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Original Article

Phenotypic characterization and antibiotic resistance in staphylococcus species associated with subclinical mastitis in goats

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Abstract

Staphylococcus species are the main cause of small ruminant's intramammary infections. This study was carried out to determine the prevalence of subclinical mastitis associated with *Staphylococcus* in goat milk and their resistance to antibiotics in Nsukka Agricultural area of Enugu State, Nigeria. Eighty-four milk samples were collected from goats randomly. White slide mastitis test was performed on the milk**.** Catalase production, coagulase production and haemolytic testing was done on the staphylococci isolates**.** Antibiotic susceptibility test was carried out using disc diffusion method. Eleven antibiotics were used. Eighty-four (100%) isolates were recovered. They were catalase positive and non-haemolytic. Forty-five (53.6%) were coagulase positive while 39 (46.4%) were coagulase negative. The isolates showed high resistance to ampicillin, cefoxitin, oxacillin, vancomycin, ciprofloxacin, kanamycin, tetracycline, and erythromycin. This study shows that there is high prevalence of *Staphylococcus* species in goat milk in Nsukka Agricultural zone of Enugu State. The high resistance to antibiotics shown by the isolates could make it difficult to treat mastitis in that area.

Keywords: subclinical mastitis, coagulase positive *Staphylococcus*, goat, milk, antibiotics resistance

1. Introduction

Goats were among the first farm animals to be reared in agriculture (Monteiro, Costa & Lima, 2017). Evidence indicates a symbiotic association with man for up to 10,000 years (Baruwa, 2013). Goats are spread all over the world because of their great adaptability to varied environmental conditions and the spectrum of nutritional regimes under which they were evolved are subsequently maintained (Miller & Lu, 2019). The Eastern part of Nigeria is an area where no religious order prevents goat husbandry. Sheep and goat form the most important groups of milk producing animals after dairy cattle in both temperate and tropical agriculture (Ferro, Tedeschi & Atzori, 2017).

Goat is the main supplier of dairy and meat products for rural people in developing countries surpassing all other farm animals (Morales-Jerrett, Mancilla-Leytón, Delgado-Pertíñez, & Mena, 2020). Monteiro *et al.*, (2017) stated that goats rank the third in global milk production among animal species. The goat being a dairy supplying resource meets the demand for home consumption of goat milk (Morales-Jerrett *et al.*, 2020).

Milk can serve as a medium for the growth of many micro-organisms including *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Staphylococcus* and *Micrococcus* species (Pukančíková, Lipničanová, Kačániová, Chmelová, & Ondrejovič, 2016). The presence of micro-organisms in milk and milk products has important implications to safety, quality, regulations, and public health (Owusu-Kwarteng, Akabanda, Agyei & Jespersen, 2020). Garedew, Mengesha, Birhanu, & Mohammed, (2015) stated that milk from the farm can become contaminated with bacteria present on teats, teat

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ends, teat canal, udder surfaces, mastitis of udders, and contaminated water used to clean the milking systems, or bacteria residing in the milking system.

Mastitis is the total or partial inflammation of the mammary gland provoked by one or more pathogenic microorganisms, and it can appear either in clinical or in subclinical forms (Martins *et al.,* 2019); Jilo, Galgalo & Mata, 2017). Caprine mastitis is caused by various bacteria such as *S. aureus* and *Escherichia coli,* which are often present in the goat's skin and in the environment (Hughes & Watson 2018). Studies have reported that *Staphylococcus* species are the most prevalent pathogens in goat's mastitis (Marogna *et al.,* 2012; Silva *et al.,* 2011). In Nigeria, 29.3% prevalence of *Staphylococcus* species was diagnosed from subclinical cases of mastitis, as reported by Tambuwal & Jibrin, 2017. Also, a 52.75% prevalence has been reported in lactating goats from Bulgaria (Hristov, Popova, Pepovich, & Nikolov, 2016).

Staphylococcus species are part of the normal skin and the main etiological agent of small ruminant's intramammary infections (IMI) (Götz, Bannerman & Schleifer, 2006). Coagulase-negative *staphylococci* (CNS) and coagulase-positive *staphylococci* (CPS) have been reported in clinical and subclinical cases of dairy goats (Bergonier *et al.,* 2014; Mahlangu, Maina & Kagira, 2018). The *Staphylococcus* species can be transmitted from doe to doe by unhygienic milking procedures (Abebe, Hatiya, Abera, Megersa & Asmare, 2016). The indiscriminate and widespread use of antimicrobials in the treatment and control of such bacterial infections has stimulated the emergence and spread of resistance genes among bacteria and between bacteria, in human and animal sources (Nelson, Moore & Rao 2019). The evaluation of the antibiotic susceptibility of *Staphylococcus* species isolated from goats with subclinical mastitis is of interest for clinical purposes, in order to decide which antibiotics should be administered, and also for monitoring the spread of multiple resistant strains on farms (Widianingrum, Windria & Salasia, 2016). Therefore, the objectives of this study were to determine the prevalence of *Staphylococcus* species in goat milk, evaluate some phenotypic properties associated with the *Staphylococcus* isolates, and determine the antibiotic resistance profile of the *Staphylococci* isolates.

2. Materials and Methods

This study was carried out on both apparently healthy and sick West African Dwarf (WAD) goats within different households, and among those brought to the market for sale in the metropolis. Simple random selection was used. Milk samples were collected from randomly selected WAD goats with subclinical mastitis (showing a slight inflammation of the udder). A total of 84 milk samples were collected from 84 WAD goats between July and December, 2019. These were used for the study.

The mammary glands of lactating do were disinfected using methyl alcohol and cotton wool following restraint. The udder was massaged to enable milk let down, then the milk samples were collected at mid-stream and were transported to the Microbiology laboratory of the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, where they were subsequently processed for bacterial isolation and characterization.

2.1 White Side Test (WST) for mastitis

This was performed as described by Kahir *et al.,* (2008), immediately when the sample was brought to the laboratory. The milk was mixed thoroughly avoiding shaking vigorously, 50 µl (five drops) of milk were placed on a glass slide with a dark background by micropipette. Then 20 µl (a drop) of 4% NaOH were added to the milk sample and the mixture was stirred rapidly with a toothpick for 20-25 seconds. A breaking up of milk in flakes, shreds, and viscid mass, was indicative of positive reaction. Milky and opaque and entirely free of precipitation was indicative of negative reaction.

2.2 Isolation and identification

The milk samples were streaked on the prepared nutrient agar and incubated for 24 hours at 37^0C. Presumptive *Staphylococcus* colonies were sub-cultured and used for further biochemical identification. Each colony was Gram stained and examined using simple light microscope. Gram-positive cocci in bunches or clusters were recorded.

The following biochemical tests was performed on Gram positive cocci in bunches.

Catalase test: A drop of 3% hydrogen peroxide solution was placed on a slide and a small portion of the bacterial colony was emulsified in the solution. The bubbles (the release of oxygen) indicate a positive test.

Coagulase test: Tube coagulase test was performed. 2ml of citrated rabbit plasma diluted 1:5 in small sterilized test tubes were inoculated with the bacterial colony. Formation of a fibrin clot within 4-24 hours of incubation indicates a positive test.

2.3 Haemolytic testing

A portion of colony from a pure culture was streaked on blood agar plate and incubated overnight at 37^0C. After incubation, the plates were examined for presence of clear zones of haemolysis around the colonies.

2.4 Antibiotic susceptibility test

This was carried out using disc diffusion method following the procedure of Clinical and Laboratory Standard Institute (Clinical and Laboratory Standards Institute, 2012). Eleven different antibiotics were used. strategically placed onto the inoculated isosensitest agar. The antibiotics tested were ampicillin (AMP), cefoxitin (FOX), oxacillin (OX), vancomycin (VA), tetracycline (TE), erythromycin (E) trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), clindamycin (DA), kanamycin (K), and gentamicin (CN). The plates were then incubated at 35^0C for 24 hours. Susceptibility to an antibiotic was observed as a clear zone of inhibition around the disc. The inhibition zone diameters (IZD) were measured and recorded as susceptible or resistant.

3. Results and Discussion

Staphylococcus readily colonizes the teat canal and normal skin, particularly the damp areas in the region of the teat orifice (Paduch & Krömker, 2011). Out of the 84 milk samples collected and examined from the various parts of the zone, all (100%) were positive for white side test. High reliability of WST for the diagnosis of subclinical mastitis has been reported by Sharma, Maiti & Pandey, (2008). The isolates were all identified to be Gram positive cocci in bunches (staphylococci). The presence of staphylococci shows that the prevalence of the *Staphylococcus* species involved in the subclinical cases of mastitis in goat is high. Previous studies carried out on dairy herd farms have also shown high prevalence of staphylococci in the milk (Wang *et al.*, 2018). Coagulase positive staphylococci are virulent strains of staphylococci. Forty-five (53.6%) of the staphylococci isolates were coagulase positive while 39 (46.4%) were coagulase negative in tube coagulase test, see Table 1. Positive and negative coagulase test cases are shown in Figure 1. The majority of the *Staphylococcus* isolates found in this study were coagulase positive with a high prevalence. High prevalence of coagulase positive staphylococci has been recorded by many authors (Kateete, Asiimwe, Mayanja, Najjuka & Rutebemberwa, 2020). The high prevalence of coagulase positive staphylococci in subclinical mastitis goat milk with potential enterotoxin genes and other virulence factors may suggest that the consumption of raw milk would present a risk for public health, and the safety of dairy products related to goat milk needs attention.

Various coagulase negative staphylococci have also been isolated from the subclinical mastitis goats (Salaberry *et al.*, 2015). Our findings show that coagulase negative staphylococci isolates were rare. This is in contrast to the findings of Omar & Mat-Kamir, (2018) who reported a high prevalence of coagulase negative staphylococci. The low incidence rate of coagulase negative staphylococci in this study, compared to other reports, may be due to differences in management of the goats as well as in the environments of the farms, since coagulase negative staphylococci could infect the udder through unhygienic teat canal. Diseases caused by staphylococci are attributed to several virulence factors. Haemolysin and catalase are very important virulence factors of the staphylococci and other bacterial species (Becker, Heilmann, & Peters, 2014). All the isolates were catalase positive. None of the isolates was identified as haemolytic on sheep blood agar.

Inappropriate use of antibiotics is one of the triggering factors for the emergence of antibiotic resistance observed in bacteria (Cantón, Horcajada, Oliver, Garbajosa &Vila, 2013). The antibiotic resistance profile of the isolates tested is shown in Table 2. The *Staphylococcus* species isolated showed high resistance to ampicillin, followed by cefoxitin and oxacillin, see Table 2. The susceptibility of the isolates to ampicillin, which is markedly low in this study, is in line with the work done by Akanbi, Njom, Fri, Otigbu & Clarke, (2017). This finding is in accordance with ampicillin sensitivity to the penicillinases, which are frequently produced by *Staphylococcus* species, particularly by coagulase negative *Staphylococcus* species (Virdis *et al.*, 2010). On the other hand, the isolates showed high resistance to cefoxitin. Previous studies have shown susceptibility to cefoxitin in

Table 1. Numbers of coagulase positive *Staphylococci* and coagulase negative *Staphylococci* isolates from goats with subclinical mastitis

Number of coagulase positive	Percentage	Number of coagulase negative	Percentage
45	53.6	39	46.4

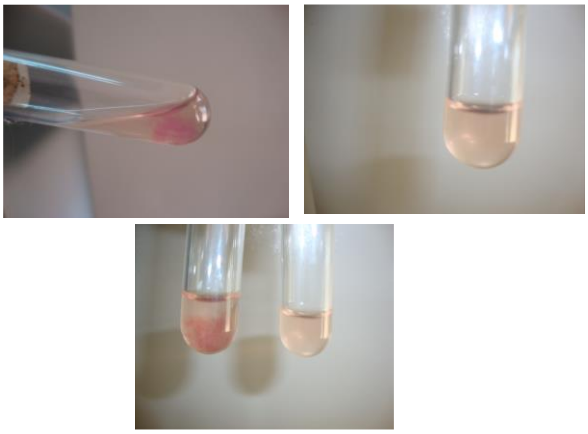

Figure 1. Photos of coagulase test showing both positive and negative cases

Table 2. Antimicrobial susceptibility of *Staphylococcus* species isolated from goats with subclinical mastitis

Antimicrobial agent	No of sample tested	Not resistant (%)	Not susceptible (%)
AMP	84	81(96.4)	3 (3.6)
FOX	84	50 (59.5)	34 (40.5)
OX	84	35 (41.7)	49 (58.3)
VA	84	19 (22.6)	65 (77.4)
TE	84	12 (14.3)	72 (85.7)
E	84	10 (11.9)	74 (88.1)
SXT	84	6 (7.1)	78 (92.9)
CIP	84	4 (4.8)	80 (95.2)
DA	84	4 (4.8)	80 (95.2)
K	84	2 (2.4)	82 (97 7)
CN	84	0 (0)	81 (96.4)

ampicillin (AMP), cefoxitin (FOX), oxacillin (OX), vancomycin (VA), tetracycline (TE), erythromycin (E) trimethoprim/ sulfamethoxazole (SXT), ciprofloxacin (CIP), clindamycin (DA), kanamycin (K) and gentamicin (CN)

coagulase negative staphylococci isolated from goats with subclinical mastitis (Martins *et al.*, 2017), which does not agree with the results obtained in this study. The result obtained in this study is, however, in line with the report of Fowoyo & Ogunbanwo (2017), which shows high resistance to cefoxitin. The resistance to beta-lactams may be due to the fact that these antibiotics are commonly used to treat various bacterial diseases in dairy animals.

The isolates showed high resistance to oxacillin. The high resistance pattern of these *staphylococci* isolates to oxacillin in this study is in line with the findings of Odogwu *et* *al.* (2019), who reported that > 50% of *Staphylococcal* isolates screened in their work were methicillin resistant *Staphylo coccus aureus*. High susceptibility to trimethoprim/ sulfamethoxazole as recorded in this study has also been previously found in some strains isolated from goats with subclinical mastitis (Wald, Hess, Urbantke, Wittek & Baumgartner, 2019). Resistance to these antibiotics by the staphylococci isolated may have developed due to horizontal transfer of resistance determinants encoded by mobile genetic elements through plasmids, transposons, and the staphylo coccal cassette chromosome; or by mutations in chromosomal genes (Foster, 2017). The staphylococci showed high susceptibility to vancomycin, clindamycin, ciprofloxacin, kanamycin, gentamycin, and erythromycin. This finding is in line with the findings of Yıldız *et al.* (2014). These antibiotics can be recommended for managing subclinical cases of mastitis.

An antibiotic resistance comparison between coagulase positive staphylococci and coagulase negative staphylococci to different antibiotic tested is shown in Table 3. Both groups showed high resistance to ampicillin, cefoxitin, oxacillin, vancomycin and erythromycin. However, the susceptibility to tetracycline by the isolates was observed to be lower in coagulase positive *Staphylococcus* when compared to coagulase negative *Staphylococcus*. A number of authors have previously reported a marked variability in the susceptibility of these microorganisms to tetracycline (El-Razik, Arafa, Hedia & Ibrahim, 2017).

4. Conclusions

This study shows that there was a high prevalence (100%) of *Staphylococcus* species in goat milk screened in Nsukka Agricultural zone of Enugu State. Most of these staphylococci are resistant to commonly used antibiotics. This can make it difficult to treat subclinical mastitis in dairy goats of the study area. The high prevalence of this group of bacteria (coagulase positive staphylococci) may pose challenges to maintaining a constant supply of goat milk in the study area. This study also showed that coagulase negative staphylococci are emerging as important minor mastitis pathogens, and can affect the quantity and quality of goat milk.

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Table 3. Comparison of antibiotic resistances in coagulase positive and negative *Staphylococci* isolates from goats with subclinical mastitis

Antibiotic	Coagulase positive			Coagulase negative		
	Number tested	Resistance %	Susceptible %	No. tested	No. resistance (%)	No. rusceptible (%)
AMP	45	45 (100)	00	39	37 (94.9)	2 (5.1)
FOX	45	23 (51.1)	22 (48.9)	39	18 (46.2)	21 (53.8)
OX	45	21 (46.7)	24 (53.3)	39	17 (43.6)	22 (56.4)
VA	45	18 (40)	27 (60)	39	12 (30.8)	27 (69.2)
TE	45	7 (15.6)	38 (84.4)	39	5 (12.8)	34 (87.2)
E	45	14 (31.1)	31 (68.9)	39	13(33.3)	26 (66.7)
SXT	45	4 (8.9)	41 (91.1)	39	4 (10.3)	33 (84.6)
CIP	45	2 (4.4)	43 (95.6)	39	3 (7.7)	36 (92.4)
DA	45	3 (6.7)	42 (93.3)	39	4 (10.3)	
K	45	3 (6.7)	42 (93.3)	39	2 (5.1)	37 (94.9)
CN	45	2 (4.4)	43 (95.6)	39	1 (2.6)	38 (97.4)

ampicillin (AMP), cefoxitin (FOX), oxacillin (OX), vancomycin (VA), tetracycline (TE), erythromycin (E) trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), clindamycin (DA), kanamycin (K) and gentamicin (CN)

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