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Research Article

ASYMPTOMATIC COLONIZATION OF *STAPHYLOCOCCUS AUREUS* WITH INTERMEDIATE RESISTANCE TO VANCOMYCIN HARBORING VANB RESISTANCE GENE

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ABSTRACT

Objective: Vancomycin has been used worldwide due to empirical therapy against methicillin-resistant Staphylococcus aureus infections. As a result a selective pressure that favors the outgrowth of vancomycin intermediate S. aureus clones will be created. This study was carried out to evaluate vancomycin resistance pattern of S. aureus in Jordan.

Methods: A total of 1179 samples, including 566 (48%) from human and 613 (52%) from animals were examined for the presence of S. aureus using standard biochemical tests and polymerase chain reaction (PCR) amplification of coa gene. Resistance to antibiotics was determined by the disk diffusion method. Methicillin resistance strains were tested for vancomycin resistance by minimal inhibitory concentration (MIC), E- test, and the results were confirmed by amplification of van genes, and Pulsed-field gel electrophoresis (PFGE).

Results: The prevalence of S. aureus among human source was: 19.35%, 14%, and 8.8% for nasal, nail, and skin, respectively, and for animal sources 27.3%, 5.51%, and 15.86% for milk, nasal, and meat, respectively. Four VISA strains (1.87%) were found to colonize human nares, nails, and skin with vancomycin MIC of 4-8 µg/ml. Van B resistance gene was detected and PFGE with SmaI-digested VISA genomic DNA revealed two different pulsotypes.

Conclusion: This is believed to be the first report of VISA strains containing vanB gene isolated from a routine carriage survey. Effective screening directed to persons colonized with VISA should therefore be a priority.

Keywords: *Staphylococcus aureus*, Vancomycin intermediate *Staphylococcus* aureus, Pulsed-field gel electrophoresis, Polymerase chain reaction, *VanB* genes.

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INTRODUCTION

Staphylococcus aureus is a common pathogen associated with serious community and hospital-acquired diseases and has for long been considered as a major problem of Public Health [1]. Both healthy people and those with underlying illness are at risk for diverse skin and soft tissue infections, endocarditis, osteomyelitis, meningitis, bacteremia, and pneumonia [2] with mortality rates ranging from 6% to 40% [3]. Several virulence factors implicated in the pathogenesis of *S. aureus* strains have been described in the literature [4] which is involved in tissue invasion of the host cells.

Increasing prevalence of methicillin-resistant *S. aureus* (MRSA) infections has led to the extensive use of vancomycin for treating these conditions. However, the overuse of this antibiotic has led to the emergence of *S. aureus* strains with reduced susceptibility to vancomycin. Hiramatsu *et al*. [5] from Japan were the first to report a clinical strain of MRSA with reduced susceptibility to vancomycin. It was due to decreased availability of the vancomycin for intracellular target molecules. As a consequence of the extensive use of vancomycin, a selective pressure was established that eventually resulted in the emergence of strains of *S. aureus* with a decreased susceptibility to vancomycin and other glycopeptides. The reports of vancomycin-intermediate *S. aureus* (VISA) and vancomycin resistance (VRSA) have been increasing from various parts of the world [6].

The emergence of vancomycin resistance strains exhibits different mechanisms. Another than plasmid-mediated vancomycin resistance gene transfer from Enterococcal species to *S. aureus* [7], laboratory studies have demonstrated that resistant to vancomycin can be produced by a step pressure procedure [8]. However, interspecies transfer of resistant genes was thought not to be responsible for intermediate resistance to vancomycin in *S. aureus* [9]. Instead, VISA strains have been observed to have lower growth rates and thicker cell walls than fully susceptible strains. The increases in cell wall turnover will lead to an increase of non-cross-linked d-alanyl-d-alanine side chains; these chains are capable of binding vancomycin outside of the cell wall, making less vancomycin available for intracellular target molecules [10]. Nevertheless, some data in the literature raise the possibility that factors in the invading bacterium may also contribute. Examination of the vancomycin minimal inhibitory concentration (MIC) value of MRSA strains that were recovered from patients with failed therapy showed that therapeutic failure correlated significantly with the vancomycin MIC value of the strains, even when this antibiotic was not used for therapy [11]. These data suggest that the vancomycin susceptibility profile of MRSA strains correlates with some aspects of the invasive potential of the bacteria.

Since the first observation of VISA, there is a steady increase in the number of cases with VISA and VRSA. In our region, a report from Jordan has been published in which a case of VISA was reported in the year 2006 by Bakri and coworkers in a patient with erythrodermic psoriasis with a persistent *S. aureus* bacteremia [12]. Another interesting report came from Oman on the treatment of infective endocarditis due to VISA [13]. Moreover, five strains of VISA were reported from North Lebanon by MIC dilution method [14]. While vancomycin is traditionally a first line and relatively effective antibiotic, its continued use is under question, as reports of resistance in *S. aureus* isolates are increasing. Detection of VISA is difficult in the laboratory, and special inquiries about susceptibility testing methods may be needed. In contrast to some reports of *S. aureus* isolates with uniform susceptibility to vancomycin [15], the importance of screening of MRSA isolates for VISA has repeatedly been emphasized. However, a survey of laboratories indicated that many did not use methods that can detect VISA strains [10]. Due to these strains appear to have developed from strains of MRSA instead of a single clone, they have often been missed by disk diffusion testing. Surveillance of patient populations,

especially in units where the probability of MRSA carriage and prolonged glycopeptide therapy is high, should be regularly done using vancomycin agar screening tests, E-test, and Hi Comb vancomycin MIC strips as per the criteria of the centers for disease control and prevention [9]. Recently, VISA and VSSA isolates are easily separated using Matrix-assisted laser desorption ionization time-of-flight mass spectrometry [16].

Although the acquired vancomycin-resistance determinants *vanA, vanB, vanD*, *vanE*, *vanF*, and *vanG* have been reported from VRSA, these resistance determinants have not previously been identified in cases with intermediate resistance [17]. However, one study showed the existing of *vanA* gene among nasal isolates [18]. VISA-infected patients had underlying illnesses, and their infections did not appear to respond well to conventional treatment. It is necessary to check the level of susceptibility of MRSA to vancomycin, especially when VISA exposed to appropriate selective step-up pressure; the strains will eventually take a resistant form. The main objective of the study was to evaluate the possible occurrence of VISA in human and animal isolates of MRSA and to characterize them genetically.

METHODS

Collection of samples

Human samples

Nail, nasal, and skin samples were obtained from Al-Balqa' Applied University students.

Nasal swabs
A total of 217 nasal specimens were obtained with sterile swabs after rotating 5 times around the inside of both nostrils while applying constant pressure. Nasal swabs were collected and stored in an ice box. The swabs were incubated into Tryptic Soy Broth (TSB) containing 7% NaCl for 24 hrs and then subcultured on mannitol salt agar (MSA) and incubated at 37°C for 24 hrs [19].

Nail swabs
A total of 217 nail specimens were obtained by swabbing the subungual space of the nails from the hands with sterile cotton swabs. Swabs were transferred to TSB containing 7% NaCl for 24 hrs and then subcultured on MSA, incubated at 37°C for 24 hrs [20].

Skin swabs
About 68 specimens were taken from two separate sites on the forehead and one site from one cheek. The swabs were incubated into TSB containing 7% NaCl. The isolates were subcultured on MSA and incubated at 37°C for 24 hrs.

Clinical samples
Different sources of clinical isolates (blood infection, urinary tract infection, abscess, and ear and eye infections, etc.) from four hospitals in Jordan (Al-Salt, Prince Hamza, Al-Basheer, and Al-Islami) were collected aseptically and transported directly to the laboratory [21]. The isolates then subcultured on MSA and BPA, and incubated at 37°C for 24-48 hrs. The suspected colonies maintained on Staphylococcal 110 media and were kept at 4°C for identification procedures.

Animal samples

Animal samples were collected from three central slaughterhouses in Jordan which represent the population of animals in Amman city (capital of Jordan). Animals were local, and imported (Romanian and Australian) including 207 sheep, 67 goats, and 16 cows. The age of the animals ranged from 1.5 to 3 years.

Raw meat
A total of 219 meat samples obtained from various muscle sites by collection of segments of the muscle of slaughtered animals. Samples were enriched in TSB containing 7% NaCl for 24-48 hrs of incubation at 37°C. A portion of the enrichment cultures was then streaked on MSA and BPA and incubated at 37°C for 24-48 hrs. The suspected colonies were maintained on *Staphylococcal* 110 media and kept at 4°C for identification.

Nasal swabs
About 290 swabs were obtained from nares of the animals before slaughtering. Swabs were transferred to the TSB containing 7% NaCl. The isolates were subcultured on MSA and incubated at 37°C for 24 hrs [22].

Milk samples
About 33 raw milk samples were taken from sheep barn belonging to Al-Balqa' Applied University. None of these animals were diagnosed with clinical mastitis and have normal mammary glands. Samples were collected into sterilized screw cap bottles as described previously [23]. 1 milk sample (10-15 ml) was taken aseptically from each mammary gland after washing with water and cleaning the teats with 70% ethanol. Milk samples were cultured on TSB containing 7% NaCl and the isolates were subcultured on MSA and BPA, and incubated at 37°C for 24-48 hrs. The suspected colonies then maintained on *Staphylococcal* 110 media and were kept at 4°C for identification.

Biochemical tests

Isolates were examined by gram stain, catalase, coagulase, clumping factor and Protein A (BBL™ Staphyloslide Latex), mannitol fermentation, oxidase, hemolysin production using human and sheep RBCs, amylase, protease, sensitivity to acriflavine [24], tellurite reduction, and DNase test. *S. aureus* strain (ATCC 25923) was used as a positive control for all biochemical tests [25].

Molecular identification of the isolates

Extraction of DNA

Bacterial culture was grown overnight in nutrient broth, and 2 mL of the culture was centrifuged for 2 minutes. The pellet was resuspended in 567 µL of TE buffer to which 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K were added, mixed gently and incubated for 1 hr at 37°C. Following this, 100 µL of 5 M NaCl was added and mixed thoroughly. After addition of 80 µL of 10% CTAB, 0.7 M NaCl solution and the tubes were incubated for 10 minutes at 65°C. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed well and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube, and an equal volume of chloroform/isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube and 0.8 volumes of isopropanol were added, mixed gently until the DNA was precipitated. The DNA was washed with 70% ethanol and resuspended in 50 µL TE buffer [26]. DNA quality was assessed by Spectrophotometric analysis (Thermoscientific Nanodrop 1000, USA) at the absorbance ratio of 260 and 280 nm.

Polymerase chain reaction (PCR) amplification

For identification of *S. aureus,* the primers (COAG2 and COAG3) (5'-ACCACAAGGTACTGAATCAACG-3' and 5'-TGCTTTCGATTGTTCG ATGC-3') specific for the *coa* gene of *S. aureus* were used (Table 1). The reaction was prepared with 10 µL of extracted DNA (20 nm), 1 µM of each primer, 200 µM dNTPs, 1 U of Taq DNA polymerase, 2 mm of MgCl2, and 4 µL of 10 X buffer - with a final volume of 40 µL. The program used consisted of 50 seconds at 95°C, 2 minutes at 55°C, and 4 minutes at 72°C for 40 cycles [27]. The separation of the amplified products was carried out using 1% (w/v) agarose gel electrophoresis stained with 0.5 g.ml of ethidium bromide in TBE buffer, 70 volts for 1 hr. UV transillumination was used for the visualization of the bands, and the size of the amplified fragments was determined by comparison with a molecular weight marker (1 kb plus DNA Ladder or 100 bp DNA Ladder).

Vancomycin susceptibility testing in *S. aureus*

Disc diffusion method

Plates of Muller-Hinton Agar were seeded by bacterial broth culture and incubated following introduction of the antibiotic disks at 37°C for

Table 1: Primer pairs specific for vancomycin resistance gene

Number of base (mere)	Primer name	Primer sequence	Melting temperature
25	*VanA*	5'-ATGAATAGAATAAAAGTTGCAATAC-3'	62°C
21	*VanA1*	5'-CCCTTTAACGCTAATACGAT-3'	62°C
23	*VanB*	5'-CCCGAATTTCAAATGATTGAAAA-3'	59°C
18	*VanB1*	5'-CGCCATCCTCCTGCAAAA-3'	59°C
23	*VanC*	5'-GCTGAAATATGAAGTAATGACCA-3'	58°C
21	*VanC1*	5'-CGGCATGGTGTTGAATTTCGTT-3'	58°C

18 hr. The following discs were used; clindamycin (2 µg), erythromycin (15 µg), gentamycin (10 µg), methicillin (10 µg), penicillin (10 µg), amikacin (30 µg), cefoxitin (30 µg), novobiocin (50 µg), tetracycline (30 µg), vancomycin (30 µg), linezolid (30 µg), teicoplanin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), tobramycin (10 µg), and trimethoprim-sulfa (25 µg). Antibiotic resistance profile was determined according to the guidelines recommended by Clinical and Laboratory Standards Institute [28]. *S. aureus* standard strain ATCC 29213 was used as reference strain for MRSA, VSSA, and *Enterococcus faecalis* ATCC 51299 was used as a positive control (VRE). Variable diameters of inhibition zone were recorded for different antibiotics.

MIC agar method

Agar dilution method was applied using different concentration of vancomycin. Briefly, gradient plates of Mueller-Hinton agar were prepared with vancomycin (0.5-128 µg/ml). Colony suspension of 0.5 McFarland was prepared in normal saline from 18 to 24 hrs agar plate culture. All strains were spotted onto gradient plates and incubated overnight at 35°C for any visible growth. *S. aureus* ATCC 29213 was used as vancomycin susceptible controls and *E. faecalis* ATCC 51299 as vancomycin-resistant control. The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is known as the MIC. Vancomycin breakpoints were defined as follows susceptible at a vancomycin agar MIC of ≤2 µg/ml, intermediate at a vancomycin MIC of 4 to 8 µg/ml, and resistant at a vancomycin MIC of ≥16 µg/ml [28].

Vancomycin E-test

The conventional vancomycin E-test strips (bioMerieux, Durham, NC) were used which consists of a printed gradient concentrations of vancomycin from 0.015 to 256 µg/ml. Bacterial suspension was adjusted to 0.5 McFarland and plates were incubated for 24 hrs at 35°C. MICs were read by a direct observation, and the results were interpreted according to CLSI breakpoints [29].

PCR amplification of *vanA*, *vanB*, and *vanC* genes

Conventional PCR amplifications were performed with crude DNA using primer pairs specific for vancomycin resistance genes (Fig. 1). The PCR conditions were as previously described, except for the annealing temperatures, which varied from 58°C to 60°C depending on the *van*-specific primers used [30]. *E. faecalis* ATCC 51299 was used as a positive control.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed using the Gene Path strain typing system (Bio-Rad, Tokyo, Japan). According to Yoshida *et al*. [31], samples of 150 µl from overnight cultures grown in 3 ml BHI broth were harvested by centrifugation at 12,000 g for 1 minute, resuspended in 150 µl cell suspension buffer and maintained at 55°C. Lysozyme/lysostaphin (0.15 mg, 6 µl) and 150 µl embedding agarose were added to this suspension, which then was poured into a plug mold. After setting for 20 minutes at room temperature, the agarose plug was placed in 500 µl lysis buffer with 20 µl lysozyme/lysostaphin (0.5 mg) and incubated at 37°C for 1 hr. The plugs were then washed in 1 ml 1× wash buffer and incubated at 50°C for 16-20 hrs in 500 µl proteinase K buffer containing 20 µl (12 U) proteinase K. Six wash steps, each continued for 30 minutes, were performed using 1 ml of 1 X washing buffer, followed by another wash in 0.1 X washing buffer and a final wash in 500 µl SmaI buffer. After the plugs were placed in 300 µl SmaI buffer, 5 µl (25 U) SmaI enzyme was added and the plugs incubated at 25°C for 16-20 hrs. After restriction digestion, the plugs were electrophoresed in 1% agarose gels using parameters preprogramed with the GenePath system using CHEF-DRIII (Bio-Rad) for *Staphylococcal* species. The resulting profiles were visualized by staining with ethidium bromide. Electrophoretic patterns were analyzed, and strains were considered identical when they shared same number and sizes of fragments. The strains varied with only 2 or 3 bands were considered closely related [32]. *S. aureus* ATCC 25923 was used as a control.

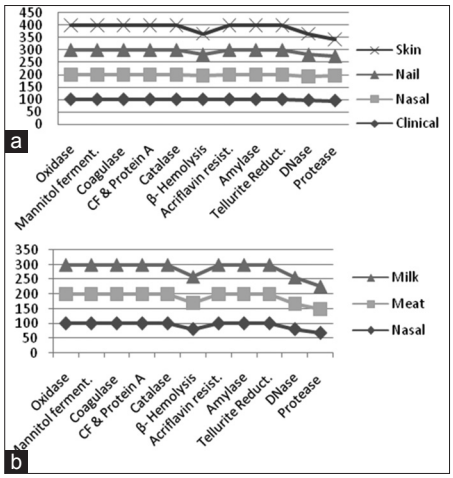

Fig. 1: Phenotypic characterization of *Staphylococcus aureus* strains isolated from (a) human and (b) animal sources

RESULTS

Isolation of *S. aureus*

A total of 1179 samples including 566 (48.0%) obtained from human and 613 (51.9%) obtained from animal sources were used in the study. Gram staining, mannitol fermentation, catalase, coagulase, staphyloslide latex test, and thermonuclease were important phenotypic identifying markers of *S. aureus*. The overall prevalence of *S. aureus* was 143 (25.6%) and 71 (11.58%) isolated from human and animal sources, respectively (Table 2).

Biochemical characterization of *S. aureus*

In this study, we found that all *S. aureus* were positive for oxidase, catalase, coagulase, clumping factor and protein A, manitole fermentation, acriflavin resistance, amylase, and tellurite reduction. However, a variation in the phenotypic characteristics of *S. aureus* isolated from human and animal sources was appeared in beta hemolysis on blood agar supplemented with 8% (v/v) human and sheep blood, protease on 2% skim milk agar, and DNase on toluidine blue- DNA Agar (Fig. 1). Human isolates obtained from clinical, nasal, nail, and skin showed 100%, 95.2%, 87.1%, and 83.3% for β- hemolysin; 96.8%, 95.2%, 90.3%, and 83.2% for DNase; 95%, 100%, 80.6%, and 66.7% for protease activity, respectively. On the other hand, animal isolates obtained from nasal, meat, and milk showed 81.25%, 89.1%, and 88.9% for β- hemolysin; 81.25%, 86.9%,

and 88.8% for DNase; 68.75, 80.5%, and 77.8% for protease activity, respectively.

Molecular identification

PCR amplification for S. aureus

The isolation of the genomic DNA from the bacterial isolates was the first step for the molecular study. The DNA that extracted from the suspected *S. aureus* isolates were subjected to PCR and loaded on 0.7% agarose gel electrophoresis and photographed under UV light. The size of *coa* gene specific for *S. aureus* gives PCR products that ranged from approximately 440 to approximately 915 bp when comparing to the DNA marker (Fig. 2).

Antibiotic susceptibility test

Disk diffusion assay

Disk diffusion assay was performed according to the protocol recommended by CLSI. All isolates showed variable sensitivity to the antibiotics that have been used (Tables 3 and 4). Multidrug resistance is defined as a complete resistance to 3 or more antimicrobial classes. A high percentage of MRSA was isolated from human as compared to animal sources (Fig. 3). All isolates proved to be susceptible to vancomycin by the disc diffusion method.

Vancomycin MIC test

Agar dilution technique was used to determine the lowest concentration of vancomycin that inhibits the growth of MRSA strains. The test was

Table 2: Numbers and percentages of *S. aureus* isolated from human and animal sources

Source n (%)	Sample sources	Number of samples	*S. aureus* n (%)	Total No. (%)
	Nasal	217	42 (19.35)	
Human sources	Nail	217	31 (14.28)	143 (25.26%)
566 (48)	Clinical	64	64 (100)	
	Skin	68	6 (8.82)	
Animal sources	Milk	33	9 (27.27)	
613 (52)	Nasal	290	16 (5.52)	71 (11.58)
	Meat	290	46 (15.86)	
Total		1179	214 (18.15)	

S. aureus: Staphylococcus aureus

Table 3: Antibiotic sensitivity test among *S. aureus* strains isolated from human sources

Type of antibiotics	Nasal No. 42 (%)			Nail No. 31 (%)			Clinical No. 64 (%)			Skin No. 6 (%)		
	S	I	R	S	I	R	S	I	R	S	I	R
Clindamycin (2 µg)	73.1	23.1	3.85	87.5	0	12.5	83.6	1.36	1.36	66.7	0	33.3
Erythromycin (15 µg)	14.3		85.7	12.9	0	87.1	6.3	0	93.7	16.7	0	83.3
Gentamycin (10 µg)	92.3	0	7.69	100	0	0	5.47	0	94.5	33.3	0	66.7
Methicillin (10 µg)	9.5	0	90.5	6.5	0	93.5	6.3	0	93.7	0	0	100
Penicillin (10 µg)	7.69	0	92.3	6.25	0	93.75	10.9	0	89	16.7	0	83.3
Amikacin (30 µg)	97.6	0	2.4	100	0	0	98.4	0	1.6	100	0	0
Cefoxitin (30 µg)	9.5	0	90.5	9.7	0	90.3	9.4	4.6	86	0	0	100
Novbiocin (50 µg)	95.2	0	4.8	100	0	0	93.7	0	6.2	100	0	0
Tetracycline (30 µg)	84.6	0	15.4	75	6.25	18.75	65.7	1.36	32.8	100	0	0
Vancomycin (30 µg)	97.6	2.4	0	100	0	0	95.3	4.7	0	100	0	0
Linezolid (30 µg)	92.3	0	7.69	93.6	3.2	3.2	93.75	1.6	4.7	83.3	16.6	0
Teicoplanin (30 µg)	71.4	4.8	23.8	87.1	0	12.9	78.2	3.1	18.7	83.3	0	16.7
Ciprofloxacin (5 µg)	85.7	0	14.3	93.5	0	6.5	43.8	0	56.2	66.6	16.7	16.7
Levofloxacin (5 µg)	7.1	2.4	90.5	16.1	0	83.9	1.6	1.6	96.8	50	0	50
Tobramycin (10 µg)	4.8	0	95.2	100	0	0	3.2	3.2	9.4	100	0	0
Trimethoprim (25 µg)	11.9	4.8	83.3	12.9	3.2	83.8	46.9	0	53.1	50	0	50

Table 4: Antibiotic sensitivity test among *S. aureus* strains isolated from animal sources

Types of antibiotics	Nasal No. 16 (%)			Meat No. 46 (%)			Milk No. 9 (%)		
	S	I	R	S	I	R	S	I	R
Clindamycin (2 µg)	46.6	33.3	20	73.17	12.19	14.63	50	0	50
Erythromycin (15 µg)	18.7	0	81.3	13.1	0	86.9	0	0	100
Gentamycin (10 µg)	86.6	13.3	0	97.56	0	2.43	50	0	50
Methicillin (10 µg)	18.7	0	81.3	13.1	0	86.9	11.1	0	88.9
Penicillin (10 µg)	13.3	0	86.6	12.19	0	87.8	25	0	75
Amikacin (30 µg)	93.7	0	6.3	82.6	0	17.4	88.9	0	11.1
Cefoxitin (30 µg)	93.7	0	6.3	95.6	0	4.4	33.3	0	66.7
Novbiocin (50 µg)	100	0	0	86.9	2.1	11	100	0	0
Tetracycline (30 µg)	66.6	20	13.3	31.7	17.07	51.21	66.7	0	33.3
Vancomycin (30 µg)	100	0	0	100	0	0	100	0	0
Linezolid (30ug)	87.5	0	12.5	86.9	2.1	10.9	55.5	11.1	33.3
Teicoplanin (30 µg)	78.5	0	12.5	97.8	2.8	0	88.9	0	11.1
Ciprofloxacin (5 µg)	93.7	0	6.3	95.6	0	4.4	100	0	0
Levofloxacin (5 µg)	93.7	0	6.3	91.4	4.3	4.3	100	0	0
Tobramycin (10 µg)	81.2	0	18.8	86.9	0	13.1	88.9	0	11.1
Trimethoprim (25 µg)	37.4	6.2	62.6	87.8	9.75	2.43	55.6	0	44.4

judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot (Fig. 4). According to CLSI recommendations, four MRSA (1.87%) exhibits vancomycin MIC of 4-8 µg/ml. These isolates belong to human (2.79%) and include 2 nasal (4.76%), 1 nail (3.2%), and 1 skin isolates (16.6%) (Table 5).

E-test

To confirm the presence of VISA detected by agar dilution MIC test, a standard E-test was performed. The concentration of vancomycin in the strips ranged from 0.015 to 256 µg/ml. The four VISA isolates found to be resistant to 4-8 µg/ml vancomycin by E-test (Fig. 5). VISA strains appeared resistant to oxacillin (100%), erythromycin (75%), and trimethoprim-sulfa (25%), while sensitive to other antibiotics that have been used (Table 6).

Detection of the van genes

The vancomycin-resistant genes - including *vanA, vanB, vanC* - were detected using a PCR as previously described. The PCR cycle was as follows: 1 cycle of 5 minutes at 94°C, then 35 cycles of 94°C, 1 minute, 58°C, 1 minute, and 72°C 1 minute; followed by a final 10 minutes extension at 72°C. *vanB* gene was found in the four isolates of *S. aureus* with intermediate resistance to vancomycin (Fig. 6).

PFGE

Molecular analysis of *Sma*I-digested DNA resulted in two distinct PFGE types (Fig. 7) which confirmed their epidemiologic relationship. Isolates in lines 1, 3, and 4 showed the same profile (profile I) and isolates in line 2 was considered different (profile II).

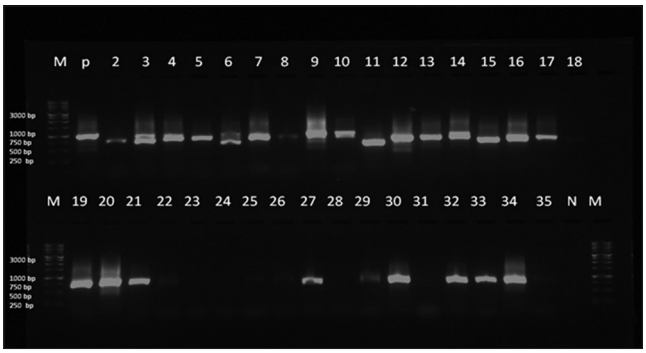

Fig. 2: Primer COAG 2, 3 for identification of *Staphylococcus aureus*, M: 100 bp marker ladder; P: Positive control of *Staphylococcus aureus* (440-915); samples from (2-22), (25-27), and (29-35) are *Staphylococcus aureus*; N: Negative control

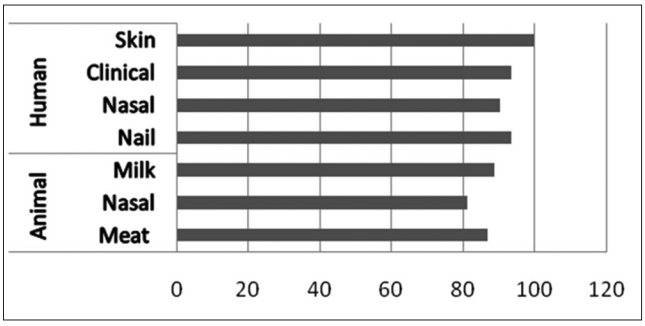

Fig. 3: Percentages of methicillin-resistant *Staphylococcus aureus* among human and animal isolates

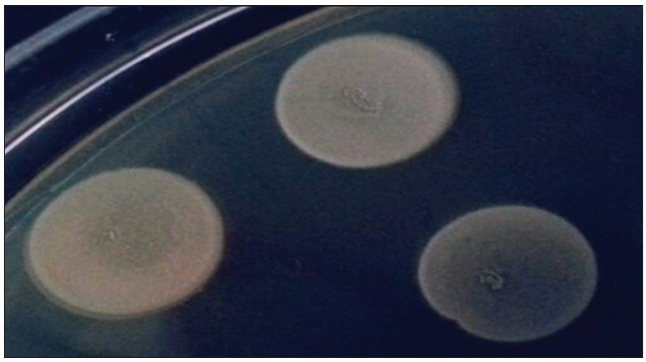

Fig. 4: Growth of *Staphylococcus aureus* on MHA containing 4 µg/ml vancomycin

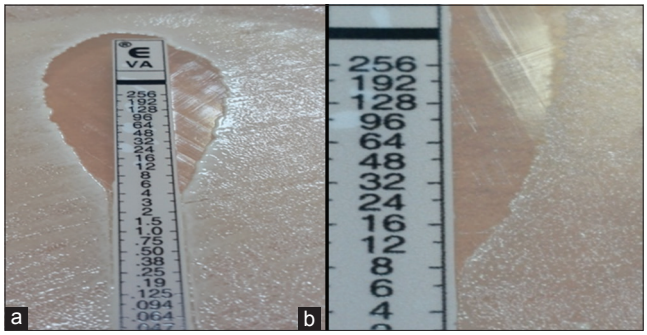

Fig. 5: E-test for vancomycin intermediate *Staphylococcus aureus* (VISA) strains (a) VISA strain with MIC 4 µg/ml, and (b) VISA strain with MIC 8 µg/ml

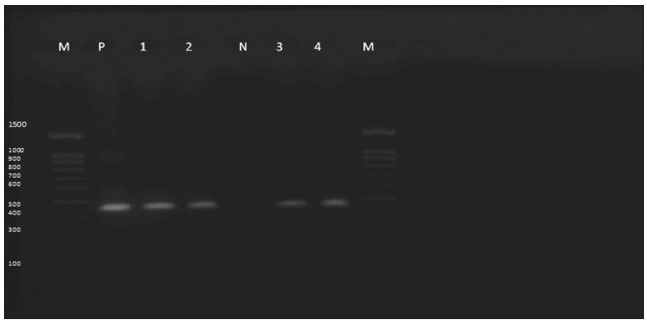

Fig. 6: Agarose gel electrophoresis of PCR- amplified *vanB* resistance gene. Lane M: 100 bp marker ladder; P: Positive control of VRE ATCC51299; samples 1,2,3, and 4 are vancomycin intermediate *Staphylococcus aureus* isolates; N: Negative control

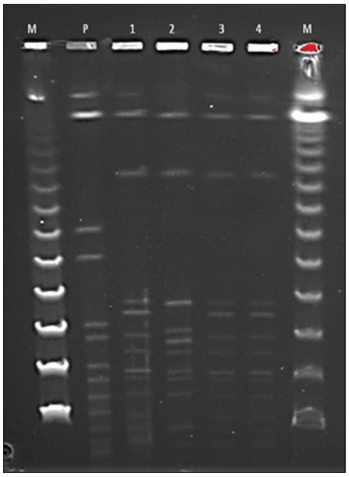

Fig. 7: Pulsed field gel electrophoresis of vancomycin intermediate *Staphylococcus aureus* isolates digested with *Sma*I. Lane M: Lambda ladder; Lane P: Positive control *Staphylococcus aureus* ATCC 25923; Lane 1: Nail isolate; Lane 2: Skin isolate; Lane 3, 4: Nasal isolate

Table 5: Prevalence of VISA as detected by MIC and E-test

Source of samples	Source (No.)	VSSA/ VISA/VRSA	Vancomycin concentration µg/ml	
			4 µg/ml	8 µg/ml
Human sources No. 143	Nasal (42)	40/02/00	1 (2.38%)	1 (2.38)
	Nail (31)	30/01/00	-	1 (3.2%)
	Skin (6)	05/01/00	-	1 (16.6)
	Clinical (64)	64/00/00	-	-
Animal sources No. 71	Nasal (16)	16/00/00	-	-
	Meat (46)	46/00/00	-	-
	Milk (9)	09/00/00	-	-

VISA: Vancomycin intermediate *S. aureus*

Table 6: Antimicrobial resistance pattern of VISA strains isolated from human specimens

Type of antibiotics	Skin	Nasal	Nasal	Nail
Clindamycin (10 µg)	S	S	S	S
Erythromycin (15 µg)	S	R	R	R
Levofloxacin (5 µg)	S	S	S	S
Methicillin (5 µg)	R	R	R	R
Penicillin (10 µg)	S	S	S	S
Trimethoprim (25 µg)	R	S	S	S
Teichoplanin (30 µg)	S	S	S	S
Ciprofloxacin (5 µg)	S	S	S	S
Amikacin (30 µg)	S	S	S	S
Gentamycin (10 µg)	S	S	S	S
Cefoxitin (30 µg)	R	R	R	R
Novbiocin (50 µg)	S	S	S	S
Tetracycline (30 µg)	S	S	S	S
Vancomycin (30 µg)	S	S	S	S
Linezolid (30 ug)	S	S	S	S
Tobramycin (10 µg)	S	S	S	S
Gentamycin (10 µg)	S	S	S	S

VISA: Vancomycin intermediate *S. aureus*

DISCUSSION

S. aureus is recognized as a cause of a wide range of infections, from minor skin infections and chronic bone infections to devastating septicemia and endocarditis [33]. The accurate identification of *Staphylococcus* species impacts directly on treatment outcomes and on the epidemiological analysis. Due to the excessive and uncontrolled use of antibiotics the organism becomes multidrug resistant leaving few therapeutic options for the treatment against it. Knowledge of selection of the antibiotics for treatment is important as antibiotic responsiveness pattern of MRSA may vary from region to region. In this study, a total of 214 isolates (18.15%) were identified as *S. aureus.* The prevalence of *S. aureus* among human was 19.35% for the nasal, 14% for nail, and 8.8% for skin. These results are in agreement with results obtained by other researchers [34,35]. Strains present in the nose often contaminate the back of hands, fingers and face and so nasal carriers can easily become skin carriers [36]. However, animal sources showed 27.3%, 5.51%, and 15.86% for milk, nasal, and meat, respectively. The biochemical tests that have been used for the primary isolation of *S. aureus* showed similar behavior concerning catalase, oxidase, coagulase, tellurite reduction, amylase, and resistance to acriflavine. However, human isolates showed an increase in their beta hemolytic activity, DNase, and protease production. These results are in agreement with Roberson *et al*., 1992 results. *S. aureus* that expresses large amounts of hemolysin, lipase, and DNase may be expected to colonize the skin and cause furuncles [37]. On the other hand, the prevalence of *S. aureus* in the meat samples could be indicative of contamination of meat from skin, mouth, and nose of butchers [38]. Although Samples of milk was obtained from sheep without any clinical mastitis and have normal mammary glands, the presence of *S. aureus* in milk samples was estimated to be 8%. This might be associated with subclinical mastitis and intramammary infection [39]. Isolates of *S. aureus* were confirmed by PCR analysis using *coa* gene primers.

MRSA infections can be very serious and are among the most frequently occurring of all antibiotic-resistant threats [40]. Our results showed that the majority of MRSA strains are associated with human isolates. This finding is supported by Boucher and Corey results, 2008. Increased prevalence of infection due to MRSA led to the use of glycopeptides for therapeutic purpose. The widespread use of vancomycin to treat infections has led to the emergence of vancomycin resistance strains. VISA appears to have developed from strains of MRSA instead of a single clone, and they have often been missed by disk diffusion testing. Such failures are widely under-reported, since most of the laboratories in the past would have considered these strains as vancomycin sensitive leading to failure with the use of antimicrobials for staphylococcal disease. Acceptable methods used to detect these strains are MIC of vancomycin and the E-test [41].

This study showed clearly the existent of VISA colonizing human nose, nails, and skin (4.76%, 3.2%, and 16.6%, respectively). Vancomycin MICs for the isolated strains ranged from 4 to 8 µg/ml. However, none of the students carrying VISA strains were on vancomycin at the time of survey. In a previous report from Jordan, a case of VISA was reported by Bakri *et al*. in a patient with erythrodermic psoriasis [12]. However, VISA has been reported from different parts of the world in which most of the reported cases were isolated from clinical samples [42]. In a study concerning nasal carriage survey for MRSA, four strains were detected with reduced susceptibility to vancomycin [18]. The isolation of VISA from nail, nasal, and skin colonization was rare, but the abused of vancomycin in our region at certain time might enhanced the presence of such strains. VISA colonizing normal human niches may be a potent source of VRSA, especially when exposed to the appropriate selective step-up pressure; these isolates may eventually take a resistant form. In addition, they may act as carriers, promoting easy transfer of drug resistance determinants. However, the complicated point is the potential for emergence of VRSA isolates from these strains during asymptomatic colonization, rather than during infection, which also might contribute to delays in the detection process [43]. Improper dosage and inadequate duration of therapy are other common causes in our country. Surveillance of populations, especially where the probability of MRSA carriage and prolonged glycopeptide therapy is high, should be regularly done by continuous monitoring of MIC levels of vancomycin.

The genetic changes that lead to the manifestation of the VISA phenotypes are varied and not easily detectible using a single gene assay. While these genetic alterations are variable, they tend to occur in regulatory genes that control cell metabolism and cell wall structure and function. Consequently, most VISA isolates share common phenotypic features such as increased cell wall thickness and slowed growth rate [44]. Increased cell wall thickness helps in resistance by sequestering vancomycin molecules in the cell wall peptidoglycan, thus reducing the susceptibility of *S. aureus* to vancomycin [10]. In this kind of cell wall structure, glycopeptides are confiscated and prevented from reaching the site of cell division. As a result, the rate of vancomycin diffusion decreases [45]. Another mechanism was identical to that seen in vancomycin-resistant *Enterococcus faecium* that harbors the *vanA* operon which contains 5 genes [46]. To determine the mechanism of acquirement of reduced vancomycin susceptibility of VISA strains, we detected the Van gene which was implicated in generation of vancomycin resistance [47]. *S. aureus* could acquire vancomycin resistance by transferring van genes which were even accomplished in the laboratory. However, none of the VISA strains appeared in previous studies showed to express any of the *van* determinants (*vanA, vanB, vanC1, vanC2,* or *vanC3*) present in VRSA [48]. In one interesting study from India including a nasal carriage survey for MRSA in an intensive care unit,

four strains with reduced susceptibility to vancomycin were reported with *vanA* gene [18]. To the best of our knowledge, this is the first report of VISA strains containing *vanB* isolated from a routine carriage survey worldwide. Resistance to vancomycin could be acquired by transferring *van* genes *in vivo*. This transfer was detected in the absence of any antibiotic pressure [49]. The absence of a selective vancomycin pressure might have resulted in reduced expression of the *vanB* resistance gene. It was well known that *vanB*-type strains have variable levels of inducible resistance to low-level vancomycin but are sensitive to teicoplanin [50]. It was indicated that recurrent MRSA infection and certain underlying illnesses might increase the risk of development of VISA [51]. It seemed that, in this case, the serious underlying illness and high chance of exposure to MRSA colonization might also contribute to VISA infection. We next examined the susceptibilities of the VISA strains to other antibiotics clinically used to treat MRSA infection. We found that the strains with intermediate resistance to vancomycin were not associated with a multidrug-resistant phenotype.

PFGE of *Sma*I restriction fragment was used as a molecular typing technique for classification and epidemiologic survey of VISA. However, two different cluster patterns were obtained. The first cluster included isolates obtained from nasal and nail suggesting that they are closely related. The second cluster carries different bands and present isolate obtained from skin. Although both clusters contained *vanB* gene, they had different antimicrobial susceptibility patterns. Cluster I was resistance to cefoxitin and erythromycin, whereas Cluster II showed resistance to cefoxitin and trimethoprim. VISA strains isolated in this study may be a potent source of VRSA with the expression of the vancomycin resistance gene. Infections due to VISA is a recipe for a full blown development into VRSA if no proper measures are put in place to control their spread.

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