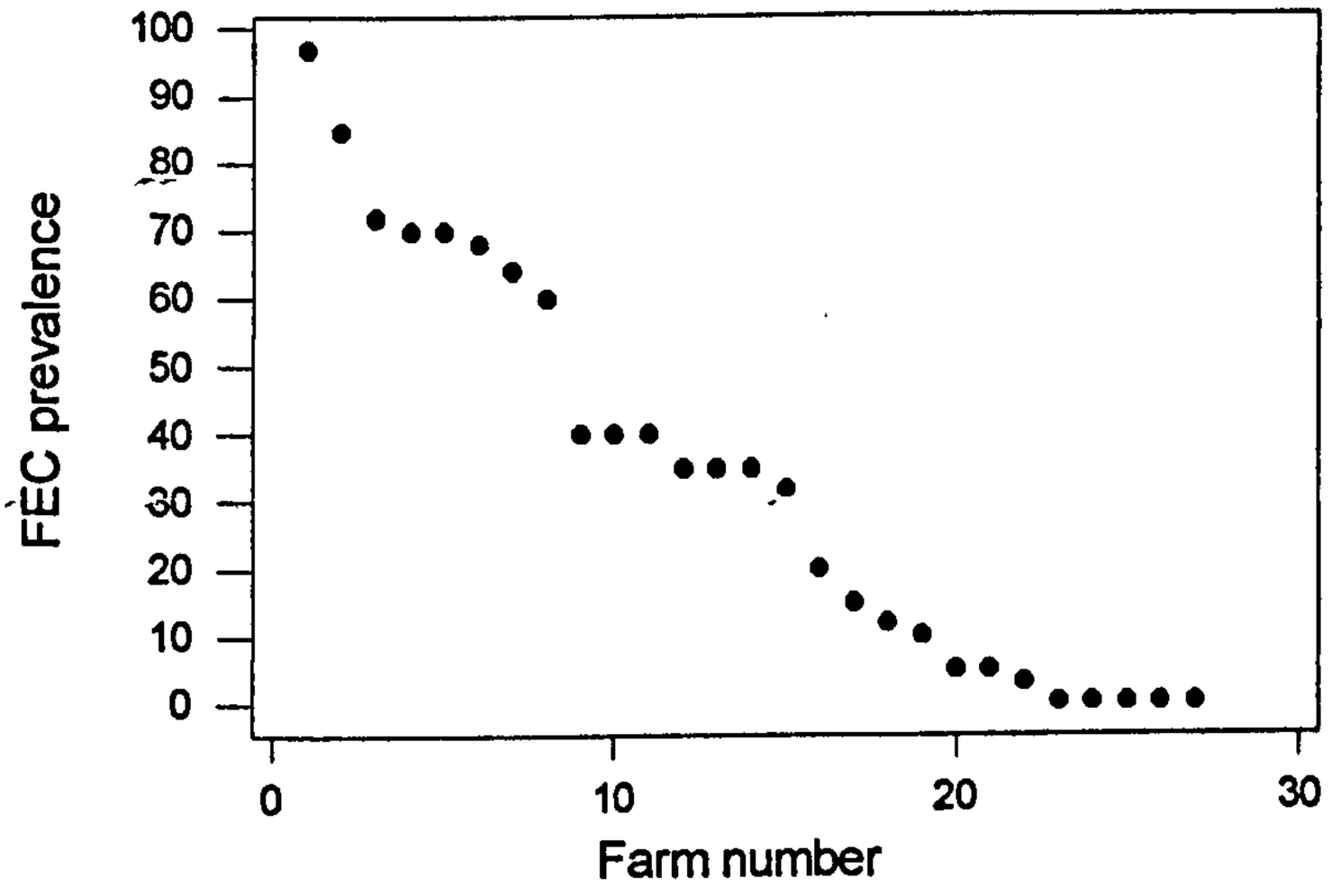

Figure 4.11. Herd prevalence measured by faecal egg count (N = 27).

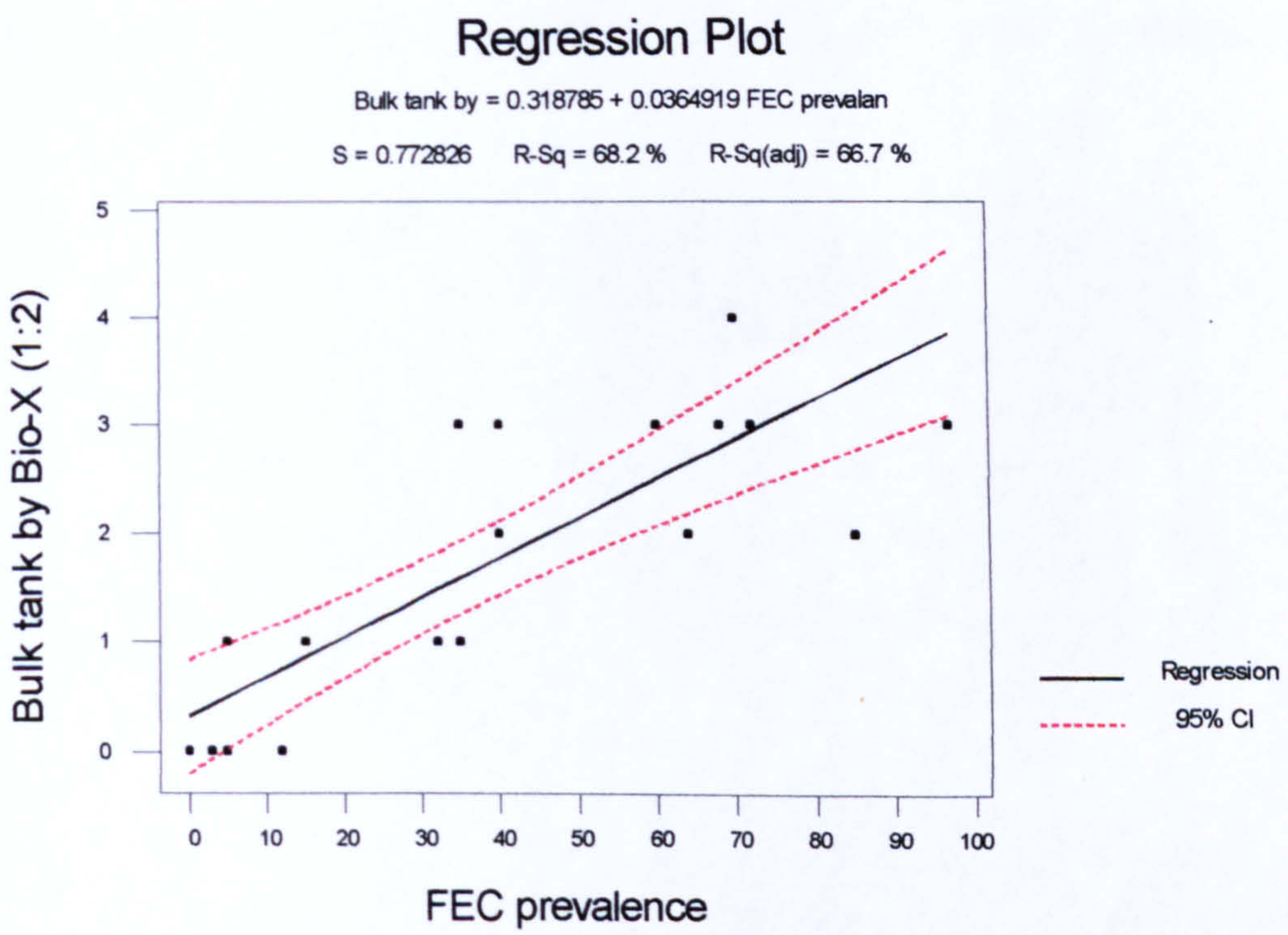

Figure 5.7. Regression plot between bulk tank milk positivity by the Bio - X test and prevalence of infection measured by faecal egg count.

5.3.2.2.2: Prevalence of infection assessed by Bio-X bulk tank milk result

The Bio–X ELISA results are interpreted on a scale of 0 (negative) to 4+ (1+ - 4+ are considered to be positive). A BTM value of 1+ or above was used to indicate a positive herd. The ranges of seroprevalence is shown in table 5.7 and milk antibody prevalence and prevalence measured by faecal egg counts compared with seroprevalence, are shown in figure 5.8. To calculation diagnostic sensitivity and specificity for the Bio-X BTM ELISA, a herd with equal to and more than 25% seroprevalence was considered to be positive and less than 25% considered negative. The results indicated that a value of 1+ had a diagnostic sensitivity of 65% (95% CI = 50% – 80%) and a diagnostic specificity of 100% (95% CI = 100% – 100%).

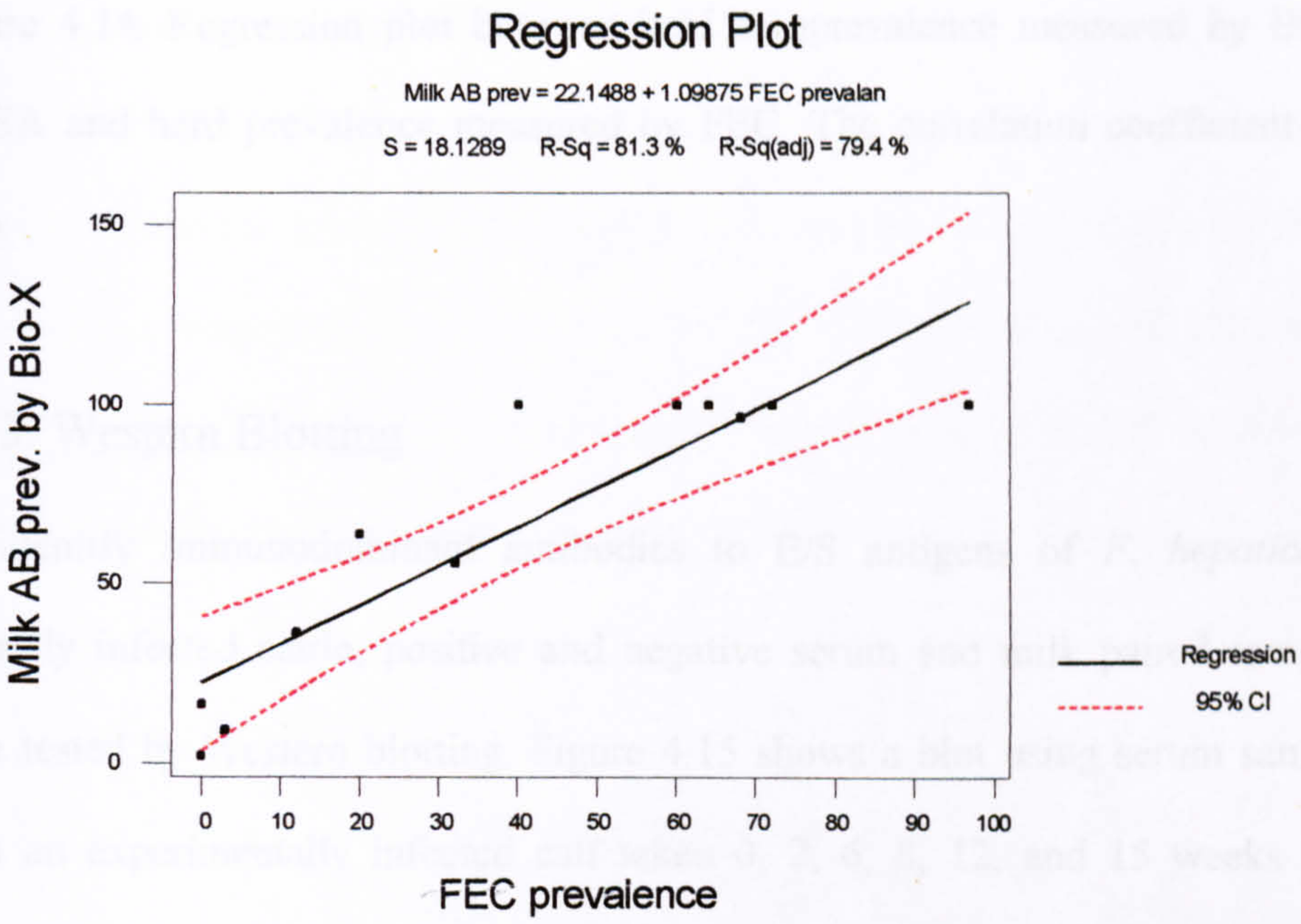

Figure 4.13. Regression plot between herd prevalence measured by Bio-X milk antibody test and herd prevalence measured by faecal egg count. The correlation coefficient was 90%.

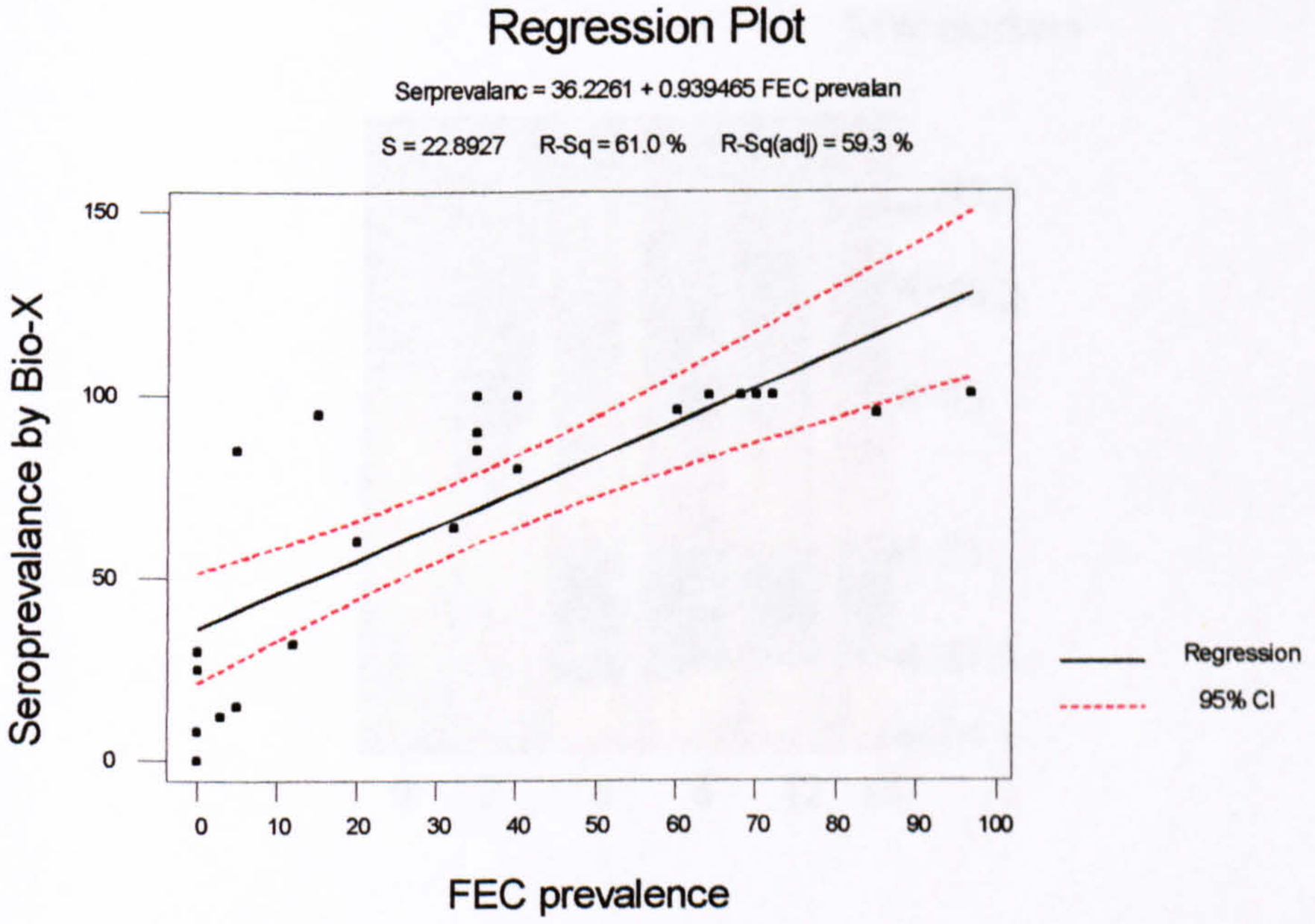

Figure 4.14. Regression plot between herd seroprevalence measured by Bio-X ELISA and herd prevalence measured by FEC. The correlation coefficient was 78%.

4.3.3. Western Blotting

To identify immunodominant antibodies to E/S antigens of *F. hepatica* in naturally infected cattle, positive and negative serum and milk paired samples, were tested by Western blotting. Figure 4.15 shows a blot using serum samples from an experimentally infected calf taken 0, 2, 6, 8, 12, and 15 weeks after infection. Bands were detected of 21, 28-30, 45, 55, 66 and 97 kDa relative molecular mass. The major immunodominant band had a relative molecular mass of 28 – 30 kDa.

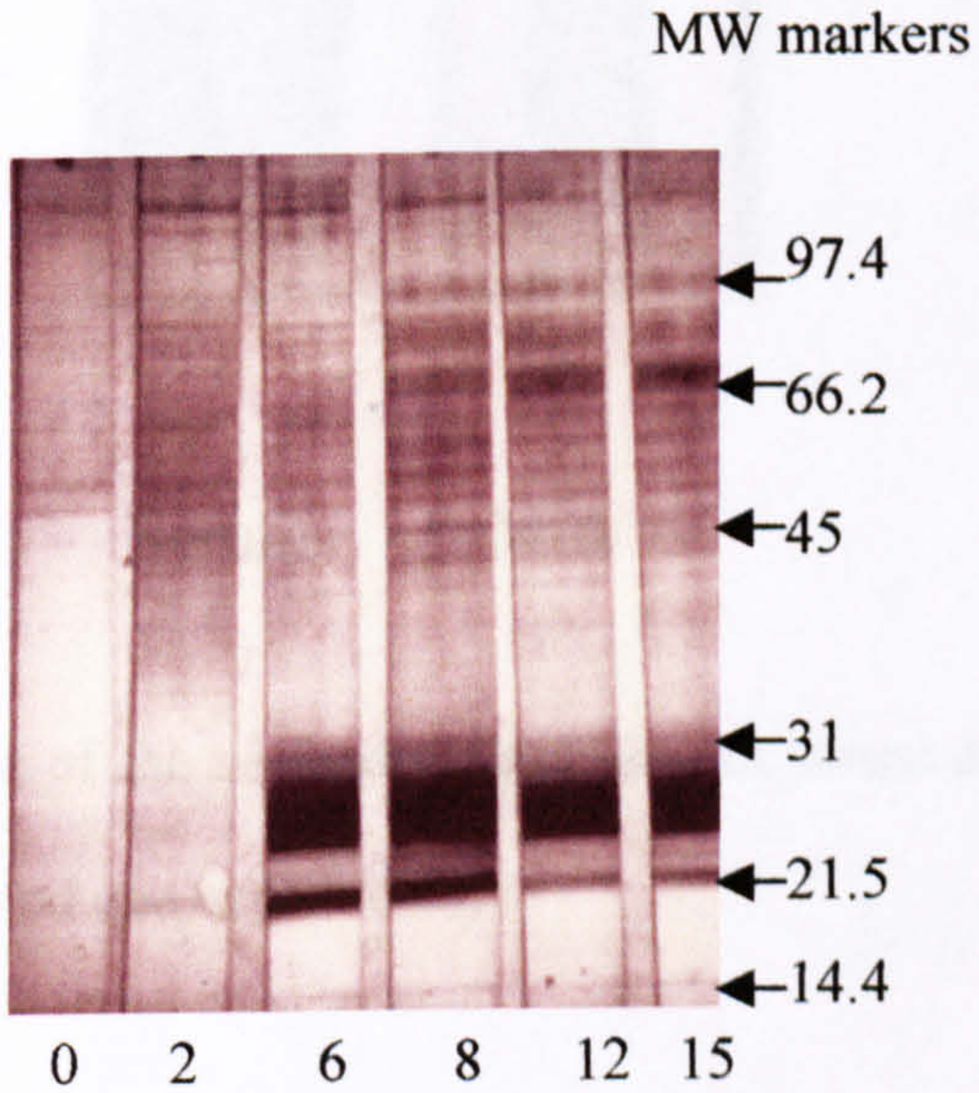

Figure 4.15. Western blotting using serum samples from an experimentally infected calf at 0, 2, 6, 8, 12, and 15 weeks after infection.

4.3.3.1. Comparison of specific anti-*F. hepatica* antibody responses in serum and milk. Figure 4.16 shows a blot using two positive and one negative serum samples compared to their corresponding milk samples. Six bands were detected of molecular weights of 14.4 – 97.4 kDa (14, 28-30, 45, 55, 66 and 97 kDa) using both milk and serum and these corresponded to the six major bands detected by the sera from the experimentally infected cow.

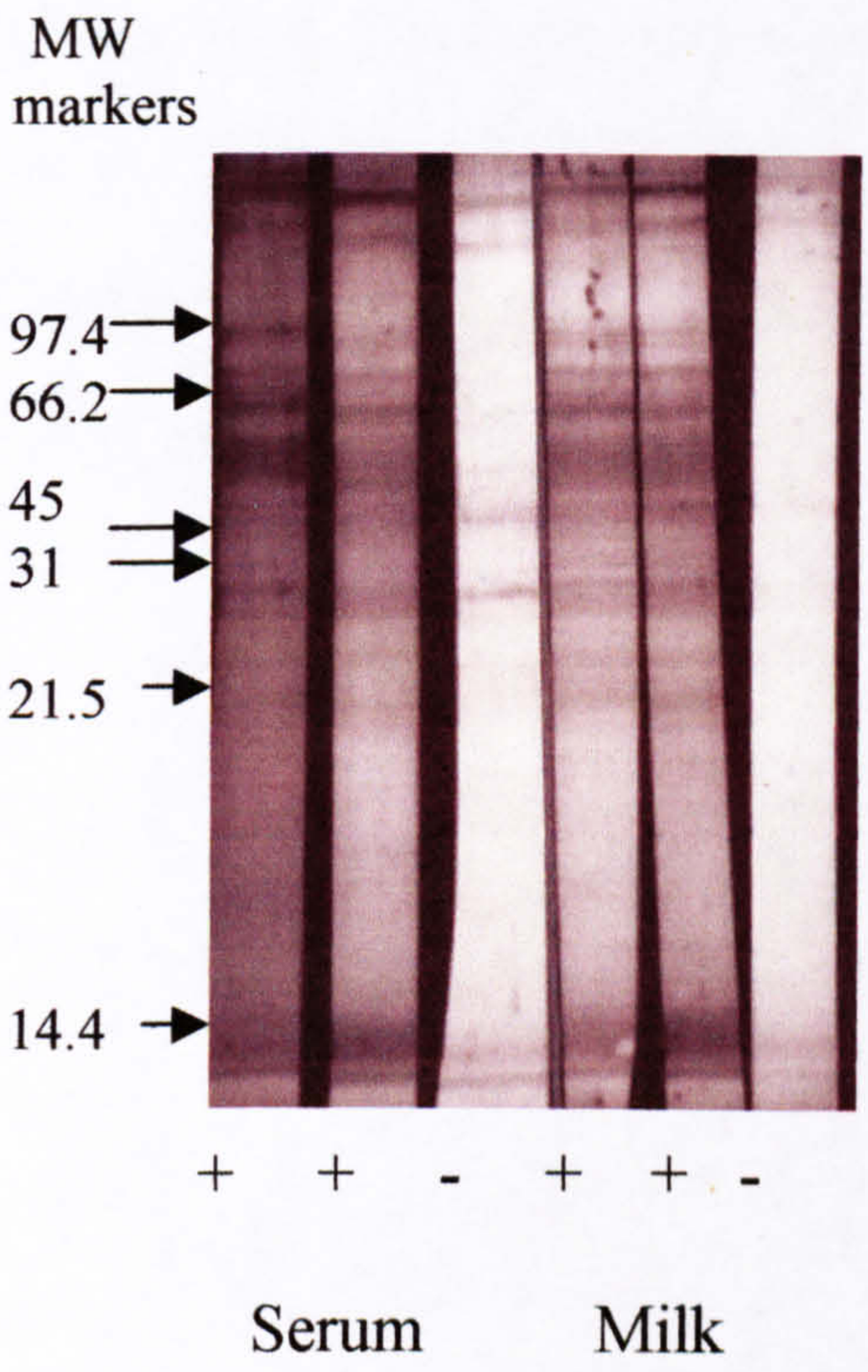

Figure 4.16. Comparison of the antibody population in serum and milk samples from two positive cows and one negative cow.

4.3.3.2. Analysis of *F. hepatica* specific antibody isotypes present in milk and serum.

To determine which antibody isotypes were present in milk, IgG1 and IgG2 specific conjugates were used in Western blots. One serum sample and the matching milk sample from a positive and a negative cow were tested and the results are shown in figure 4.17 and 4.18. Milk samples were diluted 1:2 (Figure 4.17) and serum samples were diluted 1:100 (Figure 4.18). Antibodies of both the IgG1 and IgG2 isotypes were detected in both serum and milk. Bands were detected of 21, 28-30, 45, 55, 66 and 97.4 kDa relative molecular mass. The major immunodominant band in IgG1 had a relative molecular mass of 28-30 kDa and in IgG2 had a molecular mass of 66 kDa.

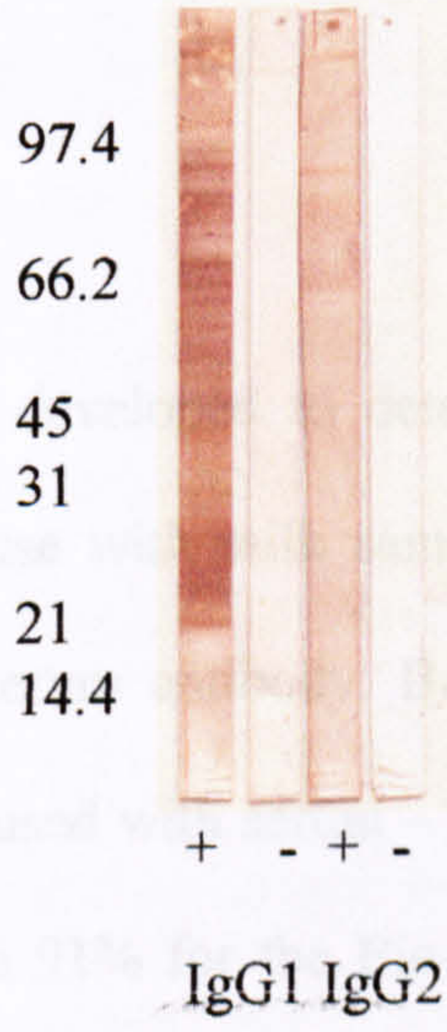

Figure 4.17. Comparison of the antibody isotype in milk samples from one positive and one negative cow.

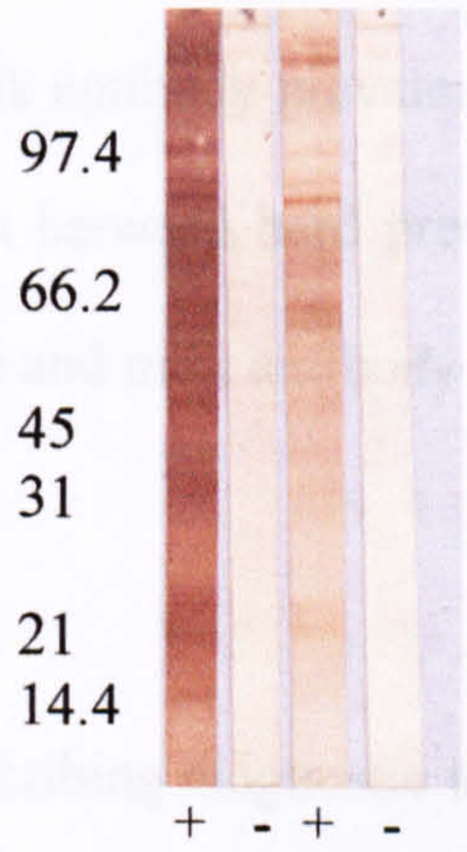

Figure 4.18. Comparison of the antibody isotype in serum samples from one positive and one negative cow.

4.4. Discussion

We have adapted two ELISAs developed to detect serum antibodies in cattle infected with *F. hepatica*, for use with milk samples and have established the correlation between milk and serum antibody. Both tests had good diagnostic sensitivity and specificity when used with serum – 98% and 96% respectively for the LSTM ELISA and 98% and 91% for the Bio-X ELISA (Chapter 3). Using LSTM milk ELISA, the diagnostic sensitivity was 92% (95% CI = 89% - 96%) and diagnostic specificity was 88% (95% CI = 85% - 91%). The correlation coefficient between milk antibody prevalence and seroprevalence was 96% and the correlation coefficient between prevalence measured by faecal egg count and both seroprevalence and milk antibody prevalence within the herd was 87%. In

the Bio-X milk ELISA, the diagnostic sensitivity was 72% (95% CI = 67% - 76%) and diagnostic specificity was 93% (95% CI = 90% - 95%). The correlation coefficient between Bio-X milk antibody prevalence and seroprevalence was 83% and the correlation coefficient between herd prevalence measured by faecal egg count and both seroprevalence and milk antibody prevalence within the herd were 78% and 90% respectively.

To our knowledge reports describing diagnostic tests for *Fasciola* infection using milk are limited. Boulard et al. (1985) found a relationship between serum and milk antibody levels for *Fasciola* infection. Kloosterman et al. (1993) reported that antibody levels in milk and serum against nematodes are correlated and the correlation is moderately strong, varying from 0.41 to 0.67, depending on the worm species, the type of samples, and milk yield. Boulard (1985) found a correlation of 0.444 between serum and milk from 23 cows for antibodies to *Hypoderma* spp. and Elvander et al. (1995) demonstrated a good correlation (96% agreement) between the ability to detect antibodies to bovine respiratory syncytial virus (BRSV) in serum and the ability to detect them in milk, although the antibody titre was generally lower in milk than in serum. The majority of IgG antibodies in milk are transported from serum to the mammary gland and the remainder is produced locally (Norcross, 1982). The concentration of IgG in milk correlates with serum levels (Caffin et al., 1983; Caffin and Poutrel, 1988; Smith et al., 1989). Thus milk is a good medium for testing animal's exposure to many pathogens although the immunoglobulin level in milk is 20 to 40 times lower than in serum (Mach and Pahud, 1971; Caffin et al., 1983). This means that milk

should be tested at lower dilutions compared to serum. In our test milk is tested at a dilution at 1:2 whereas serum is tested at 1:800.

The results of the Western blot analysis showed that the specificity of antibody responses in milk samples were similar to their corresponding serum samples, although apparently weaker. Also the antibody isotype in milk appeared to be predominantly IgG1. Research has also shown that the antibody response to *F. hepatica* in serum of both naturally and experimentally infected cattle is predominantly an IgG1 (Bossaert et al., 2000; Ortiz et al., 2000; Hoyle et al., 2003). Naive cattle experiencing a primary infection and chronically infected cattle produced specific antibodies predominantly of the IgG1 isotype (Clery et al., 1996; Mulcahy et al., 1998) and the IgG2 response is very low or even absent (Duffus and Franks, 1981; Clery et al., 1996; Mulcahy et al., 1998). Similarly Hoyle et al. (2003) found a predominantly IgG1 response to different fluke antigens irrespective of how the cattle had been sensitized.

Pritchard et al. (2002) reported that the diagnostic sensitivity and specificity of milk ELISA for infectious bovine rhinotracheitis virus (IBRV), using serum ELISA as the definitive gold standard, were 96% and 93% respectively and for the bovine viral diarrhoea virus (BVDV), the corresponding values were 97% and 93%. They noted that there was no evidence that the stage of lactation had a significant effect on the sensitivity of the test. Beaudeau et al. (2001) reported that the sensitivity and specificity of ELISA test for BVDV applied to milk, compared with serum test, were 95% and 98% respectively. Armstrong et al. (2000) reported that diagnostic sensitivity and specificity of milk ELISA for Foot-and-Mouth

Disease (FMD), using a cut off value, were 92% and 96% respectively. A relationship has been demonstrated between serum and milk antibody levels for other organisms such as *Brucella* (Nielsen et al., 1996; Vanzini et al., 1998), *Mycobacterum avium paratuberculosis* (Winterhoff et al., 2002) and FMD (Armstrong, 1997). These results suggest that milk ELISA's are an effective alternative to serum ELISA's for diagnostic and surveillance purposes but are more cost-effective since veterinarians are not required to collect milk samples and farmers can submit samples directly to regional laboratories.

In conclusion, the LSTM milk ELISA performed well and was more sensitive than the Bio-X milk ELISA. Importantly, there was no evidence that the stage of lactation had a significant effect on the sensitivity of the test. The LSTM milk ELISA is suitable for routine veterinary diagnostic use as an alternative to testing sera in lactating animals.

Chapter Five

Development of a bulk tank milk ELISA

5.1. Introduction

Fasciola hepatica (Liver fluke), is a trematode parasite, particularly of ruminants, that is found throughout the world. Disease caused by *F. hepatica* has a major impact on productivity. It is estimated that losses due to fasciolosis are approximately US$2000 million annually (Spithill et al., 1999; Mulcahy and Dalton, 2001). Accurate and early diagnosis is necessary to implement control programmes to minimise the economic cost of the disease. Diagnosis is normally based on the observation of eggs of *Fasciola* in faeces (Boray, 1985), or serological tests for the detection of parasitic antigens (Dumenigo et al., 1996) or specific antibodies in serum samples (Hillyer and Santiago de Weil 1979, Zimmerman et al., 1982, 1985). Fasciolosis in cattle in England and Wales is increasing in significance; the number of cases of fasciolosis diagnosed in cattle in submissions to the VLA has risen from 4.5% in 1993 to 14.2% in 2002 in cattle (VLA report, 2002c).

In dairy cattle clinical cases of fasciolosis are rarely reported, but outbreaks of *Salmonella dublin* frequently occur and increasingly what were considered to be metabolic diseases in dairy cattle are now being diagnosed as associated with fluke infection (Daniel and Mitchell, 2002).

Bulk tank milk (BTM) tests are widely used as a means of identifying herds infected with pathogens including BVDV (Niskanen, 1993; van Wuijckhuise et al., 1998; Pritchard, 1998), BHV-1(Hartman et al., 1997; Nylin et al., 2000; Stahl et al., 2002), IBRV (Pritchard et al., 2002), FMD (Armstrong and Mathew, 2001)

and Johne's disease (Winterhoff et al., 2002). As yet there are no reported BTM tests for *Fasciola* infection. Here we report the modification and validation of the LSTM ELISA and the commercially available Bio-X ELISA to test bulk tank milk samples.

5.2. Materials and Methods

5.2.1. Samples

A total of 1561 individual sera and milk samples from 61 different farms were collected (Chapter 4). Also 715 faeces samples from 27 of these farms were collected. Twenty – 34 cattle were sampled on each farm. The range of herd size was between 30 and 374 and the mean herd size was 107. Details of age, parity and days into lactation were recorded at the time of sampling and a bulk tank milk sample was also collected. The bulk tank milk samples were centrifuged at 1000g for 20 minutes in order to separate milk from the fat and any cream and then were aliquoted and stored at –20°C until use.

5.2.2. Statistic analysis

The Pearson correlation coefficient, regression plots, and analysis of all data were calculated by using Minitab V. 13.32 for Windows. The diagnostic sensitivity and specificity and the 95% Confidence Intervals were calculated using WinEpiscope 2.0 (N. de Blas, C. Ortega, K. Frankena, J. Noordhuzen, M. Thrusfield: http://www.clive.ed.ac.uk/winepiscope).

5.3. Results

5.3.1. Results of the validation and evaluation of the LSTM bulk tank milk (BTM) ELISA

5.3.1.1. Development of the LSTM BTM ELISA.

Sera, milk and bulk tank milk samples were initially collected from 21 farms and the correlation coefficients between seroprevalence, milk antibody prevalence and the bulk tank milk percent positivity (PP) value at three different milk dilutions (neat, 1:2 and 1:5) were calculated (Tables 5.1 and 5.2) to obtain the optimum bulk tank milk dilution.

Table 5.1. Initial results of the Pearson correlation coefficient between bulk tank milk PP value at three different dilutions and seroprevalence within the herd.

LSTM ELISA	Correlation
Seroprevalence / Bulk tank milk(neat)	0.969
Seroprevalence / Bulk tank milk(1:2)	0.945
Seroprevalence / Bulk tank milk (1:5)	0.934

Table 5.2. Initial results of the Pearson correlation coefficient between bulk tank milk PP value at three different dilutions and milk antibody prevalence within the herd.

LSTM ELISA	Correlation
Milk AB preval. / Bulk tank milk (neat)	0.969
Milk AB preval. / Bulk tank milk (1:2)	0.884
Milk AB preval. / Bulk tank milk (1:5)	0.876

The results suggest that there is a significant (P<0.001) correlation between the PP value for neat bulk tank milk and seroprevalence and milk antibody prevalence within the herds. Subsequently all bulk tank milk samples were tested neat in the LSTM ELISA.

5.3.1.2. Validation of the LSTM BTM ELISA

5.3.1.2.1. Defining a cut off value: Bulk tank milk samples from 61 different farms were collected and were tested with LSTM ELISA. To define the optimum cut off value for LSTM bulk tank milk ELISA, an in-herd seroprevalence of 25% was chosen. This means that a herd with equal to and more than a 25% seroprevalence was considered to be positive and less than 25% considered negative. This cut off was used to indicate the herds true infection status for calculation of diagnostic sensitivity and specificity. The sensitivity and specificity of different bulk tank milk PP cut off values were calculated. The results indicated that a cut off value of 27 PP had a diagnostic sensitivity of 93% and a diagnostic specificity of 81% (Table 5.3 and Figure 5.1).

Table 5.3. The diagnostic sensitivity and specificity with 95% confidence intervals for different values for 61 herds.

LSTM BTM PP value	Sensitivity (95% CI)	Specificity (95% CI)
20	100% (100 - 100)	48% (29 - 67)
25	96% (90 - 100)	78% (62 - 93)
26	93% (83 - 100)	78% (62 - 93)
27*	93% (83 - 100)	81% (67 - 96)
28	89% (78 - 100)	81% (67 - 96)
29	86% (73 – 99)	85% (72 – 99)
30	86% (73 - 99)	85% (72 - 99)
35	75% (59 - 91)	85% (72 - 99)
40	64% (47 - 82)	96% (89 - 100)
45	50% (31 - 69)	96% (89 - 100)
50	46% (28 - 65)	100% (100 - 100)

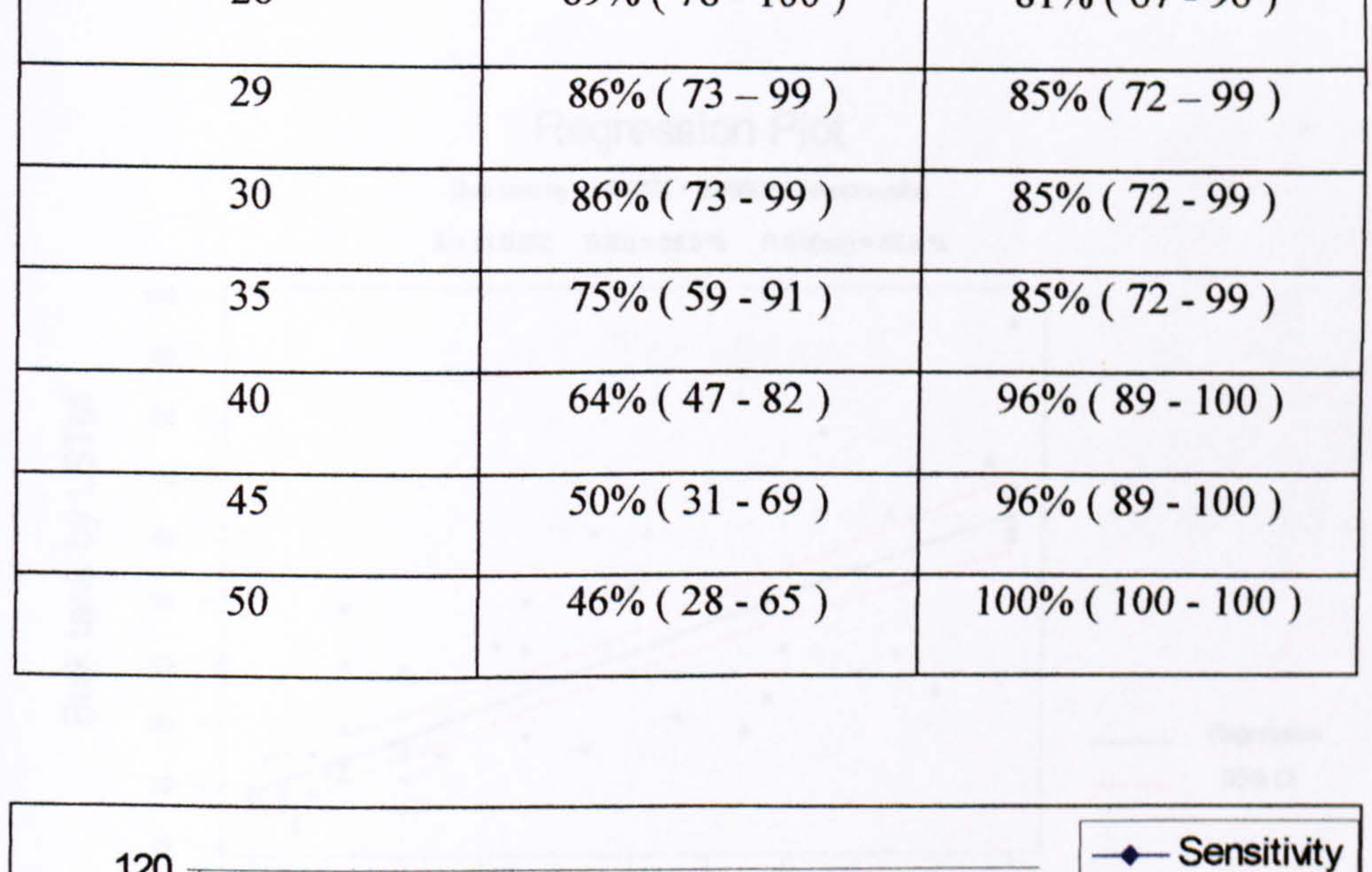

Figure 5.1. The sensitivity and specificity of different bulk tank milk PP values.

5.3.1.2.2. Comparing the prevalence of infection within each herd measured by serum, milk, and faeces with the bulk tank milk percent positivity value.

The correlation coefficient between bulk tank milk PP values and seroprevalence, milk antibody prevalence and prevalence measured by faecal egg counts for 61 herds was calculated by Minitab version 13 and the results are shown in figure 5.2, 5.3, 5.4 and summarised in Table 5.4.

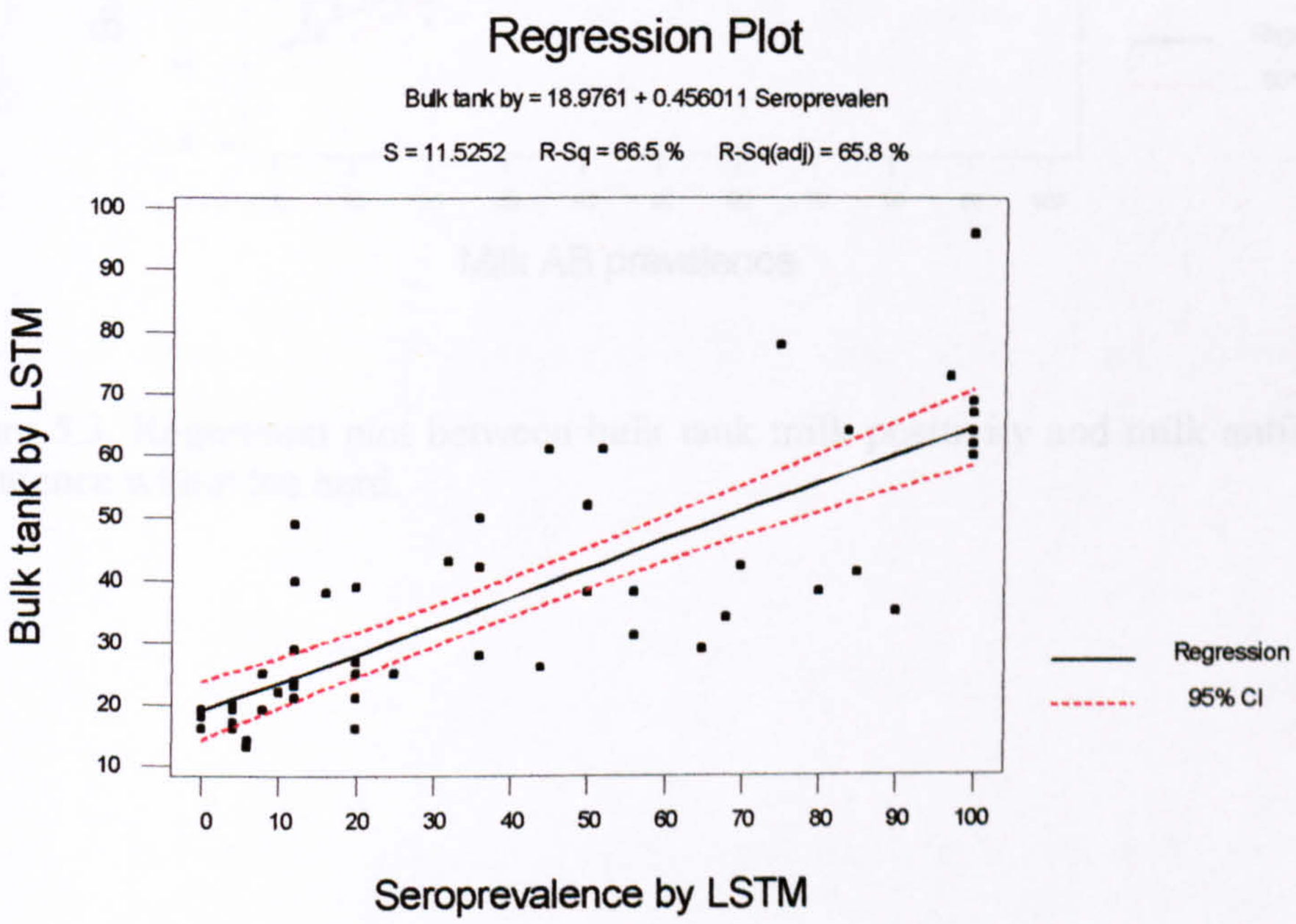

Figure 5.2. Regression plot between bulk tank milk positivity and seroprevalence within the 61 herds.

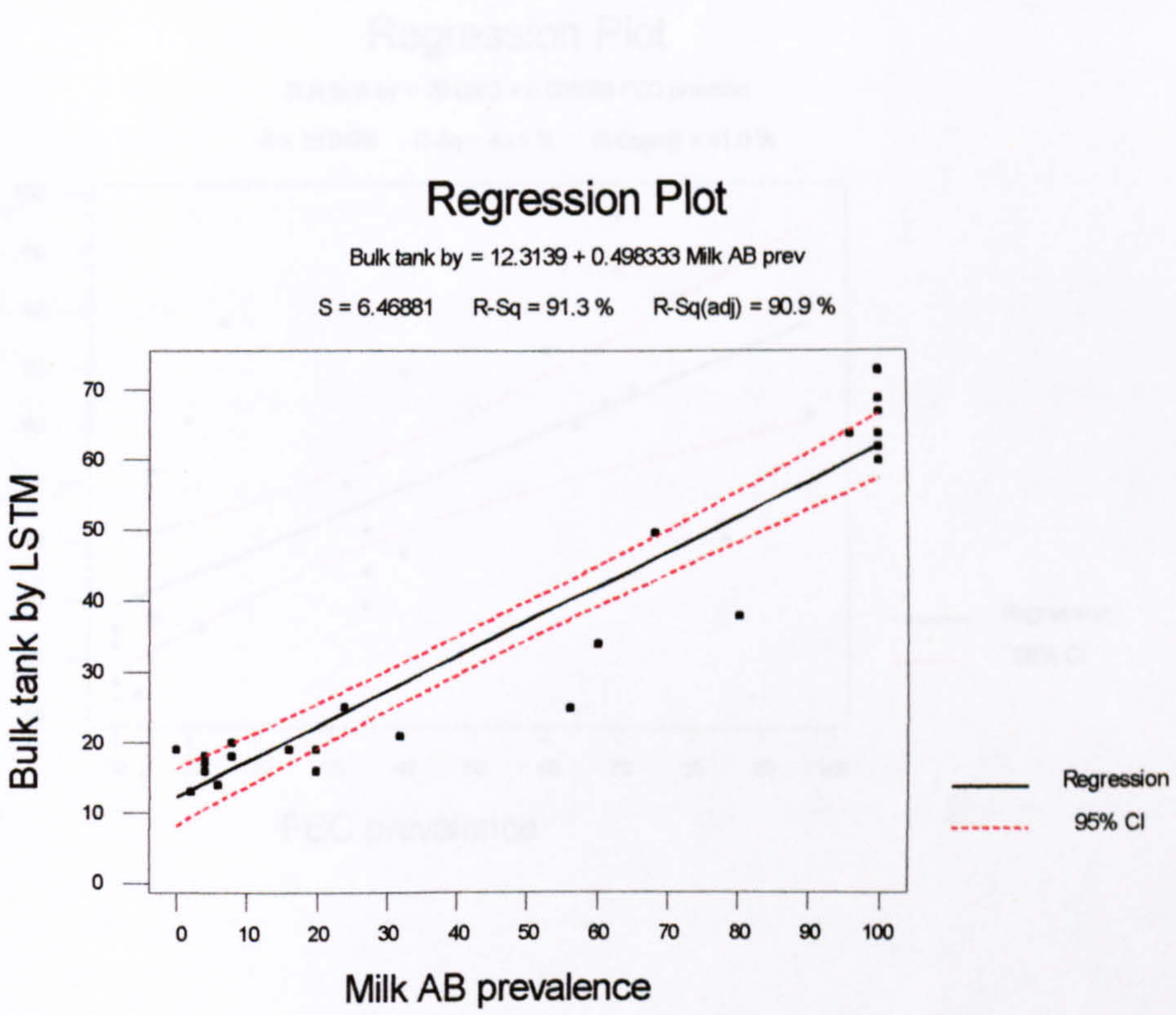

Figure 5.3. Regression plot between bulk tank milk positivity and milk antibody prevalence within the herd.

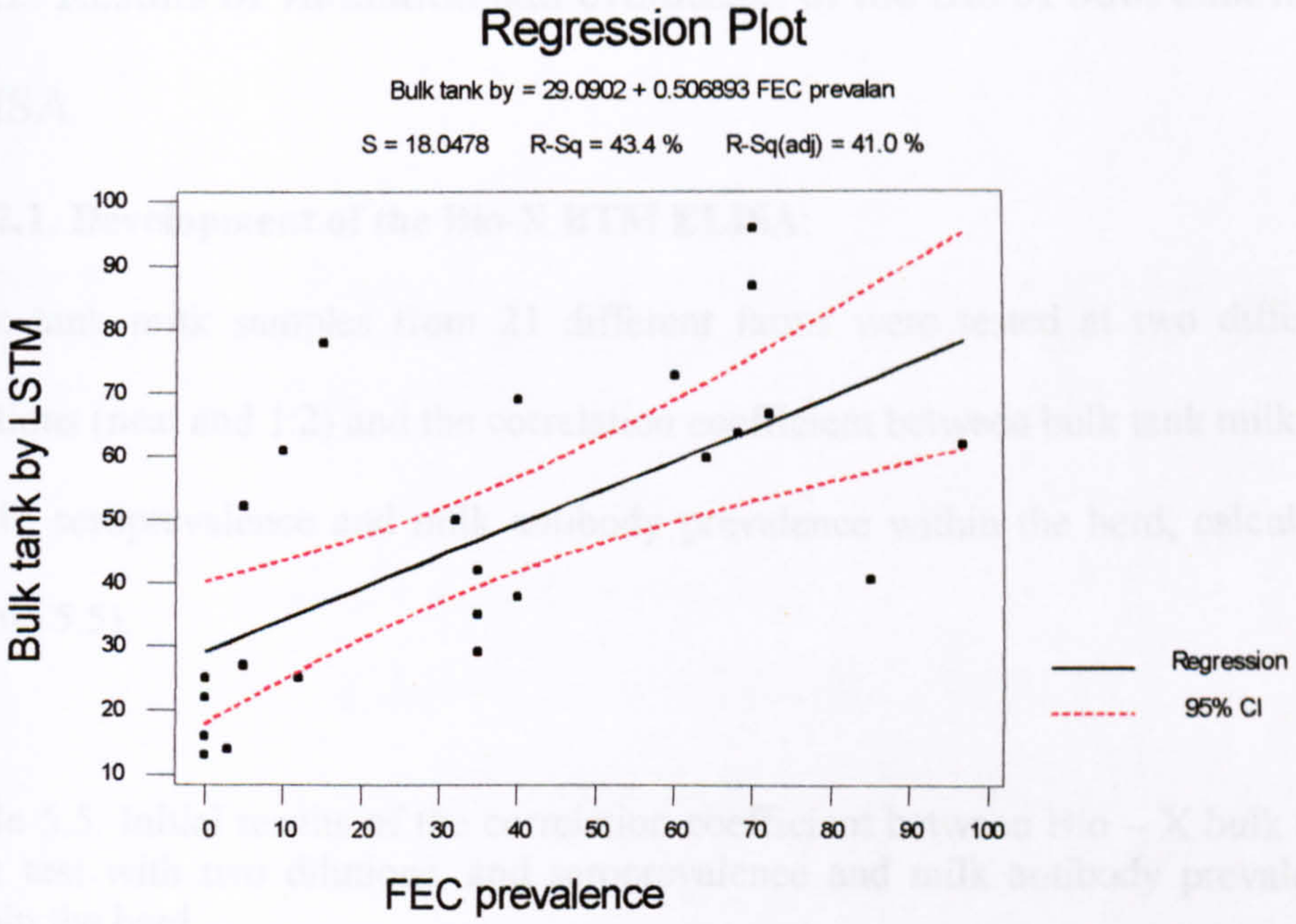

Figure 5.4. Regression plot between bulk tank milk positivity and prevalence measured by faecal egg count.

Table 5.4. The Pearson correlation coefficient between bulk tank milk positivity and seroprevalence, milk antibody prevalence, and prevalence of infection measured by faecal egg count within the herd.

LSTM ELISA	Correlation
Bulk tank milk / Seroprevalence	83%
Bulk tank milk / Milk AB prevalence	96%
Bulk tank milk / FEC prevalence	66%

5.3.2. Results of validation and evaluation of the Bio-X bulk tank milk ELISA

5.3.2.1. Development of the Bio-X BTM ELISA:

Bulk tank milk samples from 21 different farms were tested at two different dilutions (neat and 1:2) and the correlation coefficient between bulk tank milk test result, seroprevalence and milk antibody prevalence within the herd, calculated (Table 5.5).

Table 5.5. Initial results of the correlation coefficient between Bio – X bulk tank milk test with two dilutions, and seroprevalence and milk antibody prevalence within the herd.

Bio - X	Correlation
Seroprevalence / Bulk tank milk(neat)	0.793
Seroprevalence / Bulk tank milk(1:2)	0.777
Milk AB preval. / Bulk tank milk (neat)	0.922
Milk AB preval. / Bulk tank milk (1:2)	0.962

These results suggested that there is a good correlation between milk antibody prevalence and bulk tank milk PP value at a 1:2 dilution. All bulk tank milk samples subsequently were tested at a 1:2 dilution with Bio–X ELISA.

5.3.2.2. Validation of the Bio-X BTM ELISA

5.3.2.2.1: Evaluation of Bio-X bulk tank milk ELISA

The correlation coefficient between bulk tank milk positivity and the seroprevalence, milk antibody prevalence and the prevalence of infection

measured by faecal egg count for each herd were calculated and are shown in table 5.6. The regression plots are shown in figures 5.5, 5.6 and 5.7.

Table 5.6. The correlation coefficient between bulk tank milk positivity and seroprevalence, milk antibody prevalence, and prevalence of infection measured by faecal egg count within the herd.

Bio - X ELISA	Correlation
Bulk tank milk / Seroprevalence	73%
Bulk tank milk / Milk AB prevalence	95%
Bulk tank milk / FEC prevalence	83%

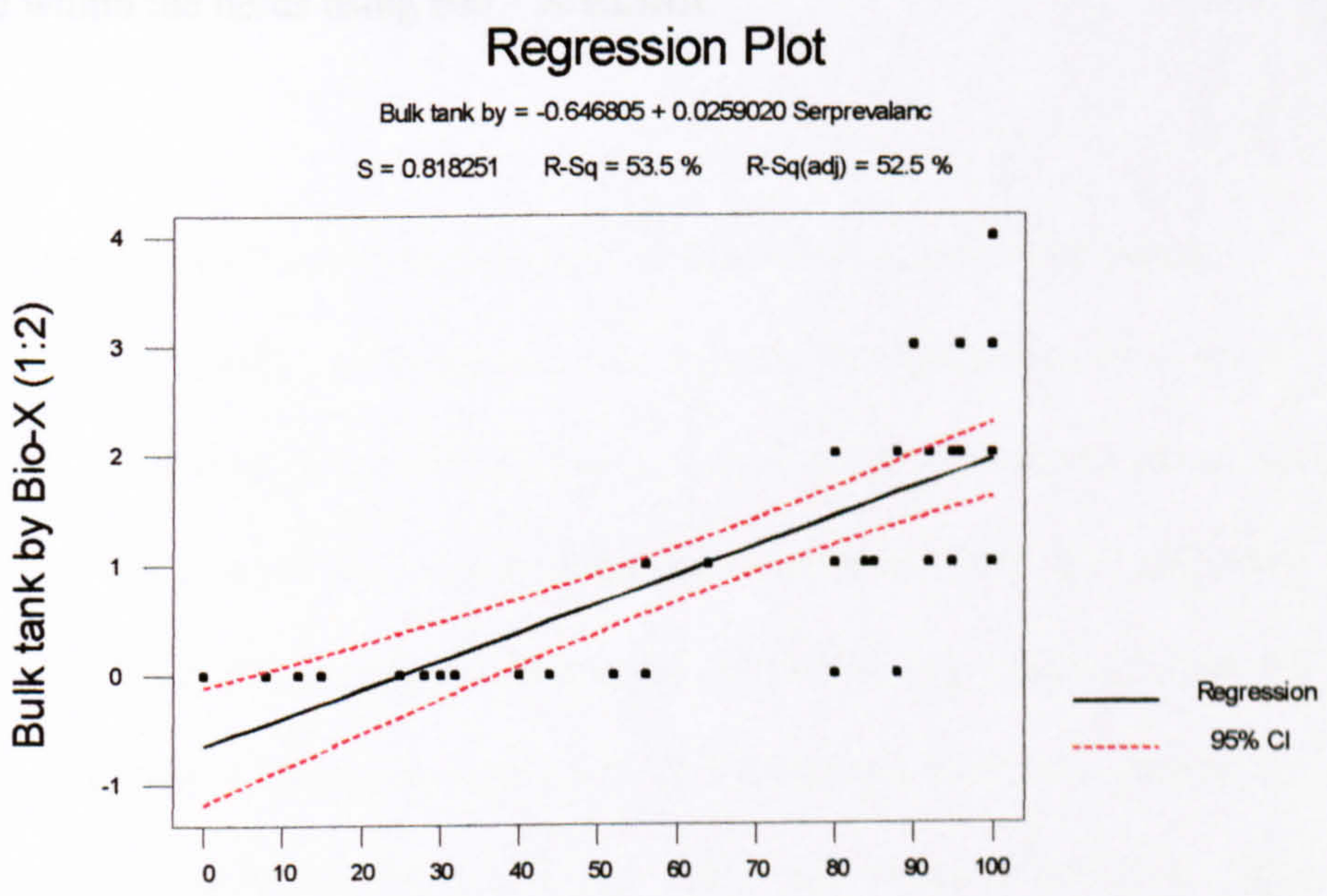

Figure 5.5. Regression plot between bulk tank milk positivity measured by Bio-X ELISA and seroprevalence within the herd.

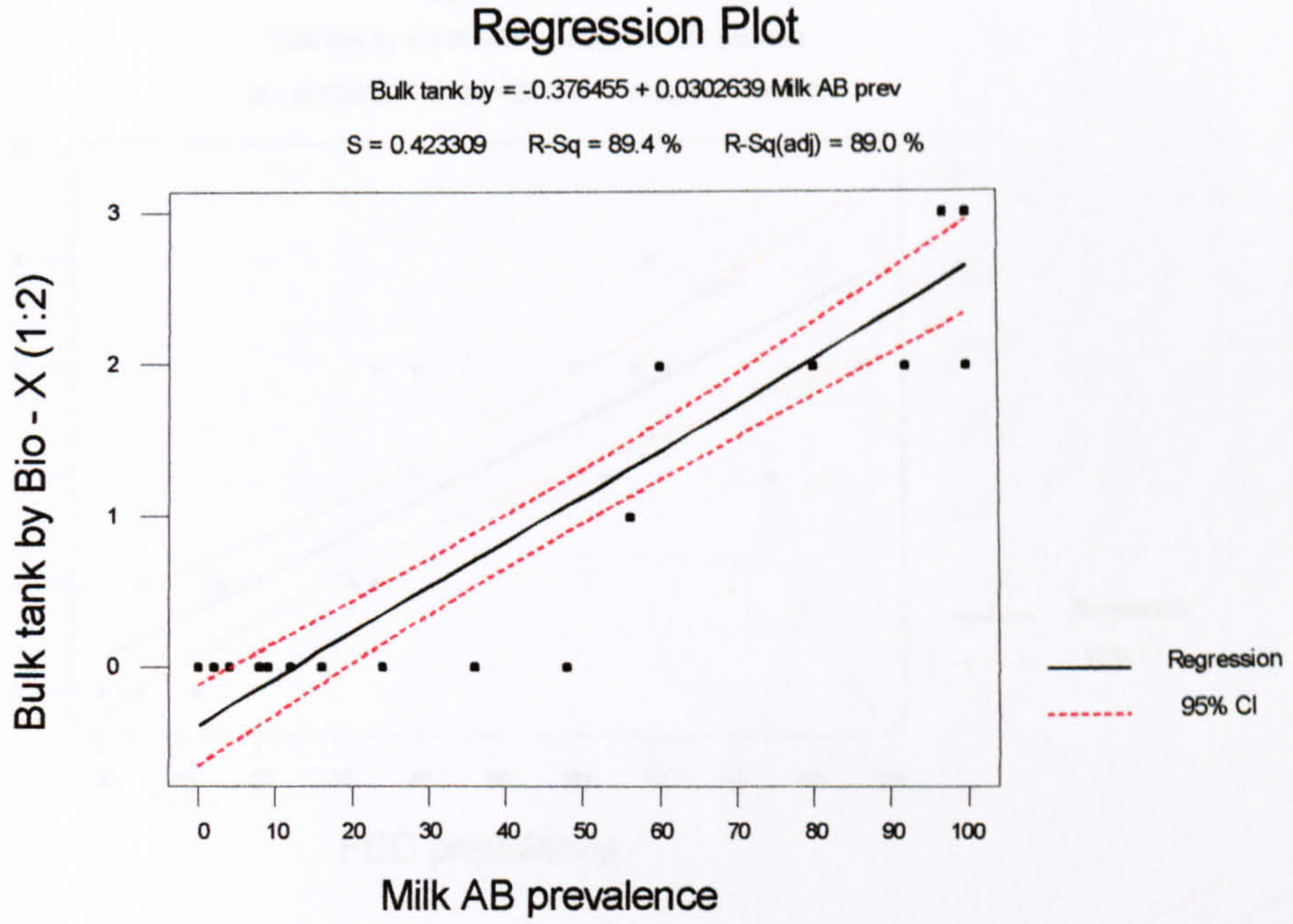

Figure 5.6. Regression plot between bulk tank milk positivity and milk antibody prevalence within the herds using Bio – X ELISA.

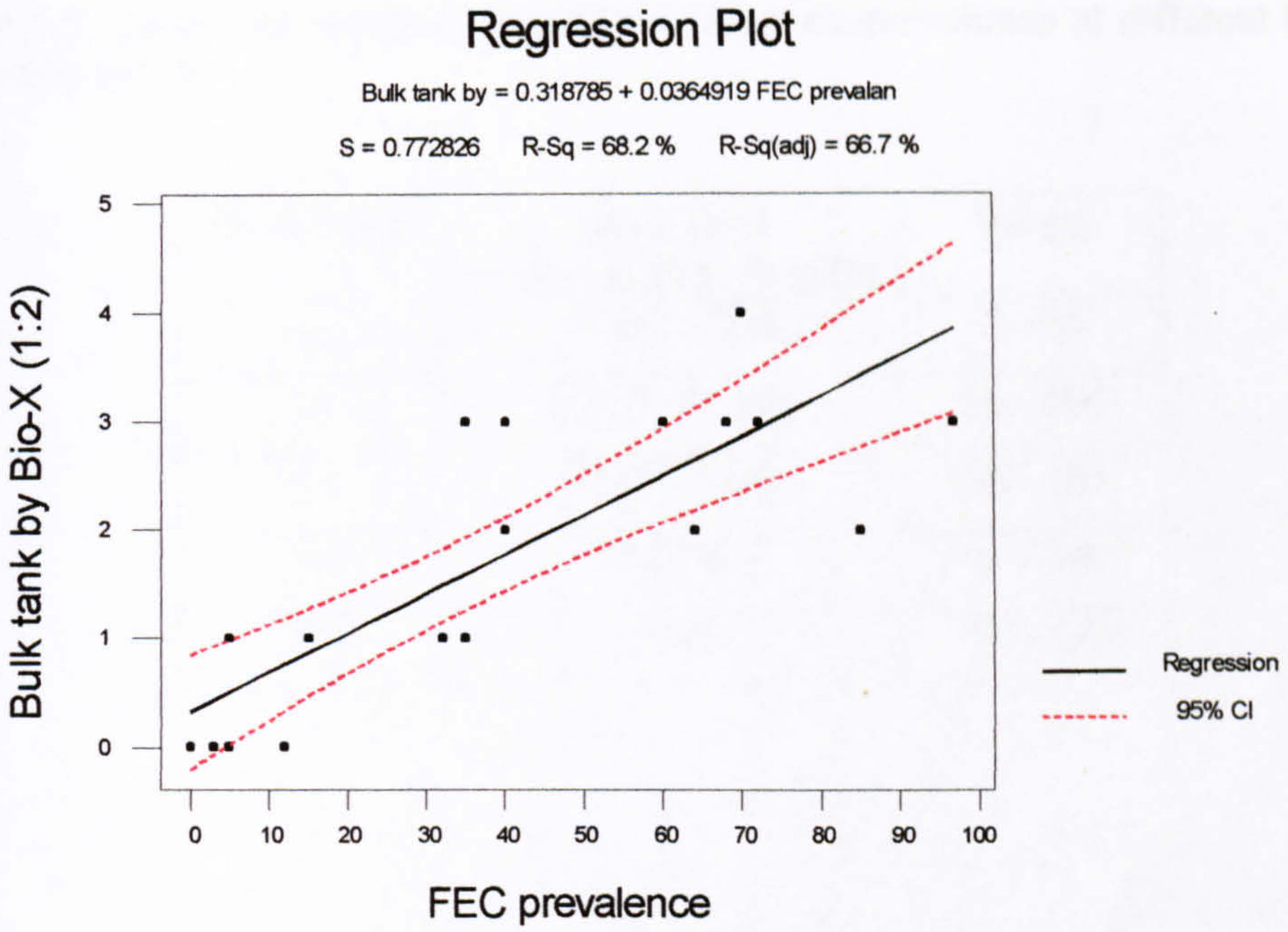

Figure 5.7. Regression plot between bulk tank milk positivity by the Bio - X test and prevalence of infection measured by faecal egg count.

5.3.2.2.2: Prevalence of infection assessed by Bio-X bulk tank milk result

The Bio–X ELISA results are interpreted on a scale of 0 (negative) to 4+ (1+ - 4+ are considered to be positive). A BTM value of 1+ or above was used to indicate a positive herd. The ranges of seroprevalence is shown in table 5.7 and milk antibody prevalence and prevalence measured by faecal egg counts compared with seroprevalence, are shown in figure 5.8. To calculation diagnostic sensitivity and specificity for the Bio-X BTM ELISA, a herd with equal to and more than 25% seroprevalence was considered to be positive and less than 25% considered negative. The results indicated that a value of 1+ had a diagnostic sensitivity of 65% (95% CI = 50% – 80%) and a diagnostic specificity of 100% (95% CI = 100% – 100%).

Table 5.7. Mean and standard deviation of herd seroprevalence at different bulk tank milk values.

BTM value	Mean herd seroprevalence % (SD)	Range
0	36.48 (27.53)	0 - 88
1+	83.73 (13.48)	56 - 100
2+	90.14 (7.84)	80 - 100
3+	98 (3.83)	90 - 100
4+	100	100 - 100

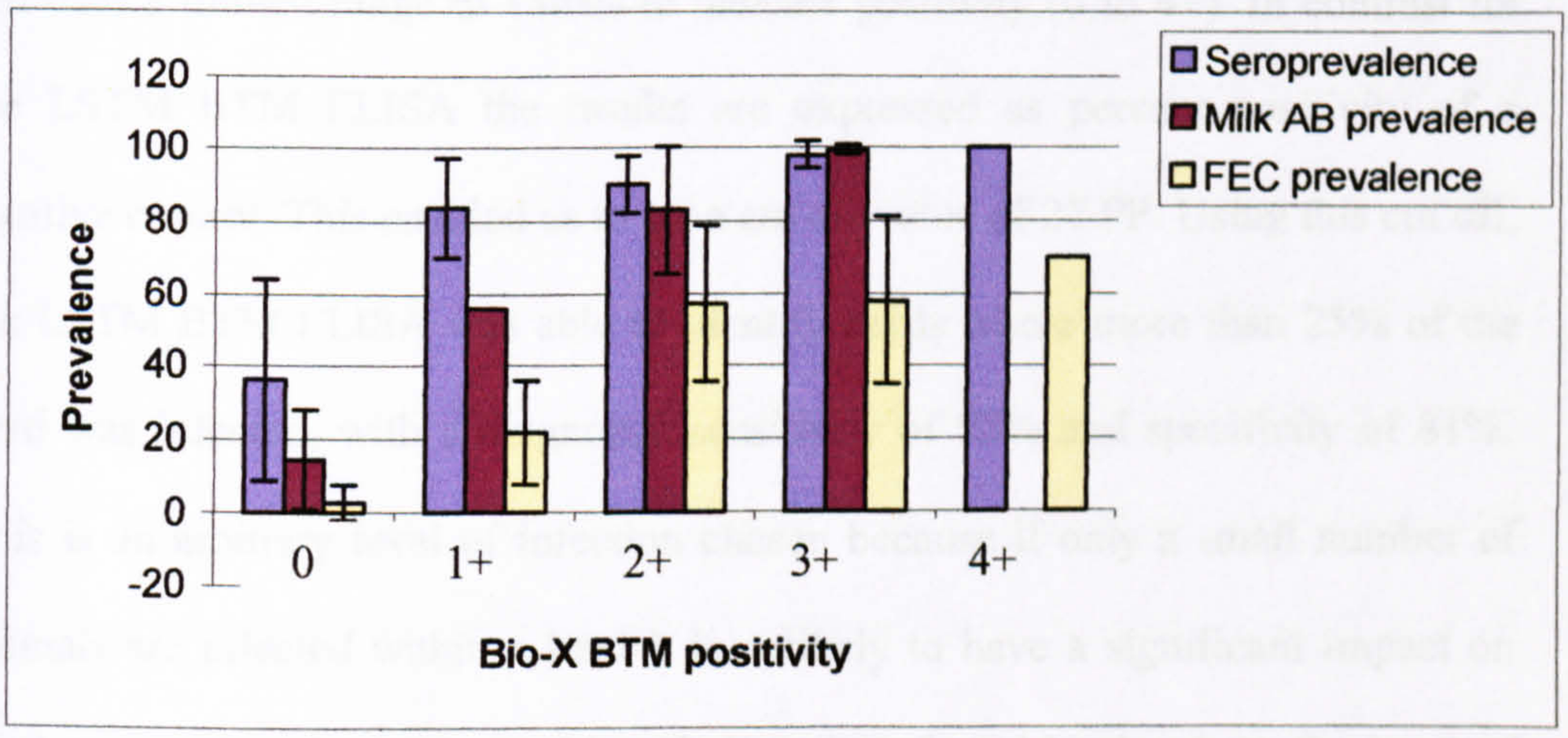

Figure 5.8. The comparison of seroprevalence, milk antibody prevalence, and prevalence of infection measured by faecal egg count within herds with different bulk tank milk values.

5.4. Discussion

We have adapted two ELISAs, initially developed to detect serum antibodies in cattle infected with *F. hepatica*, for use with bulk tank milk samples and have established the agreement between the bulk tank milk values and the proportion of positive animals within a herd. Both tests had good diagnostic sensitivity and specificity when used with serum – 98% and 96% respectively for the LSTM ELISA and 98% and 91% for the Bio-X ELISA (Chapter 3). The prevalence of infection within each of the 61 herds was established using individual serum, milk or faecal egg count. The correlation between the LSTM BTM ELISA and herd seroprevalence was higher (83%) compared to the Bio-X ELISA (73%). In addition, the mean LSTM BTM ELISA value was higher than Bio-X BTM ELISA ($P<0.001$). It is likely that the reason for this discrepancy is that the Bio-X test uses a limited range of values to indicate positivity (0 to 4+). In contrast for the LSTM BTM ELISA the results are expressed as percent positivity of a positive control. This enabled us to set a cut off value of 27 PP. Using this cut off, the LSTM BTM ELISA was able to identify herds where more than 25% of the herd was infected, with a diagnostic sensitivity of 93% and specificity of 81%. This is an arbitrary level of infection chosen because if only a small number of animals are infected within a herd it is unlikely to have a significant impact on productivity. It is important to identify heavily infected herds where the level of infection may result in disease, or loss of productivity. In this study 51% of herds (31 herds out of 61 herds) had a seroprevalence below 25%.

The difference between the results for the LSTM and Bio-X tests may also be due to the dilution of milk used in each test – neat for the LSTM ELISA and 1:2 for the Bio – X test. However these dilutions were chosen to give the optimum sensitivity and specificity using a sub sample of herds. The correlation between faecal egg count and LSTM BTM result was 66% whereas the correlation between faecal egg count and Bio-X bulk tank milk was 83%. This supports the results for diagnostic sensitivity and specificity obtained for the two tests. The diagnostic sensitivity for the Bio-X test was 65% and for the LSTM ELISA, was 93%. In contrast the specificity of the Bio-X test was 100% compared to the LSTM test of 81%. The sensitivity of faecal egg counts is generally considered to be low- 66.7% (Anderson et al., 1999) but the specificity is very high. Similarly the Bio-X test has a low sensitivity but good specificity.

To our knowledge this is the first report of the validation of a bulk tank milk test for fasciolosis. Sanchez et al. (2002) reported that a bulk tank milk ELISA could be used as a monitoring tool for *Ostertagia ostertagi* infection and showed that it was possible to relate different BTM antibody levels to different infection levels within herds. Bulk tank milk ELISAs have also been used for monitoring and immunoepidemiological surveys of *Hypoderma* spp. in cattle (Frangipane di Regalbono et al., 2003). Research on bovine viral diarrhoea virus (BVDV) infection status in cattle herds revealed that there is a relationship between the herd infection status, based on serum sampling, and the bulk tank milk ELISA result (Bitsch and Ronsholt, 1995; Houe et al., 1995; Bitsch et al., 1997). Bulk tank milk sampling is now used in several countries for screening of dairy populations for BVDV (Niskanen et al., 1991; Niskanen, 1993; Radwan et al.,

1995; Paton et al., 1998; Lindberg and Alenius, 1999; Melendez and Donovan, 2003). Bulk tank milk samples have also been used to screen for bovine herpesvirus-1 (Hartman et al., 1997; Nylin et al., 2000; Stahl etal., 2002), Foot-and-Mouth Disease (Armstrong and Mathew, 2001), bovine respiratory syncytial virus (Elvander et al., 1995) and brucellosis (Vanzini et al., 2001).

An ELISA using bulk milk samples will be a useful, cost–effective and easy means of identifying herds at risk from fasciolosis. The LSTM test, like other tests used to measure antibody levels in bulk tank milk samples has several advantages, such as reduced cost of testing samples, ease of collecting samples and the ability to estimate herd seroprevalence without the need to blood sample a large number of animals within the herd. This information may be used by veterinarians for planing preventive medicine programmes. Bulk tank milk samples tested routinely every few months provide a simple, low cost method of establishing the prevalence of fasciolosis throughout a region. However, an antibody positive bulk tank milk test does not necessarily mean that infected cattle are present, since anti-*Fasciola* antibodies in serum can persist for several months after treatment (Levieux et al., 1992b; Ibarra et al., 1998; Castro et al., 2000).

In conclusion, LSTM ELISA is a more sensitive means of identifying infected herds than Bio-X ELISA. The LSTM ELISA can be used with bulk milk samples for large surveillance schemes, to monitor the efficacy of regional or national control programmes and can provide a good guide as to whether infection is, or has recently been, active in a herd.

Chapter Six

The prevalence of *F. hepatica* infection in dairy cattle in England and Wales

6.1. Introduction

Fasciola hepatica is an important trematode parasite of mammals that is found in temperate and sub-tropical regions of the world and causes significant economic losses to farming industries. Distribution of the parasite depends on the presence or absence of the obligatory intermediate host, snails of the Lymnaeidae family. The parasite is seen commonly in Western Europe, particularly in areas with mild, damp climates that favour survival of the snail host. *F. hepatica* is also widespread across parts of Eastern Europe, Russia and Associated states, Asia, North and South America and Australia (Torgerson and Claxton, 1999).

The incidence of fasciolosis in cattle in England and Wales increased between 1993 and 2002 reaching a peak in 2000 (VLA report, 2002c). Also the number of cases of Black disease encountered in adult cattle increased in England and Wales and it seems that fasciolosis is predisposing factor to this disease (VLA report, 2002c). This increase in the number of cases of fasciolosis was measured by an increase in the number of submissions to the Veterinary Laboratory Agency. However there is a lack of information about the extent of infection within sheep and cattle nationally. A national survey to assess the prevalence of infection using faecal egg counts would be time consuming and expensive. Thus the objective of this study was to determine the herd-level prevalence of fasciolosis in England and Wales in dairy cattle, using an ELISA for detection of antibodies in bulk tank milk samples. Approximately 2200 bulk tank milk samples, collected over a nineteen-month period, were used to assess both the prevalence of fasciolosis in different regions of England and Wales and the seasonal variation in prevalence.

6.2. Materials and Methods

6.2.1. Sampling area

The survey was carried out in England and Wales where there are 18,125 dairy farms and a total of 1,490,226 cattle (DEFRA, 2001, Department for Environment, Food and Rural Affairs, UK). Thirteen Veterinary Investigation Centres (VIC) had cooperated in collecting bulk tank milk samples from each of their areas as part of a national survey of Bovine Viral Diarrhoea Virus in dairy cattle.

6.2.2. Sample collection

Bulk tank milk samples were collected monthly and were tested with LSTM ELISA. Approximately 600 samples are submitted to the Veterinary Investigation Centre at Sutton Bonington each month from the regional centres and a subset of about 100 were randomly selected and sent to LSTM for the survey between September 2001 and April 2003. The bulk tank milk samples were centrifuged at 1000g for 20 minutes in order to separate milk from the fat and any cream and then were aliquoted and stored at –20°C until use.

6.2.3. ELISA test

Undiluted bulk tank milk samples were tested in duplicate using the LSTM BTM ELISA as described in Chapter 5. Quadruplicate negative and positive controls were included on each plate. Results were only accepted if control values fell within the range described in Chapter 4. A sample was considered to be positive if its PP value was equal to or above 27 and negative if it was below 27. The 27PP

cut off value had been chosen to indicate if more than 25% of a herd had evidence of fluke infection (Chapter 5).

6.2.4. Statistical analysis

Data were analysed using Microsoft Excel, Minitab 13.2 and SPSS 11.0 for Windows. Descriptive statistics, pairwise comparison of means, chi-squared analysis and multiple comparisons were employed to interpret data. The mean difference was considered significant at the 0.05 level.

6.3. Results

6.3.1. Monthly variation in prevalence of *F. hepatica* infection

The prevalence of fasciolosis as measured by the proportion of positive samples, was calculated each month. During the period September 2001 to April 2003, the highest prevalence was in October 2002 (80%) and the lowest prevalence recorded in April 2002 (39%). Results show highly significant ($P<0.001$) differences in the prevalence of fasciolosis over these 19 months (Table 6.1 and Figure 6.1).

Table 6.1. Prevalence of *Fasciola* infection by month in England and Wales.

Month	No. of samples	Prevalence (95% CI)
Sep 01	106	42% (32 – 51)
Nov 01	229	53% (46 – 59)
Dec 01	91	40% (29 – 50)
Jan 02	113	60% (51 – 69)
Feb 02	114	52% (42 – 61)
Mar 02	87	49% (39 – 60)
Apr 02	94	39% (29 – 49)
May 02	97	51% (40 – 60)
Jun 02	88	42% (32 – 53)
Jul 02	178	66% (59 – 74)
Aug 02	88	67% (57 – 77)
Sep 02	95	65% (56 – 75)
Oct 02	188	80% (74 – 86)
Nov 02	115	67% (58 – 76)
Dec 02	90	73% (64 – 83)
Jan 03	106	70% (61 – 79)
Feb 03	99	41% (32 – 51)
Mar 03	103	44% (34 – 53)
Apr 03	97	65% (55 – 75)

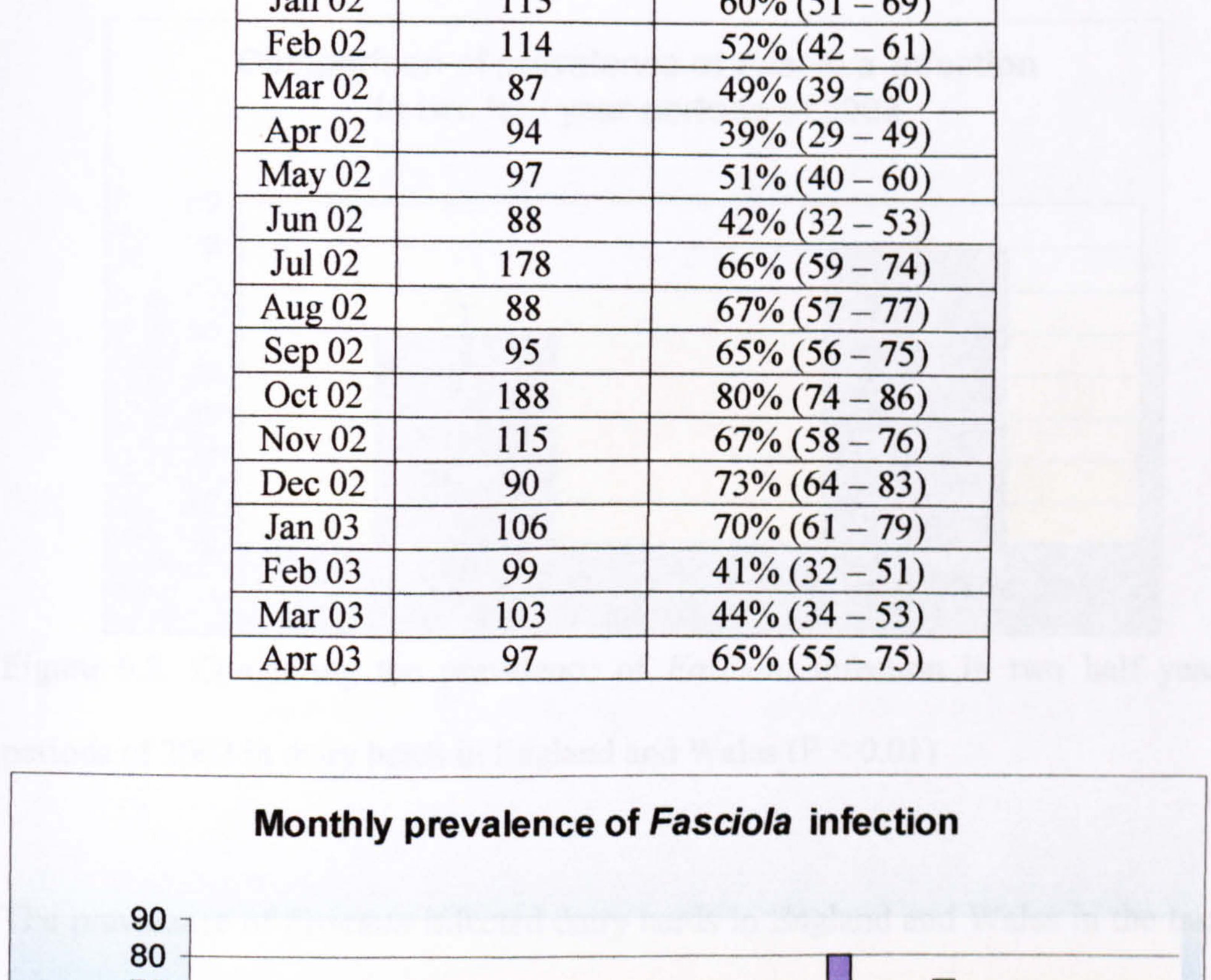

Figure 6.1. Prevalence of *Fasciola* infection in herds in England and Wales by month.

The prevalence of *Fasciola* infected herds was compared for the first (Jan – June) and the second (July – Dec) 6 month periods of the year 2002. Results indicated that prevalence of fasciolosis in second half of the year was significantly ($P < 0.01$) higher than in first half of the year (Figure 6.2).

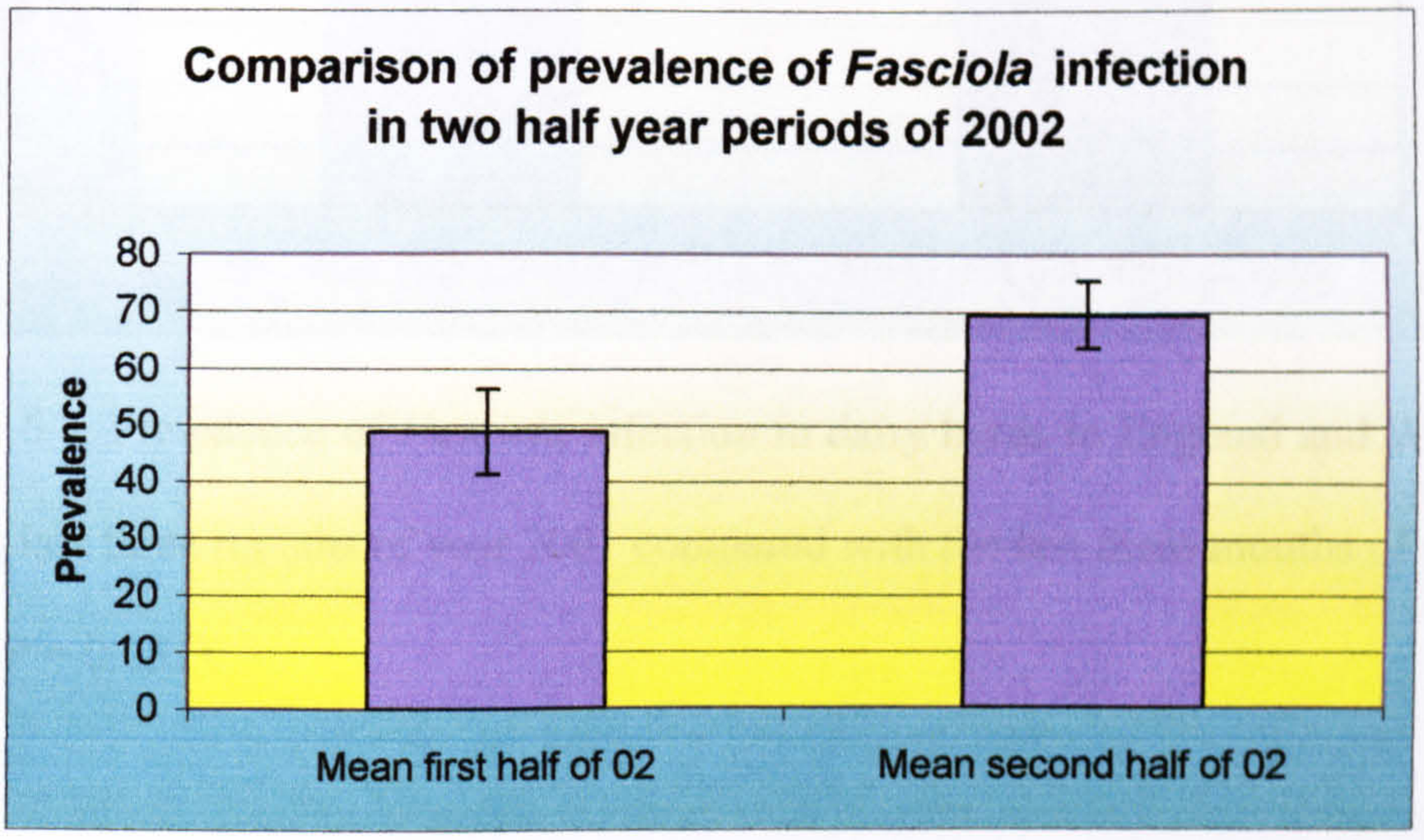

Figure 6.2. Comparing the prevalence of *Fasciola* infection in two half year periods of 2002 in dairy herds in England and Wales ($P < 0.01$)

The prevalence of *Fasciola* infected dairy herds in England and Wales in the last three months (Oct – Dec) of 2001 and 2002 and first four months (Jan – April) of 2002 and 2003 were compared to determine if the prevalence of infection during these periods increased or decreased. The results indicated that the prevalence of *Fasciola* infection in dairy herds during last three months of 2002 compared with 2001 was higher and approaching significant ($P = 0.051$) (Figure 6.3). There was no significant ($P > 0.05$) difference between the prevalence of fasciolosis in first four months of 2002 and the first four months of 2003 (Figure 6.4).

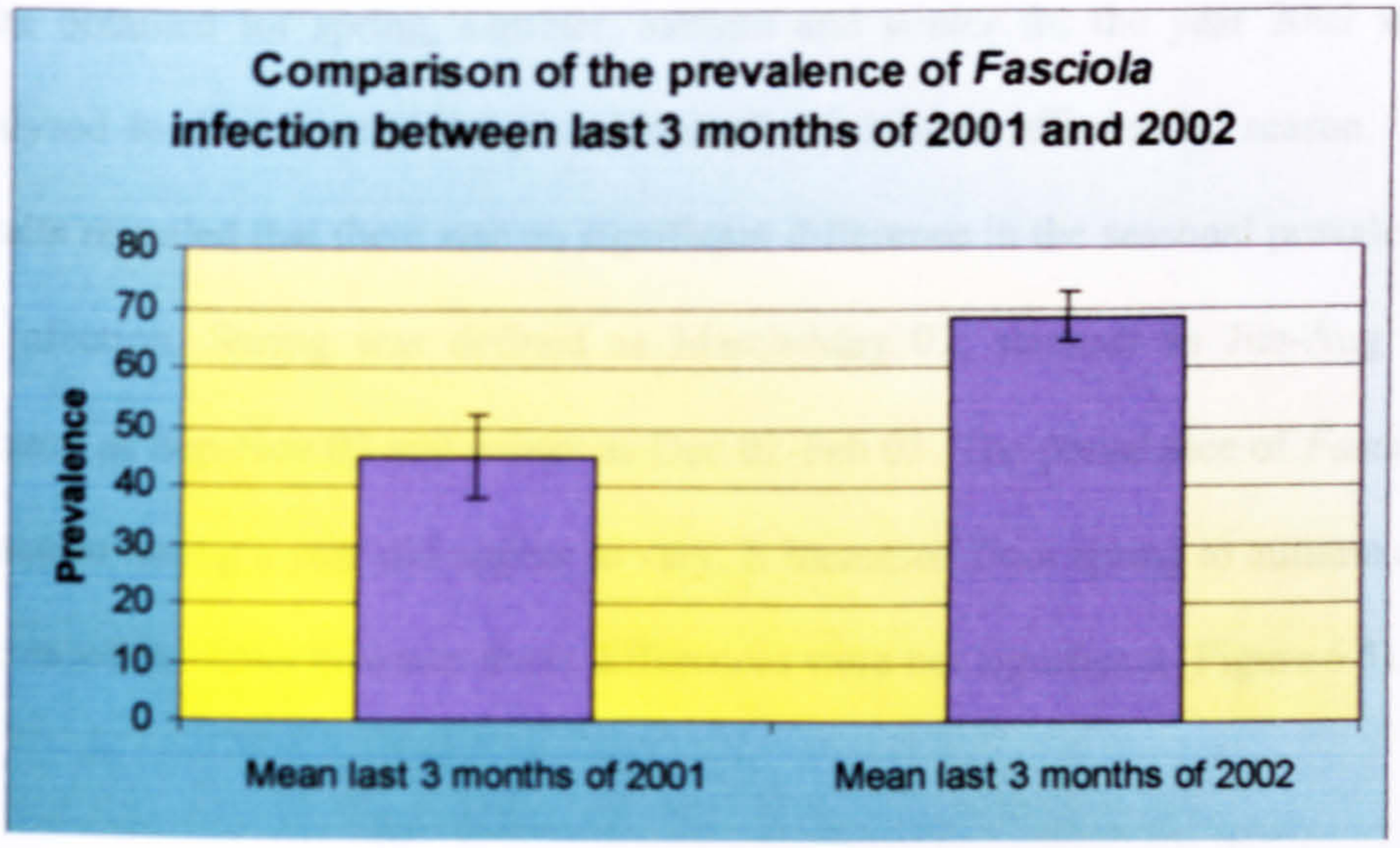

Figure 6.3. Prevalence of *Fasciola* infection in dairy herds in England and Wales during last three months of year 2001 compared with the last three months of year 2002 ($P = 0.051$).

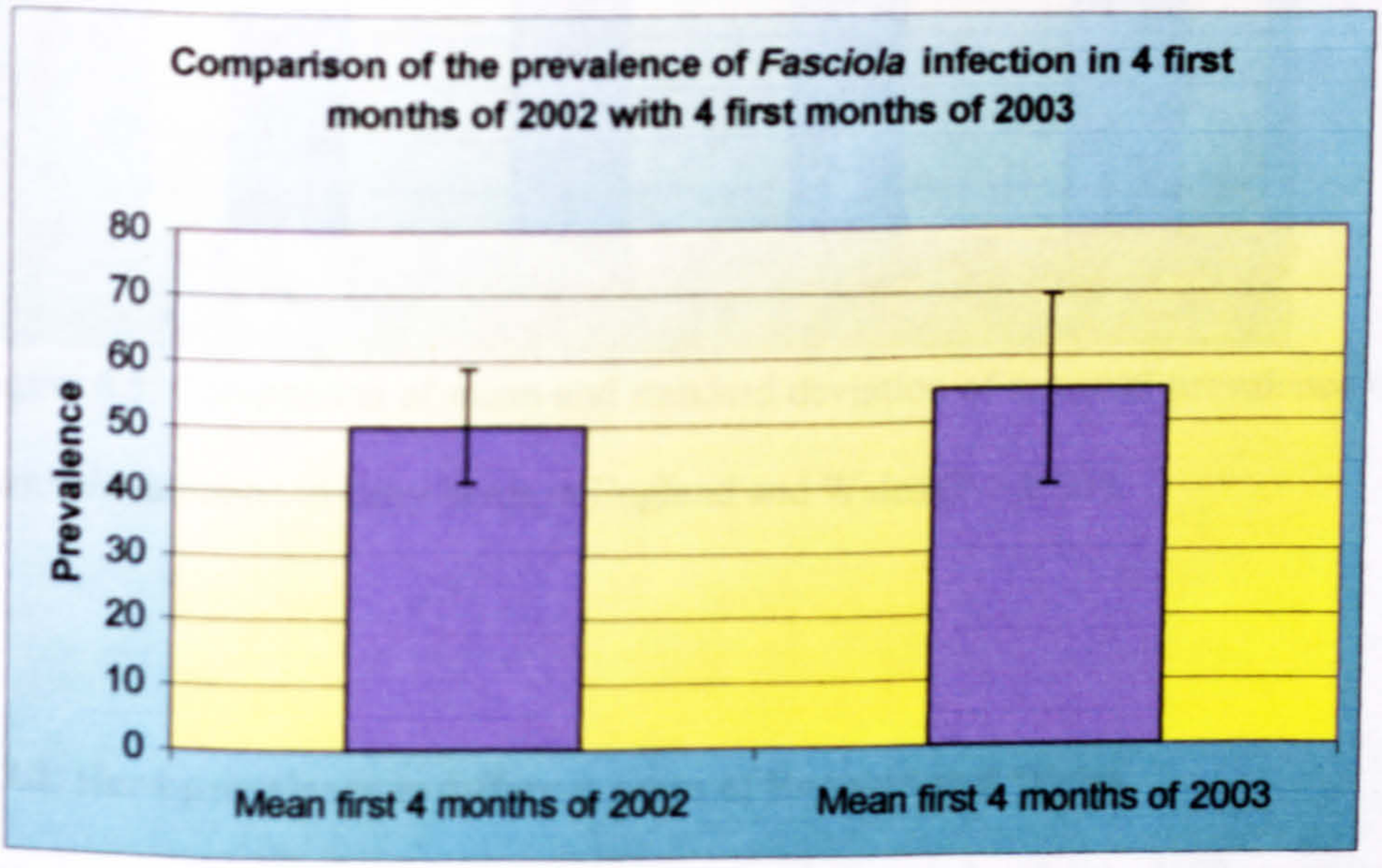

Figure 6.4. Comparison of the prevalence of *Fasciola* infection in dairy herds in the first four months of 2002 with first four months of 2003 in England and Wales ($P > 0.05$).

Data obtained for spring, summer, autumn and winter for the year 2002 were analysed to determine if the prevalence of infection is affected by season. The results revealed that there was no significant difference in the seasonal prevalence of infection. Spring was defined as March-May 02, summer as Jun-Aug 02, autumn as Sep-Nov 02 and winter as Dec 02-Feb 03. The prevalence of *Fasciola* infection during a year did appear to vary. It increased from spring to autumn and decreased in winter however these differences were not significant (Figure 6.5).

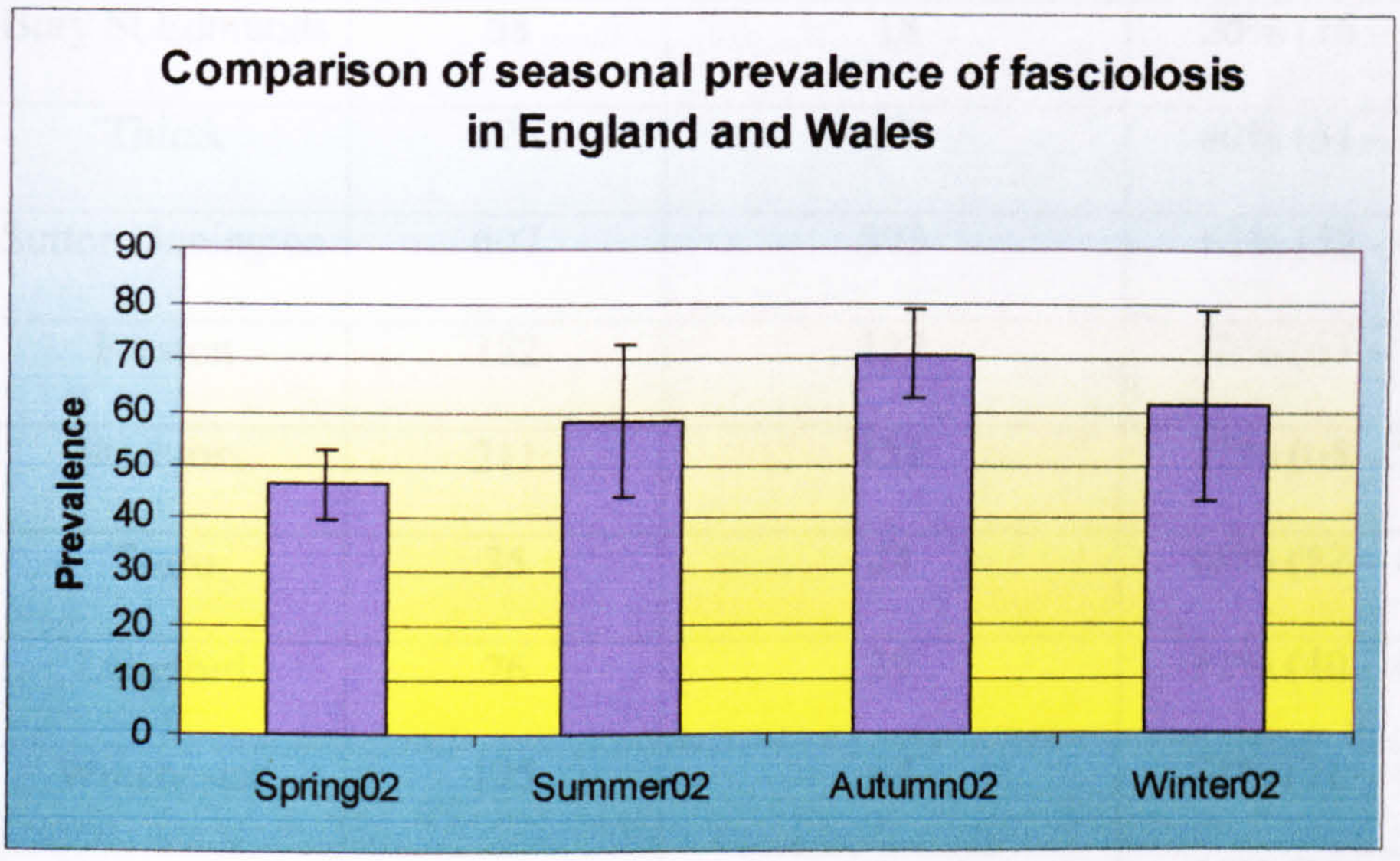

Figure 6.5. Comparison of mean and standard deviation of seasonal prevalence of *Fasciola* infection in dairy herds in England and Wales ($P > 0.05$).

6.3.2. Herd prevalence in different areas of England and Wales

Details of the originating VIC were obtained for each of the 2182 samples collected between September 2001 and April 2003. The number of positive samples obtained from each VIC over the 19-month period is shown in Table 6.2

and Figure 6.6. In total 2182 bulk tank milk samples were tested during the study and of these 1253 were positive and 929 were negative.

Table 6.2. The number of *F. hepatica* positive herds in different regions of England and Wales ($P < 0.001$).

Area	No. of samples	No. of positive herds	% Positive (95% CI)
Newcastle	23	6	26% (7 – 46)
Bury St Edmunds	68	18	26% (16 – 37)
Thirsk	125	50	40% (31 – 49)
Sutton Bonington	607	373	61% (58 - 65)
Preston	182	127	70% (63 – 77)
Starcross	211	151	72% (65 – 78)
Truro	35	24	69% (52 – 85)
Langford	76	39	51% (40 – 63)
Winchester	175	67	38% (31 – 46)
Shrewsbury	326	185	57% (51 – 62)
Luddington	124	29	23% (16 – 31)
Carmarthen	190	154	81% (75 – 89)
Aberystwyth	34	24	71% (54 – 87)

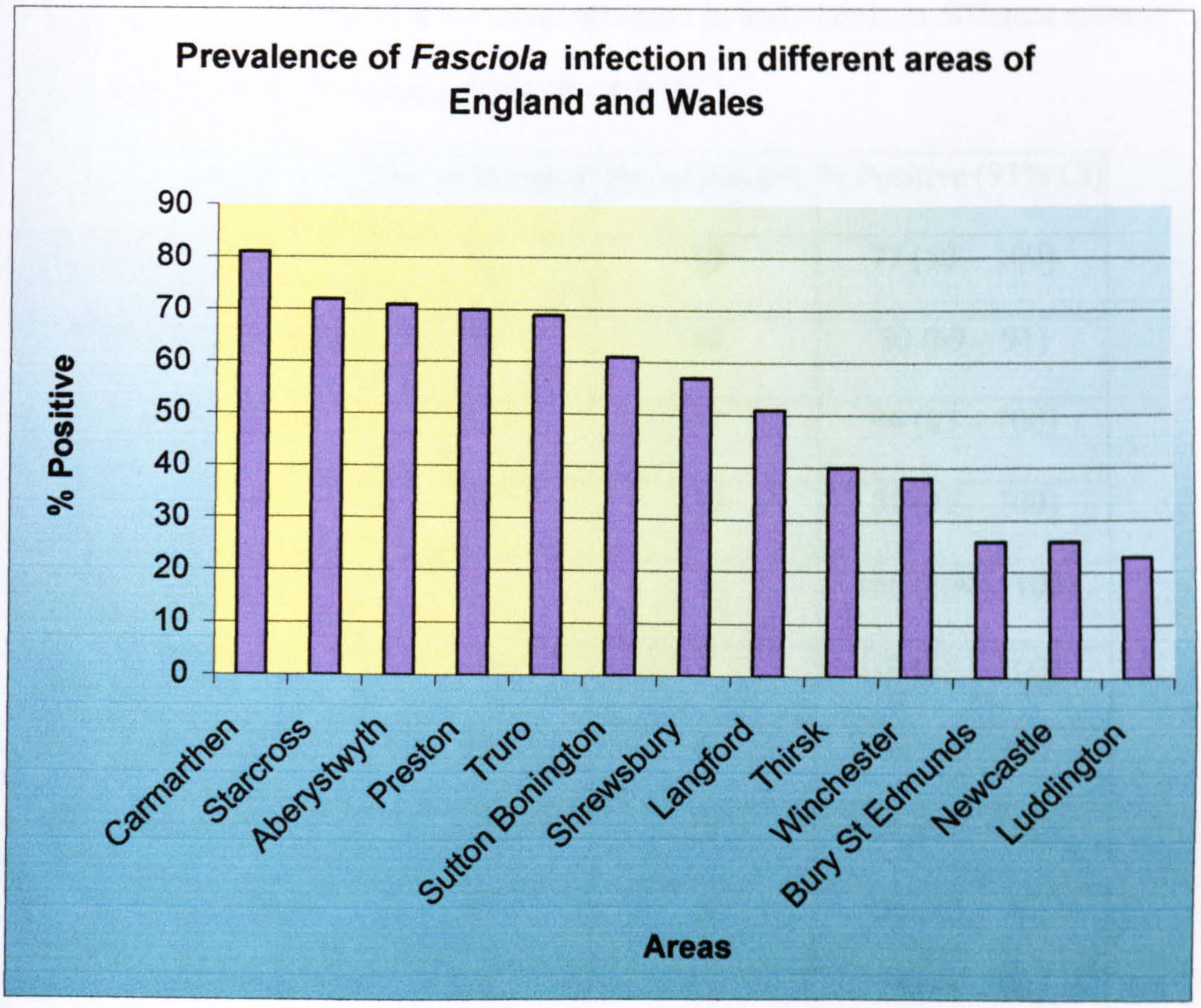

Figure 6.6. Percentage of *F. hepatica* positive herds in samples submitted by the regional VIC's in England and Wales.

It is possible that over the 19 month period, some VICs sent in repeated samples from the same farms to monitor BVDV status, thus it was not possible to use all the samples collected to determine the prevalence of *F. hepatica* infected dairy herds in England and Wales. Therefore one month, October 2002, was selected to assess the prevalence of *Fasciola* infection in different areas of England and Wales. Thus samples were unlikely to contain more than one from each farm. The results are shown in Table 6.3.

Table 6.3. The prevalence of *Fasciola* infection in dairy herds in different areas of England and Wales in October 2002 ($P < 0.001$).

Area	No. of samples	No. of positive	% Positive (95% CI)
Thirsk	13	10	77 (50 – 100)
Sutton Bonington	50	40	80 (69 – 91)
Preston	18	17	94 (83 – 100)
Starcross	31	27	87 (75 – 100)
Truro	6	6	100 (100 – 100)
Langford	10	7	70 (35 – 100)
Winchester	12	6	50 (17 – 83)
Shrewsbury	19	16	84 (66 – 100)
Luddington	9	5	56 (15 – 96)
Carmarthen	16	12	75 (51 – 99)
Aberystwyth	4	4	100 (100 – 100)

Using samples collected in October, the prevalence of *F. hepatica* infected dairy herds in England and Wales was 80% (95% CI = 74% - 86%).

6.4. Discussion

The aim of this study was to assess the seasonal prevalence of dairy herds exposed to *F. hepatica* in England and Wales, using bulk tank milk samples collected throughout England and Wales. This study was conducted between September 2001 and April 2003, and dairy farms of England and Wales were studied with the

co-operation of 13 Veterinary Investigation Centres located in different areas of England and Wales.

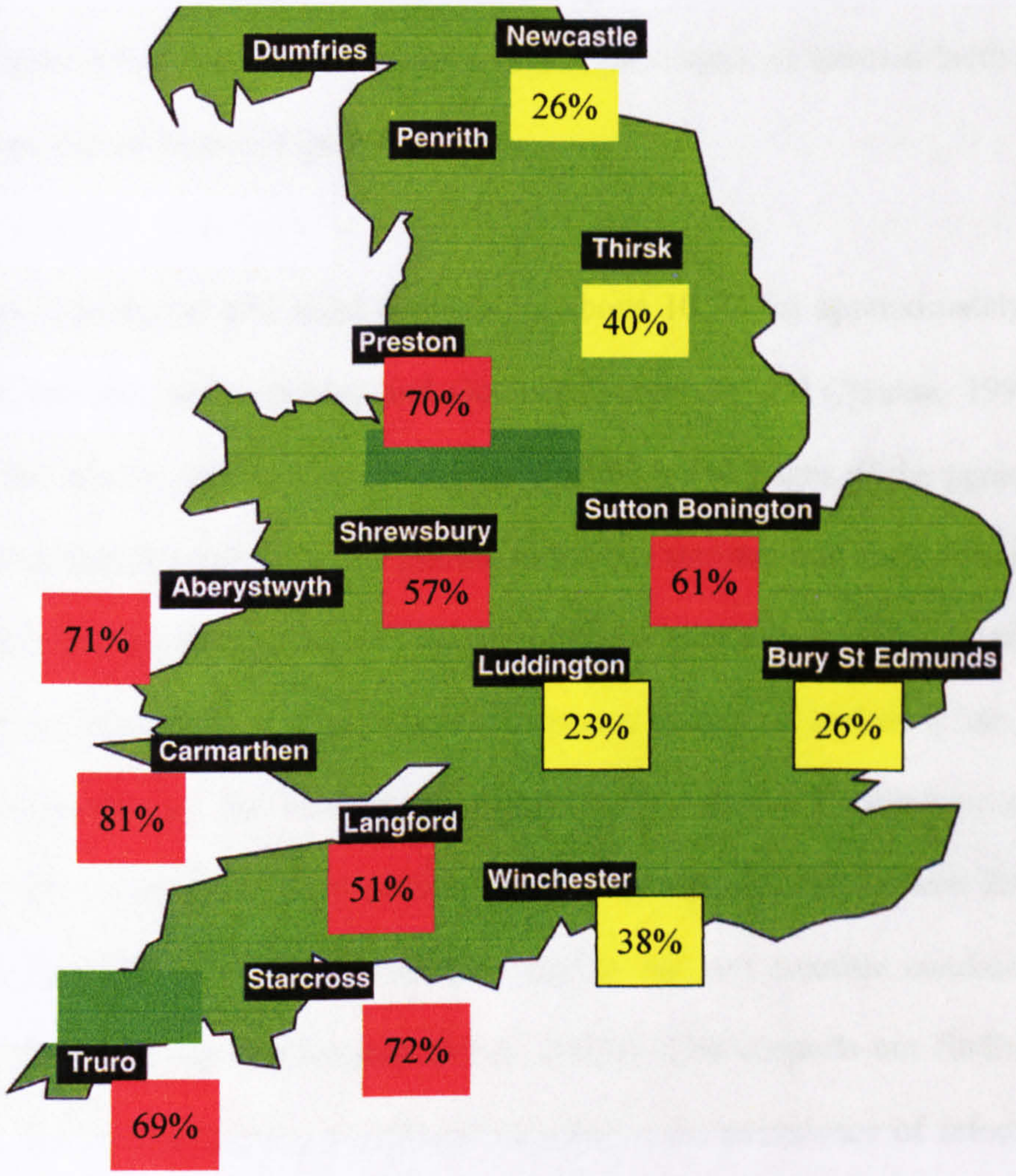

Figure 6.7. A map of England and Wales, indicating the distribution of the positive samples from different regional VICs.

This study is the first report indicating the seasonal prevalence and distribution of *Fasciola* infection in dairy herds in England and Wales. The results of the statistical analysis are consistent with of what is known about the epidemiology of *F. hepatica* in the UK, and confirms that infection increases in the autumn and winter (Figure 6.5). Moreover there was a higher prevalence of infected herds in the Western side of Britain (Figure 6.7).

Britain has a mean day and night temperature above 10 °C for approximately 6 months of the year between May and October (Torgerson and Claxton, 1999). During these months most of the development of the larval stages of the parasite occurs. Metacercariae appear on the pasture in the late summer and early autumn, the numbers present depend on the climate over the previous summer months. Therefore, the number of infected animals increases in autumn and early winter due to the increasing the number of metacercariae ingested (Torgerson and Claxton, 1999). Data from the Veterinary Laboratory Agency, for Autumn 2002 suggested that 2002 was a high risk year due to the wet weather conditions prevailing in the Spring and Summer (VLA, 2002b). This supports our findings that there was an approaching significant increase in the prevalence of infected dairy herds in the last quarter of 2002 compared to similar period in 2001. Although VLA forecasts can be used to advise farmers to treat sheep prophylactically to prevent fasciolosis, dairy cattle are rarely treated because of the long milk withdrawal times associated with flukicides. Moreover antibodies are known to persist in serum after drug treatment (Levieux et al, 1992b; Ibarra et al., 1998: Castro et al, 2000) and it is likely that they also persist in milk. Thus a

positive BTM test does not discriminate between drug treated and infected cattle and this may influence the number of positive herds recorded.

The prevalence of *F. hepatica* infection in areas such as Carmarthen and Starcross were higher than in Bury St Edmunds and Luddington. This is probably due to the fact that rainfall in the West of England and Wales is higher than in the East of England. Analysis of the climate throughout Britain revealed that over much of the East and Southeast of England it is rare for rainfall to exceed the potential transpiration for more than three of the summer months, and it is in these areas that little disease normally occurs (Ollerenshaw and Smith, 1969). Froyd (1975), also reported that fasciolosis mainly occurred in the western counties of England and Wales. An increase in case reports of fasciolosis from Carmarthen and Starcross coincident with Black disease has been reported by the VLA for 2002 (VLA report, 2002c).

This study suggested that in 2001 – 2003, 57% (55 – 60 C.I.) of dairy herds in England and Wales had evidence of exposure to *F. hepatica*. The VLA (2003) reported an increase in the prevalence of fasciolosis in sheep and cattle between 1997 – 2003. Moreover fasciolosis has recently been diagnosed in areas such as Bury St. Edmunds that are not traditionally associated with infection (VIDA, 2001; VLA report, 2003) and this means that *Fasciola* may have spread into the east part of the country probably in sheep.

Although an antibody positive bulk tank milk test does not necessarily mean that cattle with an active infection are present within the herd, nevertheless prevalence

of *F. hepatica* in dairy cattle Britain is higher than thought. Earlier reports, using liver examination at abattoirs suggested that prevalence of *F. hepatica* infection in cattle in Great Britain was 21% (Froyd, 1975). Our study of livers at the Shrewsbury abattoir suggested that the prevalence of infection was higher, around 30% (Chapter 7). There are no recent published data indicating the current level of infection in cattle in UK.

In conclusion, we have used a bulk tank milk ELISA to obtain an indication of the prevalence of infection within the dairy cow population in England and Wales. The data indicated that 57% (55 – 60, 95% C.I.) of the herds tested have been exposed to infection and that there are significant geographical differences in the prevalence of infected herds.

Chapter Seven

Evaluation of LSTM ELISA to assess intensity of *F. hepatica* infection in cattle

(Intensity of infection)

7.1. Introduction

The liver is one of the largest and most important and vital organs in the body and involved in almost all biochemical processes. One of the important diseases that affect the liver is fasciolosis, which has a world-wide distribution and causes economic losses to the animal husbandry industry particularly in cattle and sheep production. Disease, which ranges from a peracute syndrome with sudden death, to acute and chronic disease, is caused by the migration of flukes through the liver parenchyma and the presence of adults flukes in the bile ducts (Boray, 1969; Behm and Sangster, 1999). The severity of disease is proportional to the parasite burden. Losses due to animal mortality, liver condemnation, and production are the three main categories of economic loss due to fasciolosis. Reductions of 13 kg per carcass and 14% milk production have been reported for cattle (Marley et al., 1996; Behm and Sangster, 1999). Therefore diagnosis and control of the disease are very important, particularly if a method of assessing the intensity of infection could be developed that could be used to target the most heavily infected and hence most diseased animals. The LSTM ELISA was developed to diagnose *F. hepatica* infection in cattle. The aim of this study was to determine if this ELISA could be used to discriminate between different intensities of infection in cattle. Two-hundred and ninety four blood samples were collected from infected cattle at post mortem at a local abattoir, were used to determine if the LSTM ELISA can estimate the intensity of infection in cattle.

7.2. Materials and Methods

7.2.1. Blood samples

Nine hundred and sixty nine cattle livers were inspected for evidence of fluke infection on 10 separate visits during the winter of 2000 to the Shrewsbury Abattoir, England. Two hundred and ninety four blood samples were collected directly from heart or main vein of livers. On the first visit to the abattoir the extent of infection was assessed in two ways. First, the number of flukes in bile ducts following a single cut to the ventral side of the liver were noted. If more than 3 flukes were found the infection intensity was categorised as high, if 2-3 flukes were found, the infection intensity was categorised as medium and if only one fluke was found the liver was categorised as low intensity of infection. In addition the extent of liver pathology was assessed. Livers were considered to have a high severity of infection when the majority of the liver was involved with disease, and fibrosis and cirrhosis affecting the majority of liver was observable without cutting the liver. Livers were considered to be in the medium category of infection when flukes were rapidly found in the bile ducts. A low category of infection was used to describe livers with limited signs of disease including damage to the parenchyma due to migration, some thickening of bile ducts, and the presence of low numbers of flukes after extensive searching. After the first visit just the extent of liver pathology was used to indicate the intensity of infection. It was clear that there was strong association between the number of flukes observed in the liver and the extent of the pathology. Of the 294 blood samples that were collected, 152 samples classified as low, 95 samples were from cows with a medium intensity of infection, and 47 samples were considered high.

In addition 120 serum samples were collected from zero grazed cattle from a farm in Cheshire, England and used as the negative population. All samples were tested with LSTM ELISA. Twenty-seven randomly selected serum samples, 9 from the each of the three categories (low, medium and high) together with 27 samples from non-infected cattle were tested at doubling dilutions ranging from 1:800 to 1:102,400 to obtain a fail antibody titre.

The positive control serum was a pool of serum taken between 2 and 9 weeks after infection from a single cow that had been infected orally with 1000 *F. hepatica* metacercariae on three occasions (Akca, 1999). The negative control serum was from a cow, which had been kept indoors through out its life, on the Animal Husbandry Farm Leahurst, which is known to be free of *F. hepatica* and *Lymnaea trunculata* snails.

7.2.2. ELISA procedure

The ELISA was performed as described in Chapter 3 using E/S antigens at 0.5 μg/ml to coat the plates. The cattle sera were tested at 1:800. The conjugate, used at 1:6000 dilution, was a monoclonal anti-bovine IgG conjugated to horseradish peroxidase. The results are given as the mean of the optical density (OD) obtained from duplicate samples expressed as a percentage of the positive control (C+), using the following formula:

$$\text{Percent Positive (PP)} = \frac{\text{Mean OD of test sample}}{\text{Mean OD of C+}} \times 100$$

In each ELISA test, negative and positive controls were included and tested in quadruplicate. Each plate was accepted if the control values fell within a predetermined rang (page 59).

7.2.3. Statistical analysis

Data were analysed using software Minitab 13.2, SPSS 11.0 for Windows and Microsoft Excel. Descriptive statistics, tally for discrete variables, chart and graph means, repeated measure variables (general linear model = GLM) and discriminant analysis were employed to interpret the data of different categories of infection and non-infected samples. To analyse data of the titration of each of the three categories of infection, data for individual samples, which were tested at dilutions ranging from 1:800 – 1:102400 were collected and the cut off point for each sample that became negative identified. The negative cut off used was <15 PP value. Then descriptive statistics for each category of infection were calculated.

7.3. Results

7.3.1. Analysis of sera from animals with three different intensities of infection

All 294 samples from infected cattle and the 120 samples from uninfected cattle were tested in the LSTM ELISA and results are shown in Table 7.1 and Figure 7.1. Repeated measure variables (GLM), using SPSS, was employed to estimate if there were any significant differences between the three levels of infection. The

results (Table 7.2) indicated that there were significant (P<0.001) differences between the PP values of sera from cattle in the three different categories.

Category of infection	Total number	Range PP values	Mean PP value	St. Dev.	95% CI
Zero (Un-infected)	120	7 – 14	11.55	1.36	11.31 – 11.88
Low (1)	152	9 - 33	17.97	5.89	17.02 - 18.92
Medium (2)	95	26 - 53	37.45	6.36	36.16 - 38.75
High (3)	47	42 - 121	64.24	17.37	59.15 - 69.34

Table 7.1. Percent positivity of sera tested with LSTM ELISA at a 1:800 dilution, from animals with different levels of pathology resulting from *F. hepatica* infection.

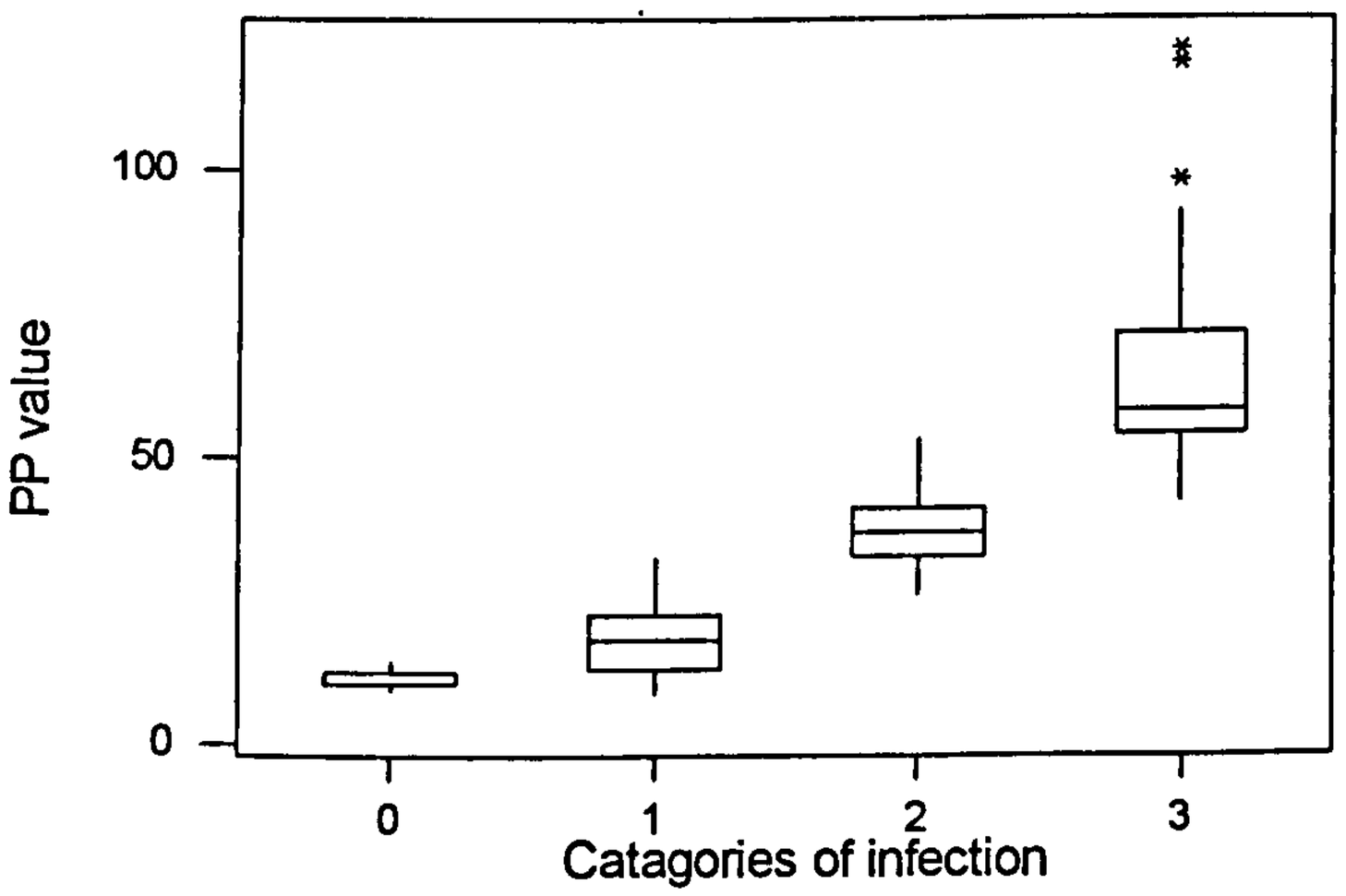

Figure 7.1. Percent positivity of sera tested with LSTM ELISA at a 1:800 dilution, from animals with different levels of pathology resulting from *F. hepatica* infection.

(I) Infection	(J) Infection	Mean Difference (I - J)	Std. Error	Sig. [a]	95% Confidence Interval for mean difference (I-J)	
					Lower level	Upper level
Low	Medium	-21.29*	1.19	0.00	-23.68	-18.89
	High	-47.29*	2.62	0.00	-52.57	-42.01
Medium	Low	21.29*	1.19	0.00	18.89	23.68
	High	-26.00*	2.76	0.00	-31.56	-20.44
High	Low	47.29*	2.62	0.00	42.01	52.57
	Medium	26.00*	2.76	0.00	20.44	31.56

Table 7.2. Multiple comparisons of three levels of infection based on estimated marginal means.

* The mean difference is significant at the 0.05 level.

[a] Adjustment for multiple comparisons: Least significant difference (equivalent no adjustments).

7.3.2. Discriminant analysis

Results of discriminant analysis revealed that 91% of category 1 (Low infection) PP values were less than 28PP with a median of 17.6; 93% of category 2 (Medium infection) PP values were between 28 – 50PP with a median of 36.8; and 87% of category 3 (High infection) all the PP values were over 51 PP with a median of 57.9. These results suggest that the LSTM ELISA can be used to assess the intensity of infection within an animal.

7.3.3. Titration of sera

Fifty-four samples, 27 from infected cows and 27 from non-infected cows, were tested with LSTM ELISA at dilutions ranging from 1:800 – 1:102,400. The PP

values are shown in Table 7.3 and Figure 7.2. Mean PP values with 95% confidence intervals for each category of infection at different dilutions were calculated separately and summarised in table 7.3. Also the data for uninfected samples that were tested at dilutions ranging from 1:800 – 1:102,400 were analysed and were summarised in Table 7.3. Data of individual samples, which were tested at ranging dilutions of 1:800 – 1:102400 were analysed to identify the cut point of each diluted sample that became negative. Then mean and standard deviation for each category of infection were calculated to determine the dilution when each infected sample become negative (Table 7.4).

Serum dilution	Mean PP value at Category 3 (95% CI)	Mean PP value at Category 2 (95% CI)	Mean PP value at Category 1 (95% CI)	Mean PP value for Uninfected (95% CI)
1:800	69 (58 – 79)	35 (32 – 38)	20 (18 – 22)	11 (10 – 12)
1:1600	52 (42 – 62)	24 (21 – 27)	15 (13 – 17)	10 (10 – 11)
1:3200	38 (28 – 47)	17 (15 – 19)	12 (10 – 13)	10 (10 – 11)
1:6400	26 (19 – 33)	12 (10 – 14)	10 (8 – 12)	10 (9 – 10)
1:12800	18 (13 – 23)	10 (8 – 11)	9 (7 – 11)	10 (9 – 10)
1:25600	12 (9 – 14)	7 (6 – 8)	8 (6 – 10)	10 (9 – 10)
1:51200	8 (7 – 10)	7 (6 – 7)	7 (6 – 9)	10 (9 – 10)
1:102400	8 (6 – 9)	7 (6 – 8)	7 (5 – 9)	10 (10 – 11)

Table 7.3. Table showing the results of the titration of 54 samples from infected and non-infected cattle. Results are expressed as PP values. The shaded areas represent PP values below the negative cut off value of 15PP.

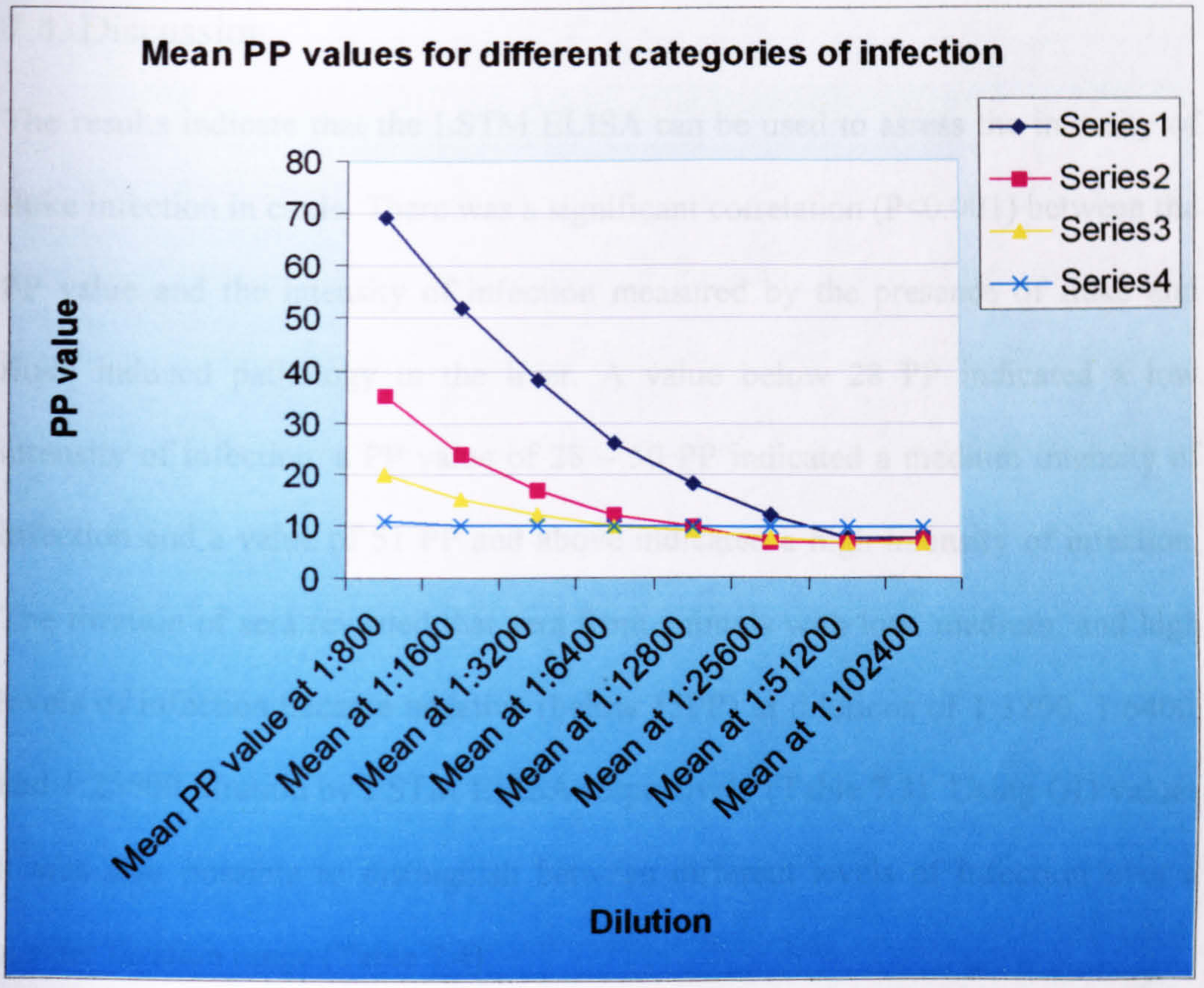

Figure 7.2. Titration of 54 sera from infected and non-infected cattle, which were tested by LSTM ELISA at dilution of 1:800 – 1:102400.

	Mean reciprocal titre for sera in the high category	Mean reciprocal titre for sera in the medium category	Mean reciprocal titre for sera in the low category
Mean	28444.44	11377.77	5777.77
SD	9487.41	2073.23	1113.77

Table 7.4. Twenty seven sera, 9 from each of the three categories of infected cattle were titrated from 1:800 to 1:102400. The last positive titre was the value that was greater than the mean OD value for the 27 negative samples. The mean reciprocal titre for each group was calculated and are shown here.

7.4. Discussion

The results indicate that the LSTM ELISA can be used to assess the intensity of fluke infection in cattle. There was a significant correlation ($P<0.001$) between the PP value and the intensity of infection measured by the presence of fluke and fluke induced pathology in the liver. A value below 28 PP indicated a low intensity of infection, a PP value of 28 – 50 PP indicated a medium intensity of infection and a value of 51 PP and above indicated a high intensity of infection. The titration of sera revealed that sera from animals with low, medium, and high levels of infection became negative (below 15PP) at dilutions of 1:3200, 1:6400, and 1:25600 titration by LSTM ELISA respectively (Table 7.3). Using OD values it was also possible to distinguish between different levels of infection over a similar titration range (Table 7.4).

Other authors reported that there was no correlation between absorbance values in an antibody detection ELISA and the rate of faecal egg excretion (Fagbemi and Gubadia, 1995; Reichel, 2002) or fluke burden (Haroun and Hillyer, 1986; Keegan and Trudgett, 1992; Reichel, 2002). However a direct correlation was reported between antigen concentration in faeces and the number of adult flukes in cattle infected with *F. hepatica* and it was suggested that antigen release may be correlated with worm burden (Espino and Finlay, 1994; Dumenigo et al., 1996). Also a positive correlation between specific IgG1 antibody titre and fluke burden was reported by Clery et al., (1996), Mulcahy et al., (1998) and Bossaert et al., (2000).

It has been reported that following infection, most of the pathological damage caused by flukes takes place when they migrate through the liver parenchyma before their establishment in the bile ducts (Sanchez-Andrade et al., 2000) and the severity of disease is related to parasite load (Soulsby, 1982). Therefore it is very important to use a diagnostic test that can detect early i.e. pre-patent infections and those animals that are most heavily parasitised. This will allow treatment and reduce the losses associated with *F. hepatica* infection in cattle. Examination of livers at the abattoir allowed an accurate assessment of the degree of pathology and level of infection. It is more representative of the field situation than samples collected from experimentally infected animals. The number of parasites that develop to maturity following an experimental challenge is variable and usually very low; 12.6 – 20.5 % (Bulgine and Anderson, 1984), 8.2 – 30% (Rose, 1965; Rose et al., 1966; Doyle, 1971, 1972) and 13.5% (Clery et al., 1995). Thus an experimental infection does not necessarily give a good indication of the intensity of infection unless the cows are killed and flukes enumerated.

Fasciolosis causes significant losses such as decreased feed conversion, and reduced milk and meat production in affected cattle (Marley et al., 1996; Behm and Sangster, 1999); and there is positive correlation between intensity of infection and economic losses. Bovine fasciolosis has recently been associated with metabolic disease in high yielding dairy cattle (Mason, 2002). It has also been reported in mice, that infection has a potential by immunosupressive effect on the host, that may compromise the host's ability to combat bacterial co-infection (Brady et al., 1999). Hence it is very important to identify animals with a high or medium intensity of infection, not only because of the economic

implications but also because they may be more susceptible to other diseases such as *Salmonella dublin*. Heavily infected animals may also increase transmission of the parasite because they will probably shed more eggs in their faeces.

In this study, 30% (294/969) of cattle livers, which were inspected, were infected with fluke. Among these, 52% had a low intensity of infection, 32% had a medium intensity of infection, and 16% had a high intensity of infection. Earlier abattoir studies revealed that 21% of all cattle livers examined were infected with fluke (Froyd, 1975). Although the Shrewsbury Abattoir receives cattle mainly from Wales and Northwest England, and assuming that the samples were collected randomly it appears that the prevalence of bovine fasciolosis was higher in 2000 than in 1970. There are several reports which suggest that the prevalence of bovine fasciolosis has increased in recent years in UK and it has been suggested that this is due to warmer, wetter summers and increased movement of stock around the country, allowing the parasite to establish in a wider geographical range than previously (Blamire et al., 1980; SAC Veterinary science division, 2000; Daniel and Mitchell, 2002; VLA, surveillance report 2000, 2002a, 2002b, 2002c, and 2003).

In conclusion, we have developed a test that can be used, not only to diagnose *F. hepatica* infection in cattle but also that can be used to assess the intensity of infection.

Chapter Eight

General Discussion

and

Conclusion

General discussion and conclusion

The study described in this thesis was concerned with developing and validating an antibody detection ELISA using serum from *F. hepatica* infected cattle. The ELISA was comparable to a commercially available test and was shown to detect early infection, before eggs would be detectable in faeces. This test was then adapted for use with milk from dairy cattle. The correlation coefficient between serum and milk was 84.3%. Also there was a highly significant correlation (96%) between the milk antibody prevalence within the herd and the seroprevalence. The number of days into lactation when the milk sample was collected did not affect the sensitivity of the test. The ELISA was also adapted for use with bulk tank milk samples. A study of 61 farms established that the agreement between the bulk tank milk value and the proportion of positive animals measured by milk antibody within a herd was 96%. When this test was used to assess the prevalence of *F. hepatica* infection in dairy herds in England and Wales we demonstrated that 57% of dairy herds had evidence of exposure to *F. hepatica*. Finally we demonstrated that the ELISA OD or PP values were proportional to the intensity of *F. hepatica* infection in cattle.

The LSTM ELISA, a serum antibody detection ELISA, was developed as a first step to developing a milk antibody detection test. The purpose of developing a milk antibody ELISA was to enable more cattle to be sampled in a cost-effective manner for epidemiological purposes. The ELISA had a diagnostic sensitivity of 98% (95% CI = 96% - 100%) and a diagnostic specificity of 96% (95% CI = 93% - 98%) at a cut off value of 15PP. This compares favourably to other ELISA's

developed to diagnose *F. heptica* infection. Anderson et al., (1999) reported that a diagnostic sensitivity of 86% and specificity of 70% for the antibody response to *F. hepatica* E/S antigens by ELISA. Ibarra et al., (1998) described the diagnostic sensitivity and specificity of 97.5% and 80% for a DIG-ELISA, 93.1% and 95.4% for a dot-ELISA and also a diagnostic sensitivity and specificity of 96.5% and 98.8% for indirect ELISA.

Results of experimentally infected calves revealed that antibodies to *F. hepatica* were detectable by LSTM ELISA between 2 and 4 weeks after infection. This is consistent with results reported by others, using a variety of sero-diagnostic tests (Farrel et al, 1981; Hughes et al, 1981; Wyckoff and Bradley, 1986; Sinclair and Wassall, 1988; Santiago and Hillyer, 1988; Fagbemi and Guobadia, 1995; Abdel-Rahman et al, 1998; Bossaert et al, 2000). The ELISA also performed well in comparison to a commercially available test, which is used by the VLA for diagnosis of *F. hepatica* in cattle, although there is no published validation data for this test.

To assess the performance characteristics of an ELISA test applied to milk, a collection of matched serum/milk samples was needed. Seven hundred and sixty five samples were collected from 26 farms and used to determine the correlation between milk and serum antibody. It is well established that the concentration of IgG in milk correlates with serum levels (Caffin et al., 1983; Caffin and Poutrel, 1988; Smith et al., 1989); the majority of IgG antibodies in milk are transported from serum to the mammary gland and the remainder is produced locally (Norcross, 1982). Thus milk is a good medium for testing an animal's exposure to

many pathogens although the immunoglobulin level in milk is 20 to 40 times lower than in serum (Mach and Pahud, 1971; Caffin et al., 1983). This means that milk is normally tested at lower dilutions compared to serum. The Pearson correlation coefficient between milk and serum antibody responses to *F. hepatica* infection was 84.3% using LSTM ELISA and 85% using Bio-X ELISA. Although to our knowledge reports describing diagnostic tests for *Fasciola* infection using milk are limited, these findings compare favourably to other diseases such as parasitological diseases (Boulard, 1985; Kloosterman et al., 1993), bacterial diseases (Nielsen et al., 1996; Vanzini et al., 1998; Winterhoff et al., 2002) and viral diseases (Elvander et al., 1995; Armstrong, 1997). In conclusion these results suggest that milk ELISA's are an effective alternative in comparison to serum ELISA's for diagnostic and surveillance purposes. Moreover milk ELISA's are more cost-effective since veterinarians are not required to collect milk samples and farmers can submit samples directly to regional laboratories.

The ELISA was developed further to test bulk tank milk samples. The aim here was to establish a test that could be used to obtain data about the prevalence of infection within a herd. The correlation between the BTM ELISA and herd seroprevalence was 83%. Discrimination of herds where more than 25% of the herd was infected, was possible using a cut off value of 27 PP with a diagnostic sensitivity and specificity of 93% (95% CI = 83% - 100%) and 81% (95% CI = 67% - 96%) for LSTM BTM ELISA. This is an arbitrary level of infection chosen because if only a small number of animals were infected this would probably not have a significant impact on productivity. In fact, the main purpose of this study was to identify the heavily infected herds where the level of infection had a

significant impact on seroprevalence. In this study 51% of herds (31 herds out of 61 herds) had seroprevalence below 25%.

To our knowledge this is the first report of the validation of a bulk tank milk test for fasciolosis. Bulk tank milk ELISAs have been used for monitoring and immunoepidemiological surveys for large number of infectious diseases in cattle; such as *Ostertagia ostertagi* infection (Sanchez et al., 2002), *Hypoderma* spp. (Frangipane di Regalbono et al., 2003), bovine viral diarrhoea virus (BVDV) infection (Niskanen et al., 1991; Niskanen, 1993; Bitsch and Ronsholt, 1995; Houe et al., 1995; Radwan et al., 1995; Bitsch et al., 1997; Paton et al., 1998; Lindberg and Alenius, 1999; Melendez and Donovan, 2003), bovine herpesvirus-1 (Hartman et al., 1997; Nylin et al., 2000; Stahl etal., 2002), Foot-and-Mouth Disease (Armstrong and Mathew, 2001), bovine respiratory syncytial virus (Elvander et al., 1995) and brucellosis (Vanzini et al., 2001).

The ELISA was used to test approximately 100 bulk tank milk samples every month collected at random from about 600 BTMs received monthly by Sutton Bonington VIC, as part of a surveillance study of BVDV in dairy cattle. The prevalence of infected herds in England and Wales was investigated and the effect of season and region determined. The results show clearly that *F. hepatica* infection is widespread in dairy cattle and the prevalence of *Fasciola* infection in England and Wales is consistent with what is known about the epidemiology of this infection in UK. Thus our results suggest that fluke infection in dairy cattle increases in Autumn and Winter. Britain has a mean day and night temperature above 10 °C for approximately 6 months of the year between May and October

(Torgerson and Claxton, 1999). Hence during these months most of the development of larval stages of parasite occurs. It is well established that the number of infected sheep and beef cattle increases in Autumn and early Winter due to the increasing number of metacercariae appearing on the pasture as a result of the summer infection of snails (Torgerson and Claxton, 1999). This is the first demonstration that a similar pattern of infection appears in mature dairy cattle. We do not yet know how changes in management practices may affect the exposure of cows to *F. hepatica* as cattle are grazed outdoors for longer in the autumn.

The number of infected dairy herds varied between regions with the highest reported prevalence in Western areas of England and Wales. This is probably because the amount of rainfall in the West is higher than in the East of England and Wales. Analysis of the climate throughout Britain revealed that over much of the East and Southeast of England it is rare for rainfall to exceed the potential transpiration for more than three of the summer months, hence little disease normally occurs in these areas (Ollerenshaw and Smith, 1969; Froyd, 1975). This study was the first national survey of the regional prevalence of *Fasciola* infection in dairy herds and the results also suggest that the prevalence of *F. hepatica* infection in dairy cattle Britain is higher than previously thought.

The results of the intensity of infection assay revealed that LSTM ELISA could be used to assess the intensity of fluke infection in cattle. There was a significant correlation ($P<0.001$) between the PP value and the intensity of infection which was categorised as a low, medium or high. Some authors reported that there was no correlation between absorbance values in an antibody detection ELISA and the

rate of faecal egg excretion (Fagbemi and Gubadia, 1995) or fluke burden (Haroun and Hillyer, 1986; Keegan and Trudgett, 1992). However a direct correlation was reported between antigen concentration in faeces and the number of adult flukes (Espino and Finlay, 1994; Dumenigo et al., 1996) and a positive correlation between specific IgG1 antibody titre and fluke burden in cattle infected with *F. hepatica* (Mulcahy et al., 1998; Bossaert et al., 2000). In adult cattle reports of chronic disease are rare and often only apparent as production losses (Behm and Sangster, 1999). However inflammatory reactions due to the presence of the fluke cause the thickening of bile ducts and calcium deposits start to form in the duct walls resulting in the typical description of fluke affected liver (Behm and Sangster, 1999). It has been suggested that in cattle few flukes reach the bile duct and few eggs are passed and most flukes are lost by 30-50 weeks after infection (Behm and Sangster, 1999). Cholangitis may take place months or years after initial infection and is due to inflammation and hyperplasia of epithelium of bile ducts and mechanical obstruction of the biliary duct but it is not clear to what extent this occurs in adult cattle. However cattle exposed to natural infection do appear to retain infection and can be re-infected indicating that resistance is slow to develop (Clery et al., 1996; Ortiz et al., 2000). Nevertheless the severity of disease is related to parasite load (Soulsby, 1982). Hence the LSTM ELISA would assist in discriminating between animals with low fluke burdens and those with high burdens that are most likely to require treatment.

In this study, 30% of the beef cattle livers which were inspected, were infected with fluke while earlier studies revealed that 21% of all cattle livers examined were infected with fluke (Froyd, 1975). This finding indicated that prevalence of

bovine fasciolosis was higher in 2000 than in 1970. There are several reports which suggest that the prevalence of bovine fasciolosis has increased in recent years in the UK and it has been suggested that this is due to wetter summers and increased movement of stock around the country allowing the parasite to establish in a wider geographical range than previously (Blamire et al., 1980; Daniel and Mitchell, 2002; SAC Veterinary science division, 2000; VLA, surveillance reports 2000, 2002a, 2002b, 2002c, and 2003).

Although this study has generated interesting and valuable results, it has been limited by some factors. The ELISA that was developed was an antibody detection ELISA, thus serum and possibly milk antibodies might be presented if the animal had previously been infected (Leclipteux et al., 1998). Antibodies might indicate past exposure to the parasite rather than the presence of an active infection (Dumenigo et al., 1996). Also ELISA methods that measure antibody levels are not always appropriate if animals are under chemotherapy (Leclipteux et al., 1998). Antibodies persist in fluke-infected cattle after treatment with triclabendazole for up to 7 months (Levieux et al., 1992b; Castro et al., 2000). However treatment of dairy cattle is rare due to long milk withdrawal times associated with flukicides. Also results of our study showed that the age of dairy cattle and the stage of lactation does not affect the sensitivity of the LSTM milk ELISA. The probability of detecting antibodies in bulk tank milk samples is related to the presence and the number of infected animals within the milking herd, the magnitude of the antibody response and the daily milk yield, i.e. the dilution of positive milk in the tank at sampling. However our results showed that

the correlation between the LSTM BTM ELISA and milk antibody prevalence within a herd was 96%.

It was difficult during this study to obtain a representative sample of dairy herds for England and Wales. Therefore we used bulk tank milk samples from a BVDV study conducted by Sutton Bonington VLA. These herds were selected, based on their veterinarian's decision to monitor the BVDV status of herds. Thus the BTM samples were not a true random sample and there could be some bias in the collection of samples. Nevertheless none of these herds was being investigated for *F. hepatica* infection. The disadvantage of using these samples is that it was not possible to determine if some herds were submitting samples on more than one occasion. For this reason, the samples collected over a 12-month period could not be used to estimate the national prevalence of infection. These samples could only be used to determine seasonal and regional variation in prevalence of infection.

Further studies are needed to determine the national prevalence of infection in the UK; the risk factors for infection, such as the presence of sheep and their treatment schedules; and also the impact of infection on the health and productivity of dairy cattle. Further studies could also be included using geographical information system (GIS) to map distribution of infection and how it is influenced by rock and soil type, hydrology, vegetation and climate. It could be suggested that in areas in which the incidence of fasciolosis is increasing or the prevalence of infection is high, control strategies, land management, grazing schedules, nutrition and existence of sheep or other reservoir hosts should be reviewed.

It is well established that fluke infection affects immune responses to other pathogens such as *Bordetella pertussis* in mice (Brady et al., 1999). The presence of fluke may therefore affect immune responses and susceptibility to other pathogens in cattle. For instance, tuberculosis is spreading in cattle in Britain in areas where fluke is common as found in this study. The prevalence of bovine tuberculosis has increased significantly in Great Britain in recent years and the number of confirmed new tuberculosis cases in dairy cattle in the West of England and Wales was significantly higher than in the East of the UK in 2003 (DEFRA, 2004). Thus it is possible that pre-existing trematode or nematode infections may diminish protective immunity to tuberculosis (Mulcahy et al., 2004). Control of fasciolosis may reduce the spread of diseases such as bovine tuberculosis.

It has been reported that *F. hepatica* infection causes reductions of 13 kg in carcass weight and 14% milk production and a decrease in feed conversion rates (Marley et al., 1996). Clinical disease is rare in adult cattle but in the subclinical form of the disease, reduced productivity is also reported (Behm and Sangster, 1999). Some metabolic disorders and increasing abortion due to *S. dublin* may be due to existence of subclinical fasciolosis which is undiagnosed. Reports relating to abortions due to *S. dublin* have increased and in 2002 the number of abortions due to *S. dublin* in Autumn and Winter was higher compared to Spring and Summer with peak in October (VIDA, 2002). These results show a similar pattern to the prevalence of fasciolosis in 2002. Therefore it could be useful to identify cattle with subclinical fasciolosis to investigate the relationship between fasciolosis and diseases caused by other pathogens.

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Appendixes

Appendix 1

LSTM ELISA

Coating (carbonate) buffer (pH 9.6)

0.795 g Sodium carbonate ($Na_2 Co_3$) and 1.465 g Sodium Bicarbonate ($NaHCo_3$) were dissolved in 100 ml distilled water and made up to 500 ml with distilled water. Adjusted the pH with 1 or 0.5 M of Sodium Hydroxide (NaOH).

Phosphate buffer saline, PBS, pH 7.2

5 X PBS: 90 g Sodium Chloride (NaCl), 18.5 g Di-Sodium Hydrogen Orthophosphate (Na_2HPo_4), and Potassium Dihydrogen Orthophosphate (KH_2Po_4) were dissolved in one litre distilled water and made up to 2.5 litres with distilled water and diluted 1/5 for use.

Stopping solution, 0.5 M of H_2So_4

2.74 ml of 98% Sulphuric acid (H_2So_4) was diluted in 97.26 ml of distilled water.

Appendix 2

Sodium Dodecyle Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

4X Resolving gel buffer (1.5 Tris-Cl, pH 8.8)

36.3g Tris base (FW 121.1) were dissolved in 150 ml distilled water, adjusted to pH 8.8 with 50% hydrochloric acid (HCL) and made up to 200 ml with distilled water. It was filtered through Whatman No. 1 filter paper and stored at 4°C for up to 3 months.

4X stacking gel buffer (0.5 M Tris-Cl, pH 6.8)

3 g Tris were dissolved in 40 ml distilled water, adjusted to pH 6.8 with hydrochloric acid (HCL) and made up to 50 ml with distilled water. It was filtered through Whatman No. 1 filter paper and stored at 4°C for up to 3 months.

10% Ammonium persulphate (APS)

1g APS was dissolved in 10 ml distilled water. Made fresh each time.

Separating Gel (10%) for SDS-PAGE Minigel

5 ml 30% acrylamide solution and 3.8 ml 4X resolving gel buffer pH 8.8 and 6 ml ultra pure water and 75µl of 10% APS were mixed and 5µl of TEMED added.

Stacking Gel (4%) for SDS-PAGE Minigel

0.88 ml 30% acrylamide solution, 1.66 ml 4X stacking gel buffer pH 6.8, 4 ml ultra pure water and 33.4µl of 10% APS were mixed and at the end 3.3µl of TEMED added.

2X sample buffer (0.125M Tris-Cl, 4% SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, 0.02% bromophenol blue, pH 6.8)

2.5 ml stacking gel buffer, 4 ml of 10% sodium dodecyl sulphate (SDS), 2 ml glycerol, 2 mg bromophenol blue and 1 ml of 2-mercaptoethanol were mixed and made up to 10 ml with distilled water. Stored 0.5 ml aliquots at –20° C for up to 6 months.

10X Tank (running) buffer (0.25M Tris pH 8.3, 1.92M glycine, 1%SDS)

30.3g Tris base, 144g glycine and 10g SDS were mixed and made up to 1 litre with distilled water. Did not adjust pH and stored at room temperature for up to 1 month and diluted 1/10 for use.

Staining solution (0.025% Coomassie Brilliant blue R 250, 40% Methanol, 7% Acetic acid)

0.25 g Coomassie Brilliant blue were dissolved in 400 ml Methanol (stir until dissolved) and then added 70 ml acetic acid and made up to 1 litter with ultra pure water. Stored at room temperature for up to 6 months.

Destaining solution I (40% Methanol, 7% Acetic acid)

400 ml methanol mixed with 70 ml of acetic acid and made up to 1 litre with ultra pure water. Stored at room temperature indefinitely.

Destaining solution II (7% Acetic acid, 5% Methanol)

70 ml acetic acid mixed with 50 ml methanol and made up to 1 litre with ultra pure water.

Transfer buffer (25mM Tris, 192mM Glycine, 20 v/v Methanol, pH 8.3)

3.03 g Tris and 14.4 g Glycine were dissolved in 100 ml of Methanol and made up to 1 litre with distilled water.

List of

Publications

Research in Veterinary Science, 70 (Supp A), (2001), 44

Development of an antibody-detection ELISA for *Fasciola hepatica* and its evaluation against a commercially available test.

Salimi-Bejestani, M.R.*[1], McGary,J.[1], Ortiz,P.[2], Claxton,J.R.[3] and Williams, D.J.L.[1]

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Abstract

An ELISA test was developed for the detection of *Fasciola hepatica* antiboby in blood of cattle. The assay was applied to sera from 284 naturally infected cattle, 243 non-infected cattle and six calves experimentally infected with *F. hepatica*. The diagnostic sensitivity and specificity of the ELISA test was 82.3%, 99.6% respectively at a cut-off value of 20 percent positivity. The results using sera from the experimentally infected calves showed that antibodies were first detected 2 – 4 weeks after infection. The ELISA test was also compared to the comercial available Bio – X Bovine *Fasciola hepatica* ELISA Kit. A subset of 44 positive sera and 45 negative sera were selected from the samples used to evaluate the in-house test. The results indicated that the agreement between the two tests was almost perfect (k=94.5%).

Research in Veterinary Science, 72 (Supp A), (2002), 34

Modification and validation of a *Fasciola hepatica* specific antibody detection ELISA for use with individual and bulk tank milk samples

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Abstract

The number of cases of fasciolosis diagnosed in cattle in England and Wales has increased significantly over the past five years. Nevertheless the majority of chronic infections in dairy cattle go undiagnosed or misdiagnosed because farmers do not associate scouring with fluke infection and often suspect that condition loss is due to trace element deficiencies. Diagnosis of fasciolosis is costly to the farmer since it involves collection of blood and faeces from suspected cases. A test that can detect antibodies in milk or bulk tank samples would assist in diagnosis and surveillance of infection. The VLA, to aid diagnosis of bovine fasciolosis uses the commercially available Bio X ELISA for the detection of serum antibodies to *F. hepatica*. This test was adapted to detect specific antibodies in milk samples. Serum, milk and faeces were collected from 250 cattle on farms with a reported outbreak of fasciolosis and 250 serum and milk samples were collected from cattle on farms with no history of fasciolosis. Using the serum to determine the true infection status of the cattle, the diagnostic sensitivity of the Bio X ELISA for milk was 73% and the diagnostic specificity was 90%. The stage of lactation when the milk sample was collected did not appear to affect the sensitivity and specificity of the test. Bulk tank samples were collected from 20 farms on which 25 cows from the milking herd were sampled for serum and faeces. The positivity of the bulk tank readings correlated with the seroprevalence of the herd ($r = 0.94$) and the prevalence of infection measured by faecal egg counts ($r = 0.85$). The results suggest that the bulk tank milk test can identify herds with a seroprevalence of 30% or greater.

Research in Veterinary Science, 74 (Supp A), (2003), 32

Development and application of an ELISA to detect anti – *Fasciola hepatica* antibodies in bulk tank milk

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Abstract

An ELISA has been developed using bulk milk tank samples, to assess exposure of dairy cattle to the parasite *Fasciola hepatica*. Fifty herds were used to validate the test. The correlation between herd seroprevalence and bulk tank ELISA value was 0.85. A percent positivity value of 27 and above was used to indicate a seroprevalence within the herd of greater than 25%.

A total of 1568 bulk tank milk samples from 13 Veterinary Investigation Centres in England and Wales, were tested between September 2001 and October 2002. The results suggest that the degree of exposure to *F. hepatica* in the UK is much higher than previously thought. In some areas, 78% of dairy farms tested had evidence of infection. There was no evidence of seasonal variation in the level of exposure.

Research in Veterinary Science, 76 (Supp A), (2004), 19

Estimation of the prevalence of *F. hepatica* infection in England and Wales

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Abstract:

The number of incidents of fasciolosis in cattle increased between 1993 and 2002 reaching a peak in 2000, in England and Wales. It is important to know the prevalence of *F. hepatica* infection in cattle in order to map the areas most affected and implement control programmes to prevent economic loss. Measuring antibodies to *Fasciola* infection by an ELISA using bulk tank milk samples provides a simple, low cost method of assessing the disease status of dairy herds in England and Wales. Approximately 2200 bulk tank milk samples from 13 Veterinary Investigation Centres in England and Wales, were tested between September 2001 and April 2003 to assess herd prevalence of fasciolosis. The results suggest that the degree of exposure to *F. hepatica* in the UK is much higher than previously thought. In some areas, 81% of dairy farms tested had evidence of infection. There was no evidence of seasonal variation in the level of exposure ($P < 0.001$).

Research in Veterinary Science, 76 (Supp A), (2004), 19

Evaluation of LSTM ELISA to assess intensity of *F. hepatica* infection in cattle

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Abstract

Fasciolosis has a worldwide distribution and causes significant losses to the animal husbandry industry, particularly for cattle and sheep production. Diagnosis and control of the disease is very important, particularly in view of the increased prevalence of *F. hepatica* infection in the UK in recent years. A method of assessing the intensity of infection would target the most heavily infected, and hence most diseased, animals. Thus an ELISA technique that was developed and shown to have diagnostic sensitivity of 98% and specificity of 96% was adapted to measure intensity of infection. A total of 294 blood samples from infected cattle were collected from the abattoir. The level of infection in each animal was assessed using a scoring system. The results indicated that there was a significant correlation ($P<0.001$) between the PP value and the intensity of infection. A value below 28 PP indicated a low intensity of infection, a value of 28 – 50 PP indicated a medium intensity of infection and a value of 51 PP and above indicated a high intensity of infection.

Research in Veterinary Science, In Press (2004)

Development of an antibody-detection ELISA for *Fasciola hepatica* and its evaluation against a commercially available test.

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Abstract:

An ELISA was developed for the detection of *Fasciola hepatica* antibody in serum of cattle. The assay was applied to sera from 258 naturally infected cattle, 256 non-infected cattle and six calves experimentally infected with *F. hepatica*. The diagnostic sensitivity and specificity of the ELISA test was 98% and 96% respectively at a cut-off value of 15 percent positivity. The results using sera from the experimentally infected calves showed that antibodies were first detected 2 – 4 weeks after infection. The ELISA test was also compared to the commercially available Bio-X bovine *Fasciola hepatica* ELISA kit. A subset of 39 positive sera and 47 negative sera were selected from the samples used to evaluate the in-house test. The results indicated that the agreement between the two tests was almost perfect (k statistic = 0.82).