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CHARACTERISTICS OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM ACUTE, SUB-ACUTE AND SUB-CLINICAL STAPHYLOCOCCOSIS IN RABBITS

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Abstract: *Staphylococcus aureus* bacteria isolated from different clinical presentations of staphylococcosis in rabbits were examined for the production of various virulence factors using biochemical and immunological tests. In the total of 106 *S. aureus* isolates; toxic shock syndrome toxin-1, staphylococcal enterotoxin-C, DNase, α-haemolysin, β-haemolysin, δ-haemolysin, protein A and clumping factor were observed with a frequency of 33.2, 16.98, 83.96, 69.81, 36.79, 100, 78.30 and 54.72 percent, respectively. No SE-A, SE-B and SE-D producing isolates were recovered in this study. All the *S. aureus* isolates from acute staphylococcosis produced TSST-1, SE-C and protein A. While δ–haemolysin and clumping factor were not detected in any acute isolates, these factors were observed at a relatively higher frequency in isolates from sub-acute and sub-clinical staphylococcosis. Coagulase type III was observed more predominantly with a frequency of 45.28%, while coagulase types V and VII were not observed in any isolate. Most of the virulence factors belonged to coagulase type III followed by type VI. TSST-1 and SE-C along with coagulase types III and VI could be correlated with the acute and sub-acute staphylococcal infections in rabbits in this study.

Key Words: *Staphylococcus aureus,* coagulase typing, virulence factors, rabbits, toxic shock syndrome toxin-1, enterotoxins.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) infections are a major problem in rabbits (Corpa *et al*., 2009). These infections are commonly presented in the form of pododermatitis, subcutaneous abscesses and mastitis in all rabbit age groups. Sporadically, purulent inflammatory lesions are observed in internal organs, predominantly in lungs, liver and uterus (Hermans *et al.*, 2003, Vancraeynest *et al.*, 2004).

S. aureus produces a wide spectrum of virulence factors and many of the diseases caused by this bacterium in livestock, including rabbits, could be attributed to the virulence factors the bacteria produce, which include haemolysins, enterotoxins, toxic shock syndrome toxin-1… (Tenover and Gaynes, 2000; Vancraeynest *et al.*, 2006; Meulemans *et al*., 2011).

The coagulase protein is an important phenotypic determinant and accepted as a major virulence factor of *S. aureus*. Based on the antigenic variability of the coagulase, staphylococci have been classified into 8 serotypes (I to VIII) which differ in pathogenicity and epidemiology (Novick, 2000).

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Though *S. aureus* contributes significantly to a variety of infections in rabbits, very little information is available on staphylococcal virulence factors in rabbit strains of staphylococci and their epidemiological relationship with staphylococcosis in rabbits. On the contrary, most of the research and epidemiological surveillance is centred on staphylococcosis in man, cattle and goats.

Thus, the purpose of this study was to characterise *S. aureus* isolates from rabbits for the production of virulence factors and to relate these virulence factors with different clinical presentations of staphylococcosis in rabbits.

MATERIALS AND METHODS

History and sample collection

A total of 106 coagulase positive *S. aureus* isolates were collected from 6 different rabbitries in India (Table 1). Rabbitries 1 and 2 experienced frequent episodes of staphylococci-associated problems with symptoms of severe mastitis in breeding does, sub-cutaneous abscesses in broiler rabbits and pododermatitis in rabbits of all ages. Some of the rabbits (does) in these rabbitries exhibited combined symptoms of mastitis, sub-cutaneous abscesses and pododermatitis. Rabbitries 3 and 4 also had history of staphylococci associated problems with symptoms of mastitis, sub-cutaneous abscesses and pododermatitis. However, severity of the infections was relatively less in these rabbitries. Rabbits from rabbitries 5 and 6 were apparently healthy and these rabbitries had no history of staphylococcosis.

For the classification, isolates from the rabbits with combined symptoms of mastitis, pododermatis and sub-cutaneous abscesses were grouped as acute staphylococcosis, whereas isolates from rabbits with symptoms of either mastitis, sub-cutaneous abscesses or pododermatis were grouped as sub-acute mastitis. Isolates from rabbits without any symptoms mentioned above were grouped as sub-clinical staphylococcosis.

Samples from rabbits with acute and sub-acute staphylococcosis were collected from respective lesions, whereas samples from rabbits without any symptoms (sub-clinical cases) were collected from anterior nares and inter-digital spaces.

Preliminary identification

The samples collected were inoculated onto Brain Heart Infusion Agar (HiMedia, India) plates containing 5% of sheep blood, followed by growth in Staphylococcus Agar No. 110 (HiMedia, India). One to two typical staphylococcal colonies from each sample were identified using

Table 1: Technical data and clinical signs observed in rabbits examined for the presence of *S. aureus* bacteria from different rabbitries.

	Clinical signs			
Rabbitry	Mastitis	Abscesses	Pododermatitis	Former diagnosis
1	P	P	P	+
2	P	P	P	+
3	P	NO	P	+
4	NO	P	P	+
5	NO	NO	NO	ND
6	NO	NO	NO	ND

P: Present; NO: Not observed; ND: Not done. +: Frequent episodes of staphylococcosis.

biochemical tests. Isolates yielding positive reactions in the catalase and tube coagulase tests and negative reactions in the oxidase test were classified as *S. aureus*.

Confirmation of isolates

After preliminary identification of the isolates as *S. aureus*, all the isolates were further confirmed by polymerase chain reaction using the following sequences of primers: 5'- CGA TTC CCT TAG TAG CGG CG-3' and 5'- CCA ATC GCA CGC TTC GCC TA-3'. The primers were designed based on the DNA sequence coding for 23S rRNA (Riffon *et al.*, 2001).

Characterisation

Detection of DNase: DNase production was determined on DNase test agar (HiMedia Laboratories, India) by the standard method.

Detection of haemolysins: The detection of staphylococcal haemolytic toxins was performed with 7% (v/v) blood agar plates containing erythrocytes from rabbit, horse, sheep or human as described by Matsunaga *et al.* (1993).

Clumping factor: Clumping factor was detected as described by Easmon and Goodfellow (1990).

Cell wall associated-protein A: Detection of protein A was performed by slide agglutination test described by Winblad and Ericson (1973).

Detection of toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEs): Production of TSST-1 and SEs (SE-A to SE-D) was tested by reverse passive latex agglutination test using TST-RPLA and SET-RPLA (Denka Seiken, Japan), respectively.

Coagulase typing: Coagulase typing was carried out using a coagulase typing kit (Denka Seiken, Japan) with neutralising rabbit antisera specific to the eight coagulase types I to VIII.

Statistical tests

Chi square test was used to analyse the frequency of various virulence factors in different forms of staphylococcosis for all rabbits sampled. A significance level of 0.05 and 0.01 was used.

RESULTS

Production of virulence factors

In the total of 106 *S. aureus* isolates, the positive rates for various virulence factors were as described in Table 2: TSST-1 (33%), SE-C (17%), α-haemolysin (84%), β-haemolysin (70%), δ-haemolysin (37%), DNase (100%), protein A (78%) and clumping factor (55%). No SE-A, SE-B and SE-D producing isolates were recovered in this study. In all, 50% of isolates showed the production of TSST-1 and SEs.

All the 12 isolates from acute staphylococcosis produced TSST-1, SE-C and protein A, whereas clumping factor and δ-haemolysin were not produced by any acute isolate. *S. aureus* isolates from sub-acute and sub-clinical staphylococcosis showed production of TSST-1 at frequencies of 26 and 23%, respectively. SE-C producers were detected at a frequency of 18% in sub-acute isolates. None of the isolates from sub-clinical cases produced any enterotoxin. Statistical analysis showed significant difference ($P<0.01$) in the production of TSST-1, SC-C and protein A by acute isolates as compared to sub-acute and sub-clinical isolates. In contrast, other factors

Table 2: Virulence factors produced by the *S. aureus* isolates in the present study.

	Acute	Sub-acute	Sub-clinical	Total
No.	12	34	60	106
Test				
TSST-1	100.0	26.5	23.3	33.0
SE-A	0	0	0	0
SE-B	0	0	0	0
SE-C	100.0	17.65	0	16.98
SE-D	0	0	0	0
α-haemolysin	83.33	88.24	81.67	83.96
β-haemolysin	75.00	76.47	65.00	69.81
δ-haemolysin	0	38.24	43.33	36.79
DNase	100.0	100.0	100.0	100.0
Protein A	100.0	70.6	78.3	78.3
Clumping factor	0	47.06	70.00	54.72

TSST-1: Toxic shock syndrome toxin-1. SE-A to SE-D: Staphylococcal enterotoxin A to D.

such as δ-haemolysin and clumping factor were more ($P<0.01$) noticeably detected in sub-acute and sub-clinical isolates than in acute isolates (Table 2).

Coagulase typing

All 106 *S. aureus* isolates were coagulase typed, of which 7 (7%) isolates were found to be non-typeable (Table 3). Overall, the most predominant type found was coagulase type III (45%), followed by type VI (15%). Four isolates (4%) showed mixed reaction to type III and VI. Coagulase types V and VII were not observed in rabbit *S. aureus* isolates in this study. Coagulase type III producers were observed in all 3 forms of staphylococcosis in rabbits; however, its frequency was observed more predominantly ($P<0.05$) in acute isolates (83%) than in sub-acute and sub-clinical isolates (Table 3).

Coagulase types and virulence factors

All the virulence factors including TSST-1 and SE-C showed the production of coagulase III and coagulase VI types (Table 4). Moreover, 80% of TSST-1 and 89% of SE-C producers (including

Table 3: Coagulase types produced by the *S. aureus* isolates (%) per classified group.

	Acute	Sub-acute	Sub-clinical	Total
No.	12	34	60	106
Coagulase Types				
I	0	8.82	13.33	10.38
II	0	0	15.00	8.50
III	75.0	67.65	26.67	45.28
IV	0	5.88	8.33	6.60
V	0	0	0	0
VI	16.67	11.76	16.67	15.10
VII	0	0	0	0
VIII	0	2.94	5.00	3.77
III & VI	8.33	2.94	3.33	3.77
NT	0	0	11.67	6.60

NT: Non-typeable.

Table 4: Virulence factors and coagulase types.

							Haemolysin				Clumping
	TSST-1	SE-A	SE-B	SE-C	SE-D	DNase	α	β	δ	Protein A	factor
No.	35	0	0	18	0	106	89	74	39	83	58
Test											
I	0	0	0	0	0	100.0	9.00	9.46	5.13	7.23	12.07
II	0	0	0	0	0	100.0	10.11	4.05	7.70	7.23	10.34
III	77.14	0	0	83.33	0	100.0	47.19	52.70	53.85	51.81	48.28
IV	0	0	0	0	0	100.0	4.50	6.76	7.70	3.61	3.45
VI	20.00	0	0	11.11	0	100.0	15.73	10.81	12.82	13.25	13.80
VIII	0	0	0	0	0	100.0	3.37	4.05	5.13	3.61	5.17
III&VI	2.86	0	0	5.56	0	100.0	3.37	2.70	2.56	4.82	3.45
NT	0	0	0	0	0	100.0	5.62	8.12	5.13	7.23	3.45

TSST-1: Toxic shock syndrome toxin-1. SE-A to SE-D: Staphylococcal enterotoxin A to D. NT: Non-typeable.

isolates which showed combined production of coagulase III and VI) belonged to coagulase III serotype (Table 3). Other virulence factors such as haemolysins (α, β and δ), protein A and clumping factor were also observed to be produced by this serotype at a higher ($P<0.05$) frequency than those of other coagulase types.

TSST-1 producers vs. non-producers

There were no recognisable differences in TSST-1 producers and TSST-1 non-producers (Table 5), except the production of SE-C in higher frequency ($P<0.01$) by TSST-1 producers (43%) than those of non-producers (4%).

Co-production of virulence factors

In all, 12 isolates showed the co-production of TSST-1+SE-C+Coagulase III; 9 from the cases of acute staphylococcosis and 3 from sub-acute staphylococcosis. Two isolates, all from acute staphylococcosis, showed the production of TSST-1+SE-C+Coagulase VI (Table 6).

Table 5: Comparative production of virulence-associated factors by TSST-1 producers and TSST-1 non-producers.

	TSST-1 producers	TSST-1 non-producers
No.	35	71
Test		
SE-A	0	0
SE-B	0	0
SE-C	42.86	4.23
SE-D	0	0
DNase	100.0	100.0
α-haemolysin	94.29	78.87
β-haemolysin	82.86	63.38
δ-haemolysin	37.14	36.62
Protein A	94.29	70.42
Clumping factor	37.14	63.38

TSST-1: Toxic shock syndrome toxin-1. SE-A to SE-D: Staphylococcal enterotoxin A to D.

Table 6: Multiple virulence factor production by *S. aureus* strains from different clinical manifestations.

Virulence factors	Acute	Sub-acute	Sub-clinical	Total
TSST-1+SE-C+III	9	3	-	12
TSST-1+SE-C+VI	2	-	-	2

TSST-1: Toxic shock syndrome toxin-1. SE-C: *Staphylococcus* enterotoxin C. III and VI: Coagulase types III and VI.

DISCUSSION

In the present investigation, we characterised *S. aureus* isolates from rabbits for the production of virulence factors and attempted to relate the virulence factors to different clinical situations of staphylococcosis in rabbits.

As a result, we observed that the properties of *S. aureus* isolates from acute staphylococcosis were notably different from those of sub-acute and sub-clinical staphylococcosis. All 12 isolates from acute staphylococcosis produced TSST-1 and SE-C. Moreover, all the isolates were positive for protein A but did not produce clumping factor and δ-haemolysin.

Of all the virulence factors produced by *S. aureus*, TSST-1 and SEs, are known to be associated with clinical syndromes (e.g. toxic shock syndrome) by exerting strong mitogenic activity in T-cells (Llewelyn and Cohen, 2002), thus triggering an excessive TH1-cytokine response, characterised by IL-2, IFN-γ and TNF-β production, leading to toxic shock (Anderson and Tary-Lehmann, 2001). This evidence and our results suggest that TSST-1 and SE-C might have contributed to the inflammatory reactions resulting into staphylococcosis in rabbits.

In this study, coagulase typing indicated the prevalence of coagulase type III at considerably higher frequency. Furthermore, most of the virulence factors including TSST-1 and SE-C belonged to coagulase types III. Thus, coagulase type III along with TSST-1 and SE-C could also be correlated with staphylococcosis in rabbits. The involvement of coagulase type III in the staphylococcal infections can further be substantiated with the fact that coagulase III serotype was found associated with the epizootics of staphylococcosis on several occasions in most rabbitries in central India (Tirpude *et al.*, 2007).

Similar kinds of observations were made by other authors in different host species. Matsunaga *et al.* (1993) showed the involvement of TSST-1 and SE-C along with coagulase type VI producers in acute mastitis in cattle. Matsuda *et al*. (1992) reported a higher frequency (>80%) of coagulase types II and III from patients with MRSA infections.

In the present study, TSST-1+SE-C+Coagulase III producers were recovered from acute and sub-acute staphylococcosis, albeit more predominantly in acute staphylococcosis. However, the severity of infection was more pronounced in acute staphylococcosis, where all the rabbits showed symptoms of severe mastitis along with sub-cutaneous abscesses and pododermatitis. It is likely that other factors might have influenced the severity of disease that determined the clinical outcome.

To our knowledge, this is the first report on production of TSST-1, enterotoxin and coagulase typing of *S. aureus* isolates in the rabbits. Very little information is available on the characterisation of *S. aureus* strains from rabbits with regard to toxin phenotypes and genotypes. Rodríguez-Calleja *et al.* (2006) reported *S. aureus* strains containing *seb* and *sec* genes from rabbit meat, whereas Vancraeynest *et al.* (2006) reported *egc* cluster, containing the enterotoxin (like) genes *seg*, *sei*, *selm*, *seln*, *selo* and *selu* in high virulence rabbit *S. aureus* strains.

In conclusion, *S. aureus* isolates from the rabbits exhibited several virulence factors. The high incidence of toxigenic *S. aureus* strains portends a serious potential threat to rabbitries, which in recent years have been gaining importance in the commercial animal industry in India.

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