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Moderate Inflammatory Reaction During Experimental *Escherichia coli* Mastitis in Primiparous Cows

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ABSTRACT

Nineteen primiparous cows were experimentally infected in 2 quarters with 1×10^4 (group A) or 1×10^6 (group B) cfu of *Escherichia coli* P4:O32 per quarter within 2 to 4 wk after parturition. Blood and milk samples were collected from all primiparous cows at regular time intervals from d –4 to d +3 relative to inoculation. Milk production rapidly decreased in both groups during *E. coli* mastitis, but recovery appeared to be faster in group B at d + 1 postinfusion (p.i.). The milk production losses in the noninfected quarters were substantial on the day of inoculation, which is probably due to pronounced systemic effects. However, on d + 2 p.i. milk production in the noninfected quarters nearly reached preinfection levels, indicating a moderate clinical severity following intramammary inoculation. None of the other severity criteria evolved towards a severe response pattern. Reticulorumen motility was inhibited in both groups during *E. coli* mastitis. The clinical episode was short lasting in both groups. Rectal temperature, heart rate, blood leukocyte count, number of colony-forming units, milk somatic cell count and several indicators for the disintegration of the blood-milk barrier returned to normal values within 24 to 72 h p.i. Primiparous cows reacted with a moderate inflammatory response following intramammary infusion with a relatively high dose of *E. coli*. Despite the use of a high inoculum dose, primiparous cows in both groups showed pronounced resistance against severe intramammary *E. coli* infection. A possible effect of the inoculum dose could be present, however, further research into the effect of the inoculum dose on the inflammatory response should be performed.
(**Key words:** primiparous dairy cow, *Escherichia coli* mastitis, inoculum size, early lactation)

Abbreviation key: BLC = blood leukocyte count, **HR** = heart rate, **MP** = milk production, **PCV** = packed cell volume, **p.i.** = postinfusion, **PIH** = postinfusion hour, **QMP** = quarter milk production, **ROS** = reactive oxygen species, **RR** = respiration rate, **RT** = rectal temperature.

INTRODUCTION

Several factors have been shown to play a role in the clinical outcome of *Escherichia coli* mastitis, namely farm management (Smith et al., 1985; Schukken et al., 1989a, 1989b; Oliver et al., 1990; Lam et al., 1995; Barkema et al., 1999), bacterial factors (Frost et al., 1980; Hill, 1981; Linton and Robinson, 1984; Sanchez-Carlo et al., 1984a; Sanchez-Carlo et al., 1984b; Todhunter et al., 1991; Hogan et al., 1992; Cross et al., 1993; Nemeth et al., 1994; Hogan et al., 1995, 1999; Nagy and Fekete, 1999), and physiological factors (Heyneman et al., 1990; Gilbert et al., 1993; Kremer et al., 1993a, 1993b; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997, 2001; van Werven et al., 1997; Mehrzad et al., 2001, 2002; Vangroenweghe et al., 2001; Burton and Erskine, 2003).

Preventive measures, which are known to be efficient against contagious mastitis, such as postmilking teat disinfection (Schukken et al., 1989a, 1989b; Barkema et al., 1999) have been shown to be inefficient in the control of *E. coli* mastitis (Smith et al., 1985; Oliver et al., 1990; Lam et al., 1995). Several epidemiological studies have demonstrated a negative correlation between SCC and the incidence of *E. coli* mastitis. Therefore, SCC is thought to be a farm-related risk factor for the susceptibility to *E. coli* mastitis (Schukken et al., 1989a, 1989b; Barkema et al., 1999).

Various bacterial virulence factors have been studied during *E. coli* mastitis (Sanchez-Carlo et al., 1984a, 1984b); however, only a few have been found to substantially influence the eventual outcome of the disease. *Escherichia coli* is of environmental origin (Nemeth et al., 1994). Although over 100 subtypes have been recognized, no specific O-serotypes have been related to bovine *E. coli* mastitis (Linton and Robinson, 1984). Nevertheless, intramammary challenge with *E. coli* 487 caused more severe clinical signs of mastitis than

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did *E. coli* 727 (Hogan et al., 1992, 1995, 1999; Todhunter et al., 1991). Moreover, a capsulated *E. coli* B117 strain appeared to cause more severe clinical symptoms of mastitis because it was more difficult to opsonize than a noncapsulated *E. coli* P4 strain (Hill, 1981). Clinical expression after challenge with low doses of *E. coli* is comparable with those after inoculation of high doses (Frost et al., 1980). The adhesion factors present in enterotoxigenic *E. coli*, which are essential during the initial steps of adhesion in pathogenesis, do not play a role in the pathogenesis of *E. coli* mastitis (Nagy and Fekete, 1999). A bacterial factor, which plays an important role in the pathogenesis of *E. coli* mastitis, is endotoxin or LPS. Lipopolysaccharide is a potent inducer of inflammatory cytokines (Shuster et al., 1993) during growth and killing (Burvenich, 1983). Clinical signs following experimentally induced *E. coli* mastitis are due to mediator shock rather than to endotoxin shock, because endotoxin mainly plays a local role (Hoeben et al., 2000; Dosogne et al., 2002).

Physiological factors have a big impact on the clinical outcome of *E. coli* mastitis (Burvenich et al., 2003), far more than farm management and bacterial characteristics that were sited above. Several markers to predict the clinical outcome of *E. coli* mastitis have been studied, such as the number of circulating leukocytes, production of reactive oxygen species (**ROS**) by neutrophils (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993), and chemotactic activity of PMN (Kremer et al., 1993a; Kremer et al., 1993b; Dosogne et al., 1997; van Werven et al., 1997). However, predictability of clinical outcome is only significant during 1 to 2 d prechallenge and does not explain any causal relationship. These markers were mainly studied in multiparous cows, varying between second lactation (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997) to second to sixth lactation (Kremer et al., 1993a, 1993b; van Werven et al., 1997). Because BHBA alters both PMN chemotactic activity and ROS production (Heyneman et al., 1990; Kremer et al., 1993a, 1993b; Vandeputte-Van Messom et al., 1993), many *E. coli* challenges that were executed at Ghent University and the University of Utrecht were performed on cows with a serum BHBA concentration <1.4 mmol/L (Kremer et al., 1993b).

Early lactating cows, infected with *E. coli*, are much more severely affected than cows after peak lactation (Hill, 1981). This is mainly due to the impairment of early lactation leukocyte function, as observed by many research groups (Burton and Erskine, 2003). During this period a decrease in cell function of the PMN, resident in the healthy mammary gland (Dosogne et al., 2001; Mehrzad et al., 2001; Vangroenweghe et al., 2001) has been observed. The decrease in cell function was mainly related to the decrease in viability, oxidative burst, and intracellular killing by PMN (Dosogne et al., 2001; Mehrzad et al., 2001; Vangroenweghe et al., 2001).

Besides energy balance and stage of lactation, cow parity was also found to be an important physiological factor that influences severity of clinical mastitis (Gilbert et al., 1993; van Werven et al., 1997; Mehrzad et al., 2002). Blood PMN function was higher in younger animals than in cows after their fourth parturition (Gilbert et al., 1993; van Werven et al., 1997). Moreover, viability and oxidative burst have been found to be significantly different between primiparous cows and multiparous cows during the periparturient period (Mehrzad et al., 2002).

In conclusion, many studies indicate that physiological factors determine the clinical outcome of *E. coli* mastitis. In this study the clinical outcome of *E. coli* mastitis was studied in primiparous cows using the same criteria for severity as in other studies using multiparous cows (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997; van Werven et al., 1997; Hoeben et al., 2000).

The purpose of the present study was to evaluate the outcome of intramammary *E. coli* inoculation in primiparous cows under identical conditions as described before with multiparous cows (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997, 2002; Hoeben et al., 2000). Moreover, the relation between preinfection parameters and the outcome of infection in term of severity was assessed. Finally, 2 high-inoculum doses, with a 100-fold difference (1×10^4 and 1×10^6 cfu), were used because the amount of LPS produced is related to the number of *E. coli* bacteria (Burvenich, 1983; Cross et al., 1993; Monfardini et al., 1999). The primiparous cows in this study were inoculated with the same strain as described before (Vandeputte-Van Messom et al., 1993; Hoeben et al., 2000; Dosogne et al., 2002).

MATERIALS AND METHODS

Experimental Animals and Study Facilities

All primiparous cows (n = 19) were in their seventh month of pregnancy on arrival at the dairy farm (CDFO—Commercial Dairy Farm Oudenaarde, Oudenaarde, Belgium) and between 24 and 30 mo of age at calving. The primiparous cows were on a system of zero grazing from arrival until the end of the inoculation trial and were fed twice daily at 0700 and 1700 h. The ration consisted of corn silage, good quality hay, and water ad libitum. Concentrates (Sandilac;

Dumoulin Voeders Sanders, Moorslede, Belgium) were distributed according to milk production.

For inclusion into the intramammary inoculation trial, treatment of clinical diseases (retentio secundinarum, mastitis, metritis, and so on) was not allowed within 10 d before the intramammary inoculation. Therefore, only healthy animals, free of major mastitis pathogens through 3 consecutive bacteriologically negative examinations with a foremilk SCC below 200,000 cells/mL at quarter level, were included for the intramammary *E. coli* challenge. Primiparous cows accepted for the intramammary challenge were inoculated between 14 and 28 d postparturition.

Machine milking was performed daily at 0800 and 1800 h using a quarter milking device (Packo & Fullwood, Zedelgem, Belgium). Daily quarter milk production (**QMP**), the yield of the evening and subsequent morning milking, expressed as liters per day, was measured at d –4, d –1, d 0 (postinfusion hour [**PIH**] 0 to 24), d1 (PIH 24 to 48), d 2 (PIH 48 to 72) and d 3 (PIH 72 to 96).

Inoculation Dose

The generation time of *E. coli* in mammary secretions can be as short as 20 min (Burvenich et al., 2003). Therefore, 2 high inoculum doses (1×10^4 [group A; n = 9] and 1×10^6 [group B; n = 10] cfu) were used in this experiment because we were interested in bacterial clearance rather than in bacterial growth in the affected mammary glands.

Intramammary Inoculation Procedure and Experimental Design

Inoculation was performed as described before (Hoeben et al., 2000). Briefly, *E. coli* P4:O32 (H37, β-glucuronidase +, haemolysin –), maintained as a stock in lyophilization medium at –20°C, was subcultured in brain-heart infusion broth (CM225; Oxoid, Nepean, ON, Canada) at 37°C during 3 consecutive days, subsequently washed 3 times with pyrogen-free PBS, and resuspended in PBS. Just before inoculation, the suspension was diluted in pyrogen-free PBS to a final concentration of 1×10^4 cfu/mL (group A) or 1×10^6 cfu/mL (group B). On d 0, 30 min after morning milking (1.5 h after feeding), the cows were inoculated in the left front and rear quarters with a total volume of 10 mL of pyrogen-free saline solution (0.9%) per quarter.

Animals were challenged on 4 different trial days. The 1×10^4 cfu inoculum group (group A) was challenged in a group of 4 and 5 animals, and the 1×10^6 cfu inoculum group (group B) was challenged in 2 groups of 5 animals.

Table 1. Severity estimation scheme, based on systemic disease signs, for the classification of primiparous cows, following an experimental inoculation with *Escherichia coli* P4:O32 (according to Wenz et al., 2001; with slight modifications). Briefly, the 4 parameters are scored, total score is calculated, and compared with respective ranges for classification into mild, moderate, or severe disease. In the present study, the total score did not exceed 5, meaning that no severe responses were observed throughout the entire experimental study period.

Variable	Criteria	Score
Rectal temperature (°C)	37.80–39.25	0
	39.30–39.80	1
	>39.80 or <37.80	2
Skin turgor	Regains normal shape in <5 s	0
	Regains normal shape in >5 s	1
Rumen motility rate (contractions/min)	3×/2 min	0
	1-2×/2 min	1
	0×/2 min	2
General attitude (signs of depression)	Alert	0
	Lethargic	1
	Depressed-unable to stand	2
	Extremely sick-recumbent	3
Total score	Mild disease	0-2
	Moderate disease	3-5
	Severe disease	6-8

Sampling Procedure

Blood and milk samples were collected at d –4, d –1, d 0, d +1, d +2 and d +3 relative to the day of challenge. On the day of challenge, blood and milk samples were collected at PIH 3, 6, 9, 12, 15, 18, and 21.

Blood samples were drawn aseptically from the external jugular vein of each cow by venipuncture in evacuated tubes (lithium heparin for plasma preparation, sodium-fluoride for glucose analysis, and coagulated for serum preparation) for subsequent use in different analyses. Foremilk was aseptically collected for diagnostic bacteriology on major pathogens before inoculation and for quantification of the number of *E. coli* (cfu) from PIH 3 onwards, determination of SCC, and preparation of skim milk. All samples were kept on melting ice (1°C) during transport and at the laboratory until analysis was performed.

Clinical Examination

Classical clinical parameters were examined. Rectal temperature (**RT**), heart rate (**HR**), respiration rate (**RR**), rumen motility, skin turgor, fecal appearance, appetite, general attitude, BCS (Edmondson et al., 1989), and aspects of the mammary gland (abnormal milk, swelling, teat relaxation, and milk leakage [Massart-Leën et al., 1988]) were recorded by a veterinarian each time blood and milk samples were collected.

Severity Determination

The severity of *E. coli* mastitis was determined based on QMP in the noninfected quarters at d +2

Table 2. Preinfection levels of blood and milk constituents (expressed as means ± SEM) in primiparous cows infused with 1×10^4 (group A; n = 9) and 1×10^6 (group B; n = 10) cfu of *Escherichia coli* P4:O32.

	(Values are means ± SEM)	
	Group A (n = 9)	Group B (n = 10)
Blood constituents		
β-Hydroxybutyric acid (mmol/L)	0.89 ± 0.20	0.66 ± 0.05
Glucose (mmol/L)	3.8 ± 0.3	3.2 ± 0.2
Blood leukocyte count (log_{10}/mL)	6.942 ± 0.024	6.903 ± 0.027
Milk constituents		
SCC (log_{10}/mL)	4.46 ± 0.10	4.18 ± 0.07
Albumin (mg/dL)	23.4 ± 3.3	27.8 ± 3.1
Milk production (l/24 h per quarter)	3.4 ± 0.4	3.8 ± 0.2
Fat (g/24 h per quarter)	155 ± 22	164 ± 29
Protein (g/24 h per quarter)	101 ± 5	110 ± 8
Lactose (g/24 h per quarter)	169 ± 18	186 ± 12

postinfusion (**p.i.**). Animals with a QMP in the noninfected quarters at d +2 higher than 50% compared with their QMP at d –1 in the same quarters were scored as moderate responders, whereas animals with a QMP at d +2 lower than 50% were considered severe responders (Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997, 1999). Moreover, severity was scored based on Wenz et al. (2001) with some slight modifications. Therefore, clinical data (RT, skin turgor, reticulorumen motility, and general attitude) obtained from PIH 9 to 48 were scored as described in Table 1, and based on their total score, primiparous cows were classified into mild, moderate, and severe responders.

SCC and Milk Composition

Somatic cell count was determined using a fluoro-opto electronic method (Fossomatic 400 cell counter; Foss Electrics, Hillerød, Denmark). Fat, protein, and lactose concentration (g/L) were determined using mid-infrared-photospectrometry (Foss Electrics). The daily production of fat, protein, and lactose (g/24 h per quarter) was calculated based on daily QMP and concentration of these parameters.

Indicators of the Disintegration of the Blood-Milk Barrier

Milk samples for the determination of serum albumin (mg/dL), sodium (Na^+), chloride (Cl^-), and potassium (K^+) concentration (mmol/L) were centrifuged at $1000 \times g$ (30 min, 4°C). Fat was removed and samples of skim milk were taken and immediately frozen at –80°C until analysis. After thawing, serum albumin was quantified using a radial immunodiffusion kit (bovine low level albumin, Bethyl VET-RID; Bethyl Laboratories, Montgomery, TX). Ion concentration was analyzed using an ion-selective electrode analyzer (Ilyte; Instrumentation Laboratories, Milan, Italy).

Packed Cell Volume, Blood Leukocyte Count, and Differentiation

Blood packed cell volume (**PCV**) (%) was determined in hematocrit-capillaries (60 μL/75 mm; Hirschmann Laborgeräte, Eberstadt, Germany) using a micro-hematocrit centrifuge (Hawksley, London, UK). Blood leukocyte count (**BLC**) (log_{10}/mL) was determined using an electronic particle counter (Coulter Counter Z2; Coulter Electronics Ltd., Luton, UK). Differential BLC was performed on blood smears. Briefly, 10 μL of homogenized whole blood was added onto a microscope slide and very thin smears were prepared, following drying and staining as described previously, differential counts were carried out (Mehrzad et al., 2001).

Colony-Forming United in the Inoculated Quarters

The number of *E. coli* (cfu/mL) after experimental inoculation was determined by appropriate 10-fold dilutions of each milk sample in PBS. Ten microliters of these dilutions was plated out on Columbia Sheep Blood agar (Biokar Diagnostics, Beauvois, France). All dilutions were performed in duplicate. Colonies were counted after a 24-h incubation at 37°C. The colony count was converted to cfu/mL based on the factor of dilution and finally expressed as log_{10}/mL for statistical analysis.

Glucose and β-Hydroxybutyrate in Serum

On arrival at the laboratory, clotted blood was incubated for 2 h at 37°C to neutralize the cryoglobulins present in bovine serum. After centrifugation ($1000 \times g$, 20 min, 20°C), serum was aliquoted and stored at

Table 3. Relationship between preinfection blood and milk constituents and the reduction in MP at d +2 (postinfusion h 48 to72) in the noninfected quarters of primiparous cows from both groups, assessed by linear regression with correction for inoculum dose. The slope value indicates the change in milk production reduction for one unit change in the blood and milk constituents.

	Slope	Standard error	*P*-value
Blood constituents			
β-hydroxybutyric acid (mmol/L)	–24	14	0.110
Glucose (mmol/L)	–11	6	0.089
Blood leukocyte count ($\log_{10}$/mL)	–44	64	0.497
Milk constituents			
SCC ($\log_{10}$/mL)	+12	20	0.529
Albumin (mg/mL)	–0.8	0.5	0.126
Milk production (l/24 h per quarter)	+0.013	0.014	0.120
Fat (g/24 h per quarter)	+0.14	0.07	0.060
Protein (g/24h per quarter)	+0.37	0.20	0.089
Lactose (g/24 h per quarter)	+0.23	0.12	0.067

–80°C until analysis of BHBA. NaF-collected blood was centrifuged (1000 × *g*, 20 min, 20°C) and plasma was collected and stored at –20°C until analysis of glucose.

Glucose concentration (mmol D-glucose/L) was determined using an UV-method (D-Glucose; Roche Diagnostics, Brussels, Belgium). β-Hydroxybutyrate (mmol/L) was determined twice in the week before challenge to check the status of the cow's energy balance. For analysis, the method with acetone oxidation, described by Williamson and Mellanby (1970), was used.

Statistical Analysis

The 2 inoculum groups were not formally compared because inoculum dose was not randomly assigned to animals. Preinfection values of blood and milk constituents of the inoculum groups (Table 2) were compared just before intramammary *E. coli* inoculation using a paired 2-sided *t*-test, assuming unequal variances (Statistix; Analytical Software, Tallahassee, FL). The relationship between preinfection concentrations of blood and milk constituents and the milk production reduction in the noninfected quarters at d +2 (PIH 48 to 72) was assessed by linear regression with an adjustment for inoculum size.

RESULTS

Relation Between Preinfection Blood and Milk Constituents and Percentage Reduction in MP at d +2 p.i.

No significant relationships between preinfection blood and milk constituents and the reduction in MP at d +2 p.i. could be demonstrated (Table 3).

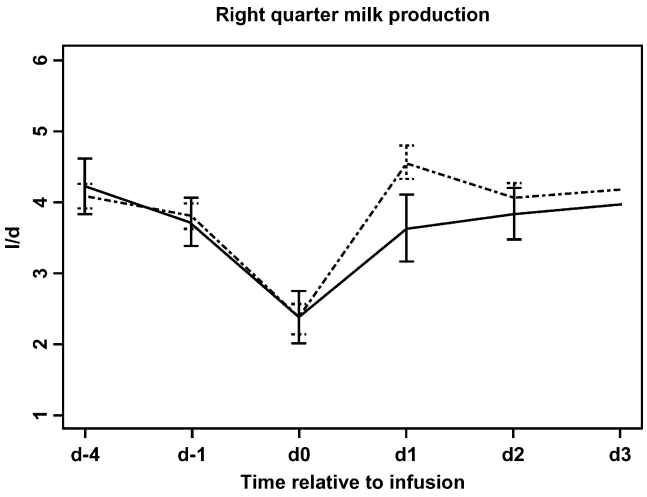

Figure 1. Milk production (L/d) of the uninfected right quarters from d –4 until d +3 relative to infusion from primiparous cows infused with 1×10^4 (—; group A; n = 9) and 1×10^6 (------; group B; n = 10) cfu of *Escherichia coli* P4:O32. Data are means (± SEM).

Clinical and Laboratory Results of Primiparous Cows in Group A Inoculated with 1×10^4 cfu

All primiparous cows in group A had a moderate clinical outcome of *E. coli* mastitis, based on their QMP of the noninfected quarters on d +2 p.i. Milk production in the noninfected quarters returned to preinfection level on d +2 p.i. (Figure 1). Rectal temperature increased from PIH 9 onwards, with peak fever reached at PIH 12 (Figure 2a). Heart rate and RR peaked at PIH 12 (Figures 2b and 2c). Rumen motility was depressed from PIH 9 onwards, resulting in a decrease in appetite. Primiparous cows returned to normal appetite and reticulorumen motility around PIH 21. Udder parameters, such as swelling and elevated quarter temperature, appeared at PIH 9 in the infected quarters. Teat relaxation, milk leakage, and diarrhea, considered as indicators for severe clinical illness, only appeared in a small number of animals. Changes to abnormal milk with clots, flakes, and a watery appearance were maximally present in all infected animals at PIH 18 (results not shown). Body condition score slightly decreased from 3.5 to 2.5 between calving and the end of the experimental challenge period.

Based on the severity scoring adapted from Wenz et al. (2001), primiparous cows in group A predominantly reacted with a mild response (n = 7) at PIH 9. At PIH 12, the clinical symptoms progressed mostly to a moderate response (n = 7). Thereafter, only one heifer with a moderate response was observed at PIH 15. No severe responses were observed at any timepoint between PIH 9 and 48 in group A, which received the 1×10^4 cfu *E. coli* inoculum dose.

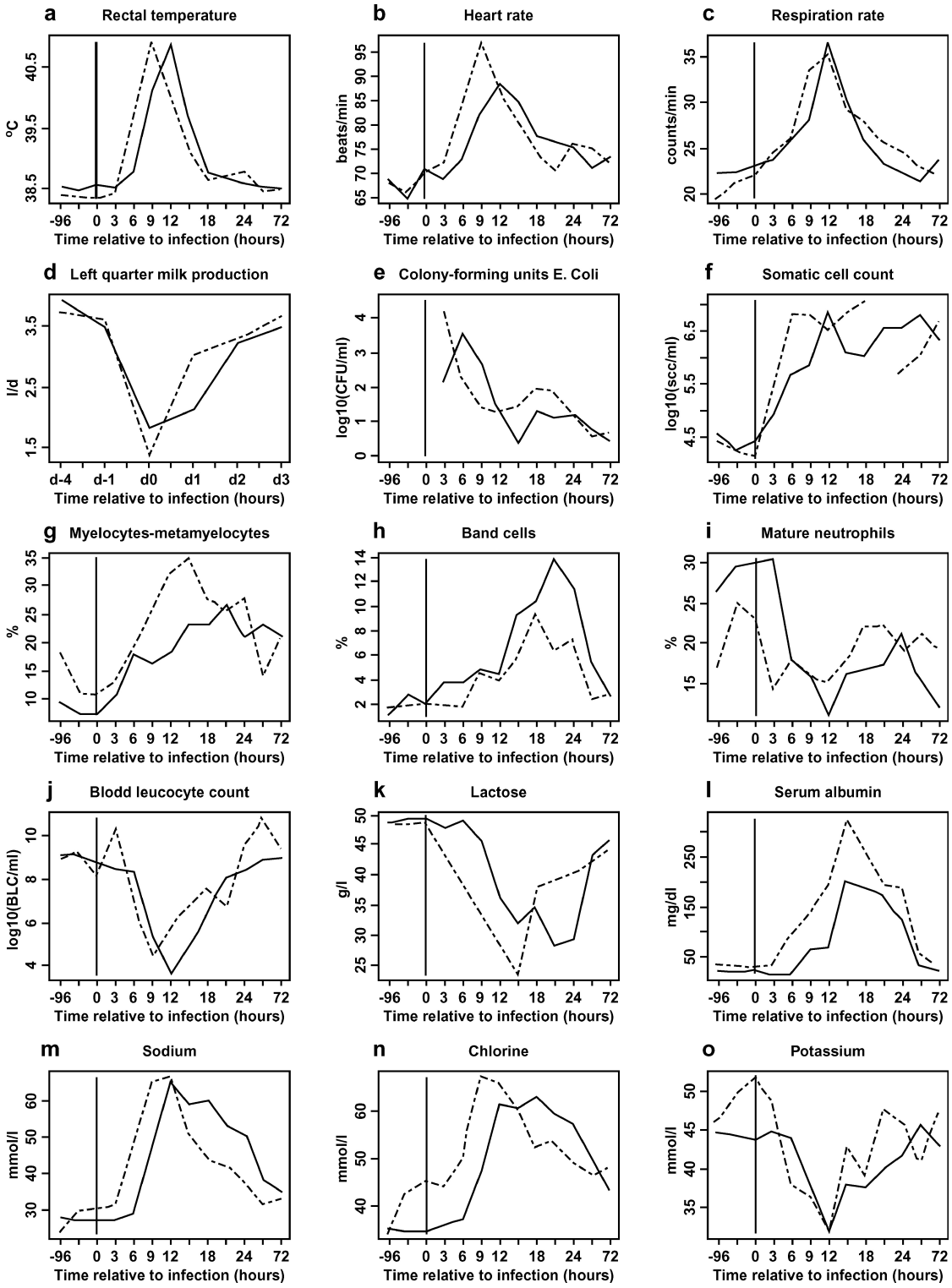

Figure 2. Rectal temperature (a), heart rate (b), respiration rate (c), left quarter milk production (d), number of colony-forming units of *E. coli* P4:O32 (e), SCC (f), percentage of myelocytes-metamyelocytes (g), percentage of band cells (h), percentage of mature neutrophils (i), blood leukocyte count (j), lactose (k), serum albumin (l), milk sodium (m), milk chlorine (n), and milk potassium (o) from postinfusion h –96 until postinfusion h 72 in infected quarters or blood, respectively, from primiparous cows infused with 1×10^4 (—; group A; n = 9) and 1×10^6 (------; group B; n = 10) cfu of *Escherichia coli* P4:O32. Data are means.

Milk production in the infected quarters maximally decreased (±55%) on the day of challenge. At d +1 p.i., MP gradually increased and almost totally recovered by d +3 p.i (Figure 2d). The number of *E. coli* in the infected quarters peaked at PIH 6, followed by a rapid clearance until PIH 15. At PIH 72, only 5 animals still had low numbers of *E. coli* in the infected quarters (Figure 2e). Somatic cell count rapidly increased from PIH 6, reached a maximum at PIH 12, and remained high until the end of the experiment at PIH 72 (Figure 2f).

Blood leukocyte count decreased to nadir at PIH 12 and recovered to preinfection level at PIH 48 (Figure 2j). The presence of early immature PMN (myelocytes-metamyelocytes) in circulation increased at PIH 6 and reached peak levels at PIH 21 (Figure 2g). Similarly, maximal percentages of late immature PMN (band cells) were reached at PIH 21 (Figure 2h). Concomitantly, circulating mature PMN decreased at PIH 6 with nadir at PIH 12 (Figure 2i).

The concentration of lactose in the infected quarters decreased from PIH 9 with a minimum at PIH 21 to 24 (Figure 2k). Serum albumin in the infected quarters increased from PIH 9 and peaked at PIH 15 (Figure 2l). Sodium and chloride concentrations peaked at PIH 12 (Figures 2m and 2n), whereas potassium concentration decreased from PIH 9 and reached its greatest reduction at PIH 12 (Figure 2o). All indicators for the disintegration of the blood-milk barrier gradually recovered to preinfection levels at PIH 72, after reaching their peak values (Figures 2k to 2o).

Clinical and Laboratory Results of Primiparous Cows in Group B Inoculated with 1×10^6 cfu

Clinical reaction and changes in laboratory parameters were similar to the previously described results for group A. However, the changes during inflammation generally appeared more rapidly (approximately 3 h) in primiparous cows from group B, which were infused with the 1×10^6 cfu *E. coli* inoculum dose, compared with group A, which were infused with the 1×10^4 cfu *E. coli* inoculum dose.

All primiparous cows in this group reacted with a moderate clinical response to intramammary *E. coli* infusion. Milk production in the noninfected quarters was only decreased until d +1 p.i. (Figure 1). Rectal temperature increased from PIH 6, peaked at PIH 9, and gradually decreased to preinfection values at PIH 18 (Figure 2a). Peak tachycardia was reached at PIH 9 (Figure 2b), whereas maximal RR was reached at PIH 12 (Figure 2c). Reticulorumen motility and all other clinical parameters seemed to change some 3 h earlier in group B. The same trend in BCS could be observed as in group A.

Clinical scores, adapted from Wenz et al. (2001), showed an earlier reaction (approximately 3 h) in group B compared with group A. At PIH 9, most of the primiparous cows reacted moderately (n = 8), gradually decreasing at PIH 12 (n = 5). Only one animal in group B had a prolonged moderate response until PIH 24. No severe responses were present in this group at any timepoint between PIH 9 and 48.

Milk production reduction in the infected quarters was maximal (±65%) on the day of infusion and recovered rapidly to preinfection production at d +3 p.i. (Figure 2d). The number of *E. coli* in the infected quarters peaked at PIH 3 (Figure 2e). A rapid influx of cells into the infected quarters was observed from PIH 3, with a peak at PIH 9 (Figure 5f).

The number of circulating blood leukocytes decreased from PIH 6, peaking at PIH 9. A pronounced rebound effect was observed between PIH 15 and 48 (Figure 2j). The increase in circulating early immature cells was pronounced and peaked at PIH 15 (Figure 2g). Band cells increased at PIH 9 and peaked at PIH 18, thereafter gradually decreasing to reach prechallenge levels at PIH 72 (Figure 2h). The greatest reduction in circulating mature PMN was reached at PIH 3, gradually increasing to preinfection values at PIH 21 (Figure 2i).

The concentrations of lactose, serum albumin, sodium, chloride, and potassium followed similar kinetics as in group A, although the initial changes appeared approximately 3 h earlier (Figure 2k to o).

DISCUSSION

In this study, the same strain and range of high inoculum dose of *E. coli* was used to induce *E. coli* mastitis as previously described (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993; Hoeben et al., 2000; Dosogne et al., 2002). In contrast to previous studies, primiparous cows were used. Similar clinical symptoms, such as quarter inflammation, fever, depression of reticuloruminal motility, loss of appetite, and general discomfort were observed as expected. In previous studies, however, the clinical responses showed large variations from mild-moderate to severe, whereas in the present study, the variation in clinical response was quite narrow. Quarter inflammation was associated with a temporary loss of MP, combined with the secretion of abnormal milk from the infected glands. Maximal decrease in MP in the infected and noninfected quarters occurred on the day of challenge, in contrast to previous observations where maximal decrease in MP was observed later (Heyneman et al.,

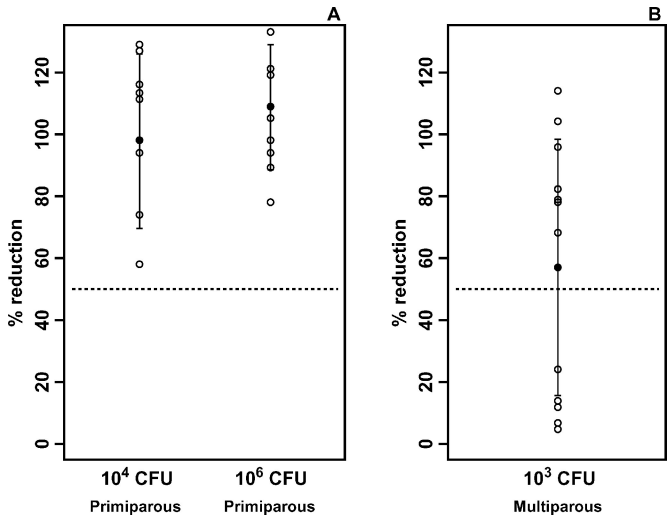

Figure 3. Percentage of initial quarter milk production (MP) in the uninfected quarters on d +2 (postinfusion h 48 to 72) relative to infection. A. Primiparous cows infused with 1×10^4 (group A; n = 9) or 1×10^6 (group B; n = 10) cfu of *Escherichia coli* P4:O32 in both left quarters (present study). B. Multiparous cows infused with 1×10^3 cfu of *Escherichia coli* O157 in both left quarters and scored into moderate (n = 7) and severe (n = 5) clinical response (Dosogne et al., 1997; historical control). Means (•) and standard deviation (I) of percentages of initial MP in the uninfected quarters were given for each group. Line (....) at 50% of initial MP arbitrarily indicates the difference between moderate and severe responders (Vandeputte-Van Messom et al., 1993).

1990; Hoeben et al., 2000). In the present study, none of the animals reacted severely following intramammary *E. coli* challenge (Figure 3). In both infected and noninfected quarters, milk production seemed to recover more rapidly on d +1 p.i. in primiparous cows from group B, which received the 1×10^6 cfu *E. coli* inoculum dose. Animals were scored as previously described (Vandeputte-Van Messom et al., 1993) and compared with the clinical score used by Wenz et al. (2001). This resulted in a similar classification of mild and moderate responses. The only difference that could be observed between group A and group B was that the time of latency was shorter when higher inoculum doses were used.

Body condition score and the concentration of BHBA and glucose indicated that the primiparous cows in this study were not ketotic at the time of challenge (Kremer et al., 1993b), although their QMP was comparable to the multiparous cows previously challenged (Heyneman et al., 1990; Vandeputte-Van Messom et al., 1993).

The number of circulating PMN, a marker to predict the clinical outcome of the disease, was similar to the levels previously observed in moderate responders (Vandeputte-Van Messom et al., 1993). Primiparous cows in this study could therefore be expected to react with a moderate clinical response. Although no PMN functionality was determined in this study, it is expected that PMN ROS production was high in these primiparous cows. Mehrzad et al. (2002) recently found that in the period from 5 wk before until 5 wk postparturition the chemiluminescence activity of PMN was higher in primiparous cows than in multiparous cows. Production of PMN ROS and chemotactic activity were inversely correlated to the clinical outcome of *E. coli* mastitis (Heyneman et al., 1990; Kremer et al., 1993a, 1993b). No significant relationship with the reduction in MP on d +2 p.i. was observed. This is possibly due to the limited number of animals in this study.

The onset of local clinical signs of mastitis, characterized by quarter swelling, coincided with the influx of PMN to the infected quarters. Somatic cell count increased rapidly in both groups, which is in accordance with earlier observations, where moderate responders had a rapidly occurring leucocytosis in the infected glands (Vandeputte-Van Messom et al., 1993). The extraction of mature PMN from the blood to the infected glands is known to result in early and late immature PMN recruitment from the bone marrow to restore the number of circulating PMN. In this study the recruitment of immature PMN was of short duration, when compared with Heyneman et al. (1990), who observed immature forms in circulation for at least 3 d in moderate responders and for almost 10 d in severe responders.

The rapid influx of PMN into the infected glands was associated with fast clearance of bacteria from the quarters. In this study, high inoculum doses were used for experimental induction of *E. coli* mastitis because we were mainly interested in bacterial clearance, rather than bacterial growth in the mammary gland. In contrast to an earlier study with the same inoculum dose in multiparous animals (Vandeputte-Van Messom et al., 1993), peak bacterial numbers were already reached around PIH 3 to 6. This peak number of bacteria was followed by a rapid clearance from the affected glands. Contrary to the induction of *E. coli* mastitis with low inoculum doses, where clearance is preceded by excessive bacterial growth, in this study, peak numbers were reached within 6 h p.i. and followed by a subsequent bacterial clearance. Therefore, PMN influx is thought to be fast and strong enough to rapidly clear the bacteria from the affected glands. It can be presumed that the bactericidal capacity of the PMN migrated to the infected quarters is high because more efficient PMN functionality was reported in primiparous cows recently (Mehrzad et al., 2002).

Several indicators (lactose, serum albumin, sodium, chloride, and potassium) for the disintegration of the

blood-milk barrier were determined in this study. Generally, the changes could be observed approximately 3 h earlier in group B. Peak levels were almost identical for both groups, however, which coincides with all other data, indicating that the animals reacted with a mild to moderate response and that little variation in the clinical response was present in this study.

From this study, it appears that the inflammatory response in primiparous cows from group B has an earlier onset compared with group A. One possible explanation for this observation could be the 100-fold difference in the number of *E. coli* infused into the mammary glands because the amount of LPS produced is related to the number of *E. coli* bacteria (Burvenich., 1983; Cross et al., 1993; Monfardini et al., 1999). A direct effect of LPS present in the inoculum can be excluded because the bacterial cultures were washed 3 times in pyrogen-free PBS before further dilutions were made. Lipopolysaccharide, known as a potent inducer of inflammatory cytokines (Shuster et al., 1993), can be produced quite rapidly during bacterial growth following intramammary infusion. Therefore, a sufficient amount of inflammatory cytokines should be produced early during inflammation in both groups, resulting in the rapid attraction of PMN from the blood into the mammary gland, with a subsequent pronounced increase of SCC in the infected glands.

Following *E. coli* mastitis, treatment with a bactericidal antibiotic at PIH 10 has been shown to be inefficient to alter local and systemic symptoms already present, although the number of bacteria in the infected quarters decreased 100-fold (Monfardini et al., 1999). Therefore, it can be suggested that early inflammatory events (first 3 h) could play an important role in the further regulation of the inflammatory response to combat the invading organisms. Experimental design in this study, particularly in relation to the number and time of samplings, however, was not suitable for unraveling these elements of early acute phase response.

CONCLUSIONS

In conclusion, despite the use of relative high inoculum doses, primiparous cows react with a moderate inflammatory response following intramammary infusion. This moderate response was evident from the preinfection number of circulating leukocytes, the concentration of BHBA and glucose, the prompt clinical response, the rapid influx of PMN into the infected quarters, the efficient bacterial clearance of the affected glands, and the fast recovery of MP in both infected and noninfected glands. To follow-up the bacterial clearance from the affected glands, the use of a high inoculum dose was of particular interest. In contrast to previous studies, preinfection parameters were not significantly related to the clinical outcome of the disease in terms of severity. The absence of a significant relation between these parameters could be due to the narrow variation in clinical response in the present study, especially compared with the large variation, ranging from mild-moderate to severe, observed in previous studies. The difference in time of latency between both inoculum doses could also be considered as an interesting observation. However, to further elucidate the effect of inoculum dose, a completely randomized study should be designed for this purpose.

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REFERENCES

Barkema, H. W., Y. H. Schukken, T. J. G. M. Lam, M. L. Beiboer, G. Benedictus, and A. Brand. 1999. Management practices associated with the incidence rate of clinical mastitis. J. Dairy Sci. 82:1643–1654.

Burton, J. L., and R. J. Erskine. 2003. Immunity and mastitis—some new ideas for an old disease. Vet. Clin. North Am. Food Anim. Pract. 19:1–45.

Burvenich, C. 1983. Mammary blood flow in conscious lactating goats in various physiological and pathological (mastitis) conditions. Ph.D. Thesis, Faculty of Veterinary Medicine, Ghent University.

Burvenich C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. Vet. Res. 34:521–564.

Cross, A. S., S. M. Opal, J. C. Sadoff, and P. Gemski. 1993. Choice of bacteria in animal models of sepsis. Infect. Immun. 61:2741–2747.

Dosogne, H., C. Burvenich, T. van Werven, E. Roets, E. N. Noordhuizen-Stassen, and B. Goddeeris. 1997. Increased surface expression of CD11b receptors on polymorphonuclear leukocytes is not sufficient to sustain phagocytosis during *Escherichia coli* mastitis in early postpartum dairy cows. Vet. Immunol. Immunopathol. 60:47–59.

Dosogne, H., E. Meyer, A. Sturk, J. van Loon, A.-M. Massart-Leën, and C. Burvenich. 2002. Effect of enrofloxacin treatment on plasma endotoxin during bovine *Escherichia coli* mastitis. Inflamm. Res. 51:201–205.

Dosogne, H., F. Vangroenweghe, B. Barrio, P. Rainard, and C. Burvenich. 2001. Decreased bactericidal activity of bovine blood and milk neutrophils against *Staphylococcus aureus* in dairy cows shortly after parturition. J. Dairy Res. 68:539–549.

Edmondson, A. J., I. J. Lean, I. D. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 72:68–78.

Frost, A. J., A. W. Hill, and B. E. Brooker. 1980. The early pathogenesis of bovine mastitis due to *Escherichia coli*. Proc. R. Soc. Lond., B 209:431–439.

Gilbert, R. O., Y. T. Gröhn, P. M. Miller, and D. J. Hoffman. 1993. Effect of parity on periparturient neutrophil function in diary cows. Vet. Immunol. Immunopathol. 36:75–82.

Heyneman, R., C. Burvenich, and R. Vercauteren. 1990. Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced *Escherichia coli* mastitis in cows. J. Dairy Sci. 73:985–994.

Hill, A. W. 1981. Factors influencing the outcome of *Escherichia coli* mastitis in the dairy cow. Res. Vet. Sci. 31:107–112.

Hogan J. S., V. L. Bogacz, M. Aslam, and K. L. Smith. 1999. Efficacy of an *Escherichia coli* J5 bacterin administered to primigravid primiparous cows. J. Dairy Sci. 82:939–943.

Hogan J. S., W. P. Weiss, D. A. Todhunter, K. L. Smith, and P. S. Schoenberger. 1992. Efficacy of an *Escherichia coli* J5 mastitis vaccine in an experimental challenge trial. J. Dairy Sci. 75:415–422.

Hogan J. S., W. P. Weiss, D. A. Todhunter, K. L. Smith, and P. S. Schoenberger. 1995. Effects of an *Escherichia coli* J5 vaccine on mild clinical coliform mastitis. J. Dairy Sci. 78:285–290.

Hoeben, D., E. Monfardini, C. Burvenich, and J. Hamann. 2000. Treatment of acute *Escherichia coli* mastitis in cows with enrofloxacin: effect on clinical signs and chemiluminescence of circulating neutrophils. J. Dairy Sci. 67:485–502.

Kremer, W. D. J., E. N. Noordhuizen-Stassen, F. J. Grommers, A. J. J. M. Daemen, P. A. J. Henricks, C. Burvenich, and A. Brand. 1993a. Preinfection chemotactic response of blood polymorphonuclear leukocytes to predict severity of *Escherichia coli* bovine mastitis. J. Dairy Sci. 76:1568–1574.

Kremer, W. D. J., E. N. Noordhuizen-Stassen, F. J. Grommers, Y. H. Schukken, R. Heeringa, and A. Brand. 1993b. Severity of experimental *Escherichia coli* mastitis in ketonemic and nonketonemic dairy cows. J. Dairy Sci. 76:3428–3436.

Lam, T. J. G. M., J. H. van Vliet, and Y. H. Schukken. 1995. Tepeldesinfectie en mastitis bij het rund: een literatuuroverzicht. Tijdschrift voor Diergeneeskunde 120:392–399.

Linton, A. H., and T. C. Robinson. 1984. Studies on the association of *Escherichia coli* with bovine mastitis. Br. Vet. J. 140:368–373.

Massart-Leën, A.-M., G. Vandeputte-Van Messom, and C. Burvenich. 1988. Oxytocin release during experimentally induced fever and inflammation in lactating ruminants. Arch. Int. Physiol. Biochim. 96:43–44.

Mehrzad, J., H. Dosogne, E. Meyer, R. Heyneman, and C. Burvenich. 2001. Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. J. Dairy Res. 68:399–415.

Mehrzad, J., L. Duchateau, S. Pyörälä, and C. Burvenich. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition, and early lactation. J. Dairy Sci. 85:3268–3276.

Monfardini, E., C. Burvenich, A.-M. Massart-Leën, E. Smits, and M. J. Paape. 1999. Effect of antibiotic induced bacterial clearance in the udder on L-selectin shedding of blood neutrophils in cows with *Escherichia coli* mastitis. Vet. Immunol. Immunopathol. 67:373–384.

Nagy B., and P. Z. Fekete. 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Vet. Res. 30:259–284.

Nemeth, J., C. A. Muckle, and C. L. Gyles. 1994. In vitro comparison of bovine mastitis and fecal *Escherichia coli* isolates. Vet. Microbiol. 40:231–238.

Oliver, S. P., S. H. King, M. J. Lewis, P. M. Torre, K. R. Matthews, and H. H. Dowlen. 1990. Efficacy of chlorhexidine as a postmilking teat disinfectant for the prevention of bovine mastitis during lactation. J. Dairy Sci. 73:2230–2235.

Sanchez-Carlo, V., J. S. McDonald, and R. A. Packer. 1984a. Virulence factors of *Escherichia coli* isolated from cows with acute mastitis. Am. J. Vet. Res. 45:1775–1777.

Sanchez-Carlo, V., R. A. Wilson, J. S. McDonald, and R. A. Packer. 1984b. Biochemical and serological properties of *Escherichia coli* isolated from cows with acute mastitis. Am. J. Vet. Res. 45:1771–1774.

Schukken, Y. H., F. J. Grommers, D. van de Geer, and A. Brand. 1989a. Incidence of clinical mastitis on farms with low somatic cell counts in bulk milk. Vet. Rec. 125:60–63.

Schukken, Y. H., D. van de Geer, F. J. Grommers, J. A. H. Smit, and A. Brand. 1989b. Intramammary infections and risk factors for clinical mastitis in herds with low somatic cell counts in bulk milk. Vet. Rec. 125:393–396.

Shuster, D. E., M. E. Kehrli, and M. G. Stevens. 1993. Cytokine production during endotoxin-induced mastitis in lactating dairy cows. Am. J. Vet. Res. 54:80–85.

Smith, K. L., D. A. Todhunter, and P. S. Schoenberger. 1985. Environmental pathogens and intramammary infection during the dry period. J. Dairy Sci. 68:402–417.

Todhunter D. A., K. L. Smith, J. S. Hogan, and P. S. Schoenberger. 1991. Intramammary challenge with *Escherichia coli* following immunization with a curli-producing *Escherichia coli*. J. Dairy Sci. 74:819–825.

Vandeputte-Van Messom, G., C. Burvenich, E. Roets, A.-M. Massart-Leën, R. Heyneman, W. D. J. Kremer, and A. Brand. 1993. Classification of newly calved cows into moderate and severe responders to experimentally induced *Escherichia coli* mastitis. J. Dairy Sci. 60:19–29.

Vangroenweghe F., H. Dosogne, J. Mehrzad, and C. Burvenich 2001. Effect of milk sampling and stage of lactation on milk composition, viability, and functions of resident cells in milk. Vet. Res. 32:565–579.

van Werven, T., E. N. Noordhuizen-Stassen, A. J. J. M. Daemen, Y. H. Schukken, A. Brand, and C. Burvenich. 1997. Preinfection in vitro chemotaxis, phagocytosis, oxidative burst, and expression of CD11/CD18 receptors and their predictive capacity on the outcome of mastitis induced in dairy cows with *Escherichia coli*. J. Dairy Sci. 80:67–74.

Wenz, J. R., G. M. Barrington, F. B. Garry, K. D. McSweeney, R. P. Dinsmore, G. Goodell, and R. J. Callan. 2001. Bacteremia associated with naturally occurring acute coliform mastitis in dairy cows. JAVMA 219:976–981.

Williamson, D. H., and J. Mellanby. 1970. D-(-)-3-Hydroxybutyrat. Pages 1772–1775 in Methoden der enzymatischen analyse. 2. Auflage—Band II. Bergmeyer H.U. (Ed.). Verlag Chemie, Weinheim, Germany.