Identification of β-lactam-resistant coding genes in the treatment plant by activated sludge process

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ABSTRACT

Wastewater treatment plants are the most important release source of antibiotic-resistance genes (ARGs) into the environment. This study aimed to identify β-lactam-resistance genes in Qom province municipal wastewater and also to determine the effect of municipal wastewater treatment plants with different processes on reducing these pollutants. Sampling was performed according to the standard protocol and at constant temperature conditions. Nine genes resistant to the six groups of β-lactam antibiotics were selected, and polymerase chain reaction (PCR) test was performed to identify the presence/absence of ARGs. At the same time, real-time PCR test was also carried out to quantitatively measure three ARGs in wastewater and effluent samples. The obtained results showed that the highest and the lowest removal were related to bla_{nps-1} (90.61%) and $bla_{CTX-M-32}$ (65.93%), respectively; however, a decreasing trend in these two genes were showed in to bla_{oxa-1} with an increase in effluent unit (45.12%). The results showed that conventional biological wastewater treatment processes not only have little potential to reduce antibiotic-resistance genes during the treatment process but also these processes sometimes increase resistant genes. The results of the _oxa-1_ gene in this study are consistent with this trend. In addition, the sludge treatment unit was based on the results of sequencing _Salmonella enterica_ and _Klebsiella pneumoniae_. Our study suggests that wastewater treatment plants using conventional chlorination do not favor the proliferation of antibiotic resistance bacteria and ARGs during wastewater treatment. Thus, equipping treatment plants with advanced processes will be efficient in reducing bacterial resistance.

Keywords: Antibiotic resistance genes; β-lactam; Bacteria; Polymerase chain reaction; Activated sludge

1. Introduction

The World Health Organization (WHO) has introduced resistance to a variety of antibiotics as one of the most important public health issues in the 21st century since antibiotics have endangered public health over the last few decades [1]. Since the 1950s, this global problem has become more dangerous due to the issue of multiple antibiotic resistance in microorganisms. The annual nine billion euro in Europe and 35 billion dollars in the United States, along with two million patients and 2,300 deaths related to antibiotic resistance have been reported [2].

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Recent studies have indicated an increase in bacterial resistance to antibiotics to such an extent that currently, 95% of *Staphylococcus aureus* species are resistant to penicillin in the United States while only 2.4% of these species were resistant in 1976 [3]. From 2000 to 2015, due to the rising use of antibiotics (by 35%), they have been released into the water and soil environment [4,5]. Aquatic environments, in particular the wastewater, are the good setting to convert bacteria and genes into antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) since they are the main recipients of gram-negative bacteria. It is worth mentioning that most of the antibiotics used by humans and animals enter the sewage unchanged through urine and feces [6,7]. Various antibiotics such as β-lactam compounds have been identified in urban and hospital wastewater samples since these antibiotics have been one of the most often used antibiotics in recent years. Penicillins, Cephalosporins, Monobactams, and Carbapenems are some of these antibiotics known by the β-lactam ring in their molecular structure. According to the WHO report, the amount of antibiotics used in Iran between 2015 and 2018 was 23% for Penicillins, 19% for macrolides, and 11% for quinolones, respectively [8]. Moreover, based on a study by Golfeshan et al. [9] in Qom City, the most commonly prescribed antibiotics were ceftriaxone (20.7%), ampicillin (11.9%), and meropenem (7.5%) all of which belong to the group of β-lactams. Resistance to β-lactam antibiotics in some bacteria is mediated by the β-lactamase enzyme through degrading the β-lactam ring in the antibiotic molecular structure. There are more than 1,300 types of β-lactamases in nature which are classified into four classes, namely, A, B, C, and D by Ambler Classification (1980) according to their molecular properties. Classes A, C, and D include serine β-lactamases and Class B are metallo-β-lactamases (MBLs) [10,11]. For instance, bla_{TEM-1}, $bla_{CTX-M-4}$, and $bla_{CTX-M-32}$ are categorized in Class A [12,13]. The genes encoding extended-spectrum β-lactamase (ESBLs) are usually present on the plasmid and transmit genes by horizontal gene transfer (HGT) [14]. MBLs include bla_{IMP} and bla_{VIM} genes [15] which are among the most common genes replicated and spread around the world. *AmpC* group is an example for Class C enzymes which are known as cephalosporinases, in which binding sites in Class C enzymes are more open [16]. In addition, some genes in Class D of β-lactamases include bla_{oxa-1}, *mecA*, and bla_{nps-1}, which hydrolyze Carbapenems, Methicillin, and Carbenicillin [17–19]. Activated sludge process is one of the oldest and the most common biological methods of wastewater treatment, and the conventional activated sludge process is the most often applied one for municipal wastewater treatment [20]. During the formation of biofilms in wastewater, due to high microbial density and nutrient availability, cell-to-cell contact would cause the transfer of resistant genes in wastewater [21]. ARGs in different environments such as wastewater can be transferred from cell to cell in three stages: first, acquire resistant genes through conjugation, transformation, and transduction (phage-mediated transfer); second, express the resistance genes, and finally, antibiotics pose the selective pressure on ARGs and consequently the bacteria that have acquired the resistant gene are selected and the antibiotic-sensitive ones will die. After bacteria acquires resistant genes, mobile genetic elements such as plasmids (R-factor), integrons, transposons, and bacteriophages release resistant genes among the bacteria. Conventional wastewater treatment is not sufficient to completely eliminate biological contaminants such as bacteria, viruses, and protozoa. Studies show that despite the disinfection process, resistant bacteria and genes are ubiquitous throughout wastewater treatment plants stages. Piotrowska et al. [22] showed the presence of bla_{TEM} and bla_{oxa} genes in the effluent of treatment plants using activated sludge process in the Netherlands and Poland. The presence of the bla_{CTX-M} gene was evaluated by Zagui et al. [23] in hospital sewage and urban wastewater treatment plants in Brazil. In Iran, this gene was reported to be positive in hospital wastewater [24]. In China, bla_{IMP-1} was identified as one of the most abundant genes in the treatment plant effluent by activated sludge process [25]. Khan et al. [26] Methicillin-resistant *mecA* was also observed in Sweden at the effluent of the treatment plant by the trickling filter process. There are two reasons for the occurrence of resistant genes in the effluent. The first is that some of them may continue to multiply during treatment due to the inefficiency of the treatment process and reach the effluent without any changes, and the second is that resistant genes are created in native bacteria due to different transmission mechanisms [27]. Fig. 1 illustrates these two causes. Facilities applied in the conventional treatment process not only are not reliable options for controlling genes encoding antibiotic resistance to the environment, but some research studies showed these facilities could be effective in quantitatively increasing bacteria and genes encoding resistance [34]. The occurrence and
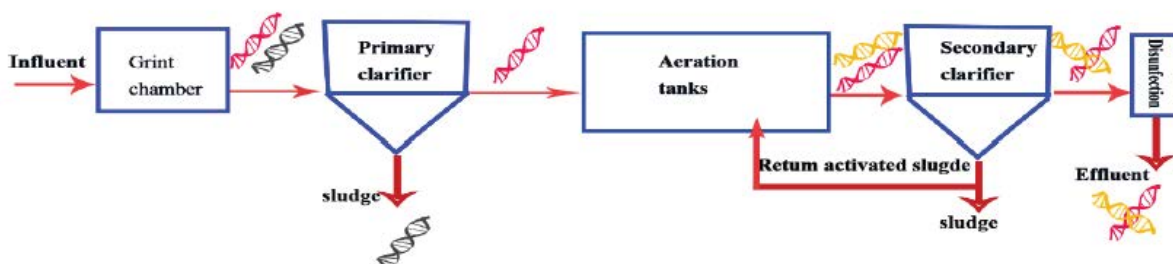

Fig. 1. Schematic transfer of ARGs through wastewater treatment process (WWTP). ARB and ARGs discharged with the effluent may have different origins. Some may be removed through the entire WWTP if the bacteria survive treatment (here, for example, the black gene), others may originate from populations of bacteria that grow in the WWTP (red and yellow).

spread of antibiotic resistance in the treatment plant need to be investigated more as a novel phenomenon. To the best of our knowledge this work is the only study conducted on β-lactam resistant genes in the wastewater treatment plant in Qom, Iran. The present study aims to investigate the abundance and occurrence of β-lactam-resistant genes through wastewater treatment plants using the activated sludge method and to determine the effect of the different processes on the presence of the aforementioned genes as well.

2. Materials and methods

2.1. Sampling locations

In this descriptive study, 24 samples of wastewater and sludge (primary, gravity thickened, and dewatered sludge) from a wastewater treatment plant (No. 3) in Qom, Iran, were obtained from May to September 2019. Sampling was performed instantaneously and the sampling tools and equipment were in accordance with the two Iranian National Standards No. 20148 and 7960 for wastewater and also the standard methods (1060 and 1080). Table 1 shows the specifications of the treatment plant.

2.2. Sample preparation

The samples were first transferred to the environmental microbiology laboratory, then 10^{-3} dilutions of each sample were prepared using sterile normal saline and 100 μL of which was transferred to plates containing nutrient agar medium. After spreading the sample by pour plate method, the culture plates were incubated at 37°C for 18–24 h. The pure culture was prepared from the grown bacteria and each bacterial colony with an apparent difference was isolated and finally transferred to the molecular and cell biology laboratory for DNA extraction and polymerase chain reaction (PCR) [28].

2.3. DNA extraction and identification of genes resistant to the antibiotic β-lactam

2.3.1. DNA extraction

Extraction and preparation of DNA from wastewater samples was performed using Wizard® Genomic DNA Purification Kit [29].

2.3.2. Identification of nine selected genes resistant to β-lactam

PCR assay was performed to determine the presence/absence of the studied genes in wastewater samples. PCR amplification done in a final volume of 15 μL including 8 μL Master Mix 2X (Ampliqon, Denmark), 0.5 μL of each primer (Metabion, Germany) (10 pmol/μL) (Table 2), 2 μL template DNA, and 4 μL deionized water. PCR amplification

Table 1
Specifications of the studied treatment plant

Type of WWTP	Conventional activated sludge
Capacity (m³/d)	52,000
Population covered	500,000
Disinfection system	Chlorination
Final output destination	Agricultural application
Geographical location	34 41 46N
	50 57 17E

Table 2
Specifications of primers related to selected β-lactam resistant genes

Primer	Primer sequence (5′ to 3′)	Amplicon size (bp)	Annealing temperature (°C)
$bla_{CTX-M-4}$	GGAGAAAAGTTCGGGAGGTC GCTTATCGCTCTCGCTCTGT	155	58
$bla_{CTX-M-32}$	CGTCACGCTGTTGTTAGGAA CGCTCATCAGCACGATAAAG	156	58
bla_{TEM-1}	CATTTTCGTGTCGCCCTTAT GGGCGAAAACTCTCAAGGAT	167	61
bla_{VIM-4}	TCCGACTTTACCAGATTGCC TTTCAATCTCCGCGAGAAGT	171	53
bla_{IMP-2}	CGGTTTGGTGGTTCTTGTAAA ATTCAGATGCATACGTGGGA	200	61
AmpC	CCTCTTGCTCCACATTTGCT ACAACGTTTGCTGTGTGACG	189	58
bla_{nps-1}	TTCTGGCCTGTAGCCTCTGT TGTTGAGCACCTTGAACGTC	188	58
bla_{oxa-1}	TATCTACAGCAGCGCCAGTG CGCATCAAATGCCATAAGTG	199	61
mecA	AAAAAGATGGCAAAGATATTCAA TTCTTCGTTACTCATGCCATACA	185	56

conditions were as follows: an initial genome denaturation step at 95°C for 5 min (one cycle), followed by 35 cycles of secondary denaturation at 95°C for 30 s, primer annealing (according to Table 2), and extension at 72°C for 30 s. The final extension was performed at 72°C for 5 min. Once the PCR process was completed, analysis with electrophoresis was performed to characterize the resulted target genes [30].

2.4. Real-time PCR (RT-PCR) for three genes

RT-PCR was performed on 96-well plates in the final volume of 10 μL in pairs. The reaction mixture consisted of 5 μL of SYBR Green qPCR Master Mix 2X (Yektatajhiz, Iran), 0.4 μL of forward and reverse primers at 10 picomolar (pM) concentration, 2.5 μL of template DNA, 0.20 μL of Rox reference dye and 2.5 μL of deionized water [31], namely, bla_{nps-1}, $bla_{CTX-M-32}$, and bla_{oxa-1} were selected for quantitatively measurement of water and wastewater samples. The device temperature program was performed in three steps. The first stage activate the FastStart Taq DNA polymerase then the one-cycle melting curve program, finally, the experiment protocol as well as optimised concentrations are shown in Table 3.

2.5. Absolute quantification in real-time RT-PCR

Absolute quantification is achieved by comparing the CT values of the test samples to a standard curve. The result of the analysis is quantity of nucleic acid (copy number/μg) per given amount of sample. In absolute quantification, the quantity (e.g., copy number or unit mass) of the unknown sample was interpolated from a range of standards of a known quantity. To construct a standard curve, a template with known concentration was required. Dilution of this template was then performed and these dilutions serve as the standards. The unknown test samples were assayed with the standards in the same experimental run. The standard curve constructed from the diluted standard template can then be used to determine the target quantity in the unknown sample by interpolation, similarly to using molecular size standards to determine the molecular size of an unknown

DNA band on an agarose gel [32]. A standard curve was constructed, with the logarithm of the initial copy number of the standards plotted along the *x*-axis and their respective CT values plotted along the *y*-axis. The equation for the linear regression line [$y = mx + b$] where y = CT, m = slope, x = log quantity, and b = *y*-intercept. Based on the equation for the linear regression, we can derive the following:

$$\text{quantity} = 10^{\frac{C_t - b}{m}} \tag{1}$$

The number of copies of each gene in wastewater samples was calculated using C_t vs. log(quantity) curves. Regression coefficient (*R*-squared) values show the linearity of the results. Table 4 shows the results related to dilution.

2.6. Phylogenetic analysis and building phylogenetic tree

For phylogenetic analysis, PCR was performed on the 16S rRNA gene using universal 27F 5'–AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-ACGGYTACCTTGTTACGACT-3' [33,34]. The amplification temperatures of 16S rRNA is shown in Table 5. 50 μL of PCR product of each sample was sequenced after purification. At first, the nucleotide sequence of positive samples was modified via Chromas Software (Technelysium Pty Ltd, Australia), and the desired sequence was analyzed and compared with the registered sequences in the BLAST software in the Nucleotide BLAST section (http://blast.ncbi.nlm.nih.gov). The sequences were then aligned with MEGA10 (Molecular Evolutionary Genetics Analysis) software [35]. Phylogenetic analysis and drawing phylogenetic tree were performed by neighbor-joining phylogeny and bootstrapping analysis (1,000 replicates) to reliably estimate the dependence of the strains [36].

2.7. Statistical analysis

McNemar's and Chi-square tests were used to determine the presence of β-lactam-resistant genes in the influent and

Table 4
Specifications for standard diagrams of three selected genes

Gene	Slope	Efficiency	R^2	Percentage of dilution
$bla_{CTX-M-32}$	−3/271	102%	0.74	2copy/μL – 2 × 10⁶ copy/μL
bla_{nps-1}	−3/255	103%	0.99	2copy/μL – 2 × 10⁶ copy/μL
bla_{oxa-1}	−3/256	90%	0.97	1copy/μL – 10⁶ copy/μL

Table 5
Programming the thermal cycler for 16S rRNA primer amplification

Cycles	Duration of cycle	Temperature	Steps
1	5 min	95°C	Initial denaturation
	45 s	94°C	Denaturation
35	1 min	55°C	Annealing
	1.30 min	72°C	Extension
1	5 min	72°C	Final extension

Table 3
Specifications of primers used in real-time PCR

Gene	Preincubation	Amplification	Melting
$bla_{CTX-M-32}$	95°C for 15 min	45 cycles of	95°C for 15 s
		95°C for 20 s	60°C for 60 s
		58°C for 30 s	59°C for 15 s
		72°C for 30 s	
bla_{oxa-1}	95°C for 15 min	40 cycles of	95°C for 15 s
		95°C for 20 s	60°C for 60 s
		61°C for 10 s	59°C for 15 s
		72°C for 30 s	
bla_{nps-1}	95°C for 15 min	40 cycles of	95°C for 15 s
		95°C for 20 s	60°C for 60 s
		58°C for 30 s	59°C for 15 s
		72°C for 30 s	

effluent, and Wilcoxon and Kruskal–Wallis tests were also applied for quantitative analysis of those genes (SPSS22). *P*-values less than 0.05 (typically ≤ 0.05) were considered statistically significant. The Spearman correlation between two environmental parameters under different terms was performed using the software SPSS 22.0.

3. Results

3.1. Identification of β-lactam-resistant genes in wastewater treatment plant and sludge treatment unit by qualitative PCR

Figs. 2 and 3 illustrate the heat map of all studied genes through the wastewater and sludge treatment units obtained by the qualitative PCR method. This study showed that all nine genes except $bla_{CTX-M-4}$ and bla_{VIM-4} were present in different units of wastewater treatment plant and in sludge treatment unit as well. The frequency of genes resistant to β-lactam antibiotic group ($bla_{CTX-M-4}$, $bla_{CTX-M-32}$, bla_{TEM-1}, bla_{VIM-4}, bla_{IMP-2}, $AmpC$, bla_{nps-1}, bla_{oxa-1}, $mecA$) was determined by PCR method. According to Fig. 4, which shows the frequency diagram of β-lactam-resistant genes through different units of the studied wastewater treatment plant, it is obvious that $bla_{CTX-M-32}$ and bla_{nps-1} genes were dominant at the highest frequency (100%) among other genes in all sampling points including influent, primary settling, aeration, secondary settling and even after the chlorination stage (in the effluent). In the next position, $AmpC$ and bla_{oxa-1} genes had high frequencies, respectively. Genes bla_{IMP-2}, $mecA$, and bla_{TEM-1} were less abundant frequency among other genes in wastewater treatment plant stages. In the case of the sludge treatment process, $bla_{CTX-M-32}$, bla_{oxa-1}, and bla_{nps-1} genes had higher frequency in primary sludge, return sludge, and digested sludge steps (Fig. 5). Genes $AmpC$ and bla_{IMP-2} also had the same amount in the three mentioned units. At the same time, the lowest frequency was for $mecA$ gene which had the lowest abundance. Gene bla_{TEM-1} was only present in the primary sludge.

Genes $bla_{CTX-M-32}$ and bla_{TEM-1} belonging to ESBLs have been found in wastewater treatment units. According to McNemar's test, no statistically significant difference was observed between nine β-lactam-resistant genes in wastewater treatment plant units as well as sludge treatment ones ($p > 0.05$). Frequencies distribution of all genes through the wastewater and sludge treatment units were depicted in Figs. 4 and 5.

3.2. Identification of β-lactam-resistant genes in wastewater treatment plant and sludge treatment unit by quantitative PCR analysis

In this study, genes $bla_{CTX-M-32}$ ESBLs, bla_{oxa-1} from carbapenem-hydrolyzing Class D β-lactamases (CHDLs), and bla_{nps-1} were detected in different units of wastewater and sludge treatment via absolute quantification method and standard graphs. Based on the results obtained from gene $bla_{CTX-M-32}$ from ESBLs had the maximum quantity (4.81E+03 copy/100 mL) in the influent of the plant. A decreasing trend was observed in the following units of the plant, but an increase occurred in the effluent (3.41E+03 copy/100 mL). A trend similar to $bla_{CTX-M-32}$ was observed for gene bla_{nps-1} with the highest quantity in the influent (5.16E+02 copy/100 mL), decreasing trend through the plant units, and an increase in the effluent (4.11E+01 copy/100 mL) in copy/100 mL. Gene bla_{oxa-1} showed the lowest quantity (9.74E+01 copy/100 mL) among others in the influent, a downward trend in the following units of the treatment plant until it reaches 1.18E+02 copy/100 mL in the effluent which indicates the increase in the effluent unit. Fig. 6 illustrates the quantitative amount of the three selected genes through the wastewater treatment plant (WWTP). According to the Wilcoxon test, there is no significant difference in the number of genes between influent and effluent units as well as other treatment processes ($p > 0.05$). Furthermore, based on this test, it was
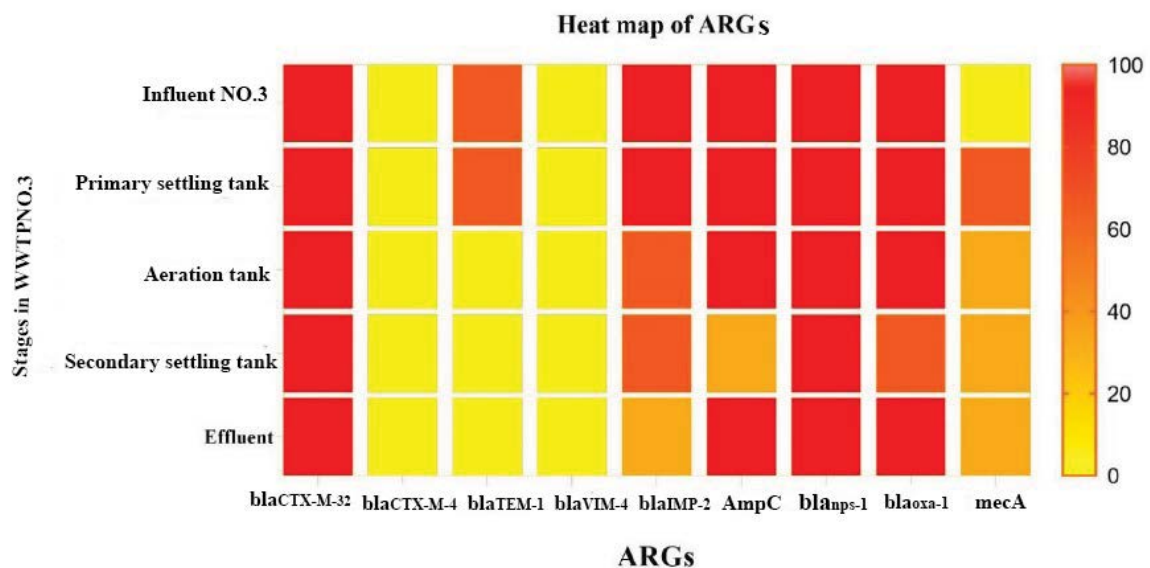

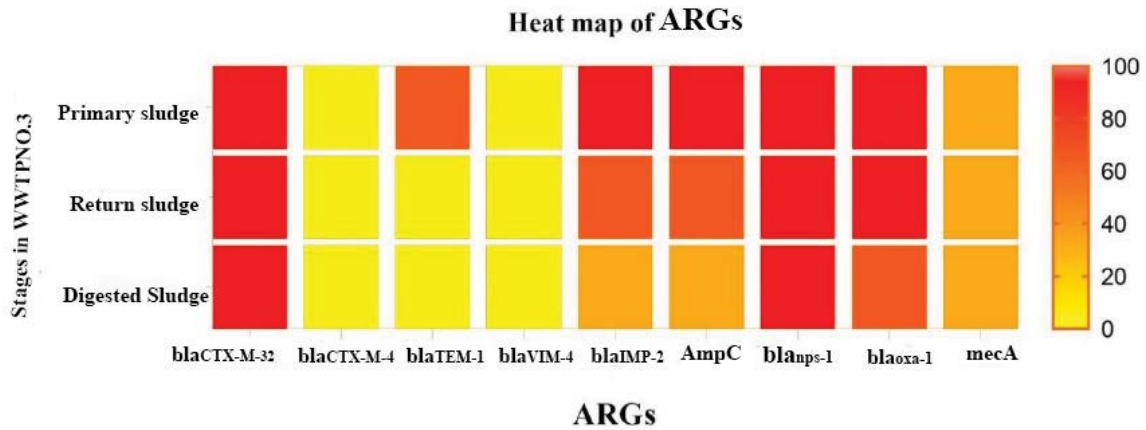Fig. 2. Heat map of β-lactam-resistant genes in different units of wastewater treatment plant.

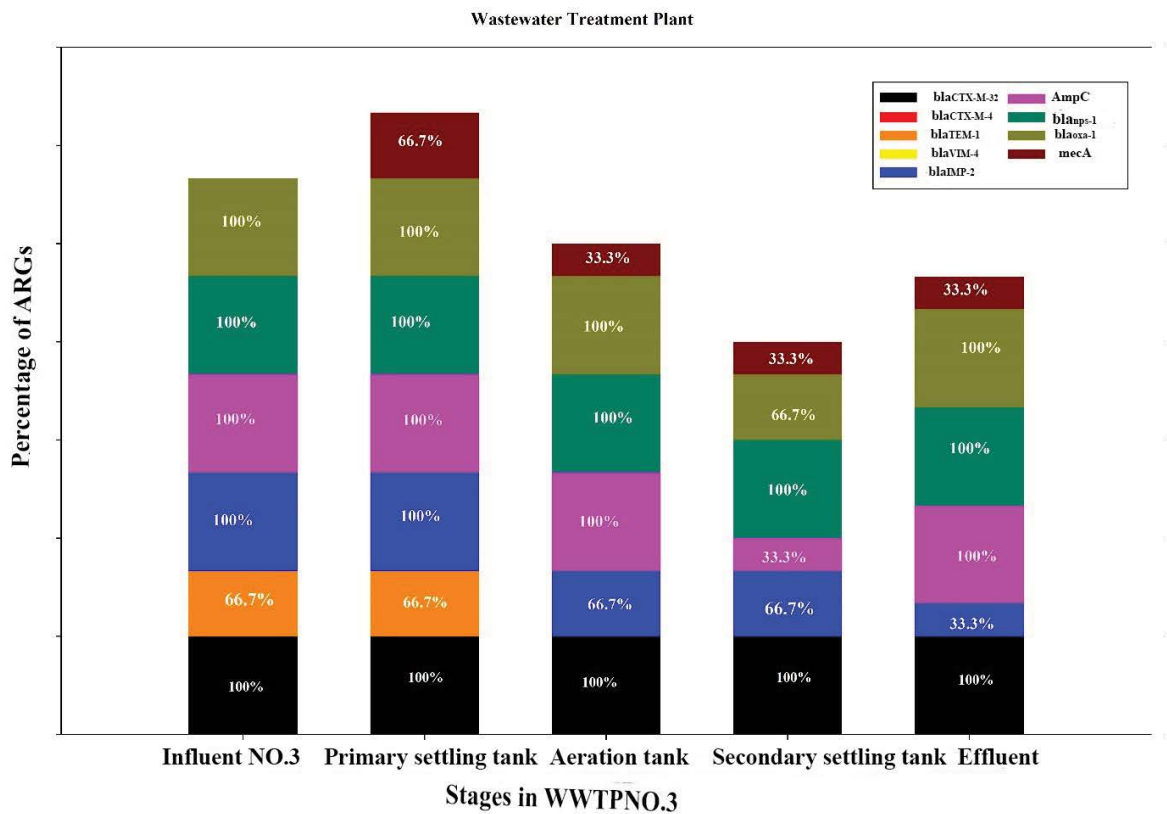Fig. 3. Heat map of β-lactam-resistant genes in primary, return, and digested sludge in wastewater treatment plant.

Fig. 4. Percentage frequency distribution of β-lactam-resistant genes in different treatment plant units.

found that genes $bla_{CTX-M-32}$ and bla_{nps-1} were more abundant while gene bla_{oxa-1} demonstrated the opposite trend.

Fig. 7 illustrates the number of β-lactam-resistant genes through the sludge treatment stages. Gene $bla_{CTX-M-32}$ had the highest amount (5.54E+03 copy/100 mL) in the primary sludge step (from the gravity settlement unit). It reduced in the return sludge unit and raised to 2.38E+03 copy/100 mL during sludge digestion, that is, anaerobic digestion and composting process. This trend was also seen for the other two genes. The amount of bla_{nps-1} genes was 6.03E+02 copy/100 mL in the primary sludge and 5.03E+02 copy/100 mL after sludge digestion. 1.70E+02 and 1.69E+02 copy/100 mL of gene bla_{oxa-1} was also found in primary sludge and sludge digestion units, respectively. In the sludge treatment process, according to the Kruskal–Wallis test, there is no significant difference between the three sludge treatment units ($p > 0.05$). According to the results obtained by this test, it was found that the average of genes $bla_{CTX-M-32}$ and bla_{nps-1} in the three stages of sludge treatment.

Wastewater Treatment Plant

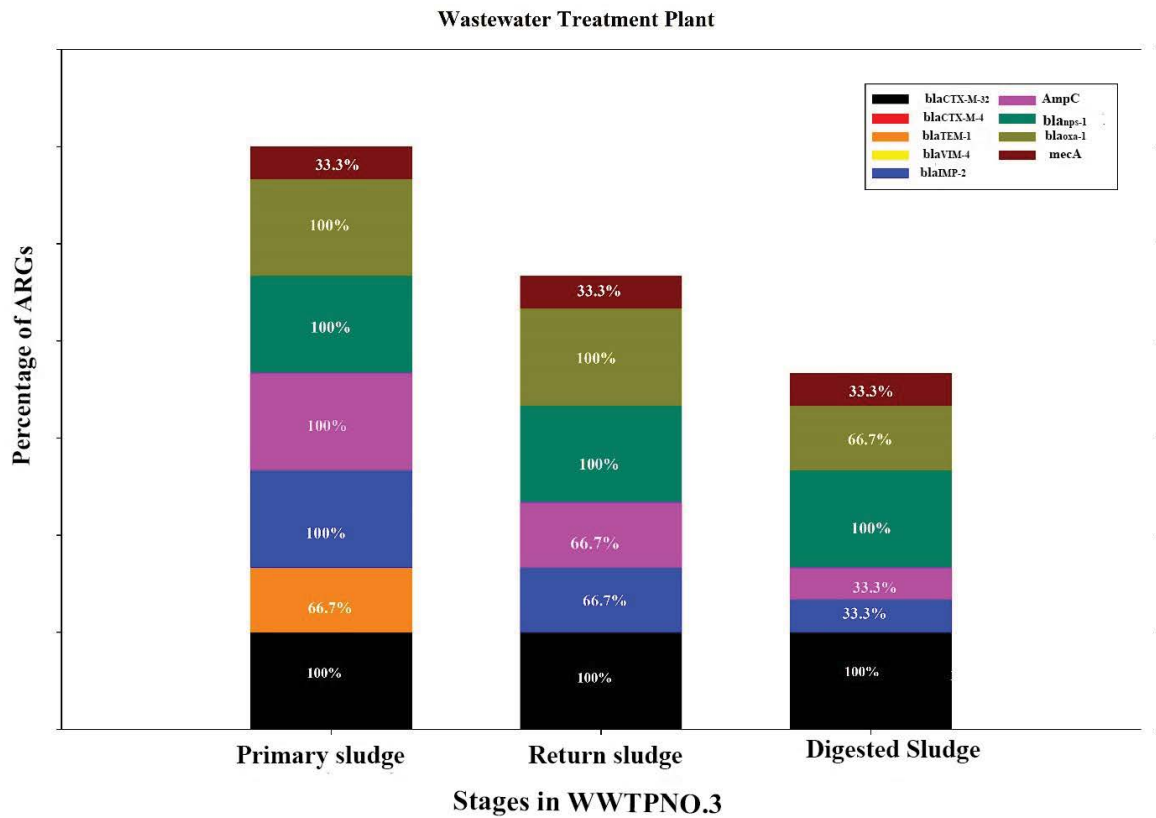
Fig. 5. Percentage frequency distribution of β-lactam-resistant genes in different sludge treatment units.
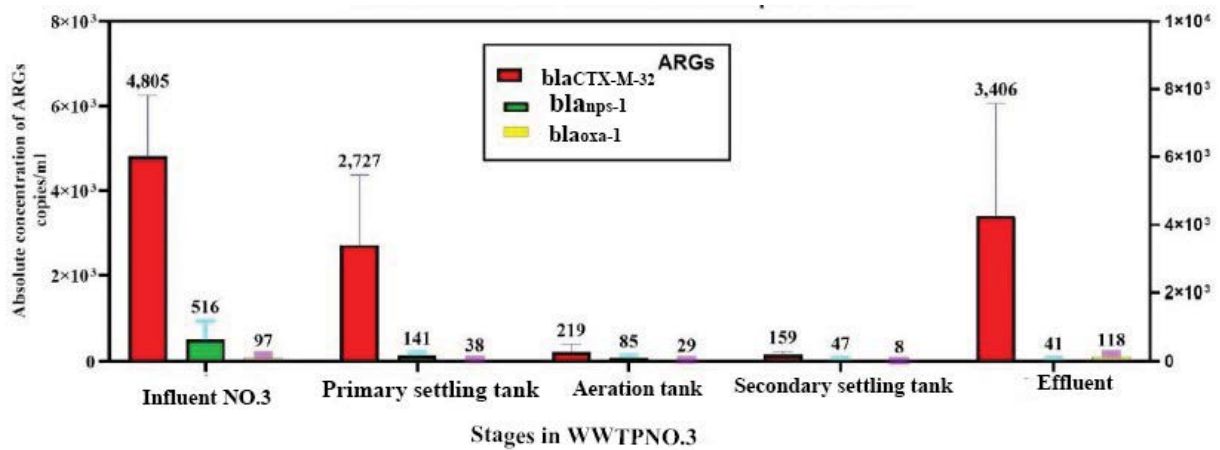

Fig. 6. Quantitative amount of β-lactam-resistant genes through different units of wastewater treatment plant.

3.3. Deletion rate of resistant genes in the influent and effluent units of treatment plant

According to the results, the highest and the lowest deletion rate was related to genes bla_{nps-1} (90.61%) and $bla_{CTX-M-32}$ (65.93%), respectively. Fig. 9 shows these reduction rates in the chlorination stage. In contrast to the decreasing trend in these two genes, bla_{oxa-1} gene was increased in the effluent unit by around 45.12% indicating the inefficiency of the chlorination unit for the removal of this gene.

3.4. Relationship between three selected genes and physicochemical parameters in wastewater treatment plant

According to Spearman's correlation test, it was concluded that the studied genes increase with the increase in chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), coliform bacteria, and residual chlorine. At the same time, there is a reverse relationship between gene bla_{oxa-1} and these physico-chemical parameters of wastewater.

3.5. Sequencing results

3.5.1. Results of wastewater sequencing analysis

Sequencing analysis and building the phylogenetic trees were performed using 16S rRNA gene sequencing. The obtained sequences were compared to the sequences in GenBank [37]. The results of the comparison showed that the isolated colony at the influent showed the highest sequence (100%) similarity with LR738964.1_*Escherichia coli* sp. (Fig. 8). In the secondary sedimentation unit, the maximum homology (100%) was with MW090899.1_*Enterobacter hormaechei* sp. (Fig. 9), but at the effluent after chlorination unit, there was 95% similarity between the isolated gene and MN582959.1_*Pseudomonas entomophila* (Fig. 10).

3.5.2. Sequencing results of sludge treatment unit in treatment plant

Sequencing analysis and the phylogenetic trees for the isolated colony in primary sludge treatment showed the maximum homology (100%) with HM548453.1 *Salmonella*

enterica subsp species (Fig. 11). For the sludge digestion unit, 55% similarity was found with MT539080.1 *Klebsiella pneumoniae* subsp species (Fig. 12).

4. Discussion

It is now proved that ARB and ARGs are ubiquitous in nature and are found in high concentration ranges in hospital, domestic, industrial, and agricultural wastewater [38]. Firstly, the presence, the persistence, and the spread of these resistant bacteria and genes in the environment lead to an increase in infection with these pathogens, and secondly, the release of these resistant bacteria and genes increase the reservoir of ARGs in the environment, thereby transferring resistant genes [39]. Numerous studies have shown that DNA encoding antibiotic resistance can be transported to susceptible organisms through conjugation and other transmission mechanisms [40]. The production of β-lactamase which hydrolyzes antibiotics is the most important mechanism of resistance to β-lactam antibiotics. Over the past 20 years, many new
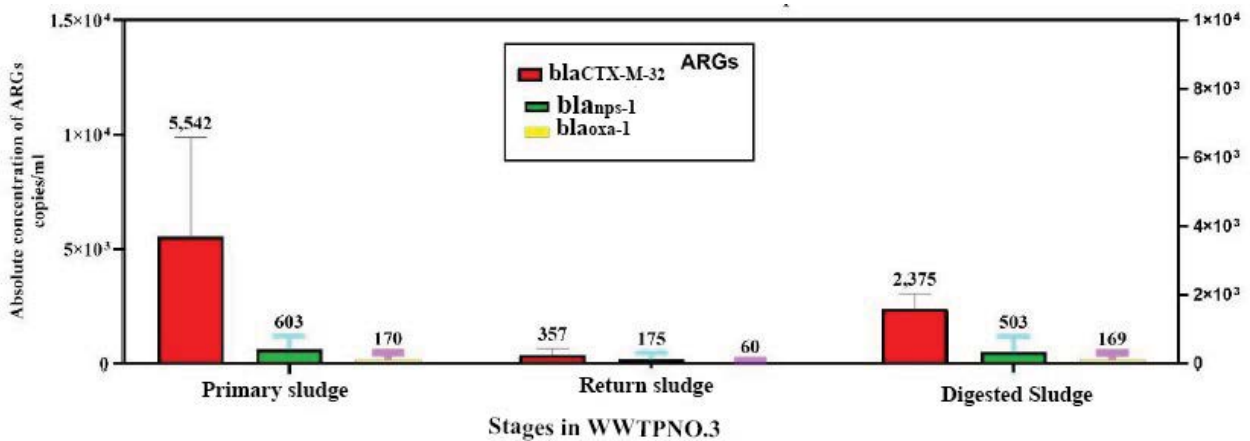

Fig. 7. Quantitative amount of β-lactam-resistant genes in sludge treatment units.
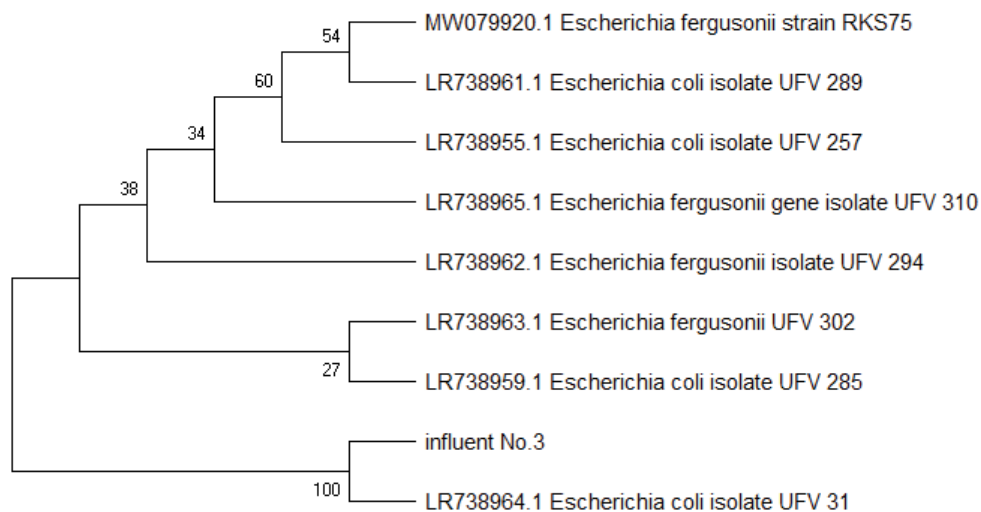

Fig. 8. The phylogenetic tree for isolated colony from influent of treatment plant based on sequencing analysis by 16S rRNA.
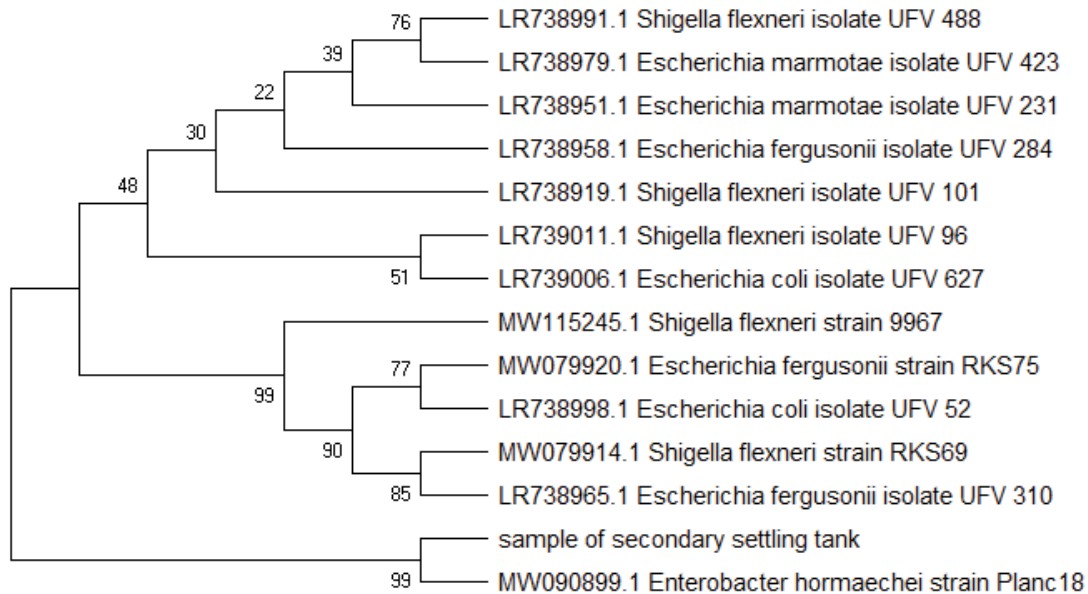
Fig. 9. The phylogenetic tree for isolated colony from secondary sedimentation stage of treatment plant based on sequencing analysis by 16S rRNA.
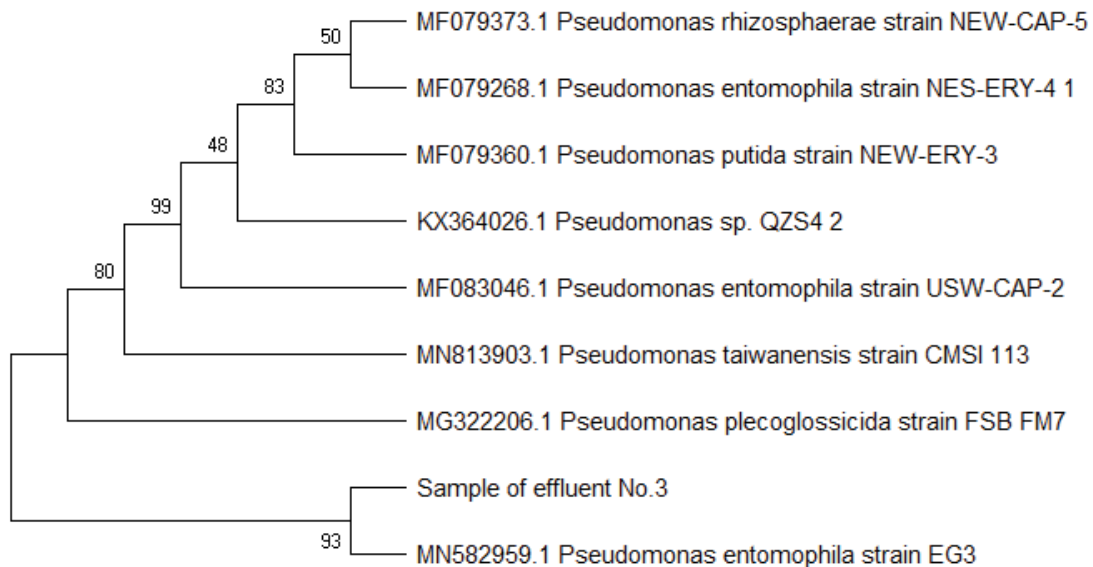

Fig. 10. The phylogenetic tree for isolated colony from effluent of treatment plant based on sequencing analysis by 16S rRNA.

β-lactam antibiotics resistant to the hydrolyzing activity of β-lactamase have been introduced. *E. coli* and *Klebsiella* spp. are the most important microorganisms for the production of ESBLs [41]. The reason for the spread of the ESBL phenotype is the high consumption of broad-spectrum Penicillins and Cephalosporins. β-lactams were the most commonly used antibiotics (23%) from 2015 to 2018 [8]. According to Abbasi et al. [9] study in Qom, the most commonly prescribed antibiotics were ceftriaxone (20.7%), ampicillin (11.9%), and meropenem (7.5%), all of which belong to the β-lactam group, indicating the high consumption of this group of antibiotics. bla_{CTX-M} and bla_{TEM-1} genes are the most common forms of β-lactamase

in *Enterobacteriaceae*, including *E. coli* [42]. In the present study, $bla_{CTX-M-32}$ belonging to the β-lactamase group was observed through water treatment plant and decreased up to 65.93% at the effluent. Jäger et al. [4] reported a 99.4% (from 2.73×10^6 to 1.5×10^4) decrease in $bla_{CTX-M-32}$ gene by activated sludge process in a treatment plant in Germany which is consistent with this study. In addition, another β-lactamase gene, bla_{TEM-1}, was completely removed at the effluent of treatment plant. In the study performed by Jäger et al. [4], this gene was decreased but not completely eliminated (1.22×10^5). In this study, *mecA* showed a declining trend and was minimal at the effluent (4.7×10^1), consistent with the study by Jäger et al. [4] in Germany. In

Table 6
Relationship between three selected genes and physico-chemical parameters of wastewater treatment plant by Spearman correlation coefficient

| Relationship between resistant genes, physical and chemical parameters in wastewater through Spearman correlation coefficient						
Sample points	Resistant genes	COD	BOD	TSS	Coliform bacteria	Remaining chlorine
Influent	$bla_{CTX-M-32}$	0.498	**$r > 0.99$	***$r > 0.99$	0.500	–
	bla_{oxa-1}	**$r > 0.99$	0.496	0.500	0.499	–
	bla_{nps-1}	0.496	**$r > 0.99$	**$r > 0.99$	0.497	–
After chlorination	$bla_{CTX-M-32}$	0.453	0.457	0.866	0.493	0.491
	bla_{oxa-1}	–0.466	–0.486	–0.866	–0.482	–0.483
	bla_{nps-1}	0.471	0.476	0.866	0.494	**$r > 0.99$

*$p < 0.05$;
**$p < 0.01$.
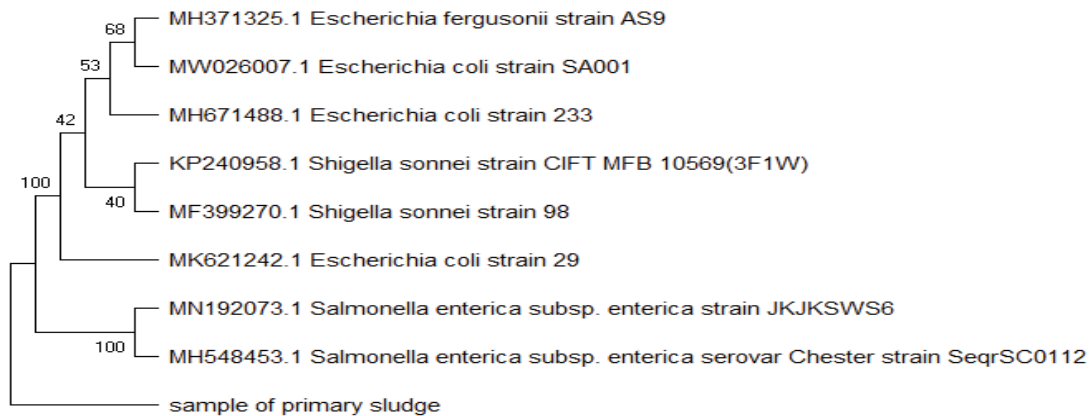
Fig. 11. The phylogenetic tree for isolated colony from primary sludge unit based on sequencing analysis by 16S rRNA.
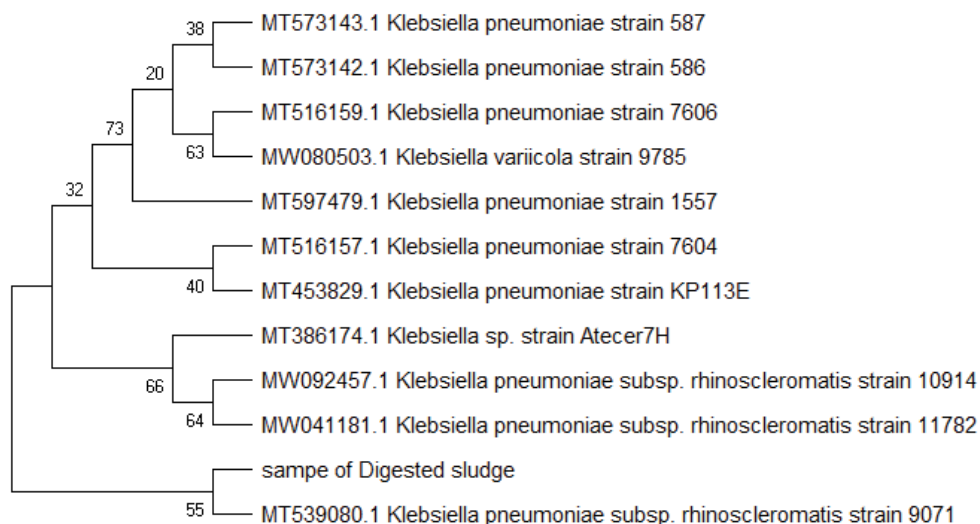

Fig. 12. The phylogenetic tree for isolated colony from sludge digestion unit based on sequencing analysis by 16S rRNA.

Thakali et al. [43], *mecA* was entirely eliminated by chlorination. In this study, the bla_{VIM-4} gene was not identified which can be attributed to the low consumption of this type of antibiotic such as meropenem in Qom. In contrast, in the study in the Netherlands, this gene was observed with a frequency of 4% at the treatment plant effluent [22]. Furthermore, the bla_{IMP-2} gene showed a decreasing trend through treatment process, while this gene was not

present at the effluent of the treatment plant studied by Tianjin et al. [25]. bla_{oxa-1} gene was observed in all treatment stages and showed 45.12% increase at the effluent of the treatment plant. This gene belongs to ESBLs and was found in *Pseudomonas* in samples collected from treatment plants in Turkey and France [44]. In this research, *Pseudomonas entomophila* was observed in the results of the phylogenetic tree interpretation at the effluent of treatment plant. The genus *Pseudomonas* belongs to the phylum *Proteobacteria*, the class Gamma *Proteobacteria*, the order *Pseudomonadales*, the family *Pseudomonadaceae*, and the bacterium species *Pseudomonas putida*. *P. putida* species are found in soil and water and are rarely isolated from human beings. According to studies, this species can develop resistance to antibiotics like β-lactams, aminoglycosides, macrolides, polyketides, and sulfonamides. This bacterium is gram-negative, rod-shaped, aerobic, nonspore-forming, motile with polar flagella, oxidase-positive and catalase-positive [45]. In a study conducted by Zagui et al. [23], the frequency of *Pseudomonas aeruginosa* in a treatment plant in Brazil was 22%. It is worth mentioning that various factors such as integrons cause the transfer of ARGs through horizontal gene transfer (HGT). In the study by Zerva et al. [46], it was found that *int1* is involved in the distribution of resistant genes such as *sul1* and *ermF*. It is worth mentioning that some resistant bacteria and genes increase after the chlorination stage. The amount of chlorine used in the treatment plant and the contact time are important factors for controlling ARBs. Studies showed that high chlorine concentration with short contact time would be beneficial to reduce ARB levels. In contrast, lower concentrations with longer contact times can help ARB regrow and be activated in the water distribution system. The contact time of 30 min is typically chosen for balancing costs and benefits in conventional treatment plants [47]. Moreover, lower levels of COD and SS, higher DO values, proper pH, and temperature would contribute to the inactivation of ARGs. Recent studies demonstrated that such factors directly affect the occurrence and diversity of ARGs [48]. Zieliński et al. [49] observed a significant, positive, and relationship between bla_{oxa}, bla_{SHV}, *sul2*, *mefA*, and *tetA* genes and factors such as COD and BOD. Results of the present study indicated that by decreasing COD, BOD, and TSS through the studied wastewater treatment, $bla_{CTX-M-32}$ and bla_{nps-1} genes decreased, but bla_{oxa-1} gene increased. The reason for this increase and the factors leading to the emergence and spread of antibiotic resistance during disinfection is unclear. The results of this study indicated that conventional biological treatment processes are not efficient enough in reducing bacteria and ARGs [50]. Zerva et al. studied different types of bacteria and showed that although chlorination reduces coliform and filamentous bacteria, it does not reduce denitrifiers, such as *Acidovorax*, *Pseudomonas*, and *Thauera*. It was also found that chlorination had no effect on *Chloroflexi* with dechlorination capability and bacteria involved in enhanced biological phosphorus removal, such as *Candidatus accumulibacter* and *Candidatus competibacter* [51]. Thus, the use of membranes and advanced oxidation processes may reduce ARGs [52].

5. Conclusion

It is reported that the amount of intracellular and extracellular ARG (plasmids) is increased which may pose a potential risk to public health. Results demonstrated that chlorination promotes the direct transformation of plasmids. Through direct transformation, ARGs are released in the form of naked DNA from dead bacteria by chlorination, and eventually, their number will be increased. According to the obtained results, the amount of ARGs, including β-lactam-resistant genes, has increased after the rapid sand filtration stage. Furthermore, the genes are likely to become resistant to antibiotics and disinfectants. Likely, the bacteria have entered the secondary growth phase during the chlorination stage, after the settling stage, thereby increasing the number of ARGs. The produced sludge contains pathogens such as *Salmonella* and *Klebsiella*. If such sludge is spread on farmland, it will increase the environmental pathogens and thus can exacerbate the risk of transmitting infection and disease to human beings and animals. Many of the pathogens potentially found in sludge are important in the biogeochemical cycle. The entry of these agents into the environment endangers public health. Therefore, equipping treatment plants with advanced processes can be useful in reducing bacterial-resistant species in the human life cycle. All in all, due to the complexity of the factors affecting drug resistance in aquatic environments, extensive research is needed to monitor the emergence and rise of antibiotic resistance in pathogenic bacteria in order to improve and maintain human health.

Ethical issues

The authors certify that all data collected during the study is presented in this manuscript, and no data from the study has been or will be published separately.

Competing interests

The authors have declared that they have no conflict of interests.

Authors' contribution

All authors contributed in the study design, data collection, analysis, and interpretation. All authors reviewed and approved the manuscript.

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