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Phenotypic Characteristics of *Staphylococcus aureus* Isolated from Bovine Mastitis in Israeli Dairy Herds

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With 1 figure and 2 tables

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Summary

A study of the characterization of the phenotypic patterns of *Staphylococcus aureus* strains isolated from bovine subclinical mastitis in Israeli dairy herds and their correlation with the severity of the disease was undertaken. A total of 400 chronically *S. aureus*-infected Israeli-Holstein cows, from 15 dairy herds were included in this study. Based on the results of the biochemical reactions, of the anti-biogram and phage typing, one major type of *S. aureus* was determined in each herd, its prevalence being between 54 and 100 % of the total isolates from that same herd. The majority of the isolates were found to be non-haemolytic (62.7 %). The most common phage type was 3/A,3/C,55,71, which was predominant in five herds. In two herds none of the isolates (24) were typable by this set of phages. All isolates were susceptible to methicillin, erythromycin, cephalotin, norfloxacin, trimethoprin-sulphamethoxazole and novobiocin. Most isolates were resistant to penicillin (96.6 %) and 52 % to oxytetracyclin. Differences in protein patterns between 50 and 36 kDa were found by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis. No correlation between any combination of the phenotypic characteristics was found when correlation was done with milk yield and somatic cell count, corresponding to the 6 months before sampling. Otherwise, a positive correlation was found between type of haemolysis and the *N*-acetyl-β-glucosaminidase (NAGase) values. In milk from quarters infected with the-non-haemolytic strains, the level of NAGase was significantly lower ($P < 0.05$) than that from quarters infected with the haemolytic strains (69.7 and 105.9, respectively). However, the level of NAGase activity in the milk of the quarters infected with the non-haemolytic strains was significantly higher ($P < 0.05$) when compared to the milk of quarters infected with coagulase-negative staphylococci (43.5).

Introduction

Mastitis is one of the major causes of economic loss in dairy cattle. *Staphylococcus aureus* is the main pathogenic species causing the subclinical form of mastitis. This type of udder infection impairs alveolar function, reduces milk yield and has a deleterious effect on milk composition, including increased milk somatic cell count (SCC) (Gudding et al., 1984; Nickerson, 1989). previous studies have evaluated the characteristics, pathogenesis and typing of *S. aureus* associated with human and animal infections. One of the most accepted techniques in epidemiological studies is bacteriophage typing. Although there are different typing sets, *S. aureus* from bovine mastitis has mainly been characterized by using the human international set of phages according to the technique of Blair and Williams (1961), (Wallace et al., 1962; Frost, 1967; Laevens et al.,

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1996). Other phenotypic characterization includes haemolytic activity, biochemical and enzymatic reactions, including activity on selective media, and antibiotic susceptibility patterns (Frost, 1967; Matsunaga et al., 1993; Aarestrup et al., 1995a; Laevens et al., 1996). These works show marked differences among the characteristics of *S. aureus* isolates, with the different methods for identification and typing used, without establishing the relationship between bacterial type and the clinicopathological reaction of the mammary gland. The aim of the current study was to characterize the phenotypic patterns of *S. aureus* isolates taken from cows with bovine subclinical mastitis in Israeli dairy herds and to correlate them with the severity of the disease as expressed by the SCC and *N*-acetyl-β-glucosaminidase (NAGase) values.

Materials and Methods

A total of 400 Israeli-Holstein dairy cows, taken from 15 herds in different regions of Israel, from diverse calvings and lactational stages, were included in this study. All cows were chronically infected with *S. aureus*. Cows were kept on coral providing 10 square m shade/cow and a 10 m^2/cow dirt yard, and milked three times a day. Food was offered in mangers located in the sheds.

Study design

Milk samples were collected from individual quarters of the cows. Teats were washed with warm water, dried with individual towels, disinfected with an individual non-woven towelette moistened with chlorhexidine, cetrimide and ethanol (Medi-Wipes, AL Baad Messuot Itzhak, Israel) and the first three squirts of milk were discarded. Milk samples (5 ml) were taken into a sterile tube and kept refrigerated (4°C) for bacteriological and NAGase activity examinations. Data on monthly SCC and milk yield during the 6-month period before sampling were received from the Israeli Cattle Breeders Association Herd Book.

Bacterial isolation and identification methods

Milk samples were cultured and bacteriological identification was conducted according to the National Mastitis Council protocols (Barnes-Pallesen et al., 1987). An aliquot of 10 μl from each sample was spread over blood agar plates (Bacto-Agar, Difco, Laboratories, Detroit, MI) containing 5% washed sheep erythrocytes and incubated at 37°C for 24 h. Colonies suspected of being staphylococci were sub-cultured on blood agar plates together with a *Streptococcus agalactiae* culture for the cyclic AMP (CAMP) test (Koneman et al., 1988). A coagulase test ([tube test] Anilab, Tal-Shachar, Israel; Koneman et al., 1988) and slide latex agglutination test (BACTI Staph, Remel, Santa Fe Drive, Lenexa, KS) were performed on all colonies suspected of being staphylococci in the sub-culture. Coagulase-negative colonies were included as 'CNS'. Coagulase-positive colonies were identified with ID-32-API STAPH test (BioMerieux S.A., Marcy L'etoile, France). Isolates were considered as being *S. aureus* if the isolate was identified $>98\%$ and $T > 65\%$. *Staphylococcus aureus* isolates were further tested on selective media: Baird Parker (BP) (Difco) for lipid activity and Toluidine Blue D'Nase (Remel), for the production of depolymerized deoxyribonuclease enzyme (Koneman et al., 1988).

Susceptibility test

The susceptibility test was performed using the disk diffusion technique on Mueller–Hinton agar (Difco) (Acar and Goldstein, 1986). Commercially available disks (Dispens-O-Disc, Susceptibility Test System, Difco) were applied as recommended, and the plates were incubated at 30°C for methicillin (5 μg/disk) and 37°C for other antibiotics, penicillin G (10 units/disk), oxytetracyclin (30 μg/disk), erythromycin (15 μg/disk), cephalotin (30 μg/disk), novobiocin (30 μg/disk), norfloxacin (10 μg/disk), trimethoprin-sulphamethoxazol (1.25–23.75 μg/disk). The interpretation of susceptibility or resistance was done as recommended. (Barnes-Pallesen et al., 1987).

Phage typing

Phage typing was performed by the technique of Blair and Williams (1961), with phages issued by the International Reference Laboratory, Colindale, UK, as modified by Samra and Gadba (1993).

Bacterial growth and supernatant harvesting for haemolysin pattern

Staphylococcus aureus was cultured in 250 ml Columbia broth (CB) supplemented with 0.1 % D-glucose, yeast extract and 0.5 % NaCl in a 3-l Ehrlemayer and incubated at 37°C for 10 h. The bacteria were separated by centrifugation (1000 ***g***, 15 min), the supernatant was collected and the pellet washed three times before storage at −20°C for electrophoresis. Supernatants of each of the isolates were tested for haemolytic activity against rabbit or sheep erythrocytes. The erythrocytes were washed and resuspended in phosphate-buffered saline (PBS) to a final concentration of 1 % (v/v). Supernatants were added to the erythrocytes at equal proportions and the mixture was incubated at 37°C for 30 min, followed by incubation at 4°C for 50 min.

Electrophoresis

One-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (1-D-SDS–PAGE) was performed according to the procedure of Laemmli (1970). Bacterial cells were disrupted with glass beads by a homogenizer (Braun Melsungen, AG, Germany) for 10–15 min. The glass beads and the remaining bacteria were removed by centrifugation (1000 ***g***, 15 min). The bacterial suspension was filtered through 0.2-μm filters. The protein concentration of the disrupted bacteria was determined with the Bio-rad protein assay, based on the method of Bradford (1976). Between 50 and 80 μl (depending on the protein concentration) of each disrupted isolate was added in 30 μl of a buffer containing 1.5 M Tris–HCl, pH 8.8, 10 % SDS, 20 % glycerol and 5 % 2-mercaptoethanol. Samples were heated for 5 min at 100°C, centrifuged (800 ***g***, 5 min) and loaded into the gel at 20 μl per lane. Gels (4 % stacking gel, 10 % resolving gels) were run at 25 mA for 45 min and stained with Coomassie brilliant blue R-250.

NAGase test

The concentration of NAGase in milk was fluorometrically determined according to the ADL MILK NAGase test (ADC Applied Diagnostics Corporation, Helsinki, Finland).

Statistical analysis

Data were analysed using the SAS general linear model (GLM) procedure (SAS/STAT® User's Guide, 1990). First the correlations were performed between the phenotypic characteristics of each isolate and haemolysis pattern, phage typing, lipase activity, D'Nase activity, susceptibility tests and protein pattern as results from the 1-D-SDS–PAGE. Correlation's were examined between and within each herd for milk yield, SCC corresponding to the 6 months before sampling and NAGase activity, and each of the phenotypic characteristics was studied.

Results

A total of 319 isolates was identified as *S. aureus*. However, some differences were recognized by the ID-32-API STAPH between the isolates considering the production of *urease,* when 84.6 % of the isolates were found to be urease positive and the remaining 15.3 % negative. The isolates were grouped according to their location (herds) and phenotypic characterization: type of haemolysis, lipid activity, DNase activity, phage typing, antibiotic susceptibility and the 1-D-SDS–PAGE SDS protein pattern. Major types of *S. aureus* were present in each herd, with a prevalence of 54–100 %, while a large variability was found between herds. No differences were found if haemolytic activity was tested on blood agar plates or the supernatant of the bacteria with rabbit or sheep erythrocytes. Of all *S. aureus* isolates, 62.7 % (200) were non-haemolytic, 23.2 % (74) $\alpha + \beta$ haemolytic, 12.5 % (40) β haemolytic and only 1.6 % (5) were α haemolytic (Table 1). The lipase activity, as tested with the BP method, resulted in 75.2 % positive and 24.7 % negative isolates. Of the isolates 83.3 % were DNase positive and 16.6 % were negative. The most common phage type was 3/A,3/C,55,71 (40 %) and it was isolated in five of the 15 herds tested. Moreover, high number of isolates from the other herds were sensitive to the 3/A,3/C phage in combination with others. In five of the herds, the isolates were sensitive to a high number of phages, primarily DH11/HK_2,90,92.24 isolates from two of the herds were not sensitive to any of the phages used.

All isolates were sensitive to methicillin, erythromycin, cephalotin, norfloxacin, novobiocin

Table 1. Distribution of 319 *S. aureus* isolates from 15 dairy herds in Israel according to haemolysin, lipase activity, DNase activity and phage typing

	Characterization	%
Haemolysis	negative	62.7 (10)
	$\alpha + \beta$	23.2 (5)
	β	12.5 (5)
	α	1.6 (2)
Lipase activity	positive	75.2 (4)
	negative	24.8 (14)
DNase activity	positive	83.3 (8)
	negative	16.7 (14)
Phage type	0 [0]	7.5 (2)
	71 [3/A]	0.6 (1)
	3/A, 3/C	4.9 (2)
	3/A, 3/C, 55	4.2 (1)
	3/A, 3/C, 55, 71	40.1 (5)
	3/A, 3/C [29, 88, 89]	3.7 (1)
	D11$^+$/HK$_2$, 84 [90]	3.5 (1)
	52/A, 53, 83/A, 90, 92, D11$^+$/HK$_2$	19.4 (1)
	6, 42/E, 52, 75, 79, 81, 88/89, 90, 92, D11$^+$/HK$_2$	5.1 (1)
	6, 42/E, 47, 53, 75, 83/A, 85, 90, D11$^+$/HK$_2$ [54, 81, 84]	1.1 (1)
	3/A,3/C,6,29,42/E,77, 81, 83/A, 84, 90, 92, D11$^+$/HK$_2$ [55, 75, 94, 96]	10.5 (2)

[a] % of isolates and number of herds which were isolated

and trimethoprin-sulphamethoxazole. Most isolates (96.6 %) were resistant to penicillin, and 52 % to oxytetracycline.

In comparing the protein patterns of the 25 representative strains with the 1-D-SDS–PAGE, differences were found between 50 and 36 kDa. Figure 1 presents seven different protein patterns, in Fig. 1(a) all bands are between 98 and 50 kDa and in Fig. 1(b) the bands are between 50 and 36 kDa. Each of the strains was coded according to these patterns.

No correlation was found between any combination of the phenotypic characteristics: haemolysis pattern, phage typing, lipase activity, DNase activity, susceptibility tests and protein pattern. No correlation was found when compression was done with milk yield and SCC corresponding to the 6 months before sampling with each of the phenotypic characteristics. Otherwise, when comparing was done between each of those phenotypic characteristics and NAGase activity, a positive correlation was found only between the type of haemolysis and the NAGase values. In milk from quarters infected with the-non-haemolytic strains, the level of NAGase was significantly lower ($P < 0.05$) than that from quarters infected with the haemolytic strain (69.7 and 105.9 nmol/min/ml/25°C, respectively) (Table 2). However, the level of the NAGase activity in the milk of the quarters infected with the non-haemolytic strains was significantly higher ($P < 0.05$) when compared to the milk of quarters infected with CNS (43.5) and significantly higher ($P < 0.001$) when compared to the milk from the quarters where no bacteria were found (18.2) (Table 2).

Discussion

Phenotypic characteristics of *S. aureus* isolated from cattle with chronic mastitis in Israeli dairy herds revealed high levels of identity within in each one of the herds, while a large variability was encountered between herds.

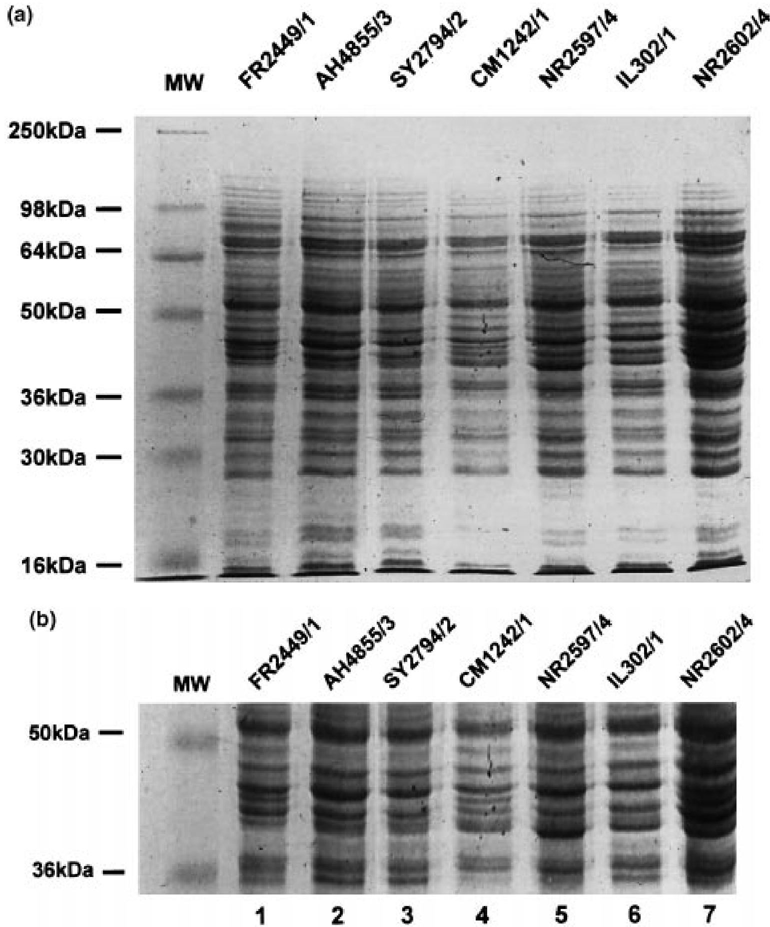

Fig. 1. One-dimensional SDS–PAGE (10 %) of disrupted *S. aureus* strains. Twenty microlitres (200–250 μg/ml protein) of disrupted *S. aureus* strain supernatant was applied to each column; (a) shows all the protein patterns of the strains and (b) shows the bands between 50 and 36 kDa. Lanes 1, FR2449/1; lanes 2, AH4855/3; lanes 3, SY2794/2; lanes 4, CM1242/1; lanes 5, NR2597/4; lanes 6, IL302/1; and lanes 7, NR2602/4. Molecular-weight markers are indicated on the right.

Table 2. Mean ± SE of NAGase activity in milk from quarters infected with haemolytic or-non-haemolytic *S. aureus*, or CNS, or healthy quarters with no bacteria found

Bacteria	Haemolysis	NAGase
S. aureus	positive	105.9 ± 9.3^{A}
S. aureus	negative	69.7 ± 7.1^{B}
CNS[a]	–	43.5 ± 6.1^{C}
No bacteria found	–	18.2 ± 2.4^{D}

^{A,B,C,D} Means with no common superscript differ significantly ($P < 0.05$); [a]CNS, coagulase-negative staphylococci

Haemolysis of sheep erythrocytes is one of the most common characterization techniques in the identification of *S. aureus* strains. In this study, over 63 % of the isolates were non-haemolytic strains. This result differ from other studies which described a high percentage of the isolates identified with haemolysis of different types (Frost, 1967; Poutrel and Ducelliez, 1979; Matsunaga et al., 1993). This difference could be explained by the initial identification in this study, i.e. all colonies suspected of being staphylococci were further identified by the coagulase test and by the ID-32-API STAPH. This suggested that in order to identify *S. aureus* from colonies suspected of being staphylococci it is necessary not only to test for coagulase activity and haemolysis but also for other parameters, such as acid production from carbohydrates.

The most prevalent phage type found in the present study was 3/A,3/C,55,77. In Sweden, Thorne and Hallander (1970) reported that phage type 3/A prevailed in 31.1 % of their isolations. However, in another report from the same country in 1975 (Holmberg, 1975) phage type 47,83A was found to be highly prevalent while 3/A represented only 1 % of isolates. In a study conducted in Northern Ireland by Mackie et al. (1987), phage type 3/A represented less than 2 % and type 55,77 less than 1 % while in a report from a Nordic country (Aarestrup et al., 1995a), 7 % (30/424) of the isolates were 3/A,3/C. Although our results differ from the others, the difference in the reports from Sweden may indicate that methods of sampling may have an important role as well as the difference in time periods, suggesting that in order to compare the epidemiology of *S. aureus*, similar examination methods should be established.

Although all strains had the same *in vitro* growing conditions, differences in the protein pattern, as shown in the 1-D-SDS–PAGE, clearly demonstrated that different proteins were produced, however, no correlation was found between the proteins band(s) nor any of the other phenotypic characteristics. Further examinations are being conducted for the identification of those proteins and their functions.

The high resistance to penicillin (96.6 %) is in agreement with results reported by Aarestrup et al. (1995b). In their study, 75 % of the strains isolated from bovine mastitis in Denmark were resistant to this antibiotic. The increasing penicillin resistance of *S. aureus* strains involved in mastitis may be related to the extensive use of this drug in mastitis treatment.

Economic losses due to udder infection are linked to the virulence of the pathogen. In this study no correlation was found between any of the isolates' characteristics and the milk yield or SCC during the 6-month period before the time of sampling. However, the comparison of the NAGase activity in the milk from the quarters infected with *S. aureus*, revealed that the non-haemolytic strains had significantly lower NAGase values than haemolytic strains and despite the difference in the absolute levels of NAGase between cows and herds, no interaction was found. These results are in agreement with those of other studies (Ward et al., 1979; Bramley et al., 1989; Calamari et al., 1992). NAGase is a lysosomal enzyme present in low levels in milk from the healthy bovine udder that originates mainly from the cytoplasm of udder tissue cells. Secretory disturbance caused by growth of pathogenic bacteria, results in tissue damage which causes an influx of cellular NAGase into the secreted milk (Kitchen et al., 1978). Nevertheless, it is important to relate to the non-haemolytic isolates differently than the CNS as shown in this study, because of the different levels of NAGase found. This could indicate that although haemolysis is related to the severity of mastitis, other proteins and/or glycoproteins are involved in the pathogenicity of the bacteria.

It is possible that although variability was found among the strains, owing to the way cows were chosen in this study (all cows chronically infected with *S. aureus*) isolation was mainly of virulent strains, while non-virulent strains, contaminating the udder and/or the environment, were not found. Further epidemiological monitoring of *S. aureus* strains and the resulting severity of mastitis episodes caused by them in dairy herds is warranted.

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