

The interaction between *Fasciola hepatica* and other pathogens naturally co-infecting dairy and beef cattle in the UK

Alison Katherine Howell

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Abstract

Liver flukes are helminth parasites of ruminants which cause economic losses and adverse effects on animal welfare. The common liver fluke *Fasciola hepatica* is prevalent in temperate regions, including the UK where up to 80% of dairy herds are exposed to the parasite, whilst the tropical liver fluke, *F. gigantica*, is found in tropical climates. Both flukes require an intermediate snail host to complete their life cycle, and this determines where infection occurs. Liver fluke is challenging to control, especially in dairy cattle.

Chronic liver fluke infection moderates the host immune system towards a non-protective T helper cell type 2 (Th2) / regulatory T cell (Treg) response, characterised by IgG, IL4 and IL10, which suppress T helper cell type 1 (Th1) cytokines such as interferon (IFN γ). Previous studies have shown that this may affect the pathogenesis and diagnosis of other diseases, particularly bovine tuberculosis (bTB). However, most studies have been performed in experimentally infected animals under laboratory conditions, and the importance of the findings have not been verified in naturally infected cattle. The aims of this thesis were to investigate the effects of fluke infection on two mycobacterial diseases, bTB and Johne's disease, and on the food poisoning bacterium *Escherichia coli* O157.

Chapter 2 describes the dynamics of *F. hepatica* exposure in UK herds as measured by antibody detection ELISA. Individual results from 5937 cattle from 30 herds and 24 bulk milk tank results are used. The distributions of the antibody percent positivity (PP) values were right-skewed for all herds. The bulk milk result correlated with individual results. A significant effect of season was seen, but age was not significantly associated with antibody levels, both of which are in agreement with other recent studies.

Chapter 3 contains the results of cross sectional and case-control studies looking at the association between liver fluke exposure with the bTB skin test. A comparison of IgG isotype ratios between fluke positive cattle testing positive and negative for bTB is also included. No significant effect was seen, but these studies were underpowered due to difficulties in obtaining samples. Overall there was a trend that fluke antibodies were associated with a decrease in the odds ratio (OR) of a positive bTB skin test.

Chapter 4 is a systematic review of the literature on co-infection with liver fluke and tuberculosis. We extracted data on the association between fluke infection and the bTB skin test, interferon gamma test, lesion detection and culture/bacterial recovery. Evidence from nine studies included in the review points to liver fluke infection having the effect of

decreasing all of the four measures of bTB diagnosis, but most studies showed a small and/or non-significant effect, and there was a high risk of bias across all studies.

In Chapter 5, the hypothesis that there is an association between *F. hepatica* and *Mycobacterium paratuberculosis* subsp. *avium* (MAP, which causes Johne's disease) was tested. The spatial distribution of MAP was examined using MAP antibody results from 885606 cows from 1245 herds, but no spatial pattern was seen. 3766 samples from 17 herds were tested for MAP antibody and *F. hepatica* antibody. Subsequently six farms were followed longitudinally for 1 year and up to four samples for each animal were obtained. No association between the two pathogens was found using any of these approaches.

Chapter 6 describes a study on co-infection between fluke and *E. coli* O157 in finishing cattle. A significant association between the log PP of the *F. hepatica* copro-antigen ELISA and *E. coli* O157 shedding was found when the fixed effects of day of sampling and the age of the youngest animal in the group, and the random effect of farm were adjusted for, although the result should be interpreted cautiously due to the many study limitations, particularly a very low level of fluke infection. The effect of this association was that a change from the 25th quartile of *F. hepatica* PP to the 75th quartile corresponded with a 6.7% increased OR of *E. coli* O157 shedding ($p = 0.01$).

Overall, these findings suggest that fluke infection may have an effect on bTB and *E. coli* O157 in naturally exposed animals, although conclusive evidence was lacking. Subtle effects may be obscured in field studies due to the large amount of natural variation between animals, and many unknown factors may introduce bias. Concentrating research on particular subgroups of animals that may be disproportionately at risk of adverse effects of co-infection, and evaluating immune profiles alongside diagnostic measures, may help to provide more certain evidence.

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List of abbreviations

ADCC	-	Antibody dependent cellular cytotoxicity
A/E	-	Attaching and effacing
AIC	-	Akaike Information Criterion
BCG	-	Bacillus Calmette–Guérin
BLV	-	Bovine leukaemia virus
BMT	-	Bulk milk tank
bTB	-	Bovine tuberculosis
BVD	-	Bovine viral diarrhoea
CD	-	Cluster of differentiation
cfu	-	Colony forming units
CI	-	Confidence interval
DEFRA	-	Department for the Environment, Food and Rural Affairs
ELISA	-	Enzyme linked immunosorbent assay
ERU	-	Epidemiology Research Unit (SAC Consulting Veterinary Services Disease Surveillance Centre, Inverness)
E/S	-	<i>Fasciola hepatica</i> excretory/secretory products
FEC	-	Faecal egg count
Fox P3	-	Forkhead box P3 molecule
FSA	-	Food Standards Agency
GLM	-	Generalised linear model
HRP	-	Horse radish peroxidase
IBR	-	Infectious bovine rhinotracheitis
IFN	-	Interferon
Ig	-	Immunoglobulin
IL	-	Interleukin
IMS	-	Immunomagnetic separation
IR	-	Inconclusive reactor to the bTB skin test
MAP	-	<i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i>
MPBST	-	PBST containing 2% skimmed milk powder (Marvel)
OD	-	Optical density
OR	-	Odds ratio
PAMP	-	Pathogen associated molecular pattern
PBMC	-	Peripheral blood mononuclear cells
PBS	-	Phosphate buffered saline
PBST	-	Phosphate buffered saline containing 0.05% Tween-20
PCR	-	Polymerase chain reaction
PP	-	Percent positivity in fluke antibody detection ELISA
PPDa	-	Purified protein derivative (avian)
PPDb	-	Purified protein derivative (bovine)
SICCT	-	Single intradermal comparative cervical tuberculin test

S/P	-	Sample to positive ratio (MAP antibody ELISA)
SRUC	-	Scotland's Rural College
TCR	-	T cell receptor
TGF	-	Transforming growth factor
Th	-	T helper cell
TGF	-	Transforming growth factor
TNF	-	Tumour necrosis factor
Treg	-	Regulatory T cell
TTL	-	To the laboratory
TTSS	-	Type three secretion system (<i>E. coli</i> O157)
WC	-	Workshop cluster

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Chapter 1: Introduction

1.1 General introduction

Animals have co-evolved with parasites, bacteria and viruses over millennia. These interactions are complex, with the effects on the host depending on many different intrinsic and extrinsic factors such as genetics, nutrition, age, reproductive state and environmental conditions. Parasites, bacterial and viral micro-organisms can alter host immunity and even host behaviour to increase their own chances of survival.

In farmed animals, intensive conditions, lack of genetic variation, large numbers of animals being kept closely confined together and the stresses of production can increase susceptibility to infectious disease. Up until recently, this problem has been dealt with by the use of antimicrobial and parasitic drug treatments combined with vaccines and mandatory control programmes for certain diseases. The growing world demand for food combined with increasing resistance to antimicrobial and antiparasitic treatments make it important to find new ways of reducing the burden of parasitic and infectious disease in livestock.

A significant proportion of animals that are infected with one pathogen are also co-infected with others, whether these are helminth parasites, viruses, bacteria or protozoa. These pathogens can have effects on each other, either directly or through the host's immune system, and can alter the transmission of disease, disease progression, clinical presentation and response to diagnostic tests (Cox, 2001; Griffiths et al., 2011). In a recent review, co-infection was found to be detrimental to the host and was associated with an increased intensity of infection in most cases (Griffiths et al., 2011). Understanding the effects of parasite infestation on other co-infecting organisms should help to develop targeted control programmes that are more effective and better for overall health than the current approach where each infectious agent is treated in isolation (Griffiths et al., 2011).

Studies on co-infections can be performed under laboratory conditions with standardised timings and doses of infectious agents, to minimise the risk of unknown biases interfering with results. However, there is the risk that the chosen conditions will not be the same as those that wild or farmed animals face. Under natural conditions, the order in which animals are exposed to each pathogen and the length of time between exposures are unknown. Repeated exposure to the same pathogens in varying doses and possibly via

different routes is likely to occur, and may affect the observed outcome. Consequently, study of naturally exposed populations is necessary to confirm laboratory observations, even though much greater variation occurs in naturally exposed populations, making subtle effects more difficult to recognise.

Recently there has been interest in co-infections with the liver fluke *Fasciola hepatica*, because evidence from studies of experimentally infected animals have suggested that fluke infection reduces the sensitivity of the skin test used in cattle to detect bovine tuberculosis (Claridge et al., 2012; Flynn et al., 2007), and may also have an effect on the pathogenesis of the disease (Garza-Cuartero et al., 2016).

In this thesis, the epidemiological association is examined between liver fluke and *Mycobacterium bovis*, and between liver fluke and two other infectious agents, *Mycobacterium paratuberculosis* subsp. *avium*, and *Escherichia coli* O157, in naturally exposed cattle.

1.2 The liver fluke *Fasciola hepatica*

1.2.1 Life cycle

Fasciola hepatica, also known as the common liver fluke, is a trematode parasite. Adult liver flukes reside in the bile ducts and gall bladder of the definitive host, usually a ruminant although other species, including man, can also become infected. Fluke eggs are produced following self- or cross-fertilisation and are passed out onto pasture. Following embryonation, each egg produces a single miracidium which must find its way into the body of the intermediate snail host, *Galba truncatula*, within 24 hours (Graczyk and Fried, 1999). Asexual replication occurs within the snail, giving rise to redia which become cercariae and are shed from the snail (Graczyk and Fried, 1999). These then encyst as metacercariae on plants, and may remain infectious for several months, depending on conditions (Andrews, 1999). The definitive host becomes infected by eating infected grass or other plants. Metacercariae excyst in the small intestine and migrate to the liver parenchyma where they take 6-8 weeks to mature (Andrews, 1999).

1.2.2 Epidemiology of *Fasciola hepatica*

Two distinct patterns of disease are seen in the UK. Large numbers of metacercariae appear on pasture in late summer and autumn, when sufficient weeks of warm weather have passed to allow completion of the development of parasite stages on the pasture and

within the snail intermediate host. Acute fasciolosis is caused by juvenile fluke migrating through the liver parenchyma, and is seen 6-8 weeks after the ingestion of large numbers of metacercariae (Behm and Sangster, 1999). Acute disease is uncommon in cattle, but when it occurs is seen in autumn and early winter (Torgerson and Claxton, 1999). Clinical signs include anaemia, lethargy, dyspnoea and sudden death.

Chronic fasciolosis is seen 4-5 months after ingestion of smaller numbers of metacercariae (Behm and Sangster, 1999) and is associated with adult fluke in the bile ducts. Typical signs include loss of condition, anaemia, submandibular oedema, ascites, decreased milk yield, and fibrosis and calcification of the bile ducts may be seen at post mortem. Chronic disease is seen in winter and early spring (Torgerson and Claxton, 1999). Additionally, sub clinical infections in cattle are common, and may result in considerable reduction in growth rates and milk production (Charlier et al., 2012, 2007; Howell et al., 2015; Mezo et al., 2011; Sanchez-Vazquez and Lewis, 2013; Schweizer et al., 2005).

Recent studies have shown that 40-80% of dairy herds across the UK are infected, depending on region (McCann et al., 2010; Salimi-Bejestani et al., 2005a). The parasite is also prevalent in beef animals with 16.5% of livers being condemned due to infection in 2015 (Ford and Hadley, 2015).

Galba truncatula requires a specific habitat of damp, neutral to mildly acidic soil which is found in specific localised areas (Torgerson and Claxton, 1999). Parasite and intermediate host survival and development is favoured by mild, damp weather (Ollerenshaw and Rowlands, 1959). Therefore, the location of suitable snail habitats governs the range and distribution of the parasite, whilst weather and season influence the annual pattern of disease and year-to-year variations in incidence (Bennema et al., 2011; Charlier et al., 2011; Graczyk and Fried, 1999). The effects of longer term climate change are difficult to predict, but it is likely that the seasonality and parasite burdens of fluke will be altered (Fox et al., 2011; Morgan and Wall, 2009).

Risk factors for *fasciola hepatica* infection in temperate climates at the herd level have been studied in dairy herds. Positive risk factors include being in an area of higher rainfall and with higher minimum temperatures, grazing on damp or boggy pasture, access to a stream or pond, increased length of grazing season or proportion of grass in the diet, grazing improved pastures with a lower soil pH, grazing non-sloping pastures and being at a higher altitude (Bennema et al., 2011; Howell et al., 2015; McCann et al., 2010). Infection risk is highest in late summer/autumn and the highest numbers of diagnostic submissions occur in

winter (VIDA). However, antibody levels may remain high all year on farms with high selection pressure (Duscher et al., 2011). These risk factors reflect the risk of exposure directly or by intermediate host habitats. Presence of beef cattle on farm, smaller herd sizes and buying in cattle have also been associated with increased risk (Howell et al., 2015; McCann et al., 2010; Olsen et al., 2011). These are probably a reflection of general disease risks in different management systems. Treatment for liver fluke is strongly associated with presence of antibodies at the herd level, reflecting the fact the farmers are more likely to treat their animals if fluke is a problem (Broughan et al., 2009; Howell et al., 2015). Individual risk factors are not fully understood. Increasing age is not thought to be protective against fluke (Gonzalez-Lanza et al., 1989; Yildirim et al., 2007). Breed is difficult to separate from management effects, but one study suggested that dairy cattle are more likely to have fluke infection than beef cattle (DEFRA, 2005). Females have been found to be more likely to have liver fluke, but again this could be due to management differences (Affroze et al., 2013; Yildirim et al., 2007). Treatment for fluke should be protective at the individual level, although the antibody levels take several weeks to reduce and the risk of re-infection may be high, meaning that antibody levels may be high in treated animals (Salimi-Bejestani et al., 2005b). The effects of pregnancy and lactation are not yet known.

1.2.3 Diagnosis

Fasciola hepatica can be diagnosed by faecal egg count, antibody detection in milk or serum, faecal antigen detection and post mortem by liver inspection. On dairy farms, bulk milk antibody detection is a convenient way of screening the whole herd (Bennema et al., 2009; Duscher et al., 2011; Salimi-Bejestani et al., 2005b). Tests which detect antibodies can detect infection at an early stage, from 2-4 weeks post infection (Salimi-Bejestani et al., 2005b), whereas eggs in faeces are only detectable after the pre-patent period which lasts around 7-10 weeks. Antibody tests are more sensitive than egg detection, so are more useful as a screening tool, but do not necessarily indicate a current infection, as antibody levels can remain high for several weeks after treatment. Table 1.1 shows the relative merits of each type of diagnostic test.

Table 1.1 A summary of the performance of some of the tests commonly used to diagnose liver fluke infection in cattle

Test	Sensitivity	Specificity	Comments	References
Faecal egg count	43-65%	90-100%	Does not become positive until 7-10 weeks following infection. Sensitivity depends on egg count and weight of faeces used. Most methods are based on sedimentation	(Charlier et al., 2008)
Serology	79-95%	80-93%	Can detect infection 2-4 weeks post infection Remains positive for several weeks after cure	(Charlier et al., 2008; Salimi-Bejestani et al., 2005b)
Milk antibody detection	92% 96%	88% 80%	Individual Herd	(Salimi-Bejestani et al., 2007, 2005b)
Copro-antigen ELISA	40-98%	92-94%	Detects infection 6-8 weeks post infection, Returns to negative 1-2 weeks post treatment	(Brockwell et al., 2013; Charlier et al., 2008; Duscher et al., 2011)
Post mortem diagnosis	63-93%	100%	Sensitivity varies: Lower at meat inspection, higher if liver is sliced up and soaked	(Rapsch et al., 2006)

For all methods, sensitivity tends to be worse in animals harbouring only a light infection, where a missed diagnosis is likely to be of least importance (Vercruysse and Claerebout, 2001). Specificity is good for all fluke diagnostic tests, with the milk antibody ELISA being the worst at around 80-88% specificity.

1.2.4 Treatment and control

Control of *F. hepatica* is aimed at limiting parasite burden in individual animals, and reducing contamination of pasture. Drugs active against *F. hepatica* licensed for use in cattle include albendazole, oxclozanide, clorsulon, closantel and triclabendazole, all of which are at least partially active against the adult stages. Of these, only triclabendazole is active against all stages of the parasite whilst nitroxylnil and closantel are active against the adult and juvenile stages. Treatment of dairy cattle is restricted to three drench options: triclabendazole which cannot be used within 45 days of calving, closantel which cannot be used within 60 days of calving, or oxclozanide or albendazole, which have a 72 and 60 hour milk withhold respectively and are not effective against juvenile stages. Cattle should be

treated at housing to prevent immediate re-infection, making things difficult if this does not co-occur with drying off (COWS, 2016). Fluke that are resistant to triclabendazole and albendazole have been identified, and triclabendazole resistance in particular is a major problem as there is no alternative drug available to treat acute fasciolosis (Gordon et al., 2012; Sanabria et al., 2013; Sargison et al., 2010; Sargison, 2012; Sargison and Scott, 2011).

Snail control is not practised in the UK due to the lack of an environmentally suitable and effective agent. Pasture management measures can be effective on many farms, although programmes need to be designed on a farm-specific basis (Knubben-Schweizer et al., 2010). Methods include avoiding risky pastures at certain times of year and fencing off snail habitats (Knubben-Schweizer et al., 2010). These can be difficult to implement, depending on the range of grazing available on individual farms. There are currently many unknowns concerning the distribution of metacercariae on pasture, the limits of snail habitats and other snail-related aspects of liver fluke transmission. Refinement of pasture management methods may be possible in the future, as our understanding of these areas improves (Knubben-Schweizer et al., 2010; Roberts and Suhardono, 1996).

1.2.5 Immunology

1.2.5.1 General immunology

The innate immune system is the first line of defence against invading pathogens. The cells of the innate immune system, including macrophages, mast cells and dendritic cells, recognise conserved pathogen associated molecular patterns (PAMPs), and initiate an inflammatory response by releasing the cytokines tumour necrosis factor (TNF) α , interleukin (IL)1 and IL6. This serves to enable other immune cells present in peripheral circulation, such as neutrophils, monocytes and eosinophils, to access the site by increasing blood flow and the permeability of blood vessels. Chemokines produced by macrophages attract these immune cells to the area. Activation of the complement system stimulates phagocytosis of the pathogen by neutrophils and macrophages. These responses are generic and act to protect the host during the first few hours or days of infection, before the adaptive immune system is fully activated. However, the response is not appropriate for every type of pathogen, and in some cases can assist the pathogen in invading the body (Tizard, 2013).

B and T lymphocytes are the cells of the adaptive immune system. The innate immune system is required to activate these cells, and this is done by the antigen presenting cells

including macrophages and dendritic cells. Antigen is presented to helper T (Th) cells, which then activate and regulate other parts of the adaptive immune response. Th cells express CD4 and CD25. Each Th cell has a unique antigen specific receptor, and when activated, undergoes clonal expansion. Th cells act within lymph nodes to activate other T cells and B cells, and also travel via the vascular system around the rest of the body (Tizard, 2013).

Two main types of Th cell response occur, depending, amongst other things, on the type of pathogen and the chronicity of infection: Th1 and Th2. Th1 cells are primarily responsible for pro-inflammatory and cell mediated immune responses, and produce IL2 and interferon (IFN) γ . These cytokines activate CD8+ cytotoxic T cells, which induce the death of pathogen-infected cells, and, in cattle, stimulate IgG2 production by B cells. They also induce the classical activation of macrophages (Spellberg and Edwards, 2001). Classically activated macrophages attack pathogens by phagocytosis and production of oxidative free radicals, and secrete pro-inflammatory cytokines including TNF α , IL12 and IL18. Th1 responses are generally referred to as cell-mediated immunity, and through the upregulation of IFN γ and induction of cytotoxic T cells, are more effective against intracellular pathogens (Tizard, 2013).

Th2 cells produce IL4 and IL5 which induce eosinophils and activate humoral immunity by stimulating B cells to produce antibodies including IgG1, IgA and IgE (Falcon et al., 2010; Moreau and Chauvin, 2010). Antibodies bind to antigens, enabling complement fixation and killing of the pathogen by neutrophils, macrophages or eosinophils, termed antibody dependent cellular cytotoxicity (ADCC). Macrophages become activated via an alternative pathway in the presence of IL4 (Anthony et al., 2006). These alternatively activated macrophages exhibit reduced free radical and inflammatory cytokine production, and suppress Th1 responses through the production of IL10 (Flynn and Mulcahy, 2008). They have strong anti-inflammatory effects and contribute to fibrosis and repair of tissue damage. The Th2 response also mediates changes in the epithelial barrier which contributes to immunity to helminth parasites. Goblet cells increase mucus production and epithelial cells increase their permeability so that more fluid is excreted into the gut lumen. These combined with the increased contractility of smooth muscle aid in removing parasites from the gut (Gause et al., 2003).

Th2 immune responses are associated with infection with extracellular pathogens and are also seen in chronic infections, where they are associated with the failure of immune control and escalation of clinical disease in some conditions (Begg et al., 2011).

Although either a Th1 or Th2 response usually predominates, they are not mutually exclusive and interact together and with other T cell types. $\gamma\delta$ T cells constitute around 20% of circulating lymphocytes in adult cattle (McGill et al., 2014). These have both innate and adaptive immune roles, and can present antigen and upregulate Th cells (Price and Hope, 2009). $\gamma\delta$ T cells expressing workshop cluster (WC) 1.1 produce inflammatory cytokines and can be directly cytotoxic as part of the adaptive immune response (Rogers et al., 2005). WC 1.2+ $\gamma\delta$ T cells have a regulatory function which is mediated through the production of IL10 and transforming growth factor (TGF) β , as well as through other independent pathways which suppress Th1, Th2 and Th17 responses (Hoek et al., 2009; Moreau and Chauvin, 2010). This reduces tissue damage by regulating pro-inflammatory responses which otherwise would be deleterious to the host in chronic conditions (McCole et al., 1999). These WC 1.2+ $\gamma\delta$ T cells have the same function as human or mouse regulatory T cells expressing CD25, CD4 and a specialised transcription factor, FoxP3. In cattle, CD25+CD4+FoxP3+ cells are found, but do not have a regulatory function (Hoek et al., 2009). Th17 cells have pro-inflammatory effects, secrete IL17, require TGF β for activation, and are involved in defence against extracellular microparasites, regulation of immune tolerance, and are also associated with immunopathology (Ma et al., 2014; Weaver et al., 2006). Cattle have two types of IL17 producing cells, as in addition to Th17 cells found in mice and humans, their WC1.1+ $\gamma\delta$ T cells can also be induced to secrete IL17 (Peckham et al., 2014).

Other factors can also affect the Th1/Th2 balance, for example high progesterone levels in pregnant cattle have been shown to suppress Th1/Th17 immunity and upregulate Th2 immunity (Maeda et al., 2013). Similarly, high levels of glucocorticoids, whether of endogenous origin due to stress or resulting from exogenous corticosteroid administration, can also lead to a Th2 shift (Elenkov, 2004).

1.2.5.2 Fluke specific immunology

Cattle can become infected with fluke at any age (Clery et al., 1996; McCole et al., 1999). In early infection in experimentally infected animals, the host has a mixed Th1 and Th2 immune response, characterised by production of IFN γ , TGF β , IL2 and IL4 (Flynn and Mulcahy, 2008). The immunoglobulins IgE, IgG and IgA are produced by B cells to activate ADCC (Piedrafita et al., 2001). As a result, neutrophils, eosinophils and classically activated macrophages surround and attack the migrating juvenile fluke (Chauvin and Boulard, 1996; Sibille et al., 2004).

After about 3-5 weeks, the Th1 response tails off and a Th2 response predominates initially, with increasing regulatory influence probably mediated by WC1.2+ $\gamma\delta$ T cells (Bossaert et al., 2000; McCole et al., 1999; Rogers et al., 2005). Th2/regulatory responses are likely to be a host adaptation to chronic infections, to avoid excessive tissue damage resulting from inflammatory Th1 cytokines, but are also induced by fluke antigens (Moreau and Chauvin, 2010; Spellberg and Edwards, 2001). IgG1 is the predominant antibody isotype with IgG2 present at lower concentrations (Bossaert et al., 2000; Clery et al., 1996). Antibodies are detectable from 2-3 weeks after infection and levels remain high throughout the period of infection (Ortiz et al., 2000; Salimi-Bejestani et al., 2005b). Antibodies do not appear to have any protective function against liver fluke, and the magnitude of the antibody response correlates with the burden of infection (Bossaert et al., 2000; Clery et al., 1996). Th2 cells produce IL5, which causes increased eosinophil production, and IL4 and IL13, which induce alternatively activated macrophages, associated with healing and fibrosis (Kreider et al., 2007; MacDonald et al., 2002). The fibrosis associated with this response may limit the number of metacercariae that develop into adults and limits egg production by the fluke (Moreau and Chauvin, 2010). After around 5 weeks, a regulatory response is seen, as upregulation of IL10 and TGF β cause the decline of IL2 and IFN γ levels (Flynn and Mulcahy, 2008; Gazzinelli et al., 1992; McCole et al., 1999).

However, these Th2/regulatory responses do not give protective immunity against liver fluke. A study in comparing Indonesian thin tailed sheep, which are inherently resistant to *F. gigantica*, with susceptible sheep, found that the resistant sheep had a predominant Th1 response in early infection, whereas the susceptible sheep had a mixed Th1 and Th2 type response (Zhang et al., 2005). Vaccine trials in cattle showed that the immunity induced in response to cathepsin L cysteine proteases was Th1-mediated (Mulcahy and Dalton, 2001). This evidence suggests that a fluke-specific Th1 response could potentially be protective against liver fluke (McNeilly and Nisbet, 2014).

Most of the above findings have been shown in experimentally infected animals. In a recent study on the immune responses of naturally infected dairy heifers, Graham-Brown (2016) reported that there was no evidence of a Th1 response in early infection in these animals, with little IFN γ produced, and the Th2 type response described above was dominant from early infection onwards. Other studies report higher serum IgG1 levels in animals infected with a trickle dose as opposed to a single larger dose of metacercariae (Bossaert et al., 2000), and in naturally infected compared to experimentally infected (Oblitas, 1997). This

could be because the Th1 response only occurs after high doses of metacercariae are ingested, which rarely occurs under natural conditions.

1.2.5.3 Evasion and modulation of the host immune system by fluke

Flukes have evolved various ways of evading the host immune response. The fast turnover of their protective outer tegument and glycocalyx reduces antibody binding and subsequent damage to the parasite surface by immune cells (Hanna, 1980). Around 80% of fluke ES products are cathepsin L cysteine proteases (Dalton et al., 2013). Cathepsins cleave IgE and IgG required for ADCC (Smith et al., 1993). ES also contains several antioxidants: superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, peroxiredoxin and fatty acid binding proteins (Jefferies et al., 2001). These inhibit ADCC by neutralising the free radicals produced by immune cells (Cervi et al., 1999; Ganga et al., 2007; Mulcahy et al., 1999; Sibille et al., 2004).

Modulation of the immune system is mediated by both ES products and tegument antigens. Cathepsins suppress lymphoproliferation (Moreau et al., 2002; Prowse et al., 2002) and induce eosinophil apoptosis (Serradell et al., 2007). Cathepsins also inhibit the release of pro-inflammatory cytokines from macrophages (Donnelly et al., 2010), and can interfere with T cell function by cleaving CD4 (Prowse et al., 2002). Peroxiredoxin has been shown to induce alternatively activated macrophages (Cervi et al., 1998). Additionally, the ES contain products that mimic cytokines, for example, a molecule that acts like IL5 and attracts eosinophils to the site (Milbourne and Howell, 1993). Juvenile fluke have been shown to produce a TGF β -like molecule that can bind to mammalian TGF β binding sites, although further study is required to determine the effect on the host (Japa et al., 2015).

Fluke tegument antigen induces TGF β production which suppresses production of pro-inflammatory cytokines TNF α , IL6 and IFN γ by innate immune cells. This prevents the induction of Th1 and Th17 responses (Flynn and Mulcahy, 2008; Vukman et al., 2013; Walsh et al., 2009). Tegument antigen also induces alternatively activated macrophages (Haçarız et al., 2011). Taken together, these immunomodulatory mechanisms show the complexity of the host-parasite relationship.

1.3 Bovine tuberculosis

1.3.1 Epidemiology of bovine TB in the UK

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, is a serious animal health problem affecting UK farms, and £500 million was spent by the government in trying to

control it during the last decade (Department for Environment Food and Rural Affairs, 2014a). In spite of this considerable investment, the number of TB-restricted herds in England and Wales has continued to rise (Department for Environment Food and Rural Affairs, 2014b). South west England and South Wales are considered endemic for bTB, with an increasing number of cases being seen in the midlands and mid Wales. Scotland currently has Officially TB Free status, with approximately 50 new TB breakdowns each year (Department for Environment Food and Rural Affairs, 2014c).

The bTB control programme is compulsory in the UK, and all cattle over the age of 6 weeks are tested at regular intervals of between one and four years depending on whether they are in a high or low risk area. Cattle that test positive are culled from the herd, restrictions are applied to the herd to limit movement of animals, and more regular testing is carried out until no further positive cases are found. Many farms in the worst affected areas are more or less permanently under restriction, with infected badgers being the main reservoir of infection (Bessell et al., 2012; Biek et al., 2012; Donnelly and Nouvellet, 2013; Griffin et al., 2005; Johnston et al., 2011).

Clinical bTB in cattle is rarely seen in the UK, as the disease progression is slow and the testing programme removes most infected animals before they become symptomatic. Pasteurisation of milk prevents virtually all zoonotic transmission, with only a handful of human *M. bovis* infections seen annually, most of which originate outside the UK (de la Rua-Domenech, 2006).

Risk factors at the herd level include location, with herds in the endemic areas being at higher risk. Contact with badgers, buying in cattle, large herds and previous incidence within the herd also increase the risk (Bessell et al., 2012; Johnston et al., 2011). Dairy cattle are more likely to test positive than beef cattle (Brooks-Pollock et al., 2013). Risk increases with age up to around 3 years, after which it plateaus or decreases slightly (Brooks-Pollock et al., 2013). Vaccination against, Johne's disease is known to decrease the sensitivity of the SICCT (Whelan et al., 2011), so it is possible that Johne's infection could also have an effect, although this has not been confirmed. The effects of lactation and pregnancy have not been established.

Amongst reactor cattle, those with antibodies to *Mycobacterium avium* paratuberculosis are more likely to have confirmed bTB, whereas the opposite is true for those cattle without these antibodies. Meanwhile, beef cattle were more likely to have confirmed bTB than dairy cattle (DEFRA, 2005).

1.3.2 Immunology

Mycobacterium bovis is a slow growing intracellular bacterium with a lengthy pre-clinical phase lasting months or years. *M. bovis* is taken up by macrophages but survives the acidic phagosome (Podinovskaia et al., 2013). Th1 cells produce pro-inflammatory cytokines including IFN γ and TNF α . $\gamma\delta$ T cells also produce IFN γ and are thought to bridge innate and adaptive immunity in *M. bovis* infection (McGill et al., 2014). A strong IFN γ response is associated with lower pathology and better control of bTB (Welsh et al., 2005). IFN γ and TNF α induce classically activated macrophages and activate CD8+ T cells and $\gamma\delta$ T cells to produce factors which can directly kill mycobacteria within cells (Kaufmann, 2002). Macrophages form granulomas around the infected cells (Buddle et al., 2013; Flynn et al., 1995), which prevents further spread of infection, but also protects the mycobacteria from attack by the immune system (Giacomini et al., 2001). In this way an equilibrium is reached where the infection is controlled but not eliminated.

In chronic infection, Th2/regulatory responses increase (Flynn et al., 1995; Wangoo et al., 2001; Welsh et al., 2005). Pathogenic mycobacteria induce regulatory T cells to produce IL-10 and TGF- β and prevent macrophage apoptosis, hindering elimination of the pathogen (Balcewicz-Sablinska et al., 1998; Lee et al., 2009; O'Garra et al., 2013). In experimentally infected cattle, strong cell mediated immunity was observed at 16 weeks post infection, and by 25-34 weeks post infection Th1 immunity had declined to the extent that the cattle failed to respond to PPD_b stimulation in the IFN γ test (Welsh et al., 2005). As the protective Th1 declines and is superseded by a humoral (Th2) type response, control over the infection is lost and active, disseminated disease occurs, together with increased antibody production (Welsh et al., 2005). Later in the course of disease, necrotic foci in the lungs enable extracellular survival of mycobacteria and this is when the disease becomes infectious (Lee et al., 2009).

1.3.3 Diagnosis

The single intradermal comparative cervical tuberculin test (SICCT, also known as the skin test) is the main element of the UK bTB control programme. This detects a cellular immune response to *M. bovis*, which in most bTB cases is the predominant immune response. The SICCT involves injecting bovine and avian tuberculin purified protein derivative (PPD) respectively into two sites on an animal's neck, following measurement of skin thickness. If the animal has been exposed to the antigens in the PPD, a type IV hypersensitivity reaction occurs. This is mediated by Th cells, macrophages and monocytes, and causes an

inflammatory response with localised swelling at the injection site after two to three days. Any reactions are measured 72 hours later and compared between the two sites. A chart is used to interpret the results, but in general, at the standard interpretation, a swelling at the bPPD site that is 4mm or more greater than the swelling at the aPPD site indicates that the animal is a bTB reactor (APHA, 2014a, 2014b). The aPPD is used as a control to avoid false positives due to exposure to environmental mycobacteria.

In the UK, testing is usually carried out by private veterinary surgeons on the government's behalf, but all bTB testing is under the control of the government, and veterinary surgeons or researchers are not allowed to perform the SICCT or any other bTB test of their own volition. The sensitivity of the SICCT has been variously estimated at between 56% and 90% (Ameni et al., 2000; Clegg et al., 2011; Costello et al., 1997; Gonzalez Llamazares et al., 1999; Karolemeas et al., 2012). As the SICCT detects a cellular immune response, low sensitivity may result from the a lack of response in the early stages of disease before the immune response is functional, lack of response due to predominating humoral immunity during advanced disease, and factors such as the peri-partum period, corticosteroid treatment, production related stress, and, possibly, co-infection with other pathogens (Strain et al., 2011).

Cattle that test positive on the SICCT are then slaughtered and infection status is confirmed by visualisation of lesions, culture and histopathology. However, small lesions are difficult to detect. The sensitivity of visible lesion detection varies between 20% and 95%, depending on the post mortem procedure and stage of infection (Corner, 1994; Frankena et al., 2007; Liebana et al., 2008). The pathogen can be cultured from approximately 70-90% of lesions (Department for Environment Food and Rural Affairs, 2014b; Liebana et al., 2008). In TB test negative animals, between 53-67% of TB-like lesions seen in the slaughterhouse are later confirmed on culture (Liebana et al., 2008; Shittu et al., 2013).

Other tests for bTB are commercially available and are useful in specific situations. The interferon gamma (IFN γ) ELISA (Bovigam[®]) is more sensitive than the SICCT. It is used in some circumstances as part of the mandatory bTB control programme in the UK, with the aim of identifying a higher proportion of infected cattle on farms where bTB is already known to be present (Strain et al., 2011). A blood sample is taken and peripheral blood mononuclear cells (PBMCs) stimulated with PPDa or PPDb, and the quantity of IFN γ produced is then measured using an ELISA. Blood must be maintained at specific temperatures following extraction from the cow, and must be tested within a few hours of

collection, making it challenging in field situations. It is expensive and difficult to perform, and the lower specificity makes it unsuitable for routine use (Strain et al., 2011). However, this test can detect animals exposed to lower doses of *M. bovis* and at earlier stages of infection (Dean et al., 2005; Pollock et al., 2005) and is therefore a useful adjunct to the SICCT. As the IFNY test detects cellular immunity, similar to the SICCT, its sensitivity is likely to be affected by the same factors that affect the SICCT. An antibody ELISA is not currently used in the UK, but may be useful for detecting cattle in the later stages of disease, when they become anergic to the bTB skin test but antibody levels rise.

The difficulty in determining the true bTB infection status of naturally exposed cattle, in the absence of a gold standard test, is a challenge both for disease control programmes and epidemiological studies.

1.4 Johne's disease

1.4.1 Epidemiology

Johne's disease is a contagious, chronic condition mainly affecting ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP is found worldwide, and is able to survive for lengthy periods in the environment. In a recent UK-wide study, up to 65% (95% CI 56-74%) of dairy herds were found to contain infected animals as determined by a MAP-antibody ELISA (Caldow et al., 2009).

Cattle normally acquire infection as new born calves, although older animals are also susceptible if infection pressure is high (Mortier et al., 2014). Infected animals are not infectious to others unless they progress to the later stages of disease, characterised by a failing Th1 immune response and the onset of clinical signs. This usually does not occur until they are over two years of age (Nielsen and Ersbøll, 2006).

Control measures are intended to avoid contact between the faeces of infectious cattle and new born calves, and also to keep the infection pressure generally low, and include selective culling, removal of calves from their dams shortly after birth and maintenance of hygiene in calving pens (Mortier et al., 2015). Costs are associated with deaths or culling as a result of disease, which may reach 1-5% of cattle in a herd annually, and subclinical effects such as reduced milk yield, reduced feed conversion and increased susceptibility to other diseases (Hasonova and Pavlik, 2006).

Risk factors for infection with MAP include poor farm hygiene and other management practices which increase infection pressure and lead to calves being exposed, such as feeding pooled colostrum (Mortier et al., 2015). The age at which the infection becomes clinical depends partially on the initial infective dose (Mortier et al., 2015). Poor nutrition, stress related to transport, lactation, parturition, and immunosuppression by co-infecting agents have been implicated in hastening the start of clinical Johne's disease (McKenna et al., 2006).

1.4.2 Immunology

MAP primarily targets the ileum, where the mycobacteria survive and multiply within macrophages. During the early stages of infection, a T-helper (Th)1 type profile characterised by high levels of interferon gamma (IFN- γ) keeps infection under control (Begg et al., 2011; Cooper et al., 1993; Flynn et al., 1993). In most cases, the infection is self-limiting although animals are thought to remain infected for life (Nielsen and Toft, 2008). In a proportion of animals, progression to clinical disease occurs (Arsenault et al., 2014). At this time, a Th2/regulatory response predominates, IFN- γ levels decline and clinical signs appear (Begg et al., 2011). Proliferation of mycobacteria is facilitated by increased production of IL-10, an anti-inflammatory cytokine induced by MAP (Weiss et al., 2005). Inflammation of the intestinal tract results in reduced nutrient absorption, weight loss and diarrhoea. Once symptomatic, Johne's disease is eventually fatal in most cases.

1.4.3 Diagnosis

Diagnostic tests include faecal culture (individual or pooled), serum or milk antibody ELISA, and faecal polymerase chain reaction (PCR). Antibody detection by ELISA is the most sensitive test (Caldow et al., 2009; Collins et al., 2005; Pinedo et al., 2008). In the early stages of infection, cattle shed MAP infrequently and antibody levels often do not rise to detectable levels until the later stages of disease, so infected animals are rarely detected before 2-3 years of age (Nielsen and Ersbøll, 2006). Hence for every detectable infected animal in a herd, there are likely to be several others which are infected and will progress to the clinical stage at a later time (Whitlock and Buergelt, 1996).

1.5 *E. coli* O157

1.5.1 Epidemiology

Escherichia coli O157 is a zoonotic bacterium that causes haemorrhagic diarrhoea in humans and occurs worldwide. It is highly virulent with a low infectious dose of less than 700 cfu (Tuttle et al., 1999) and is a considerable public health problem. Cattle are considered the main source of human infection. Recent studies have shown that 4-7% of UK finishing cattle are shedding *E. coli* O157. (Ellis-Iversen et al., 2007; Gunn et al., 2007; Paiba et al., 2003; Pearce et al., 2009).

Cattle do not show clinical signs of disease as a result of *E. coli* O157, because they lack receptors for Shiga toxins (Pruimboom-Brees et al., 2000). Within groups of cattle housed together, infection is likely to spread rapidly through the group (Ellis-Iversen et al., 2007). A 'super shedding' event is defined as $>10^4$ colony forming units (cfu) *E. coli* O157 being excreted at once. This occurs as a result of a biofilm of bacteria being shed from the distal gastro-intestinal tract. A small number of super shedding events can be responsible for up to 90% of *E. coli* O157 excretion (Chase-Topping et al., 2008). Most infected cattle probably 'super shed' intermittently; there is more variation within individuals than between them (Paiba et al., 2003; Pearce et al., 2004; Williams et al., 2014). The intermittent nature of shedding and the uneven distribution of bacteria within faeces may hinder diagnosis (Pearce et al., 2004).

Season has been found to affect the risk of *E. coli* O157 shedding, but the highest risk time of year was found to be either winter or summer, depending on the study (Milnes et al., 2009; Smith et al., 2016). For calves, being housed in pens significantly increased the risk compared to grazing cattle (Gunn et al., 2007; Smith et al., 2016), particularly if bedding was wet (Ellis-Iversen et al., 2007). One study found that calving was associated with a decreased risk of shedding (Synge et al., 2003). Older cattle are less likely to shed or carry *E. coli* O157 (Gunn et al., 2007; Milnes et al., 2009). Stress was found to increase shedding in finishing cattle (Chase-Topping et al., 2007).

1.5.2 Immunology

Although cattle do not become ill as a result of *E. coli* O157 infection or colonisation, they do mount an immune response against it (Munns et al., 2015). *E. coli* O157 flagellin induces an innate immune response leading to the production of pro-inflammatory cytokines including IL8, IL1 and TNF α (Miyamoto et al., 2006). Lipopolysaccharide, a highly conserved

pathogen associated molecule and a component of *E. coli*, is also recognised by the innate immune system. *E. coli* produces anti-inflammatory effector proteins which interrupt the immune response, enabling its survival (Walle et al., 2010).

It is not clear whether a protective adaptive immunity is induced (Walle et al., 2010). A humoral response is elicited, and IgG is protective in calves (Fröhlich et al., 2009). A variety of *E. coli* O157 antibodies have been found in experimentally infected cattle, however these alone are not protective against colonisation (Bretschneider et al., 2007). A cellular response has also been demonstrated with antigen specific lymphocyte proliferation and upregulation of IFN γ (Corbishley et al., 2014). In one study it was found that animals with higher level of antibodies to *E. coli* O157 were more likely to shed large numbers, presumably as a result of the ability to slough off the biofilm (Nart et al., 2008).

1.6 Co-infections with *F. hepatica*

1.6.1 Laboratory studies

Experimentally infected cattle and rats have been used to investigate the effects of *F. hepatica* on animals infected with *Salmonella dublin*, *Bordetella pertussis* and *M. bovis*. Cattle infected with *F. hepatica* and *S. dublin* and took longer to clear infection, and shed higher numbers of bacteria, than fluke free animals, although survival was not affected (Aitken et al., 1981) and this was thought to be attributable to impaired cell mediated responses (Aitken et al., 1979). A similar effect was seen in rats (Aitken et al., 1980).

Mice infected with *F. hepatica* had a suppressed Th1 response and delayed clearance of *B. pertussis* from the lungs, and had significantly higher bacterial loads (Brady et al., 1999). In a study on rats those infected with *M. bovis* and *F. hepatica* had fewer liver lesions from fluke than those infected with *F. hepatica* alone (Sadzikowski and Gundlach, 1985).

Experimental infection of *M. bovis* infected cattle with *F. hepatica* was shown to result in a decreased response to the bTB skin test, although overall results were still all positive (Claridge et al., 2012). A much more marked effect was seen in cattle infected with Bacillus Calmette–Guérin (BCG) (Flynn et al., 2007). IFN γ levels were shown to be decreased in co-infected cattle in three studies, although the observed effects were small (Flynn et al., 2009, 2007; Garza-Cuartero et al., 2016). The numbers of fluke recovered from co-infected cattle were found to be lower than in fluke only infected animals, although again the differences were small (Flynn et al., 2009). Mycobacterial uptake by macrophages and

mycobacterial recovery from infected tissues was also found to be lower in co-infected animals (Garza-Cuartero, 2014; Garza-Cuartero et al., 2016). The ability of liver fluke to induce alternative macrophage activation could delay the uptake of *M. bovis*, and reduce tubercle formation (Garza-Cuartero, 2014).

1.6.2 Field studies

Studies on co-infection with liver fluke in naturally infected animals are concentrated on *S. dublin* and *M. bovis*. Salmonella infection was found to be strongly associated with *F. hepatica* infection in cattle at the herd level in the Netherlands (Vaessen et al., 1998), although another study found no effect (Taylor and Kilpatrick, 1975).

An abattoir study of 200 TB reactors and 200 in-contact animals found that fluke positive TB test reactors were less likely to have confirmed bTB by lesions detection, histopathology or culture compared to the TB skin test positive, fluke negative cattle (DEFRA, 2005). A herd level study of 3000 UK dairy farms found that herds infected with *F. hepatica* were one third less likely to have a TB breakdown. However, in a 'Fluke infected' or 'TB infected' herd, there are usually only a minority of animals actually infected with either pathogen, so at the herd level, this is not conclusive evidence of an effect.

Several early field studies on co-infection with fluke and bTB looked at presence of bTB lesions and the presence of fluke in the liver, and none of the authors were convinced of any significant effect (Keller, 1952; Ljesevic, 1957; Meyer, 1963).

Of two studies of cattle naturally infected with the tropical liver fluke, *F. gigantica*, one found that fluke infected cattle had smaller reactions to the bTB skin test (Ameni and Medhin, 2000), whereas another found a strong positive relationship between fluke and bTB in an abattoir study (Munyeme et al., 2012). However, cattle in this study were likely to be at more advanced stages of diseases, as there is no regular bTB testing programme in this setting.

1.6.3 Summary of co-infections with *F. hepatica*

In general, it seems that Th1 responses are down regulated in fluke infected animals, which leads to reduced clearance of the bacterial pathogen in the case of *S. dublin* and *B. pertussis*, but in the case of *M. bovis*, there is an inverse relationship between the two pathogens. The reduced bacterial uptake and reduced granuloma formation can be explained by the effect of fluke on macrophage activation. The appearance of false positives in the fluke infected cattle in DEFRA (2005) could also reflect reduced granuloma

formation as a result of alternatively activated macrophages. However, the observation that there are fewer flukes in *M. bovis* infected cattle is more difficult to explain. The results of the field studies were generally less consistent than those of the laboratory studies, which is likely to reflect both the increased risk of bias and the greater level of natural variation in naturally exposed populations. Various risk factors may overlap between the pathogens being studied, and these are summarised in Table 1.3.

Table 1.2. Risk factors for the four pathogens studied, highlighting in purple those that may cause confounding

	<i>Fasciola</i>	<i>M. bovis</i>	MAP	<i>E. coli</i> O157	References
Herd size	Smaller herds higher risk	Larger herds higher risk	Larger herds higher risk	Unknown	Howell et al., 2015; McCann et al., 2010; Olsen et al., 2011; Bessell et al., 2012; Johnston et al., 2011
Buying in cattle	Increased risk	Increased risk	Increased risk	Increased risk	Howell et al., 2015; McCann et al., 2010; Olsen et al., 2011; Bessell et al., 2012; Johnston et al., 2011
Grazing vs. housed	Probably no risk of infection whilst housed but antibodies could still be high	Effect depends on risk area (whether main risk is badgers or cattle)	Probably no effect on clinical onset	Increased risk if housed	Bennema et al., 2011; Howell et al., 2015; McCann et al., 2010; Gunn et al., 2007; Smith et al., 2016
Age	Risk increases up to two years and then plateaus	Risk increases up to 2 – 3 years then declines slightly	Infected at young age but highest risk of becoming clinical at 2-5 years then declines slightly	Older age is protective	Gonzalez-Lanza et al., 1989; Yildirim et al., 2007; Brooks-Pollock et al., 2013, Mortier et al., 2015, Gunn et al., 2007; Milnes et al., 2009
Indoor hygiene	No effect	Unknown	Poor hygiene increases both infection and clinical onset	Poor hygiene increases shedding risk	Mortier et al., 2015, Ellis-Iversen et al., 2007
Calving	Unknown	Unknown	Possibly hastens clinical onset	Associated with decreased shedding	McKenna et al., 2006, Synge et al., 2003
Lactation	Unknown	Unknown	Possibly hastens clinical onset	Unknown	McKenna et al., 2006
Stress	Unknown	Unknown	Associated with clinical onset	Associated with increased shedding	McKenna et al., 2006, Chase-Topping et al., 2007
Location	Western areas most at risk	Southern/western areas most at risk	UK-wide	UK-wide	McCann et al., 2010, Claridge et al., 2012
Rainfall	Rainy areas most at risk	Probably no effect	Probably no effect	Probably no effect	Howell et al., 2015; McCann et al., 2010
Season	Infection levels highest in late winter, but antibodies may stay high throughout the year	Unknown	Probably no effect	An effect exists but studies disagree on whether winter or summer are the highest risk times	Duscher et al., 2011, VIDA, Milnes et al., 2009; Smith et al., 2016
Breed/type	Unknown	Breed differences exist but not fully understood	Unknown	Unknown	Brooks-Pollock et al., 2013, DEFRA, 2005

1.7 Thesis aims

The aim of this thesis was to investigate the interaction between liver fluke and other pathogens, to answer the question of whether fluke infection makes cattle more susceptible to certain bacterial pathogens. Following on from previous studies on co-infection with liver fluke and *M. bovis* in experimentally infected animals, the aim of Chapter 3 was to investigate whether exposure to liver fluke affects the bTB skin test in naturally infected animals. In Chapter 4 we present a systematic review of the literature on co-infection with liver fluke and mycobacteria. In Chapters 5 and 6 we investigate the association of liver fluke infection with two further pathogens, MAP and *E. coli* O157.

Chapter 2: Dynamics of *Fasciola hepatica* exposure in whole herds of naturally infected cattle, and comparison of milk and serum ELISAs

Abstract

Fasciola hepatica, or the common liver fluke, a parasite affecting welfare and causing economic losses in ruminants, is estimated to affect around 70-80% of UK dairy herds. A total of 5937 cattle from 30 purposively selected UK beef and dairy herds were tested using an ELISA to detect antibodies to *F. hepatica* excretory-secretory products, applied to either a milk or serum sample. Bulk milk tank samples were also tested from 24 out of the 30 farms. The percentage of animals testing positive for fluke in each herd ranged between 2 and 89%, with a median of 46%. The distributions of the antibody percent positivity (PP) values were right-skewed for all herds. The bulk milk tank results were well correlated with individual results, although the results of both individual and bulk milk tank ELISAs tended to be higher than the serum ELISA results. This could have been due to the lower specificity of the individual and bulk milk tank ELISAs compared to the serum ELISA. Using multi-level models, a significant effect of season was seen, which is in agreement with other recent studies, but age was not significantly associated with antibody levels.

2.1 Introduction

Fasciola hepatica, or the common liver fluke, is a widespread parasite affecting welfare in ruminants and causing economic losses in farming, and around 70-80% of UK dairy herds are reported to be affected (Claridge et al., 2012; Howell et al., 2015; McCann et al., 2010). Cattle do not develop protective immunity to fluke infection and can become re-infected throughout life (Clery et al., 1996), although liver fluke do have a shortened lifespan in cattle compared to sheep, with most fluke surviving up to about 10 months compared with several years' survival in sheep (Ross, 1967a, 1967b). In cattle older than 24 months, fluke prevalence tends to increase with age, approaching a maximum prevalence after a number of years (Gonzalez-Lanza et al., 1989).

It has been noted that the typical distribution of parasite burdens within herds tends to be skewed, with a minority of animals having high parasite burdens, and the majority having a

low burden or being parasite free (Chaparro et al., 2016; Charlier et al., 2008). The reasons for this phenomenon are not fully explained. Some possible reasons include genetic variation, metabolic stresses or co-infecting pathogens making some individuals more susceptible to infection. Due to the limited habitat of the intermediate host, parasite distribution on pasture is very uneven (Graczyk and Fried, 1999). Individual cattle preferences for certain types of grazing, or variations in social status within the group might mean that some animals are more likely to graze certain areas, and hence be exposed to infection.

Liver fluke requires an intermediate snail host, *Galba truncatula*, for the completion of its life cycle. Wet habitats such as river banks and boggy ground are the required habitat for *G. truncatula*, and, in the UK, the snail proliferates during the milder weather of late spring and summer. Following a period of intra-snail development, infectious metacercariae are deposited on pasture in high numbers during late summer and autumn. Therefore grazing ruminants are most likely to become infected at this time, although metacercariae are able to survive for several months given suitable weather conditions. The usual management practice in the UK of housing cattle over winter reduces their exposure to the parasite from October or November onwards.

Antibody detection ELISAs have been widely used for epidemiological studies on *F. hepatica* (Charlier et al., 2005; Claridge et al., 2012; Howell et al., 2015, 2012; McCann et al., 2010). An ELISA to detect antibodies to excretory-secretory fluke products was developed at the University of Liverpool by Salimi-Bejestani et al. (2005b), and other ELISAs which detect antibodies to different fluke proteins are also commonly used. ELISAs are efficient compared to other fluke detection methods, and are versatile, as they can be performed on individuals or herds, on blood or milk samples.

The aim of this chapter was to investigate the dynamics of fluke infection in dairy and beef herds, in order to determine the effect of seasonality and animal age on sero-positivity. This information was useful for the interpretation of further studies on the interaction between fluke infection and other pathogens, reported in later chapters.

2.2 Methods

2.2.1 Samples

Thirty herds were sampled altogether. Thirteen herds were visited as part of a study on co-infection between liver fluke and bovine TB (bTB). These farms were selected by their

private vets as they were thought likely to be exposed to both bTB and liver fluke. Ten farms were visited once, two farms were visited twice and one farm was visited three times. During each visit, all animals over the age of 24 months that were presented for the TB test were blood sampled from the tail vein. This was done under Home Office licence PIL 40/3621 and was approved by the University of Liverpool Ethics Committee ref VREC290. At the University of Liverpool, the samples were centrifuged at 1000 g for 20 minutes and the serum separated off for testing. Age and sex data were obtained from 12 of these farms. One also provided drying off dates. On one of the farms, all animals were faecal sampled per rectum at the same time as being blood sampled.

In addition, seventeen different herds with evidence of fluke exposure on ELISA were selected from those sampled as part of a whole-herd disease screening programme. The samples were submitted to a commercial milk recording company for routine analysis and the remaining sample was sent to the University of Liverpool for fluke testing, following the addition of bronopolnatamycin (MSI, Nottingham) as preservative. On arrival samples were centrifuged at 1000 g for 20 minutes and aliquots of skimmed milk were taken for testing. Of these herds, 11 farms were sampled once, two farms were sampled three times and four farms were sampled four times. All lactating animals were sampled at each visit.

2.2.2 Testing

The blood and milk samples were all tested using an ELISA to detect antibodies to *Fasciola hepatica* excretory-secretory products developed by Salimi-Bejestani et al. (2005b).

Briefly, 0.5 µg/ml ES antigen in 0.1M carbonate buffer (pH 9.6) was used to coat Immulon-2 ELISA plates (Dynatech Laboratories, VA, USA). Plates were incubated for 1 h at room temperature (approx. 24° C) then refrigerated overnight (4° C).

The plates were then washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST): 2 short and 1 five-minute washes, repeated once. Blocking solution (200 µl per well of 2% skimmed milk powder (Marvel, Premier Beverages, Stafford, UK) in PBST (MPBST) was added and plates incubated for 1 h at 37° C before washing as before.

Previously prepared negative, medium and strong positive serum controls diluted 1 in 800 in MPBST were added in quadruplicate at 100 µl per well. The positive control consisted of a pool of serum samples taken from known positive animals. The negative control came from a cow kept indoors and from a farm known to be free of *F. hepatica* and *Galba truncatula*. Samples were diluted as follows: serum samples, 1 in 800 in MPBST; individual

milks, 1 in 2 in MPBST; bulk tank milks, neat. One hundred µl of each diluted sample was added to duplicate wells. Two wells were left as blanks containing only 100 µl MPBST. Following incubation for 1 h at 37° C and washing, 100 µl per well of conjugate (IL2-A monoclonal anti-bovine IgG conjugated to horse radish peroxidase, sensitive to IgG1 and IgG2 (Williams et al., 1990)) diluted 1:45,000 in MPBST was added. The concentration of conjugate was first optimised by a checkerboard titration. After incubation for 1 hour at 37° C, the plate was washed and 100 µl tetramethylbenzidine (TMB) aqueous substrate (Uptima, Interchim) was added to every well. Following incubation for 30 min at room temperature, 100 µl stop solution (0.5 M HCL) was added and the colour change was read at 450 nm on an automatic ELISA reader (Infinite F50, Tecan, Salzburg, Austria). The ELISA was considered valid if the optical density (OD) of the positive control was between 1 and 3 and if the negative controls and blanks were less than 0.1 OD.

Results were expressed as percent positivity (PP) of the strong positive control. Individual results were considered valid if the duplicate PP values were within 10 % of each other. A PP of above 15 and 20 for individual serum and milk respectively, defines a positive result. For bulk milk tank samples, a PP of 27 or above is considered positive and corresponds with more than 25% of the herd being infected (Salimi-Bejestani et al., 2007).

The faecal samples were tested using Flukefinder® (Richard Dixon, ID, USA, www.flukefinder.com), a kit used for sedimentation of faecal samples. Three g of stool was homogenised in water before being tipped into the Flukefinder unit, which consists of two sieves of different sized mesh (exact sizes proprietary). The unit was then washed through with copious amounts of water, until the water ran clear. Remaining sediment was backwashed into a 40 ml beaker which was refilled with water. After 2 mins, most of the water was poured off, being careful not to disturb the sediment. The beaker was then refilled and the process was repeated several times, until the water in the beaker looked clean. The remaining sediment was collected in a 5 cm petri-dish, stained with a few drops of 10% methylene blue solution and viewed under the dissecting microscope at x40 magnification. Eggs were categorised as *F. hepatica* based on morphological characteristics (Valero et al., 2009), and counted.

2.2.3 Analysis

Although a positive/negative cut off is defined for each ELISA, the analysis was performed mostly using the fluke PP values. This was because most false positives and false negatives

occur in values very close to the cut off, and the PP values give an indication of the severity of the parasite burden (Charlier et al., 2008; Salimi-Bejestani et al., 2008).

2.2.3.1 Descriptive analysis

R (R Core Team, 2011) with ggplot2 (Wickham, 2009) were used to perform descriptive analysis and draw maps and plots of the data.

2.2.3.2 Comparison of bulk milk tank and individual results

Plots were drawn of the individual and bulk milk tank results. Faecal egg count data and lactation status data, which were both available from two of the farms, were used to investigate this relationship further.

2.2.3.3 Seasonality

The data had a nested structure of visit within cow within farm. Cow and farm were inputted as random effects at level 2 and 3 respectively, to account for non-independence of observations clustered within farm or when sampling the same individual repeatedly. A variable intercept model was built with log of fluke ELISA PP (log PP) as the outcome variable. The month of sample collection was modelled as a fixed effect using a sin/cosine function:

$$\cos\left(2\pi \times \frac{month}{12}\right) + \sin\left(2\pi \times \frac{month}{12}\right)$$

Whether the sample tested was a milk or serum sample was included as a fixed effect.

The model equation for an individual PP result was:

$$\log PP = \text{intercept} + \text{month function} + \text{sample type} + \text{farm effect} + \text{cow effect}$$

Other information such as type of cattle was not included in models, as the farms were purposively rather than randomly chosen, and so biased effects were likely. The Lme4 package was used for multilevel modelling in R (Bates et al., 2015).

2.2.3.4 Age

A multi-level model was created using data for a subgroup of the farms. A similar approach was taken to that described for seasonality, with age in months added as an additional fixed effect.

2.3 Results

2.3.1 Characteristics of the cattle and farms

A total of 5937 cattle were sampled, from thirty UK dairy and beef herds (Table 2.1).

Table 2.1 A summary of the characteristics of the animals and herds tested for fluke

	Blood sampled		Milk sampled	
	Farm information	Cow-level information	Farm information	Cow-level information
Number sampled	13 farms	1584 cattle	17 farms	4355 cattle
Number sampled per herd	Range: 27-192 Median:133		Range: 80-438 Median:202	
Beef	4 beef farms	263 cattle of beef breeds	No beef farms	Unknown
Dairy	9 dairy farms	1321 cattle of dairy breeds	17 dairy farms	Unknown
Age (months)	Data available for 12 farms	Range: 24-208 Median: 58	No data	Unknown
Sex	Data available for 12 farms	F:1485 M:21 Unknown:78		All female

Cattle from thirteen herds had blood samples taken, whilst cattle from 17 herds had individual milk samples taken. Two farms were visited twice, three farms were visited three times, and four farms were visited four times. In total, 9673 individual samples and 24 bulk milk tank samples were taken. Figure 2.1 shows the locations of the sampled herds.

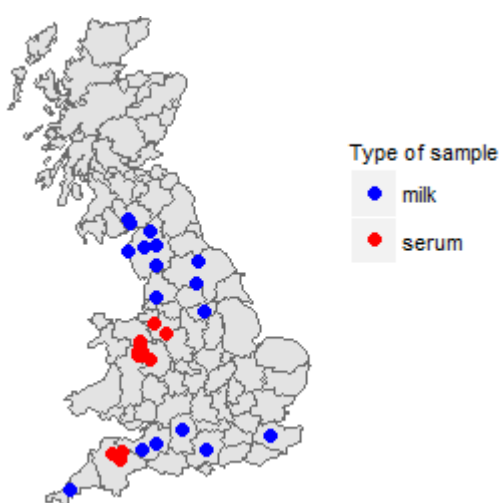


Figure 2.1. The locations of the herds sampled for liver fluke, jittered randomly by 10 km to preserve anonymity. The type of sample taken from cattle on each farm is shown by the colour of the point

2.3.2 Distribution of the fluke results

4465 of samples (46%) tested positive for fluke on the ELISA. The overall distribution of fluke ELISA PP values was log normal (Fig. 2.2)

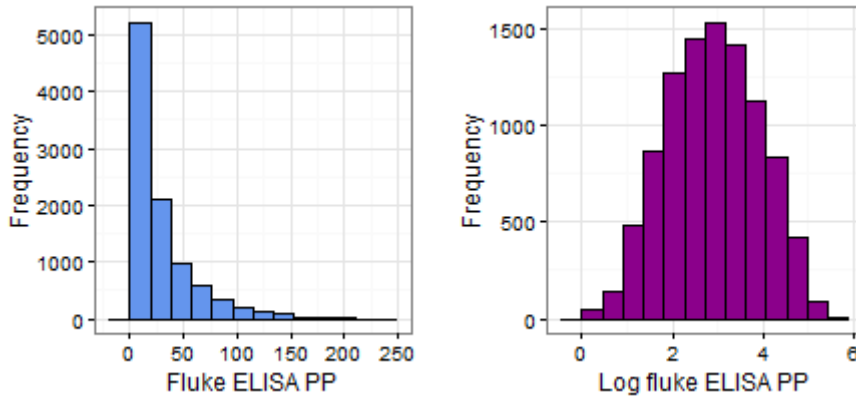


Figure 2.2. Distributions of the individual fluke ELISA PP results for 9763 samples from 5937 cattle from 30 UK beef and dairy farms

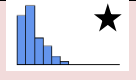
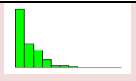
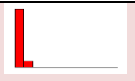
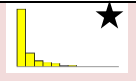

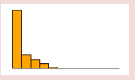
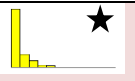

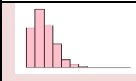

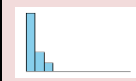
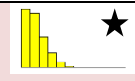

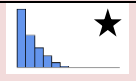

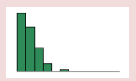

The PP results obtained by testing milk and serum had a similar median, but the range of PP values resulting from testing the milk samples was considerably greater (Table 2.2). The difference between the two groups was statistically significant (Wilcoxon signed rank test, $W = 8322500$, $p = 0.0004$).

Table 2.2 Summary statistics for the PP values obtained by testing milk and serum samples using a *Fasciola hepatica* excretory-secretory antibody detection ELISA

Method	Median	Mean	Range
Milk	17.0	29.4	0.0-229
Serum	16.8	24.1	0.7-125

The percentage testing positive for fluke in each herd ranged between 2 and 89%, with a median of 46%. Figure 2.3 shows the distribution of fluke ELISA PP results on each farm at each visit. All of the farms showed a right skewed distribution. The tails were longer in those farms where milk samples were tested, with the maximum values reaching 229 PP, whereas for serum samples the maximum was 135 PP.

Figure 2.3. Histograms showing the distributions of fluke ELISA PP results for all farms.

	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
2013												
2014						 	 		  	 	  	

(Continued overleaf)

	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
2015												
2016												

Legend

Enlarged histogram (left): The x axis was the same scale for all charts, whilst the y axis represents density.

Each farm had either milk samples taken from lactating cattle or blood samples from cattle > 24 months.

Farms where serum samples are taken were visited in 2013-14 and have a pink background. Farms where milk samples were taken were visited in 2015-16 and have a blue background.

Each farm is a different colour. Farms which were sampled more than once are shown by ★

2.3.3 Comparison of the bulk milk tank and individual results

A positive bulk milk tank ELISA indicates that at least 25% of individuals in the herd are positive, which corresponds to the cow at the 75th percentile being positive. When bulk milk tank results were plotted against the ELISA PP result of the cow at the 75th percentile, a difference was seen between milk and serum samples. The bulk milk tank result, if positive, indicates that at least 25% of the herd are infected. In figure 2.4A it can be seen that there is a high level of agreement between the individual and the bulk milk tank results for milk samples. For serum samples however, the bulk milk tank result is higher than would be expected given the individual results. A similar effect is seen for the percentage of fluke positives on the farm for three of the serum-tested farms (Fig. 2.4B). In spite of this, in all but one serum-tested farm, use of the bulk milk tank result would have given the correct result in terms of whether or not the herd was positive.

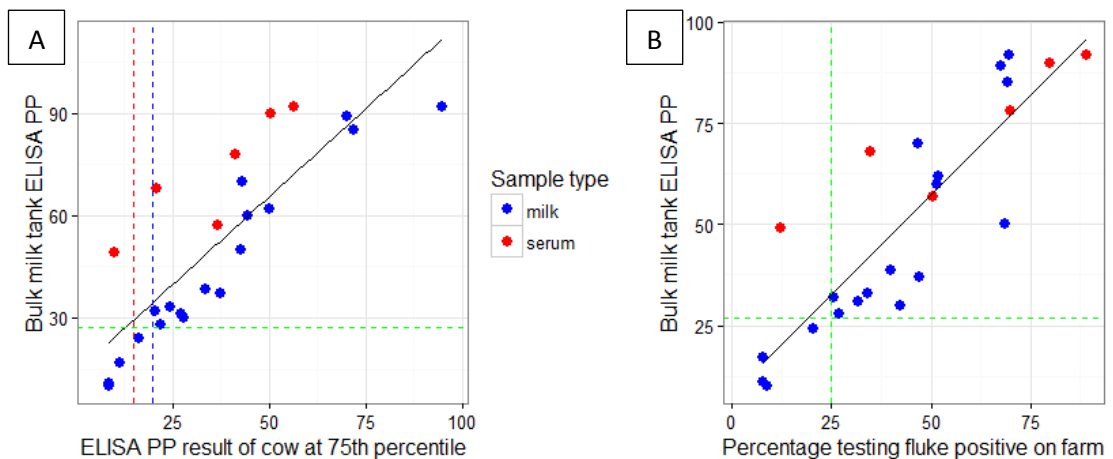


Figure 2.4. The result of the bulk milk tank ELISA is an accurate predictor for defining farms where at least 25% of cattle test positive for fluke on milk ELISA, but tends to over-estimate the number testing positive on serum ELISA. The sloped line is a regression line, the green dotted line indicates the cut off for the bulk milk tank ELISA, the red dotted line shows the cut off for the serum ELISA and the blue dotted line is the cut off for the milk ELISA. (A) shows the ELISA PP of the cow at the 75th percentile on each farm (as a positive BMT ELISA indicates that this cow should be positive). (B) shows the percentage of cattle testing fluke positive on each farm

One possible explanation for this discrepancy is that the herds that were serum-tested included non-lactating animals such as dry cows, heifers that had not yet calved and bulls, and that the lactating animals contributing to the tank had higher fluke PP values than the dry animals. Lactation information was available for one farm, so non-lactating animals were removed from the analysis. However, this did not alter the results as the PP values were similar for the dry and lactating groups of animals, and in any case only a small proportion of the cattle were not part of the milking herd.

Another possibility is that the serum ELISA is consistently under-diagnosing fluke-infected animals. Faecal egg count data were available to compare with ELISA data taken on the same day from one of the farms, so these were used to compare the two sets of results (Table 2.3). This showed that only 2.3% of cattle that were positive on faecal egg count were negative on ELISA. Therefore it does not seem likely that the serum ELISA is making a large number of false negative diagnoses.

Table 2.3. Results of fluke ELISA and faecal egg counts for cattle from one of the farms

	Faecal +	Faecal -
ELISA +	29	73
ELISA -	4	67

2.3.4 Seasonality

A variable intercept model was built with log of fluke ELISA PP (log PP) as the outcome variable. The model was as follows:

Seasonality model				
Outcome: Log PP				
Fixed effects: Month, sample type				
Random effects: Farm, cow				
	Co-efficient	Std Error	t value	<i>p</i>
Intercept	2.795	0.141	19.86	<0.00001
Cosmonth	-0.009	0.012	-0.76	0.45
Sinmonth	-0.281	0.014	-20.12	<0.00001
Serum sample	-0.154	0.215	-0.72	0.47
AIC = 23566, deviance = 23552, df=9666.				
Random effects: Variance between farms = 0.33				
Intraclass correlation within farms = 0.35				
Variance between cows = 0.38				
Intraclass correlation within cows = 0.31				

The predicted values were slightly lower for serum than for milk (Fig. 2.5), although the difference in the observed values was not statistically significant in the model ($p = 0.47$).

Month was a highly significant predictor for log PP. The predicted PP values were plotted along with the median observed values for each month (Fig. 2.5). The observed results were somewhat distorted by the fact that all animals from each farm were all sampled at once, for example, several farms with little fluke infection were tested in June, resulting in the relatively low median seen for June, whereas only one very flukey farm was sampled in August causing a high median result. Nonetheless, the model is an approximation for the data, with the highest PP values occurring in late summer/early autumn, and the lowest values in late winter/early spring.

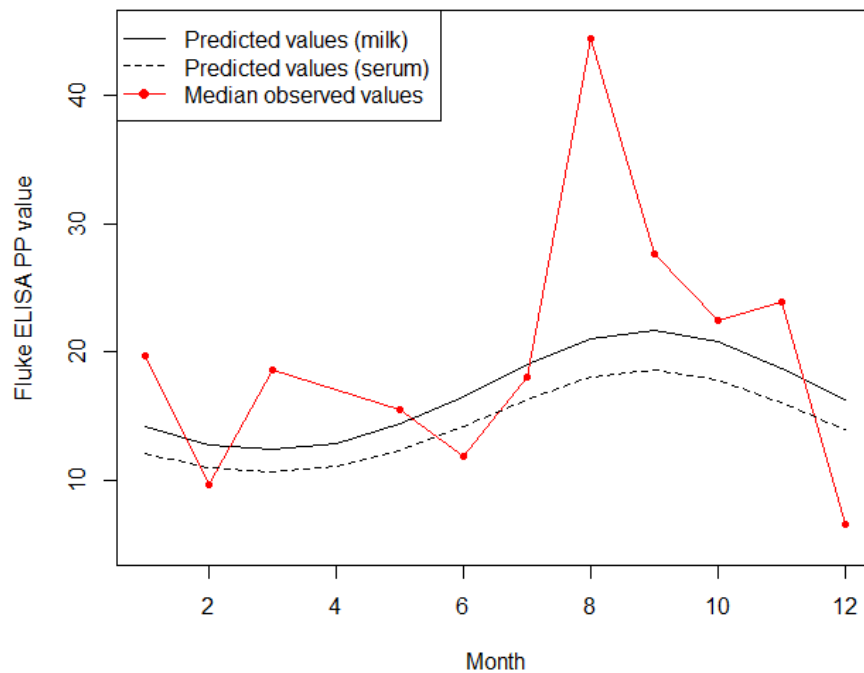


Figure 2.5. The predicted log fluke ELISA PP values from the multi-level linear regression model and the observed median PP values for each month. The type of sample tested is shown. The difference between serum and milk samples was non-significant.

2.3.5 Age

Ages of cattle were available for 12 of the serum tested farms, which had a total of 2020 animals tested. Ages were missing for 171 of these cattle, and these were spread throughout the farms. The age distribution was right skewed, with the oldest cow being over 17 years of age (Fig. 2.6).

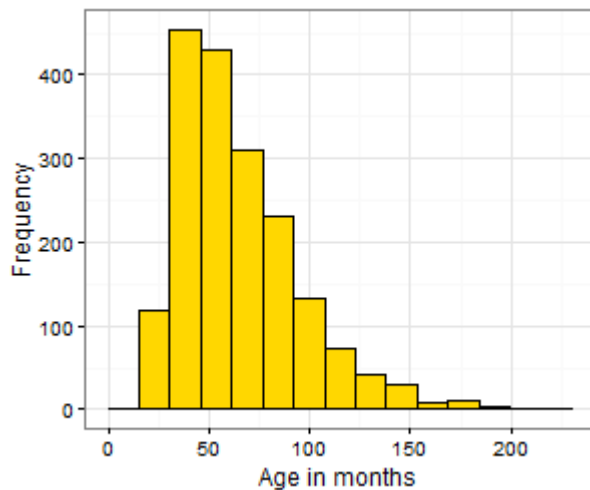


Figure 2.6. The age distribution of cattle tested for fluke.

Age was categorised into five groups to check for a linear relationship with fluke. There appeared to be a linear relationship, with higher ages associated with a higher fluke PP value, but this was non-significant.

A mixed model was built with log PP as the outcome variable, age in months as a fixed effect, and cow within farm as level 2 and level 3 random effects. Month was also included as a fixed effect. The age of the cow in months was not a significant predictor for fluke PP ($p = 0.05$).

Age model

Outcome: Log PP

Fixed effects: Age, month

Random effects: Farm, cow

	Co-efficient	Std Error	t value	<i>p</i>
Intercept	2.68	0.21	12.64	<0.00001
Cosmonth	0.50	0.0007	1.11	0.27
Sinmonth	0.11	0.05	9.82	<0.00001
Age (months)	0.0007	0.06	1.96	0.05

AIC = 4200, deviance = 4186, df=1842.

Random effects: Variance between farms = 0.51

Intraclass correlation within farms = 0.26

Variance between cows = 0.28

Intraclass correlation within cows = 0.46

2.4 Discussion

The distributions of PP values overall and within each herd were skewed to the right, with most animals having a low PP value and only a small number having a high value. This was seen across all herds, even in farms with 60-80% of animals testing positive. PP value and fluke burden are correlated (Charlier et al., 2008; Salimi-Bejestani et al., 2008), so the observed distribution may reflect the fact that even in herds where most animals are infected, most animals have only a light parasite burden. This is supported by other research on fluke numbers at post mortem (Byrne et al., 2016; Charlier et al., 2008) and using faecal egg counts (Chaparro et al., 2016). Some caution is needed in interpreting very high antibody levels because there are many other factors that may affect them, particularly in milk. However, fluke antibody levels have been shown to be negatively associated with milk yield (Charlier et al., 2005; Mezo et al., 2011), so provide an indication of the relative effect that the parasite is having on the cow's health. Animals with high and low parasite burdens may not be affected in the same way, and a quantitative assessment of antibody levels may give an indication of this, which is potentially useful in interpreting the results of co-infection studies.

The distribution was significantly different between the milk and serum tested cattle due to the higher maximum values seen in the milk test results, although the median values were very similar for both tests. The concentration of antibodies in serum is much higher than in milk, and this is reflected in the different dilutions used for the samples in the ELISA, so the results are not directly comparable. In addition, antibody levels in milk vary depending on the time of lactation and milk yield, which may contribute to the extreme high PP values.

The finding that the bulk milk tank ELISA PP was higher than expected given the individual serum ELISA results was interesting. During the development of the bulk milk tank ELISA it was compared against serum ELISA results and faecal egg counts (Salimi-Bejestani et al., 2005a). We did not have paired milk and serum samples or paired milk and faecal samples for any of the farms in this study, but comparison with the faecal egg count data available from one of the study farms indicates that the serum ELISA has high sensitivity. It is known that the specificity of both of the milk ELISAs are lower than that of the serum ELISA (80-88% compared to 96%), whilst the sensitivity of all three tests is similar (Salimi-Bejestani et al., 2007, 2005a). The larger range of PP values and more extreme high values seen in individuals from the milk tested farms will affect the bulk milk tank result and are likely to

lead to some false positives, since a positive herd result is interpreted as more than 25% of the herd being infected.

For the purposes of screening herds, all except one herd fell into the correct positive/negative category. For epidemiological purposes, if a single test is used for all study subjects, the slight difference between the ELISA results should not introduce bias, however it does highlight the importance of being aware of potential differences of performance between tests.

The pattern of seasonality seen in condemnations of cattle livers at slaughter shows a peak in around January each year, with a trough in July (Skuce and Zadoks, 2013), and juvenile fluke are found only in autumn, indicating that this is when infections are occurring (Charlier et al., 2008). The peak seen here is a little earlier, and this could be partly because the ELISA detects infections from two weeks post infection (Salimi-Bejestani et al., 2005b), before the fluke become large enough to cause condemnation of the liver, which may not occur until 1-2 months post infection.

The seasonal variability seen in ELISA PP in the current study is relatively small, with a difference between the predicted peak and trough of around 10 PP. This may reflect the lag period between decrease in liver fluke numbers (occurring due to treatment and fluke deaths without re-infection) and decrease in antibody levels, which remain elevated for some weeks after cure, by which time some animals may have become re-infected (Skuce and Zadoks, 2013). In high fluke areas, practitioners have observed that all grazing animals have a constantly elevated antibody level as a result of ongoing exposure, regardless of current infection status (Skuce and Zadoks, 2013). In addition there is considerable noise in the data due to the variation between individuals which may depend on other factors such as production stresses, food sources, grazing rotations and flukicide treatments.

Fluke antibody levels showed an increase with age, which approached significance. Other studies report increasing fluke burdens with age (Chaparro et al., 2016; Gonzalez-Lanza et al., 1989), and this is likely to reflect ongoing exposure without the development of protective immunity (Torgerson and Claxton, 1999).

Test performance and differences between tests, differing ages of cattle within a herd, the time of year that samples are collected and the variation in the effects of fluke infection between individuals should all be considered when interpreting the results of epidemiological studies on fluke.

Chapter 3: Can the effect of *Fasciola hepatica* infection on the bovine TB skin test be evaluated in naturally infected animals using on farm studies?

Abstract

Bovine tuberculosis is one of the most challenging diseases faced by the UK cattle industry. Laboratory studies have shown that liver fluke infection may reduce the sensitivity of the bovine TB skin test, the main component of the mandatory control programme. The aim of this study was to determine whether infection with liver fluke, as measured by antibody detection ELISA, affects the outcome of the skin test, in naturally exposed animals on UK farms. Whole herds were blood sampled at the same time as the statutory TB test was performed, and in a separate study, milk samples were obtained from TB reactors and controls in dairy herds. No significant difference in fluke ELISA results was seen between TB reactors and controls. However, in this study it was difficult to recruit farmers willing to take part in the study, and as a result the study was underpowered.

3.1. Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease caused by *Mycobacterium bovis*. It is one of the most costly diseases affecting the UK cattle farming industry (DEFRA, 2015). A mandatory bTB control programme has been in operation in the UK since the 1950s, and initially this was successful in bringing bTB under control, with the nadir of bTB cases occurring in the early 1980s, and most bTB cases concentrated in the south west of England (TB Free England, 2015). Since then, bTB has been on the increase and is now endemic in South Wales, most of Southern England and the Midlands (Department for Environment Food and Rural Affairs, 2014c). However, clinical cases of bTB in cattle are rarely seen in the UK due to the control programme, and the public health risk is very low, as pasteurisation of milk kills *M. bovis* (de la Rúa-Domenech, 2006).

Changes in the cattle industry, such as larger herd sizes, more intensive conditions and more cattle movement around the country (particularly as a result of restocking following

the foot and mouth epidemic of 2001) are likely to have contributed to this rise (Gilbert et al., 2005; Griffin et al., 1993). The badger is the main wildlife reservoir, and the increasing protection of badgers in law from the late 1980s onwards is also likely to have had a significant adverse effect on the control of bTB, as this has led to a large increase in the badger population, inevitably leading to more contact with farm animals (Bessell et al., 2012; Biek et al., 2012; Donnelly and Nouvellet, 2013; Griffin et al., 2005; Johnston et al., 2011).

The control programme involves compulsory testing of all cattle over 6 weeks of age at intervals of 1-4 years depending on whether they are in a low or high risk area. The high risk areas are in the South West and West Midlands in England, with an edge area bordering this region, where regular testing is also performed. Annual testing is also performed in the whole of Wales although most bTB is diagnosed in South Wales and the border areas of Mid Wales (TB Hub, 2016). Tests are performed by private veterinary surgeons acting on behalf of the government. The single intradermal comparative cervical tuberculin test (SICCT) is used, which entails injecting bovine and avian tuberculin purified protein derivative (PPD) respectively into two sites on an animal's neck, following measurement of skin thickness. The avian tuberculin increases the specificity of the test by acting as a control, as exposure to environmental mycobacteria strains can cause a non-specific reaction to the test. The skin thickness is measured again 72 hours later, and animals are classed as TB reactors, inconclusive reactors (IRs) or clear, based on the difference in skin thickness at the two sites. Tests can be read as 'standard' or 'severe'; which interpretation to use is decided by DEFRA depending on the situation. A smaller difference in skin thickness is required to classify an animal as a reactor when using the severe interpretation. The severe interpretations also differ between England and Wales, with the Welsh version being more stringent (APHA, 2014a, 2014b).

The SICCT can be technically challenging to administer due to fractious animals, poor handling facilities and time pressure. It is also a subjective test, depending on the consistency of the operator in measuring the skin thickness (Northern Ireland Audit Office, 2009; Strain et al., 2011). The sensitivity of the SICCT has been variously estimated at between 56% and 90% at the standard interpretation, with a specificity of 99.5-100% (Ameni et al., 2000; Clegg et al., 2011; Costello et al., 1997; Gonzalez Llamazares et al., 1999; Karolemeas et al., 2012). At the severe reading, sensitivity is increased to around 77-85% whilst specificity is decreased to 99.2% (Clegg et al., 2011; Karolemeas et al., 2012). Low sensitivity in the SICCT can result from a lack of response in the early and advanced

stages of disease, interference from exposure to environmental mycobacterial species, and factors leading to immunosuppression such as the peri-partum period, corticosteroid treatment, production related stress, and co-infection with other pathogens (de la Rua-Domenech et al., 2006; Pollock and Neill, 2002; Strain et al., 2011).

Cattle that test positive on the SICCT are slaughtered and infection status is confirmed by visualisation of lesions, culture and histopathology. In the UK, no TB testing may be carried out unless under the direction of the Animal and Plant Health Agency (APHA). Thus we were not able to use ancillary TB tests (such as interferon gamma or antibody tests) for this study.

Fasciola hepatica (liver fluke) is a parasite of cattle, causing health and welfare problems as well as financial losses. Immunity to *M. bovis* requires a T helper (Th) 1 type immune response, with interferon gamma production (Flynn et al., 1993). This cellular response is also required for diagnosis of bTB using the SICCT, and animals become non-responsive to the SICCT during active disseminated bTB when a Th2 response predominates (Strain et al., 2011). *F. hepatica* has been shown to modify the immune response of cattle infected with *M. bovis* or BCG towards a Th2 type response, with decreased production of interferon gamma and interleukin 4 (Flynn et al., 2009, 2007; Garza-Cuartero et al., 2016). This has implications for the SICCT, and an effect has been demonstrated in experimentally infected calves, where animals co-infected with fluke and *M. bovis* or BCG had a smaller response to the SICCT than those infected with *M. bovis* only (Claridge et al., 2012; Flynn et al., 2007).

These findings show that *F. hepatica* has an effect both on the immune system and on the SICCT, under controlled conditions. In naturally exposed cattle, the balance of the cellular and humoral immune responses in those co-infected with bTB and fluke is likely to be variable (Strain et al., 2011), and to depend on factors such as the magnitude of the infectious dose, the effect of ongoing exposure, other undiagnosed infections, and production or reproductive stresses. Therefore, it is desirable to determine whether the effect of fluke on the SICCT observed under experimental conditions is also seen in naturally exposed cattle. Field studies have shown a significant negative association between herds diagnosed with *F. hepatica* and bTB (Claridge et al., 2012), but these findings have not been confirmed in individual animals. The aim of this study was to determine whether infection with liver fluke, as measured by antibody detection ELISA, affects the outcome of the SICCT, in naturally exposed animals on UK farms.

3.2 Methods

3.2.1 Cross sectional study

3.2.1.1 Selection of farms and sample collection

A sample size calculation showed that to have 80% power and 95% confidence to detect an odds ratio of 0.5 for a fluke infected cow having TB compared to a fluke uninfected cow, and with a TB prevalence within herds of 5% and a fluke prevalence of 30%, 71 bTB positive animals and 1420 bTB negative animals would be required

(<http://powerandsamplesize.com/Calculators>, Table 3.1). This assumed that the animals were not clustered within herds, however, as bTB is a relatively rare event and positive animals are removed from the herd prior to the next test, this is a reasonable assumption as long as herds all have a recent history of bTB, and other factors such as herd size and month of sample collection are taken into consideration. From previous discussions with veterinary surgeons in high risk areas, it was estimated that reaching this sample size might require testing 10-20 purposively selected herds.

Table 3.1. Details of the sample size calculation

Odds ratio of TB in fluke infected vs. fluke uninfected	0.5
Expected percentage of controls exposed to fluke	30%
Expected bTB prevalence	5%
Power	80%
Alpha	5%
Number of bTB negative animals required	1420
Number of bTB positive animals required	71

Veterinary surgeons with practices in high risk areas for bTB were asked to identify farmers who might be willing to participate in the study. Farmers were then contacted and given information about the study, before they decided whether to take part. Participating farms were visited between November 2013 and December 2014, at the time of the herd TB test, and all cattle over the age of 24 months were blood sampled from the tail vein. This was done under Home Office licence PIL 40/3621 and was approved by the University of Liverpool Ethics Committee ref VREC290. The TB test results (clear, reactor or IR) were obtained from the veterinary surgeon. For the purposes of this study, all of the results were used according to the interpretation (standard or severe) applied at the time of the test. Due to the lower than expected prevalence of bTB in the sampled cattle, the required

number of bTB cases was not reached, as time and funds did not allow further herds to be tested.

3.2.1.2 Testing of samples

At the University of Liverpool, the samples were centrifuged at 1000 g for 20 minutes and the serum separated off for testing. Serum was used to test for *F. hepatica* antibodies using an excretory-secretory ELISA (Salimi-Bejestani et al., 2005, see Chapter 2 for details).

Results obtained were a percent positivity (PP) of a known positive control, and PP values of 15 or above were considered to be positive.

An IgG isotype ELISA was used to compare *F. hepatica* specific IgG1 and IgG2 ratios in four reactors and four control (clear) animals. This was done using the same method as for the serum ELISA, with the following modifications: a separate ELISA plate was used for each isotype, and samples were added to the plate in decreasing concentrations. Polyclonal IgG1 and IgG2 conjugates were used (Sheep anti-bovine IgG1 horse radish peroxidase (HRP) and Sheep anti-bovine IgG2 HRP, Biorad, catalogue nrs AAI21 and AAI22). The conjugate concentrations were optimised using a checkerboard, and were 1 in 20,000 for IgG1 and 1 in 2500 for IgG2.

The mean optical density (OD) of the negative control was subtracted from the OD of the test samples, and the concentration of the last positive titre was recorded for each sample on each plate. This was used to calculate the ratio of IgG1 to IgG2 for each of the samples.

3.2.1.3 Analysis

Analysis was performed on both reactor and IR animals using R (R Core Team, 2011). Plots, summary statistics and logistic regression models were used to compare bTB diagnoses between fluke infected and uninfected animals. The month of sample collection modelled as a sinusoidal function ($\cos(2\pi \cdot \text{month}/12) + \sin(2\pi \cdot \text{month}/12)$), the age, sex and breed of cattle, and herd size and type were included as explanatory variables. These were added in different combinations and the best model was considered to be that with the lowest AIC. A longitudinal analysis was performed on data from one farm which was sampled three times.

Bootstrapping analysis was performed on the collected data to estimate the power of the current study and the sample size that would have been required under the observed bTB prevalence:

y_i (the probability of a cow i being diagnosed with bTB) was simulated for each cow in the dataset as follows:

y_i was drawn from a Bernoulli distribution of π_i , where $\text{logit}(\pi_i) = \alpha + x_i\beta$

Logit (π_i) is the log odds of cow i having bTB, α is the log odds of a fluke negative cow having bTB, x_i is the fluke positivity (0 = negative, 1 = positive) of cow i , and β is the coefficient for fluke infection.

A generalised linear regression model was constructed using these simulated values of Y_i . The simulation and model were run 1000 times and the p value of β recorded each time. The proportion of significant β values is an estimate of the power.

Values of α and β were varied. α depends on the prevalence of bTB whilst β depends on the magnitude of the effect of fluke.

This method was first used to calculate the power of the dataset to detect a significant difference with the values of α and β as found in the observed data (the output of the logistic regression model). Next, the effect size that could be detected by the sample size reached in the current study was estimated, and finally, by sampling with replacement from the collected dataset, the required sample size needed to detect the observed effect size at the observed prevalence was calculated.

3.2.2 Case control study

3.2.2.1 Selection of farms and sample collection

In a separate study, milk samples were obtained from reactor cattle and an equal number of controls from the same herd. Due to the difficulty recruiting participants for the cross sectional study, a different approach was taken to obtain samples for this study. Obtaining milk samples through a milk recording company meant that the participating farmers would remain anonymous to the research team, which, it was hoped, would remove one of the barriers to recruitment. Although it is unlikely that any of the farms in the cross sectional study sent in samples for this study, the anonymous nature of the recruitment means that it is a possibility.

The study was publicised through the milk recording company newsletter and through collection staff visiting farms. Interested farmers were then sent a pack containing consent form, sample collection tubes, bronopolnatamyin (MSI, Nottingham) tablets to add to

samples, and were asked to take samples from reactor cattle at or shortly after the next TB test, plus from a control animal, which should be the next one to enter the crush or race. Alternatively, if recording staff were visiting farms shortly after a TB test whilst reactor cattle were still on the farm, they took the samples then.

From the sample size calculation in Claridge (2012), 167 matched pairs were required. Due to difficulty recruiting farmers, the required sample size was not reached.

Samples were tested at University of Liverpool using an ELISA to detect antibodies to *F. hepatica* excretory-secretory products (Salimi-Bejestani et al. (2007), see chapter 2 for details).

3.2.2.2 Analysis

Following exploratory analysis, a logistic regression model was built with TB test result as the outcome measure, and month of sample collection (modelled as a sinusoidal function ($\cos(2\pi \cdot \text{month}/12) + \sin(2\pi \cdot \text{month}/12)$) and log fluke ELISA PP or fluke positivity as explanatory variables.

3.3. Results

3.3.1 Cross sectional study

1582 cattle from 13 UK dairy and beef herds were sampled. Of these, two farms were visited twice and one farm was visited three times. In total, 2089 samples were taken.

There were a total of nine reactor cattle from two farms, one of which was visited twice. Twenty-seven IRs were found on six different farms, one of which was visited three times. The characteristics of the farms are shown in Table 3.2.

Table 3.2. Characteristics of the study farms

	Number (%)
Total samples tested	2089
Number of herds	13
Median herd size	133
Type	9 dairy farms 4 beef farms
Reactors	9 (0.43%)
IRs	27 (1.3%)
Number testing positive for fluke	1101 (53%)
Median fluke PP	16.8

The distribution of the fluke ELISA PP results for the clear, IR and reactor cattle is shown in Figure 3.1. The median of the clear, IR and reactor cattle are 17, 7.5 and 12.8 respectively.

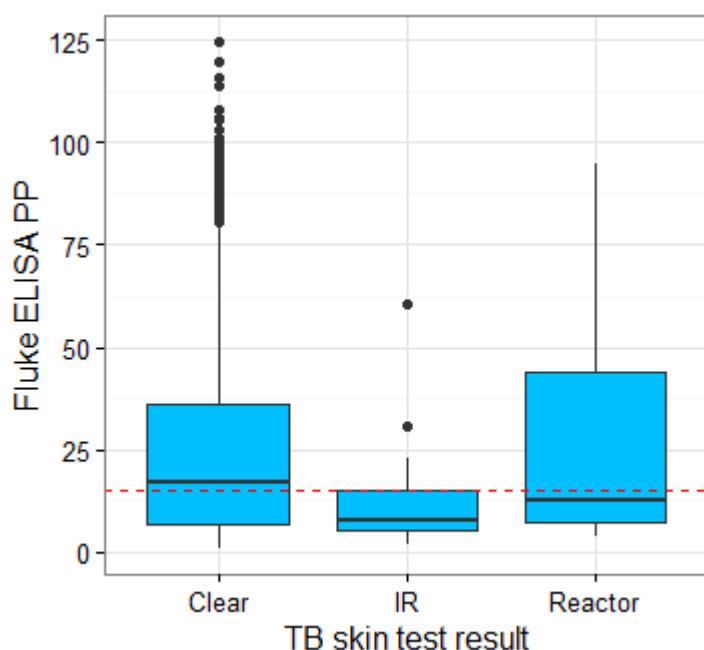


Figure 3.1. The distributions of fluke ELISA PP results for cattle that tested clear, IR and reactor for bTB, on farms where all animals over 2 years of age were sampled. The red dotted line shows the positive/negative cut off

The best logistic regression models in all four combinations of reactor, IR, fluke positivity and log fluke ELISA PP included the month of sample collection as a significant explanatory variable, but fluke was not a significant predictor for either R or IR in any model once the month of sample collection was adjusted for (Table 3.3). Inclusion of other variables such as age, breed and sex of cattle and type and size of herd did not improve model fit.

Table 3.3. Results of logistic regression models for TB reactor and IR status. All models are adjusted for month of sample collection, modelled as a sinusoidal function ($\cos(2\pi \cdot \text{month}/12) + \sin(2\pi \cdot \text{month}/12)$)

Outcome variable	Explanatory variable	Odds ratio	p value
TB reactor	Fluke positivity (positive = 1)	0.53	0.38
TB reactor	Log fluke ELISA PP	1.05	0.89
IR	Fluke positivity	0.54	0.18
IR	Log fluke ELISA PP	0.74	0.11

2.4.1.1 Longitudinal analysis

Follow up of cattle with a reactor or IR diagnosis from one farm which was visited three times, during May, July and October 2014, showed that for most animals, fluke status did not change. Four of the animals with a change in bTB status during the study period had a

change in fluke status. Of these, two occurred at the same time as a change in bTB status: one animal went from fluke positive to negative and the other went from fluke negative to positive, whilst both changed from IR to clear. Two other cattle went from negative to positive but this did not coincide with a change in fluke status. The other cattle remained either positive or negative for fluke throughout (Fig. 3.2).

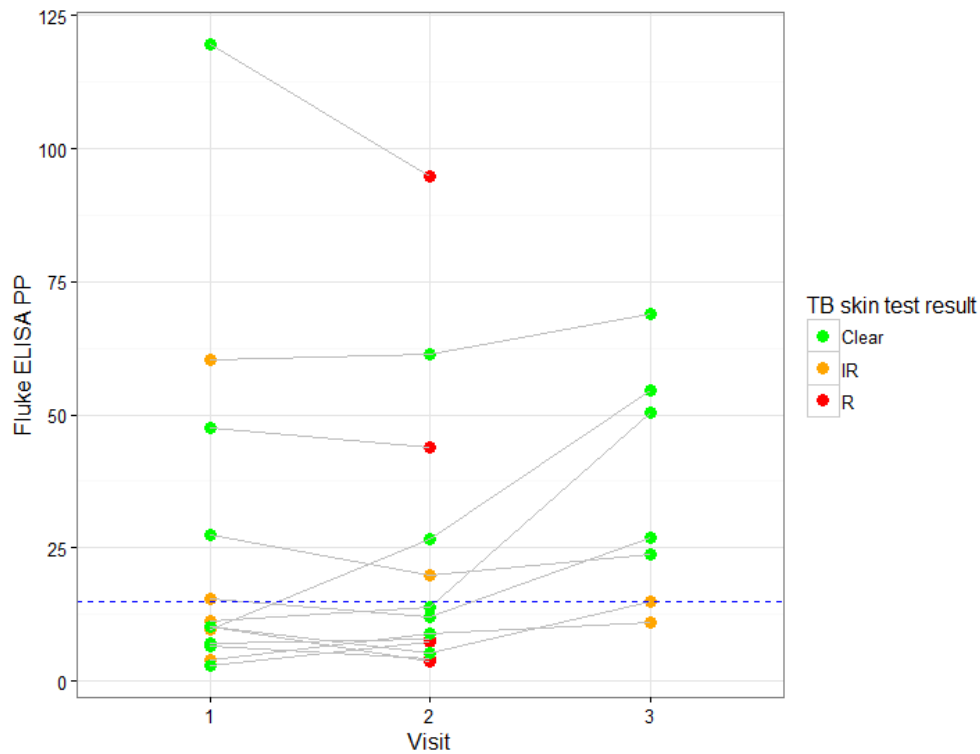


Figure 3.2. Fluke ELISA PP results for individual animals that were reactors or IRs at one of the TB tests, from one farm sampled three times on visits 60-90 days apart, during May, July and October 2014. The blue dotted line shows the positive/negative cut off

Similarly, fluke ELISA PP results did not change substantially between visits. Between the first two visits, mean PP values fell for the herd as a whole and also for most of those having a change in bTB status, with the exception of the group of cattle which went from IR to C (n=5), whose PP values rose slightly (Fig. 3.3). Between the second two visits mean fluke PP increased for all the groups.

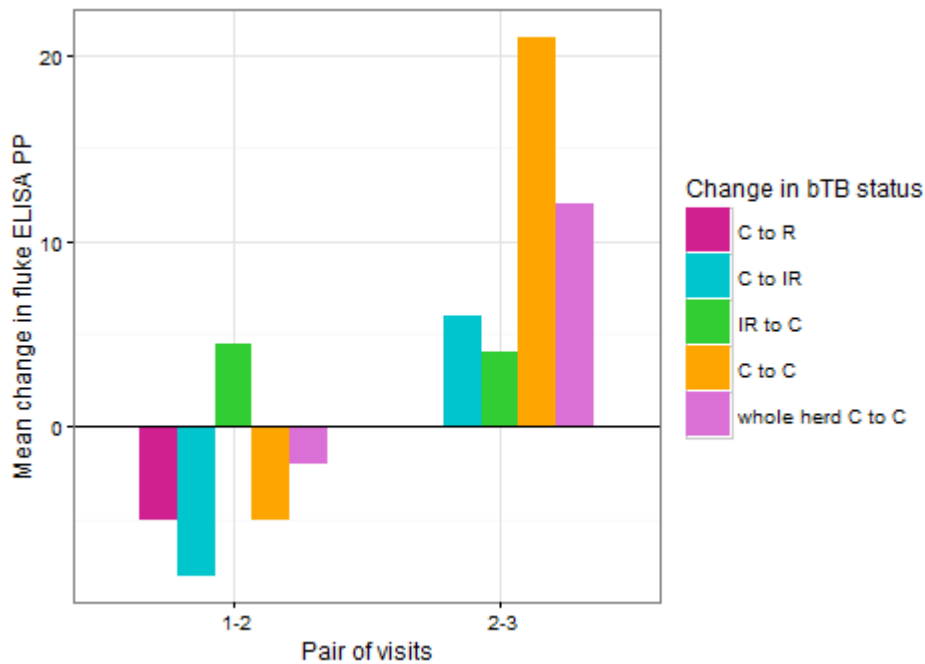


Figure 3.3. The mean change in fluke ELISA PP values between each pair of visits, for cattle with a change in bTB status and for the rest of the herd. R=reactor, I=inconclusive reactor, C=clear

2.4.1.2 Bootstrapping analysis

Bootstrapping analysis performed on the collected data showed that the study had an 11% power to detect an odds ratio of 0.44 with an apparent bTB prevalence of 0.44%, which were the values derived from the logistic regression coefficients of $\alpha = -5.08$, $\beta = -0.82$.

Next, the effect size that would be required to be detectable by a sample size of 2089, with an apparent prevalence of 0.44% was estimated. However, in this scenario, it was not possible to reach 80% power to detect even an odds ratio of 0.05, and the maximum power possible was around 50%.

A sample size of around 10,000 cattle would be needed for 80% power for the observed effect size and prevalence.

3.3.1.2 IgG isotype comparison

Four TB reactor animals were each matched with an animal from the same farm with a similar fluke ELISA PP result. Three of the pairs were fluke positive and the fourth was fluke negative because there were no further fluke positive reactors. They were also matched as closely as possible on age. The IgG1 titres were the same for each pair of reactor and control, but the IgG2 titres were higher in the controls than in the reactors apart from in

the fluke negative pair. This meant that in all cases the IgG1:IgG2 ratio was higher in the reactors than in the controls, except for the fluke negative pair (Table 3.4).

Table 3.4. Details of the cattle and samples used in the IgG isotype comparison

Samples	Fluke ELISA PP	Age (months)	IgG1 titre	IgG2 titre	IgG1:IgG2 ratio
TB R 1	44	50	1 in 12800	1 in 6400	2
Control 1	45	unknown	1 in 12800	1 in 12800	1
TB R 2	95	74	1 in 25600	1 in 6400	4
Control 2	94	56	1 in 25600	1 in 25600	1
TB R 3	44	36	1 in 3200	1 in 3200	1
Control 3	44	36	1 in 3200	1 in 12800	0.25
TB R 4	13	45	1 in 1600	1 in 1600	1
Control 4	14	40	1 in 1600	1 in 1600	1

3.3.2 Case control study

Seven dairy farms took part in the study (Fig. 3.4). Seventy one samples were obtained, 36 from reactors and 35 from TB test negative controls. Twenty nine (41%) of cattle tested positive for fluke on the ELISA, and this varied between 0 and 100% across the farms (Table 3.5).

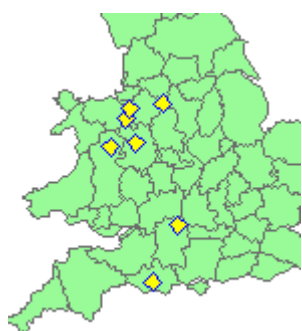


Figure 3.4. The approximate locations of the seven farms which took part in the study

The distributions of the fluke ELISA PP results were similar for the reactor and control groups (Fig. 3.5). The median fluke ELISA PP of the reactors was 12.3 and the median of the controls was 9.6.

Table 3.5. The numbers of cattle testing positive for fluke and bTB on each farm

Farm	Number of TB reactors that tested positive for fluke	Number of controls that tested positive for fluke	Number of samples submitted
1	7 (78%)	8 (89%)	18
2	1 (50%)	0 (0%)	4
3	0	0	12
4	2 (40%)	2 (50%)	9*
5	3 (100%)	3 (100%)	6
6	1 (50%)	2 (100%)	4
7	0	2 (20%)	20
All farms	14 (39%)	17 (49%)	71

*One sample was lost in transit so farm 4 had 5 cases and only 4 controls. Other farms had equal numbers

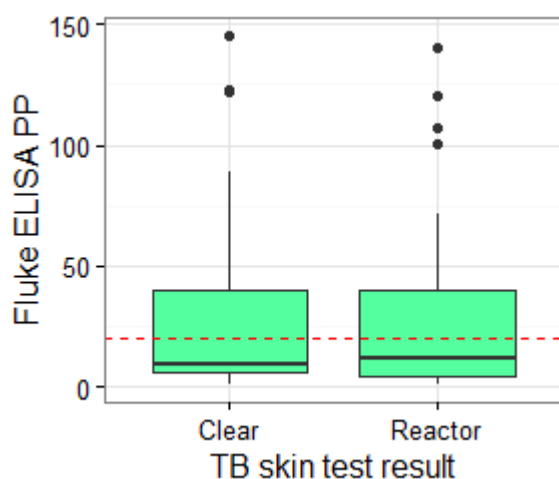


Figure 3.5. Fluke ELISA PP values for cattle testing positive or negative in the bTB skin test. The red dotted line shows the cut off for the ELISA

Logistic regression models showed that neither fluke ELISA PP nor fluke positivity were significant predictors for bTB when month of sampling was adjusted for (Table 3.6).

Table 3.6. Results of logistic regression models for TB reactor status. Both models are adjusted for month of sample collection, modelled as a sinusoidal function ($\cos(2\pi \cdot \text{month}/12) + \sin(2\pi \cdot \text{month}/12)$)

Outcome variable	Explanatory variable	Odds ratio	<i>p</i> value
TB reactor	Fluke positivity (1=positive)	0.64	0.38
TB reactor	Log fluke ELISA PP	0.93	0.70

3.4 Discussion

In this study we attempted to use SICCT results and fluke antibody ELISA results from naturally exposed animals to test the hypothesis that fluke infection reduces the response to the SICCT in bTB infected animals. No significant difference was seen in bTB test outcome between animals with different fluke results once month of sample collection was adjusted for. It was important to adjust for this because season has an effect on both bTB diagnosis (Moustakas and Evans, 2016) and fluke infection and failing to do so could lead to confounding.

Although our study was underpowered, if there had been a strong effect of fluke infection on bTB diagnosis, we would expect a consistent trend to be seen. In all but one model, the odds ratio for a cow with fluke antibodies to be diagnosed as a reactor or IR compared to a cow without fluke (or with a lower antibody titre) was less than one (Tables 3.3 and 3.6). Although none of the models were statistically significant, the results do suggest that fluke infected cows may be less likely to be reactors to the SICCT, although a larger sample size is required for this effect to become statistically significant. This finding is also supported by other studies which found subtle effects, for example, in a study where cattle were experimentally infected with controlled doses of *M. bovis* and fluke, the reduction in skin reactions observed in fluke infected cattle, although consistent, was not sufficient to lead to any of the cattle being reclassified from reactor to clear (Claridge et al., 2012).

The longitudinal study did not provide much useful information because the fluke ELISA PP values of most of the cattle did not change much between visits. Only four cattle changed fluke status across the three visits, and two of these did not have a coincidental change in bTB status. Due to the milk withdrawal times for flukicidal drugs, treatment during the dry period is the most practical approach for controlling fluke in dairy cows, and this was practised on the farm from which these samples came. As it happened, none of the reactor or IR cattle were treated during the study period, although two were treated shortly before the start of the study period and their fluke results remained negative throughout. Treating infected cattle with a flukicide to ensure fluke PP values change before the next bTB test, together with a longer follow up period to allow PP values to rise again, may give more useful results in such a study in future, although this would only be possible for IRs as reactors would be removed from the herd. Using non-lactating cattle would allow more choice in the timing of flukicide administration for this purpose.

There was a difference in *F. hepatica* IgG isotype ratios with IgG1:IgG2 ratios being higher in the reactors than the controls. The anti-fluke IgG1 titres were the same in the reactors and fluke only infected cows and in 2/3 fluke only animals, the IgG1:IgG2 ratio was 1 (Table 3.4). But the IgG2 titres were consistently lower in the fluke infected reactors compared to the fluke only infected cattle. Although we did not test sufficient samples to reach statistical significance, these data suggest that there was an interaction between fluke and TB infection and a difference in the cytokine milieu in the co-infected animals that warrants further investigation.

In *Fasciola* infected cattle, there has been shown to be an increase in both IgG1 and IgG2, with IgG1 the dominant isotype, and the rise in IgG1 occurring earlier in the course of infection than the rise in IgG2 (Phiri et al. 2006; Bossaert et al. 2000). In vaccine studies, high levels of IgG2 were associated with protective immunity to fluke (Mulcahy et al., 1998). Conversely, in a study of cattle experimentally infected with *M. bovis*, there was a strong IgG1 response but only 1/6 animals showed any increase in IgG2 levels (Welsh et al., 2005). Similarly, in humans with TB caused by *M. tuberculosis*, IgG1 has been shown to be the predominant isotype, with no IgG2 upregulation evident (Hussain et al., 1995; Macedo et al., 2011). It appears that being infected with both *M. bovis* and liver fluke may downregulate the Th1 response even further than being infected with fluke alone.

Legal restrictions on bTB testing and the rapid removal of reactors from the farm for culling, meant that the only way to approach the study was to blood sample at the same time as the bTB test. Unfortunately, the sensitive nature of bTB, which carries a considerable stigma for farmers and can often mean economic ruin and even suicide, made it very difficult to recruit farmers to take part in the study (Spedding, 2009). The low uptake was in contrast to that seen in Chapter 5 for a study on Johne's disease, where all farmers who were approached agreed to take part. We relied on private veterinary surgeons to assist in recruiting farmers for the cross sectional study, and some stated that they did not want to add extra stress for their farming clients at a potentially difficult time. Reasons given by farmers for declining to take part in the study included not wanting to delay the TB test or cause additional stress to animals, and also confidentiality concerns and a feeling that the test procedure might be altered in the presence of researchers. These factors may have led to farms that were less likely to have bTB being included in the study, despite them being in high risk areas and having a recent history of bTB. Abattoirs dealing with reactors were also approached, but declined to be involved because of lack of time and health and safety concerns.

Since both bTB and fluke are chronic infections, they both, separately, bias the immune response towards a T-regulatory response (Bossaert et al. 2000; Bossaert et al. 2000; Wangoo et al. 2001; Flynn et al. 1995). There is also evidence that both liver fluke and pathogenic mycobacteria exert immunomodulatory effects on the host immune system (Arsenault et al., 2014; Balcewicz-Sablinska et al., 1998; Moreau and Chauvin, 2010). Our results, despite their limitations, suggest that fluke infection may reduce the sensitivity of the SICCT, and that the immune modulation seen in experimental studies is also observable in naturally exposed animals. This could have important effects both on the bTB control programme and on the overall health and welfare of cattle. Future research on this topic is still required, in particular to investigate the large effect reported by Claridge et al. (2012), who estimated that bTB was undetected by up to one third on farms with liver fluke. However, this may need to be done via official channels in order to gain access to the required information and samples. It would also be beneficial to look at post mortem examination results and alternative bTB tests such as the interferon gamma test, to understand any effect of fluke infection on the SICCT.

Chapter 4: A systematic review of the literature on co-infection with *Fasciola hepatica* and *Mycobacterium bovis*

Abstract

Liver fluke and bovine tuberculosis (bTB) are both common pathogens responsible for significant economic losses to farmers. Some research suggests that infection with liver fluke can decrease the sensitivity of the bTB skin test, but other studies have found the opposite effect. We undertook a systematic review of the literature on co-infection with these two pathogens and extracted data on the association between fluke infection and the bTB skin test, interferon γ test, lesion detection and culture/bacterial recovery. Of a large body of literature dating from 1950 to 2016, only nine studies met the inclusion criteria. These included studies of experimentally infected calves, case control studies on adult cows, cross sectional abattoir studies and a herd level study. All the studies had a medium or high risk of bias and there was a suggestion of reporting bias. Results should be interpreted cautiously as many of the results come from a small number of studies from the same group of authors. Much of the evidence points to liver fluke having the effect of decreasing all of the four measures of bTB diagnosis, but most studies showed a small and/or non-significant effect.

4.1 Introduction

4.1.1 Description of fluke

Fasciola hepatica, also known as the common liver fluke, and *F. gigantica* (the tropical liver fluke) are helminth parasites which have adverse effects on cattle health, welfare and production. The common fluke affects up to 80% of UK dairy herds (McCann et al., 2010) whereas the tropical fluke is widespread in tropical regions (Abunna et al., 2010; Howell et al., 2012; Malone et al., 1998). Fluke infection may cause illness, subclinical effects such as reduced milk yield and growth rates, and occasionally, acute deaths. All ages of cattle are affected as protective immunity does not seem to develop (Clery et al., 1996). Treatment is restricted in lactating animals, and re-infection will occur unless animals are housed or grazed on safe pastures. Animals grazing damp and boggy pastures which provide habitats for the respective intermediate host snails, *Galba truncatula* and *Lymnaea natalensis* are at

risk. In many respects the two flukes have a similar biology and pathology, but there are thought to be some differences in the immune response to infection (Roberts et al., 1997; Toet et al., 2014; Zhang et al., 2005).

Early infections with liver fluke are characterised by a mixed T helper (Th) 1 and 2 type immune response with upregulation of interferon (IFN) γ , immunoglobulin (Ig)G1, and interleukin (IL)4. The Th1 response fades later in infection and a Th2/regulatory T cell (Treg) response predominates, with upregulation of the cytokines IL4, IL5, IL13, transforming growth factor (TGF) β and IL10. It is thought that fluke have evolved to be able to moderate the host immune response in order to facilitate their own survival within the host (Altmann, 2009; Moreau and Chauvin, 2010). An anti-inflammatory response also has some benefits for the host, as a strong pro-inflammatory reaction would lead to tissue damage. Low level infections are well tolerated by cattle, although little is known about the impact on the host immune response.

4.1.2 Diagnosis of fluke

Diagnostic methods for fluke include faecal egg count, serum or milk antibody detection ELISA or detection of fluke at post mortem examination (Table 4.1).

Visualisation of fluke in the liver is considered the gold standard for fluke diagnosis. Specificity is excellent and if performed carefully, sensitivity should be high as well. In a commercial abattoir setting sensitivity may be considerable lower as the liver is not examined in as much detail (Rapsch et al., 2006). The antibody ELISAs are generally highly sensitive and specific. Serum testing is generally thought to be more reliable than milk testing, due to the fluctuations in milk antibody levels that occur throughout lactation (Chanlun et al., 2006; Schares et al., 2004). The bulk milk tank ELISA can be used to screen the herd for fluke exposure. Faecal egg count is a highly specific but relatively labour intensive diagnostic method. For most methods of fluke detection, sensitivity increases considerably as worm burden increases (Charlier et al., 2008). The sensitivity and specificity also vary depending on the population, and are usually lower in mixed groups of infected and uninfected animals than in the separated populations often used to evaluate the tests (Leeflang and Bossuyt, 2005; Ransohoff and Feinstein, 1978).

Table 4.1. A summary of some of the different diagnostic tests used for *Fasciola* spp.

	Sensitivity	Specificity	Comments	Ref
Faecal egg count	43-65%	90-100%	Does not become positive until 7-10 weeks following infection. Sensitivity depends on intensity of infection and weight of faeces used.	Charlier et al., 2008
Serum antibody ELISA	79-95%	80-93%	Can detect infection 2-4 weeks post infection Remains positive for several weeks after treatment	Charlier et al., 2008; Salimi-Bejestani et al., 2005b
Milk antibody ELISA	92-95%	88-98%		Reichel et al., 2005; Salimi-Bejestani et al., 2007
Bulk milk antibody ELISA	96%	80%	Convenient method for whole herd screening	Salimi-Bejestani et al., 2005a
Post-mortem detection of fluke in liver	63% - 93%	100%	Sensitivity varies widely depending on protocol	Rapsch et al., 2006

4.1.3 Description of bovine TB

Bovine tuberculosis (bTB) is caused by *Mycobacterium bovis* and occurs in cattle throughout the world. *M. bovis* can be transmitted to humans, although the risk is low if milk is pasteurised and meat is cooked. Test and cull control programmes for cattle, combined with abattoir surveillance, have been successful in eliminating bTB as a public health problem in most European countries, although some countries are still seeing many test-positive cases in cattle, particularly the UK and Ireland. Clinical cases are rarely seen because most cattle are culled well before reaching the clinical stage of disease. Herds are usually tested between every 1 and 4 years, with more frequent retesting if there are positive results within a herd. Positive animals are compulsorily slaughtered and attempts are made to confirm bTB infection by lesion detection, histopathology and culture. Outside the EU, many countries do not have control programmes and cattle are more frequently seen with clinical bTB.

M. bovis is a slow growing intracellular bacterium with a lengthy pre-clinical phase lasting months or years. Infection with *M. bovis* induces a cell-mediated immune response with the production of IFN γ and TNF α (Buddle et al., 2013; Flynn et al., 1995, 1993). Later in the course of disease a humoral (Th2) type response predominates with increased antibody

production. Latency is not reported as a feature of bTB infection in cattle, although it is widely recognised in humans (Flynn et al., 1993).

4.1.4 Diagnosis of bTB

BTB can be diagnosed pre-mortem by the single intradermal comparative cervical tuberculin test (SICCT) and by the Bovigam® TB Kit (Thermo Fisher Scientific Inc., MA, USA) which measures IFN γ response to bovine and avian purified protein derivative (PPDb and PPDa). Post-mortem diagnosis is by finding lesions in affected tissues, most commonly the lungs and lymph nodes, and also by culture and/or histopathology of tissues.

No gold standard for the detection of bTB exists, with all tests having a relatively poor sensitivity (Strain et al., 2011) (Table 4.2). Currently in the UK, all bTB testing is under government control and cannot be carried out other than as part of the mandatory control scheme. The SICCT is the standard test used for screening of animals in the UK. It entails injecting bovine and avian PPD into two sites on the neck following measurement of skin thickness using calipers. If the animal is infected with *M. bovis*, a hypersensitivity reaction occurs and a swelling results. The injection sites are checked 72 hours later and any swellings are measured. Different interpretations are used depending on the sizes of the swellings and whether or not oedema is present, but in general, if the increase in skin thickness at the PPDb is more than 4 mm greater than the increase in thickness at the PPDa site, the test is considered positive and the cow is described as a 'reactor'. If there is a difference of 2-4 mm the cow is designated an 'inconclusive reactor' (IR). The PPDa is used as a control to avoid false positives due to exposure to environmental *Mycobacterium* spp. The SICCT is subjective, and relies on the operator's judgement, and there are operational difficulties due to time pressure, poor handling facilities and safety (de la Rua-Domenech et al., 2006; Strain et al., 2011). In the UK, testing is usually carried out by private veterinary surgeons on the government's behalf, and a considerable conflict of interest exists (Northern Ireland Audit Office, 2009). Some countries now recognise this and quality control their testers (Duignan et al., 2012). Poor sensitivity is also due to the fact that immune responses in infected animals are variable and often lacking during early or late stages of disease (de la Rua-Domenech et al., 2006; Pollock and Neill, 2002; Strain et al., 2011). A non-comparative test omitting the PPDa is used in some countries where environmental mycobacteria are less of a problem (Strain et al., 2011).

The IFN γ (Bovigam®) test is used in the UK as an additional test in specific situations such as a known infected herd, where a test with higher sensitivity is required (Strain et al.,

2011). A blood sample is taken and peripheral blood mononuclear cells (PBMCs) stimulated with PPDa or PPDb, and the IFN γ produced is then measured using an ELISA. Blood must be maintained at specific temperatures following extraction from the cow, and must be tested within a few hours of collection, making it challenging in field situations. It is expensive and difficult to perform, and the lower specificity makes it unsuitable for routine use (Strain et al., 2011). However, this test can detect animals exposed to lower doses of *M. bovis* and at earlier stages of infection (Dean et al., 2005; Pollock et al., 2005) and is therefore a useful adjunct to the SICCT.

Post-mortem examination at slaughter is a part of bTB surveillance in many countries. Between 7 and 50% of new herd breakdowns are detected this way in the UK (Broughan et al., 2014). Lesions can be visualised by slicing into the lungs, lymph nodes and other organs. The sensitivity of lesion detection can be poor, especially in a commercial abattoir where inspection is limited to a couple of slices into the lungs and lymph nodes (Liebana et al., 2008). In experimental situations, the whole lung can be sliced into tiny pieces, and all lymph nodes and sundry other tissues examined, vastly improving sensitivity (Corner, 1994).

Lesion detection can also be poorly specific, especially in older cattle or those with concurrent diseases such as liver fluke, as the bTB lesions can be confused with those due to other conditions (Shittu et al., 2013). Under controlled conditions, using animals which are unlikely to have other lesion-causing conditions, the specificity is likely to be far higher. Culture and/or histopathology can be carried out to confirm the cause of lesions. However, culture of *M. bovis* is difficult and the failure to culture anything does not rule out bTB. In the field, culture is normally dependent on finding lesions whereas in experimental settings, tissues may be cultured even if no lesions are seen.

Table 4.2. A summary of some commonly used methods of bTB detection

	Sensitivity	Specificity	Comments	Ref
SICCT	50-100%	99.9%	Sensitivity adversely affected by metabolic stresses, concurrent infection with <i>M. avium</i> subsp. <i>paratuberculosis</i>	de la Rua-Domenech et al., 2006; Karolemeas et al., 2012; Neill et al., 2001; Strain et al., 2011
Interferon γ test	67-100%	85-96%	Able to detect early infection	de la Rua-Domenech et al., 2006; Dean et al., 2005
Lesion detection	20 – 95%	53-90%	Test accuracy much poorer in commercial abattoir setting	Corner, 1994; Department for Environment Food and Rural Affairs, 2014; Frankena et al., 2007; Liebana et al., 2008
Culture	53-90%	>99%		Department for Environment Food and Rural Affairs, 2014; Liebana et al., 2008; Strain et al., 2011

4.1.5 Co-infection with parasites and other pathogens

Several studies have shown that infection with parasites alters host response to co-infecting pathogens. For example, children exposed to filarial nematodes or blood flukes have a reduced Th1 immune response to mycobacterial infections following BCG vaccination (Malhotra et al., 1999). Also in children, immunity to *M. tuberculosis* and *M. leprae* is reduced by infection with *Onchocerca volvulus* (Stewart et al., 1999). In the case of *F. hepatica*, there is evidence that infection can increase susceptibility to *Bordetella bronchiseptica* in mice (Brady et al., 1999), and to *Salmonella dublin* in cattle (Aitken et al., 1979). In these examples the effect of the parasite is to decrease immunity to co-infecting pathogens; these responses, however, should not be viewed in isolation, as parasites have co-evolved with their hosts over a long time. In some cases, treatment for parasites has been found to worsen symptoms of concurrent disease (Fenton, 2013). At the population level, there is a trade-off between the effects on the individual and the effects on the population, and co-infection may help to keep the population stable (Ezenwa and Jolles, 2014; Fenton, 2008; Jolles et al., 2008). Ecological studies show that the relationship between parasites and host immunity is dynamic and context specific, depending on

infection pressure, host genetics, and environmental factors (Friberg et al., 2013; Graham et al., 2007).

4.1.6 Reasons for doing the review

For a long time there has been a concern that liver fluke may affect the outcome of the bTB skin test, and that this may hamper control programmes for bTB. Two small scale experimental studies and one herd-level study reported that fluke decreased the sensitivity of the SICCT (Claridge et al., 2012; Flynn et al., 2009, 2007). However, other research reported the opposite effect (Broughan et al., 2014). In addition, the poor sensitivity and specificity of diagnostic tests for bTB and the absence of a gold standard make it difficult to interpret findings of studies. In spite of this, the findings of the former three studies are now widely reported (Bovine TB info, 2016; Strain et al., 2011). There is a need to examine the evidence to try to better understand the complexities of the relationship between liver fluke and bTB diagnosis and disease.

4.1.7 Objective

To review primary research to find out whether liver fluke infection affects the diagnosis of bTB.

4.2 Methods

The recommendations for systematic reviews from the Cochrane Collaboration were followed (The Cochrane Collaboration, 2011).

4.2.1 Type of studies

All types of study were considered for inclusion, as long as they included animals co-infected with liver fluke and tuberculosis, and a control group testing negative for one or both pathogens to compare them with. We searched for studies concerning *F. hepatica*, *F. gigantica*, *M. bovis* and *M. tuberculosis*, in any species of host. Observational studies and experimental studies were considered.

4.2.2 Fluke measures

Any method of herd or individual fluke diagnosis was considered.

4.2.3 TB measures

The outcomes of interest were TB diagnostic measures, whether pre- or post-mortem. Some studies had looked at various other measures of immune response, but for the purposes of this study, we only included those measures that were directly related to

diagnosis: SICCT response, IFN γ test, lesion detection, culture or bacterial recovery, and other commercially available tests such as antibody assays.

4.2.4 Search methods

Searches were carried out in Google Scholar, Scopus, Web of Science and Pub Med using combinations of the following search terms: Fasciola, liver fluke, tuberculosis, tuberculin, mycobacterium and BCG. Searching was performed separately by two researchers. Further searches were carried out in Google and in the conference proceedings of the World Association for Advances in Veterinary Parasitology, Society for Epidemiology and Preventive Veterinary Medicine, the International *Mycobacterium bovis* Conference, International Conference of Parasitology and British Society for Parasitology. Hand searches of reference lists from recovered papers were performed. Finally, personal contacts from other research institutions were approached to ask for any unpublished studies.

4.2.5 Data collection and analysis

4.2.5.1 Selection of studies

Search results were merged and duplicates removed. Titles and abstracts were examined and obviously irrelevant papers removed. The full text of reports was then obtained where possible, through the British Library or by contacting the main research institutions in the countries of origin. For foreign language studies, these were initially screened by one researcher able to read the language. Where papers were felt to be of interest, another colleague was then sought for a second opinion. For the English language papers, these were all read independently by two researchers initially. Included papers were then read by two additional colleagues.

Papers were discarded at this stage according to the following criteria: a review or letter rather than an original study, a different research question addressed, no control group, did not separate fluke effect from other parasites, did not mention co-infections between the two parasites, or did not include both pathogens.

4.2.5.2 Data extraction and management

Studies were then read in detail by at least two researchers to assess the quality of evidence and extract the data (data extraction forms can found in the appendix). In the case of disagreement, a consensus was reached by discussion or by seeking a third opinion. Studies were numbered in order of date of publication. Where a single study was reported in more than one place, the reports were grouped together and given a single number. A small number of studies were rejected at this stage due to inability to extract useful data.

4.2.6 Assessment of the risk of bias

This was a difficult assessment, given the heterogeneity of the studies. There was much discussion between team members and further details of aspects of the decision making process are given in the results. The risk of bias was categorised as low, medium or high for each study, based on consideration of the following:

4.2.6.1 Study design

Low: Randomised experiment

High: All other study designs including observational studies, cohort, case control and cross sectional designs.

4.2.6.2 Sampling bias

Low: Animals were randomly selected from the population

High: Study design made bias likely, eg. selection from different populations or selected based on features related to the outcomes of interest

4.2.6.3 Randomisation

This is applicable only to studies which included an intervention.

Low: Animals were randomly allocated to a control or intervention group, with a suitable method of randomisation.

High: No appropriate method of randomisation was reported, or randomisation was not used

4.2.6.4 Blinding

Low: There was blinding at all of the following stages: administration of intervention (if possible), data collection, diagnoses or measurements taken, laboratory work, and statistical analysis

High: No blinding was used at one or more of these stages

4.2.6.5 Comparability of groups

Low: Groups were compared and found to have similar characteristics with regard to relevant factors such as age of animals, breed, region of origin

High: Groups were not compared, or were compared and seemed different

4.2.6.6 Confounders

The main confounders were considered to be age, breed, use, region, and previous treatment

Low: Confounders were adjusted for at the analysis stage e.g. by inclusion in a regression model

High: Confounders were not adjusted for

4.2.6.7 Incomplete outcome data

This refers to animals that were selected to be part of the study but for which some or all results were not reported.

Low: All data was complete, or reasons for incomplete data were analysed and found to be unlikely to introduce bias

High: Incomplete information for a proportion of study subjects with no explanation, or for reasons likely to introduce bias

4.2.6.8 Selective reporting

Low: All aspects of the study that were mentioned in the methods were fully reported in the results. Interpretation of results and discussion of the implications fitted what the results showed

High: Some procedures that were mentioned in the methods were missing from the results. Results were over interpreted or claims were made that were not supported by the results.

4.2.7 Data synthesis

As the studies were heterogenous in their methods and outcome measures, as well as being few in number, a meta-analysis was not possible. Instead a harvest plot and narrative synthesis was used to synthesise the results (Ogilvie et al., 2008). For the harvest plot, each outcome measure for bTB was plotted against the 'effect' of fluke infection/exposure: decreased, no difference or increased. Studies were plotted with a box showing effect size, and colour coded for quality of evidence. This is to be interpreted alongside the narrative synthesis, which gives further details about the evidence.

4.3 Results

4.3.1 Results of search

A total of 67 studies were identified through database searches, eight through hand searching reference lists of other papers, four through personal contacts, two through searching the internet using Google, and one from conference proceedings. Full details of the sources of the papers can be found in figure 4.1. Following removal of duplicates and initial screening, 54 papers remained. These were published between 1950 and 2016, were published in seven languages, and included peer reviewed published studies, PhD theses and unpublished data.

A total of eight studies were excluded because the full text could not be obtained. Five full texts could not be obtained; these were Russian or Eastern European studies dating from the 1950s-70s. Two studies were not used because they were unfinished at the time this review was carried out. One study was omitted because it was reported in conference proceedings with insufficient details.

The full text was screened for 46 studies. Thirty-five of these were excluded because they did not meet the study requirements, or because they were a letter or review (further details can be found in figure 4.1). Six reports were combined into three because they were found to be using the same animals with different aspects reported in various places (Table 4.3 and DEFRA 2005/Broughan et al, 2009).

4.3.2 Included studies

Nine studies were included in the final analysis, and are summarised in table 4.4. Their publication dates ranged from 1962 to 2016. Eight were published in peer reviewed journals, one was reported in a thesis, and part of one study was unpublished.

Three studies used experimentally infected calves, and were performed by the same research group in Ireland. BCG was used to infect calves in one experiment (Flynn et al., 2007) and *M. bovis* was used in the other two. The latter two experiments were reported in three different papers (see table 4.3).

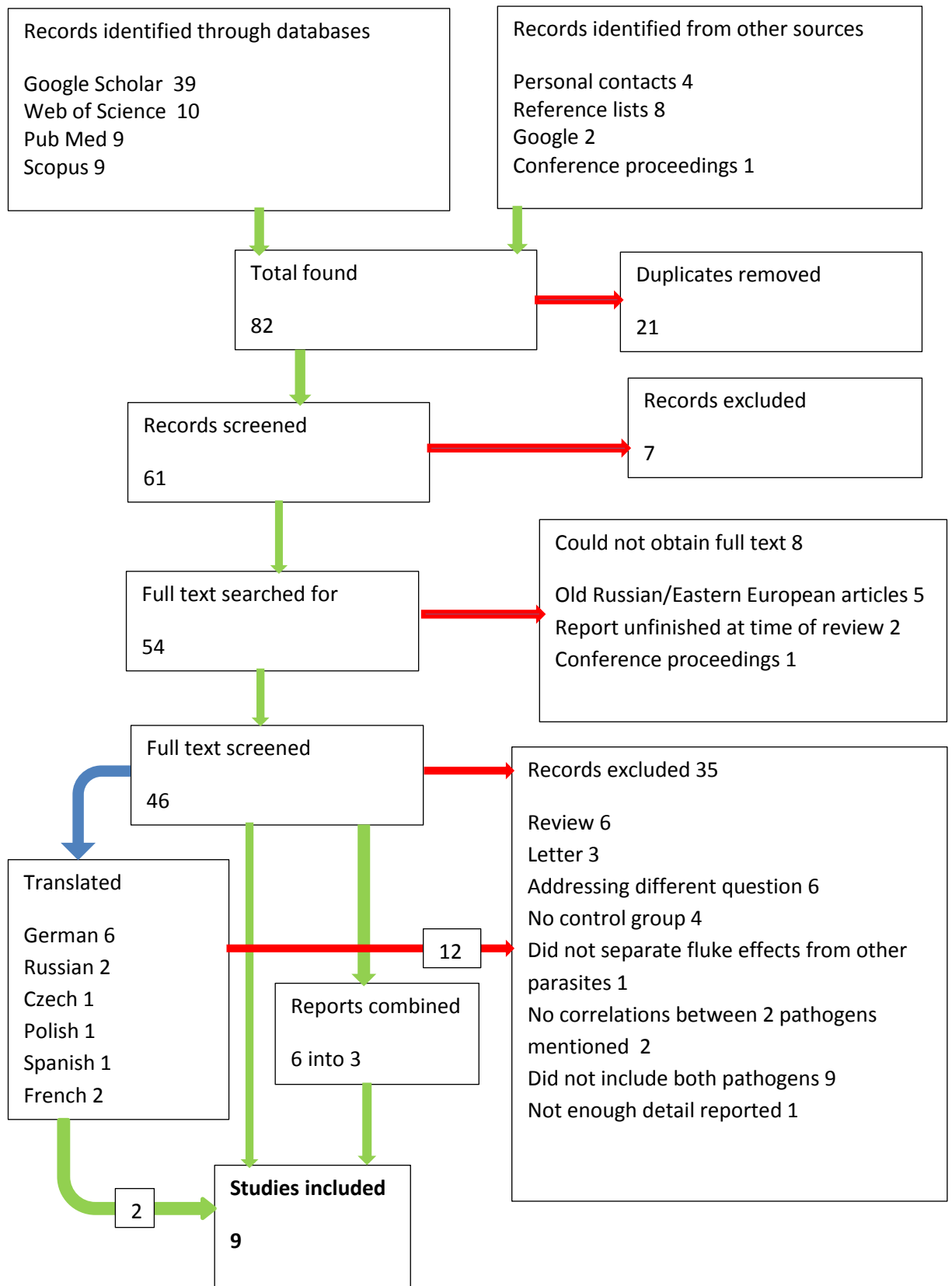


Figure 4.1. Numbers of studies found and removed at each stage of the systematic review

Table 4.3. A summary of where the outcomes from experimental studies of two groups of calves infected with *M. bovis* were published

Experiment	Aspect of study	Reported in
1	SICCT	Unpublished
	Lesions	Flynn et al., 2009
	Bacterial recovery	Garza-Cuartero et al., 2016
	IFN γ	Flynn et al., 2009
2	SICCT	Claridge et al., 2012
	Lesions	Garza-Cuartero et al., 2016
	Bacterial recovery	Garza-Cuartero et al., 2016
	IFN γ	Garza-Cuartero et al., 2016

Claridge et al. (2012) investigated the association between bTB herd breakdown and the fluke antibody level in a bulk milk tank sample. A case control study looking at the fluke antibody levels of adult cattle and their TB test result was reported in a PhD thesis by Claridge (2012). These were both UK studies.

There were three cross-sectional abattoir studies, one from Czechoslovakia (Schanzel and Stolarik, 1962), one from Germany (Meyer, 1963) and one from Zambia (Munyeme et al., 2012). The Czech and German papers compared numbers of cattle with liver fluke and bTB lesions at abattoir between those testing positive and negative on the bTB skin test. The Zambian study was on the tropical liver fluke *F. gigantica*, and investigated the association between bTB lesions and presence of fluke in the liver.

Finally there was a case control study carried out by DEFRA in the UK. This was a large study looking at many aspects of bTB infection in cattle, of which liver fluke was one small part. The population looked at was different compared to other studies, as there were two groups, reactors and 'in contacts' (cattle which were in contact with bTB reactors but not from the same farm as the bTB reactors), and no 'TB free' cattle were included. Fluke exposure was measured by antibody ELISA and bTB was defined as lesions confirmed by either culture or histology (DEFRA, 2005). Some aspects of this study were written up in a poster (Broughan et al., 2009).

Table 4.4. A summary of the studies meeting the criteria for inclusion in the systematic review

Study	Reported in	Sample size	Country	Type of study	Species/type	Findings
1	Schanzel and Stolarik, 1962	10,711	Czechoslovakia	Cross-sectional, bTB tested cattle slaughtered and examined for bTB lesions and fluke	Cattle, slaughtered. Different ages included	No difference in the sensitivity or specificity of the bTB skin test in fluke-infected compared to fluke-free cattle
2	Meyer, 1963	320	East Germany	Cross sectional, bTB tested cattle slaughtered and examined for bTB lesions and fluke	Cattle, slaughtered. Details not given	The authors report slightly fewer lesions seen in TB test positive cattle in the fluke positive group (more 'false positives'). Statistical significance not reported, but the authors did not consider it clinically important
3	Broughan et al., 2008; DEFRA, 2005	400	UK	Case control 200 TB reactors, 200 in contacts (partially matched but not from same farm). Detailed post mortem, culture from lesions and antibody ELISA for fluke	Adult cattle, beef and dairy	Cattle with fluke antibodies less likely to have confirmed bTB in both SICCT positive and negative animals. Significant in dairy reactors only (adjusted for confounders)
4	Flynn et al., 2007	18	Ireland	Experimental, infected with BCG and/or fluke. 4 groups: Fluke only, Fluke first then BCG 4 weeks later, BCG then fluke 4 weeks later, and BCG only. SICCT carried out 13w after BCG infection	Calves	<ol style="list-style-type: none"> 1. Co-infected calves more likely to test negative on both Bovigam test and SICCT than BCG only – in both the BCG first and the fluke first groups. 2. No difference in IFN γ response to bPPD 3. Fluke only calves had higher fluke numbers than co-infected, and fluke-BCG had more fluke than BCG-fluke (non-significant)
5	Flynn et al., 2009; Garza-Cuartero et al., 2016, unpublished	18	Ireland	Experimental. 3 groups: fluke only, fluke and <i>M. bovis</i> and <i>M. bovis</i> only	Calves.	<ol style="list-style-type: none"> 1. Fluke only calves had higher fluke numbers than co-infected (non-significant) 2. Co-infected had lower IFN γ production than <i>M. bovis</i> only (non-significant) 3. Co-infected group had fewer TB lesions (non-significant) 4. Co-infected group had fewer culture positive lesions 5. Bacterial recovery was lower in co-infected (significant) 6. Fewer SICCT positives in the co-infecteds than in <i>M. bovis</i> only (non-significant)
6	Munyeme et al., 2012	1680	Zambia (Fasciola gigantica)	Cross sectional, abattoir study, gross TB lesions and presence of fluke	Adult cattle of various breeds	Cattle infected with fluke were significantly more likely to have bTB lesions
7	Claridge, 2012	80	UK	Case control. 40 matched pairs, 20 each of reactors and inconclusive reactors.	Dairy cattle	No difference between groups in terms of fluke antibody levels, $p=0.5$
8	Claridge et al., 2012[obs]	3026 herds	UK	Cross sectional, bulk milk tank (BMT) sample taken from each herd and tested against herd bTB breakdown status using logistic regression. Confounders controlled for: environmental, herd size, nearest neighbour with bTB	Dairy cattle, whole herd	<ol style="list-style-type: none"> 1. Positive fluke test is significant protective predictor for TB breakdown 2. Fluke antibodies and TB breakdown are spatially separated
9	Claridge et al., 2012[exp]; Garza-Cuartero et al., 2016	12	Ireland	Experimental: two groups: <i>M. bovis</i> only and fluke and <i>M. bovis</i>	Calves	<ol style="list-style-type: none"> 1. Co-infected had smaller response to SICCT (all still positive)(significant) 2. <i>M. bovis</i> bacterial load lower in co-infected (significant) 3. No difference in lesions between groups 4. IFN γ response lower in co-infected (non-significant)

4.3.3 Risk of bias in included studies

4.3.3.1 Study design

Of the nine studies considered, three were laboratory studies, and there were two case control studies and four cross sectional studies.

4.3.3.2 Sampling bias

This did not apply to the experimental studies, where animals were selected to be similar and there were small numbers included. Convenience sampling was used in five of the other studies, which could introduce bias. This was suspected in Schanzel and Stolarik (1962) because the rate of bTB was nine times higher in the fluke infected than the fluke uninfected animals, suggesting that there are other differences between the groups. One study (DEFRA, 2005) used matched TB reactors and controls, although the matched pairs came from different farms, so there is a risk that other differences between farms, such as location or management, could introduce confounders.

4.3.3.3 Randomisation

None of the experimental trials gave a method of randomisation.

4.3.3.4 Blinding

Many of the studies included blinding of the SICCT administrator, but none of the studies mentioned blinding at the analysis stage.

4.3.3.5 Comparability of groups

This did not apply to the experimental trials as the small numbers of animals included were all of a similar age, sex and breed and kept under controlled conditions. For the other studies, only one had thorough comparisons available (DEFRA, 2005). Often information was not available to researchers due to the nature of sample collection.

4.3.3.6 Confounders

The main confounders were considered to be age, breed, use, region, and previous treatment, and again, these data were often not available to researchers.

Two of the studies controlled for the main confounders in the analysis (Claridge et al., 2012; DEFRA, 2005).

4.3.3.7 Incomplete outcome data

Only one of the studies (Munyeme et al., 2012) explicitly mentioned missing data. Some of this related to the region of origin of the cattle and could have introduced bias. One of the

other studies had unexplained differences between sampling times between the different groups of calves (Flynn et al., 2007).

4.3.3.8 Selective reporting

The reporting of studies 5 and 9 shows possible confirmation bias in the way in which the results have been interpreted (i.e. the authors believed that fluke infection could affect the TB test, and this has led them to interpret the results to confirm this). There is a statistically significant difference between the mean IFN- γ results of the groups only at 3 out of 8 (study 5) or 2 out of 15 time points (study 9), yet this is interpreted as being likely to affect the outcome of diagnostic tests. These studies did not report qualitative Bovigam[®] results although use of the test was reported.

In study 9, the mean skin thickness was significantly different between the two groups of calves but the qualitative result of the SICCT was still the same (all were still considered positive under the normal interpretation of the test), yet this is reported as likely to cause under diagnosis of bTB. The skin measurements for study 5 were not statistically significantly different, and have never been published.

4.3.3.9 Other sources of bias

Study 8 used smoothed fluke ELISA PP values for each farm as independent variables in a logistic regression model. Smoothing is a statistical process which aims to capture patterns in data whilst reducing noise. It is assumed that all variability in bulk milk tank (BMT) results between nearby farms is due to error. If, however, a proportion of this variability is real, for example if it is due to differences in fluke exposure on different farms, then the regression co-efficients will appear greater than they are, artificially enhancing the observed effect of fluke exposure.

4.3.3.10 Summary of potential for bias

None of the studies met the required criteria for avoiding bias. This is not surprising for the observational studies, but even for the experimental studies, missing information in both the methods and results made it difficult to interpret the validity of the findings. Most of the recent studies were done by the same group of collaborators and some also on the same calves, increasing the risk of confirmation bias, as authors who have previously reported one result are probably more likely to publish similar findings in the future. None of the studies accounted for the poor sensitivity of the tests.

Experimental studies may provide more consistent evidence due to similarity of infectious doses and the animals used, whereas in naturally infected animals it may be difficult to be

sure whether an animal is infected at all. In the three laboratory studies included here, the infectious doses of the pathogens and routes of infection were fairly similar to what animals might experience in the field (Dean et al., 2005). However, experiments are generally to maximise the chance of finding an effect by infecting with each pathogen in a particular order and measuring outcomes at optimal time points. Table 4.5 summarises the assessments of bias for each study.

Table 4.5. Summary of bias for the different studies. Red denotes a high risk of bias, yellow, medium, and green, a low risk of bias. NA (not applicable) refers to measures which do not apply to the study due to its design.

		Study design	Sampling bias	Random allocation	Blinding	Comparability of groups	Confounders considered	Incomplete outcome data	Selective reporting
1	Schanzel and Stolarik, 1962	Red	Yellow	NA	Red	Red	Red	Green	Yellow
2	Meyer, 1963	Red	Yellow	NA	Red	Red	Red	Green	Yellow
3	Broughan et al., 2008; DEFRA, 2005	Red	Red	NA	Red	Green	Green	Green	Green
4	Flynn et al., 2007	Green	NA	Red	Red	NA	NA	Red	Red
5	Flynn et al., 2009; Garza-Cuartero et al., 2016, unpublished	Green	NA	Red	Red	NA	NA	Green	Yellow
6	Munyeme et al., 2012	Red	Red	NA	Red	Red	Red	Red	Green
7	Claridge, 2012	Red	Yellow	NA	Red	Red	Red	Green	Green
8	Claridge et al., 2012[obs]	Red	Yellow	NA	Red	Yellow	Yellow	Green	Green
9	Claridge et al., 2012[exp]; Garza-Cuartero et al., 2016	Green	NA	Red	Red	NA	NA	Green	Red

4.3.4 Effects of co-infection

Overall, most of the studies found that liver fluke exposure was associated with a decreased response to all of the four aspects of bTB diagnosis assessed: SICCT, IFN γ , lesion detection and mycobacteria cultured or recovered. Most of the studies found only a small effect. Those showing a clinically significant effect were those where the evidence was deemed of poorer quality.

Four of the studies which contribute the most to figure 4.2 are from the same group of authors. Therefore these results should be viewed with caution, as there is a possibility of confirmation bias.

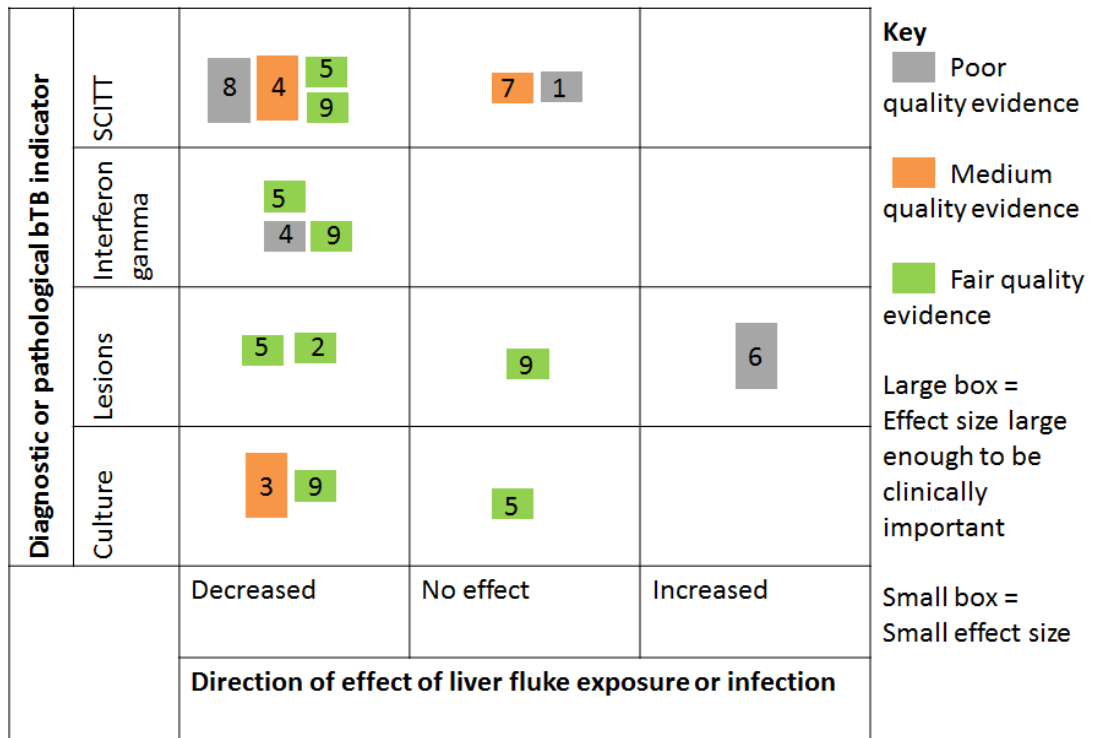


Figure 4.2. A harvest plot showing the results from the nine included studies. The numbers correspond to study numbers given in Tables 4.4 and 4.5. Studies which cover more than one aspect are included more than once. It should be noted that studies 4, 5, 8 and 9 were all by the same collaborating group of authors. Quality of evidence relates to the study design and likelihood of bias. Poor quality evidence is that which is dubious and unreliable, either because of the study design, bias in reporting, or a poor standard of reporting. Medium quality evidence has some merit, but is regarded with caution due again to study design causing a high risk of bias, errors in the report or the study being underpowered. Fair quality evidence is from studies which are overall good. The size of the box is an assessment of the likely importance of the finding, if it were true. Large boxes are shown for results where the effect size would be large enough to matter in a clinical situation. Small boxes are shown where either the effect size is too small to make any difference in practice. This includes some results considered statistically significant.

4.3.4.1 SICCT

Five studies investigated the effect of fluke on SICCT. Study 1 and study 7 found no effect. The nine-fold higher bTB prevalence in the fluke-infected versus fluke-free cattle in study 1

seems likely to indicate that there are fundamental differences between the groups so this result is interpreted with caution. Study 7 was under-powered and because all samples were taken during one unusually wet season from south west Wales, most animals were positive for fluke antibody.

Studies 4, 5 and 9 found that the response to the SICCT was reduced in fluke-infected calves. Study 4 had the largest effect, with 4/5 BCG infected calves testing positive whilst 1/9 co-infected calves tested positive. Study 4 also investigated the relative timing of the infections, and found that the greatest effect was seen when the animal was infected with *F. hepatica* before *M. bovis*, but the effect was still observed when the animal was infected with *M. bovis* first. Studies 5 and 9 had similar experimental designs, using *M. bovis* to infect the calves. In study 5 (unpublished results), 6/6 of the *M. bovis* calves tested positive whilst only 4/6 of the co-infected calves did. In study 9, all *M. bovis* and co-infected calves tested positive but there was a significantly greater reaction in the *M. bovis* only group compared to the co-infected group (raw skin measurements unpublished but differences between avian and bovine reactions published in Claridge et al. (2012)).

Study 8 showed that fluke infected herds were one third less likely to have a cow test positive for bTB on the skin test. However, the effect size may be inflated by the assumption that all variation in fluke ELISA PP result between nearby farms is due to error. Secondly, fluke infection levels vary widely between individuals and usually only a very small proportion of animals test positive for bTB at any herd breakdown, so we cannot tell whether the cattle testing positive for bTB are the same ones that have fluke. Therefore the large effect seen in this study should be interpreted cautiously.

Overall, our interpretation is that fluke infection probably reduces the response to the SICCT, although whether this effect is large enough to be of clinical significance in naturally infected adult cattle on farms is far from certain.

4.3.4.2 Interferon γ

Three studies looked at IFN γ , all under experimental conditions. Study 4 reported both Bovigam[®] qualitative results (positive/negative) and the quantitative IFN γ response to bPPD stimulation for individuals. However, some of those reported as testing positive using Bovigam[®] had lower IFN γ levels than those reported as testing negative. This was because the response to the PPDa was much higher in the co-infected animals (R. Flynn, personal communication), but this was not seen in any other study, so could be an artefact resulting from the use of BCG. In studies 5 and 9 the mean IFN γ was consistently higher in the *M.*

bovis group than in the co-infected group, but this is a small difference and is only statistically significant at a small number of time points. The qualitative interpretation is not reported, and overall, the effect of fluke on the Bovigam® test is probably not clinically important.

4.3.4.3 Lesions

Four studies reported on lesions. In experimentally infected animals, study 5 reported reduced numbers of lesions in co-infected animals compared to *M. bovis* only, although this was not a statistically significant difference, whilst study 9 reported no difference between the groups.

Study 2, a cross sectional abattoir study, reported that fluke-infected animals were around 5% less likely to have bTB lesions whether or not they had a positive bTB skin test (although the authors considered the difference too small to be of practical importance). They were also more likely to be negative on both bTB skin test and lesion detection than fluke-free animals. These were naturally occurring infections, and thus the result could have arisen by chance or due to bias. In naturally occurring infections, we cannot tell whether the absence of lesions is due to the animal not being infected or because it did not produce any lesions in spite of infection.

Study 6, also a cross sectional study, reported that fluke infected cattle were five times more likely to have bTB lesions than those without fluke. This is a big difference from the other studies, for which there are several possible reasons. This is an observational study and bias is likely, moreover, there are major errors in the analysis of the results, and inconsistencies as to what is defined as a fluke-infected liver, so the result could simply be wrong. Nonetheless, from the raw data shown there appears to be a large difference in bTB prevalence between fluke-infected and fluke-free cattle. The study is in a tropical setting, where *F. gigantica* is the endemic species of fluke. *F. gigantica* induces a different immune response from *F. hepatica*, for example certain species of sheep are immune to *F. gigantica* but not *F. hepatica*, and IL10 is upregulated more strongly in *F. hepatica* infected sheep (Roberts et al., 1997; Zhang et al., 2005). The most intriguing possibility is that, due to there being no routine bTB testing programme in Zambia, bTB infections in this study were more advanced, altering the apparent effect.

In conclusion, the evidence for the effect of fluke on the development of TB lesions is not clear cut. Still, the data suggest that fluke infected animals may, on average, have fewer visible lesions, at least in the early stages of infection.

4.3.4.4 Culture

Three studies looked at culture/bacterial recovery. Studies 5 and 9 were experimental studies, and all lung and lymph node tissues with or without lesions were examined, rather than culturing only from lesions as would be the case in the bTB control programme. A greater number of bacteria were recovered from *M. bovis* only calves compared to co-infected calves in study 9, whilst no difference was reported in study 5. There were only six calves per group in each study and the data were skewed with most calves having low numbers of bacteria. Study 3 was a case control study with bTB reactors and in contact animals. Fluke was found to be a significant negative predictor for confirmed bTB in both reactors to the SICCT and non-reactors, but only in dairy animals. The authors interpreted these findings as fluke causing false positives to the SICCT. However, an alternative explanation would be that fluke decreases likelihood of finding visible lesions. The study design and absence of a gold standard diagnostic test for bTB makes it impossible to say which is the true interpretation.

4.4 Discussion

4.4.1 Summary of main findings

This review aimed to bring together and synthesise the literature on co-infection with bTB and liver fluke to answer the question of whether liver fluke infection has an effect on the diagnosis of bTB. There was a variety of different study designs and types of animals studied, making interpretation difficult, and all of the studies were considered to have a high or medium risk of bias.

The overall consensus from the nine studies included here is that liver fluke-infected animals are likely to have fewer visible lesions, fewer bacteria recovered/cultured from their lesions, and a reduced response to both the SICCT and the Bovigam® test, however the clinical and practical importance of this effect is likely to be small. The effect size may vary depending on the relative timings of the infections (as seen in Flynn et al., 2007), and magnitude of infection, so the effect may be considerable in some individuals.

Studies of co-infection with fluke and other pathogens including *Bordetella pertussis* and *Salmonella dublin* have found that fluke increases the susceptibility of the host to these pathogens (Aitken et al., 1979; Brady et al., 1999). The fact that fluke induces a Th2 type response whilst dampening down the Th1 response required for immunity to bTB makes it surprising that fewer visible lesions and lower burdens of mycobacteria were seen in fluke-infected cattle.

If we accept that there is an inverse relationship between fluke and bTB (at least in the early stages of disease) there are several possible explanations.

It is possible that fluke is protective against infection with bTB, and this would explain the negative relationship seen with all measures of bTB. This was suggested by Garza-Cuartero (2014), who found reduced mycobacterial uptake by macrophages from fluke infected animals, which would effectively increase resistance to bTB. There is some evidence that there is a genuine inverse association between the two pathogens as two of the studies reported that co-infected animals had fewer flukes at post mortem than those infected with *F. hepatica* only (Flynn et al., 2009, 2007).

Alternatively, it could be that fluke infected animals are equally (or even more) likely to be infected with bTB, but that progression of disease is altered. A fluke-induced modulation of the immune system may affect the SICCT by reducing the reaction, whilst also limiting the formulation of granulomatous lesions making lesion detection less likely. The decreased mycobacterial recovery from lung and lymph nodes reported by Garza-Cuartero et al. (2016) would not support this hypothesis, although this result was only seen in a small proportion of animals examined. A further possibility is that *M. bovis* may colonise atypical locations in fluke infected animals, with small numbers of mycobacteria in widely disseminated sites making it more difficult to detect. This phenomenon has been suggested in human TB infection (Parrish et al., 1998).

A third possibility suggested by Garza-Cuartero (2014) is that helminths may induce latency, a phenomenon not reported in cattle but widely seen in humans (Pollock and Neill, 2002). Latency occurs when the animal has become infected and mounted an immune response which keeps the infection from progressing (Parrish et al., 1998). In this case, the immune modulation caused by fluke infection could reduce mycobacterial phagocytosis, thereby limiting the ability of the *M. bovis* to enter and replicate within macrophages, which is required for infection to progress (Podinovskaia et al., 2013). However, humans with latent TB still react to the skin test (Parrish et al., 1998), so this would not explain the effect of fluke on the SICCT. In humans, immunosuppressive events lead to reactivation of latent infections (Parrish et al., 1998), so it seems more likely that fluke infection would end the latent state rather than cause it.

4.4.2 Findings in the excluded studies

Many of the earlier studies stated a hypothesis that fluke caused false positives on the SICCT, and many were designed to prove or disprove this, and therefore did not include bTB

infected animals. However, several studies showed that fluke infected animals did not have false positives on the TB skin test (El-Ahwal, 1969; Hartwig and El-Ahwal, 1968; Kokurichev and Karabainov, 1957; Manukyan, 1955), and so it seems more likely that, in the absence of a gold standard test for bTB, so-called 'false positives' to the SICCT seen in populations where bTB is endemic are actually due to a lack of lesions rather than a lack of bTB infection. Interestingly, none of the studies published since 2000 made reference to the earlier ones.

4.4.3 Completeness and applicability of the evidence

The review includes data from a variety of different situations and study types, and four different aspects of bTB diagnosis have been covered. There is a lack of information about the use of the Bovigam test in naturally co-infected animals, which probably reflects the fact that this test is not widely used, and where it is used this is in conjunction with the SICCT (for example, in the UK the Bovigam test is only used in herds already under TB restrictions (DEFRA, 2016)). Due to the bTB control programme in the UK and other developed nations, the later stages of bTB are rarely seen, so most of the available evidence only relates to the early stages of bTB infection.

4.4.4 Quality of the evidence

There is a medium to high risk of bias in all of the included studies. Blinding at the analysis stage, whilst potentially challenging to arrange, would greatly reduce the risk of bias. The poor sensitivity of the SICCT is also an issue, as we do not know which animals really are bTB free. The effect of a number of bTB infected animals being undetected for reasons other than parasite co-infection may be to confuse and hide any small effect of fluke infection. Studies where animals are infected with standardised doses avoid this problem, but may have the effect of inducing a larger effect than would be seen in naturally infected animals. Nonetheless, in this review, the results of the different studies were generally consistent, indicating that experimentally infected animal models are likely to be reasonably representative of naturally infected cattle.

4.4.5 Potential biases in the review process

The authors of some of the included studies have collaborated with the research group at the University of Liverpool. This meant that we were able to obtain some extra unpublished information which improved our understanding of these studies. This is likely to have improved the review by enabling us to include more complete data.

Conversely, due to the difficulties associated with obtaining and reading some of the older foreign language studies, and the fact that methods were reported very briefly and authors were not contactable, some studies had to be excluded. This may have biased the conclusions reached in this analysis.

4.5 Author conclusions

4.5.1 Implications for research

Evidence shows that different animals are differently affected by co-infection, with a much greater effect seen in dairy cows than beef cows (Broughan et al., 2008; DEFRA, 2005), and large variations in immune responses seen even between individual calves infected with identical doses of the two pathogens (Flynn et al., 2007). Genetics, management factors and metabolic stress may play a role. Therefore, for studies of naturally infected animals, targeting particular types of animal that may be at higher risk of immunosuppression, for example those with heavy parasite burdens or under great metabolic stress, may help to avoid effects getting lost in the background noise.

Longer follow up of experimentally infected animals would be useful to understand how fluke infection affects the progression of bTB, and whether the effect on diagnostic tests varies throughout the course of infection. Related to this, methods of following up naturally infected animals are required, which would need to involve either research in settings without routine bTB testing, or an ecological model, to avoid the compulsory culling of infected animals and the restrictions which apply under EU law.

When interpreting results, the poor sensitivity of the bTB diagnostic tests and the poor specificity of fluke serology or milk testing should be considered. Blinding at the analysis stage would greatly help to reduce the potential for bias, and results for individual animals should be reported where available.

4.5.2 Implications for practice

The current evidence suggests that fluke-infected animals are likely to have a decreased response to the commonly used diagnostic methods for bTB, although this difference is probably only rarely great enough to affect the quantitative results of the tests. Caution should be used when the results of laboratory studies are applied to clinical situations, as the interaction of co-infections within naturally exposed populations is much more complex and dynamic. The effects of the relative timings and intensity of infection are likely to be important, but currently very little is known about this. Nevertheless, fluke status should be

borne in mind as a factor that may contribute towards the occurrence of false negative bTB tests.

4.6 References

4.6.1 Included studies

Broughan, J.M., Durr, P., Clifton-Hadley, R., Colloff, A., Goodchild, T., Sayers, R., Williamson, S., Downs, S.H., 2009. Bovine tuberculosis and *Fasciola hepatica* infection. [Poster]. Proceedings of the annual meeting of the Society for Epidemiology and Preventive Veterinary Medicine, London, UK

Claridge, J.A., 2012. Does *Fasciola hepatica* infection increase the susceptibility of cattle to infection with other pathogens normally controlled by a Th1 or pro-inflammatory response? [PhD Thesis]. University of Liverpool, UK

Claridge, J., Diggle, P., McCann, C.M., Mulcahy, G., Flynn, R., McNair, J., Strain, S., Welsh, M., Baylis, M., Williams, D.J.L., 2012. *Fasciola hepatica* is associated with the failure to detect bovine tuberculosis in dairy cattle. *Nat. Commun.* 3. doi:10.1038/ncomms1840

Flynn, R.J., Mannion, C., Golden, O., Hacariz, O., Mulcahy, G., 2007. Experimental *Fasciola hepatica* Infection Alters Responses to Tests Used for Diagnosis of Bovine Tuberculosis. *Infect. Immun.* 75, 1373–1381. doi:10.1128/IAI.01445-06

Flynn, R.J., Mulcahy, G., Welsh, M., Cassidy, J.P., Corbett, D., Milligan, C., Andersen, P., Strain, S., McNair, J., 2009. Co-Infection of Cattle with *Fasciola hepatica* and *Mycobacterium bovis* - Immunological Consequences. *Transbound. Emerg. Dis.* 56, 269–274. doi:10.1111/j.1865-1682.2009.01075.x

Garza-Cuartero, L., Blanco, A., McNair, J., Flynn, R.J., Williams, D., Diggle, P., Cassidy, J., Mulcahy, G., 2016. *Fasciola hepatica* Infection Reduces *Mycobacterium* and *Mycobacterial* Uptake Suppresses the Pro-inflammatory Response. *Parasite Immunol.* 38, 387–402. doi:10.1111/pim.12326

Meyer, W., 1963. [Hetero-allergy to the tuberculin reaction in cattle]. *Monatsh. Veterinarmed.* 18, 325–328. (German)

Munyeme, M., Munang'andu, H.M., Nambota, A., Muma, J.B., Phiri, A.M., Nalubamba, K.S., 2012. The Nexus between Bovine Tuberculosis and Fasciolosis Infections in Cattle of the Kafue Basin Ecosystem in Zambia: Implications on Abattoir Surveillance. *Vet. Med. Int.* 2012, 921869. doi:10.1155/2012/921869

Schanzel, H., Stolarik, L., 1962. [The influence of liver flukes on the specificity of the intradermal tuberculin test in cattle]. *Vet Med Praha* 7, 39. (Czech)

4.6.2 Excluded published studies on co-infection with liver fluke and TB

Abdul-Fattah, M.M., El-Motayam, M., El-Shami, E.A., Salem, G.A., Soliman, A.M., Khorshed, S.E., 2008. Effect of Toxoplasma co-infected with intestinal helminths on cell mediated immunity to tuberculosis patients. *J. Egypt. Soc. Parasitol.* 38, 895–902.

Aleksandrov, N.A., 1969. [Specificity of the tuberculin test in fascioliasis and echinococcosis]. *Veterinariia* 10, 45–6. (Russian)

Ameni, G., Medhin, G., 2000. Effect of gastro-intestinal parasitosis on tuberculin test for the diagnosis of bovine tuberculosis. *J. Appl. Anim. Res.* 18, 221–224.
doi:10.1080/09712119.2000.9706347

Anishchenko, A.K., 1970. [Influence of fascioliasis on the tuberculin test in cattle]. *Nauchnye Tr. Nauchno-Issledovatel'skogo Vet. Instituta, Minsk* 8, 31–37. (Russian)

Bachvarova, Y., Kostov, G., Lilkova, N., Savova, S., Bardarov, I., Baitchev, Z., 1999. [Occurrence of the non-specific tuberculin reactions in cattle and nature of the accompanying pathomorphological and bacterial findings]. *Bulg. J. Vet. Med.* 2, 33–39. (Bulgarian)

Belloli, A., Agosti, M., Proverbio, D., Avezza, F., 1995. Interference of bovine *Fasciola hepatica* on the tuberculin intradermal test reaction. *Bov. Pract.* 0, 141–142.

Bratanovic, U.E., 1954. Tuberculine et tuberculation. *Bull. l'Office Int. des Epizoot.* 42, 278. (French)

Brito, R., Perez, Y., Cotrina, N., 1979. [Correlation of fasciola Hepatica findings in animals reacting to the tuberculin test (bovines)]. [Conference proceedings]. National Day of the Cuban Society of Epizootiology and Anthroozoonosis, La Habana (Cuba), 23-24 Nov 1979. Centro de Informacion y Documentacion Agropecuario. (Spanish)

El-Ahwal, A.M.A., 1969. [Effect of experimental fascioliasis on the results of the intradermal tuberculin test in the guinea pig]. *Berliner und Münchener tierärztliche Wochenschrift* 82, 484–5. (German)

Feldjusin, W., 1952. No Title. *Veterinarija* 29, 32. (Russian)

Hartwig, H., El-Ahwal, A.M.A., 1968. Untersuchungen ueber die bedeutung der Fasciolose als Ursache positiver Tuberkulinreaktionen beim Rind. *Berliner und Muenchener Tieraerztliche Wochenschrift* 81, 315–316. (German)

Hejj, L., Nyiredy, I., Tuboly, S., 1969. [Role of *Fasciola hepatica* in inducin tuberculin allergy in cattle]. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infekt. und Hyg.* 210, 387-. (German)

Keller, H., 1952. [Relationship between tuberculosis of the liver and liver flukes]. [PhD thesis] University of Munich. (German)

Kochnev, P.N., 1950. [*Fasciola* in the lungs of cattle]. *Veterinariya* 27. (Russian)

- Kokurichev, P.I., Karabainov, M.A., 1957. [Specificity of the tuberculin test in cattle with fascioliasis]. Sb. Nauchnikh Tr. Leningr. Inst. Uovershenstvovaniya Vet. Vrachei 11, 81–85. (Russian)
- Ljesevic, Z., 1957. [Effect of parasitic allergy on the tuberculin test in cattle]. Acta Vet. Brno. 7, 81–91. (Russian)
- Losieczka, K., 1960. Periodic occurrence of non-specific tuberculin reactions in cattle. Med. Weter. 16, 720–723. (Polish)
- Manukyan, Z.K., 1955. [Non-specific tuberculin reactions in cattle with fascioliasis]. Tr. Armyanskogo Nauchno-Issledovatelskogo Vet. Instituta. 8, 25–28. (Russian)
- Merlen, H., 1950. F. hepática and the tuberculin reaction. Vet. Rec. 62.
- Nyiredy, I., Hej, L., Tuboly, S., 1966. [The role of saprophytic mycobacteria in inducing tuberculin sensitivity in cattle]. Magy. Allatorvosok Lapja 21, 433–439. (Hungarian)
- Parra Florez, A.D., 1982. Prevalencia de la tuberculosis bovina en la sabana de Bogota como base para un programa de control y erradicacion. Programa Univ. Nacional de Colombia. (Spanish)
- Paterson, A.B., 1957. The tuberculin test in tuberculosis-free cattle. Proc. R. Soc. Med. 50, 253–5.
- Piñeiro-Pérez, R., García-Hortelano, M., José Mellado, M., García-Ascaso, M., Medina-Claros, A., Fernández, N., Subirats, M., José Cilleruelo, M., 2012. Is there interference in the interpretation of the tuberculin skin test in children with intestinal parasitic infestation? Pathog. Glob. Health 106, 172–6. doi:10.1179/2047773212Y.0000000023
- Quarante, 1970. Les reactions parasitaires distomiennes en interference avec les reactions tuberculiniques (intra-dermo reactions). Rev. Path.Comp. Med. Exp. 70, 29. (French)
- Soulsby, E.J.L., 1954. Skin hypersensitivity in cattle infested with Fasciola hepatica. J. Comp. Pathol. Ther. 64, 267–274. doi:http://dx.doi.org/10.1016/S0368-1742(54)80029-4
- Strelchenok, K., 1953. [Non-specificity of tuberculin reactors in cattle with fascioliasis]. Veterinarija 30, 26. (Russian)
- Thompson, R.C.A., Howell, M.J., 1979. Effect of BCG on the resistance of rats to infection with Fasciola hepatica. Zeitschrift fur Parasitenkd. Parasitol. Res. 61, 93–98. doi:10.1007/BF00927090
- Yamaguchi, M., Azuma, R., Kawanishi, Y., 1955. Studies on no visible lesion reactor 1. Correlation of fasciolaiasis to tuberculin rection. Japanese J. Vet. Sci. 17, 133–137.
- Zorawski, H., Lipiec, M., Skwarek, P., Cegielski, A., 1987. Occurence of acid-fast bacilli in the liver of cattle infected with Fasciola hepatica. Bull. Vet. Inst. Pulawy 30–31, 1–5.

4.6.3 General references

See reference list at the back of this thesis

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Chapter 5: Cross-sectional and longitudinal study of co-infection with *Fasciola hepatica* and *Mycobacterium avium* subsp. *paratuberculosis*

Abstract

Johne's disease is a contagious, chronic condition mainly infecting ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). *Fasciola hepatica* is a common parasite of ruminant livestock which is able to modulate the host immune response. The hypothesis that there is an association between *F. hepatica* and MAP was tested. The spatial distribution of MAP was examined using MAP antibody results from 885606 cows from 1245 herds, but no spatial pattern was seen. 3766 samples from 17 herds were tested for MAP antibody and *F. hepatica* antibody. No association between the two pathogens was found at either the herd or individual level, once farm level effects were adjusted for. Subsequently six farms were followed longitudinally for 1 year and up to four samples for each animal were obtained. Data were analysed using a 3-level nested model of sample within cow within herd, and also by categorising cows into red, orange and green based on the combination of their MAP results. No association between the two pathogens was found using any of these approaches.

5.1 Introduction

Johne's disease is a contagious, chronic condition mainly infecting ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP is found worldwide, and is able to survive for lengthy periods in the environment. *Fasciola hepatica*, or the common liver fluke, is a common parasite of ruminant livestock, affecting around 80% of UK dairy herds (McCann et al., 2010). *F. hepatica* is able to modulate the host immune response, altering susceptibility to other infections (Cox, 2001; Moreau and Chauvin, 2010).

In a recent UK-wide study, up to 65% (95% CI 56-74%) of dairy herds were found to contain infected animals as determined by a MAP-antibody ELISA (Caldow et al., 2009). In individual herds, the culling/mortality rate may be between 1-5%; however, subclinical losses such as

reduced milk yield, reduced feed conversion and increased susceptibility to other diseases add further to costs (Hasonova and Pavlik, 2006). Apart from the economic and welfare effects on cattle farming, MAP is of importance because it has been associated with Crohn's disease in humans (Feller et al., 2007; Waddell et al., 2015).

New born calves are most susceptible to MAP, which is thought to be due to their immune system being immature and therefore less able to mount the cellular immune response that is required for protection from MAP (Mortier et al., 2014). Older cattle including adults can also acquire infection, especially if infection pressure is high, although this is less frequent (Mortier et al., 2014).

MAP primarily targets the ileum, where the mycobacteria survive and multiply within macrophages, facilitated by increased production of IL10, an anti-inflammatory cytokine (Weiss et al., 2005). During the early stages of infection, a T-helper (Th)1 type profile characterised by high levels of interferon gamma (IFN γ) keeps infection under control (Begg et al., 2011; Cooper et al., 1993). In most cases, the infection is self-limiting although animals are thought to remain infected for life (Nielsen and Toft, 2008). There is much variation between the immune responses of individuals even under controlled experimental conditions, suggesting that genetics contributes to resistance (Begg et al., 2011; van Hulzen et al., 2012). Reactivation may be due to immunosuppression (Nielsen et al., 2013), although other factors such as initial MAP dose, continued exposure and age at first infection are also important and the reasons are not fully understood (Coussens, 2001; Espejo et al., 2013; Nielsen and Toft, 2006; R. L. Smith et al., 2016). In a proportion of animals, progression to clinical signs occurs, usually after at least 2-3 years of age (Arsenault et al., 2014). At this time, a Th2 response predominates, IFN γ levels decline and clinical signs appear (Begg et al., 2011). Inflammation of the intestinal tract results in reduced nutrient absorption, weight loss and diarrhoea. Once symptomatic, Johne's disease is eventually fatal in most cases.

Diagnostic tests for MAP include faecal culture (individual or pooled), serum or milk antibody ELISA, and faecal PCR. ELISA is the most sensitive test (Caldow et al., 2009; Collins et al., 2005; Pinedo et al., 2008). In the early stages of infection, cattle shed MAP infrequently and antibody levels often do not rise to detectable levels until the later stages of disease, so infected animals are rarely detected before 2-3 years of age (Nielsen and Ersbøll, 2006). Hence for every detectable infected animal, there are likely to be several

others which are infected and will progress to the clinical stage at a later time (Whitlock and Buergelt, 1996).

Nonetheless, for the purposes of limiting spread of MAP within the herd, it may be sufficient to identify cattle in the later stages of infection, as this is when high level MAP shedding occurs. Control measures are intended to avoid contact between the faeces of infectious cattle and new born calves, and also to keep the infection pressure generally low, and include selective culling, removal of calves from their dams shortly after birth and maintenance of hygiene in calving pens (Mortier et al., 2015). In naturally infected cattle, rising antibody levels correspond with shedding high levels of MAP, so individual milk antibody ELISA is a relatively low cost and convenient method of identifying infectious cattle (Collins et al., 2005; Kudahl et al., 2008). Antibodies are ineffective at fighting intracellular pathogens (Stabel, 1996) and rising antibody levels in mycobacterial infections signify a dysfunctional immune response (Begg et al., 2011). Several milk recording companies offer a 3-monthly individual MAP antibody testing scheme in the UK.

Infection with helminth parasites moderates the cow's immune response towards a Th2 and T-regulatory type response, characterised by the cytokines interleukin (IL)4, IL5, IL10, IL3 and TGF β , IgE, mast cells and eosinophils, which are aimed at attacking large extracellular parasites and limiting tissue damage (Maizels et al., 2004). It has been shown that in fluke-infested hosts this polarising effect is not just limited to the immune response to the parasite, but also affects the host response to other co-infecting pathogens, down regulating the Th1 response that is generally required for immunity to intracellular pathogens such as mycobacteria (Brady et al., 1999; Cox, 2001; Kullberg et al., 1992). Because a Th2 response is associated with the failure of cellular immunity seen in the later stages of MAP infection, it is hypothesized that liver fluke infestation may hasten or cause the onset of the clinical phase of infection.

Two previous studies investigated co-infection of cattle with fluke and MAP. One study found that the number of suspected Johne's disease cases on a farm was significantly inversely related to the *F. hepatica* antibody ELISA PP of individuals on that farm, once farm-level random effects were adjusted for, although there was no association with the number of confirmed Johne's disease cases (Claridge, 2012). Another found no association, when farm level bulk milk tank (BMT) PP and presence or absence of Johne's cases on farm were modelled (Howell et al., 2015). Neither of these studies looked at the MAP status of individual animals.

The aims of this study were, firstly, to map the distribution of MAP infection across the UK to look for spatial patterns; and secondly, to determine whether there is any association between exposure to liver fluke and increased MAP antibody levels in individual cattle.

5.2 Methods

5.2.1 Data sources

Samples and data were obtained from a UK commercial milk recording company which provides services to dairy herds, including quarterly MAP antibody testing. MAP results reported here are the results of the *Mycobacterium paratuberculosis* Antibody ELISA (IDEXX, part nr P07130-10) (manufacturer reported sensitivity and specificity of 80.7% and 99.7% respectively), which was performed at the laboratories of the milk recording company. The results were given as S/P (sample to positive ratio) interpreted in relation to negative and positive controls as follows:

$$\frac{S}{P} = 100 \times \frac{OD_{450} \text{ sample} - OD_{450} \text{ negative control}}{OD_{450} \text{ positive control} - OD_{450} \text{ negative control}}$$

Samples with S/P ratio ≥ 0.3 are considered to be from a MAP infected cow, and for the purposes of this study are defined as MAP positive. An S/P ≤ 0.2 are considered to be from a cow which has not been infected by MAP. Samples with an S/P ratio between 0.2 and 0.3 are considered to be suspect for MAP antibodies and a second sample from the animal would be required to confirm its status, and so these results were excluded from the analysis. Locations of herds by county level were provided, but due to the confidentiality requirements of the commercial company, no further information was available for the farms or animals.

5.2.2 Spatial study

MAP test results were obtained from every animal tested by the milk recording company between January 2014 and October 2015. Data were aggregated using R (R Core Team, 2011), to give a code of 'positive' if one or more tests per county were positive, and 'negative' if all tests in that county were negative. The results were mapped in R using the ggplot2 package (Wickham, 2009). Two maps were produced: one showing all the counties with at least one positive farm, and one showing the proportion of positive farms per county. For the latter map, only the most recent result for each farm was included.

5.2.3 Cross-sectional study

Milk samples were obtained from animals tested as part of a quarterly MAP testing scheme, between May and September 2015. A sample size calculation showed that to detect a 20% difference in MAP prevalence between fluke positive and fluke negative animals, with 90% power and 95% confidence, 121 test positive animals and 2420 controls were needed (the 20% difference between groups was chosen as a fairly small difference, due to the lack of literature) (<http://powerandsamplesize.com/Calculators/Compare-2-Proportions/2-Sample-Equality>). Seventeen herds that had either 5% of a sample of cows positive for fluke antibody ELISA or a positive bulk milk tank test, and at least one MAP ELISA positive case, were used in the study. For these herds, milk samples from all animals were then tested at University of Liverpool using an ELISA to detect antibodies to *F. hepatica* excretory-secretory products (Salimi-Bejestani et al., 2007), see chapter 2 for details).

Results were plotted for initial analysis and data cleaning in R (R Core Team, 2011). Percentage of animals testing positive for MAP was modelled against percentage testing positive for fluke to investigate herd level associations. For individual animals, both fluke PP and binary fluke result (positive/negative) were used sequentially as explanatory variables in multi-level regression models. Fluke PP was logged to improve model fit. MAP positivity was used as a positive/negative to avoid outliers having too influential an effect, as the distribution of the data was very skewed. The effect of using log MAP S/P as outcome variable was tested. MAP S/P was also divided into five categories (≤ 0.025 , 0.025-0.05, 0.05-0.1, 0.1-0.3 and > 0.3) and modelled against fluke PP in order to see what effect changing the test cut off would have. Further analysis was performed using a classical regression model with MAP positivity as the outcome measure and fluke exposure as the explanatory variable. Multi-level models were then built using the lme4 package (Bates et al., 2015), with MAP positivity as the outcome measure, fluke exposure as a level one fixed effect, day of testing as a level 2 fixed effect and farm as a random effect, to allow for clustering within farms.

5.2.4 Longitudinal study

For the longitudinal study, the six farms with the most MAP-positive individuals and at least 5% of cattle positive for fluke were chosen from the original 17, and followed for a year, with repeat testing every quarter for all cows in milk, for both MAP and fluke, using the assays described above. Plots of the data were used to look for patterns in results from

individual animals. Those with only a single result were excluded from further analysis. Modelling was approached as for the cross sectional study, with classical models and then a multi-level model constructed with MAP positivity as the outcome measure, fluke exposure as a fixed effect, date of sampling as a level 2 fixed effect, and farm and cow as random effects. Date was coded as a number with 1st January as 1. The effects of using log MAP S/P and of MAP S/P divided into five categories were also tested. To analyse the possibility of cattle being affected differently by being consistently high for MAP antibody as opposed to just a single positive test, cattle were then separated into three groups by visually looking at their repeated MAP results. Those with all or the most recent three MAP results as positive were classed as red, those with fluctuating results were classed as orange, and those with consistently negative results were classed as green (Nielsen and Toft, 2008). The mean fluke PP was then calculated for each animal, and a multilevel logistic regression model was constructed with farm as a level 2 random effect and MAP category (red or green) as the outcome measure.

5.3 Results

5.3.1 Spatial analysis

MAP results from 885606 cows from 1245 herds were used. Out of 72 counties where at least one individual sample was tested, 71 had at least one positive test (Fig. 5.1A). The percentage of farms in each county with at least one MAP positive cow at the last test ranged between 0 and 40, with no discernable geographical pattern (Fig. 5.1B). These results should not be taken as a measure of prevalence as they are inherently biased towards those farms sending samples for MAP testing, and only a small number of farms were tested in most counties; however, they do demonstrate that MAP is widespread across GB.

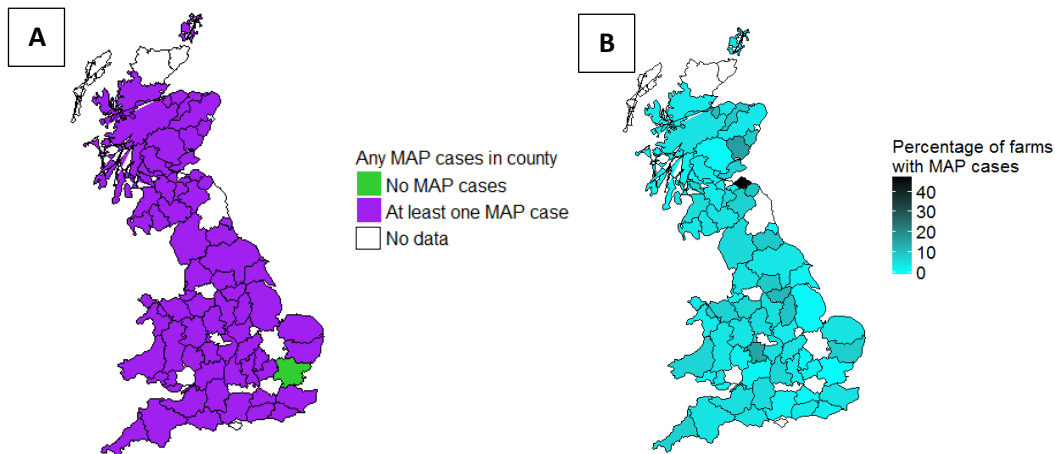


Figure 5.1. A: Map of GB showing counties where at least one cow tested positive for MAP between January 2014 and December 2015. B: Map showing the percentage of farms with at least one cow testing positive for MAP. Antibody ELISA MAP results from 885606 cows from 1245 herds were used.

5.3.2 Cross-sectional study

3766 samples from 17 herds were tested for MAP antibody and fluke antibody. Overall, 139 (3.7%) samples tested positive for MAP and 1516 (40%) tested positive for fluke. A summary of the herd level information is shown in table 5.1.

Table 5.1. Summary of herd information for the cross sectional MAP and fluke co-infection study

Information for each herd	Range (%)	Median	Mean
Nr samples tested	80-403	190	222
Nr MAP positive (%)	1-34 (0.41-10.0)	4 (2.2)	8.2 (4.7)
Nr fluke positive (%)	7-272 (6.2-78.9)	81 (33.6)	89.2 (37.3)
Median fluke PP	4-44	15	18.9
Date samples collected	May 2015 to July 2015		

The farms were located mainly in Northern and Southern England. There was no obvious pattern of higher herd level prevalence of the MAP positives, whereas for fluke, a greater number of positives per herd were seen in Northern England. (Fig. 5.2)

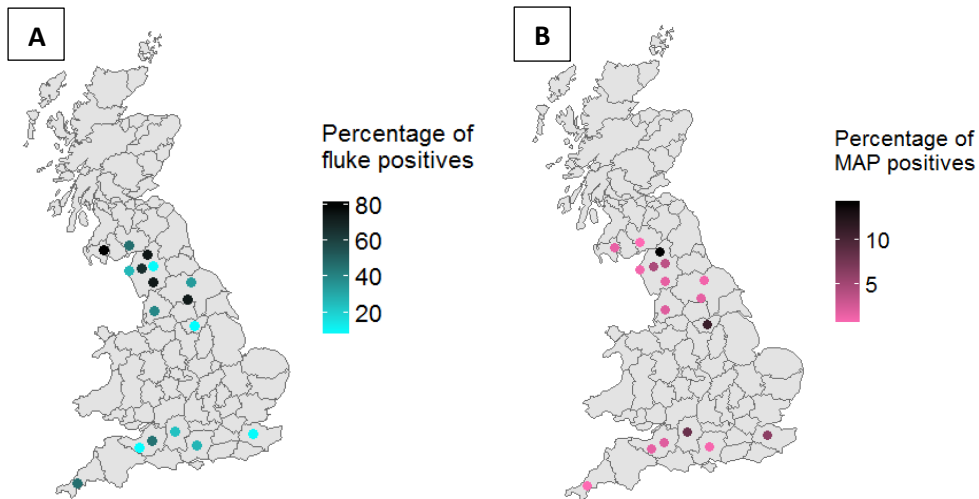


Figure 5.2. Approximate farm locations (randomly placed within county) and colour coded for the percentage of animals per herd testing positive for fluke antibody ELISA (A) and MAP antibody ELISA (B).

The individual fluke PP results followed a log normal distribution (Fig. 5.3A&B) and the individual MAP results were strongly positively skewed (Fig. 5.3 C&D).

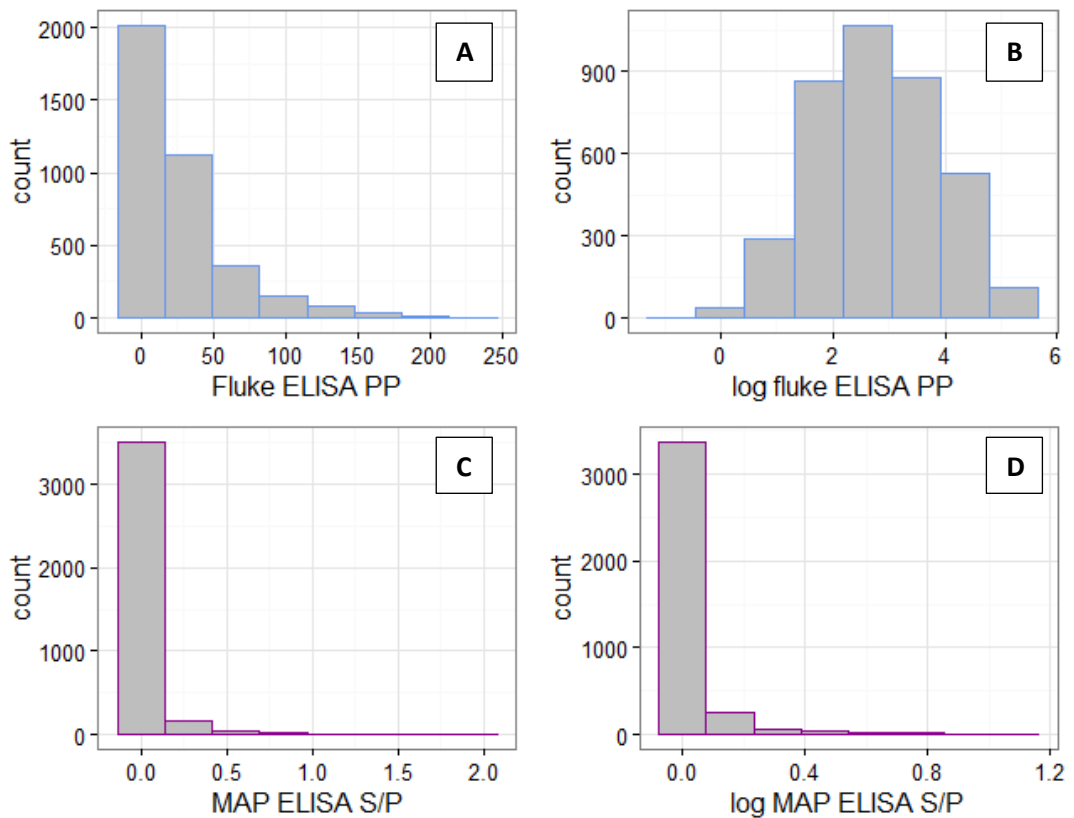


Figure 5.3. The distributions of the individual fluke and MAP ELISA results for 3766 cattle tested from 17 farms between May and September 2015. A & B show fluke ELISA results. C & D show MAP ELISA results.

The percentage of cattle testing positive for each pathogen, by herd, showed no association (Fig. 5.4). Percentage testing positive for fluke had a high correlation with median herd fluke PP ($R = 0.97, p < 0.00001$).

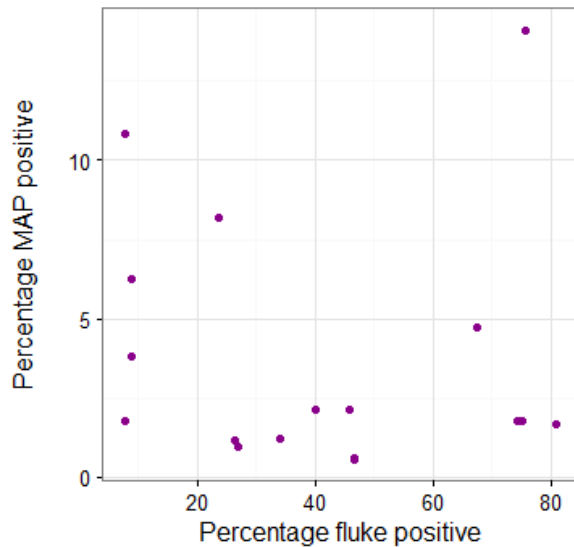


Figure 5.4. No association was seen between percentage of individuals testing positive for MAP antibody and fluke antibody. Each point represents one farm.

5.3.2.1 Models

The only information available was farm, fluke and MAP results. Spatial data were not included as there was not thought to be a spatial element to MAP risk. At the herd level, there was no association between percentage testing positive for each pathogen. At the individual level, log fluke PP was not significant in the classical logistic regression model. The null model with farm as random effect but no fixed effects was a better fit than the classical model. However, adding log fluke PP, or fluke positivity to this model did not improve model fit (Table 5.2). Incorporating random slopes did not change the model fit. Using log MAP S/P or MAP S/P divided into five categories as the outcome measure did not give an improved model fit over using MAP positivity.

Table 5.2. Outcome of regression models for the cross sectional study on co-infection with MAP and fluke. Data from all 17 farms were used for all models. Farm was used as a level 2 random effect

	Model	Response variable	Fixed effects in best model	Random effects	Outcome
1	Classical linear regression - herds	Percent testing positive for MAP	Percent testing positive for fluke	None	$R^2=0.004$ Fluke percent $p=0.8$
2	Classical logistic regression - individuals	MAP antibody positivity	Log fluke PP	None	AIC = 1191.3 df = 3728 Deviance = 1187.3 Log fluke $p=0.8$
3	Null multi-level model	MAP antibody positivity	None	Farm	AIC = 1115.3 df = 3728 Deviance = 1111.3 Inter-farm variance = 0.78 Intraclass correlation = 0.19
4	Multi-level model with fluke	MAP antibody positivity	Log fluke PP	Farm	AIC = 1116.9 df = 3727 Deviance = 1110.9 Inter-farm variance = 0.77 Intraclass correlation = 0.19 Log fluke $p=0.5$
5	Multi-level model with fluke	MAP antibody positivity	Fluke positivity	Farm	AIC = 1117.2 df = 3727 Deviance = 1110.9 Inter-farm variance = 0.78 Intraclass correlation = 0.19 Fluke $p=0.8$

5.3.3 Longitudinal study

A total of 5213 individual milk samples were tested from 6 farms, between May 2015 and May 2016. Four of the farms were visited four times and two were visited three times. 4852 cows had two or more samples taken. Overall, 6.1% and 51% of samples tested positive for MAP and fluke respectively.

For the colour coded analysis, where cows were categorised as red, orange or green according to the pattern of their MAP results over all visits, 1907 were coded green, 61 red and 101 orange.

When looking at the median fluke ELISA PP results over time, there were no significant differences either between farms or between time points. The proportion testing positive for MAP on each farm showed no significant difference between time points for each single farm, although there was a difference in the overall proportion testing positive between farms (Fig. 5.5).

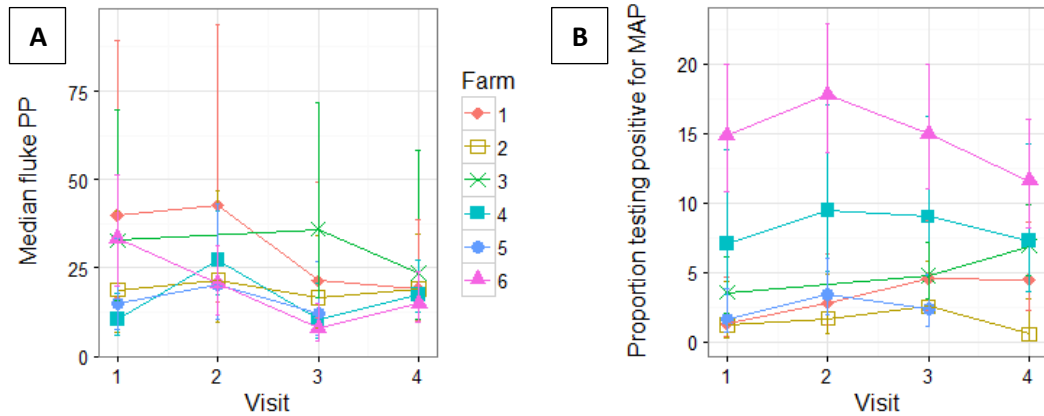


Figure 5.5. A: the median fluke ELISA PP for each farm at each visit. The error bars represent the interquartile range. B: the proportion of cows testing positive for MAP on each farm at each visit. The error bars represent 95% confidence intervals.

When cows were categorised as red, orange or green for MAP, there were no overall differences in fluke PP value between the categories (Fig. 5.6).

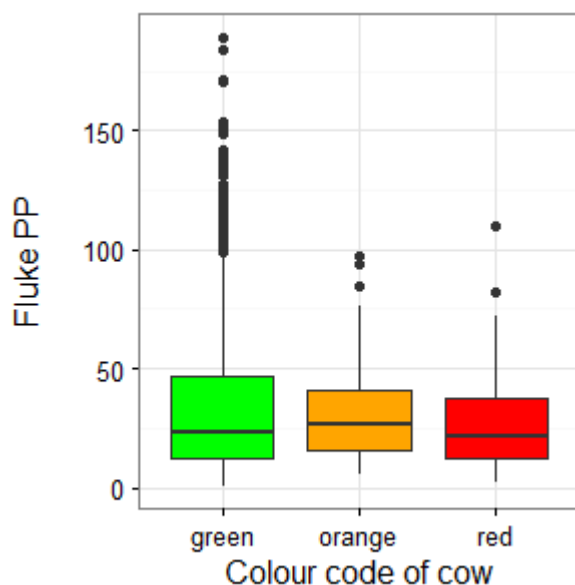


Figure 5.6. The distribution of fluke ELISA PP results for cows colour coded green, orange and red for MAP antibody. Green cows are those which have never tested positive for MAP, orange are those with fluctuating results, and red cows are those which consistently test positive.

5.3.3.1 Models

Models were built using all animals with more than one submitted sample. Two null models were constructed using all of the data, to compare a two- and three-level model. The addition of farm level random effects did not explain any extra variation, therefore only cow level effects were used. This was not surprising given that the farms were purposively selected to be similar in terms of their fluke and MAP results, and there were no farm-level explanatory factors. It is likely that there would be significant inter-farm variation if randomly selected farms were chosen. The addition of fluke to the two-level model including date of sample collection did not improve the model fit. Visit number and date of sampling were highly correlated (because the first visits for each farm took place within a three month period) so could not be included in the same model. When visit was included instead of date, it was seen that cows were significantly more likely to test positive for MAP at visits 2, 3 and 4 rather than visit 1. This could be a seasonal effect, as the effect was not consistent between visits. Adding varying slopes prevented the model from converging (Table 5.3, Models 7-10).

Use of log MAP S/P instead of MAP positivity as the outcome measure did not improve model fit.

For the analysis of colour coded cows, the null model included farm level random effects and a single outcome for each cow (a red or green colour code assigned based on the repeated MAP results: red for consistently high, and green for consistently negative). The addition of the mean of fluke PP to this model did not improve model fit (Table 5.3, Models 9 and 10).

Table 5.3. Summary of the regression models used to investigate interaction between fluke and MAP infection. Sine and cosine functions were used to model day of sample collection. Random intercepts only were used.

	Logistic regression model	Response variable	Fixed effects in best model	Random effects	Outcome
6	Null multi-level model	MAP antibody positivity	None	Cow	AIC = 1274.3 df = 4850 Deviance = 1270.3 Variance between cows =116.4 Intraclass correlation (within cow) = 0.97
7	Null multi-level model	MAP antibody positivity	None	Cow within farm	AIC = 1276.4 df = 4849 Deviance = 1270.4 Variance between cows =116.3 Variance between herds =0.0 Intraclass correlation (within herd)= 0.00 (within cow) =0.97
8	Multi-level model	MAP antibody positivity	Log fluke PP, day of sample collection	Cow	Log fluke $p=0.28$ AIC = 1245.6 df = 4847 Deviance = 1235.6 Variance between cows =131.3 Intraclass correlation = 0.97
9	Multi-level model	MAP antibody positivity	Log fluke PP, visit	Cow	Log fluke $p=0.28$ AIC = 1256.8 df = 4846 Deviance = 1244.8 Variance between cows =126.6 OR visit 2= 5.3 OR visit 3=4.1 OR visit 4=3.0 Intraclass correlation = 0.97
10	Null multi-level model	Red colour code for MAP	None	farm	AIC = 558.8 df = 1640 Deviance = 554.8 Variance between cows =0.58 Intraclass correlation = 0.15
11	Multi-level model	Red colour code for MAP	Log fluke PP	farm	Log fluke $p=0.14$ AIC = 558.7 df = 1639 Deviance = 552.7 Variance between cows =0.58 Intraclass correlation = 0.15

day was included as “ $\cosine(2\pi(date/365)) + \sin(2\pi*(date/365))$ ”

5.4 Discussion

The question of interest was whether fluke infected animals are more likely to have increased MAP antibody levels, as these correspond to clinical Johne's disease. Hence they are of importance in controlling the spread of MAP within herds and reducing economic losses (Coussens, 2001). The analyses performed included a spatial study, a cross sectional study to ascertain whether animals with MAP antibodies were more or less likely to have fluke antibodies, and a longitudinal study to ascertain whether levels of antibodies to the two pathogens interacted over time. Analyses were performed at both herd and individual level, and overall, no association between MAP positivity and fluke PP was seen.

There are many reasons why activation of MAP occurs in some animals whilst others seem to be resistant. These include the initial dose of MAP, the age at infection, genetic variation, metabolic stresses, the gut microbiome and current age (Espejo et al., 2013; Mortier et al., 2015; Nielsen et al., 2013; Nielsen and Toft, 2006; Weber et al., 2010). Our models showed that there was far more variation between individual cattle than between farms, which indicates the importance of cow-level factors. This information, if we had been able to obtain it, would probably have considerably improved model fit.

Only a small proportion of cattle tested MAP positive, which is to be expected even in herds with a large proportion of infected animals, given the low sensitivity of the test (McKenna et al., 2005). It has been estimated that as few as 20% of infected animals can be detected at two years of age, rising to 50-60% by five years of age (Nielsen et al., 2013; Nielsen and Ersbøll, 2006). There are likely to be a large number of latently infected cattle present in each herd, but for the purposes of this study, as we are interested in the later stages of infection when antibodies rise, this was not thought to affect the results.

The MAP antibody ELISA cut off has been set to maximise specificity at the expense of sensitivity (Begg et al., 2011). Lowering the cut offs, to capture animals with lower level of antibodies which are likely to be MAP-infected but in the early stages of infection (with the trade-off of reduced specificity), was investigated in this study by dividing MAP results into five categories. However, no association was seen with fluke PP.

A larger problem is that we cannot distinguish the latently MAP-infected cattle from the uninfected and this is important when attempting to model factors affecting disease progression (Groenendaal et al., 2002; Marcé et al., 2010). An added complication is that a small number of those in the 'latent' group may have high antibody levels, making them

appear to be in the active infection group, although they still have cellular immunity that protects them from disease. It has been shown that many infected animals will produce antibodies right from the start of infection, albeit at a lower level than during the later stages (Begg et al., 2011; Coussens, 2004; Koets et al., 2001; Mortier et al., 2015; Stabel, 1996; Waters et al., 2003).

To aid in determining which animals are infected in the absence of a gold standard test, longer follow up of animals to find out which eventually become positive (Eisenberg et al., 2015; R. L. Smith et al., 2016) and using several tests in conjunction are suggested. Use of IFN- γ results to indicate the decline of cellular immunity (Begg et al., 2011), faecal culture and additional MAP antibody ELISA, alongside clinical signs such as reduced milk yield could be added into the models, as agreement between tests is poor (Collins et al., 2005; Huda and Jensen, 2003; Koets et al., 2001; Nielsen and Toft, 2008).

Given the known immunomodulatory effects of *F. hepatica*, it seems surprising that it has no effect on the susceptibility of cattle to active MAP infection. However, the complexities of MAP epidemiology combined with the limitations of the MAP antibody ELISA make it likely that a small effect of fluke infestation on MAP reactivation would not be apparent against background noise. Nonetheless, our data suggest that infection with *F. hepatica* does not have a major effect on the reactivation of MAP in adult dairy cattle.

Chapter 6: Investigation into co-infection with *Fasciola hepatica* and *Escherichia coli* O157 in finishing cattle on UK farms

Abstract

Escherichia coli O157 is a zoonotic bacterium which causes haemorrhagic diarrhoea in humans and occurs worldwide. Cattle are considered the main source of human infection. *Fasciola hepatica* is a helminth parasite of ruminant livestock which is able to modulate the host immune response, altering susceptibility to other infections. The current study obtained *E. coli* O157 results and fluke copro-antigen results from 334 cattle from 14 UK farms sampled between January and October 2015. A significant association was found between the log percent positivity (PP) of the *F. hepatica* copro-antigen ELISA and *E. coli* O157 shedding when the fixed effects of day of sampling and the age of the youngest animal in the group, and the random effect of farm were adjusted for. The effect of this association was that a change from the 25th quartile of *F. hepatica* PP to the 75th quartile corresponded with a 6.7% increased risk of *E. coli* O157 shedding ($p = 0.01$). The result should be interpreted cautiously due to the many study limitations, particularly a very low level of fluke infection in the sampled animals, and studies involving longitudinal sampling of cattle are required to investigate this relationship further.

6.1 Introduction

Escherichia coli O157 is a zoonotic bacterium which causes haemorrhagic diarrhoea in humans and occurs worldwide. In a proportion of cases, mainly in young children, haemolytic uraemic syndrome may occur, and is potentially fatal (Chase-Topping et al., 2008). Disease is caused by shiga toxins which block host protein production by damaging host cell RNA (Endo et al., 1988). Cattle are considered the main source of human infection (Gyles, 2007; Strachan et al., 2006). *Fasciola hepatica*, or the common liver fluke, is a common parasite of ruminant livestock, occurring world-wide in temperate regions. *F. hepatica* is able to modulate the host immune response, altering susceptibility to other infections (Cox, 2001; Moreau and Chauvin, 2010).

The annual reported incidence of human *E. coli* O157 is 1.8 cases per 100,000 population in England and Wales, and 4.5 cases per 100,000 in Scotland (Health Protection Network, 2013; Public Health England, 2013). Up to 85% of human cases are sporadic, with the remainder occurring as part of identified outbreaks. Contact with farm animals, their faeces and farm environments are major risk factors for sporadic cases (Locking et al., 2001). Infection also occurs via ingestion of contaminated food or water, with items such as salads, raw vegetables, beef products and unpasteurised milk being involved in recent outbreaks (Health Protection Network, 2013; Strachan et al., 2006). *E. coli* O157 is highly virulent with a low infectious dose of less than 700 colony forming units (cfu) (Tuttle et al., 1999) and is a considerable public health problem.

Two recent studies of groups of finishing cattle showed that 20% of Scottish herds had at least one animal positive for *E. coli* O157 (Gunn et al., 2007; Pearce et al., 2009). Four to seven percent of animals were shedding, which equated to around 25% of animals within *E. coli* positive herds. In England and Wales, a cross sectional study of young stock revealed that 30-40% of herds contained *E. coli* O157 positive animals, whilst within-herd prevalence was 4% (Ellis-Iversen et al., 2007; Paiba et al., 2003).

Escherichia coli O157 infections in cattle are usually asymptomatic as cattle lack receptors for the pathogenic shiga toxins (Pruimboom-Brees et al., 2000). Within groups of cattle housed together, infection is likely to spread rapidly to all or most of the group, particularly where cattle are housed in wet bedding (Ellis-Iversen et al., 2007), although the intermittent nature of shedding and the uneven distribution of bacteria within faeces may hinder diagnosis (Pearce et al., 2004). In a proportion of cattle, the distal rectum becomes temporarily colonised by *E. coli*, and this may lead to the formation of a biofilm (Munns et al., 2015). A 'super shedding' event is defined as $>10^4$ cfu *E. coli* being excreted at once, and occurs as a result of the shedding of the biofilm, although the factors that cause the shedding are not understood (Munns et al., 2015). Only a small number of super shedding events can be responsible for up to 90% of *E. coli* O157 excretion (Chase-Topping et al., 2008). Most infected cattle probably 'super shed' intermittently; there is more variation within individuals than between them (Paiba et al., 2003; Pearce et al., 2004; Williams et al., 2014).

Both cellular and humoral immune responses are induced by *E. coli* O157 infection in cattle, and a characteristic inflammation of the mucosa occurs (Nart et al., 2008; Nataro and Kaper, 1998; Naylor et al., 2007). Epidemiological studies show that prevalence is higher in

weaned calves than in adult cattle, suggesting that immunity develops with age (Renter et al., 2004). This is supported by evidence from experimental studies showing that a partially and transiently protective immunity results from exposure, with re-infected cattle showing reduced shedding after a second challenge with the same strain (Naylor et al., 2007). *E. coli* O157 uses a type three secretion system (TTSS) to export effector proteins to host cells (Jarvis and Kaper, 1996). Amongst these are proteins which attach to and damage host cells, causing so-called attaching and effacing (A/E) lesions (Nataro and Kaper, 1998). Cattle produce antibodies which are protective against elements of the TTSE and A/E lesions (Asper et al., 2011; Pirro et al., 1995; Vilte et al., 2008), but these wane a few weeks after the end of infection (Naylor et al., 2007). A localised cellular response characterised by the induction of IFN γ and antigen-specific CD4+T cell proliferation, and an absence of IL4 and IL13, was observed in experimentally infected cattle (Corbishley et al., 2014). However, *E. coli* O157 shiga toxins are thought to be capable of suppressing cellular responses (Hoffman et al., 2006). It is thought that both cellular and humoral responses are required for immunity to *E. coli* O157 (Corbishley et al., 2016, 2014).

Fasciola hepatica is a common parasite of ruminant livestock, with recent estimates showing around 50-80% of UK dairy farms have been exposed (Howell et al., 2015; McCann et al., 2010; Pritchard et al., 2005). These studies were done by testing a single bulk tank milk sample per farm with an antibody detection ELISA based on fluke excretory-secretory antigens. The distribution within farms tends to be right skewed, with most animals having a lower antibody level, and a minority having higher levels (chapter 2).

Prevalence studies on fluke in beef cattle have not been performed in the UK. These studies would require a much more labour intensive diagnostic method, involving collection of multiple individual animal samples from each farm, followed by either faecal egg counting or serum or copro-antigen ELISA. Therefore it is unknown whether the prevalence in beef suckler herds, or indeed amongst the non-milking animals in dairy herds, is similar to that of milking cows in dairy herds. Figures released by the Food Standards Agency report that 16.5% of cattle livers were condemned due to liver fluke during 2015 (Ford and Hadley, 2015).

Infestation with *F. hepatica* induces a Th2 and T-regulatory response, with increased production of IL4, IL5, IL10, IL3 and IGF β , whilst Th1 responses are down regulated (Flynn et al., 2010; Graham-Brown, 2016; Maizels et al., 2004). Various studies have shown that helminth parasites can affect host immunity to other pathogens (Moreau and Chauvin,

2010), including by making the host more susceptible to infection (Aitken et al., 1981, 1979; Brady et al., 1999), changing the pathogenesis of disease (Garza-Cuartero et al., 2016), interfering with diagnostic tests (DEFRA, 2005; Flynn et al., 2007) and vaccination (van Riet et al., 2007). The Th2 response also mediates changes in the epithelial barrier which contributes to immunity to helminth parasites. Goblet cells increase mucus production and epithelial cells increase their permeability so that more fluid is excreted into the gut lumen. These combined with the increased contractility of smooth muscle aid in removing parasites from the gut (Gause et al., 2003). It is possible that these effects could also alter the ability of *E. coli* to colonise the intestinal mucosa, or increase the potential for shedding. Co-infection is complex due to the many other factors that may affect pathogen interactions, including host genetics, other possibly undetected pathogens and the stresses that animals may be under (Moreau and Chauvin, 2010). Accurate diagnosis of disease and interpreting the meaning of multiple different tests can also be challenging.

To date, to our knowledge, there has been little research to determine if there is an interaction between co-infection of *E. coli* O157 and *F. hepatica*. The present study aims to gather pilot data to determine whether shedding of *E. coli* O157 is associated with *F. hepatica* infection in cattle.

6.2 Methods

This study was designed as an add on to an existing larger study on *E. coli* O157 in beef cattle intended for human consumption by the UK Food Standards Authority (FSA; Project FS101055). For the FS101055 study, sample size calculations showed that 110 Scottish farms and 160 farms from England and Wales were required, to give 95% confidence that the true herd prevalence would fall within a tolerance of 16.8%, given an estimated prevalence of 20%.

A sample size calculation to assess the feasibility of the current study was performed by Hickey et al. (2015) using simulated datasets. The estimated prevalence of *E. coli* O157 was set at 4% of cattle and 20% of farms (Pearce et al., 2009) whilst the estimated prevalence of *F. hepatica* was set at 20% of cattle and 80% of farms (McCann et al., 2010; Salimi-Bejestani et al., 2005b). 100% sensitivity and specificity of both tests were assumed. The result of these parameters was that 50 farms and 1645 individual samples were required, and the study would have a power of 87% to detect an odds ratio of 2 (i.e. cattle with fluke are twice as likely to shed *E. coli* O157).

6.2.1 Data collection

Two hundred and seventy farms were sampled in the FS101055 study, and included a variety of types of enterprise and breeds of cattle. Of these, 110 were Scottish farms. These were randomly selected from all Scottish farms that had participated in either of two earlier studies, which in turn were selected by random sampling stratified by region (Gunn et al., 2007). The inclusion criterion was that there was at least one male or female cow without calves on the farm, as these farms were most likely to contain animals that would end up in the food chain. In addition, 160 farms for England and Wales were recruited from a randomly selected subset with either a male of any breed aged over 1 year, or a female of a non-dairy breed aged over 1 year. Farmers were initially notified by letter and given the chance to opt out, and were then contacted by phone in a randomised order to enrol them in the study.

Farms were visited once between September 2014 and October 2015. The unit of sampling was fresh faecal pats as this was comparable to previous research, and accurately represents environmental on-farm contamination. Individual faecal pat samples were taken from the floor or ground, for a group of cattle from each farm. The number of samples collected from each group was determined by a protocol assuming 8% of animals to be positive, there would be a 0.9 probability of identifying groups containing at least one positive animal (Chase-Topping et al., 2007). The group of cattle chosen was one containing animals closest to going off the farm for slaughter. These were then sent to the Epidemiology Research Unit (ERU) microbiological facilities, SAC Consulting Veterinary Services Disease Surveillance Centre, Inverness, within 48 hours of collection, and tested for *E. coli* O157. The recruitment and visits were done by members of Scotland's Rural College (SRUC) project team in Scotland, and the ADAS UK Ltd project team in England and Wales.

For farms for which samples were submitted to SRUC's ERU laboratory on or after 5th January 2015, and which consented to taking part in this further study, 2g of faecal material for all animals from the sampled group was frozen at -20° C in polypropylene tubes and retained for liver fluke testing. Delays due to funding and contractual issues meant that samples received prior to this date were not retained.

Of 39 farms with one or more cattle testing positive for *E. coli* O157, two declined to take part in further research and samples from two farms were delayed in transit and were therefore no longer suitable for analysis. Therefore, samples from 35 herds were sent to

Moredun Research Institute (MRI), Edinburgh where they were tested for fluke. MRI staff members were blinded to the *E. coli* O157 status of the samples.

In addition, information on animal characteristics and farm management was collected from the livestock keeper or farm manager on each farm, via a questionnaire administered by the survey staff. The information was collected in an electronic format and was a shortened version of a questionnaire used in a previous study (Chase-Topping et al., 2007). The questionnaire was piloted with several farmers before use. The finalised questionnaire was approved by the FSS Survey Control team (see Appendix). The questionnaire was conducted in Welsh for Welsh-speaking respondents.

The information was at the farm level, for example the age of animals was given as a range for the group, and all animals were treated as having been managed the same in terms of housing, feeding and treatments given. The information relevant for the fluke study was identified and extracted. As the aim was to develop a model to determine the presence of an association between fluke and *E. coli* O157, rather than a predictive model, only management information relevant to fluke was taken for use in the model, to control for possible confounders which may be linked to both fluke and *E. coli* O157. A summary of these is shown in Table 6.1.

6.2.2 *E. coli* testing

One gram (1g) of faeces was added to 20 ml of buffered peptone water (BPW, Thermo Scientific, UK). The BPW was incubated for six hours at 37°C ($\pm 1^\circ$) then subjected to immunomagnetic separation (IMS). Briefly, a 1 ml aliquot from each 20 ml BPW sample was added to 20 μ l paramagnetic beads coated with polyclonal antibody for *E. coli* O157 lipopolysaccharide (Lab M Ltd., UK). The aliquots were mixed on a rotary mixer for 30 minutes before being washed three times in PBS with 0.05% Tween 20 (PBST, Sigma-Aldrich Co. Ltd.). After the third wash, the beads were re-suspended in 100 μ l PBST and cultured onto MacConkey agar containing sorbitol, cefixime (0.05 mg/l) and tellurite (2.5 mg/l) (CT-SMac, Thermo Scientific, UK).

Following overnight incubation at 37°C ($\pm 1^\circ$) plates were examined for non-sorbitol-fermenting colonies and any suspect colonies were subcultured onto Chromocult coliform agar (Merck KGaA., Germany). After a further overnight incubation at 37°C ($\pm 1^\circ$) any resulting red colonies were tested with anti-*E. coli* O157 latex (Thermo Scientific, UK) for

agglutination. Colonies that agglutinated were identified as presumptively positive and enumerated by limiting dilution.

Polymerase Chain Reaction (PCR) was used to confirm the serogroup of the isolates as *E. coli* O157 (Jenkins et al., 2003). For all positive samples, the number of *E. coli* O157 were enumerated by culturing 10-fold dilutions of faeces in minimum recovery diluent, starting from 1:10, on duplicate CT-SMac plates. Typical colonies were counted after overnight incubation at 37°C ($\pm 1^\circ$) and counts expressed as colony forming units (cfu) per gram of faeces.

IMS is considered to be a highly sensitive method of identifying *E. coli* O157, and has a lower limit of detection of 50 cfu/g (Aydin et al., 2014). For the positive/negative analysis, an *E. coli* O157 positive cow was defined as one that tested positive by IMS. The limit of accurate enumeration was 100 cfu/g of faeces (Pearce et al., 2004), and samples from which too few *E. coli* were cultured to be enumerated were assigned a cfu/g of 10.

6.2.3 Fluke testing

The remaining samples were frozen at -80° C and transported to MRI where they were tested using a copro-antigen ELISA according to the manufacturer's instructions (Bio-X Diagnostics, Jemelle, Belgium). The effect of freezing the samples on ELISA results was tested beforehand and was found to make no difference to the sensitivity or specificity of the test (Personal communication, Dr Philip Skuce).

The result was determined by calculating the percentage positivity (PP) of each sample relative to the optical density (OD) of the positive control, after subtracting the OD of the negative control (provided in the kit). The positive/negative cut off was determined by the quality control insert supplied with the kit, and was either 7 or 8 for all the kits used for this study.

6.2.4 Statistical analysis

For each animal, the following results were obtained: *E. coli* O157 positive/negative, *E. coli* O157 cfu count, *F. hepatica* positive/negative derived by applying the cut off to the copro-antigen ELISA results, and *F. hepatica* PP result (on a continuous scale). Farms without a single fluke positive animal were excluded from further analysis, because we wanted to be sure that cattle at least had a possibility to be infected by fluke, which would not necessarily be the case if there was no fluke on the farm. R (R Core Team, 2011) was used, with the lme4 (Bates et al., 2015) and ggplot2 (Wickham, 2009) packages. Due to

restrictions for confidentiality, figures or data relating to groups of fewer than five farms cannot be shown.

6.2.5 Spatial analysis

Maps were drawn showing the locations of the farms and the proportions testing positive for the two pathogens on each farm.

6.2.6 Multilevel model

Correlations between the numerical explanatory variables were checked to ensure highly correlated variables were not entered simultaneously into the model. All models were fitted using maximum likelihood. Either a positive *E. coli* result, for logistic regression, or $\log_{10} E. coli$ cfu (for linear regression) was used as the outcome variable. The organisation of the data is shown in figure 6.1. The fluke result (either positive/negative or log PP) was the only level 1 explanatory variable, and all other animal and farm management information was at level 2.

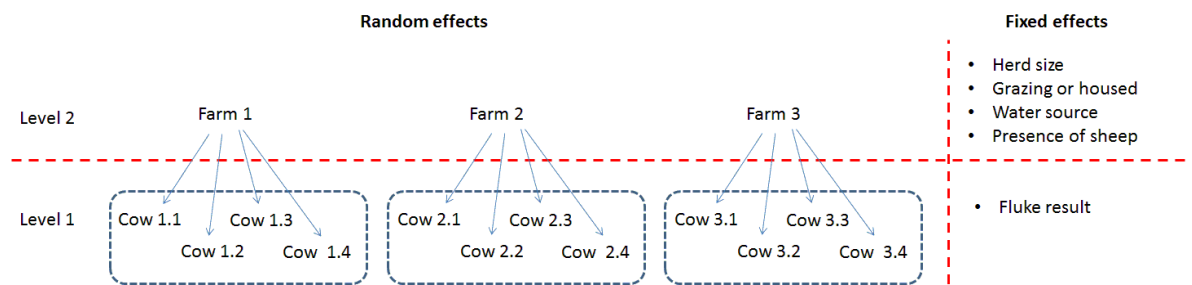


Figure 6.1. Structure of the data. Only the fluke result is known for individual animals, all other variables were the same for each animal from a single farm. Some examples of fixed effects farm level data are shown.

For a variable intercept mixed model, where all farms have the same slope for the relationship between fluke and *E. coli*, the log odds of any animal having *E. coli* is represented by the following equation:

$$\text{Log}(\text{odds}(\text{Positive } E. coli \text{ result})) = \text{intercept} + \beta \times \text{fluke} + \text{farm effect} + \text{error}$$

Where *intercept* is the mean log odds when all other variables are set to zero, β is the coefficient of the slope, *fluke* is the animal's fluke test result, *farm effect* is the same for all animals on the farm, and the *error* is made up of individual differences between animals, which could be accounted for by variables not included in the model or currently unknown.

And, for example:

$$\text{farm effect} = \text{Intercept} + \gamma \times \text{herd size} + \alpha \times \text{grazing} + \text{random effect of farm}$$

Where *intercept* is the grand mean of the $\log(\text{odds}(\text{positive } E. coli \text{ result}))$ when all explanatory variables = 0. γ and α are coefficients for the slopes which correspond with the relationship between *herd size* or *grazing* and $\log(\text{odds}(\text{positive } E. coli \text{ result}))$. The coefficients are the same for all farms, whilst the magnitude of the variables (e.g. *Herd size*) will vary between farms. Additional explanatory variables can be added in. *Random effect of farm* is different for every farm and is made up of unknown variables or those not included in the model.

The starting point was a variable intercept model including a positive fluke result as a level 1 explanatory variable and farm as a level 2 random effect. Management variables which met the inclusion criteria were then added one at a time. The same process was repeated with fluke PP as the explanatory variable. The process was then repeated again with $\log_{10} E. coli$ cfu as a continuous outcome variable (equation as above, except the outcome is simply $\log_{10} E. coli$ cfu). Variable slopes were also tested. The Akaike information criterion (AIC) was used to compare models, with a lower AIC considered better than a higher one.

6.3 Results

Between 13th January and 19th October 2015, 35 farms that consented for further testing had at least one *E. coli* O157 positive sample, and of these, sufficient sample remained for testing using the *F. hepatica* copro-antigen test for 810 animals (there was insufficient sample for 5 cows). Of these, 14 farms had at least one cow testing positive for *F. hepatica*. The 334 animals from these farms were included in the following analysis. Between 7 and 40 cattle were sampled from each farm (median = 22).

6.3.1 Descriptive statistics

The characteristics of the farms are shown in Table 6.1. The data were also examined to find out whether groups of cattle were housed or grazing, how long they had been housed or grazing for, and whether they had received a worming or flukicide treatment within the past 3 months. However, even in groups which had received a flukicide, fluke cases were still present, and similarly some groups of cattle which had been housed for several months still had significant numbers of fluke cases. Therefore treatment history was not used to exclude farms and all animals which came from groups with at least one fluke case were included, on the basis that they would all have had the chance to become fluke infected.

6.3.1.1 Animal level

Overall, 50.9% of cattle tested positive for *E. coli* O157 and 13.2% tested positive for *F. hepatica*. The distributions are shown in figure 6.2.

Table 6.1. Characteristics of the animals and farms in the fluke and *E. coli* O157 study

	Farm-level (n=14)	Individual animals (n=334)
Day of sample collection (Day 1=1st Jan 2014)	Range = 20-293 Median = 126	
Grazing Housed	3 (21.43 %) 11 (78.57 %)	
Dairy Suckler beef Finisher Other	2 (14.29 %) 8 (57.14 %) 2 (14.29 %) 2 (14.29 %)	
Youngest in group (months) Oldest in group	Range = 6-26 Median = 14.5 Range = 11-48 Median = 20	
Total number of cattle on farm	Range = 41-516 Median = 117	
Total number of cows on farm (Females that have had a calf)	Range = 0-208 Median = 33	
Total number of heifers on farm	Range = 0-65 Median = 6	
Total number of cattle under 1 year on farm	Range = 0-215 Median = 30	
Total number of ewes on farm	Range = 0-700 Median = 0	
Total number of sheep overwintering on farm	Range = 0-433 Median = 0	
Water supply from mains Water supply from spring or well Water supply from natural source	10 (71.43 %) 6 (42.86 %) 11 (78.57 %)	
Median percentage of fluke positive cows ¹ Range of positive cows Fluke positive Median PP Range PP	6.55% 2.13-100%	44 (13.17%) 0.82 -1.07-73.74
Median percentage of <i>E. coli</i> O157 positive cows ² Range of positive cows <i>E. coli</i> positive Median cfu/g ³ Range cfu/g ³	43.10% 4.00-100%	170 (50.9%) 10 0-1.45 x 10 ⁵

¹Fluke positive' refers to an animal with a copro-antigen ELISA result of 7PP or above. 'Negative' is below 7 PP.

²*E. coli* O157 positive' refers to an animal with a positive IMS *E. coli* test

³Samples from which *E. coli* numbers fell below the limit of enumeration were assigned cfu = 10

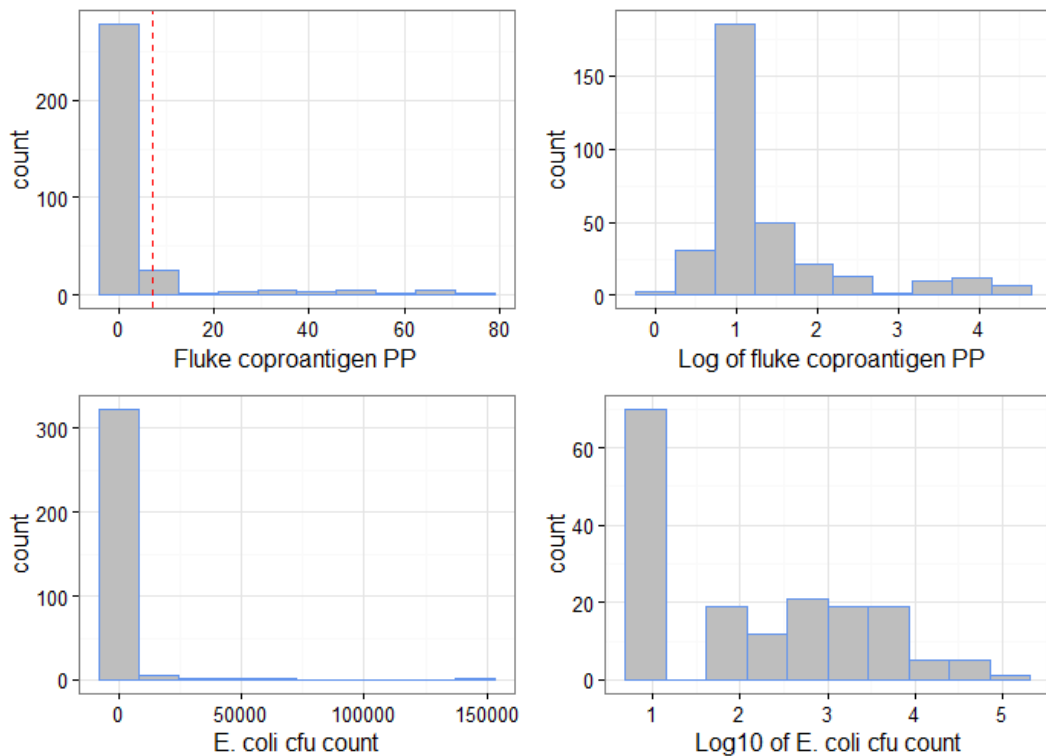


Figure 6.2. Distribution of *Fasciola hepatica* coproantigen PP results and *E. coli* O157 cfu counts for animals across all farms

6.3.1.2 Farm level

Within farms, between 4 and 100% of cattle tested positive for *E. coli* (mean = 43.5%, median = 43.1%) whilst for *F. hepatica* the range was 2.1 to 100% (mean = 14.7%, median = 6.5%). The distribution of log *E. coli* O157 cfu varied between farms, but in general it was right skewed in ten farms whilst four farms showed a more symmetrical platykurtic distribution. For fluke PP, all except one farm had a right skewed distribution. The natural log of fluke PP value was used for multi-level models.

6.3.1.3 Spatial analysis

The farms were spread throughout Great Britain with 6 from Scotland 4 from England and 4 from Wales. North Wales, South Wales, the Welsh borders, Northern England and a variety of Scottish locations were represented. There was no spatial trend visible in terms of either fluke or *E. coli* distribution (maps not shown for confidentiality reasons).

6.3.2 Associations between fluke and *E. coli* O157

6.3.2.1 Animal level

Inspection of scatterplots revealed no visible association between the fluke PP and log *E. coli* O157 cfu (data not shown).

6.3.2.2 *E. coli* O157 super shedders

Ten cattle from six farms were classed as super shedders (had a cfu > 10⁴). Of these, only one had fluke. The sample size was too small to formally test the difference whilst adjusting for farm effects, however, there did not appear to be any association between fluke PP and being a super shedder (fig. 6.3)

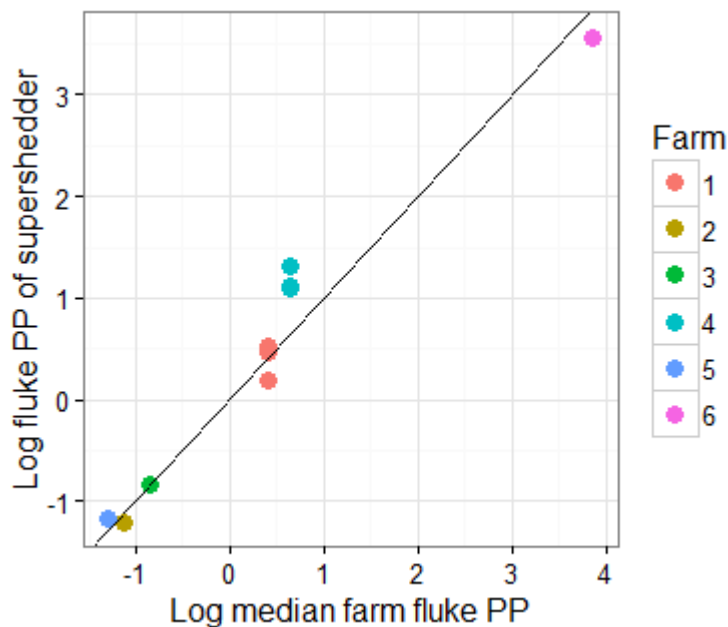


Figure 6.3. The relationship between the log PP of the super shedder and the median log PP of the farm, showing that fluke PP is similar for super shedders and other cattle on each farm. The diagonal line shows equivalence.

6.3.2.3 Between farms

Inspection of scatterplots revealed no visible association between the proportion of *E. coli* O157 and fluke cases on a farm, or between the median fluke PP and median log *E. coli* O157 cfu.

6.3.2.4 Within farms

More detailed inspection of three farms with more than 10% cases of fluke revealed that there was no consistent pattern with regard to which individuals were positive for which

pathogen. In one farm all of the fluke positive animals were also *E. coli* positive, in a second farm all of the fluke positives were *E. coli* negative, and in a third farm all animals had fluke and the PP values were evenly spread between the *E. coli* positive and negative animals.

6.3.3 Multi-level models

The starting point was a variable intercept logistic regression model with *E. coli* O157 positive as the outcome measure. Compared to the null model containing only farm level random effects and no fixed effects (Model AB), the addition of fluke to the model (Model A1) did not improve the model fit, or explain any of the variance between the farms. However, a model including a random effect for farm was a better fit for the data than the classical logistic regression model (Model A2) indicating that there were important differences between farms.

Model AB: null multi-level logistic regression model

Outcome: Log odds *E. coli* O157 positive

Fixed effects: None

Random effects: Farm

	Co-efficient	Std Error	Z value	<i>p</i>
Intercept	-0.28	0.58	-0.47	0.64

AIC = 317.5, deviance = 313.5, df=332.

Random effects: Variance between farms = 4.34

Intraclass correlation = 0.57

Model A1: multi-level logistic regression model

Outcome: Log odds *E. coli* O157 positive

Level 1 fixed effects: Fluke positive

Random effects: Farm

	Co-efficient	Std Error	Z value	<i>p</i>
Intercept	-0.28	0.59	-0.47	0.64
Fluke positive	0.01	0.59	0.02	0.98

AIC = 319.5, deviance = 313.5, df=331.

Random effects: Variance between farms = 4.33

Intraclass correlation = 0.57

Model A2: classical logistic regression modelOutcome: Log odds *E. coli* O157 positive

Level 1 fixed effects: Fluke positive

Random effects: None

	Co-efficient	Std Error	Z value	<i>p</i>
Intercept	-0.028	0.12	-0.23	0.81
Fluke positive	0.49	0.33	1.48	0.14

AIC = 464.7, deviance = 460.7, df=332.

Using log fluke PP instead of the binary fluke result showed that log PP was highly significant when interfarm variation was not accounted for (Model B2), but became non significant when this was included (Model B1).

Model B1: multi-level logistic regression modelOutcome: Log odds *E. coli* O157 positive

Level 1 fixed effects: Log fluke PP

Random effects: Farm

	Co-efficient	Std Error	Z value	<i>p</i>
Intercept	-0.74	0.66	-1.12	0.26
Log fluke PP	0.33	0.23	1.44	0.15

AIC = 317.5, deviance = 311.5, df=331.

Random effects: Variance between farms = 4.14

Intraclass correlation = 0.56

Model B2: classical logistic regression modelOutcome: Log odds *E. coli* O157 positive

Level 1 fixed effects: Log fluke PP

Random effects: None

	Co-efficient	Std Error	Z value	<i>p</i>
Intercept	-0.46	0.21	-2.23	0.03
Log fluke PP	0.37	0.13	2.79	0.005

AIC = 458.49, deviance = 454.5, df=332.

The null linear regression model (Model CD) for the outcome variable log *E. coli* cfu had a much lower farm level variance than null model AB. This showed that adding fluke to this

null model did not improve the model fit (Model C1). There was no association between *E. coli* O157 positivity and fluke PP.

Model CD: null multi-level linear regression model

Outcome: Log *E. coli* cfu

Fixed effects: None

Random effects: Farm

	Co-efficient	Std Error	Z value	<i>p</i>
Intercept	0.95	0.25	3.77	0.0002

AIC = 953.1, deviance = 947.1, df=331.

Random effects: Variance between farms = 0.84

Intraclass correlation = 0.49

Model C1: multi-level linear regression model

Outcome: Log *E. coli* cfu

Level 1 fixed effects: Fluke positive

Random effects: Farm

	Co-efficient	Std Error	t value	<i>p</i>
Intercept	0.96	0.25	3.78	0.001
Fluke positive	-0.07	0.21	-0.37	0.72

AIC = 954.9, deviance = 946.9, df=330.

Random effects: Variance between farms = 0.85

Intraclass correlation = 0.49

Model C2: classical linear regression model

Outcome: Log *E. coli* cfu

Level 1 fixed effects: Fluke positive

Random effects: None

	Co-efficient	Std Error	t value	<i>p</i>
Intercept	1.11	0.08	13.84	<0.00001
Fluke positive	0.06	0.22	-0.25	0.80

AIC = 1163.1, deviance = 624.9, df=332.

Model D1: multi-level linear regression modelOutcome: Log *E. coli* cfu

Level 1 fixed effects: Log fluke PP

Random effects: Farm

	Co-efficient	Std Error	t value	<i>p</i>
Intercept	0.86	0.28	3.08	0.002
Log fluke PP	0.07	0.09	0.78	0.54

AIC = 954.4, deviance = 946.4, df=330.

Random effects: Variance between farms = 0.83

Intraclass correlation = 0.49

Model D2: classical linear regression modelOutcome: Log *E. coli* cfu

Level 1 fixed effects: Log fluke PP

Random effects: None

	Co-efficient	Std Error	t value	<i>p</i>
Intercept	0.92	0.14	6.78	<0.00001
Log fluke PP	0.15	0.08	1.78	0.08

AIC = 1160, deviance = 619.2, df =332.

The plotting of management variables against \log_{10} *E. coli* O157 cfu did not reveal any non-linear relationships. No correlations of >0.7 were seen between any of the explanatory variables, except between numbers of animals of different ages/types. It was not possible to add more than two extra explanatory variables at once because of the relatively small number of fluke cases which led to non-convergence of the model, due to perfect partitioning. The explanatory variables shown in Table 6.1 were each tested. A seasonal pattern was expected for *E. coli* (Ferens and Hovde, 2011), so day was modelled as a sinusoidal function to allow for this. The model fitted better with day as a linear variable, which could be due to the possible true seasonal pattern being hidden by the relatively few farms and all animals from each being sampled on the same day.

The best models for each combination of *E. coli* O157 positive and \log_{10} *E. coli* O157 cfu, and log fluke PP and fluke positive are shown (Models A3-D3). Day and youngest in group were included in all of the models. The higher the age of the youngest animal in the group, the lower the risk of infection with *E. coli* O157. Risk of *E. coli* O157 was found to decrease

throughout the year, from January until October . Log fluke PP was significant when modelled against positive *E. coli* O157 result (Model B3), but not in any other combination.

Model A3: multilevel logistic regression model

Outcome: Log odds *E. coli* O157 positive

Level 1 fixed effects: Fluke positive

Level 2 fixed effects: Day, Youngest in group

Random effects: Farm

	Co-efficient	Std Error	Z value	<i>p</i>
Intercept	4.27	0.72	5.98	<0.00001
Fluke positive	0.50	0.52	0.96	0.34
Youngest in group	-0.18	0.04	-4.85	<0.00001
Day	-0.02	0.003	-5.65	<0.00001

AIC = 298.3, deviance = 288.3, df=329.

Random effects: Variance between farms = 0.34

Intraclass correlation = 0.09

Model B3: multilevel logistic regression model

Outcome: Log odds *E. coli* O157 positive

Level 1 fixed effects: log fluke PP

Level 2 fixed effects: Day, Youngest in group

Random effects: Farm

	Co-efficient	Std Error	Z value	<i>p</i>	Effect on risk of being <i>E. coli</i> positive of changing from 25 th to 75 th percentile
Intercept	3.6	0.67	5.40	<0.0001	
Log fluke PP	0.48	0.19	2.57	0.010	Odds increases by 0.067 (6.7%)
Youngest in group	-0.18	0.033	-5.5	<0.0001	Protective*
Day	-0.02	0.002	-6.13	<0.0001	Protective

AIC = 293.2, deviance = 83.2, df=329.

Random effects: Variance between farms = 0.19

Intraclass correlation = 0.05

* The higher the age of the youngest animal in the group, the lower the risk of infection with *E. coli* O157

Model C3: multilevel linear regression model

Outcome: Log *E. coli* O157 cfu

Level 1 fixed effects: Fluke positive

Level 2 fixed effects: Day, Youngest in group

Random effects: Farm

	Co-efficient	Std Error	t value	<i>p</i>
Intercept	2.92	0.38	7.64	<0.00001
Fluke positive	-0.02	0.20	-0.12	0.90
Youngest in group	-0.08	0.02	-3.53	0.0004
Day	-0.006	0.001	-4.52	<0.00001

AIC = 941.5, deviance = 929.5, df=328.

Random effects: Variance between farms = 0.22

Intraclass correlation = 0.20

Model D3: multilevel linear regression model

Outcome: Log *E. coli* O157 cfu

Level 1 fixed effects: log fluke PP

Level 2 fixed effects: Day, Youngest in group

Random effects: Farm

	Co-efficient	Std Error	t value	<i>p</i>
Intercept	2.80	0.38	7.32	<0.00001
Log fluke PP	0.09	0.08	1.14	0.26
Youngest in group	-0.08	0.02	-3.71	0.00024
Day	-0.006	0.001	-4.64	<0.00001

AIC = 940.2, deviance = 928.2, df=328.

Random effects: Variance between farms = 0.20

Intraclass correlation = 0.18

The introduction of random slopes worsened the model fit in each case so this was not pursued.

To investigate whether one particular farm with higher than average fluke levels might be having an excessive influence on the model, the model was run again without this one farm. Fluke PP was no longer significant although the co-efficients for the other two explanatory variables were almost unaltered. However, a logistic regression model including animals from just this one farm did not show fluke PP as a significant predictor for

E. coli shedding, indicating that the other farms are also contributing to the effect seen in model B3.

6.4 Discussion

E. coli O157 results from 334 cattle from 14 farms were modelled against their fluke results. Of the four combinations tested, a significant association between the log PP of the *F. hepatica* copro-antigen ELISA and *E. coli* O157 shedding was found when the fixed effects of day of sampling and the age of the youngest animal in the group, and the random effect of farm were adjusted for, on farms where both *E. coli* O157 and *F. hepatica* are present. The effect of this association was that a change from the 25th quartile of *F. hepatica* PP to the 75th quartile corresponded with a 6.7% increased odds of *E. coli* O157 shedding ($p = 0.010$).

The effect of this change in odds depends on the propensity of each animal that sheds to contaminate others. It is known that 5% of high shedding animals infect 80% of the others (Matthews et al., 2006). Therefore, a small increase in the number of cattle shedding would probably have little effect unless they were shedding large quantities of bacteria. Our study found no effect of fluke infection on the quantity of *E. coli* O157 shed.

E. coli O157 shedding varies widely from day to day (Robinson et al., 2009), and sensitivity may be as low as 40% for a one-off faecal sample (Echeverry et al., 2005). However, enumeration of *E. coli* cfu is likely to be even more subject to error so the binary measure (*E. coli* O157 positive or negative) which was derived by IMS could be expected to be a more accurate measure to use in a model. With the fluke copro antigen ELISA, the cut-off has been subject to debate, with some studies setting their own cut-off to increase sensitivity (Brockwell et al., 2014). However, this would have an unknown effect on the specificity and in the absence of an alternative fluke test to give an indication of the likely effect, we decided against arbitrarily reducing the cut off. A continuous measure of PP avoids this problem and gives a more reliable variable for modelling. Antigen level is correlated with fluke burden (Kamaludeen, 2016), so this is also a more biologically meaningful measure.

Day of sample collection and age of youngest animal in the group are relevant to both fluke and *E. coli* O157 levels. Younger animals are thought to have both a higher level of *E. coli* infection and to shed more (Chase-Topping et al., 2007). The reported seasonality of *E. coli* shedding varies between studies, with housed or feedlot cattle seeing a greater seasonality than cattle which are grazed in the summer (Ellis-Iversen et al., 2007). The linear

relationship between day and *E. coli* shedding seen here may be due to the fact that farms were sampled between January and October, with no samples taken during November and December. The fact that all animals from each farm were sampled on the same day is likely to contribute to the strong effects of the farm level variables. Geographical factors such as rainfall which may affect fluke were not included because there was no reason to suspect that *E. coli* was spatially clustered in this study or previously (Gunn et al., 2007).

The addition of further variables was prevented by insufficient variability within the data; the reasons for this are discussed below. Therefore there may be other explanatory or confounding variables that are not included in these models. The result should therefore be interpreted with caution, as the effect size is small and it is only seen in one of the model combinations.

There were 60 *E. coli* O157 positive farms in the FS101055 survey, thus it should have been possible to reach the 50 farms required. However, samples from only 35 farms were available for this study. This was mainly due to it being an add-on to a larger project. The study was also under powered due to the very low levels of fluke seen. *F. hepatica* only occurred in 43% of farms which was considerably less than the predicted 80%, meaning that only 14 farms were included in the analysis. This was despite a feasibility study having been conducted and published (Hickey et al., 2015).

Two references (McCann et al., 2010; Salimi-Bejestani et al., 2005a) were used to estimate a herd-level fluke prevalence of 70-80% for the sample size calculations in the feasibility study, and the effect of lower prevalence was not considered (Hickey et al., 2015). The type of animals tested differed between the fluke reference studies, which targeted dairy animals mainly because they are easy to test for the presence of *F. hepatica* presence via Bulk Milk Tank (BMT) samples, and the current study, in which animals due to enter the food chain were targeted. This meant that most animals sampled were beef animals. There are no current prevalence estimates for fluke in beef cattle, and there are various differences between beef and dairy animals. Dairy cows are under a great deal of metabolic stress and this may induce a Th2 shift in immune response (Elenkov, 2004), which may make them more susceptible to fluke infection. Treatment for fluke is more difficult in dairy animals due to the long milk withhold times of flukicides. Even though not all farms mentioned it in the questionnaire (only the previous three months of worming history was requested), it is likely that beef animals would be treated for fluke at housing if in a fluke risk area, particularly those that are due to go for slaughter soon.

Fluke prevalence varies by season and most prevalence studies take this into account and collect samples during the peak seasons of late summer and autumn, whereas in the current study the collection times were determined by other factors and were throughout the year. Partly due to season but also the nature of farming units tested, of the 35 groups of cattle sampled, only 9 were currently grazing, and of these, 3 had been turned out within the past 3 weeks. It is likely that some of the groups were permanently housed, which would put them at low risk for fluke exposure; however, this information was not detailed in the questionnaire.

The copro-antigen test has a similar diagnostic sensitivity to faecal egg count (FEC) at around 50%-60% sensitivity in sheep and lower in cattle, presumably due to lower fluke burdens and a greater volume of faeces (Duscher et al., 2011; Gordon et al., 2012), whereas the BMT antibody ELISA used to get prevalence estimates in previous studies has a sensitivity of 96% (Salimi-Bejestani et al., 2005a). An antibody ELISA can be expected to return more positive results as it can detect cases 2 weeks post-infection, whereas the copro-antigen ELISA does not reliably detect pre-patent infections (Gordon et al., 2012; Valero et al., 2009). Residual antibodies persisting for weeks or months after treatment will also result in false positives on the antibody ELISA. Some authors state that in high prevalence areas, all cattle can be expected to be positive on antibody ELISA regardless of current infection status (Skuce and Zadoks, 2013). The difference in sensitivity and specificity between the two types of test were not taken into consideration in the feasibility study (Hickey et al., 2015).

Biologically, the shedding of *E. coli* O157 is related both to colonisation, and also to triggers for a shedding event. Immune stress could be a reason for increased colonisation and also possibly for a shedding event although the mechanism for this has not been established (Munns et al., 2015). Stressful events and poor hygiene have been found to contribute to high level shedding (Chase-Topping et al., 2007; Ellis-Iversen et al., 2007). It is possible that infection with *F. hepatica*, which is known to modulate the immune response, may affect the propensity of an individual to be either infected with or shed *E. coli* O157. However as fluke levels do not change dramatically from day to day, it is difficult to relate the fluke infection of an individual to a single shedding event. There were insufficient samples in the study to test for an association between fluke infection and super shedding, but there did not appear to be a relationship.

The results of this study are not conclusive, but indicate that further research is required to investigate the possible association between *E. coli* O157 shedding and *F. hepatica* infection. A better approach would be to use a smaller number of herds which are known to have fluke, but to recruit a larger number of animals from each, thereby avoiding some of the inter-herd variation and ensuring fluke exposure, and to follow these animals up over a longer time frame and test a larger volume of faeces, thereby avoiding the issues of intermittent shedding and uneven distribution of bacteria within the faeces.

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Chapter 7: General Discussion

7.1 Background to this thesis

The growing world demand for food, combined with increasing resistance to antimicrobial and antiparasitic treatments, make it important to find new ways of reducing the burden of parasitic and infectious disease in livestock. Infection with liver fluke is known to modulate the immune system of co-infected hosts, altering the diagnosis and disease progression of other infections such as bovine tuberculosis (bTB), caused by *Mycobacterium bovis*.

bTB is a serious problem for the livestock industry, and much research has focussed on the effect of liver fluke on the bTB skin test (see chapter 4). Interest in this topic has persisted over the past 70 years and it has been investigated in countries all over the world. In earlier times it was thought that liver fluke infection caused false positives on the skin test, because bTB lesions were not always observed in skin test positive cattle (e.g. Manukyan, 1955; Merlen, 1950; Strelchenok, 1953). Suggested mechanisms for this hypothesis included the introduction of saprophytic mycobacteria by the migrating fluke (Keller, 1952; Nyireddy et al., 1966; Zorawski et al., 1987), or that migration of liver fluke through the lungs led to a false positive in the absence of bTB infection (Kochnev, 1950). However, the majority of cross sectional studies and all of studies on experimentally infected animals known to us, did not confirm that fluke infection led to false positives (El-Ahwal, 1969; Meyer, 1963; Yamaguchi et al., 1955).

More recently, improved knowledge of the immune system has changed our understanding of the interaction between fluke and bTB. Chronic liver fluke infection has been shown to induce a non-protective T helper cell type 2 (Th2) / regulatory response, characterised by IgG1, IL4 and IL10, which suppress T helper cell type 1 (Th1) cytokines such as interferon (IFN) γ (Bossaert et al., 2000; Bossaert et al., 2000; Graham-Brown, 2016). This has been shown to alter disease progression and diagnosis of bovine tuberculosis (bTB) in experimentally infected calves: co-infected calves had weaker responses to the bTB skin test, to the IFNY test, and their macrophage function was altered leading to less bacterial uptake (Flynn et al., 2009, 2007; Garza-Cuartero et al., 2016). A field study at the herd level found that fluke infection was associated with a bTB under-ascertainment rate of about one-third using the single intradermal comparative cervical tuberculin test (SCITT), compared to the expected rate in the absence of fluke (Claridge et al., 2012). One of the

aims of this thesis was to establish whether this effect on the bTB skin test was seen in individual cattle naturally exposed to *M. bovis* and *F. hepatica*.

Other previous work has shown that fluke infection alters immunity to *Salmonella dublin* and *Bordetella pertussis* (Aitken et al., 1981; Brady et al., 1999), and we therefore asked if immunomodulation due to liver fluke has an effect on susceptibility to other pathogens of cattle. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is of particular interest because of its similarities to *M. bovis* in terms of chronicity and immunomodulatory effects, although there are also some important differences, for example, the fact that very young calves in the first two weeks of life are the most susceptible, which is not the case for *M. bovis*. MAP is a common pathogen in the UK, and currently is not subject to restrictions, which makes it easier to study than *M. bovis*. We therefore investigated the effect of fluke in cows infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), to find out whether fluke infection was associated with the onset of clinical signs of Johne's disease.

Finally, we tested for an association between fluke infection and the shedding of the food poisoning bacterium *Escherichia coli* O157 in finishing cattle. *E.coli* O157 does not usually cause disease in cattle, but around 4-7% are colonised (Ellis-Iversen et al., 2007; Gunn et al., 2007; Paiba et al., 2003; Pearce et al., 2009), and it is a cause of severe disease in people (Chase-Topping et al., 2008; Strachan et al., 2006). This study came about through collaboration with the Moredun Research Institute and Scotland's Rural College. Samples and data were obtained through a prevalence study undertaken by the Food Standards Agency (FSA).

7.2 Summary of findings

7.2.1 Bovine tuberculosis

In chapter 3, we report on cross sectional and case control studies looking for an association between antibodies to liver fluke and the result of the bTB skin test. No significant association was found, but in 5/6 models the odds ratio for a cow with fluke antibodies to be diagnosed as a reactor or IR compared to a cow without fluke/with a lower antibody titre was less than one. This suggests that fluke infected cows may be less likely to be reactors to the SICCT. However, both the cross sectional and case control studies were under-powered, due to difficulty obtaining samples, so these results are not conclusive.

In the systematic review (chapter 4), the evidence from the nine included studies showed that liver fluke infection was associated with a reduction in the sensitivity of the bTB skin test, and was negatively associated with other measures of bTB disease progression including IFN γ production, lesions and bacterial recovery. These findings serve to confirm the results of chapter 3, however, associations were relatively small and the clinical significance in naturally exposed animals has not been adequately established. Moreover, the reason for the association has not been determined, and it is as yet unclear whether fluke infected animals are less likely to be infected with bTB, or whether they are only less likely to test positive for it.

7.2.2 Johne's disease

In chapter 5, no association was found between MAP antibodies and fluke antibody ELISA percent positivity (PP) values in dairy cattle, in either cross sectional or longitudinal studies. To our knowledge this is the first research study to examine the relationship between these two co-infecting pathogens, and there have been few studies of co-infection with MAP and other bovine pathogens. It is known that liver fluke infection causes the down-regulation of pro-inflammatory immunity with bystander effects on co-infecting pathogens, and that a decrease in Th1-type immunity is associated with the onset of clinical Johne's disease (Begg et al., 2011; Moreau and Chauvin, 2010). It has been suggested that within-herd prevalence is increased in the presence of pathogens that cause immunosuppression, such as bovine leukaemia virus (BLV) and bovine viral diarrhoea virus (BVDV) (Tiwari et al., 2009). Therefore, it would seem plausible that fluke could have an effect on MAP infection, but that this was not detected in our study. Possible reasons for this are discussed below.

7.2.3 *E. coli* O157

In chapter 6, a significant association between increasing *F. hepatica* copro-antigen ELISA result and *E. coli* O157 shedding was found, in a model controlling for the effects of season, farm and animal age. The effect of this association was that a change from the 25th quartile of *F. hepatica* copro-antigen ELISA PP to the 75th quartile corresponded with a 6.7% increased risk of *E. coli* O157 shedding ($p = 0.01$). This implies that fluke-infected cattle are more likely to shed *E. coli* O157. To our knowledge there is no previous research on co-infection with liver fluke and *E. coli* O157. Further research is required to support these findings, especially given the limitations of the study. However, it is logical that the immunomodulation induced by liver fluke would have an effect on the immune response to *E. coli* O157, because a Th1 type immune response with upregulated IFN γ is associated with

clearance of *E. coli* from the gut of cattle (Corbishley et al., 2016, 2014). If liver fluke does increase the likelihood of cattle being colonised with or shedding *E. coli* O157, this has public health implications.

7.3 Interpreting co-infection studies in naturally exposed populations

It is widely acknowledged that the interactions between pathogens seen in observational co-infection studies may not be consistent with those seen in laboratory studies under controlled conditions (Bradley and Jackson, 2008; Fenton et al., 2014). Interactions between co-infecting pathogens are changeable, depending on the relative timings and infectious burdens (Fenton, 2013), and vary between subsets of the population such older animals, males or females, and the immunocompromised (Fenton et al., 2014). In the case of bTB, the obvious effect of fluke in reducing the response to the bTB skin test reported in experimentally infected calves (Claridge et al., 2012; Flynn et al., 2009, 2007) was not always seen in cross sectional studies, some of which reported no association or an interaction in the opposite direction (Claridge, 2012; Munyeme et al., 2012; Schanzel and Stolarik, 1962).

In addition, the results may be difficult to interpret, as the finding that fluke positive animals are less likely to have visible lesions could be due to false positives on the bTB skin test, or because lesions are atypical in fluke infected animals (e.g. DEFRA, 2005). In diseases of wild animals, similar observations of inconsistencies between studies have been made (Fenton, 2013; Fenton et al., 2014). Interactions between pathogens at the individual level can appear to be cancelled out by the effects of other pathogens which come into play in the absence of one of the co-infecting pathogens of interest (Bradley and Jackson, 2008). These factors indicate why supporting evidence from experimental studies is required to increase confidence in the results of the *E. coli* and MAP studies.

The method used for analysis can also enhance or obscure the true effects (Fenton et al., 2014; Haukialmi and Henttonen, 1998). Comparing statistical methods in a simulated cross-sectional dataset, Fenton et al. (2014) reported that different approaches led to statistically significant results of the opposite sign. In general, generalised linear models (GLMs) were thought to be the most reliable method as these are able to include adjustments for confounders. Nonetheless, there are always likely to be other confounders which are not controlled for, because it is difficult to collect detailed enough information

about large numbers of naturally infected animals. Examples of variables that are particularly difficult to measure include variation in exposure risk due to behavioural differences, and intrinsic immune variation between individuals. Longitudinal GLMs are the best tool of statistical analysis as these control for the intra-individual variation that cannot easily be measured (Fenton et al., 2014). In this thesis, GLMs and longitudinal GLMs were used but the information required to control for confounders was not available in most cases, due to the anonymous nature of the data collection. For example, the MAP study was compromised by the lack of data on the animals' age, and for the *E. coli* study data on grazing and treatment history was incomplete.

Inherent immunity varies, but immune responses are also acquired as a result of exposure to pathogens, and the order in which pathogens occur is important. This was seen experimentally when the effects of liver fluke infection on *M. bovis* immunity were greater in cattle infected with *F. hepatica* before *M. bovis*, than in those which were infected with *M. bovis* first (Flynn et al., 2007). However, in naturally exposed populations it is difficult to know the order that infections occurred. Some assumptions may be justified: for example, young calves are likely to be infected with MAP before they are exposed to liver fluke. This could be one possible explanation for the lack of association observed between liver fluke and MAP antibodies in chapter 5. In the case of bTB, exposure to fluke may precede exposure to bTB, particularly if the herd has no previous history of bTB. However, in some persistently infected herds or where badgers are a source of infection, young calves could be exposed to *M. bovis* first. This could mean that the effect of fluke on the bTB skin test may be greater in herds with new bTB breakdowns, because fluke infection precedes exposure to *M. bovis*.

Ecological interference occurs when individuals are removed from the pool of susceptibles due to mortality (Rohani et al., 2003). In herds with persistent bTB, this could cause individuals with strong immune reactions to bTB being removed, and could inadvertently select for individuals that are more susceptible to fluke, if these are indeed less likely to respond to the bTB skin test. Treatment options for fluke-infected dairy cattle are limited, and even if treated, complete and lasting parasite clearance is unlikely as re-infection will occur unless the animals are housed. Over time, this could explain why, within an area of fluke prevalence, herds with high levels of fluke are less likely to have a bTB breakdown, as reported by Claridge et al., (2012).

7.4 Difficulties encountered

For various reasons, the bTB and *E. coli* studies were under-powered. There were also limitations relating to the sensitivity and interpretation of the diagnostic tests used. Consideration of some of the difficulties encountered may increase the likelihood of gaining useful results in future studies.

7.4.1 Reaching the required sample size

The bTB case control and cross sectional studies both failed to reach the required sample size, in spite of considerable efforts made to recruit participants (chapter 3). A previous case control study on naturally exposed animals in the UK also failed to reach the required sample size (Claridge, 2012). The restrictions applied under the bTB control programme and the stigma surrounding this made recruitment of farmers difficult. Other research on co-infection with liver fluke and bTB in individuals has either been performed in laboratory animals (Flynn et al., 2009, 2007; Garza-Cuartero et al., 2016) or in settings where restrictions do not apply such as in developing countries (Ameni et al., 2000; Munyeme et al., 2012) or prior to the introductions of bTB restriction in Europe (e.g. Ljesevic, 1957; Meyer, 1963). The exception was a study performed by DEFRA (2005), where access to information on bTB infected herds and samples from bTB test reactor animals were available due to the fact that the study was performed by the state animal health service.

The *E. coli* study was under powered due to the very low levels of fluke seen. This was despite a sample size calculation having been conducted and published (Hickey et al., 2015). Difficulties resulting from adding our study onto the FSA study meant that resources were not ready in time, meaning that 35% of the samples were not available to us.

Secondly, fluke prevalence in the tested samples was much lower than anticipated, at least partly because the feasibility study did not take into consideration the low sensitivity of the copro-antigen ELISA compared to the antibody ELISAs on which prevalence estimates were based (Duscher et al., 2011; Salimi-Bejestani et al., 2005a). Another reason was that the study population was finishing cattle, which as a group are less likely to graze outside than other groups, and may be more likely to be proactively treated for parasites to maximise weight gain, and therefore may be less likely to have fluke than the populations of dairy cattle on which prevalence studies have been done (Howell et al., 2015; McCann et al., 2010). This study was intended to be a pilot study, using samples obtained through another study in order to save resources. However, the problems encountered demonstrate the importance of designing a study to answer the particular question of interest.

7.4.2 Diagnostic test sensitivity

In chapter 3 and 5, diagnosis of fluke, MAP and bTB was via the immune response rather than direct identification of the pathogen: antibodies in the case of MAP and fluke, and the cellular response in the bTB skin test. The antibody titres give an indication of the burden of infection, but vary widely between individuals (Graham-Brown, 2016). They may give a more accurate indication of the immune response of the animal than absolute parasite numbers, but it may not be straightforward to tell the difference between protection and exposure (Bradley and Jackson, 2008). Direct identification of bTB and fluke is difficult in live animals, as faecal egg count (FEC) has low sensitivity and bTB infected cattle, in the early stages, are often not infectious and therefore the diagnostic methods used in humans, such as examination of a sputum smear, are not useful (DEFRA, 2005).

In general, error in the independent variable is likely to bias the effect size seen in a regression model towards zero, so determining the true infection status of an animal is important to accurately estimate the effect of risk factors (Schennach and Schennach, 2012). The relatively poor sensitivity of the bTB skin test can be mitigated to some extent by performing different tests in parallel, but as bTB testing in the UK is controlled by DEFRA, this was not possible in our study.

The main limitation of the MAP study concerns the poor sensitivity of the test used to detect MAP, although this is considered to be the most sensitive test available (Caldow et al., 2009). The ELISA is only suitable for detecting cattle in the later, clinical stages of disease. In the clinical situation, the test is used alongside other information such as age and clinical signs, and tests are interpreted as one of a sequence, with the animal coded red, orange or green, depending on the results of the entire series. In our study, follow-up was only possible for 1 year, but given the long incubation period of MAP, a longer follow period would be preferable.

In the *E. coli* study, direct diagnosis of both pathogens was performed, with no measures of immune response. Due to the fact that the study was an add-on to another study, the samples available were faecal samples, which were tested for fluke using the copro-antigen ELISA. This has a similar, or according to some studies, lower sensitivity than FEC (Brockwell et al., 2014, 2013; Duscher et al., 2011). Both only detect current, patent infections, which accounts for some of the reduced sensitivity compared to antibody tests that become positive early during infection and remain positive for some weeks afterwards (Brockwell et al., 2013). However, even when used on cows with patent infections, test sensitivity is low.

One study reported that only 30% of seropositive animals were identified, compared to 45% of animals that were detected by faecal egg count (Duscher et al., 2011). There has also been debate about the cut-offs used. Some studies reported a higher sensitivity for the copro-antigen ELISA but used lower cut-offs than recommended by the manufacturer (Brockwell et al., 2013; Charlier et al., 2008). In our study, in the absence of any alternative method of fluke diagnosis, we did not alter the cut-off.

Whilst use of a poorly sensitive test may be acceptable in clinical cases, where results are interpreted in conjunction with farm history and clinical signs, a sensitivity this low is probably not appropriate for epidemiological studies where no supporting information is available, as the increased error is likely to cause an unacceptable loss of power.

The pattern of *E. coli* O157 shedding is known to be intermittent, and in groups of animals where one is found to be shedding, one or most are thought to be infected (Robinson et al., 2009). The original FSA study was designed to determine whether a group was infected, and thus the intermittent nature of shedding was not a problem, but a single negative *E. coli* test does not determine that an animal is uninfected.

7.4.3 Other confounding factors

In these studies, the amount of data available on confounding factors was limited. This was due to the anonymous nature of the sample collection in the MAP study and the bTB case control study. For the *E. coli* study, information was available but only at the group level. For the bTB cross sectional study, information was collected but the dataset was not big enough to allow its inclusion in the model.

7.5 Ways forward

7.5.1 Optimising study design

As bTB is a rare disease occurring in only a small proportion of cattle in most infected herds, it is relatively inefficient to study using a cohort study, however, longitudinal follow up of individuals maximises the likelihood of genuine interactions being detectable (Fenton et al., 2014). Confounding factors include age, risk of exposure, lactation stage, whether pregnant, whether home bred or originating from another farm, body condition score, treatment history, general health, time of year and area of the country, and it is difficult to adjust for all of these (Fenton et al., 2014). Acquired immunity to previous infection may also affect the current 'co-infecting' pathogen (Lello et al., 2008), and individual differences

in risk of infection result from genetic and behavioural variation. Longitudinal follow-up allows this individual variation to be controlled for. However, gaining co-operation of farmers to participate in a long and involved research project may be difficult. Longitudinal studies also need to be timed with reference to the changes in parasite status, and their effects, and the dataset needs to be large enough to allow for potential confounders to be included in a model (Fenton et al., 2014). A longer period of follow up would ideally be required, over at least two grazing seasons to allow for inter-seasonal variation. Treatment with a flukicide would also be useful to ensure a change in fluke levels, although the limitations of treating dairy cattle mean that the effects of flukicide treatment may be difficult to separate from immunological changes around calving.

Similar considerations should be made for study of MAP. Longer follow up, possibly lifelong, would give a more accurate estimate of whether a cow was in the red/clinical phase, or whether it was in the orange/intermittent phase of infection.

Finite resources lead to trade-offs between immune responses, with one type of response being dominant (Bradley and Jackson, 2008). This trade-off has been found to be more marked in resource limited situations in wild animals (Ezenwa et al., 2010; Hayward et al., 2014; Nussey et al., 2014), and this may also apply to farmed animals under metabolic stress. Selective breeding for characteristics such as milk yield, growth rate and carcass conformation may have caused decreased genetic variation in immune response between individuals within a herd and between herds. There was evidence of differences between beef and dairy cattle in a study that showed an association between fluke and bTB in dairy cattle but not in beef cattle which may have been due to inherent differences between different types of cattle (DEFRA, 2005). In the same study, dairy cattle were significantly less likely to have visible bTB lesions than other cattle, demonstrating a difference in pathology. Other sub-populations may be more susceptible to infection due to age, pregnancy, production stresses or immunosuppressive disease. Fluke infection generally only affects a small proportion of the herd, with 20% of animals having 80% of the fluke burden (see chapter 2). Focusing on these 20% could be a more effective way to concentrate resources than testing the whole herd (Lloyd-Smith et al., 2005; Woolhouse et al., 1997).

7.5.2 Choice of diagnostic tests

As none of the tests used here are perfect in terms of both sensitivity and specificity, utilisation of more than one type of test will add certainty to the results. This may involve

getting more than one type of sample. We found that individual cattle fluke results did not change significantly over three sampling sessions three months apart. There is a time lag of at least 11 weeks after parasitological cure before antibody levels return to negative using the ELISA (Salimi-Bejestani et al., 2005b), and this may take up to 6 months (Castro et al., 2000). Thus another method of fluke diagnosis would be useful to confirm diagnosis. The relatively poor sensitivity of FEC could be increased by using large volumes of faeces and testing over several days, although this is time consuming (Charlier et al., 2008; Rapsch et al., 2006).

For bTB, use of ancillary tests such as the IFNY test and antibody test would add much useful information. The antibody test would have been of particular interest to discover any effect of fluke in enhancing Th2 responses. Ideally this would be followed by post mortem examination combined with culture and histopathology. An investigation into cattle-to-cattle transmission of bTB (DEFRA, 2005) took the approach of keeping SCITT-negative in-contact cattle for seven weeks to see whether these animals would become test positive and develop lesions eventually. This method, although logistically difficult, could be useful to improve the sensitivity of the two tests, as 11.5% of the initially SCITT-negative cattle were confirmed to have bTB at post mortem examination. In view of the restrictions surrounding bTB cases and the resulting difficulty in obtaining samples, collaboration with DEFRA is probably required for the effective study of bTB in the UK.

Due to the poor sensitivity of the MAP antibody test, parallel testing of faecal samples to confirm shedding would be very useful. In the case of the *E. coli* study, given the intermittent nature of shedding, re-sampling over several days is required to confirm whether or not an individual animal is infected, and determining the proportion of days that an individual sheds could be used to provide an indication of its infectivity to others.

7.5.3 Measurement of immune profiles

Different types of response are characterised by specific cytokine production, and measurement of these can be used to categorise the immune response and can aid causal inference (Bradley and Jackson, 2008). This is because the broad consequences of infection (Th1/Th2/Th17/regulatory) are fairly consistent for certain types of pathogen, even though the consequences in terms of pathogen interaction may differ for every co-infecting combination and are difficult to predict (Graham et al., 2007).

A recent study by Graham-Brown (2016) defined the cytokine profiles of cattle naturally infected with liver fluke. IL4, IL5 and eosinophilia were observed, although these were also seen with nematode infected, fluke-negative cattle, showing the importance of obtaining as much information about co-infecting pathogens as possible. There was also considerable variation between individuals. In bTB infected cattle, IFN γ levels were raised initially, and bTB specific IgG1 antibodies were detected later in the course of disease, from 60-100 days post infection in experimentally infected animals, although this varied depending on antigen used (Hanna et al., 1992). In naturally infected cattle, IFN γ and IL10 were higher in cattle with confirmed bTB than in those without lesions (DEFRA, 2005). Measurement of cytokine production from infected host cells stimulated with pathogen specific and non-specific antigens can provide information about the effect on the host of the co-infecting pathogens, and on the overall immune environment (Bradley and Jackson, 2008).

A pitfall of measuring only the infection status without any idea of the underlying immune responses is that any correlations seen may have multiple explanations. There may be external factors related to transmission that cause an apparent correlation, for example underlying host susceptibility that is independent of both pathogens, or a spatial correlation (Fenton et al., 2014). In a study of two co-infecting parasites in children, measurement of the immune response showed that cross-protective immunity was induced, whereas analysis of parasite burden alone gave the impression that infection with the two species were correlated. This was due to spatial correlation resulting from where the study subjects lived, which was not easily controlled for (Booth, 2006). If two pathogens compete within the host but the host is susceptible to both, whereas other hosts are resistant to one, the infections could be correlated and the competitive aspect obscured, unless immune responses are measured (Bradley and Jackson, 2008). Cytokine measurement can also give information about interactions when altered outcomes are not apparent until a later time. For example Ezenwa and Jolles (2014) measured IFN γ alongside the bTB skin test to identify a difference in immune response between animals co-infected with bTB and nematodes, and singly infected animals. The risk of bTB infection was no different in the nematode-infected and nematode-free cattle, but further evidence that a difference existed was later shown by the finding that mortality in co-infected animals was more than nine times as likely. These examples show how an appreciation of the immune response can aid interpretation of infection status as well as facilitating causal inference in co-infection studies.

7.5.4 Other research priorities

Experimentally infected animals are the most reliable way of detecting interactions of co-infecting pathogens in the first instance (Fenton et al., 2014). This has been done for fluke and bTB, but not, as yet, for fluke plus *E. coli* O157 or MAP.

Other common pathogens of cattle which would be of interest to investigate in terms of co-infections include nematodes, leptospirosis, BVDV and infectious bovine rhinotracheitis (IBR).

Also yet un-investigated are the production effects of co-infections. For example, clinical Johne's disease and fluke infections have both been shown to decrease milk yield (Charlier et al., 2007; Hasonova and Pavlik, 2006; Howell et al., 2015), but their combined effect is unknown.

7.6 Conclusion

Co-infections are complex and difficult to study in naturally exposed populations. Carefully planned studies with longitudinal follow up and consideration given to the most effective use of diagnostic tests are required. Despite the problems encountered during the completion of this research, we have found evidence for an effect of fluke on the bTB skin test and on *E. coli* O157 shedding. This adds to the body of evidence that liver fluke infection has bystander effects on immunity to other pathogens. Future research in naturally exposed populations should include cytokine measurement to aid understanding of underlying immune mechanisms.

APPENDIX: Bovine TB and Liver Fluke Systematic Review data collection form

Study ID	Author(s)	Year
Report ID	Reviewed by	Date

Methods:

Age	
Number	
Study population	
Selection method	
Duration	
Setting	
Country	
Fluke detection methods	
TB detection methods	
Details of intervention/dose rate etc	
Design	Retrospective/prospective Data source of retrospective

Risk of bias

Comparability of groups assessed? How?	No Groups materially different
Selection bias No	Yes/possible
Response bias No	Yes/possible
Random allocation (RCT only) Yes	No
Allocation concealment (RCT only) Yes	No
Detection bias No	Yes/possible
Blinding Yes (Who, At which stage of study)	No and may cause bias

No but probably not causing bias	
Measurement bias No	Yes/possible
Recall bias No	Yes/possible
Ascertainment bias No	Yes/possible
Confounders considered? Which ones? [Age, herd size, region,	No
Missing data: None Present, accounted for, not causing bias	Present but not accounted for Present, accounted for causing bias or unclear Not mentioned
All methods performed and reported as described	Some results missing or analysed differently to methods
Overall risk of bias Low	High
Quality of study High	Low

Analysis:

Described fully?

Results:

Data

Author conclusions:

Funding source

References to be followed up:

Author correspondence required

Any other comments

References

- Abunna, F., Asfaw, L., Megersa, B., Regassa, A., 2010. Bovine fasciolosis: coprological, abattoir survey and its economic impact due to liver condemnation at Soddo municipal abattoir, Southern Ethiopia. *Trop. Anim. Health Prod.* 42, 289–92. doi:10.1007/s11250-009-9419-3
- Affroze, S., Begum, N., Islam, M., Rony, S., Islam, M., Mondal, M., 2013. Risk Factors and Gross Pathology of Bovine Liver Fluke Infection at Netrokona District, Bangladesh. *J. Anim. Sci. Adv* 3, 83–9083. doi:10.5455/jasa.20130219031948
- Aitken, M.M., Hughes, D.L., Jones, P.W., 1980. Effects of *Fasciola hepatica* infection on responses of rats to reinfection with *Salmonella dublin*. *Res. Vet. Sci.* 28, 250–3.
- Aitken, M.M., Hughes, D.L., Jones, P.W., Hall, G.A., Smith, G.S., 1979. Immunological responses of fluke-infected and fluke-free cattle to *Salmonella dublin* and other antigens. *Res. Vet. Sci.* 27, 306–12.
- Aitken, M.M., Jones, P.W., Hall, G.A., Hughes, D.L., Brown, G.T., 1981. Responses of fluke-infected and fluke-free cattle to experimental reinfection with *Salmonella dublin*. *Res. Vet. Sci.* 31, 120–6.
- Altmann, D.M., 2009. Review series on helminths, immune modulation and the hygiene hypothesis: nematode coevolution with adaptive immunity, regulatory networks and the growth of inflammatory diseases. *Immunology* 126, 1–2. doi:10.1111/j.1365-2567.2008.03006.x
- Ameni, G., Medhin, G., 2000. Effect of Gastro-intestinal Parasitosis on Tuberculin Test for the Diagnosis of Bovine Tuberculosis. *J. Appl. Anim. Res.* 18, 221–224. doi:10.1080/09712119.2000.9706347
- Ameni, G., Miorner, H., Roger, F., Tibbo, M., 2000. Comparison between comparative tuberculin and gamma-interferon tests for the diagnosis of bovine tuberculosis in Ethiopia. *Trop. Anim. Health Prod.* 32, 267–276. doi:10.1023/a:1005271421976
- Andrews, S.J., 1999. The Life Cycle of *Fasciola hepatica*, in: Dalton, J.P. (Ed.), *Fasciolosis*. CABI Publishing, New York, pp. 1–29.
- Anthony, R.M., Urban, J.F., Alem, F., Hamed, H.A., Rozo, C.T., Boucher, J.-L., Van Rooijen, N., Gause, W.C., 2006. Memory TH2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat. Med.* 12, 955–960. doi:10.1038/nm1451
- APHA, 2014a. TB64A(W).
- APHA, 2014b. TB64A(ES).
- Arsenault, R.R.J., Maattanen, P., Daigle, J., Potter, A., Al, E., 2014. From mouth to macrophage: Mechanisms of innate immune subversion by *Mycobacterium avium* subsp. *Paratuberculosis*. *Vet. Res.* 45, 1–15. doi:10.1186/1297-9716-45-54
- Asper, D.J., Karmali, M.A., Townsend, H., Rogan, D., Potter, A.A., 2011. Serological response of Shiga toxin-producing *Escherichia coli* type III secreted proteins in sera from vaccinated rabbits, naturally infected cattle, and humans. *Clin. Vaccine Immunol.* 18, 1052–7. doi:10.1128/CVI.00068-11
- Aydin, M., Herzig, G.P.D., Jeong, K.C., Dunigan, S., Shah, P., Ahn, S., 2014. Rapid and sensitive detection of *Escherichia coli* O157:H7 in milk and ground beef using magnetic bead-based immunoassay coupled with tyramide signal amplification. *J. Food Prot.* 77, 100–5. doi:10.4315/0362-028X.JFP-13-274
- Balcewicz-Sablinska, M.K., Keane, J., Kornfeld, H., Remold, H.G., 1998. Pathogenic *Mycobacterium*

tuberculosis evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-alpha. *J. Immunol.* 161, 2636–41.

- Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* 67. doi:10.18637/jss.v067.i01
- Begg, D.J., de Silva, K., Carter, N., Plain, K.M., Purdie, A., Whittington, R.J., 2011. Does a th1 over th2 dominance really exist in the early stages of mycobacterium avium subspecies paratuberculosis infections? *Immunobiology* 216, 840–846. doi:10.1016/j.imbio.2010.12.004
- Behm, C.A., Sangster, N.C., 1999. Pathology, Pathophysiology and Clinical Aspects, in: Dalton, J.P. (Ed.), *Fasciolosis*. CABI Publishing, New York, pp. 185–224.
- Bennema, S., Vercruyse, J., Claerebout, E., Schnieder, T., Strube, C., Ducheyne, E., Hendrickx, G., Charlier, J., 2009. The use of bulk-tank milk ELISAs to assess the spatial distribution of *Fasciola hepatica*, *Ostertagia ostertagi* and *Dictyocaulus viviparus* in dairy cattle in Flanders (Belgium). *Vet. Parasitol.* 165, 51–57. doi:10.1016/j.vetpar.2009.07.006
- Bennema, S.C., Ducheyne, E., Vercruyse, J., Claerebout, E., Hendrickx, G., Charlier, J., 2011. Relative importance of management, meteorological and environmental factors in the spatial distribution of *Fasciola hepatica* in dairy cattle in a temperate climate zone. *Int. J. Parasitol.* 41, 225–233. doi:10.1016/j.ijpara.2010.09.003
- Bessell, P.R., Orton, R., White, P.C.L., Hutchings, M.R., Kao, R.R., 2012. Risk factors for bovine Tuberculosis at the national level in Great Britain. *BMC Vet. Res.* 8, 51. doi:10.1186/1746-6148-8-51
- Biek, R., O’Hare, A., Wright, D., Mallon, T., McCormick, C., Orton, R.J., McDowell, S., Trewby, H., Skuce, R.A., Kao, R.R., 2012. Whole Genome Sequencing Reveals Local Transmission Patterns of *Mycobacterium bovis* in Sympatric Cattle and Badger Populations. *Plos Pathog.* 8. doi:10.1371/journal.ppat.1003008
- Booth, M., 2006. The role of residential location in apparent helminth and malaria associations. *Trends Parasitol.* 22, 359–362. doi:10.1016/j.pt.2006.06.007
- Bossaert, K., Farnir, F., Leclipteux, T., Protz, M., Lonneux, J.F., Losson, B., 2000. Humoral immune response in calves to single-dose, trickle and challenge infections with *Fasciola hepatica*. *Vet. Parasitol.* 87, 103–23.
- Bossaert, K., Jacquinet, E., Saunders, J., Farnir, F., Losson, B., 2000. Cell-mediated immune response in calves to single-dose, trickle, and challenge infections with *Fasciola hepatica*. *Vet. Parasitol.* 88, 17–34. doi:10.1016/S0304-4017(99)00200-9
- Bovine TB info, 2016. Accuracy of the TB test for cattle [WWW Document]. URL <http://www.bovinetb.info/testing.php> (accessed 9.1.16).
- Bradley, J.E., Jackson, J. a, 2008. Measuring immune system variation to help understand host-pathogen community dynamics. *Parasitology* 135, 807–823. doi:10.1017/S0031182008000322
- Brady, M.T., O’Neill, S.M., Dalton, J.P., Mills, K.H.G., 1999. *Fasciola hepatica* suppresses a protective Th1 response against *Bordetella pertussis*. *Infect. Immun.* 67, 5372–5378.
- Bretschneider, G., Berberov, E.M., Moxley, R.A., 2007. Isotype-specific antibody responses against *Escherichia coli* O157:H7 locus of enterocyte effacement proteins in adult beef cattle following experimental infection. *Vet. Immunol. Immunopathol.* 118, 229–238. doi:10.1016/j.vetimm.2007.06.005
- Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster, N.C., 2014. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. *Int. J. Parasitol. Drugs Drug Resist.* 4, 48–54.

doi:10.1016/j.ijpddr.2013.11.005

- Brockwell, Y.M., Spithill, T.W., Anderson, G.R., Grillo, V., Sangster, N.C., 2013. Comparative kinetics of serological and coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. *Vet. Parasitol.* 196, 417–426. doi:10.1016/j.vetpar.2013.04.012
- Brooks-Pollock, E., Conlan, A., Mitchell, A., Blackwell, R., McKinley, T., Wood, J., 2013. Age-dependent patterns of bovine tuberculosis in cattle. *Vet. Res.* 44, 97.
- Broughan, J., Brouwer, A., Upton, P., Downs, S., Goodchild, T., Brunton, L., Blissitt, M., Government, S., De La Rua-Domenech, R., 2014. Analysis of bovine tuberculosis surveillance at routine slaughter of cattle in Great Britain (2009-2013).
- Broughan, J.M., Durr, P., Clifton-Hadley, R., Colloff, A., Goodchild, T., Sayers, R., Williamson, S., Downs, S.H., 2009. Bovine tuberculosis and *Fasciola hepatica* infection. *Soc. Epidemiol. Prev. Vet. Med.*
- Broughan, J.M., Durr, P., Colloff, A., Goodchild, T., Sayers, R., Williamson, S., Downs, S.H., 2008. Bovine tuberculosis and *Fasciola hepatica* infection. *Test* 123, 2008–2008.
- Buddle, B.M., Wilson, T., Luo, D., Voges, H., Linscott, R., Martel, E., Lawrence, J.C., Neill, M.A., 2013. Evaluation of a Commercial Enzyme-Linked Immunosorbent Assay for the Diagnosis of Bovine Tuberculosis from Milk Samples from Dairy Cows. *Clin. Vaccine Immunol.* 20, 1812–1816. doi:10.1128/cvi.00538-13
- Byrne, A.W.A., McBride, S., Lahuerta-Marin, A., Guelbenzu, M., McNair, J., Skuce, R.A., McDowell, S.S.W.J., 2016. Liver fluke (*Fasciola hepatica*) infection in cattle in Northern Ireland: a large-scale epidemiological investigation utilising surveillance data. *Parasit. Vectors* 9, 209. doi:10.1186/s13071-016-1489-2
- Caldow, G.L., Burns, F., Mackay, E., 2009. SB4022 An Integrated Strategy to Determine the Herd Level Prevalence of Johne's Disease in the UK Dairy Herd, Chapter 2: Results of serological screening of the sample herds.
- Castro, E., Freyre, A., Hernandez, Z., 2000. Serological responses of cattle after treatment and during natural re-infection with *Fasciola hepatica*, as measured with a dot-ELISA system. *Vet. Parasitol.* 90, 201–208. doi:10.1016/s0304-4017(00)00228-4
- Cervi, L., Rossi, G., Cejas, H., Masih, D.T., 1998. *Fasciola hepatica*-induced immune suppression of spleen mononuclear cell proliferation: role of nitric oxide. *Clin. Immunol. Immunopathol.* 87, 145–54.
- Cervi, L., Rossi, G., Masih, D.T., 1999. Potential role for excretory–secretory forms of glutathione-S-transferase (GST) in *Fasciola hepatica*. *Parasitology* 119, 627–633.
- Chanlun, A., Emanuelson, U., Aiumlamai, S., Björkman, C., 2006. Variations of *Neospora caninum* antibody levels in milk during lactation in dairy cows. *Vet. Parasitol.* 141, 349–55. doi:10.1016/j.vetpar.2006.05.010
- Chaparro, J.J., Ramírez, N.F., Villar, D., Fernandez, J.A., Londoño, J., Arbeláez, C., López, L., Aristizabal, M., Badel, J., Palacio, L.G., Olivera, M., 2016. Survey of gastrointestinal parasites, liver flukes and lungworm in feces from dairy cattle in the high tropics of Antioquia, Colombia. *Parasite Epidemiol. Control* 1, 124–130. doi:10.1016/j.parepi.2016.05.001
- Charlier, J., Bennema, S.C., Caron, Y., Counotte, M., Ducheyne, E., Hendrickx, G., Vercruyse, J., 2011. Towards assessing fine-scale indicators for the spatial transmission risk of *Fasciola hepatica* in cattle. *Geospat. Health* 5, 239–245.
- Charlier, J., Claerebout, E., Duchateau, L., Vercruyse, J., 2005. A survey to determine relationships

- between bulk tank milk antibodies against *Ostertagia ostertagi* and milk production parameters. *Vet. Parasitol.* 129, 67–75. doi:10.1016/j.vetpar.2004.11.024
- Charlier, J., De Meulemeester, L., Claerebout, E., Williams, D., Vercruyse, J., 2008. Qualitative and quantitative evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. *Vet. Parasitol.* 153, 44–51. doi:10.1016/j.vetpar.2008.01.035
- Charlier, J., Duchateau, L., Claerebout, E., Williams, D., Vercruyse, J., 2007. Associations between anti-*Fasciola hepatica* antibody levels in bulk-tank milk samples and production parameters in dairy herds. *Prev. Vet. Med.* 78, 57–66. doi:10.1016/j.prevetmed.2006.09.010
- Charlier, J., Hostens, M., Jacobs, J., Van Ranst, B., Duchateau, L., Vercruyse, J., 2012. Integrating Fasciolosis Control in the Dry Cow Management: The Effect of Closantel Treatment on Milk Production. *PLoS One* 7. doi:10.1371/journal.pone.0043216
- Chase-Topping, M., Gally, D., Low, C., Matthews, L., Woolhouse, M., 2008. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nat. Rev. Microbiol.* 6, 904–12. doi:10.1038/nrmicro2029
- Chase-Topping, M.E., McKendrick, I.J., Pearce, M.C., MacDonald, P., Matthews, L., Halliday, J., Allison, L., Fenlon, D., Low, J.C., Gunn, G., Woolhouse, M.E.J., 2007. Risk factors for the presence of high-level shedders of *Escherichia coli* O157 on Scottish farms. *J. Clin. Microbiol.* 45, 1594–1603. doi:10.1128/JCM.01690-06
- Chauvin, A., Boulard, C., 1996. Local immune response to experimental *Fasciola hepatica* infection in sheep. *Parasite* 3, 209–15.
- Claridge, J.A., 2012. Does *Fasciola hepatica* infection increase the susceptibility of cattle to infection with other pathogens normally controlled by a Th1 or pro-inflammatory response ? This thesis has been submitted in accordance with the requirements of the University of Li.
- Claridge, J., Diggle, P., McCann, C.M., Mulcahy, G., Flynn, R., McNair, J., Strain, S., Welsh, M., Baylis, M., Williams, D.J.L., 2012. *Fasciola hepatica* is associated with the failure to detect bovine tuberculosis in dairy cattle. *Nat. Commun.* 3. doi:10.1038/ncomms1840
- Clegg, T.A., Duignan, A., Whelan, C., Gormley, E., Good, M., Clarke, J., Toft, N., More, S.J., 2011. Using latent class analysis to estimate the test characteristics of the gamma-interferon test, the single intradermal comparative tuberculin test and a multiplex immunoassay under Irish conditions. *Vet. Microbiol.* 151, 68–76. doi:10.1016/j.vetmic.2011.02.027
- Clery, D., Torgerson, P., Mulcahy, G., 1996. Immune responses of chronically infected adult cattle to *Fasciola hepatica*. *Vet. Parasitol.* 62, 71–82. doi:10.1016/0304-4017(95)00858-6
- Collins, M.T., Wells, S.J., Petrini, K.R., Collins, J.E., Schultz, R.D., Whitlock, R.H., 2005. Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. *Clin. Diagn. Lab. Immunol.* 12, 685–92. doi:10.1128/CDLI.12.6.685-692.2005
- Cooper, A.M., Dalton, D.K., Stewart, T.A., Griffin, J.P., Russell, D.G., Orme, I.M., 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med.* 178, 2243–7.
- Corbishley, A., Ahmad, N.I., Hughes, K., Hutchings, M.R., McAteer, S.P., Connelley, T.K., Brown, H., Gally, D.L., McNeilly, T.N., 2014. Strain-dependent cellular immune responses in cattle following *Escherichia coli* O157:H7 colonization. *Infect. Immun.* 82, 5117–31. doi:10.1128/IAI.02462-14
- Corbishley, A., Connelley, T., Wolfson, E., Ballingall, K., Beckett, A., Gally, D., McNeilly, T., 2016. Identification of epitopes recognised by mucosal CD4+ T-cell populations from cattle experimentally colonised with *Escherichia coli* O157:H7. *Vet. Res.* 47.
- Corner, L.A., 1994. Post mortem diagnosis of *Mycobacterium bovis* infection in cattle. *Vet. Microbiol.*

40, 53–63. doi:10.1016/0378-1135(94)90046-9

- Costello, E., Egan, J.W.A., Quigley, F.C., O'Reilly, P.F., 1997. Performance of the single intradermal comparative tuberculin test in identifying cattle with tuberculous lesions in Irish herds. *Vet. Rec.* 141, 222–224.
- Coussens, P.M., 2001. Mycobacterium paratuberculosis and the bovine immune system. *Anim. Health Res. Rev.* 2, 141–61.
- Coussens, P.M., 2004. Model for immune responses to Mycobacterium avium subspecies paratuberculosis in cattle. *Infect. Immun.* 72, 3089–3096. doi:10.1128/IAI.72.6.3089
- COWS, 2016. Liver fluke and cattle [WWW Document]. Control Worms Sustain. URL <http://www.cattleparasites.org.uk/fluke.html>
- Cox, F.E., 2001. Concomitant infections, parasites and immune responses. *Parasitology* 122 Suppl, S23–S38. doi:10.1017/S003118200001698X
- Dalton, J.P., Robinson, M.W., Mulcahy, G., O'Neill, S.M., Donnelly, S., 2013. Immunomodulatory molecules of Fasciola hepatica: candidates for both vaccine and immunotherapeutic development. *Vet. Parasitol.* 195, 272–85. doi:10.1016/j.vetpar.2013.04.008
- de la Rúa-Domenech, R., 2006. Human Mycobacterium bovis infection in the United Kingdom: Incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. *Tuberculosis (Edinb).* 86, 77–109. doi:10.1016/j.tube.2005.05.002
- de la Rúa-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H., Clifton-Hadley, R.S., 2006. Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. *Res. Vet. Sci.* 81, 190–210. doi:10.1016/j.rvsc.2005.11.005
- Dean, G.S., Rhodes, S.G., Coad, M., Whelan, A.O., Cockle, P.J., Clifford, D.J., Hewinson, R.G., Vordermeier, H.M., 2005. Minimum infective dose of Mycobacterium bovis in cattle. *Infect. Immun.* 73, 6467–6471. doi:10.1128/iai.73.10.6467-6471.2005
- Department for the Environment, Food and Rural Affairs. Pathogenesis and diagnosis of tuberculosis in cattle - complementary field studies. Final report. Veterinary Laboratories Agency, Surrey, UK; 2005 Sept. Project No.: SE3013.
- DEFRA, 2015. 2010 to 2015 government policy: bovine tuberculosis (bovine TB) [WWW Document]. URL <https://www.gov.uk/government/publications/2010-to-2015-government-policy-bovine-tuberculosis-bovine-tb/2010-to-2015-government-policy-bovine-tuberculosis-bovine-tb#issue> (accessed 9.20.16).
- DEFRA, 2016. Official Veterinarian Instructions [WWW Document]. URL http://ahvla.defra.gov.uk/External_OV_Instructions/TB_Instructions/Active_Surveillance/index.htm (accessed 9.21.16).
- Department for Environment Food and Rural Affairs, 2014a. The Strategy for achieving Officially Bovine Tuberculosis Free status for England.
- Department for Environment Food and Rural Affairs, 2014b. Monthly publication of National Statistics on the Incidence of Tuberculosis (TB) in Cattle to end May 2014 for Great Britain.
- Department for Environment Food and Rural Affairs, 2014c. Latest statistics on the incidence of tuberculosis (TB) in cattle in Great Britain [WWW Document]. URL <https://www.gov.uk/government/statistics/incidence-of-tuberculosis-tb-in-cattle-in-great-britain>
- Donnelly, C.A., Nouvellet, P., 2013. The contribution of badgers to confirmed tuberculosis in cattle in

- high-incidence areas in England. *PLoS Curr.* 5.
doi:10.1371/currents.outbreaks.097a904d3f3619db2fe78d24bc776098
- Donnelly, S., O'Neill, S.M., Stack, C.M., Robinson, M.W., Turnbull, L., Whitchurch, C., Dalton, J.P., 2010. Helminth cysteine proteases inhibit TRIF-dependent activation of macrophages via degradation of TLR3. *J. Biol. Chem.* 285, 3383–92. doi:10.1074/jbc.M109.060368
- Duignan, A., Good, M., More, S.J., 2012. Quality control in the national bovine tuberculosis eradication programme in Ireland. *Rev. Sci. Tech.* 31, 845–60.
- Duscher, R., Duscher, G., Hofer, J., Tichy, A., Prosl, H., Joachim, A., 2011. *Fasciola hepatica* - Monitoring the milky way? The use of tank milk for liver fluke monitoring in dairy herds as base for treatment strategies. *Vet. Parasitol.* 178, 273–278. doi:10.1016/j.vetpar.2011.01.040
- Echeverry, A., Loneragan, G.H., Wagner, B.A., Brashears, M.M., 2005. Effect of intensity of fecal pat sampling on estimates of *Escherichia coli* O157 prevalence. *Am. J. Vet. Res.* 66, 2023–7. doi:10.2460/ajvr.2005.66.2023
- Eisenberg, S.W.F., Veldman, E., Rutten, V.P.M.G., Koets, A.P., 2015. A longitudinal study of factors influencing the result of a *Mycobacterium avium* ssp. *paratuberculosis* antibody ELISA in milk of dairy cows. *J. Dairy Sci.* 98, 2345–55. doi:10.3168/jds.2014-8380
- El-Ahwal, A.M.A., 1969. [Effect of experimental fascioliasis on the results of the intradermal tuberculin test in the guinea pig]. *Berliner und Münchener tierärztliche Wochenschrift* 82, 484–5.
- Elenkov, I.J., 2004. Glucocorticoids and the Th1/Th2 Balance. *Ann. N.Y. Acad. Sci.* 1024, 138–146. doi:10.1196/annals.1321.010
- Ellis-Iversen, J., Smith, R.P., Snow, L.C., Watson, E., Millar, M.F., Pritchard, G.C., Sayers, A.R., Cook, A.J.C., Evans, S.J., Paiba, G.A., 2007. Identification of management risk factors for VTEC O157 in young-stock in England and Wales. *Prev. Vet. Med.* 82, 29–41. doi:10.1016/j.prevetmed.2007.05.004
- Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T., Igarashi, K., 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur. J. Biochem.* 171, 45–50. doi:10.1111/j.1432-1033.1988.tb13756.x
- Espejo, L.A., Kubat, N., Godden, S.M., Wells, S.J., 2013. Effect of delayed exposure of cattle to *Mycobacterium avium* subsp. *paratuberculosis* on the development of subclinical and clinical Johne's disease. *Am. J. Vet. Res.* 74, 1304–10. doi:10.2460/ajvr.74.10.1304
- Ezenwa, V.O., Etienne, R.S., Luikart, G., Beja-Pereira, A., Jolles, A.E., 2010. Hidden Consequences of Living in a Wormy World: Nematode-Induced Immune Suppression Facilitates Tuberculosis Invasion in African Buffalo. *Am. Nat.* 176, 613–624. doi:10.1086/656496
- Ezenwa, V.O., Jolles, A.E., 2014. Opposite effects of anthelmintic treatment on microbial infection at individual versus population scales. *Science* (80-.). 347, 175–7.
- Falcon, C., Carranza, F., Martinez, F.F., Knubel, C.P., Masih, D.T., Motran, C.C., Cervi, L., 2010. Excretory-secretory products (ESP) from *Fasciola hepatica* induce tolerogenic properties in myeloid dendritic cells. *Vet. Immunol. Immunopathol.* 137, 36–46. doi:10.1016/j.vetimm.2010.04.007
- Feller, M., Huwiler, K., Stephan, R., Altpeter, E., Shang, A., Furrer, H., Pfyffer, G.E., Jemmi, T., Baumgartner, A., Egger, M., 2007. *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis. *Lancet Infect. Dis.* 7, 607–613. doi:10.1016/S1473-3099(07)70211-6

- Fenton, A., 2008. Worms and germs: the population dynamic consequences of microparasite-macroparasite co-infection. *Parasitology* 135, 1545–1560. doi:10.1017/S003118200700025X
- Fenton, A., 2013. Dances with worms: the ecological and evolutionary impacts of deworming on coinfecting pathogens. *Parasitology* 140, 1119–32. doi:10.1017/S0031182013000590
- Fenton, A., Knowles, S.C.L., Petchey, O.L., Pedersen, A.B., 2014. The reliability of observational approaches for detecting interspecific parasite interactions: Comparison with experimental results. *Int. J. Parasitol.* 44, 437–445. doi:10.1016/j.ijpara.2014.03.001
- Ferens, W.A., Hovde, C.J., 2011. *Escherichia coli* O157:H7: Animal Reservoir and Sources of Human Infection. *Foodborne Pathog. Dis.* 8, 465–487.
- Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A., Bloom, B.R., 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 178, 2249–54.
- Flynn, J.L., Goldstein, M.M., Chan, J., Triebold, K.J., Pfeffer, K., Lowenstein, C.J., Schreiber, R., Mak, T.W., Bloom, B.R., 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* 2, 561–72.
- Flynn, R.J., Mannion, C., Golden, O., Hacariz, O., Mulcahy, G., 2007. Experimental *Fasciola hepatica* Infection Alters Responses to Tests Used for Diagnosis of Bovine Tuberculosis. *Infect. Immun.* 75, 1373–1381. doi:10.1128/IAI.01445-06
- Flynn, R.J., Mulcahy, G., 2008. Possible Role for Toll-Like Receptors in Interaction of *Fasciola hepatica* Excretory/Secretory Products with Bovine Macrophages. *Infect. Immun.* 76, 678–684. doi:10.1128/IAI.00732-07
- Flynn, R.J., Mulcahy, G., 2008. The roles of IL-10 and TGF-beta in controlling IL-4 and IFN-gamma production during experimental *Fasciola hepatica* infection. *Int. J. Parasitol.* 38, 1673–80. doi:10.1016/j.ijpara.2008.05.008
- Flynn, R.J., Mulcahy, G., Elsheikha, H.M., 2010. Coordinating innate and adaptive immunity in *Fasciola hepatica* infection: Implications for control. *Vet. Parasitol.* 169, 235–240. doi:10.1016/j.vetpar.2010.02.015
- Flynn, R.J., Mulcahy, G., Welsh, M., Cassidy, J.P., Corbett, D., Milligan, C., Andersen, P., Strain, S., McNair, J., 2009. Co-Infection of Cattle with *Fasciola hepatica* and *Mycobacterium bovis* - Immunological Consequences. *Transbound. Emerg. Dis.* 56, 269–274. doi:10.1111/j.1865-1682.2009.01075.x
- Ford, F., Hadley, P., 2015. Minimising carcass losses for Better Returns. Beef and sheep BRP manual 9.
- Fox, N.J., White, P.C.L., McClean, C.J., Marion, G., Evans, A., Hutchings, M.R., 2011. Predicting Impacts of Climate Change on *Fasciola hepatica* Risk. *PLoS One* 6. doi:10.1371/journal.pone.0016126
- Frankena, K., White, P.W., O’Keeffe, J., Costello, E., Martin, S.W., van Grevenhof, I., More, S.J., 2007. Quantification of the relative efficiency of factory surveillance in the disclosure of tuberculosis lesions in attested Irish cattle. *Vet. Rec.* 161, 679–684.
- Friberg, I.M., Little, S., Ralli, C., Lowe, A., Hall, A., Jackson, J.A., Bradley, J.E., 2013. Macroparasites at peripheral sites of infection are major and dynamic modifiers of systemic antimicrobial pattern recognition responses. *Mol. Ecol.* 22, 2810–2826. doi:10.1111/mec.12212
- Fröhlich, J., Baljer, G., Menge, C., 2009. Maternally and naturally acquired antibodies to Shiga toxins in a cohort of calves shedding Shiga-toxigenic *Escherichia coli*. *Appl. Environ. Microbiol.* 75, 3695–704. doi:10.1128/AEM.02869-08

- Ganga, G., Varshney, J.P., Patra, R.C., 2007. Activity of antioxidant enzymes in excretory-secretory fluid and somatic extracts of *Fasciola gigantica*. *J. Vet. Parasitol.* 21, 51–52.
- Garza-Cuartero, L., 2014. Effect of *Fasciola hepatica* on the Response of Bovine Macrophages to *Mycobacterium bovis*. University College Dublin.
- Garza-Cuartero, L., Blanco, A., Mcnair, J., Flynn, R.J., Williams, D., Diggle, P., Cassidy, J., Mulcahy, G., 2016. *Fasciola hepatica* Infection Reduces *Mycobacterium* and *Mycobacterial* Uptake Suppresses the Pro-inflammatory Response. *Parasite Immunol.* 38, 387–402. doi:10.1111/pim.12326
- Gause, W.C., Urban, J.F., Staderker, M.J., 2003. The immune response to parasitic helminths: insights from murine models. *Trends Immunol.* 24, 269–77.
- Gazzinelli, R., Oswald, I., James, S., 1992. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages. *J.*
- Giacomini, E., Iona, E., Ferroni, L., Miettinen, M., Fattorini, L., Orefici, G., Julkunen, I., Coccia, E.M., 2001. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J. Immunol.* 166, 7033–41.
- Gilbert, M., Mitchell, A., Bourn, D., Mawdsley, J., Clifton-Hadley, R., Wint, W., 2005. Cattle movements and bovine tuberculosis in Great Britain. *Nature* 435, 491–496. doi:10.1038/nature03548
- Gonzalez-Lanza, C., Manga-Gonzalez, Y., Del-Pozo-Carnero, P., Hidalgo-Argüello, R., 1989. Dynamics of elimination of the eggs of *Fasciola hepatica* (Trematoda, Digenea) in the faeces of cattle in the Porma Basin, Spain. *Vet. Parasitol.* 34, 35–43.
- Gonzalez Llamazares, O.R., Gutierrez Martin, C.B., Alvarez Nistal, D., de la Puente Redondo, V.A., Dominguez Rodriguez, L., Rodriguez Ferri, E.F., 1999. Field evaluation of the single intradermal cervical tuberculin test and the interferon-gamma assay for detection and eradication of bovine tuberculosis in Spain. *Vet. Microbiol.* 70, 55–66.
- Gordon, D., Zadoks, R., Skuce, P., Sargison, N., 2012. Confirmation of triclabendazole resistance in liver fluke in the UK. *Vet. Rec.* 171, 159–160. doi:10.1136/vr.e5381
- Gordon, D.K., Zadoks, R.N., Stevenson, H., Sargison, N.D., Skuce, P.J., 2012. On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*. *Vet. Parasitol.* 187, 436–444. doi:10.1016/j.vetpar.2012.02.009
- Graczyk, T.K., Fried, B., 1999. Development of *Fasciola hepatica* in the intermediate host, in: J.P., D. (Ed.), *Fasciolosis*. CABI Publishing, New York, pp. 31–45.
- Graham-Brown, J., 2016. Immune responses in dairy cattle naturally exposed to *fasciola hepatica*. University of Liverpool.
- Graham, A.L., Cattadori, I.M., Lloyd-Smith, J.O., Ferrari, M.J., Bjørnstad, O.N., 2007. Transmission consequences of coinfection: cytokines writ large? *Trends Parasitol.* 23, 284–291. doi:10.1016/j.pt.2007.04.005
- Griffin, J.M., Haehy, T., Lynch, K., McCarthy, J., Hurley, T., 1993. The association of cattle husbandry practices, environmental factors and farmer characteristics with the occurrence of chronic bovine tuberculosis in dairy herds in the Republic of Ireland. *Prev. Vet. Med.* 17, 145–160.
- Griffin, J.M., Williams, D.H., Kelly, G.E., Clegg, T.A., O'Boyle, I., Collins, J.D., More, S.J., 2005. The impact of badger removal on the control of tuberculosis in cattle herds in Ireland. *Cattle Pract.* 13, 381–399.

- Griffiths, E.C., Pedersen, A.B., Fenton, A., Petchey, O.L., 2011. The nature and consequences of coinfection in humans. *J. Infect.* 63, 200–206. doi:10.1016/j.jinf.2011.06.005
- Groenendaal, H., Nielen, M., Jalvingh, A.W., Horst, S.H., Galligan, D.T., Hesselink, J.W., 2002. A simulation of Johne's disease control. *Prev. Vet. Med.* 54, 225–245. doi:10.1016/S0167-5877(02)00027-2
- Gunn, G.J., McKendrick, I.J., Ternent, H.E., Thomson-Carter, F., Foster, G., Synge, B.A., 2007. An investigation of factors associated with the prevalence of verocytotoxin producing *Escherichia coli* O157 shedding in Scottish beef cattle. *Vet. J.* 174, 554–564. doi:10.1016/j.tvjl.2007.08.024
- Gyles, C.L., 2007. Shiga toxin-producing *Escherichia coli*: an overview. *J. Anim. Sci.* 85. doi:10.2527/jas.2006-508
- Haçarız, O., Sayers, G., Mulcahy, G., 2011. A preliminary study to understand the effect of *Fasciola hepatica* tegument on naïve macrophages and humoral responses in an ovine model. *Vet. Immunol. Immunopathol.* 139, 245–9. doi:10.1016/j.vetimm.2010.08.007
- Hanna, J., Neill, S.D., O'Brien, J.J., 1992. ELISA tests for antibodies in experimental bovine tuberculosis. *Vet. Microbiol.* 31, 243–249. doi:10.1016/0378-1135(92)90082-5
- Hanna, R.E.B., 1980. *Fasciola hepatica*: An immunofluorescent study of antigenic changes in the tegument during development in the rat and the sheep. *Exp. Parasitol.* 50, 155–170. doi:10.1016/0014-4894(80)90017-x
- Hartwig, H., El-Ahwal, A.M.A., 1968. Untersuchungen ueber die bedeutung der Fasciolose als Ursache positiver Tuberkulinreaktionen beim Rind. *Berliner und Muenchener Tieraerztliche Wochenschrift* 1 81, 315–316.
- Hasonova, L., Pavlik, I., 2006. Economic impact of paratuberculosis in dairy cattle herds: a review. *Vet. Med. (Praha)*. 51, 193–211.
- Haukialmi, V., Henttonen, H., 1998. Analysing interspecific associations in parasites: alternative methods and effects of sampling heterogeneity. *Oecologia* 116, 565–574. doi:10.1007/s004420050622
- Hayward, A.D., Garnier, R., Watt, K.A., Pilkington, J.G., Grenfell, B.T., Matthews, J.B., Pemberton, J.M., Nussey, D.H., Graham, A.L., 2014. Heritable, Heterogeneous, and Costly Resistance of Sheep against Nematodes and Potential Feedbacks to Epidemiological Dynamics. *Am. Nat.* 184, S58–S76. doi:10.1086/676929
- Health Protection Network, 2013. Guidance for the Public Health Management of Infection with Verotoxigenic *Escherichia coli* (VTEC).
- Hickey, G.L., Diggle, P.J., McNeilly, T.N., Tongue, S.C., Chase-Topping, M.E., Williams, D.J.L., 2015. The feasibility of testing whether *Fasciola hepatica* is associated with increased risk of verocytotoxin producing *Escherichia coli* O157 from an existing study protocol. *Prev. Vet. Med.* doi:10.1016/j.prevetmed.2015.02.022
- Hoek, A., Rutten, V.P.M.G., Kool, J., Arkesteijn, G.J.A., Bouwstra, R.J., Van Rhijn, I., Koets, A.P., 2009. Subpopulations of bovine WC1(+) gammadelta T cells rather than CD4(+)CD25(high) Foxp3(+) T cells act as immune regulatory cells ex vivo. *Vet. Res.* 40, 6. doi:10.1051/vetres:2008044
- Hoffman, M.A., Menge, C., Casey, T.A., Laegreid, W., Bosworth, B.T., Dean-Nystrom, E.A., 2006. Bovine immune response to shiga-toxigenic *Escherichia coli* O157:H7. *Clin. Vaccine Immunol.* 13, 1322–7. doi:10.1128/CVI.00205-06
- Howell, A., Baylis, M., Smith, R., Pinchbeck, G., Williams, D., 2015. Epidemiology and impact of *Fasciola hepatica* exposure in high-yielding dairy herds. *Prev. Vet. Med.* 121, 41–48. doi:10.1016/j.prevetmed.2015.05.013

- Howell, A., Mugisha, L., Davies, J., LaCourse, E.J., Claridge, J., Williams, D.J.L., Kelly-Hope, L., Betson, M., Kabatereine, N.B., Stothard, J.R., 2012. Bovine fasciolosis at increasing altitudes: Parasitological and malacological sampling on the slopes of Mount Elgon, Uganda. *Parasit. Vectors* 5. doi:10.1186/1756-3305-5-196
- Huda, A., Jensen, H., 2003. Comparison of Histopathology, Cultivation of Tissues and Rectal Contents, and Interferon-gamma and Serum Antibody Responses for the Diagnosis of Bovine Paratuberculosis. *J. Comp. Pathol.* 129, 259–267. doi:10.1016/S0021-9975(03)00042-2
- Hussain, R., Dawood, G., Abrar, N., Toossi, Z., Minai, A., Dojki, M., Ellner, J.J., 1995. Selective increases in antibody isotypes and immunoglobulin G subclass responses to secreted antigens in tuberculosis patients and healthy household contacts of the patients. *Clin. Diagn. Lab. Immunol.* 2, 726–32.
- Japa, O., Hodgkinson, J.E., Emes, R.D., Flynn, R.J., 2015. TGF- β superfamily members from the helminth *Fasciola hepatica* show intrinsic effects on viability and development. *Vet. Res.* 46, 29. doi:10.1186/s13567-015-0167-2
- Jarvis, K.G., Kaper, J.B., 1996. Secretion of Extracellular Proteins by Enterohemorrhagic *Escherichia coli* via a Putative Type III Secretion System. *Infect. Immun.* 64, 4826–4829.
- Jefferies, J.R., Campbell, A.M., van Rossum, A.J., Barrett, J., Brophy, P.M., 2001. Proteomic analysis of *Fasciola hepatica* excretory-secretory products. *Proteomics* 1, 1128–32. doi:10.1002/1615-9861(200109)1:9<1128::AID-PROT1128>3.0.CO;2-0
- Jenkins, C., Pearce, M.C., Smith, A.W., Knight, H.I., Shaw, D.J., Cheasty, T., Foster, G., Gunn, G.J., Dougan, G., Smith, H.R., Frankel, G., 2003. Detection of *Escherichia coli* serogroups O26, O103, O111 and O145 from bovine faeces using immunomagnetic separation and PCR/DNA probe techniques. *Lett. Appl. Microbiol.* 37, 207–12.
- Johnston, W.T., Vial, F., Gettinby, G., Bourne, F.J., Clifton-Hadley, R.S., Cox, D.R., Crea, P., Donnelly, C.A., McInerney, J.P., Mitchell, A.P., Morrison, W.I., Woodroffe, R., 2011. Herd-level risk factors of bovine tuberculosis in England and Wales after the 2001 foot-and-mouth disease epidemic. *Int. J. Infect. Dis.* 15, E833–E840. doi:10.1016/j.ijid.2011.08.004
- Jolles, A.E., Ezenwa, V.O., Etienne, R.S., Turner, W.C., Olf, H., 2008. Interactions between macroparasites and microparasites drive infection patterns in free-ranging African buffalo. *Ecology* 89, 2239–2250. doi:10.1890/07-0995.1
- Kamaludeen, J., 2016. The Detection and Occurrence of Anthelmintic Resistance in *Fasciola hepatica* in Sheep in the United Kingdom. University of Liverpool.
- Karolemeas, K., de la Rúa-Domenech, R., Cooper, R., Goodchild, A. V., Clifton-Hadley, R.S., Conlan, A.J.K., Mitchell, A.P., Hewinson, R.G., Donnelly, C.A., Wood, J.L.N., McKinley, T.J., 2012. Estimation of the Relative Sensitivity of the Comparative Tuberculin Skin Test in Tuberculous Cattle Herds Subjected to Depopulation. *PLoS One* 7. doi:10.1371/journal.pone.0043217
- Kaufmann, S.H.E., 2002. Protection against tuberculosis: cytokines, T cells, and macrophages. *Ann. Rheum. Dis.* 61 Suppl 2, ii54-8. doi:10.1136/ARD.61.SUPPL_2.II54
- Keller, H., 1952. Relationship between tuberculosis of the liver and liver flukes., *Parasites, Vectors, Pathogens and Biogenic Diseases of Animals.*
- Knubben-Schweizer, G., Ruegg, S., Torgerson, P.R., Rapsch, C., Grimm, F., Hassig, M., Deplazes, P., Braun, U., 2010. Control of bovine fasciolosis in dairy cattle in Switzerland with emphasis on pasture management. *Vet. J.* 186, 188–191. doi:10.1016/j.tvjl.2009.08.003
- Kochnev, P.N., 1950. *Fasciola* in the lungs of cattle. *Veterinariya* 27.
- Koets, A.P., Rutten, V.P., de Boer, M., Bakker, D., Valentin-Weigand, P., van Eden, W., 2001.

Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infect. Immun.* 69, 1492–8. doi:10.1128/IAI.69.3.1492-1498.2001

- Kokurichev, P.I., Karabainov, M.A., 1957. Specificity of the tuberculin test in cattle with fascioliasis. *Sb. Nauchnikh Tr. Leningr. Inst. Uovershenstvovaniya Vet. Vrachei* 11, 81–85.
- Kreider, T., Anthony, R.M., Urban, J.F., Gause, W.C., 2007. Alternatively activated macrophages in helminth infections. *Curr Opin Immunol* 19, 448–453.
- Kudahl, A.B., Nielsen, S.S., Østergaard, S., 2008. Economy, efficacy, and feasibility of a risk-based control program against paratuberculosis. *J. Dairy Sci.* 91, 4599–609. doi:10.3168/jds.2008-1257
- Kullberg, M.C., Pearce, E.J., Hieny, S.E., Sher, A., Berzofsky, J.A., 1992. Infection with *Schistosoma mansoni* alters Th1/Th2 cytokine responses to a non-parasite antigen. *J. Immunol.* 148, 3264–70.
- Lee, J., Hartman, M., Kornfeld, H., 2009. Macrophage apoptosis in tuberculosis. *Yonsei Med. J.* 50, 1–11. doi:10.3349/ymj.2009.50.1.1
- Leeflang, M.M.G., Bossuyt, P.M.M., 2005. Test accuracy is likely to vary depending on the population it is used in. *Vet. Parasitol.* 134, 189. doi:10.1016/j.vetpar.2005.08.002
- Lello, J., Norman, R.A., Boag, B., Hudson, P.J., Fenton, A., 2008. Pathogen Interactions, Population Cycles, and Phase Shifts. *Am. Nat.* 171, 176–182. doi:10.1086/525257
- Liebana, E., Johnson, L., Gough, J., Durr, P., Jahans, K., Clifton-Hadley, R., Spencer, Y., Hewinson, R.G., Downs, S.H., 2008. Pathology of naturally occurring bovine tuberculosis in England and Wales. *Vet. J.* 176, 354–360. doi:10.1016/j.tvjl.2007.07.001
- Ljesevic, Z., 1957. Effect of parasitic allergy on the tuberculin test in cattle. *Acta Vet. Brno.* 7, 81–91.
- Lloyd-Smith, J.O., Schreiber, S.J., Kopp, P.E., Getz, W.M., 2005. Superspreading and the effect of individual variation on disease emergence. *Nature* 438, 355–359. doi:10.1038/nature04153
- Locking, M.E., O'Brien, S.J., Reilly, W.J., Wright, E.M., Campbell, D.M., Coia, J.E., Browning, L.M., Ramsay, C.N., 2001. Risk factors for sporadic cases of *Escherichia coli* O157 infection: the importance of contact with animal excreta. *Epidemiol. Infect.* 127, 215–20.
- Ma, X., Wang, L., Zhao, H., Pang, N., Zhang, F., Jiang, T., Liu, X., Mamuti, W., Wen, H., Ding, J., 2014. Th17 cells are associated with the Th1/Th2-cell balance during *Echinococcus multilocularis* infection. *Mol. Med. Rep.* 10. doi:10.3892/mmr.2014.2170
- MacDonald, A.S., Araujo, M.I., Pearce, E.J., 2002. Immunology of parasitic helminth infections. *Infect. Immun.* 70, 427–33. doi:10.1128/IAI.70.2.427-433.2002
- Macedo, G.C., Bozzi, A., Weinreich, H.R., Bafica, A., Teixeira, H.C., Oliveira, S.C., Macedo, G.C., Bozzi, A., Weinreich, H.R., Bafica, A., Teixeira, H.C., Oliveira, S.C., 2011. Human T cell and antibody-mediated responses to the *Mycobacterium tuberculosis* recombinant 85A, 85B, and ESAT-6 antigens. *Clin. Dev. Immunol.* 2011, 351573. doi:10.1155/2011/351573
- Maeda, Y., Ohtsuka, H., Tomioka, M., Oikawa, M., 2013. Effect of progesterone on Th1/Th2/Th17 and Regulatory T cell-related genes in peripheral blood mononuclear cells during pregnancy in cows. *Vet. Res. Commun.* 37, 43–49. doi:10.1007/s11259-012-9545-7
- Maizels, R.M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M.D., Allen, J.E., 2004. Helminth parasites - Masters of regulation. *Immunol. Rev.* 201, 89–116. doi:10.1111/j.0105-2896.2004.00191.x

- Malhotra, I., Mungai, P., Wamachi, a, Kioko, J., Ouma, J.H., Kazura, J.W., King, C.L., 1999. Helminth- and Bacillus Calmette-Guérin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. *J. Immunol.* 162, 6843–6848.
- Malone, J., Gommers, R., Hansen, J., Yilma, J., Slingenberg, J., Snijders, F., Nachtergaele, F., Ataman, E., 1998. A geographic information system on the potential distribution and abundance of *Fasciola hepatica* and *F. gigantica* in east Africa based on Food and Agriculture Organization databases. *Vet. Parasitol.* 78, 87–101. doi:10.1016/S0304-4017(98)00137-X
- Manukyan, Z.K., 1955. Non-specific tuberculin reactions in cattle with fascioliasis. *Tr. Armyanskogo Nauchno-Issledovatelskogo Vet. Instituta.* 8, 25–28.
- Marcé, C., Ezanno, P., Weber, M.F., Seegers, H., Pfeiffer, D.U., Fourichon, C., 2010. Invited review: modeling within-herd transmission of *Mycobacterium avium* subspecies paratuberculosis in dairy cattle: a review. *J. Dairy Sci.* 93, 4455–70. doi:10.3168/jds.2010-3139
- Matthews, L., Low, J.C., Gally, D.L., Pearce, M.C., Mellor, D.J., Heesterbeek, J.A.P., Chase-Topping, M., Naylor, S.W., Shaw, D.J., Reid, S.W.J., Gunn, G.J., Woolhouse, M.E.J., 2006. Heterogeneous shedding of *Escherichia coli* O157 in cattle and its implications for control. *PNAS* 103, 547–552.
- McCann, C.M., Baylis, M., Williams, D.J.L., 2010. Seroprevalence and spatial distribution of *Fasciola hepatica*-infected dairy herds in England and Wales. *Vet. Rec.* 166, 612–617. doi:10.1136/vr.b4836
- McCann, C.M., Baylis, M., Williams, D.J.L., 2010. The development of linear regression models using environmental variables to explain the spatial distribution of *Fasciola hepatica* infection in dairy herds in England and Wales. *Int. J. Parasitol.* 40, 1021–1028. doi:10.1016/j.ijpara.2010.02.009
- McCole, D.F., Doherty, M.L., Baird, A.W., Davies, W.C., McGill, K., Torgerson, P.R., 1999. T cell subset involvement in immune responses to *Fasciola hepatica* infection in cattle. *Parasite Immunol.* 21, 1–8. doi:10.1046/j.1365-3024.1999.00188.x
- McGill, J.L., Sacco, R.E., Baldwin, C.L., Telfer, J.C., Palmer, M. V, Waters, W.R., 2014. The role of gamma delta T cells in immunity to *Mycobacterium bovis* infection in cattle. *Vet. Immunol. Immunopathol.* 159, 133–43. doi:10.1016/j.vetimm.2014.02.010
- McKenna, S.L.B., Keefe, G.P., Tiwari, A., VanLeeuwen, J., Barkema, H.W., 2006. Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. *Can. Vet. J.* 47, 1089–99.
- McKenna, S.L.B.L.B., Keefe, G.P.P., Barkema, H.W.W., Sockett, D.C.C., 2005. Evaluation of three ELISAs for *Mycobacterium avium* subsp. paratuberculosis using tissue and fecal culture as comparison standards. *Vet. Microbiol.* 110, 105–111. doi:10.1016/j.vetmic.2005.07.010
- McNeilly, T.N., Nisbet, A.J., 2014. Immune modulation by helminth parasites of ruminants: implications for vaccine development and host immune competence. *Parasite* 21, 51. doi:10.1051/parasite/2014051
- Merlen, H., 1950. *F. hepática* and the tuberculin reaction. *Vet. Rec.* 62.
- Meyer, W., 1963. Hetero-allergy to the tuberculin reaction in cattle. *Monatsh. Veterinarmed.* 18, 325–328.
- Mezo, M., Gonzalez-Warleta, M., Antonio Castro-Hermida, J., Muino, L., Ubeira, F.M., 2011. Association between anti-*F. hepatica* antibody levels in milk and production losses in dairy cows. *Vet. Parasitol.* 180, 237–242. doi:10.1016/j.vetpar.2011.03.009
- Milbourne, E.A., Howell, M.J., 1993. Eosinophil differentiation in response to *Fasciola hepatica* and its excretory/secretory antigens. *Int. J. Parasitol.* 23, 1005–9.

- Milnes, A.S., Sayers, A.R., Stewart, I., Clifton-Hadley, F.A., Davies, R.H., Newell, D.G., Cook, A.J.C., Evans, S.J., Smith, R.P., Paiba, G.A., 2009. Factors related to the carriage of Verocytotoxigenic *E. coli*, Salmonella, thermophilic *Campylobacter* and *Yersinia enterocolitica* in cattle, sheep and pigs at slaughter. *Epidemiol. Infect.* 137, 1135–48. doi:10.1017/S095026880900199X
- Miyamoto, Y., Iimura, M., Kaper, J.B., Torres, A.G., Kagnoff, M.F., 2006. Role of Shiga toxin versus H7 flagellin in enterohaemorrhagic *Escherichia coli* signalling of human colon epithelium in vivo. *Cell. Microbiol.* 8, 869–879. doi:10.1111/j.1462-5822.2005.00673.x
- Moreau, E., Chauvin, A., 2010. Immunity against Helminths: Interactions with the Host and the Intercurrent Infections. *J. Biomed. Biotechnol.* doi:10.1155/2010/428593
- Moreau, E., Hervé, S., Yu, Z.W., Alain, C., 2002. Modulation of sheep lymphocyte responses by *Fasciola hepatica* excretory-secretory products. *Vet. Parasitol.* 108, 207–15.
- Morgan, E.R., Wall, R., 2009. Climate change and parasitic disease: farmer mitigation? *Trends Parasitol.* 25, 308–313. doi:10.1016/j.pt.2009.03.012
- Mortier, R.A.R., Barkema, H.W., De Buck, J., 2015. Susceptibility to and diagnosis of *Mycobacterium avium* subspecies paratuberculosis infection in dairy calves: A review. *Prev. Vet. Med.* 121, 189–198. doi:10.1016/j.prevetmed.2015.08.011
- Mortier, R.A.R., Barkema, H.W., Wilson, T.A., Sajobi, T.T., Wolf, R., De Buck, J., 2014. Dose-dependent interferon-gamma release in dairy calves experimentally infected with *Mycobacterium avium* subspecies paratuberculosis. *Vet. Immunol. Immunopathol.* 161, 205–210. doi:10.1016/j.vetimm.2014.08.007
- Moustakas, A., Evans, M.R., 2016. Regional and temporal characteristics of Bovine Tuberculosis of cattle in Great Britain. *Stoch Env. Res Risk Assess* 989–1003.
- Mulcahy, G., Dalton, J.P., 2001. Cathepsin L proteinases as vaccines against infection with *Fasciola hepatica* (liver fluke) in ruminants. *Res. Vet. Sci.* 70, 83–6. doi:10.1053/rvsc.2000.0425
- Mulcahy, G., Joyce, P., Dalton, J.P., 1999. Immunology of *Fasciola hepatica* infection, in: Dalton, J.P. (Ed.), *Fasciolosis*. CABI Publishing.
- Mulcahy, G., O'Connor, F., Mcgonigle, S., Dowd, A., Clery, D.G., Andrews, S.J., Dalton, J.P., 1998. Correlation of specific antibody titre and avidity with protection in cattle immunized against *Fasciola hepatica*. *Vaccine* 16, 932–939.
- Munns, K.D., Selinger, L.B., Stanford, K., Guan, L., Callaway, T.R., McAllister, T.A., 2015. Perspectives on Super-Shedding of *Escherichia coli* O157:H7 by Cattle. *Foodborne Pathog. Dis.* 12, 89–103. doi:10.1089/fpd.2014.1829
- Munyeme, M., Munang'andu, H.M., Nambota, A., Muma, J.B., Phiri, A.M., Nalubamba, K.S., 2012. The Nexus between Bovine Tuberculosis and Fasciolosis Infections in Cattle of the Kafue Basin Ecosystem in Zambia: Implications on Abattoir Surveillance. *Vet. Med. Int.* 2012, 921869. doi:10.1155/2012/921869
- Nart, P., Naylor, S.W., Huntley, J.F., McKendrick, I.J., Gally, D.L., Low, J.C., 2008. Responses of Cattle to Gastrointestinal Colonization by *Escherichia coli* O157:H7. *Infect. Immun.* 76, 5366–5372. doi:10.1128/IAI.01223-07
- Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11, 142–201.
- Naylor, S.W., Flockhart, A., Nart, P., Smith, D.G.E., Huntley, J., Gally, D.L., Low, J.C., 2007. Shedding of *Escherichia coli* O157:H7 in calves is reduced by prior colonization with the homologous strain. *Appl. Environ. Microbiol.* 73, 3765–7. doi:10.1128/AEM.02670-06
- Neill, S.D., Bryson, D.G., Pollock, J.M., 2001. Pathogenesis of tuberculosis in cattle. *Tuberculosis* 81,

79–86. doi:10.1054/tube.2000.0279

- Nielsen, S.S., Ersbøll, A.K., 2006. Age at Occurrence of Mycobacterium avium Subspecies paratuberculosis in Naturally Infected Dairy Cows. *J. Dairy Sci.* 89, 4557–4566. doi:10.3168/jds.S0022-0302(06)72505-X
- Nielsen, S.S., Toft, N., 2006. Age-Specific Characteristics of ELISA and Fecal Culture for Purpose-Specific Testing for Paratuberculosis. *J. Dairy Sci.* 89, 569–579. doi:10.3168/jds.S0022-0302(06)72120-8
- Nielsen, S.S., Toft, N., 2008. Ante mortem diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon- γ assay and faecal culture techniques. *Vet. Microbiol.* 129, 217–235. doi:10.1016/j.vetmic.2007.12.011
- Nielsen, S.S., Toft, N., Okura, H., 2013. Dynamics of Specific Anti-Mycobacterium avium Subsp. paratuberculosis Antibody Response through Age. *PLoS One* 8. doi:10.1371/journal.pone.0063009
- Northern Ireland Audit Office, 2009. *The Control of Bovine Tuberculosis in Northern Ireland*. Belfast.
- Nussey, D.H., Watt, K.A., Clark, A., Pilkington, J.G., Pemberton, J.M., Graham, A.L., McNeilly, T.N., 2014. Multivariate immune defences and fitness in the wild: complex but ecologically important associations among plasma antibodies, health and survival. *Proc. R. Soc. London B Biol. Sci.* 281.
- Nyiredy, I., Hejz, L., Tuboly, S., 1966. The role of saprophytic mycobacteria in inducing tuberculin sensitivity in cattle. *Magy. Allatorvosok Lapja* 21, 433–439.
- O'Garra, A., Redford, P.S., McNab, F.W., Bloom, C.I., Wilkinson, R.J., Berry, M.P.R., 2013. The Immune Response in Tuberculosis. *Annu. Rev. Immunol.* 31, 475–527. doi:10.1146/annurev-immunol-032712-095939
- Oblitas, P.L.O., 1997. Humoral immune response to *Fasciola hepatica* in experimentally infected calves and in cattle naturally exposed to fasciolosis in Cajamarca, Peru. University of Liverpool.
- Ogilvie, D., Fayter, D., Petticrew, M., Sowden, A., Thomas, S., Whitehead, M., Worthy, G., Deeks, J., Khan, K., Song, F., Popay, J., Nixon, J., Kleijnen, J., Lewis, S., Clarke, M., Slavin, R., Jackson, N., Waters, E., Popay, J., Roberts, H., Sowden, A., Petticrew, M., Arai, L., Rodgers, M., Britten, N., Roen, K., Duffy, S., Lucas, P., Baird, J., Arai, L., Law, C., Roberts, H., Tugwell, P., Petticrew, M., Robinson, V., Kristjansson, E., Maxwell, L., Macintyre, S., Chalmers, I., Horton, R., Smith, R., Evans, T., Brown, H., Briss, P., Zaza, S., Pappaioanou, M., Fielding, J., Agüero, L.W.-D., Truman, B., Hopkins, D., Dolan-Mullen, P., Thompson, R., Woolf, S., Carande-Kulis, V., Anderson, L., Hinman, A., McQueen, D., Teutsch, S., Harris, J., Thomas, H., Chaloupka, F., Wechsler, H., Lewit, E., Hyland, A., Kerrebrock, N., Cummings, K., Miles, M., Huberman, A., Fienberg, S., Wainer, H., Jackson, N., Tufte, E., 2008. The harvest plot: A method for synthesising evidence about the differential effects of interventions. *BMC Med. Res. Methodol.* 8, 8. doi:10.1186/1471-2288-8-8
- Ollerenshaw, C.B., Rowlands, W.T., 1959. A method of forecasting the incidence of fascioliasis in Angelsey. *Vet. Rec.* 71, 591–598.
- Olsen, A., Frankena, K., Bødker, R., Toft, N., Thamsborg, S.M., Enemark, H.L., Halasa, T., 2011. Prevalence, risk factors and spatial analysis of liver fluke infections in Danish cattle herds. doi:10.1186/s13071-015-0773-x
- Ortiz, P.L., Claxton, J.R., Clarkson, M.J., McGarry, J., Williams, D.J., 2000. The specificity of antibody responses in cattle naturally exposed to *Fasciola hepatica*. *Vet. Parasitol.* 93, 121–34.
- Paiba, G.A., Wilesmith, J.W., Evans, S.J., Pascoe, S.J.S., Smith, R.P., Kidd, S.A., Ryan, J.B.M., McLaren,

- I.M., Chappell, S.A., Willshaw, G.A., Cheasty, T., French, N.P., Jones, T.W.H., Buchanan, H.F., Challoner, D.J., Colloff, A.D., Cranwell, M.P., Daniel, R.G., Davies, I.H., Duff, J.P., Hogg, R.A.T., Kirby, F.D., Millar, M.F., Monies, R.J., Nicholls, M.J., Payne, J.H., 2003. Prevalence of faecal excretion of verocytotoxigenic *Escherichia coli* O157 in cattle in England and Wales. *Vet. Rec.* 153, 347–353. doi:10.1136/vr.153.12.347
- Parrish, N., Dick, J., Bishai, W., 1998. Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol.* 6, 107–112.
- Pearce, M.C., Chase-Topping, M.E., McKendrick, I.J., Mellor, D.J., Locking, M.E., Allison, L., Ternent, H.E., Matthews, L., Knight, H.I., Smith, A.W., Synge, B. a, Reilly, W., Low, J.C., Reid, S.W.J., Gunn, G.J., Woolhouse, M.E.J., 2009. Temporal and spatial patterns of bovine *Escherichia coli* O157 prevalence and comparison of temporal changes in the patterns of phage types associated with bovine shedding and human *E. coli* O157 cases in Scotland between 1998-2000 and 2002-2004. *BMC Microbiol.* 9, 276. doi:10.1186/1471-2180-9-276
- Pearce, M.C., Fenlon, D., Low, J.C., Smith, A.W., Knight, H.I., Evans, J., Foster, G., Synge, B.A., Gunn, G.J., 2004. Distribution of *Escherichia coli* O157 in bovine fecal pats and its impact on estimates of the prevalence of fecal shedding. *Appl. Environ. Microbiol.* 70, 5737–5743. doi:10.1128/AEM.70.10.5737-5743.2004
- Peckham, R.K., Brill, R., Foster, D.S., Bowen, A.L., Leigh, J.A., Coffey, T.J., Flynn, R.J., 2014. Two distinct populations of Bovine IL-17+ T-cells can be induced and WC1+IL-17+ $\gamma\delta$ T-cells are effective killers of protozoan parasites. *Sci. Rep.* 4. doi:10.1038/srep05431
- Phiri, I.K., Phiri, A.M., Harrison, L.J.S., 2006. Serum antibody isotype responses of *Fasciola*-infected sheep and cattle to excretory and secretory products of *Fasciola* species. *Vet. Parasitol.* 141, 234–242. doi:10.1016/j.vetpar.2006.05.019
- Piedrafita, D., Parsons, J.C., Sandeman, R.M., Wood, P.R., Estuningsih, S.E., Partoutomo, S., Spithill, T.W., 2001. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile *Fasciola hepatica* in vitro is mediated by reactive nitrogen intermediates. *Parasite Immunol.* 23, 473–82.
- Pinedo, P.J., Rae, D.O., Williams, J.E., Donovan, G.A., Melendez, P., Buergelt, C.D., 2008. Association among Results of Serum ELISA, Faecal Culture and Nested PCR on Milk, Blood and Faeces for the Detection of Paratuberculosis in Dairy Cows. *Transbound. Emerg. Dis.* 55, 125–133. doi:10.1111/j.1865-1682.2007.01009.x
- Pirro, F., Wieler, L.H., Failing, K., Bauerfeind, R., Baljer, G., 1995. Neutralizing antibodies against Shiga-like toxins from *Escherichia coli* in colostrum and sera of cattle. *Vet. Microbiol.* 43, 131–141. doi:10.1016/0378-1135(94)00089-F
- Podinovskaia, M., Lee, W., Caldwell, S., Russell, D.G., 2013. Infection of macrophages with *Mycobacterium tuberculosis* induces global modifications to phagosomal function. *Cell. Microbiol.* 15, 843–59. doi:10.1111/cmi.12092
- Pollock, J.M., Neill, S.D., 2002. *Mycobacterium bovis* infection and tuberculosis in cattle. *Vet. J.* 163, 115–127. doi:10.1053/tvjl.2001.0655
- Pollock, J.M., Welsh, M.D., McNair, J., 2005. Immune responses in bovine tuberculosis: Towards new strategies for the diagnosis and control of disease. *Vet. Immunol. Immunopathol.* 108, 37–43. doi:10.1016/j.vetimm.2005.08.012
- Price, S.J., Hope, J.C., 2009. Enhanced secretion of interferon-gamma by bovine $\gamma\delta$ T cells induced by coculture with *Mycobacterium bovis*-infected dendritic cells: evidence for reciprocal activating signals. *Immunology* 126, 201–8. doi:10.1111/j.1365-2567.2008.02889.x
- Pritchard, G.C., Forbes, A.B., Williams, D.J.L., Salimi-Bejestani, M.R., Daniel, R.G., 2005. Emergence of

- fasciolosis in cattle in East Anglia. *Vet. Rec.* 157, 578–582.
- Prowse, R.K., Chaplin, P., Robinson, H.C., Spithill, T.W., 2002. *Fasciola hepatica* cathepsin L suppresses sheep lymphocyte proliferation in vitro and modulates surface CD4 expression on human and ovine T cells. *Parasite Immunol.* 24, 57–66.
- Pruimboom-Brees, I.M., Morgan, T.W., Ackermann, M.R., Nystrom, E.D., Samuel, J.E., Cornick, N. a, Moon, H.W., 2000. Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10325–10329. doi:10.1073/pnas.190329997
- Public Health England, 2013. *Escherichia coli* (E. coli) O157 annual totals in England and Wales [WWW Document]. URL <https://www.gov.uk/government/publications/escherichia-coli-e-coli-o157-annual-totals/escherichia-coli-e-coli-o157-annual-totals-in-england-and-wales> (accessed 11.8.16).
- R Core Team, 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing.
- Ransohoff, D.F., Feinstein, A.R., 1978. Problems of spectrum and bias in evaluating the efficacy of diagnostic tests. *N. Engl. J. Med.* 299, 926–30. doi:10.1056/NEJM197810262991705
- Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. *Int. J. Parasitol.* 36, 1153–1158. doi:10.1016/j.ijpara.2006.06.001
- Reichel, M.P., Vanhoff, K., Baxter, B., 2005. Performance characteristics of an enzyme-linked immunosorbent assay performed in milk for the detection of liver fluke (*Fasciola hepatica*) infection in cattle. *Vet. Parasitol.* 129, 61–66. doi:10.1016/j.vetpar.2004.12.013
- Renter, D.G., Sargeant, J.M., Hungerford, L.L., 2004. Distribution of *Escherichia coli* O157:H7 within and among cattle operations in pasture-based agricultural areas. *Am. J. Vet. Res.* 65, 1367–1376. doi:10.2460/ajvr.2004.65.1367
- Roberts, J.A., Estuningsih, E., Widjayanti, S., Wiedosari, E., Partoutomo, S., Spithill, T.W., 1997. Resistance of Indonesian thin tail sheep against *Fasciola gigantica* and *F. hepatica*. *Vet. Parasitol.* 68, 69–78.
- Roberts, J.A., Suhardono, 1996. Approaches to the control of fasciolosis in ruminants. *Int. J. Parasitol.* 26, 971–981. doi:10.1016/0020-7519(96)00081-1
- Robinson, S.E., Brown, P.E., Wright, E.J., Hart, C.A., French, N.P., 2009. Quantifying within- and between-animal variation and uncertainty associated with counts of *Escherichia coli* O157 occurring in naturally infected cattle faeces. *J. R. Soc. Interface* 6, 169–77. doi:10.1098/rsif.2008.0183
- Rogers, A.N., Vanburen, D.G., Hedblom, E.E., Tilahun, M.E., Telfer, J.C., Baldwin, C.L., 2005. Gammadelta T cell function varies with the expressed WC1 coreceptor. *J. Immunol.* 174, 3386–93. doi:10.4049/JIMMUNOL.174.6.3386
- Rohani, P., Green, C.J., Mantilla-Beniers, N.B., Grenfell, B.T., 2003. Ecological interference between fatal diseases. *Nature* 422, 885–888. doi:10.1038/nature01542
- Ross, J.G., 1967a. The lifespan of *Fasciola hepatica* in cattle. *Vet. Rec.* 82, 567–589.
- Ross, J.G., 1967b. Studies of immunity to *Fasciola hepatica*. Acquired immunity in cattle, sheep and rabbits following natural infection and vaccine procedures. *J. Helminthol.* 41, 393–339.
- Sadzikowski, A., Gundlach, J.L., 1985. Specific and unspecific stimulation of protective mechanisms against invasion of *Fasciola hepatica* in the rat. *Med. Weter.* 41, 146–149.

- Salimi-Bejestani, M.R., Cripps, P., Williams, D.J.L., 2008. Evaluation of an ELISA to assess the intensity of *Fasciola hepatica* infection in cattle. *Vet. Rec.* 162, 109–111.
- Salimi-Bejestani, M.R., Daniel, R., Cripps, P., Felstead, S., Williams, D.J.L., 2007. Evaluation of an enzyme-linked immunosorbent assay for detection of antibodies to *Fasciola hepatica* in milk. *Vet. Parasitol.* 149, 290–293. doi:10.1016/j.vetpar.2007.08.008
- Salimi-Bejestani, M.R., Daniel, R.G., Felstead, S.M., Cripps, P.J., Mahmood, H., Williams, D.J.L., 2005a. Prevalence of *Fasciola hepatica* in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. *Vet. Rec.* 156, 729–731.
- Salimi-Bejestani, M.R., McGarry, J.W., Felstead, S., Ortiz, P., Akca, A., Williams, D.J.L., 2005b. Development of an antibody-detection ELISA for *Fasciola hepatica* and its evaluation against a commercially available test. *Res. Vet. Sci.* 78, 177–181. doi:10.1016/j.rvsc.2004.08.005
- Sanabria, R., Ceballos, L., Moreno, L., Romero, J., Lanusse, C., Alvarez, L., 2013. Identification of a field isolate of *Fasciola hepatica* resistant to albendazole and susceptible to triclabendazole. *Vet. Parasitol.* 193, 105–110. doi:10.1016/j.vetpar.2012.11.033
- Sanchez-Vazquez, M.J., Lewis, F.I., 2013. Investigating the impact of fasciolosis on cattle carcass performance. *Vet. Parasitol.* 193, 307–311. doi:10.1016/j.vetpar.2012.11.030
- Sargison, N., 2012. Diagnosis of triclabendazole resistance in *Fasciola hepatica*. *Vet. Rec.* 171, 151–152. doi:10.1136/vr.e5357
- Sargison, N., Wilson, D., Penny, C., Bartley, D., 2010. Unexpected production loss caused by helminth parasites in weaned beef calves. *Vet. Rec.* 752–754.
- Sargison, N.D., Scott, P.R., 2011. Diagnosis and economic consequences of triclabendazole resistance in *Fasciola hepatica* in a sheep flock in south-east Scotland. *Vet. Rec.* 168, 159–U22. doi:10.1136/vr.c5332
- Schanzel, H., Stolarik, L., 1962. The influence of liver flukes on the specificity of the intradermal tuberculin test in cattle. *Vet Med Praha* 7, 39.
- Schares, G., Bärwald, A., Staubach, C., Wurm, R., Rauser, M., Conraths, F.J., Schroeder, C., 2004. Adaptation of a commercial ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. *Vet. Parasitol.* 120, 55–63. doi:10.1016/j.vetpar.2003.11.016
- Schweizer, G., Braun, U., Deplazes, P., Torgerson, P.R., 2005. Estimating the financial losses due to bovine fasciolosis in Switzerland. *Vet. Rec.* 157, 188–193.
- Serradell, M.C., Guasconi, L., Cervi, L., Chiapello, L.S., Masih, D.T., 2007. Excretory-secretory products from *Fasciola hepatica* induce eosinophil apoptosis by a caspase-dependent mechanism. *Vet. Immunol. Immunopathol.* 117, 197–208. doi:10.1016/j.vetimm.2007.03.007
- Shittu, A., Clifton-Hadley, R.S., Ely, E.R., Upton, P.U., Downs, S.H., 2013. Factors associated with bovine tuberculosis confirmation rates in suspect lesions found in cattle at routine slaughter in Great Britain, 2003–2008. *Prev. Vet. Med.* 110, 395–404. doi:10.1016/j.prevetmed.2013.03.001
- Sibille, P., Tliba, O., Boulard, C., 2004. Early and transient cytotoxic response of peritoneal cells from *Fasciola hepatica*-infected rats. *Vet. Res.* 35, 573–84. doi:10.1051/vetres:2004033
- Skuce, P.J., Zadoks, R.N., 2013. Liver fluke A growing threat to UK livestock production. *Cattle Pract.* 21, 138–149.
- Smith, A.M., Dowd, A.J., Heffernan, M., Robertson, C.D., Dalton, J.P., 1993. *Fasciola hepatica*: a secreted cathepsin L-like proteinase cleaves host immunoglobulin. *Int. J. Parasitol.* 23, 977–83.
- Smith, R.L., Gröhn, Y.T., Pradhan, A.K., Whitlock, R.H., Kessel, J.S. Van, Smith, J.M., Wolfgang, D.R.,

- Schukken, Y.H., 2016. The effects of progressing and nonprogressing *Mycobacterium avium* ssp . paratuberculosis infection on milk production in dairy cows. *J. Dairy Sci.* 99, 1383–1390. doi:10.3168/jds.2015-9822
- Smith, R.P., Pollitt, W.J., Paiba, G.A., 2016. A longitudinal study of risk factors for shedding of VTEC O157 by young cattle in herds with known *E. coli* O157 carriage. *Epidemiol. Infect.* 144, 1818–1829. doi:10.1017/S095026881600008X
- Spedding, A., 2009. Stress and loss – the impact of bovine TB on farming families.
- Spellberg, B., Edwards, J.E., 2001. Type 1/Type 2 immunity in infectious diseases. *Clin. Infect. Dis.* 32, 76–102. doi:10.1086/317537
- Stabel, J.R., 1996. Production of gamma-interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis. *J. Vet. Diagn. Invest.* 8, 345–50.
- Stewart, G.R., Boussinesq, M., Coulson, T., Elson, L., Nutman, T., Bradley, J.E., 1999. Onchocerciasis modulates the immune response to mycobacterial antigens. *Clin. Exp. Immunol.* 117, 517–23.
- Strachan, N.J.C., Dunn, G.M., Locking, M.E., Reid, T.M.S., Ogden, I.D., 2006. *Escherichia coli* O157: Burger bug or environmental pathogen? *Int. J. Food Microbiol.* 112, 129–137. doi:10.1016/j.ijfoodmicro.2006.06.021
- Strain, S.A.J., McNair, J., McDowell, S.W.J., 2011. Bovine tuberculosis: A review of diagnostic tests for *M. bovis* infection in cattle.
- Strelchenok, K., 1953. Non-specificity of tuberculin reactors in cattle with fascioliasis. *Veterinarija* 30, 26.
- Synge, B.A., Chase-Topping, M.E., Hopkins, G.F., McKendrick, I.J., Thomson-Carter, F., Gray, D., Rusbridge, S.M., Munro, F.I., Foster, G., Gunn, G.J., 2003. Factors influencing the shedding of verocytotoxin-producing *Escherichia coli* O157 by beef suckler cows. *Epidemiol. Infect.* 130, 301–12.
- Taylor, S.M., Kilpatrick, D., 1975. The relationship between concurrent liver fluke infection and salmonellosis in cattle. *Vet. Rec.* 96, 342–3. doi:10.1136/VR.96.15.342
- TB Free England, 2015. History of bovine TB [WWW Document]. URL <http://www.tbfreeengland.co.uk/assets/4148> (accessed 9.20.16).
- TB Hub, 2016. Government Policy [WWW Document]. URL <http://www.tbhub.co.uk/tb-policy/> (accessed 10.14.16).
- The Cochrane Collaboration, 2011. *Cochrane Handbook for Systematic Reviews of Interventions*, Version 5. ed.
- Tiwari, A., Vanleeuwen, J.A., Dohoo, I.R., Keefe, G.P., Haddad, J.P., Scott, H.M., Whiting, T., 2009. Risk factors associated with *Mycobacterium avium* subspecies paratuberculosis seropositivity in Canadian dairy cows and herds. *Prev. Vet. Med.* 88, 32–41. doi:10.1016/j.prevetmed.2008.06.019
- Tizard, I.R., 2013. *Veterinary Immunology*, 9th ed. Elsevier Saunders, St Louis, Mo.
- Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccines in ruminants: Strategies, progress and future opportunities. *Int. J. Parasitol.* 44, 915–927. doi:10.1016/j.ijpara.2014.07.011
- Torgerson, P., Claxton, J., 1999. Epidemiology and Control, in: Dalton, J.P. (Ed.), *Fasciolosis*. CABI Publishing, New York, pp. 113–149.

- Tuttle, J., Gomez, T., Doyle, M., Wells, J., Zhao, T., Tauxe, R., Griffin, P., 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol. Infect.* 122, 185–192. doi:10.1017/S0950268898001976
- Vaessen, M.A.M., Veling, J., Frankena, K., Graat, E.E.A.M., Klunder, T., 1998. Risk Factors for *Salmonella* Dublin Infection on Dairy Farms. *Vet. Q.* 20, 97–99. doi:10.1080/01652176.1998.9694848
- Valero, M.A., Ubeira, F.M., Khoubbane, M., Artigas, P., Muiño, L., Mezo, M., Pérez-Crespo, I., Periago, M.V., Mas-Coma, S., 2009. MM3-ELISA evaluation of coproantigen release and serum antibody production in sheep experimentally infected with *Fasciola hepatica* and *F. gigantica*. *Vet. Parasitol.* 159, 77–81. doi:10.1016/j.vetpar.2008.10.014
- van Hulzen, K.J.E., Schopen, G.C.B., van Arendonk, J.A.M., Nielen, M., Koets, A.P., Schrooten, C., Heuven, H.C.M., 2012. Genome-wide association study to identify chromosomal regions associated with antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in milk of Dutch Holstein-Friesians. *J. Dairy Sci.* 95, 2740–8. doi:10.3168/jds.2011-5005
- van Riet, E., Hartgers, F.C., Yazdanbakhsh, M., 2007. Chronic helminth infections induce immunomodulation: Consequences and mechanisms. *Immunobiology* 212, 475–490. doi:10.1016/j.imbio.2007.03.009
- Vercruyse, J., Claerebout, E., 2001. Treatment vs non-treatment of helminth infections in cattle: defining the threshold. *Vet. Parasitol.* 98, 195–214. doi:10.1016/s0304-4017(01)00431-9
- VIDA, n.d. Veterinary Investigation Diagnostic Analysis Report [WWW Document]. URL https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/308233/pub-vida12-cattle.pdf (accessed 3.10.17).
- Vilte, D.A., Larzábal, M., Cataldi, A.A., Mercado, E.C., 2008. Bovine colostrum contains immunoglobulin G antibodies against intimin, EspA, and EspB and inhibits hemolytic activity mediated by the type three secretion system of attaching and effacing *Escherichia coli*. *Clin. Vaccine Immunol.* 15, 1208–13. doi:10.1128/CVI.00027-08
- Vukman, K. V., Adams, P.N., Metz, M., Maurer, M., O’Neill, S.M., 2013. *Fasciola hepatica* tegumental coat impairs mast cells’ ability to drive Th1 immune responses. *J. Immunol.* 190, 2873–9. doi:10.4049/jimmunol.1203011
- Waddell, L.A., Rajic, A., Staerk, K.D.C., McEwen, S.A., 2015. The zoonotic potential of *Mycobacterium avium* ssp *paratuberculosis*: a systematic review and meta-analyses of the evidence. *Epidemiol. Infect.* 143, 3135–3157.
- Walle, K.V., Vanrompay, D., Cox, E., 2010. Bovine innate and adaptive immune responses against *Escherichia coli* O157:H7 and vaccination strategies to reduce faecal shedding in ruminants. *Vet. Immunol. Immunopathol.* doi:10.1016/j.vetimm.2012.09.028
- Walsh, K.P., Brady, M.T., Finlay, C.M., Boon, L., Mills, K.H.G., 2009. Infection with a Helminth Parasite Attenuates Autoimmunity through TGF- β -Mediated Suppression of Th17 and Th1 Responses. *J. Immunol.* 183, 1577–1586. doi:10.4049/jimmunol.0803803
- Wangoo, A., Sparer, T., Brown, I.N., Snewin, V.A., Janssen, R., Thole, J., Cook, H.T., Shaw, R.J., Young, D.B., 2001. Contribution of Th1 and Th2 cells to protection and pathology in experimental models of granulomatous lung disease. *J. Immunol.* 166, 3432–9.
- Waters, W.R., Miller, J.M., Palmer, M. V, Stabel, J.R., Koistinen, K. a, Steadham, E.M., Hamilton, M.J., Davis, C., Bannantine, J.P., Jones, D.E., 2003. Early Induction of Humoral and Cellular Immune Responses during Experimental *Mycobacterium avium* subsp . *paratuberculosis* Infection of Calves Early Induction of Humoral and Cellular Immune Responses during Experimental

- Mycobacterium avium* subsp. *paratuberculosis*. *Infect. Immun.* 71, 5130–5138.
doi:10.1128/IAI.71.9.5130
- Weaver, C.T., Harrington, L.E., Mangan, P.R., Gavrieli, M., Murphy, K.M., 2006. Review Th17: An Effector CD4 T Cell Lineage with Regulatory T Cell Ties. *Immunity* 24, 677–688.
doi:10.1016/j.immuni.2006.06.002
- Weber, M.F., Kogut, J., de Bree, J., van Schaik, G., Nielen, M., 2010. Age at which dairy cattle become *Mycobacterium avium* subsp. *paratuberculosis* faecal culture positive. *Prev. Vet. Med.* 97, 29–36. doi:10.1016/j.prevetmed.2010.07.004
- Weiss, D.J., Evanson, O.A., de Souza, C., Abrahamsen, M.S., 2005. A critical role of interleukin-10 in the response of bovine macrophages to infection by *Mycobacterium avium* subsp. *paratuberculosis*. *Am. J. Vet. Res.* 66, 721–726. doi:10.2460/ajvr.2005.66.721
- Welsh, M.D., Cunningham, R.T., Corbett, D.M., Girvin, R.M., McNair, J., Skuce, R.A., Bryson, D.G., Pollock, J.M., 2005. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. *Immunology* 114, 101–111.
doi:10.1111/j.1365-2567.2004.02003.x
- Whelan, A.O., Coad, M., Upadhyay, B.L., Clifford, D.J., Hewinson, R.G., Vordermeier, H.M., 2011. Lack of correlation between BCG-induced tuberculin skin test sensitisation and protective immunity in cattle. *Vaccine* 29, 5453–5458. doi:10.1016/j.vaccine.2011.05.057
- Whitlock, R.H., Buergelt, C., 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). *Vet. Clin. North Am. Food Anim. Pract.* 12, 345–56.
- Wickham, H., 2009. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.
- Williams, D.J.L., Newson, J., Naessens, J., 1990. Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. *Vet. Immunol. Immunopathol.* 24, 267–283.
doi:10.1016/0165-2427(90)90042-Q
- Williams, K.J., Ward, M.P., Dhungyel, O.P., Hall, E.J.S., Van Breda, L., 2014. A longitudinal study of the prevalence and super-shedding of *Escherichia coli* O157 in dairy heifers. *Vet. Microbiol.* 173, 101–109. doi:10.1016/j.vetmic.2014.07.001
- Woolhouse, M.E., Dye, C., Etard, J.F., Smith, T., Charlwood, J.D., Garnett, G.P., Hagan, P., Hii, J.L., Ndhlovu, P.D., Quinnell, R.J., Watts, C.H., Chandiwana, S.K., Anderson, R.M., 1997. Heterogeneities in the transmission of infectious agents: implications for the design of control programs. *Proc. Natl. Acad. Sci. U. S. A.* 94, 338–42.
- Yamaguchi, M., Azuma, R., Kawanishi, Y., 1955. Studies on no visible lesion reactor 1. Correlation of fascioliasis to tuberculin reaction. *Japanese J. Vet. Sci.* 17, 133–137.
- Yildirim, A., Ica, A., Duzlu, O., Inci, A., 2007. Prevalence and risk factors associated with *Fasciola hepatica* in cattle from Kayseri province, Turkey. *Rev. Méd. Vét* 158, 613–617.
- Zhang, W.Y., Moreau, E., Hope, J.C., Howard, C.J., Huang, W.Y., Chauvin, a., 2005. *Fasciola hepatica* and *Fasciola gigantica*: Comparison of cellular response to experimental infection in sheep. *Exp. Parasitol.* 111, 154–159. doi:10.1016/j.exppara.2005.06.005
- Zorawski, H., Lipiec, M., Skwarek, P., Cegielski, A., 1987. Occurrence of acid-fast bacilli in the liver of cattle infected with *Fasciola hepatica*. *Bull. Vet. Inst. Pulawy* 30–31, 1–5.