

**‘Investigation of the diagnostic value of ELISAs using
colostrum derived immunoglobulins for targeted production
animal diseases’**

by

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Abstract

Due to the process of colostrogenesis, maternal antibodies are selectively transferred from the serum and are concentrated in the colostrum. The concentration of IgG in cow colostrum can be up to 10 times greater in concentration compared to serum, while the concentration of IgG in the sow and the ewe may be up to 2.5-3 times greater than serum. In dairy cows and sows in farrowing crates, the collection of colostrum is simple and non-invasive. Following fractionation of colostrum into whey and curds, the concentrations of Igs in the whey are often higher than in the colostrum. Due to these higher concentrations of Igs, colostrum and colostrum whey may improve the diagnostic utility of antibody ELISAs used for the diagnosis of important infectious production animal diseases.

There are a number of commercially available antibody-ELISAs for important production animal diseases. These tests are inexpensive and easy to perform, and are used extensively. Tests with a high sensitivity correctly identify a higher proportion of infected animals within a population, increasing the assurance of absence or presence of disease. Tests with a low sensitivity correctly identify a lower proportion of infected animals, increasing the incidence of false-negative test results.

This thesis investigated the diagnostic value of colostrum derived immunoglobulins for targeted production animal diseases. Using vaccinated animals, the diagnostic sensitivities of commercially available ELISAs for erysipelas (*Erysipelas rhusiopathiae*) and enzootic pneumonia (*Mycoplasma hyopneumoniae*) in pigs, Bovine Viral Diarrhoea Virus in dairy cattle and Johne's disease (*Mycobacterium avium* subspecies *paratuberculosis*) in sheep were determined when using colostrum compared

to serum. Additionally, the diagnostic specificity was also determined for Bovine Viral Diarrhoea Virus in dairy cattle using samples collected from an unvaccinated, bulk tank milk negative herd. The diagnostic utility of the ELISA for Johne's disease was also further investigated using samples collected from a dairy cattle herd with a history of previous infection.

A model using sow disease-specific antibody levels for the prediction of piglet serum disease-specific antibody levels was also developed, as well as the use of colostrum in ELISA for the detection of heifers carrying a calf persistently infected (PI) with Bovine Viral Diarrhoea Virus. The relationship between rennet dilution and IgG concentration in colostrum whey and the benefit of oral rennet supplementation during the neonatal period on the serum globulin concentration of piglets was also investigated.

Overall, the diagnostic sensitivities of the ELISAs were improved when using colostrum compared to serum, whilst also maintaining diagnostic specificity. Sow colostrum and serum were useful predictors of piglet serum disease-specific antibody levels, while colostrum collected from heifers carrying PI calves had significantly higher disease-specific antibody levels. The experiments that explored the coagulation potential of rennet were unable to demonstrate any improvements in Ig concentrations.

Colostrum may be a useful sample type for the detection of other important livestock diseases. The sensitivity of the ELISA for bovine tuberculosis when using serum is too low for control policies to be effective, while the analytical sensitivity of the ELISA for *Neospora caninum* when using bulk tank milk could still be improved.

Declaration of Originality

I certify that this work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or tertiary institution without the prior approval of the University of Adelaide and, where applicable, any partner institution responsible for the joint award of this degree.

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Caitlin J. Jenvey

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Chapter 1: The diagnostic value of colostrum

1.1 Introduction

The mammary gland

The role of the mammary gland

The mammary gland contains two main protective components: humoral immunity, which comprises soluble components such as immunoglobulins (Igs), and cellular immunity, which comprises leukocytes. Together, these two components provide immunological protection to the neonate. Additionally, the mammary gland provides protection for the neonate and the mammary gland against infections by pathogens (Beer et al., 1974; Bourne, 1977). One of the mechanisms by which the mammary gland facilitates this function is by the transfer of immunoglobulins (Ig) from the circulation of the dam into the mammary gland where it is secreted in the colostrum. Mammary gland secretions have two distinct functions: to provide a rich source of Ig to the neonate in the early post-partum period and to provide a continuous supply of antibody which acts locally in the gut (mucosal immunity) during the milk dependent period (Bourne, 1977). The importance of the role that the mammary gland plays in the protection of the neonate depends upon the physiology of the placenta which is species specific. There are three main methods in which the dam can provide protection to the neonate: placental transfer of Ig, lacteal transfer of Ig or combined placental and lacteal transfer. In animals that have placental transfer of Ig (e.g. humans and rabbits), the neonate is born with circulating Ig concentrations similar to or equal to maternal serum antibody concentrations (Marnila and Korhonen, 2011b). In species that have a lacteal transfer of Ig (e.g. cows, sows and ewes), the neonate is born with minimal circulating

Ig derived from the dam. This is due to the anatomy and physiology of the placenta in these species, which does not allow trans-placental transfer of maternal Ig to the foetus during gestation (Enders and Carter, 2004; Nechvatalova et al., 2011). Despite this, the neonate is still able to mount an immune response when exposed to antigens (Cortese, 2009). In species with lacteal transfer of Ig, the neonate relies upon the absorption of maternal Ig through ingestion of colostrum and milk (Wagstrom et al., 2000). However, as the circulations of the dam and foetus are isolated from each other, this provides immunological separation and protection for the dam and the neonate against immunological incompatibility, such as the dam mounting an immune response against the foetus (Enders and Carter, 2004). In species which have placental and lacteal transfer of antibodies (e.g. dogs, mice and rats), maternal antibodies are transferred to the foetus during gestation and additionally absorbed by the neonate through ingestion of colostrum and milk in the early post-partum period. (Marnila and Korhonen, 2011b).

The local immune system

The immune system of the mammary gland has two main functions: to protect the glands against local infection and to provide immune components to the neonate via colostrum and milk (Wagstrom et al., 2000).

Local protection

Due to the composition of nutrients found within mammary secretions which will support pathogen growth, the mammary gland is relatively susceptible to infection if pathogens are introduced into the gland (Stelwagen et al., 2009). The mammary gland has a number of physical and physiological adaptations which create a highly effective

barrier against pathogens (Stelwagen et al., 2009). There is a link between the immune systems of the mammary gland and the gastrointestinal system (Bourne, 1977; Wagstrom et al., 2000). The adaptive immune system is mediated by the T and B lymphocytes in response to previous antigenic stimulation of the mammary gland experienced in the past. Mammary plasma cells form due to the transferral of lymphocytes from the intestinal mucosal immune system. Exposure to antigens results in the activation of B lymphocytes found in the gut-associated lymphoid tissue (GALT), which includes Peyer's patches, lymphoid and myeloid cells (Hurley, 2003). Some of these lymphocytes are transferred to the mammary gland where they become mammary IgA-secreting plasma cells, providing a link between the intestinal and mammary immune systems (Hurley, 2003). The porcine mammary immune system is considered to also be a part of the mucosal immune system, which includes intestinal, respiratory, reproductive and mammary lymphoid tissues (Wagstrom et al., 2000). However, the mammary immune system of the ruminant may not be as closely linked to the mucosal immune system. Immunisation of the ewe intestinal tract has shown an enhanced response to antigen in the mammary gland, however, this was due to the release of IgA molecules from the mammary gland that were originally derived from GALT, and not due to the transfer of lymphocytes from GALT to the mammary gland during lactation (Sheldrake and Husband, 1985).

The mammary gland epithelium also plays an active role in the defence of the mammary gland as it synthesises a number of immune factors which are components of the innate immune system. These components include: lactoferrin, β -defensin and lipopolysaccharide-binding protein (Stelwagen et al., 2009). Other components of the innate immune system include: neutrophils, macrophages, complement system,

oligosaccharides and immunoglobulins (Stelwagen et al., 2009). Immunoglobulins are particularly important as they function as flexible adaptors that link various parts of the immune system together (Korhonen et al., 2000a).

Neonatal protection

Immune factors in colostrum and milk not only play an important role in the host defence of the mammary gland, but also provide the neonate with protection against certain pathogens to which the dam has been exposed. Local stimulation of the mammary gland via vaccination can stimulate mammary gland production of antibodies (Bourne, 1977; Lee and Lascelles, 1970). In a study by Newby and Bourne (1977) intra-mammary infusion with antigen in late pregnancy stimulated production of antigen specific-antibody by the mammary gland for at least the first 30 days of lactation. However, the proportion of Igs produced locally by the mammary gland in response to stimulation in cows and sows is different. Stimulation of the cow mammary gland can significantly increase antibody production; however, antibody production in the sow when stimulated under the same circumstances remains unchanged (Newby and Bourne, 1977). It should be noted that production of antibodies in the mammary gland may be affected by stage of lactation. A rapid increase in IgG concentration occurs in ruminants in the first week after drying off and last few weeks of gestation (Sordillo et al., 1997). After parturition, the concentration of Igs rapidly declines during the transition of colostrum to mature milk.

Immunoglobulins

Immunoglobulins are produced by B lymphocytes. Most of the Igs act as receptors on the surface of B lymphocytes, while other Igs float freely in body fluids (Marnila and Korhonen, 2011b). Dependent upon the species, each Ig can also consist of a number of subclasses e.g. cow IgG has two subclasses; IgG₁ and IgG₂. The basic molecular structure of all Igs is very similar. All Ig molecules consist of two light chains and two heavy chains which are bound together by a disulphide bond. The Ig molecule has two antigen-binding sites which comprise an antigen-binding fragment (Fab) (light chain) and a constant fragment (heavy chain). The constant fragment (Fc) is present at the opposite end of the Ig molecule to the Fab and genes encoding the Fc determines the identity of an Ig as a particular subclass (Hurley and Theil, 2011). It is the Fc region of the Ig molecule that binds to Fc receptors on mammary epithelial cells, enabling transport into the mammary gland (Hurley and Theil, 2011; Marnila and Korhonen, 2011b). Contact between B lymphocytes and antigen is required for the B lymphocytes to develop into antibody-forming and secreting plasma cells (Marnila and Korhonen, 2011b). In the ruminant, these cells mainly produce IgA, but some IgG and IgM can also be synthesised by these cell types in the mammary gland (Marnila and Korhonen, 2011b). The immunologic function of Igs depends upon the Ig class, and they can be both immunostimulatory and immunosuppressive (Bell, 2000). The most important function of an Ig is to protect the host by agglutinating bacteria to prevent adhesion to epithelial surfaces (Marnila and Korhonen, 2011b). They can also block enzymes and receptors which inhibits bacterial metabolism and reduces the ability of bacteria to adhere to surfaces (Marnila and Korhonen, 2011b). As Igs represent the specific immune response of the dam to antigens encountered in the past, normal colostrum and milk contain agglutinating Igs specific for a larger number of pathogenic and non-

pathogenic organisms (Marnila and Korhonen, 2011b). These Igs can also benefit the neonate by binding and inactivating toxins produced by pathogenic microbes and neutralising viruses (Marnila and Korhonen, 2011b). The concentration of Igs varies dependent upon species, breed, age, health status, and stage of lactation (Marnila and Korhonen, 2011b). Immunoglobulins in maternal serum are selectively transferred to the mammary gland and into colostrum prior to parturition, causing a marked decline in Ig concentration in maternal serum and a marked increase in Ig concentration in colostrum. This transfer results in significantly higher Ig concentrations in colostrum compared to serum. Concentrations of Igs in colostrum, when compared to serum, can be up to 5 (Beer et al., 1974) to ten times (Baumrucker et al., 2010) greater in cow colostrum, up to 2.5-3 times greater in sow colostrum (Curtis and Bourne, 1971), and up to 3 times greater in ewe colostrum (Campbell et al., 1977). Concentrations of total IgG, IgG₁, IgG₂, IgA and IgM in serum, milk and colostrum for cows, ewes and sows are presented in Table 1.1. It should also be noted that the rate at which the mammary gland is emptied influences the rate of decline of antibodies post parturition.

Table 1.1 Mean, range and/or S.E. Total IgG, IgG1, IgG2, IgA and IgM concentrations for colostrum, milk and serum for cows, ewes and sows. Concentrations measured in mg/ml.

	Colostrum			Milk			Serum		
	Cow	Ewe	Sow	Cow	Ewe	Sow	Cow	Ewe	Sow
Total IgG	15-180 ⁷ 112 (13-256) ²⁴	60 (52-64) ⁹ 98.8 ³ 101.2 ± 7.24 (50-164) ¹² 115 ± 10.1 ¹¹	5.87 ¹⁸ 53.67 ± 1.1 ¹⁰ 62.3 ± 20.1 ¹⁰ 95.6 ⁵	14.2 ⁵		10.2-20.5 ¹⁰ 11.83 ± 14.2 ⁵ 16.8 ± 9.7 ¹⁰		18.82 (11-30) ¹² 21 (18-24) ⁹ 21.3 ± 1.5 ¹¹ 32.7 ± 4.5 ¹³	24.33 ± 0.94 ¹ 28.2 ¹⁷
IgG1	11.2 ⁷ 34.96 ⁴ 47.60 ¹⁴ 64.9 ²⁴	21-126 ²⁰ 69 ± 1.0 ²¹		0.11-0.90 ⁸ 47.60 ¹⁴	2.88 ± 0.18 ²⁶		0.32-8.30 ⁸ 3.0 ⁶ 11.2 ⁷	18.02 ± 1.84 ²⁶ 18.9 ± 1.5 ¹⁵ 95.1 ¹⁹	
IgG2	2.2 ²⁴ 2.90 ¹⁴ 6 ⁴ 9.2 ⁷	0.15 ± 0.03 ²⁶		0.05 ²⁴ 0.27-1.7 ⁸ 2.90 ¹⁴	0.22 ± 0.03 ²⁶		0.15-17.0 ⁸ 0.4 ⁶ 9.2 ⁷	5.8 ± 0.8 ¹⁵ 6.74 ± 0.72 ²⁶	14 ²⁵ 14.08 ± 0.49 ¹
IgA	0.4 ⁷ 0.56-2.5 ⁸ 1.66 ⁴ 3.5 ²⁴ 3.90 ¹⁴	2 (0.9-3) ⁹ 6.24 ± 0.96 (1.53-17.28) ¹²	1.87 ¹⁸ 8.6-11.9 ²² 21.2 ⁵	0.13 ²⁴ 0.14 ¹⁴	2.29 ± 0.67 ¹⁶ 6.58 ± 1.29 ²⁶	6.3 ⁵	0.20-1.10 ⁸ 0.34 ²⁴ 0.4 ⁷	0.17 ± 0.02 ¹³ 0.25 (0.05-0.1) ⁹ 0.309 (0.08-0.84) ¹² 0.8 ± 0.3 ¹⁵	0.2 ± 0.02 ²⁷ 2.0 ¹⁷ 2.37 ± 0.20 ¹
IgM	1.4-5.1 ⁸ 3.1 ⁷ 3-5 ² 4.20 ¹⁴ 4.32 ⁴ 8.7 ²⁴	2.91±2.2 (0.82-4.45) ¹² 4.1 (1.8-5.6) ⁹	0.32 ¹⁸ 9.1 ⁵	0.04-0.1 ² 0.04 ²⁴ 0.05 ¹⁴	0.21 ± 0.02 ²⁶	2.7 ⁵	1.0-3.6 ⁸ 3.1 ⁷	1.20 (0.8-1.8) ⁹ 1.72 ± 0.14 ¹³ 1.9 ± 0.2 ¹⁵ 2.01 (0.95-4.7) ¹²	1.1 ± 0.07 ²⁷ 2.92 ± 0.18 ¹ 4.8 ¹⁷

1.Curtis and Bourne 1971; 2.Elfrstrand et al. 2002; 3.Halliday 1974; 4.Kehoe et al. 2007; 5.Klobasa et al. 1987; 6.Korhonen et al. 2000b; 7.Marnila and Korhonen 2011b; 8.Newby and Bourne 1977; 9.Pahud and Mach 1970; 10.Quesnel 2011; 11.Reneau et al. 1973; 12.Smith et al. 1975; 13.Smith et al. 1976; 14.Stelwagen et al. 2009; 15. Watson and Lascelles 1973; 16. Husband 1987; 17. Klobasa et al. 1985; 18. Porter 1969; 19. Halliday 1978; 20. McGuire et al. 1983; 21. Gilbert et al. 1988; 22.Loisel et al. 2013; 23. Jackson et al. 1995; 24. Conneely et al. 2013; 25.Bokhout et al. 1986; 26.Cripps et al. 1985; 27.Bianchi et al. 1995.

Immunoglobulin G (IgG)

In ruminants and swine, IgG has two subclasses: IgG₁ and IgG₂ (Bourne, 1977; Campbell et al., 1977; Korhonen et al., 2000b; Lee and Lascelles, 1970). The predominant Ig of ruminant and swine colostrum is IgG₁, which is derived from the maternal circulation in all species (Bourne, 1977; Campbell et al., 1977). However, there is some evidence to suggest that local synthesis of IgG can occur in the stimulated mammary gland (Lee and Lascelles, 1970). The predominant subclass of ruminant milk is IgG₁ (serum derived) but IgG₂, IgA and IgM are also present in smaller amounts (Newby and Bourne, 1977). The hormonal interactions of oestrogen, progesterone and prolactin cause the mammary epithelial cells to bind to the heavy chain of IgG₁; resulting in its selective transport from maternal circulation over other Ig classes (Wagstrom et al., 2000). Immunoglobulin G has a number of functions: activation of complement-mediated bacteriolytic reactions, opsonisation of bacteria, prevention of adhesion of bacteria to surfaces, inhibition of bacterial metabolism, agglutination of bacteria and neutralisation of toxins and viruses (Korhonen et al., 2000b). The concentration of IgG changes throughout lactation as colostrum transitions into milk. These changes in concentration are presented in Table 1.1.

In swine colostrum, IgG accounts for 80% of the total Ig content found in colostrum (Curtis and Bourne, 1971) and is the predominant Ig of colostrum (Wagstrom et al., 2000). In sow milk, IgG accounts for 20-30% of total Ig content (Curtis and Bourne, 1971). Where IgG₁ is almost entirely derived from maternal circulation for colostrum, this is not the case for milk. There is no local synthesis of IgG₁ for milk (Newby and Bourne, 1977). Immunoglobulin G₂ is derived in equal amounts from the maternal circulation and synthesised locally in the mammary gland. A 5-fold reduction in

colostral IgG concentration occurs in the first 24 hours of lactation and a 30-fold reduction in concentration during the first week of lactation (Bourne, 1977; Curtis and Bourne, 1971). A study by Curtis and Bourne (1971) suggest that this rapid decrease in IgG concentration is due to the equilibration of absorbed Ig between the intra- and extra-vascular fluids. Although preferential transport of IgG₁ has been shown to occur in both the cow and the ewe, Curtis and Bourne (1971) could not demonstrate this in the sow, as both IgG₁ and IgG₂ were present throughout lactation. A study by Klobasa et al. (1987) determined that sow parity, induction of farrowing, litter size and gestation length did not significantly influence colostral IgG concentrations. However, they did find that IgG concentrations were greater in older sows (Klobasa et al., 1987). IgG is the predominant Ig class found in cow colostrum and milk and is mostly derived from maternal circulation (Hurley and Theil, 2011). Immunoglobulin G₁ accounts for 50-80% of Igs found in both colostrum and milk (Marnila and Korhonen, 2011b). In a study by Elfstrand et al. (2002) IgG₁ concentrations remained at the same level during the first 10 hours post calving, reducing to 20% of the initial concentration after 2 days post calving.

Additionally, IgG₂ concentrations reduced by 30% during the first 10 hours post calving but remained at that level for the following 10 hours post calving (Elfstrand et al., 2002). The half-life of IgG in the cow is 1-3 weeks (Hurley and Theil, 2011) and in the pig is 12-14 days (Yokoyama et al., 1993). The half-life of cow IgG₁ and IgG₂ is approximately 10-22 days (Cervenak and Kacskoviks, 2009), however the half-life of IgG₂ is slightly longer compared to IgG₁ due to a stronger binding ability with the FcRn (Cervenak and Kacskoviks, 2009; Hurley and Theil, 2011). The lactational yield of IgG produced by cows in their first lactation is approximately half or less than cows in later

lactations. This is due to lower IgG concentrations and smaller colostrum volumes (Marnila and Korhonen, 2011b). Concentrations of IgG, IgG₁ and IgG₂ as found in previous studies for cows, ewes and sows is presented in Table 1.1.

Immunoglobulin A (IgA)

Monomeric IgA has a similar structure to IgG, except for the addition of a C-terminal octapeptide to the heavy chains (Korhonen et al., 2000b; Marnila and Korhonen, 2011b). Immunoglobulin A can occur as a monomer or a dimer. The dimer structure consists of two IgA molecules joined together by a J-chain and a secretory component (SC) (Korhonen et al., 2000b; Marnila and Korhonen, 2011b). The SC is a portion of a poly-Ig receptor (pIgR) where it complexes with a J-chain of dimeric IgA for transport to the luminal membranes where the receptor is enzymatically cleaved to become SC (Marnila and Korhonen, 2011b; Stelwagen et al., 2009). The SC protects the hinge region of secretory IgA from protease cleavage, therefore extending its survival time in mucosal environments (Bourne, 1977; Korhonen et al., 2000b; Stelwagen et al., 2009). The main function of IgA is to protect mucosal barriers, including those within the mammary gland (Curtis and Bourne, 1971). IgA does this by agglutinating antigens, neutralising toxins and preventing adhesion of bacteria to mucosal surfaces (Bell, 2000; Hurley and Theil, 2011; Korhonen et al., 2000b; Stelwagen et al., 2009). In ruminants, IgA is the dominant Ig of all secretions except lacteal secretions (Korhonen et al., 2000b) and there is evidence to suggest that IgA synthesis occurs in the mammary gland in the absence of stimulation by antigen (Bourne, 1977; Lee and Lascelles, 1970; Newby and Bourne, 1977). Secretory IgA is produced and secreted by plasma cells in the mammary tissue, which were formerly a part of GALT (Hurley and Theil, 2011). As mentioned previously, some lymphocytes from GALT are transferred to the mammary

gland, where they become mammary IgA-secreting plasma cells and provide a direct link between the intestinal and mammary immune systems (Bourne, 1977; Wagstrom et al., 2000).

Immunoglobulin A is the predominant Ig of sow milk (Wagstrom et al., 2000).

Immunoglobulin A plasma cells predominate in the mammary gland at all stages of lactation with 60% of colostral IgA produced by the mammary gland, although it is only a minor component of sow colostrum (Bourne, 1977). Although it can be synthesised locally in the mammary gland, serum IgA does make a significant contribution to IgA concentrations in sow colostrum (Newby and Bourne, 1977). This is also true of colostrum and milk of ruminants (Marnila and Korhonen, 2011b). The mammary gland of the sow can produce up to 30g IgA per day (Wagstrom et al., 2000). The concentration of IgA in sow colostrum is four times higher when compared to serum and during the first 24 hours post-partum, which is followed by a 3-fold drop in concentration (Curtis and Bourne, 1971). In a study by Curtis and Bourne (1971) concentrations of IgA in sow colostrum at parturition was 9.66 mg/ml, which declined to 3.04 mg/ml 8-35 days post parturition. A study by Newby and Bourne (1977) found concentrations of IgA in cows to be elevated during the first few days of lactation which they suggested may be due to the continued transfer of serum IgA to the udder during this time. In the cow, 9% of colostrum IgA and 10% of mature milk IgA is involved in the local immune response. This proportion is higher in the sow, with 50-60% of IgA involved in the local immune response (Newby and Bourne, 1977). After the first week of lactation, IgA accounts for 50-60% of the Igs found in sow milk (Curtis and Bourne, 1971). The half-life of IgA is 1-2 days (Hurley and Theil, 2011). Concentrations of IgA as found in previous studies for cows, ewes and sows is presented in Table 1.1.

Immunoglobulin M (IgM)

Monomeric IgM has the same basic molecular structure as IgA, and can be a monomer or a pentamer (Korhonen et al., 2000b; Marnila and Korhonen, 2011b). The pentamer structure consists of five monomers linked together in a circular mode by disulphide bonds and a J-chain (Korhonen et al., 2000b; Marnila and Korhonen, 2011b). Secretory IgM also occurs similarly to secretory IgA, where pIgR is enzymatically cleaved to become SC (Hurley and Theil, 2011). The size of pentameric IgM (900kDa) prevents significant transfer from the serum of this Ig class; therefore it is locally produced in the mammary gland (Lee and Lascelles, 1970; Newby and Bourne, 1977). Immunoglobulin M is the first response when a primary infection occurs (Bell, 2000; Hurley and Theil, 2011), and is specific to bacteria and viruses (Bell, 2000). Immunoglobulin M has a low specificity and lower potency to combat infection (Hurley and Theil, 2011). Although it is produced in lower quantities than IgG, it can be more efficient at complement fixation, opsonisation and agglutination of bacteria (Korhonen et al., 2000b; Marnila and Korhonen, 2011b).

Immunoglobulin M in cow colostrum is derived from serum, however local synthesis in the mammary gland can account for more than 50% of the total IgM found in colostrum (Newby and Bourne, 1977). Immunoglobulin M accounts for only a small proportion of the total Ig content of colostrum. Concentrations in sow colostrum are only slightly higher when compared to serum and during the first 24 hours of lactation this concentration is halved (Curtis and Bourne, 1971). In a study by Elfstrand et al. (2002) the concentration of IgM in cow colostrum was 3-5g/L and in milk was 0.04-0.1g/L. Their study also observed a reduction in IgM concentration of 50% between 11-20 hours post calving, which was reduced by a further 10% in the following 10 hours. By

31-80 hours post calving, the concentration of IgM had reached 17% of the initial concentration (Elfstrand et al., 2002). Concentrations of IgM as found in previous studies for cows, ewes and sows is presented in Table 1.1.

Secretions of the mammary gland

Colostrum

Colostrogenesis

The production of colostrum, termed ‘colostrogenesis’ is the production of a protein-rich secretion of the mammary gland which provides maternally derived Igs to the neonate for protection against pathogens and toxins in the first days and months of life. Colostrogenesis is regulated by lactogenic hormones. Lactogenesis I, which occurs prior to birth, begins by a decrease in progesterone concentration, which promotes an increase in prolactin concentrations. This increase in prolactin concentrations is essential for colostrogenesis (McGuirk and Collins, 2004). Transport of maternal Igs from the serum to the mammary gland occurs several weeks before birth, and in sows begins from day 90 of gestation (Quesnel et al., 2012). This process continues during late gestation but stops abruptly at birth. Uptake and transfer of Igs across the mammary epithelial barrier occurs via an Fc-receptor mediated process (Hurley and Theil, 2011; Marnila and Korhonen, 2011b). Mammary gland secretory epithelial cells contain Fc-specific receptors which can be found on the basolateral surfaces of the cell. Once bound to a mammary epithelial cell, Igs are transported intra-cellularly by endocytosis and transported to the apical end of the cell and then released into the alveolar lumen (Beer et al., 1974; Hurley and Theil, 2011; Marnila and Korhonen, 2011b).

The majority of Igs transported into colostrum and milk by this process are either derived from the maternal circulation or are produced in situ by intra-mammary plasma cells (Van de Perre, 2003). Colostrum excretion, or Lactogenesis II, begins shortly before the beginning of parturition and lasts for approximately 24 hours post parturition (Quesnel et al., 2012). The transition from colostrum to milk is characterised by two main events: a reduction in the permeability of the mammary epithelium to Igs due to the development of tight junctions between mammary epithelial cells, and an increase in the synthesis of lactose and excretion into the mammary alveoli (Quesnel, 2011). Following birth, low progesterone concentrations, an increase in serum corticoids and high prolactin concentrations act as a trigger to increase milk secretions and suppress colostrum production (Wagstrom et al., 2000). In most species, the cessation of colostrum secretion occurs between 12-48 hours after birth (Devillers et al., 2007; Quesnel, 2011; Quesnel et al., 2012).

Characteristics

Colostrum is characterised by high concentrations of Igs and lower concentrations of lactose and lipids (Marnila and Korhonen, 2011b) and a lower pH compared to mature milk (Hurley and Theil, 2011; Klimes et al., 1986). Colostrum dry matter is approximately 2 times higher than milk due to the concentrations of proteins, fats and minerals (Klimes et al., 1986; Marnila and Korhonen, 2011b). Colostrum contains many factors that influence cell growth and differentiation. Non-nutrient colostrum components stimulate jejunal and skeletal muscle development in the neonate, while non-peptide components (glutamine and polyamines) maintain gastrointestinal mass and modulate the neonatal immune system (Marnila and Korhonen, 2011b). The major growth factors found within colostrum are transforming growth factor (TGF) and

insulin-like growth factor (IGF) (Elfstrand et al., 2002). In cow colostrum, 85-95% of TGF is TGF- β 2. Although more than 90% of TGF- β 2 in colostrum is inactive, it can be activated by proteolytic enzymes or acidification (Elfstrand et al., 2002). In cow colostrum, the major form of IGF is free form IGF-1 which accounts for 73% of IGF. It is also bound to binding proteins (IGFBP) in all biological fluids, including milk, which can inactivate IGF-1 or enhance activity (Elfstrand et al., 2002; Marnila and Korhonen, 2011b).

Colostrum also contains immune components which reduce the risk of mammary gland infection (Klimes et al., 1986; Marnila and Korhonen, 2011b). These components include: defensins, Igs, lactoferrin, lysozyme and lactoperoxidase (Elfstrand et al., 2002; Marnila and Korhonen, 2011b; Nechvatalova et al., 2011) and carry non-specific antimicrobial and immune-enhancing properties (Marnila and Korhonen, 2011b). For example, lactoferrin is synthesised in the mammary gland and has a broad bacteriostatic and bactericidal spectrum, and can act synergistically with lysozyme to become antibacterial (Marnila and Korhonen, 2011b). Lactoperoxidase is an antimicrobial oxidoreductase enzyme with has a broad spectrum antimicrobial effect (Marnila and Korhonen, 2011b). Colostrum also contains large numbers of cells such as leukocytes including neutrophils and lymphocytes (Nechvatalova et al., 2011). These cells protect the mammary gland against pathogens and are important for the maturation of neonatal cellular immunity (Marnila and Korhonen, 2011b).

Immunoglobulin specific trans-cellular transport

Immunoglobulin G

The receptor responsible for the transport of IgG into colostrum is the FcRn receptor, or neonatal Fc receptor (Hurley and Theil, 2011; Quesnel, 2011; Stelwagen et al., 2009).

The FcRn receptor is a heterodimer composed of a membrane bound α -chain similar to MHC class I molecules and a smaller MHC class I protein (β 2-microglobulin) (Hurley and Theil, 2011). Binding of IgG to FcRn is pH dependent, with high affinity binding occurring at acidic pH and weak binding occurring at neutral pH (Hurley and Theil, 2011). Korhonen et al. (2000a) suggested that approximately 50-100g per day, or up to 500g per week, of IgG₁ may be transferred from the maternal circulation and into colostrum. Dairy cows transport the highest mass of IgG from serum to the mammary gland compared to other species, however the concentration of IgG in colostrum in dairy cattle is diluted due to the high volumes produced (Weaver et al., 2000). Hurley and Theil (2011) have suggested that the presence of specific high-affinity receptors for IgG₁ and IgG₂ on the mammary acinar epithelium corresponds to the concentrations of these subclasses in colostrum and milk at the time of maximal IgG transport.

Additionally, they have also suggested that the difference in concentrations between IgG₁ and IgG₂ in colostrum could be due to the recycling of IgG₂ back into the extracellular fluid instead of into the alveolar lumen (Hurley and Theil, 2011). In the cow, oestradiol-17 β stimulates the transfer of IgG from serum to colostrum (Devillers et al., 2007). In ruminants, prolactin inhibits expression of the FcRn receptor, which suppresses transfer of IgG into colostrum and simultaneously stimulates lactose synthesis and closure of tight junctions (Quesnel, 2011).

Immunoglobulins A and M

The receptor responsible for the transport of IgA and IgM into colostrum is the polymeric Ig receptor, or pIgR (Hurley and Theil, 2011). Expression of pIgR in the mammary gland is controlled by the same hormones that are responsible for the regulation of lactation (Hurley and Theil, 2011). The pIgR binds dimeric IgA and pentameric IgM in the mucosal tissues, but only IgA/IgM that contain a J-chain have a high affinity for pIgR (Hurley and Theil, 2011). The bound IgA/IgM is transported to the apical end of the mammary epithelial cell. This is where pIgR is cleaved and IgA/IgM is released into the alveolar lumen with a receptor fragment (secretory component, SC) bound to the Ig molecule (Hurley and Theil, 2011; Stelwagen et al., 2009).

Para-cellular transport

Para-cellular transport is the direct and bi-directional movement, between the alveolar lumen and interstitial space, of low-molecular-weight substances and macromolecular solutes between the epithelial cells (tight junctions) of the mammary gland (McManaman and Neville, 2003; Nguyen and Neville, 1998). Para-cellular transport occurs during gestation, involution, or periods of inflammation (i.e. mastitis) (McManaman and Neville, 2003; Nguyen and Neville, 1998) due to leaky tight junctions. It is not clear whether leakiness of the tight junctions is due to the state of the tight junctions, or due to dead/dying cells which leave holes in the epithelium (Nguyen and Neville, 1998). This pathway is closed during lactation due to the closure of tight junctions between epithelial cells (McManaman and Neville, 2003), which prevents inter-diffusion of milk with interstitial fluid (Nguyen and Neville, 1998). Closure of the tight junctions occurs around the time of parturition changes the permeability of the

paracellular transport pathway (Shennan and Peaker, 2000), which is thought to be due to glucocorticoids and prolactin receptor activation (Nguyen and Neville, 1998; Shennan and Peaker, 2000).

Milk

The change from colostrum to mature milk occurs in the first few days following parturition and continues at a reduced rate for 5-7 days (Marnila and Korhonen, 2011b). The composition of milk changes throughout lactation. For example, concentrations of fats, lactose, vitamins and minerals, and proteins are higher in early lactation compared with late lactation (Marnila and Korhonen, 2011a). The Ig concentration in milk declines rapidly following parturition from, on average, 60 mg/ml IgG in colostrum to 0.47 mg/ml IgG in milk (Marnila and Korhonen, 2011b). The Igs found in milk accounts for 1-2% of total protein (Devillers et al., 2007; Korhonen et al., 2000b).

Immune components

Milk contains similar immune components to colostrum (Hurley and Theil, 2011).

Immune components which can be found within milk include: lactoferrin (iron-binding antimicrobial protein), lactoperoxidase (antibacterial enzyme), lysozyme (antibacterial and lytic enzyme), oligosaccharides (analogues of microbial ligands on mucosal surfaces) defensins (antimicrobial heat stable peptides), soluble CD 14 (Hurley and Theil, 2011) and other cytokines. Milk Igs have been found to act synergistically with non-specific antimicrobial components of milk, such as lactoferrin and lysozyme (Marnila and Korhonen, 2011b). Milk also contains leukocytes; activated neutrophils, macrophages and lymphocytes (Hurley and Theil, 2011). The presence and

concentrations of immune components in milk vary depending upon the stage of lactation, but concentrations increase during late lactation (Stelwagen et al., 2009).

Whey

The milk protein system consists of two families of proteins: caseins (insoluble) and whey (soluble) (Madureira et al., 2007). After ingestion and passage into the stomach, the caseins within the milk clot to form a curd under the influence of an acidic environment and proteolytic activity of the enzyme chymosin (rennet). The casein curd separates from the milk, leaving behind a watery liquid called whey (Madureira et al., 2007). Casein is retained in the stomach of the neonate longer than whey proteins, including IgG, as it requires the acidity of the stomach to digest it completely (Hurley and Theil, 2011). In milk from dairy cows and sheep, caseins account for approximately 80% of the protein content of milk (Madureira et al., 2007; Park et al., 2007), approximately 50% of the milk solids (Smithers, 2008) and approximately 20% of the milk protein appear in the whey (Park et al., 2007; Smithers, 2008). The concentrations of whey proteins depends on a number of factors: the type of whey (acidic or sweet), source of milk (cow, sow, ewe), the time of year, the type of feed, stage of lactation and quality of processing (Smithers, 2008). Acid whey can be created by direct acidification of milk and has a pH equal to or less than 5.1. Sweet whey can be created by rennet coagulation of milk and has a pH equal to or greater than 5.6 (Smithers, 2008). It is possible that similar conditions could also affect the curd and whey production in the stomach of the neonate.

Clotting enzymes

The abomasum of the young mammal contains milk-clotting enzymes (chymosin and pepsin) which are designed to clot milk rapidly. Chymosin has sufficient proteolytic activity to coagulate milk, but it is too weak to extensively damage Igs (Andren, 2011). Clotting slows down the flow rate of milk into the small intestine, and delays the casein and fat in the abomasum until enough pancreatic juice is secreted to achieve optimal digestibility in the small intestine (Andren, 2011). Chymosin and pepsin are produced as inactive zymogens (prochymosin and pepsinogen) by the chief cells in glands of the fundic region of the abomasal mucosa. They are activated to chymosin and pepsin at low pH due to hydrochloric acid secreted by the parietal cells in the fundic glands (Andren, 2011). Chymosin is the dominant proteinase in the abomasum of calves from birth and the proportion of chymosin and pepsin in the abomasal mucosa of calves is dependent upon the feeding regime and age of the calf (Andren, 2011). If a calf is suckled or milk-fed, the milk-clotting activity of chymosin at a pH of 6.5 is approximately 90%. The amount of chymosin decreases with age and only trace amounts can be found in adult cattle (Andren, 2011). The optimal proteolytic pH of chymosin is approximately 3.8, but it also has a high specific milk-clotting activity at the pH of milk (pH 6.7). The optimal proteolytic pH of pepsin is 2 (Andren, 2011).

Whey proteins

Whey proteins are globular molecules which contain a high content of α -helix motifs (Hurley and Theil, 2011). Whey proteins include: β -lactoglobulin, α -lactalbumin, Igs, bovine serum albumin, lactoferrin and lactoperoxidase. The whey protein β -lactoglobulin is synthesised in the mammary gland and accounts for 58% of whey protein. It plays a role in passive transfer and regulation of phosphorus metabolism of

the mammary gland (Hurley and Theil, 2011). The whey protein α -lactalbumin accounts for 20% of whey protein and acts as a co-enzyme for the biosynthesis of lactose. It is synthesised in the mammary gland (Hurley and Theil, 2011). Lactoferrin is synthesised by glandular epithelial cells and neutrophils and provides antibacterial and anti-viral activity. Lactoperoxidase accounts for 1% of the protein content of whey and is an important part of the natural defence system of the host by providing protection against invading microorganisms (Hurley and Theil, 2011). The proportion of whey proteins in colostrum is substantially higher when compared to milk (Marnila and Korhonen, 2011b), and the concentration of total protein in colostrum whey from the first milking is 1.5-2 times higher than the concentration of the maternal serum (Klimes et al., 1986). In cow and sow colostrum, Igs account for the majority of the whey proteins (Klobasa et al., 1987; Marnila and Korhonen, 2011b). In a study by Klobasa et al. (1987) the concentrations of IgG, IgA and IgM in sow whey progressively declined post-partum, with concentrations of 95.6mg/ml, 21.2mg/ml and 9.1mg/ml, respectively, at 0 hours (colostrum) and 14.2mg/ml, 6.3mg/ml and 2.7mg/ml, respectively, at 24 hours post-partum. These concentrations declined even further by 42 days post-partum (end of sample collection), the study recording concentrations of 0.8mg/ml, 9.4mg/ml and 1.8mg/ml, respectively (Klobasa et al., 1987). In a study by Frenyo et al. (1986) 2.2 μ g of iodinated Igs was incubated with bovine milk, which was separated into fat, casein and whey fractions. The study showed that 520ng/ml IgG1 (90%), 470ng/ml IgG2 (86%), 440ng/ml sIgA (78%) and 437ng/ml IgM (69%) were associated with the whey fraction. The remaining Igs were associated with the fat and casein fractions (Frenyo et al., 1986).

Passive transfer

Neonatal protection

Due to the placental anatomy and physiology of ruminants and swine, the neonate requires transfer of passive immunity via colostrum in order to provide protection against infection (Beer et al., 1974). Colostrum ingested by the neonate is important for neonatal health as it can impact on the immunological development of the neonate, as well as provide passive immunity until the immune system of the neonate can mature (Bourne, 1977; Hurley and Theil, 2011; Marnila and Korhonen, 2011a). The immune system of the neonate is fully developed by parturition; however, it is immature in regards to exposure to pathogens (Cortese, 2009). Therefore, susceptibility to infection is not due to the inability of the neonate to mount an immune response, but rather due to the lack of exposure of its immune system to infection.

Dependent upon the species, the absorption of Igs across intestinal cells can be specific or non-specific. However, for species with lacteal passive transfer, absorption of Igs is selective (Beer et al., 1974; Stelwagen et al., 2009). The neonatal intestinal epithelium has the ability to allow unrestricted passage of large macromolecules (Igs) via epithelial cells of the small intestine and the action of pinocytosis (Campbell et al., 1977; Stelwagen et al., 2009; Wagstrom et al., 2000). The globules enlarge and coalesce to form a small number of large vacuoles which fill the apical region of the epithelial cell, pressing the nucleus to the base. The vacuoles switch position, moving basal to the nucleus (Campbell et al., 1977). Most macromolecules are degraded by digestive enzymes; however, some portions of macromolecules including proteins can be transported across the intestinal tract intact, (Hurley and Theil, 2011). Pepsin is a major

proteolytic enzyme produced by the stomach, and its digestion of IgG produces an F(ab)₂ fragment, which includes two antigen-binding sites (Fab) of the IgG molecule (Hurley and Theil, 2011). In the small intestine, trypsin preferentially digests cow IgG₁ over IgM and chymotrypsin preferentially hydrolyses IgM over IgG. Immunoglobulin G₁ is more susceptible to hydrolysis by pepsin than IgG₂, but IgG₂ is more sensitive to trypsin (Hurley and Theil, 2011). It should be noted however, that a study by Foltmann et al. (1980) determined that up to five days of age, milk clotting activity in the stomach of piglets was almost entirely derived from chymosin.

Immunoglobulins are transported across the cell and into the lymphatic system by exocytosis, where the Igs gain access to the circulatory system via the thoracic duct (Campbell et al., 1977; Marnila and Korhonen, 2011b; Wagstrom et al., 2000).

Immunoglobulin transfer is optimal in the first four hours post parturition (Wagstrom et al., 2000). Additionally, the vacuole containing cells become increasingly restricted to the apical region of the villi after parturition and the mitotic rate of the crypts of Lieberkuhn increases, which results in an increased rate of cell movement up the villi (Campbell et al., 1977). The intestine loses the ability to absorb Igs from 12, 36 to 48 hours post parturition dependent upon the species (Campbell et al., 1977; Marnila and Korhonen, 2011b; Stelwagen et al., 2009; Wagstrom et al., 2000) and corresponds with the highest IgG concentration colostrum (Marnila and Korhonen, 2011b). Campbell et al. (1977) and Wagstrom et al. (2000) hypothesised that the replacement of intestinal cells after birth by mature epithelial cells which are incapable of absorption is the mechanism by which gut closure occurs.

IgG specific transport

The Fc γ receptors on enterocytes of the small intestine mediate the selective binding and transcytosis of IgG until weaning (Marnila and Korhonen, 2011b; Van de Perre, 2003). The Fc γ receptor preferentially binds and transports IgG₁, as well as IgG_{2a} and IgG_{2b}, which depress endogenous production of IgG via a negative feedback system (Van de Perre, 2003). Transport of IgG across the enterocyte is bi-directional and is involved in immune surveillance and protection of the mucosal lining against pathogens (Hurley and Theil, 2011). After closure, uptake of IgG can occur via the FcRn receptor. Intestinal FcRn deliver IgG-antigen immune complexes to the lamina propria for immune processing which enhances the local mucosal immune response (Hurley and Theil, 2011). The IgG Fc binding protein blocks the passage of IgG-antigen complexes to the enterocyte surface, blocking uptake and transport to the lamina propria. This allows complexes to be degraded in the intestinal lumen and excreted (Hurley and Theil, 2011).

Monitoring of disease

Since the Igs found in colostrum are derived from the dam and are specific to those antigens experienced by the dam in the past, it is possible that the testing of colostrum for disease-specific antibodies could be an additional method for the monitoring of disease exposure within livestock populations. Additionally, neonate serum could be tested for disease-specific antibodies acquired through passive transfer. In a study conducted by Bandrick et al. (2008), a *Mycoplasma hyopneumoniae* (Mhyo) vaccine model was used to demonstrate whether specific colostrum-derived cellular immunity occurred in piglets. In piglets that did not ingest colostrum or were derived from non-vaccinated sows, no antigen-specific antibodies were detected. However, piglets

derived from vaccinated sows had high serum IgG and IgA levels, as well as significantly greater Mhyo-specific responses when challenged with Mhyo-specific antigen (Bandrick et al., 2008). The study also demonstrated significantly more Mhyo-specific proliferation of lymphocytes in sow colostrum collected from vaccinated sows compared to non-vaccinated sows (Bandrick et al., 2008).

Measures of diagnostic utility

There are many different measures of diagnostic utility that can be used to assess the effectiveness of tests used for the diagnosis of disease. Ultimately, these measures of diagnostic utility are used to determine how well a test can discriminate between individuals that are either diseased or non-diseased. The prevalence of the targeted disease and the prevalence of other diseases within a population are important considerations in measures of the diagnostic utility of a test.

Sensitivity and specificity

There are two ways in which sensitivity and specificity can be described: analytical and diagnostic. Analytical sensitivity refers to the smallest amount of a substance in a sample that can be accurately measured by an assay. Analytical specificity refers to the ability of an assay to measure one particular organism or substance in a sample, and not another (Crowther, 2009; Saah and Hoover, 1997). Diagnostic sensitivity is the percentage of animals with a disease that are identified as positive by the assay. Diagnostic specificity is the percentage of animals without a disease that are correctly identified by the assay as negative (Crowther, 2009; Saah and Hoover, 1997). It is diagnostic sensitivity and specificity which are the focus of this discussion. It should be

noted that the specificity should be derived from a population that is free from the disease of interest, but which contains the range and prevalence of other diseases which are in the target population for which the test is intended (Smith, 2006).

A perfect diagnostic test has the ability to identify categorically, animals with and without disease. However, such a test is rare, therefore in most tests, animals with values above a cut-off threshold do not always indicate disease, as animals without the disease may also have high values (false positives) (Simundic, 2008). Additionally, although the majority of animals which have values below a cut-off threshold are disease-free, some may also have the disease (false negatives) (Simundic, 2008).

Therefore, the use of a cut-off threshold divides the population into four subgroups:

- i. True Positives (TP): animals with the disease with a value above the cut-off threshold
- ii. False Positives (FP): animals without the disease with a value above the cut-off threshold.
- iii. True Negatives (TN): animals without the disease with a value below the cut-off threshold.
- iv. False Negatives (FN): animals with the disease with a value below the cut-off threshold.

Table 1.2 2x2 contingency table comparing proportion of animals classified as positive (true positive and false positive) or negative (true negative and false negative) determined by a reference (gold standard) test and alternative test.

Alternative test	Reference (gold standard) test	
	Positive	Negative
Positive	362 (TP)	30 (FP)
Negative	96 (FN)	512 (TN)

Using the example in Table 1.2, the number of true positives and true negatives in a hypothetical population is 362 and 512, respectively, and the number of false positives and false negatives is 30 and 96, respectively. Therefore, the diagnostic sensitivity and specificity of the alternative test, relative to the reference test, is 79% and 94%, respectively.

It is often quoted in the literature that the values of sensitivity and specificity are independent of disease prevalence, and can therefore be used for any population. This statement is subject to the condition that the prevalence and clinical stages of other diseases within the sample populations used to calculate the specificity are identical to the population on which the test is being used. If this condition is not satisfied, the value of the specificity may not be accurate for the target population. This condition is rarely met or considered in practice. Both diagnostic sensitivity and specificity can be influenced by the severity of the disease within a population. Additionally, the sensitivity and specificity can be determined for each stage of clinical infection, and would be more precise with an increase in clinical severity (Eusebi, 2013; Simundic, 2008).

Likelihood ratio

The Likelihood ratio is a useful measure of diagnostic utility and is independent of the prevalence of the disease of interest in the target population. It is defined as the ratio of the probability of an expected test result in animals with the disease, to the probability in animals without the disease (Deeks and Altman, 2004; Eusebi, 2013; Simundic, 2008). Likelihood ratios are divided into likelihood ratio of a positive test result (LR+) and likelihood ratio of a negative test result (LR-). The likelihood ratio indicates the change in odds for a given result. This can be used to generate the post-test probability of disease from the pre-test probability of disease.

The LR+ indicates how many times more likely it is that a positive test result will be observed in animals with the disease, than in those without the disease (Eusebi, 2013; Simundic, 2008). The higher the LR+ value is above 1, the stronger the evidence is for the presence of disease. If the LR+ value is equal to 1, the test does not provide any additional information to differentiate between animals with the disease and animals without the disease (Deeks and Altman, 2004; Eusebi, 2013; Simundic, 2008). The LR+ is calculated using the following equation: $\text{Sensitivity}/(1-\text{Specificity})$.

The LR- tells us how many times less likely it is that a negative test result will occur in animals with the disease, than in those without the disease (Deeks and Altman, 2004; Eusebi, 2013; Simundic, 2008). The LR- value is usually less than 1, as it is less likely that a negative test result will occur in animals with the disease, and the lower the LR- value is below 1, the stronger the evidence for the absence of disease (Deeks and

Altman, 2004; Eusebi, 2013; Simundic, 2008). The LR- is calculated using the following equation: $(1 - \text{Sensitivity}) / \text{Specificity}$. Using the example in Table 1.2, the LR+ value is 13.2 and the LR- value is 0.22.

Good diagnostic tests have a LR+ more than 10 and LR- less than 0.1, however if LR+ and LR- are equal, then the test is of no diagnostic value (Deeks and Altman, 2004; Simundic, 2008). As LR+ and LR- are calculated using diagnostic sensitivity and specificity, these measures are independent of the prevalence of the disease of interest, except where the prevalence of other diseases within the populations used to calculate specificity are different. Additionally, the LR from one study can be used in another, as long as the definition of disease (specificity) remains unchanged (Simundic, 2008).

Diagnostic odds ratio

The diagnostic odds ratio (DOR) is used for the general estimation of the discriminative power of diagnostic tests, as well as the comparison of diagnostic accuracies between two or more diagnostic tests (Eusebi, 2013; Simundic, 2008). The DOR is defined as the ratio of the odds of positivity in animals with the disease, to the odds in animals without the disease (Eusebi, 2013; Glas et al., 2003; Simundic, 2008). The DOR can be calculated using the following equation: $\text{LR+} / \text{LR-}$. The value of the DOR may range from 0 to infinity, with higher values indicating better test discrimination (Glas et al., 2003). A DOR value of 1 indicates the test cannot discriminate between animals with the disease and animals without the disease, while DOR values less than 1 indicate improper test interpretation e.g. false negatives and false positives (Glas et al., 2003). Using the example in Table 1.2, the DOR is 60.

The DOR is highly dependent upon the sensitivity and specificity, as a test with high sensitivity and specificity, with low rates of false positives and false negatives will have a high DOR (Eusebi, 2013; Glas et al., 2003; Simundic, 2008). Also, as the DOR uses LR, which is derived from the diagnostic sensitivity and specificity, this measure is also not dependent upon disease prevalence, except where the prevalence of other diseases within the populations used to calculate specificity are different, therefore the DOR does depend upon the definition used to define disease (specificity) (Eusebi, 2013; Glas et al., 2003; Simundic, 2008).

Predictive values

Knowing the probability of an animal having or not having a disease given a test result is important in decision making. The predictive values are the parameters that indicate the probabilities.

Predictive values can be represented by positive predictive values (PPV) and negative predictive values (NPV). The PPV is defined by the equation: $\text{True Positive} / (\text{True Positive} + \text{False Positive})$ (Eusebi, 2013; Simundic, 2008). The PPV indicates the proportion of animals that test positive that have the disease. The NPV is defined by the equation: $\text{True Negative} / (\text{True Negative} + \text{False Negative})$ (Eusebi, 2013; Simundic, 2008). The NPV indicates the proportion of animals that test negative that do not have the disease. A PPV equal to 1 indicates that all positive animals have the disease of interest (100% specificity and no false positives). A NPV equal to 1 indicates that all negative animals do not have the disease of interest (100% sensitivity and no false negatives). Using the example in Table 1.2, the PPV is 0.92 and the NPV is 0.84.

The PPV and NPV can also be calculated from the sensitivity and specificity using the following equations:

$$\text{PPV} = \frac{\text{sensitivity} \times \text{prevalence}}{\text{sensitivity} \times \text{prevalence} + (1 - \text{specificity}) \times (1 - \text{prevalence})}$$
$$\text{NPV} = \frac{\text{specificity} \times (1 - \text{prevalence})}{(1 - \text{sensitivity}) \times \text{prevalence} + \text{specificity} \times (1 - \text{prevalence})}$$

The PPV and the NPV determine the probability of being diseased (PPV) or not diseased (NPV) for an animal with a positive or negative test result. If the sensitivity and the specificity are both 100%, then the PPV and NPV are independent of disease prevalence and can be transferred to other studies. If, however, the sensitivity or specificity is below 100%, the PPV and NPV can be influenced by disease prevalence and should not be transferred to another study. The PPV increases and the NPV decreases with an increase in disease prevalence (Eusebi, 2013; Simundic, 2008). It should be noted that the changes in PPV is more substantial when compared to NPV (Simundic, 2008).

Receiver Operating Characteristic (ROC) curve and Area Under the Curve (AUC)

There is a pair of sensitivity and specificity values for every individual cut-off threshold, which are also inversely related dependent upon the cut-off threshold used (Greiner et al., 2000). Due to this inverse relationship, selection of a cut-off threshold is based upon the particular parameter that is attempting to be optimised. For example, due to the zoonotic ability of Bovine Spongiform Encephalopathy, prevention of false negative results is important to prevent infected meat entering the food chain. This is

achieved by selecting a cut-off threshold which optimises a high sensitivity (true positives) and low specificity (true negatives). In comparison, tests used to detect Johne's disease have selected a cut-off threshold which optimises a high specificity and low sensitivity. These tests ensure that animals without Johne's disease are not slaughtered unnecessarily (high specificity). However, the low sensitivity will mean that some false negative animals are retained in the herd, thus perpetuating the disease.

Additionally, the performance of a test can be assessed by calculating the sensitivity and specificity at multiple cut-off thresholds (Greiner et al., 2000). To construct a Receiver Operating Characteristic (ROC) curve, pairs of values are plotted on a graph, with the x-axis representing 1-Specificity and the y-axis representing Sensitivity (Eusebi, 2013; Greiner et al., 2000; Simundic, 2008). The closer the curve is to the upper left hand corner of the graph, the better the test is at discriminating against animals with the disease and animals without the disease (Eusebi, 2013; Simundic, 2008).

The area under the curve (AUC) also provides an index of the diagnostic accuracy i.e. $\frac{TP + TN}{All\ animals}$. The AUC can have any value between 0 and 1. A perfect diagnostic test has an AUC of 1, while a test that cannot discriminate between animals with the disease and animals without the disease, has an AUC of 0.5 (Eusebi, 2013; Greiner et al., 2000; Simundic, 2008). A higher AUC indicates a higher sensitivity and specificity for all available cut-off thresholds (Eusebi, 2013; Simundic, 2008). Arbitrary guidelines for AUC are as follows:

Table 1.3 Guidelines for area under the curve (AUC) to determine test utility Adapted from Greiner et al. (2000).

AUC	Test utility
AUC=0.5	Not informative
0.5<AUC<0.7	Less accurate
0.7<AUC<0.9	Moderately accurate
0.9<AUC<1	Highly accurate
AUC=1	Perfect

The AUC provides a summative index of diagnostic utility, but does not define the individual parameters, such as sensitivity, specificity and predictive values, or the contribution of the test to ruling-in and ruling-out disease. However, the comparison of two AUC values for two separate ROC curves can determine which test is more suitable for distinguishing between animals with disease and animals without disease (Greiner et al., 2000; Simundic, 2008). However, a study by Wald and Bestwick (2014) determined that the AUC was an unreliable measure of the performance of diagnostic tests, as they found that a larger AUC did not necessarily indicate better test performance. However, this study was not trying to optimise a test for the detection of a particular disease, or to optimise either sensitivity or specificity, therefore the AUC may in fact be a useful indicator of diagnostic utility when a particular cut-off threshold is selected in order to optimise either sensitivity or specificity.

Youden's index

The Youden's index provides a measure of the overall discriminative power of a diagnostic test, enabling a comparison to be made with other tests. It can be calculated using the following equation: sensitivity + specificity – 1 (Simundic, 2008). Tests that have poor diagnostic utility have a Youden's index = 0, while tests that have a perfect diagnostic utility have a Youden's index = 1 (Simundic, 2008; Youden, 1950). Using the example in Table 1.2, the Youden's index is 0.68. The main disadvantage of the Youden's index is that it is not receptive to differences in test sensitivity and specificity (Simundic, 2008). For example, two tests that have differing sensitivities and specificities can have the same Youden's index. If an assessment of diagnostic effectiveness of two tests with differing sensitivities and specificities was based only upon the Youden's index, it would be inaccurately concluded that the two tests are comparable and equally effective. The Youden's index is not affected by disease prevalence, except where the prevalence of other diseases within the populations used to calculate specificity are different. Therefore, the Youden's index can be affected by the spectrum of disease within a population (Simundic, 2008).

Diagnostic accuracy

Diagnostic accuracy estimates the 'effectiveness' of a test and is defined as the proportion of correctly classified animals (true positive + true negative) among all the animals in the population (true positive + true negative + false positive + false negative) (Eusebi, 2013; Simundic, 2008). Using the example in Table 1.2, the diagnostic accuracy is 0.87. The diagnostic accuracy is affected by the prevalence of the disease of interest and is unlikely to be transferrable between populations.

Table 1.4 Measures of diagnostic utility used to assess the effectiveness of the alternative test compared to the reference test in the example used in Table 1.2. Measures of diagnostic utility used include diagnostic sensitivity (DSe) and specificity (DSp), positive predictive value (PPV) and negative predictive value (NPV), likelihood ratio positive (LR+) and likelihood ratio negative (LR-), diagnostic odds ratio (DOR), Youden's index and diagnostic accuracy.

DSe	DSp	LR+	LR-	DOR	PPV	NPV	Youden's index	Diagnostic accuracy
79%	94%	13.2	0.22	60	0.92	0.84	0.68	0.87

As previously discussed, there are many measures of diagnostic utility available for the assessment of diagnostic tests. Using the example in Table 1.2, a hypothetical test was assessed using the measures of diagnostic utility discussed previously. Table 1.4 demonstrates that this hypothetical test has quite a high specificity, while the sensitivity is a lot lower. The LR indicates that for animals that test positive, they are 13.2 times more likely to have the disease of interest, while for animals that test negative, these animals are 0.22 times less likely to have the disease of interest. The DOR, Youden's index and diagnostic accuracy indicate that the test can discriminate well between animals with and without the disease.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA is a highly versatile and flexible testing system for the diagnosis of disease. In general, the procedure of ELISA involves the binding of specific antibody to antigen on a solid phase (generally a 96-well microtitre plate). After washing, an enzyme-conjugate is added, which binds to the antibody, resulting in a colour reaction. This

colour reaction directly relates to the amount of antibody within the initial sample and is quantified using a spectrophotometer. The ELISA is highly suited to disease surveillance and large-scale testing, as it can test a large number of samples at one time, is relatively inexpensive, easy to perform, and rapid (Crowther, 2009).

Types of ELISA

Direct

The Direct ELISA is considered to be one of the simplest types of ELISA, where antigen is attached to the solid phase by passive adsorption (Crowther, 2009).

Antigen is added to a solid phase, and following incubation, the antigen adsorbs to the solid phase (“coating”) and remains bound after a washing step. Antigen-specific antibodies labelled with enzyme conjugate are then added to the solid phase, directed specifically against the antigenic sites on the solid phase. Antibody in the sample binds to the antigen, and any unbound conjugate is then washed away. A substrate solution is added to the solid phase, causing a colour reaction to occur. This reaction is terminated by the addition of a stop solution and the amount of colour that has developed is quantified using a spectrophotometer (Crowther, 2009). Although the simplest assay, the Direct ELISA has severe limitations as it is limited to single antibody preparations. However, the assay is still important as it is the basis for competition and inhibition assays.

Indirect

The Indirect ELISA is very similar to the Direct ELISA, as the antigen coating step is the same for both assays. The Indirect ELISA is different to the Direct ELISA as antibodies from a specific species react with antigen attached to the solid phase (Crowther, 2009).

Following the initial adsorption of antigen to the solid phase, and after washing, antibodies in the sample are incubated with the solid phase. Antibodies that are specific for the antigen will bind, while all non-specific antibodies will be washed away in the next step. Antibodies labelled with an antibody-specific enzyme conjugate are then added to the solid phase, which binds to the antibodies already attached to the antigen. Following a period of incubation, excess conjugate is washed away. A substrate solution is added to the solid phase, causing a colour reaction to occur. This reaction is terminated by the addition of a stop solution and the amount of colour that has developed is quantified using a spectrophotometer (Crowther, 2009). The advantage of the Indirect ELISA is that any number of anti-sera can be examined for binding to a given antigen, and is therefore utilised greatly for large-scale screening. However, the assay does experience varying degrees of non-specific binding, which increases result variability, thus more samples need to be tested (duplicates).

Sandwich

The Sandwich ELISA can be divided into two systems; direct and indirect. They can also be described as capture ELISAs.

Direct Sandwich ELISA

This assay involves the passive attachment of antibodies to the solid phase by incubation in a buffer solution. Any unbound antibodies are then washed away. Antigen is then added to the solid phase and becomes bound by coating the antibodies during an incubation process. Antigen-specific antibodies labelled with enzyme conjugate are then added to the solid phase, but they do not have to be species specific. The conjugated antibodies bind to the antigen and excess conjugate is washed away. A substrate solution is added to the solid phase, causing a colour reaction to occur. This reaction is terminated by the addition of a stop solution and the amount of colour that has developed is quantified using a spectrophotometer (Crowther, 2009). The Direct Sandwich ELISA is limited to the specificities of the antigen-specific antibodies being used, which limits the versatility of the assay. Additionally, antigens must have at least two antigenic sites as both the capture antibodies and antigen-specific antibodies both need to bind.

Indirect Sandwich ELISA

This assay is similar to the Direct Sandwich ELISA, where antibodies are passively attached to the solid phase and antigen is bound by coating antibodies during the incubation process. A second antibody (not the same as previously bound antibodies) binds to the antigen, and unbound antibodies are washed away. Anti-species conjugate is then added, which specifically binds to the second antibodies added to the solid phase. It should be noted that the anti-species conjugate should also not react with the initially bound antibodies. Following incubation, excess conjugate is washed away. A substrate solution is added to the solid phase, causing a colour reaction to occur. This

reaction is terminated by the addition of a stop solution and the amount of colour that has developed is quantified using a spectrophotometer (Crowther, 2009). The advantage of the Indirect Sandwich ELISA is the ability to add a number of different sources of antibodies (samples) to the captured antigen, but only as long as the species of the antibodies being added is not the same as the capture antibody.

Competition and inhibition assays

Competition and inhibition assays involve the quantification of a substance by its ability to interfere with an established system, which includes the previously described ELISA assays, and can also be referred to as a blocking ELISA. These assays can also either be used for the measurement of antibody or antigen (Crowther, 2009). The advantage of competition and inhibition assays is that they rely upon antigen-capture, therefore the assays can be readily adapted to measure either antibody or antigen.

Disease-specific ELISAs

Many ELISA systems have been developed, and are available commercially, for the detection of disease-specific antibodies or antigen. The following discussion briefly describes some important diseases of livestock and the assessment of the diagnostic utility, in particular sensitivity and specificity, of their disease-specific ELISAs by previously published studies. A gold standard (reference) test refers to the results of a particular method which indisputably classifies animals as infected and not infected (Crowther, 2009). Most reference tests represent the current best method, with the highest sensitivity and specificity, but most are imperfect. The current reference tests for the following diseases are bacterial faecal culture (Johne's disease) (Gwozdz, 2010), virus isolation or real-time polymerase chain reaction (Bovine Viral Diarrhoea Virus)

(Lanyon et al., 2014a), lung tissue culture during late stage infection (Enzootic Pneumonia) (Sibila et al., 2009), and pulsed-field gel electrophoresis (Erysipelas) (Wang et al., 2010), however these methods are more time-consuming and costly compared to ELISA.

Johne's disease (Mycobacterium avium subspecies paratuberculosis)

Johne's disease is an enteric and chronic wasting disease of ruminants caused by *Mycobacterium avium subspecies paratuberculosis* (MAP). There are two main strains of MAP; an S (sheep) strain and a C (cattle) strain. Cross-species transmission of the S strain and the C strain has been demonstrated experimentally and under natural conditions (Greig, 2000; Whittington et al., 2001). The disease is characterised by a long subclinical incubation period, and infected animals often shed low numbers of mycobacteria in their secretions (colostrum and milk) and excretions (faeces), spreading infection to susceptible animals (Bakker et al., 2000; Lugton, 2004). There are species differences in the age susceptibility to infection. Young animals, usually less than 30 days old, are the most susceptible to infection (Bakker et al., 2000; Nielsen et al., 2008; Stewart et al., 2007). Cattle over 12 months of age are considered to be fairly resistant to infection (Windsor and Whittington, 2010), whereas adult sheep remain susceptible (McGregor et al., 2012). In cattle, clinical signs can develop over a period of weeks or months, and are usually observed in animals between 3-5 years of age (Gwozdz, 2010). The onset of clinical signs is sometimes associated with a stressful event, such as calving. Once chronic enteritis is established, observed clinical signs can include decreased milk production, progressive weight loss, diarrhoea, and death. (Gwozdz, 2010). It is also possible that there may be links to Crohn's disease in humans. A study in 1984 isolated MAP from the tissues of 11 patients with Crohn's disease (Chiodini,

1989), while MAP has also been isolated from the milk of lactating mothers (Burrells et al., 1998). The disease is common and occurs worldwide. In Australia, Johne's disease was first reported in cattle in 1925 and in grazed sheep in 1980. The prevalence of Johne's disease in Victorian dairy cattle populations is between 17% and 41% of herds (Lombard et al., 2006), while sheep flocks in the central and southern tablelands of New South Wales have a prevalence of approximately 60% (Lugton, 2004). The number of infected sheep within a flock varies between 1% and 15% (Gwozdz, 2010).

Historically, the early ELISAs for the detection of Johne's disease suffered from poor specificity. Studies by Yokomizo et al. (1985) and Milner et al. (1990) developed an indirect ELISA system with an additional absorption step using *Mycobacteria phlei*, which reduced non-specific binding and cross-reactions, thereby increasing specificity. Since then, a number of studies have utilised both serum and milk to assess ELISA sensitivity, as sensitivity is influenced by animal age, stage of infection and level of MAP infection (Gwozdz, 2010). The ELISA is currently the most sensitive serological method for the detection of Johne's disease, detecting approximately 30-40% of cows identified as infected by faecal culture (Gwozdz, 2010). In a study by Hope et al. (2000) ELISA sensitivity ranged between 35% and 54% when using serum by pre-selecting sheep with low body condition scores. Additionally, studies in dairy cattle have demonstrated ELISA sensitivities of 21.2% and 23.5% when using serum and milk, respectively (Lombard et al., 2006). However, the detection of animals that are in the subclinical incubation phase is often difficult, as these animals shed low numbers of mycobacteria, and often demonstrate a weak antigenic stimulation of the humoral response. A study by Reichel et al. (1999) detected 8.9% of low MAP shedding cows using a commercial serum ELISA, which increased to 75% for high MAP shedding

cows. In comparison, a study by Hendrick et al. (2005) detected 51% and 62% of low shedding cows using a serum and milk ELISA, respectively, which increased to 84% and 95% for serum and milk for high shedding cows.

Bovine viral diarrhoea virus (BVDV)

Bovine viral diarrhoea virus (BVDV) is a pestivirus within the family Flaviviridae and is considered one of the most economical important infectious diseases of cattle worldwide (Houe, 2003; Lanyon et al., 2012). There are two genotypes of BVDV: BVDV-1 and BVDV-2, of which there are approximately 12 (a-l) BVDV-1 sub-genotypes and 2 (a,b) BVDV-2 sub-genotypes (Ridpath et al., 2010). However, the vast majority of infections which occur in Australia are caused by BVDV-1c (Mahony et al., 2005; Ridpath et al., 2010). The disease is characterised by three infective states: acute infection, foetal infection and persistent infection. Acute infection, although mostly subclinical, can include some clinical signs including fever, diarrhoea, inappetence, and thrombocytopenia (Brownlie et al., 1998; Campbell, 2004; Nettleton and Entrican, 1995). Foetal infection occurs when a susceptible, pregnant dam is infected, causing mild or subclinical infection. Infection of the foetus can result in reproductive failures which can include mummification, abortion, stillbirth, and congenital deformities (McGowan and Kirkland, 1995; Nettleton and Entrican, 1995; Traven et al., 1991). Calves born with persistent infection (foetal infection between 40-120 days gestation) are born virus-positive and antibody-negative (Nettleton and Entrican, 1995). Although these calves can suffer from growth retardation, they can be born clinically normal (Houe, 2003). Persistently infected (PI) calves are the main reservoir of infection in susceptible cattle populations, and can account for 1-2% of the population (Lindberg and Alenius, 1999). Approximately, 76% of Australian beef herds have shown some

evidence of infection with BVDV (Taylor, 2010), while 97% of dairy and 85% of beef herds in South Australia have shown some evidence of infection with BVDV (Lanyon et al., 2012).

Some of the first antibody ELISAs for the detection of BVDV-antibody were unreliable due to high background readings or difficulties experienced in attaching the appropriate antigen to the solid phase (Lanyon et al., 2014a). Current antibody ELISAs possess high diagnostic sensitivity and specificity, which may vary dependent upon the sample being tested and the reference (gold standard) test to which it is being compared. Using serum, the sensitivity of the antibody ELISA can range from 96.7% to 97.6%, and specificity may range from 97.1% to 97.8% (Beaudeau et al., 2001; Durham and Hassard, 1990; Lanyon et al., 2013). Using individual milk, the sensitivity of the ELISA may range from 96.6% and 100%, and specificity between 89.2% and 94% (Lanyon et al., 2014b; Weir et al., 2013). Using bulk-tank milk, the sensitivity of the ELISA has been reported to range from 64% to 81%, and specificity between 64% and 91% (Lanyon et al., 2014b; Thobokwe et al., 2004).

Antigen-ELISAs also exist for the detection of BVD virus; these can be used to detect antigen in a range of sample types, including serum, milk and ear notches. Current antigen ELISAs also demonstrate high sensitivity and specificity. Studies by Sandivk (2005) and Saliki et al. (2006) demonstrated ELISA diagnostic sensitivity and specificity, relative to virus isolation, of 95% and 98.8%, and 93.6% and 100%, respectively.

Enzootic Pneumonia (Mycoplasma hyopneumoniae)

Enzootic pneumonia is a chronic respiratory disease of swine caused by *Mycoplasma hyopneumoniae*. The disease causes high morbidity and low mortality, and is an economically important disease for the commercial pig industry worldwide (Djordjevic et al., 1994; Frey et al., 1994; Maes et al., 2008; Sibila et al., 2009). Infection occurs via direct contact with the respiratory secretions of carrier animals. The disease is characterised by a chronic, non-productive cough, which appears within 10-16 days following infection, and ceasing 6-8 weeks after initial infection (Maes et al., 2008). Other clinical signs can include macroscopic lesions, reduced weight gain and reduced feed conversion efficiency (Maes et al., 2008). If the disease is compromised by a secondary infection, clinical signs can also include laboured breathing, pyrexia and death (Sibila et al., 2009). Due to a low antigenic challenge presented by the infecting mycoplasma, the antibody response of the infected animal is often low and difficult to detect (Howard and Taylor, 1985).

The ELISA is a common tool for the monitoring of the health status of swine populations for Enzootic Pneumonia. Both indirect and blocking ELISAs are available commercially for the detection of *M. hyopneumoniae* antibodies. The sensitivity and specificity of the ELISA varies depending upon the stage of infection. Studies by Sørensen et al. (1997), Ameri-Mahabadi et al. (2005) and Erlandson et al. (2005) demonstrated ELISA diagnostic sensitivities ranging from 60-100% for serum collected during early infection, and 13-100% for serum collected during mid to late infection, whilst still maintaining a high specificity. All the studies found decreasing sensitivity with an increase in days post infection (Ameri-Mahabadi et al., 2005; Erlandson et al.,

2005; Sørensen et al., 1997), indicating that the sensitivity of the ELISA was influenced by stage of infection.

Erysipelas (Erysipelothrix rhusiopathiae)

Erysipelas is a bacterial infection caused by two main bacterial species; *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum*. Erysipelas has a worldwide distribution and infection has been documented in humans and in up to 50 different species of animals (Brooke and Riley, 1999; Eriksson et al., 2009; Wang et al., 2010), including both domestic and wild species. The highest incidence of infection with erysipelas occurs in swine (Brooke and Riley, 1999; Wang et al., 2010). The disease is economically important to the commercial pig industry and is ranked as one of the top ten causes for swine carcass condemnations (Bender et al., 2011; Colavita et al., 2006). Healthy swine, with no outward appearance of infection, are the major source of infection for swine herds (Brooke and Riley, 1999; Wang et al., 2010). Transmission occurs via direct contact with infected animals, or indirectly via the environment. Swine erysipelas has three clinical presentations; acute, subacute and chronic. Acute erysipelas is characterised by septicaemia, which occurs 24 hours following exposure, and sudden death of apparently healthy animals (Colavita et al., 2006; Wang et al., 2010). Presentation of acute erysipelas at the herd level can also include depression and inappetance caused by fever, and reproductive disorders in sows (Wang et al., 2010). Subacute erysipelas is characterised by less severe clinical signs, or a subclinical period with no obvious signs of infection or lesions (Wang et al., 2010). Skin lesions can appear 2-3 days following infection (Colavita et al., 2006), and the intensity of the lesions is directly related to the diagnosis (Wang et al., 2010). Chronic erysipelas may

follow an acute or subacute infection, and is characterised by arthritis in the hock, stifle and carpal joints, as well as endocarditis (Wang et al., 2010).

The majority of ELISAs that exist for the detection of erysipelas specifically detect antibodies to *E. rhusiopathiae*. A number of studies (Eamens et al., 1989; Imada et al., 2003; Kirchhoff et al., 1985; Sato et al., 1998) have assessed the effectiveness of the ELISA for the detection of these antibodies in swine populations, as well as improving the ability of the ELISA to detect these antibodies by using surface protective antigen (Spa) as the coating antigen (Imada et al., 2003). In a study by Eamens et al. (1989), an indirect antibody ELISA was used to determine the ability of the ELISA in diagnosing swine erysipelas arthritis. The study used two different cut-off thresholds for two trial groups and found a diagnostic sensitivity and specificity for group 1 of 92% and 100%, respectively (cut-off threshold 0.212) and diagnostic sensitivity and specificity of 66% and 83%, respectively, for group 2 (cut-off threshold 0.815). The study also determined that specific IgG antibody concentrations did not correlate well with pathological changes in the joints (Eamens et al., 1989).

Utilisation of colostrum and whey in ELISA

As previously discussed, colostrum and whey contain relatively higher concentrations of Igs compared to serum. In cows, these concentrations can be up to 10 times greater (Baumrucker et al., 2010) in concentration, while in ewes and sows, these concentrations can be up to 3 times greater in concentration when compared to serum (Campbell et al., 1977; Curtis and Bourne, 1971). Also previously discussed, some disease-specific ELISAs suffer from poor diagnostic utility, particularly in regards to

diagnostic sensitivity. It is possible that the testing of colostrum and/or whey may be able to improve the utility of these ELISAs by improving diagnostic sensitivity, as well as other measures of diagnostic utility. Such improvements in diagnostic utility could have broader implications, such as for the monitoring and surveillance of disease within herds and flocks and biosecurity at farm, state, national and international levels. As ELISA is relatively inexpensive and capable of large-scale testing, the monitoring and surveillance of disease within herds and flocks is generally accomplished through bulk or pooled herd and flock testing of samples. Pooled colostrum/whey testing could provide an improvement in the detection of animals at the herd/flock level by improving analytical sensitivity. For example, *Neospora caninum* infection can only be detected in bulk-milk samples when there are at least 5-7.5% seropositive cows in the lactating herd (Chanlun et al., 2002). Also, diagnostic sensitivity of the ELISA for the detection of *Mycobacterium bovis* could also be improved, as although having a high specificity, the ELISA has a variable sensitivity (between 18 and 73%) due to a variable humoral immune response of infected animals (Casal et al., 2014). Tests which have a high sensitivity improve the assurance of absence of disease, as animals that test positive are more likely to be true positives and are culled. Such an improvement in sensitivity, provided a high test specificity can also be maintained, ensures that through continued herd/flock testing, absence of disease is preserved. Absence of disease, as well as improvement in test sensitivity, is important for biosecurity at all levels. Biosecurity should begin at the farm level, where producers use regular monitoring and surveillance techniques to test their herd/flock for disease. Using colostrum/whey could improve the detection of infected animals by improving diagnostic test sensitivity, ensuring that these infected animals are removed from the herd/flock. If these animals remain undetected, they can spread infection to other susceptible animals within the herd/flock,

which could lead to the spread of infection to other herds/flocks at state, national and international levels through the regular sale and movement of livestock.

Thesis outline

The following thesis investigates the usefulness of colostrum derived immunoglobulins to improve the diagnostic utility of ELISAs used for the detection of some important diseases of production animals. In Chapter 2, the coagulation potential of rennet in the production of colostrum whey was investigated in two independent experiments. The aim of the first experiment was to determine the relationship between dilution of rennet dosage and the IgG concentration of colostrum whey, while the aim of the second experiment was to determine whether oral supplementation of piglets with rennet increases the piglet immunoglobulin absorption and serum globulin concentrations. In Chapter 3, the sensitivities of two commercially available disease-specific antibody ELISAs (*E. rhusiopathiae* and *M. hyopneumoniae*-specific antibodies) were investigated using serum and colostrum from sows vaccinated against these diseases. An additional aim of this experiment was to determine if maternal *E. rhusiopathiae* and *M. hyopneumoniae*-specific antibody OD values were correlated with specific-antibody serum OD values in the piglet. In Chapter 4, two experiments investigated the sensitivity of a commercial BVDV-antibody ELISA when using colostrum. The first experiment used BVDV vaccinated dairy cows as a model of antibody responses to infection, and the second experiment used beef heifers experimentally infected with BVDV-type 1c. The aim of the first experiment was to determine the comparative performance characteristics of a commercially available ELISA for BVDV antibodies when using colostrum and serum, while the aim of the second experiment was to determine the diagnostic value of colostrum BVDV antibody concentrations in

identifying PI calves following an experimental infection of the dam in the first trimester of pregnancy. In Chapter 5, two experiments investigated the diagnostic value of colostrum as a sample for the detection of MAP-specific antibody using a commercial Johne's disease ELISA. The aim of the first experiment was to investigate the sensitivity of a MAP-specific antibody ELISA when using colostrum and whey samples compared to serum using vaccinated sheep as a model of infection. In the second and final experiment presented in this thesis, the diagnostic utility of a commercial MAP-specific antibody ELISA was determined for a Johne's infected dairy herd. The performance of the ELISA was determined for colostrum and serum, using faecal culture as the reference test. The high-throughput Johne's polymerase chain reaction (HT-J PCR) assay was also compared.

The chapters in this thesis are presented in published manuscript format and publications resulting from these chapters have been inserted immediately following each chapter.

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1.2 Letter: Role for colostrum and whey in testing for bovine TB and Johne's disease?

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Chapter 2: The coagulation potential of rennet

Occurring naturally in the stomachs of newborn mammals, rennet or chymosin, has been readily used in the commercial cheese making industry for many years. The process of curd formation using rennet is a relatively simple process. Production of curds is not the only by-product of this process, but also liquid whey, which contains very high concentrations of immunoglobulins. In this chapter, the coagulation potential of rennet was investigated in two experiments. The aim of the first experiment, presented in Chapter 2.1, was to determine the relationship between rennet dilution and IgG concentration in colostrum whey. The aim of the second experiment, presented in Chapter 2.2, was to investigate whether oral supplementation of neonatal piglets with rennet would improve piglet serum globulin concentrations.

2.1 Improving the performance of disease antibody tests: Immunoglobulin G concentrations in colostrum and whey produced using different rennet coagulation protocols

Abstract

Whey contains higher concentrations of immunoglobulins (Igs) compared to colostrum. Using whey instead of colostrum when testing by ELISA for disease specific antibodies may increase test sensitivity. Separation of whey from colostrum is easily achieved by rennet coagulation, where saline is used as a medium in which rennet is added to the colostrum. The aim of this study was to determine the relationship between dilution of rennet and the IgG concentration of colostrum whey, as measured by an RID assay. In addition, a BVDV Ab ELISA was used to measure the relative concentrations (S/P ratios) of disease specific antibodies in colostrum and whey. Colostrum was mixed with either 5 different dilutions of a fixed dose of microbially derived rennet in saline (rennet treatment group), and the same 5 dilutions of saline (saline treatment group). All these samples were incubated for 60 minutes at 37°C, followed by centrifugation. The study also investigated the impact of incubation and centrifugation on colostrum IgG concentrations. These treatments consisted of colostrum with: no centrifugation and no incubation, centrifugation and no incubation, no centrifugation and incubation, and centrifugation and incubation. The IgG concentrations of the whey produced from the rennet treatments were significantly higher compared to the saline dilutions and the no centrifugation and no incubation colostrum, but this was not observed for all dilutions. A dilution effect was also observed.

Introduction

The production of colostrum is a selective process. Maternal immunoglobulins (Igs) are transferred to the mammary gland, from the dam's bloodstream, prior to parturition and in the early post-partum period. The concentration of Igs in cattle colostrum have been found to be five (Beer, et al. 1974) to ten times (Baumrucker, et al. 2010) higher than serum. Colostrum and milk are comprised of two main groups of proteins: caseins (insoluble) and whey proteins (soluble). Concentrations of Igs in the whey are dependent upon a number of factors which includes dietary and physiological factors of the animal, as well as the method of extraction (Madureira, et al. 2007; Marnila and Korhonen 2011b). Although the casein accounts for 80% of the total protein in colostrum/milk, the majority of the Igs are contained within the whey (Marnila and Korhonen 2011a). A study by Neave, et al. (2013) found the concentration of IgG in colostrum (26.2g/100g) to be lower than the concentration of IgG in colostrum whey (48.6g/100g). A review by Smithers (2008) identified that the content of Igs in cheese whey is approximately 300-600 mg/L, thus its separation from colostrum should produce a sample which has a higher concentration of Igs. Production of whey from milk in cheese making is achieved through the coagulation of casein by incubating milk with rennet. Rennet is composed of a group of enzymes that occur in the stomach of the newborn mammal; the active enzyme is chymosin, but can also be referred to as rennin. Chymosin assists in the release of Igs from the colostrum and milk ingested by coagulating the casein, resulting in formation of a solid curd and Ig-rich whey (Andren 2011). It is possible that the rennet coagulation of colostrum to produce an Ig-rich whey, could improve the detection of disease relative to colostrum when using ELISA. The aim of this study was to determine the relationship between dilution of rennet dosage and the IgG concentration of colostrum whey. The outcomes were measured

using a BVDV-specific antibody ELISA and a bovine specific IgG radial immunodiffusion (RID) assay.

Materials and methods

Colostrum dilutions and incubation procedure

A representative herd sample was created by pooling equal volumes of bovine colostrum from six dairy cows (BVD vaccinated dairy herd). The following protocols were investigated (Table 2.1):

Table 2.1 The variables and protocols investigated in this study, including incubation time and temperature, centrifugation speed and time, saline volume and rennet volume.

Variable	Protocol
Incubation Time and Temperature	37°C for 60 minutes
Centrifugation	3,000 rpm for 10 minutes
Saline (0.9%)	40mcL diluted in 5 different volumes of R/O water
Rennet (8 IMCU/mL)	40mcL diluted in 5 volumes of R/O water as per saline

The pooled colostrum was used in all the protocols. Microbially derived vegetarian rennet (8 IMCU/mL) (Cheeselinks, Little River, VIC), which is suspended in 0.9% saline, was pre-diluted with reverse osmosis (R/O) water, as per Table 2. Five dilutions were used. An identical quantity of saline was diluted using the same dilutions as a control (Table 2.2). All samples required to be incubated were incubated in a water

bath for 60 minutes at 37°C. Following incubation, all samples required to be centrifuged were centrifuged for 10 minutes at 3000rpm. All samples were stored at -80°C until testing could be performed.

Further samples of colostrum were subjected to 4 protocols: incubation and centrifugation, no incubation and centrifugation, incubation and no centrifugation and no incubation and no centrifugation. The incubation and centrifugation parameters are indicated in Table 1. There were three replicates used for each treatment.

Table 2.2 Details of dilutions for rennet and saline added to colostrum prior to incubation and centrifugation.

Dilution	Volume of rennet in saline (mL)	Volume of saline (mL)	Volume of R/O water (mL)	Volume rennet/saline dilution added to colostrum (mL)	Volume of colostrum (mL)	Total volume (mL)
1:200	40	40	0	40	8000	8040
1:16	40	40	460	500	8000	8500
1:8	40	40	960	1000	8000	9000
3:16	40	40	1460	1500	8000	9500
1:4	40	40	1960	2000	8000	10000

IgG RID assay

All samples were pre-diluted 1:10 to ensure they would fall within the range of the standards (300-3000 mg/dL). The bovine IgG concentration of the samples was tested using a commercial RID assay kit (Kent Laboratories, Bellingham, WA, USA) and was performed as per the manufacturer's instructions for overnight readings (18 hour incubation). The plates were read independently by two technicians, who were blinded to the identification of the samples. The mean of the two readings was calculated and

multiplied by the dilution factor to determine the final IgG concentration, measured in mg/dL.

BVDV ELISA

All samples were tested using a commercial BVDV antibody ELISA (IDEXX, Rydalmere, NSW). The samples were tested as per the manufacturer's instructions for milk (undiluted). Results were expressed as sample-to-positive (S/P) ratio.

Statistical analysis

The mean \pm standard error was calculated for each treatment protocol. A one-way analysis of variance with a post-hoc Tukey's test was performed to determine statistical differences between the treatments, for each test (ELISA, RID).

Results

IgG RID assay

The IgG concentration was highest in samples with smaller dilutions; however the colostrum without rennet and/or saline added had the lowest IgG concentrations (Figure 2.1a). Each of the five dilutions of the rennet treatments and their saline controls were compared with the IgG concentration of the no centrifugation and no incubation colostrum. Within the 1:200 dilution, the IgG concentration of the saline and rennet (whey) treatments were not significantly different (Table 2.3). The IgG concentration of the saline ($P < 0.001$) and rennet (whey) ($P < 0.01$) treatments were significantly higher

compared to the colostrum with no centrifugation and no incubation colostrum (Table 2.3). Within the 1:16 dilution, the IgG concentration of the saline and rennet (whey) treatments were not significantly different. The IgG concentration of the saline ($P < 0.05$) and rennet (whey) ($P < 0.001$) treatments were significantly higher compared to the no centrifugation and no incubation colostrum (Table 2.3). Within the 1:8 dilution, the IgG concentration of the rennet (whey) treatment was significantly higher ($P < 0.01$) compared to the saline treatment. The IgG concentration of the rennet (whey) ($P < 0.05$) treatment was significantly higher compared to the no centrifugation and no incubation colostrum. There was no significant difference between the saline treatments and the no centrifugation and no incubation colostrum (Table 2.3). Within the 3:16 dilution, there was no significant difference between the IgG concentration of the saline and rennet (whey) treatments. There was also no significant difference in IgG concentration between the saline and rennet (whey) treatments and the no centrifugation and no incubation colostrum (Table 2.3). Within the 1:4 dilution, there was no significant difference in IgG concentration between the saline and rennet (whey) treatments. The IgG concentration of the rennet (whey) treatment ($P < 0.05$) was significantly higher compared to the no centrifugation and no incubation colostrum. There was no significant difference in IgG concentration between the saline treatment and the no centrifugation and no incubation colostrum (Table 2.3).

The IgG concentration of the no centrifugation and no incubation colostrum was also compared to IgG concentrations of three centrifugation and incubation protocols: no centrifugation and incubation, centrifugation and no incubation, and centrifugation and incubation. There were no significant differences in IgG concentration between any of the centrifugation and incubation protocols (Table 2.3).

BVDV ELISA

The S/P ratio was highest in samples with smaller dilutions (Figure 2.1b). The S/P ratios of each of the rennet (whey) treatment dilutions and their saline controls were compared with the S/P ratio of the no centrifugation and no incubation colostrum. Within the 1:200 dilution, there was no significant difference in S/P ratio between the saline and rennet (whey) treatments (Table 2.4). The S/P ratio of the saline treatment ($P < 0.001$), and of the rennet (whey) treatment ($P < 0.001$), were significantly higher compared to the S/P ratio of the no centrifugation and no incubation colostrum (Table 2.4). Within the 1:16 dilution, there was no significant difference in S/P ratio between the saline and rennet (whey) treatments (Table 2.4). The S/P ratio of the saline treatment ($P < 0.001$), and of the rennet (whey) treatment, were significantly higher compared to the S/P ratio of the no centrifugation and no incubation colostrum (Table 2.4). Within the 1:8 dilution, there was no significant differences in S/P ratio between the saline and rennet (whey) treatments (Table 2.4). The S/P ratio of the saline treatment ($P < 0.001$), and of the rennet (whey) treatment, were significantly higher compared to the S/P ratio of the no centrifugation and no incubation colostrum (Table 2.4). Within the 3:16 dilution, there was no significant difference in S/P ratio between the saline and rennet (whey) treatments (Table 2.4). The S/P ratio of the saline treatment ($P < 0.001$), and of the rennet (whey) treatment, were significantly higher compared to the S/P ratio of the no centrifugation and no incubation colostrum (Table 2.4). Within the 1:4 dilution, there was no significant difference in S/P ratio between the saline and rennet (whey) treatments. The S/P ratio of the saline treatment ($P < 0.001$), and of the rennet (whey) treatment, were significantly higher compared to the S/P ratio of the no centrifugation and no incubation colostrum (Table 2.4).

The S/P ratio of the no centrifugation and no incubation colostrum was also compared to the S/P ratios of three centrifugation and incubation colostrum protocols: no centrifugation and incubation, centrifugation and no incubation, and centrifugation and incubation. The S/P ratios of the centrifugation and incubation colostrum ($P < 0.001$) and of the centrifugation and no incubation colostrum ($P < 0.001$) were significantly higher compared to the S/P ratio of the no centrifugation and no incubation colostrum (Table 2.4). All other comparisons within the colostrum protocols were not significant.

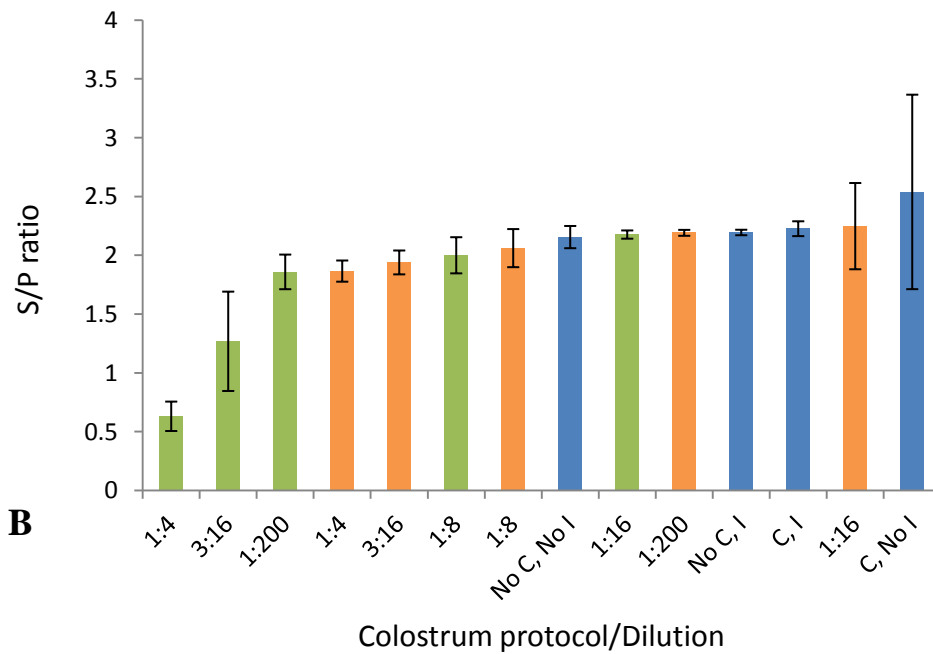
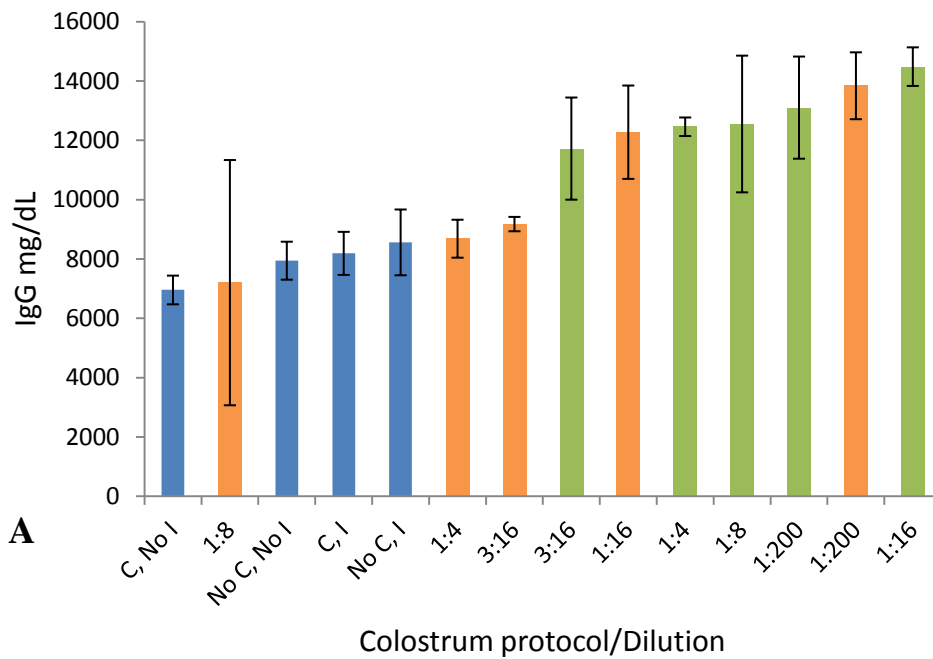


Figure 2.1 Mean (A) IgG concentration (mg/dL) and mean (B) Sample-to-Positive (S/P) ratio (b) \pm 95% confidence intervals for the Rennet (whey) treatment (green), saline treatment (orange) and colostrum protocols without rennet or saline added (blue- No C, I: no centrifugation and incubation; C, I: centrifugation and incubation; C, No I: centrifugation and no incubation; No C, No I: no centrifugation and no incubation) created from a pooled colostrum sample of six dairy cows.

Discussion

The aim of this study was to determine the relationship between the dilution of rennet dosage and the IgG concentration of colostrum whey. All rennet (whey) dilutions, excluding the 3:16 dilution, had a significantly higher IgG concentration compared to the no centrifugation and no incubation colostrum. Comparison of all the rennet (whey) dilutions were only compared to the no centrifugation and no incubation colostrum to determine whether the IgG concentration of whey was higher than whole colostrum. Studies by Elfstrand, et al. (2002) and Neave, et al. (2013) found colostrum whey IgG concentrations of 1800mg/dL and 3120 mg/dL, respectively, which were both lower than the colostrum whey concentrations found in the current study (12866 mg/dL). These results also confirm previous studies where whey contained higher Ig concentrations than colostrum. However, as previously mentioned, the Ig concentration of whey can be determined by a number of dietary and physiological animal factors, as well as the method of extraction (Madureira, et al. 2007; Marnila and Korhonen 2011b). In the study by Elfstrand, et al. (2002) colostrum was collected from Swedish Friesian cows in lactation stages 1, 2 and ≥ 3 . Both age, breed and diet of the cow influences the IgG concentration of the colostrum. Younger cows (lactation stage 1 or 2) typically produce colostrum which has lower concentrations of IgG, while different breeds also produce colostrum which differs in Ig content. Due to the location of the Elfstrand, et al. (2002) study compared to the current study, it can be assumed that the feeding of the sampled cows would also cause differences in colostrum IgG concentration.

Saline controls were used to determine whether any significant results seen in the rennet (whey) dilutions were due to the effect of the rennet and not of the saline carrier. Only two saline dilutions (1:200 and 1:16) had IgG concentrations significantly higher

compared to the no centrifugation and no incubation colostrum. However, when the S/P ratio results of the ELISA were compared to the IgG concentrations of the RID assay, all the saline dilutions, as well as the rennet (whey) dilutions, had significantly higher S/P ratios compared to the no centrifugation and no incubation colostrum. Considering that the saline dilutions were diluted whole colostrum, these results were expected to be lower than what was found for the no centrifugation and no incubation colostrum. It is possible that the opacity of the whole colostrum is improved through dilution, resulting in a better performance in the ELISA.

The effect of centrifugation and incubation on the IgG concentration of colostrum was also investigated. No significant differences were found between the no centrifugation and no incubation colostrum and the other three methods. However, the ELISA showed significantly higher S/P ratios in the centrifugation and incubation colostrum and the centrifugation and no incubation colostrum compared to the no centrifugation and no incubation colostrum. The results observed in the ELISA may be due to the opacity of the colostrum being improved through dilution.

A trend consistent with a dilution effect on the IgG concentration was also observed. Samples that had small dilutions exhibited higher IgG concentrations and S/P ratios, however, the pattern of the dilution effect was anomalous and did not follow the expected pattern of the dilutions investigated.

Conclusions

In conclusion, this study provided inconsistent evidence for the hypothesis that the IgG concentrations of the whey produced from the rennet dilutions (whey) would be significantly higher compared to both the saline dilutions and the no centrifugation and no incubation colostrum. The saline controls yielded higher IgG concentrations than expected. The rennet (whey) treatments did however yield significantly higher concentrations than any of the colostrum only protocols. The BVDV ELISA results yielded inconsistent results. The authors acknowledge that further research into the optimisation of IgG concentration in colostrum whey still needs to be investigated. A study where the power of the experiment is increased and focus is placed on a single rennet and saline dilution could solve the questions that have arisen from this study.

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Table 2.3 Comparisons between mean \pm standard error (SE) IgG concentrations (mg/dL) for Rennet (whey) treatment, saline treatment and colostrum protocols (NC: no centrifugation; NI: no incubation; C: centrifugation; I: incubation) using a one-way ANOVA with post-hoc Tukey's test. Stars indicate level of significance: ***P< 0.001, **P< 0.01, *P< 0.05. NS: not significant.

Protocol/Dilution (Mean \pm SE)	Colostrum protocols				Saline treatment					Rennet (whey) treatment				
	NC+I	NC+NI	C+I	C+NI	1:200	1:16	1:8	3:16	1:4	1:200	1:16	1:8	3:16	1:4
(8563 \pm 566)	(8563 \pm 566)	(7946 \pm 327)	(8193 \pm 370)	(6959 \pm 247)	(13842 \pm 575)	(12277 \pm 803)	(7206 \pm 2109)	(9180 \pm 123)	(8686 \pm 327)	(13106 \pm 878)	(14487 \pm 332)	(12553 \pm 1176)	(11725 \pm 878)	(12461 \pm 159)
	Colostrum protocols													
NC+I (8563 \pm 566)	NS													
NC+NI (7946 \pm 327)	NS	NS												
C+I (8193 \pm 370)	NS	NS	NS											
C+NI (6959 \pm 247)	NS	NS	NS	NS										
	Saline treatment													
1:200 (13842 \pm 575)	**	***	*	***	NS									
1:16 (12277 \pm 803)	NS	*	NS	**	***	NS								
1:8 (7206 \pm 2109)	NS	NS	NS	NS	***	**	NS							
3:16 (9180 \pm 123)	NS	NS	NS	NS	*	NS	NS	NS						
1:4 (8686 \pm 327)	NS	NS	NS	NS	**	NS	NS	NS	NS					
	Rennet (whey) treatment													
1:200 (13106 \pm 878)	*	**	**	***	NS	NS	***	NS	*	NS				
1:16 (14487 \pm 332)	***	***	***	***	NS	NS	***	***	**	NS	NS			
1:8 (12553 \pm 1176)	NS	*	*	**	NS	NS	**	NS	NS	NS	NS	NS		
3:16 (11725 \pm 878)	NS	NS	NS	*	NS	NS	*	NS	NS	NS	NS	NS	NS	
1:4 (12461 \pm 159)	NS	*	*	**	NS	NS	**	NS	NS	NS	NS	NS	NS	NS

Table 2.4 Comparisons between mean \pm standard error (SE) sample-to-positive (S/P) ratios (mg/dL) for Rennet (whey) treatment, saline treatment and colostrum protocols (NC: no centrifugation; NI: no incubation; C: centrifugation; I: incubation) using a one-way ANOVA with post-hoc Tukey's test. Stars indicate level of significance: ***P< 0.001, **P< 0.01, *P< 0.05. NS: not significant.

Protocol/Dilution (Mean \pm SE)	Colostrum protocols				Saline treatment					Rennet (whey) treatment				
	NC+I	NC+NI	C+I	C+NI	1:200	1:16	1:8	3:16	1:4	1:200	1:16	1:8	3:16	1:4
	(1.27 \pm 0.22)	(0.63 \pm 0.06)	(1.87 \pm 0.05)	(1.94 \pm 0.05)	(1.86 \pm 0.08)	(2.25 \pm 0.19)	(2.18 \pm 0.02)	(2.00 \pm 0.08)	(2.06 \pm 0.08)	(2.16 \pm 0.05)	(2.54 \pm 0.42)	(2.19 \pm 0.01)	(2.23 \pm 0.03)	(2.19 \pm 0.01)
	Colostrum protocols													
NC+I (1.27 \pm 0.22)	NS													
NC+NI (0.63 \pm 0.06)	NS	NS												
C+I (1.87 \pm 0.05)	NS	***	NS											
C+NI (1.94 \pm 0.05)	NS	***	NS	NS										
	Saline treatment													
1:200 (1.86 \pm 0.08)	NS	***	NS	NS	NS									
1:16 (2.25 \pm 0.19)	**	***	NS	NS	NS	NS								
1:8 (2.18 \pm 0.02)	*	***	NS	NS	NS	NS	NS	NS						
3:16 (2.00 \pm 0.08)	NS	***	NS	NS	NS	NS	NS	NS	NS					
1:4 (2.06 \pm 0.08)	*	***	NS	NS	NS	NS	NS	NS	NS	NS				
	Rennet (whey) treatment													
1:200 (2.16 \pm 0.05)	**	***	NS	NS	NS	NS	NS	NS	NS	NS	NS			
1:16 (2.54 \pm 0.42)	***	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		
1:8 (2.19 \pm 0.01)	**	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
3:16 (2.23 \pm 0.03)	**	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
1:4 (2.19 \pm 0.01)	**	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

2.2 Investigation of the impact of oral rennet supplementation on the serum globulin concentration in neonatal piglets

The objective of this experimental trial reported in the following published manuscript was to determine whether oral supplementation of piglets with vegetable rennet would improve the serum globulin concentration in neonatal piglets. Additionally, a linear model was also developed to determine whether there were any sow or pig factors that could also influence piglet serum globulin concentrations.

Original Article: Investigation of the impact of oral rennet supplementation on the serum globulin concentration in neonatal piglets

CJ Jenvey, WHEJ Wettere, MP Reichel, PD Cockcroft (2014)

Journal of Swine Health and Production Vol. 22(6), Pp. 282-286

Statement of Authorship

Title of Paper	Investigation of the impact of oral rennet supplementation on serum globulin concentrations in neonatal pigs
Publication Status	<input checked="" type="radio"/> Published, <input type="radio"/> Accepted for Publication, <input type="radio"/> Submitted for Publication, <input type="radio"/> Publication style
Publication Details	CJ Jenvey, WHEJ Wettene, MP Reichel, PD Cockcroft (2014) Journal of Swine Health and Production, vol. 22(6), pp.282-286.

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Caitlin J Jenvey	
Contribution to the Paper	Helped design animal trial, managed animal trial, collected samples, conducted sample analysis, data analysis and interpretation, drafted and edited manuscript, acted as corresponding author.	
Signature		Date 17/3/15

Name of Co-Author	William HEJ Wettene	
Contribution to the Paper	Assisted with animal work, helped to interpret data, helped to edit manuscript.	
Signature		Date 17/3/2015

Name of Co-Author	Michael P Reichel	
Contribution to the Paper	Helped to design animal trial, helped to interpret data, helped to edit manuscript.	
Signature		Date 16/3/2015

Name of Co-Author	Peter D Cockcroft	
Contribution to the Paper	Secured project funding, helped to design animal trial, assisted with animal work and sample collection, helped interpret data, helped to edit manuscript.	
Signature		Date 17/05/2015

Jenvey, C.J., Wettere, W.H.E.J., Reichel, M.P. & Cockcroft, P.D. (2014) Investigation of the impact of oral rennet supplementation on the serum globulin concentration in neonatal piglets
Journal of Swine Health and Production, v. 22 (6), pp. 282-286

NOTE:

This publication is included on pages 99 - 103 in the print copy of the thesis held in the University of Adelaide Library.

Chapter 3: Swine Erysipelas and Enzootic Pneumonia

The aim of the experiment presented in Chapter 3.1 was to compare the sensitivities of two commercially available ELISAs for the detection of *E. rhusiopathiae* and *M. hyopneumoniae*-specific antibodies in sow colostrum and serum. Also, the correlation of these maternal disease-specific antibody levels with piglet serum disease-specific antibody levels was also investigated.

3.1 *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae*: The sensitivities of enzyme-linked immunosorbent assays for detecting vaccinated sows of unknown disease status using serum and colostrum, and the correlation of the results for sow serum, colostrum, and piglet serum

Abstract

Due to the high concentrations of immunoglobulins, colostrum has the potential to improve the sensitivity of diagnostic tests for diseases in pigs. The sensitivity of a commercially available ELISA for the detection of *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae*-specific antibodies in sow colostrum in comparison to serum was investigated, and to determine the correlation of maternal *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae*-specific antibody levels with specific-antibody serum levels in the piglet. Blood and colostrum samples were collected from 20 sows (10 PP and 10 MP), and blood samples collected from the piglets of each sow, 48-72 hours post farrowing. The samples were tested using commercial ELISAs for *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae* (blocking ELISA). The mean *Erysipelothrix rhusiopathiae* sow colostrum OD values were significantly higher when compared to the mean serum OD values. The mean *Mycoplasma hyopneumoniae* sow colostrum OD values were significantly lower when compared to the mean serum OD values. The DSe of both ELISAs were significantly improved when using colostrum compared to serum. For *Erysipelothrix rhusiopathiae*, mean sow OD values for serum and colostrum were significantly associated with piglet serum OD levels. For *Mycoplasma hyopneumoniae*, mean sow colostrum OD values were associated with piglet serum OD values. The improvement in ELISA DSe observed when using

colostrum increases the proportion of infected animals which can be detected. This is especially important when monitoring for disease status within a susceptible herd.

Introduction

The production of colostrum is a selective process during which maternal antibodies that are present in the bloodstream of the dam are transferred into the mammary gland prior to parturition. This process is especially important in sows, as there is minimal transfer of maternal antibodies to the piglet during gestation (Marnila and Korhonen 2011; Nechvatalova, et al. 2011). Piglets rely upon colostrum to provide protection against infections in the first few weeks and months of life. *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae* are two such infections. Infection with *Erysipelothrix rhusiopathiae* causes urticarial lesions, septicaemia and chronic endocarditis (Brooke and Riley 1999), while infection with *Mycoplasma hyopneumoniae* causes retarded growth and a predisposition to other bacterial pulmonary infections (Sibila, et al. 2009). Both diseases cause significant production losses to the pig industry worldwide due to poor production, thus tests which accurately and efficiently identify infected animals are required. The provision of cellular immune response transfer from sow colostrum to piglets has previously been demonstrated in sows vaccinated against *Mycoplasma hyopneumoniae* (Bandrick, et al. 2008). Piglets from vaccinated sows had significantly greater *Mycoplasma hyopneumoniae*-specific responses when challenged with *Mycoplasma hyopneumoniae*-specific antigen, while significantly more *Mycoplasma hyopneumoniae*-specific proliferation of lymphocytes in sow colostrum was observed in vaccinated sows compared to non-vaccinated sows (Bandrick, et al. 2008). However, there is little research into whether colostrum would be suitable to increase diagnostic test sensitivity in pigs. The aim of this study was to

investigate the sensitivity of a commercially available ELISA for the detection of *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae*-specific antibodies in sow colostrum in comparison to serum, from a herd of vaccinated sows, and to determine if maternal *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae*-specific antibody OD values were correlated with specific-antibody serum OD values in the piglet.

Materials and methods

All experimental procedures were conducted at the University of Adelaide's Roseworthy piggery, South Australia, with approval from The University of Adelaide Animal Ethics Committee.

Sow selection

The piggery was selected based upon the vaccination of the herd with the *Mycoplasma hyopneumoniae* vaccine Respire One (Zoetis) and the *Erysipelothrix rhusiopathiae* vaccine Eryvac (Zoetis). Ten primiparous (PP) and 10 multiparous (MP) sows were selected from the herd based on farrowing dates.

Sample collection

The sow colostrum samples were collected from randomly selected teats within 12 hours of farrowing and the sow blood samples were collected from the jugular vein at weaning (28 days post farrowing). Piglet blood samples were collected by venepuncture of the anterior vena cava 48 to 72 hours post parturition. All the blood samples were centrifuged to separate the serum. The blood and colostrum samples were stored at -80°C until testing could be performed.

Erysipelothrix rhusiopathiae antibody ELISA

The serum and colostrum samples were tested using a commercial antibody ELISA (Ingezim Mal Rojo Indirect ELISA, Ingenasa, Spain). The sow samples were diluted 1:200, 1:400 and 1:800, and the piglet serum samples were diluted 1:200 using the diluent supplied with the ELISA kit. All samples were tested in triplicate and results expressed as corrected optical density (OD).

Mycoplasma hyopneumoniae antibody ELISA

The serum and colostrum samples were tested using a commercial antibody blocking ELISA (Ingezim M.Hyo Compac ELISA, Ingenasa, Spain). The sow samples were diluted 1:2, 1:50 and 1:100, and the piglet serum samples were diluted 1:2 using the diluent supplied with the ELISA kit. All samples were tested in triplicate and results expressed as corrected optical density (OD).

Diagnostic sensitivity

Diagnostic sensitivity in the context of this study is the ability to identify vaccinated sows (model of infection). The recommended cut-off OD value for each ELISA was used to identify vaccinated sows. Diagnostic sensitivity (DSe) \pm 95 per cent confidence intervals was determined for each sample type (serum, colostrum) at each of 15 OD threshold values, using the following equation:

$$\text{DSe} = \frac{\text{Number of positive sows detected}}{\text{Total number of vaccinated sows}} \times 100$$

Statistical analysis

Descriptive statistics to determine normal distribution were performed. Mean, standard error and 95 per cent confidence intervals were calculated for each sample (serum, colostrum), and each dilution. A Student's paired t-test was used to identify any significant differences between dilutions. The variables of sow serum OD and sow colostrum OD were assessed for significance in relation to piglet serum OD using a multi-variable linear regression analysis with a backwards stepwise elimination of non-significant factors ($P > 0.05$). Predictive models for piglet serum OD concentrations were developed using the results of the regression analysis. All statistical analyses were performed using the statistics program R (version 3.0.2).

Results

Erysipelothrix rhusiopathiae

Sow serum and colostrum. The mean sow colostrum OD was significantly higher when compared to mean sow serum OD, for all dilutions ($P < 0.05$). Within the PP sows, 1:400 serum ($P < 0.05$) was significantly higher compared to 1:800 serum, 1:200 colostrum was significantly higher compared to 1:400 colostrum ($P < 0.05$) and 1:800 colostrum ($P < 0.01$), and 1:200 ($P < 0.05$), 1:400 ($P < 0.05$) and 1:800 ($P < 0.05$) serum, 1:400 colostrum was significantly higher compared to 1:400 ($P < 0.05$) and 1:800 ($P < 0.05$) serum, and 1:800 colostrum was significantly higher compared to 1:800 ($P < 0.05$) serum (Table 3.1). Within the MP sows, colostrum OD values were significantly higher when compared to serum, for all combinations, except for 1:200 serum and 1:800 colostrum ($P = 0.08$). Comparing PP and MP sow serum and colostrum, 1:200 PP serum was significantly higher compared to 1:800 MP serum ($P < 0.05$), 1:200 MP serum was significantly higher compared to 1:800 PP serum ($P <$

0.05), and all MP colostrum dilutions were significantly higher compared to PP serum, except for 1:200 PP serum and 1:800 MP colostrum ($P = 0.12$) (Table 3.1). There were no observed significant differences between PP colostrum and MP serum and colostrum.

Diagnostic sensitivity. The DSe of both serum and colostrum decreased with an increase in OD threshold. Using the recommended dilution for serum (1:200), the diagnostic sensitivity (DSe) for colostrum (PP and MP sows) was significantly higher compared to serum (PP and MP sows), from an OD threshold of 0.2 (Figure 3.1a). The DSe of serum reached 0 per cent by an OD threshold of 0.8. At this same threshold, the DSe of colostrum was 25 per cent, and did not reach 0 per cent until an OD threshold of 1.4 (Figure 3.1a). PP sow colostrum reached 0 per cent DSe at an OD threshold of 1.2, while MP sow colostrum reached 0 per cent DSe at an OD threshold of 1.4 (Figure 3.1b). MP sow colostrum DSe was significantly higher compared to PP sow colostrum DSe, but only at OD thresholds of 0.3, 0.5, 0.6, 0.7, 1.1, 1.2, 1.3 and 1.4 (Figure 3.1b). The largest difference in DSe between MP sow colostrum and PP sow colostrum was at an OD threshold of 0.3, where there were observed DSe's of 90 per cent and 60 per cent, respectively (Fig. 1b). PP sow serum reached 0 per cent DSe at an OD threshold of 0.6, while MP sow serum reached 0 per cent DSe at an OD threshold of 0.8. MP sow serum DSe was significantly higher compared to PP sow serum DSe for all thresholds except for an OD threshold of 0.4 (Figure 3.1b).

Association with piglet serum. Sow colostrum OD ($P < 0.001$) and sow serum ($P < 0.05$) OD were significantly associated with piglet serum OD. The multiple R-squared

and P-value for the model were 0.64 and < 0.001 , respectively. The linear regression analysis was used to develop a model to predict piglet serum OD, using the equations:

$$\text{Piglet serum OD} = 0.51 \times \text{Sow serum OD} + 0.42 \text{ (S. E. } \pm 0.23\text{)}$$

$$\text{Piglet serum OD} = 0.83 \times \text{Sow colostrum OD} + 0.42 \text{ (S. E. } \pm 0.08\text{)}$$

For a combined increase in sow serum and colostrum of 0.2 OD, the model predicted an increase of 0.69 OD in piglet serum (Figure 3.3a).

Mycoplasma hyopneumoniae

Sow serum and colostrum. The detection of *Mycoplasma hyopneumoniae*-specific antibodies was determined using a blocking ELISA. The mean sow colostrum OD values were significantly lower when compared to sow serum OD values, for all dilutions ($P < 0.05$). Within the PP sows, the mean colostrum OD values were significantly lower when compared to mean serum, for all combinations ($P < 0.05$) (Table 3.2). Within the MP sows, the mean colostrum OD values were significantly lower when compared to mean serum ($P < 0.05$), for all combinations, except for 1:50 serum and 1:100 serum ($P = 0.08$), and 1:50 colostrum and 1:100 colostrum ($P = 0.15$) (Table 3.2). When comparing PP and MP sow serum and colostrum, the mean colostrum OD values were significantly lower when compared to mean serum, for all combinations, except for the following comparisons; 1:2 PP serum and 1:2 MP colostrum ($P = 0.18$), 1:50 PP serum and 1:50 MP colostrum ($P = 0.06$) and 1:100 MP colostrum ($P = 0.09$), 1:100 PP serum and 1:50 MP serum ($P = 0.77$), and 1:100 PP colostrum and 1:50 MP colostrum ($P = 0.67$) (Table 3.2).

Diagnostic sensitivity. The DSe of both serum and colostrum increased with an increase in OD threshold. Using the recommended dilution for serum (1:2), the DSe for sow

colostrum (PP and MP sows) was significantly higher compared to sow serum (PP and MP sows) for all OD thresholds from a OD threshold of 0.2 (Figure 3.2a). The DSe of colostrum reached 100 per cent at an OD threshold of 0.7, while the DSe of serum reached 100 per cent at an OD threshold of 0.9 (Figure 3.2a). The largest difference in DSe between sow serum and sow colostrum was at an OD threshold of 0.4, where the observed DSe was 20 per cent and 55 per cent, respectively (Figure 3.2a). PP sow serum and MP sow serum both reached 100 per cent DSe at an OD threshold of 0.9. PP sow colostrum reached 100 per cent DSe at an OD threshold of 0.6, while MP sow colostrum reached 100 per cent DSe at an OD threshold of 0.7 (Figure 3.2b). PP sow colostrum had a significantly higher DSe compared to MP sow colostrum only at OD thresholds of 0.2 and 0.6, while MP sow serum had a significantly higher DSe compared to PP sow serum at OD thresholds of 0.4 and 0.6 (Figure 3.2b).

Association with piglet serum. Sow colostrum OD ($P < .001$) was significantly associated with piglet serum OD. The multiple R-squared and P-value for the model were 0.53 and < 0.001 , respectively. The linear regression analysis was used to develop a model to predict piglet serum OD, using the following equation:

$$\text{Piglet serum OD} = 0.84 \times \text{Sow colostrum OD} + 0.10 \text{ (S. E. } \pm 0.15\text{)}$$

For an increase in sow colostrum of 0.2 OD, the model predicted an increase of 0.27 OD in piglet serum (Figure 3.3b).

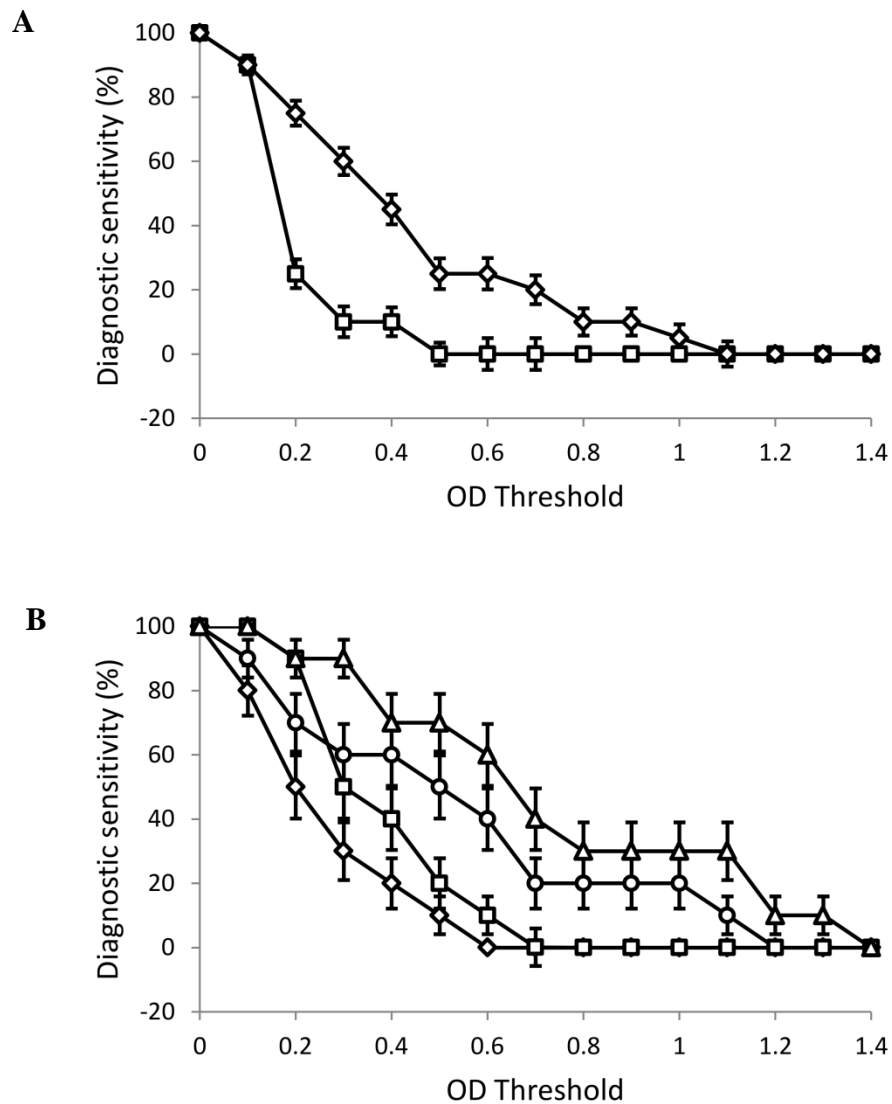


Figure 3.1 Diagnostic sensitivity \pm 95 per cent confidence intervals for (A) All Serum (square) and All Colostrum (diamond); and (B) PP serum (diamond), PP colostrum (circle), MP serum (square) and MP colostrum (triangle) collected from 10 primiparous (PP) and 10 multiparous (MP) sows vaccinated against *Erysipelothrix rhusiopathiae*.

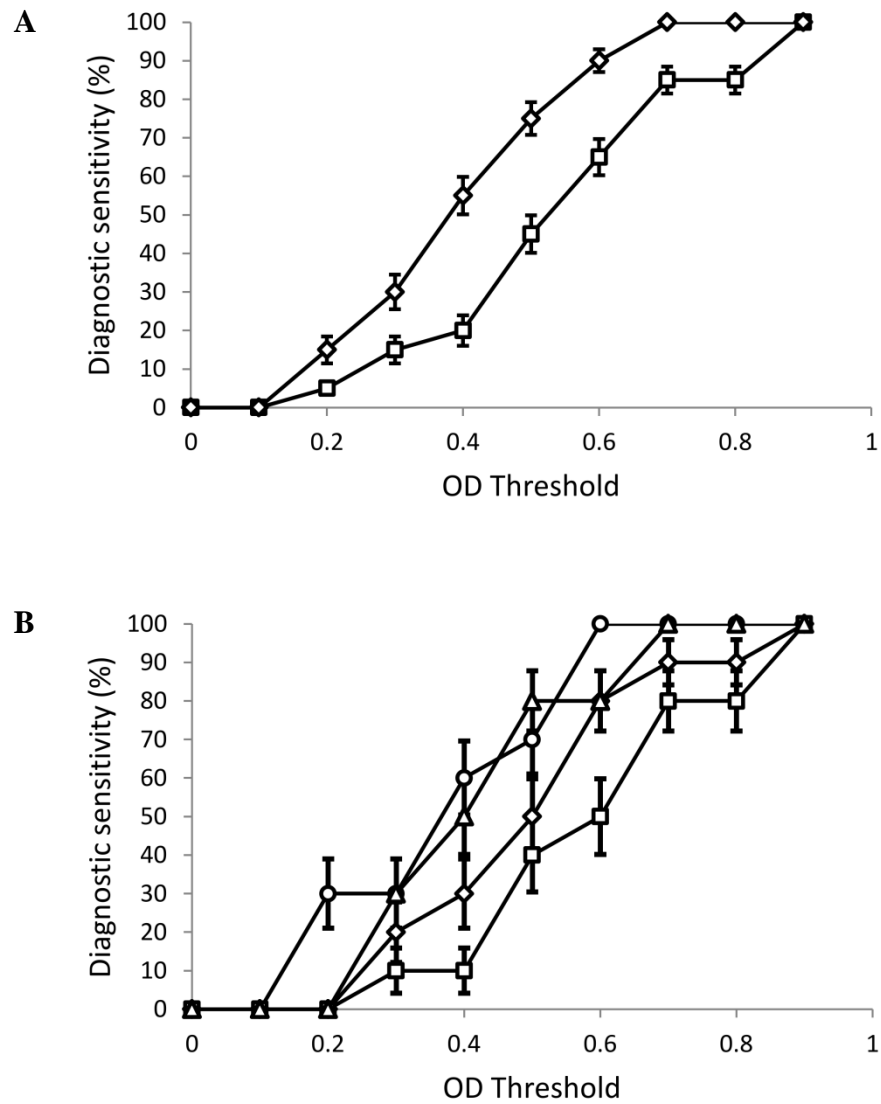


Figure 3.2 Diagnostic sensitivity \pm 95 per cent confidence intervals for (A) All Serum (square) and All Colostrum (diamond); and (B) PP Serum (diamond), PP Colostrum (circle), MP Serum (square) and MP Colostrum (triangle) collected from 10 primiparous (PP) and 10 multiparous (MP) vaccinated against *Mycoplasma hyopneumoniae*.

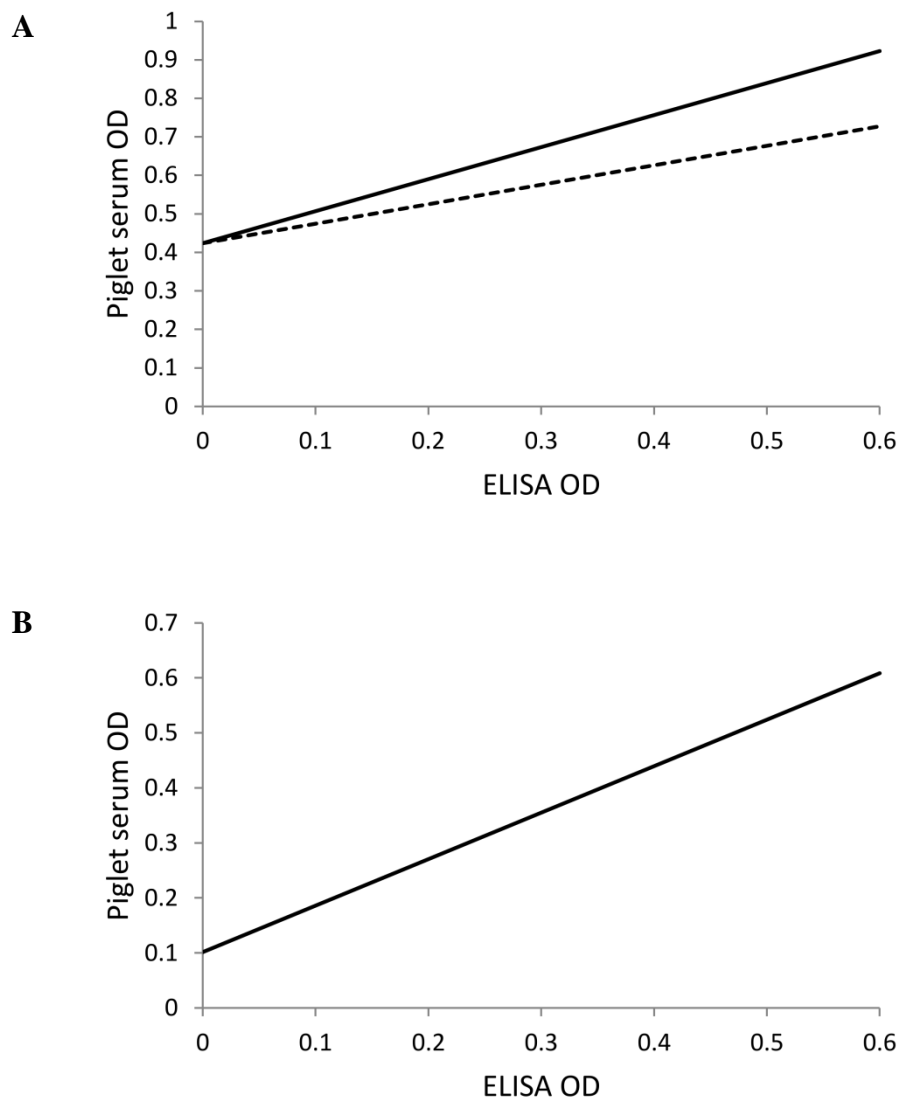


Figure 3.3 (A) Association of piglet serum OD concentrations with sow serum (dotted line) and sow colostrum (solid line) OD concentrations for multiparous sows vaccinated against *Erysipelothrix rhusiopathiae* (R-squared = 0.64, $P < 0.001$), (B) Association of piglet serum OD concentrations with sow colostrum OD concentrations for multiparous sows vaccinated against *Mycoplasma hyopneumoniae* (R-squared = 0.53, $P < 0.001$).

Discussion

The aim of this study was to investigate the sensitivity of a commercially available ELISA for the detection of vaccinated sows using the recommended cut-off OD value for positive animals. An additional aim of this study was to determine if maternal *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae* specific-antibody levels were associated with specific-antibody serum levels in the piglet. Mean sow colostrum OD values (PP and MP) for *Erysipelothrix rhusiopathiae* was significantly higher when compared to mean sow serum OD values, and mean sow colostrum OD values (PP and MP) for *Mycoplasma hyopneumoniae* were significantly lower when compared to mean sow serum OD values. Within the PP and MP sows, all colostrum dilution OD values for *Erysipelothrix rhusiopathiae* were significantly higher when compared to their corresponding serum dilution OD values, and all colostrum OD values for *Mycoplasma hyopneumoniae* were significantly lower when compared to their corresponding serum dilution OD values. However, no differences were observed between corresponding PP and MP sow colostrum dilutions, for both ELISAs. The concentration of IgG in sow colostrum can range from 5870 mg/dL (Marnila and Korhonen 2011) to 6180 mg/dL (Curtis and Bourne 1971) and 6230 mg/dL (Quesnel 2011), which is significantly higher than the concentrations that can be found in sow serum and milk. Sows with two parities and over tend to have a greater colostrum yield (Devillers, et al. 2007). A study by Klobasa, et al. 1987 found piglets from MP sows (4 parities and over) had higher serum IgG concentrations than piglets from PP sows. In the current study, there were no observed significant differences in the specific-antibody colostrum concentrations between PP and MP sows. This is likely due to the low numbers of animals used in the current study.

The DSe of both ELISAs in this study were increased when using colostrum compared to serum. When the sows were split by parity (PP and MP), colostrum still increased the DSe of both ELISAs, at a given OD threshold. A previous study by Bandrick, et al. 2008 observed significantly more *Mycoplasma hyopneumoniae*-specific proliferation of lymphocytes in *Mycoplasma hyopneumoniae* vaccinated sow colostrum compared to non-vaccinated sows. However, there is little research into whether colostrum would be suitable to increase diagnostic test sensitivity in pigs. A study by Levonen (1994) used an ELISA to test colostrum collected from confirmed and suspected *Mycoplasma hyopneumoniae* infected pig herds. The study determined the specificity and sensitivity of the ELISA at the herd level to be 91.5 per cent and 66.7 per cent, respectively. In the current study, the observed DSe of the ELISA (recommended positive OD threshold 0.5) when testing colostrum for *Mycoplasma hyopneumoniae*-specific antibodies was 75 per cent, and was significantly higher compared to the DSe for serum (45 per cent). The difference in sensitivity between the studies is most likely due to the collection of samples during the incubation period and presentation of early clinical signs in the study by Levonen (1994). When testing the same samples for *Erysipelothrix rhusiopathiae*-specific antibodies in the current study, the observed DSe of the ELISA (recommended positive OD threshold 0.3) when testing colostrum was 60 per cent, which was also significantly higher compared serum (10 per cent). What both studies highlight is the improved detection of infected animals and the possible benefits of pooled colostrum testing. Levonen (1994) noted that strong *Mycoplasma hyopneumoniae* reactions started to decline between dilutions of 1:2,000 and 1:3,200. In the current study, at dilutions of 1:800 (*Erysipelothrix rhusiopathiae*) and 1:100 (*Mycoplasma hyopneumoniae*), the observed diagnostic sensitivity of the ELISAs were 60 per cent and 0 per cent,

respectively (Data not shown). This may also be of benefit for other diseases of pigs where detection of infected animals is difficult.

Piglet serum OD was significantly associated with sow serum OD and sow colostrum OD (*Erysipelothrix rhusiopathiae*), and with sow colostrum OD (*Mycoplasma hyopneumoniae*). During colostrogenesis, concentrations of immunoglobulins (Igs) in colostrum are significantly higher than what can be found in circulating maternal blood. There is evidence to suggest that the concentration of Igs found in piglet circulation post-suckling is non-selective, as piglet concentrations mimic the concentrations found in sow serum and colostrum (Bandrick, et al. 2014). Therefore, the significant association found in the current study between piglet serum OD and sow colostrum OD was anticipated. However, the use of predictive models is incredibly important for the commercial pig industry, as piglets at risk of low serum specific-antibody concentrations can be identified before they become susceptible to infection, and subsequent measures, such as colostrum supplementation, can be taken. It must also be noted that piglet concentrations of specific-antibody, as well as Ig, does not solely depend upon the concentrations found in sow colostrum, but also the amount of colostrum that is ingested by the piglet and the timing of gut closure (Bandrick, et al. 2014).

Conclusion

The improvement in diagnostic sensitivity observed in this study when using colostrum is particularly important for the monitoring of disease within herds, as well as pooled colostrum monitoring, which will enable a greater proportion of infected animals to be identified, or increase the assurance of absence of disease within a population.

Additionally, sow colostrum OD concentrations were predictive of piglet serum OD concentrations. This promotes the identification of piglets 'at risk' of low serum OD concentrations, allowing early intervention with methods such as supplementation to improve piglet serum antibody concentrations. The specificity of the ELISA when using colostrum still needs to be investigated.

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Table 3.1 Comparisons between mean \pm standard error optical density (OD) concentrations for the serum and colostrum collected from primiparous and multiparous sows vaccinated against *Erysipelothrix rhusiopathiae* using a Student's paired t-test. Stars indicate level of significance: ***P< 0.001, **P< 0.01, *P< 0.05. NS: not significant.

Dilution (Mean \pm SE)	Primiparous sows						Multiparous sows					
	Serum			Colostrum			Serum			Colostrum		
	1:200 (0.27 \pm 0.05)	1:400 (0.23 \pm 0.03)	1:800 (0.18 \pm 0.03)	1:200 (0.49 \pm 0.12)	1:400 (0.41 \pm 0.09)	1:800 (0.38 \pm 0.10)	1:200 (0.36 \pm 0.05)	1:400 (0.26 \pm 0.03)	1:800 (0.23 \pm 0.03)	1:200 (0.71 \pm 0.13)	1:400 (0.55 \pm 0.09)	1:800 (0.48 \pm 0.09)
Primiparous sows												
Serum												
1:200 (0.27 \pm 0.05)	NS											
1:400 (0.23 \pm 0.03)	NS											
1:800 (0.18 \pm 0.03)	NS											
Colostrum												
1:200 (0.49 \pm 0.12)	*											
1:400 (0.41 \pm 0.09)	NS											
1:800 (0.38 \pm 0.10)	NS											
Multiparous sows												
Serum												
1:200 (0.36 \pm 0.05)	NS											
1:400 (0.36 \pm 0.05)	NS											
1:800 (0.23 \pm 0.03)	NS											
Colostrum												
1:200 (0.71 \pm 0.13)	NS											
1:400 (0.55 \pm 0.09)	NS											
1:800 (0.48 \pm 0.09)	NS											

Table 3.2 Comparisons between mean \pm standard error optical density (OD) concentrations for the serum and colostrum collected from primiparous and multiparous sows vaccinated against *Mycoplasma hyopneumoniae* using a Student's paired t-test. Stars indicate level of significance: ***P< 0.001, **P< 0.01, *P< 0.05. NS: not significant.

Dilution (Mean \pm SE)	Primiparous sows						Multiparous sows					
	Serum			Colostrum			Serum			Colostrum		
	1:2 (0.50 \pm 0.06)	1:50 (1.01 \pm 0.02)	1:100 (1.07 \pm 0.02)	1:2 (0.36 \pm 0.06)	1:50 (0.87 \pm 0.03)	1:100 (0.93 \pm 0.02)	1:2 (0.58 \pm 0.06)	1:50 (1.06 \pm 0.02)	1:100 (1.10 \pm 0.02)	1:2 (0.40 \pm 0.05)	1:50 (0.94 \pm 0.02)	1:100 (0.97 \pm 0.02)
Primiparous sows												
Serum												
1:2 (0.50 \pm 0.06)	NS											
1:50 (1.01 \pm 0.02)	***	NS										
1:100 (1.07 \pm 0.02)	***	***	NS									
Colostrum												
1:2 (0.36 \pm 0.06)	*	***	***	NS								
1:50 (0.87 \pm 0.03)	***	***	***	***	NS							
1:100 (0.93 \pm 0.02)	***	***	***	***	**	NS						
Multiparous sows												
Serum												
1:2 (0.58 \pm 0.06)	NS	***	***	*	**	***	NS					
1:50 (1.06 \pm 0.02)	***	NS	NS	***	**	**	***	NS				
1:100 (1.10 \pm 0.02)	***	**	NS	***	***	***	***	NS	NS			
Colostrum												
1:2 (0.40 \pm 0.05)	NS	***	***	NS	***	***	***	***	***	NS		
1:50 (0.94 \pm 0.02)	***	NS	**	***	NS	NS	***	***	***	***	NS	
1:100 (0.97 \pm 0.02)	***	NS	**	***	**	NS	***	**	***	***	NS	NS

Short Communication: *Erysipelothrix rhusiopathiae* and *Mycoplasma hyopneumoniae*: The sensitivities of enzyme-linked immunosorbent assay for detecting vaccinated sows of unknown disease status using serum and colostrum, and the correlation of the results for sow serum, colostrum, and piglet serum

CJ Jenvey, MP Reichel and PD Cockcroft (2015)

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Caitlin J Jenvey		
Contribution to the Paper	Helped design animal trial, managed animal trial, collected samples, conducted sample analysis, data analysis and interpretation, drafted and edited manuscript, acted as corresponding author.		
Signature		Date	17/3/15

Name of Co-Author	Michael P Reichel		
Contribution to the Paper	Helped to design animal trial, helped to interpret data, helped to edit manuscript.		
Signature		Date	17/3/2015

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Contribution to the Paper	Secured project funding, helped to design animal trial, assisted with animal work and sample collection, helped interpret data, helped to edit manuscript.		
Signature		Date	17/03/2015

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

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Journal of Veterinary Diagnostic Investigation, v. 27 (2), pp. 211-216

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It is also available online to authorised users at:

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Chapter 4: Bovine Viral Diarrhoea Virus

In this chapter, a commercial ELISA for BVDV antibodies was assessed when using colostrum for two experimental trials. The aim of the first experiment, presented in Chapter 4.1, was to identify the performance characteristics of the ELISA using colostrum and serum collected from a BVD-vaccinated dairy herd in South Australia and a bulk-tank milk, antibody and PCR negative dairy herd in New Zealand. The aim of the second experiment, presented in Chapter 4.2, was to determine the diagnostic value of colostrum in ELISA for the detection of beef heifers carrying a persistently infected calf, following experimental infection.

4.1 Performance characteristics of ELISA to detect Bovine Viral Diarrhoea Virus (BVDV) antibodies in colostrum

Abstract

Colostrum contains substantially higher concentrations of immunoglobulins (Igs) when compared to serum or milk, which might improve the diagnostic sensitivity of an antibody ELISA when using colostrum. In this study, BVD was used as a model to identify the performance characteristics of colostrum and to assess the potential for increased ELISA sensitivity when compared to serum. Blood and colostrum samples were collected from cows within two dairy cattle herds; a previously infected and BVD-vaccinated Holstein-Friesian (positive herd) herd, and a bulk-tank milk antibody negative (negative herd) Jersey herd. All samples were tested using a commercial BVDV antibody ELISA. Median sample-to-positive (S/P) colostrum ratios were significantly higher than their respective serum counterparts, and positive herd S/P ratios were significantly higher than the respective negative herd values ($P < 0.001$). Using the manufacturer's recommended serum dilution (1:5) and colostrum dilution (undiluted), and a cut-off threshold S/P ratio of 0.2, diagnostic sensitivity (DSe) and diagnostic specificity (DSp) for colostrum was 100% and 70%, respectively. These values increased to 100% DSe and 100% DSp with an increase in cut-off threshold S/P to 0.5. At a sample dilution of 1:100, the DSe of colostrum was 90% and significantly higher compared to serum (DSe 17%). Colostrum has the potential to improve identification of previously infected animals, either individually, or when using pooled samples.

Introduction

The production of colostrum is a selective process. Maternal immunoglobulins (Igs) are transferred to the mammary gland, from the dam's bloodstream, prior to parturition and in the early post-partum period. The concentration of these Igs in cattle colostrum have been found to be five (Beer, et al. 1974) to ten times (Baumrucker, et al. 2010) higher than serum. Colostrum, therefore, has the potential to improve the sensitivity of ELISAs.

Bovine viral diarrhoea virus (BVDV) is a pestivirus in the family Flaviviridae and is considered one of the most economically important infectious diseases of cattle worldwide (Houe, et al. 2003; Gunn, et al. 2004). Infection with the virus may cause abortion and other clinical signs including fever and diarrhoea. Clinical severity is dependent upon the viral subtype but acute infections are often accompanied by mild clinical signs. The virus is economically detrimental to both the beef and dairy industries due to an 18-40% (McGowan and Kirkland 1995) increase in abortion rates and a 22% (Brownlie, et al. 1974) decrease in conception rates in infected cattle. Acutely infected animals mount an immune response and remain antibody positive for an extended period. Antibody (Ab)-ELISA is the test of choice for routine diagnosis of exposure to BVDV, as it is rapid, relatively inexpensive to perform and more suitable for large scale testing (Beaudeau, et al. 2001). The ELISA has previously been used, not only for the testing of serum (Durham and Hassard 1990; Gonzalez, et al. 2014; Lanyon, et al. 2014), but also, milk (Beaudeau, et al. 2001; Thobokwe, et al. 2004; Humphry, et al. 2012).

Although the testing of individual and bulk tank milk samples has been established, there is currently no research into whether colostrum would be superior to both serum and milk. Due to the high concentration of Igs found in colostrum, the testing of colostrum should improve the sensitivity of the Ab-ELISA and therefore increase confidence in the absence of disease, or improve identification of exposure at the herd level when using pooled samples. The collection and testing of serum and colostrum samples from a BVD vaccinated dairy herd and a BVD bulk-milk negative dairy herd was a 'proof-of-concept' study to determine the performance characteristics of a commercially available ELISA for BVDV antibodies when applied to colostrum, as compared to serum.

Materials and Methods

Animal selection

Positive herd. A Holstein-Friesian dairy herd was selected based upon the vaccination of the herd with the BVD vaccine Pestigard® (Zoetis), which had previously been infected with BVDV. The first 30 dairy cows (first calving heifers were not included) to calve during March to May 2012 were sampled.

Negative herd. A Jersey dairy herd was selected on the basis of three consecutive bulk-tank milk negative tests, over a 10 month period, prior to sample collection. The first 20 dairy cows to calve (first calving heifers were not included) during the period of August to October 2013 were sampled.

Owner informed consent and project approval was obtained from the University of Adelaide Animal Ethics Committee.

Sample collection and testing

Positive herd. The colostrum samples were collected within 12 hours of calving and the blood samples were collected from the coccygeal vein 30-60 days post calving. Blood samples were centrifuged for 10 minutes at 1,157g to separate the serum. All samples were stored at -80°C until testing was performed. The samples were then tested using a commercial BVDV Total Antibody ELISA (IDEXX). Individual samples were diluted (1:2, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:100) using the diluent supplied with the ELISA kit. All samples were tested in triplicate and results expressed as sample-to-positive (S/P) ratio.

Negative herd. Colostrum samples were collected within 24 hours of calving and prior to the first milking and the blood samples were collected from the coccygeal vein between 30-60 days post calving. The samples were tested by Gribbles Pathology (Palmerston North, NZ) using a commercial BVDV Total Antibody ELISA (IDEXX). Individual samples were tested as per the manufacturer's instructions for milk (undiluted) and serum (1:5 diluted), and expressed as sample-to-positive (S/P) ratio.

Statistical analyses

Frequency histograms and Q-Q plots determined that the data was non-normally distributed. Minimum, median and maximum Serum/Positive (S/P) ratios for all dilutions for serum and colostrum were used to create box-and-whisker plots (Microsoft Excel 2010). Wilcoxon signed rank test was used to identify any significant differences (R version 3.0.2). A ROC curve analysis was performed to determine DSe in relation to sample dilution for both serum and colostrum using the data from the positive herd.

Diagnostic sensitivity (DSe, from the positive herd) and diagnostic specificity (DSp; from the negative herd) were calculated by the following equations:

$$DSe = \frac{\text{Number infected/vaccinated cows testing serum positive}}{\text{Total number infected/vaccinated cows}} \times 100$$

$$DSp = \frac{\text{Number of bulk – tank negative cows testing serum negative}}{\text{Total number bulk – tank milk negative cows}} \times 100$$

Results

The median S/P ratio for colostrum collected from the positive herd was significantly higher when compared to the corresponding positive serum samples, at all dilutions ($P < 0.001$) (Table 4.1.1). The median S/P ratio for colostrum collected from the negative herd was significantly higher compared to negative serum ($P < 0.001$) (Table 4.1.1). Comparison of samples at a 1:5 dilution showed the median S/P ratio for the positive herd colostrum was significantly higher compared to negative herd colostrum and positive herd serum ($P < 0.001$); negative herd colostrum was significantly higher compared to negative herd serum ($P < 0.001$); positive herd colostrum was significantly higher compared to negative herd colostrum ($P < 0.001$); positive herd colostrum was significantly higher compared to negative herd serum ($P < 0.001$); positive herd serum was significantly higher compared to negative herd colostrum ($P < 0.001$); and positive herd serum was significantly higher compared to negative herd serum ($P < 0.001$) (Table 4.1.1).

The DSe of both serum and colostrum decreased with an increase in sample dilution. Serum DSe decreased more rapidly with an increase in dilution, reaching a DSe of just 17% at a sample dilution of 1:100 (Figure 4.1.1). The DSe of colostrum also decreased

with an increase in sample dilution, but did not fall below 90% (at 1:100 dilution) (Figure 4.1.1). Differences in DSe between serum and colostrum were statistically significant for dilutions 1:20, 1:40, 1:80 and 1:100 ($P < 0.05$; Figure 4.1.1).

Two-graph receiver operating characteristic (TG-ROC) analysis for the serum and colostrum samples is presented in Figure 4.1.2. The TG-ROC analysis indicates the specificity (using the negative herd data) and sensitivity (using the positive herd data) at various cut off S/P thresholds. Using the recommended serum dilution and cut-off S/P ratio of 0.2, both DSp and DSe for serum was 100% (Figure 4.1.2). Using undiluted colostrum and the same cut-off threshold, DSp and DSe was 70% and 100%, respectively (Figure 4.1.2). An increase in cut-off S/P to 0.5 increased colostrum DSp to 100%, while DSe for colostrum remained at 100% (Figure 4.1.2). From a cut-off S/P ratio of 0.5 and upwards, colostrum showed an increasingly and significantly higher DSe compared to serum ($P < 0.05$), whilst maintaining high DSp comparable to that of serum (Figure 4.1.2).

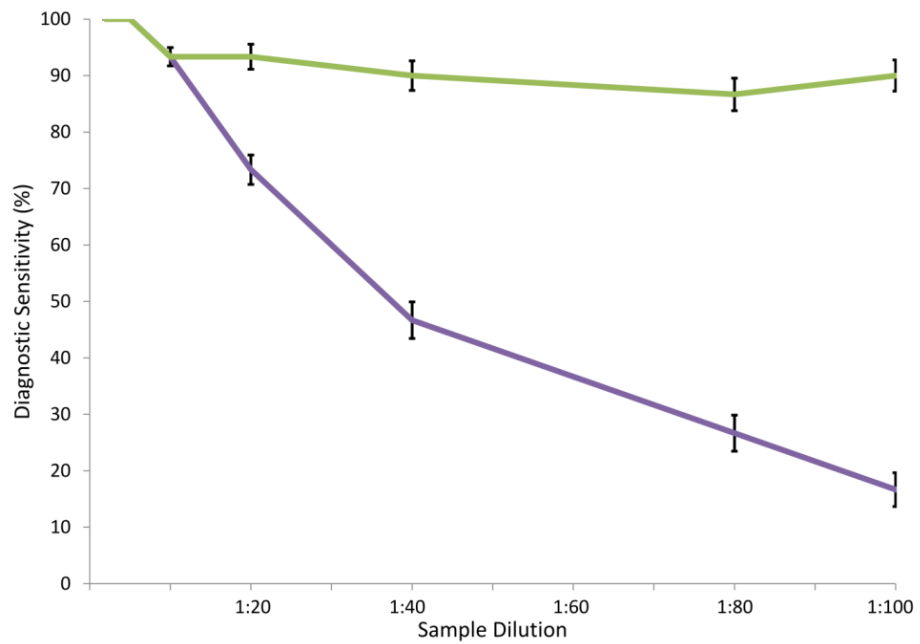


Figure 4.1.1 Diagnostic sensitivity (DSe) \pm 95% confidence intervals, expressed as a percentage, for serum (purple) and colostrum (green) collected from the positive herd (BVD vaccinated), diluted 1:2, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:100.

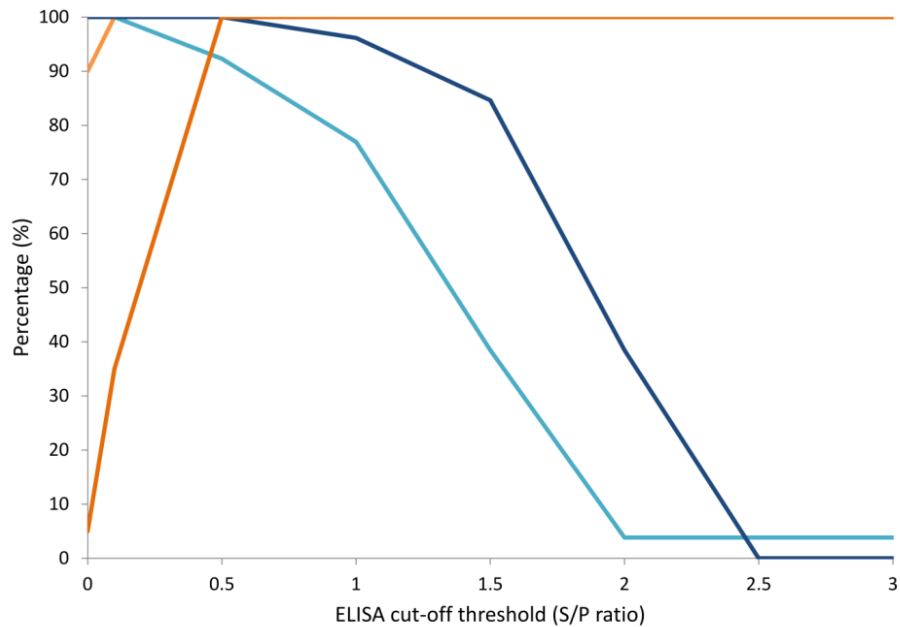


Figure 4.1.2 Two-graph receiver operating characteristic analysis of serum and colostrum using a commercial ELISA for the detection of antibodies specific to BVDV. Diagnostic sensitivity (serum- light blue; colostrum- dark blue) was calculated using sample-to-positive (S/P) ratio results from the positive herd (BVD vaccinated) and diagnostic specificity (serum- light orange; colostrum- dark orange) was calculated using sample-to-positive (S/P) ratio results from the negative herd (bulk-tank milk negative).

Discussion

In the current study, colostrum demonstrated a significantly higher signal (S/P ratios) and DSe compared to serum, in particular with an increase in sample dilution. For example, at a sample dilution of 1:100 the diagnostic sensitivity of the ELISA using serum samples was just 17% compared to 90% for colostrum at the same dilution. The DSe of colostrum did not fall below 90% for all the dilutions investigated. Although the cattle in the current study were vaccinated against BVDV, this vaccination occurred following a previous outbreak of BVDV in the milking herd; therefore, the ELISA S/P results in this study are likely to be higher than what would be found in herds that had only been vaccinated. When using pooled samples, colostrum would be able to detect a lower prevalence of BVDV exposure when compared to milk or serum.

Using a positive cut-off of ≥ 0.5 , the DSe and DSp of ELISA testing colostrum was 100%. Using the same commercial ELISA, a study by Weir and others (2013) recommended a decrease in the positive ELISA cut-off value from ≥ 0.3 to ≥ 0.13 when testing for BVDV using milk. When using the new milk cut-off value, the study found a relative sensitivity and specificity of 100% and 94%, respectively (Weir and others 2013). A similar study by Lanyon and others ('In press') found a relative sensitivity and specificity of 96.6% and 89.2%, respectively, which was lower than what was observed in the study by Weir and others (2013) but most likely due to the use of the unadjusted positive cut-off value of ≥ 0.3 . Both the studies by Weir and others (2013) and Lanyon and others ('In press') concluded that milk was an appropriate alternative to the testing of serum using ELISA, as the agreement between the two samples was high. However, in our study, colostrum has an even better performance than reported for milk or serum

with regards to the diagnostic sensitivity at higher dilutions due, most likely, to the higher concentrations of Igs in colostrum.

Although, in our study, we achieved similar performance characteristics for both serum and colostrum after the ELISA cut-off threshold had been adjusted, the DSe for colostrum continued to be significantly higher compared to serum when the samples are diluted. This indicates that in BVD monitoring programmes, particularly where eradication is under consideration, colostrum samples could provide an improved diagnostic utility compared to serum or milk samples when using the antibody ELISA on larger pools of samples.

This observation of improved test performance with colostrum compared to serum or milk with regards to sensitivity may also find application in the control of other important livestock diseases, such as *Mycobacterium bovis* (Bovine Tuberculosis), *Neospora caninum*, Leptospirosis and Johne's disease; both at an individual level and when using pooled samples. A study by Casal, et al. (2014) used serology for the diagnosis of bovine tuberculosis after intradermal skin testing. The study found that the serological response of animals infected with *Mycobacterium bovis* can vary dependent upon the infecting antigen and although serological tests for *M. bovis* have a high specificity, the sensitivity is quite variable, and has been reported to be between 18 and 73% (Casal, et al. 2014). *N. caninum* and Leptospirosis are the cause of significant numbers of abortions in cattle worldwide (Reichel and Pfeiffer 2002; Schares, et al. 2004; Picardeau 2013). A study which inoculated three groups of cattle with three *L. interrogans* serovars were found to be IgG and IgM positive at week 1 post inoculation,

and were still IgG positive 12 to 13 weeks later (Cousins, et al. 1985), however the IgM response was negative by 3 to 6 weeks post inoculation (Cousins, et al. 1985). Abortion is most likely to occur middle to late gestation, when this antibody response has declined (Hines 2014). The ELISA for the detection of *N. caninum* has both a high specificity and sensitivity (Reichel and Pfeiffer 2002) and the sensitivity has also shown to be improved when using milk samples compared to serum samples (Schaes, et al. 2004). However, *N. caninum* infection can only be detected in bulk-milk samples when there are at least 5-7.5% seropositive cows in the lactating herd (Chanlun, et al. 2002). The ELISA for detection of Johne's disease has high specificity, but sensitivity is low during the subclinical disease period, and has been shown to be as low as 8.9% when attempting to detect low MAP shedding animals (Reichel and others 1999). Recently, the testing of colostrum using ELISA was investigated by Zervens, et al. (2013) to assess the occurrence of non-specific reactions when testing colostrum for MAP-specific antibodies. Zervens, et al. (2013) found only 4% non-specific reactions when testing colostrum collected at 0 DIM (days-in-milk). The study concluded that since few non-specific reactions occur in the ELISA using colostrum samples, colostrum would be a useful diagnostic sample for the detection of MAP-specific IgG.

Conclusion

In conclusion, the increase in diagnostic sensitivity that may be able to be achieved when testing colostrum provides an opportunity to improve the identification of infected animals, either individually, or by using pooled samples. This observation of improved test performance using colostrum compared with serum or milk with regards to diagnostic sensitivity may also find application in the diagnosis of other important

(dairy) cattle diseases, such as *Mycobacterium bovis*, *Neospora caninum*, Leptospirosis and Johne's disease.

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Table 4.1.1 Comparisons between median serum and median colostrum sample-to-positive (S/P) ratios collected from the positive herd and the negative herd using a Wilcoxon signed rank test. Stars indicate level of significance: ***P<0.001, **P<0.01, *P<0.05. NS: not significant.

Dilution	Positive herd														Negative herd		
	Serum							Colostrum							Serum	Colostrum	
	1:2	1:5	1:10	1:20	1:40	1:80	1:100	0	1:2	1:5	1:10	1:20	1:40	1:80	1:100	1:5	0
Median (Range)	1.38 (0.44-4.41)	1.31 (0.36-3.34)	1.05 (0.03-2.80)	0.60 (-0.05-2.19)	0.28 (-0.07-1.13)	0.05 (-0.13-0.90)	0.05 (-0.15-0.84)	1.95 (0.74-2.25)	1.81 (0.42-4.75)	1.95 (0.21-4.76)	1.91 (0.08-4.48)	1.60 (-0.02-4.02)	1.41 (-0.15-3.36)	1.27 (-0.09-2.73)	1.21 (-0.09-2.69)	-0.03 (-0.10-0.05)	0.16 (-0.05-0.62)
Positive herd																	
Serum																	
1:2	NS																
1:5	NS																
1:10	***	***	NS														
1:20	***	***	NS	NS													
1:40	***	***	***	***	NS												
1:80	***	***	***	***	***	NS											
1:100	***	***	***	***	***	**	NS										
Colostrum																	
0	***	***	***	***	***	***	***	NS									
1:2	***	***	***	***	***	***	***	NS	NS								
1:5	***	***	***	***	***	***	***	NS	NS	NS							
1:10	***	***	***	***	***	***	***	NS	NS	*	NS						
1:20	*	*	***	***	***	***	***	NS	***	***	***	NS					
1:40	NS	NS	*	***	***	***	***	**	***	***	***	***	***	NS			
1:80	NS	NS	*	***	***	***	***	***	***	***	***	***	***	***	NS		
1:100	NS	NS	NS	***	***	***	***	***	***	***	***	***	***	**	NS		
Negative herd																	
Serum																	
1:5	***	***	***	***	***	**	NS	***	***	***	***	***	***	***	***	***	NS
Colostrum																	
0	***	***	***	**	NS	NS	NS	***	***	***	***	***	***	***	***	***	NS

Short Communication: Performance characteristics of ELISA to detect bovine viral diarrhoea virus (BVDV) antibodies in colostrum

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Contribution to the Paper	Secured project funding, helped to design animal trial, assisted with animal work and sample collection, helped interpret data, helped to edit manuscript.	
Signature		Date 17/03/2015

Performance Characteristics of ELISA to Detect Bovine Viral Diarrhea Virus (BVDV) Antibodies Using Colostrum

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Abstract

Colostrum contains substantially higher concentrations of immunoglobulins (Igs) when compared with serum or milk, which may improve the diagnostic sensitivity of an antibody ELISA when using colostrum. In this study, BVD was used as a model to identify the performance characteristics of colostrum and to assess the potential for increased ELISA sensitivity when compared with serum. Blood and colostrum samples were collected from cows within two dairy cattle herds: a previously infected and BVD-vaccinated Holstein-Friesian (positive herd) herd, and a bulk-tank milk antibody negative (negative herd) Jersey herd. All samples were tested using a commercial BVDV antibody ELISA. Median sample-to-positive (S/P) colostrum ratios were significantly higher than their respective serum counterparts, and positive herd S/P ratios were significantly higher than the respective negative herd values ($P < 0.001$). Using the manufacturer's recommended serum dilution (1:5) and colostrum dilution (undiluted), and a cut-off threshold S/P ratio of 0.2, diagnostic sensitivity (DSe) and diagnostic specificity (DSp) for colostrum were 100% and 70%, respectively. These values increased to 100% DSe and 100% DSp with an increase in cut-off threshold S/P to 0.5. At a sample dilution of 1:100, the DSe of colostrum was 90% and significantly higher compared with serum (DSe 17%). Colostrum has the potential to improve identification of previously infected animals, either individually, or when using pooled samples.

Keywords

BVDV, Colostrum, ELISA, Sensitivity, Specificity

1. Introduction

The production of colostrum is a selective process. Maternal immunoglobulins (Igs) are transferred to the

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mammary gland, from the dam's bloodstream, prior to parturition and in the early post-partum period. The concentration of these Igs in cattle colostrum has been found to be five [1] to ten times [2] higher than serum. Colostrum, therefore, has the potential to raise the analytical (as well as diagnostic) sensitivity of serological assays for the detection of antibodies against specific diseases.

Bovine viral diarrhoea virus (BVDV) is a pestivirus in the family Flaviviridae and is considered one of the most economically important infectious diseases of cattle worldwide [3] [4]. Infection with the virus may cause abortion and other clinical signs including fever and diarrhoea. Clinical severity is dependent upon the viral subtype, but acute infections are often accompanied by mild clinical signs. The virus is economically detrimental to both the beef and dairy industries due to an increase of 18% - 40% [5] in abortion rates and a 22% [6] decrease in conception rates in infected cattle. Acutely infected animals mount an immune response and remain antibody positive for an extended period. Antibody (Ab)-enzyme-linked immunosorbent assay (ELISA) is the test of choice for routine diagnosis of exposure to BVDV, as it is rapid, relatively inexpensive to perform and more suitable for large scale testing [7]. The ELISA has previously been used, not only for the testing of serum [8]-[10], but also milk [7] [11]-[13].

Although the testing of individual and bulk tank milk samples has been established, there is currently little research into whether colostrum will be superior to both serum and milk. A study by Jenvey *et al.* [14] demonstrated a significant increase in diagnostic sensitivity when comparing colostrum with serum in Johnes' disease vaccinated sheep. It is possible that using colostrum can also improve the sensitivity of a commercial BVDV Ab-ELISA and therefore increase confidence in the absence of disease, or improve identification of exposure at the herd level when using pooled samples. The collection and testing of serum and colostrum samples from a BVD vaccinated dairy herd and a BVD bulk-milk negative dairy herd were a "proof-of-concept" study to determine the performance characteristics of a commercially available ELISA for BVDV antibodies when applied to colostrum, as compared with serum.

2. Materials and Methods

2.1. Animal Selection

Positive herd. A Holstein-Friesian dairy herd was selected as the positive herd based upon a history of previous BVDV infection, as well as the vaccination of the herd with the BVD vaccine Pestigard® (Zoetis). The herd was vaccinated annually since 2009, and most recent vaccination was done in April 2012. Sample collection was performed in March to May 2012. The first 30 dairy cows to calve were sampled (excluding first calving heifers).

Negative herd. A Jersey dairy herd was selected as the negative herd based upon consistent low bulk tank milk antibody levels (*i.e.* herd was sampled 3 times per year for 6 years prior to sample collection and consistently recorded S/P ratio results < 0.25). In addition, a bulk tank milk antibody S/P ratio of 0.0 was recorded several months after sample collection had concluded, indicating that the herd was not exposed to BVDV at the time of sampling. Sample collection was performed in August to October 2013. The first 20 dairy cows to calve were sampled (excluding first calving heifers).

Owner informed consent and project approval was obtained from the University of Adelaide Animal Ethics Committee.

2.2. Sample Collection and Testing

Positive herd. The colostrum samples were collected within 12 hours of calving and the blood samples were collected from the coccygeal vein 30 - 60 days post calving. Blood samples were centrifuged for 10 minutes at 1157 g to separate the serum. All samples were stored at -80°C until testing was performed. The samples were then tested using a commercial BVDV Total Antibody ELISA (IDEXX). Individual samples were diluted (1:2, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:100) using the diluent supplied with the ELISA kit. All samples were tested in triplicate and results expressed as sample-to-positive (S/P) ratio.

Negative herd. Colostrum samples were collected within 24 hours of calving and prior to the first milking and the blood samples were collected from the coccygeal vein between 30 - 60 days post calving. The samples were tested by Gribbles Veterinary (Palmerston North, NZ) using a commercial BVDV Total Antibody ELISA (IDEXX). Individual samples were tested as per the manufacturer's instructions for milk (undiluted) and serum (1:5 diluted), and expressed as sample-to-positive (S/P) ratio.

2.3. Statistical Analysis

Frequency histograms and Q-Q plots determined that the data was non-normally distributed. A Wilcoxon signed rank test was used to identify any significant differences (R version 3.0.2). A receiver operating characteristic (ROC) curve analysis was performed to determine DSe in relation to sample dilution for both serum and colostrum using the data from the positive herd. A two graph-receiver operating characteristic (TG-ROC) analysis was performed for both colostrum and serum, for a range of S/P cut-off thresholds. Diagnostic sensitivity (DSe, from the positive herd) and diagnostic specificity (DSp; from the negative herd) were defined and calculated as follows:

$$\text{DSe} = \frac{\text{Number infected/vaccinated cows testing serum positive}}{\text{Total number infected/vaccinated cows}} \times 100$$

$$\text{DSp} = \frac{\text{Number of bulk tank milk negative cows testing serum negative}}{\text{Total number bulk tank milk negative cows}} \times 100$$

3. Results

The median S/P ratio for colostrum collected from the positive herd was significantly higher when compared to the corresponding positive serum samples, at all dilutions ($P < 0.001$) (Table 1). The median S/P ratio for colostrum collected from the negative herd was significantly higher compared to negative serum ($P < 0.001$) (Table 1). Comparison of samples at a 1:5 dilution showed the median S/P ratio for the positive herd colostrum was significantly higher compared to negative herd colostrum and positive herd serum ($P < 0.001$); negative herd colostrum was significantly higher compared to negative herd serum ($P < 0.001$); positive herd colostrum was significantly higher compared to negative herd colostrum ($P < 0.001$); positive herd colostrum was significantly higher compared to negative herd serum ($P < 0.001$); positive herd serum was significantly higher compared to negative herd colostrum ($P < 0.001$); and positive herd serum was significantly higher compared to negative herd serum ($P < 0.001$) (Table 1).

The DSe of both serum and colostrum decreased with an increase in sample dilution. Serum DSe decreased more rapidly with an increase in dilution, reaching a DSe of just 17% at a sample dilution of 1:100 (Figure 1). The DSe of colostrum also decreased with an increase in sample dilution, but did not fall below 90% (at 1:100 dilution) (Figure 1). Differences in DSe between serum and colostrum were statistically significant for dilutions 1:20, 1:40, 1:80 and 1:100 ($P < 0.05$; Figure 1).

Two-graph receiver operating characteristic (TG-ROC) analysis for the serum and colostrum samples is presented in Figure 2. The TG-ROC analysis indicates the specificity (using the negative herd data) and sensitivity (using the positive herd data) at various cut off S/P thresholds. Using the recommended serum dilution and cut-off S/P ratio of 0.2, both DSp and DSe for serum was 100% (Figure 2). Using undiluted colostrum and the same cut-off threshold, DSp and DSe was 70% and 100%, respectively (Figure 2). An increase in cut-off S/P to 0.5 increased colostrum DSp to 100%, while DSe for colostrum remained at 100% (Figure 2). From a cut-off S/P ratio of 0.5 and upwards, colostrum showed an increasingly and significantly higher DSe compared to serum ($P < 0.05$), whilst maintaining high DSp comparable to that of serum (Figure 2).

4. Discussion

Diagnostic sensitivity is defined as the percentage of subjects with a disease that are identified by the assay as positive for the disease [15]. This study does not attempt to identify true positive and true negative animals, but rather, it attempts to determine whether colostrum can improve the diagnostic sensitivity of an ELISA relative to serum samples. In this study, the use of colostrum as a sample demonstrated a significantly higher signal (S/P ratios) and DSe compared to serum, in particular with increasing sample dilution. Using a sample dilution of 1:100; the DSe of the ELISA when using colostrum samples compared to serum samples was 90% and 17%, respectively. The DSe of the ELISA when using colostrum did not fall below 90% for all the dilutions investigated. Although the cattle in the current study were vaccinated against BVDV, this vaccination occurred following a previous outbreak of BVDV in the milking herd; therefore, the ELISA S/P results in this study are higher than what would be found in herds that had only been vaccinated (Lanyon, 2014, personal communication). When using pooled samples, colostrum would be able to detect a lower prevalence of BVDV exposure when compared to milk or serum.

Table 1. Comparisons between median serum and median colostrum sample-to-positive (S/P) ratios collected from the positive herd and the negative herd using a Wilcoxon signed rank test. Stars indicate level of significance: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Dilution Median (Range)	Serum			Positive herd			Colostrum			Negative herd		
	S/P	95% CI	95% CI	S/P	95% CI	95% CI	S/P	95% CI	95% CI	S/P	95% CI	95% CI
1:2	1:5	1:10	1:40	1:100	0	1:2	1:10	1:20	1:40	1:80	1:100	1:200
1:38 (0.44-4.41)	1:31 (0.36-3.34)	1:05 (0.03-2.80)	0.28 (-0.07-1.13)	0.05 (-0.13-0.90)	1.95 (0.74-2.25)	1.81 (0.42-4.75)	1.94 (0.08-4.48)	1.60 (-0.02-4.02)	1.41 (-0.15-3.36)	1.24 (-0.09-2.73)	1.24 (-0.09-2.69)	1.24 (-0.05-0.62)
1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)	1:105 (0.03-2.80)
1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)	1:40 (-0.05-2.19)
0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)	0.28 (-0.07-1.13)
0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)	0.05 (-0.13-0.90)
1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)	1:100 (-0.15-0.84)
0	0	0	0	0	0	0	0	0	0	0	0	0
1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)	1:95 (0.74-2.25)
1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)	1:2 (0.42-4.75)
1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)	1:81 (0.42-4.75)
1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)	1:95 (0.21-4.76)
1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)	1:10 (0.08-4.48)
1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)	1:91 (-0.02-4.02)
1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)	1:20 (-0.15-3.36)
1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)	1:60 (-0.09-2.73)
1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)	1:40 (-0.15-3.36)
1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)	1:41 (-0.09-2.73)
1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)	1:96 (-0.09-2.73)
1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)	1:27 (-0.09-2.73)
1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)	1:100 (-0.09-2.69)
1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)	1:21 (-0.09-2.69)
0	0	0	0	0	0	0	0	0	0	0	0	0
1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)	1:5 (-0.10-0.05)
0	0	0	0	0	0	0	0	0	0	0	0	0
0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)	0.16 (-0.05-0.62)

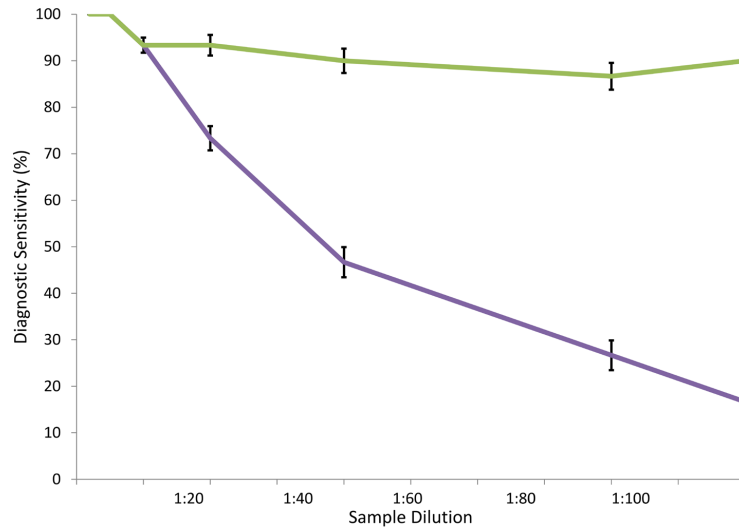


Figure 1. Diagnostic sensitivity (DSe) \pm 95% confidence intervals, expressed as a percentage, for serum (purple) and colostrum (green) collected from the positive herd (BVD vaccinated), diluted 1:2, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:100.

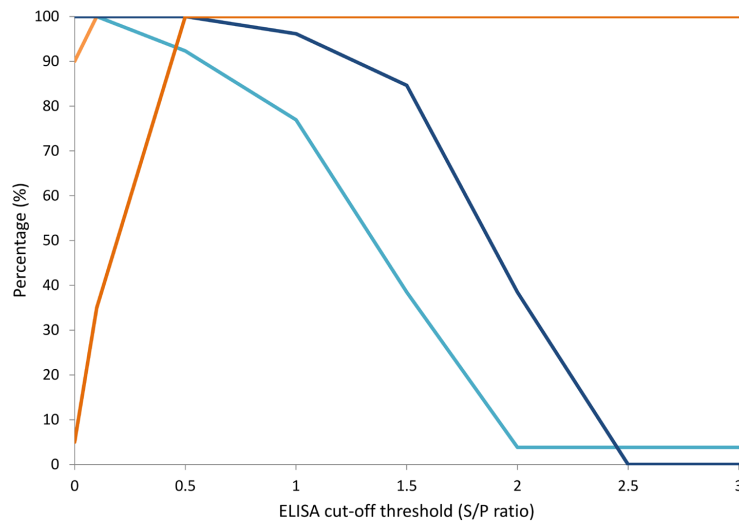


Figure 2. Two-graph receiver operating characteristic analysis of serum and colostrum using a commercial ELISA for the detection of antibodies specific to BVDV. Diagnostic sensitivity (serum: light blue; colostrum: dark blue) was calculated using sample-to-positive (S/P) ratio results from the positive herd (BVD vaccinated) and diagnostic specificity (serum: light orange; colostrums: dark orange) was calculated using sample-to-positive (S/P) ratio results from the negative herd (bulk tank ilk negative).

Using a positive cut-off of ≥ 0.5 , the DSe and DSp of ELISA testing colostrum was 100%. Using the same commercial ELISA, a study by Weir *et al.* [13] recommended a decrease in the positive ELISA cut-off value

from ≥ 0.3 to ≥ 0.13 when testing for BVDV using milk. When using the new milk cut-off value, the study found a relative sensitivity and specificity of 100% and 94%, respectively [13]. A similar study by Lanyon *et al.* [16] found a relative sensitivity and specificity of 96.6% and 89.2%, respectively, which was lower than what was observed in the study by Weir *et al.* [13] but most likely due to the use of the unadjusted positive cut-off value of ≥ 0.3 . Both the studies by Weir *et al.* [13] and Lanyon *et al.* [16] concluded that milk was an appropriate alternative to the testing of serum using ELISA, as the agreement between the results for both sample types was high. In our study, however, colostrum has an even better performance than reported for milk or serum with regards to the diagnostic sensitivity at higher dilutions due, most likely, to the higher concentrations of Igs in colostrum. Although, in our study, we achieved similar performance characteristics for both serum and colostrum after the ELISA cut-off threshold had been adjusted, the DSe for colostrum continued to be significantly higher compared to serum when the samples were diluted. This would suggest that in BVD monitoring programs, particularly where eradication is under consideration, colostrum samples could provide an improved diagnostic utility compared to serum or milk samples when using the antibody ELISA on larger pools of samples.

Recently, the testing of colostrum using ELISA was investigated by Zervens *et al.* [17] to assess the occurrence of non-specific reactions when testing colostrum for *Mycobacterium avium* subspecies *paratuberculosis* (MAP)-specific antibodies. Zervens *et al.* [17] found only 4% non-specific reactions when testing colostrum collected at 0 DIM (days-in-milk). The study concluded that since few non-specific reactions occur in the ELISA using colostrum samples, colostrum would be a useful diagnostic sample for the detection of MAP-specific IgG.

5. Conclusion

In conclusion, the increase in diagnostic sensitivity that may be able to be achieved when testing colostrum provides an opportunity to improve the identification of infected animals, either individually, or by using pooled samples. This observation of improved test performance using colostrum compared with serum or milk with regards to diagnostic sensitivity may also find application in the diagnosis of other important (dairy) cattle diseases, such as *Mycobacterium bovis* (Bovine Tuberculosis), *Neospora caninum*, Leptospirosis and John's disease.

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4.2 Optimising the measurement of colostrum antibody concentrations for identifying BVDV persistently infected calves

In the following experimental trial, a small herd of beef heifers were experimentally infected with BVDV-type 1c. The aim of this experimental trial was to determine the diagnostic value of colostrum when using ELISA for the detection of beef heifers carrying a persistently infected calf.

Short Communication: Optimising the measurement of colostrum antibody concentrations for identifying BVDV persistently infected calves

CJ Jenvey, MP Reichel, SR Lanyon and PD Cockcroft (2015)

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Contribution to the Paper	Collected samples, conducted sample analysis, data analysis and interpretation, drafted and edited manuscript, acted as corresponding author.	
Signature		Date 17/3/15

Name of Co-Author	Sasha R Lanyon	
Contribution to the Paper	Helped to design animal trial, managed animal trial, helped to interpret data, helped to edit manuscript.	
Signature		Date 19/3/2015

Name of Co-Author	Michael P Reichel	
Contribution to the Paper	Secured project funding, helped to design animal trial, helped to interpret data, helped to edit manuscript.	
Signature		Date 16/3/2015

Name of Co-Author	Peter D Cockcroft	
Contribution to the Paper	Helped to interpret data, helped to edit manuscript.	
Signature		Date 17/03/2015

Communication

Optimizing the Measurement of Colostrum Antibody Concentrations for Identifying BVDV Persistently Infected Calves

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Abstract: Colostrum contains substantially higher concentrations of immunoglobulins compared to serum, which may help to improve the utility of diagnostic tests. The aim of this study was to determine the diagnostic value of colostrum antibody concentrations in identifying Bovine Viral Diarrhoea Virus (BVDV) PI (persistently infected) calf carrying beef heifers following an experimental infection. Colostrum was collected within 12 hours of parturition and tested in undiluted, 1:5, 1:10, 1:100, 1:200, and 1:500 dilutions using an enzyme-linked immunosorbent assay (ELISA) for BVDV antibody. Cows were determined to be carrying a PI calf based on positive quantitative Real Time-Polymerase Chain Reaction and antigen ELISA result on pre-colostral serum and ear notch samples collected from their calf. The median ELISA sample-to-positive (S/P) ratio for colostrum collected from heifers that carried a PI calf were significantly higher than the median ELISA S/P ratio for colostrum collected from heifers that did not carry a PI calf at dilutions of 1:100, 1:200, and 1:500. This study provides further evidence for increased antigenic stimulation *in utero* by the BVDV viraemic PI calf, which can also be identified with 100% diagnostic sensitivity when using 1:500 dilution colostrum.

Keywords: BVDV; colostrum; ELISA; PI calves

1. Introduction

Colostrum contains high concentrations of maternal antibodies that are selectively transferred into the mammary gland from the serum of the dam prior to calving. Concentrations of antibodies in bovine colostrum can be up to five [1] to 10 times [2] greater when compared to serum. Due to these high antibody concentrations, colostrum could improve the utility of diagnostic tests by increasing the magnitude of detectable differences between cohorts of individual animals, including, but not limited to, cows infected with Bovine Viral Diarrhoea Virus (BVDV). Dams that are infected with BVDV between 40 and 120 days of gestation may produce persistently infected (PI) offspring that test negative for BVDV-specific antibodies [3]. These PI animals are important reservoirs of infection due to their high persistent levels of viral excretion and infectivity for susceptible cattle [4]. There is evidence to suggest that the BVDV antibody concentration of dams carrying PI calves are elevated during late gestation when compared to previously infected dams carrying non-PI calves [5–7], but this difference has not been convincing enough to be utilised in practice (early detection of PI calves). These studies found that, on average, the antibody levels of cows carrying PI calves and cows not carrying PI calves tended to overlap, and although samples should be collected in the last few months of gestation, there is larger variation in antibody levels between these two groups at parturition compared with early pregnancy [5–7]. There is little research into whether this increase in BVD specific antibody concentrations may also be found in colostrum collected from dams carrying PI calves. It is possible that concentrations found in colostrum may be quantitatively greater compared to serum, thus also allowing identification of the PI-carrying dam using colostrum. The aim of this study was to determine the diagnostic value of colostrum BVDV antibody concentrations in identifying PI calves following an experimental infection of a group of beef heifers.

2. Methods

2.1. Heifers

Twelve beef heifers were experimentally infected with BVDV-type 1c by co-mingling with a PI dairy cow from day 90, post artificial insemination [8]. All cows seroconverted within 28 days of exposure (antibody enzyme-linked immunosorbent assay), at which time the PI cow was removed from further direct or indirect contact with the heifers. Heifers were determined to have been carrying a PI calf based on positive quantitative real-time polymerase chain reaction and antigen ELISA result on serum and ear notch samples collected from their calf prior to colostrum ingestion.

2.2. Antibody ELISA

Colostrum was collected within 12 hours of calving and stored at -80°C until testing could be performed. The colostrum samples were tested as undiluted samples, as well as at dilutions of 1:5, 1:10, 1:100, 1:200, and 1:500. Samples were diluted using the diluent supplied with the ELISA kit and tested in triplicate using the IDEXX BVDV Total Ab Test. Results were expressed as sample-to-positive (S/P) ratios.

2.3. Statistical Analysis

A Mann-Whitney U test was performed to identify significant differences between colostrum from heifers carrying PI calves and heifers that did not carry PI calves at each dilution, with a p -value < 0.01 considered significant. A two-graph receiver operating characteristic analysis (TG-ROC) was performed to determine DSe, DS_p, and 95% confidence interval (CI) of the antibody ELISA for the detection of a PI calf for each dilution, or no dilution. The diagnostic sensitivity (DSe) and diagnostic specificity (DS_p) were calculated using the following equations:

$$DSe = \frac{\text{Number of heifers carrying PI calves that tested ELISA positive}}{\text{Number of heifers carrying PI calves}} \times 100 \quad (1)$$

$$DSp = \frac{\text{Number of heifers carrying non-PI calves that tested ELISA negative}}{\text{Number of heifers carrying non-PI calves}} \times 100 \quad (2)$$

3. Results

Of the twelve beef heifers that were experimentally infected, the calves of three heifers tested virus positive by RT-PCR and antigen positive by ELISA in their serum prior to colostrum ingestion. The calves from the remaining eight heifers tested virus negative and antigen negative and one heifer aborted her fetus 253 days post-AI. The median ELISA S/P ratio for colostrum collected from heifers carrying a PI calf were significantly higher compared to colostrum collected from heifers that were not carrying a PI calf for dilutions of 1:100 ($p < 0.05$), 1:200 ($p < 0.01$), and 1:500 ($p < 0.01$) (Table 1). For undiluted and 1:5 diluted colostrum, the median ELISA S/P ratio for colostrum samples collected from heifers carrying and not carrying PI calves did not differ significantly (Table 1).

Table 1. Median (Range) optical density value for colostrum collected from cows carrying and not carrying PI calves for dilutions of 0, 1:5, 1:100, 1:200, and 1:500.

Dilution	Heifers carrying PI calf	Heifers not carrying PI calf
0	2.08 (1.56–2.13) ^a	2.22 (1.63–2.52) ^a
1:5	2.55 (2.58–2.51) ^b	2.55 (2.38–2.66) ^b
1:100	2.21 (2.31–2.15) ^c	1.81 (1.14–2.19) ^d
1:200	1.98 (2.07–1.89) ^e	1.50 (0.85–1.85) ^f
1:500	1.81 (1.83–1.67) ^g	1.00 (0.37–1.60) ^h

^a Different superscript letters in the same row are statistically different (Mann-Whitney U Test).

The TG-ROC for DSe and DS_p for each dilution are shown in Figure 1. The value of the DSe and DS_p at the intercept increased with the dilution of the sample, reaching 100% at a colostrum dilution of 1:500. The DSe, DS_p, and S/P threshold at the intercept for each dilution were: 33% at an S/P ratio threshold of 2.10 for undiluted colostrum (Figure 1a), 56% at a 1:5 dilution colostrum at an S/P ratio threshold of 2.60 (Figure 1b), 93.9% at a 1:100 dilution colostrum at an S/P ratio threshold of 2.20 (Figure 1c), 93.9% at a 1:200 dilution colostrum at an S/P ratio threshold of 1.90 (Figure 1d), and 100% at a 1:500 dilution colostrum at an S/P ratio threshold of 1.65 (Figure 1e).

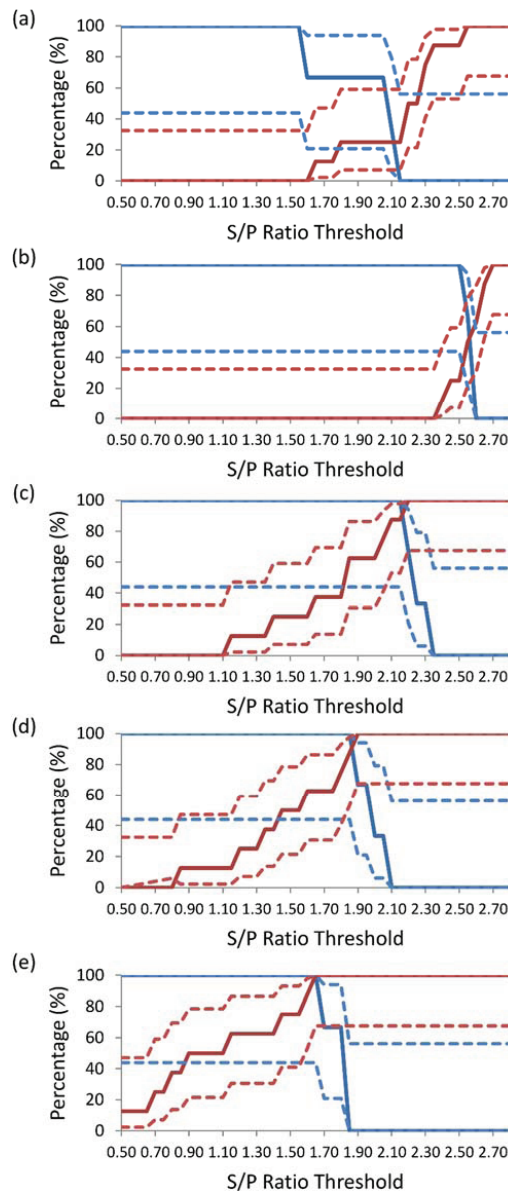


Figure 1. Two-graph receiver operating characteristic analysis of (a) undiluted colostrum, (b) 1:5 dilution colostrum, (c) 1:100 dilution colostrum, (d) 1:200 dilution colostrum, and (e) 1:500 dilution colostrum, using a commercial enzyme-linked immunosorbent assay for the detection of antibodies specific to BVDV. Diagnostic sensitivity (solid blue line) \pm 95 percent confidence interval (dotted blue line) was calculated using sample-to-positive (S/P) ratio results for colostrum collected from heifers carrying PI calves ($n = 3$) and diagnostic specificity (red line) \pm 95 per cent confidence interval (dotted red line) was calculated using S/P ratio results for colostrum collected from heifers carrying non-PI calves ($n = 8$).

4. Discussion

The purpose of this study was to determine the diagnostic value of colostrum BVDV antibody concentrations in identifying PI calves following an experimental infection of beef heifers. Previous studies [5–7] have demonstrated higher BVDV antibody concentrations in serum collected from seropositive cows carrying PI calves when compared to cows that do not carry a PI calf. However, there is little research into whether this same claim can be made with regards to colostrum samples. These previous studies also demonstrated some overlapping of antibody levels from cows that carried a PI calf and cows that did not carry a PI calf [5–7]. This result was also demonstrated in this study as no significant differences were observed in the median ELISA S/P ratios for heifers that carried a PI calf and heifers that did not carry a PI calf when colostrum was tested undiluted or diluted 1:5. However, the median ELISA S/P ratio for colostrum collected from heifers that carried a PI calf were significantly higher compared to the median ELISA S/P ratio for colostrum collected from heifers that did not carry a PI calf for dilutions of 1:100, 1:200 and 1:500. In addition, DSe and DSp of the ELISA was 100% when using colostrum diluted at 1:500. The results of this study indicate that colostrum, when diluted at 1:500, could be used to identify PI calves immediately following calving. It is possible that further dilution of the serum collected in these previous studies (1:10 [6] and 1:100 [6,7]) may have further increased the differences in antibody levels observed between cows that carried a PI calf and cows that did not carry a PI calf, thus, reducing the overlapping of antibody levels observed between the two groups.

This study provides further evidence for increased antigenic stimulation *in utero* by the BVDV viraemic PI calf. This study does not make any recommendations regarding the replacement of other PI detection methods with colostrum antibody testing, but rather, this study highlights the fact that colostrum can be used for PI calf detection and may be used in conjunction with other methods of detection.

5. Conclusions

The improvement in DSe observed in this study when colostrum was diluted, highlights the ability to improve the diagnostic utility of a test when disease-specific antibody concentrations (*i.e.*, BVDV antibody concentrations) supersede the ability of the ELISA to measure beyond the maximal S/P threshold. Additionally, colostrum may also benefit tests that demonstrate poor test characteristics, such as the poor diagnostic sensitivity experienced when testing for Johne's disease. This approach, while tested here in beef heifers, could most readily be applied in dairy cows, where colostrum is routinely and easily collected by the producer.

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Author Contributions

Caitlin J. Jenvey is a PhD candidate from the University of Adelaide and was involved in sample collection, data analysis and writing the manuscript. Michael P. Reichel and Sasha R. Lanyon designed the experimental plan and assisted with data analysis and manuscript editing. Peter D. Cockcroft was the primary supervisor and assisted with data analysis and manuscript editing.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 5: Johne's disease

In this chapter, a commercial ELISA for the detection of *Mycobacterium avium* subspecies *paratuberculosis* (Johne's disease) was assessed when using colostrum compared to serum for two experimental trials. The aim of the first experiment, presented in Chapter 5.1, was to investigate the sensitivity of the ELISA when using colostrum, whey and serum samples collected from vaccinated ewes. The aim of the second experiment, presented in Chapter 5.2, was to determine whether colostrum could improve the diagnostic accuracy of the ELISA when compared to serum, collected from a Jersey dairy herd with a history of previous infection. Faecal culture and the HT-J PCR were also compared.

5.1 Investigation of the comparative diagnostic sensitivity of serum, colostrum and whey for the detection of specific antibodies in a flock of South Australian Merino-cross ewes vaccinated against Johne's disease

Abstract

Johne's disease is characterised by a long incubation period and the sensitivity of serological tests against *Mycobacterium avium* subspecies *paratuberculosis* (MAP) are low during the subclinical stage of infection. Due to the relatively high concentration of immunoglobulins present in colostrum, testing of this sample might improve diagnostic test sensitivity and detection of infected animals. Blood, colostrum and whey samples were analysed from 26 vaccinated Merino-cross ewes and were tested for antibodies against MAP by indirect enzyme-linked immunosorbent assay (ELISA). At the recommended dilution for serum (1:20), the diagnostic sensitivity (DSe) of colostrum and whey were significantly higher compared to serum, but not significantly different from each other. The significant improvement in DSe when using colostrum compared to serum suggests that it might be possible to detect a greater proportion of infected animals using this sample.

Introduction

Johne's disease is an enteric and chronic wasting disease of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease is characterised by a long-subclinical incubation period, followed by a clinical period resulting in decreased production and severe economic losses to the livestock industries (Benedictus, et al. 1987; Johnson-Ifeorunlu, et al. 1997). During the subclinical incubation period, infected animals shed MAP in their faeces. However, generally low levels of MAP are excreted during this period and the sensitivity of diagnostic tests used to diagnose Johne's disease is also low during this period. The enzyme-linked immunosorbent assay (ELISA) is one type of diagnostic test that can be used for detection of MAP. The ELISA is rapid and low-cost (Hendrick, et al. 2005) and is therefore more suitable for large-scale testing. The ELISA can be used for the testing of samples of serum (Reichel, et al. 1999; Stabel, et al. 2001; Hendrick, et al. 2005; Lombard, et al. 2006; Goodridge, et al. 2013; Angelidou, et al. 2014), plasma (Milner, et al. 1990; Goodridge, et al. 2013), or milk (Hendrick, et al. 2005; Lombard, et al. 2006; Sorge, et al. 2010; Angelidou, et al. 2014; Lavers, et al. 2014; Nielsen, et al. 2014).

In low, medium and high MAP shedding animals, the sensitivity of a commercially available ELISA for detection of MAP in cattle serum was 8.9%, 47.1% and 75.0%, respectively (Reichel, et al. 1999). It is apparent that the sensitivity of the ELISA for Johne's disease is low when testing low MAP shedding animals, but it is possible that the testing of colostrum may improve the sensitivity of the ELISA, particularly for the detection of low MAP shedding animals. The production of colostrum is a selective process, where maternal immunoglobulins are transferred to the mammary gland prior

to parturition. Concentrations of immunoglobulins in cattle colostrum have been found to be five (Beer, et al. 1974) to ten times (Baumrucker, et al. 2010) greater than serum, while concentrations in sheep colostrum can be up to three times greater (Campbell, et al. 1977). Colostrum consists of two main components; the casein (curds) and the liquid whey. The whey contains the majority of the immunoglobulins (Marnila and Korhonen 2011), therefore its separation from colostrum may further improve ELISA sensitivity. The testing of colostrum using ELISA has only recently been investigated to assess the occurrence of non-specific reactions when testing colostrum for MAP-specific antibodies (Zervens, et al. 2013). It is possible that absorbance readings for ELISA may be affected by the opacity of colostrum. A study by Zervens, et al. (2013) found that non-specific reactions accounted for only 4% of the binding in the ELISA when colostrum was tested, thus suggesting that this sample was suitable for detection of MAP-specific IgG. Separation of the whey from the colostrum might also improve test sensitivity, as the majority of the immunoglobulins are contained within this fraction (Marnila and Korhonen 2011).

The inactivated Ovine Johne's disease vaccine, Gudair®, has been used by Australian sheep producers since 2002 and is highly regulated, as the serological ELISA has difficulty distinguishing between vaccinal antibodies and antibodies elicited by MAP infections. The present study was performed as proof-of-concept to investigate the sensitivity of an ELISA when using colostrum. Samples from vaccinated sheep were used to model the antibody status of animals exposed to the pathogen, comparing use of colostrum, whey or serum samples, on the diagnostic sensitivity of a commercially available ELISA for detection of MAP-specific IgG.

Materials and methods

Animal selection

Twenty six pregnant Merino-cross ewes were selected based on the presence of a JD vaccine ('V') ear tag, indicating that these animals were vaccinated with the JD vaccine, Gudair[®] (Pfizer, West Ryde, NSW) within their first six months of life. All the ewes were selected and sampled, following informed owner's consent and approval of the study protocol by the University of Adelaide Animal Ethics Committee (S-2012-045).

Sample collection and whey separation

Sample collection was performed from March to May 2012. The blood samples were collected (from the jugular vein) two weeks prior to the predicted lambing date and the colostrum was collected within 12 hours of lambing. The blood samples were centrifuged for 10 mins at 1,157g to separate the serum. The whey was extracted by incubating colostrum as a 1:4 dilution with vegetarian rennet (200 IMCU/mL) (Cheeselinks, Little River, VIC), which had been pre-diluted 1:50 with R/O water. The incubation was performed in a water bath at 37 °C for 60 mins. All samples were stored at -80 °C until testing was performed.

Antibody ELISA

The samples of serum, colostrum and whey were tested using the Parachek[®] 2 ELISA kit (Prionics AG, Switzerland), which uses *M.phlei* pre-absorption for the specific detection of MAP-specific IgG. All samples were diluted (1:2, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:100) using the diluent supplied with the ELISA kit. Each sample was tested in triplicate using 100mcl aliquots. Absorbance (optical density; OD) was measured at 250 nm (Benchmark Plus Microplate Spectrophotometer, BioRad) and the values for

each sample determined using the recommended endpoint detection protocol for ovine samples (mean negative control + 0.2).

Statistical analysis

Frequency histograms and Q-Q plots determined the data was non-normally distributed. Minimum, median and maximum OD values for all dilutions and sample values for all dilutions and sample types were used to create box-and-whisker plots using Microsoft Excel 2010. A Wilcoxon signed rank test was used to identify any significant differences using the statistical program R (version 3.0.2).

Diagnostic sensitivity (DSe) \pm 95% CI was determined for each dilution, for each sample type, using the following equation:

$$\text{DSe} = \frac{\text{Number of positive animals detected}}{\text{Total number of animals}} \times 100$$

Results

Median optical density (OD) values for both whey and colostrum were significantly higher than serum, at all dilutions ($P < 0.01$) (Table 5.1.1). At the recommended dilution for serum of 1:20, the OD range for colostrum and whey were wider compared to serum (Figure 5.1.1). There was no significant difference between median OD for colostrum and whey (Table 5.1.1).

The diagnostic sensitivity (DSe) for colostrum and whey was significantly higher than for serum ($P < 0.05$) (Figure 5.1.2). At the recommended dilution for serum (1:20), the DSe of colostrum and whey were 77% and 81%, respectively. The DSe of serum was 23% (Figure 5.1.2). As sample dilution increased, the DSe of colostrum and whey

remained significantly higher than serum, and the DSe of colostrum remained significantly higher than whey for dilutions 1:10, 1:40 and 1:100 (Figure 5.1.2). At a sample dilution of 1:100, colostrum had a significantly higher DSe (35%) compared to both serum (8%) and whey (4%) (Figure 5.1.2).

Table 5.2.1 Median OD values of anti-MAP IgG for serum, whey and colostrum collected from 26 vaccinated Merino-cross ewes.

Sample Dilution	Serum	Whey	Colostrum
1:2	2.23 ^a	3.58 ^b	3.52 ^b
1:5	0.45 ^a	2.54 ^b	2.39 ^b
1:10	0.33 ^a	1.51 ^b	1.70 ^b
1:20	0.29 ^a	0.79 ^b	0.72 ^b
1:40	0.26 ^a	0.43 ^b	0.58 ^b
1:80	0.26 ^a	0.31 ^b	0.37 ^b
1:100	0.26 ^a	0.28 ^b	0.29 ^b

^{a,b} Different superscript letters in the same row are statistically different ($P < 0.001$).

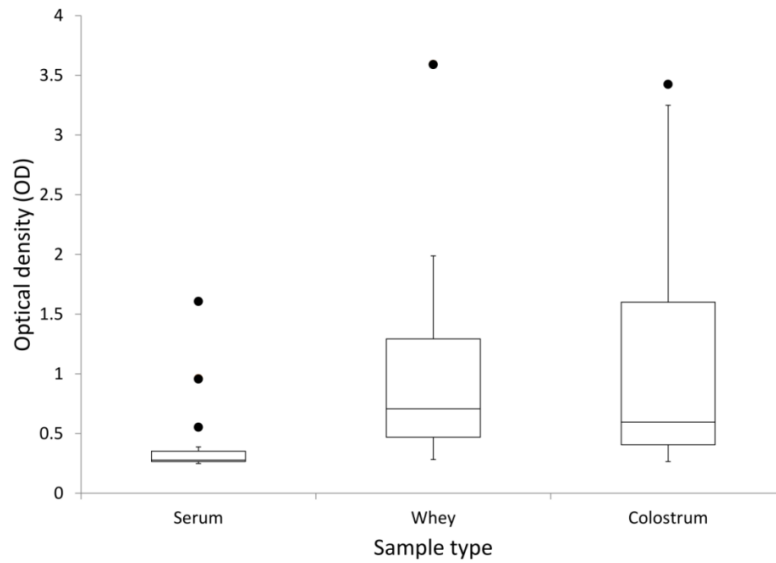


Figure 5.1.1 Boxplots for serum (median 0.12, range 0.05-1.42), whey (median 0.59, range 0.08-3.39) and colostrum (median 0.54, range 0.07-3.22) diluted 1:20 collected from 26 Merino-cross ewes, measured in optical density (OD).

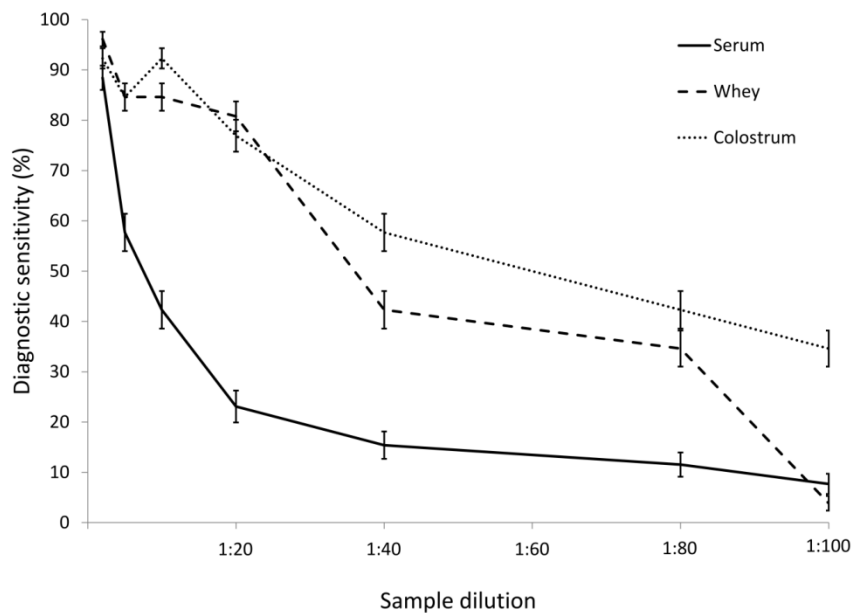


Figure 5.1.2 Diagnostic sensitivity (DSe) \pm 95% confidence intervals plotted against sample dilution for serum, whey and colostrum collected from 26 Merino-cross ewes.

Discussion

The DSe of the Parachek® 2 ELISA for detection of MAP-specific antibodies was significantly increased when testing colostrum and whey samples when compared to serum. A similar study by Hendrick, et al. (2005) in dairy cattle found milk to have a reduced sensitivity compared to serum. The sensitivity of milk remained reduced when cattle were split into low, medium and high shedding categories. Lombard, et al. (2006) found no difference in the relative sensitivities of milk and serum when compared to different shedding levels, but the ability of both the milk and serum ELISAs to detect cows shedding MAP increased as MAP shedding level increased. In the current study, colostrum always maintained a higher DSe when compared to serum, regardless of dilution. At a sample dilution of 1:100, colostrum was able to correctly identify 35% of the vaccinated ewes, a significantly higher DSe compared to both serum and whey. A study by Zervens, et al. (2013) showed the estimated pre-calving prevalence of MAP in milk to be 11%, which increased when testing colostrum to 97% on the calving date. The results of the current study, and in that of Zervens, et al. (2013), are likely due to the relatively high concentrations of immunoglobulins found in colostrum around parturition (Marnila and Korhonen 2011).

This study was also designed to determine whether whey would have higher test sensitivity when compared to colostrum. Although the whey contains a higher concentration of Igs than colostrum (Marnila and Korhonen 2011), a dilution effect caused by the rennet incubation procedure caused the whey to record OD results slightly lower and not significantly different than colostrum. The use of a 1:4 dilution was to promote the thorough mixing of the rennet with the colostrum sample. Instead, this procedure caused dilution of the Igs in the colostrum by as much as 20%. This

eliminated any improvement in DSe that may have been provided by testing whey. In retrospect, the 1:50 pre-dilution of the rennet, in addition to the 1:4 dilution of the rennet with colostrum, may not be required to promote thorough mixing and curd formation. However, a study by Van Brandt, et al. (2009) added 0.03% rennet to create colostrum whey and found that the ELISA readings for colostrum and colostrum whey corresponded perfectly; therefore it is possible that a significant difference in Ig concentration is not achievable when comparing colostrum and whey.

For sheep with confirmed MAP infection, based on histopathology of jejunum, colon and associated mesenteric lymph nodes (Hope, et al. 2000), the median MAP ELISA OD value for serum samples has been reported to be 0.183 (J. Gwozdz, personal communication), which is similar to the median value for serum (0.12), but much lower than the median value for colostrum (0.72) reported in the current study (Table 1; dilution 1:20). These results highlight the benefits of testing colostrum, as animals with equivocal ELISA results with serum could potentially exhibit a stronger signal using colostrum, thereby improving test sensitivity. At a 1:100 sample dilution, colostrum was able to identify 35% of vaccinated ewes, which was a significantly greater proportion compared to both serum and whey. The specificity was not determined as this study was a proof-of-concept to investigate sensitivity only. If no improvement in ELISA sensitivity could be demonstrated by this study, there would be no use in also investigating the specificity of the ELISA when using colostrum. Now that the potential improvements in DSe when using colostrum have been established, a full evaluation of DSe and DSp, based on naturally (or experimentally, or both) infected animals can now be investigated.

Conclusion

Using the recommended dilution for serum, the DSe of colostrum and whey were significantly higher compared to serum, but not significantly different from each other. If these study findings can be replicated in MAP-infected sheep and cattle, it might enable a greater proportion of animals with subclinical infection to be identified. Pooled colostrum samples would potentially be superior to pooled serum or milk samples for herd surveillance, and monitoring for Johne's disease presence and prevalence. It would be necessary to repeat this study, comparing different commercial ELISAs to determine whether sensitivity remains high when using colostrum in a variety of assays.

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Short Communication: Investigation of the comparative diagnostic sensitivity of serum, colostrum and whey for the detection of specific antibodies in a flock of South Australian Merino-cross ewes vaccinated against Johne's disease

CJ Jenvey, MP Reichel and PD Cockcroft (2015)

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Caillin J Jenvey		
Contribution to the Paper	Helped to design animal trial, managed animal trial, collected samples, conducted sample analysis, data analysis and interpretation, drafted and edited manuscript, acted as corresponding author.		
Signature		Date	17/3/15

Name of Co-Author	Michael P Reichel		
Contribution to the Paper	Helped to design animal trial, helped to interpret data, helped to edit manuscript.		
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5.2 The diagnostic performance of an antibody enzyme-linked immunosorbent assay using serum and colostrum to determine the disease status of a Victorian Jersey dairy herd infected with *Mycobacterium avium* subspecies *paratuberculosis*

Abstract

Colostrum may have the ability to improve the diagnostic accuracy of some tests when compared to serum for important livestock diseases due to the high concentrations of immunoglobulins present within this sample type. The enzyme-linked immunosorbent assay (ELISA) for Johne's disease is one such test, as it suffers from low sensitivity when testing serum samples collected during the subclinical stage of infection. Blood and colostrum samples were collected from 34 Jersey dairy cows and tested for antibodies against *Mycobacterium avium* subspecies *paratuberculosis* (MAP) by ELISA. Faecal samples were also collected and tested by a high throughput-Johne's polymerase chain reaction (HT-J PCR) and faecal culture (FC), which was used as the reference test. The diagnostic accuracy (DA) and area under the curve (AUC) were calculated. The HT-J PCR and faecal culture results were also compared. Of the 34 cows in this study, 4 had FC results consistent with MAP infection. The HT-J PCR did not identify any FC positive cows. Using a 1:20 dilution and S/P ratio cut-off threshold of 0.15, both serum (DA 0.88; AUC 0.44) and colostrum (DA 0.85; AUC 0.37) were unable to identify any of the FC positive cows. With a decrease in sample dilution, serum was still unable to identify any FC positive cows, but colostrum was able to identify 3/4 FC positive cows at a dilution of 1:5 and S/P ratio cut-off threshold of 0.15. The resulting DA and AUC values for colostrum were 0.88 and 0.73, respectively.

Colostrum may provide improved identification of animals in the early stages of infection with MAP.

Introduction

Due to the selective transfer of maternal immunoglobulins into colostrum prior to parturition, colostrum contains higher concentrations of immunoglobulins than serum. Concentrations of immunoglobulins in cow colostrum can be up to five (Beer, et al. 1974) to 10 times (Baumrucker, et al. 2010) greater when compared to serum. The high concentrations of immunoglobulins found in colostrum should improve the utility of diagnostic tests by improving the sensitivity, provided specificity is maintained. Such an improvement would be useful for diseases such as Johne's disease, where infected animals shed low numbers of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) during the subclinical stage of infection. Animals initially respond with a strong cell-mediated immune (CMI) response, rather than a humoral immune response (Stabel 2000). Although the enzyme-linked immunosorbent assay (ELISA) is a relatively inexpensive and easy-to-perform tool for the diagnosis of Johne's disease, it suffers from poor test sensitivity during the subclinical stage of infection (Reichel, et al. 1999), as the ELISA measures the humoral response to infection. There is some evidence to suggest that colostrum could improve diagnostic test sensitivity. A previous study (Jenvey, et al. 2015) has demonstrated a significant increase in diagnostic sensitivity when comparing colostrum and serum in sheep vaccinated against Johne's disease. The aim of this study was to determine whether colostrum could achieve a better diagnostic accuracy when compared to serum for the detection of MAP-specific antibodies using a commercial ELISA in a commercial dairy herd.

Materials and Methods

Herd selection and sample collection

A dairy herd of Jersey cows located in the state of Victoria, Australia was selected for testing. This herd was classified by the Department of Environment and Primary Industries as infected following confirmatory tests for Johne's disease in the recent past. Samples of colostrum, serum and faeces were collected from 34 Jersey cows, which ranged in age from 2 to 7 years old. The samples of colostrum were collected within 12 hours of parturition and the serum and faecal samples were collected 30-60 days post parturition. The samples of colostrum were initially stored at -20°C until all colostrum samples had been collected. This was then followed by storage of the colostrum and serum samples at -80°C until testing of both colostrum and serum samples could be performed together.

Faecal culture and HT-J PCR

The faecal samples were tested using faecal culture (reference test) (Gwozdz 2010) and high throughput-Johne's polymerase chain reaction (HT-J PCR) assay (Gwozdz 2013) which were performed by the Biosciences Research Division (Department of Environment and Primary Industries, Bundoora, Victoria). Cows were determined to be infected with MAP based upon a positive faecal culture result.

Antibody ELISA

The serum and colostrum samples were tested using a MAP antibody ELISA (Parachek 2, Prionics). All samples were diluted 1:2, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:100 using the diluent supplied with the ELISA kit. Each sample was tested in triplicate using 100µl aliquots. Absorbance (optical density; OD) was measured at 250nm (Benchmark Plus Microplate Spectrophotometer, BioRad). The results were expressed as sample-to-positive (S/P) ratios.

Statistical analysis

Various measures of diagnostic accuracy were calculated using the diagnostic sensitivity and diagnostic specificity were calculated at each of 6 S/P ratio cut-off thresholds (0.04, 0.05, 0.1, 0.15, 0.2 and 0.25). These measures of diagnostic accuracy were generated as per the following:

$$\text{Diagnostic sensitivity (DSe)} = \frac{\text{number of faecal culture (FC) positive cows that tested ELISA positive}}{\text{total number of FC positive cows}}$$

$$\text{Diagnostic specificity (DSp)} = \frac{\text{number of faecal culture (FC) negative cows that tested ELISA negative}}{\text{total number of FC negative cows}}$$

$$\text{Area Under the Curve (AUC)} = \frac{U}{\text{\# of FC positive cows} + \text{\# of FC negative cows}}$$

Where U =

$$\frac{\text{\# FC positive cows} \times \text{\# FC negative cows} + \text{\# FC negative cows} \times (\text{\# FC negative cows} + 1)}{2 - R}$$

Where R = the rank sum of the negative samples

(CMDT version 1.0; URL: <http://www.cmdt.f2s.com>)

$$\text{Overall Diagnostic Accuracy (DA)} = \frac{\text{\# of FC positive cows} + \text{\# of FC negative cows}}{\text{total \# of cows}}$$

$$= \frac{DSe}{1 - DSe}$$

Likelihood Ratio Positive (LR+)

$$\text{Likelihood Ratio Negative (LR-)} = \frac{1 - \text{DSp}}{\text{DSp}}$$

$$\text{Odd's Ratio (OR)} = \frac{\text{LHR+}}{\text{LHR-}}$$

$$\text{Youden's index} = \text{DSe} + \text{DSp} - 1$$

Results

Faecal culture and HT-J PCR

Using faecal culture as the reference test, faecal samples from 4 cows (12%) had results consistent with MAP infection, and were determined to be positive, while faecal samples from 30 cows (88%) had no results consistent with MAP infection and were determined to be negative. Faecal culture and the HT-J PCR showed concordant results for 88% of the samples, with both tests determining the same 30 cows to be negative. The HT-J PCR did not detect any cows as having results consistent with MAP.

Numbers of FC positive and negative, area under the curve and diagnostic accuracy

Using the manufacturer's recommended dilution for serum of 1:20 and recommended S/P ratio cut-off threshold of 0.15, the number of FC positive cows and the number of FC negative cows were 0 and 30, respectively, with an associated DA of 0.88 and AUC of 0.44 (Table 5.2.1). With a decrease in serum dilution to 1:5 and S/P ratio cut-off threshold of 0.15, the number of FC positive cows and the number of FC negative cows were also 0 and 30, respectively, with an associated DA of 0.88 and AUC of 0.45 (Table 5.2.1). With a further decrease in serum dilution to 1:2 and same S/P ratio cut-off threshold of 0.15, the number of FC positive and FC negative cows was the same as

dilution 1:5, but with a slightly lower DA of 0.82 and slightly higher AUC of 0.62 (Table 5.2.1).

Using the 1:20 dilution and S/P ratio cut-off threshold of 0.15, the number of FC positive cows and the number of FC negative cows for colostrum were 0 and 29, respectively, with an associated DA of 0.85 and AUC of 0.37 (Table 5.2.1). With a decrease in colostrum dilution to 1:5 and S/P ratio cut-off threshold of 0.15, the number of FC positive cows and the number of FC negative cows were 3 and 27, respectively, with an associated DA of 0.88 and AUC of 0.73 (Table 5.2.1). Using the manufacturer's recommended S/P ratio cut-off threshold for milk of 0.1 and dilution 1:5, the number of FC positive cows and the number of FC negative cows were 3 and 20, respectively, with an associated DA of 0.68 (Table 5.2.1).

Likelihood ratios, Odds ratio and Youden's index

Using the manufacturer's recommended dilution for serum of 1:20 and S/P ratio cut-off threshold of 0.15, the associated LR+, LR-, Odds ratio and Youden's index for serum was 0, 1, 0 and 0, respectively (Table 5.2.2). The values for LR+, LR-, Odds ratio and Youden's index did not change dramatically with changes in dilution and S/P ratio cut-off threshold.

Using the 1:20 dilution and S/P ratio cut-off threshold of 0.15, the associated LR+, LR-, Odds ratio and Youden's index for colostrum was 0, 1.03, 0 and 0, respectively (Table 5.2.2). With a decrease in colostrum dilution to 1:5, the associated LR+, LR-, Odds ratio and Youden's index were 7.50, 0.28, 27 and 0.65, respectively (Table 5.2.2).

Discussion

Faecal culture was used as the reference test for the faecal samples in this study and compared to the HT-J PCR assay. In this study, only 4 of the 34 cows had FC results consistent with MAP infection; however the HT-J PCR determined all animals tested in this study to be negative. The HT-J PCR was originally based upon research performed using direct quantitative PCR, where 8/13 experimentally infected FC positive sheep, 24/40 MAP exposed FC negative sheep, and 68/69 MAP exposed FC positive sheep tested positive by PCR (Kawaji, et al. 2007). More recently, a study which investigated the HT-J PCR using Australian cattle and sheep populations found that in 870 cattle, 111 were FC positive and 124 were HT-J PCR positive, while in 507 sheep, 111 were FC positive and 117 were HT-J PCR positive (Plain, et al. 2014). However, the study also attempted to identify faecal samples which were known to contain very low numbers of MAP, as the identification of subclinically infected animals is of particular importance in regards to maintenance of infection. The study found that replicate qPCR assays had variable detection rates when using samples that contained low MAP numbers (Plain, et al. 2014). The study also showed that the agreement between the HT-J PCR and BACTEC culture decreased with decreasing numbers of MAP in the original sample, as culture was performed on a separate aliquot to what was used for HT-J PCR (Plain, et al. 2014). It is possible that the low numbers of FC positive cows identified in this study was due to the presence of subclinical infection in the study herd, therefore the faecal samples tested contained very low numbers of MAP, reducing the identification of infected animals. As such, the HT-J PCR has been recommended as a flock and herd test, rather than individual animal testing (Plain, et al. 2014).

Using the recommended S/P ratio cut-off threshold and serum dilution of 1:20, both serum and colostrum were unable to identify any of the FC positive cows. It has been well-established that the dilution of serum at 1:20 is optimal for the detection of MAP by ELISA (Yokomizo, et al. 1985; Milner, et al. 1990). However, this study demonstrated that serum at this dilution was no more diagnostically accurate than colostrum, and the AUC score for both samples at this dilution were deemed 'not informative' (Greiner, et al. 2000). Also, with a decrease in sample dilution to 1:5, serum was also unable to identify any FC positive cows. As mentioned previously, it is likely that the herd selected for testing in this study was subclinically infected. During subclinical infection, animals respond with a strong cell-mediated immune (CMI) response. As the disease progresses, the CMI response decreases and is replaced by a strong humoral immune response (Stabel, 2000). Therefore, as the ELISA is detecting specific-antibody (humoral immune response), the detection of subclinically infected animals is impaired.

However, using a 1:5 dilution, colostrum identified 3/4 FC positive cows and 27/30 FC negative cows. Although both serum and colostrum had the same DA (0.88), this improvement in the identification of FC positive and FC negative cows was reflected in the AUC, as the AUC for colostrum was higher compared to serum and deemed 'moderately accurate' (Greiner, 2000). Also, the LR+ value for colostrum at this dilution and S/P ratio cut-off threshold indicates that animals that test ELISA positive are 7.5 times more likely to be infected with MAP, as well as being 0.28 times less likely that a negative ELISA result will be observed in animals infected with MAP (Simundic, 2008). Additionally, the odds ratio and Youden's index for colostrum at this dilution and 0.15 S/P ratio cut-off threshold indicates that the discriminative power of

the ELISA is increased. Considering the potential subclinical status of the herd tested in this study, it is possible that colostrum may provide improved identification of animals in the early stages of MAP infection when compared to serum. However, the results warrant further investigation due to the small sample size used in this study.

Conclusion

In conclusion, using the recommended serum dilution of 1:20, colostrum was no more diagnostically accurate than serum for the identification of FC positive cows. However, colostrum was able to identify 3/4 FC positive cows when using a 1:5 dilution, which was reflected with a 'moderately accurate' AUC score. Colostrum may provide improved identification of animals in the early stages of MAP infection. Considering the small sample size of this study, further research testing samples from herds where MAP is more highly prevalent is warranted.

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Table 5.2.1. Number of faecal culture positive (FC +) and faecal culture negative (FC -) cows testing positive and negative in serum (S) and colostrum (C) by enzyme-linked immunosorbent assay (ELISA) based upon S/P ratio cut-off thresholds of 0.04, 0.05, 0.1, 0.15, 0.2 and 0.25, and the associated diagnostic accuracy (DA) and area under the curve (AUC).

Dilution		AUC	S/P Ratio Cut-off Threshold																	
			0.04			0.05			0.1			0.15			0.2			0.25		
			FC +	FC -	DA	FC +	FC -	DA	FC +	FC -	DA	FC +	FC -	DA	FC +	FC -	DA	FC +	FC -	DA
1:2	S	0.62	0/4	26/30	0.76	0/4	27/30	0.79	0/4	28/30	0.82	0/4	28/30	0.82	0/4	29/30	0.85	0/4	30/30	0.88
	C	0.73	4/4	3/30	0.21	3/4	3/30	0.18	3/4	9/30	0.35	3/4	12/30	0.44	3/4	18/30	0.62	3/4	20/30	0.68
1:5	S	0.45	0/4	29/30	0.85	0/4	29/30	0.85	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.73	3/4	14/30	0.5	3/4	15/30	0.53	3/4	20/30	0.68	3/4	27/30	0.88	1/4	28/30	0.85	1/4	28/30	0.85
1:10	S	0.44	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.48	1/4	20/30	0.62	1/4	22/30	0.68	1/4	28/30	0.85	1/4	28/30	0.85	1/4	28/30	0.85	0/4	28/30	0.82
1:20	S	0.44	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.37	1/4	27/30	0.82	1/4	28/30	0.85	0/4	28/30	0.82	0/4	29/30	0.85	0/4	29/30	0.85	0/4	29/30	0.85
1:40	S	0.46	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.31	0/4	29/30	0.85	0/4	29/30	0.85	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
1:80	S	0.48	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.36	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
1:100	S	0.46	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.38	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88

Table 5.2.2. Likelihood ratio positive (LR+) and negative (LR-), Odds ratio (ODD) and Youden's index (YOU) calculated for serum (S) and colostrum (C) collected from faecal culture positive and negative cows testing positive and negative by ELISA based upon S/P ratio cut-off thresholds of 0.04, 0.05, 0.1, 0.15, 0.2 and 0.25.

Dilution		S/P Ratio Cut-off Threshold																							
		0.04				0.05				0.1				0.15				0.2				0.25			
		LR+	LR-	ODD	YOU	LR+	LR-	ODD	YOU	LR+	LR-	ODD	YOU	LR+	LR-	ODD	YOU	LR+	LR-	ODD	YOU	LR+	LR-	OD	YOU
1:2	S	0	1.16	0	0	0	1.11	0	0	0	1.07	0	0	0	1.03	0	0	0	1.03	0	0	0	1	0	0
	C	1.11	0	0	0.10	0.83	2.5	0.33	0	1.07	0.83	1.29	0.05	1.25	0.63	2	0.15	1.88	0.42	4.5	0.35	2.25	0.38	6	0.42
1:5	S	0	1.03	0	0	0	1.03	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
	C	1.41	0.54	2.63	0.22	1.5	0.50	3.00	0.25	2.25	0.38	6	0.42	7.50	0.28	27	0.65	3.75	0.80	4.67	0.18	3.75	0.80	4.67	0.18
1:10	S	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
	C	0.75	1.13	0.66	0	0.94	1.02	0.92	0	3.75	0.8	4.67	0.18	3.75	0.80	4.67	0.18	3.75	0.80	4.67	0.18	0	1.07	0	0
1:20	S	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
	C	2.5	0.83	3	0.15	3.75	0.80	4.66	0	0	1.07	0	0	0	1.03	0	0	0	1.03	0	0	0	1.03	0	0
1:40	S	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
	C	0	1.03	0	0	0	1.03	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
1:80	S	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
	C	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
1:100	S	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0
	C	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0

Short Communication: The diagnostic performance of an antibody enzyme-linked immunosorbent assay using serum and colostrum to determine the disease status of a Victorian Jersey dairy herd infected with *Mycobacterium avium* subspecies *paratuberculosis*

CJ Jenvey, MP Reichel and PD Cockcroft (2015)

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Contribution to the Paper	Helped to design animal trial, managed animal trial, conducted sample analysis, data analysis and interpretation, drafted and edited manuscript, acted as corresponding author.		
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Short Communication

The diagnostic performance of an antibody enzyme linked immunosorbent assay using serum and colostrum to determine the disease status of a Victorian Jersey dairy herd infected with *Mycobacterium avium* subspecies *paratuberculosis*

Caitlin J. Jenvey, Michael P. Reichel and Peter D. Cockcroft

Abstract. Colostrum may have the ability to improve the diagnostic accuracy of some tests when compared to serum for important livestock diseases due to the high concentrations of immunoglobulins present within this sample type. The enzyme-linked immunosorbent assay (ELISA) for Johne's disease is one such test, as it suffers from low sensitivity when testing serum samples collected during the subclinical stage of infection. Blood and colostrum samples were collected from 34 Jersey dairy cows and tested for antibodies against *Mycobacterium avium* subspecies *paratuberculosis* (MAP) by ELISA. Faecal samples were also collected and tested by a high throughput-Johne's polymerase chain reaction (HT-J PCR) and faecal culture (FC), which was used as the reference test. The diagnostic accuracy (DA) and area under the curve (AUC) were calculated. The HT-J PCR and faecal culture results were also compared. Of the 34 cows in this study, 4 had FC results consistent with MAP infection. The HT-J PCR did not identify any FC positive cows. Using a 1:20 dilution and S/P ratio cut-off threshold of 0.15, both serum (DA 0.88; AUC 0.44) and colostrum (DA 0.85; AUC 0.37) were unable to identify any of the FC positive cows. With a decrease in sample dilution, serum was still unable to identify any FC positive cows, but colostrum was able to identify 3/4 FC positive cows at a dilution of 1:5 and S/P ratio cut-off threshold of 0.15. The resulting DA and AUC values for colostrum were 0.88 and 0.73, respectively.

Colostrum may provide improved identification of animals in the early stages of infection with MAP.

Due to the selective transfer of maternal immunoglobulins into colostrum prior to parturition, colostrum contains higher concentrations of immunoglobulins than serum. Concentrations of immunoglobulins in cow colostrum can range from five² to 10 times¹ greater when compared to serum. The higher concentrations of immunoglobulins found in colostrum should improve the utility of diagnostic tests by improving the sensitivity, provided specificity is maintained. Such an improvement would be useful for diseases such as Johne's disease, where infected animals shed low numbers of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) during the subclinical stage of infection. Animals initially respond with a strong cell-mediated immune (CMI) response, rather than a humoral immune response.¹¹ Although the enzyme-linked immunosorbent assay (ELISA) is a relatively inexpensive and easy-to-perform tool for the diagnosis of Johne's disease, it suffers from poor test sensitivity during the subclinical stage of infection¹⁰, as the ELISA measures the humoral response to infection. There is some evidence to suggest that colostrum could improve diagnostic test sensitivity. A previous study⁶ has demonstrated a significant increase in diagnostic sensitivity when comparing colostrum and serum in sheep vaccinated against Johne's disease. The aim of this study was to determine whether colostrum could achieve a better diagnostic accuracy when compared to serum for the detection of MAP-specific antibodies using a commercial ELISA in a commercial dairy herd.

A dairy herd of Jersey cows located in the state of Victoria, Australia was selected for testing. This herd was classified by the Department of Environment and Primary

Industries as infected following confirmatory tests for Johne's disease in the recent past. Samples of colostrum, serum and faeces were collected from 34 Jersey cows, which ranged in age from 2 to 7 years old. The samples of colostrum were collected within 12 hours of parturition and the serum and faecal samples were collected 30-60 days post parturition. The samples of colostrum were initially stored at -20°C until all colostrum samples had been collected. This was then followed by storage of the colostrum and serum samples at -80°C until testing of both colostrum and serum samples could be performed together.

The faecal samples were tested using faecal culture (reference test)⁴ and high throughput-Johne's polymerase chain reaction (HT-J PCR) assay⁵, which were performed by the Biosciences Research Division (Department of Environment and Primary Industries, Bundoora, Victoria). Cows were determined to be infected with MAP based upon a positive faecal culture result. The serum and colostrum samples were tested using a MAP antibody ELISA^a. All samples were diluted 1:2, 1:5, 1:10, 1:20, 1:40, 1:80, and 1:100 using the diluent supplied with the ELISA kit. Each sample was tested in triplicate using 100µl aliquots. Absorbance (optical density; OD) was measured at 250 nm^b. The results were expressed as sample-to-positive (S/P) ratios.

An area under the curve (AUC) analysis was performed for each sample and each dilution (CMDT version 1.0; URL: <http://www.cmdt.f2s.com>), while diagnostic accuracy (DA) was performed for each sample and each S/P cut-off threshold (0.04, 0.05, 0.1, 0.15, 0.2 and 0.25).

Area under the curve (AUC) and diagnostic accuracy (DA) were generated as per the following:

$$\text{Area Under the Curve (AUC)} = \frac{U}{\# \text{ of FC positive cows} + \# \text{ of FC negative cows}}$$

Where U =

$$\frac{\# \text{ FC positive cows} \times \# \text{ FC negative cows} + \# \text{ FC negative cows} \times (\# \text{ FC negative cows} + 1)}{2 - R}$$

Where R = the rank sum of the negative samples

$$\text{Diagnostic Accuracy (DA)} = \frac{\# \text{ of FC positive cows} + \# \text{ of FC negative cows}}{\text{total \# of cows}}$$

Using faecal culture as the reference test, faecal samples from 4 cows (12%) had results consistent with MAP infection, and were determined to be positive, while faecal samples from 30 cows (88%) had no results consistent with MAP infection and were determined to be negative. Faecal culture and the HT-J PCR showed concordant results for 88% of the samples, with both tests determining the same 30 cows to be negative. The HT-J PCR did not detect any cows as having results consistent with MAP.

Using the manufacturer's recommended dilution for serum of 1:20 and recommended S/P ratio cut-off threshold of 0.15, the number of FC positive cows and the number of FC negative cows were 0 and 30, respectively, with an associated DA of 0.88 and AUC of 0.44 (Table 1). With a decrease in serum dilution to 1:5 and S/P ratio cut-off threshold of 0.15, the number of FC positive cows and the number of FC negative cows were also 0 and 30, respectively, with an associated DA of 0.88 and AUC of 0.45 (Table 1). With a further decrease in serum dilution to 1:2 and same S/P ratio cut-off threshold of 0.15, the number of FC positive and FC negative cows was the same as dilution 1:5, but with a slightly lower DA of 0.82 and slightly higher AUC of 0.62 (Table 1).

Using the 1:20 dilution and S/P ratio cut-off threshold of 0.15, the number of FC positive cows and the number of FC negative cows for colostrum were 0 and 29, respectively, with an associated DA of 0.85 and AUC of 0.37 (Table 1). With a decrease

in colostrum dilution to 1:5 and S/P ratio cut-off threshold of 0.15, the number of FC positive cows and the number of FC negative cows were 3 and 27, respectively, with an associated DA of 0.88 and AUC of 0.73 (Table 1). Using the manufacturer's recommended S/P ratio cut-off threshold for milk of 0.1 and dilution 1:5, the number of FC positive cows and the number of FC negative cows were 3 and 20, respectively, with an associated DA of 0.68 (Table 1).

Faecal culture was used as the reference test for the faecal samples in this study and compared to the HT-J PCR assay. In this study, only 4 of the 34 cows had FC results consistent with MAP infection; however the HT-J PCR determined all animals tested in this study to be negative. The HT-J PCR was originally based upon research performed using direct quantitative PCR, where 8/13 experimentally infected FC positive sheep, 24/40 MAP exposed FC negative sheep, and 68/69 MAP exposed FC positive sheep tested positive by PCR.⁷ More recently, a study which investigated the HT-J PCR using Australian cattle and sheep populations found that in 870 cattle, 111 were FC positive and 124 were HT-J PCR positive, while in 507 sheep, 111 were FC positive and 117 were HT-J PCR positive.⁹ However, the study also attempted to identify faecal samples which were known to contain very low numbers of MAP, as the identification of subclinically infected animals is of particular importance in regards to maintenance of infection. The study found that replicate qPCR assays had variable detection rates when using samples that contained low MAP numbers.⁹ The study also showed that the agreement between the HT-J PCR and BACTEC culture decreased with decreasing numbers of MAP in the original sample, as culture was performed on a separate aliquot to what was used for HT-J PCR.⁹ It is possible that the low numbers of FC positive cows identified in this study was due to the presence of subclinical infection in the study herd, therefore the faecal samples tested contained very low numbers of MAP, reducing

the identification of infected animals. As such, the HT-J PCR has been recommended as a flock and herd test, rather than individual animal testing.⁹

Using the recommended S/P ratio cut-off threshold and serum dilution of 1:20, both serum and colostrum were unable to identify any of the FC positive cows. It has been well-established that the dilution of serum at 1:20 is optimal for the detection of MAP by ELISA.^{8,12} However, this study demonstrated that serum at this dilution was no more diagnostically accurate than colostrum, and the AUC score for both samples at this dilution were deemed 'not informative'.³ Also, with a decrease in sample dilution to 1:5, serum was also unable to identify any FC positive cows. As mentioned previously, it is likely that the herd selected for testing in this study was subclinically infected. During subclinical infection, animals respond with a strong cell-mediated immune (CMI) response. As the disease progresses, the CMI response decreases and is replaced by a strong humoral immune response.¹¹ Therefore, as the ELISA is detecting specific-antibody (humoral immune response), the detection of subclinically infected animals is impaired.

However, using a 1:5 dilution, colostrum identified 3/4 FC positive cows and 27/30 FC negative cows. Although both serum and colostrum had the same DA (0.88), this improvement in the identification of FC positive and FC negative cows was reflected in the AUC, as the AUC for colostrum was higher compared to serum and deemed 'moderately accurate'.³ Considering the potential subclinical status of the herd tested in this study, it is possible that colostrum may provide improved identification of animals in the early stages of MAP infection when compared to serum. However, the results warrant further investigation due to the small sample size used in this study.

In conclusion, using the recommended serum dilution of 1:20, colostrum was no more diagnostically accurate than serum for the identification of FC positive cows. However, colostrum was able to identify 3/4 FC positive cows when using a 1:5 dilution, which was reflected with a ‘moderately accurate’ AUC score. Colostrum may provide improved identification of animals in the early stages of MAP infection. Considering the small sample size of this study, further research testing samples from herds where MAP is more highly prevalent is warranted.

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- a. Parachek 2, Prionics
- b. Benchmark Plus Microplate Spectrophotometer, BioRad

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Table 1. Number of faecal culture positive (FC +) and faecal culture negative (FC -) cows testing positive and negative in serum (S) and colostrum (C) by enzyme-linked immunosorbent assay (ELISA) based upon S/P ratio cut-off thresholds of 0.04, 0.05, 0.1, 0.15, 0.2 and 0.25, and the associated diagnostic accuracy (DA) and area under the curve (AUC).

Dilution	AUC	S/P Ratio Cut-off Threshold																		
		0.04			0.05			0.1			0.15			0.2			0.25			
		FC +	FC -	DA	FC +	FC-	DA	FC +	FC -	DA	FC +	FC -	DA	FC +	FC -	DA	FC +	FC -	DA	
1:2	S	0.62	0/4	26/30	0.76	0/4	27/30	0.79	0/4	28/30	0.82	0/4	28/30	0.82	0/4	29/30	0.85	0/4	30/30	0.88
	C	0.73	4/4	3/30	0.21	3/4	3/30	0.18	3/4	9/30	0.35	3/4	12/30	0.44	3/4	18/30	0.62	3/4	20/30	0.68
1:5	S	0.45	0/4	29/30	0.85	0/4	29/30	0.85	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.73	3/4	14/30	0.5	3/4	15/30	0.53	3/4	20/30	0.68	3/4	27/30	0.88	1/4	28/30	0.85	1/4	28/30	0.85
1:10	S	0.44	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.48	1/4	20/30	0.62	1/4	22/30	0.68	1/4	28/30	0.85	1/4	28/30	0.85	1/4	28/30	0.85	0/4	28/30	0.82
1:20	S	0.44	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.37	1/4	27/30	0.82	1/4	28/30	0.85	0/4	28/30	0.82	0/4	29/30	0.85	0/4	29/30	0.85	0/4	29/30	0.85
1:40	S	0.46	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.31	0/4	29/30	0.85	0/4	29/30	0.85	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
1:80	S	0.48	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.36	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
1:100	S	0.46	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88
	C	0.38	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88	0/4	30/30	0.88

Chapter 6: Discussion and Conclusions

Many important diseases of production animals cause significant economic losses for producers. It is important that diagnostic tests are able to accurately identify infected animals, thereby reducing the spread of infection and maintaining biosecurity status. However, for diseases such as Johne's disease, swine erysipelas and enzootic pneumonia, detection of infected animals is often difficult due to subclinical stages of the disease, product of a weak humoral immune response. Although a number of reference (gold standard) tests are available for the detection of these diseases, which represent the current and best test available, these tests are often imperfect, labour intensive and expensive to perform. The ELISA is highly suited to the surveillance and monitoring of disease within livestock populations for the detection of humoral antibodies. It is usually relatively inexpensive in comparison to the reference test, is easy to perform and can be performed quickly. However, in some diseases, the ELISA can also suffer from poor test sensitivity due to the weak antigenic stimulation. However, disease-specific humoral antibodies can be found in a range of sample types including serum, plasma, saliva, milk and colostrum. Although colostrum is routinely collected in the dairy industry, it has not previously been utilised as a sample type for the detection of disease using ELISA. Colostrum contains relatively higher concentrations of Igs compared to serum, which can be up to 10 times greater in concentration in cattle. This thesis hypothesised that due to the high concentrations of Igs found in colostrum; that this sample type would improve the detection of animals which have seroconverted to a specific disease. Diagnostic tests that have a high sensitivity more accurately identify animals that have been exposed to a particular

disease, whilst diagnostic tests that have a high specificity more accurately identify animals that have not been exposed to a particular disease, increasing the assurance of absence of disease.

This thesis has presented three ways in which colostrum can be utilised in ELISA to improve the detection of animals that have been exposed to some important diseases of production animals.

Firstly, the diagnostic sensitivity of the antibody ELISAs used for the detection of pigs vaccinated Erysipelas and Enzootic Pneumonia (Chapter 3.1), dairy cattle vaccinated against BVDV (Chapter 4.1) and sheep vaccinated against Johne's disease (Chapter 5.1) were significantly improved when using colostrum samples compared to serum samples. Such a significant improvement in sensitivity indicates that a higher proportion of vaccinated animals can be accurately identified. Additionally, the ELISA still maintained a significantly higher diagnostic sensitivity when compared to serum even with an increase in sample dilution to 1:100 (Chapters 4.1 and 5.1). This result indicates that colostrum will improve diagnostic utility when using pooled samples when compared to serum, thus enabling larger groups to be combined for testing. This is of particular importance for producers, as the surveillance and monitoring of disease within livestock populations is more economically achieved at the group (herd or flock) level, rather than at the individual animal level. Additionally, the diagnostic utility of the ELISA for the detection of Johne's disease was also improved when using colostrum compared to serum, which was collected from a dairy cattle herd with a history of previous infection (Chapter 5.2). The impact that colostrum had on ELISA specificity was only investigated in Chapters 4.1 and 5.1, and was only investigated on a

small number of animals. It is therefore difficult to make recommendations for the use of colostrum in ELISA for commercial purposes until the impact on the specificity of the ELISA has been investigated in larger cohorts of animals.

Secondly, colostrum was able to improve the differentiation of exposed and non-exposed individual animals within a single population. In Chapter 4.2, colostrum was collected from heifers that were experimentally infected with BVDV. As dilution of the colostrum increased, the diagnostic sensitivity of the ELISA was also increased, achieving 100% diagnostic sensitivity and specificity at a 1:500 dilution. This result is also of particular importance when using ELISA, as this method has limitations regarding the detectable range of the assay. By diluting colostrum in this way, the diagnostic utility of the ELISA may be further improved when the disease-specific antibody concentrations being tested, supersede the ability of the ELISA to measure beyond the maximal range of the assay. As the experimental infection of the heifers used in this experiment was not conducted entirely within the optimal time of gestation for the production of PI calves (40-120 days gestation), only 3 PI calves were produced. This result reduced the statistical power of the study; therefore, repeating this comparison using more PI calves would be of benefit.

Thirdly, colostrum disease-specific antibody concentrations in the sow were determined to be a useful predictor of serum disease-specific antibody concentrations in the piglet (Chapter 3.1). A linear regression analysis was used to develop a model to predict piglet serum disease-specific antibody concentrations using the disease-specific vaccinal antibody concentrations in sow serum and colostrum. In the cases of both diseases,

colostrum disease-specific ELISA OD values were significantly associated with the disease-specific ELISA OD value in piglet serum. These results are not only useful for the commercial pig industry, but also other intensive livestock industries, as these results highlight the importance of optimising vaccination protocols in order to achieve high disease-specific antibody concentrations in maternal colostrum, which will be passed to the neonate via passive transfer following parturition, enhancing the disease-specific antibody concentrations in the neonate.

This thesis also investigated the coagulation potential of the enzyme rennet. In Chapter 2.1, the production of Ig-rich whey from colostrum by rennet incubation was investigated. This experiment provided inconsistent evidence for production of colostrum whey with a higher Ig concentration than colostrum. Although there is evidence to suggest that colostrum whey has higher Ig concentrations when compared to whole colostrum and serum (Klimes et al., 1986; Marnila and Korhonen, 2011), this experiment was unable to unequivocally demonstrate this hypothesis. In Chapter 2.2, the ability of oral rennet supplementation of piglets to improve serum globulin concentrations was investigated. Although the supplementation of calves with rennet-incubated colostrum positively influence calf GGT activity (Gregory, 2003), which has been shown to be associated with serum globulin concentrations, this experiment was unable to provide further evidence to support this observation. Further investigations are required to compare the concentrations of disease-specific IgG in colostrum whey compared to colostrum. If whey does contain significantly higher concentrations of disease-specific IgG compared to colostrum, then the sensitivity of the ELISA can be further increased by the use of colostrum whey. Similarly with the oral rennet supplementation experiment in piglets, further investigations are warranted. Using

piglets separated at birth would allow the variance of the serum concentrations to be reduced by controlling the frequency, volume and timing of the colostrum ingested by the piglet post-farrowing, thus increasing the power of the experiment.

Evidence provided by this thesis supports the hypothesis that colostrum can significantly improve the sensitivity of ELISA for a number of important diseases of production animals. The impact on both the sensitivity and specificity was examined in three experiments (Chapter 4.1, 4.2 and 5.2), which indicated an overall increase in the diagnostic utility of ELISA when using colostrum compared to serum. Although this thesis has only addressed a limited number of important diseases of production animals, there is potential to improve the diagnostic utility of other important diseases, such as Neosporosis (*Neospora caninum*), and Bovine Tuberculosis (*Mycobacterium bovis*).

Neosporosis is an infectious disease of the placenta and fetus in cattle which is caused by the protozoan parasite *Neospora caninum*. Infection with *N.caninum* causes clinical disease in dogs, which is also the definitive host of infection. Infection with *N. caninum* infection in cattle is characterised by increased abortions, which can occur as three main types: sporadic, endemic and epidemic (Reichel et al., 2013). Epidemic abortions are the most devastating to cattle populations, causing significant economic losses to producers. Transmission from the cow to the fetus is very efficient, occurring at rates between 75-100% (Reichel et al., 2013). Diagnosis of Neosporosis is based upon histopathology and immunohistochemistry of the fetus, or serology. Following infection, the infected animal will seroconvert, which is generally considered to persist for the animals' lifetime. Cows that are seropositive have a higher risk of abortion and

are more likely to produce a persistently infected calf (Byrem et al., 2012; Reichel et al., 2013). In a study by Reichel and Pfeiffer (2002), the sensitivity and specificity of an ELISA when testing serum was 77% and 95%, respectively, when using an indirect fluorescent antibody test (IFAT) ≥ 600 . For milk samples, the sensitivity of the ELISA has been demonstrated to range between 60% and 90% , while the specificity has been demonstrated to range between 85% and 99%, which is dependent upon the type of milk being tested and the cut-off threshold being used (Schaes et al., 2004; Byrem et al., 2012). However, the analytical sensitivity of the ELISA could be effected when testing bulk milk samples, as *N. caninum* antibodies can only be detected in bulk milk samples when at least 5-7.5% of cows in the lactating herd are seropositive (Chanlun et al., 2002).

Bovine TB is an economically important zoonotic disease that has a worldwide distribution (Pollock et al., 2005). Bovine TB is caused by intra-cellular infection with *M.bovis* resulting in chronic disease and the development of granulomatous pathological changes in the lower and upper respiratory tract (Pollock et al., 2005). Following initial infection, the cell mediated immune (CMI) response is activated, where T-cells act as anti-mycobacterial macrophages. As infection progresses, the response shifts from a CMI response to a humoral immune response, which involves the development of anti-mycobacterial B-cell antibody responses, which increases with an increase in *M. bovis* shedding from the respiratory tract (Pollock et al., 2005). The current primary screening test for Bovine TB is the intradermal tuberculin test, which measures dermal swelling produced by the CMI response following injection with purified protein derivative (PPD) (Bezoz et al., 2014). The test is relatively inexpensive to perform, however there is no standardisation of the technique and interpretation in highly subjective (Bezoz et

al., 2014). In a study by Alvarez et al. (2012), the sensitivity of the intradermal tuberculin test ranged between 53% and 69.4%, while the specificity of the test was 99%. A number of ELISAs are available for the detection of Bovine TB. Benefits of the ELISA over the intradermal tuberculin test include the standardisation of the method, as well as being rapid. Although the specificity of the ELISA is high (Silva, 2001), the sensitivity of the ELISA is much the same, if not lower, than the intradermal tuberculin test due to the variability of the humoral immune response in individual animals and among different animal species (Bezoz et al., 2014). However, a study by Casal et al. (2014) was able to improve the sensitivity of a commercial antibody ELISA and a multiplex chemiluminescent immunoassay by performing the intradermal tuberculin test prior to testing. The sensitivity of the ELISA improved from 16.7% to 66.7% when comparing samples collected prior to and 72 hours post intradermal tuberculin test with samples collected 15 days post intradermal tuberculin test, while the sensitivity of the multiplex chemiluminescent immunoassay was also improved from 0% to 83.8% (Casal et al., 2014).

It is apparent from this discussion that improvements in ELISA sensitivity could still be made for the detection of Neosporosis and Bovine TB. The detection of Neosporosis in bulk milk is only achievable when at least 10-15% of cows in the lactating herd are seropositive. It is possible that the use of pooled colostrum for the detection of this disease could improve the analytical sensitivity due to the increased antibody concentrations found in colostrum, thereby decreasing the number of seropositive cows that need to be in the lactating herd in order for the ELISA is test positive. Also, previous research has been able to increase the sensitivity of the ELISA and multiplex immunoassay for Bovine TB by performing the intradermal tuberculin test 15 days prior

to sample collection. However, greater improvements in diagnostic sensitivity may also be achieved by the testing of colostrum samples rather than serum (Cockcroft et al., 2014).

Despite the findings of the research presented in this thesis, some questions still remain unanswered. The experiment presented in Chapter 2.1 regarding the production of Ig-rich colostrum whey by rennet coagulation was not fully explored. The literature on colostrum whey does suggest that the production of such an Ig-rich product is achievable; therefore further experiments should investigate the use of even smaller rennet dilutions and optimise a protocol that produces a whey product more richly concentrated in Igs when compared to colostrum. It should also be noted that the Ig concentration of colostrum changes markedly during the first 24 hours following parturition. The use of colostrum collected at different times following parturition will affect the sensitivity of the ELISA, therefore the sensitivity of ELISA when using colostrum collected at different times following parturition, as well as from different species and different dam parities should be investigated. A predictive model of ELISA sensitivity could then be devised based upon the ELISA result and the time post-partum that the colostrum sample was collected. Finally, the Johne's disease infected dairy cattle herd sampled in the experiment presented in Chapter 5.2 were not tested by pooled faecal culture prior to sample collection, therefore the prevalence of infection within this herd was lower than expected and resulted in the confirmation of only 4 cows with faecal culture results consistent with MAP infection. Due to this observed low prevalence, a larger study should be performed that uses samples from herds in Western Australia (Johne's disease-free) to calculate specificity, positive herds should be tested by pooled faecal

culture prior to inclusion in the study, and a larger number of positive herds should be tested to increase the statistical power.

In conclusion, this thesis presents evidence for increased diagnostic utility of ELISAs used for the detection of some important production animal diseases when using colostrum samples. It is evident that ELISA protocols need to be adjusted in order to achieve a diagnostic utility comparable to that of serum and milk when using colostrum. It is also possible that the use of colostrum in ELISA could also apply to other important production animal diseases, such as Neosporosis and Bovine TB, where improvements in ELISA sensitivity (both analytical and diagnostic) could still be made.

Appendix 1: Supporting Publications- Conference Papers

The following supporting publications are oral and poster contributions of the work presented in this thesis which has been presented at a national and international scientific conferences. The presenting author is highlighted in bold.

Oral Conference Presentations

CJ Jenvey, MP Reichel, and PD Cockcroft (2014) Investigation of the comparative sensitivity values of serum, colostrum and whey using an enzyme-linked immunosorbent assay for detection of specific antibodies in a Johne's disease vaccinated flock of Merino-cross ewes. *XXVIII World Buiatrics Congress*. 28th-31st July, Cairns, Australia.

CJ Jenvey, AM Weir, MP Reichel, and PD Cockcroft (2014) Performance characteristics of an ELISA for the detection of Bovine Viral Diarrhoea Virus (BVDV) antibodies when using bovine colostrum compared to serum samples. *XXVIII World Buiatrics Congress*. 28th-31st July, Cairns, Australia.

CJ Jenvey, MP Reichel, and PD Cockcroft (2013) Investigation of the comparative sensitivity of serum, colostrum and whey using an enzyme-linked immunosorbent assay for detection of specific antibodies against the Johne's disease (JD) vaccine, Gudair®, in a flock of Merino-cross ewes in South Australia. *Australian Association of Veterinary Laboratory Diagnosticians Conference*. 28-29th November, Geelong, Australia.

CJ Jenvey, PD Cockcroft, and MP Reichel (2013) Investigation of the comparative sensitivity of serum, colostrum and whey using an enzyme-linked immunosorbent assay for detection of specific antibodies against the Bovine Viral Diarrhoea Virus (BVDV) vaccine, Pestigard®, in a herd of South Australian Holstein-Friesian dairy cows. *16th International Symposium of the World Association of Veterinary Laboratory Diagnosticians*. 5-8th June, Berlin, Germany.

Poster Conference Presentations

CJ Jenvey, MP Reichel, SR Lanyon and PD Cockcroft (2014) Investigation of the diagnostic value of colostrum BVDV antibody concentrations in identifying PI calves following experimental infection of beef heifers. *XXVIII World Buiatrics Congress*. 28th-31st July, Cairns, Australia.

CJ Jenvey, MP Reichel and PD Cockcroft (2013) Investigation of the comparative sensitivity values of serum, colostrum and whey using an enzyme-linked immunosorbent assay for detection of specific antibodies in a Johne's disease vaccinated flock of Merino-cross ewes. *16th International Symposium of the World Association of Veterinary Laboratory Diagnosticians*. 5-8th June, Berlin, Germany.

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