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ORIGINAL PAPER

Gold nanoparticles: An offer to control of vancomycin-resistant enterococci in wastewater

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ABSTRACT

Industrial wastewater is one of the most dangerous and important sources of bacterial pathogens. This study aimed to determine the frequency of vancomycin-resistant enterococci (VRE) in samples taken from wastewater plants of Golestan Province, Iran, and evaluate the antimicrobial effect of gold nanoparticles (AuNPs) in combination with vancomycin on the growth of isolates resistant to vancomycin. Samples were taken from three plants in Gorgan, Kordkuy and Bandar Torkaman. Enterococcal species were identified based on the most probable number (MPN), filtration, microbiological and biochemical tests. Susceptibility to six antibiotics with monitoring of vancomycin was investigated using the Kirby-Bauer method, according to the CLSI-2015 guidelines. The antibacterial effect of AuNPs was evaluated using agar well diffusion method. More than 60% of wastewater samples were positive for enterococcal species, 65% of which were found in raw effluent, while the remaining 35% were found in the treated effluent. Based on the results, 88.2% of the isolates were resistant to ampicillin. The frequency of vancomycin-resistant enterococci was 47.1%. Our findings indicate the presence of multi-drug resistant enterococci and high rate of vancomycin resistance in wastewater samples from Golestan Province, Iran. Results show good antibacterial effects of AuNPs in combination with vancomycin in high densities against all the drug-resistant enterococci strains.

Keywords: *Enterococci*, Wastewater, Gold nanoparticles, Vancomycin

Introduction

Sewage is a suitable place for the growth of harmful pathogenic microorganisms with multidrug resistance genes. The spread of these microorganisms in nature endangers public health. Although enterococci are part of the normal flora of the human gastrointestinal tract, as opportunistic pathogens, they can cause urinary tract infection, bacteremia, septicemia and intra-abdominal infection (IAI). *Enterococcus faecalis* and *Enterococcus faecium* are two important coexisting species of faecal enterococci.[1,2]

When drought conditions put pressure on water resources, the use of treated wastewater and recycled water is increased for applications such as landscaping, irrigation and artificial groundwater recharge.[3] Those who use or come in contact with recycled water during these processes are more likely to be exposed to bacteria that may survive in recycled water sources. Recent reports indicate that the rate of antibiotic-resistant enterococci has increased in the last two decades, especially vancomycin-resistant enterococci strains.[4, 5] These resistant bacterial strains are also found in environmental samples, including sewage.[6] Therefore, antibiotic-resistant enterococci can be found in wastewater treatment plants, hospital wastewater, raw municipal sewage,[7, 8] surface waters, coastal waters, and treated wastewater

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used for irrigation.[9-12] In addition to their ability to cause severe infections, VRE strains are important public health concerns due to their inherent ability to transfer drug resistance genes.[13, 14] The spread of vancomycin-resistance genes has been observed not only among enterococcal species but also among other types of bacteria, including Staphylococcus aureus.[15] On the other hand, the increasing rate of antibiotic resistance indicates the need for the discovery of novel antibacterial agents, such as nanoparticles. Nanotechnology, as a hotspot in the science of materials, is used increasingly in researches and applications related to human health. Gold nanoparticles (AuNPs) have selective toxicity and could be used as a high potential antimicrobial agent instead of some antibiotics. AuNPs are effective in preventing antimicrobial activities on prosthesis and catheter surfaces due to the antimicrobial activities of gold coating materials. In marine environments, the stability of AuNPs in high ionic strength media is highly dependent on the surface coating.[16] Antimicrobial properties of AuNPs increase with their size. Its mechanism of action is size- and dose-dependent so that at a size of 6-40 nm, it has the greatest effect on bacteria.[17,18] In biomedicine, AuNPs have been proved to be a significant revolution for drug delivery and cancer therapy[19] leading to an economic justification. Infrared spectroscopy of antibiotics coated with AuNPs has shown that AuNPs have a strong affinity for the structural rings of some antibiotics. Researchers showed the presence of vancomycin bound gold nanoparticle (VBGNP) in abundance on the cell wall surface of vancomycin-resistant S. aureus (VRSA). The antibacterial mechanism may be due to VBGNP binding non-specifically to transpeptidases, instead of terminal peptidases of the glycopeptide precursors on the cell surface of test strain.[20,21] This study aimed to determine the frequency of VRE in wastewater samples from Golestan Province and compare the antimicrobial effects of AuNPs in combination with the vancomycin on these isolates.

Materials and Methods

Sampling

Pre-treated (raw wastewater) and treated samples were taken twice from three treatment plants located in Golestan Province (Gorgan, Kordkuy and *Bandar* Torkaman) from February 2018 to May 2019. Samples (250 mL) were collected in sterile tubes by placing them at a depth of 50-70 cm. The samples were transferred to the microbiology laboratory of the Islamic Azad University of Gorgan, on ice, and were examined within 3 h. Physicochemical analysis, including measurement of the amount of dissolved oxygen, pH, turbidity and temperature, was performed by calibrated portable devices at the sampling site.

Bacterial isolation

After filtering suitable volumes of water, the filters were placed on *Enterococcus* agar medium (Merck, Germany) and incubated at 37 °C. To isolate enterococci, each wastewater sample was inoculated into brain heart infusion (BHI) broth and sodium azide broth media (Merck, Germany). After 2 h of incubation at 37 °C, the samples were cultured on 5% sheep blood agar at 37 °C and in 5% CO_2 for 24 h. Enterococci strains were identified based on microbiological tests such as Gram staining, catalase test, SXT disk susceptibility test, growth on sodium azide agar and in sodium chloride 6.5%, sodium hypochlorite hydrolysis, methyl red test, Voges–Proskauer test, oxidation/fermentation test, indole test, citrate test and hydrolysis of lactose, inositol, glucose, maltose, mannitol, mannose, ribose, sorbitol and sucrose. Most probable number testing of water samples was also performed as described previously.[22] For this purpose, water samples were poured into tubes containing 10 mL of selenite F broth. Then, 1, 0.1 and 0.01 mL of the mixtures were inoculated into the first, second and third tubes, respectively. The results were recorded after incubation at 37 °C for 24-48 h. In the confirmation and supplementary phases, samples from positive tubes in the presumptive phase were cultured in the confirmation culture media of azide agar and bile esculin agar (BEA). After 24 h of incubation at 37 °C, the confirmatory and differential tests for enterococci species including catalase, salt tolerance, growth in alkaline medium, tolerance to temperatures of 10, 45, and 60 °C, and carbohydrate fermentation test were performed.

Determination of antibiotic susceptibility by Kirby-Bauer method

A 24-hour culture was prepared from confirmed enterococci isolates. The spread culture was carried out on Mueller Hinton agar (Merck, Germany) from the bacterial suspension with turbidity equal to half McFarland standard. Next, the following antibiotic disks were placed on the culture medium inoculated with the bacteria using sterile forceps: ciprofloxacin (CP5), ampicillin (AM10), tetracycline (TE30), teicoplanin (TEC30), vancomycin (V30) and chloramphenicol (C30). After incubation for 16-18 hours at 37 °C, the diameter of growth inhibition zone around antibiotic disks was measured and the isolates were classified as resistant, intermediate and sensitive according to the standard CLSI-2015 guidelines.[23] Moreover, *E. faecalis* (ATCC 29212) and *E. faecalis* (ATCC 51299) were used as control strains.

Antibacterial properties of AuNPs in combination with vancomycin

The antibacterial effect was evaluated using agar well diffusion method. Firstly, AuNPs with a size of 28 nm as shown in Fig. 1 (Nanopishgaman, Iran) and concentration of 100 mg/L were diluted to 50, 25 and 12.5 mg/L. Afterwards, drug stock (concentration = 100 µg/mL) was prepared by adding vancomycin powder (Sigma-Aldrich, USA) with distilled water. Then a mixture of vancomycin-AuNPs dilutions (50/50) placed in a shaker incubator for 4 h. After centrifugation at 3800 rpm, the resulting precipitate was dissolved in distilled water.

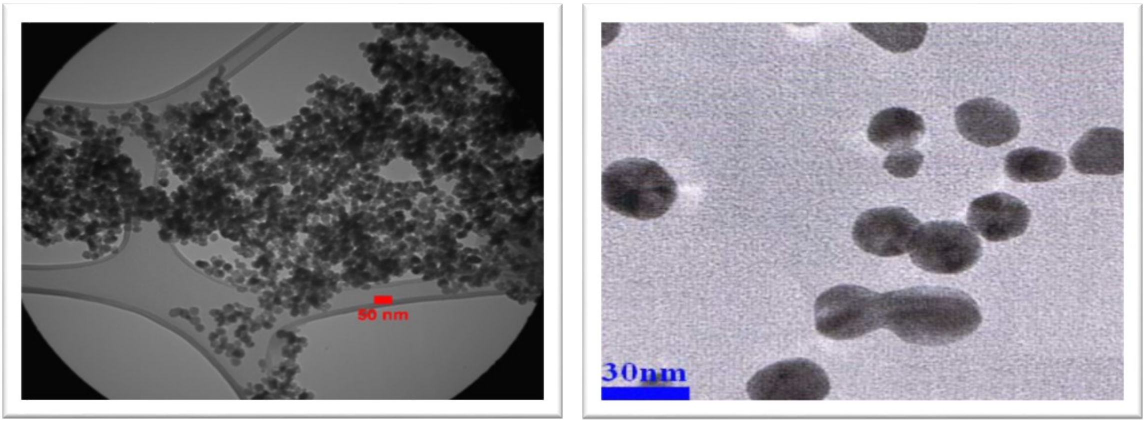

Fig. 1. Transmission electron microscopy image of AuNPs

Absorption spectra in the range of 600-700 nm were detected using spectroscopy to ensure binding. Bacterial suspension with turbidity equal to half McFarland standard was prepared from all vancomycin-resistant strains and was cultured in the form of a spreadsheet on the Mueller Hinton agar medium. Then, wells with the diameter of 7 mm were drilled in the medium using sterile pipette Pasteur, and 100 µL of AuNPs- vancomycin solution (each dilution separately) were inoculated into the well. Two wells containing vancomycin and AuNPs were considered as controls. Plates were incubated in 37 °C. After 24 h, the diameter of the inhibition zone created around each well was measured and recorded in mm. Zones with a diameter of over 12 and 10 mm were considered as susceptible and resistant to this combination respectively.

Data analysis

The mean of quantitative parameters was compared with the three groups using t-test. Charts were drawn in the Microsoft Excel 2010 software. Statistical analysis of data was carried out in IBM SPSS Statistics 23 at a significance level of 0.01.

Results and Discussion

Demographic characterization

Seventeen samples out of 28 wastewater samples (60.7%) were positive for enterococcal species, the majority of which were *E. faecalis* (70.6%). Table 1 shows the results of the MPN method for 12 *E. faecalis* isolates from effluent samples that were confirmed in the phenotypic tests. *Enterococcus asini* (17.6%) and *E. faecium* (11.8%) were other species detected in the wastewater samples. Among these bacteria, 11 isolates (64.7%) were from raw/untreated effluent samples and six isolates (35.3%) were from treated effluent of the final *pond* of the treatment plants.

As expected, raw effluents in all treatment plants had more enterococcal contamination than treated effluents. *E. faecium* was only present in raw and treated effluent samples from Kordkuy. The relative frequency of *E. faecalis* isolates was identical between all samples. Besides, E. asini isolates were present in raw effluents collected from Kordkuy and Gorgan, but they were not detectable in the treated samples (Fig. 2).

Result of antibiotic susceptibility testing

As shown in Table 2, the frequency of VRE isolates was 47.1%. Ampicillin and teicoplanin were the least and most effective antibiotics against enterococci isolates (Table 2).

Results of the antibacterial effect of gold nanoparticle

The results of the antibacterial effects of AuNPs showed that the best and most effective dose of nanoparticles in combination form was 100 mg/L (Table 3). The mean diameter of the growth inhibition zone of the combined form of vancomycin-AuNPs was much larger (2.5-fold) than the single form of AuNPs and 2-fold higher than the single form of vancomycin (P=0.001).

Table 1. Frequency of *E. faecalis* species isolated from wastewater samples by the MPN method

Sample number	MPN (100 mL)
1	2
2	2
3	2
4	0
5	2
6	2
7	0
8	2
9	0
10	2
11	0
12	0

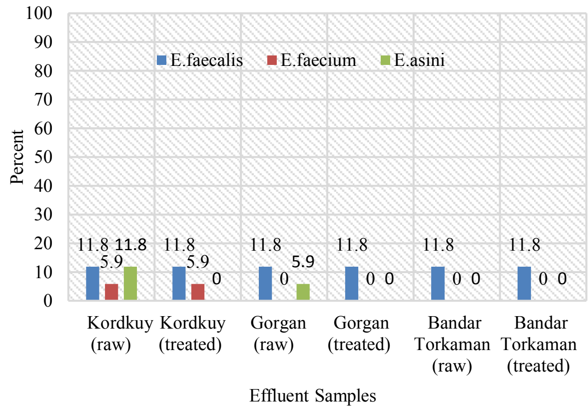

Fig. 2. Relative frequency of enterococci species based on wastewater type of each plant

Enterococcus isolates are opportunistic pathogens that can persist in the environment for a long time and endanger both environmental and public health. Since wastewater is used for irrigation and enterococci can serve as a reservoir of antibiotic resistance genes, the presence of VRE in wastewater can be a serious threat to public health[24] which requires special attention.

In our study, more than 60% of wastewater samples were positive for *enterococci* species, 65% of which were found in raw effluent and the remaining 35% in the treated effluent. This indicates a reduction in the microbial load of the effluent following the treatment process.

Table 2. Antibiotic resistance pattern of *enterococci* isolates

Sampling site	No.	Species		Antibiotic resistance pattern						Sensitivity/resistance of *enterococci* (%)		
				CP	AM	TE	TEC	V	C	S	I	R
Kordkuy (raw)	1	*E. faecalis*		R	R	R	S	S	S	50	0	50
Kordkuy (raw)	2	*E. faecalis*		R	S	R	S	R	S	50	0	50
Kordkuy (treated)	3	*E. faecalis*		R	R	R	S	R	R	16.7	0	83.3
Kordkuy (treated)	4	*E. faecalis*		S	R	R	S	S	S	66.7	0	33.3
Gorgan (raw)	5	*E. faecalis*		R	R	R	S	R	R	16.7	0	83.3
Gorgan (raw)	6	*E. faecalis*		I	R	R	S	S	S	50	16.7	33.3
Gorgan (treated)	7	*E. faecalis*		S	R	R	S	R	R	33.3	0	66.7
Gorgan (treated)	8	*E. faecalis*		S	R	S	S	S	I	66.6	16.7	16.7
Bandar Torkaman (raw)	9	*E. faecalis*		R	R	R	S	S	S	50	0	50
Bandar Torkaman (raw)	10	*E. faecalis*		S	S	S	S	S	S	100	0	0
Bandar Torkaman (treated)	11	*E. faecalis*		R	R	R	R	R	R	0	0	100
Bandar Torkaman (treated)	12	*E. faecalis*		R	R	R	R	R	R	0	0	100
Kordkuy (raw)	13	*E. faecium*		R	R	S	S	R	R	33.3	0	66.7
Kordkuy (treated)	14	*E. faecium*		S	R	R	S	R	R	33.3	0	66.7
Kordkuy (raw)	15	*E. asini*		S	R	R	S	S	S	66.7	0	33.3
Kordkuy (raw)	16	*E. asini*		S	R	R	S	S	S	66.7	0	33.3
Gorgan (raw)	17	*E. asini*		S	R	R	S	S	R	50	0	50
Growth inhibitory potential (%)			S	47.1	11.8	17.7	88.2	52.9	47.1			
			I	5.8	0	0	0	0	5.8			
			R	47.1	88.2	82.3	11.8	47.1	47.1			

S: Sensitive; I: Intermediate; R: Resistant

Table 3. The diameter of inhibition zone due to the effects of AuNPs-vancomycin concentrations

Standard deviation	Inhibition zone diameter (mm)	Concentrations (mg/L)
4.7	16.00	100
3.8	11.10	50
0.3	9.00	25
0.0	7.00	12.5

P-Value <0.01

Previous studies also reported the effectiveness of aerobic processes, especially purification, in eliminating bacteria and even spores from sludge.[25] Among the three treatment sites studied, samples from Kordkuy and Gorgan had higher enterococci contamination compared to those taken from Bandar Turkaman (35% vs. 30%), which might be due to the recent flood in the area and accumulation of water in some areas of Bandar Torkaman.

In general, identifying major environmental pathogens and determining the exact pattern of antibiotic resistance is essential for establishing an accurate and effective treatment/control plan. Most antibiotic resistance studies have been focused on clinical isolates, while a limited number of studies have investigated the impact of environmental microbes as reservoirs of resistance genes and their distribution in the environment via hospital, domestic, industrial and agricultural effluents.[26-28]

In the present study, about 47.1% of the enterococci isolates were VRE. Overuse of vancomycin for treatment of enterococcal infections may have led to the emergence of VRE strains.[15] Two previous studies in Iran[10] and Austria[29] reported high rates of VRE contamination in wastewater samples. In the study by Reinthaler *et al.*, the highest rate of antibiotic resistance was against ampicillin and piperacillin (among penicillins), followed by tetracycline (57%).[29] We also observed that the highest rate of antibiotic resistance was against ampicillin. (88.2%) and tetracycline (82.3%). Multi-drug resistance in bacteria isolates from various wastewaters is reported frequently.[29,30] According to the results of studies in Canada[31] and North America[32], the identified VRE isolates were resistant to several antibiotics.[31-32] In our study, all VRE isolates except for one isolate was multi-drug resistant.

One of the promising strategies for confronting drug resistance is to use metal nanoparticles. Williams *et al*. studied their antibacterial properties on S. aureus and showed that AuNPs don't have antibacterial effects on their own, but in combination with gentamicin inhibit *S. aureus* isolates well.[33] Das *et al.* synthesized and studied the antimicrobial effect of AuNPs against Escherichia coli, Pseudomonas aeruginosa and Bacillus subtilis. They found out that this nanoparticle has a good effect on bacteria in conjugation with Rhizopus oryzae mycelium.[34] Perhaps the reason for this ability is the action of nanoparticles on various vital parts of the cell such as energy production, microbial cell movement and protein synthesis.[34]

Conclusion

Our findings indicate the presence of multi-drug resistant enterococci and the high rate of vancomycin resistance in wastewater samples from the Golestan Province, Iran. Thus, it is essential to develop fully-equipped wastewater treatment systems that are capable of eliminating these strains to prevent the distribution of antibiotic resistance genes in the environment. Similar to previous findings, our results demonstrate that wastewater can be considered as one of the main causes of environmental infections. Therefore, there is a need for continuous and simultaneous monitoring of environmental and clinical samples to assess antibiotic resistance patterns. Furthermore, appropriate control measures should be taken to prevent the spread of resistant isolates and overuse of vancomycin in antibiotic therapy. In the present study, $AuNP_s$ show good antibacterial effects in combination with vancomycin, and their antimicrobial effect increases upon increasing the concentration of the nanoparticles. Hence, these nanoparticles can be hopefully used in future for treating strains resistant to vancomycin.

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