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Molecular and Bacteriological Study of Vancomycin Resistant *Staphylococcus Aureus* (VRSA) Isolated from Buffalo Raw Milk in Baghdad

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ABSTRACT

One hundred buffalo raw milk samples from different locations in Baghdad governorate, Iraq, were tested for Vancomycin-resistant *Staphylococcus aureus* (VRSA) by streaking on a plate of mannitol salt agar plate and incubated in 37℃ for 24hr then inoculated on blood agar. The isolates were confirmed according to colony morphology, Gram staining and conventional biochemical techniques and polymerase chain reaction (PCR) to detect vanB gene. The disc diffusion testing was done to determine antimicrobial resistance including VRSA. *Staphylococcus aureus* (*S. aureus*) was found in 46 (46%) of raw buffalo milk. The isolates exhibited high resistance (80%) to methicilin, 75% to Oxacillin and Cefoxitin, 55% to gentamycin, and 50% to vancomycin. The phenotypic analyses revealed that out of 46 S. aureus, 23 (50%) were VRSA. While the genotypic identification by PCR showed that out of the 23 VRSA, 18 (78.2%) isolates had vanB genes. Results highlight the potential zoonotic risk of buffalo milk as a potential source of VRSA, which may be transmitted to human beings working in close contact with the buffalos.

KEYWORDS: buffalo milk, *Staphylococcus aureus*, VRSA, antimicrobial susceptibility, vanB gene.

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INTRODUCTION

Staphylococci are Gram-positive, catalase-positive, non-motile, non-spore forming aerobic coccid (with an average diameter 0.5 – 1.0 µm), arranged as pairs, tetrads or oftentimes irregular grape-like clusters [1]. *S. aureus* is the most important human and animals opportunistic pathogen, among the staphylococci species and is characterized by the production of golden pigment. The coagulase test is the simplest laboratory method to distinguish *S. aureus* from coagulase-negative staphylococci; also *S. aureus* can be detected or confirmed by PCR technique [2]. Many articles have shown that several *S. aureus*, isolated from humans, animals or food processing, it is associated with healthcare-associated community-acquired outbreaks worldwide [3]. The main diseases in the animals are caused by the pathogenic *Staphylococcus spp.* include mastitis, abscesses, and dermatitis [4, 5].

The pathogenicity of *S. aureus* is related to its capability of producing toxins and enzymes, and thereby leading to serious diseases, such as bacteremia, myocarditis, acute endocarditis, pneumonia, osteomyelitis and meningitis [6]. It has the ability to emerge and spread new antimicrobial resistance, such as VRSA [1]. vancomycin is a semisynthetic beta-lactamase–resistant penicillin that was introduced in 1959 to treat bacterial infection; shortly thereafter, vancomycin-resistant isolates of *S. aureus* was detected, the first strain of MRSA was recorded in 1961 in the United Kingdom, it is now associated with healthcare infections. Since its first isolation in 1961 until now, VRSA has been considered as one of the main pathogens of healthcare-associated infections at hospitals [7].In the livestock, the VRSA was first, isolated from mastatic cow milk in Belgain [8], after that, it was reported in several (wild and domestic) animals [9, 10]. It is

increasingly recognized in the animal world [11]. Horizontal transmission of VRSA between livestock on farms and in veterinary institutions is well recognized [12]. We report the molecular characterization and antimicrobial susceptibility of VRSA isolated from buffalo raw milk.

MATERIALS AND METHODS

This study was approved by The Committee of Ethics Research of the College of Veterinary Medicine, Baghdad University, Ministry of High Education and Scientific Research-Iraq. *S. aureus* was isolated from buffalo raw milk samples in Baghdad governorate, Iraq from October 2019 to March 2020. One hundred buffalo milk samples were collected in sterile condition from (Abu ghraib villages) in Baghdad and streaked on manitol salt plates and incubated at 37°C for 24 hr, confirmation of the isolates was depended on the morphology of the colony, gram staining and biochemical tests [1]. The antimicrobial susceptibility test for *S. aureus* isolates was performed according to previous methods [13].

The *S. aureus* isolates were subjected to PCR analysis. Its genomic DNA was extracted by the Genomic DNA Minni Kit (Geneaid, Thailand) according to the manufacturer's instructions. The concentrations and purity of extracted DNA were quantified using a Nanodrop spectrophotometer. Gel electrophoresis was used to detect the DNA bands stained with red safe dye visualized under UV light [14]. Agarose 1 g was used for DNA extraction and 2 g in the case of PCR products was added to 100 ml 1XTBE buffer, then boiled and left to cool at 50°C, after 2µl of Red Safe dye was poured on the preparing tray. Post polymerization (30 min at room temperature) the comb was removed post-hardening of agarose in wells. Gels were placed into a gel chamber filled with 1 X TBE buffer. The gel pocket was completely covered with buffer. Then 10 µl of each sample was mixed with 5 µl of loading dye buffer and loaded into wells in the gel. The obtained DNA products were screened for the presence of *vanB* gene genes by PCR methods as shown in Table 1, these primers were supplied by Bionneer Company, Korea and prepared according to the recommendation of the manufacturer.

The polymerase chain reaction mixture was set up in a volume of 20 µl which included: 2 µl of PCR premix (contains: *Taq* DNA polymerase, MgCl2, dNTPs, KCl, stabilizer tracking dye and tris-HCl), 1 µl of each primer (final concentration was 10 picomole /µl) and 2 µl of DNA template then the volume was completed with sterile distal water.

Table 1: Primers used for amplification of vanB gene gene in *Staphylococcus aureus.*

Genes	Primer	Sequence	Product size(bp)	Reference
vanB	*vanB* -F	5' GTG ACA AAC CGG AGG CGA GGA 3′	430	[15].
	vanB-R	5′ CCG CCA TCC TCC TGC AAA AAA-3'		

The PCR product tubes were mixed, and placed in a programmed thermocycler for amplification according to conditions shown in Table 2 below.

Table 2: Cyclic conditions of polymerase chain reactions

Gene	Initial denaturation	No.of cycles	Denaturation	Annealing	Extension	Final Extension
vanB	94ºC for 10min.	30	94ºC, 30 sec.	50ºC, 45 sec.	70 ºC, 30 sec.	72 ºC, 10min.

Before electrophoresis, PCR products for each well were loaded with 5 µl sample. DNA ladders 100bp and 1kb (KAPA Biosystem/USA) were run concurrently with each electrophoresis run to detect the size of the amplified PCR product. Electrophoresis was performed at 70V for 1.5 hours for extracting genomic DNA and PCR products, then the DNA bands were visualized by UV transilluminator and photographed using a digital camera. All steps of PCR assays were performed in the Department of Internal and Preventing Veterinary Medicine, College of Veterinary Medicine, University of Baghdad.

RESULTS

The total isolation rate of *S. aureus* was 46% (46/100) from buffalo raw milk samples. Suspected colonies of *S. aureus* isolates appeared on mannitol salt agar as round, smooth,

fermented mannitol and convert colour from red to yellow (Figure 1)**.**

Gram staining of *S. aureus* under light microscope (Figure 2). Microscopically, suspected *S. aureus* isolates appeared in the light microscope as Gram-positive, cocci, single cell pairs, grape-like clusters. The isolates were positive for catalase (figure 3), gelatinase tests (Figure 4), hemolysis, DNase and coagulase (Table 3)**.**

Figure 1- *Staphylococcus aureus* on Mannitol Salt Agar show mannitol fermentation.

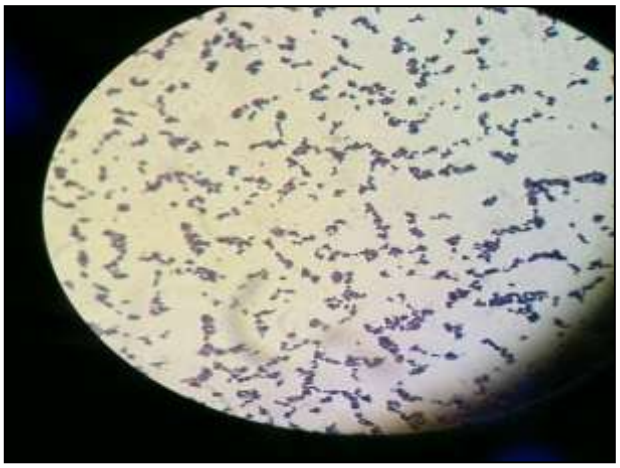

Figure 2- Gram staining of *S. aureus* under the light microscope, Gram-positive, cocci, single cells pairs.

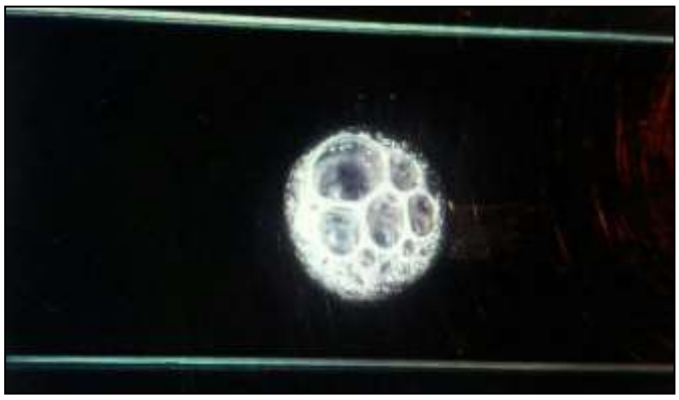

Fig3. -*S. aureus* positive catalase test. *S. aureus* positive catalase test.

Figure 4 - *Staphylococcus aureus* positive result for gelatinase test

Table 3: Biochemical tests of *Staphylococcus aureus* isolates

Test	Result
Catalase	+
Coagulase	+
Gelatin liquefaction	+
Hemolysis	+
Mannitol fermentation	+
Oxidase	-
DNAse	+

The *S. aureus* isolates of buffalo raw milk showed high resistance to methicilin (80%), to Oxacillin, and Cefoxitin (75%) and gentamycin (55%) and vancomycin (Table 4).

Table 4: Percentage of resistant and susceptible isolates of *Staphylococcus aureus* to antibiotics in buffalo raw milk.

Antibiotic (mg)	Sensitive	Intermediate	Resistant
Methicillin(5)	9(15.6%)	1(2%)	38(80%)
Oxacillin(1)	7(14%)	4(8%)	36(75%)
Cefoxitin(30)	5 (12%)	5(10%)	36(75%)
Gentamycin(10)	19(40%)	4(8%)	23(55%)
Chloramphenicol(30)	22(47%)	10(20%)	14(30.25%)
Trimethoprim(5)	25(54%)	8(16%)	13(28%)
Clindamycin(2)	17(37%)	11(22%)	18(39%)
Vancomycin(30)	20(50%)	3(4%)	23(50%)

Genomic DNA of *S. aureus* was extracted from all isolates, detected and visualized by 1% gel electrophoresis as compact bands (Figure 5) and the purity of DNA ranged from 1.8 to 2 at absorbance 260/280 nm.

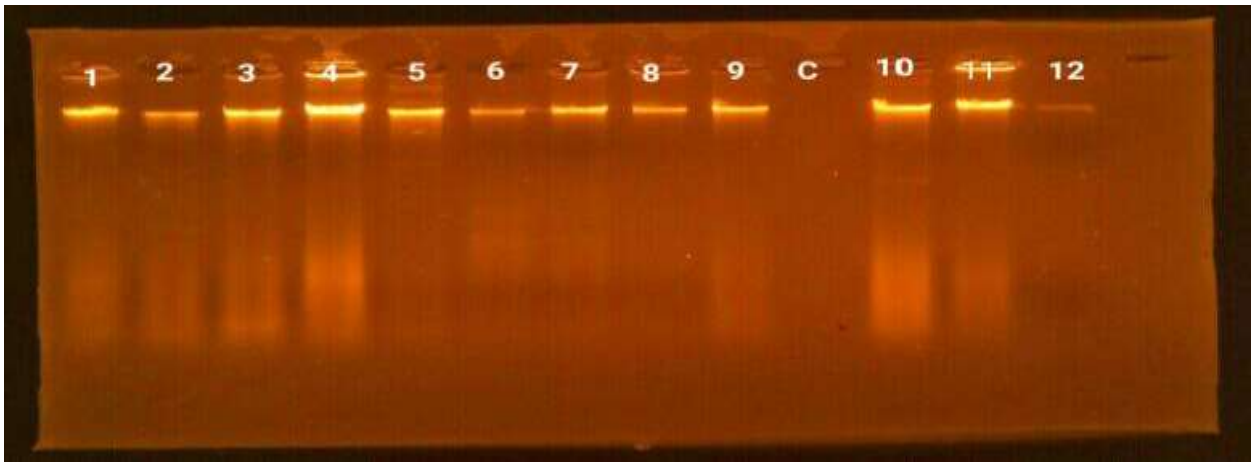

Figure 5: Agarose gel electrophoresis (1 % agarose, 70V, for 1.5 hours) of genomic DNA of *S. aureus* isolated from buffalo raw milk showed bands of DNA.

PCR employed for the detection of ß lactamase enzyme encoded by the *vanB* gene showed that 18 (50%) of *S. aureus* isolates gave positive results to *vanB* (430bp) gene (Figure 6).

Figure 6- showing the PCR product analysis of *vanB* gene (430bp) in *S. aureus* isolates on agarose gel electrophoresis image. Where M: marker (2000-100bp), lane (20-22) negative isolates and lane (23-31) positive isolates from buffalo raw milk samples at PCR product

DISCUSSION

Our results reveal similar phenotypic characters for *S. aureus* as reported previously [1]. We found an incidence of *S. aureus* of 46 (46%) from buffalo raw milk samples, which is higher than that has been observed in the Basra governorate, South of Iraq, where the incidence was 22.2 % from buffalo milk samples [17]. Also, our finding was higher than those reported by others [18, 19]. Buffalo raw milk could act as a vehicle for MRSA transmitted to humans [20]. The comparatively high rates of MRSA recorded in our study may be contributed to unhygienic milking practices and the rearing of buffaloes in the study area (Abo ghraib villages). The majority of buffalo herds in Iraq are household reared, and their milk and dairy products are sold in unofficial markets. Many practices such as manual milking, unhygienic milking equipment, and milk storage and transportation may participate to the high contamination rate [21].

Our findings regarding the susceptibility and resistance of *S. aureus* to antimicrobial agents confirm previous findings [18, 19, and 22]. Several studies have shown that *S. aureus* is resistant to one or more antimicrobial agents [23, 24]. The improper usage of antimicrobial drugs poses great hazards to both animal and human health due to the emergence of antimicrobial resistance [25]. The extensive use of antimicrobial agents in food animals' production, where they are often applied sub-therapeutically for growth production and routine disease prevention, often gives rise to multidrug-resistant and VRSA strains in animals [26]. Monitoring the emergence of multidrug-resistant strains of *S. aureus* in human and animals are essential to control the spreading of this pathogen and the related zoonotic hazard [27]. During the last years, VRSA in animals has acquired special awareness from public health authorities, it is known that the use of antimicrobials for animal treatment can promote survival or emergence of MRSA in animals [28].

Several techniques were developed for the detection of MRSA including phenotypic and genotypic analysis, the *vanB* gene, which is considered a genetic marker used for the rapid and direct identification of VRSA [29]. The phenotypic analysis revealed that out of 46 *S. aureus* isolated from buffalo milk 23 (50%) were VRSA. While the genotypic identification of 39 MRSA isolated from buffalo raw milk, showed that 18(50%) isolates had *vanB* gene. The genotypic identification rate of *vanB* in the current study is nearly similar to that has been observed previously [30, 31]. Monitoring the development of multidrug-resistant *S. aureus* in humans and animals is essential to prevent or control the spreading of this pathogen and the related zoonotic hazard [27].

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