Short communication

Bovine immune response to papillomatous digital dermatitis (PDD)-associated spirochetes is skewed in isolate reactivity and subclass elicitation

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1. Introduction

Papillomatous digital dermatitis (PDD) is a major cause of lameness in dairy cattle worldwide. Farms with PDD-afflicted cows experience economic loss due to treatment costs, decreased milk production, lower reproductive efficiency and premature culling. Cows exhibit both humoral and cellular immune responses to PDD-associated spirochetes. This study was undertaken to further characterize the bovine humoral response to PDD-associated spirochetes. Forty-seven sera samples collected from cattle (Field cattle) on three different dairy operations in Iowa were analyzed. In addition, sera were obtained from six young steers (Test cattle) that received a mixed inoculum of four previously isolated Treponema phagedenis-like spirochetes (1A, 3A, 4A and 5B) on two separate occasions. Relative levels of total IgG, IgG1, IgG2 and IgM reactive to each individual spirochete were determined. Field cattle had a higher mean antibody response to 5B compared to the other isolates and T. phagedenis. Test cattle reacted most strongly with 4A following initial exposure, shifting to a greater reactivity with 5B and a reactivity profile similar to field cattle following secondary exposure. No measurable IgM was detected. IgG1 was produced predominately in all cattle. Low to moderate levels of total IgG reactivity to T. phagedenis occurred with sera from all cattle.

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spirochetes isolated from PDD lesion material would produce similar or different results. Thus, the aim of the present study was to investigate the bovine humoral response to a mixed inoculation of PDD-associated treponemes and compare that to the humoral response of naturally exposed cattle (Field cattle). In addition, we wanted to evaluate the immune response elicited by each individual spirochete and determine the IgG isotype produced in response to primary inoculation in naïve animals (Test cattle) vs. secondary inoculation in the same animals. These data were compared to sera from dairy cattle kept on farms where PDD is present. We found differences existed between the humoral response in naturally exposed Field cattle and the humoral response to the primary inoculation of Test cattle with regards to which spirochete elicited the strongest antibody response. The humoral response of Test cattle to the secondary exposure resulted in a similar reactivity profile as seen in the Field cattle. In addition, contrary to previously published results, IgG1, not IgG2, was the primary Ig isotype detected in this study.

2. Materials and methods

2.1. Bacterial strains and media

The PDD-associated treponema strains 1A, 3A, 4A, and 5B were used in this study (Trott et al., 2003). The PDD isolates were grown in pre-reduced, anaerobic oral treponeme isolation (OTI) broth containing 10% heat inactivated fetal bovine sera, without antibiotics prepared as described (Trott et al., 2003). Treponema phagedenis (ATCC #27087) was grown in PY medium with cocarboxylase and serum (ATCC media 1828). All bacteria were incubated under anaerobic conditions in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) under an atmosphere of 85% N2, 5% CO2, and 10% H2. All bacterial manipulations were carried out under anaerobic conditions.

2.2. Cattle inoculations

Spirochete cultures were harvested at 0.6 OD620 during the late exponential phase of growth. Culture viability was confirmed by the observation of active motility and the absence of spherical bodies using dark-field microscopy. Cell counts were performed using a Petroff–Hausser bacterial counting chamber prior to harvesting by centrifugation (6000 × g for 20 min). A mixed inoculum was prepared containing all four of the PDD isolates in a ratio of 1:1:1:1. For example, 2.5 × 1010 spirochetes of each isolate were pooled, mixed, pelleted by centrifugation and resuspended in 0.2 mL of sterile PBS for each inoculum.

For Test cattle (three Holstein and three Jersey) exposure, a small area of the lateral cervical region on each steer was shaved, swabbed with 70% ethanol, and inoculated with a single subcutaneous injection of bacterial suspension. All inoculations of live spirochetes were performed within 15 min of inoculum preparation. Cattle were observed approximately 1 and 6 h following inoculation and then every few days for signs of swelling, abscess formation and/or lesion development at the inoculation site. Secondary inoculations were performed 6 months later in an identical manner at the original injection site. The use of these experimental animals was in accordance with the institutional guidelines.

2.3. Field and Test cattle sera samples

Sera from two groups of cattle were used for this study. For the Field cattle (naturally exposed), banked sera samples (47 total, Jersey and Holstein) were used. These sera were collected from three Iowa dairy farms, with a history of PDD, during the years 1999–2001. Forty percent of the Field cattle sera samples came from cattle with known lesions. The lesion status of the remaining cattle was not recorded at the time the sera samples were collected. Preliminary ELISAs were run on each Field cattle sera sample (see Section 2.4) to determine the presence of anti-treponema antibodies as evidence of exposure. Three times the S.D. of the mean OD of the negative samples run (Test cattle d0 and 2 known negative samples) was used as a negative cut off value to determine if the samples were positive (above that value) or negative (below that value) for antibodies to PDD-associated spirochetes. Based on this criteria, all of the sera samples used in this study were positive for each of the isolates at a 1:100 dilution. Sera were maintained at –80 °C during storage. For Test cattle (experimentally exposed, see Section 2.2), blood was drawn from the jugular vein 2 weeks post-inoculation and allowed to clot at 25 °C for 1 h. Sera were collected and stored at –80 °C until use.

2.4. PDD-spirochete and serum Ig ELISAs

Standard ELISA methods were utilized for the determination of antibody production. Briefly, 96-well microtiter plates (Immulux by Dynex, Chantilly, VA) were coated with formalin-killed PDD isolates (either 1A, 3A, 4A, or 5B) or formalin-killed T. phagedenis at 108 spirochetes/mL in a volume of 100 μL of 0.1 M carbonate buffer (pH 9.6) per well, then incubated overnight at 4 °C. The plates were washed three times with a solution consisting of 50 mM Tris, 0.14 M NaCl and 0.05% Teen 20 (Tris-T) and blocked with Tris-T with a 1:100 dilution and then diluted 1:3 down columns of the plate (ending dilution 1:72,900). Plates were incubated for 1 h at RT followed by washing five times with Tris-T. Secondary antibodies were diluted in Tris-T and then added to the plate. Total IgG was detected using a Rabbit anti-bovine HRP-conjugated F(ab′)2 fragment at 1:25,000 (Rockland, Pennsylvania, USA). IgM, IgG1 and IgG2 were detected using HRP conjugated sheep anti-bovine IgG1, IgG2 and IgM (Fitzgerald Industries International, Inc., Concord, MA) diluted in Tris-T 1:5000 for the determination of total IgG and antibody isotypes produced. Plates were incubated for 1 h at RT. Following incubation, plates were washed five times with Tris-T and TMB (Sigma) was used to visualize bound antibody. The reaction was stopped using 0.18 M sulfuric acid and the absorbance read.
at 450 nm. Positive and negative control sera were run for each set. ELISA units were calculated by multiplying the OD value \times dilution factor of the highest dilution to fall on the linear portion of the graphed data. Inter-plate variation was normalized to the positive control sample.

ELISAs to determine serum levels of IgG1 and IgG2 in all serum samples were conducted using reagents from Bethyl for this purpose according to the product company protocols. Sera IgG2 levels were also determined with Fitzgerald sheep anti-bovine IgG1 and IgG2 in place of Bethyl sheep anti-bovine IgG1 and IgG2 to determine if a difference was present in the reactivity of the secondary antibodies to IgG2 in the sera of Field and Test cattle.

2.5. Statistical analysis

Differences in sera reactivity in each group of cattle for total IgG, IgG1 and IgG2 to each isolate were analyzed by one-way ANOVA (GraphPad Prism) using the Tukey–Kramer test as a post-test. Differences in relative levels of antibody produced between the primary and secondary exposure in Test cattle to each isolate were analyzed using a two-tailed, unpaired \( t \)-test (GraphPad Prism). A 0.05 value of significance was used for all data. Data are expressed as the mean \( \pm \) S.E.M.

3. Results and discussion

We sought to determine if the relative reactivity of antibodies produced in response to individual treponemes isolated from PDD lesions would vary in cattle naturally exposed to PDD and to Test cattle inoculated with a mixed culture containing all four isolates (1A, 3A, 4A, and 5B). As our isolates appear most closely related to \( T. phagedenis \) (Trott et al., 2003), we included measurements of serological reactivity to \( T. phagedenis \) to determine to what degree, if any, antigenic-cross-reactivity exists between it and the isolates used in this study.

The mode of test exposure in this study is taken from the mouse abscess model utilized previously to investigate pathogenic potential of these PDD-associated spirochetes (Elliott et al., 2007). We chose to investigate a mixed inoculum due to the number of different spirochetes cattle are exposed to at the same time during a natural infection. The inoculation site was shaved in order that any inflammation or gross changes in this area would be easily noted. No visible swelling, redness or gross pathological changes occurred at any time during or after the inoculation. The skin surrounding the inoculation site appeared perfectly healthy and normal throughout the observation period. For this reason, no histopathology was performed.

As in agreement with previous studies (Demirkan et al., 1999; Dhawi et al., 2005; Murray et al., 2002), no measurable IgM was present for any group (data not shown) while IgG anti-treponema antibodies were detected in all cattle sera tested (Fig. 1). Sera from Field cattle contained antibodies with a higher mean reactivity to antigens from \( T. phagedenis \) than was present in sera from Test cattle. Field cattle (Fig. 1-A) had a significantly higher relative level of antibodies reacting with 5B compared to other isolates. The lowest relative level of antibody detected was to 3A. This result may indicate that of the four isolates tested, 5B is the most antigenic and 3A is the least. Alternatively, the differences may be linked to exposure in the Field.

Although it is not certain that each Field cow was exposed to all or any of these specific isolates, the likelihood is high given that the spirochetes used in this study were isolated from lesions obtained from cattle on the farm from which the majority of the sera samples were collected. In addition to differing levels of exposure, isolates are likely not evenly represented in lesions or situations where exposure occurs. As new data become
available, the diversity of the population of spirochetes detected in PDD lesions continues to grow (Evans et al., 2008; Klitgaard et al., 2008; Nordhoff et al., 2008). Competition for resources among the treponemes, and other bacteria, present in a lesion during infection may also affect the numbers of each population represented in any given lesion.

Another explanation for the high level of reactivity to 5B may be due to its antigenic nature. Our murine study gave evidence that of the four isolates, 5B showed the greatest heterologous cross-reactivity with sera raised to each of the other isolates (Elliott et al., 2007). We felt this was likely due to 5B expressing antigens found on the each of the other isolates. For this reason, sera from a mixed infection would show a higher reactivity with 5B due not only to antibodies produced in response to 5B specifically, but with the added antibody binding from cross-reactive antibodies produced in response to other PDD-associated spirochetes. Sera from cattle exposed to individual isolates will be needed to determine if this is the case.

Primary inoculation of Test cattle resulted in a higher relative level of antibody reactive with 4A (Fig. 1-B) with the lowest level reactivity occurring with 1A, a different trend then seen with Field cattle. This may indicate the antigenicity of each isolate upon primary exposure, as each isolate was represented equally in the inoculation. As expected, secondary exposure resulted in a significant increase in the amount of antibody to each isolate \((p < 0.001, \text{total IgG levels Fig. 1-B vs. -C, all isolates})\). Interestingly, following secondary exposure, the levels of reactivity to each isolate changed with the greatest reactivity occurring with 5B, instead of 4A, and the least occurring with 3A in place of 1A (Fig. 1-C). The result was an antibody reactivity profile resembling that seen in naturally exposed cattle, although the differences did not reach statistical significance, as occurred with the Field cattle sera.

Evaluation of IgG1 and IgG2 antibodies reactive for each isolate revealed that relative levels of anti-treponema IgG1 were significantly higher than levels of IgG2 for each isolate in both Field and Test cattle \((p < 0.01; \text{Fig. 2})\). Although not exactly the same, anti-spirochete IgG1 from Field cattle followed a similar reactivity pattern as total IgG with sera reacting most strongly with 5B (Fig. 2-A) and mean antibody binding to 3A being significantly lower compared to antibody binding to all other isolates \((p < 0.05)\).

Primary inoculation of the Test cattle (Fig. 2-B) resulted in the greatest reactivity to 3A and the lowest to 1A \((p > 0.001 3A \text{ vs. } 1A)\), differing from the results obtained with total IgG measurements (Fig. 1-B). Although anti-treponema IgG1 in the sera following the secondary inoculation showed the highest mean reactivity with 5B (Fig. 2-C), there is notably less difference in the reactivity between the isolates when comparing relative levels of IgG1 (Fig. 2-C) to total IgG (Fig. 1-C). This may be due to the presence of IgG3. Despite a proposed low transcription level for IgG3 (Zhao et al., 2003) it cannot be ruled out that this IgG isotype is produced in response to PDD-associated spirochetes in this model of exposure. IgG3 may contribute to the total IgG reactivity measured and the lack of detection when the sera are assayed for IgG1 results in differing levels of reactivity compared to that seen with total IgG.

The induction of IgG1 in the absence of IgG2 may impart an advantage to the introduction and persistence of PDD-associated treponemes within a lesion. Although both IgG1
and IgG2 activate complement. IgG2 had been shown to work effectively as an opsonin while IgG1 does not (McGuire et al., 1979). As lesions generally require treatment and do not spontaneously heal, or recur in successfully treated cattle (Berry et al., 2002), it may be that the induction of IgG1, and presumably a T-helper type 2 immune response, may suppress the production of IgG2 (Estes and Brown, 2002) resulting in reduced clearance of PDD-associated spirochetes, imparting a survival advantage to those spirochetes during infection. Production of IgG2 would potentially increase the number of spirochetes phagocytosed, while an IgG1 dominated response would result in less efficient opsonization.

The findings in this study differ from previously published reports (Demirkan et al., 1999; Dhawi et al., 2005; Murray et al., 2002) that indicate measurable levels of bovine IgG2 are produced to PDD-associated treponemes while anti-treponema IgG1 is not detected. One reason may be due to different secondary antibodies being used (polyclonal sheep anti-bovine IgG1 and IgG2 vs. monoclonal murine anti-bovine IgG1 and IgG2). We felt the use of polyclonal sera for detection would yield the most accurate measure of antibodies present due to the multiple antigenic specificity of the preparation, increasing the likelihood of binding to the target Ig in the experimental sample. There is evidence that the allotypes of IgG2 represent co-dominant alleles and may not be equally represented in cattle breeds or individual cattle of a given breed (Kacskovics et al., 1995). Allotype bias in detection reagents can occur in anti-bovine IgG2 polyclonal anti-sera raised in rabbits and goats (Butler and Heyermann, 1986) with IgG2a (formerly IgG2A1) being more readily detected than IgG2b (formerly IgG2A2) due to an immunodominant determinant on the Fc portion of IgG2a (Blakeslee et al., 1971; Butler and Heyermann, 1986). Although we do not believe this is the case based on the sera level IgG2 in the Field and Test cattle in this study being comparable to published levels (data not shown) and the treponema-specific IgG2 being very low when the same polyclonal anti-sera was used for detection in each case. We cannot rule out, however, that both of the commercial sheep antibovine IgG2 sera used are biased toward IgG2a and detecting only IgG2a and anti-treponema IgG2b present is going undetected, despite the fact that this bias toward allotype reactivity has not been shown to occur in sheep.

An alternate explanation of the low IgG2 levels is that in the assay used in this study, the antigen-specific IgG1 antibodies bind the treponema with greater affinity, out competing antigen-specific IgG2, resulting in a lower relative level of antigen-specific IgG2 detected than is actually present in each sample. Another condition that may factor into the resulting data is that our ELISA is measuring the response to exterior antigens using formalin-killed spirochetes. The amount of internal antigen available in an assay measuring reactivity to sonicated whole cell preparations may allow for the detection of antigens not available in our assay, raising the possibility that different antigenic determinants induce the production of different isotypes of Ig. For example, outer membrane antigens elicit a primarily IgG1 response while cell wall, inner membrane, and cyttoplasmic antigens elicit primarily IgG2.

4. Conclusions

From this study, we conclude that cattle produce a serological response to inoculation of live cultures of PDD-associated treponemes that changes with regard to isolate reactivity upon secondary exposure to a defined set of PDD-associated treponemes. Measurable amounts of both IgG1 and IgG2 anti-treponema antibodies are present in the sera of naturally exposed and experimentally inoculated cattle with IgG1 being the predominant isotype detected in this study.

With the persistence of PDD in dairy operations around the world, and its emergence in the beef cattle industry, the need for effective vaccines and affordable treatments becomes critical. Imperative to the development of these tools is a thorough understanding of the role spirochetes play in lesion initiation and progression. In conjunction with etiology, understanding the immune response elicited by PDD-associated spirochetes infecting cattle is an important first step in designing efficacious vaccines.

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References


