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Enterotoxigenicity and Antibiotic Resistance of *Staphylococcus aureus* Isolated from Sub-clinical Bovine Mastitis Milk in Plateau State, Nigeria

[1]A.B. Suleiman, [3]V.J. Umoh, [2]J.K.P. Kwaga and [1]S.J. Shaibu
[1]National Veterinary Research Institute, Vom, Nigeria
[2]Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria
[3]Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria

Corresponding Author: A.B. Suleiman, National Veterinary Research Institute, Vom, Nigeria Tel: +234-8036127111

ABSTRACT

The aim of the study was to determine the prevalence, enterotoxigenicity and antibiotic resistance of *S. aureus* from mastitic milk using standard procedures. The results showed that out of the 339 quarter milk samples tested 105(30.9%) had subclinical mastitis and only two out of 105 mastitic milk was without *S. aureus* isolates. Seventy three of the isolates tested for enterotoxin production showed that 20 isolates produced Staphylococcal Enterotoxin A (SEA) either singly or in combination with other enterotoxins 22 SEB, 24 SEC and 17 SED. One isolate produced all four enterotoxins and two out of the 20 isolates tested had enterotoxin gene encoding SEA. The study also revealed high resistance patterns for both enterotoxin (range 20-87%) and non enterotoxin (range 29-69%) producers. None of the isolates was sensitive to all antibiotics tested. The findings are of public health importance since raw milk is frequently consumed by pastoralists and their children at farm stead's. There is need to create awareness through outreach education.

Key words: *Staphylococcus aureus*, enterotoxins, antibiotics, resistance, bovine mastitis

INTRODUCTION

Staphylococcus aureus is recognized all over the world as a frequent cause of subclinical intramammary infection in dairy cows (Akineden *et al*., 2001). A good number of these strains are enterotoxigenic (Holeckova *et al*., 2002; Joffe and Barrnovics, 2006). *Staphylococcus aureus* is therefore, considered to be one of the major pathogens causing outbreaks of food poisoning. Among the foods implicated in Staphylococcal Food Poisoning (SFP), dairy products play an important role. Staphylococcal Food Poisoning (SFP) is a mild intoxication occurring after the ingestion of food containing Staphylococcal Enterotoxins (SEs). Enterotoxins are a group of heat-stable, pepsin-resistant exotoxins belonging to a large family of pyrogenic toxin superantigens (PTSAgs) encoded on phages, pathogenicity islands, chromosomes, or plasmids (Balaban and Rasooly, 2000; Dinges *et al*., 2000). The genome of staphylococci may encode multiple enterotoxins located on various mobile genetic elements (Jaroslaw *et al*., 2009). Eighteen types of SEs are currently known (SEA to SEE, SEG to SEQ, SER and SEU), the role of some newly described enterotoxins in SFP is still unclear (Orwin *et al*., 2001; Yarwood *et al*., 2002).

Many researchers have shown occurrence of enterotoxin-forming staphylococcus in bovine mastitic milk (Joffe and Barrnovics, 2006). High prevalence of enterotoxin forming *S. aureus* strains has been reported in Germany-58.7% (Zschock *et al*., 2005), Trinidad-53.6% (Adesiyun, 1995) and Japan-67.8% (Katsuda *et al*., 2005). In other countries with relatively low prevalence of enterotoxigenic *S. aureus* strains were reported Korea-22.3% (Lim *et al*., 2004) and USA-28.6% (Kenny *et al*., 1993; Cenci-Goga *et al*., 2003). In the countries aforementioned antibiotics are commonly used in the control of mastitis. Administration of antibiotics agents to mastitis cows exposes the microbial agents in the milk and consumers to sub-therapeutic levels of these drugs. The study is aimed at investigating the prevalence and serotypes of enterotoxigenic *S. aureus* from mastitic milk, their susceptibility to commonly used antibiotic agents and presence of genes encoding staphylococcal enterotoxin.

MATERIALS AND METHODS

The study was conducted in the Northern parts of Plateau State, which lies between latitude 80°24^{1} N and longitude 80°32^{1} E. The area comprises of Jos South, Barkin Ladi, Jos North, Jos East, Riyom and Bassa Local Government Areas. The study involved 339 quarter milk samples collected from 98 lactating cows. Prior to sampling, the udder, teats and adjacent flank areas were thoroughly washed and dried with single-service sanitary paper towel. The teats were disinfected with 70% ethanol.

Milk sampling and California mastitis test (CMT): Each quarter milk sample was obtained and tested for subclinical mastitis using California mastitis reagent according manufacturers instructions. Subsequently 15 mL of milk sample were then collected in sterile containers from CMT positive cases (≥1+). All CMT negative and trace were excluded because they were assessed as having originated from cows free of subclinical mastitis. Milk samples were transported in ice pack to the laboratory for microbiological analysis.

Isolation and identification of *S. aureus*: *Staphylococcus aureus* were isolated from 103 CMT positive milk samples following the procedures by Henning *et al*. (2004). Briefly an aliquot of 100 µL from each sample was spread-inoculated on blood agar plates (Bacto-Agar, Difco MI) containing 5% washed sheep erythrocytes and incubated at 37°C for 24 h. Typical colonies that were Gram positive cocci in clusters were sub-cultured on Baird Parker Agar plates and the haemolytic reaction recorded. The *S. aureus* isolates were subjected to the following biochemical test: coagulase, catalase, Dnase, Voges-Proskauer and mannitol fermentation. Isolates were then confirmed serologically using Staphytect plus slide agglutination system (Oxoid).

Enterotoxin detection: Seventy-three of the serologically confirmed *S. aureus* isolates were tested for their ability to elaborate enterotoxin by inoculation into tryptone soya broth and incubation at 37°C for 20 h with shaking. Detection of the enterotoxins in the supernatant was by reverse latex agglutination test technique using staphylococcal enterotoxin A, B, C, D detection kit (Oxoid). Agglutination was observed against a dark background and results recorded.

Antibiotic susceptibility: The seventy-three isolates were tested for susceptibility to ten antibiotics agents frequently used in the treatment of human and animal infections. The disk diffusion method of Kirby-bauer was employed (Quinn *et al*., 1999). The antibiotic agents and

concentrations used were: erythromycin (5 μg), oxacillin (1 μg), chloramphenical (30 μg), amikacin (30 μg), lincomycin (5 μg), gentamycin (10 μg), trimethoprim-sulfamethazole (1.25 μg), compound sulphonamide (5 μg), tetracycline (30 μg) and kanamycin (30 μg). Each isolate to be tested was diluted in Trypticase soy broth to a 0.5 Mcfarland standard and incubated at 37°C for 6 h. The diluted bacterial suspension was transferred onto Mueller-Hinton agar (Merck, Darmstadt, Germany) plates using sterile cotton swabs. The respective plates were seeded uniformly by rubbing the swab against the entire agar surface. Each antibiotic impregnated disk was applied onto the surface of the inoculated plate using a disk dispenser (Oxoid, Basingstoke, UK). The plates were incubated at 37°C for 16 to 18 h. The interpretation of the growth inhibition zones and classification of isolates as susceptible or resistant followed the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). *Staphylococcus aureus* ATCC 25923 reference strain was used as a quality control standard.

Detection of enterotoxin gene: PCR amplification of sea, seb, sec and sed were carried out. The sequences of the primers used were *sea* (AAAGTCCCGATCAATTTATGGCTA and GTAATTAACGGAAGGTTCTGT) with an amplicon size of 566 bp, seb (TCGCATCAAACTGACAAACG and GCAGGTACTCTATAAGTGCC with 424 bp, sec (GACATAAAGCTAGGAATTT and AAATCGGATTAACATTATCC) with 315 bp and sed (CTAGTTTGGTAATATCTCCT and TAATGCTATATCTTATAGGC with 401 bp .The thermocycler programmes and gel electrophoresis were as reported by Tsen and Chen (1992) and Johnson *et al.* (1991).

RESULTS

CMT Reaction test: The results of the CMT from the areas studied showed a prevalence of 30.9% and 25.7 to 30.8% between herd location, are as presented in Table 1.

Isolation and biochemical reactions of *S. aureus*: The 103 isolates obtained were catalase positive, showed typical growth on Baird Parker medium, coagulase and clumping factor positive, fermented mannitol and other tests as shown in Table 2.

Enterotoxigenicity: The results of the detection of enterotoxins of the *Staphylococcus aureaus* isolates indicated that enterotoxin A is the most predominant, while enterotoxin D was the least as presented on Table 3.

Table 1: California mastitis test reaction of milk from bovine mastitis

		CMT results		
Herd location	No. of samples tested	Negative	Positive	Prevalence (%)
Jos South	61	44	17	27.9
Barkin Ladi	61	43	18	29.5
Bassa	47	30	17	36.2
Riyom	66	44	22	33.3
Jos East	34	21	13	38.2
Jos North	70	52	18	25.7
Total (%)	339	234 (61.1)	105 (30.9)	30.9

Table 2: Biochemical test of *Staphylococcus aureus* isolates from bovine mastitis milk

	Test (n = 103)	No. (%) positive
No. of samples with growth	105	(100)
No. of samples with staph strains	103	(98)
Alpha haemolysis	40	(39.0)
Beta haemolysis	20	(19.0)
Gamma haemolysis	43	(42.0)
Coagulase positive	103	(100)
Clumping factor	103	(100)
DNase	80	(78.0)
Voges-proskauer	90	(87.0)

Table 3: Distribution of *Staphylococcal enterotoxins* (SEs) produced by *Staphylococcus aureus* isolated from bovine mastitis

SE production	No. of isolates	%
A	6	13.3
B	4	8.8
C	4	8.8
D	2	4.4
AB	4	8.8
AC	1	2.2
AD	5	11.1
BC	7	15.5
CD	5	11.1
ABC	2	4.4
BCD	3	6.6
ABCD	2	4.4
NIL	28	0.0
Total	73	100.0

Table 4: Resistance pattern of enterotoxigenic and non enterotoxigenic staphylococcal strains from mastitis milk

Antimicrobial agents (conc.)	SES n = 45 No. (%)	Non SES n = 28 No. (%)
Oxacillin (1 µg)	30 (66.7)	16 (57.1)
Amikacin (30 µg)	17 (37.8)	11(39.3)
Erythromycin (5 µg)	21 (46.7)	11(39.3)
Tetracycline (30 µg)	9 (20.0)	8 (28.6)
Gentamycin (10 µg)	13 (28.9)	8 (28.6)
Trimethoprim-sulfamethoxazole (1.25 µg)	39 (86.7)	17 (60.7)
Chloramphenicol (30 µg)	28 (62.2)	14 (50.0)
Sulphonamide (5 µg)	23 (51.1)	13 (46.4)
Kanamycin (30 µg)	16 (35.6)	16 (57.1)
Lincomycin (5 µg)	36 (80.0)	16 (57.1)

Antibiogram: The results of the antibiogram on the enterotoxigenic and non-enterotoxigenic strains of *Staphylococcus aureus* revealed that the enterotoxigenic strains were more resistant as compared to the non-enterotoxigenic strains, as presented in Table 4.

DISCUSSION

The CMT score of 1+ corresponding to 500,000 somatic cell counts has been accepted as the threshold index of Intra-Mammary Infection (IMI) (IDF, 1979; Henning *et al.*, 2004). In this study,

105 (30.9%) milk samples tested were CMT positive (≥1+). It could be predicted that these milk samples are likely to come from infected quarters. Therefore the CMT test and the pure culture of isolates on blood agar plates are both reliable indicators of IMI in the dairy herds studied this can be seen in the close relation between the 105 CMT positive quarters and the 103 *S. aureus* isolated from the same quarters. Studies elsewhere, reported higher prevalence of sub-clinical mastitis (Omore *et al*., 1996; Karimuribo, 2002). The difference is probably due to the number of infected quarters.

All the *S. aureus* isolates confirmed using conventional biochemical tests were also positive with the Staphytect test kit. Reports from other studies gave a 62.7% positive results for *S. aureus* from bovine mastitis and 98% for *S. aureus* of human origin (Smole *et al*., 1998; Boerlin *et al*., 2003) using latex agglutination test kit. This finding may link most of the *S. aureus* isolated in this study to be of human origin. This is because of the high percentage (100%) as seen in this study. *Staphylococcus aureus* strains of animal origin coagulate bovine plasma, produced beta-haemolysis and are less often enterotoxigenic than human strains (Devriese *et al*., 1985), similar to our findings.

A study by Joffe and Barrnovics (2006) also confirmed high prevalence of 77.3% of enterotoxigenic *S. aureus* from bovine mastitic milk. Previously many other authors have reported the occurrence of high prevalence of enterotoxigenic *S. aureus* from herds with mastitis infection (Cenci-Goga *et al*., 2003). This probably indicates that mastitis quarters could be reservoir for enterotoxigenic *S. aureus*. The wide distribution of enterotoxigenic strains in this study area could be attributed to milking hygiene, close association of lactating animals with other animals and humans. The high prevalence of staphylococcal enterotoxin A is of public health concern because it is regarded as a major cause of staphycoccal intoxication (Yamashita *et al*., 2003; Ikeda *et al*., 2005). This study is significant because milk is consumed raw without prior heat treatment or fermented as nono. Enterotoxins are known to be stable at low pH and at pasteurization temperature. The high prevalence of enterotoxins, in mastitic milk could serve as a vehicle of milk borne intoxication in the study area. Various reports have indicated that SEA producing strains are mainly from humans, whereas SEC are from animals (Orden *et al*., 1992), indicating a possible transmission from humans during milking. The ability of several strains of *S. aureus* to elaborate more than one type of enterotoxins have been reported (Joffe and Barrnovics, 2006). In this study, a significant percentage (64.4%) of the enterotoxigenic strains produced more than one enterotoxin. This probably means more virulence could be expressed by few enterotoxins forming *S. aureus* strains present in the milk. However, the detection of enterotoxigen genes by PCR failed to correlate with the detection using reverse passive latex agglutination assay kit. The study revealed the presence of only sea gene in 2 of the 20 isolates tested. Larsen *et al*. (2002) also reported only one of 414 *S. aureus* isolates from Danish cattle with mastitis as carrying a toxin gene. The reason for this could best be speculated.

In this study both staphylococcal enterotoxin producers and non-producers showed high resistance ranging from 20% for tetracycline to 87% for trimethoprim. The commonly used drugs in human and animal practice. It was however, observed that tetracycline the most commonly used antibiotic in Nigeria livestock industry had the least resistance recorded. The increase in resistance is attributed to the different practices of administering antimicrobial products (dosage, timing frequency, type and frequent change of bacteria types) (Aarestrup *et al*., 1995). The extend and use of same antibiotics in livestock farming and in the treatment of human diseases are the cause of increasing resistance of bacteria to antimicrobial agents in the study area.

In conclusion, the high prevalence of mastitis and wide distribution of enterotoxigenic *S. aureus* in bovine mastitic milk in the study area are probably due to milking hygiene and herd management which allows transmission via contact. The high resistant pattern of strains points to the indiscriminate administration of the commonly used antimicrobial agents by herd owners. The findings are of public health concern that could be controlled by outreach education.

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