Prevalence and identification of systemic markers of sub-clinical endometritis in postpartum dairy cows

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ABSTRACT

The prevalence of sub-clinical endometritis (scEndo) and identification of serum markers of scEndo was investigated in pasture-fed, postpartum dairy cows. Mixed age lactating dairy cows (n = 169) were examined for uterine health status on day (D) 21 postpartum (± 3 days) by Metricheck classification. On D42 in a sub-population (n = 47; 32 clean, 15 scEndo) Metricheck assessment was repeated and uterine cytology undertaken on D21 and D42 [scEndo = polymorphicnuclear (PMN) cells >18%]. Uterine bacteriology, as well as haematological, biochemical and milk parameters were also measured. The prevalence of scEndo was 15/169 (8.9%) and 4/47 (8.5%) cows on D21 and D42 respectively. A high incidence of self-resolving scEndo (11/15, 73%) was revealed. Numerous species of pathogenic bacteria were isolated from both scEndo and clean cows. Despite the uterine infection being sub-clinical, systemic markers were detectable as on D21 and D42 haematological and biochemical parameters differed. Irrespective of the scEndo classification method used, the mean blood neutrophil concentration was higher ($P < 0.05$) and plasma albumin lower (<0.05) in scEndo cows compared to clean cows. Milk fat and protein % also differed ($P < 0.05$) between scEndo and clean cows. The monitoring in early lactation of these markers could aid identification of scEndo, thereby preventing potentially detrimental effects on subsequent fertility.

Keywords: sub-clinical; bacteriology; cytology; haematology; milk.

INTRODUCTION

An early resumption of oestrous cycles and rapid uterine involution during postpartum are required to maintain a 365-day calving interval in New Zealand dairy cows. The success of this process is negatively affected by uterine infection specifically endometritis that can manifest itself either sub-clinically or clinically. Causes of endometritis are extensive and commonly include dystocia, twinning and retained foetal membranes (Williams *et al.*, 2005; McDougall *et al.*, 2007). Whilst the prevalence of clinical endometritis varies, it is estimated to be ~15% (Le Blanc *et al.*, 2002; Williams *et al.*, 2005; Azawi, 2008). However, the prevalence of sub-clinical endometritis is thought to be much greater at 30 to 55%, yet its impact on subsequent milk yield and reproduction is poorly understood (Le Blanc, 2008).

Studies of sub-clinical endometritis (scEndo) have commonly relied upon uterine cytological analysis and the identification of polymorphonuclear (PMN) cells, depending on the time of sampling postpartum, above a prevalence of approximately 15% (Kasimanickam *et al.*, 2005; Barlund *et al.*, 2008). PMN are primarily responsible for phagocytosis of bacteria in the uterine lumen (Klucinski *et al.*, 1990; Mateus *et al.*, 2002). Recently in New Zealand, a Metricheck system has been used to identify the occurrence of endometritis (McDougall *et al.*, 2007). Although this device accurately identified clinical endometritis, its reliability diagnosing sub-clinical endometritis has yet to be tested. In addition to uterine cytology and bacteriology, systemic markers for scEndo have been investigated. These diagnostic markers include the release of pro-inflammation cytokines and acute phase proteins in the blood (Williams *et al.*, 2005).

The aim of this study was to determine, via the Metricheck system, the prevalence of scEndo, the identity of common pathogens and to investigate the existence of systemic markers of scEndo and its impact on follicular dynamics (see Back *et al.,* 2009) in New Zealand pasture-fed postpartum dairy cows. The ultimate goal is the early diagnosis and treatment of scEndo to alleviate negative impacts upon reproduction and milk production.

MATERIALS AND METHODS

This study was approved by the Ruakura Animal Ethics Committee and conducted during August and September 2008. Mixed age lactating dairy cows (n = 169) were grazed on ryegrass (*Lolium perenne L.*) – white clover (*Trifolium repens L.*) pasture and offered sufficient fresh pasture to allow intakes of approximately 15 kg dry matter (DM)/cow/d at the start of the experimental period, increasing to 18 kg DM/cow/d by end of the experimental period. Pasture intake was estimated using visual assessment of pre- and post-grazing mass. Cows were milked twice daily at approximately 06:30 and 15:00 h. Milk yield was

TABLE 1: Identification of bacterial species found on day 21 postpartum in cows diagnosed as either having sub-clinical endometritis (scEndo; n = 15) or not (Clean; n = 32) based on uterine status classified by Metricheck or %PMN. Bacterial species categorised by their pathogenic potential as: (1) Recognised uterine pathogens; (2) Potential uterine pathogens; and (3) Opportunist uterine contaminants. Within each bacterial category, prevalence is shown for each group (clean vs scEndo), with the relative proportion found in the combined aerobic and anaerobic culture systems in parenthesis. PMN = polymorphicnuclear cells.

Bacterial pathogenic potential	Uterine status		Most prevalent matched species
	Clean	scIUI	
Metricheck classification			
1	8/15 (53%)	7/15 (47%)	*Arcanobacterium pyogenes, Proteus* spp., *Prevotella melninogenica, Fusobacterium necrophorum*
2	9/13 (69%)	4/13 (31%)	*Bacillus licheniformis, Enterocossus faecalis,* Non-haemolytic Streptoc
3	10/19 (53%)	9/19 (47%)	*Providencia stuartii, Staphlococcus* spp., *Clostridium perfigens*
%PMN classification			
1	9/15 (60%)	6/15 (40%)	*Arcanobacterium pyogenes, Proteus* spp., *Escherichia coli, Prevotella melninogenica, Fusobacterium necrophorum*
2	5/7 (71%)	2/7 (29%)	*Bacillus licheniformis, Enterocossus faecalis*
3	12/19 (63%)	7/19 (37%)	*Providencia stuartii, Streptococcus* spp., *Staphlococcus* spp.

TABLE 2: Mean haematological and biochemical measures ± standard error of mean for day postpartum and uterine status (mean of day (D) 21 and D42 data) classified via Metricheck or %PMN in dairy cows (n = 47) diagnosed as either having sub-clinical endometritis (scEndo) or not (Clean) at D21 and D42 postpartum. PMN= polymorphicnuclear cells.

Measurement	Day			Uterine status classified by Metricheck			Uterine status classified by %PMN		
	D21	D42	P value	Clean	scIUI	P value	Clean	scIUI	P value
Haematological analyte (Normal reference range)									
Red blood cells (5.0 – 7.7 x 10^{12}/L)	6.2 ± 0.1	6.0 ± 0.1	0.05	6.2 ± 0.1	6.0 ± 0.2	0.26	6.2 ± 0.1	6.1 ± 0.2	0.33
Haemoglobin (85 – 130 g/L)	113 ± 2	107 ± 2	0.005	111 ± 1	109 ± 3	0.33	110 ± 1	109 ± 4	0.26
Haematocrit (0.24 – 0.40 L/L)	0.30 ± 0.01	0.28 ± 0.01	0.004	0.29 ± 0.01	0.29 ± 0.01	0.22	0.30 ± 0.01	0.29 ± 0.01	0.15
Platelets (220 – 640 x 10^9/L)	454 ± 19	454 ± 18	0.23	447 ± 14	479 ± 32	0.76	447 ± 17	462 ± 29	0.81
White blood cell (3.8 – 11.0 x 10^9/L)	7.0 ± 0.3	7.8 ± 0.3	0.05	7.4 ± 0.3	7.4 ± 0.5	0.15	7.5 ± 0.3	7.0 ± 0.5	0.43
Fibrinogen (2 – 7 g/L)	5 ± 1	5 ± 1	0.82	5 ± 1	5 ± 1	0.36	5 ± 1	5 ± 1	0.55
Neutrophils (0.7 – 4.9 x 10^9/L)	2.7 ± 0.2	3.1 ± 0.2	0.03	2.8 ± 0.1	3.1 ± 0.4	0.05	2.9 ± 0.1	2.9 ± 0.4	0.12
Lymphocytes (1.5 – 8.0 x 10^9/L)	3.5 ± 0.2	3.9 ± 0.2	0.09	3.8 ± 0.1	3.4 ± 0.3	0.72	3.8 ± 0.1	3.4 ± 0.3	0.64
Monocytes (0.0 – 0.9 x 10^9/L)	0.5 ± 0.1	0.3 ± 0.1	0.71	0.3 ± 0.1	0.7 ± 0.3	0.07	0.3 ± 0.1	0.6 ± 0.3	0.16
Eosinophils (0.0 – 1.9 x 10^9/L)	0.4 ± 0.1	0.5 ± 0.1	0.86	0.5 ± 0.1	0.3 ± 0.1	0.08	0.4 ± 0.1	0.4 ± 0.1	0.96
% Neutrophils	35.6 ± 1.4	40.0 ± 1.2	0.02	37.5 ± 1.0	38.8 ± 2.9	0.20	37.4 ± 1.0	39.2 ± 2.9	0.05
% Lymphocytes	51.8 ± 1.4	49.3 ± 1.2	0.17	50.7 ± 1.0	49.9 ± 2.5	0.62	51.7 ± 0.9	49.3 ± 2.8	0.04
% Monocytes	5.5 ± 0.4	4.2 ± 0.3	0.04	4.6 ± 0.3	5.9 ± 0.7	0.03	4.5 ± 0.3	5.0 ± 0.7	0.85
% Eosinophils	6.6 ± 0.7	5.9 ± 0.7	0.29	6.6 ± 0.6	4.7 ± 0.6	0.05	5.8 ± 0.6	5.7 ± 1.0	0.80
Biochemical analyte in blood (Normal reference range)									
Total protein (60 – 86 g/L)	81 ± 1	80 ± 1	0.50	81 ± 1	79 ± 1	0.04	80 ± 1	80 ± 1	0.98
Albumin (25 – 40 g/L)	36 ± 1	36 ± 1	0.86	36 ± 1	34 ± 1	0.04	37 ± 1	35 ± 1	0.05
Globulin (28 – 53 g/L)	45 ± 1	44 ± 1	0.65	44 ± 1	44 ± 1	0.91	44 ± 1	45 ± 1	0.44
Albumin/Globulin ratio	0.82 ± 0.02	0.82 ± 0.02	0.97	0.84 ± 0.02	0.80 ± 0.04	0.30	0.85 ± 0.02	0.78 ± 0.03	0.14
B-hydroxybutyrate (0.2 – 1.0 mmol/L)	0.59 ± 0.05	0.55 ± 0.03	0.19	0.59 ± 0.04	0.50 ± 0.04	0.20	0.59 ± 0.04	0.48 ± 0.05	0.32

measured using in-line meters and composition determined on an infrared milk analyser (FT120, Foss Electric, Hillerød, Denmark) from one herd test in September. Somatic cell count (SCC) was measured using an automated cell counter (Fossomatic 5000, Foss Electric).

The uterine health status of the lactating dairy cows (n = 169) was assessed on day (D) 21 (± 3d) postpartum by examination of vaginal discharge using a Metricheck device (Simcrotech, Hamilton, New Zealand). This device had been previously validated to identify the presence of uterine infection (McDougall *et al.*, 2007). Exclusion criteria for cows selected for this trial are described by Back *et al.* (2009). Cows were assigned to groups based on their Metricheck score (0-1 = Non-infected, 2-3 = Sub-clinical, 4-5 = Clinical). Animals that exhibited signs of clinical infection were treated and excluded from the trial.

In a sub-population (n = 47; 32 clean and 15 scEndo), uterine endometrial samples were taken on D21 and D42 post-partum (± 3d) for bacteriological and cytological analysis. Bacteriological samples to identify aerobic and anaerobic pathogens (Sheldon *et al.*, 2002) were collected using a cytobrush technique (Kasimanickam *et al.*, 2005; Barlund *et al.*, 2008). Bacteria were isolated in aerobic and anaerobic culture at the Animal Health Centre, Morrinsville and categorised on the basis of expected pathogenic potential within the uterus (Sheldon *et al.*, 2002; Williams *et al.*, 2007). The three categories were: (1) pathogens known to cause endometrial lesions; (2) other recognised uterine pathogens and (3) bacteria not recognised as uterine pathogens.

A second cytobrush was collected and percentage of polymorphonuclear neutrophils (%PMN) determined at the Animal Health Centre, Morrinsville. Animals were classified as scEndo if %PMN was greater than 18% (Kasimanickam *et al.*, 2005).

Blood samples were taken by venipuncture on D21 and D42 postpartum and analysed for haematological and biochemical parameters by the New Zealand Veterinary Pathology, Hamilton. Cows were assessed for body condition score (BCS) at D21 and 42 postpartum using a 10-point scale, where 1 is emaciated and 10 is obese (MacDonald & Roche, 2004). Data were analysed using a MIXED procedure employing Tukey's post-hoc test where appropriate, in SAS version 9.1 (SAS, 1997). Change in BCS between D21 and D42 were normality tested and used with a diagonal covariate structure in the model.

RESULTS

The proportion of cows with scEndo using the Metricheck classification on D21 was 15/169 (8.9%) and on D42, 4/47 (8.5%) cows. The incidence of self-resolving scEndo cows by D42 was 11/15 (73%). Classification via %PMN was undertaken on 43 and 46 cows on D21 and D42 respectively. In the sub-population, 16/43 cows (37%) and 3/46 cows (6.5%) were classified as having scEndo by %PMN on D21 and D42 respectively. The proportion of self-resolving scEndo cows by D42 was 13/16 (81%). Only 50% agreement was found between the two methods of classification. Consequently, only 8/15 cows determined scEndo by Metricheck were also determined by %PMN and a further 8 cows were identified from the remaining sub-population as having scEndo.

Bacteria isolated from uterine samples taken on D21 in a sub-population (n = 47) of clean and scEndo cows are shown in Table 1. Based on the D21 Metricheck results, in the scEndo group (n = 15) 10 cows had bacterial species from the classification isolated, two had non-classified bacteria only, isolated and three had no bacteria cultured/species identified. Whereas in the cows identified as clean (n = 32), 16 cows had classified bacteria isolated, two had non-classified bacteria only, isolated and 14 had no bacteria

TABLE 3: Mean daily ± standard error of mean milk parameters shown for uterine status classified via Metricheck or %PMN in dairy cows (n = 47) diagnosed as either having sub-clinical endometritis (scEndo) or not (Clean) at day (D) 21 postpartum. PMN= polymorphicnuclear cells.

Milk constituent	Uterine status classified by Metricheck			Uterine status classified by %PMN		
	Clean	scIUI	P value	Clean	scIUI	P value
Daily milk production (L)	21.0 ± 0.8	21.1 ± 0.8	0.99	21.0 ± 0.8	20.6 ± 0.8	0.84
Daily fat production (kg)	1.05 ± 0.07	1.14 ± 0.09	0.79	1.12 ± 0.08	0.97 ± 0.09	0.17
Daily fat percentage (%)	4.95 ± 0.20	5.47 ± 0.26	0.08	5.39 ± 0.18	4.62 ± 0.34	0.03
Daily protein production	0.75 ± 0.03	0.75 ± 0.03	0.93	0.76 ± 0.02	0.70 ± 0.05	0.22
Daily protein percentage (%)	3.57 ± 0.09	3.54 ± 0.06	0.80	3.66 ± 0.06	3.39 ± 0.15	0.07
Fat:Protein ratio	1.39 ± 0.04	1.55 ± 0.06	0.02	1.47 ± 0.05	1.38 ± 0.06	0.22
Log_{10} somatic cell count (x 1,000 cells/mL)	1.83 ± 0.09	2.02 ± 0.18	0.54	1.69 ± 0.06	1.95± 0.11	0.04

cultured/species identified Similar values were found using the %PMN classification (P.J. Back, Unpublished data).

Of the three categories of pathogen that potentially cause uterine infection, all of the five species in Category 1 and three of the species in Category 2 were isolated (Table 1). Bacterial species that are not included in the classification were also isolated. These included *Pseudomonas* and *Lactobacillus* species. By D42, in agreement with the high self-resolve rate found in these cows, the number of different species present and their incidence had decreased dramatically. In total, only *Fusobacterium necrophorum* (1 cow), *E. coli* (1 cow) and non-haemolytic *Streptococci* (4 cows) were isolated.

All haematological and plasma biochemical parameters were within normal ranges (Table 2) and cell morphologies were found to be normal. Irrespective of how scEndo was classified, either by Metricheck or %PMN, red blood cell count, haemoglobin and haematocrit concentrations were higher ($P < 0.05$) on D21 than on D42 (Table 2). On D42 white blood cell count, neutrophil concentration and the percentage of neutrophils were significantly ($P < 0.05$) elevated compared to D21.

For both Metricheck and %PMN classification, scEndo cows compared to clean cows had higher ($P < 0.05$) neutrophil parameters and lower ($P < 0.05$) plasma albumin concentration. Moreover when classified via Metricheck only, plasma total protein concentration was lower ($P < 0.05$), whilst monocyte and eosinophil concentrations differed ($P < 0.05$) between scEndo and clean cows (Table 2).

Cows identified as scEndo by %PMN classification were found to have lower mean total milk fat % ($P = 0.03$) and tended to have lower milk total protein % ($P = 0.07$), whilst SCC was higher ($P = 0.04$) compared to clean cows (Table 3). When classified via Metricheck results scEndo cows had an increased fat/protein ratio compared to clean cows.

DISCUSSION

Using the Metricheck system, the prevalence of scEndo was approximately 9% at D21 with a high self-resolve rate of 73% by D42 post-partum in New Zealand pasture-fed dairy cows. Comparable estimates were also identified via %PMN classification, although only 50% agreement existed between the two classification methods, with regards to which animals were identified as having scEndo. This is not surprising, since the two methods can not be compared directly, as they rely on vastly different measures to assess infection. The former estimates mucus content of the vagina and the latter inflammation of the endometrium in the uterus. The prevalence rate is considerably lower than that reported for clinical endometritis (Le Blanc *et al.*, 2002; Williams *et al.*, 2005; McDougall *et al.*, 2007; Azawi, 2008) or even that estimated for sub-clinical endometritis (Le Blanc, 2008). In contrast, the self-resolve rate is higher compared with the range of 30 to 45% in clinical endometritis (Parkinson *et al.,* 2007). Both these disparities can be attributed predominantly to the selection criteria used in the current trial and that sub-clinical rather than clinical endometritis was being investigated.

Isolation of bacterial species known to cause endometritis was similar between clean and scEndo cows in the present study. This was expected, since Sheldon *et al.* (2002) found no influence between the pathogenic group present and severity of endometritis. Instead, the bacterial load of the specific pathogens present was found to correlate directly to infection severity. Correlation between the pathogens present and occurrence of scEndo could not be estimated in the current study. However, Williams *et al.* (2005) identified a relationship between the severity of infection, in terms of physical mucus present, and the bacterial load of the uterine lumen. Interestingly, Williams *et al.* (2005) found cows with small amounts of uterine mucus, equivalent to Metricheck Score 1 in the current study, had similar bacterial profiles to those deemed as clean, a finding supported by the present data. These authors also found that animals with flecks of mucus displayed a longer calving to conception interval, similar to that for cattle with mild endometritis reported elsewhere (Kasimanickam *et al.*, 2004) highlighting the impact of scEndo on reproduction.

Haematological parameters, irrespective of uterine health status, showed significant changes between D21 and D42. Decreased concentrations of red blood cell count, haemocrit concentration and haemoglobin concentration were found at D21 compared to D42, whilst whilte blood cell count and lymphocyte % were elevated on D42. Fluctuations in these parameters, although unrelated to infection status, could reflect adaptation after parturition, combined with negative energy balance and increased metabolic stress due to peak milk production.

When haematological parameters were analysed with respect to endometritis, white blood cell count was not influenced by the uterine status, in agreement with Subandrio *et al.* (2000). These authors however found elevated neutrophil concentrations in animals that had induced endometritis. Acute endometrial inflammation is evidenced by increased numbers and cellular infiltration of immune cells, predominantly neutrophils, into the uterus (Tizard, 1996; Mateus *et al.*, 2002). In the present study, increased plasma neutrophil and monocyte concentrations were found

in scEndo cows, yet in clinical endometritis cases plasma neutrophil concentrations are reported as being decreased in circulation, suggesting cells may be leaving and entering the site of infection in the uterus (Tizard, 1996). Postpartum changes in plasma monocyte populations of cows spontaneously recovering from endometritis have also been reported (Mateus *et al.*, 2002). Detection of mild infection such as scEndo by epithelial and stromal uterine cells may therefore be sufficient to induce an immune response, as demonstrated in elevated plasma concentrations of these immune cells, but not great enough to lead to substantial infiltration from the blood into the uterus.

Somatic cell count was found to be elevated in scEndo cows identified by the %PMN classification only. To our knowledge this is a finding not previously associated with scEndo. In the current study, no differences in milk production were evident between clean and scEndo cows in agreement with Le Blanc (2008). Compositional changes in the milk were however identified, with a decreased fat % and tendency for a decreased protein % in scEndo cows identified by the %PMN classification, and an increased fat:protein ratio in scEndo cows identified by the Metricheck classification. Reduced milk protein concentration has been associated with endometritis (Bell & Roberts, 2007). A decrease in protein % and an increased fat:protein ratio has also been found to be associated with a reduction in conception rates and prolonged calving to conception intervals (Buckley *et al.*, 2003), although association with reproductive success in the present animals to date remains to be established. All these current findings are in contrast to those of McDougall *et al.* (2007), although the timing of measurement relative to calving, as well as the current study including only scEndo may account for these discrepancies. Notably, only minimal agreement was evident between the Metricheck and %PMN classification regarding significant differences in milk constituents for scEndo and clean cows. This agreement with previous studies suggests that %PMN classification is perhaps a more sensitive method in identifying markers of endometritis in milk.

In summary, Metricheck classification was found to be a useful method for determining the prevalence of scEndo in postpartum dairy cows when used in conjunction with normal farm health monitoring.

Despite endometritis being sub-clinical, a systemic haematological marker, like increased neutrophil populations, and systemic biochemical markers like lower plasma albumin and total protein, as well as lower milk protein and milk fat percentages were evident. This is the first study to identify easily detectable markers in scEndo cows. Consequently the monitoring in early lactation of these markers of reproductive health, in conjunction with an early Metricheck examination, prior to that normally undertaken at planned start of mating, could aid detection of sub-clinical endometritis, thereby preventing potentially detrimental effects on fertility in the subsequent breeding season.

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