International Journal of Molecular and Clinical Microbiology 6(2) (2016) 693-699

International Journal of
Molecular and Clinical Microbiology

A Study on the Antimicrobial Effect of Zinc Oxide Nanoparticles on Clinical Strains of *Staphylococcus aureus* Resistant to Vancomycin

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ARTICLE INFO

Article history:
Received 25 august 2016
Accepted 19 october 2016
Available online 1 December 2016

Keywords:
Staphylococcus aureus,
Vancomycin,
Zinc oxide, Nanoparticle,
Drug resistance

ABSTRACT

Staphylococcus aureus, as one of the main agentsfor hospital infections,is considered as highly important because they show resistance to a wide range of antibiotics. Resistance to selective antibiotics such as vancomycin is a serious problemin the medical community; thus it seems rational to use alternative substances for treating these bacteria. The aim of this study is to investigate the prevalence of resistance to vancomycin in clinical isolates of *S. aureus* as well as the antimicrobial effects of zinc oxide nanoparticles on them. In this study, 70 samples of wound, boil, abscess and urine were isolated.*Staphylococcus aureus* strains resistant to vancomycin were then identified through routine laboratory tests using Broth Micro dilution test. The antibacterial effect of ZnO nanoparticles (20 nanometer) was investigated at concentrations of 100, 50, 25, 12.5 and 6.25 mg/ml using agar well diffusion method over strains resistant to vancomycin. From the total of 70 samples, 30 samples were identified as *S. aureus* out of which 23.3% showed resistance to vancomycin. During this study it was found that ZnO nanoparticles in concentrations of 50 and 100 mg/ml have a good antibacterial effect and can be a good alternative for controlling *S. aureus* resistant to vancomycin. Considering the increasing trend in drug resistance, the growth of pathogenic bacteria can be inhibited by increasing the concentration of zinc oxide nanoparticles.

1. Introduction

Staphylococcus aureus is the second most important agent causing hospital infections after *Pseudomonas aeruginosa*. This community-acquired agenthas now turned to be one of the major health problems in the world due to its virulence potential and increasing resistance to antibacterial agents. Moreover, these bacteria cause many serious infections such as septicemia (blood poisoning), endocarditis and osteomyelitis in hospitalized patients, and are among common causes of mortality in patients undergoing dialysis (CDC-VISA-VRSA; Kluytmans et al., 2008).

In addition to being widely distributed in the nature, *S. aureus* also inhabits human body and is found on the skin and in the mucosa of healthy persons'bodies. Many people (20%–40%) also carry the bacteria in the anterior part of the nasal cavity. In some hospitals, these bacteria are more prevalentin the ICU ward, causing mortality in patients (Ostopark et al., 2006).

Methicillin is one of the penicillinase-resistant penicillins which was introduced in 1960.It was only a year after that the first case of drug-resistant *S. aureus* (MRSA) was detected.

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Vancomycin is a glycopeptide drug naturally produced by *Streptomyces orientalis*. However, today another form of resistanceto vancomycin is seen in *S. aureus* strains which is formed through a different mechanism. Vancomycin is the most important drug of choice for treating infections caused by MRSA strains. Therefore, existence of intermediate strains resistant to vancomycin should be regarded as a warning for the medical community and treatment. Ancestral strains of *S. aureus* resistant to vancomycin, known as hVISA (hetro VRSA) were first reported in Japan. Upon using vancomycin in their treatment, these strains may also become resistant to Vancomycin (Hiramatsu et al., 1997).

Some studies have reported vancomycin as a stimulating factor for production of resistant strains. VRSA and VISA strains grow slower and have thicker cell walls compared to sensitive strains. It seems that the thickness of the cell wall leads to the increase of MIC in VRSA and VISAspecies (Liu et al., 2003; Sancak et al., 2005).

Zinc oxide (ZnO) nanoparticles have selective toxicity and can be used as antibacterial substances with an ideal potential to replace some antibiotics and can even affect the spores that are resistant to temperature and pressure.These compounds are highly active against bacteria and can be used as an antimicrobial agent. These nanoparticles destroy bacteria walls, and are effective in prevention of antimicrobial activities through the antimicrobial activity of ZnO coating materials over prosthesis and catheter surfaces. So far, several mechanisms have been proposed for the antimicrobial behavior of ZnO nanoparticles, such as the release of metal ions, generating active oxygen, hydrogen peroxide and protein leakage (Livin et al., 2007; Wang et al., 2012).

This study aims to investigate the antimicrobial activity of ZnO nanoparticles on clinical isolates of *S. aureus* resistant to vancomycin.

2. Materials and Methods

2.1. Collecting S. aureus samples

This descriptive cross-sectional study was conducted in three hospitals and 70 samples of urine, wounds, boilsand abscess were separated. The samples were cultured on Mannitol salt agar medium and were then incubated at 35°C for 24–48 hours. The yellow colonies (fermenter of Mannitol and suspected to contain *S. aureus*) were cultured on nutrient agar mediumfor the purpose of next tests.

According to the instruction by Bergey, *S.aureus* is detected according to colony morphology tests inclusing Gram stain, hemolysis, catalase, slide and tube coagulase and DNase.

2.2. *Determining sensitivity to vancomycin using Broth Microdilution method*

In this study, in order to reach the target drug concentration, 10 µl of vancomycin solution (produced by SigmaCo.) with final concentration of 10,000 µg/l were added to 90 µl of Mueller Hinton Broth containing sodium chloride. The final concentration of 64 µg/ml was achieved upon addition of 4.6 µl of the said solution to 93.6 µl of Mueller Hinton Broth (produced by Merck Co). Then, upon using 96-well microplate wells, 50 µl of Mueller Hinton Broth was poured into the first well added by 50 µl of the concentration of 64 µg, and dilution was then performedin all wells. Accordingto the CLSI, the final concentration ofbacteria in each well should be 5×10^5µl/ml. With the addition of 50 µl of bacterial suspension with the final concentration of 5×10^6µl/ml to each well (containing 50 µl of culture medium with antibiotics), the final concentration of bacteria will reach 5×10^5µl/ml. Finally microplates were incubated at 37°C for 24 hours. After incubation, the first well without turbidity was set as the MIC (minimum inhibitory concentration of growth), and the results of sensitivity and resistance to vancomycin were reported based on CLSI guidelines (CLSI, 2008). In this method, the standard *S. aureus* strains of ATCC25923 were used.

2.3. Determining the antibacterial effect of ZnO nanoparticles

To prepare suspensions of ZnO nanoparticles, 0.2g of powder (purchased from Iranian Nano-materials Pioneers Company) with thesize of 20 nmwas poured in a sterile tube containing 2 ml of distilled water and dimethyl sulfoxide 20%, and a shaker machine was used for 30 minutes to dispersethem well (Fig.1).

From theprimary concentration of ZnO nanoparticles (100 mg/ml), serial dilutions with concentrations of 50, 25, 12.5 and 6.25 mg/ml were prepared in thetube. Then, bacterial suspension of *S. aureus* strains resistant to vancomycin, equivalent to 0.5 McFarland, was prepared, and upon diluting it with the ratio of 1 to 100, a suspension with concentration of $1/5\times10^6$ bacteria/ml was produced.

To investigate the antimicrobial effect of ZnO nanoparticles using Agar Well Diffusion method, first a bacterial suspension was cultured on Mueller Hinton agar medium containing NaCl (Merck Co.) by means of swaps.Then, by using the Pasteur pipette on Mueller Hinton agar medium, 6 wells with a diameter of 6 mm were created. Afterwards, 100µl ofeach ZnO nanoparticle concentration (with concentrations of 100, 50, 25, 12.5 and 6.25 mg/ml) were added to each well. Well No.6, which contained distilled water, was used as the negative control.The plates were finally incubated for 24 hours at 37°C and were evaluated after 24 hours in terms of creation inhibition zone.

Each stage of the test was repeated three times. To verify the existence of significant differences in the results, variance analysis and Chi-square test were applied and the significance level was considered at P<0.001.

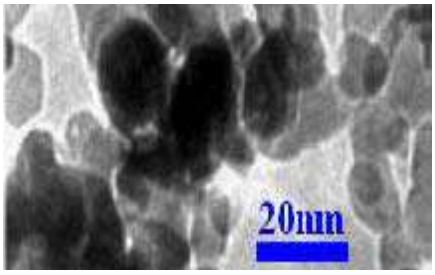

Fig.1. Transmission Electron Microscopy Image of Zinc Oxide Nanoparticles

3. Results

From the total 70 clinical samples, 30 samples (42.85% of samples) were identified and isolated as *S. aureus* of all samples, abscess (36.7%) and urine (10%) were the most and least clinical forms of samples respectively in this study (Table1). The samples were then evaluated using Broth Micro dilution method to determine their sensitivity to vancomycin. The MIC range obtained for Vancomycin compared to the range of reference strain effect (the control strain) was 0.5–4 µg/ml, and 63.3% of *S. aureus* were in this MIC range.The vancomycin with MIC of 32 µg/ml showed the best inhibitory effect. 7 samples (23.3% of all samples) in the dilution of 1.5×10^5 cell/ml, showed the MIC range of 16–32µg/ml and were considered as VRSA (Fig.2).

Table 1: Distribution of absolute and relative abundance *S.aureus* strainsisolated from Patients

Relative abundance	Absolute abundance	Clinical forms
20%	6	Boils
33.3%	10	Wound
36.7%	11	Abscess
10 %	3	Urine
100	30	Total

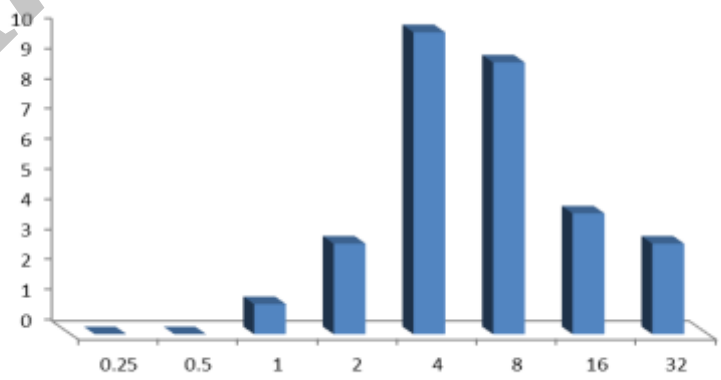

Fig. 2: Sucseptibility testing of 30 clinical isolates to vancomycin by Microdilution Broth test

In this study, upon assessing the antimicrobial effect of ZnO nanoparticles on vancomycin resistant *S. aureus* strains, the antibacterial activity of the same was confirmed.

The results of evaluating ZnO nanoparticleswith concentration of 100, 50, 25, 12.5 and 6.25mg/mlusing Agar Well Diffusion method which was carried out through variance analysis test showed a significant relationship between the concentration of ZnO nanoparticles with inhibition zone diameter ($P<0.001$). In other words, the antimicrobial effects of ZnO nanoparticles are dose-dependent (Fig.3).

In this study, the best and most effective dose of nanoparticles were 100 and 50mg/ml (Fig.4.).

Figure 3. Antibacterial activity of Zno (20nm) against *S. aureus* in different concenterations (mg/ml)

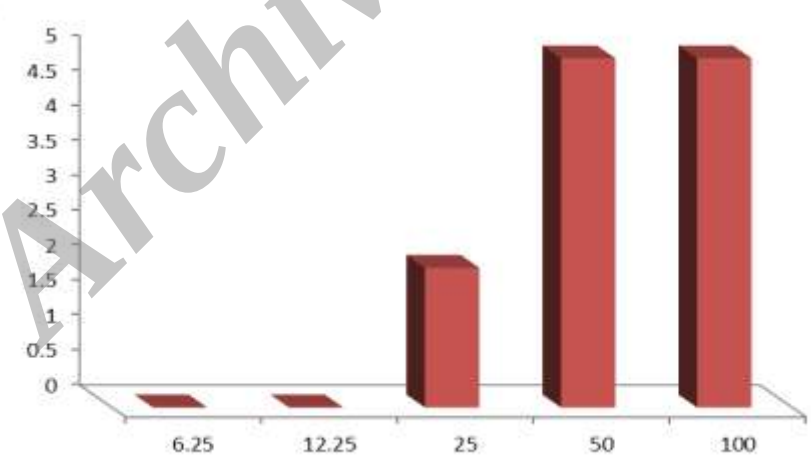

Fig 4. Antimicrobial activity of different ZnO Nanoparticle concentrations against Strains of *S. aureus* resistant to vancomycin

4. Discussion

Today, due to the increasing use of antibiotics, we are facing a dramatic growth in resistance to antibiotics. In Iran, too, antibiotic resistance among pathogenic bacteria has become an important challenge for the medical community in the treatment of infectious

diseases. Previous studies have shown that gram-positive coccisuch as coagulase negative staphylococci, *S. aureus* and Enterococci are amongst the main causes of infection in hospitals (Moza et al., 2007).

Vancomycin is the antibiotic that is greatly used in hospital environments, mainlyto treat *S.aureus* resistant to methicillin. Upon reporting and spreading cases ofVRSA, treatment problem of MRSA is raised; and although a few cases of *S. aureus* resistant to vancomycinhave so far been reported, it is likely that more cases be seen in near future.

Various studies have been carried out worldwide on the prevalence of *S. aureus* resistant to vancomycin. The most cases of VRSA were reported in the United States (Michigan 1997, New Jersey 1997, New York 1998, Illinois1999, Minota 2000, Maryland 2000, and Ohio 2000). In this study, 7 cases of VRSA and 4 cases of VISA were isolated (CDC-VISA-VRSA). Moradi et al studied on the clinical stains of *S. aureus* and observed in 3.8% resistant of VISA form (Moradi et al., 2011).

In their study, Tiwari et al reported 6 VISA strains (Tiwari et al., 2009) which is consistent with the results of the present study to some extent.

Naderi Nasab et al reported 4 cases of MIC in Imam Reza Hospital's burn patients and 1 case of MIC, equal to 12.5 µg/ml, in Ghaem Hospitalin Mashhad (Naderi Nasab et al., 2004).

Shajari et al studied on drug resistance of 76 *Staphylococcus aureus* strains isolated from patients that referred to Kashan's central Laboratory .They confirmed the highest range of drug resistance respectively Oxacilin (96.1%), Cloxacilin (63.2%), Cephalotin (23.7%), Vancomycin (18.4%) which almost consistent with our results about vancomycin resistance (Shajari et al., 2002).

Tabarraie et al studied 1193 healthy primary school students in Gorgan and found out that 194 cases (equal to 16.3%) are carriers of *S.aureus*. They reported 1.7% of the strains as resistant to Vancomyc in (Tabbarai et al., 2001). while in the presentstudy 23.3% of strains were resistant to vancomycin.

Ghana'at and Sadeghian showed that resistance of hospital strains especially *S. aureus* to various drugs is growing (Ghanaat and Sadeghian, 2001).

Leonard et al conducted a study on the effect of vancomycinon *S. aureus* in the US, and reported the MIC of vancomycin at 0.25–2 µg/ml (Leonard et al., 2008) while the present study has reported 11 strains with MIC $\leq$ 2 µg/ml.

In the study carried out by Safari et al in Kashanon *S. aureus* resistance to vancomycin, the MIC of Vancomycin was reported between 0.5–4µg/ml (Safari et al., 2010) while in the present study, the highest rate was MIC $\leq$ 2 µg/ml.

Zhang et al examined various factors such as the size and concentration of ZnO nanoparticles over the impact of antimicrobial against *Escherichia coli*. They concluded that the concentration of nanoparticles plays a more important role than their size, and the antimicrobial effect increases asthe concentration of nanoparticles increases (Zhang et al., 2007). In the present study, the best inhibitory effect was observed by increasing nanoparticle concentration to an amount between 50 to 100 µg/ml.

Ramani et al in India examined antibacterial effect of several zinc oxide nanoparticles with different structures on four gram positive and gram negative bacterial strains. They confirmed that spherical zinc oxide nanoparticles have antibacterial effect better than others (Ramani et al., 2012).

Sinha et al investigated the antimicrobial effects of ZnO nanoparticles and came to the conclusion that the gram-negative Enterobacterspecies are more sensitive in comparison to gram-positive *Bacillus subtilis* nanoparticles (Sinha et al., 2011).

Li et al studied the antimicrobial effect of ZnO nanoparticles coated on the polyvinyl chloride film overgram-positive *S. aureus* and *E.coli* bacteria, and reported that ZnO nanoparticles are more effective against gram-positive bacteria than against gram-negative ones (Li et al., 2009).

According to studies and reports by Makhlouf et al perforation of the bacteria wall by metal oxide nanoparticles and entry of nanoparticles into cells could beinvolved in the antimicrobial effect it has over the bacteria (Makhluf et al., 2005).

Humberto et al studied the inhibitory effect of silver nanoparticles on bacteria which show great drug resistance, and found out that silver

nanoparticles have a considerable bacteriostatic effect on the bacteria (Humberto et al., 2010), which is in line with the results of the effects of ZnO nanoparticles in this study on *S. aureus.*

Due to the increase in *S. aureus* strains resistant to vancomycin in the world, *Staphylococcus aureus* isolated from patients, especially hospitalized patients and medical staff, should be precisely controlled in terms of resistance and sensitivity to vancomycin.

VISA and VRSA are pathogens that have the potential for prevalence. As resistance to vancomycin is growing in our country, accurate methods for determining vancomycin resistance should be provided in clinical laboratories, and laboratory staff should be trained in this regard and be informed on the importance of the issue.

The increased use of dialysis, complex surgeries and medical methods that are associated with theuse of Vancomycin have made there search on VISA and VRSA strains a state of emergency. There are reports on the rapid spread of resistant strains in hospitals; thus, in order to prevent and control, physicians and infection specialists are recommended to consider the importance of identifying VSSA, VISA and VRSA in infections caused by *S.aureus*, and try to examine patients from this point of view, and then treat them upon performing sensitivity identification tests on the bacteria isolated from patients.Furthermore, researches should be conducted based on rapid determination of VISA and VRSA infections, monitoring VISA and VRSA isolates and elaborating on the use of new and old antimicrobial agents in controlling VISA and VRSA infections.

Acknowledgments

This work was supported by the Research Council of the Islamic Azad University, Gorgan Branch, Iran.We wish to thank the Department of Medical Microbiology, Shahid Beheshti University for supplying the clinical isolates of *S. aureus.*

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