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 nase, nails, and skin with vancomycin MIC of 4-8 μg/ml. Van B resistance gene was detected and PFGE with Smal-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.

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 the possibility 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

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 maintained on MSA and BPA, and incubated following introduction of the antibiotic disks at 37°C for 24 hrs [22].

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 Staphylococal 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].

About 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 Staphylococal 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 protease 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'-ACCACAGGTTACTGATAACGG-3' and 5'-GCTTTGATTGTCGATGC-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 (mer) | Primer name | Primer sequence | Melting temperature |
|----------------------|-------------|-----------------|---------------------|
| 25                   | VanA        | 5'-ATGAATTGATAGAAATTGGAATAC-3' | 62°C |
| 21                   | VanA1       | 5'-CCCTTTAAGGCTAAATAGTTACF-3' | 57°C |
| 23                   | VanB        | 5'-CCTAATTGACCTTTGAAAAA-3' | 59°C |
| 18                   | VanB1       | 5'-GGGATCTCTGTGGAAA-3' | 59°C |
| 23                   | VanC        | 5'-GCTGAAATATGAAGTAATGACCA-3' | 58°C |
| 21                   | VanC1       | 5'-GCCCAGTGCGTTGATATTGGT-3' | 58°C |

18 hr. The following discs were used; clindamycin (2 µg), erythromycin (15 µg), gentamycin (10 µg), methicillin (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 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 MIC of ≤2 µg/ml, intermediate at a vancomycin MIC of 2 to 8 µg/ml, and resistant at a vancomycin MIC of ≥16 µg/ml [28].

Vancomycin E-test
The conventional vancomycin E-test strips (bioMérieux, 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 primer and PCR conditions. The resulting profiles were visualized by staining with ethidium bromide. The strains varied with only 2 or 3 bands were considered closely related [32]. S. aureus ATCC 29213 was used as a control.

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 (11.5%) 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, manitol 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- hemolysis; 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- hemolysis; 81.25%, 86.9%, 89.1%, and 80.6% for DNase.

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

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**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. (%) |
|-------------|----------------|-------------------|-------------------|---------------|
| Human sources | Nail | 217 | 42 (19.35) | 143 (25.26%) |
|              | Skin  | 68  | 6 (8.82)  | 66.7 (18.15) |
| Animal sources | Milk | 33  | 9 (27.27)  |               |
|              | Meat  | 290 | 16 (5.52)  | 71 (11.58)   |
| Total        |       | 1119 | 214 (18.15) |               |

*S. aureus: Staphylococcus aureus*

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**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 0        | 6.3 0 93.7         | 16.7 0 83.3   |
| Gentamicin (10 µg) | 92.3 0 7.69     | 100 0 0        | 5.4 0 94.5         | 33.3 0 66.7   |
| Methicillin (10 µg) | 9.5 0 90.5 6.5  | 0 93.5 0        | 6.3 0 93.7         | 0 0 100       |
| Penicillin (10 µg) | 7.6 0 92.3 6.25  | 0 93.75 0       | 10.9 0 89          | 16.7 0 83.3   |
| Amikacin (30 µg) | 97.6 0 2.4 100  | 0 0 0          | 98.4 0 1.6         | 100 0 0       |
| Carfotin (30 µg) | 9.5 0 90.5 9.7  | 0 90.3 0        | 9.4 4.6 86         | 0 0 100       |
| Novobiocin (50 µg) | 95.2 0 4.8 100  | 0 0 0          | 93.7 0 6.2         | 100 0 0       |
| Tetracycline (30 µg)| 84.6 0 15.4 75   | 62.5 18.75 0    | 65.7 1.36 32.8     | 100 0 0       |
| Vancomycin (30 µg) | 97.6 2.4 0 100 | 0 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   |
| Telcoparin (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 1.6 96.8 50 | 0 0 50       |
| Tobramycin (10 µg) | 4.8 0 95.2 100  | 0 0 0        | 3.2 3.2 94         | 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       |

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**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 10     | 73.1 12.19 14.63 | 50 0 50 |
| Erythromycin (15 µg)| 18.7 0 81.3      | 13.1 0 86.9 0   | 0 0 100       |
| Gentamicin (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     | 889 0 11.1    |
| Cefotin (30 µg) | 93.7 0 6.3       | 95.6 0 4.4      | 33.3 0 66.7   |
| Novobiocin (50 µg) | 100 0 0          | 86.9 2.1 11     | 100 0 0       |
| Tetracycline (30 µg)| 66.6 20 13.3     | 31.7 17.0 51.21 | 66.7 0 33.3   |
| Vancomycin (30 µg )| 100 0 0          | 100 0 0        | 100 0 0       |
| Linezolid (30µg) | 87.5 0 12.5      | 86.9 2.1 10.9   | 55.5 11.1 33.3 |
| Telcoparin (30 µg) | 78.5 0 12.5      | 97.8 2.8 0      | 889 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     | 889 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 Smal-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).
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. 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 MIC for the isolated strains ranged from 4 to 8 µg/ml. However, 46.6% 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 erythrodernmic 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 enhance the presence of such strains. VISA colonizing normal human niches may be a potent source of VISA, 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 VISA 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 detectable 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 conjugated 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 which harbors the vanA operon which contains 5 genes [46]. To determine the mechanism of acquisition 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 genes (vanA, vanB, vanC1, vanC2, or vanC3) present in VISA [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 [9]. The absence of a selective vancomycin pressure might have resulted in reduced expression of the $vanB$ resistance gene. It was well known that $vanA$-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 Smal 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 $vanA$ 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 VISA with the expression of the vancomycin resistance gene. Infections due to VISA is a recipe for a full blown development into VISA if no proper measures are put in place to control their spread.

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