The effect of *Fasciola hepatica* infection on respiratory vaccine responsiveness in calves

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**ABSTRACT**

*Fasciola hepatica* is a common parasite in cattle, and bovine fasciolosis causes significant production losses, as well as being a zoonotic disease of global importance. *F. hepatica* has been shown to have immunoregulatory effects and the aim of this research was to establish whether *F. hepatica* infection influences the response to vaccination against respiratory pathogens in calves. A total of 48 calves were randomly and equally allocated to two groups. The experimental group was infected with *F. hepatica*, while the other group was used as a control. At week 2 and 6 after infection calves from both groups were administered a vaccine containing inactivated PI-3, BRSV and *Mannheimia haemolytica*, pathogens commonly associated with bovine respiratory disease. Blood samples were taken weekly over 12 weeks to measure specific antibodies against all vaccine antigens and against *F. hepatica*, as well as IgG1 and IgG2 isotypes for PI-3 and BRSV specific antibodies. Faecal samples were examined for *F. hepatica* eggs and routine haematology and liver enzyme biochemistry were performed and cytokine production *in vitro* measured. Liver enzymes (GGT and GLDH) and eosinophils were significantly higher in the experimental group, whereas neutrophil numbers were higher in the control group. There was no significant difference between the groups in terms of vaccine-specific total responses to PI-3, BRSV and *M. haemolytica*. IgG1 levels were higher in comparison to IgG2 levels in both PI-3 and BRSV specific antibodies. IL-4 levels from stimulated and unstimulated PBMC were significantly higher in the control group. IFN-γ levels were significantly higher in PBMC from the control group when cultured in medium only. No significant differences were noted in the levels of other cytokines measured. In this work, no effect of early *F. hepatica* infection on the antibody responses to a range of respiratory vaccine antigens in calves was shown. However, differences in cytokine responsiveness of PBMC between control and infected groups were observed, indicating that further work in measuring the effect of *F. hepatica* infection response to challenge infection following vaccination would be warranted.

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1. Introduction

Vaccines are an important tool in livestock production, not only as a means of maintaining health and freedom from clinical diseases, but also in some cases as a means of preventing zoonotic disease and thus enhancing food safety and public health.
Bovine respiratory disease (BRD) in calves and young cattle is a significant source of morbidity and mortality, and is a major contributing factor for economic losses in cattle industry (Snowder et al., 2006). BRD is a multifactorial disease (Babiuk et al., 1988); the most common bacterial pathogens associated with it are Mannheimia haemolytica, Pasteurella multocida, Mycoplasma bovis as well as various viral pathogens; bovine respiratory syncytial virus (BRSV), parainfluenza virus type 3 (PI-3), bovine viral diarrhea virus (BVDV) and bovine herpes virus type 1 (BHV-1). Even though there is limited published information that definitively establishes the efficacy of respiratory vaccines in the field in reducing the burden of bovine respiratory disease (Perino and Hunsaker, 1997), vaccinations against one or more of the respiratory pathogens are commonly used.

Helminth infections can influence the immune response to unrelated antigens (Kullberg et al., 1992). Furthermore, they have been shown to decrease the response to vaccinations in various host species (Elias et al., 2001; Urban et al., 2007). Fasciola hepatica, a helminth parasite, causes fasciolosis in cattle and sheep and fasciolosis is also a zoonosis. It is a common parasite, especially in the temperate climate of the UK and Ireland, where the prevalence in cattle is as high as 84% (McCann et al., 2010). F. hepatica has been proven to have immunoregulatory effects in mice (Brady et al., 1999). In cattle, it can alter the response to immune-mediated diagnostic tests (Flynn et al., 2007) but its effect on vaccine responsiveness in this species has not yet been studied.

The aim of this study was to establish whether a concurrent F. hepatica infection influences the immune response induced by vaccination against respiratory pathogens in calves. This study was undertaken in an effort to measure the extent to which helminth-mediated immunoregulation may have a demonstrable effect on the host’s ability to control other diseases which are economically important in livestock.

2. Materials and methods

2.1. Experimental design

Castrated male Holstein–Friesian calves (n = 48), aged 3–7 months, were used in the study. The calves were kept indoors at University College Dublin Lyons Research Farm under normal husbandry conditions. All experimental procedures were approved by the UCD Animal Ethics Committee and under licence from the Department of Health, Dublin, Ireland (reference number B100/4399).

All animals were administered ivermectin (Noromectin, Norbrook) prior to the commencement of the trial (as per manufacturer’s guidelines). Initial serological and faecal analyses, performed two weeks prior to commencement of the trial were used to determine any previous exposure to the viral respiratory pathogens (PI-3 and BRSV) and F. hepatica, respectively. The calves with no previous exposure to liver fluke infection were then randomly allocated to one of two groups—an experimental group which was infected with F. hepatica by the administration of 150 viable metacercariae (Ridgeway Research, Lydney, Gloucestershire, UK) resuspended in distilled water per os at the commencement of the study (week 0 of the trial), and a second group used as a control. Four animals which were seropositive for liver fluke at the beginning of the trial were automatically assigned to the experimental group.

Two weeks later (week 2 of the trial), following the establishment of a fluke infection, calves from both groups were administered 5 ml of Bovipast RSP vaccine (MSD Animal Health) containing inactivated PI-3, BRSV and M. haemolytica in accordance with the manufacturer’s guidelines. A booster vaccination was administered four weeks later (week 6 of the trial).

2.2. Faecal egg count (FEC)

Faeces were sampled from the rectum of each animal prior to commencement of the study, and at weeks 4 and 12 of the trial. Identification and counts of F. hepatica eggs in 3 g of faecal material were carried out using the sedimentation technique (Cawdery and Ruane, 1971).

2.3. Haematology and biochemistry

Blood sampling of all animals was carried out weekly from week 2 of the trial; 2 weeks post infection, using EDTA vaccutainers for haematology and uncoated vaccutainers for biochemistry. Total cell counts were measured using an Advia 2120 Analyzer (Siemens). Serum gamma glutamyl-transferase (GGT) and glutamate dehydrogenase (GLDH) levels were measured using an Imola Clinical Chemistry Analyzer (Randox).

2.4. Serological analysis

Blood samples from all animals were collected into uncoated tubes for serological examination (ELISA and serum neutralisation test), in accordance with the procedures outlined below. Sampling was carried out on a weekly basis starting at week 2 (the day of 1st vaccination).

2.4.1. ELISA for detection of F. hepatica antibodies

Blood samples were centrifuged at 1900 × g for 5 min and the serum removed. The specific antibody response to the F. hepatica infection was measured by ELISA using rmFhCL1 as antigen and a monoclonal anti-bovine IgG1 as previously described by Golden et al. (2010) with some modifications. Briefly, 96-well plates were coated with 1 μg/well of rmFhCL1 overnight at 37 °C. Phosphate buffered saline with tween (0.05% PBST) was used as blocking buffer and 1% bovine serum albumin (BSA) in 1% BSA–PBS was used as blocking and dilution buffer.

Serum dilutions (1:20) were added in duplicate to the plate (100 μl per well) and incubated for 30 min at 37 °C. Bound antibody was detected by addition of a monoclonal anti-bovine IgG1–HRP conjugated antibody (Prionics), diluted 1:100, followed by 3,3,5,5-tetramethylbenzidine (TMB-Sigma). Absorbances were read at 450 nm on an Expert 96 Microplate reader (Biochrom).

2.4.2. Serum neutralisation test (SNT) for BRSV and PI-3

Double dilutions of inactivated sera were made in a 96-well flat-bottomed cell-culture grade microtitre
plate. After incubation with virus for 24 h at 37 °C a cell suspension of foetal bovine kidney cells was added. After incubation for 3–5 days at 37 °C, the plates were read microscopically for cytopathic effects (CPE). The test serum results were expressed as the reciprocal of the dilution of serum that neutralised the virus in 50% of the wells. If 50% of the wells with undiluted serum neutralised the virus, the (initial dilution) titre was read as 1 (1:2 using the final dilution convention). If all the undiluted and 50% of the wells with 1:2 diluted serum neutralised the virus, the (initial dilution) titre was 2 (final dilution 1:4).

2.4.3. ELISA for detection of PI-3 and BRSV specific antibodies

Serum antibody levels to BRSV and PI-3 were evaluated using the commercial indirect IgG ELISAs Svanovir BRSV-Ab and Svanovir PI-3-Ab, respectively (Svanova Biotech, Uppsala, Sweden). Both the ELISA testing procedure and the interpretation of results were performed according to the manufacturer’s instructions. Sera were tested at a dilution of 1:25 and results reported as percent positivity values (PP), calculated in respect to a common positive control.

2.4.4. ELISA for detection of IgG1 and IgG2 isotypes

Antibody isotypes for PI-3 and BRSV were measured at week 2, 4, 7, 9 and 12 using bovine parainfluenza virus type 3 (Svanovir PIV3-Ab) and bovine respiratory syncytial virus (Svanovir BRSV-Ab) antibody test kits from Boehringer-Ingelheim Svanova (Uppsala, Sweden). Sera were diluted 1:25 (total volume was 100 μl) in PBST (0.01%), and plates were incubated at 37 °C for 1 h. Plates were washed three times in PBST, and tapped to remove excess liquid. Conjugate (100 μl) was added and plates were incubated at 37 °C for 1 h. For the IgG1 ELISA, the anti-bovine HRP conjugate was used as provided. For the IgG2 ELISA, a mouse anti-bovine IgG2 HRP conjugate (ABD Serotec, Kidlington, UK) was substituted for the kit reagent and used at a dilution of 1:500. Plates were washed three times in PBST and TMB substrate was added, with incubation at room temperature for 10 min. Development of the solution was stopped and the plates were read at 450 nm using an LED96 Detect plate reader (Dynamica Scientific, Milton Keynes, UK). Corrected optical density readings were calculated by subtracting the plate background reading.

2.4.5. ELISA for detection of M. haemolytica specific antibodies

Antibodies specific for M. haemolytica were measured at week 2, 6, 10 and 12 with an in-house ELISA at the laboratory of MSD Animal Health (Boxmeer, Netherlands). Microtitre plates were coated with M. haemolytica antigen. Serum was diluted and incubated and bound antibodies were detected after incubation with an anti-bovine serum-peroxidase conjugate as previously described (Assié et al., 2009).

2.5. Cytokine expression

At weeks 8 and 12, blood samples from a subset of calves (six randomly selected animals from each group) were collected into heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated by centrifuging over Histopaque (Sigma) and recovered in RPMI 1640 medium (supplemented with 10% heat-inactivated foetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine). Viable cells were counted by Trypan blue exclusion and resuspended at a concentration of 1 × 10^6 cells/ml. One millilitre of this suspension was added in duplicate to a 24-well plate. Cells were stimulated with F. hepatica excretory-secretory antigen (ES; 10 μg/mL), lipopolysaccharide (LPS; Escherichia coli 0111:B4, Alexis; 5 μg/mL) as a bacterial antigen and toll-like receptor ligand 7/8 (TLR7; Resiquimod R848, Invivogen; 1 mg/mL) as a viral antigen. Medium and Con A (Sigma; 5 μg/mL) were used as negative and positive controls, respectively. Cell cultures were incubated for 72 h at 37 °C before supernatants were collected and stored at −20 °C for cytokine analysis. Cytokine levels were measured using commercially available ELISA kits, or antibody pairs. Interleukin 4 (IL-4) was measured using a kit from Pierce Biotechnology in accordance with the manufacturer’s guidelines. Transforming growth factor beta (TGF-β) was measured using a human ELISA (Promega) as previously described (Abbott et al., 2005). Nitric oxide (NO) was measured using the Griess Reagent Kit (Promega). Interleukin 10 (IL-10) and Interferon gamma (IFN-γ) were measured using commercially available antibody pairs as previously described (Flynn and Mulcahy, 2008).

2.6. Statistical analysis

Regression procedures were employed to comparatively assess the effect of liver fluke infection status on each of the vaccination, biochemical and cytokine variables, over a 12-week period.

Prior to the analysis, the stability of the variance for each parameter was assessed, and where normality was not identified, a transformation step was applied (log transformed for PI-3 and BRSV SNT as well as GLDH and square root transformed for GGT variables). In the case of IFN-γ and BRSV, where the variance for these dependent variables could not be stabilised, a non-parametric quartile (median 50) regression model was applied.

In addition, a pre-infection comparison of all variables was carried out using an independent student *t*-test to ensure a group effect was not present prior to commencement of the trial. In cases where a *P* value of <0.20 was identified (indicating the potential for a pre-infection group effect), the pre-infection values were subtracted from all subsequent data points in order to standardise the group comparison. This adjustment was applied to both BRSV ELISA and BRSV SNT variables.

Regression models were constructed (8 for haematology and biochemistry and 9 for vaccine data), each assessing the combined effect of group, duration, and group by duration interaction on the dependent variable. *P* values of <0.05 were considered statistically significant. All statistical analysis was performed using Stata/SE v12.1 (StataCorp, Texas, USA).
3. Results

3.1. Faecal analysis

The analysis prior to the trial and at week 4 of the trial revealed no fluke eggs present in any of the animals. At week 12 post infection (p.i.), four of the 24 calves in the experimental group had a positive faecal egg count, 2 eggs were identified in one sample (3 g) and 1 egg each in the remaining three. None of the four animals that were positive for fluke antibodies before the trial had a positive faecal egg count. All animals in the control group were negative for F. hepatica eggs throughout the experiment.

3.2. Haematology and biochemistry

The results of the haematology analysis showed a significant difference between the groups for absolute eosinophil and absolute neutrophil numbers, as presented in Fig. 1A and B. Absolute eosinophil numbers were significantly higher \((p \leq 0.001)\) in the infected group at 4, 5, 6, 8 and 10 weeks p.i. (one animal was identified as an outlier because of significantly higher values throughout the experiment and was excluded from the analysis). Peak eosinophil counts occurred at week 5 p.i. Mean neutrophil numbers of all calves \((n=48)\) were above the reference range \((0.6–4 \times 10^9 \text{L}^{-1})\) prior to the experiment and declined thereafter. Absolute neutrophil counts were significantly higher in the control group at 2, 7, 9 and 10 weeks p.i., with the overall model construct, accounting for group and time effects, indicating a significant difference \((p=0.004)\) between the groups. No significant differences were measured in the number of lymphocytes, basophils or monocytes (data not shown).

The results of the biochemistry analysis indicated significantly elevated liver specific enzymes, GLDH \((p \leq 0.001)\) and GGT \((p < 0.001)\) in the experimental group, as presented in Fig. 1C and D. The difference between the groups for GLDH was apparent by week 5 p.i., and persisted throughout. For GGT, the difference was apparent by week 8 p.i., and also persisted throughout.

3.3. Serological analysis

3.3.1. ELISA for detection of F. hepatica antibodies

Prior to the commencement of the experiment, 4 out of 24 of 48 calves were positive for anti F. hepatica antibodies (above the cut-off value of 20 PP), which were therefore allocated to the experimental group. A total of 21 of the 24 animals in the experimental group were positive by 4 weeks p.i., with all 24 animals seroconverted by week 6 and remained positive until the end of the study. All of the animals in the control group were negative for F. hepatica antibodies throughout the experiment.
3.3.2. Serum neutralisation test for PI-3 and BRSV specific antibodies

The results of the SNT for PI-3 and BRSV are presented in Fig. 2A and B. At the initial examination (week −2), the mean log titre of PI-3 and BRSV specific antibodies of all calves from both groups was 1.7 ± 0.08 and 0.45 ± 0.07, respectively. The PI-3 SNT profile indicated a comparable antibody profile between both groups, with no significant difference identified (p = 0.18). Antibody levels increased rapidly for both groups at week 3 p.i. (one week following 1st vaccination) and remained elevated with minor variations throughout. The BRSV SNT profile also indicated no differences between the groups (p = 0.62). Antibody titres fluctuated similarly for both groups, with rising titres mainly correlating with the vaccination protocol with the exception of low values at week 9.

3.3.3. ELISA for detection of PI-3 and BRSV specific antibodies

ELISA results showed all animals were positive for PI-3 antibodies (cut-off value at 10 PP) with a mean value of 109.8 ± 6.7 PP and 36 (75%) were positive for BRSV.
antibodies (cut-off value at 10 PP) with a mean value of 56.3 ± 7.1 PP prior to the vaccination.

ELISA results for PI-3 are presented in Fig. 2C and these indicate that antibody titres were not significantly different between the groups, and the profile of antibody changes over time were identical. There was an increase in antibody levels following primary vaccination (week 2 p.i.), with a steady decline following week 8. The ELISA results for BRSV (Fig. 2D) indicate increasing antibody levels following primary vaccination (week 2 p.i.), a steady increase to week 10 and then a decline. There was no difference between groups in either case, with a p-value of 0.81 for BRSV and 0.55 for PI-3.

3.3.4. ELISA for detection of M. haemolytica specific antibodies
Antibody titres specific to M. haemolytica are presented in Fig. 2E, which shows there was no difference between the groups (p = 0.26). Animals from both groups showed high antibody titres on the day of vaccination, which increased over time with a peak at week 10.

3.3.5. ELISA for IgG1 and IgG2 isotypes
Levels of IgG2 were lower in comparison to IgG1 for both PI-3 (Fig. 3A) and BRSV (Fig. 3B). There was a significant difference (p = 0.01) between the groups for BRSV IgG1 antibodies at week 4 but the difference was not evident thereafter. Levels of IgG1 for BRSV reached their peak at week 9, whereas levels of IgG1 for PI-3 peaked at week 4 and gradually declined thereafter. On the other hand, levels of IgG2 for BRSV were lowest at week 4 and at week 2 for PI-3 but no group effect was identified for any of the IgG2 levels.

3.4. Cytokine expression

In vitro cytokine analysis indicated that the production of IFN-γ by PBMC was significantly higher (p = 0.048 for
the overall model) in calves from the control group when the cell culture was not stimulated with antigens (Med) as shown in Fig. 4A. At week 8, IL-4 production was significantly higher \((p = 0.03)\) in the control group from unstimulated cells (Fig. 4B). At week 12 the concentration of IL-4 was significantly higher \((p = 0.04)\) in the control group from cells stimulated with TLRL 7/8 (Fig. 4C) as well as from cells stimulated with LPS \((p = 0.06)\) as shown in Fig. 4D.

No significant differences between groups were noted in IL-10, TGF-β and NO production, although there was a response to all antigens in these cytokines, as well as production from unstimulated PBMC (data not presented).

4. Discussion

The purpose of this study was to establish if the immunomodulatory effects of liver fluke infection in cattle can affect the host response to bacterial and viral vaccination. The effects of helminth infection on the host immune system have been well characterised, and can be summarised by an increase in the production of Th2 and regulatory T cell cytokines, with an associated negative feedback on Th1 cells (McSorley and Maizels, 2012). *F. hepatica* infection in cattle has a similar effect on the host immune system as other helminths (Flynn et al., 2010), and in previous studies in mice (O’Neill et al., 2000) and cattle (Aitken et al., 1978) the immunosuppressive effects on the response to other concurrent infections have been noted.

This study identified no alteration in the specific total antibody response to BRSV, PI-3, and *M. haemolytica* within the first 12 weeks of an experimental infection with *F. hepatica*. In both groups the antibody profiles to BRSV, PI-3, and *M. haemolytica* vaccination over time were similar. This finding delivers reassurance that vaccination elicits a comparable immune response despite concurrent infection. A single exception identified for IgG1 levels against BRSV at week four indicates a potential group effect. However, this difference is no longer evident by week 7 and thereafter, indicating that on a wider timescale, there is no difference between the groups. Additionally, despite the identification of pre-existing antibodies to BRSV, PI-3 and *M. haemolytica*, derived from either a maternal source or previous exposure to the antigens, the calves from both groups showed an initial increase in antibody levels after vaccination. In contrast to convention that such antibodies can have an inhibitory effect on the vaccine response (Kimman and Westenbrink, 1990), this study demonstrates a serological response to vaccination in the presence of pre-existing antibodies. It remains to be determined whether the antibody levels were sufficient to prevent clinical outbreaks of disease.

While our findings relate to *F. hepatica* infection, a similar study also concluded that there were no effects of *Ostertagia ostertagi* and *Cooperia* spp. infection on the respiratory vaccine response in calves (Schutz et al., 2012). However, these results are in contrast to other host species. For example, helminth infection in the pig reduced the
efficacy of vaccines against *Mycoplasma hyopneumoniae* (Steenhard et al., 2009).

Adjuvants can have an important role in the success of a vaccine. QuilA, the adjuvant used with this vaccine has been previously shown to offset the immunomodulatory effects of the fluke, and to offer protective effect against *F. hepatica* in sheep (Haçariz et al., 2009). Similarly in this study in cattle, it is plausible that the QuilA in the vaccine may provoke an adequate immune response to viral and bacterial infection despite concurrent infection with *F. hepatica*.

It is also noteworthy that the immunoregulatory effects described by Flynn et al. (2007) relates to the suppressive effect of *F. hepatica* on the type IV delayed hypersensitivity (antibody independent) reaction, as modelled on the intradermal skin test. The vaccine used in this trial has a combination of bacterial and viral antigens, which is likely to induce a different immune response to that caused by *Mycobacterium bovis* and therefore may relate to the difference between immune mechanisms involved in an antibody independent hypersensitivity reaction and an antibody dependent immune response to vaccination.

Relative to other comparable studies (Waldvogel et al., 2004) where a higher dose of *F. hepatica* metacercariae was used, the low dose used in this study could have resulted in a parasite burden too low to have a demonstrable effect on vaccine responsiveness. However, all animals in the experimental group were infected, and did mount an immunological reaction to liver fluke infection as indicated by seroconversion and increased liver enzymes. Low number of calves with a positive faecal egg count reflects the fact that the analysis was conducted early in the patent period. Also, detection of *F. hepatica* eggs in bovine faeces is a relatively insensitive diagnostic method (Anderson et al., 1999).

The increase in eosinophils as well as liver enzymes was significantly higher in the experimental group relative to the non-infected group, where no or little increase in these parameters was identified. In contrast, neutrophil numbers, which were elevated relative to the reference range at the start of the experiment, followed by a decline in both groups, were significantly lower in the infected group relative to the non-infected group. This observation in the neutrophil count was in contrast to other studies (Egbru et al., 2013) where a significant increase was identified. A reasonable explanation to account for this response is not forthcoming. Stress, as a cause of the initial elevated numbers, is an unlikely explanation, given the animals were on the farm for 2 months prior to the commencement of the experiment. However, it is possible that an unknown sub-clinical infection may account for the unusual neutrophil profile, with the concurrent *F. hepatica* infection potentially hampering the neutrophil response in the infected group.

The expected cytokine profile following liver fluke infection of elevated Th2 and inhibited Th1 profiles was not identified. In contrast to previous research, we found IL-4 concentrations produced by unstimulated and stimulated PBMC to be higher in the control group. However, the levels were very low, which could be partially due to the fact that the peak production of IL-4 is at 4 to 6 weeks after infection (Flynn and Mulcahy, 2008) whereas our measurements only took place at week 8 and 12 p.i. BRSV has the ability to induce IL-4 production (Gershwin, 2012). If cells from liver fluke infected animals were hyporesponsive, it might explain the higher IL-4 production in the control group. The increased production of IFN-γ in the control group would be indicative of a Th1 response, whereas calves infected with *F. hepatica* had a lower Th1 response. However, the surprisingly low IgG2 values after vaccination would be indicative of decreased Th1 response in all animals regardless of the liver fluke infection. This is in contrast to other studies done with BRSV vaccination, where IgG2 levels were as high as IgG1 (West and Ellis, 1997) even though claims have been made that BRSV vaccination induces a Th2 response in cattle (Kalina et al., 2004). IL-10 and TGF-β produced by regulatory T cells can play an important role in maintaining parasite infections (Beiting et al., 2007). In our case no significant production of these cytokines in the fluke infected group was detected.

5. Conclusion

This study demonstrated that *F. hepatica* does not alter the immunocompetence of calves to mount an immune response to respiratory vaccination. Further studies including challenge experiments would be required to establish the impact of liver fluke infection (acute as well as chronic) on vaccination efficacy and its effects on clinical signs, survival rate and potentially economic impact.

Conflict of interest

The authors declare there are no conflicts of interest.

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