Original article

Emergence of vancomycin-intermediate resistant *Staphylococcus aureus* in north of Palestine

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Abstract

Objective: This study was conducted to update the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates among human clinical *S. aureus* isolates recovered from Northern Palestine, to evaluate the possible presence of vancomycin-Resistant *S. aureus* (VRSA) and vancomycin- intermediate resistant *S. aureus* strains (VISA) and to determine the antimicrobial susceptibilities of these clinical isolates. **Methods**: The *in vitro* activities of 11 antibiotics against 204 non-duplicate *S. aureus* isolates from clinical samples in North of Palestine were determined by the disk-diffusion method. These samples were isolated between June 2006 and December 2007. The minimum inhibitory concentration (MIC) of vancomycin for 115 methicillin resistant *Staphylococcus aureus* (MRSA) strains was carried out using the agar dilution method. **Results**: One hundred and fifteen (56.4 %) of these isolates were MRSA and according to their antibiotic profile these are multidrug resistant (resistant to three or more non-β-lactam antibiotics). Ninety nine (43.6 %) isolates were methicillin sensitive *S. aureus* (MSSA), forty four of MSSA isolates (44.4 %) were multidrug resistant, while forty five (45.6 %) were non multidrug resistant. Our results showed that the most common resistance (95.6 %) was to penicillin. Two strains of MRSA have shown to be vancomycin- intermediate resistant, had MIC of 4 μg/mL and 8 μg/mL and these vancomycin- intermediate resistant *S. aureus* strains (VISA) are resistant to all antibiotics tested. **Conclusion**: According to our information this is the first study report about VISA in Palestine.

Keywords: Methicillin resistant *Staphylococcus aureus* (MRSA); Vancomycin- intermediate resistant *Staphylococcus aureus* (VISA); *Staphylococcus aureus*; Multidrug resistant *Staphylococcus aureus*; Palestine

INTRODUCTION

Staphylococcus aureus is a major human pathogen responsible for serious community-acquired and nosocomial infections worldwide. It is considered as one of the most important pathogen because of both the diversity and the severity of the infections it causes, including endocarditis, and bacteremia, as well as a variety of toxin-mediated diseases such as gastroenteritis, staphylococcal scalded-skin syndrome, and toxic shock syndrome[1].

Indeed, over 90 % *S. aureus* strains are resistant to penicillin[2]. Currently one of the most serious aspects is concerned, resistance to methicillin, and essentially resistance to all other beta-lactam antibiotics. Epidemic strains are more prevalent and can spread within or be-

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tween hospitals and between countries. Methicillin-resistant *S. aureus* (MRSA) isolates are genetically heterogeneous[3], and conferred resistance by carriage of the *mecA* gene encoding an alternate penicillin binding protein (PBP2). This gene is located on a genetic element called the staphylococcal cassette chromosome mec (SCC*mec*)[4]. Once *mecA* gene introduced into a microbial population, it may be transferred horizontally and recombined among methicillin-sensitive *S. aureus* (MSSA) cells. This has led to the global spread of MRSA in association with increasing geographic mobility of infected patients and carriers.

Infections caused by MRSA are associated with significant adverse outcomes and higher health care costs than infections caused by MSSA[5]. In addition to that, MRSA infections are associated with significant morbidity and mortality, especially in patients with bacteremia[5,6].

The increased incidence of MRSA has led to more frequent use of vancomycin in early 1990s, the drug commonly relied on for treating MRSA infections. As a consequence, selective pressure was established that eventually led to the emergence of strains of *S. aureus* with decreased susceptibility to vancomycin. The first report on an infection with *S. aureus* exhibiting reduced susceptibility to glycopeptides came from Japan in 1997[7], and that decreased susceptibility to vancomycin became a clinical reality. The first two clinical Vancomycin-Resistant *S. aureus* (VRSA) isolates were reported in 2002 from USA[8,9].

Microbiological and epidemiological studies are of crucial importance due to the growing incidence of MRSA infections worldwide, their multidrug resistance, several reservoirs of resistant strains, facility to spread outside hospitals and to cause outbreaks requires efficacious infection control measures. This study was designed to update the prevalence of MRSA isolates among human clinical *S. aureus* isolates recovered from Northern Palestine, to evaluate the possible presence of VRSA and vancomycin- intermediate resistant *S. aureus* strains (VISA) and to determine the antimicrobial susceptibilities of these clinical isolates.

MATERIALS AND METHODS

Bacterial strains and identification

A total of 204 *S. aureus* were investigated for the period between June 2006 and December 2007. The strains were collected from various clinical specimens including pus, urine, wound, surgical infection, ear, diabetic foot, vaginal swabs, blood, sputum, and semen from the patients of different inpatient and outpatient of hospitals and from some private medical laboratories in the North of Palestine. All isolates were identified by routine laboratory procedures in microbiology laboratories of An-Najah National University, Palestine, using Gram stain, culture properties on nutrient agar and mannitol salt agar, detection of hemolysis on 5 % sheep blood agar, and coagulase reaction.

Antimicrobial susceptibility testing

S. aureus strains were tested for antibiotic resistance using the disc diffusion method[10]. Antibiotic disks (Oxoid) used were penicillin G (10 U), Gentamicin (10 μg), Tetracycline (15 μg), Norfloxacin(10μg), sulfonamides compound (300 μg), kanamycin(30μg), erythromycin (15 μg), nalidixic acid (30 μg), oxacillin (1 μg), amoxicillin-clavulanic acid(30μg) and methicillin (5 μg). Inhibition zones were determined in accordance with procedures of the Clinical and Laboratory Standards Institute (formerly, the NCCLS)[11], isolates were categorized as susceptible and resistant. According to methicillin and oxacillin, *S. aureus* isolates considered susceptible if inhibition zones are ≥14 mm and ≥13 mm, respectively. MRSA ATTC 43300 and MSSA ATCC29213 were included in this assessment as references.

Assessment of VISA by agar dilution method

All MRSA strains isolated between June 2006 and December 2007 were screened for VISA using Mueller-Hinton agar containing 4 μg of vancomycin per mL (MH-V4). MRSA strains grew on MH-V4 were subjected to determine minimal inhibitory concentration (MIC) testing against vancomycin. MIC of vancomycin was determined by agar dilution method recommended by the Clinical and Laboratory Standards Institute[12]. Briefly, Vancomycin (Sigma) was incorporated into Mueller-Hinton agar plates in serial dilution from 32 μg/mL to a final concentration 4 μg/mL using twofold dilutions. By direct colony suspension method 0.5 McFarland equivalent inoculum were prepared in normal saline from 18-24 h agar plate culture. 10 μL of bacterial suspension (inoculum size of 10^5 CFU/spot) were

inoculated on Mueller-Hinton agar containing vancomycin. The inoculated plates were incubated overnight at 35 ℃ for any visible growth. Strains that showed MIC 4-8 μg/mL were considered VISA[13].

DNA extraction

Staphylococcal DNA was isolated from about 10 colonies of the bacteria as described previously[14]. Bacterial cells were washed once with 1.0 mL of 0.02 M sodium phosphate ($Na_2HPO_4.2H_2O$) pH 7.4 in 0.9 % NaCl and centrifugation at 12 000 × g for 10 min. The pellet was resuspended in 200 μL of lysis buffere (1 mM EDTA, 10 mM Tris-chloride, pH8) with 12 U lysostaphin (Sigma) and incubated for 45-60 min at 37℃. Then 4.5 U of proteinase K (MO BIO) were added and incubated for 45 min at 60 ℃, then for 10 min at 95 ℃. The total DNA was spinned at 12 000 × g for 15 s. and kept at −20 ℃ for DNA amplification. Enterococcus faecium ATCC 51559 is included in this assessment as reference.

Detection of *vanA* and *vanB* genes by PCR

PCR assay was carried out using oligonucleotide primers for vanA (A1 5′-ATG AAT AGA ATA AAA GTT GCA ATA C-3′ and A2 5′ -CCC CTT TAA CGC TAA TAC GAT-3′) and vanB (B1 5′-CCC GAA TTT CAA ATG ATT GAA AA-3′ and B2 5′-CGC CAT CCT CCT GCA AAA-3′) genes as described previously with some modification[15]. For PCR amplification, the reaction mixture (50 μL) included 75 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate (PeQLab), 1 × PCR reaction buffer (PeQLab), 2 mM $MgCl_2$ (PeQLab), and 1.25 U of Taq DNA polymerase (PeQLab), and finally 5 μL of DNA template was added to each 0.2 mL reaction tube. The amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf) with the PCR program consisted of an initial denaturation step at 94 ℃ for 5 min; this was followed by 30 cycles of DNA denaturation at 94 ℃ for 60 s, primer annealing at 62 ℃ and 59 ℃ for 50 s for *Van A* and *Van B* primer, respectively, and DNA extension at 72 ℃ for 90 s. After the last cycle, the reaction was terminated by incubation at 72 ℃ for 5 min. The PCR products were separated by electrophoresis in 1.0 % agarose gel and stained with ethidium bromide. Enterococcus faecium ATCC 51559 and Enterococcus faecalis ATCC 51299 were used vanA and vanB positive control strains respectively.

RESULTS

One hundred and fifteen (56.4 %) *S. aureus* isolates were MRSA and according to their antibiotic profile these are multidrug resistant isolates (resistant to three or more non-β-lactam antibiotics). Ninety nine (43.6 %) isolates were MSSA, forty four of MSSA isolates (44.4 %) were multidrug resistant MSSA, while forty five (45.6 %) were non multidrug resistant MSSA. Using the disk diffusion method, our results showed that most isolates (95.6 %) were resistance to penicillin. Two strains of MRSA have shown to be vancomycin- intermediate resistant, had MIC 4 and 8 μg/mL and these VISA are resistant to all antibiotics tested. However, none of theses two VISA strains could demonstrate the presence of *vanA* and or *vanB* gene by PCR These strains were isolated from diabetic foot swab and urine sample of patients visiting outpatient departments.

DISCUSSION

The results of this study indicated that methicillin resistance has become a serious problem in Palestine as well as in other countries. MRSA is currently the most commonly identified antibiotic-resistant pathogen in hospitals in many parts of the world, including Europe, the Americas, North Africa, and the Middle-and Far-East[16]. The prevalence of MRSA in human infections reported for countries such as USA, southern Europe, northern Europe, Iran, Korea, Japan, Pakistan and Israel has ranged from <1 % - 60 % [16-25]. In 1998/1999 the prevalence of MRAS among *S. aureus* isolated from clinical isolates from North Palestine was 8.7 %[26]. Our results showed high prevalence of MRSA, which is higher than previous report 6.5 folds. The prevalence of MRSA is believed to be increasing internationally. In the United States, MRSA prevalence among all hospital *S. aureus* isolates has increased from 2.4 % in 1975 to 29 % in 1991 [27]. Between 1992 and 2003, the proportion of *S. aureus* isolates from patients in intensive care units that were methicillin-resistant rose from 35.9 % to 64.4 % [28]. In England and Wales, the proportion of *S. aureus* bacteraemia due to MRSA increased from 1 % to 2 % in 1990-1992 to ap-

proximately 40 % in 2000 [29].

MRSA is typically resistant to multiple classes of antibiotics including aminoglycosides, Macrolides, chloramphenicol, tetracycline, and fluoroquinolones [30]. Therefore, treatment options for the management of serious MRSA infections are limited. Glycopeptides have historically been the drugs of choice for the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, the continued selective pressure has led to the emergence of non-susceptible strains including vancomycin- intermediate resistant *S. aureus* strain and this is of great concern. Several research groups have addressed the prevalence VISA[31-36], and the frequency of VISA in these publication is vary. This may be due to that in most of these publication surveillance was not performed routinely, the historical isolates were analyzed together with current ones, the sample size was small, and methods for identifying VISA differed between studies. This result is in contrast to our previous report which showed that all MRSA were vancomycin susceptible[26]. Higher rates of MRSA in Palestine and a concomitant increase in the use of vancomycin would increase emergence of glycopeptide resistance. These two patients had been treated with vancomycin for a long time before the resistant strains emerged. The absence of *vanA/B* genes in the present isolates dose not rule out that these strains are not VISA (36-37). These findings suggested that, with prolonged vancomycin exposure, VISA organisms produce a cellular modification including a thickened cell wall matrix, limiting drug penetration under antibiotic pressure rather than by acquisition of a resistance gene from other strains, such as vancomycin-resistant enterococci.

In conclusion, our study confirms the high prevalence of MRSA in the North of Palestine and this is the first study report about VISA in North of Palestine. The emergence of VISA might also be prevalent in other parts of Palestine as antibiotic misuse is equally common there. Nationwide surveillance program should be carried out to map the vancomycin susceptibility pattern in this country. The current vancomycin resistant staphylococci in hospitals as well as in community are alarming situation to the clinicians. Hence, there should be an immediate response from the concerned authorities to check further emergence and spreading of these strains. A strict regulation on irrational antibiotic usages might be an appropriate and effective approach in this direction and enhancing the specificity of the routine laboratory identification of MRSA, VRSA, and VISA is important in hospitals with a high prevalence of this organism.

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