Vet.Med.J.,Giza. Vol.53, No.2. (2005) :253-265

STUDIES ON VIRULENCE FACTORS OF STAPHYLOCOCCUS AUREUS ISOLATED FROM MASTITIC BUFFALOES

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Received: 10.11.2004

Accepted:28.2. 2005.

SUMMARY

Staphylococci were isolated from 103 out of 393 mastitic quarters of buffaloes (26.2%), of which 62 isolates (36.7%) were recovered from clinical cases and 41 (18.3%) from subclinical cases. *S. aureus* was isolated from 23.7% and 13.7% of quarters of clinical and subclinical mastitic cases, respectively. Studies of virulence factors of *S. aureus* revealed that the majority of S. aureus isolates recovered from clinical mastitis (65%) grew as diffuse colonies on serum soft agar, indicating the presence of capsules, whereas almost the same percent of isolates from subclincal mastitis (64.3%) grew as compact colonies, which means they lacked capsules. Out of 40 *S. aureus* isolates obtained from clinical mastitic milk samples, 14 (35.0%) and 32 (80%) were positive for clumping factor and tube coagulase respectively, while out of 14 isolates recovered from subclinical mastitic milk samples, 9 (64.3%) and 10 (71.4%) isolates were positive for the same tests. 85.2% of the examined *S. aureus* isolates were positive for haemolysin production and the rate was higher among isolates detected from the clinical cases (90%) in comparison to that of subclinical cases (71.4%). 36 isolates (66.7%) were found to be toxigenic. Enterotoxin C was the most common as it was detected in 21 isolates (38.9 %), followed by enterotoxin A from 8 (14.8%) and enterotoxin B from 7 (13.0%). Mortality rate was higher among mice infected I/P with *S. aureus* recovered from clinical mastitic milk samples (73.3%), when compared with those infected with isolates obtained from subclinical mastitic milk samples (52.4%). The encapsulated isolates that produced toxins were highly virulent to mice. 100% mortality was caused by 12 isolates, of

which 9 isolates produced enterotoxin C, 1 isolate produced B and 2 produced A.

INTRODUCTION

Mastitis caused by *Staphylococcus aureus* is the most significant cause of economic losses of dairy industry; once it is established in the mammary gland, it is difficult to eradicate (Stringfellow et al., 1991). To establish an infectious process, S. aureus must overcome the different host defense mechanisms (Phagocytosis, elimination by milking etc) in order to reach and colonize the ductular and alveolar mammary gland region (Aguilar et al., 2001).

Bacterial binding to host cells is likely to be an important early event in the pathogenesis of several infections (Opdebeeck et al. , 1987). The interaction of the microorganism with the host is strongly dependent on its cell surface properties especially the presence of the capsular polysaccharide containing layer, which appears to play an important role in virulence (Norcross and Opdebeeck, 1983). *Staphylococcus aureus* is able to produce capsular polysaccharide (CP) in vivo or under defined culture conditions (Tollersurd et al. , 2000), encapsulated *Staphylococcus aureus* strains are more resistant to phagocytic uptake than non-capsulated strains (Sutra et al. , 1990).

The definition of the capsule in Staphylococcus aureus is much debate matter because of the diversity of the tests used for demonstrating encapsulation and the variation of expression of the capsule according to culture condition. Rather et al. (1985) used serum soft agar (SSA.) technique to investigate the presence of capsule in bovine milk *Staphylococcus aureus* isolates. In this technique, encapsulated strains show diffuse colonies, whereas compact colonies indicate the absence of capsule. Moreover, additional method to detect capsule, based on the masking of cell surface antigens, have proposed negative clumping factor (Calvinho and Dodd , 1994).

The aim of the present work was to determine the virulence factors of *Staphylococcus aureus* isolated from mastitic milk of buffaloes and to evaluate their correlation with pathogenicity to mice.

MATERIAL AND METHODS

Milk samples

A total of 157 dairy buffaloes in 4 herds from different farms were examined for mastitis. According to clinical observation and California mastitis test (Schalm et al., 1971), the mastitic cases (103) were classified into clinical and subclinical cases. A total of 393 individual quarter milk samples were collected from the mastitic buffaloes, 169 quarter milk samples from clinically infected animals (47 buffaloes) and 224 samples from subclinically mastitic animals (56 buffaloes).

Isolation procedure

Isolation of bacteria was carried out according to Quinn et al. (1994). Milk samples were incubated aerobically at 37°C for 24 h, then centrifuged at 3000 r.p.m. for 20 minutes. The cream and supernatant were discarded. A loopful from the sediment was streaked onto the surface of blood agar, nutrient agar, MacConkey's agar, EMB, mannitol salt agar, Edward`s medium and Sabouraud dextrose agar (Carter and Cole, 1990). The inoculated plates were incubated at 37°C for 24-48 h and examined for bacterial growth. Sabouraud dextrose agar plates were incubated for one week. Suspected colonies, appearing on different media were subcultured, purified, and preserved in semisolid agar for further identification. Only staphylococci were identified to the species level in this study.

Identification of Staphylococcus species:

Colonies of staphylococci were selected on the base of their strong catalase positive reaction. Further identification was carried out using the following tests: coagulase test using slide and tube methods, mannitol fermentation and salt tolerance using mannitol salt agar, haemolytic activity in 7 % sheep blood agar and fermentation of sugars viz. sucrose. maltose. Acetoin, urease, oxidase, nitrate and dnase tests were also applied (Cruickshank et al., 1975, MacFaddin, 1980 and Carter and Cole, 1990)

Detection of virulence factors of *Staphylococcus aureus:*

For detection of virulence factors of *Staphylococcus aureus,* the following tests were done: serum soft agar technique, coagulase tests, determination of haemolysin titre and detection of enterotoxins.

Serum soft agar technique (Opdebeeck et al., 1987): All isolates were grown in 18 ml of mod. Staphylococcus 110 broth at 37°C for 18 h., about 100 µl were transferred in 2 ml volume of mod. Staphylococcus 110 broth and incubated at 37°C for 2 h, of which one loopful was used to inoculate serum soft agar (SSA). The colony morphology was recorded as diffuse or compact after incubation at 37°C for 24 h.

Coagulase test (Marchlewicz and Duncan, 1980): All isolates were cultured in mod. Staphylococcus 110 broth for 5 days at 37°C , coagulase activity was determined either by the tube or slide methods. Positive and negative control were included.

Detection of haemolysin titre (Feder et al., 1994): Single colonies of isolates grown on tryptic soy agar (TSA) plates containing 5% sheep blood (24 h at 37°C in 6% CO_2) were subcultured onto TSA plates and incubated at 6% CO_2 for 16 h at 37°C . Colonies were harvested with cold RPMI 1640 medium and were transferred to an ice chilled flask. the RPMI suspension was in-

cubated aerobically for 1 h at 4°C , then centrifuged at 12.000 xg for 10 min. at (4°C) and 25 µl amount of supernatant were added to 25 µl of PBS in the first well of a microtiter plate and serial two fold dilutions in PBS were made in subsequent wells; 25 µl amount of 1% sheep erythrocytes suspension and 50 µl of PBS were added to each well and the plates were then incubated at 37°C for 30 min., followed by incubation for 2 h at 4°C .The titre was defined as the reciprocal of highest dilution showing complete haemolysis. Titre > 128 was considered positive.

Detection of enterotoxins (Igarashi et al., 1986): *S. aureus* enterotoxins were detected by the latex test using RPLA kit (Sanko, Tokyo, Japan). The isolates were grown in 5 ml brain heart infusion broth and incubated at 37°C for 18 h; culture supernatant was diluted five folds and placed into microtitre plate wells. An equal volume of latex particles sensitized with specific anti- enterotoxin of *S. aureus* (a, b, c, d, e and f immunoglobulins) was added to each well. Normal rabbit globulin sensitized latex particles were used as a control. After thorough mixing, the plates were incubated at room temperature for 16 h and the agglutination reactions with anti-enterotoxin immunoglobulin sensitized latex particles were observed.

Pathogenicity in mice (Yoshida and Takuchi, 1970): S. aureus cell suspensions for injection into mice were prepared from cultures grown on mod. Staphylococcus 110 agar so as to contain 1x109 cfu/ml. 0.5 ml was injected i/p in each mouse, where each group consisted of 6 mice. The number of mice that died during 2 weeks after challenge was recorded. Those isolates that caused death of 60% or more of the animals were regarded as virulent.

RESULTS

Microorganisms isolated from mastitic buffaloes

As shown in Table (1) 393 isolates of bacteria and fungi could be recovered from tested quarters, of which 211 were obtained from clinical cases and 182 from subclinical cases. The majority of isolates were bacteria (65.1%) , followed by yeasts (25.4%) and moulds (9.5%). *Staphylococci* were isolated from 103 quarters (26.2%), of which 62 isolates (36.7%) were recovered from clinical cases and 41 (18.3%) from subclinical

Table (1) : Microorganisms isolated from mastitic milk of buffaloes

Number of quarters tested	Bacteria			Yeasts	Moulds	Total no. of isolates
	Staphylo cocci	Other bacteria	Total			
Clinical mastitis (169 quarters)	62 (36.7%)	85 (50.3%)	147 (87.0%)	48 (28.4%)	16 (9.5%)	211 (124.9%)
Subclinical mastitis (224 quarters)	41 (18.3%)	68 (30.4%)	109 (48.7%)	52 (23.2%)	21 (9.4%)	182 (81.3%)
Total (393 quarters)	103 (26.2%)	153 (38.9%)	256 (65.1%)	100 (25.4%)	37 (9.5%)	393 (100%)

% calculated according to the total number of quarters

Prevalence of staphylococcal species isolated from mastitic buffaloes:

Results given in Table (2) show that *S. aureus* was isolated from 23.7% and 13.7% of quarters of clinical and subclinical mastitic cases, respectively.

Table (2) : Prevalence of staphylococcal species isolated from mastitic buffaloes.

Staphylococcal species	Clinical mastitis 169 quarters)		Subclinical mastitis (224 quarters)		Total (393 quarters)	
	No	%	No	%	No	%
S.aureus	40	23.7	14	6.3	54	13.74
S. epidermidis	11	6.5	13	5.8	24	6.11
S.intermedius	8	4.7	11	4.9	19	4.83
S. hyicus	3	1.8	3	1.3	6	1,53
Total	62	36.7	41	18.3	103	26.21

% calculated according to the total number of quarter

Determination of virulence factors of Staphylococcus aureus:

1. Growth pattern

As shown in Table (3), the majority of *S. aureus* isolates recovered from clinical mastitis (65%) grew as diffuse colonies in SSA, whereas almost the same percent of isolates obtained from subclincal mastitis (64.3%) grew as compact colonies.

Table (3): Growth pattern of S. aureus isolates recovered from clinical and subclinical mastitic milk in SSA

Source of isolates	Total No. of isolates	Growth patterns			
		Diffuse growth		Compact growth	
		No.	%	No.	%
Clinical mastitis	40	26	65.0	14	35.0
Subclinical mastitis	14	5	35.7	9	64.3
Total	54	31	57.4	23	42.6

% calculated according to the total number of isolates

2. Coagulase reaction

Out of 40 *S. aureus* isolates recovered from clinical mastitic milk samples, 14 (35.0%) and 32 (80%) were positive for clumping factors (CF) and tube coagulase, respectively, while from 14 isolates recovered from subclinical mastitic milk samples, 9 (64.3%) and 10 (71.4%) were positive for the same tests (Table, 4).

Table (4): Coagulase reaction (clumping factor , tube coagulase) of *S. aureus* isolates from clinical and subclinical mastitic milk samples

Source of isolates	No. of isolates	Clumping factor				Clumping factor			
		Positive		Negative		Positive		Negative	
		No	%	No	%	No	%	No	%
Clinical mastitis	40	14	35.0	26	65.0	32	80.0	8	20.0
Subcinical mastitis	14	9	64.3	5	35.7	10	71.4	4	28.6
Total	54	23	42.6	31	57.4	42	77.8	12	22.2

% calculated according to the total number of quarter

Haemolysin titres of *S. aureus*:

As shown in Table (5), 85.2% of all *S. aureus* isolates were positive for haemolysin production. The rate was higher (90%) in isolates recovered from the clinical cases in comparison to that of subclinical cases (71.4%).

Table (5): Haemolysin titre of S. aureus isolates recovered from clinical and subclinical mastitic milk samples

Source of isolates	Haemolysin titre						
	1/32	1/64	1/128*	1/256	1/520	1/1024	Total positive
Clinical mastitis	1/40	3/40	7/40	8/40	9/40	12/40	36/40 (90.0%)
Subclinical mastitis	1/14	2/14	5/14	4/14	1/14	0/14	10/14 (71.4%)
Total	2/54	5/54	12/54	12/54	10/54	12/54	46/54 (85.2%)

* Titre ≥ 128 was considered positive

Toxigenicity of *S. aureus*

Results obtained in Table (6) showed that out of 54 *S. aureus* isolates , 36 were found to be toxigenic with an incidence of 66.7%. Enterotoxin C was the most common, it was detected in 21 isolates (38.9 %), followed by enterotoxin A from 8 (14.8%) and enterotoxin B from 7 (13.0%).

Table (6): Toxigenic *S. aureus* isolates recovered from clinical and subclinical mastitic milk samples

Source of isolates	No. of isolates	Toxigenic isolates	Haemolysin titre		
			A	B	C
Clinical mastitis	40	28 (70.0%)	7 (17.5%)	5 (12.5%)	16 (40.0%)
Subclinical mastitis	14	8 (57.1%)	1 (7.14%)	2 (14.29%)	5 (35.7%)
Total	54	36 (66.7%)	8 (14.8%)	7 (13.0%)	21 (38.9%)

% calculated according to the total number of S. aureus isolates

Pathogenicity of *S. aureus* in mice

As shown in Table (7), mice were infected i/p with isolates recovered from clinical and subclinical mastitic milk samples. Mortality rate was higher among mice infected with S. aureus isolates recovered from clinical mastitic milk samples (73.3%), when compared with those infected with isolates obtained from subclinical mastitic milk samples (52.4%).

Table (7) : Pathogenicity of ***S. aureus*** isolates recovered from clinical and subclinical mastitic milk samples in mice

Source of isolates	No. of isolates	No. of inoculated mice	No. of dead mice per day					No. of dead mice	Mortality rate
			1-3d	3-6d	6-9d	9-12d	12-14d		
Clinical mastitis	40	240	34	74	53	15	15	176	73.3%
Subcinical mastitis	14	84	12	23	9	0	0	44	52.4%
Total	54	324	46	97	62	15	15	220	67.9%

d= day, % calculated according to the total number inoculated mice

Relationship of virulence factors and mortality rate in mice of S. aureus isolates recovered from clinical and subclinical mastitic milk

Tables (8) and (9) show that all isolates developing diffuse colonies (encapsulated) exhibited negative clumping factor and positive tube coagulase, with the exception of eight isolates in case of clinical mastitis and 4 in subclinical mastitis; which were also tube coagulase negative. From the results it was found that diffuse strains that produced toxins had been found to be highly virulent to mice, particularly when they were positive for haemolysin and coagulase enzymes. 100% mortality was caused by 11 isolates, of which 8 isolates produced enterotoxin C, one B and 2 A. Only one isolate of the subclinical cases caused 100% mortality and it produced enterotoxin C. All these isolates developed diffuse colonies.

Table (8) : Relationship of pathogenicity attributes and mortality rates in mice of *S.aureus* isolates recovered from clinical mastitic milk

Isol.no.	Colony morph	C.F.	Tube coagulase	Haemolysin	Toxin types	Mortality %
1	Diffuse	-	-	+	B	83.3
2	Diffuse	-	-	+	C	100
3	Diffuse	-	-	+	C	100
4	Diffuse	-	-	-	-	66.6
5	Diffuse	-	-	+	C	83.3
6	Diffuse	-	-	-	C	83.3
7	Diffuse	-	-	+	C	100
8	Diffuse	-	-	+	A	83.3
9	Diffuse	-	+	+	C	100
10	Diffuse	-	+	+	B	100
11	Diffuse	-	+	+	A	100
12	Diffuse	-	+	+	-	83.3
13	Diffuse	-	+	+	C	100
14	Diffuse	-	+	+	A	100
15	Diffuse	-	+	+	C	83.3
16	Diffuse	-	+	+	A	83.3
17	Diffuse	-	+	+	B	83.3
18	Diffuse	-	+	+	A	83.3
19	Diffuse	-	+	-	C	83.3
20	Diffuse	-	+	+	-	83.3
21	Diffuse	-	+	+	A	83.3
22	Diffuse	-	+	+	C	100
23	Diffuse	-	+	+	B	83.3
24	Diffuse	-	+	+	C	100
25	Diffuse	-	+	+	C	100
26	Diffuse	-	+	+	C	100
27	Compact	+	+	+	A	66.6
28	Compact	+	+	+	B	50
29	Compact	+	+	+	-	50
30	Compact	+	+	+	-	33.3
31	Compact	+	+	+	-	33.3
32	Compact	+	+	+	-	16.6
33	Compact	+	+	+	-	16.6
34	Compact	+	+	+	-	16.6
35	Compact	+	+	+	-	33.3
36	Compact	+	+	+	C	83.3
37	Compact	+	+	+	C	66.6
38	Compact	+	+	+	-	33.3
39	Compact	+	+	+	-	16.6
40	Compact	+	+		C	66.6

Isol.=Isolate, C.F. = Clumping factor

Table (9): Relationship of pathogenicity attributes and mortality rates in mice of ***S.aureus*** isolates recovered from subclinical mastitic milk.

Str..no.	Colony morph	C.F.	Coagulase	Haemolysin	Toxin	Mortality %
1	Diffuse	-	-	+	B	66.6
2	Diffuse	-	-	+	C	100
3	Diffuse	-	-	+	A	66.6
4	Diffuse	-	-	+	C	83.3
5	Diffuse	-	+	+	C	83.3
6	Compact	+	+	+	-	16.6
7	Compact	+	+	-	-	33.2
8	Compact	+	+	+	C	83.3
9	Compact	+	+		-	16.6
10	Compact	+	+	-	-	16.6
11	Compact	+	+	+	B	66.6
12	Compact	+	+	+	-	33.3
13	Compact	+	+	-	-	0
14	Compact	+	+	+	C	66.6

Isol.=Isolate, C.F. = Clumping factor

DISCUSSION

In the present study, results of characterization of S. aureus recovered from clinical and subclinical mastitis showed that 26 (65%) and 14 (35%), respectively produced diffuse colonies on SSA after cultivation in mod Staphylococcus 110 medium. This proportion is nearly similar to those obtained by Opdebeeck and Norcross (1983) and Opdebeeck et al. (1987) with field isolates directly inoculated from bovine milk in SSA. Han et al. (2000) reported that 83.1% S. aureus were diffuse in SSA. Diffuse colony morphology in SSA was considered a certain evidence of encapsulation, which seems to be a characteristic of most S. aureus strains isolated from domestic animal milk either freshly isolated or cultivated under suitable condition (Sutra et al. , 1990).

It is commendable to mention that Tollersrud et al. (2000) suggested a correlation between capsule production and the clinical severity of mastitis, where > 90% of isolates from cows with clinical mastitis were encapsulated. Aguilar et al. (2001) stated that some *S. aureus* strains are able to produce these capsular polysaccharides, which may preclude the interaction between opsonins and phagocytes. The majority of *S. aureus* strains involved in mastitis are surrounded by the this layer, a poorly immunogenic and labile structure which is lost or partially lost on in

vitro sub-culture (Sordelli et al., 2000).

For several decades, toxins and extracellular enzymes as coagulase produced by *S. aureus* were considered directly related to the establishment and progress of staphylococcal infection (Colque-Navarro et al. 2000). The 54 isolates of S. aureus isolated from mastitic milk samples were examined for slide coagulase (CF) and tube coagulase tests and results revealed that 23(42.6%) and 42 (77.8%) gave positive reaction with CF and tube coagulase, respectively. Of the rest 31 negative CF isolates, 12 gave negative tube coagulase and these results were in accordance with that mentioned by Smith et al. (1971), who found that of negative 40 CF isolates 22 (55%) gave negative tube coagulase. Anyhow coagulase tests correlate well with pathogenicity as some pathogenic *S.aureus* can be negative to slide coagulase test (CF) but positive to tube test (Quinn et al., 1994).

One of the virulence factors under examination in this study was haemolysin activity and the importance of this factor was also demonstrated by Feder et al. (1994). Results obtained revealed that the majority of the isolates of *S.aureus* (85.2%) were haemolysin positive. Cifrian et al. (1996) reported that lyses of bovine erythrocytes was due primarily β toxin but the presence of α toxin in culture supernatant from S.aureus didn't increase the lyses of bovine erythrocytes.

In the present investigation, the reverse passive latex agglutination (RPLA) test was used in this study as a recent technique for detection of staphylococcal enterotoxins. Schumacher et al. (1995) confirmed the accuracy of these commercially available kits for detection of enterotoxins. The obtained results in this study showed high incidence of type C, followed by type A and then type B. These results agree with that mentioned by Soriano et al. (2002).

Correlation between the various virulence factors and lethality in mice was evaluated in this study. The results obtained showed that encapsulated isolates of *S. aureus* that grew as diffuse colonies in S.S.A. and had negative clumping factors showed increased mouse virulence. This substantiates observation reported by Thakker et al. (1998), who indicated that optimal expression of capsule enhances bacterial virulence in the mouse model of bacterimia, probably by rendering the organisms resistant to opsonophagocytic killing by leukocytes. However, the maximum rate of mortality was achieved with isolates that had diffuse colonies and were able to produce enterotoxins, particularly of the C type. Dinges et al. (2000) suggested that the exotoxin exhibits lethal activity, pyrogenicity, super-antigenicity and the capacity to induce lethal hypersensitivity to endotoxin. Aguilar et al. (2001) suggested that the slime layer nearly covers the CF on the cell surface and also made the organisms less susceptible to phagocytosis in

vivo. The haemolysin seems also to play a role in association with encapsulation. It was noted that isolates, which developed diffuse colonies and produced haemolysin but no enterotoxins caused also high mortality (83.3%). This study suggested that most cases of severe S. aureus disease can not be explained by the action of a single virulence determinant and it is likely that a number of factors act in combination during infective process, a conclusion that agrees with the opinion of Peacock et al. (2002).

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