Comparison of host-parasite relationships of *Fasciola gigantica* infection in cattle (*Bos indicus*) and swamp buffaloes (*Bubalus bubalis*)

Thesis submitted by

ELIZABETH C. MOLINA

for the degree of Doctor of Philosophy
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and Biomedical Sciences
James Cook University

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STATEMENT ON THE CONTRIBUTION OF OTHERS

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The experimental animals for the experimental infection were kept at the Philippine Carabao Center at the University of Southern Mindanao (USM), Kabacan, Cotabato, Philippines. Some of the laboratory analyses undertaken for this work were conducted at the Veterinary Hospital of the College of Veterinary Medicine at USM.

This project has the approval of the Animal Ethics Subcommittee (Permit Number A778_02).

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ABSTRACT

The host-parasite interactions in *Fasciola gigantica* infection in cattle and swamp buffaloes have not been extensively investigated. Designing of future approaches for the control of tropical fasciolosis requires an understanding of the host-parasite relationships. This study was therefore undertaken to look at and compare the host-parasite interactions of *F. gigantica* infection between cattle (*Bos indicus*) and swamp buffaloes (*Bubalus bubalis*). This study compared the prevalence of infection, clinico-pathological and parasitological manifestations, sequential production of antibody isotypes and of Th1 and Th2 cytokines, local immune responses, and the histopathology of the infection between cattle and swamp buffaloes during infection with *F. gigantica*.

The study shows that cattle and buffaloes are both susceptible to infection with *F. gigantica* in the Philippines with the infection affecting young and old animals. However, there are indications that these animals differ in their responses to infection.

The red blood cell (RBC) count was significantly higher in infected than in non-infected swamp buffaloes ($P<0.05$) while there was no significant difference in packed cell volume (PCV) and haemoglobin values between infected and non-infected buffaloes ($P>0.05$). Red blood cell count was significantly higher in buffaloes with high fluke burdens (>70 flukes) than those with no flukes or with medium fluke burden (21-70 flukes) ($P<0.05$). Significantly higher PCV value was also observed in buffaloes with high fluke burdens compared with those with low or medium worm loads ($P<0.05$). Haemoglobin values did not differ significantly between buffaloes with low, medium, high or no fluke burdens ($P>0.05$). On the other hand, infected cattle showed significantly lower RBC counts than non-infected cattle ($P<0.05$) and these counts were negatively related to fluke burden. Packed cell volume was also significantly lower in cattle with high fluke burden than those with fewer flukes ($P<0.05$). These findings showed that swamp buffaloes were not as severely affected by *F. gigantica* compared with cattle suggesting that they can cope with infection much better than cattle. From these observations, it was concluded that swamp buffaloes are more resilient to *F. gigantica* infection than cattle.

There was a trend of a lower fluke burden and faecal egg counts in naturally infected swamp buffaloes than in cattle. Fluke burdens were also lower in buffaloes than cattle at 3, 7, 12 and 16 weeks post- experimental infection with 1000 metacercariae. Sixteen weeks after the experimental infection, eggs were already seen in cattle but none in buffaloes and only immature flukes were present in buffaloes at this time, indicating that the prepatent period of *F. gigantica* in cattle is shorter than in swamp buffaloes. These findings support a conclusion that swamp buffaloes are more resistant than cattle to *F. gigantica*.

An indirect ELISA was done to assess the sequential production of antibody isotypes IgG1, IgG2 and IgE reacting to *F. gigantica*. Infected cattle and buffaloes showed increased levels of these isotypes relative to the controls. No marked increase in IgG1 and IgG2 occurred in cattle except during the later part of infection. In buffaloes, the elevations of these two isotypes showed a pattern of increasing trend. IgG1 and IgG2 values in buffaloes were higher than in cattle. It is proposed that IgG2 may be associated with resistance against *F. gigantica* in these species, higher IgG2 in buffaloes being related to the higher resistance observed in these animals compared with that in cattle.

The levels of IFN-γ, IL-6 and IL-8 in serum of cattle and buffaloes were assessed by a sandwich ELISA. IFN-γ was not present in detectable levels in the serum of these animals suggesting that this cytokine may not be important in the immune response against *F. gigantica* in cattle and swamp buffaloes. Serum IL-6 levels were higher in infected than in non-infected animals from one to 16 weeks post-infection and higher in cattle than in buffaloes. This suggests that IL-6 is not important in resistance against *F. gigantica* in these animals. Higher serum IL-8 levels were observed in infected buffaloes than in cattle suggesting that this cytokine is associated with the higher degree of resistance against *F. gigantica* in swamp buffaloes than in cattle.

The local immune response in the liver of infected animals was assessed by immunohistochemistry and histology. T and B lymphocytes, plasma cells, eosinophils and mast cells were present in hepatic lesions. A progressive increase in T cell numbers occurred after infection in buffaloes whereas these continuously declined in

cattle after a sharp rise at three weeks post-infection. The numbers of B lymphocytes and plasma cells increased from 3-16 weeks post-infection in both species. Eosinophils were also present in hepatic lesions, which may be partly a consequence of the degranulation of mast cells in hepatic lesions as a result of antigenic stimulation from the flukes. It is concluded that both cellular and humoral responses are induced in the liver of cattle and swamp buffaloes infected with *F. gigantica*. The T cell response in cattle was apparently suppressed after week 3 of infection which may be due partly to the rapid migration of flukes or to a suppression of the local immune response in the liver of cattle by *F. gigantica*. The increasing responsiveness in buffaloes represented by the gradually increasing numbers of T lymphocytes may have contributed to the suppression of development of flukes or delayed their migration in these animals. This difference in the expression of the hepatic T cell response between cattle and swamp buffaloes may be related to the observed differences in their level of resistance against *F. gigantica*.

The percentage of eosinophils in the blood increased in infected animals. The eosinophilia observed may have resulted from the generalized inflammation following liver fluke infection and may not be protective as migrating flukes or dead flukes with surrounding eosinophils were not seen in the liver. Eosinophilia also indicates a stimulation of a Th2-type of immune response in these animals during infection with *F. gigantica*. The kinetics of eosinophilia differed between hosts. A rapid eosinophilia was observed within 1-3 weeks post-infection in cattle whereas this was considerably delayed in buffaloes to weeks 6-11. The slower eosinophil response in buffaloes may be associated with the increased resistance to *F. gigantica* in this host, i.e eosinophils are not an effector cell involved in killing immature *F. gigantica* during the first five weeks of infection.

Histopathology of liver and hepatic lymph nodes revealed some differences in the extent of lesions between cattle and swamp buffaloes at different periods of infection. At three weeks post-infection, focal necrosis was present in cattle but not in buffaloes. The hepatic lymph node (HLN) of cattle showed stronger follicular and parafollicular hyperplasia compared with buffaloes. Lymphocytic infiltration in portal areas was more marked in cattle than buffaloes at seven weeks post-infection and more plasma

cells were present in the medullary cords of HLN of cattle than buffaloes. Marked portal reaction, bile duct hyperplasia and severe cirrhosis were seen in cattle at 12 weeks post-infection. Only moderate cirrhosis was observed in buffaloes at the same time post-infection. At 16 weeks post-infection in cattle necrosis of bile ducts was seen with mostly eosinophils in the inflammatory infiltrate. In buffaloes, most of the inflammatory cells were lymphocytes. These results imply that there was milder damage and inflammatory response in the liver and milder stimulation of the HLN at some stages of infection in buffaloes compared with cattle which could be due to their lower fluke burden or to the delayed migration or suppressed development of flukes in buffaloes.

Results of this study showed that there were similarities and differences in the immune responses of cattle and buffaloes during infection with F. *gigantica*. These varying responses to *F. gigantica* infection represent differences in host-parasite relationships of *F. gigantica* infection between cattle and swamp buffaloes and may be linked to the observed varying levels of resistance and resilience to infection between these hosts.

List of Published Papers

1. Prevalence of Infection with *Fasciola gigantica* and Its Relationship to Carcase and Liver Weights, and Fluke and Egg Counts in Slaughter Cattle and Buffaloes in Southern Mindanao, Philippines (E. C. Molina, E. A. Gonzaga and L. A. Lumbao, 2005, Tropical Animal Health and Production, 37, 215-221)

2. Cellular and humoral responses in liver of cattle and buffaloes infected with a single dose of *Fasciola gigantica* (Elizabeth C. Molina and Lee F. Skerratt, 2005, Veterinary Parasitology, 131, 157-163

3. Serum interferon-gamma and interleukins-6 and -8 during infection with *Fasciola gigantica* in cattle and buffaloes (Elizabeth C. Molina, 2005, Journal of Veterinary Science, 6, 135-139)

4. Clinico-pathological indications of resilience of swamp buffaloes (*Bubalus bubalis*) to infection with *Fasciola gigantica* (E. C. Molina, E. O. Sinolinding and Peralta A., 2005, Tropical Animal Health and Production, 37 (6), 451-455)

5. Differences in Susceptibility Between Cattle and Swamp Buffaloes to Infection with *Fasciola gigantica* (E. C. Molina, E. A. Gonzaga, E. O. Sinolinding, L. A. Lumbao, A. Peralta and A. Barraca, 2005, Tropical Animal Health and Production, 37 (8), 611-616)

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LIST OF ABBREVIATIONS

ABTS	2,2-Azino-di-[3-ethylbenthizolin sulfonat (6)]
ADCC	Antibody-dependent cell-mediated cytotoxicity
DAB	Diaminobenzidine
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory-secretory
FABP	Fatty-acid binding protein
FEC	Faecal egg count
GGT	Gamma-glutamyl transferase
GLDH	Glutamate dehydrogenase
GST	Glutathione *S*-transferase
HRP	Horseradish peroxidase
HLN	Hepatic lymph node
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
ITT	Indonesian thin-tail
mAb	Monoclonal antibody
MCH	Mean corpuscular haemoglobin
NEJ	Newly excysted juvenile
OD	Optical density
pAb	Polyclonal antibody
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCV	Packed cell volume
PMN	Polymorphonuclear
TBS	Tris buffered saline
Th	Helper T
TMB	Tetramethylbenzidine
Tris-EDTA	Tris-ethylenediamine tetra-acetic acid

CHAPTER 1

GENERAL INTRODUCTION

Tropical fasciolosis is a significant disease of domestic livestock, particularly in cattle and buffaloes causing losses to agricultural and livestock production (Spithill, Smooker and Copeman 1999). It is caused by the tropical liver fluke, *Fasciola gigantica*. *Fasciola gigantica* is a digenetic leaf-like trematode that requires a lymnaeid snail, *Lymnae rubiginosa auricularia*, as an intermediate host (Soulsby 1982). The parasite invades the liver and maturity occurs in the bile ducts.

Fasciola gigantica is generally restricted to the tropics and having been recorded in Africa, the Middle East, eastern Europe and south and eastern Asia (Torgerson and Claxton 1999). Reports about *F. gigantica* are limited despite its being a common disease among livestock. The host-parasite interactions of the infection in cattle (*Bos indicus*) and swamp buffaloes (*Bubalus bubalis*) have not been extensively studied hence information that may be used as a basis to indicate possibilities for the control of the disease in these animals is lacking.

The aim of this project was to look at differences of the host-parasite relationships between cattle and swamp buffaloes. Abattoir data on the prevalence of infection, clinico-pathological and parasitological parameters from animals naturally infected with *F. gigantica* were compared between cattle and buffaloes to provide an indication on their resilience and resistance to infection. An experimental infection was undertaken to further examine these aspects and to look at the immune responses of infected animals. The sequential production of IgG1, IgG2 and IgE isotypes, profile of serum cytokines and cellular and hepatic immune responses were evaluated.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

In this chapter there is a general description of *Fasciola gigantica* and the disease the parasite causes, fasciolosis. Most of the information describes *F. gigantica* infection in cattle since studies in buffaloes are few and limited. Information about *F. hepatica* infection is also provided to serve as a basis for comparison with *F. gigantica* infections. Emphasis is given to the immunology of fasciolosis such as the specific humoral and cell-mediated immune responses caused by infection with both *F. gigantica* and *F. hepatica*. Recent advances in understanding the role of T-cells and/or the cytokines they produce during *Fasciola* infection is also described with emphasis mainly on infection with *F. hepatica* since published reports dealing with this aspect are limited only to infection caused by this species.

2.2 *Fasciola gigantica*

Fasciola gigantica is a liver fluke that is a leaf-shaped digenetic trematode that invades the liver before entering the bile ducts where maturity occurs (Soulsby 1982). It is a parasite of domestic and wild animals and humans but is most important in cattle and buffaloes (Losos, 1986). It causes the disease tropical fasciolosis (Soulsby 1982). Research in Indonesia has established that infection with *F. gigantica* reaches a high prevalence in cattle and buffaloes in irrigated rice-producing areas where the snail intermediate host thrives (Copeman 1999). The common practice by farmers of using faeces from buffaloes and cattle as fertilizer in rice fields, feeding the rice straw after harvest and allowing these animals access to newly harvested fields to graze promotes infection and facilitates the continuation of the life-cycle of *F. gigantica* (Suhardono et al., personal communication; Copeman 1999).

Soulsby (1982) and Losos (1986) outlined the life cycle of *F. gigantica* and it will be briefly described here. Adults in bile ducts shed eggs after 12-20 weeks post-

infection. The eggs are carried by the bile, excreted via the faeces of infected animals and hatch in water after 10 to >100 days depending on the temperature. Fully-developed miracidia leave the eggs and swim actively to penetrate lymnaeid snails such as *Lymnaea auricularia rubiginosa* and develop for 25 to >100 days into sporocyts, rediae and then cercariae. The cercariae are shed from the snails and develop as metacercariae, which encyst on immersed herbage near the surface, or remain in water as "floating cysts". When the metacercariae are ingested by a definitive host, excystment occurs in the duodenum and flukes migrate from here into the peritoneal cavity, across the liver capsule and into the liver parenchyma. The flukes wander in the liver parenchyma for about 7-8 weeks after infection. They then enter the bile ducts from about 9 weeks post-infection where they mature.

2.3. Economic Significance

Fasciolosis is a major constraint to agricultural and animal production in most countries located in the wet tropics (Spithill et al 1999). It causes a significant reduction in meat and milk production, growth rate, fertility and draught power in infected animals (Dargie 1987). Other causes of economic loss are condemnation of infected livers at slaughter and the cost of control measures. It is difficult to estimate the global economic losses due to fasciolosis due to the paucity of available information on the effect of worm burden, nutritional level and breed on the extent to which infection adversely affects animal production. From 1975 to 1997, it has been estimated that economic losses in tropical countries reached more than US$3200 million (Spithill et al 1999). Significant economic losses occur in tropical regions where prevalence of infection is high. The disease has been reported to cause losses amounting to US$20 million to US$107 million in countries where it is prevalent such as the Philippines, Cambodia and Indonesia (Spithill et al 1999). However, these estimates are conservative as they are usually based on loss caused by only one or two production indices such as liver condemnation or lower carcass weight at slaughter, whereas infection also reduces draught and reproductive performance and milk production.

2.4 Pathogenesis and pathology

The pathogenesis of *F. gigantica* has been regarded as similar with that of *F. hepatica* since both flukes have similar life cycles in the definitive hosts (Soulsby 1982). In addition, migrating immature flukes of both species similarly cause damage to the hepatic parenchyma and the mature flukes cause damage to the bile ducts (Soulsby 1982; Losos 1986). However, information regarding the pathogenesis of *F. gigantica* is relatively limited to support or refute this hypothesis. It was considered that the infectivity between the two *Fasciola* species differs, with *F. gigantica* having greater infectivity than *F. hepatica* in cattle due to its longer duration of infection (Hammond and Sewell 1975). It is also considered more pathogenic due to its ability to cause more damage because of its larger size and longer migration in the liver than *F. hepatica* (Ogunrinade 1984).

2.4.1 Prepatent period and infection in the definitive host

The time for larval *F. gigantica* to develop in the hepatic parenchyma and enter the bile ducts in cattle takes about 89 days after infection (Guralp, Ozcan and Simms 1964). The prepatent period may vary from 12 to 16 weeks (Grigoryan 1958; Guralp et al 1964; Sewell 1966; Prasitirat, Thammasart, Chompoochan, Nithiuthai and Taira 1966). In buffaloes, reports of the prepatent period vary. Sanyal (1996) observed a prepatency period of 63 days whereas Yadav et al (1999) observed a longer prepatent period of 92-97 days. Variations in the prepatent period may be due to the sensitivity of the egg detection method used, the size of the infecting dose, breed of host and strain of *F. gigantica* (Spithill et al 1999).

Egg output increases during the first 4 to 12 weeks after eggs appear in the faeces (Sewell 1966; Prasitirat et al 1996). Survival of adult *F. gigantica* in cattle is usually less than a year but in some cases reaches 3 to 4 years (Hammond and Sewell 1975).

2.4.2 Clinical signs

Animals infected with *F. gigantica* show poor condition. Effects such as weight loss and emaciation (Haroun and Hussein 1975; Dargie 1987) may reach significant levels

and a reduction of 75 kg is possible during the growing years in infected buffaloes and cattle (Roberts, Bakrie, Copeman and Teleni 1991). Similarly, infection with *F. hepatica* is also characterised by significant weight loss in sheep (Coop and Sykes 1977) and cattle (Ross 1970; Hope-Cawdery, Stickland, Conway and Crowe 1977). The effects of fasciolosis on growth performance in cattle are manifested during the biliary stage of the infection. Decreased growth rate, poor body condition, very poor appetite, dullness and lethargy were observed 10 weeks after infection in young cattle experimentally infected with 2000 metacercariae of *F. gigantica* (Hammond and Sewell, 1974). At this time flukes are migrating into the bile ducts. Meanwhile, reduction in growth rate of buffaloes infected with *F. gigantica* may occur earlier when flukes are still in the hepatic parenchyma. Yadav et al (1999) reported that inappetence was first observed from 5 weeks of infection in riverine buffaloes infected with 1000 metacercariae of *F. gigantica*. This continued and poor weight gain became significant from 7 weeks after infection.

Other manifestations of infection with *F. gigantica* are decreased work capacity, reproductive performance and milk production. Reduction in work performance could be a major concern especially in the farming rural communities where buffaloes and cattle are the major source of draught power. As much as 27-35% more time to work in the field is required in infected buffaloes and cattle with a further 15% additional working time in animals that are anaemic as a result of infection (Roberts et al 1991). Infected Ongole cows in Indonesia had longer intercalving intervals (31.5 months) compared to those treated with triclabendazole (18.5 months) (Suhardono, Widjajanti, Stevenson and Carmichael 1991). Infertility due to *F. hepatica* has been encountered in infected sheep and cattle. Lower fertility rates were noted in infected or inadequately treated cattle (Oakley, Owen and Knapp 1979; Hope-Cawdery, 1984) and in ewes (Hope-Cawdery 1976) infected with *F. hepatica*. Heavy infections of *F. hepatica* may result in abortion and stillbirths in sheep (Sinclair 1972; Hope-Cawdery 1976). A study on the fertility of rural cows indicated that poor reproductive performance among infected animals is influenced by anaemia that occurs during infection (Kumar and Sharma 1991). This study showed that anoestrus cows and repeat breeders had significantly lower haemoglobin levels than cows with normal cycles. A lower packed cell volume (PCV) was also observed in *F. gigantica*-infected Ongole cows in Indonesia which manifest longer intercalving

interval than those cows treated with triclabendazole (Suhardono et al 1991). Thus, the effects of fasciolosis have a great impact on the productivity of the animals, not only in terms of weight gain but also in other aspects of production such as reproductive performance which is also economically important in the farming communities in the rural tropical areas.

An increase in milk yield by a mean of about 5.5 liters per buffalo cow per week occurred after treatment with triclabendazole (Kumar and Pachauri 1989). Horschner et al (1970) and Randell and Bradley (1980) (cited by Dargie 1987) reported a decrease of 90-300 kg/lactation in cattle infected with *F. hepatica*. However, there are also reports that indicate that infection with *F. gigantica* does not adversely affect milk production. The weaning weight of calves from cows infected with *F. gigantica* did not differ significantly from those calves from cows that were treated every 8 to 12 weeks (Needham 1977). Reliable estimates of the loss of milk production caused by fasciolosis cannot be made due to this limited information.

2.4.3 Clinical Pathology

The common haematological features in fasciolosis are anaemia and hypoproteinaemia. Anaemia is considered as the single most important factor contributing to disease caused by liver fluke infections (Behm and Sangster 1999). It may be due to ingestion of blood by flukes (Ogunrinade and Bamgboye 1980), loss through haemorrhage in bile ducts (Behm and Sangster 1999), toxic components, or failure of the blood-forming tissues to function properly (Blood, Henderson and Radostits 1983). In cattle naturally infected with *F. gigantica*, total erythrocyte counts, haemoglobin level, packed cell volume (PCV) and mean corpuscular haemoglobin (MCH) were significantly lower compared with values observed in apparently healthy cattle (Taimur, Halder, Chowdhury, Akhter, Islam, Kamal and Islam 1993). Anaemia occurs as flukes establish in the bile ducts indicating that adult flukes are haematophagous. It was observed as early as 12-14 weeks (Ogunrinade and Anosa, 1981) or later at 16 weeks (Ening Wiedosari et al, personal communication) or at 20-25 weeks post-infection in cattle infected with *F. gigantica* (Ogunrinade 1983a). Similar observations were encountered in sheep infected with *F. gigantica* and *F. hepatica* (Behm and Sangster 1999). These however, differ from

observations in riverine buffaloes infected with *F. gigantica* in which a marked fall in haemoglobin levels commenced during the early prepatent period at 45 days post-infection (Yadav et al 1999). These authors considered that the migrating juvenile flukes cause hepatic haemorrhage which continues until flukes establish in the bile ducts.

The number of eosinophils in peripheral blood of infected animals is elevated during *F. gigantica* infection. Eosinophilia commences early in the infection in cattle, buffaloes and sheep infected with *F. gigantica* (Hansen, Clery, Estuningsih, Widjajanti, Partoutomo and Spithill 1999; E. Wiedosari et al, personal communication). *Fasciola hepatica* infections are also manifest by high levels of eosinophilia which increases rapidly during the time that flukes are migrating in the parenchyma and persisting until the biliary stage of infection (Ross, Todd and Dow 1966; Sinclair 1973, 1975; Poitou, Baeza and Boulard 1992, 1993).

The role of eosinophils during infection with *F. gigantica* remains to be investigated. It could be associated with resistance against *F. gigantica* in some species as resistant animals manifest a trend for a higher level of eosinophilia. Percentage of eosinophils in the blood of *F. gigantica*-resistant ITT sheep was higher compared to that found in the more susceptible Merinos (Hansen et al 1999). Some authors reported that eosinophils may be associated with the expression of resistance to *F. hepatica* as these cells infiltrate the intestine and selectively adhere to newly-excysted juveniles (NEJs) (Doy, Hughes and Harness 1980; Van Milligen, Cornelissen, Hendriks, Gaasenbeek and Bokhout 1998) where they degranulate causing severe damage and perhaps death to the young flukes (Davies and Goose, 1981; Burden, Bland, Hammet and Hughes 1983).

Destruction of the hepatic tissue during migration of larval flukes and the presence of adult flukes in the bile ducts cause changes in the levels of serum proteins and enzymes of infected animals. Proteins associated with liver functions either increase or decrease during infection causing hypoalbuminaemia and hyperglobulinaemia which are common features in liver fluke infections (Behm and Sangster 1999). This was seen in buffaloes and cattle infected with *F. gigantica* (Haroun and Hussein, 1975; Kumar, Pathak and Pachauri 1982) and in calves with experimental infection of

F. hepatica (Anderson, Berret, Brush and Patterson 1978). The decline in plasma albumin concentrations is partly due to a decreased synthesis and partly to an expansion of the plasma volume following the destruction of the hepatic parenchyma during fluke migration (Anderson et al 1977; Dargie 1981; Symons 1989).

Hepatic enzymes are liberated into the blood due to the destruction of hepatic tissue (Behm and Sangster 1999). These result in increased levels of serum enzyme associated with liver function. Levels of glutamate dehydrogenase (GLDH) increase due to inflammation of the liver induced by the migrating juvenile flukes during the first stages of fasciolosis (Galtier, Larrieu, Tufenkji and Franc 1986; Benchaoui and McKellar, 1993). Damage to bile ducts induced by adult flukes cause an increase in gamma-glutamyl transferase (GGT) (Sandeman and Howell 1981; Wensvoort and Over 1982). Levels of these enzymes in the serum or plasma could be useful indicators of hepatic damage during fasciolosis (Kramer 1980). They also give an indication about the progression of infection as GLDH provides information on the migration of immature flukes in the hepatic parenchyma, whereas GGT indicates establishment of flukes in the bile ducts (Sykes, Coop and Robinson 1980; Galtier et al 1986; Ferre, Ortega-Mora and Rojo-Vazquez 1997).

2.4.4 Gross pathology and histopathology

Most of the pathological manifestations of infection with *F. gigantica* are seen in the livers of infected animals and are related to the migration of juvenile flukes in the hepatic parenchyma and the presence of adult flukes in the bile ducts. The initial lesion consists of haemorrhages in the liver induced by migrating flukes with migratory tracts enlarging and necrosis occurring as young flukes grow (Losos 1986). This initial necrosis is followed by proliferation and calcification of the bile duct (Losos 1986). Severe infections are characterised by fibrosis, hyperplasia and calcification of the bile ducts, with liver fibrosis being the most significant lesion in chronic infection (Losos 1986; Yadav et al 1999). Fibrosed bile ducts may be seen beneath the liver capsule in buffaloes infected with *F. gigantica* (Yadav et al 1999). *Fasciola hepatica* infection in cattle is also characterised by thickening of the bile ducts but in sheep it does not usually occur (Hammond 1973; Behm and Sangster 1999).

Histopathological lesions are evident in the liver and bile ducts in cattle and buffaloes infected with *F. gigantica*. These lesions are similar with those observed in cattle infected with *F. hepatica.* The lesions described below were noted in cattle infected with *F. gigantica* (Ogunrinade 1983a) and similar microscopic lesions could be observed in buffaloes and sheep infected with *F. gigantica* (Losos 1986). Inflammatory cells surrounded haemorrhagic tracts in the liver. The portal and hepatic arteries contained many thrombi and the bile duct epithelium had mild portal cellular inflammation and desquamation. There was marked inflammatory cell infiltration of the portal triad, which was surrounded by fibroplasia. Hepatocytes showed degeneration resulting from fibrosis and calcification of the liver.

Some of the pathological changes such as intense fibrosis and calcification of the bile ducts in cattle infected with *F. gigantica* are a result of the immune response that the host mounts against the flukes (Hammond and Sewell 1975). T lymphocytes mediate the cellular inflammatory response against the migrating flukes in the hepatic parenchyma inducing fibrosis (Hughes 1987). This response is beneficial to the host as it partially protects it against the tissue damage caused by the flukes (Behm and Sangster 1999). It may also contribute to resistance against secondary infections with *F. hepatica* in cattle (Ross 1967; Boray 1967) as cited by Mulcahy, O'Connor, Clery, Hogan, Dowd, Andrews and Dalton (1999b) and Doyle (1971) since fibrosis limits the movement of the flukes in the liver (Behm and Sangster 1999).

The above studies only described the general pathology and histopathology during *F. gigantica* infection in cattle and swamp buffaloes. No definitive comparison can be made on their pathology at different stages of infection because of the absence of sufficient information comparing the pathological lesions between cattle and buffaloes in relation to the time of infection.

2.4.5 Resistance to infection

Resistance to *Fasciola* infection is usually indicated by reduced worm burden, egg production and damage in the liver. Resistance and the degree of susceptibility to infection to *F. gigantica* vary among mammalian species (Spithill et al 1999). In fasciolosis, the outcome of an infection depends on the

interaction between the host and the parasite (Piedrafita, Raadsma, Prowse and Spithill 2004).

Few published data describe resistance to *F. gigantica* in cattle and none in buffaloes. From these studies it can be surmised that non-immune and immune factors are associated with resistance to *F. gigantica* in cattle. Non-immune resistance involves the development of fibrosis in the liver which would create a physical barrier to invading flukes (Bitakaramire and Bwangamoi 1970). Reduction in fluke burden in cattle challenged with *F. hepatica* was attributed to fibrosis that was induced by primary infection (Doyle 1973; Kendall and Parfitt 1975; Doy and Hughes 1984; Piedrafita et al 2004). However, a long infective period from a primary infection is necessary before this can be induced (Piedrafita et al 2004). Fibrosis from a primary infection that became diffuse at 21 weeks prevented significant liver damage in cattle from a secondary infection with *F. gigantica* except at the ventral lobe at 16 weeks post-infection (Bitakaramire and Bwangamoi 1970). Younis, Yagi, Haroun, Gameel and Taylor (1986) observed that eight weeks after administration of irradiated metacercariae in zebu cattle, a 65 % reduction in fluke burden after challenge with *F. gigantica* was induced. As the eight-week period between primary and secondary challenge was not sufficient for extensive fibrosis to occur, these authors suggested that the resistance shown by these vaccinated zebu cattle could not be due to fibrosis but rather was acquired. An acquired immune resistance mechanism was also demonstrated in an unpublished study involving Bali, Ongole and buffalo calves given a trickle infection of 15 metacercariae of *F. gigantica* for 32 weeks (E. Wiedosari et al, personal communication). This study showed that Bali cattle which manifest an increasing IgG2 response from week 4 of infection, showed a declining GLDH value after week 24 and a progressive reduction in egg count after week 26. E. Wiedosari et al (personal communication) proposed that this apparent manifestation of resistance in Bali cattle was due to their high specific IgG2 response as it was suggested that protection induced by vaccination against bovine fasciolosis caused by *F. hepatica* has been linked to IgG2 (Mulcahy, O'Connor, McGonigle, Dowd, Clery, Andrews and Dalton 1998). However these data are limited and prevent an informed conclusion being made. Vaccination of naïve cattle with defined *F. hepatica* antigens protected these animals from *F. hepatica* infection indicating that acquired immune responses protect animals from infection (reviewed in Spithill

and Dalton 1998; Mulcahy and Dalton 2001). The above observations suggest that resistance to liver fluke infection in cattle may thus be associated with both immune and non-immune factors as both fibrosis and immune mechanisms appear to confer protection against subsequent infections. No conclusions can be made for swamp buffaloes as there is insufficient information describing resistance against fasciolosis in these animals.

Resistance to *F. gigantica* is also demonstrated in sheep (Ogunrinade 1984; Wiedosari and Copeman 1990; Roberts, Estuningsih, Widjajanti, Wiedosari, Partoutomo and Spithill 1997a). ITT sheep demonstrated a remarkable resistance to this parasite (Wiedosari and Copeman, 1990). This resistance is immunologically-based as it was suppressed by dexamethasone (Roberts, Estuningsih, Wiedosari and Spithill 1997b).

Resilience to *F. gigantica* may also vary as differences in response to infection occur among different breeds or species. One study compared responses of buffaloes, Bali and Ongole cattle to a trickle infection with *F. gigantica* (E. Wiedosari et al, personal communication). Weight loss per fluke was similar in buffaloes and Ongoles but significantly higher in Bali cattle suggesting that resilience of buffaloes and Ongoles to *F. gigantica* is similar and the effect of infection on Bali cattle is comparatively much more severe. However, because Bali cattle showed a decline in GLDH value after week 24 and a progressive reduction in egg count after week 26 despite a trickle infection with *F. gigantica* for 32 weeks it is apparent that this breed manifest a degree of resistance to *F. gigantica* (E. Wiedosari et al, personal communication). These observations indicate differences in responses between the Ongole and Bali breeds of cattle and between these breeds and buffaloes to infection with *F. gigantica*. Cattle also appeared to be less susceptible to *F. gigantica* than sheep and goats (Sewell 1966) as infection with 500 or 1000 metacercariae of *F. gigantica* did not induce clinical signs of fasciolosis in these animals (Hammond and Sewell 1974). Signs of infection such as reduced appetite and lethargy could be manifest only when these animals were infected with 2000 metacercariae (Hammond and Sewell 1974). Indonesian thin-tailed (ITT) sheep are more resistant to *F. gigantica* compared with Merinos (Roberts et al 1997b). West African Dwarf sheep were considered to be more resistant to *F. gigantica* than the West African Dwarf goats because the former

showed longer mean survival period than the goats during a heavy infection (Ogunrinade 1984). Contrary to observations of infections with *F. gigantica* in sheep, these animals do not develop resistance against *F. hepatica* (Roberts, Widjajanti and Estuningsih 1996; Sandeman and Howell 1981; Chauvin, Bouvet and Boulard 1995) except the St. Croix sheep which had some resistance against *F. hepatica* (Boyce, Courtney and Loggins 1987) but did not have resistance against *F. gigantica*. Breed may therefore influence the development of resistance against *F. gigantica* and the establishment and severity of the infection. Roberts et al (1996) proposed that *F. gigantica* adults contain an antigen that stimulates a protective response and that there is no analogue to that antigen in *F. hepatica*. All these observations suggest that differences in resistance may be due to the breed of animal and also species of fluke.

2.5 Antigenicity of *F. gigantica*

2.5.1 Somatic and excretory-secretory (ES) antigens

Fasciola gigantica possesses antigenically diverse materials that are capable of stimulating the host immune responses. These antigens have been utilised to investigate the immune response of the host and as possible sources of vaccine materials. These antigens include the somatic antigens (Hanna and Jura 1977; Ogunrinade 1983b; Yoshihara and Goto 1993) and excretory-secretory (ES) antigens (Guobadia and Fagbemi 1995; Fagbemi, Aderibigbe and Guobadia 1997; Velusamy, Singh, Sharma and Chandra 2004). Excretory-secretory antigens include the 28-kDa cysteine protease (Fagbemi and Guobadia 1995). Excretory-secretory antigens (27-kDa to 60-kDa) are also present in *F. hepatica* (Irving and Howell 1982; Mulcahy et al 1999b).

2.5.2. *Fasciola* antigens as vaccine candidates

Protection against liver fluke infection has been demonstrated utilising a number of antigens produced by *Fasciola* spp. Vaccination of zebu calves with irradiated metacercariae of *F. gigantica* gave a 45 to 68% protection against *F. gigantica* (Younis et al 1986). Irradiated metacercariae of *F. hepatica* protected cattle and rats

from *F. hepatica* (Rickard and Howell 1982; Hughes 1987). Glutathione-*S* transferase (GST) and *F. gigantica* cathepsin L did not significantly protect cattle against *F. gigantica* (Estuningsih, Smooker, Wiedosari, Widjajanti, Vaiano, Partoutomo and Spithill 1997). These proteins were protective against *F. hepatica* where a higher degree of protection is conferred in vaccinated cattle and sheep (Hillyer, Haroun, Hernandez and De Galanes 1987, Sexton, Milner, Panaccio, Waddington, Wijffels, Chandler, Thompson, Wilson, Spithill, Mitchell and Campbell 1994; Morrison, Colin, Sexton, Bowen, Wicker, Friedel and Spithill 1996). This varying response of animals to vaccination with FABP, GST and cathepsin L of *F. gigantica* and *F. hepatica* suggests that these two parasites differ in their host-parasite relationships. Estuningsih et al (1997) suggested that *F. gigantica* may differ from *F. hepatica* in being resistant to bovine immune responses induced by vaccination with GST.

2.6 Recent Advances in Understanding the Role of T Cells in Parasitic Infection

2.6.1 T Cells

Mature T cells are classified according to the antigen they express on their surface (Tizard 1996). CD4 and CD8 are surface glycoproteins, which serve as specific markers for T lymphocytes. They are used to identify lymphocyte subpopulations. T lymphocytes that recognise exogenous processed antigen have CD4 on their surface (Tizard 1996). These CD4+ T cells are of two kinds, inflammatory T cells and helper T cells. Helper T cells activate macrophages and stimulate production of antibodies by B cells (Sehgal and Berger, 2000). These cells promote immune responses (Tizard et al., 1996). CD8 is only found on cytotoxic T cells, which attack and kill abnormal cells and may suppress the immune response (Tizard 1996).

The cytokine produced by the helper T cells determine whether the immune response induced is Th1 or Th2 response (Tizard 1996). T helper lymphocytes are the major cells involved in an immune response when any infectious agent infects an immunologically intact host (Cox 2001). Initially, T cells are at first uncommitted but they gradually differentiate into Th1 and Th2 cells, each characterised by the cytokines they produce and eventually, once they have differentiated they mediate either a humoral or cell-mediated immune response through their production of

cytokines (Cox 2001). Th1 cells produce T1 cytokines, particularly IL-2 that stimulates the production of cytotoxic T cells, and interferon-γ (IFN-γ) that activates macrophages. Th1 response is normally manifested during infection with intracellular bacteria and viruses (Abbas, Murphy and Sher 1996; Finkelman, Pearce, Urban and Sher 1991; O'Garra and Murphy, 1994) whereas Th2 response is induced following allergy and infection with helminth parasites (Abbas et al 1996; Finkelman et al 1991; O'Garra and Murphy 1994) and is manifest by IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 which activate B cells leading to production of antibody (Cox 2001; Abbas et al 1996; Mosmann and Sad, 1996; O'Garra, 1998). The Th1 response represents the cell-mediated arm of the immune response and the Th2 component mediates a humoral response (Cox 2001). In some cases however, the response during infection is non-polarized (Th0 response) when there is a simultaneous expression of cytokines characteristic of Th1 and Th2 responses. This was demonstrated in cattle with chronic *F. hepatica* infection where either IL-4, IL-2 and IFN-γ or combinations of IL-2 and IL-4 or IL-4 and IFN-γ were present (Brown, Davis, Dobbelaere and Rice-Ficht 1994a).

It may thus be concluded that the nature of the disease is influenced by the type of immune response induced during infection and that cytokines direct the type of response. Hence, the response could either be Th1, Th2 or Th0 response. An immune response that is characteristic of either of these types is influenced by a number of factors, among which are the cytokines produced following infection. Cells of the Th1 and Th2 type elaborate cytokines that down-regulate the differentiation and functions of the reciprocal subtype. For example, IL-4 is important in inducing a Th2 response and has also been observed to be involved in the reciprocal down-regulation of Th1 responses (Brady, O'Neill, Dalton and Mills 1999) whereas IL-12 and IL-18 produced by dendritic cells and macrophages lead to a Th1 response (Bohn, Singh, Zumbihl, Bielfeldt, Okamura, Kurimoto, Heeseman and Autenriech 1998; Trincheiri, 1994). A Th0 response is characterised by a production of cytokines characteristic of both Th1 and Th2 responses (Brown, Woods, Chitko-Mcknown, Hash and Rice-Ficht 1994b). Because bovine helper T cells are considered to be a mixture of three subsets: Th1, Th2 and Th0 (Brown, Rice-Ficht and Estes 1998), it is possible that the cytokines that will be present during infection will manifest considerable crossover among these three subsets. The sum of

each subset response over the course of an immune reaction will determine the outcome of an infection.

In addition to the cytokine responses, the outcome of the infection will also be determined by the properties of the parasite. It was observed that sheep peritoneal macrophages did not exert an ADCC against *F. hepatica* (Piedrafita, Parsons, Sandeman, Wood, Estuningsih, Partoutomo and Spithill 2001) but these cells killed *F. gigantica in vitro* (Estuningsih, Widjajanti, Partoutomo and Spithill 1999). This indicates that *F. gigantica* and *F. hepatica* differ in biochemical traits that determine susceptibility to sheep immune effector mechanisms, such as defence enzyme expression (Piedrafita et al 2004).

2.6.2 The role of T helper cells in parasitic infections

There is evidence that various infections caused by parasites can cause polarization of T cell responses into CD4+ T cell subset response (Scott, Natovitz, Coffman, Pearce and Sher 1988). These authors considered that the cytokines produced by these different Th cell responses tend to have opposing effects on the parasites, which subsequently results in either control of infection or promotion of disease. In addition, the cytokines of one CD4+ T helper subset tend to downregulate the production and/or activity of the cytokines produced by the other subset resulting in the establishment and survival of parasites (Sher and Coffman 1992).

However, manifestation of cytokines during infection does not always follow the Th1/Th2 theory. Kelso (1995) proposed that Th1 and Th2 cells are just among the heterogeneous population of clonal T cells expressing different levels of a variety of cytokines and that there are other T cell populations which cannot be detected by current techniques because of their lack of sensitivity. Cattle may express heterogeneous subsets of cytokines but it is also possible that either a type 1 or type 2 response dominates during infection or following antigen exposure (Brown et al 1998). It is also possible that switches may occur in the profiles of Th1 and Th2 cytokines during an infection (Allen and Maizels 1997).

2.6.2.1 Th1 cytokines are responsible for protection against intracellular parasites

CD4+ T cells play a dual regulatory and effector roles in parasitic infections. This is best exhibited during infection with *Leishmania major* in mice wherein a Th1 response characterised by the production of IFN-γ, was necessary for the control of the infection while a Th2 response manifested by IL-4 production resulted in the unrestrained growth of the parasite (Scott et al 1988, 1989). IFN-γ - and TNF-α induced macrophages to release nitrites which killed amastigotes (Liew, Li and Millot 1990). In the presence of IL-4 however, macrophage activation was prevented resulting in a disseminated and fatal infection whereas mice given a single injection of anti-IL-4 antibody during infection secreted IFN- γ and eliminated the parasite (Locksley and Scott 1991). Thus, progressive infection with this protozoan was manifested by IL-4 production while an infection with spontaneous healing was associated with a decreased Th2 and an increased Th1 response (Reiner and Locksley 1995).

IFN-γ is also important in the development of protective immunity against other intracellular parasites, such as *Toxoplasma* sp. (Gazzinelli, Hieny, Wynn, Wolf and Sher 1993), *Eimeria* sp. (Rose, Wakelin and Hesketh 1989) and *Cryptosporidium* sp. (Ungar, Kao, Burris and Finkelman 1991).

2.6.2.2 Th2 cytokines are responsible for protection against enteric parasites

Some studies on nematode-infected mice demonstrated that Th2 cytokines are associated with protective immunity against helminths. Type 2 responses are induced by helminths, and expulsion of helminth parasites are induced largely by these responses rather than by the type 1 responses (Sher and Coffman 1992; Urban, Katona, Paul and Finkelman 1991; Svetic, Madden, Zhou, Lu, Katona, Finkelman, Urban and Gause 1993). Protection against nematode parasites is associated with cytokines characteristic of the Th2 response such as IL-3, IL-4, IL-5 and IL-9 and antiparasite IgE and IgG1 antibody responses (Else, Finkelman, Maliszewski and Grencis 1994; Grencis, Hultner and Else 1991; Urban, Madden, Cheever, Trotta, Katona and Finkelman 1993). On the other hand, a Th1 response is manifested by susceptible hosts (Finkelman, Shea-Donohue, Goldhill, Sullivan, Morris, Madden,

Gause and Urban 1997; Else et al 1994). For example, resistance to *Trichuris muris* and *Trichinella spiralis* in mice was reduced following the administration of anti-IL-4 or anti-IL-4 receptor antibodies while IL-4 knockout mice were not able to resist infections with these parasites (Bancroft, McKenzie and Grencis 1998). Protective immunity was also blocked in mice with challenge infection of *Heligmosomoides polygyrus* when anti-IL-4 and anti-IL-4 receptor antibodies were given. This was manifest by their inability to limit adult worm survival and fecundity (Urban et al 1991). Th2 responses characterized by high levels of IgE and eosinophilia were significantly correlated with reduced parasite weight and fecundity in necatoriasis (Pritchard, Quinnell and Walsh 1995).

Immune responses induced by helminth parasites among livestock are also indicated by stimulation of Th2 cells where a more pronounced type 2 response could result in a greater ability to control the infection. This was demonstrated during infection with *Dictyocaulus viviparus* in calves (Scott, McKeand and Devaney 1996) and *Haemonchus contortus* in sheep where isolated lymphoid cells showed a strong production of IL-5 indicating a stimulation of the Th2 type of immune response (Gill, Altmann, Cross and Husband 2000).

Although several studies showed that protection against most gastrointestinal helminths is associated with Th2 stimulation (Urban, Maliszewski, Madden, Katona and Finkelman 1995; Finkelman et al 1997; Else et al 1994; Grencis et al 1991; Urban et al 1993), some studies have demonstrated that a Th1 response is also capable of protection against some GI helminths and that chronicity of disease is due to a Th2 response (Scott, Pearce, Cheever, Coffman and Sher 1989). Mice were protected against *Schistosoma mansoni* after adoptive transfer of CD4+ Th1 clones obtained from mice protectively immunized against the parasite (Jankovic, Aslund, Oswald, Caspar, Champion, Pearce, Coligan, Strand, Sher and James 1996). Invasive larval stages of some human parasites appear to be controlled by a Th1 response (Pritchard and Wilson 1997). A stronger stimulation of the Th1 response has also been demonstrated in cattle following vaccination with cathepsin-L and haemoglobin of *F. hepatica* when higher IgG2 levels were induced compared to IgG1 in vaccinated cattle (Mulcahy et al 1998). IgG2 production is stimulated by IFN-γ which is associated with a Th1 response (Estes 1996).

2.6.2.3 Cytokine profile during fasciolosis

There is no published report regarding cytokine profiles of cattle and buffaloes infected with *F. gigantica* hence no conclusion can be derived regarding differences in cytokine responses between these species. The few published studies on cytokine profile during fasciolosis are on *F. hepatica* infection in cattle and sheep. Most of these studies have demonstrated a dominant Th2-type immune response during the chronic phase of infection. But contrasting observations have been noted during the early phase of infection. Some studies observed a non-polarized Th0 cell response during the early phase of infection wherein IFN-γ, IL-2 and IL-4 were produced (Clery and Mulcahy 1998; Mulcahy and Dalton 2001; Hoyle and Taylor 2003). A non-polarized Th0 response has also been demonstrated during the chronic phase of *F. hepatica* infection in cattle (Brown et al 1994a) and in rats infected with *F. hepatica* (Cervi, Cejas and Masih 2001). However, a recent study in calves infected with *F. hepatica* described an enhanced IL-4 expression between 10 and 28 days post-infection but not of IFN-γ, suggesting an early stimulation of the Th2-type immune response (Waldvogel, Lepage, Zakher, Reichel, Eicher and Heussler 2004). This suggests that in these animals there was a down-regulation of IFN-γ expression early in the infection, which was not observed in the studies of Clery and Mulcahy (1998), Hoyle and Taylor (2003) and Brown et al (1994a). It can be inferred from these studies that the T cell response of animals during liver fluke infection varies particularly during the early phase of the infection. This variation may be due to differences in the level of stimulation induced by varying levels of inoculation. The calves in the study of Waldvogel et al (2004) were given a single dose while calves in the study of Clery and Mulcahy (1998) were infected with trickle doses of *F. hepatica*. Bossaert, Farnir, Leclipteux, Protz, Lonneux and Losson (2000a) demonstrated a stronger IgG1 response (a Th2 response) in calves with a single infection compared to calves with trickle infection of *F. hepatica*. Hence, it is likely that an early Th2-biased response could be induced by a single dose whereas a trickle infection would commence with a Th1 or Th0 response with polarization towards Th2 response as the infection progresses.

Clery and Mulcahy (1998) concluded that the presence of IFN-γ during early *F. hepatica* infection and its absence during the chronic course of the infection demonstrates the ability of *F. hepatica* to modulate the immune responses of cattle during infection and change the responsiveness of Th subsets as the parasites mature. A number of factors have also been incriminated in the shift from Th1 to Th2 response. Oldham (1985) noted that in cattle infected with *F. hepatica*, IL-2 levels decreased from five weeks post-infection while levels of IL-2-producing lymphocytes remained stable, suggesting that IL-2 production by this population of lymphocytes was suppressed. She proposed that the production of a non-specific suppressor of the immune response by T cells might have a role in this suppression. Down-regulation of Th1 response could be induced by Th2 cytokines such as IL-10 and IL-4 (Brown et al 1994b). IL-10 inhibited nitric oxide production by recombinant IFN-γ -activated monocytes in sheep infected with *F. hepatica* (Moreau, Chauvin and Boulard 1998). Brady et al (1999) demonstrated that IL-4 induced in mice against *F. hepatica* suppressed a Th1 response against *Bordetella pertussis* leading to a predominantly Th2 response. Brown et al (1994a) on the other hand, observed clones of T cells in cattle that expressed both unrestricted (Th0) and Th2-like phenotypes in response to stimulation by *F. hepatica* antigens. They did not observe the presence of Th1 cells, suggesting that the Th1 response is down-regulated during infection with *F. hepatica* in cattle.

A number of studies suggested that ES products of *F. hepatica* inhibit T cell proliferation in sheep (Jeffries et al, 1996, 1997; Moreau, Herve, Yu and Alain 2002). The major component in the ES product that is responsible for suppression of host lymphocytes is cathepsin L (Prowse, Chaplin, Clem Robinson and Spithill 2002). Cathepsin-L may either down-regulate or poorly stimulate the immune response of cattle during early infection with *F. hepatica* (Hoyle and Taylor 2003). It also suppresses the Th1 response to concurrent infections in mice infected with *F. hepatica* or inoculated with ES or cathepsin-L by down-regulating IFN-γ production (O'Neill, Brady, Callanan, Mulcahy, Joyce, Mills and Dalton 2000; O'Neill, Mills and Dalton 2001).

The response of cattle to infection with *F. hepatica* is primarily a Th2 or a humoral response. This is shown by a down-regulation of IFN-γ production after

five weeks post-infection in cattle infected with *F. hepatica* (Clery and Mulcahy 1998), an absence of IFN-γ production in chronically infected cattle (Clery, Torgerson and Mulcahy 1996), and the presence of an enhanced IL-4 expression in calves starting at 10 days until 70 days post-infection in calves infected with *F. hepatica* (Waldvogel et al 2004). Sheep infected with *F. hepatica* also had a predominantly Th2 response as IL-10 was produced throughout the first six weeks of infection causing the inhibition of IFN-γ production as the infection progressed (Moreau et al 1998).

Th2 response is not protective against *F. hepatica*. Cows with a predominantly Th2 response were still superinfected with trickle challenge of *F. hepatica* metacercariae (Clery et al 1996). In mice, the down-regulation of Th1 response results in the survival of *F. hepatica* since this results in evasion of the cellular or Th1 immune defenses by the fluke (O'Neill et al 2000). This phenomenon was supported by the finding of these authors wherein mice with more pronounced Th1 responses and (reduced Th2 responses) showed higher resistance during a challenge infection. This observation has also been noted in cattle vaccinated with cathepsin L proteinases of *F. hepatica* (Mulcahy et al 1998; Mulcahy and Dalton 2001). These data show that protection against *F. hepatica* may be induced by a response other than a Th2 response. More studies are needed to support this hypothesis. Immunization of cattle with a vaccine containing cathepsin L proteinases and fluke haemoglobin of *F. hepatica* formulated in Freund's adjuvant induced high level of protection against the parasite. IgG1 and IgG2 were 8 times and 200 times higher, respectively, compared with those in non-immunized cattle (Dalton, McGonigle, Rolph and Andrews 1996; Mulcahy et al 1998). IgG2 titres and fluke burden in these cattle were negatively correlated (Mulcahy et al 1998). In cattle IgG2 is driven by a Th1 response as it is associated with the cytokine IFN-γ (Estes, Closser and Allen 1994). More pronounced Th1 responses and reduced Th2 responses in mice resulted in their being more resistant during a challenge infection with *F. hepatica* (O'Neill et al 2000). This is in contrast with the observation in sheep infected with *F. gigantica* wherein resistant ITT sheep have a down-regulated IgG2 response (Hansen et al 1999). Spithill et al (1999) suggested that this manifests differences in resistance mechanism against *Fasciola* between cattle and sheep with Th1 immune responses being involved in cattle and Th2 responses in sheep.

2.6.3 T cell responses to fasciolosis

There is no published report on the involvement of CD4+ and CD8+ T lymphocytes in the immune response of cattle and buffaloes infected with *F. gigantica*. Published observations on the inflammatory response during *Fasciola* infection are limited to *F. hepatica*. Information indicating the stimulation of local immune responses in the liver, spleen and hepatic lymph nodes during *F. gigantica* infection in cattle and buffaloes is lacking therefore no comparison can be made on their T cell responses. Both CD4+ and CD8+ T lymphocytes are involved in the immune response of cattle against *F. hepatica*. Bovine peripheral blood lymphocyte populations depleted of CD4+ or CD8+ T lymphocytes had reduced or essentially absent proliferative response during days 21-56 when cultured in the presence of *F. hepatica* antigen, leading McCole, Doherty, Baird, Davies, McGill and Torgerson (1999) to conclude that both CD4+ and CD8+ T lymphocytes are involved during the acute stages of infection with *F. hepatica* in cattle. Brown et al. (1994a) had previously demonstrated that during the acute stage of infection in cattle, CD4+ T lymphocytes are primarily involved in the immune response as evidenced by a vigorous proliferation of cell lines when cultured with *F. hepatica* antigen.

CD4+ and CD8+ T lymphocytes are also involved during infection with *F. hepatica* in sheep and goats. Acute lesions in the hepatic parenchyma in sheep and hepatic lymph nodes in goats were predominated by CD4+ T lymphocytes (Meeusen, Lee, Rickard and Brandon 1995; Perez, de las Mulas, De Lara, Gutierrez-Palomino, Becerra-Martel and Martinez-Moreno 1998). CD4+ T cells and B cells increased significantly in the local draining lymph nodes of sheep infected with *F. hepatica* (Meeusen et al 1995). These authors noted that B cells were present in high numbers in hepatic lymph nodes in sheep during primary and secondary infections, but their numbers declined to control levels during chronic primary infection. As the infection became chronic, predominantly CD8+ T lymphocytes cells were present in fibrotic areas of the liver of sheep (Meeusen et al 1995) and goats infected with *F. hepatica* (Perez et al 1998). The predominance of CD4+ T lymphocytes during the acute stages of *F. hepatica* infection in sheep and goats indicates that humoral response operates during the acute phase of infection with *F. hepatica* in these animals

whereas a cell-mediated immune response occurs when adult flukes have established in the bile ducts. The change from a primarily CD4+ T lymphocyte-mediated response to a primarily CD8+ T cell-mediated response might be associated with the ability of *F. hepatica* to down-regulate the level of CD4 expression on T lymphocytes (Prowse et al 2002). Recently, these authors demonstrated that ES products of *F. hepatica* cleaved CD4 from the surface of sheep T lymphocytes resulting in the suppression of lymphocyte proliferation. They considered this as a probable mechanism used by *F. hepatica* to modulate the T cell responses of sheep during infection thus contributing to the absence of resistance to infection with *F. hepatica* in these species.

The presence of B cells, IgG+ plasma cells and CD3+ T lymphocytes has also been used to describe the local inflammatory and immune responses during infection with *F. hepatica.* To my knowledge, studies describing these responses in cattle and buffaloes infected with *F. gigantica* have not been undertaken. Hence, no information can be obtained regarding the stimulation of the local humoral and cellular immune responses in tissues of infected animals and how they differ between these species. Perez, Ortega-Moreno, Morrondo, Lopez-Sandez and Martinez-Moreno (2002) observed intense local cellular and humoral immune responses, represented by hepatic infiltration with CD3+ T cells, B cells and IgG+ plasma cells, in sheep infected with *F. hepatica.* Also, studies in goats infected with *F. hepatica* noted that acute haemorrhagic tracts and chronic lesions were surrounded with neutrophils, eosinophils, macrophages, IgG+ plasma cells and CD3+ T lymphocytes (Martinez-Moreno, Jimenez-Luque, Moreno, Redondo, de las Mulas and Perez 1999; Perez, Martin de las Mulas, Carrasco, Gutierrez, Martinez-Cruz and Martinez-Moreno 1999). However, these authors observed that these responses differed between acute and chronic lesions with only few CD3+ T lymphocytes in the former while numerous CD3+ T cells were seen in chronic lesions. This result led them to suggest that *F. hepatica* inhibits migration of these cells into acute migratory tracts. They also observed numerous IgG+ plasma cells in acute lesions in the liver, indicating that a strong local humoral immune response occurred along these sites. This agrees with the observation of Meeusen et al (1995) in sheep and Perez et al (1998) in goats infected with *F. hepatica.* Furthermore, both local cellular inflammatory and humoral responses also occurred in the chronic lesions in the liver

since there was a marked infiltration of CD3+ T lymhocytes, along with neutrophils, eosinophils and macrophages and IgG+ plasma cells (Martinez-Moreno et al 1999; Perez et al 1999).

Perez et al (2002) noted that the hepatic lymph nodes of sheep infected with *F. hepatica* were markedly activated with numerous IgG+ plasma cells. This is similar to the observation in goats infected with *F. hepatica* (Perez et al 1999). These results indicate that during *F. hepatica* infection in sheep and goats, the local immune response in the hepatic lymph node is primarily humoral. This is in accordance to the findings of Meeusen et al (1995) and Perez et al (1998).

2.7 Humoral immune responses of *Fasciola*-infected animals

2.7.1 IgG1 response in cattle and buffaloes

Only few studies which investigated the IgG1 response against *F. gigantica* in infected cattle and buffaloes have been reported. Results indicated that IgG1 response is induced by both early larval stages migrating in the hepatic parenchyma and adults in the bile ducts (Ogunrinade 1983b; E. Wiedosari, et al personal communication). Serum IgG1 rose progressively starting at 4 weeks to reach a plateau at about week 20 in infected buffaloes, Ongole and Bali calves given a trickle infection with 15 metacercariae of *F. gigantica* twice weekly over 32 weeks (E. Wiedosari et al, personal communication). A single infection with *F. gigantica* in White Fulani cattle was manifest by IgG1 response which progressively increased, starting at 2 weeks, which reached a peak value at 25 weeks post-infection and thereafter declined terminally by 52 weeks post-infection (Ogunrinade 1983b). The difference in the IgG1 response against *F. gigantica* between single and trickle infections may be due to the result of the chronicity of exposure to infection, or to the nature of a chronic infection itself. Ferre et al (1997) stated that differences in peak levels in antibody response against *F. hepatica* could be associated with differences in metacercariae dosing.

The plateau in IgG1 response observed by E. Wiedosari et al (personal communication) despite continued trickle infection with *F. gigantica* suggests that antibody response may be suppressed by the presence of adult flukes in the bile ducts.

A plateau in IgG1 response occurred at about 15 weeks and 6 weeks in cattle infected with *F. hepatica* (Clery et al 1996). Chauvin et al (1995) demonstrated an apparent suppression of the IgG antibody response to challenge infection with *F. hepatica* in sheep when serum IgG levels decreased after secondary infection to significantly lower levels than seen after primary infection. This also occurs in cattle infected with *F. hepatica* as challenge infections did not cause any significant change in their specific IgG1 response (Clery et al 1996; Bossaert et al 2000a).

An apparent absence of resistance to reinfection with *F. gigantica* may occur in buffaloes and cattle as these animals can still be reinfected with the parasite as shown by E. Wiedosari et al (personal communication). Clery et al (1996) noted that cattle infected with *F. hepatica* were still susceptible to reinfection. This could be partly due to this insufficient IgG1 response but it is more likely that IgG1 is being produced in response to excretory/secretory (ES) antigens that are not important in inducing resistance. Bossaert et al (2000a) noted that in cattle infected with *F. hepatica*, recognition of a group of antigens present in ES extracts of *F. hepatica* occurred as early as 6-8 weeks post-infection with maximum recognition between weeks 11-16 during patency. These authors observed that thereafter, this intensity of recognition decreased and became faint from week 20-24.

Serum IgG1 level in cattle infected with *F. gigantica* declines after week 25 as this is lost into the bile as infection progressed. Ogunrinade (1983b) noted the appearance of IgG1 in the bile of infected cattle in his study. This was also observed in sheep infected with *F. hepatica* (Movsesijan and Jovanovic 1975). The increase and decrease in bile IgG occur at the same time with that of serum IgG suggesting that IgG in the bile is directly derived from the bloodstream (Hughes, Hanna and Symonds 1981; Jackson and Cooper 1981).

The decline in serum IgG1 level after week 25 after a single infection in cattle with *F. gigantica* (Ogunrinade 1983b) may also indicate that when flukes have established in the bile ducts there are relatively fewer antigens that are available to stimulate the immune response than during the parenchymal phase. Velusamy et al (2004) observed that in bovine calves experimentally infected with *F. gigantica*, circulating antigen levels declined from 12th or 13th week post-infection until the 26th week when

observations ceased. A decrease in circulating immune complex at 10 weeks post-infection occurred in cattle infected with *F. hepatica* (Langley and Hillyer 1989). Serum immunoglobulin levels in sheep infected with *F. hepatica* declined after flukes had invaded the biliary system since its ES antigens are channelled away into the alimentary canal once *F. hepatica* has established in the bile ducts (Hanna 1980a; 1980b).

Peak IgG1 response in cattle and buffaloes given trickle infection with *F. gigantica* (E. Wiedosari et al, personal communication) occurred later than the 3-week peak level observed by Clery et al (1996) and the 6-week peak level observed by Bossaert et al (2000a) in cattle with trickle infection of *F. hepatica*. This might be due to the lower dose of *F. gigantica* metacercariae given by E. Wiedosari et al (personal communication) than of *F. hepatica* given by Clery et al (1996) and Bossaert et al (2000a), or this might also suggest that *F. gigantica* may have the ability to suppress immune responses in infected cattle and buffaloes to a greater extent than occurs with *F. hepatica* in cattle. However, suppression of immune responses by *F. gigantica* remains to be investigated (Spithill et al 1999).

2.7.2 IgG2 response in cattle and buffaloes

To my knowledge only one study has investigated IgG2 response in cattle and buffaloes infected with *F. gigantica*. IgG2 levels began to rise 4 and 12 weeks post-infection in Bali and Ongole cattle, respectively during a trickle infection with 15 metacercariae of *F. gigantica* twice weekly over 32 weeks (E. Wiedosari et al, personal communication) indicating that stimulation of this response in Bali cattle started when immature flukes were still migrating in the liver parenchyma. This also shows that the response in Ongole cattle was induced by adult flukes in the bile ducts. Cattle infected with *F. hepatica* manifest an early specific IgG2 response starting at 2-4 weeks post-infection (Clery et al 1996; Bossaert *e*t al 2000a). The later IgG2 response observed in Ongole cattle infected with *F. gigantica* than the IgG2 response seen in Bali cattle infected with *F. gigantica* or in cattle infected with *F. hepatica* may be due to a suppression of immune responses by *F. gigantica* in Ongoles to a greater extent than occurs in Bali cattle or by *F. hepatica* in cattle.

Differences in IgG2 response occur between Bali and Ongole cattle and between these breeds and buffaloes in their response to trickle infection with *F. gigantica*. Bali cattle developed high levels of IgG2 peaking at 24 weeks post-infection whereas only a modest rise in serum IgG2 was seen in Ongole cattle from about week 12 (E. Wiedosari et al, personal communication). They did not detect IgG2 from the serum of infected buffaloes throughout the infection. It is not known whether the absence of IgG2 production in buffaloes in the previous study was a true result or an artefact. They proposed that the bovine reagents used failed to bind to IgG2 antibodies in buffaloes.

The high level of IgG2 in Bali cattle may be an indication of the development of a degree of resistance against *F. gigantica* in these animals. Bali cattle had declining levels of GLDH after week 24 indicating that the level of parenchymal damage declined (E. Wiedosari et al, personal communication). This author also observed a progressive decline in egg count after about week 26. The declining GLDH levels may indicate a development of resistance in Bali cattle possibly because of the elimination of immature flukes. The decline in egg count is also an indication of a development of resistance either through elimination of flukes or suppression of their egg-laying. There is also evidence that IgG2 may play a role in protective immune responses to *F. hepatica* in cattle as shown by Mulcahy et al *(*1998) who found that IgG2 titres and fluke burden were negatively correlated in cattle vaccinated with cathepsin L2 and haemoglobin from *F. hepatica*.

2.7.3 IgM response in cattle and buffaloes

There is no published information regarding IgM response in cattle and buffaloes infected with *F. gigantica* thus, its specific role in the immune response of cattle and buffaloes to infection with *F. gigantica* remains to be determined. However, evidence from cattle and sheep infected with *F. hepatica* suggests that IgM is unlikely to have any significant contribution to the immune responses during *F. gigantica* infection in cattle and buffaloes. In cattle infected with *F. hepatica* Clery et al (1996) found a small but not significant increase in specific IgM response 3 weeks after infection. Similarly, in both ITT and Merino sheep infected with *F. gigantica* IgM titres declined after an initial non-significant rise at

day 25-30 post-infection (Hansen et al 1999).

2.7.4 IgE response in cattle and buffaloes

There is not enough information regarding IgE response from cattle and buffaloes infected with *F. gigantica* to make informed conclusions. Only one study has been conducted. It demonstrated an immediate (type 1) hypersensitivity reaction in the skin of *F. gigantica*-infected buffaloes in response to intradermal injection of crude antigen from *F. gigantica*, indicating the presence of parasite-specific IgE-like antibodies in infected buffaloes (El-Said 1997). The author detected that sera of infected buffaloes passively sensitized skin mast cells of non-infected buffaloes. A similar response was observed in cattle infected with *F. hepatica* where antigen from both larval and adult flukes induced significant IgE-like dermal response from 2 weeks post-infection (Bossaert et al 2000a).

IgE-like response appears to be important in inducing resistance in cattle to *F. hepatica*. In the review by Howell and Boray (1994) IgE-like antibodies in cattle infected with *F. hepatica* reach a peak level at 20-24 weeks post-infection coinciding with the expulsion of flukes. Destruction of helminths by IgE-dependent eosinophil antibody dependent cell-mediated cytotoxicity (ADCC) was regarded by Tizard (1996) as perhaps the most significant mechanism of resistance to helminths although other immunoglobulins also play a protective role.

The role of IgE against *F. hepatica* in cattle has not been confirmed but it may contribute to resistance to *F. hepatica* as substantial infiltration of eosinophils during challenge infection indicates an IgE-like response (Doy et al 1978). However, interpretation of the importance of eosinophils in killing invading larval flukes in cattle must be considered with caution as there is an evidence that bovine eosinophils do not kill larval *F. hepatica* in vitro in an ADCC reaction (Duffus, Thorne and Oliver 1980).

2.8 Killing of larval *Fasciola*

Resistance to *Fasciola* may be related partly to the ability of host cells, particularly monocytes/macrophages, to destroy migrating larval flukes. Newly-excysted

juveniles are believed to be destroyed by a cytotoxic mechanism exerted by some cells of the immune system and macrophages have been associated with this destruction. Estuningsih et al (1999) demonstrated that ITT sheep peritoneal macrophages exerted an ADCC against juveniles of *F. gigantica in vitro*. This ADCC may also occur *in vivo* and may be one of the means by which ITT sheep manifest resistance to *F. gigantica* (Hansen et al 1999). In *F. hepatica* infection in the resistant rat, the killing of NEJs was attributed to monocytes/macrophages (Smith, Ovington and Boray 1992; Piedrafita and Liew 1998) through the release of free radicals of nitric oxide at the fluke surface (Spithill, Piedrafita and Smooker 1997**;** Piedrafita and Liew 1998**;** Piedrafita et al 2001)**.** This mechanism is apparently enhanced in the presence of sera from *F. hepatica*-infected animals suggesting the involvement of antibodies in this mechanism of immune killing. When immune serum was administered to rats infected with *F. hepatica* large numbers of cells attached to the juvenile flukes and levels of nitrite in culture were high whereas in cultures without sera or with sera from naive rats, few cells attached to the juvenile flukes, despite similar levels of nitrite in the culture supernatants (Spithill et al 1997**;** Piedrafita and Liew 1998**;** Piedrafita et al 2001). Hansen et al (1999) suggested that killing of larval *F. gigantica* in the resistant ITT sheep occurs 3-4 weeks post-infection and is dependent on parasite-specific IgG1. *In vitro* killing of NEJs of *F. gigantica* was exhibited when these were incubated with post-infective anti-sera and macrophages from ITT sheep infected with *F. gigantica* (Estuningsih et al 1999). These authors suggested that the presence of homologous antibodies is important in the killing mechanism and considered that ADCC may also happen *in vivo* in the resistant ITT sheep. However, this ADCC does not extend to *F. hepatica* infections in sheep as peritoneal lavage cells from naive ITT sheep did not kill juvenile *F. hepatica* (Piedrafita et al 2001). This suggests that the absence of resistance to *F. hepatica* in sheep may be partly related to the absence of ADCC against juvenile *F. hepatica*. Furthermore, it shows that these two species of liver fluke differ in their host-parasite relationships.

An ADCC reaction involving eosinophils has also been reported as a mechanism of killing NEJs of *F. hepatica*. Immunohistochemistry studies among rats infected with *F. hepatica* observed that NEJs were killed by an eosinophil-mediated cytotoxic response involving IgG antibodies that infiltrated the gut after challenge (Van

Milligen et al 1998). These studies demonstrated that NEJs were coated with IgG1 and IgG2 antibodies in the submucosa and surrounded by eosinophils.

The observations made on the killing of juvenile *Fasciola* suggest the involvement of both type 1 and type 2 immune responses. The involvement of type 1 immune response is indicated by the role of nitric oxide in the killing of juvenile flukes. IFN-γ (a type 1 response) induces the production of nitric oxide synthase, which in turn enhances the ability of macrophages to produce nitric oxide (James, Sher, Lazdins and Meltzer 1982). Meanwhile, the killing of juvenile *F. hepatica* observed in rats, which involves eosinophils and IgG1 (Van Milligen et al 1998) indicates the involvement of a type 2 response. Eosinophils are associated with a type 2 response (Pritchard et al 1995) as well as IgG1 (Estes 1996).

2.9 Mechanism of immune evasion in fasciolosis

A number of mechanisms are employed by liver flukes to evade the immune response of their hosts. Their residence in the bile ducts protects them from an immune attack (Mulcahy, Joyce and Dalton 1999a). This is indicated by a decline in antibody levels as flukes reach the bile ducts (Meeusen and Brandon 1994; Clery et al 1996; E. Wiedosari, personal communication). The flukes' ability to shed their glycocalyx enables them to escape destruction by the host's immune responses (Duffus and Franks 1980). Distinct differences in the protein profiles of the NEJ and the juvenile parasite stages were observed in *F. hepatica* (Tkalcevic, Brandon and Meeusen 1996) indicating that glycocalyx turn-over occurs as the fluke grows. The shedding of antibodies and immune cells from the glycocalyx also protects the liver flukes from immune destruction (Spithill et al 1999) and products of the shed glycocalyx may mask antibodies against the flukes preventing them from participating in ADCC (Duffus and Franks, 1980).

Liver flukes, by secreting substances, suppress leukocyte functions as a means of immune evasion (El-Ghaysh, Turner, Brophy and Barett 1999). Glutathione *S*-transferase (GST) secretion by *F. hepatica* can impair the toxic reactive oxygen products of the respiratory burst of leukocytes, neutrophils and macrophages or the reactive nitrogen intermediates generated by macrophages (Doy et al 1980; Doy and Hughes 1982). Glutathione *S*-transferase detoxifies the secondary products of lipid

peroxidation produced via immune-mediated free radical attack (Brophy, Patterson, Brown and Pritchard 1995) and by acting to reduce the impact of reactive radicals at the immune cell-parasite interface, could enable parasites to evade ADCC and degranulation by eosinophils and mast cells (Riffkin, Seow, Jackson, Brown and Wood 1996).

Liver flukes also secrete substances that cause suppression of host immune responses. Proteinases of *F. hepatica* destroy host immunoglobulins (Chapman and Mitchell, 1982; Smith, Dowd, Heffernan, Robertson and Dalton 1993; Berasain, Carmona, Frangione, Dalton and Goni 2000). The absence of antibody-mediated attachment of eosinophils to the NEJs of *F. hepatica* might be a result of this degradation (Berasain et al 2000). Cathepsin L found in the ES product of *F. hepatica* also cleaves CD4 on T cells of sheep resulting in suppression of lymphocyte proliferation thus evading the host's immune response (Prowse et al 2002).

The rapid movement of flukes through the hepatic parenchyma into the bile ducts prevents their destruction. Meeusen et al (1995) noted that although the cellular immune response during a secondary infection with *F. hepatica* was markedly increased, this response was not observed around the flukes but only around the portal tracts and lesion sites. They proposed that the challenge flukes moved more rapidly through the hepatic parenchyma to the bile ducts than during the primary infection thus evading destruction. This may be the reason why sheep do not develop resistance against reinfection with *F. hepatica*. Hence, it can be concluded that resistance may occur if the cellular response manifested by infected animals are able to destroy the invading flukes.

2.10 General conclusions of literature review

The past years have seen progress in studies of fasciolosis particularly in the fields of immunology, diagnosis and immunoprophylaxis. However, disease caused by *F. gigantica* is still a significant problem affecting cattle and buffaloes in the tropics.

The above review illustrates that there is limited information describing host-parasite relationships of *F. gigantica* infection in cattle and swamp buffaloes. There is limited information regarding differences in the host responses to infection with *F. gigantica* between these species. This information is required as it represents the basis for setting up priorities for control of infection in these animals. With the information available at present, it is not possible to point out the similarities and differences in the host responses between cattle and swamp buffaloes to infection with *F. gigantica*. Thus, further studies comparing the host-parasite relationships in cattle and buffaloes are needed.

CHAPTER 3

EPIDEMIOLOGICAL AND CLINICAL OBSERVATIONS IN CATTLE AND SWAMP BUFFALOES INFECTED WITH *FASCIOLA GIGANTICA*

3.1 Introduction

Abattoir studies were undertaken to assess the importance of *F. gigantica* infection in cattle and swamp buffaloes. In addition, some clinical observations were assessed to see whether these differ between cattle and buffaloes. The information from this study may assist in understanding the importance of *Fasciola gigantica* infection and the need to implement control. This will also provide baseline information regarding differences in host responses and/or susceptibility between cattle and swamp buffaloes to infection with *F. gigantica*. Differences in responses and susceptibility to infection with *F. gigantica* between cattle and buffaloes have been observed in a previous study (E. Wiedosari et al, personal communication). It was reported that resilience to infection with *F. gigantica* differs between cattle and buffaloes with buffaloes more resilient than cattle as infected buffaloes did not show significant reduction in weight gain, whereas weight gain in infected Bali and Ongole cattle was significantly reduced. This study also demonstrated that the fluke burden in buffaloes was also significantly lower than in Ongole cattle suggesting that buffaloes are more resistant than the Ongoles. Hence, responses to infection with *F. gigantica* may vary among different species and breeds of animals.

3.2 Materials and methods

3.2.1 Epidemiological study

3.2.1.1 Experimental animals

Thirty-two swamp buffaloes and 252 cattle were examined in Kabacan abattoir. These animals came from different municipalities of Cotabato Province, Philippines.

3.2.1.2 Collection of data

The following were measured: prevalence of infection, faecal egg counts, worm counts, and carcass and liver weights. The age, sex and place of origin of animals were recorded.

3.2.1.3 Recovery of flukes

Recovery of flukes was done using the technique of Sinclair as described by Ogunrinade and Bamgboye (1980). The bile ducts of the affected livers were carefully dissected and flukes removed. The liver was cut into slices about 5 mm thick and suspended in normal saline while being squeezed manually to macerate the parenchyma. The flukes were then collected by washing the macerated liver through a sieve. Flukes were classified as mature and immature and their numbers were determined by counting the intact flukes and fluke heads that were collected.

3.2.1.4 Statistical analysis

Chi-square test was used to compare prevalence between cattle and buffaloes, between male and female and between age groups. Statistical comparisons of liver and carcass weights and fluke and faecal egg counts were done using analysis of variance (ANOVA). The relationship between fluke and faecal egg counts and age of animals were determined using Pearson's correlation. General linear model (GLM) analysis was used to test whether sex influenced the prevalence of infection according to age groups.

3.2.2 Clinico-pathological study

3.2.2.1 Experimental animals

One-hundred and seventy five swamp buffaloes and 61 cattle slaughtered at Davao abattoir in Southern Mindanao, Philippines were used in the study.

3.2.2.2 Blood collection and recovery and counting of flukes

Blood samples were collected by jugular venipuncuture in Vacutainers (Beckton Dickinson, Franklin Lakes, New Jersey, USA) containing ethylenediamine tetra-acetic acid (EDTA) as anticoagulant. To recover the flukes, the technique described above was employed. Worm burdens were categorized as none, low (1-20 flukes), medium (21-70 flukes) and high (>70 flukes).

3.2.2.3 Haematology

Red blood cell (RBC) counts were done using a Neubauer haemocytometer. Packed cell volume (PCV) was determined by the microhaematocrit method by centrifugation at 10,000-12,000 rpm for 3-5 minutes. The haemoglobin value was determined using the acid-haematin method (Coles, 1986).

3.2.2.4 Statistical analysis

The statistical differences between groups were estimated using analysis of variance using SPSS version 11. Multiple comparisons (Tukey HSD) were used to compare mean values of RBC, PCV and haemoglobin according to fluke burdens.

3.2.3 Experimental infection

Sixteen seven month to one year old swamp buffaloes were purchased from local farmers in Cotabato province, Mindanao, Philippines after confirming by faecal examinations that they were free from infection with *F. gigantica*. Sixteen cattle of the same age group were obtained from a cattle ranch in Kiblawan, Davao del Sur, Philippines. Buffaloes and cattle were treated with ivermectin (Ivomec, Merial, UK) and triclabendazole (Fasinex 240, Novartis, Switzerland) on arrival and kept in clean pens and allocated at random into infected (8) and control (8) groups for each species. Clean napier grass and drinking water were provided ad libitum and concentrate feeds were given at 2 kg/head/day. Mineral lick and water were also provided ad libitum. After a period of 2 weeks to allow animals to adjust to their new circumstances, infection with the metacercariae of *F. gigantica* commenced as described below. Sequential slaughter was done at 0, 3, 7, 12 and 16 week post-infection. Two animals for each species were slaughtered at each period except at pre-infection and three weeks post-infection when one cattle and one buffalo from the control group were slaughtered.

The animals were cared for in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Approval Number A778_02).

3.2.3.1 Collection of the metacercariae

The parasite, *F. gigantica*, was collected from snails, *Lymnae auricularia rubiginosa* from known infected areas in Cotabato province, Philippines. Snails were crushed between two glass plates and observed under a stereomicroscope. Snails that had mature cercariae were placed in cellophane bags containing distilled water and left overnight at room temperature. The following day, the cellophane bags were washed with distilled water and the crushed snails removed. Distilled water was placed inside the bags to cover the metacercariae that had attached to the cellophane and kept overnight at room temperature. The following day, the bags with the metacercariae were kept at 4° C until use. The metacercariae were kept for not more than one week prior to their use. To assess their viability, the outer coat of 5-10 representative cysts from each batch was removed by rubbing on a fine nylon mesh and the metacercariae were observed under a dissecting microscope. Viability was based on the presence of a clear transparent cyst, active movement of the fluke and the presence of a clear outline of cellular components.

3.2.3.2 Infection with *F. gigantica*

Eight buffaloes and eight cattle were experimentally infected orally with 1000 viable metacercariae of *F. gigantica* wrapped in moistened filter paper using a balling gun. Eight buffaloes and eight cattle served as uninfected controls.

3.2.3.3 Faecal eggs examination

Commencing at six weeks post-infection and weekly thereafter until 16 weeks, faecal samples were collected from each animal and examined by the sedimentation method for the presence of *Fasciola* eggs. Three grams of faeces from each animal were weighed. To this, about 30 mL of tap water was added, the faeces stirred and the mixture was sieved through a tea strainer with an aperture of about 1mm to a conical plastic glass. To this, 0.5% Tween-20 solution was added to almost fill the glass and allowed to settle for three minutes. The mixture was then passed through a 2-layer sieve of varying sizes (450 µm and 200 µm) into a conical plastic glass and 0.5% Tween-20 solution was added to almost fill the glass and allowed to stand for three minutes. It was sieved again as before into another conical glass, added with Tween-20

solution and left to stand for three minutes. The supernatant fluid was discarded after three minutes through a 50 µm sieve and the sediment was washed off from the sieve with tap water into a plastic petri-dish using a pasteur pipette. Two drops of methylene blue were added prior to examining the sample under a dissecting microscope and the fluke eggs were then counted.

3.2.3.4 Fluke count

Livers from cattle and buffaloes were collected at slaughter at 3, 7, 12 and 16 weeks post-infection. Flukes were recovered as described in Section 3.2.1.3.

3.3 Results

In Fig. 3.1, it appears that swamp buffaloes had higher prevalence of infection with *F. gigantica* than cattle. However, when the data were stratified according to age and sex (Figs. 3.2 and 3.3), it was shown that prevalence did not differ between these animals. Prevalence differed significantly between age groups of animals. Animals more than 6 years of age had the highest prevalence, followed by those between 3 and 6 years old. The lowest prevalence was observed among young cattle and buffaloes from 7 months to 3 years of age. Sex did not significantly affect the prevalence. This observation was clearly demonstrated among cattle but not in buffaloes. This is because there were only few buffaloes and most of them were females.

There was a trend to a higher count of mature *F. gigantica* in cattle than in buffaloes (Fig. 3.4). This trend was similarly observed with regards to the number of immature flukes and faecal egg counts. Animals more than 6 years of age had more mature and immature flukes compared to younger animals (Fig. 3.5). Adult fluke numbers and faecal egg counts were highly correlated in animals more than six years of age (Pearson coefficient, $P<0.01$, $n=39$) but there was no significant relationship between egg counts and age of the animals ($P=0.88$).

Carcass weight was significantly lower in infected buffaloes more than 6 years of age than non-infected buffaloes in the same age groups (Table 3.1). No comparison can be made between infected and non-infected buffaloes in other age groups as data on their carcass weights were not collected. Infected cattle in all age groups had higher carcass

weights than non-infected cattle. Liver weight was significantly higher in infected than in non-infected animals ($P<0.01$).

In cattle, RBC counts were significantly lower in infected than non-infected animals ($P<0.05$) (Fig. 3.6). Erythrocyte counts were negatively related to worm counts ($P<0.05$) (Fig. 3.7). Packed cell volume was also significantly lower in cattle with high fluke burden (>70 flukes) than those with medium worm loads (21-70 flukes) ($P<0.05$).

Red blood cell counts were significantly higher in infected than in non-infected buffaloes ($P<0.05$) while there was no significant difference in PCV and haemoglobin values between infected and non-infected buffaloes (Fig. 3.6). When these parameters were compared according to fluke burden, it was observed that RBC was significantly higher in buffaloes with high fluke burden (>70 flukes) than those with no flukes or with medium fluke burden (21-70 flukes) ($P<0.05$) (Fig. 3.8). Significantly higher PCV value was also observed in buffaloes with high fluke burdens compared to those with low (1-20 flukes) or medium worm loads ($P<0.05$). Haemoglobin values did not differ significantly between buffaloes with low, medium, high or no fluke burdens.

During the experimental infection, buffaloes had consistently lower fluke burden than cattle at weeks 3, 7, 12 and 16 weeks post-infection (Fig. 3.9). No mature flukes were seen in buffaloes at 16 weeks post-infection while mature flukes were already present in cattle at this period. Immature fluke numbers were constant in buffaloes from weeks 3-16 whereas in cattle the number was higher. *Fasciola* eggs first appeared in the faeces of cattle at 16 weeks post-infection whereas no eggs were observed in buffaloes at the same time post-infection.

3.4 Discussion

Cattle and buffaloes are both susceptible to infection with *F. gigantica*. Animals more than 6 years of age had the highest prevalence compared with other age groups which is similar to the finding of Soesetya (1975) in Indonesia where a higher prevalence rate of infection with *F. gigantica* occurred in older cattle compared to younger cattle. This shows that cattle and swamp buffaloes are becoming more exposed to infection with *F. gigantica* as they get older. This may be due to the fact that older animals are utilised more often in farming activities, resulting in greater exposure to infection.

The effect of infection with *F. gigantica* on carcass weight was evident among buffaloes but not cattle. This contrasts with the finding of E. Wiedosari (personal communication) who observed no significant effect on weight gain in buffaloes infected with *F. gigantica*. One explanation for the varying observations in these two studies could be the different conditions under which the experimental animals were exposed. The study of Wiedosari and colleagues was under experimental conditions where buffaloes were given good nutrition while the present study involved animals under field conditions. Under these conditions, buffaloes have limited time for grazing as they are utilised for draught hence their nutritional status may not be as good as in cattle which spend most of their time grazing as they are raised mainly for breeding and meat. Hence, this observation more likely reflects the influence nutrition has on the effect of infection on live weight gain of infected animals. Buffaloes are also more exposed to environmental stress as they do heavier work than cattle. Nutrition influences the effect infection with *F. gigantica* has on weight gain (Graber 1971; Nour, Abou, Badr and El 1979) while an animal's metabolism and its feed efficiency are adversely affected by stress (Burns 2001). Adequate diet was important in preventing weight loss, cachexia, and death in cattle infected with *F. gigantica* (Graber 1971). The effects of infection with *F. gigantica* in sheep were only minimal when infected animals were given high-protein diet whereas weight gain and haemoglobin level in infected sheep fed only a basal diet were severely depressed (Nour et al 1979).

Liver weight was higher in infected than in non-infected animals. This is because of the necrotic and calcified lesions due to compensatory hypertrophy of liver parenchyma caused by infection with *F. gigantica.* Soesetya (1975) had the same observation in cattle infected with *F. gigantica.*

Cattle and swamp buffaloes are both susceptible to *F. gigantica*. Although the level of infecting dose of *F. gigantica* cannot be determined in naturally infected animals, the observation that there was a trend of a lower number of mature and immature flukes as well as faecal egg counts in swamp buffaloes compared with cattle suggests that swamp buffaloes are more resistant to infection with *F. gigantica* than cattle. This is supported by the findings during the experimental infection in which swamp buffaloes had consistently lower fluke burdens than cattle at 3, 7, 12 and 16 weeks post-infection.

Fasciola eggs were not observed from these buffaloes at week 16 of infection and the flukes in buffaloes were still immature while mature flukes were already present in cattle, suggesting that there was either delayed migration or suppressed development of flukes in the buffaloes. E. Wiedosari et al (personal communication) observed during a trickle infection of *F. gigantica* in buffaloes, Bali and Ongole cattle that buffaloes had lower fluke burden and egg count than cattle. Both Prasitirat et al (1996) and E. Wiedosari et al (personal communication) observed that faecal egg counts were lower in buffaloes than in cattle with the same infecting dose of *F. gigantica* metacercariae. In addition, immature fluke numbers were constantly lower in buffaloes from weeks 3-16 than in cattle. This suggests that, relative to cattle, elimination of immature flukes in buffaloes occurs before week 3 post-infection, indicating the possibility that a mechanism of resistance is operating against juvenile *F. gigantica* in these animals. This potential mechanism of resistance manifested by buffaloes apparently early in the infection may be associated with an ADCC against immature flukes similar to what was observed by Estuningsih et al (1999) where ITT sheep peritoneal macrophages exerted an ADCC against juveniles of *F. gigantica in vitro*. Hansen et al (1999) suggested that this ADCC may also occur *in vivo* and may be one of the means by which ITT sheep manifest resistance to *F. gigantica*.

There was no significant relationship between egg counts and age of the animals and between number of mature flukes and age. But there was a high correlation between mature *F. gigantica* and egg counts in animals more than six years of age. This suggests that adult flukes in older animals produce fewer eggs than they do in younger animals, indicating that older cattle and buffaloes may be more resistant to *F. gigantica* than younger ones.

Swamp buffaloes were not as severely affected by *F. gigantica* compared with cattle. Even in cases where fluke counts were high no adverse clinico-pathological effects occurred in buffaloes. Infected buffaloes were able to tolerate infection without showing significant depressions on RBC, PCV and haemoglobin. In contrast, significant alterations in RBC counts of cattle were observed and their PCV was also significantly reduced with high worm load. Anaemia is one of the effects of liver fluke infection and the usual sign of disease in infected animals (Behm and Sangster 1999). The absence of significant alterations in these blood parameters in swamp buffaloes

naturally infected with *F. gigantica* indicates that these animals can cope with infection much better than cattle hence they are more resilient to infection with *F. gigantica* than cattle. There may be a possibility of bias in this result as the haematological picture could be affected by sex, age, and presence of other infections. However the number of animals observed could overcome the possibility of bias occurring. There were also non-infected animals that were used in the comparison which would serve as control animals. Also, a previous study reported that swamp buffaloes are more resilient to *F. gigantica* than cattle (E. Wiedosari et al, personal communication), supporting this present observation.

The resilience of buffaloes to *F. gigantica* has been demonstrated by E. Wiedosari et al (personal communication) in which a trickle infection of 15 metacercariae of *F. gigantica* for 32 weeks did not affect weight gains of buffaloes but caused significant reduction in weight gains of Bali and Ongole cattle. She also observed that buffaloes exhibited the least decline in PCV compared with Bali and Ongole calves, reflecting the higher resilience of buffaloes to infection. The observation on the PCV of buffaloes in this study agrees with Wiedosari's and collegues' observation. However, Yadav et al (1999) noted that Murrah buffaloes experimentally infected with *F. gigantica* had significant reduction in haemoglobin. These contrasting observations might be due to breed differences in response to *F. gigantica* infection, with Murrah buffaloes more susceptible than swamp buffaloes. Spithill et al (1999) stated that the clinical effects of infection with *F. gigantica* vary between breeds and among individuals within a breed due to variation in resistance and resilience to infection. Breed differences in susceptibility to *F. gigantica* have been reported to exist between Friesian and Boran cattle (Wamae, Hammond, Harrison and Onyango-Abuje 1998), between Indonesian thin-tailed sheep, Merinos and St. Croix sheep (Widjajanti, Estuningsih, Partoutomo, Roberts and Spithill 1999), and between Red Masai and Dorper sheep (Waweru, Kanyari, Mwangi, Ngatia and Nansen 1999). These present observations show that swamp buffaloes are more resilient than cattle to *F. gigantica* and support previous observations that these cattle and swamp buffaloes differ in their susceptibility to *F. gigantica* infection.

The findings from the epidemiological and clinical observations reflect the importance of *F. gigantica* infection in cattle and swamp buffaloes such that an understanding of

the host-parasite relationship of *F. gigantica* infection in cattle and buffaloes is important for setting up priorities for control of the infection. These studies also revealed that these animals apparently differ in their susceptibility to *F. gigantica* with buffaloes showing higher resilience and probably resistance than cattle. This difference in susceptibility to infection between these animals may be a result of the host-parasite relationships differing between them. No substantive literature is available to explain this phenomenon hence studies that will investigate and compare the immune responses of cattle and buffaloes to infection with *F. gigantica* infection may provide insights to understanding the host-parasite relationships of *F. gigantica* in these animals. Therefore these studies were undertaken as described in the following chapters. It is anticipated that these will assist in understanding the apparent differences in host responses between cattle and swamp buffaloes to infection with *F. gigantica*.

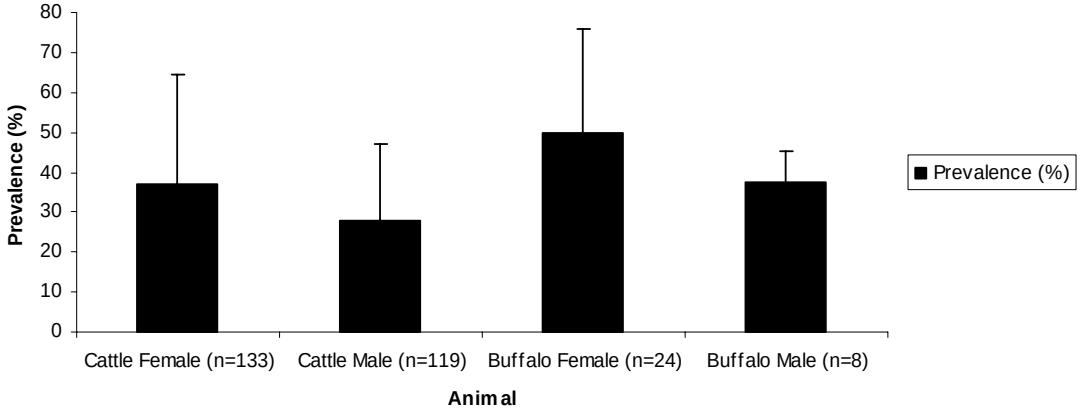

Fig. 3.1 Prevalence of fasciolosis in cattle and buffaloes in Cotabato province, Mindanao, Philippines. May-September 1999. Error bars = 95% confidence limits (n, cattle= 252; n, buffaloes= 32).

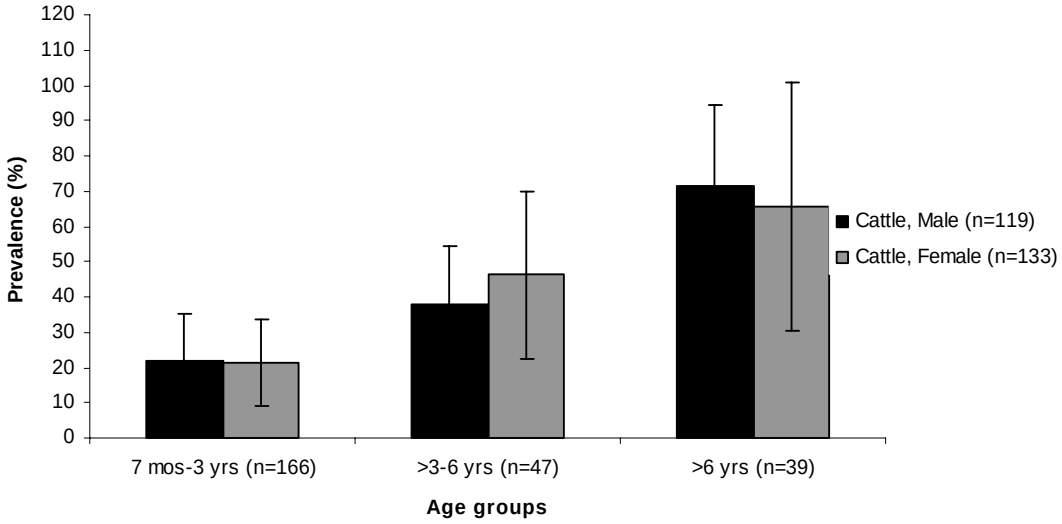

Fig. 3.2 Prevalence of fasciolosis in cattle according to age in Cotabato province, Mindanao, Philippines. May-September 1999. Error bars = 95% confidence limits.

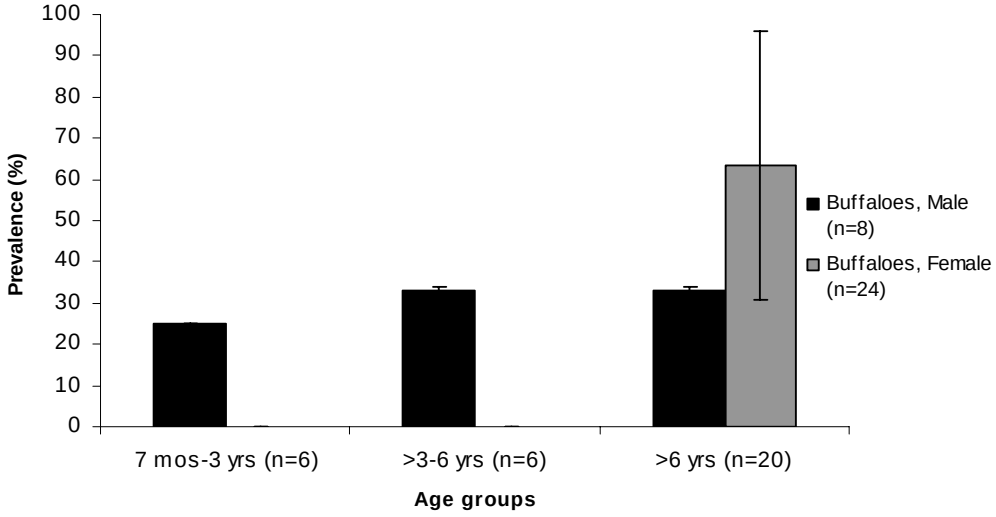

Fig. 3.3 Prevalence of fasciolosis in swamp buffaloes according to age in Cotabato province, Mindanao, Philippines. May-September 1999. Error bars = 95% confidence limits.

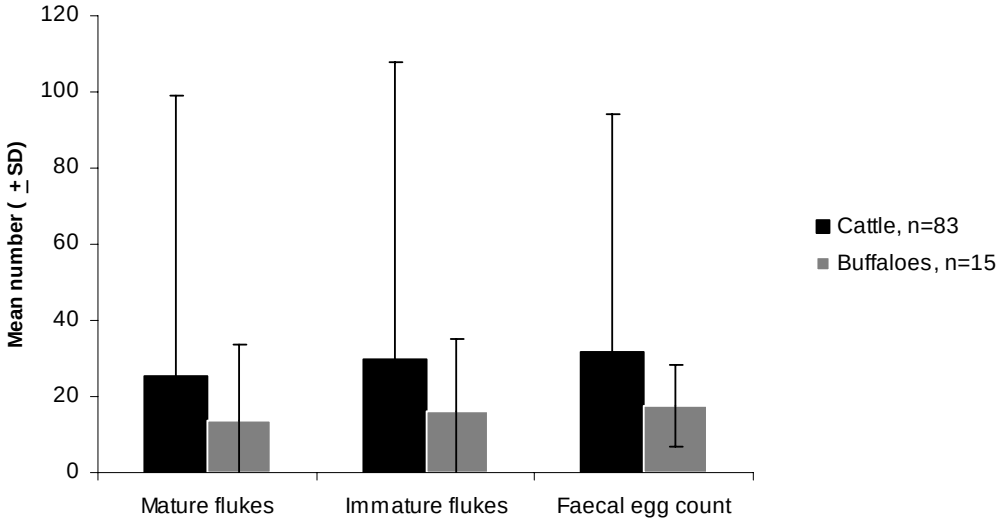

Fig. 3.4 Mean number of mature and immature flukes and faecal egg counts in cattle and buffaloes infected with *F. gigantica*. Error bars = standard deviation (SD)

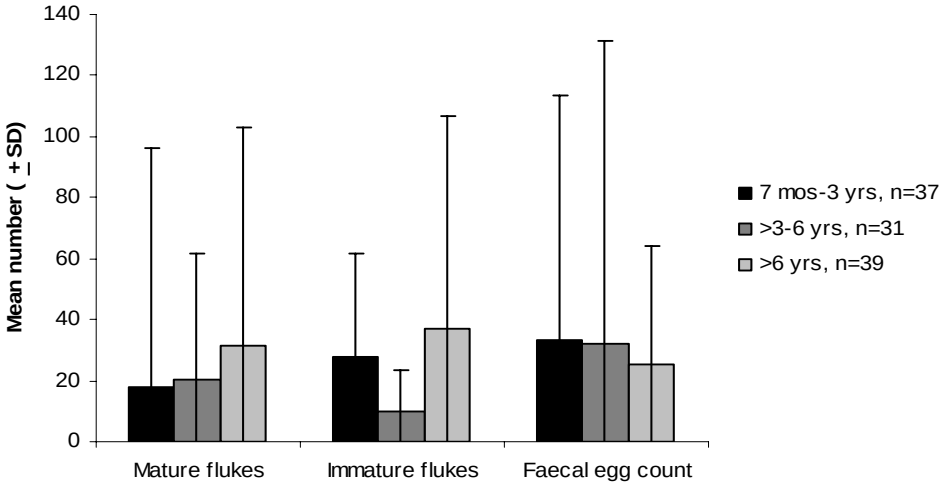

Fig. 3.5 Mean number of mature and immature flukes and faecal egg counts in cattle and buffaloes infected with *F. gigantica* according to age groups. Error bars = SD

Table 3.1 Mean (± SD) carcass and liver weights in cattle and buffaloes infected with *F. gigantica* and non-infected animals according to age in Cotabato province, Mindanao, Philippines, May-September 1999.

Age	Carcass weight (kg)[1]				Liver weight (kg)[2]			
	Cattle		Buffaloes		Cattle		Buffaloes	
	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected
<1 year to 3 years	124.4 (± 38.88) (n=22)	115.9 (±43.25) (n=74)	-[3]	100 (±34.64) (n=3)	4.79 (±1.2) (n=17)	3.24 (±1.05) (n=52)	-[3]	5.25 (±2.47) (n=2)
3 years to 6 years	161.4 (± 62.59) (n=14)	117.4 (±38.69) (n=17	-[3]	150 (±0) (n=1)	5.14 (±1.43) (n=11)	3.96 (±1.16) (n=13)	-[3]	[3]
> 6 years	146.3 (+ 48.1) (n=19)	119.7 (±40.0) (n=8)	138.8 (±39.59) (n=10)	150.2 (±25.34) (n=5)	5.44 (±1.34) (n=16)	5.0 (±0) (n=5)	7.44 (n=9)	5.44 (±1.26) (n=3)

[1] Significantly higher in non-infected than infected buffaloes more than 6 years old ($P<0.05$)

[2] Significantly higher in infected than non-infected animals ($P<0.05$); [3] Data not available

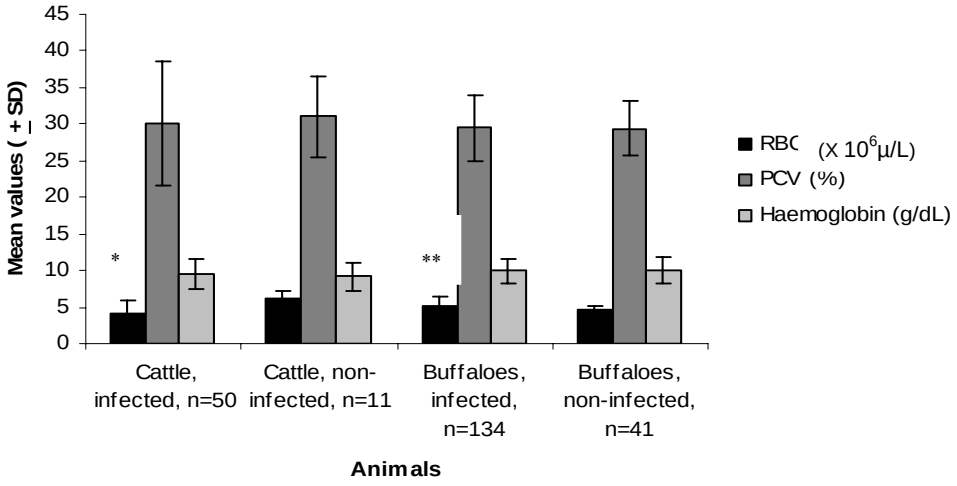

Fig. 3.6 Mean RBC, PCV and haemoglobin values in cattle and buffaloes infected with *F. gigantica*. * significantly lower in infected than in non-infected cattle ($P<0.05$); ** significantly higher in infected than in non-infected buffaloes ($P<0.05$). Error bars = SD

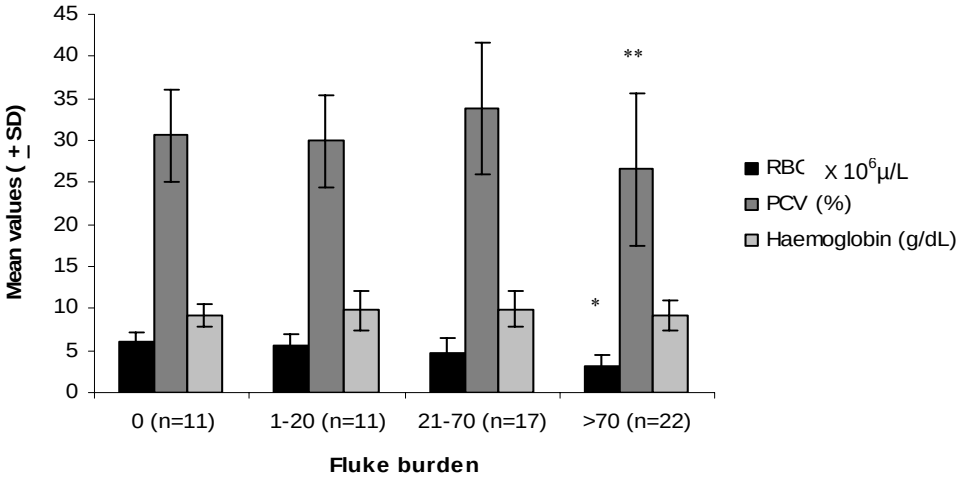

Fig. 3.7 Mean RBC, PCV and haemoglobin values in cattle according to fluke burden. * significantly lower in cattle with >70 flukes than those with lower burdens or non-infected cattle ($P<0.05$); ** significantly lower in cattle with >70 flukes than those with 21-70 flukes ($P<0.05$). Error bars = SD

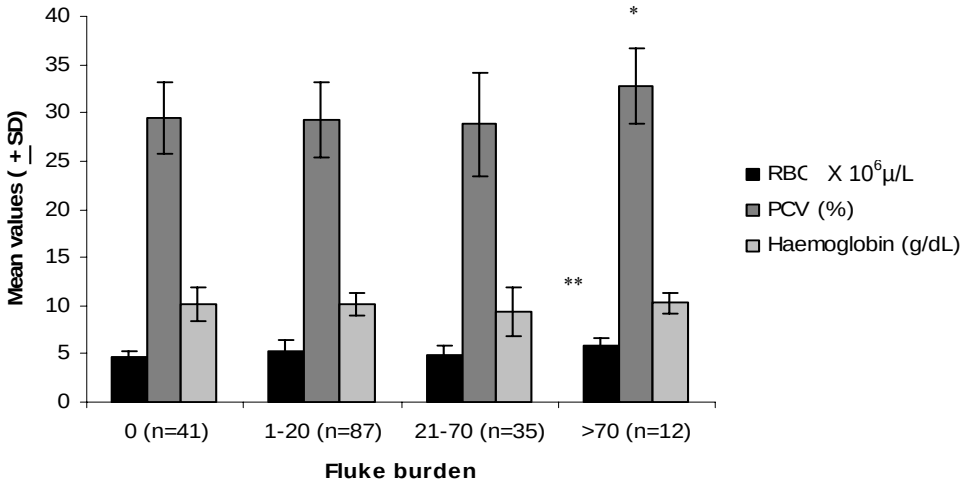

Fig. 3.8 Mean RBC, PCV and haemoglobin values in swamp buffaloes according to fluke burden. * significantly higher in buffaloes with >70 flukes than those with lower burdens ($P<0.05$); ** significantly higher in buffaloes with >70 flukes than those non-infected or those with 21-70 fluke ($P<0.05$). Error bars = SD

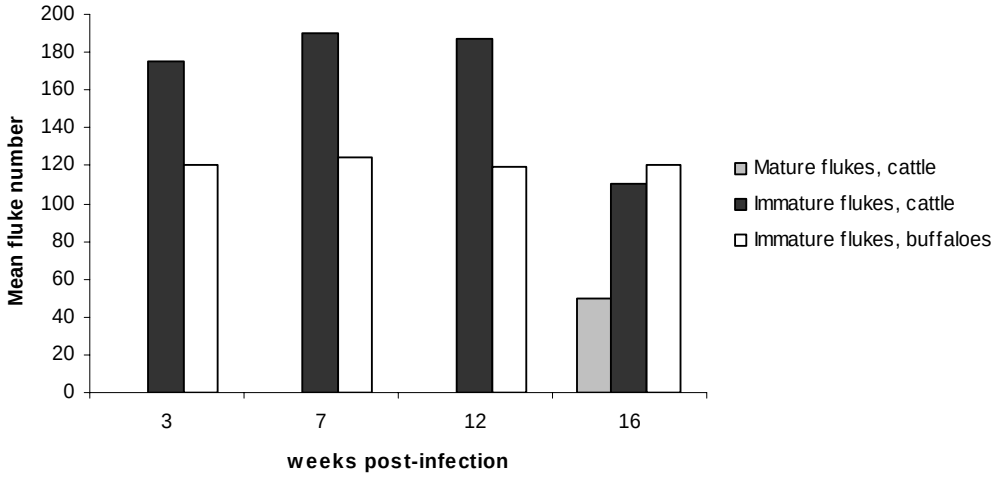

Fig. 3.9 Mean number of flukes in cattle and swamp buffaloes experimentally infected with 1000 metacercariae of *F. gigantica*. Means were from two animals at each time point of infection.

CHAPTER 4

ANTIBODY ISOTYPE PRODUCTION IN CATTLE AND BUFFALOES INFECTED WITH *FASCIOLA GIGANTICA*

4.1 Introduction

A number of studies have demonstrated humoral stimulation in cattle and buffaloes infected with *F. gigantica* but most of these studies only investigated specific IgG1 or IgG production (Ogunrinade 1983; Dixit, Yadav and Sharma 2004; Velusamy et al 2004). One study examined Bali, Ongole and buffalo calves infected with *F. gigantica* to measure their IgG1 and IgG2 responses (E. Wiedosari et al, personal communication) but IgG2 was not detected in infected buffaloes. Information regarding specific IgE response from cattle and buffaloes infected with *F. gigantica* is limited as only one study has indicated the presence of IgE-like antibodies in infected buffaloes (El-Said 1997). There was no published report comparing the IgG1, IgG2 and IgE responses between cattle and swamp buffaloes infected with *F. gigantica*. Therefore the present study was undertaken to determine and compare the production of antibody isotypes IgG1, IgG2 and IgE antibodies during infection with *F. gigantica* between these animals. Investigation of specific antibody isotypes will give an indication of a stimulation of a Th1 or Th2 response during infection. Generally, helminth infections are manifest by a Th2 response (Finkelman et al 1991) with the production of cytokines such as IL-4 and IL-10 which causes the stimulation of IgG1 (Estes 1996) and IgE responses (Pritchard et al 1995). In *F. hepatica* infection, there is a predominance of IgG1 (Clery et al 1996; Bossaert et al 2000a) indicating that the T cell response is predominantly a Th2 response.

4.2 Materials and Methods

The animals experimentally infected with *F. gigantica* (Chapter 3) were used in this study.

4.2.1 Collection and preparation of serum

Samples of jugular blood were collected weekly starting at pre-infection until 16 weeks post-infection using sterile vacutainers and needles. These were centrifuged and the sera collected were kept in cryovials and stored at -20° C until analysis.

4.2.2 Assay for mouse anti-bovine IgG2 on buffalo serum

In a previous study (E. Wiedosari et al, personal communication) no IgG2 was detected in buffalo calves infected with *F. gigantica*. It was suggested that this may be because the mouse anti-bovine IgG2 used in their study did not recognize IgG2 produced by buffaloes. This observation prompted an investigation on the reactivity of buffalo serum to mouse anti-bovine IgG2 by an indirect ELISA to confirm this previous observation.

Bovine and mouse sera were obtained from the Microbiology and Immunology laboratory at JCU, Townsville, Queensland, Australia. The buffalo serum was from one of the uninfected buffaloes used during the study.

4.2.3 Assessment of the reactivity of buffalo serum to mouse anti-bovine IgG2 reagent

Indirect ELISA was performed to determine the reactivity of buffalo serum to mouse anti-bovine IgG2 (Serotec Ltd, Kidlington, Oxford, England). ELISA plates were coated with bovine and buffalo serum diluted 1/2000, 1/5000 and 1/10,000 in carbonate/bicarbonate (JCU Tropical Biotechnology Pty Ltd., Townsville, Queensland, Australia). Mouse serum diluted in the same dilutions was used as a control. The plates were incubated overnight at 4° C. After three washes with TEN-Tween 20 buffer (pH 8.0), 50 µL of mouse anti-bovine IgG1 or IgG2 diluted in 1/200 in ELISA diluent was added to the wells. The plates were incubated for one hour at 37° C. After six washes with the buffer, 50 µL of horseradish peroxidase-conjugated anti-mouse IgG was added at a dilution of 1/500 and 1/1000. The plates were incubated for one hour at 37° C and were washed six times with the buffer. 2,2-Azino-di-[3-ethylbenthizolin sulfonat (6)] (ABTS) (Kirkegaard and Perry Laboratories, 2 Cessna Court, Gaithersburg, Maryland, USA) was added to the plates as a chromogen. The plates were kept for two hours at 37° C then the optical density (OD) was measured by a spectrophotometer at 414nm with a reference wavelength of 494nm.

4.2.4 Enzyme-linked immunosorbent assay (ELISA) for antibody isotype determination

4.2.4.1 Preparation of antigen for detection of antibody isotypes

Adult flukes of *F. gigantica* were collected from the liver of cattle and buffaloes at Maa abattoir, Davao City, Philippines, rinsed with saline and stored at -20° C until use. On thawing, flukes were washed twice in phosphate buffered saline (Appendix 3) and were ground by a glass tissue grinder with 1 mL per fluke of worm solubilization buffer (Appendix 3). The fine homogenate was centrifuged at 4° C for 30 minutes. The supernatant fluid was stored at -10° C. The protein concentration of the supernatant was measured using the BCA™ protein assay kit (Pierce, Rockford, Illinois, USA).

4.2.4.2 ELISA for IgG1 and IgG2

Sequential production of antibody isotypes (IgG1 and IgG2) reacting to *F. gigantica* antigens in the sera of infected cattle and buffaloes were evaluated by indirect ELISA. The format of the ELISA used is briefly described below. The optimal antigen and conjugate working dilutions were determined by ELISA in checkerboard titration using positive and negative sera diluted 1/200. The optimum reagent concentrations were those that gave the maximum ratio between the absorbance values of wells containing positive and negative sera. The optimum dilutions of antigen and conjugate are listed in Table 4.1**.** They were subsequently used in ELISA to determine the presence and estimate levels of IgG1 and IgG2 in serum. As there were no positive and negative controls, variations from plate to plate were controlled by running samples together. Sera from the abattoir were not used as controls as they may come from animals infected with other parasites whose antigens might cross-react with antigens of *F. gigantica*.

The 96-well microtitre plate was coated with somatic fluke antigen (1.3 mg/mL) diluted in carbonate-bicarbonate buffer (pH 8.0) (50 µL/well) and incubated at 4° C overnight. The following day, the plate was washed three times with TEN-Tween 20 buffer (pH 8.0) and sera diluted in ELISA diluent were added (50 µL/well) and incubated at 37° C for one hour. After three washes with the buffer, anti-bovine supernatants (mouse anti-bovine IgG1 (Serotec) or sheep anti-bovine IgG2 (Bethyl

Laboratories, Montgomery, Texas)) diluted in ELISA diluent were added (50µL/well). After one hour at 37° C, the plate was washed again six times with the buffer, and then a conjugate was added (50 µL/well) and incubated for one hour at 37° C. After six washes with the buffer, the substrate, ABTS, was added (50 µL/well) and incubated at 37° C for two hours. The absorbance was read at 414 and 494 nm using an ELISA plate reader.

4.2.4.3 IgE ELISA

IgE against *F. gigantica* somatic antigen was determined during infection using an ELISA described by Kooyman, Yatsuda, Ploeger and Eysker (2002) with some modifications. Briefly, plates were coated with the somatic antigen (1.3 mg/mL of protein concentration) in coating buffer. Heat treated sera were applied undiluted to the plates followed by rabbit anti-bovine IgE (kindly provided by Department of Parasitology and Tropical Veterinary Medicine, Utrecht University, Utrecht, The Netherlands). Goat anti-rabbit Ig/HRPO conjugate (JCU Tropical Biotechnology Pty, Ltd, Townsville, Queensland, Australia) was used in a 1:120 dilution. ABTS was used as substrate and plates were read at 414 nm with a reference wavelength of 492 nm after 2 hours incubation at 37° C.

4.3 Results

4.3.1 IgG2 response of buffaloes using mouse anti-bovine IgG2

The graph (Figure 4.1**)** shows that buffaloes do not react to mouse anti-bovine IgG2. Absorbance values (OD) observed ranged from only 0.031-0.067 compared to the ODs of bovine serum which reached a mean of 3.09 using a conjugate dilution of 1/1000.

4.3.2 Antigen and conjugate working dilutions

Table 4.1 Optimal dilutions of parasite antigen and conjugates in ELISA

Antibody Isotype to be Detected	Ag Dilution	Conjugate Dilution
IgG1	1/64	1/500
IgG2	1/10	1/3000

4.3.3 Sequential production of antibody isotypes reacting to *F. gigantica*

IgG1 and IgG2 levels in cattle and buffaloes infected with *F. gigantica* increased relative to the controls (Figs. 4.2 and 4.3). Both IgG1 and IgG2 levels in infected cattle did not markedly increase during the observation period except after weeks 12. In contrast, levels of both isotypes were markedly increased with a pattern of increasing trend in infected buffaloes. Values for IgG1 and IgG2 in buffaloes tended to be higher than those in cattle (Figs. 4.5 and 4.6).

IgE response to *F. gigantica* antigens was elevated in both infected cattle and buffaloes (Fig. 4.4). The increase in IgE level in both species commenced at one week post-infection and remained higher than IgE levels in uninfected controls until the end of the experiment. The IgE response did not markedly differ between cattle and swamp buffaloes (Fig. 4.7).

4.4 Discussion

Fasciola gigantica induces the production of IgG1, IgG2 and IgE in cattle and swamp buffaloes. This is the first time that IgG2 reacting to *F. gigantica* antigens was demonstrated in swamp buffaloes and the first time that IgE was demonstrated in serum of cattle and swamp buffaloes infected with *F. gigantica*.

IgG1 response to immature and mature fluke antigens in cattle infected with *F. gigantica* was demonstrated previously (Ogunrinade 1983b; E. Wiedosari et al, personal communication). IgG1 response in Ongole, Bali and buffalo calves infected with *F. gigantica* rose progressively until week 20 when it reached a plateau (E. Wiedosari et al, personal communication). They also observed that the IgG2 response in Ongoles occurred after week 12 and started at week 4 in Bali cattle with a sharp rise after week 12. No IgG2 was detected in buffaloes which she suggested could be due to a lack of binding of the monoclonal anti-bovine IgG2 used to measure IgG2 in buffaloes. In the present study, a polyclonal anti-bovine IgG2 was used to detect IgG2. Cattle infected with *F. hepatica* showed specific IgG2 response starting at 2-4 weeks post-infection (Clery et al 1996; Bossaert et al 2000a). The sharp rise in IgG1 and IgG2 in cattle at the later stage of infection was probably induced by stage-specific antigens released by maturing and mature flukes. Both cattle and buffaloes manifest increased levels of IgG1 and IgG2 however the pattern of production of these isotypes differed between these

species. IgG1 and IgG2 in buffaloes showed a pattern of gradually increasing levels while there was no marked increase in these responses in cattle. This may be an indication of a lesser stimulation of these responses in cattle or a suppression of immune response in cattle by *F. gigantica* to a greater extent than occurs in buffaloes. The significance of the differing pattern of IgG1 production between cattle and buffaloes is not known. It may suggest differences in resistance to *F. gigantica* between these animals as there were indications that they differ in their resistance and resilience to the parasite. Swamp buffaloes manifest an apparently higher resistance than cattle as evidenced by their lower fluke burdens and egg counts observed in the abattoir study and lower fluke burden and longer prepatent period during the experimental infection (Chapter 3). However, previous studies showed that IgG1 does not appear related to protection or level of resistance in cattle to *F. gigantica* (E. Wiedosari et al, personal communication) or *F. hepatica* (Clery et al 1996; Bossaert et al 2000a). Cattle with a dominant IgG1 response induced by infection with *F. hepatica* were not protected from subsequent infection (Clery et al 1996). These authors also observed that in these cattle IgG2 responses were either absent or much lower than their IgG1 responses. Specific IgG1 titres and fluke burden in cattle infected with *F. hepatica* were not correlated (Haroun and Hillyer 1986; Keegan and Trudgett 1992; Bossaert et al 2000a). E. Wiedosari et al (personal communication) and Mulcahy et al (1998) however considered that a protective response could be associated with IgG2. The increase in IgG2 in Bali cattle was probably related to development of a degree of resistance to *F. gigantica* (E. Wiedosari et al, personal communication). They observed that Bali cattle showed lower fluke burden than Ongoles, a declining level of GLDH after week 24 and a declining egg count after about week 26. A decreased GLDH suggests reduced parenchymal damage induced by larval flukes while a declining egg count suggests suppression of egg production by adult flukes or a reduction in adult fluke numbers. Both of these are indications of a development of resistance. In cattle vaccinated with cathepsin L2 and haemoglobin from *F. hepatica*, IgG2 titres and fluke burden were negatively correlated (Mulcahy et al 1998).

IgG2 may be associated with resistance against *F. gigantica* in cattle and swamp buffaloes; higher levels in buffaloes indicating a higher level of resistance against *F. gigantica* than in cattle. At 16 weeks of infection, no fluke eggs were detected in the faeces of infected buffaloes whereas at this period eggs were first seen in cattle

(Chapter 3). This suggests that there was a slower development of flukes in buffaloes resulting in a longer prepatent period compared with cattle. The latter assumption is supported by the presence of only immature flukes in buffaloes at 16 weeks post-infection, at a time when flukes are expected to be present in the bile ducts as maturing and mature flukes (Chapter 3). Moreover, buffaloes had consistently lower fluke burdens than cattle when counts were made at 3, 7, 12 and 16 weeks post-infection (Chapter 3). In the abattoir study, a trend of lower burdens of mature and immature fluke and faecal egg counts in buffaloes than in cattle was observed (Chapter 3). These observations support a conclusion that swamp buffaloes are more resistant than cattle to *F. gigantica*. This may be linked to their higher IgG2 response than in cattle as demonstrated in Bali cattle infected with *F. gigantica* (E. Wiedosari et al, personal communication) or in cattle vaccinated with *F. hepatica* cathepsin L and haemoglobin (Mulcahy et al 1998).

There is no report on the role of IgE during *F. gigantica* infection but its detection in infected cattle and buffaloes in this study shows its association with *F. gigantica* infection in these animals. This result confirms previous report of an involvement of IgE-like antibody in buffaloes and specific IgE response in sheep infected with *F. gigantica* (El Said 1997; Hansen et al 1999). An IgE-like dermal response which occurred from 2 weeks post-infection in response to both larval and adult fluke antigen has also been reported in cattle infected with *F. hepatica* (Bossaert et al 2000a). In the present study, *F. gigantica* stimulated similar levels of IgE between these animals but they differed in their resistance to infection (Chapter 3). Hence, this immune response may not be associated with protection or resistance to *F. gigantica* in cattle and swamp buffaloes. However, in *F. gigantica* infection in sheep it was suggested that IgE may be involved with the clearing of migrating flukes in Indonesian thin-tailed (ITT) sheep as it occurred early post-infection coinciding with the time when killing of *F. gigantica* occurs (Hansen et al 1999) but this phenomenon has not been confirmed. It was also considered that a *Fasciola*-specific IgE like response appears to be important in inducing resistance in cattle to *F. hepatica*. IgE-like antibodies in cattle infected with *F. hepatica* reached a peak level at 20-24 weeks post-infection coinciding with the expulsion of flukes (reviewed by Howell and Boray 1994).

The role of IgE against *F. hepatica* in cattle has not been confirmed but it may contribute to resistance to *F. hepatica* as substantial infiltration of eosinophils during challenge infection indicates an IgE like response (Doy et al 1978). However, interpretation of the importance of eosinophils in killing invading larval flukes in cattle must be considered with caution as there is evidence that bovine eosinophils do not kill larval *F. hepatica* in vitro through antibody dependent, cell mediated cytotoxicity (Duffus et al 1980).

Alternatively, the IgE response demonstrated in this study may be involved in the inflammatory response seen in the infected livers where infiltration of eosinophils and mast cells occurred (Chapter 7) as eosinophil-mediated inflammatory responses constitute an interaction between antigen, IgE and mast cells (Prussin and Metcalfe 2003; Shakoory, Fitzgerald, Lee, Chi and Krishnaswamy 2004) .

IgG1 and IgE production in the animals in this study is an indication that there was a stimulation of a Th2-type of immune response in cattle and buffaloes infected with *F. gigantica* as IgG1 and IgE responses are associated with a Th2 response (Romagnani, Maggi, Parronchi, Macchia and Piccini 199; Estes 1996; Borish and Steinke 1997). However, this response may not be associated with protection during *F. gigantica* in these animals. The increase in IgG2 indicates a stimulation of a Th1-type of response as IgG2 is associated with a Th1 response (Estes et al 1994). This response may be associated with resistance against *F. gigantica* in these species, as it was in cattle vaccinated with *F. hepatica* cathepsin L and haemoglobin (Dalton et al 1996; Mulcahy et al 1998).

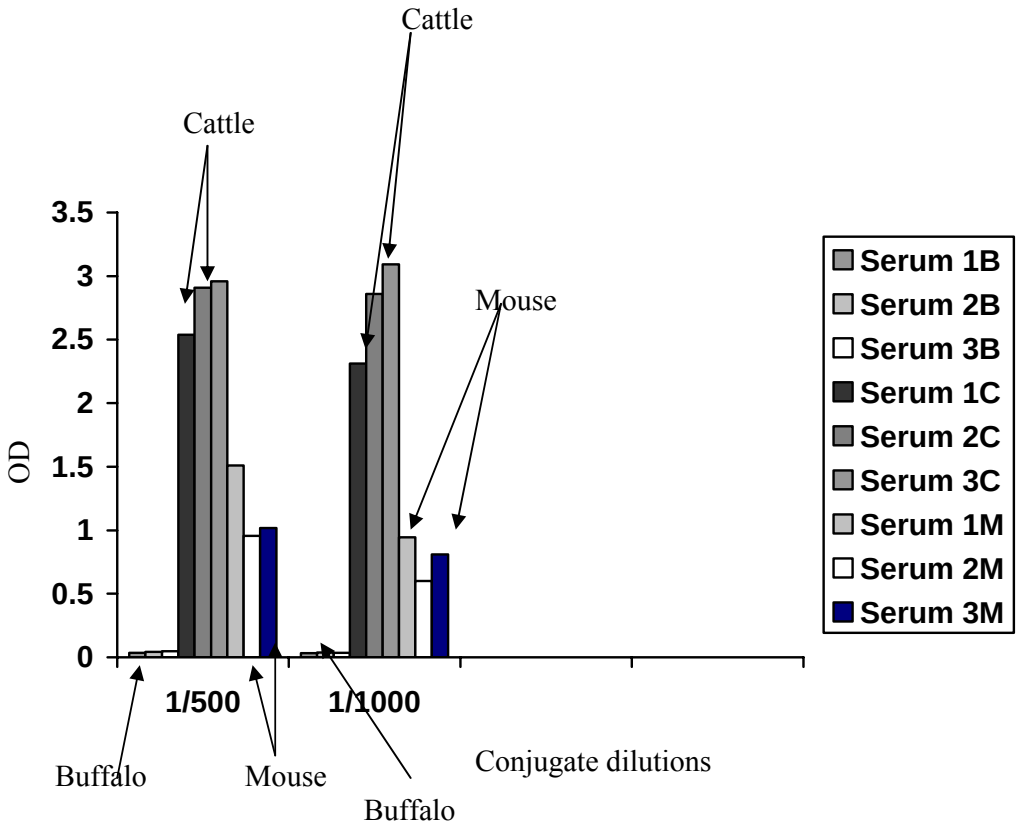

Fig. 4.1 Mean OD (IgG2) of buffalo (B) and cattle (C) sera using mouse anti-bovine IgG2 supernatant. Mouse serum (M) was used as the negative control. Serum dilutions were 1/2000 (Serum 1), 1/5000 (Serum 2) and 1/10000 (Serum 3).

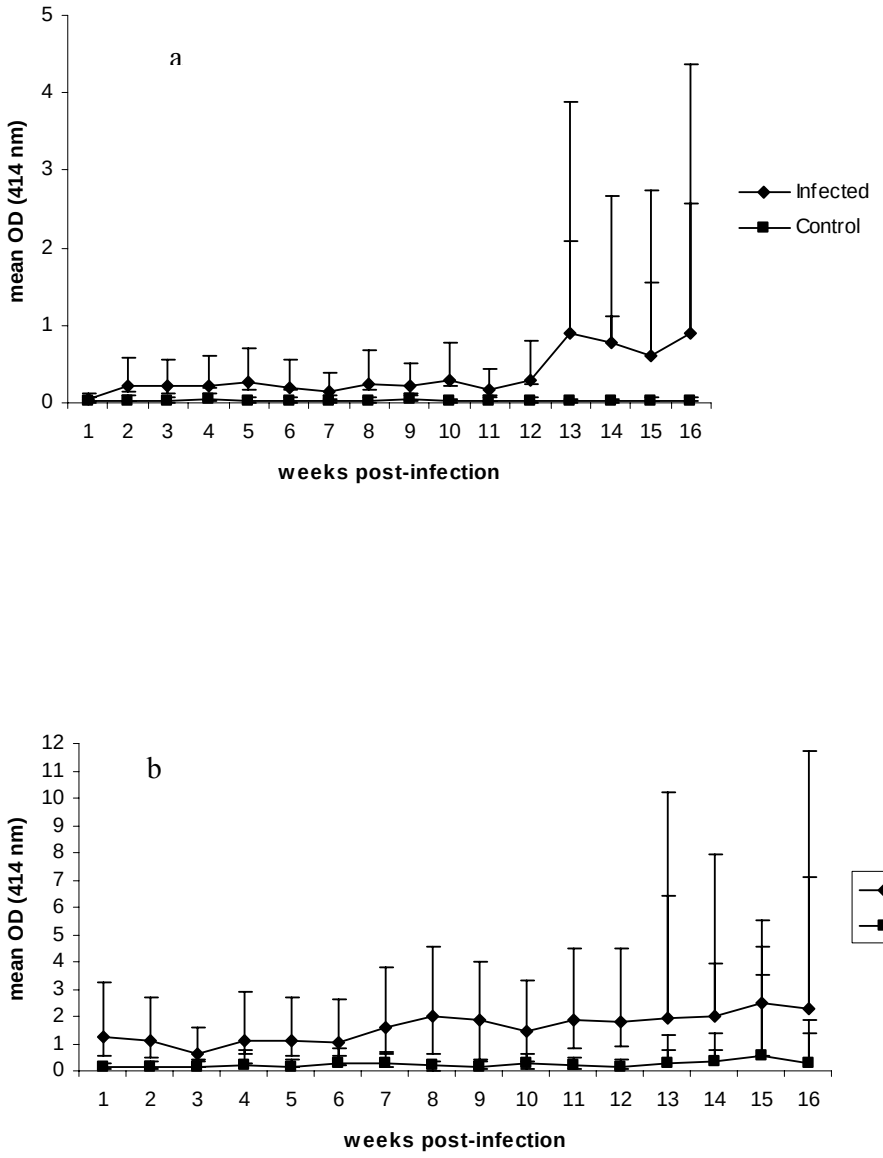

Fig. 4.2 Mean IgG1 in cattle (a) and buffaloes (b) infected with *F. gigantica*. Values are means of absorbance values at each time point of infection. Error bars = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

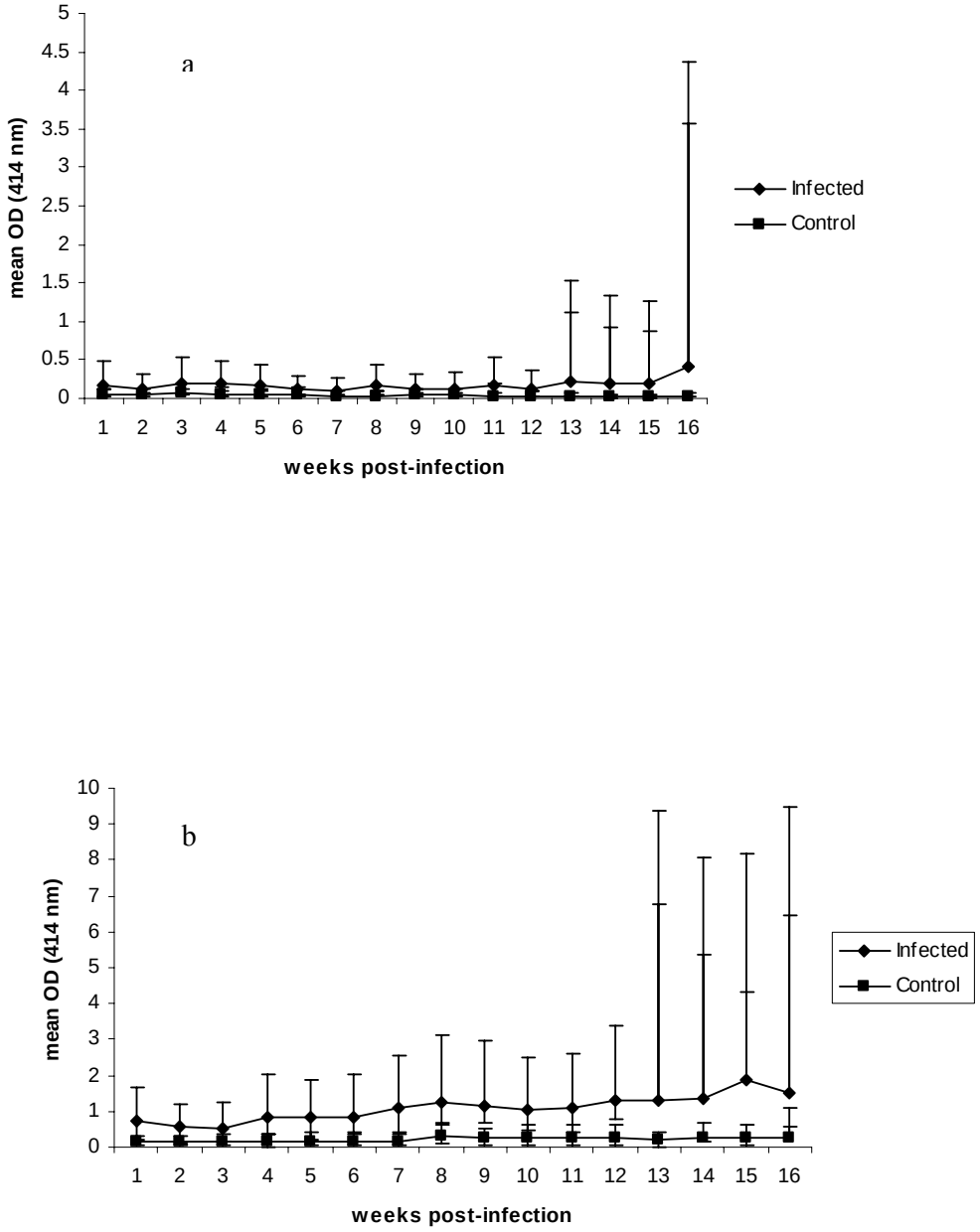

Fig. 4.3 Mean IgG2 in cattle (a) and buffaloes (b) infected with *F. gigantica*. Values are means of absorbance values at each time point of infection. Error bars = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

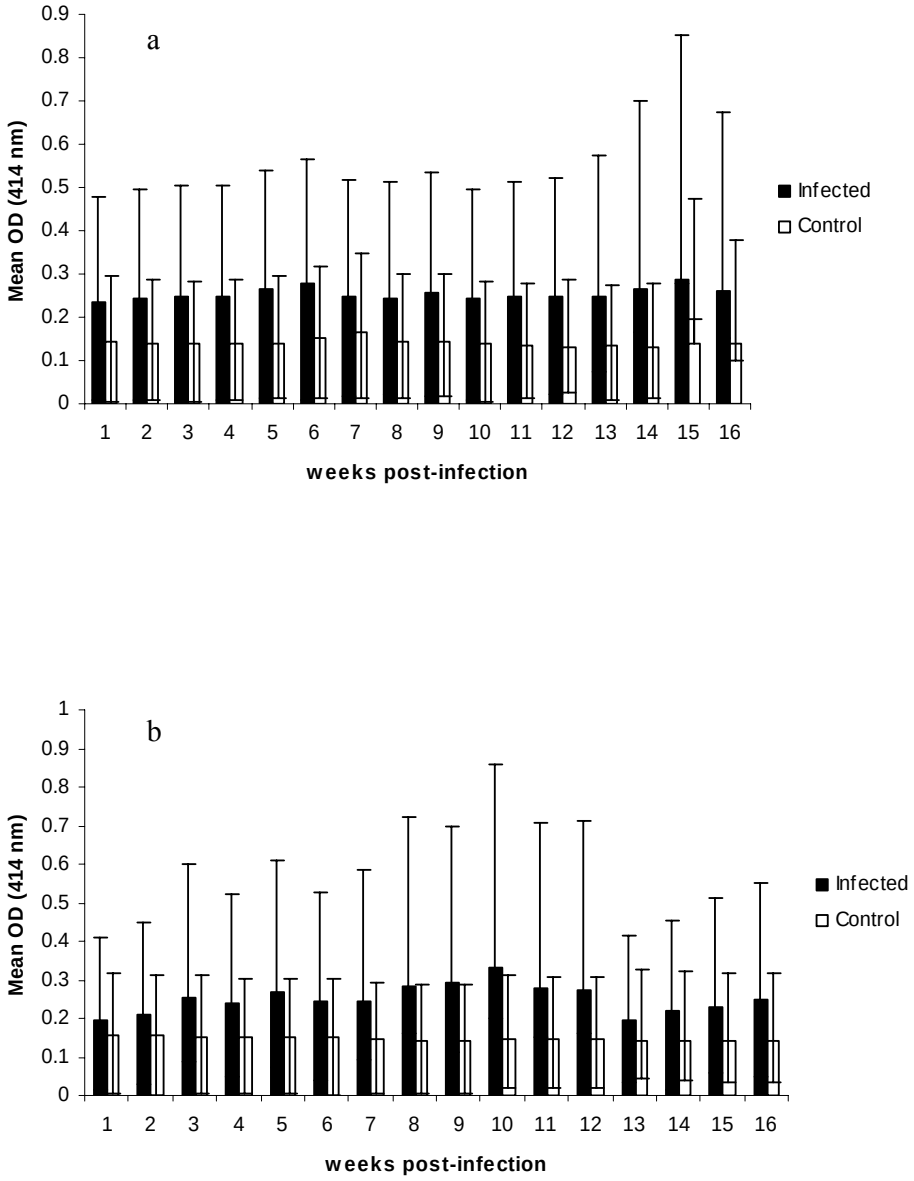

Fig. 4.4 Mean IgE in cattle (a) and buffaloes (b) infected with *F. gigantica*.
Values are means of absorbance values at each time point of infection. Error bars = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

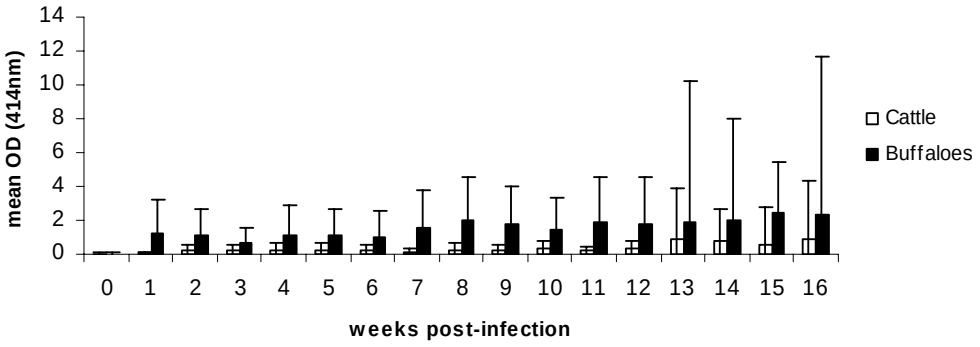

Fig. 4.5. Comparison of IgG1 responses between cattle and buffaloes infected with *F. gigantica*. Values are means of absorbance values at each time point of infection. Error bar = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

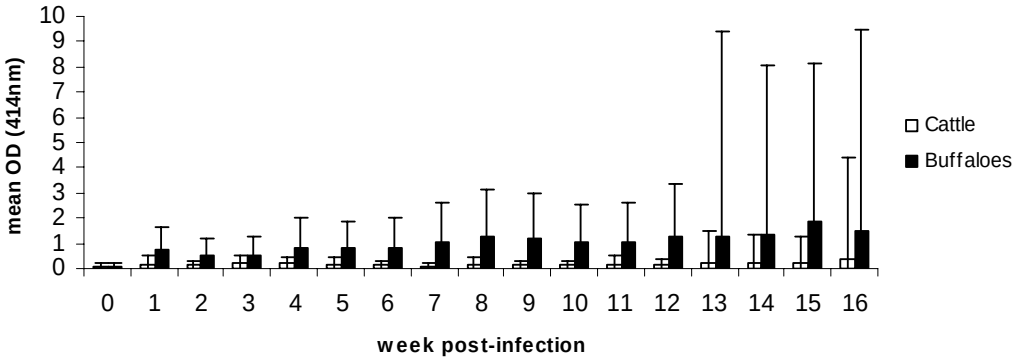

Fig. 4.6. Comparison of IgG2 responses between cattle and buffaloes infected with *F. gigantica*. Values are means of absorbance values at each time point of infection. Error bar = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

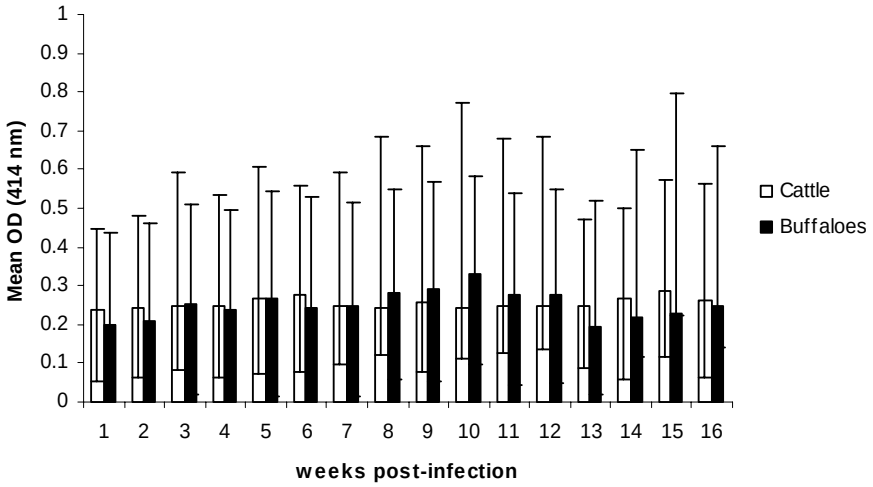

Fig. 4.7 Comparison of IgE responses between cattle and buffaloes infected with *F. gigantica*. Values are means of absorbance values at each time point of infection. Error bars = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

CHAPTER 5
TH1 AND TH2 CYTOKINE RESPONSES IN CATTLE AND BUFFALOES INFECTED WITH *FASCIOLA GIGANTICA*

5.1 Introduction

Immune responses to parasitic infections are regulated by T helper cells via their production of cytokines (Mosmann and Coffman 1989). Development of protective immunity to infection is influenced by these cells and their cytokines (Sher and Coffman 1992). Description of T helper cell responses during fasciolosis is limited to *F. hepatica*. The aim of the present study was to observe and compare T helper cell responses by measuring different types of T helper cell cytokines in cattle and swamp buffaloes infected with *F. gigantica*. This will give an indication of the T cell response during *F. gigantica* infection and may assist in understanding host-parasite relationships in *F. gigantica* infection in cattle and swamp buffaloes.

5.2 Materials and methods

5.2.1 Analysis of serum cytokines

Analysis of levels of the cytokines IFN-γ, IL-6 and IL-8 was done using the sera collected weekly from the experimental cattle and buffaloes as described in the previous chapter.

5.2.1.1 IFN-gamma Assay

The levels of IFN-γ in serum were determined using a commercial bovine gamma interferon test kit (Bovigam, CSL Limited, Victoria, Australia). The procedures were provided in the kit and are described below.

Freeze-dried components (positive and negative bovine IFN-γ controls and conjugate) were reconstituted following the instructions provided in the kit. Other reagents needed were equilibrated to room temperature during reconstitution. Fifty µL of the green diluent were added to the required wells followed by the addition of 50 µL of test and control samples to the appropriate wells containing green diluent. Control samples were added after test samples, as specified in the kit. The green diluent and controls or

samples were mixed thoroughly by pipetting up and down 5 times. Each plate was covered with a lid and incubated at room temperature for 60 ± 5 minutes. After incubation, the contents of the plate were shaken out and the wells were washed 6 times at room temperature. After the 6th wash, the plates were tapped face down several times on absorbent paper to remove as much remaining wash buffer as possible. One-hundred µL of freshly prepared conjugate reagent were then added to the wells. The plates were covered and incubated at room temperature for 60 ± 5 minutes. The plates were washed as before and excess buffer removed. One-hundred µL of freshly prepared enzyme substrate solution (chromogen) were added to the wells and mixed thoroughly by pipetting up and down 5 times. The plates were covered and incubated as before for 30 minutes. Fifty µL of enzyme stopping solution were then added to each well, care being observed to avoid transferring chromogen from well to well, then mixed by gentle agitation. The absorbance is then read within 5 minutes of terminating the reaction using a 450 nm filter.

The mean absorbance of negative and positive controls was determined. The acceptable range of means for negative bovine IFN-γ control was <0.130 and positive IFN-γ control >0.700. The positive control replicates must not deviate by more than 30% from their mean absorbance.

5.2.1.2 ELISA for IL-6 and IL-8

The levels of IL-6 and IL-8 in serum of infected and non-infected cattle and buffaloes were determined by ELISA. The assay made use of mouse anti-ovine IL-6 or IL-8 as the coating antibody and rabbit anti-ovine IL-6 (Center of Animal Biotechnology, University of Melbourne, Victoria, Australia) or IL-8 (Epitope Technologies, Victoria, Australia) as the detector antibody since reagents for cattle and buffaloes are unavailable commercially. Recombinant ovine IL-6 and IL-8 (DPI, Geelong, Australia) were used as the positive control. The procedure of the assay was adopted from the ovine IL-6 cytokine ELISA of DPI, courtesy of Dr. Peter McWaters and is described below.

Anti-IL-6 or IL-8 monoclonal antibody was diluted to5 µg/mL in coating buffer (Tropbio, JCU, Townsville, Queensland, Australia) and 100 µL was added to each well

of an ELISA plate. This was incubated overnight at 4°C. The solution from the wells was flicked and 200 µL of blocking buffer (Appendix 4) was added to each well. After one hour incubation at room temperature, the plates were washed once with the washing buffer (Tropbio, JCU, Townsville, Queensland, Australia). The recombinant ovine cytokine standard was diluted to 1 µg/mL in diluting buffer (Appendix 4). One hundred µL of diluting buffer was added to wells A2 to A12 and 100 µL of diluted cytokine standard was added to well A1. Fifty µL of standard was added to well A2 and the contents were mixed and 50 µL were transferred across the plate to well A12. This produced the standard curve against which all unknown samples were compared. To the remaining wells (B1-H12), 50 µL of diluting buffer were added and 50 µL of unknown sample was added to each well. Each sample was replicated twice. The plates were incubated for 1 hour at room temperature. After incubation the plates were washed 4 times and 100 µL of rabbit anti-ovine IL-6 diluted 1:5000 in diluting buffer were added to each well. After 1 hour incubation at room temperature, the plates were washed 4 times and 100 µL of anti-rabbit Ig HRP (Tropbio, JCU, Townsville, Australia) conjugate diluted in diluting buffer at 1:120 were added to each well. The plates were incubated at room temperature for 1 hour and then washed 5 times. Tetramethylbenzidine (TMB) substrate solution (Appendix 4) was prepared just before use and 100 µL was added to each well. The plates were incubated for 20 minutes and then the reaction was stopped by the addition of 50 µL/well of 0.5M sulfuric acid. The plates were then read on a micro-plate reader at 450 nm. The standard curve was graphed and the unknowns were read off against the curve, allowing for the dilution factor.

5.3 Results

IFN-γ was not present in detectable levels in serum of cattle and buffaloes (Figs. 5.1 and 5.2).

Serum IL-6 levels were higher in infected cattle and buffaloes than in those non-infected (Figs. 5.3 and 5.4). Increased serum IL-8 levels were observed in infected buffaloes (Fig. 5.5) but not in infected cattle (Fig. 5.6). Levels of serum IL-6 were higher in cattle than in buffaloes (Fig. 5.7) while those of IL-8 were higher in buffaloes than in cattle (Fig. 5.8).

5.4 Discussion

The present study is the first to investigate levels of IL-6 and IL-8 during infection with *F. gigantica* in cattle and buffaloes. These cytokines were demonstrated in humans infected with *F. hepatica* (Khalil, Abou Shousha, Farahat and Rashwan 1999) but there is no published information regarding their role in the immune response during liver fluke infection.

The presence of IL-6 indicates a stimulation of a Th2-type of response in these animals during infection with *F. gigantica*. IL-6 is one of the cytokines produced by Th2 cells (Abbas et al 1996; Cox 2001) and it participates in the polarization of the immune response towards a Th2 response (Angeli, Faveeuw, Delerive, Fontaine, Barriera, Franchimont, Staels, Capron and Trottein 2001). Clery et al (1996) did not detect IFN-γ in cattle during a chronic infection with *F. hepatica* indicating a predominantly Th2 response in these animals. A Th2 response, indicated by IL-10 was observed throughout the first six weeks of infection in sheep infected with *F. hepatica* while IFN-γ production occurred only until the first 2 weeks of infection (Moreau et al 1998). These authors proposed that IL-10 inhibited monocytes from the 35th day post-infection, resulting in the inhibition of IFN-γ production. More recently, Waldvogel et al (2004) observed that peripheral blood mononuclear cells (PBMC's) of calves experimentally infected with *F. hepatica* expressed high amounts of IL-4 but not of IFN-γ mRNA early in the infection indicating a Th2 biased immune response commencing early in the infection. In the present study, there were increased IgG1 and IgE (Chapter 4) and eosinophilia (Chapter 7) in infected animals. IgG1, IgE and eosinophilia are features associated with a Th2 response (Estes 1996; Pritchard et al 1995). Clery et al (1996) observed that IgG1 was the dominant isotype present in cattle infected with *F. hepatica*, with IgG2 occurring at much lower levels. The IL-6 that commenced early in the infected cattle and buffaloes in this study may have inhibited the production of detectable levels of IFN-γ as this cytokine was not detected in the infected animals. IL-6 inhibits IFN-γ receptor-mediated signals preventing autoregulation of IFN-γ gene expression by IFN-γ during CD4+ Th2 activation preventing Th1 differentiation (Diehl et al 2000). La Flamme, MacDonald and Pearce (2000) and Angeli et al (2001) reported that IL-6 participates in the polarization of the immune response towards a Th2 response. Alternatively, the absence of IFN- γ in infected cattle and buffaloes suggests that this cytokine may not be important in the

immune response against *F. gigantica* in cattle and swamp buffaloes.

IL-6 may not only be involved in influencing a Th2-type of response but it may also participate in an inflammatory innate response of infected cattle and buffaloes against *F. gigantica*. Its involvement in an innate inflammatory response includes the upregulation of acute-phase protein production in the liver (Akira, Taga and Kishimoto 1993; Steel and Whitehead 1994). A build-up of the concentrations of these proteins in the serum results in their accumulation at the site of inflammation where they act as opsonins providing additional factors that aid in the destruction of pathogenic agents, especially extracellular bacteria, yeasts and parasites (Wood 2001). Borish, Rosenbaum, Albury and Clark (1989) observed that although IL-6 was not chemotactic for neutrophils, it stimulates these cells to secrete toxic components thereby enhancing their toxicity via ADCC. IL-6 was found to manifest an inhibitory activity on hepatic stages of *Plasmodium* during the early phase of infection and during the subsequent maturation of the schizonts through an oxidative burst (Pied, Renia, Nussler, Miltgen and Mazier 1991). Recently, Nadeu, Pistole and McCormic (2002) showed that IL-6 released from epithelial cells of intestines primes polymorphonuclear (PMN) cells to release their granules and stimulates them to kill *Salmonella typhimurium*. However, since IL-6 levels were higher in cattle than in buffaloes in this study while there were indications that buffaloes are more resistant to *F. gigantica* than cattle, the inflammatory response induced by IL-6 may not be protective against *F. gigantica* in cattle and swamp buffaloes. This suggests that this response is not associated with resistance or protection against *F. gigantica* in these species.

Studies have shown that IL-8 participates in the innate immune response particularly by enhancing the oxidative burst and phagocytic responses of neutrophils (Reali, Spisani, Gavioli, Lanza, Moretti and Traniello 1995; Gougerot-Podicalo, Elbim and Chollet-Martin 1996; Mitchell, Betty and Caswell 2003). When neutrophils were exposed to IL-8, they were activated to produce oxygen radicals thus enhancing their phagocytic ability through ADCC (Reali et al 1995). IL-8 is one of the cytokines that interact with PMN activities during the immune and inflammatory responses to pathogens, and IL-8, TNF and GM-CSF strongly prime the oxidative burst of PMN's against bacterial peptides in whole blood suggesting that these cytokines may be involved in the destruction of bacteria (Gougerto-Podicalo et al 1996). Similarly, Mitchell et al (2003) demonstrated

that the prolonged exposure of bovine neutrophils to IL-8 and/or GM-CSF enhances their subsequent oxidative burst and phagocytic responses. IL-8 is the most potent chemoattractant for neutrophils and it stimulates neutrophil degranulation and the respiratory burst (Borish and Steinke, 2003). Its presence early in the host response to *M. tuberculosis* may contribute to the success of the immune response in destroying the pathogen since recombinant IL-8 induced significant chemotaxis in neutrophils from both non-vaccinated and BCG-vaccinated guinea pigs (Lyons, Yoshimura and McMurray 2004). Because chemokines such as IL-8 generate oxygen radicals in phagocytes, including reactive oxygen intermediates and reactive nitrogen intermediates (Goldsby, Kindt and Osborne 2000), which have been reported to be involved in killing flukes (Spithill et al 1997; Piedrafita and Liew 1998; Piedrafita et al 2001), the increased serum IL-8 in buffaloes may operate in a defense mechanism against *F. gigantica* in these animals.

In fasciolosis, ADCC has been considered to be a mechanism by which flukes are destroyed. In *F. hepatica*-resistant rats, larvae of *F. hepatica* were coated with antibody and host cells, including eosinophils, neutrophils, macrophages and mast cells, before they were destroyed within the peritoneal cavity (Hughes 1987). The killing of flukes in the *F. gigantica*-resistant Indonesian thin-tailed (ITT) sheep may be due to an ADCC reaction (Hansen et al 1999), a mechanism also supported by Estuningsih et al (1999) who observed *in vitro* that macrophages of ITT sheep demonstrated an ADCC against *F. gigantica*. In rats which are resistant to *F. hepatica*, the mechanism of killing juvenile flukes was identified as the release of high levels of nitric oxide by peritoneal lavage cells (Spithill et al 1997; Piedrafita and Liew 1998; Piedrafita *et* al 2001). Since IL-8 can participate in an oxidative burst (Reali et al 1995; Borish and Steinke 2003; Mitchell 2003), which has been considered to be a mechanism in killing flukes (Spithill et al 1997; Piedrafita and Liew 1998; Piedrafita et al 2001), its increased serum levels in buffaloes may have a role in an immune mechanism against *F. gigantica* in these animals. The higher levels in infected buffaloes than in cattle suggest that it may be associated with resistance or protection against *F. gigantica* in these animals.

Evidence from the present trial is not sufficient to make definitive conclusions. However from the present findings, it can be proposed that IL-6 and IL-8 are induced during infection with *F. gigantica* in cattle and swamp buffaloes. IL-6 may induce a non-protective inflammatory response during infection and therefore not important in protection against *F. gigantica* in these species, but IL-8 may be important in protection as it may be associated with a higher innate immune response against the flukes in buffaloes than in cattle. Several authors reported that IL-8 is important in an innate response against pathogens ((Reali et al 1995; Gougerot-Podicalo et al 1996; Borish and Steinke; Mitchell et al 2003).

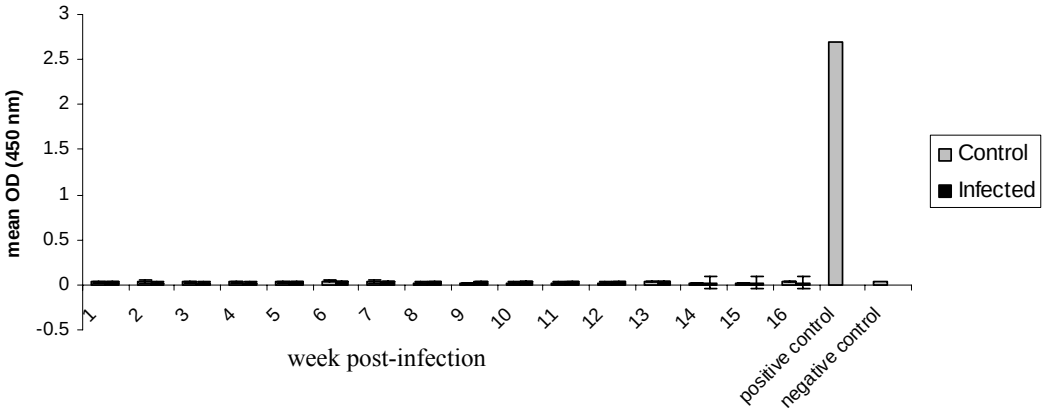

Fig. 5.1 Serum IFN-γ in cattle infected with *F. gigantica*. Error bars= 95% confidence level for means (n=8-2).

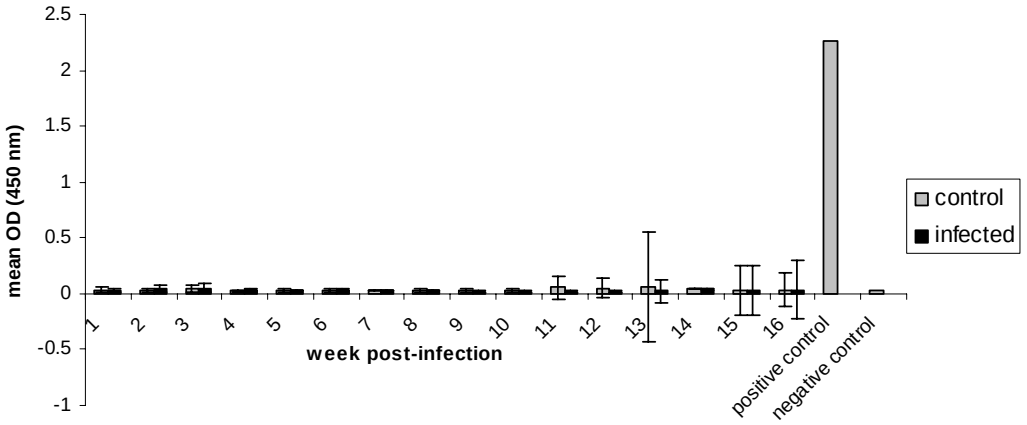

Fig. 5.2 Serum IFN-γ in buffaloes infected with *F. gigantica*. Error bars= 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

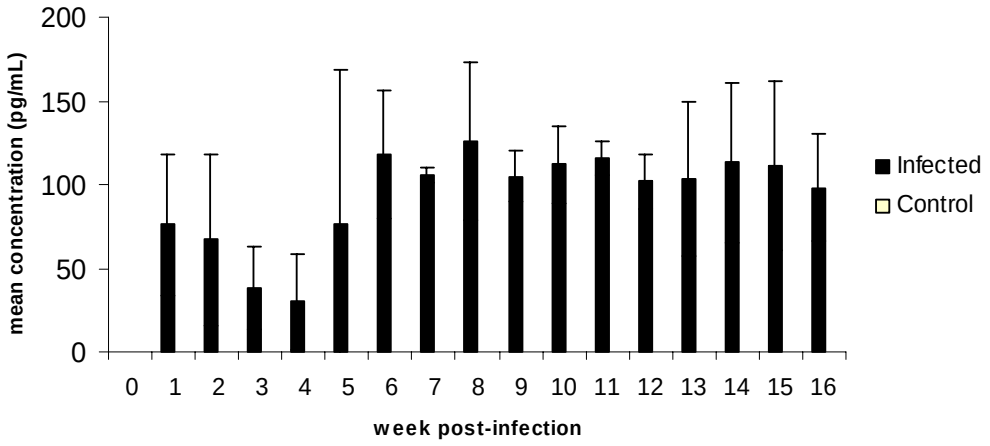

Figure 5.3 Serum IL-6 levels in cattle infected with *F. gigantica*. Error bars= 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

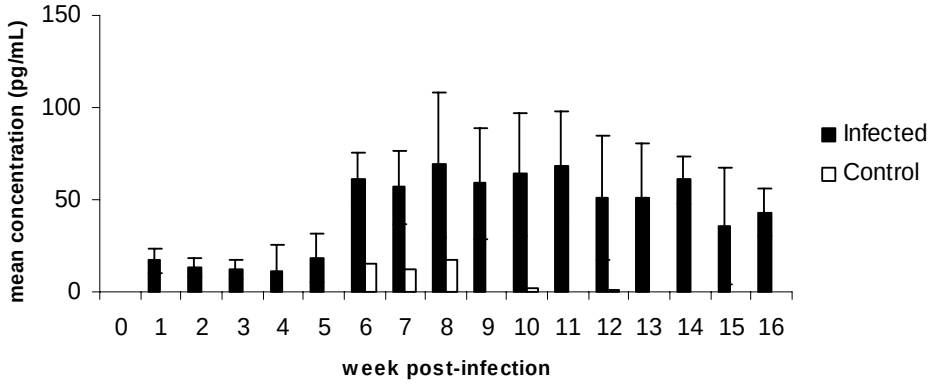

Fig. 5.4 Serum IL-6 levels in buffaloes infected with *F. gigantica*. Error bars = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

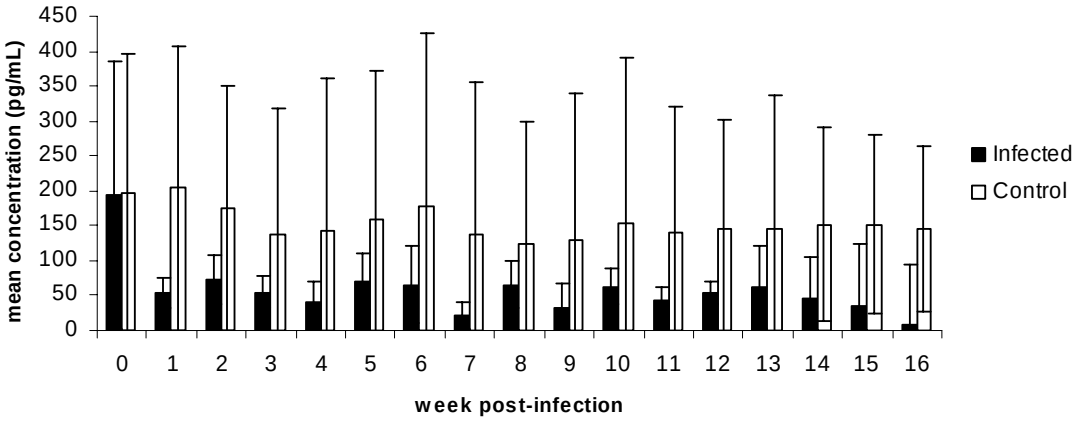

Fig. 5.5 Serum IL-8 in cattle infected with *F. gigantica*. Error bars= 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

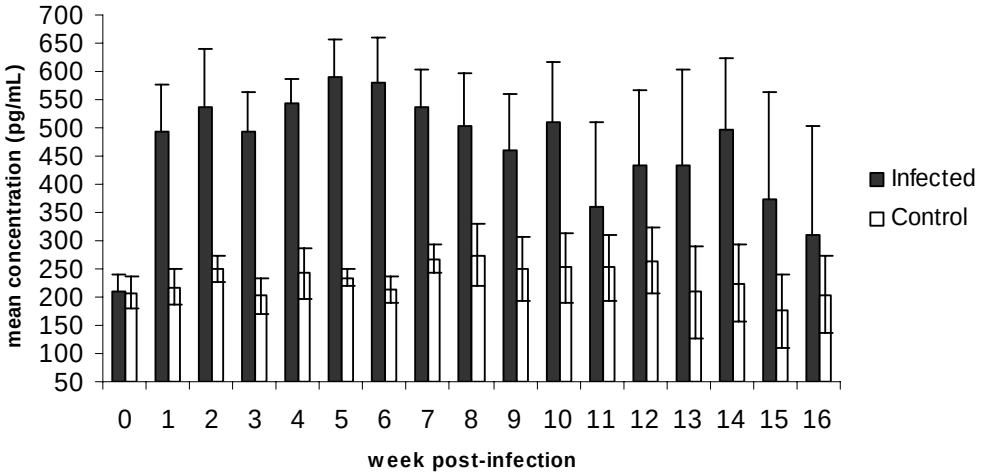

Fig. 5.6 Serum IL-8 in buffaloes infected with *F. gigantica*. Error bars= 95% confidence level for means. Statistical analysis performed at weeks 1-12 when the number of animals allowed for a valid comparison showed that values were significantly higher in infected buffaloes than in controls (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

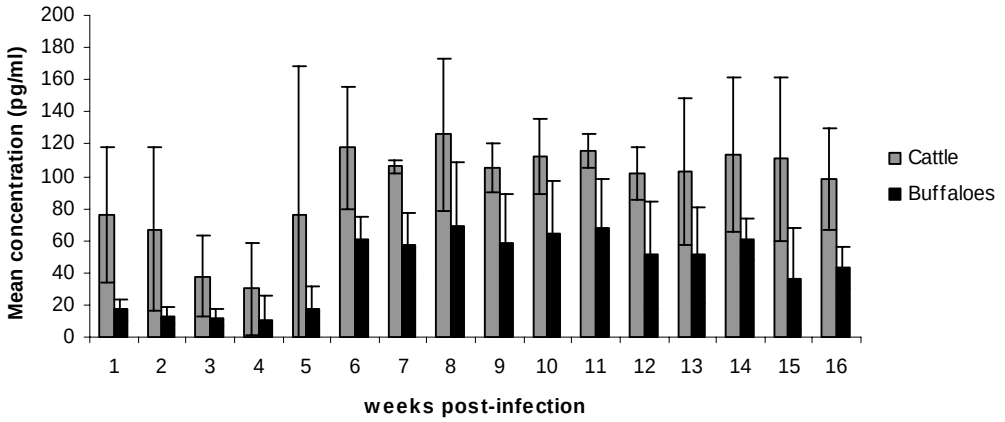

Fig. 5.7 Comparison of serum IL-6 levels between cattle and buffaloes infected with *F. gigantica*. Error bars = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

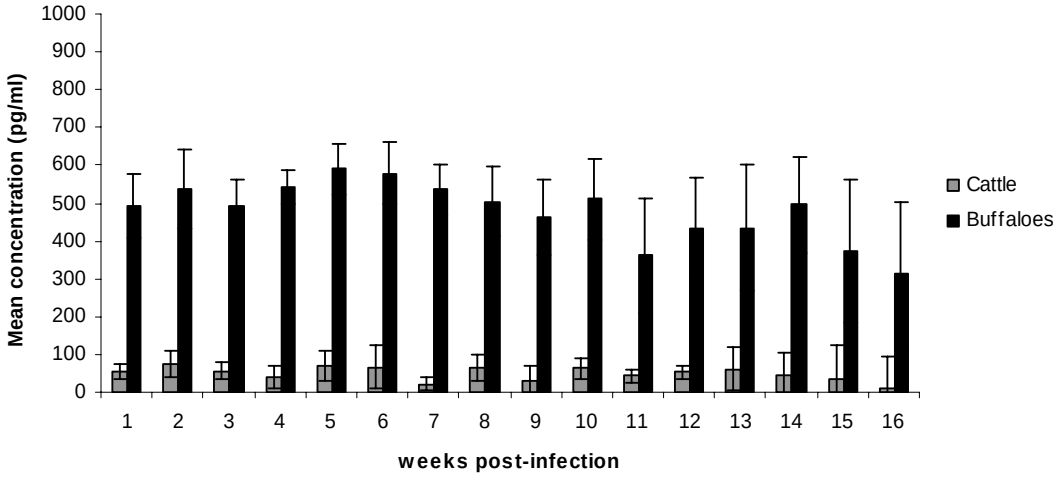

Fig. 5.8 Comparison of serum IL-8 levels between cattle and buffaloes infected with *F. gigantica*. Error bars = 95% confidence level for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

CHAPTER 6

LOCAL IMMUNE RESPONSES OF CATTLE AND SWAMP BUFFALOES INFECTED WITH *FASCIOLA GIGANTICA*

6.1 Introduction

A description of the cellular components in hepatic lesions in cattle and buffaloes infected with *F. gigantica* will provide an indication of the local immune response in the liver of infected animals and give additional insight regarding the pathogenesis of *F. gigantica* infection in cattle and buffaloes. An analysis of CD3+ T cells and CD79+ B cells would show the presence of local cellular and humoral responses, respectively, in the liver of infected animals during infections with *F. gigantica* in cattle and buffaloes. In *F. hepatica* infections, cell mediated immune response has been described in terms of *in vitro* proliferative response of peripheral blood lymphocytes (PBL) to fluke antigens and as the local cellular response occurring in the hepatic parenchyma (Meeusen et al 1995). Immunohistological studies of hepatic lesions have also been used to demonstrate the local immune response in goats infected with *F. hepatica* (Martinez-Moreno et al 1999; Perez et al 1999; Perez et al 2002). No such studies have been undertaken with *F. gigantica* infection in cattle and buffaloes.

6.2 Materials and methods

6.2.1 Experimental animals, collection and processing of tissue samples

Animals used in the study were the same as those described in Chapter 3. At slaughter at 0, 3, 7, 12 and 16 weeks post-infection, sections of the ventral lobe of the liver from each animal were obtained and fixed in 10% neutral buffered formalin for 12 hours. These were processed and embedded in paraffin.

6.2.2 Immunohistochemistry for CD3+ T lymphocytes

Sections from liver tissues were cut to 5μm thickness, mounted onto adhesive glass slides (Superfrost^{R} Plus, Menzel GmbH & Co KG, Saarbruckener Str., 248, D-38116 Braunschweig) and dried overnight at 37° C. They were then deparaffinized at 60°C oven for one hour and dried again at 37° C for about two hours to ensure adhesion of tissues to the slides. The sections were outlined with diamond pencil. They were

then immersed in two changes of xylene for five minutes each and dehydrated in three changes of ethanol. They were rinsed with tap water and washed with PBS, pH 7.4 (Appendix 1). Antigen retrieval consisted of boiling sections in citrate buffer, pH 6.0 (Appendix 1) in microwavable pressure cooker two times for five minutes each cycle. The buffer was allowed to cool down at room temperature for 20 minutes before taking out the sections. The sections were washed with TBS-Tween 20, pH 7.4 (Appendix 1) three times, two minutes each, followed by a quick rinse with TBS, pH 7.4 (Appendix 1) and incubated with 5% normal goat serum in PBS for 30 minutes. A rabbit anti-human CD3 polyclonal antibody (pAb) (Dakocytomation, Carpenteria, California, USA) diluted 1:300 in PBS was applied to the sections and incubated at 4° overnight. The anti-CD3 pAb has previously shown cross-reactivity with caprine T lymphocytes (Ramos-Vara, Miller, Lopez, Prats and Brevik 1994; Martinez-Moreno *et al*., 1999). The sections were then rinsed with TBS-Tween 20 three times, with two minutes each rinse followed by a quick rinse with TBS. A ready-to use biotinylated goat anti-rabbit immunoglobulin G (Zymed, South San Francisco, California) was applied as secondary antibody for one hour at room temperature. After three rinses in TBS-Tween 20 for two minutes each, endogenous peroxidase was quenched by incubating the sections in 3% hydrogen peroxide in PBS for 10 minutes at room temperature. Horseradish peroxidase (HRP)-streptavidin complex (Zymed) was applied to the sections after rinsing and incubated for one hour at room temperature. The reaction was developed with diaminobenzidine (DAB) (Zymed) for 15 minutes. Tissue sections were then rinsed in distilled water, counterstained with Mayer's haematoxylin for 2 minutes, dehydrated and mounted. Tissue sections in which the specific primary antibody was substituted with PBS, or which were incubated with only the HRP-streptavidin or chromogen were used as negative controls. Positive controls were lymph nodes from cattle and buffaloes.

6.2.3 Immunohistochemistry for CD79b+ B lymphocytes

Sections from liver tissues were processed as above. Antigen retrieval consisted of boiling sections in Tris-EDTA, pH 9.0 (Appendix 1) in microwavable pressure cooker for 20 minutes each. The buffer was allowed to cool down at room temperature for 20 minutes before taking out the sections. The sections were washed with TBS-Tween 20, pH 7.4 three times, two minutes each, followed by a quick rinse

with TBS, pH 7.4 and incubated with 5% normal goat serum in PBS for 30 minutes. A mouse anti-human CD79b monoclonal antibody (mAb) (LRF Immunodiagnostics Unit, Department of Clinical Biochemistry and Cellular Science, University of Oxford, Oxford, U.K.) diluted 1:10 and 1:50 in PBS was applied to the bovine and bubaline sections, respectively and incubated at 4° C overnight. The anti-CD79b mAb reacts with intracytoplasmic part of B29 protein (Mason, van Noesel, Cordell, Comans-Bitter, Micklem, Tse, van Lier, van Dongen and Borst 1992). Serial dilutions of these antibodies (1:10, 1:50, 1:100) were initially tested to determine the dilution that minimized background staining but differentially stained target cells. The sections were then rinsed with TBS-Tween 20 three times, with two minutes each rinse, which was followed by a quick rinse with TBS. A ready-to use biotinylated goat anti-rabbit immunoglobulin G (Zymed) was applied as secondary antibody for one hour at room temperature. After three rinses in TBS-Tween 20 for two minutes each, endogenous peroxidase was quenched by incubating the sections in 3% hydrogen peroxide in PBS for 10 minutes at room temperature. Horseradish peroxidase (HRP)-streptavidin complex (Zymed) was applied to the sections after rinsing and incubated for one hour at room temperature. The reaction was developed with diaminobenzidine (DAB) (Zymed) for 15 minutes. Tissue sections were then rinsed in distilled water, counterstained with Mayer's haematoxylin for 2 minutes, dehydrated and mounted. Tissue sections in which the specific primary antibody was substituted with PBS, or which were incubated with only the HRP-streptavidin or chromogen were used as negative controls. Positive controls were lymph nodes from cattle and buffaloes.

6.2.4 Plasma cell histology

Sections of liver tissue were cut, deparaffinized and dehydrated as described in Section 6.2.2. They were stained with methyl green-pyronin (Appendix 1) for 15 minutes, blotted dry with tissue paper, rinsed quickly with acetone, cleared in xylene and mounted. Positive control tissue (plasma cell tumor) was included in each run.

6.2.5 Immunohistochemistry for CD4+ and CD8+ T lymphocytes

Sections from liver, spleen and hepatic lymph nodes were cut and processed as above. Antigen retrieval consisted of boiling sections in a microwavable pressure cooker using

either Tris-EDTA, pH 9, 10 mM citrate buffer, pH 6 or 0.01 M EDTA, pH 8 (Appendix 1) for 10 minutes, 5 minutes each cycle for three cycles, or 8 minutes, respectively. The buffer was allowed to cool down at room temperature for 20 minutes before taking out the sections. Washing of sections was done with PBS, pH 7.4. The same washing buffer was used to wash the sections between each step. Sections were incubated with 5% normal goat serum in PBS for 30 minutes. Endogenous biotin blocking was performed immediately after incubating with the normal serum using the avidin/biotin kit (Vector Laboratories, Inc., Burlingame, California, USA). The avidin solution was applied and the sections were incubated for 15 minutes at room temperature. After a brief wash with the washing buffer, the biotin solution was applied and sections were incubated for 15 minutes at room temperature. A mouse anti-bovine CD4 or anti-bovine CD8 (VMRD, Inc., Pullman, Washington, USA) was applied to the sections and incubated either overnight at 4°C, overnight at room temperature, one hour at 37°C or two hours at 37°C in a humid chamber. A number of dilutions were used (1:10, 1:20, 1:25, 1:30; 1:50; 1:100). A ready-to-use biotinylated goat anti-mouse immunoglobulin G (Zymed) was applied as secondary antibody for one or two hours at room temperature. Endogenous peroxidase was quenched by incubating the sections in 3% hydrogen peroxide in PBS for 10 minutes at room temperature. Horseradish-peroxidase (HRP-streptavidin complex (Zymed) was applied and the sections were incubated for one hour at room temperature. The reaction was developed with either 3-amino-9-ethylcarbazole (AEC) or DAB as chromogen for 15 minutes, rinsed in distilled water, counterstained with Mayer's haematoxylin for two minutes and washed in tap water. They were put into PBS until blue (approximately 30 seconds) and rinsed in distilled water. Sections in which DAB was used were dehydrated, cleared in xylene and mounted. Those with AEC were immediately mounted onto slides using GVA-mount (Zymed).

Alternatively, mouse monoclonal antibody anti-human CD4 and rabbit monoclonal antibody anti-human CD8 (Lab Vision Corporation, Westinghouse Dr. Fremont, California) were also tested as these two antibodies are indicated for formalin-fixed, paraffin-embedded tissues. Dilutions used were 1:10, 1:20, 1:50 and 1:100.

6.2.6 Counting of cells

Immunoreactive cells and positively-stained cells (plasma cells) were counted with an ocular 10X10 square grid at 400X magnification using a compound microscope (Ernst Leitz GMBH Wetzlar, Germany). For each section all stained cells that fall within the grid from each lesion site were counted.

6.2.7 Statistical analysis

The mean number of cells from each section was obtained and compared using multiple T test (SPSS version 12). Differences were compared between infected and control animals at each time point and among infected animals with time.

6.3 Results

6.3.1 CD3+ T lymphocytes

T lymphocyte numbers in the liver of cattle infected with *F. gigantica* were significantly higher than those in non-infected cattle (Fig. 6.1). The number of these cells was significantly higher at three weeks post-infection than at weeks 0, 7, 12 or 16. However, after the sharp increase at three weeks post-infection, T cell numbers continuously declined until week 16 when observation ceased (Figs. 6.1, 6.7 and 6.8). The number of T cells at week 16 was significantly lower than at 12 weeks post-infection.

In buffaloes, significantly higher T cell numbers in infected animals compared to non-infected controls were observed starting at 7 to 16 weeks post-infection (Figs. 6.2, 6.9 and 6.10). A progressive increase in T cell numbers occurred after pre-infection in infected buffaloes. T cell numbers at 7, 12 and 16 weeks post-infection were significantly higher than at week 3, and those at week 12 were significantly higher than at week 7. No significant difference in numbers between 12 and 16 weeks was observed.

6.3.2 B lymphocytes in liver

From seven to 16 weeks post-infection, infected cattle and buffaloes had significantly higher number of CD79b+ B lymphocytes in hepatic lesions than non-infected controls wherein no B cells were observed in their liver portal spaces and sinusoids

(Figures 6.3 and 6.4). The numbers of CD79b+ B cells increased as infection progressed. Figures 6.11 and 6.12 show the infiltration of CD79+ B lymphocytes in hepatic lesions in cattle and buffaloes, respectively.

6.3.3 Plasma cells

There was an increasing number of plasma cells in both infected cattle and buffaloes from week 0 to 16 post-infection (Figure 6.5 and 6.6).

6.3.4 CD4+ and CD8+ T lymphocytes

The tests for CD4 and CD8 markers on T lymphocytes failed to detect them in liver, spleen and hepatic lymph nodes of cattle and buffaloes.

6.4 Discussion

The presence of CD3+ T lymphocytes in hepatic infiltrates of cattle and buffaloes infected with *F. gigantica* reflects the involvement of T cells in the immune response of these animals during infection with *F. gigantica*. T cells play a role during infection with *F. hepatica* (Chauvin and Boulard 1996; Perez et al 1999; Martinez-Moreno et al 1999). T lymphocytes are probably involved in helping and selecting specific antibody responses, in generating cell-mediated responses and in recruiting and activating macrophages and granulocytes in bovine and bubaline *F. gigantica* infection. T lymphocytes were present in areas where eosinophils were also observed suggesting that T cells play an important role in the activation of eosinophils as reported in other diseases with chronic tissue parasitism such as *Ascaris suum* infection (Perez, Garcia, Mozos, Bautista and Carrasco 2001). The production of specific antibodies (Chapter 4) by infected animals may also be influenced by T cell activation during infection.

Cattle and buffaloes showed different patterns in their T cell responses in the liver. While there was a progressive increase in buffaloes, T cell numbers in cattle decreased after week 3. This shows that there was increasing host responsiveness to the antigenic products released by flukes and to the stimulus induced by necrotic tissue in the liver in buffaloes whereas in cattle the T cell response may be depressed to a certain extent after week 3. Although these results are preliminary, they may

indicate that cattle had a decreasing capacity to develop an immune response against *F. gigantica* while buffaloes had an increasing capacity to develop an immune response against *F. gigantica*. Proliferative responses of lymphocytes from infected sheep to concanavalin A were reduced after four weeks of infection (Zimmerman, Kerkvliet, Brauner and Cerro 1983). This present result may represent a lowered responsiveness of cattle to *F. gigantica* and a reduction in the local immune response during the growth and migratory phases of the parasite. The ability of parasites to lower host responsiveness may induce chronic disease by prolonging their existence at the site of infection (Gomez-Munoz, Canals-Caballero, Almeria, Pasquali, Zarlenga and Gasbarre 2004). *Fasciola gigantica* may have either inhibited the proliferation of T lymphocytes or suppressed the local cellular response to a certain degree in cattle to facilitate their migration through the hepatic parenchyma. The local inflammatory and immune responses in the liver of sheep and goats infected with *Fasciola hepatica* may be depressed to facilitate their migration through the hepatic parenchyma as infiltration of leucocytes was hindered (Chauvin et al 1995; Chauvin and Boulard 1996; Martinez-Moreno et al 1999). The rapid migration of *F. hepatica* in goats was thought of as a possible mechanism of immune evasion by the parasite (Martinez-Moreno et al 1999). It is possible that *F. gigantica* migrated more rapidly within the hepatic tissue in cattle than in buffaloes resulting in less immune stimulation and T lymphocyte infiltration. As flukes reached the bile ducts, immune stimulation was further reduced. The observation that eggs were already present in faeces at 16 weeks post-infection in cattle and none in buffaloes and the presence of mature flukes in the bile ducts of cattle at this period are indications that flukes in cattle migrated faster than they did in buffaloes.

The presence of B lymphocytes and plasma cells in the liver of infected cattle and buffaloes shows that a local humoral response is induced during infection with *F. gigantica* in these animals. An intense local humoral response occurred in fibrous areas of hepatic lesions and gallbladder of goats infected with *F. hepatica* as shown by increased number of CD79b+ B cells (Perez et al 1998). Plasma cells were observed in the cellular inflammatory infiltrate in the liver of goats infected with *F. hepatica* indicating a local humoral response (Martinez-Moreno et al 1999; Perez et al 1999). Sheep trickle-infected with *F. hepatica* showed increased B cells and plasma cells suggesting a strong local humoral response (Perez, Ortega, Bravo, Diez-

Banos, Morrondo, Moreno and Martinez-Moreno 2004). A local humoral immune response is of importance during infections where there is chronic tissue parasitism as in *F. hepatica* infection in sheep (Chauvin and Boulard, 1996) and goats (Perez et al 1999) and *A.* suum infection (Perez et al 2001). These B cells and plasma cells may be linked with the increased serum antibodies reacting to *F. gigantica* antigens in infected animals. A strong local humoral response in sheep (Meeusen et al 1995; Chauvin and Boulard 1996; Chauvin, Moreau and Boulard 2001) and goats (Perez et al 1998; Perez et al 1999) chronically infected with *F. hepatica* correlated with the high levels of serum IgG that was observed in sheep (Chauvin and Boulard 1996; Sanchez et al 2001) and goats (Martinez-Moreno, Martinez-Cruz, Martinez, Gutierrez and Hernandez 1996; Martinez-Moreno, Martinez, Acosta, Gutierrez, Becerra and Hernandez 1997).

Both cellular and humoral immune responses are induced during infection with *F. gigantica* in cattle and buffaloes. Cattle and buffaloes showed different patterns in their local T cell response, indicating host differences in responses to infection with *F. gigantica* between these animals, which may be related to differences between these two species in their resistance and resilience to infection with *F. gigantica*.

The failure to detect CD4+ and CD8+ lymphocytes was apparently due to the primary antibodies used not reacting with these markers in the fixed tissues. Although the VMRD antibodies are specific for bovine CD4 and CD8, these are intended for use in frozen tissues hence using it in formalin-fixed, paraffin-embedded tissues was not successful in detecting the antigens despite unmasking the epitopes using different antigen retrieval buffers. Using the LabVision's antibodies was intended to overcome this problem. However these also failed as these antibodies are indicated for human CD4 and CD8. Specific reagents to demonstrate CD4 and CD8 in fixed bovine and bubaline tissues are not commercially available at present. Only fixed tissues were available for analysis as the samples were brought from the Philippines. Quarantine regulations in Australia specify that frozen tissues are not allowed to be taken in to the country.

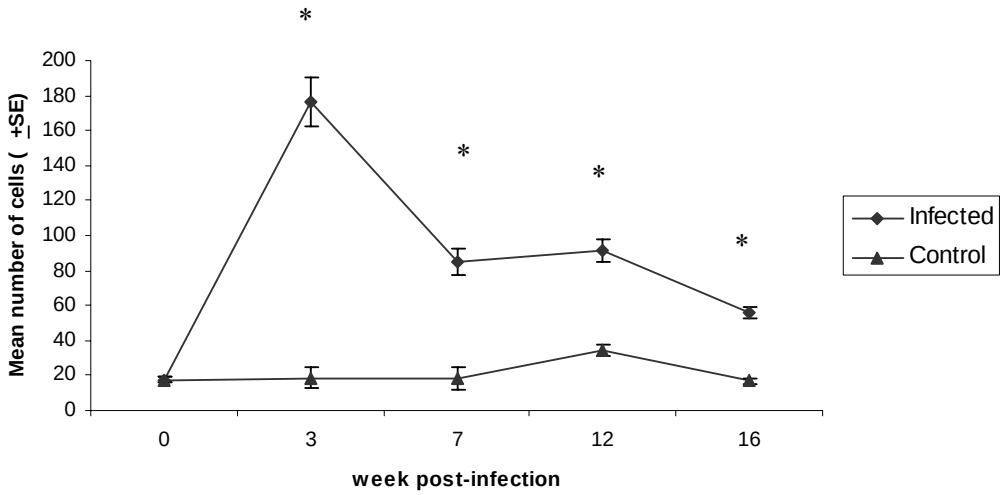

Fig. 6.1 CD3+ T lymphocyte infiltration in liver of cattle infected with *F. gigantica*. *Values were significantly higher in infected cattle than in controls ($P<0.05$). (Number of T cells of infected animals changed with time except between 7 and 12 weeks).

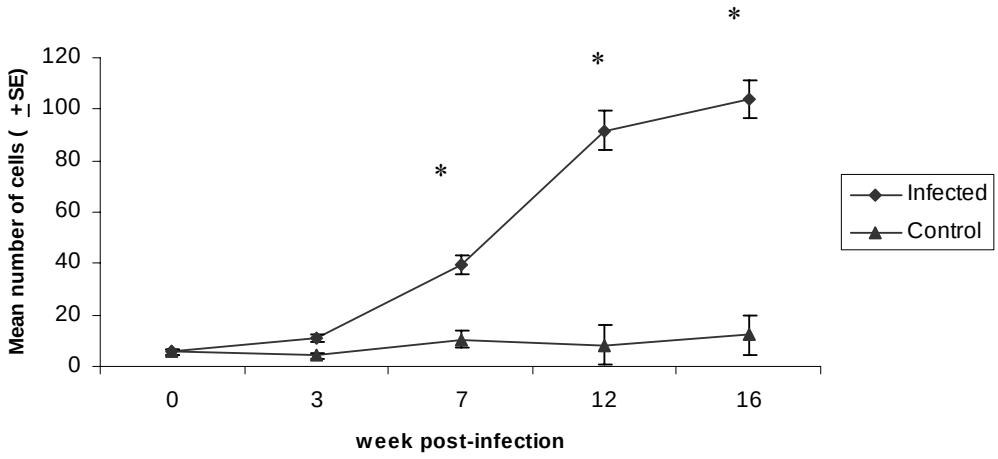

Fig. 6.2. CD3+ T lymphocyte infiltration in liver of buffaloes infected with *F. gigantica*. * Values were significantly higher in infected buffaloes than controls ($P<0.05$). (Number of T cells of infected animals changed with time except between 0 and 3 weeks and between 12 and 16 weeks).

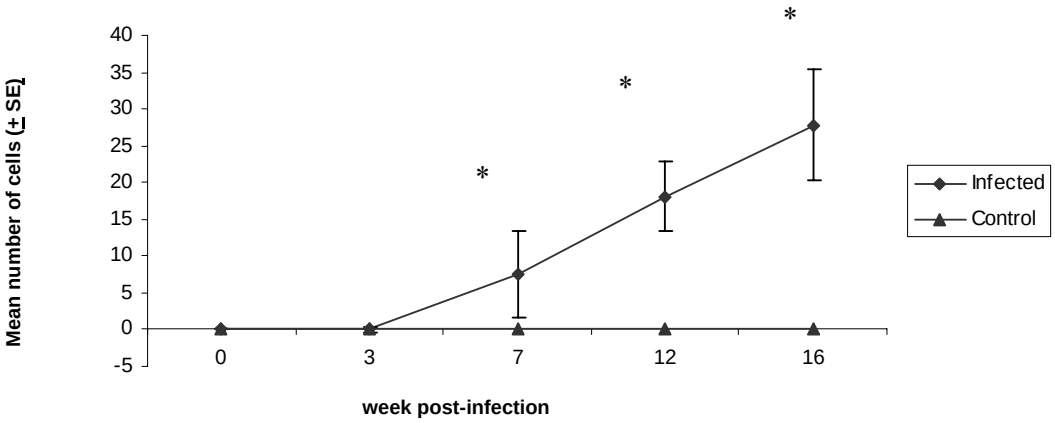

Fig. 6.3 CD79b+ B lymphocytes in liver of cattle infected with *F. gigantica*. * Values were significantly higher in infected cattle than in controls (P<0.05).

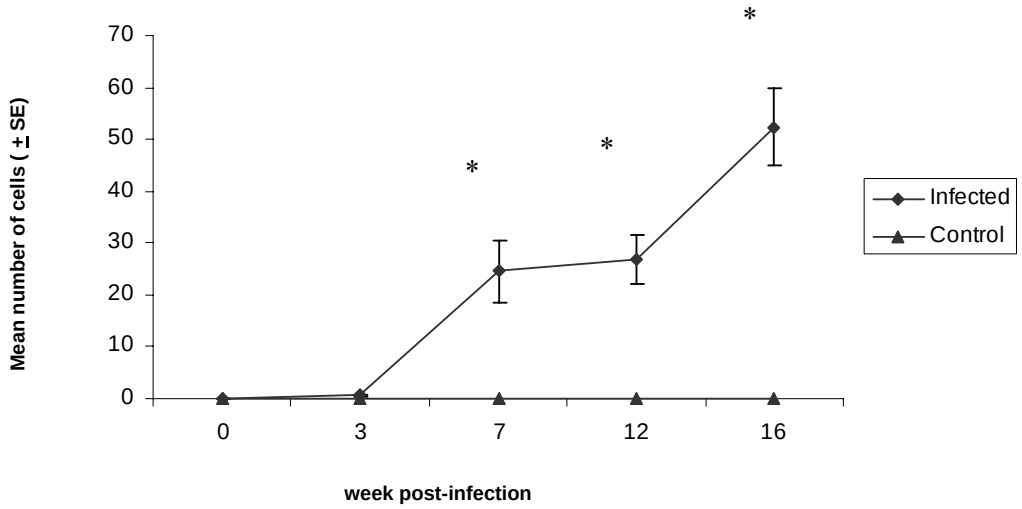

Fig. 6.4 CD79b+ B lymphocyte infiltration in liver of buffaloes infected with *F. gigantica*. * Values were significantly higher in infected buffaloes than in controls (P<0.05).

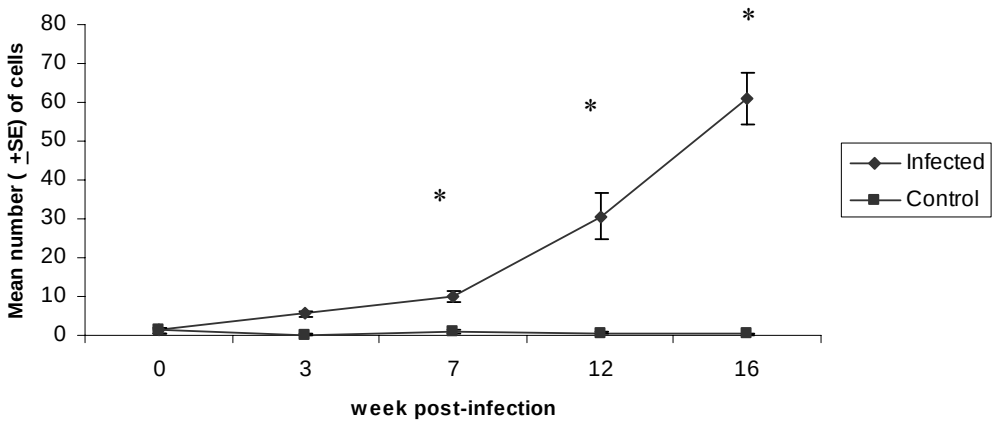

Fig. 6.5 Plasma cell counts in hepatic lesions in cattle infected with *F. gigantica*. *Values were significantly higher in infected cattle than in controls (P<0.05).

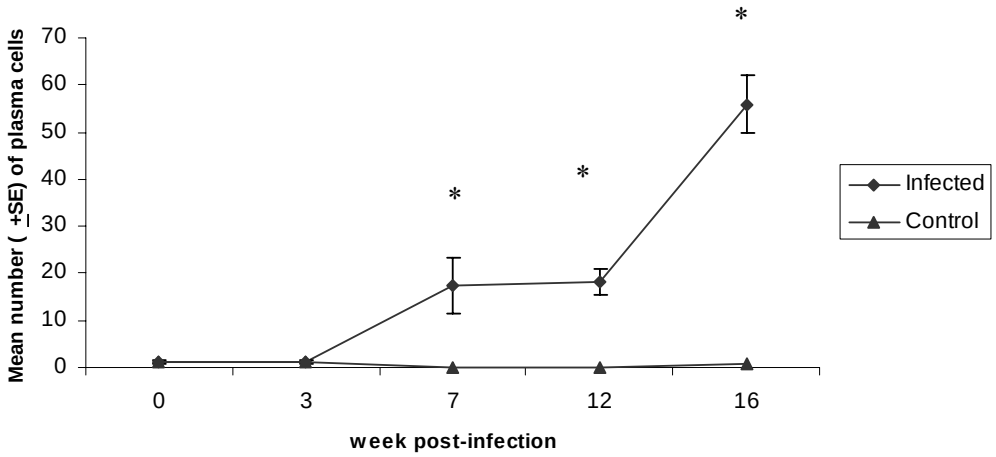

Fig. 6.6 Plasma cell counts in hepatic lesions in buffaloes infected with *F. gigantica*. * Values were significantly higher in infected buffaloes than in controls (P<0.05).

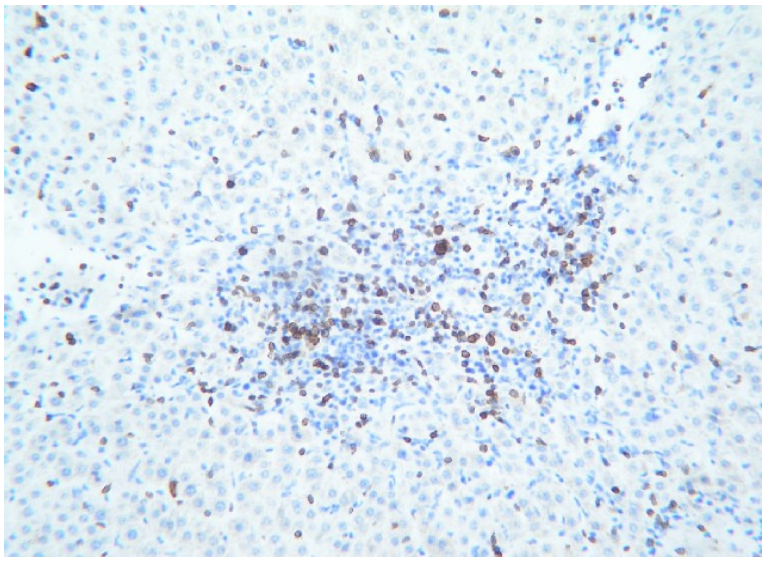

Fig. 6.7. Histological section of portal triad in the liver of cattle infected 3 weeks previously with 1000 *F. gigantica* metacercariae. Brown staining denotes CD3+ T lymphocytes (arrow). Streptavidin-biotin-peroxidase stain, x200.

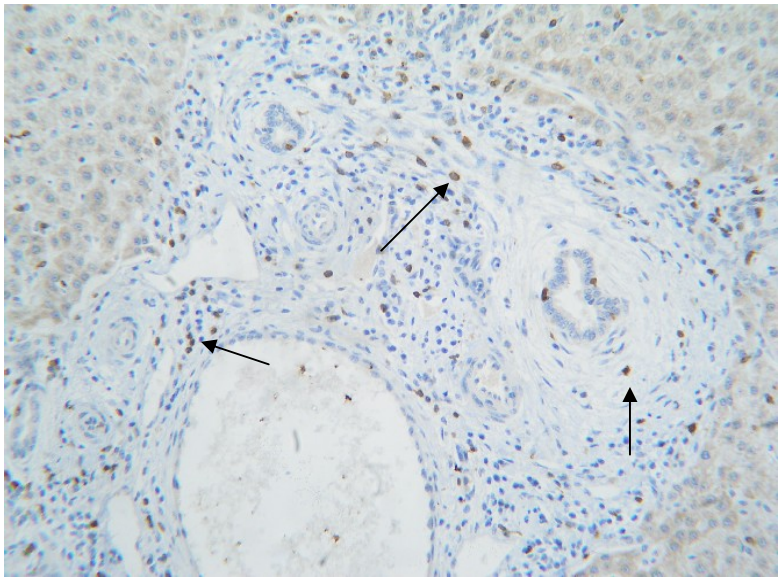

Fig. 6.8 Histological section of portal triad in the liver of cattle infected 16 weeks previously with 1000 *F. gigantica* metacercariae. Brown staining denotes CD3+ T lymphocytes (arrow). Streptavidin-biotin-peroxidase stain, x200.

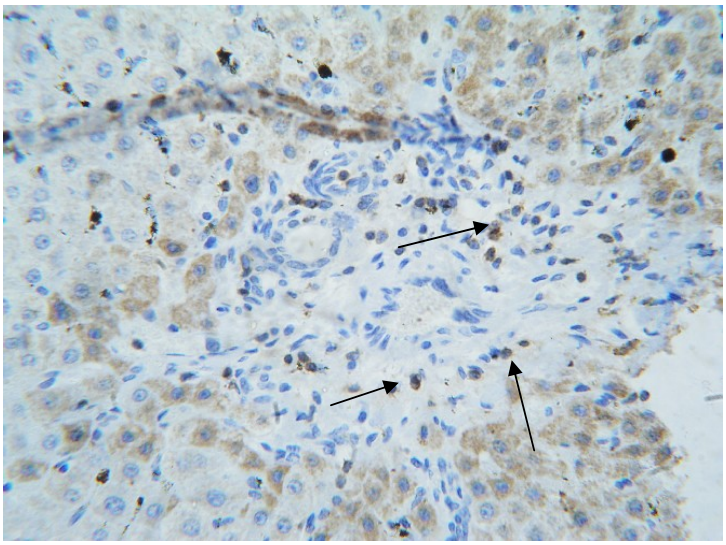

Fig. 6.9 Histological section of portal triad in the liver of buffalo infected 3 weeks previously with 1000 *F. gigantica* metacercariae. Brown staining denotes CD3+ T lymphocytes (arrow). Streptavidin-biotin-peroxidase stain, x200.

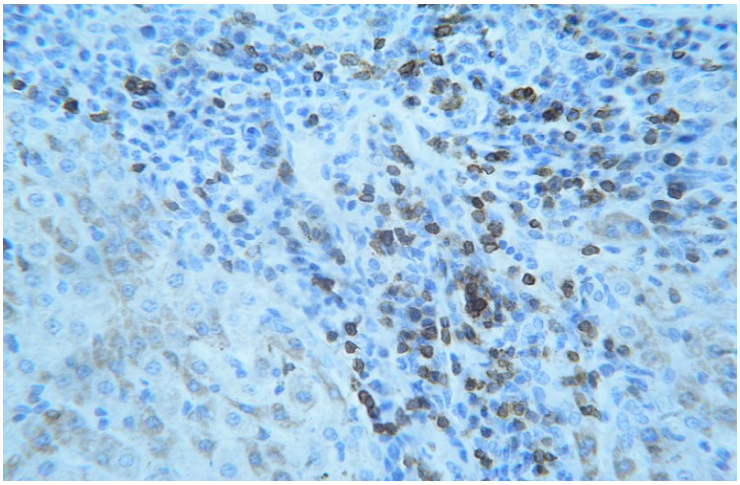

Fig. 6.10 Histological section of portal triad in the liver of buffalo infected 16 weeks previously with 1000 *F. gigantica* metacercariae. Brown staining denotes CD3+ T lymphocytes. Streptavidin-biotin-peroxidase stain, x400.

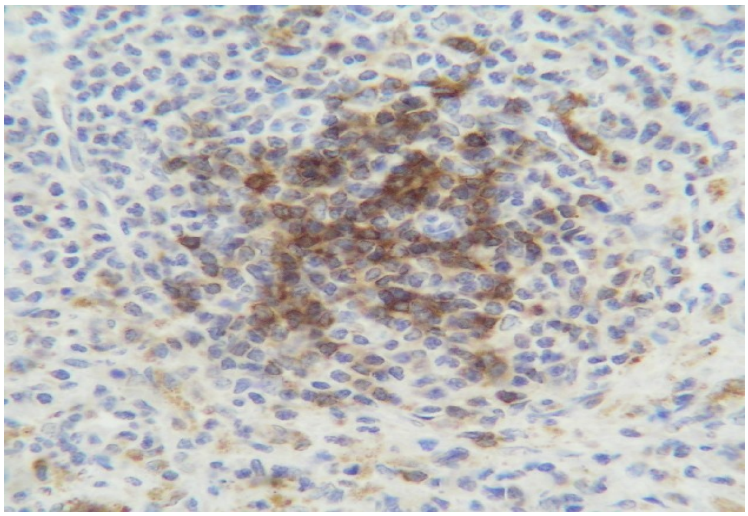

Fig. 6.11 Histological section of liver of cattle infected 16 weeks previously with 1000 *F. gigantica* metacercariae. Brown staining denotes CD79b+ B lymphocytes. Streptavidin-biotin peroxidase stain, x400.

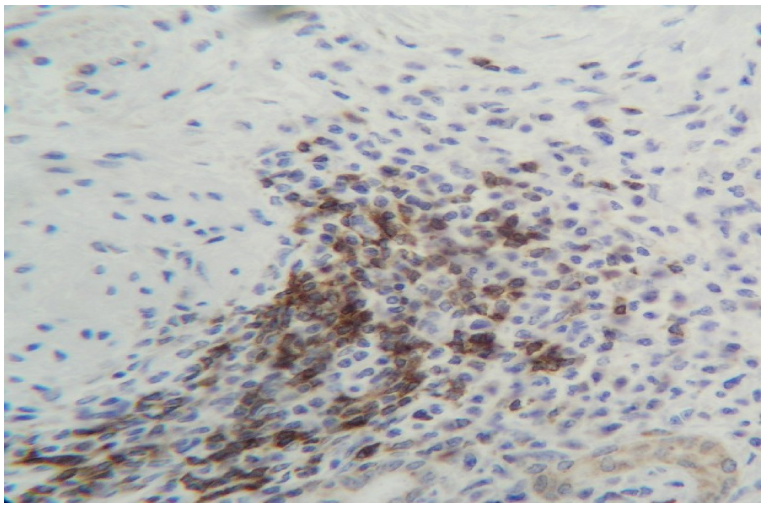

Fig. 6.12 Histological section of liver of buffalo infected 16 weeks previously with 1000 *F. gigantica* metacercariae. Brown staining denotes CD79b+ B lymphocytes. Streptavidin-biotin-peroxidase stain, x400.

CHAPTER 7
EOSINOPHILS AND MAST CELL RESPONSES OF CATTLE AND SWAMP BUFFALOES INFECTED WITH *FASCIOLA GIGANTICA*

7.1 Introduction

Increased eosinophilia is a characteristic of fasciolosis and occurs in both infections caused by *F. hepatica* and *F. gigantica* (Ross et al 1966; Haroun and Hussein 1975; Poitou et al 1992, 1993; Chauvin et al 1995; Mulcahy et al 1999a; Hansen et al 1999). Infiltration of eosinophils and mast cells also occurs in livers of infected animals (Meeusen, Lee, Rickard and Brandon 1995; Chauvin and Boulard 1996; Bossaert et al 2000b; Tliba et al 2000; Perez et al 2002). Although eosinophils are considered to be important in inducing resistance during parasitic infections (Butterworth 1984) and immature *F. hepatica* in sheep were destroyed by eosinophils (Davies and Goose 1981), there is no clear indication as to their role in bovine and bubaline fasciolosis. In this study, eosinophil responses in the blood and livers of cattle and buffaloes infected with *F. gigantica* were monitored.

7.2 Materials and Methods

7.2.1 Blood collection

Blood smears were made immediately during blood collection from the same experimental animals used in Chapter 4. Smears were stained with May-Grumwald Giemsa after air-drying.

7.2.2 Peripheral blood eosinophil count

Blood eosinophil number was obtained after counting 100 leucocytes from stained smears.

7.2.3 Histology

Sections of liver tissue were cut to 5μm thickness, mounted onto glass slides, dried and deparaffinized. A modified haematoxylin-eosin (H and E) staining was done to

demonstrate eosinophils in the sections. This involved the same steps as the standard staining procedure except that sections were immersed in H and E stains for 2 and 8 minutes, respectively. After the staining procedure, sections were dehydrated with ethanol, cleared with xylene and mounted. To determine eosinophil infiltration, the slides were examined under the high-power objective of the microscope.

Sections from the same blocks of liver tissues were also stained with toluidine blue to demonstrate mast cells. After deparaffinization, sections were immersed in toluidine blue for 15 minutes, quickly rinsed with tap water, dehydrated in ethanol, cleared in xylene and mounted. The slides were examined under the high-power objective of the microscope to determine the presence of mast cells.

7.3 Result:

7.3.1 Eosinophilia

The percentage of leucocytes in the blood which were eosinophils increased in infected cattle and buffaloes (Fig. 7.1). A clear difference between hosts was observed with the kinetics of eosinophilia in blood. A rapid eosinophilia was observed within 1-3 weeks post-infection in cattle whereas this was considerably delayed in buffaloes to weeks 6-11. In both infected cattle and buffaloes, some fluctuations in eosinophil counts occurred but still remained higher than counts in uninfected controls.

7.3.2. Eosinophils and mast cells in liver lesions

Eosinophil infiltration of hepatic lesions was observed in cattle and swamp buffaloes Mast cell degranulation was also found in these lesions.

7.4 Discussion

The increased eosinophilia seen in infected cattle and buffaloes constitutes a response of their immune systems to the presence of flukes in the hepatic tissue of infected animals. The presence of eosinophils in hepatic lesions may be considered consistent with eosinophilic hepatitis induced by the presence of flukes in the liver. It is possible that at least some of these cells are a part of the immune cell populations in the location. Although eosinophils have been incriminated in killing parasites (Butterworth

1984; Milbourne and Howell, 1990) and have been associated with protection against *F. hepatica* in the rat (Van Milligen et al 1998; Van Milligen, Cornelissen and Bokhout 1999), it is unlikely that they functioned similarly in the hepatic inflammatory response in the present experiment. Neither migrating flukes nor dead flukes with surrounding eosinophils were observed from the infected animals. The eosinophilia present may not be a protective response and may result from the inflammation induced following fluke infection. It was proposed that eosinophils were not involved with protective resistance during *F. gigantica* infection in buffaloes, Bali and Ongole cattle as buffaloes and Ongoles had similar levels of eosinophilia but their fluke burdens were significantly different (E. Wiedosari et al, personal communication). There were also reports that bovine eosinophils were ineffective in killing larval flukes *in vitro* (Duffus et al 1980). In addition, irreversible damage to *F. hepatica* larvae did not occur when incubated with sera and eosinophils from rats infected with *F. hepatica* (Doy and Hughes 1982). The role of eosinophils has not been described in *F. gigantica* infections hence it is not known if eosinophils are involved in killing of *F. gigantica*. However, Hansen et al (1999) observed that Indonesian thin-tail (ITT) sheep, which manifest resistance to *F. gigantica*, had significantly higher percentage of eosinophils than the more susceptible Merinos on days 8, 14 and 25 post-infection. In the present study there is no evidence that eosinophils are involved in protection during *F. gigantica* infection in cattle and buffaloes. The slower eosinophil response in buffaloes may be associated with the increased resistance to *F. gigantica* in these hosts, suggesting that eosinophils are not an effector cell involved in killing immature *F. gigantica* during the first 5 weeks of infection.

Intense tissue and peripheral blood eosinophilia are often induced by helminths which invade tissues or which manifest migration (Dent, Daly, Maystrofer, Zimmerman, Hallet, Bignold, Creaney and Parsons 1999; Neves, Starke-Buzetti and Castro 2003). Circulating eosinophils could have been drawn to sites of infection in the livers by mediators released from mast cells which were present in the hepatic infiltrates. During the first three weeks of infection, migratory tracts were smaller than tracts later in the infection and infiltrated with a lower density of eosinophils. As infection progressed, antigenic products released from flukes could be expected to have immunologically enhanced the extent of degranulation of mast cells. Eosinophil and mast-cell infiltrations were observed in livers of cattle infected with *F. hepatica* (Bossaert et al

2000b). Mast cells participate in eosinophil-mediated inflammatory responses when they are activated by antigen and IgE via the high-affinity receptor for IgE (Shakoory et al 2004). Furthermore, *F. gigantica*-specific IgE which was detected in sera of infected animals (Chapter 4) could also be involved in the activation of mast cells. This specific serum IgE could be produced partly by plasma cells present in the hepatic lesions. It may also be partly produced by plasma cells in hepatic lymph nodes and circulated to the liver. It may therefore be proposed that infection with *F. gigantica* in cattle and buffaloes involves the activation of eosinophil/IgE/mast cell component of the immune system.

The occurrence of eosinophilia as well as mast cells in cattle and buffaloes in this study indicates a stimulation of a Th2-type of immune response. Eosinophilia and mast cells are manifestations of a Th2 response (Pritchard *et al*., 1995; Borish and Steinke 2003). Th2 cells produce IL-4 and IL-5, cytokines essential for IgE and eosinophilia, respectively (Romagnani, Maggi, Parronchi, Macchia and Piccinni 1991; Gurish, Bryce, Tao, Kisselgof, Thornton, Miller, Friend and Oettgen 2004). Although not evaluated in this study, IL-5 may have contributed to the eosinophil response of infected cattle and buffaloes to *F. gigantica* since IL-5 is associated with eosinophils (Borish and Steinke 2003; Hirai, Miyamasu, Takaishi and Morita., 1997; Okayama, Kobayashi, Ashman, Holgate, Church and Mori 1997). IL-5 is the most important cytokine that stimulates eosinophil production and also attracts eosinophils and activates mature eosinophils, inducing eosinophil secretion and enhanced cytotoxicity (Borish and Steinke 2003). These authors also reported that mast cells and eosinophils themselves also produce IL-5. It has been confirmed that IL-5 is one of the cytokines produced by mast cells in response to IgE-dependent stimulation (Okayama et al 1997) and it is also the cytokine involved in the recruitment, activation and survival of eosinophils (Hirai et al 1997).

Milbourne and Howell (1993) showed that *F. hepatica* produces a substance that functions similarly to that of IL-5. It is possible that *F. gigantica* may also express this substance but since there was a slower eosinophil response in buffaloes this may indicate that buffalo eosinophils do not respond to this molecule. Alternatively, it is possible that *F. gigantica* does not express this molecule hence there is no immediate recruitment of eosinophils.

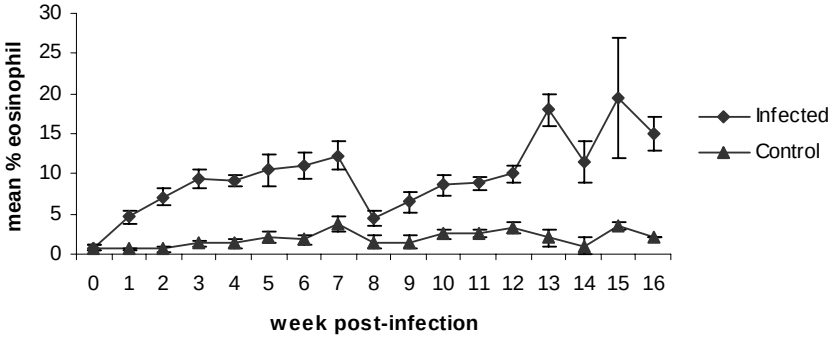

Fig. 7.1 Mean eosinophil percentage in blood of cattle infected with 1000 *F. gigantica* metacercariae. Error bars = 95% confidence interval for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

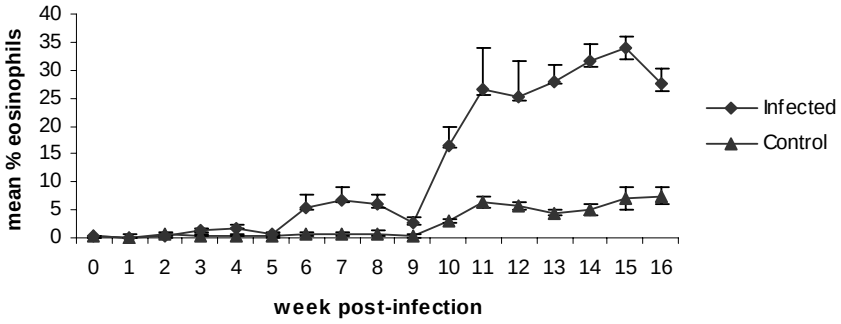

Fig. 7.2 Mean eosinophil percentage in blood of buffaloes infected with 1000 *F. gigantica* metacercariae. Error bars = 95% confidence interval for means. (n= 8 at 1-3 wpi; 6 at 4-7 wpi; 4 at 8-12 wpi; 2 at 13-16 wpi).

CHAPTER 8

HISTOPATHOLOGY OF LIVER AND HEPATIC LYMPH NODE OF CATTLE AND BUFFALOES INFECTED WITH *FASCIOLA GIGANTICA*

8.1 Introduction

It has long been recognized that fasciolosis produces severe pathological effects in the liver of infected animals (Kadhim 1976). The degree of such changes depends mainly on intensity and stage of infection and susceptibility of the host (Reid, Armour and Jennings 1970; Losos 1986). The pathology of *F. gigantica* infection in large ruminants has not been extensively studied and there has been no comparison between cattle and swamp buffaloes, particularly with regards to the extent of pathological changes at different periods of infection. Because previous trials associated with this study have shown that these animals differ in their responses to *F. gigantica*, there may also be differences in their pathology as a result of infection. Hence, this study was undertaken.

8.2 Materials and methods

Sections of liver and hepatic lymph nodes of cattle and buffaloes described in Chapter 3 were cut to 5µm thickness and processed as stated previously (Chapter 3). They were then stained with haematoxylin and eosin and observed under a compound microscope. Examination was done at 3, 7, 12 and 16 weeks post-infection.

8.3 Results

8.3.1 Cattle

At three weeks post-infection, small necrotic tracts were observed in the liver (Table 8.1). These showed leukocytic reaction consisting of eosinophils, lymphocytes and macrophages. The hepatic lymph nodes (HLN) showed follicular and parafollicular hyperplasia and the medullary areas consisted mainly of lymphocytes with few plasma cells.

Lymphocytic infiltration was present in portal tracts at week 7 of infection in liver and the hepatic lymph nodes exhibited moderate follicular and parafollicular reaction and the medullary cords were prominent with more plasma cells than in buffaloes at the same time post-infection.

A marked portal reaction with an increase in connective tissue was seen at 12 weeks post-infection. Lymphatic vessels were dilated (thin-walled) and there was bile duct hyperplasia and severe cirrhosis. There was a residual tract with extensive areas of necrosis filled with fibrinous material and eosinophils. The hepatic lymph nodes showed marked follicular and parafollicular reaction and a marked increase in plasma cells in the medullary area. Large numbers of macrophages were present in medullary sinuses. The medullary cords had only a few lymphocytes and eosinophils.

At 16 weeks post-infection, the bile duct was necrotic and the small bile ducts were fibrosed with inflammatory cells, most of which were eosinophils. There was bile duct hyperplasia with lymphocytes, macrophages and plasma cells. The liver was necrotic with haemorrhagic tracts. Follicular hyperplasia was seen in hepatic lymph nodes. There was also marked dilatation of the sinuses of the subcortical part of the lymph nodes. The medullary cords contained many plasma cells.

8.3.2 Buffaloes

No focal necrosis was seen and the liver showed very slight cellular infiltration in portal areas with lymphocytes at week 3 of infection. The HLN were less reactive than bovine HLN in follicular and parafollicular areas. The medullary areas had numerous plasma cells.

At week 7, only slight lymphocytic infiltration was seen in portal tracts and there was only a slight increase in connective tissue leading to early cirrhosis. There was moderate follicular reaction with quite well-developed mantles showing that new lymphocytes were being produced. The parafollicular reaction was quite marked. There were less plasma cells in the medulla compared with cattle.

A moderate increase in connective tissue causing moderate cirrhosis was present at 12

weeks post-infection. There was a strong cellular reaction in portal areas composed mostly of lymphocytes. This was observed to be moving into the stage of portal cirrhosis. The HLN showed moderately strong follicular reaction and quite marked parafollicular reaction. The medullary cords were prominent and sinuses contained a large number of macrophages, a few lymphocytes and eosinophils. There was an increase in the number of plasma cells.

At week 16, the bile duct lining was infiltrated with inflammatory cells composed mainly of lymphocytes. Portal areas showed increased fibrous tissue and developing cirrhosis. Early moderate cirrhosis and intense bile duct hyperplasia were present. There was a concentrated area of inflammatory cells adjacent to bile duct. There was hyperplasia of the follicular and parafollicular areas of the HLN and the medullary cords contained a large number of plasma cells and lymphocytes.

8.4 Discussion

The histopathological lesions observed in cattle and buffaloes in the present study were similar to previous observations (Ogunrinade 1983a; Losos 1986; Yadav et al 1999). The migration of flukes in the hepatic parenchyma and the presence of flukes in the bile ducts induced these pathological changes. The progression of the changes observed in the liver and hepatic lymph nodes reflect the development of flukes in these animals. Early stages of the infection were manifested by slight changes in the liver and hepatic lymph nodes. These changes gradually became pronounced as infection progressed probably due to the increased antigen load provided by the developing flukes.

Although cattle and buffaloes manifested similar lesions, the degree and onset of manifestation of some of these lesions differed. Focal necrosis was absent in buffaloes and there was only very slight cellular infiltration in their portal areas at three and seven weeks post-infection. Their hepatic lymph nodes also showed milder follicular and parafollicular hyperplasia at three weeks post-infection than in cattle. Cirrhosis was mild or moderate at 12 and 16 weeks post-infection respectively in buffaloes. The mild damage and inflammatory response in the liver and mild stimulation in the hepatic lymph nodes at some stages of infection in buffaloes and delayed onset of cirrhosis in these animals could be due to the delayed or suppressed development of flukes in

buffaloes (Chapter 3). Delayed development of flukes was evident in buffaloes since at 16 weeks post-infection, no eggs were detected in their faeces and only immature flukes were present in these animals. Also, the lower fluke burden in buffaloes compared with cattle could have partly contributed to this mild pathology. This is because the degree of liver damage and inflammatory response increases with the increasing size of the track as migratory flukes grow and with the intensity of infection (Behm and Sangster 1999). This study has given some indications of differences in host responses to infection with *F. gigantica* between cattle and swamp buffaloes, which may have important implications regarding differences in their resistance and/or resilience to the parasite.

Table 8.1. Comparison of histopathological lesions in liver and hepatic lymph nodes of cattle and buffaloes experimentally infected with a single dose of *F. gigantica*.

	Cattle				Buffaloes			
	3 wpi	7 wpi	12 wpi	16 wpi	3 wpi	7 wpi	12wpi	16wpi
LIVER								
Focal necrosis	1	1	3	3	0	0	0	2
Portal infiltration	1	1	3	3	1	1	3	3
Cirrhosis	0	1	3	3	0	1	2	2
HLN								
Follicular hyperplasia	2	2	3	3	1	2	2	3
Parafollicular hyperplasia	2	2	3	3	1	2	2	3

0 = no lesion; 1= slight; 2= moderate; 3= severe

wpi = weeks post-infection

HLN= hepatic lymph node

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

9.1 Discussion

The present study demonstrated that cattle and swamp buffaloes manifest similarities as well as differences in their immune responses during infection with *F. gigantica*. Differences in the clinico-pathological and parasitological manifestations during infection were also observed. These varying responses to *F. gigantica* infection may represent differences in host parasite relationships of *F. gigantica* infection between cattle and swamp buffaloes and may be linked to the observed varying levels of resistance and resilience to infection between these hosts.

Similarities:

Acquired immune responses commence early in cattle and buffaloes infected with *F. gigantica*. These responses are sustained as infection progresses. *Fasciola gigantica* induces immune responses in cattle and swamp buffaloes that are characteristic of Th1 and Th2 responses. A Th1-type of response was indicated by the increased IgG2 and a Th2-type of response was manifested by IgG1 and IgE responses as well as the eosinophil and mast cell responses and presence of plasma cells in hepatic lesions. IgG1 is associated with a Th2 response as it is regulated by IL-4 (Estes 1996). It was the dominant isotype in cattle, sheep and rats chronically infected with *F. hepatica* (Movsesijan et al 1975; Poitou et al 1984; Sexton et al 1994; Clery et al 1996) indicating a dominant non-Th1 response in these animals (Spithill et al 1997). IgE is linked with a Th2 response (Else et al 1994; Pritchard et al 1995; Finkelman et al 1997).

The local immune response in the liver characterised by the infiltration of T and B lymphocytes, plasma cells and eosinophils in hepatic lesions also suggest a Th2-type of immune response. An infiltration of CD4+ T cells, B cells and inflammatory cells (neutrophils, eosinophils and macrophages) in the liver (Meeusen et al 1995; Chauvin and Boulard 1996) and the systemic eosinophilia observed in sheep infected with *F. hepatica* and *F. gigantica* (Chauvin et al 1995; Roberts et al 1997a) indicate that a Th2-type of response is manifested in animals during fasciolosis (Spithill et al 1997). In addition, the presence of IL-6 in infected cattle and buffaloes in the present trial suggest

that Th2-like immune responses were induced in these animals. IL-6 is one of the cytokines produced by Th2 cells (Abbas et al 1996; Cox 2001). Along with IL-4 and IL-5, IL-6 stimulates the production of IgG1, IgE and eosinophils (Estes et al 1995) which were present in increased levels in the infected animals in this study. It is likely that IL-6 occurred with other Th2 cytokines in stimulating these responses. IL-5 may have been present in the infected animals since they exhibited increased eosinophil numbers in the blood and in hepatic lesions and eosinophils are stimulated by IL-5 (Hirai et al 1997; Borish and Steinke 2003). Moreover, it is also possible that mast cells which were seen in hepatic lesions also produced IL-5 upon IgE activation. The role of IgE-stimulated mast cells in IL-5 production was confirmed by Okayama et al (1997). The present scenario is similar with *F. hepatica* infection in which the cytokine profiles in cattle and sheep infected with *F. hepatica* are characteristic of a Th2 response (Clery et al 1996; Moreau et al 1998; Wadvogel et al 2004). Sheep infected with *F. hepatica* produced IL-10 throughout the first six weeks of infection (Moreau et al 1998). Infected cattle did not show production of IFN-γ (Clery et al 1996) while an enhanced IL-4 expression in hepatic lymph nodes and peripheral blood mononuclear cells commenced at 10 days post-infection (Waldvogel et al 2004). In the present study, interferon-γ did not occur in detectable levels in infected cattle and buffaloes when this cytokine was measured from their serum, suggesting that this cytokine is not important in the immune response against *F. gigantica* in these animals.

The relationship between a Th2-type of immune response and resistance to *F. gigantica* has not been established at present. With *F. hepatica* infection, this type of response does not confer protection as shown by previous studies which demonstrated that despite a high serological response, specific IgG1 has little apparent protective effect due to the lack of correlation between antibody titres and fluke burden (Haroun and Hillyer 1986; Keegan and Trudgett 1992). Mulcahy et al (1998) also reported a positive correlation between specific IgG1 antibody titre and fluke burden. Moreover, cattle with higher IgG1 response compared with IgG2 response were not protected against subsequent infections (Clery et al 1996). This may also apply with *F. gigantica* infection in cattle and swamp buffaloes in as much as these animals manifest Th2-like immune responses. The abattoir studies showed that these animals were still susceptible to subsequent infections as immature flukes were seen in animals that harboured adult flukes. Hence, it is suggested that the Th2-type of immune responses that cattle and buffaloes

elaborated during infection with *F. gigantica* does not confer protection against reinfection. It is likely that this response regulates infection in these animals resulting more to tolerance rather than resistance to reinfection.

Continuous stimulation by antigens released by the flukes and the damage caused by the flukes during their migration induced the accumulation of T and B lymphocytes, plasma cells and eosinophils in the hepatic lesions. It is possible that these cells are part of the immune cell populations reacting towards liver flukes in the liver. The T lymphocytes are probably involved in helping and in selecting specific antibody responses, in generating cell-mediated responses and in recruiting and activating macrophages. T cells are present in hepatic lesions in animals infected with *F. hepatica* (Chauvin and Boulard 1996; Perez et al 1999; Martinez-Moreno et al 1999). In diseases with chronic tissue parasitism such as *Ascaris suum* infection, T cells play an important role in the activation of eosinophils (Perez et al 2001) and they are also involved in the differentiation of B lymphocytes into plasma cells (Ochensberger et al 1996). The B lymphocytes and plasma cells indicate a local humoral response in the liver. Their main effector function is likely the secretion of antibodies; this is indicated by the presence of increased levels of antibodies reacting to *F. gigantica* in the infected animals. Sheep and goats chronically infected with *F. hepatica* manifest a strong local humoral response which correlated with the high levels of serum IgG (Chauvin and Boulard 1996; Martinez-Moreno et al 1996; 1997; Perez et al 1999; Sanchez et al 2001). The eosinophilia observed may not be protective since there was no indication of dead flukes with surrounding eosinophils. It is likely that it may be a result of the inflammation induced by the flukes in the liver. The accumulation of eosinophils in hepatic lesions as well as the eosinophilia may be associated with stimulation of the eosinophil/IgE/mast cell component of the immune system. Cells which express receptors for IgE such as eosinophils (Tagboto 1995) are important in protective mechanisms of mammalian hosts infected with multicellular parasites (Maizels et al 1993). Antigenic products released from flukes could be expected to have immunologically enhanced the extent of degranulation of mast cells present in the hepatic lesions resulting in the accumulation of eosinophils. Mast cells participate in eosinophil-mediated inflammatory responses when they are activated by antigen and IgE via the high-affinity receptor for IgE Shakoory et al 2004).

Differences:

The study revealed that cattle and swamp buffaloes manifest differences in their host-parasite relationships during infection with *F. gigantica* and substantial differences in susceptibility to infection exist between cattle and swamp buffaloes. Buffaloes are more resistant and resilient to *F. gigantica* than cattle. This is evident in their lower fluke burdens and faecal egg counts and in the absence of significant alterations in their RBC, PCV and haemoglobin values. The prepatent period of *F. gigantica* in swamp buffaloes is likely to be longer than in cattle as only immature flukes were present in these animals at 16 weeks post-infection. This may be due to delayed migration or suppressed development of flukes in buffaloes. Incoming immature flukes in buffaloes may have also been killed within a few weeks post-infection as immature fluke counts were lower within 3-16 weeks of infection in these animals compared with those in cattle. This is similar with the observation in Indonesian sheep infected with *F. gigantica* in which juvenile flukes were killed within 3-4 weeks of infection (Roberts et al 1997a; Piedrafita et al 2004). This observation in buffaloes suggests that a potential mechanism of resistance is operating against immature *F. gigantica* which may be responsible for the elimination of the juvenile flukes early in the infection in these hosts.

The mechanism for this apparently higher degree of resistance to *F. gigantica* in swamp buffaloes has not been described previously. It might be due to the induction of gradually increasing levels of IgG2 response as was seen in this study and has been proposed in Bali cattle infected with *F. gigantica* (E. Wiedosari et al, personal communication). Cattle and buffaloes in the present trial showed elevations in their IgG2 responses with higher levels observed in buffaloes than in cattle. A trickle infection of *F. gigantica* induced an increase in IgG2 in Bali cattle from week 4 which progressively rose after week 12. These animals also showed a progressive reduction in their egg counts and level of GLDH after week 24 (E. Wiedosari et al, personal communication). They suggested that these were indications of resistance to *F. gigantica* in Bali cattle which could be linked with their IgG2 response. There is also evidence that IgG2 is related to protection following vaccination in cattle with *F. hepatica* cathepsin L and haemoglobin as high levels of IgG2 negatively correlated with fluke burden (Dalton et al 1996; Mulcahy et al 1998). From these observations it was suggested that the immune resistance mechanism in cattle is associated with Th1-like

responses (Spithill et al 1999; Mulcahy et al 1999b). This scenario was not observed during infection with *F. hepatica* in cattle as the IgG2 response in these animals was either absent or much lower than their IgG1 response (Clery et al 1996). It is therefore probable that the stimulation of IgG2 response in cattle and swamp buffaloes infected with *F. gigantica* may result in the development of resistance to the parasite. However, at present there is no evidence to suggest that this response results in an immunological resistance to *F. gigantica* infection in cattle and buffaloes except that presented by E. Wiedosari et al (personal communication) for Bali cattle and results of this study.

The buffaloes in this study also showed a progressive increase in T cell numbers in hepatic lesions in contrast to cattle which showed reduction in their T cell numbers after week 3 of infection. This developing response in buffaloes may have partly contributed to the suppression of development of flukes or delay in fluke migration and could be related to their higher resistance to *F. gigantica* than cattle. In cattle, the reduction in their T cell numbers may possibly be associated with suppression of their immune responses induced by the flukes to hasten their migration to the bile ducts. The prepatent period of *F. gigantica* in cattle in this study was shorter than in buffaloes showing that the flukes migrated faster in cattle than in buffaloes.

In buffaloes, the slower eosinophil response, early after infection, may be associated with the increased resistance to *F. gigantica* infection. Killing of juvenile flukes in buffaloes may have occurred early in the infection as their numbers were constantly lower from 3-16 weeks post-infection than those in cattle. However, eosinophils may not be associated with this killing during the first 5 weeks of infection since eosinophilia was considerably delayed in buffaloes to weeks 6-11. Therefore these cells may not be involved in host protection but perhaps are related to host susceptibility where they act as a "blocking cell" in cattle. The absence of resistance of cattle to *F. hepatica* may be related to this mechanism wherein the parasite expresses an IL-5-like activity (Milbourne and Howell1993) to recruit eosinophils which block the cell-mediated effector arm of the immune response involving macrophages (Piedrafita et al 2004). Buffaloes infected with *F. gigantica*, having manifested a slower eosinophil response, may not respond to this substance, or *F. gigantica* does not express this molecule, hence there is no immediate recruitment of eosinophils.

The degree and onset of manifestations of histopathological lesions differed to some extent between cattle and swamp buffaloes. There was mild damage and less inflammatory response in the liver and mild stimulation in the hepatic lymph nodes at some stages of infection in buffaloes and a delay in the onset of cirrhosis in these animals. These were attributed to the lower fluke burden and delayed migration of flukes in these animals.

Higher IL-6 levels were observed in cattle than in buffaloes suggesting that this response is not associated with a protective immune response against *F. gigantica* in these animals. Increased IL-8 levels were observed in infected buffaloes but not cattle. The role that IL-8 plays during infection with *F. gigantica* is not known. In other infections IL-8 is a manifestation of an innate immune response (Reali et al 1995; Gougerot-Podicalo et al 1996; Mitchell et al 2003). The elevated levels during infection in buffaloes suggests that it is involved in the immune response against *F. gigantica* in these animals and may be associated with a higher level of innate resistance in swamp buffaloes than in cattle. Possibly, IL-8 may stimulate innate inflammatory responses in the liver causing destruction of flukes since in other infections such as *Listeria*, IL-8 is considered to have a role in focusing innate inflammatory responses to the liver resulting in the control of the infection (Cousens and Wing, 2000).

Genetics may also play a role in influencing the differences in the resistance and resilience to *F. gigantica* between cattle and swamp buffaloes as there are genetically determined differences by which animals express protective immune responses to parasite infections (Wakelin 1992; Gray and Gill 1993). The higher resistance of swamp buffaloes to *F. gigantica* than cattle may be an innate characteristic of this species.

Infection with *F. gigantica* reaches high levels in cattle and swamp buffaloes in the Philippines. Prevalence differed according to age, with prevalence higher in animals more than six years of age compared to younger animals. This may be because older animals are more exposed to infection since they are utilised more often in farming activities than younger animals. Soesetya (1975) had the same observation in Indonesia. There is an indication that older animals may be more resistant to *F. gigantica* than younger ones as adult flukes in older animals produce fewer eggs than they do in

younger ones in this study.

The observed lower carcass weight of buffaloes compared with that in cattle was considered to be not solely caused by infection under field conditions. It is considered that this more likely reflects the influence that nutrition has on the effect of infection on live weight gain. These buffaloes were naturally infected with *F. gigantica* and under field conditions their nutritional status may not be as good as in cattle since they have limited time for grazing. Cattle, meanwhile, spend most of their time grazing as they are kept mainly for breeding and meat. E. Wiedosari et al (personal communication) did not observe any significant effect on weight gain in swamp buffaloes experimentally infected with *F. gigantica*. Infection with *F. gigantica* also increases the liver weight in infected animals due to both the necrotic and calcified lesions and the resulting compensatory hypertrophy of liver parenchyma.

9.2 Major Observations, Conclusions, Implications and Future Investigations

Investigations of the host parasite interactions during infection with *F. gigantica* in cattle and swamp buffaloes demonstrated that these animals manifest similarities and differences in their responses during infection with *F. gigantica*. Infection with *F. gigantica* in these species induces immune responses characteristic of a Th-2 type of response as well as a Th-1 type of response. Cattle and swamp buffaloes show substantial differences in their host-parasite interactions with *F. gigantica* infection which are indicated by differences in the susceptibility and resistance to infection with *F. gigantica* between these species. Although cattle and swamp buffaloes are both susceptible to infection, swamp buffaloes have a higher degree of resilience and resistance to infection with *F. gigantica* than cattle. These differences may be due to varying manifestations of an innate immune response and their acquired immune responses during infection. Also, the higher resistance and resilience of swamp buffaloes could be an innate characteristic of the species that is genetically determined.

The results of this study may be useful as basis to control *F. gigantica* infection particularly in cattle. For instance, cattle may be induced by vaccination to develop higher IgG2 responses to enhance their resistance against *F. gigantica*. Cytokine therapy incorporating IL-8 may also be a possible strategy as control against the

infection. However, before any form of control could be established against tropical fasciolosis in cattle and swamp buffaloes, an understanding of the host-parasite relationships would be essential. It is hoped that this study has contributed to that knowledge.

A deeper understanding of the pathogenesis of infection with *F. gigantica* is needed to fully elucidate the mechanism of resistance against *F. gigantica* in these animals. Therefore, further studies and more detailed characterization of the T cell responses of cattle and swamp buffaloes infected with *F. gigantica* should be undertaken to really point out whether resistance is due to an innate immune response or to a specific immune response. Studies that would investigate strategies to boost the immune responses of animals to enhance their resistance may also be conducted to further indicate possibilities for control of the infection.

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APPENDICES

Appendix 1

Reagents/Buffers Used in Immunohistochemistry

50 mM TBS:
6.06 g Tris base
8.76 g NaCl
Adjust pH to 7.5 with HCl
Add distilled water up to 1000 mL

1X PBS:
8 g NaCl
1.15 g Na2HPO4
0.2 g KCl
0.2 g KH2PO4
qs to 1 liter
Adjust pH to 7.4 with HCL

0.01M EDTA, pH 8.0:
0.37 g EDTA
1 liter water
Adjust pH to 8.0

10X TBS (0.5M Tris base, 9% NaCl, pH 7.6)
61 g Tris base
90 g NaCl
1 liter distilled water
Mix to dissolve and adjust pH to 7.6 using concentrated HCl. Store at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary.

10 mM citrate buffer
1.21 g citric acid
1 liter distilled water
Adjust pH to 6.0

10X TBS-Tween 20 (0.5M Tris base, 9% NaCl, 0.5% Tween 20, pH 7.6)
61 g Trizma base
90g NaCl
1 liter distilled water
Mix to dissolve and adjust pH to 7.6 using concentrated HCl, then add 5 mL Tween 20. Store at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary.

Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0)

1.21 g Tris base
0.37 g EDTA
1 liter distilled water
Mix to dissolve. pH is usually at 9.0 and then add 0.5 mL of Tween 20 and mix well.

Methyl Green-Pyronin Solution

2 % aqueous Pyronin Y 1.5 mL
2% aqueous Methyl Green 3.0 mL
Tap water to 50 mL

Appendix 2

Reagents used in ELISA for antibody isotypes

10 X Phosphate buffered saline (PBS) pH 7.2

NaCl	80 g
KCl	2 g
$Na_2 HPO_4$	6.40 g
Distilled water	up to 1000 mL

1 X PBS was made with 100 mL of stock solution and 900 mL of distilled water added

Worm solubilization buffer

- 0.1M Tris {Tris (Hydroxymethyl) aminomethane} (Sigma Chemicals) pH 7.2
- 0.14 M NaCl (Ajax Chemicals, Sydney, Australia)
- 1% Sodium deoxycholate (BDH Chemicals Ltd, Boronia, Australia)
- 1 mM PMSF (Phenylmethylsulphonyl fluoride) (Sigma Chemicals) in isopropanol
- 0.5% (w/v) NaN_3 (Sodium Azide) (Sigma Chemicals).

Appendix 3

Reagents used in cytokine ELISA

3.1 Blocking buffer (PBS/0.1% Na casein)
0.1 g Na casein
100 mL 1x PBS

3.2 Diluting buffer (PBS/0.5% Tween 20/1% Na casein)
0.5 mL Tween 20
100 mL 1x PBS
1 g Na casein

3.3 TMB substrate stock
0.2 M sodium acetate (13.6 g/500 mL H_2O)
0.6 M citric acid (12.6 g/100 mL H_2O)
0.1mL TMB/10 mL DMSO. Store in the dark at room temperature.
30% H_2O_2 (100 vols.)
0.5 M H_2SO_4 (5.4 mL/200 mL H_2O)
This substrate stock was used as the TMB substrate. Per plate used the following:
5 mL Na acetate
5 mL regular H_2O
150 μL citric acid
1.2 μL 30% H_2O_2
100 μL TMB

Appendix 4

Data on mean fluke counts and faecal egg counts
in cattle and buffaloes (abattoir study)

Species	Mature *F. gigantica*	SD	Immature *F. gigantica*	SD	Faecal egg count	SD
Cattle (n=83)	25.6	73.6	30	77.8	31.7	62.6
Buffaloes (n=15)	13.8	19.9	15.9	19.1	17.57	10.7

Appendix 5
Data on mean haematological values in
cattle and buffaloes

Species	Status	RBC (x 10^6µ/L)	SD	PCV (%)	SD	Haemoglobin (g/dL)	SD
Cattle	Infected (n=50)	4.13	1.85	30.00	8.5	9.58	2.06
	Non-infected (n=11)	6.09	1.01	31.00	5.5	9.15	1.94
Buffaloes	Infected (n=134)	5.16	1.20	29.47	4.4	9.95	1.67
	Non-infected (n=41)	4.75	0.54	29.44	3.71	10.01	1.77

Appendix 6
Data on mean haematological values in cattle and buffaloes according to fluke burdens

Species	Fluke #	RBC (x 10^6µ/L)	SD	PCV (%)	SD	Haemoglobin (g/dL)	SD
Cattle	0 (n=11)	6.09	1.01	30.64	5.5	9.15	1.29
	1-20 (n=11)	5.5	1.38	29.91	5.5	9.80	2.35
	21-70 (n=17)	4.63	1.82	33.82	7.89	9.95	2.09
	>70 (n=22)	3.05	1.46	26.55	9.1	9.18	1.91
Buffaloes	0 (n=41)	4.74	0.54	29.4	3.7	10.1	1.8
	1-20 (n=87)	5.2	1.20	29.3	3.9	10.1	1.2
	21-70 (n=35)	4.8	1.10	28.8	5.3	9.4	2.6
	>70 (n=12)	5.8	0.87	32.8	3.9	10.3	1.1

Appendix 7

Data on ELISA for antibody isotypes in cattle and buffaloes

7.1 Mean IgG1 reacting to *F. gigantica* in cattle

Week post-infection	Infected	Control
1	0.056	0.032
2	0.213	0.036
3	0.211	0.036
4	0.209	0.046
5	0.265	0.029
6	0.193	0.031
7	0.148	0.025
8	0.245	0.028
9	0.208	0.039
10	0.288	0.025
11	0.175	0.028
12	0.284	0.024
13	0.902	0.024
14	0.785	0.024
15	0.604	0.025
16	0.890	0.025

7.2 Mean IgG1 reacting to *F. gigantica* in buffaloes

Week post-infection	Infected	Control
1	1.343	0.107
2	1.096	0.131
3	0.611	0.142
4	1.114	0.230
5	1.070	0.133
6	1.044	0.290
7	1.594	0.257
8	1.978	0.175
9	1.833	0.171
10	1.464	0.257
11	1.849	0.193
12	1.799	0.142
13	1.913	0.271
14	2.011	0.317
15	2.460	0.548
16	2.290	0.257

7.3 Mean IgG2 reacting to *F. gigantica* in cattle

Week post-infection	Infected	Control
1	0.181	0.047
2	0.128	0.049
3	0.201	0.063
4	0.197	0.054
5	0.177	0.049
6	0.120	0.054
7	0.107	0.031
8	0.166	0.035
9	0.121	0.043
10	0.129	0.043
11	0.174	0.032
12	0.133	0.033
13	0.212	0.023
14	0.196	0.022
15	0.192	0.022
16	0.406	0.025

7.4 Mean IgG2 reacting to *F. gigantica* in buffaloes

Week post-infection	Infected	Control
1	0.710	0.140
2	0.550	0.136
3	0.535	0.169
4	0.827	0.179
5	0.835	0.178
6	0.817	0.176
7	1.070	0.175
8	1.272	0.293
9	1.163	0.240
10	1.037	0.279
11	1.070	0.282
12	1.297	0.270
13	1.305	0.209
14	1.360	0.263
15	1.871	0.286
16	1.508	0.254

7.5 Mean IgE reacting to *F. gigantica* in cattle

Week post-infection	Infected	Control
1	0.236	0.145
2	0.244	0.140
3	0.247	0.138
4	0.248	0.140
5	0.265	0.141
6	0.277	0.151
7	0.250	0.167
8	0.245	0.144
9	0.258	0.142
10	0.243	0.141
11	0.249	0.133
12	0.250	0.130
13	0.248	0.134
14	0.267	0.132
15	0.287	0.140
16	0.261	0.138

7.6 Mean IgE reacting to *F. gigantica* in buffaloes

Week post-infection	Infected	Control
1	0.197	0.157
2	0.210	0.155
3	0.254	0.153
4	0.237	0.150
5	0.268	0.150
6	0.242	0.150
7	0.247	0.144
8	0.280	0.143
9	0.291	0.141
10	0.330	0.148
11	0.277	0.145
12	0.275	0.144
13	0.193	0.142
14	0.220	0.143
15	0.228	0.142
16	0.249	0.143

Appendix 8

Data of IFN-γ Assay

8.1 BUFFALOES

Positive Control Absorbance: 2.266

Negative Control Absorbance: 0.033

Week post-infection	Mean absorbance (infected)	Mean absorbance (control)
1	0.027	0.036
2	0.045	0.058
3	0.049	0.051
4	0.035	0.025
5	0.029	0.030
6	0.036	0.029
7	0.029	0.029
8	0.034	0.032
9	0.018	0.030
10	0.021	0.032
11	0.021	0.021
12	0.019	0.049
13	0.024	0.057
14	0.029	0.045
15	0.031	0.034
16	0.037	0.032

8.2 CATTLE

Positive Control OD: 1.982

Negative Control OD: 0.028

Week post-infection	Infected OD	Control OD
1	0.025	0.027
2	0.024	0.034
3	0.024	0.028
4	0.025	0.029
5	0.030	0.030
6	0.038	0.036
7	0.030	0.015
8	0.040	0.020
9	0.032	0.024
10	0.028	0.023
11	0.028	0.023
12	0.030	0.024
13	0.030	0.026
14	0.025	0.024
15	0.025	0.028
16	0.025	0.026

Appendix 9

Data on ELISA for IL-6 and IL-8 in cattle and buffaloes

9.1 Mean IL-6 concentration (pg/mL) in serum taken from cattle

Week post-infection	Infected	Control
1	76	-90
2	67	-91
3	38	-106
4	30	-109
5	76	-126
6	118	-96
7	106	-164
8	126	-55
9	105	-80
10	112	-70
11	116	-182
12	102	-177
13	103	-167
14	113	-167
15	111	-148
16	98	-235

9.3 Mean IL-6 concentration (pg/mL) in serum taken from buffaloes

Week post-infection	Infected	Control
1	17	-13
2	13	-91
3	12	-65
4	11	-80
5	18	-59
6	61	15
7	57	12
8	69	17
9	59	-3
10	64	2
11	68	0.2
12	51	0.8
13	51	-16
14	61	-25
15	36	-6
16	43	-17

9.4 Mean IL-8 concentration (pg/mL) in serum taken from cattle

Week post-infection	Infected	Control
1	55	204
2	73	175
3	55	138
4	41	143
5	71	159
6	66	177
7	22	137
8	66	123
9	31	129
10	63	153
11	44	141
12	54	146
13	62	146
14	45	152
15	34	152
16	8	146

9.5 Mean IL-8 concentration (pg/mL) in serum taken from buffaloes

Week post-infection	Infected	Control
1	493	218
2	538	250
3	495	202
4	542	242
5	590	235
6	579	214
7	536	268
8	504	275
9	461	250
10	511	252
11	361	252
12	435	264
13	435	209
14	496	224
15	373	176
16	311	205

Appendix 10

Summary of Counts for CD3+ T Lymphocytes in Liver of Cattle and Buffaloes

10.1 CD3+ T cell counts in Cattle

Week post-infection	Status	Mean Number	Standard Error of Mean
0		16.2	1.6
3	Infected	162.3	14.0
7	Infected	76.9	7.8
12	Infected	84.6	6.8
16	Infected	52.3	3.4
3	Control	10.7	1.6
7	Control	16.6	2.0
12	Control	16.6	1.6
16	Control	30.7	4.0

10.2 CD3+ T cell counts in Buffaloes

Week post-infection	Status	Mean number	Standard Error of Mean
0		4.4	1.1
3	Infected	9.7	1.4
7	Infected	35.7	3.7
12	Infected	84.4	7.4
16	Infected	96.8	7.3
3	Control	3.4	0.9
7	Control	8.0	2.6
12	Control	7.2	0.9
16	Control	10.9	1.2

Appendix 11

Summary of Counts for Plasma Cells in Liver of Cattle and Buffaloes

11.1 Plasma Cell Counts in Cattle

Week post-infection	Status	Mean number	Standard Error of Mean
0		1.3	0.8
3	Infected	5.5	0.9
7	Infected	9.8	1.4
12	Infected	30.7	5.9
16	Infected	61.0	6.8
3	Control	0.1	0.1
7	Control	1.0	0.4
12	Control	0.7	0.2
16	Control	0.4	0.2

11.2 Plasma Cell Counts in Buffaloes

Week post-infection	Status	Mean number	Standard Error of Mean
0		1.1	0.4
3	Infected	1.1	0.5
7	Infected	17.5	5.9
12	Infected	18.2	2.8
16	Infected	56.0	6.3
3	Control	1.2	0.5
7	Control	0	0
12	Control	0.1	0.1
16	Control	0.6	0.4

Appendix 12

Summary of Counts for CD79+ B Lymphocytes in
Liver of Cattle and Buffaloes

12.1 B cell counts in Cattle

Week post-infection	Status	Mean	Standard Error of Mean
3	Infected	0	0
7	Infected	7.5	2.8
12	Infected	18.1	2.2
16	Infected	27.8	3.9
3	Control	0	
7	Control	0	
12	Control	0	
16	Control	0	

12.2 B cell counts in Buffaloes

Week post-infection	Status	Mean	Standard Error of Mean
3	Infected	0.6	0.3
7	Infected	24.5	5.9
12	Infected	26.9	4.8
16	Infected	52.4	7.6
3	Control	0	
7	Control	0	
12	Control	0	
16	Control	0	

Appendix 13
Percentage of eosinophils in blood of
cattle and swamp buffaloes

13.1 Cattle

Week post-infection	Infected	Control
1	4.6	0.6
2	7.1	0.6
3	9.4	1.3
4	9.2	1.3
5	10.5	2.2
6	11.0	1.8
7	12.3	3.7
8	4.5	1.5
9	6.5	1.5
10	8.6	2.5
11	8.6	2.5
12	10.0	3.3
13	18.0	2.0
14	11.5	1.0
15	19.5	3.5
16	15.0	2.0

13.2 Buffaloes

Week post-infection	Infected	Control
1	0.1	0
2	0.4	0.7
3	1.3	0.5
4	1.8	0.5
5	0.8	0.2
6	5.3	0.7
7	6.8	0.6
8	6.0	0.8
9	2.8	0.3
10	16.5	3.0
11	26.5	6.3
12	25.3	5.8
13	28.0	4.5
14	31.5	5.0
15	34.0	7.0
16	27.6	7.5