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Distribution and Antibiotic Resistance of *Staphylococcus aureus* in raw milk of apparently healthy lactating Ewes and Does in Maiduguri, Nigeria

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ABSTRACT

Staphylococcus aureus are ubiquitous and widely distributed in nature, particularly on the skin surface of animals. They are regarded as the most important agents of intra-mammary infections in small ruminants. The presence of S. aureus in apparently healthy lactating ewes and does in Maiduguri was determined. A total number of 120 samples of raw milk were collected from lactating does and ewes. Furthermore, 60 samples each from does and ewes, and 20 samples from each of the three sampling points namely; Kasuwan Shanu, Pasture Farm, University of Maiduguri and College of Agriculture Farms were sampled. Of the 40 samples collected from each of the three sampling points, 20 each were collected from does and ewes. Purposive sampling technique was employed, and samples were processed according to standard bacteriological protocol. The number of positive isolates for Staphylococcus aureus from Kasuwan Shanu, College of Agriculture and Pasture Farms were found be 4 (15.3%), 4 (16.0%) and 7 (31.3%) respectively. The highest percentage distribution of 31.80% was obtained for Staphylococcus aureus in milk samples from College of Agriculture. The overall percentage of distribution of staphylococci and Staphylococcus aureus in milk samples from does and ewes were found to be 60.83% and 12.50% respectively. Penicillin and Cefoxitin had the highest resistance of 15 (100.0%) to isolates of Staphylococcus aureus, followed by Cefpodoxime, 7 (46.7%), Erythromycin, 6 (40.0%), Rifampin, 6 (40.0%), Nalidixic acid, 5 (33.3%), Ceftriaxone and Cefotaxime, 3 (20.0%) each and Vancomycin, 2 (13.3%). It is recommended that adequate hygienic measures and indiscriminate use of antibiotics should be discouraged.

Key words: Antibiotics, *Staphylococcus aureus*, distribution, milk, resistance

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INTRODUCTION

Staphylococcus aureus are the most pathogenic species of the genus staphylococci. They are facultative anaerobes with fermentative metabolism, catalase-positive and oxidase-negative. It is gram positive in nature, non motile, non-spore-forming cocci occurring singly, in pairs or irregular clusters, bunches or grape-like [1].

Macroscopically, colonies are opaque and may be white or cream, and occasionally yellow or orange. They are usually non-capsulated or may have a limited amount of capsule [2]. Microscopically, the organism appears in clusters or bunches of grapes. Several methods such as gram's staining, cell morphology, production of catalase and coagulase enzymes, pigment production, susceptibility to lysostaphin and lysozyme and anaerobic production of acid from glucose are used for the identification of *Staphylococcus aureus* [3].

Staphylococcus aureus are widely distributed and are ubiquitous in nature, particularly on the skin of humans and animals. About 60% of human population was estimated to be colonized by *Staphylococcus aureus,* while 20% remain as persistent carriers [4]. *Staphylococcus aureus* is one of the most important aetiological agents of intra-mammary infections in small ruminants. Infected udders represent the main source of bacterial contamination of raw milk and milk products. Mastitis is an infection of multi-etiological complex which is characterized by physical, chemical and bacteriological changes in milk and pathological changes in glandular tissues of the mammary gland [5]. In small ruminants, *Staphylococcus aureus* is frequently isolated from clinical mastitis often in acute forms. In cases of sub-clinical or chronic mastitis different authors reported the prevalence to be between 3 and 37% and between 2.4% and 11.4% in sheep and goats respectively [6]. However, presence of inflammation adversely affects the level of production or the quality of milk products that is significant to sheep and goats industry [7].

In lactating dairy goats, the inflammation of mammary infection is one of the most common infectious diseases. Furthermore mammary infections are the primary causes of 'milk-drop' syndrome in ewes (>85% of all causes) characterized by reduced milk yield of lactating ewes, with no clinical signs specific to a disease [8, 9]. The infection caused by *Staphylococcus aureus* is still prevalent in Nigeria. The prevalence of subclinical mastitis range from 9-50% and clinical mastitis is usually below 5%. This work is focused on the distribution of *Staphylococcus aureus* in raw milk of apparently healthy lactating ewes and does.

MATERIALS AND METHODS

Study Area

This study was conducted in Maiduguri metropolis, Borno State, located in the North-eastern Nigeria. Maiduguri is the Borno State capital. It lies between Latitude 11° 50′ 42″ N and Longitude 13° 9′ 36″ E. Maiduguri metropolis shares border with Konduga Local Government to the Northeast and Jere Local Government to the south and northwest. It occupies an area of 50,778 square kilometers. The climate of Maiduguri has a mean annual rainfall and temperature of about 650 mm and 32°C respectively. Maiduguri metropolitan area has a population of 521, 492 people (NPCC, 2006). The vegetation is characterized by Sahel savannah.

Sample collection, labeling, packaging and transportation

A total number of one hundred and twenty (n=120) samples from apparently healthy lactating does and ewes were sampled. Sixty samples were obtained, each from does and

ewesrespectively. Forty samples were collected from each of the three sampling points; Kasuwan Shanu (KS) (Maiduguri Cattle Market), Pasture Farm, University of Maiduguri (PF) and College of Agriculture Farm (CA). Of the 40 samples collected from each of the three sampling points, 20each were collected from does and ewes. Samples were collected using purposive sampling technique [10]. The inclusion criteria were lactating ewes and does, while the exclusion criteria were ewes and does that failed to show evidence of lactation. The udder teats were disinfected with 70% alcohol, the first few streams of milk were discarded to avoid contamination. Physical examination such as observation and palpation of the udder for symmetry and size, milk consistency and visible abnormalities were noted and recorded. About 10 ml of milk sample was collected in each sterile sample bottle, labeled appropriately, placed in a cool box containing ice packs and then transported to Veterinary Microbiology Laboratory, Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Maiduguri for immediate processing.

Preparation of laboratory media for inoculation of samples

The media (Sheep blood agar, mannitol salt agar, Nutrient agar and broth, Mueller-Hinton agar and broth and DNase agar) were prepared by dissolving the required quantity in recommended volume of distilled water followed by heating in autoclave based on manufacturer's instructions.

Processing of samples for presumptive axenic colonies of *Staphylococcus aureus*

One loopful each of 20 samples of raw milk from does (n=20) and ewe (n=20) from KS, PF and CA were inoculated onto sheep blood agar containing 5% sheep blood and incubated at 37ºC for 24-48 hours to observe for the typical growth indicative of *Staphylococcus*. The isolates obtained were then sub-cultured onto mannitol salt agar to examine for the characteristic growth of *Staphylococcus aureus*.

Pure colonies of staphylococci were picked from sheep blood agar plates with bacteriological loop and then examined using Gram's staining technique, under the microscope with (x100) objective [11].

Biochemical characterization

Isolates of staphylococci from blood agar were tested for the presence of catalase, while *S. aureus* on mannitol salt agar were subjected to coagulase and DNase tests.

Antibiotic susceptibility tests for isolates of *Staphylococcus aureus*

The susceptibility of*Staphylococcus aureus*to nine different antibiotics was performed using single disc diffusion method to determine their resistance pattern [12]. The following antibiotics: Vancomycin (30 µg), Cefoxitin (30 µg), Cefotaxime (30 µg), Penicillin (10 µg), Erythromycin (15 µg), Cefpodoxime (10 µg), Nalidixic acid (30 µg), Ceftriaxone 30 µg) and Rifampin (5 µg) were used. *Staphylococcus aureus*were sub-cultured onto Mueller-Hinton broth (OXOID-CM0405) and then incubated at 37ºC for 24 hours. Serial dilution was performed to 10% of broth with distilled water to reduce the degree of turbidity of broth culture. Thereafter, 1 ml was dispensed onto Mueller-Hinton agar and the excess discarded. Antibiotic discs were applied aseptically to the surface of the plate with the help of sterile forceps. The plates were incubated at 37ºC for 24 hours under aerobic condition to observe for zones of inhibition, which were measured with the aid of a ruler.

Data Analyses

The data was summarized into Tables using descriptive statistics to determine the

distribution of *S. aureus*, and the relationship between the organism and the hosts.

RESULTS

Distribution of staphylococci and *Staphylococcus aureus* in raw milk of lactating ewes and does in Kasuwan Shanu, Pasture and College of Agriculture Farms in Maiduguri metropolis

The number of positive isolates for staphylococci from Kasuwan Shanu, College of Agriculture and Pasture Farms were found to be 26 (65.0%), 22 (62.5%) and 25 (55.5%) respectively. The positive isolates of *S. aureus* from Kasuwan Shanu, College of Agriculture and Pasture Farms were, found to be 4 (15.3%), 4 (16.0%) and 7 (31.3%). The highest percentage distribution of 31.80% was obtained for *Staphylococcus aureus* in milk samples from College of Agriculture Farms, followed by 16.0% from Pasture Farm and 15.3% associated with Kasuwan Shanu Market. The overall percentage of distribution of staphylococciand *Staphylococcus aureus* in milk samples from does and ewes were found to be 60.8% and 12.5% respectively (Table 1).

Colonial identification of staphylococci in raw milk of lactating ewes and does in Kasuwan Shanu, Pasture and College of Agriculture Farms in Maiduguri metropolis

The total number of positive samples of staphylococci showing growth on sheep blood agar and mannitol salt agar tested for catalase were found to be 35.8% (Table 2).

Phenotypic characterization of *Staphylococcus aureus* in raw milk of lactating ewes and does in Kasuwan Shanu, Pasture and College of Agriculture Farms in Maiduguri metropolis

Eight (20.0%) isolates were found to be positive for both coagulase and DNase tests, indicative of the presence of *Staphylococcus aureus* (Table 3).

Antibiotic susceptibility tests for isolates of *Staphylococcus aureus*

The resistance and sensitivity pattern were displayed by fifteen *Staphylococcus aureus* isolates to nine different antibiotics. The isolates were graded based on whether they are sensitive or resistant. Single, double and triple plus symbols (+, ++, +++) were indicative of moderately, sensitive, sensitive and highly sensitive respectively, while negative sign (-) is indicative of resistance (Table 4a). Additionally all isolates of *Staphylococcus aureus* were resistant to Penicillin and Cefoxitin and had the highest resistance of 15 (100.0%), followed by Cefpodoxime, 7 (46.7%), Erythromycin, 6 (40.0%), Rifampin, 6 (40.0%), Nalidixic acid, 5 (33.3%), Ceftriaxone and Cefotaxime, 3 (20.0%) each and Vancomycin, 2 (13.3%) (Table 4b).

The phenotypic identification of *staphylococci* and *Staphylococcus aureus*

The colonies of staphylococci on blood agar appeared round, smooth and opaque (white), while on mannitol salt agar, isolates of *Staphylococcus aureus* produced golden yellowish colonies. Biochemically, the isolates were catalase and DNAse positive (Figure 1).

Table 1: Distribution of *Staphylococcus aureus* in raw milk of lactating ewes and does in Kasuwan Shanu, Pasture and College of Agriculture Farms in Maiduguri metropolis

Study location	No. of samples collected	No. of positive isolates for staphylococci (%) on BA	No. of positive isolates for *Staphylococcus aureus* (%) on MSA
Kasuwan Shanu (KS)	40	26 (65.0%)	4 (15.3%)
Pasture Farm, University of Maiduguri (PF)	40	25 (62.5%)	4 (16.0%)
College of Agriculture Farm (CAF)	40	22 (55.0%)	7 (31.3%)
Total	120	73 (60.8%)	15 (12.5%)

BA = Blood agar supplemented with 5% sheep blood, MSA = Mannitol salt agar with 7.5%Nacl

Table 2: Colonial Identification of staphyloccoci in raw milk of la ctating ewes and does in Kasuwan Shanu, Pasture and College of Agriculture Farms in Maiduguri metropolis

Study Location	No. of Samples Collected [n= 120]	No. positive for staphylococci	No. Positive for Catalase Test
KS	40	26	16 (40.0%)
CAF	40	25	14 (35.0%)
PF40	22		13 (32.5%)
Total	120	73	43 (35.8%)

KS = Kasuwan Shanu, PFU = Pasture Farm, CAF = College of Agric. Farm

Table 3: Phenotypic characterization of *Staphylococcus aureus* in raw milk of lactating ewes and does in Kasuwan Shanu, Pasture and College of Agriculture Farms in Maiduguri metropolis

Study Location [n = 40 per location]	No. Positive for *Staphylococcus aureus*	No. Positive for Coagulase	No. Positive for DNase
KS	4	3 (7.5%)	3 (7.5%)
PFU	4	3(7.5%)	3(7.5%)
CAF	7	2(5.0%)	2(5.0%)
Total = 120	15	8 (20.0%)	8 (20.0%)

KS = Kasuwa Shanu Milk PFU = Pasture Farm Milk, CAF = College of Agric. Milk

Table 4a: Antibiotic susceptibility test for *Staphylococcus aureus* isolates

	(n = 15)	Ceftriaxone (30 µg)	Vancomycin (30 µg)	Erythromycin (15 µg)	Penicillin (10 µg)	Rifampin (15 µg)	Cefoxitin (30 µg)	Cefpodoxime (10 µg)	Nalidixic Acid (30 µg)	Cefotaxime (30 µg)
KS	KS5	++	++	-	-	+++	-	+++	+	+
	KS7	-	+	-	-	-	-	+	+	-
	KS16	+	++	++	-	+++	-	-	+	+
	KS22	+++	++	-	-	+	-	++	-	+++
	PFU4	+++	+++	++	-	++	-	+++	+	++
PFU	PFU9	+	-	+	-	+	-	-	-	+
	PFU13	-	+	-	-	-	-	++	-	-
	PFU20	++	+++	-	-	+	-	-	+	+
	CAF7	+++	++	+++	-	+	-	++	+	++
CAF	CAF9	+++	+++	++	-	-	-	-	+	++
	CAF18	-	++	+	-	+++	-	-	+	-
	CAF21	++	-	++	-	-	-	-	-	+
	CAF22	+++	++	++	-	++	-	++	+++	+
	CAF32	++	+++	++	-	-	-	-	+	++
	CAF38	+++	++	-	-	-	-	++	-	+

KS = Kasuwan Shanu, PFU = Pasture Farm, CAF = College of Agric. Farm

+++ = Highly sensitive ++ = Sensitive + = Moderately sensitive - = Resistant

Table 4b: Sensitive and resistance percentages for isolates of *Staphylococcus aureus*

Antibiotic Agents	Sensitive (%)	Resistance (%)
	(n = 15)	
Ceftriaxone (30 µg)	12 (80.0)	3 (20.0)
Vancomycin (30 µg)	13 (86.7)	2 (13.3)
Erythromycin (15 µg)	9 (60.0)	6 (40.0)
Penicillin (10 µg)	0 (0.0)	15 (100.0)
Rifampin (15 µg)	9 (60.0)	6 (40.0)
Cefoxitin (30 µg)	0 (0.0)	15 (100)
Cefpodoxime (10 µg)	8 (53.3)	7 (46.7)
Nalidixic acid (30 µg)	10 (66.7)	5 (33.3)
Cefotaxime (30 µg)	12 (80.0)	3 (20.0)

n = Number of isolates tested. The numbers outside the bracket refer to isolates that are either sensitive or resistant to the antibiotics, while the percentages in brackets refer to the percentages that are sensitive or resistant to the antibiotics. All were based on the measures of zones of inhibition.

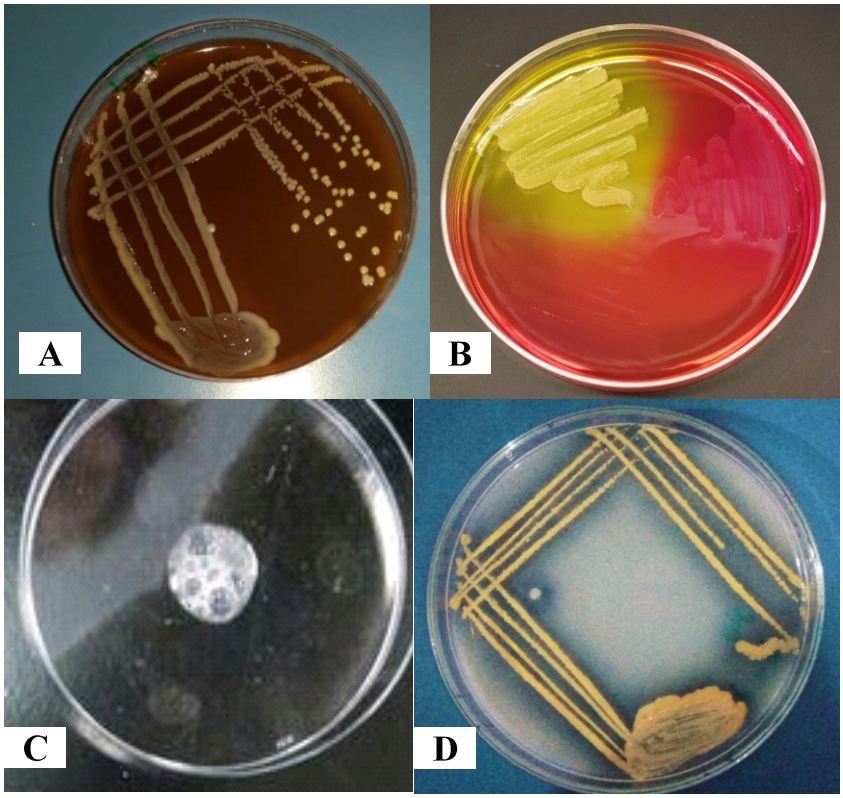

Figure 1: The colonial growth of the *Staphylococcus* on sheep blood agar (**A**), *Staphylococcus aureus* on mannitol salt agar showing the distinctive golden yellow colouration (**B**), catalase test for *Staphylococcus aureus* indicating gas bubbles after the addition of hydrogen peroxide (**C**) and DNAse positive *Staphylococcus aureus* on DNase agar showing zone of inhibition around the bacterial growth (**D**)

DISCUSSION

Staphylococcus aureus is one of the most important aetiological agents associated with intra-mammary infections in small ruminants [6]. The distribution of *S. aureus* in raw milk of lactating ewes and does varies from one location to another.*S. aureus*was detected in 33% of ovine and 32% of caprine milk samples in Switzerland [13], which is lower than the percentage of *S. aureus* identified on mannitol salt agar in raw milk of ewes (46.7%) and does (53.3%) in this study. Similarly, coagulase positive staphylococci were detected in 66% of caprine and 15% of ovine bulk tank milk samples in Spain [14, 15]. This may likely be due to variation with respect to prevalence of *S. aureus* from one area to another, and the possible existence of sub-clinical mastitis which was not detected in this study. The phenotypic characterization of *S. aureus*in milk samples of ewes and does associated with coagulase test indicated that the number of *S. aureus* was 8 (20.0%), which was lower than those reported previously in other parts of the world like Switzerland and Spain. This suggests that the presence of *S. aureus*as a potential agent of mastitis in small ruminant herds in Maiduguri metropolis might have been underestimated. No single case of mastitis was determined in this study.This could be attributed to the fact that *S. aureus* can persist in the host for a long time without causing an apparent inflammation and/or clinical infection as earlier reported [2].Similarly, this could be of particular relevance, where staphylococcal infections have been associated with consumption of raw milk from small ruminants [16], resulting in limited success of antibiotic therapy due to the ability of *S. aureus* to invade and survive within different cell types found in the mammary gland [2].

The antibiotic susceptibility test for isolates of *S. aureus* to 9 commonly used antibiotics revealed that fifteen isolates displayed 100% resistance to Penicillin and Cefoxitin. This finding is in consonance with earlier report of 100% resistance of *Staphylococcus aureus* to Penicillin, but a limited occurrence of resistance to other antimicrobial agents [17]. Similarly, higher levels of resistance of 91.5% and 100% to penicillin were reported [18, 19]. The observed level of resistance against penicillin may not be unconnected to natural resistant to penicillin group of antibiotics by *S. aureus* due to its ability to synthesize β-lactamase enzyme that inactivates penicillin. The resistance against erythromycin may likely be attributed to indiscriminate use of this agent in the study area. Additionally, [20] reported higher resistance of 85.19% to ceftriaxone than the 20.0% found in this study.

Conclusion

The study has demonstrated the occurrence and antimicrobial resistance among isolates of *S. aureus*in milk samples from ewes and does. However, no cases of clinical mastitis were recorded in this study. *Staphylococcus aureus* recorded the highest resistance to Penicillin and Cefoxitin and lower resistance to ceftriaxone. It is therefore recommended that unhygienic practices among flocks of sheep and goats and an indiscriminate use of antibioticsshould be discouraged.

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