

Original Research Article

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Isolation, Characterization and Sensitivity Pattern of *Staphylococcus aureus* Isolated from Milk Samples in Khartoum State, Sudan

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ABSTRACT

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Milk is considered as an important food material for consumers of all age groups. Albeit that, it can harbor a number of pathogens including *Staphylococcus aureus* which causes a big problems in the dairy industry by affecting milk quality and production. The imprudent use of antibiotics by farmers led to the appearance of resistant bacterial strains. The objective of this study was to isolate, identify *Staphylococcus aureus* from different farms in Khartoum State and to study its antibiotic sensitivity pattern. A total of 186 milk samples were collected from three areas in Khartoum State. After isolation and identification using Manitol salt agar media and biochemical test, 25.3 % of the samples were tested positive for *Staphylococcus aureus*. The identification of the isolated bacteria was also confirmed using PCR technique targeting *nuc* gene. Regarding sensitivity tests, the bacteria were found to be resistant to Penicillin while susceptible to Sulphamethoxazole/Trimethoprim and Ciprofloxacin.

Introduction

Milk and dairy products constitute the major source of protein for the majority of the population in underdeveloped countries. It is also considered an important health risk factor as it represents a source of some pathogens and drug residues (Bradley, 2002; Ruegg, 2003). Cattle and buffaloes contribute to a large proportion of the total milk production worldwide.

One of the most frequent diseases occurring within lactating animals is mastitis. It is a bacterial disease that causes the infection and

inflammation of udder tissue which ultimately affects the quality of milk consumed and leads to reduced milk production (Preethirani *et al.*, 2015). Among different mastitogens frequently reported are *Streptococcus agalactiae*, *Staphylococcus aureus*, *Escherichia Coli*, *Klebsiella pneumoniae*, and *Streptococcus uberis*. Two most problematic bacteria are

S. agalactiae and *S. aureus* which cause persistent infections, although the infection with *S. agalactiae* has decreased in well-developed milking industries during the past years, the *S. aureus* infection remains an important problem (Keefe, 2012, Preethirani *et al.*, 2015).

The *Staphylococcus* group belongs to the family Staphylococcaceae, which include 47 subspecies. It is a gram-positive bacteria of 0.8-1 mm in diameter, non-motile non-sporulating and grows in pairs or cluster. Staphylococci present low guanine-cytosine content. These microorganism are mesophiles, with optimum growth temperature between 35 and 37°C, aerobic or facultative anaerobic and show a respiratory and fermentative metabolism (De Gouveia Caria, 2010).

The major reservoirs of *Staphylococcus aureus* are infected udders, teat canals, and teat lesions, but these bacteria also have been found on teat skin, muzzles, and nostrils. The bacteria can spread to uninfected quarters by teat cup liners, milkers' hands, wash cloths, and flies (Radostitis *et al.*, 1994; Capurro *et al.*, 1999; Abera *et al.*, 2013).

Staphylococcus aureus is usually identified using traditional biochemical methods (Tenover *et al.*, 1994). Using molecular biology techniques is nowadays becoming widely used for identification of bacteria in general (Shuiep *et al.*, 2009; Kate, 2012; Preethirani *et al.*, 2015; Jibril *et al.*, 2018).

Antibiotics are commonly used in dairy farms for treatment of mastitis. Recently, the imprudent use of antibiotics led to the appearance of resistant bacteria towards these drugs. This has become a major problem for the treatment of mastitis (Moroni *et al.*, 2004; Tiwari *et al.*, 2013).

In Sudan, *Escherichia Coli* and *Staphylococcus aureus* are among the major causes of mastitis in Khartoum State, (Suliman and El Tigani, 2010). Farmers and animal owners are using different types of antibiotic for treatment, among the commonly used drugs are Penicillin, Oxytetracycline, Enrofloxain and Gentamycine.

The aim of this study was to investigate the

prevalence of *Staphylococcus aureus* in dairy cows in Khartoum State. Identification and characterization will be done using biochemical tests and PCR technique using species specific primer. Also to evaluate its antibiotics sensitivity pattern towards three of the commonly used antibiotics.

Materials and Methods

Study area

This study was conducted in different areas of Khartoum State – Sudan (Khartoum, Khartoum North and Omdurman). The State is located between 15-36 N and 32-33 E. The attitude is 380 M above sea level. The mean minimum and maximum temperatures range between 18.5-33.9 C°, respectively.

ii) Sample collection

A total of 186 milk samples were collected randomly from small scale dairy cows in Khartoum State. The samples were collected aseptically for bacteriological culture. Before sampling the first stream of milk was discarded and the teat ends were disinfected with cotton swabs soaked in 70 % alcohol and allowed to dry. The samples were stored in ice till transported to the laboratory at the College of Veterinary Medicine, University of Bahri for further analysis.

Isolation and Biochemical identification of bacteria

Isolation of bacteria

Samples were cultured in Manitol salt agar medium as selective and differential media used for the isolation of *Staphylococcus aureus* (Finegold, and Sweeney, 1961). The isolates were identified from their morphology and the development of a yellow colour of the red/pink media which indicates a positive result.

Identification of the bacteria

Staphylococcus aureus were identified using standard biochemical tests and further confirmed using Polymerase Chain Reaction (PCR) technique.

Biochemical methods

These included staining with crystal violet, Coagulase test and oxidase tests (Raus and Love, 1983)

Molecular identification

PCR technique was used. DNA was extracted using Guanidine – Chloride method (modified from Ghatak *et al.*, 2013). Briefly 1-3 colonies of freshly grown culture were mixed with 2ml of lysis buffer (50 mM Tris HCL, 25 mM NaCL, 25 mM EDTA, 1% SDS pH 8.0), 5µl of proteinase K, 300µl of NH₄ Acetate and 2 ml of guanidine chloride. After incubation overnight at 37° C, an equal volume of chloroform was added and then centrifuged at 6000 rpm for 5 min. The supernatant was aspirated into a clean tube. Two volumes of cold absolute ethanol were added and kept overnight at -20°C. The mixture was centrifuged at 6000rpm for 5 min. The supernatant was discarded then the DNA pellet was washed twice with 70% ethanol. Dried DNA was re-suspended in ddH₂O and finally stored at -20° C

PCR

PCR amplification was performed with a pair of primers specific for the gene nuc (genes encoding staphylococcal thermostable nuclease) according to (Preethirani *et al.*, 2015). The following primers were used:

Forward primer: 5'-GCG ATT GAT GGT GAT ACG GTT-3',

Reverse primer: 5'-AGC CAA GCC TTG

ACG AAC TAA AGC-3'

The PCR protocol was as follows: denaturation at 94°C, annealing at 55°C, and extension at 72°C. The amplification was performed for 35 cycles. The PCR products were visualized by electrophoresis in a 2 % agarose gel.

Susceptibility testing

The Susceptibility of the isolates against three antibiotics (commonly available drugs used for treatment of mastitis) was tested using agar disc diffusion method (National Committee for Clinical Laboratory Standards, 2001). Isolates that were previously confirmed as *Staphylococcus aureus* were grown in nutrient agar plate with discs of the tested antibiotics (Penicillin, Combination of Sulphamethoxazole/ Trimethoprim and Ciprofloxacin) placed in the plate. Following 16 – 18 hours of aerobic incubation the plates were examined and the diameter of the inhibition zone was measured. The zone diameters were expressed as resistant, intermediate or susceptible according to (Chengappa, 1990; CLSI document 2008).

Results and Discussion

This study was conducted to investigate the prevalence and antibiotic sensitivity pattern of *Staphylococcus aureus* in different dairy farms in Khartoum State. A total of 186 milk samples were collected randomly from the three localities.

After growth in the specific media and carrying out the biochemical tests (staining with grams stain, examination for catalase production, oxidase and coagulase tests), 47 (25.3%) of the samples were identified as *Staphylococcus aureus* (Table 1).

Molecular techniques are nowadays regarded as an important tool for identification of

bacteria. Confirmation of isolates was carried out using PCR. A band size appeared at 186 bp. Similar band size were reported by Preethirani *et al.*, (2015) (Fig. 1).

The percentage of positive samples from different localities was similar as shown in Figure 2.

A similar percentage of isolated bacteria was reported by (Liu *et al.*, 2017; Lili *et al.*, 2018), 26 % and 27% respectively. The *S. aureus* was detected in 42% of the total examined milk samples in Egypt (Awad *et al.*, 2017). Lower percentage (10.16%) was reported in raw milk samples (Patel *et al.*, 2018). The prevalence rate of *Staphylococcus aureus* shows variable variation ranging from 40% down to 10% (Patel *et al.*, 2018)

Several studies reported that *Staphylococcus aureus* is the main cause of mastitis (Kerro and Tareke, 2003; Hundera *et al.*, 2005; Mekonnen *et al.*, 2005). A study carried by Abera *et al.*, (2013) in Ethiopia, showed the percentage of *Staphylococcus aureus* isolated from mastitis cows was 42.1 % which renders it as one of the problem causing in dairy farms,

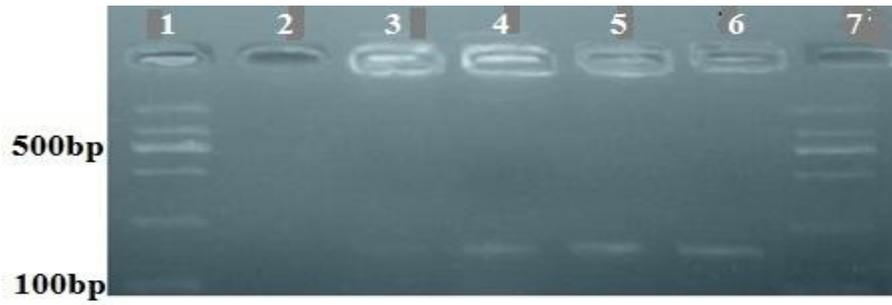
The variability in prevalence of mastitis results may be affected by different factors such as the season, farm management practices sampling procedures, method of isolation and hygienic practice of milking and selling (Abera *et al.*, 2013; Lili *et al.*, 2016; Patel *et al.*, 2016). An important feature of *Staphylococcus aureus* is it that it usually survives in the udder resulting in clinical or sub clinical mastitis, but it also can shed into the milk and it becomes a source for infection to other healthy cows during milking- process and become a main source of contaminants (Patel *et al.*, 2018)

Regarding the sensitivity tests, the previously isolated and identified *Staphylococcus aureus* was tested against three commonly used antibiotics namely Penicillin, Sulpamethoxazole / Trimethoprim and Ciprofloxacin. The isolates were reported as sensitive, intermediate or resistant after measuring the inhibition zone around the specific disc and then classified according to (Chengappa, 1990; CLSI document 2008). All sample (47 isolates) were found to be resistant to Penicillin while susceptible to Sulpamethoxazole /Trimethoprim and Ciprofloxacin (Fig. 3).

Table.1 Biochemical tests for identification of *Staphylococcus aureus*

Test	Result
Gram stain	Gram positive cocci
Catalase	Positive
Oxidase	Negative
Coagulase	Positive

Fig.1 Agarose gel for *Staphylococcus aureus* specific primer



Lane 1 and 7 are DNA marker. Lanes 2 –ve control. Lane 3, 4, 5 and 6 are *Staphylococcus aureus* 186 bp +ve samples

Fig.2 Percentage of positive samples from the three localities

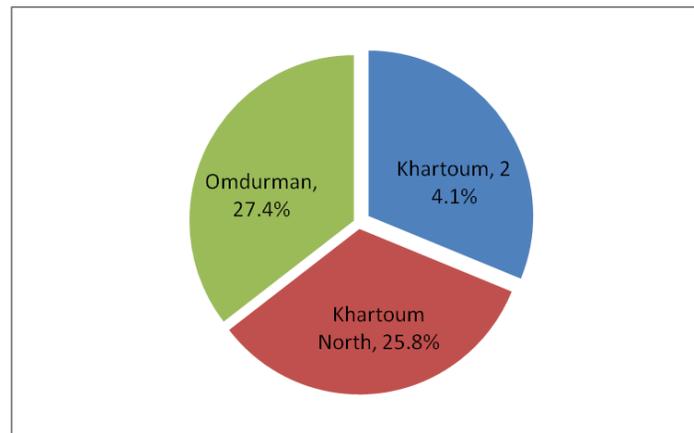
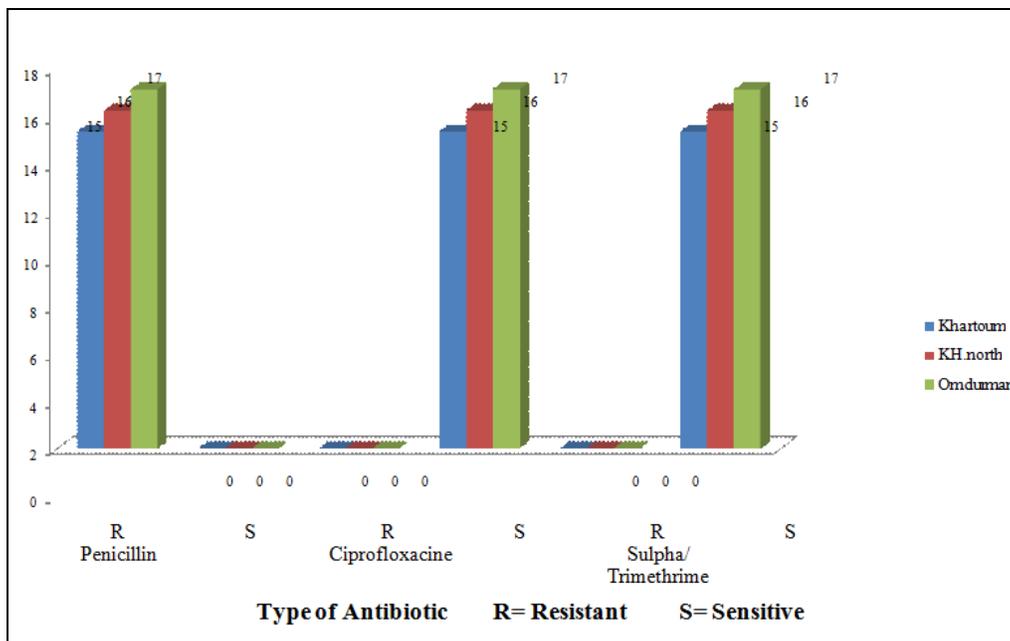


Fig.3 Antibiotic pattern of different isolates



Due to the importance of *Staphylococcus aureus* in dairy farms, its sensitivity was investigated towards a large number of drugs by different studies (Ahmed, 2003; Abera *et al.*, 2013; Mihael *et al.*, 2010; Lili *et al.*, 2016; Anueyiagu and Isiyaku, 2015). In the study carried by Abera *et al.*, (2013), *Staphylococcus aureus* was found to be highly susceptible to Chloramphenicol, followed by Gentamycin, Kanamycin and Streptomycin, while in this study the isolated *Staphylococcus aureus* was found to be susceptible to Sulpamethoxazole / Trimethoprim and Ciprofloxacin. However these isolates were resistant to Penicillin. Similar results for- penicillin resistance - were reported by (Lili *et al.*, 2016; Abera *et al.*, 2013 and Liu *et al.*, 2017) and although the latter study showed that the isolates were also resistant to Trimethoprim/ Sulpamethoxazole followed by amoxicillin.

Another study showed that *Staphylococcus aureus* isolates were sensitive also to: oxacillin, gentamicin, tylosin, tetracycline, kanamycin, novobiocin, ampicillin and erythromycin while it showed resistance to penicillin, clindamycin, collxacillin and ampicillin (Mihael *et al.*, 2010). The study carried by (Awad *et al.*, 2018) revealed a high resistance of the bacteria against penicillin and ampicillin and a lower resistance was observed against gentamicin, amikacin and ciprofloxacin.

The resistance of *Staphylococcus aureus* to the penicillin family is believed to be due to production of beta lactamase enzyme which destroys penicillin and related drug family. It is believed that around 50% of *Staphylococcus aureus* strains that cause mastitis have the ability to produce beta lactamase (Green and Bradely, 2004).

Another problem with *Staphylococcus aureus* is that due to its resistance to various to

antibiotics, this results in persistent non-curable intra-mammary infection leading to culling of infected animals. It is also causing a problem in human medicine as it has the ability to acquire resistance to the commonly used as well as last resort antimicrobials such as methicillin and vancomycin leading to multidrug-resistant strains (Abdi *et al.*, 2018)

The classic method of classification of bacteria into resistant, intermediate or resistant is now being diverted to an alternative method of “wild type” and “non-wild type” population based on the distribution of inhibition zone diameters (Supre *et al.*, 2014).

Investigating and identifying the antimicrobial resistance or sensitivity of bacteria towards the drug is a crucial issue as to advice veterinarian or farmers to the suitable treatment in order to minimize the misuse of drugs.

It is important to reduce risk of reintroduction of *S. agalactiae* or the addition of new, potentially more virulent strains of *S. aureus* to the herds through maintaining a closed herd or, at minimum, adhering to clearly defined biosecurity protocols (Keefe, 2012).

In conclusion, this study revealed the high resistance of *S. aureus* – which was present in all dairy farms investigated - to penicillin. Thus the commonly used Penicillin will be ineffective for treatment of infections caused by this bacteria especially mastitis.

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