Evaluation of subclinical endometritis and consequences on fertility in piedmontese beef cows

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Subclinical endometritis effect on Piedmontese cows

Contents

Subclinical endometritis (SEM) is poorly investigated in beef cows, as stated in the literature. This project aims to evaluate the rate and the consequences of SEM in Piedmontese cows, with a focus on bacteriological findings and fertility parameters. Uterine cytology was performed for 97 subjects; a total of 31% of the cows were diagnosed as being positive for SEM and as having an 8% neutrophil (PMN) presence on the slide, which is considered as the best cutoff to diagnose the pathology. Only 13% of the cows positive for SEM were pregnant within 130 dpp and generally showed increases of 40 days in the partum to conception interval compared with the negative cows (142 vs 182, P=0.01). Cows positive for both bacteriology and cytology showed a lower fertility than cows with only inflammation or only a bacterial presence (P=0.0004). Bacterial isolation detected different species, but no difference in regard to the impact of these bacteria on SEM was shown. Parity, presence of calves, hygiene condition, age and number of service did not affect whether a cow was positive for subclinical endometritis (P<0.05). The housing system (free stalls vs tie stalls) used seems to affect the SEM rate in Piedmontese cows; cows bred in tie stalls were more likely to be positive for SEM (OR=2.2; P=0.04). In conclusion, cytology seems to be a good technique for the diagnosis of subclinical endometritis in beef cows, and as in dairy cows, subclinical endometritis has a detrimental

effect on fertility, causing an increase in partum to conception and a decrease in the rate of cows who become pregnant within 130 dpp, particularly for those cows housed in a tie stall.

Keywords: beef cow, subclinical endometritis, cytology, bacteriology, tie stall, infertility

Introduction

Piedmontese is the most important beef cattle breed in Italy. It is a white, 'double-muscled' breed that is characterized by a posterior muscular hypertrophy, a heritable condition that primarily results from an increase in the number of posterior muscle fibers (hyperplasia) rather than an enlargement of individual muscle fibers (hypertrophy), and this condition is due to a mutation in the myostatin gene (Hanset et al., 1982). The skin and skeleton of the breed are thin, the content of fat in the meat is reduced (Shahin and Berg., 1985; Hanset, 1982), and the proportion of fat is increased in the most valuable cuts of meat (Casas et al., 1997). The superior meat quality and carcass characteristics have been responsible for Piedmontese cattle becoming widespread worldwide, both as a pure breed and as a crossbreed. With more than 41,000 frozen semen doses produced (ANABORAPI 2012), Piedmontese cow has is now widespread, with the presence of nuclei of these animals recorded in the USA (for example, Eataly Grocery Store in NY), Latin and Central America, Canada, North Africa, Australia, and many countries of Europe. However, white and double-muscled animals are more likely to experience malformations of the uterine trait, reduction in stress tolerance, diminished fertility and dystocia. (Arthur et al., 1995).

Inflammation of the genital tract is a common pathology in dairy cows; in fact, 20% of cows are diagnosed as having clinical endometritis within 3 weeks of calving (Sheldon et al., 2009). Subclinical endometritis (**SEM**) has also been accurately studied and has proven to be detrimental to fertility in dairy cows. Subclinical endometritis is defined as an inflammatory condition of the endometrium in the absence of clinical signs of pathology, with or without the presence of bacteria in the uterus, with a significant effect on reproductive performance (Sheldon et al., 2006). Diagnosis of SEM can be performed by uterine cytology, by uterine histology of biopsy samples and by transrectal

ultrasonography. Uterine cytology is considered the most reliable and accurate diagnostic technique for evaluating the presence of subclinical endometritis, and diagnostic samples can be obtained by uterine flushing or by the cytobrush method (Barlund et al., 2008; Kasimanickam et al., 2004; Kasimanickam et al., 2005a; Kasimanickam et al., 2005b). Endometrial cytology is the most important practice for evaluating the health status of the uterus, because it is quick, specific and inexpensive (Gilbert et al., 1998, 2005). SEM is diagnosed based on the proportion of polymorphonuclear cells (**PMN**) in endometrial samples: an increased proportion of PMN is associated with a decrease in reproductive performance (Barlund et al., 2008; Gilbert et al., 2005; Kasimanickam et al., 2004). The threshold value for the proportion of PMN needed to define subclinical endometritis is still controversial, and a range of 4% to 18% is reported in the literature (Barlund et al., 2008; Gilbert et al., 2005; Kasimanickam et al., 2004). Cows with subclinical endometritis do not have uterine discharge; however, the consequences of an inflammatory condition in the uterus are still considered sufficient to impair reproductive performance (Sheldon et al., 2006); SEM is associated with reduced pregnancy rates: median time to conception increases from 30 to 88 days and the proportion of cows that fail to become pregnant by 300 DIM increases by 20% (Kasimanickam et al., 2004; and Gilbert et al., 2005; LeBlanc et al. 2008). In postpartum, the uterus of a cow is often contaminated by a number of bacterial species, but this contamination does not always develop into a clinical disease. Infection and disease are dependent on the ability of pathogenic organisms to adhere to the mucosa, the tendency of bacteria to colonize or penetrate the epithelium and/or the release of toxins that facilitate the establishment of uterine disease (Janeway et al., 2001). The development of endometritis also depends on a balance between the cow's immune response and the species and number of bacteria involved. This balance can be shifted in favor of disease by risk factors such as metabolic stress, retained placenta, dystocia, twins and stillbirth (Grohn and Rajala-Schultz, 2000; Kim and Kang, 2003). *Escherichia coli* and *Trueperella pyogenes* are traditionally considered the most relevant species involved in the pathogenesis of metritis because

of their persistence in the uterus (Sheldon et al., 2009; Williams et al., 2005; Azawi, 2008), but other bacteria, such as *Fusobacterium necrophorum, Prevotella melaninogenicus, Bacteroides spp., Pseudomonas spp., Streptococcus spp., and Staphylococcus spp.*, in a variety of combinations, have been isolated from the infected uteri of cows and are also thought to be responsible for uterine disease. Other authors of metagenomic studies on postpartum uterine content have stated that in both healthy and affected cows, there are a variety of microbial populations that cannot be identified with routine isolation procedures and that these populations continually evolve in each subject differently. (Santos et al., 2011;Santos and Bicalho, 2012). Additionally, some authors failed to isolate bacteria in cows positive for SEM (McDougall et al., 2011; Barański et al., 2012; Madoz et al., 2014). To our knowledge, very few studies have been conducted on endometritis in beef cows (Santos et al., 2009), and this project aims to evaluate the rate and consequences of SEM in Piedmontese cows, with a focus on bacteriological findings and fertility parameters.

Material and methods

Following an anamnestic exam of 300 parturitions, cows with a history of dystocia, retained placenta, uterine pathologies, metabolic or locomotor affections, or mastitis were excluded from this study. Furthermore, all animal previously submitted for insemination or animals treated with antibiotics or anti-inflammatory drugs were not included in the study. Each cow underwent a transrectal ultrasound and a mucous vaginal examination, on the basis of suggested classification found in the literature (Sheldon et al., 2006), to exclude cows in case of abnormal uterine discharge and to detect any clinical uterine/cervical pathology. A total of 112 healthy Piedmontese cows from 6 different farms in the Piedmont Region of northern Italy have been enrolled in this project.

Only 11 cows were not suckled, with the calves being removed just after colostrum subministration, and 68 cows were suckled and remained with their calves for at least 5 months; both groups were housed in tie stalls (33 cows) or free stalls (64 cows) with free access to water and food (hay, bent grass, corn flour, and soy), vitamins (A and E) and mineral supplementation (Ca, P, and Mg). All

cows were vaccinated against IBR and BVDV virus. For each cow, suckling, hygiene condition, housing system, parity, and the number of times serviced were recorded. Partum to conception period **(PC)**, conception rate **(CR)** and days at first service, were also recorded.

Cytologic sampling

Each cow was sampled by cytological examination of endometrial samples once in the interval from 28 to 68 days postpartum **(dpp),** depending on the availability of the farm and based on the first insemination time. Endometrial cells that were sampled for diagnosis of subclinical endometritis were obtained by an infusion of 20 ml of sterile saline solution into the uterus with a plastic infusion catheter (53.5 cm, Bovivet uterine catheter®, Kruuse, Langeskov, Denmark), followed by uterine massage and aspiration of approximately 5 or more ml of fluid into a 50-ml syringe (Terumo®, Rome, Italy). Recovered fluid was transferred to two sterile plastic tubes and placed on ice in a portable cooler. One tube was immediately transported to the I.Z.S laboratories (Torino, Italy) to be analyzed for bacteriology, and the other tube was processed within 4 h after collection as described in Santos et al, 2009. Briefly, the sample was vortexed and centrifuged onto a glass slide in a cytocentrifuge (Shandon Cytospin®, Tharmac GmbH, Waldsolms Germany). Slides were air dried and stained with a Hemacolor® rapid staining kit (Merck KGaA, Germany), and each slide was examined using 400x magnification. Two different examiners each counted a minimum of 200 cells (endometrial cells, PMNs and squamous cells) in 10 fields on the slide.

Bacteriology

Bacterial characterization and an antibiogram were performed at the I.Z.S. laboratories (Istituto Zooprofilattico, Torino Italy) for each cow. Selected blood, chocolate, and Gassner agar media were used for aerobes, anaerobes, and total microbial growth for bacteria such as *Trueperella spp.*, *Prevotella spp.* and *Fusobacterium spp.* Gassner media was used for Enterobacteriaceae species, Baird Parker media was used for *Staphylococcus spp. and Streptococcus spp.*, PPLO agar media was used for *Mycoplasma spp*., and Sabouraud dextrose chloramphenicol agar was used for the isolation

of yeasts and molds; all plates were incubated at 37°C for 24/48 hours. After direct inoculation in selected media, a Brain-Heart-Infusion (BHI) broth media was used for final microbial growth. Bacterial colony phenotypic analysis with API bacterial identification columns was used to identify the different bacterial colonies. For long-growing bacterial species (>24/48 hrs), however, genotypic identification with PCR for rDNA 16S rRNA gene sequence analysis was used; the resulting gene sequences were compared with an online database to find a matching species (Benedetto et al., 2007).

Statistical analysis

To measure the degree of agreement between the two examiners of the cytological PMN percentage evaluation, Cohen's kappa coefficient was used. The Shapiro-Wilk test was used to determine normal distribution of the variables. For each cow, suckling (**yes** or **no**), hygiene (**poor or good**), housing system (**tie** or **free**), parity (**one**, **>one**), number of times serviced (**one**, **two**, **>two**) were recorded and analyzed along with the presence of subclinical endometritis and bacteriology with general logistics regression (GLM). Bacteriology (**BACT**) was categorized as positive (**1**) or negative (**0**) and when associated with SEM, four categories were created: "**None**" for healthy cows, "**S**" for only SEM-positive cows, "**B**" for cows positive only to bacteriology, and "**S-B**" for cows positive to SEM and bacteriology. Additionally, bacteriology species were divided into two groups: "**main**" indicating the most relevant species based on the literature and "**secondary**" indicating other species not so frequently reported in the literature. The proportion of continuous to categorical variables was analyzed with the Kruskal-Wallis method. Cox's proportional hazard ratio was used to assess the association between endometritis and the interval to conception for all cows and to evaluate the persistence of bacteria during postpartum. The optimal cutoff point for determining positivity for SEM with pregnancy status at 130 days postpartum was calculated using Receiver Operating Characteristic (ROC) curves. Statistical calculations were performed using the R ver.2.15.2 and SPSS Vers. 19 software packages.

Results

Fifteen cows were excluded in the final stages of the protocol: 7 cows developed a clinical pathology during the project; 3 cows were sold during the protocol; and for 5 cows, was not possible to insert the catheter beyond the cervix. Ultimately, 97 cows were analyzed: 73 were multiparous and 24 were primiparous. From the ROC curves, the correct diagnostic cutoff for PMN percentage was 8% (Sp 0.77, Se 0.53 with AUC 0.66; CI 95% 0.54-0.79) (Graph 1). The weighted kappa for agreement between the two examiners was k=0,82 (P =0.001).

SEM was found in 31% of the cows (30/97), 30% in the 1st quartile (28 to 30 days postpartum), 26% in the 2nd quartile (31 to 33 days postpartum), 18% in the 3rd quartile (34 to 48 days postpartum) and 26% in the 4th quartile (49to 68 days postpartum) (P<0.0005). Twenty-three (77%) cows were positive before 50 dpp, and seven (23%) were positive at >50 dpp. The percentage of SEM-negative cows that became pregnant was higher than SEM-positive cows at 130 dpp (57% vs 13%). Survival analysis (Graph 2) shows that by day 142, the 50% of SEM-negative cows were pregnant, compared with the 182 days necessary for positive cows to become pregnant (log rank test, $P < 0.004$).

However, most SEM cows (54%) became pregnant later than 130 days postpartum, with a median PC of 184 days postpartum (P=0.01). Cows in the tie stalls (42% ,14/33) were more likely to be positive for SEM than cows in the free stalls (25%,16/64) (P=0.02; OR=0.34). Parity did not affect the prevalence of SEM (P=0.23); 23% of the cows were primiparous, with an average PC of 111days compared with the 76% multiparous cows, with a PC of 106 days. Presence of calves, hygiene conditions, age, and the number of times serviced did not significantly influence positivity for SEM (P>0.05).

Bacteriology was slightly associated with increasing days of postpartum (90 vs 100, P=0.05), but when bacteriology and SEM were associated and divided into the 4 categories: None (28%), S (8%), B (41%), and S-B (22%), median PC times were 88.0 97.5, 95.0, 142.5 days postpartum, respectively (P=0.0004) (Graph 3). No difference in PC was shown between cows with the most relevant bacteria compared to those with secondary bacteria (146 vs 160, P>0.05). Calf presence, age, and the number

of times serviced did not show any effect on PC or on positivity for SEM. Interestingly, tie stalls seem to have a greater impact on the rate of SEM (OR=2.2; P=0.04): cows in the tie stalls had an increased PC compared to cows in the free stalls (133 vs 94 days).

Discussion

This study confirms that the low volume flushing technique is a practical method for obtaining samples from Piedmontese cows for cytological and microbiological analyses, because only 5 (4%) cows of the total 112 cows had to be excluded due to the difficulty of passing the catheter through the cervix. Different diagnostic methods for collecting good cytological uterine samples have been reported in the literature: the cytobrush technique (Kasimanickam et al., 2004; McDougal et al., 2007; Couto et al., 2013) and uterine flushing (Gilbert et al., 2005; Cheong et al., 2012) were the most accurate ones (Barlund et al., 2008). A lack of a gold standard for evaluating the correct % PMN cutoff for SEM evaluation is still notable in the literature; most papers report a data-collection period in dairy cows from 18 to 62 dpp with PMN thresholds that range from 5% to 18% (Kasimanickam et al., 2004; Gilbert et al.,2005; Barlund et al., 2008; Galvao et al., 2009; Madoz et al., 2013; Sens and Heuwieser et al., 2013). Many different methods have been used to calculate the most accurate PMN cutoff; some authors (Hammon et al., 2006; Plontzke et al., 2010; Sens and Heuweiser, 2013) used previous literature data to justify the application of a particular cutoff point. Other authors (Galvao et al., 2009, Kasimanickam et al., 2004) used Receiver Operator Characteristic (ROC) statistical analysis to establish a cutoff of 6.5% PMN at 35 dpp, and for samples taken at other times, a range of 18% to 10%, depending on the dpp. Santos et al. (2009) is the only author who analyzed SEM prevalence in beef cows. She used low-volume uterine flushing to collect cytology samples from 2 to 87 dpp and used the ROC statistic curve to evaluate the PMN percentage (5.5% PMN, Se 78%, Sp 100%, P=0.0001). In contrast to previous work, our work using the application of ROC statistical analysis resulted in a diagnostic value of 8% PMN cutoff for the interval from 30±2 and 65±3 dpp.

Weighted kappa agreement between the two examiners resulted in an average (k=0.82) that was similar to those stated in previous works, with a range from 0.77 to 0.97 (Dubuc et al., 2010; Santos et al., 2009), suggesting that the method is also repeatable for beef cows. The percentage of affected beef cows was 31%, similar to the 34% reported by Santos et al., but between 25 and 57 dpp and still in the average interval of 13% to 44% that has been reported for dairy cows (Kasimanickam et al., 2004; Barlund et al., 2008; Ribeiro et al., 2013; Madoz et al., 2013) depending on the time after parturition and the threshold used. As expected, and shown in previous literature (Kasimanickam et al., 2004; Sheldon et al., 2009), SEM-positive cows show a higher PC than healthy cows with an increased median of 40 days (graph 2). According to Santos et al. (2009) the rate of cows positive for SEM after 50 dpp was lower (23%) than for cows positive before 50 dpp (77%), thus appearing to be similar to the proportion showed for dairy cows (Gilbert et al., 2004): in this study, 76% of the SEM cows were diagnosed as being positive before 50 dpp. However, survival analysis (graph 1) shows that the SEM cows (54%) were pregnant after 130 dpp with a median PC of 184 days (P=0.01).

As the main objective of the work was to evaluate the impact of subclinical endometritis on partum to conception in beef cows, 7 cows that developed clinical endometritis, according to Sheldon et al 2006, were excluded from the study to avoid confounding the direct effect on partum to conception. Although recent research shows that cytological endometritis and purulent vaginal discharge are known to be distinct manifestations of reproductive tract disease with their own sets of risk factors (Dubuc et al., 2010 [3]), we preferred to exclude all animals that did not meet he predetermined criteria.

According to Gilbert et al. (2005) and Santos et al. (2009), parity did not influence the prevalence of SEM (P=0.23). Additionally, the presence of calves, hygiene condition, and the number of times serviced did not influence the rate of positivity for SEM. (P>0.05).

As expected, some of the most significant species of bacteria described in literature (Williams et al., 2005, Sheldon et al., 2009, Sens and Heuweiser, 2013) were isolated, and although the

bacteriological analysis were performed with classical bacteriological technique instead of DNA sequencing (Santos et al., 2011; Santos and Bicalho, 2012), a relevant number of other species were also isolated (table 1). However, no difference was shown between these two groups (main vs secondary) regarding the effect on increasing partum to conception; for both groups, PC increased dramatically when associated with SEM positivity. In accordance with Madoz et al., (2014), who failed to isolate bacteria in cows positive for SEM, in this study, 8% of cows were SEM positive without bacteria in the uterus. Madoz et al. (2014) suggested that, by the time of SEM diagnosis, bacteria might have been cleared from the uterus by host defense mechanisms restoring the normal uterine environment. This would be in agreement with a study (McDougall et al., 2011) that showed a low level of agreement between the presence of intrauterine pathogens and the percentage of neutrophils: some cows resulted positive according to cytology, but no bacteria were isolated from uterus.

The presence of bacteria in the postpartum uterus slightly affects fertility, but when associated with SEM (22%), increases the median PC of cows to 202 dpp (Graph 3).

The presence of calves, parity, and the number of times serviced, did not seem to affect SEM or PC, whereas PC and SEM rate (OR=2.2, P=0.04) was increased for cows in tie stalls.

Sawa et al. (2011) showed a significant interaction between the housing system and productivity levels or fertility-related parameters in dairy cows. In another study of 3,192 lactating cows, Popescu S. et al. (2013) showed that welfare of investigated cows was significantly better on farms that allow exercise for cows than on those that do not. The impact of tie stalls on fertility and SEM in our study, although in beef cows, seems to be in accord with these studies.

In conclusion, SEM is an important uterine pathology in Piedmontese cows that affects fertility by causing an increase in partum to conception time, mainly in cows kept in tie-stall housing. Cytology is a good diagnostic method for SEM diagnosis, and 8% PMN is a good diagnostic cutoff value that enables researchers to discriminate between healthy and affected cows. The presence of bacteria in

the uterus is detrimental to fertility but only when associated with the presence of PMN, i.e., SEM. Tie-stall housing systems without the possibility of movement have a significant impact on fertility, and their use is an important risk factor for subclinical endometritis in Piedmontese cows. Finally, a variety of bacteria, primary and secondary, have been isolated by accurate, traditional laboratory methods.

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Figure 1: Receiving Operating Characteristic (ROC) Curve assessing the cut-off point for percentage of PMN to evaluate the positivity to SEM.

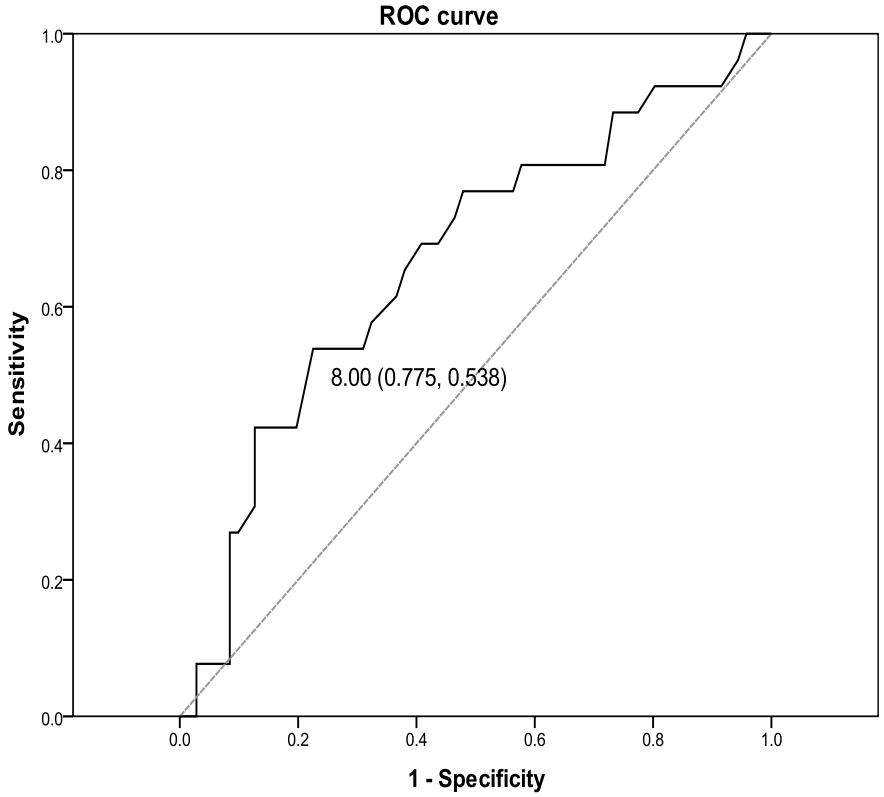

Figure2 : Kaplan-Meier survival analysis for cows with subclinical endometritis (SCE; dashed line) compared with healthy cows (thin line). SEM cows = 182 median day, Healthy cows =142 median day, (P<0.004)

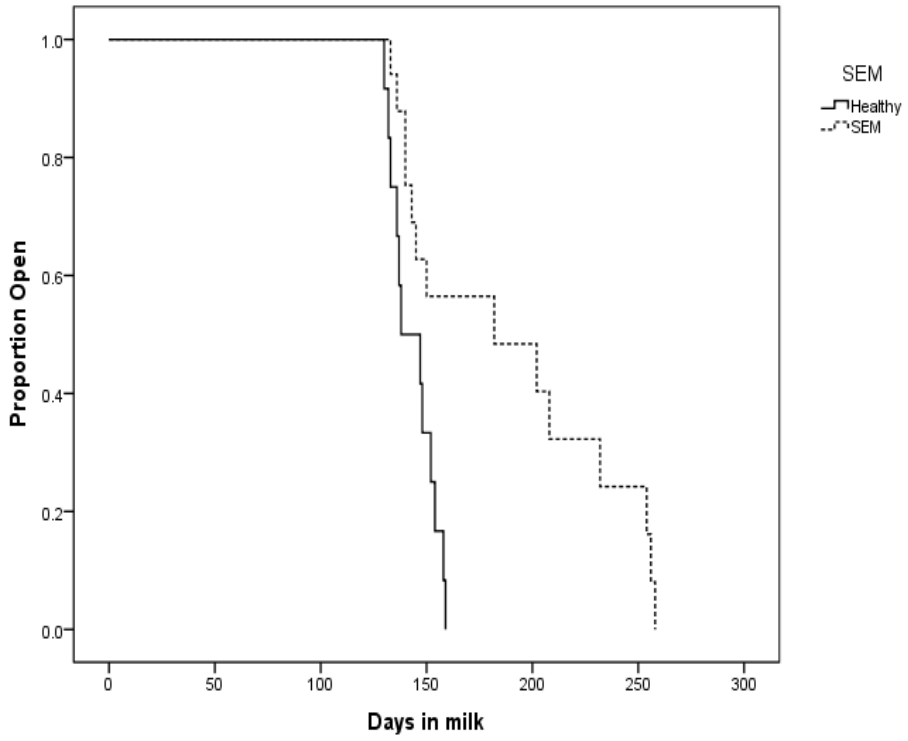

Figure 3: Box plot for the different categories of cows, None= negative for SEM and bacteriology, S= positive for SEM but not for bacteriology, B= positive for bacteriology but not for SEM, B-S= Positive either for bacteriology and SEM vith respective medians of 88.0, 97.5, 95.0, 142.5.

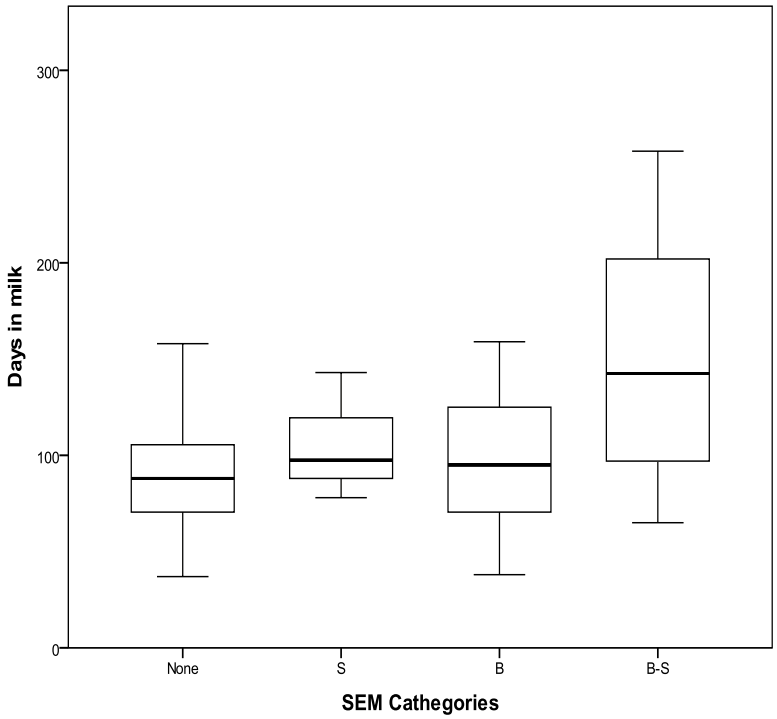

Table 1 : Bacterial species of bacteriology (main* and secondary‡) from uterine flushing .

Tipo	Freq	%
ACINETOBACTER	2	3
AEROCOCCUS VIRIDANS	5	8
BACILLUS SPP	12	19
BREVIBACTERIUM NON REACTIVE	2	3
DERMACOCCUS SPP	3	5
DIETZIA MARIS	2	3
DOLOSIGRANULUM PIGRUM	1	2
ENTEROCOCCUS SPP	2	3
ENTEROACTER	1	2
ESCHERICHIA COLI	**5**	**8**
FACKLAMIA HOMINIS	3	5
GLOBICATELLA SANGUINIS	3	5
GRANULICATELLA ADIACENS	1	2
HELCOCOCCUS KUNZII	1	2
HISTOPHYLUS SOMNI	2	3
KOCURIA ROSEA	7	11
LACTOCOCCUS LACTIS	1	2
LEIFSONIA AQUATICA	1	2
MICROCOCCUS	4	6
MORGANELLA	1	2
PANTOEA SPP	3	5
PASTEURELLA CANIS	3	5
POLIMICROBISMO	1	2
RHODOCOCCUS EQUI	1	2
SERRATIA MARCESCENS	5	8
SPHINGOMONAS PAUCIMOBILIS	5	8
STAPHYLOCOCCUS SPP	**10**	**16**
STREPTOCOCCUS	**10**	**16**
TRUEPERELLA PYOGENES	**20**	**32**

***Main** species (bold) are the species mostly referred from literature

‡**Secondary** (italics) are the species less indicated or not referred from literature.