Antibacterial activity of amino- and amido- terminated poly (amidoamine)-G6 dendrimer on isolated bacteria from clinical specimens and standard strains

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Abstract
Background: Nanoscale poly (amidoamine) dendrimers have been investigated for their biological demands, but their antibacterial activity has not been widely discovered. Thus, the sixth generation of poly (amidoamine) dendrimer (PAMAM-G6) was synthesized and its antibacterial activities were evaluated on Gram-negative bacteria; P. aeruginosa, E. coli, A. baumannii, S. typhimurium, S. dysenteriae, K. pneumoniae, P. mirabilis, and Gram-positive bacteria, and S. aureus and B. subtilis, which were isolated from different clinical specimens and standard strains of these bacteria.

Methods: In this study, 980 specimens including urine (47%), blood (27%), sputum (13%), wounds (8%), and burns (5%) were collected from clinical specimens of 16 hospitals and clinics in city of Sabzevar, Iran. Then, the target bacteria were isolated and identified using standard methods. Minimum inhibitory concentration and minimum bactericidal concentrations against Gram-positive and Gram-negative bacteria were determined according to guidelines described by clinical and laboratory standards institute (CLSI). Standard discs were prepared using 0.025, 0.25, 2.5, and 25 μg/mL concentrations of PAMAM-G6 on Mueller-Hinton agar plates to determine the zone of inhibition. The cytotoxicity of PAMAM-G6 dendrimer was evaluated in HCT116 cells by MTT assay.

Results: The most important isolated bacteria were E. coli (23.65%), S. aureus (24.7%), P. aeruginosa (10.49%), B. subtilis (7.7%), S. typhimurium (8.87%), A. baumannii (7.02%), K. pneumoniae (7.1%), P. mirabilis (6.46%), and S. dysenteriae (3.6%). Moreover, it was found that poly (amidoamine)-G6 exhibited more antibacterial efficacy on standard strains than isolated bacteria from clinical samples (p<0.05). The cytotoxicity of PAMAM-G6 to the cells showed that cytotoxicity depended on the concentration level and exposure time.

Conclusion: The PAMAM-G6 dendrimer showed a positive impact on the removal of dominant bacterial isolated from clinical specimens and standard strains.

Keywords: Antimicrobial resistance, Cytotoxicity, Poly (amidoamine)-G6, Health care-associated infections, Nosocomial pathogens, Novel antibacterial, Synthesis

Introduction
Health care-associated infections (HCAI) are a common cause of morbidity and mortality and are considered to be one of the most adverse events in health care. Also, these infections prolong hospitalization, require further extensive diagnostics and treatment, and are associated with additional costs (1). The failure of treatment for these in-
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Infections is primarily due to the emergence of multidrug-resistant strains (MDR), which is a worldwide health care concern (2). Approximately 90% of HCAI are caused by bacteria (3). The most important MDRs associated with these infections are methicillin resistant *S. aureus* (4), extended-spectrum beta-lactamase-producing *E. coli* (5), carbapenemase resistant *A. baumannii* (2), and *P. aeruginosa* (6). Therefore, considering the increasing prevalence of nosocomial infection by strains of MDR, the discovery and development of novel antibacterial agents, particularly those with structures and mechanisms of action different from traditional antibiotics, and a low potential to induce antibiotic resistance, now more than ever there is a need to control and treat health care-associated infections. Recently, the rapid growth in nanotechnology has spurred significant interest in the environmental applications of nanoparticles (NPs). NPs are excellent adsorbents, catalysts, and sensors due to their large specific surface area and high reactivity (7). More recently, several natural and engineered NPs including dendrimer NPs (8, 9), copper oxide (10), and zinc oxide (11) NPs have also been shown to have strong antimicrobial properties. However, dendrimers are a relatively new class of regularly branched macromolecules with unique structure and topological features that have emerged as strong antibacterial agents.

A typical dendrimer consists of a core molecule, monomeric branches called dendrons and surface functional groups that are able to react with other compounds (12). PAMAM dendrimers have been thoroughly explored for drug delivery and antimicrobial applications and have illustrated promising results, with amino-terminated dendrimers showing high antibacterial efficacy (13, 14). Their high antibacterial activity potential is attributed to the electrostatic interaction between the cationic dendrimer and the anionic bacteria cell surface with resultant disruption of the lipid bilayer, consequent cell lysis, and death. Thus, dendrimer biocides may be beneficial with respect to activity, localization in specific organs, reduced toxicity, and increased duration of action (13). An increase in the generation of PAMAM dendrimers is followed by a double increase in the number of functional amine groups in the structure of dendrimer (15). We designed a conceptional scheme of PAMAM-G6 dendrimer, which shows the number of functional amine groups in each generation (Fig. 1). Considering the fact that bacteria cause hospital infections and also considering the problem of MDR in bacteria, evaluating possible antibacterial properties in dendrimers and taking advantage of their ability as an antibacterial and antiseptic can be a research priority. The present study was conducted to determine the antibacterial properties of PAMAM-G6 dendrimer to remove the isolated bacteria from clinical specimens and standard strains bacteria. Overall, all the dominant bacteria related to HCAI have been investigated in the current study.

**Methods**

**Sampling and target bacteria**

In this study, sampling was performed in 16 hospitals and clinics in the city of Sabzevar, Iran, during August 2015 and May 2016. In total, 980 clinical samples including urine (47%), blood (27%), sputum (13%), wounds (8%), and burns (5%) were collected from hospitals and clinics. Then, the samples were transferred to medical microbiology laboratory. Several smears were prepared from each sample and Gram and Giemsa staining were done simultaneously. Urine Samples were cultured in Mac Conkey Agar, blood agar, and Eosin methylene blue agar (EMB). Blood cultures of tryptic soy broth (TSB), Cas-taneda medium, and lysis centrifuge method were used. Also, Mac Conkey Agar and blood agar were used for sputum culture, and wounds samples were cultured in blood agar and chocolate agar. After 24 hours of incubation at 37°C, differential catalase, oxidase, coagulase, indole, MRVP, TSI, SIM, citrate, and urease tests were per-
formed to identify the bacterial species. The isolated bacteria included Gram-negative bacteria; *P. aeruginosa*, *E. coli*, *A. baumannii*, *S. typhimurium*, *S. dysenteriae*, *K. pneumoniae*, *P. mirabilis*, *Gram-positive bacteria*, and *S. aureus* and *B. subtilis*. Standard strains used in this study were *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *A. baumannii* ATCC 17957, *S. typhimurium* ATCC 19430, *S. dysenteriae* ATCC 13313, *K. pneumoniae* ATCC 1705, *P. mirabilis* ATCC 29906, *S. aureus* ATCC 25923, and *B. subtilis* ATCC 23857, which were provided by Iranian Research Organization for Science and Technology (IROST) and Pasteur Institute of Iran. Before use, all bacteria were cultured on specific media.

**Synthesis and properties of antimicrobial agent**

Tomalia’s divergent growth approach is the most common method to synthesize ethylenediamine core PAMAM dendrimers (16). PAMAM-G6 was synthesized following a 2-step process, involving Michael addition of a suitable amine initiator core with methyl acrylate (MA), and exhaustive amidation of the resulting esters with large excess of ethylenediamine (EDA), reported elsewhere (17). Briefly, ethylenediamine (10.0 g, 0.166 mol) was dissolved in 100 mL methanol in a 1-L round-bottomed flask. Methyl acrylate (94.6 g, 0.751 mol) was added at 40°C and stirred for 24 hours in the presence of nitrogen exposure. Excess methyl acrylate was removed under vacuum condition at room temperature. A Michael addition between the amine and the acrylate yielded a product bearing 4 terminal methyl ester groups, defined as the G0.5 PAMAM. Subsequently, ethylenediamine (120 g, 2.00 mol) was dissolved in methanol and added to the G0.5 PAMAM. Then, a product bearing 4 terminal amino groups was obtained and defined as the G1 PAMAM after stirring for 48 hours in the presence of nitrogen. Moreover, by removing excess reactants using vacuum distillation, seventh generation PAMAM dendrimers was synthesized through repeating the above cycle. The chemical formula of PAMAM-G6 is C<sub>2542</sub>H<sub>5088</sub>N<sub>1018</sub>O<sub>508</sub>, molecular weight equal to 60713 g/mol, and the number of terminal amine groups is 400 to 4000 cm<sup>-1</sup>. Pure KBr acted as blank. Morphology and size distribution of PAMAM-G6 was analyzed using transmission electron microscopy (TEM, Philips CM 30). For the TEM investigations, the samples were dispersed in ethanol and deposited by placing 2 drops of NPs suspension onto carbon-covered copper-grids, followed by drying at room temperature.

**Antimicrobial susceptibility testing**

Serial dilution of antimicrobial agent was prepared with using sterile distilled water to assess the antimicrobial activity of dendrimer. Clinical and Laboratory Standards Institute (CLSI) determined the antimicrobial activity of dendrimer by calculating minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) using the serial dilution method (18). Also, disc diffusion method was used to determine the zone of inhibition.

**Disc diffusion method**

A bacteria culture that has been adjusted to 0.5 McFarland standard was evenly applied to Mueller-Hinton agar (MHA) plates using a sterile swab. The volume of 25 μL of antibacterial agent in different concentrations (0.025, 0.25, 2.5 and 25 μg/mL) was impregnated to standard blank disc (6 mm in diameter). The discs were dried for 30 minutes and placed on the MHA seeded with the target culture and were incubated at 37°C for 24 hours.

**Experiments of MIC and MBC**

To determine the exact amount of MIC and MBC of bacteria, experiments with different concentrations (0.025, 0.25, 2.5, 25, 50, 100 and 200 μg/mL) of antimicrobial agent were conducted. Tubes containing 10 mL of nutrient broth (consisting of 10<sup>5</sup>CFU/mL of bacteria and different concentrations of dendrimer) incubated in optimal conditions.

The positive control contained bacteria, with no dendrimers. Also, the negative control contained dendrimers in the absence of bacteria. A tube with the lowest concentration of antimicrobial agent and no bacterial growth was reported as MIC. To determine MBC, a loop of each tube with no growth transferred to nutrient agar plates and incubated in optimal conditions. A plate with no bacterial growth was taken as the MBC value.

**Cell cytotoxicity detection by MTT assay**

Cytotoxicity assessment was performed by MTT assay in HCT116 cells (19) (human intestinal cancer cell line), which was purchased from the Pasteur Institute Cell Bank of Iran (http://ncbi.pasteur.ac.ir/). Briefly, 5000 cells suspended in 96-well plates diluted in 100 μL RPMI 1640 media (Invitrogen, Carlsbad, CA) were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), and 100 mg/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After 24 hours that all cells were attached to the baseline, 100 μL of medium containing different concentration of PAMAM-G6 (5, 10, 20, 40, 60, 80 and 100 μg/mL) were added to each determined well and incubated in above conditions for 48 and 72 hours. One of the seeded wells in each repeat was used as a control in the absence of PAMAM-G6 dendrimer. After incubation, 20 μL containing 5 mg/mL MTT were added to the wells and incubated again for 4 hours. During this time, the MTT (yellow tetrazolium salt) was enzymatically converted into the purple formazan precipitate by viable cells, and the concentration of formazan showed the proportion of viable cells. Subsequently, all media were aspirated from the cells, then, 150 μL of DMSO was added to dissolve the formazan. Finally, absorbance was detected at 490 nm wavelength by ELISA plate reader.
Data analysis

Statistical analysis was performed using Mann-Whitney U test analysis. Statistical significance was set at p<0.05. Every experiment was repeated at least 3 times.

Results

As demonstrated in Fig. 2, the isolated bacteria were E. coli (23.65%), S. aureus (24.7%), P. aeruginosa (10.9%), B. subtilis (7.7%), S. typhimurium (8.87%), A. baumannii (7.02%), K. pneumoniae (7.1%), P. mirabilis (6.46%), and S. dysenteriae (3.6%). Fig. 2 displays that the frequently isolated bacteria with the highest percentage (46.4%) were E. coli, S. aureus, and P. aeruginosa from urine and blood samples. In addition, these 3 bacteria were present in most urine, blood, sputum, wounds, and burns samples. In other words, 59.25% of all isolated species belong to these 3 bacteria. FTIR analysis of PAMAM-G6 NPs was performed to confirm the existence of characteristic amides, terminal amino, and etc. (Fig. 3). Results in Table 1 demonstrate the corresponding functional groups of the wavelengths, which are indicated in Fig. 3. The morphology and diameter of PAMAM-G6 NPs were studied by TEM as shown in Fig. 4. PAMAM-G6 NPs were shown to have a spherical shape with a mean diameter size of 20 nm. The results of different concentration of PAMAM-G6 dendrimer effect on isolated bacteria and standard strain (using disk diffusion method) are presented in Tables 2 and 3, respectively. According to the obtained results, PAMAM-G6 dendrimer actively inhibited the growth of isolated Gram-negative and Gram-positive bacteria and standard strains. However, the antibacterial activity of PAMAM-G6 on the isolated bacteria was less than that on the standard strains, whose difference was statistically significant (p<0.05). The most sensitivity was related to P. mirabilis ATCC 29906, S. typhimurium ATCC 19430, S. dysenteriae ATCC 13313, and S. aureus ATCC 25923 at the concentration of 25 (µg/mL) PAMAM-G6 with the inhibition zone of 35, 32, 31, and 30 mm, respectively. In addition, the least sensitivity was...
related to *A. baumannii* at 25 (µg/mL) concentration of dendrimer, with 10 mm zone of inhibition. The concentration of 0.025 (µg/ml) dendrimer had no effect on the studied bacteria, except *S. typhimurium, P. mirabilis*, and *S. aureus*. Comparison between MIC and MBC values of PAMAM-G6 for isolated bacteria and standard strains are presented in Tables 4 and 5, respectively. As can be seen in the tables, the maximum amount of MIC and MBC were related to isolated bacteria, ie, *E. coli* and *A. baumannii* at 25 and 200 µg/mL concentrations of dendrimer, respectively. Also, the minimum amount of MIC and MBC were related to *S. typhimurium* ATCC 19430 at 0.025 and 25 µg/mL concentrations, respectively. The effect of PAMAM-G6 on cell viability was assessed using MTT assay. This assay measured optical density of different concentrations in comparison with the rate of viable cells. As displayed in Fig. 5, there were little cytotoxicity effects of PAMAM-G6 with different concentrations in HCT 116 cells during 48 hours when no IC50 value was obtained (Graph pad prism 6, USA). The cytotoxicity effects after 72 hours (Fig. 5) were more than 48 hours, with no measurable IC50. However, high concentration in 72 hours had more effects compared to the same concentration in 48 hours.

### Table 1. Band position of PAMAM-G6 spectrum

| Wavenumber (cm⁻¹) | Spectral assignments |
|-------------------|----------------------|
| 1025.38           | C–O stretching vibration |
| 1654.88           | C=O stretching (amide I) |
| 1546.62           | N-H bending/C-N stretching (amide II) |
| 1456.85           | H-C-H scissor |
| 1365.17           | H-C-H asymmetric |
| 2827.55 and 2942.81 | C-H stretching vibrations |
| 3416.22, and 3277.82 | N-H stretching mode of aminal and amide groups |

### Table 2. The Mean Diameter of Inhibition Zone of isolated bacteria VS Different PAMAM-G6 Dendrimer Concentrations

| Dendrimer concentration, µg/ml | Zone of inhibition, mm |
|-------------------------------|------------------------|
|                               | *E. coli* | *S. typhimurium* | *A. baumannii* | *K. pneumoniae* | *S. dysenteriae* | *P. aeruginosa* | *P. mirabilis* | *B. subtilis* | *S. aureus* |
| 0.025                         | 0         | 10              | 0              | 0              | 0              | 0              | 9              | 0              | 9              |
| 0.25                          | 0         | 19              | 0              | 13             | 0              | 19             | 9              | 10             |
| 2.5                           | 0         | 21              | 0              | 16             | 13             | 21             | 9              | 11             |
| 25                            | 20        | 31              | 10             | 26             | 27             | 22             | 32             | 17             | 21             |

### Table 3. The Mean Diameter of Inhibition Zone Of Standard Strain Bacteria VS Different Concentrations of PAMAM-G6 Dendrimer

| Dendrimer concentration, µg/ml | Zone of inhibition, mm |
|-------------------------------|------------------------|
|                               | *E. coli* | *S. typhimurium* | *A. baumannii* | *K. pneumoniae* | *P. aeruginosa* | *P. mirabilis* | *B. subtilis* | *S. aureus* |
| 0.025                         | 0         | 14              | 0              | 0              | 0              | 11             | 0              | 11             |
| 0.25                          | 0         | 22              | 9              | 19             | 14             | 0              | 21             | 9              | 11             |
| 2.5                           | 9         | 23              | 13             | 21             | 18             | 9              | 22             | 9              | 17             |
| 25                            | 24        | 32              | 20             | 27             | 30             | 22             | 35             | 21             | 30             |
Discussion

A well-known problem in modern-day hospitalization is the occurrence of nosocomial or health care acquired infections caused by multi-resistant pathogens (3). PAMAM dendrimers have been investigated for their biological applications, but antibacterial activity has not been extensively explored (20). The 980 samples were tested. Fig. 2 shows the isolated bacteria with the highest frequency belonging to *E. coli* (23.65%), *S. aureus* (24.7%), and *P. aeruginosa* (10.9%). These 3 bacteria were mostly isolated from urine and blood samples as 46.4% of these bacteria were isolated from these samples; and of these 9 isolated strains, the highest frequency belonged to these 3 bacteria (59.25%). Similar results were observed by Salamat et al. (21), as they reported that the most common pathogenic organisms were *Enterobacter* (27%), *S. aureus* (21%), and *E.coli* (14%). Other studies also reported that the most common nosocomial infections were associated with *E.coli* (22), *P. aeruginosa*, and *S. aureus* (23). In a study conducted by Mazloomi et al. (24), on 172 specimens collected at a children medical center, the most frequent isolated bacteria were *S. aureus* and *P. aeruginosa*. Ferreira et al. (25) investigated patients’ urine and blood specimens and observed that the highest frequency of bacteria in urine and blood specimen belonged to *E. coli* and *S. aureus*, respectively. These pathogens, especially *E. coli*, *P. aeruginosa*, *S. aureus*, *A. baumannii*, and *K. pneumoniae*, are often HCAIs such as Bacterimia, UTIs,
This study evaluated the antibacterial effects of PA-MAM-G6 on the isolated bacteria from clinical specimens. Previous studies have shown that antimicrobial agents cause bacterial cell membrane damage, spatial deformation, degradation of bacterial enzymes, and damage of chromosomes and bacteria cell wall damage. This character refers to end amine groups in dendrimer structure, which interact with the negative charge of the membrane or cytoplasm microorganism, causing bacterial cell wall damage, and finally, inactivation of bacteria (27). In FTIR spectrum of PAMAM-G6 dendrimer (Fig. 3), 9 main peaks are detectable at 1025 cm⁻¹, 1365 cm⁻¹, 1456 cm⁻¹, 1546 cm⁻¹, 1654 cm⁻¹, 2827 cm⁻¹, 2942 cm⁻¹, 3277 cm⁻¹, and 3416 cm⁻¹, with the last peak being related to N-H stretching vibration of primary amine. Other main band positions, based on wave number and their assignments are presented in Table 1. Overall, PAMAM-G6 dendrimer is an efficient antibacterial agent against both Gram-negative and Gram-positive bacteria. Our results are also consistent with those of Lopez et al. (28) study. They found that the antimicrobial effect of PAMAM dendrimers was modified with amine groups on Gram-positive and Gram-negative bacteria. Likewise, Xue et al. and Charles et al. found that amino-terminated PAMAM G2 and G3 dendrimers possess significant antibacterial effects against multi-resistant strains (12, 29). As shown in Tables 2 to 5, E. coli, P. aeruginosa, and A. baumannii had a higher resistance than other studied bacteria. Intrinsic and acquired resistance of these bacteria against the antibacterial agent can be attributed to the possible effect of lower concentrations of dendrimer PAMAM-G6 on the bacteria such as E. coli, P. aeruginosa (30), and A. baumannii (31) than other target bacteria. Mihani et al. indicated that P. aeruginosa were resistant against ciprofloxacin (67%), ceftazidime (71%), and imipenem (41%) (32). Mohammadimehr et al. studied cytotoxicity in different generations of PAMAM dendrimers with variety of doses. They found that increasing the dose of these dendrimers cause a decrease in the percentage of healthy and early apoptotic cells. They also demonstrated that the systematic mechanism in mammalian cells leads to cytotoxicity in various exposures of different generations of dendrimers. In line with a recent study, our data revealed a reduction in cell viability although the dose and exposure time are 2 important factors. This result is consistent with a study that found PAMAM could lead to the formation of nanoscale holes in eukaryotic membranes at high concentrations (39). Increasing the number of surface amino groups in the dendrimers and zeta potential may give rise to toxicity after increasing generation and diameter (19).

**Conclusion**

This study evaluated the antibacterial effects of PAMAM-G6 on the isolated bacteria from clinical specimens.

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and standard strains using MIC, MBC, and disc diffusion method. The highest number of isolated bacteria was related to E. coli (23.65%), S. aureus (28.7%), and P. aeruginosa (10.9%). In this study, we found that amineterminated PAMAM-G6 dendrimer is an effective antimicrobial agent against common Gram-negative and Gram-positive pathogens. Bacteria origination is one of the important variables affecting the performance of PAMAM-G6. The antibacterial activity of PAMAM-G6 on the isolated bacteria was less than that on the standard strains. Although increasing the concentration of PAMAM-G6 improves removal efficiency, their cytotoxicity in mammalian cells has to be considered at higher concentrations. However, the low levels have relatively high antibacterial effects on Gram-negative and Gram-positive bacteria. A. baumannii has the least sensitivity to PAMAM-G6, where P. mirabilis ATCC 29906, S. typhimurium ATCC 19430, S. dysenteriae ATCC 13313, and S. aureus ATCC 25923 have the highest sensitivity. These findings indicated that PAMAM-G6 could be an excellent candidate for a new class of antimicrobial compounds and could be incorporated to combat dominant bacteria in health care centers.

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Conflict of Interests
The authors declare that they have no competing interests.

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