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Staphylococcus aureus Exosecretions and Bovine Mastitis

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Summary

The role of *Staphylococcus aureus* and coagulase-negative staphylococcal exosecretions in bovine udder infection was tested by monitoring the cows' response to *in vivo* inoculation of bacterial exosecretions into udder quarters. Twenty Israeli-Holstein dairy cows were included in the study; two or three of the udder quarters of each cow were intracisternally inoculated with 0.04–0.05 mg/quarter (total proteins) of the various sterile bacterial exosecretions in a sterile pyrogen-free saline. Each udder was inoculated with two or three different bacterial exosecretions or placebo (Columbia Broth). Cows were monitored for 96 h post-inoculation for rectal temperature, heart and respiratory rates, alimentary tract activity (rumen contraction), udder temperature, pain, oedema and udder size. Milk samples were examined bacteriologically and for somatic cell count, *N*-acetyl-D-glucosaminidase (NAGase) activity and somatic cell differentiation. No enterotoxins (β–G) or toxic shock syndrome toxin-1 were detected in response to any of the bacteria tested. Control quarters or those inoculated with Columbia Broth, showed similar NAGase and somatic cell count values throughout the experiment. Twelve of the 18 strains tested, induced inflammation in the inoculated quarters while six did not. Of the 12 strains causing local inflammation, only six were found significantly different from the control and were considered as high response (group 1). The other six that caused a local inflammation did not differ significantly from the control, and were considered to be moderate response (group 2). The six *S. aureus* isolates that did not cause an inflammatory response were considered to have low response (group 3). In all quarters inoculated with *S. aureus* bacterial exosecretions belonging to groups 1 and 2, the polymorphonuclear cells and macrophages were proportionally increased while CD4$^+$ and CD8$^+$ T-lymphocyte populations decreased. One-dimensional NuPAGE (7%) Tris–acetate gel electrophoresisof the bacterial exosecretions revealed four different bands appearing between 36 and 31 kDa, marked from top to bottom as A, B, C and D. An association was found between the combinations of expressed bands and the cow responses: the majority of the cases could be linked to the expression of bands B and C.

Introduction

Staphylococcus aureus (*S. aureus*) is among the most important aetiological agents of bovine mastitis, a disease widely recognized as being of great public health importance and economic significance (Magalhaes-Lopes et al., 1990). The mechanism by which *S. aureus* evades immune elimination, and causes disease, remains elusive. Colonization by *S. aureus* and other staphylococci typically occurs in the external area of the teat duct prior to the illness (Cullen and Hebert, 1967). After colonization, mastitis results in severe damage to the mammary gland epithelial cell (Almeida et al., 1996), consequently increasing the inflammatory response in the gland. This phenomenon is probably a result of the various bacterial exonerations, which exhibit superantigen (SAg) activity and are potentially responsible for the severity of the disease. Evidence suggests that *S. aureus* SAgs have a suppressive effect by activation of lymphocytes with immunosuppressive activity (Park et al., 1992, 1993; Davis et al., 1996). One large family of SAgs is pyrogenic toxins (PTs) produced by *Staphylococcus* spp. and *Streptococcus pyogenes* (Bohach et al., 1990). Classical PTs produced by staphylococci isolated from humans include seven antigenically distinct enterotoxins (SEA, SEB, SEC1, SEC2, SEC3, SED and SEE), enterotoxin F (SEF) as later renamed toxic shock syndrome toxin-1 (TSST-1) (Bergdoll et al., 1981) and pyrogenic exotoxins A and B (Barsumian et al., 1978; Bohach and Schlievert, 1987). More recently, additional enterotoxins (SEG, SEH, SEI, and SEJ) have been identified (Zhang et al., 1988; Munson et al., 1998). In *S. aureus* isolates from ruminant mastitis, SEC was identified in 80% of the strains from sheep and in 6–16% of those from cattle; SED was also common among the bovine isolates (Hajek et al., 1978; Kenny et al., 1993). Moreover, TSST-1, SEC, and α and β-haemolysin contribute to the peracute bovine mastitis syndrome (Matsunaga et al., 1993). Enterotoxins and other PTs are potent T-cell activators (Peavy et al., 1970); major findings that have provided insight into the basis for their mitogenic activity are that enterotoxins and other PTs bind to major histocompatibility class II molecules (MHC-II) and to the $\alpha\beta$ T-cell receptor (TCR) (Fraser et al., 1989; White et al., 1989). Recently our group phenotypically characterized over 400 *S. aureus* strains (Younis et al., 2000) and several dozen coagulase- negative staphylococci (CNS) isolated from bovine mastitis in Israeli dairy herds. At the time of isolation, cows had subclinical mastitis, however, many of the cows recorded before examination, acute inflammation processes later became subclinical. In an attempt to find a correlation between the severity of the infection and any combination of the phenotypic characteristics (milk yield and somatic cell count (SCC) corresponding to the 6 months before sampling, or *N*-acetyl-β-D-glucosaminidase (NAGase) values at the day of

sampling), only positive correlation was found between the NAGase activity and the type of haemolysins (Younis et al., 2000). From that study, representative *S. aureus* strains that showed differences in their phage types and haemolytic patterns, and in the protein patterns revealed by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (1-D-SDS–PAGE), were selected for further study. In this study, an attempt was made to determine the severity of the cows' response to the bacterial exosecretions and moreover, to identify virulent factors. Bacteria were grown *in vitro*, and known enterotoxins and TSST-1 were tested. The role of the bacterial exosecretions was tested by monitoring the cows' responses to *in vivo* inoculation into their udders.

Materials and Methods

Animals

Twenty Israeli-Holstein dairy cows in mid-lactation with milk yields > 25 l/day were included in this study. The cows had at least three quarters free of infection with SCC $< 150 \times 10^3$ cells/ml and NAGase activity < 10 units. All cows were free of *S. aureus* udder infection. Udder conditions were tested by three consecutive daily examinations of quarter milk samples. Cows were kept in an open shelter providing for each cow 10 m^2 shaded slatted floor and 10 m^2 concrete-surfaced yard, and were milked three times daily. Food was offered in mangers located in the sheds.

Study Layout

The cows were brought to the stalls 1–2 h after milking and their teats were washed with warm water, dried, disinfected with individual non-woven towelettes moistened with chlorhexidine, cetrimide and ethanol (Medi-Wipes, AL Baad, Messuot Itzhak, Israel). The first three milk squirts were discarded and samples (50 ml/quarter) were then taken into a sterile tube for bacteriology, SCC, NAGase activity and somatic cell differentiation tests. Following this stage, the teats were again

disinfected and 0.04–0.05 mg/quarter (total proteins) of one of the sterile bacterial exosecretions in 5 ml pyrogen-free saline was inoculated intracisternally. Each cow was inoculated with two or three different bacterial exosecretions (each in different quarter), Columbia Broth (see bacterial exosecretions) or was left as a control. Each of the bacterial exosecretions was inoculated into two to four different cows with a different combination. Cows were monitored for rectal temperature, heart and respiratory rate, alimentary tract activity (rumen contraction) and udder temperature, pain, oedema and udder size every 4 h up to days post-inoculation (PI). Data on milk bacteriology, SCC and NAGase activity were recorded at 0, 4, 8, 12, 24, 48, 72 and 96 h PI. Milk somatic cell differentiation was performed only at 0, 24 and 48 h PI.

Bacteria

Eighteen strains of *S. aureus* (Younis et al., 2000), one strain of *Staphylococcus intermedius* (M2) (Chaffer et al., 1998) and one *Staphylococcus xylosus* (M6) were included. The bacterial identification is summarized in Table 1.

Bacterial exosecretions

Time of exosecretion harvesting was based on bacterial curve growth of various isolate. Each of the isolateswas cultured in parallel in: **1** 100 ml of non-pyrogenic Columbia broth (CBs) supplemented with 0.1% D-glucose, yeast extract and 0.5% NaCl, incubated at 37°C for 12 h, and the number of bacteria were determined (1–2.2×10^9/ml); **2** 100 ml of brain heart infusion broth (BHI), incubated at 37°C for 24 h. Bacteria were separated by centrifugation ($1000 \times$ **g** for 15 min). The crude supernatants, which included the exosecretions were collected and filtered through 0.2-μm pore-size membranes. Protein concentrations were determined with the Protein Assay kit (Bio-Rad, Munchen, Germany), after Bradford (1976). The bacterial exosecretions were concentrated tenfold in 5 ml of 7500 MWCO membrane Vivapore concentrator (Vivascience, Lincoln, UK)

Bacteria	Coagulase	Phage type[1]	Haemolysins
Staphylococcus aureus			
AL32/4	+	3/A,3/C, [29,88/89]	$\alpha + \beta$
AL302/1	+	3/A,3/C, [29,88/89]	α
AN51/3	+	85,55[3/A,29,52,52A,92]	β
AU3133	+	0 [0]	−
AZ/1	+	3/A,3/C, D11$^+$/HK$_2$	β
BH4792/1	+	3/A,3/C,55,71	$\alpha + \beta$
BS1	+	81	$\alpha + \beta$
CM1242/1	+	3/A,3/C	$\alpha + \beta$
FR780/4	+	0 [0]	−
FR2449/1	+	0 [0]	$\alpha + \beta$
GL62	+	52/A,53,83A,90,92,D11$^+$/HK$_2$	−
MH784/3	+	0 [0]	χ
NR888/2	+	3/A,3/C,71 [55]	−
NR2063/2	+	3/A,3/C,71 [55]	−
NR2597/4	+	3/A,3/C,71 [55]	−
NR2602/4	+	3/A,3/C,71 [55]	α
SY2794/2	+	3/A,3/C,55,71	β
SY2846/4	+	3/A,3/C,71 [55]	β
Staphylococcus intermedeus (M2)	−	−	−
Staphylococcus xylosus (M6)	−	−	−

Table 1. Coagulase production, phage type and haemolysin patterns of 18 *S. aureus*, *S. intermedius* and *S. xylosus* strains

[1]NI = no identification.

in order to achieve protein concentrations of 0.4 to 0.5 mg/ml, and were stored at −20°C. Sterility was tested by incubation of the bacterial exosecretions (1 ml) at 37°C for 6 days followed by culture on a blood agar plate for 24 h at 37°C.

Electrophoresis

One-dimension *NuPAGE* 10% Bis-Tris gel for small proteins and *NuPAGE* 7% Tris–acetate gel for large proteins (NOVEX-Electrophoresis, San Diego, CA) were used. The samples (bacterial exosecretions) were adjusted to 0.5 mg/ml protein concentration. To 62 μl of each sample were added 25 μl of 4 × NuPAGE sample buffer, 3 μl of ultrapure water and 10 μl of reducing agent. Sample mixtures were heated for 10 min at 70°C and loaded into the gel at 30 μl per lane. Unstained marker (NOVEX Mark 12, wild range standard) with a molecular weight of 200 to 31 kDa was used for the 7% gel and See Blue™ Pre-Stained Standard (lx) having a molecular weight of 191 to 14 kDa for the 10% gel.

Enterotoxin identification

Enterotoxins were identified by means of the 'microslide-immuno-diffusion-gel' technique (Su and Wong, 1997) at the Department of Food Hygiene, Ministry of Public Health, Haifa, Israel. Bacteria were cultured in BHI medium for 24 h at 37°C and in CBs medium for 10 h. at 37°C. Bacterial exosecretions were produced, collected and stored at −20°C. In addition, American Type Culture Collection (ATCCs) of different enterotoxins were compared with the various *S. aureus* bacterial exosecretions by means of one-dimensional NuPAGE 10% Bis-Tris gel electrophoresis.

Antibodies and conjugates

The monoclonal antibodies (VMRD Inc., Pullman, WA) used for the detection of the various leucocytes were: **1** anti-lymphocyte, anti-CD4$^+$: CACT 138A, anti-CD8$^+$: CACT 80C; **2** anti-monocyte/macrophage: BAQ151A; **3** anti-granulocyte: CH138A (G1) and **4** anti-CD11a/CD18: BAT75A. All monoclonal antibodies were species-reactive with bovine cells. The polyclonal antibodies used were: goat F(ab')2 anti-mouse IgG (H + L) conjugated with fluorescein isothiocyanate that exhibited minimal cross-reaction to human-, bovine- and horse-serum proteins, (Jackson Immuno Research Lab., West Grove, PA) and goat anti-mouse IgG-1 conjugated with TRI-COLOR affinity isolated (CALTAG Laboratories, Burlingame, CA).

Somatic cell count

The SCC was determined with a Coulter cell counter (CC), (Z1 model, Coulter Electronics Limited, Luton, UK).

NAGase test

The concentration of NAGase in milk was fluorometrically determined according to the ADLMILK NAGase test, (ADC Applied Diagnostics Corporation, Helsinki, Finland) with a computerized microplate setting. A value of 100 units corresponds to a release of about 5 μmol of product per litre per minute at 25°C.

Flow cytometry procedure

Cell differentiation was determined with a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) (Leitner et al., 2000). Milk samples were kept at room temperature and analyzed up to 3 h after collection. The laser beam was set at a wavelength of 488 nm. The number of somatic cells in each sample was determined by CC, and the volume of milk containing approximately 1×10^6 cells was split into 15-ml test tubes, one tube for each monocloral antibody (mAb) and one for the control. To calculate the percentages of the various leucocytes, 10 000 events were read per sample. The absolute cell number for each cell type was calculated from the SCC determined by CC.

Statistical analysis

The level of NAGase in each quarter pre-inoculation (time zero) subtracted from the value measured in each subsequent time. The resulting differences were obtained at 4, 8, 12, 24 and 48 h post-inoculation, and were termed 'NAGase Delta'. These data were subject to ANOVA (SAS, 2000) according to the following repeated-measurements mixed-model:

$$Y_{ijk} = M + \mathrm{BAC}_i + \mathrm{COW}[\mathrm{BAC}]_{ij} + T_k + \mathrm{BAC} * T_{ik} + E_{ijk}$$

where Y_{ijk} is the NAGase Delta value obtained from quarter of *j*-th cow (COW$_j$) inoculated with the *i*-th bacterial exosecretions (BAC$_i$), at the *k*-th time of monitoring (T_k). M is the grand mean, BAC$_i$ is the main fixed effect of the *i*-th bacterial exosecretions, COW[BACI]$_{ij}$ is the random effect of the *j*-th cow among those receiving the *i*-th bacterial exosecretions, T_k is the main fixed effect of the *k*-th time, BAC $* T_{ik}$ is the interaction between the *i*-th bacterial exosecretions and the *k*-th time, and E_{ijk} is the residual. In the ANOVA, COW[BAC]$_{ij}$ was the error term for the *F*-test of BAC$_i$, whereas E_{ijk} was the error term in the *F*-tests of the other effects. The mean of each bacterial exosecretions was tested versus the control by *t*-test contrast.

Results

Enterotoxins (A–G) and TSST-1 were not detected in any of the *S. aureus* and CNS tested by the microslide-immuno-diffusion-gel technique. Moreover, an attempt to detect the presence of the cleaved monomeric proteins released by standard enterotoxins ATCC strains, which range in size from 25.2 to 28.3 kDa, in comparison with our *S. aureus* bacterial exosecretions, showed no bands that matched at that molecular weight. An example for a gel with AZ/1 and FR2449/1 is presented in Fig. 1.

Control quarters, which received no inoculation, maintained similar NAGase and SCC values throughout the experiment. NAGase values were lower than 10 and SCC populations were lower than 150×10^3 cells/ml. No significant difference was found when comparing control quarters with quarters inoculated with the pyrogen-free CBs, indicating that the inoculation of CBs did not change either NAGase values or SCC populations. Therefore, the responses to the various bacterial exosecretions were compared with those of the non-inoculated and CB-inoculated quarters combined. Furthermore, the SCC values could not be analysed because many of the inoculated samples showed increases above the sensitivity of the CC
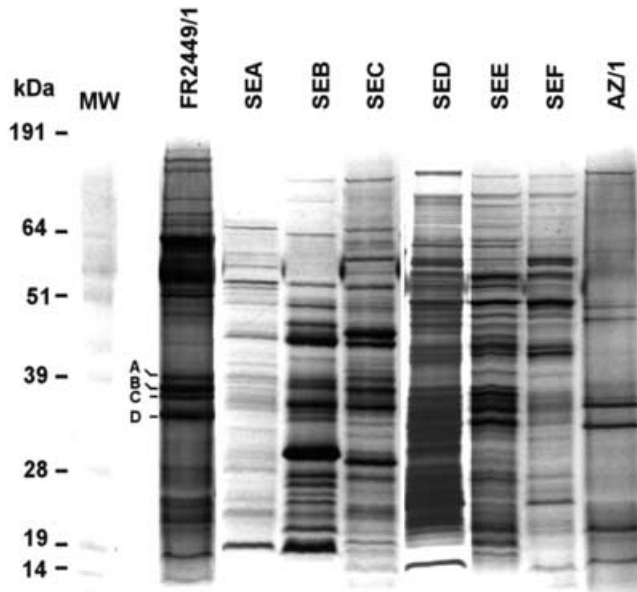
Fig. 1. Bis-Tris MOPS (3 [N-Morpholino] propansed fonic acid) proteins 10% of cleaved monomeric proteins released by standard enterotoxin ATCC strains, which range in size from 25.2 to 28.3 kDa and two representative *S. aureus* bacterial exosecretions (AZ/1 and FR2449/1).

(5000 × 10³ cells/ml). Therefore, later analysis was performed only with the NAGase Delta.

Inoculation of three bacterial exosecretions: *S. aureus* FR2449/1, GL62 and AN51/3, caused local and systemic clinical symptoms. Local symptoms of the udder included swelling, rigidity, pain and increased sensitivity, and decreased milk yield. Systemic symptoms were tachycardia (70–80 beats/min) tachypnoea (80–100 breaths/min), elevated rectal temperature (39–41°C) and low rumen activity (one to two contractions per minute). Clinical symptoms appeared as soon as 4 h PI, peaked at 10–14 h and disappeared at 24–48 h PI. The systemic symptoms appeared in all the cows inoculated with one of these three bacterial exosecretions, regardless of what other bacterial exosecretions were administered to the same cow.

Of the *S. aureus* bacterial exosecretions inoculated, 12 of 18 strains, including the three *S. aureus* mentioned above, induced a local inflammation of the quarters: SCC and NAGase activity increased 4–8 h PI and decreased to original levels within 48–72 h PI. In the other six bacterial exosecretions, no such changes could be recorded. Figure 2 compares one such cow's (1973) responses to quarter's inoculation with CBs, bacterial exosecretions of *S. aureus* AZ/1, or AL32/4, or left as non-inoculated quarter. In that cow, no significant difference in NAGase activity was observed among the quarters inoculated with CBs, inoculated with bacterial exosecretions from AL32/4 and the non-inoculated ones. However, inoculation with bacterial exosecretions of AZ/1 affected the NAGase activity, which increased from 8 h PI, peaked at 12 h and decreased to its original level at 24 h PI.

Significant differences were found between different time of monitoring and between bacterial exosecretions. Analysis of the NAGase Delta, showed that only six of the 12 *S. aureus* bacterial exosecretions that induced inflammation did so to an
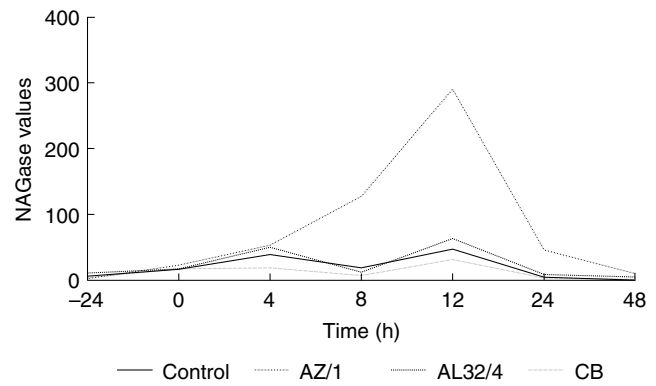

Fig. 2. Cow-1973 Delta NAGase values: response to quarter inoculation with CB, bacterial exosecretions of *S. aureus* AZ/1 or AL32/4, and values in non-inoculated quarter.
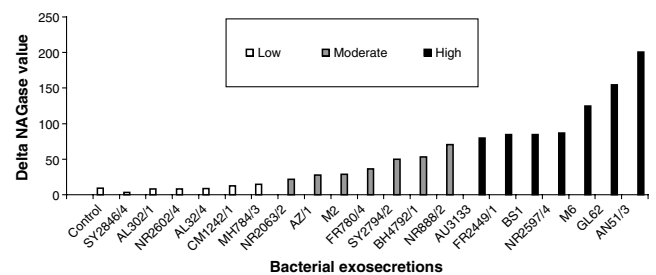

Fig. 3. *In vivo* determination of *S. aureus* and CNS bacterial exosecretions response according to milk Delta NAGase values after intracisternal inoculation of 0.04–0.05 mg/quarter (total proteins) in 5 ml pyrogen-free saline.

extent that was significantly different ($P < 0.001$) from the controls. Therefore, the 18 *S. aureus* isolates were divided into three groups according to the responses they elicited: **1** high response: comprised the six *S. aureus* isolates, AN51/3, GL62, NR2597/4, BS1, FR2449/1 and AU3133, that induced the highest inflammation responses which were found significantly different from the control (Fig. 3): **2** moderate response: comprised the six *S. aureus* isolates, NR888/2, BH4792/1, SY2794/2, FR780/4, AZ/1 and NR2063/2, that induced inflammation responses *in vivo* which did not differ significantly from the control and **3** low response: comprised the six *S. aureus* isolates, MH784/3, CM1242/1, AL32/4, NR2602/4, AL302/1 and SY2846/4 that did not induce an inflammatory response. Of the bacterial exosecretions of the two CNSs, *S. intermedius* (M2) was ranked as moderate and *S. xylosus* (M6) as high response.

In all quarters inoculated with the high and moderate response bacterial exosecretions (groups 1 and 2) or with the CNS strains, the proportions of polymorphonuclear cells and macrophages increased and those of CD4⁺ and CD8⁺ T lymphocytes decreased. In contrast, the proportion of leucocytes did not change in the quarters inoculated with CBs or with any of the six *S. aureus* bacterial exosecretions from group 3. As a result, the counts of total leucocytes (CD18⁺), PMN and CD8⁺ were found significantly different ($P < 0.001$) among the three virulent groups, but the effect of the time of sampling was not found to be significant. In order to analyse the actual change in the absolute number of each

Table 2. Mean log$_2$ and probability (F) values from twoway ANOVA of total leucocytes, Polymorphonuclear neutrophils, CD14$^+$ (Mo) and CD4$^+$ and CD8$^+$ T lymphocytes in milk from quarters inoculated with the various *S. aureus* bacterial exosecretions groups (low, moderate, high). Each value is the mean of all the bacterial exosecretions inoculated according to their virulence group and time of testing

bacterial exosecretions Inoculation	CD18$^+$	PMN	CD14$^+$	CD4$^+$	CD8$^+$
CB	16.18[b]	15.79[b]	13.79[b]	13.69[b]	13.74[b]
Low	15.89[b]	15.98[b]	12.81[b]	13.06[b]	13.20[b]
Moderate	17.76[a]	17.37[a]	15.42[a]	15.29[a]	15.54[a]
High	19.04[a]	18.68[a]	16.85[a]	16.53[a]	16.16[a]
Pr > F					
Groups	< 0.0003	< 0.0004	< 0.002	< 0.002	< 0.04
Time	< 0.01	< 0.04	< 0.008	0.074	NS
Groups/time	NS	NS	NS	NS	NS

[a,b]Mean log$_2$ within column with no common superscript differ significantly ($P < 0.05$) Groups = virulence of the *S. aureus*, 1–3; Time = time of sampling after inoculation, 0–96 h; NS = not significant.
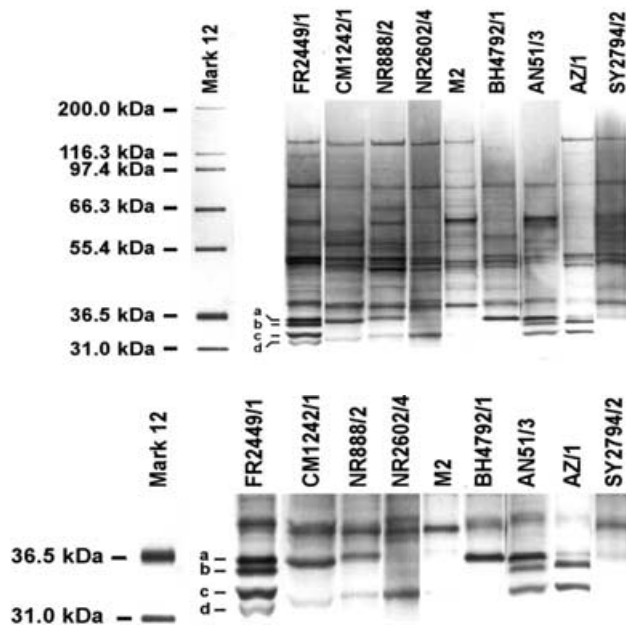

Fig. 4. Tris–acetate 7% gel. Top: full band patterns of various *S. aureus* and one CNS (M2) bacterial exosecretions. Bottom: section of the gel between 36.5 and 31.0 kDa, showing the expression of the various band (a, b, c, d) combinations.

type of cells, the percentages were multiplied by the SCC and then transformed into log$_2$. In some samples at peak response, SCC reached 5000×10^3, the limiting sensitivity of the CC and, therefore, log$_2$ was calculated from this number. Because of the increases of SCC in groups 1 and 2, the number of each of the leucocytes tested increased significantly relative to the control value, and the effects of time was also significant (Table 2). This phenomenon was not observed with bacterial exosecretions from group 3.

One-dimensional NuPAGE (7%) Tris–acetate gel electrophoresis of the bacterial exosecretions revealed four different bands between 36 and 31 kDa; Fig. 4 represents one such gel. These four bands are marked from top to bottom as a, b, c and d. The bacterial exosecretions of *S. aureus* FR2449/1 produced all four bands, five bacterial exosecretions of *S. aureus*, AN51/3, GL62, BS1 and AZ/1, NR2597/4 had combinations of bands, A, B and C (Table 3). Combinations of bands A and C were detected in bacterial exosecretions from three *S. aureus*

Table 3. Expression of combinations of the four bands in the various supernatants

Bands	Isolates	Cow responses group
A, B, C, D	FR2449/1	High
A, B, C	AN51/3, BS1, GL62, NR2597/4	High
	AZ/1	Moderate
A, C	AU3133	High
	BH4792/1, NR888/2	Moderate
A	AL32/4, AL302/1, CM1242/1, MH784/3, SY2846/4	Low
	SY2794/2	Moderate
B	FR780/4, NR2063/2	Moderate
	M6	High
C	M2	Moderate
	NR2602/4	Low

strains: AU3133, NR888/2 and BH4792/1. Band A was detected in bacterial exosecretions from *S. aureus* strains SY2794/2, MH784/3, CM1242/1, AL32/4, AL302/1 and SY2846/4, of which all except SY2794/2 belonged to group 3. Band B was detected in bacterial exosecretions from *S. aureus* strains FR780/4 and NR2063/2, and band C in bacterial exosecretions from *S. aureus* NR2602/4. Bacterial exosecretions from M6 andM2 had bands B and C, respectively (Fig. 4).

Association between combination of bands and virulence in the *in vivo* inoculation could be attributed to the expression of bands B and C in the majority of cases. Moreover, in some bacterial exosecretions, band C or B alone was associated with a moderate response, however, in one case (NR2602/4) the bacterial exosecretions had band C but the *in vivo* inoculation revealed no response.

Discussion

The severity of mastitis resulting from *S. aureus* infection is partly associated with thebacterial exosecretions, which exhibit SAg activity. In the present study, all *S. aureus* strains were isolated from field events of subclinical chronic bovine mastitis, and severity varied among the isolates. Attempts to correlate betweenphage type, *lipase* activity, DNase activity or haemolysin type and the severity of the infection as demonstrated by NAGase activity on the day of sampling showed

only a low-significance correlation with the type of haemolysins (Younis et al., 2000). Much attention has been focused on SEC (Deringer et al., 1997; Ferens et al., 1998), the predominant SEC associated with the disease in ruminants. However, no enterotoxins that included SEC or TSST-1 were detected in any of the isolates tested by the microslide-immuno-diffusion-gel technique. Moreover, no such bands could be found when the bacterial exosecretions were compared with the cleaved monomeric proteins released by standard enterotoxins of ATCC strains. It is possible however, that some of the *S. aureus* strains used in this study carried genes for SEs, but under the growth conditions used in this experiment, the microslide-immuno-diffusion-gel method did not detect it. With some of the bacterial exosecretions, four bands were detected between 36 and 31 kDa, where bands B and C were found to be positively associated with the severity of the cow's response and may be indicative of the bacterial virulence. At present, the nature of these proteins is not known, however, their molecular weights are different from those of the enterotoxins, which lie between 29 and 26 kDa, suggesting that these two proteins are not enterotoxins. Moreover, the results suggest that bands B and C are PTs, as the bacterial exosecretions containing these proteins induced inflammation in the udder. The inoculation model used in the present study is similar to that used for *Escherichia coli* inoculation lipopolysaccharide endotoxin (Heald, 1979; Guidry et al., 1983; Saad and Ostensson, 1990). Furthermore, the cows' responses to the high and moderate bacterial exosecretions were similar to their responses to intramammary inoculation of *S. aureus* (Shoshani et al., 2000) during the first 24–48 h, whereas their responses in the field trial persisted for up to 48 days because of the live bacteria present in the udder. The degree of bacteria virulence to the udder included penetration of the bacteria, resistance to phagocytosis and killing, toxicity, etc. However, the severity of the cows' response to bacteria after their establishment in the udder was the actual event studied. *S. aureus* and other CNS have long persistence periods in the cow's udder after penetration, while the severity of the cow's response: increased SCC, NAGase activity, varied among cows infection (Leitner et al., 2000). Therefore, the cow's responses to the various bacterial exosecretions could point to the mechanisms by which bacteria stimulates the immune system to recruit leucocytes into the udder, with the consequent increase of SCC. In conclusion, this study showed an association between two proteins within the bacterial exosecretions and the severity of the cow's response. Further studies are in progress, intended to purify, sequence, clone and characterize the role and mode of action of these proteins.

Acknowledgements

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