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Original Article

The rate of resistance to tetracyclines and distribution of *tetA, tetB, tetC, tetD, tetE, tetG, tetJ* and *tetY* genes in *Enterobacteriaceae* isolated from Azerbaijan, Iran during 2017

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Abstract

Introduction: *Enterobacteriaceae* are the heterogeneous group of Gram-negative bacteria, which cause different infections. The incidence of resistance to antibiotics among the *Enterobacteriaceae* is growing. This study investigated antibiotic resistance features and tetracycline resistance genes distribution in *Enterobacteriaceae* isolates from Hospitals of Azerbaijan, Iran.

Methods: The disc diffusion agar and agar dilution methods were used for assessment of antibiotics susceptibility patterns and minimum inhibitory concentration determination of tetracycline and minocycline. To detect eight tetracycline resistance genes (*tetA, tetB*, *tetC, tetD, tetE, tetG, tetJ, and tetY*), the PCR was performed in tetracycline-resistant isolates.

Results: The resistance rate to tetracycline, minocycline, doxycycline, and tigecycline by the disc diffusion agar method were 58.8%, 24%, 43.6% and 0.4%, respectively. Fifty-one (20.4%) isolates were multiple drugs resistant. The minimum inhibitory concentration results showed 52% resistance to tetracycline and 22% for minocycline. The percentage of *tet* genes distribution was *tetA* (14.4%), *tetB* (18.4%), *tetC* (2%) and *tetD* (4.4%). However, *tetE, tetG, tetJ* and *tetY* genes were not detected in the present study.

Conclusion: There is a moderate-high resistance rate to tetracycline among *Enterobacteriaceae* in Azerbaijan. The most effective antibiotic against *Enterobacteriaceae* was tigecycline followed by fosfomycin, imipenem and meropenem. The *tet* genes family especially *tetA* and *tetB* were prevalent among tetracycline-resistant isolates.

Keywords:
Enterobacteriaceae;
Tetracycline;
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Introduction

Enterobacteriaceae are the most common Gram-negative bacteria in microbiology laboratories and have an important role in the incidence of nosocomial infections, pneumoniae and local infections after surgery and septicemia (Sadeghi et al., 2016). The

most prominent pathogenic genera to human are included *Escherichia coli*, *Klebsiella pneumoniae,* Citrobacter spp.*,* Proteus spp. and Morganella spp. (Stecher et al., 2012). *E. coli* and *K. pneumoniae* are more isolated from infections caused by *Enterobacteriaceae* in particular bloodstream infections (Kim et al., 2002). Common treatments for Gram-negative bacterial infections are aminoglycosides, beta-lactams, fluoroquinolones and tetracyclines (Hawkey and Finch, 2007).

Tetracyclines are bacteriostatic antibiotics and can attach to the ribosome 30s subunit and prevent protein synthesis. They were discovered in the 1940s and were rigorously active against Gram-positive and Gram-negative bacteria and protozoan parasites (Chopra and Roberts, 2001). Amon the most well-known tetracyclines, it can be referred to tetracycline, minocycline, doxycycline and tigecycline (Connell et al., 2003). Doxycycline as a second generation tetracycline has high absorbance rate and lipophilic properties (Sloan and Scheinfeld, 2008). Minocycline is a bacteriostatic agent as well. Its performance mechanism is similar to other tetracyclines (Ritchie and Garavaglia-Wilson, 2014). Tigecycline as a new tetracycline is obtained from the combination of 9-t-butylglycylamido side chain to minocycline due to a high affinity to ribosome (Deng et al., 2014). Tigecycline is more used against the Gram-negative bacteria (Livermore, 2005). The prescription range of tetracyclines is too wide including human and animals infections and even plants (Aminov et al., 2001). The price of tetracyclines is declining due to pharmaceutical advancement, so they are considered to be the most favorite and cost-effective antibiotics around the world. Tetracyclines alone or in part with other antimicrobial agents administrate to the treatment of infections caused by *Enterobacteriaceae* (Hirsch and Tam, 2010). The infections caused by *E. coli* have been treated with tetracycline, doxycycline and minocycline (Cunha, 2012).

Before the mid-1950s, most bacteria were sensitive to tetracycline. The mechanisms of resistance to tetracycline include the decreased penetration, efflux pumps, ribosomal protection, target alternation and enzymatic modifications. Several plasmid genes contribute to resistance, which is known as *otr* and *tet* genes. Twenty-nine *tet* genes are known that to be named in the English alphabet and belong to a major facilitator superfamily which encodes the dependent membrane proteins. These proteins drove out the tetracyclines and protect ribosome (Chopra and Roberts, 2001). All detected *tet* genes are related to efflux pump and ribosomal protection mechanisms except the *tetX* gene, which belongs to the enzymatic alteration mechanism (Ng et al., 2001). Tetracyclines are not the first-line prescribed drugs for the treatment of *Enterobacteriaceae* infections, but increasing resistance to first-line drugs has turned them into an alternative treatment for these infections (Horcajada et al., 2014).

In the present study, the antibiotic resistance features and the distribution of tetracycline resistance genes in *Enterobacteriaceae* isolated from Azerbaijan Hospitals were investigated.

Materials and methods

Bacterial isolation and identification

A total of 250 *Enterobacteriaceae* isolates were gathered from clinical specimens. The organisms were identified by the microscopic feature and the differential tests such as indole production, urease, phenylalanine deaminase, glucose and lactose fermentation, motility test, methyl red, Voges Proskauer, citrate consumption and H_2S production (Hansen et al., 2004). Finally, they were stored in tryptic soy broth medium including glycerol and preserved at -70°C freezers (Rohman et al., 2013). Informed consent was obtained from all human adult participants and from the parents or legal guardians of minors. The Ethic Committee of Tabriz University of Medical Sciences approved this study (Number: Ir.tbzmed.rec.1396.638).

The disk diffusion agar method

The disk diffusion susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Jorgensen and Turnidge, 2015). Antimicrobial disks that were used in this study, purchased from “Mast” (UK) included ampicillin, piperacillin/tazobactam, imipenem, co-amoxiclav, cefepime, ciprofloxacin, ceftazidime, nitrofurantoin, meropenem, aztreonam, cotrimoxazole, fosfomycin, gentamicin, amikacin, nalidixic acid, cefazolin, tetracycline, doxycycline, minocycline and tigecycline (Hudzicki, 2009). *E. coli* ATCC (American type culture collection) 25922 was used as a quality control strain.

Table 1: Distribution of resistance rates of isolates by the DDA method

Antibiotics	Sensitive	Intermediate	Resistant
AMP	3%	0%	97%
PTZ	60.4%	25.2%	14.4%
IMP	95.6%	0.8%	3.6%
AUG	43.2%	27.6%	29.2%
FEP	48.9%	11.9%	39.3%
CIP	41.6%	8.4%	50%
CAZ	58.4%	8.4%	33.2%
FM	69.2%	5.2%	25.6%
MEP	95.2%	0.8%	4%
ATM	46.8%	8.4%	44.8%
SXT	27.4%	0%	72.6%
FOS	98%	0.4%	1.6%
GN	62.4%	4%	37.2%
AN	87.4%	3.7%	8.9%
NA	30%	3.6%	66.4%
CFZ	17.2%	13.2%	69.6%
TE	40.4%	0.8%	58.8%
DTX	38.4%	18%	43.6%
MN	58.4 %	17.6%	24%
TGC	97.6%	2%	0.4%

AMP: ampicillin, PTZ: piperacillin/tazobactam, IMP: imipenem, AUG: augmentin (amoxicillin/clavulanate), FEP: cefepime, CIP: ciprofloxacin, CAZ: Ceftazidime, FM: nitrofurantoin, MEP: meropenem, ATM: aztreonam, SXT: cotrimoxazole, FOS: fosfomycin, GN: gentamicin, AN:amikacin, NA: Nalidixic acid, CFZ: cefazolin, TE: tetracycline, DTX: doxycycline, MN: minocycline, TGC: tigecycline.

Minimum inhibitory concentration (MIC) determination

In order to achieve MIC rate in *Enterobacteriaceae* isolates to tetracycline and minocycline, the agar dilution method was conducted. The results were interpreted according to the CLSI guidelines (Andrews, 2001). *E. coli* ATCC 25922 was used as a quality control strain.

PCR

To detect tetracycline resistance genes (*tetA, tetB,tetC, tetD, tetE, tetG, tetJ,* and *tetY)*, DNA of organisms, which were resistant to tetracycline by the MIC method, were extracted by the boiling method (Zhang and Stewart, 2000). Then, the PCR was performed for screening of eight *tet* genes as previously described (Aminov et al., 2002). PCR products were evaluated by electrophoresis for 60 min on a 1.5% agarose gel at 85 Vand after staining with 0.5µg/ml ethidium bromide visualized under UV light (Akhi et al., 2017).

Statistical analysis

The data were evaluated by q2, Fisher exact test and qualitative statistics (percentages) using the SPSS software 24 (Washington, the USA), version 22.

Results

In the present study, 250 non-duplicated *Enterobacteriaceae* isolates were obtained from 104 males (41.6%) and 146 females (58.4%). The mean

Table 2: The MIC ranges, MIC_{50}, and MIC_{90} of antibiotics

Antibiotic	MIC range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Tetracycline	1-64	32	64
Minocycline	1-64	4	16

Table 3: Tetracyclines resistance patterns in identified isolates by the DDA method

Antibiotics / Bacteria	Tetracycline	Doxycycline	Minocycline	Tigecycline
***Escherichia coli,* n (%)**	117 (65)	116 (64.4)	68 (37.8)	1 (0.5)
***Klebsiella pneumoniae,* n (%)**	19 (41.30)	25 (54.34)	25 (54.34)	4 (8.69)
***Enterobacter cloacae,* n (%)**	5 (45.45)	7 (63.63)	4 (36.36)	0 (0)
***Proteus mirabilis,* n (%)**	3 (100)	3 (100)	3 (100)	0 (0)
***Shigella sonnei,* n (%)**	1 (50)	1 (50)	0 (0)	0 (0)
***Shigella flexneri,* n (%)**	2 (100)	1 (50)	1 (50)	0 (0)
***Klebsiella oxytoca* n, (%)**	0 (0)	0 (0)	1 (50)	0 (0)
***Proteus vulgaris,* n (%)**	1 (100)	0 (0)	0 (0)	0 (0)
***Morganell amorganii,* n (%)**	0 (0)	0 (0)	1 (50)	1 (50)
***Citrobacter ferundii,* n (%)**	1 (100)	1 (100)	1 (100)	0 (0)

Escherichia coli, 180 isolates; *Klebsiella pneumoniae,* 46 isolates; *Enterobacter cloacae,* 11 isolates; *Proteus mirabilis,* 3 isolates, *Shigella sonnei,* 2 isolates; *Shigella flexneri,* 2 isolates; *Morganella morganii,* 2 isolates; *Klebsiella oxytoca*, 2 isolates ; *Citrobacter ferundii,* 1 isolate, *Proteus vulgaris,* 1 isolate

age of patients was 52+13 years. Overall, isolates were collected from internal ward (61.1%) intensive care unit (11.6%), surgery ward (15.2%), pediatrics ward (6%) and the burn ward (5.6%). The isolates were collected from urine (71.6%), blood (14%), wound (7.6%), fecal (1.6%), trachea (1.6%), sputum (1.6%), peritonea (1.2%) and cerebrospinal fluid (0.8%). The most common bacterial isolates was *E. coli* (72%) and followed by *K. pneumoniae* (18.4%), *Enterobacter cloacae* (4.4%), *Proteus mirabilis* (1.2%), *Shigella sonnei* (0.8%), *Shigella flexneri* (0.8%), *Klebsiella oxytoca* (0.8%), *Morganella morganii* (0.8%), *Proteus vulgaris* (0.4%) and *Citrobacter ferundii* (0.4%). According to the disk diffusion agar, the highest resistance rate was observed to ampicillin (97%), cotrimoxazole (72.6%), cefazolin (69.6%), nalidixic acid (66.4%), tetracycline (58.8%), ciprofloxacin (50%), aztreonam (44.8%), doxycycline (43.6%), cefepime (39.3%), gentamicin (37.2%), ceftazidime (33.2%), amoxicillin/clavulanate (29.2%), nitrofurantoin (25.6%), minocycline (24%), piperacillin/tazobactam (14.4%), amikacin (8.9%), meropenem (4%), imipenem (3.6%), fosfomycin (1.6%) and tigecycline (0.4%). Table 1 shows antibiotic resistance patterns of *Enterobacteriaceae* isolates. Fifty-one (20.4%) isolates were multidrug resistance. The MIC_{50} and MIC_{90} were respectively 32µg/ml and 64µg/ml for tetracycline and 4µg/ml and 16µg/ml for minocycline (Table 2). According to MICs value, the frequency of resistance to tetracycline and minocycline was 52% and 22%, respectively. The frequency of resistance to tetracyclines was shown in Table 3. To find the tetracycline resistance genes (*tetA, tetB, tetC, tetD, tetE, tetG, tetJ* and *tetY*), the

Table 4: Distribution of *tet* genes in resistant tetracycline isolates

Bacteria, no	*tetA* n (%)	*tetB* n (%)	*tetC* n (%)	*tetD* n (%)
***Escherichia coli*, 117**	29 (24.78)	37 (31.62)	2 (1.70)	5 (4.27)
***Klebsiella pneumoniae*, 19**	6 (31.57)	8 (42.10)	3 (15.78)	4 (21.05)
***Enterobacter cloacae*, 5**	0 (0)	1 (20)	0 (0)	1 (20)
***Proteus mirabilis*, 3**	1 (33.33)	0 (0)	0 (0)	1 (33.33)

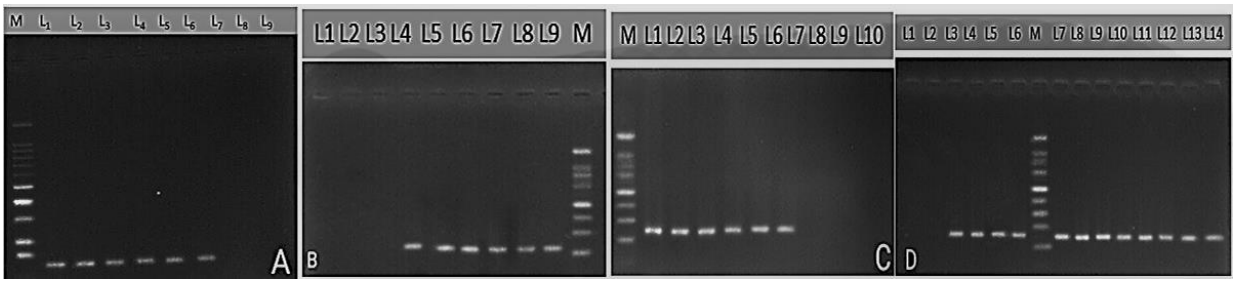

Fig.1. The PCR patterns for *Enterobacteriacae*, all PCR products separated in 1.5% agarose gel. Lane M contained the DNA size marker (100 bp). A: *tetA* gene in (164 bp), L1: positive control, $L_{2, 3, 4, 5, \text{and } 6}$: positive isolates, L_7: negative control, $L_{8 \text{ and } 9}$: negative isolates. B: *tetB* gene, (206 bp),L_1: negative control, $L_{2 \text{ and } 3}$negative isolates, $L_{4, 5, 6, 8, \text{and } 9}$: positive isolates, L_7: positive control. C: *tetC* gene, (207 bp), L_1: positive control, $L_{2, 3, 4, 5, \text{and } 6}$: positive isolates, $L_{7, 8, \text{and } 9}$ negative isolates, L_{10}: negative control. D: *tetD* gene, (187 bp), L_1: negative control, L_2: negative isolate, $L_{3,4,5,7,8,9,10,11,12,13,\text{and } 14}$: positive isolates, L_6: positive control.

isolates which were resistant to tetracycline by the MIC method were tracked to find these genes through the PCR method. The distribution rate of *tet* genes was found to be *tetA* (14.4%), *tetB* (18.4%), *tetC* (2%) and *tetD* (4.4%) (Table 4); however, there was no trace of *tetE, tetG, tetJ* and *tetY* genes (Fig. 1).

Discussion

Enterobacteriaceae are important causes of urinary tract infections, bloodstream infections, hospital and healthcare-associated pneumonias as well as various intra-abdominal infections. The emergence and spread of resistance in *Enterobacteriaceae* are complicating the treatment of serious nosocomial infections and threatening to create species resistant to all currently available agents (Paterson, 2006).

According to the results of the antibiotic susceptibility patterns, the high frequency of resistance was found to some β-lactams, sulfamethoxazole, tetracyclines, quinolones and aminoglycosides. This finding is similar to another study from Iran (Sadeghi et al., 2016) and other countries (Akram et al., 2007; Yadav et al., 2015). Tigecycline, amikacin, carbapenems and fosfomycin were most effective antimicrobial agents in this study. Carbapenems have considered as an important option for the treatment of resistant *Enterobacteriaceae* particularly MDR and ESBLs-producing isolates. The emergence of carbapenem-resistant *Enterobacteriaceae* was reported following the increased use of carbapenems (Gupta et al., 2011). In our study, the resistance to imipenem and meropenem was observed in 3.6% and 4% of isolates, respectively. Other studies reported carbapenem-resistant isolates from Iran and other countries (Hu et al., 2014; Sadeghi et al., 2016). The intensity of tetracycline resistance in *Enterobacteriaceae* has been changed during the past decades (Yezli et al., 2014). In the present study, the resistance rate to tetracycline, doxycycline and minocycline by DDA assay were 58.8%, 43.6% and 24%, respectively. The different frequency of tetracyclines resistance was reported in different countries. Tetracycline resistance rate was reported 93% in *E. coli* isolates from Pakistan (Hussain et al., 2014). Yezli et al. from Saudi Arabia were reported resistance tetracycline from 27.5% to 50% among *Enterobacteriaceae* isolates (Yezli et al., 2014). Differences in the frequency of resistance to

tetracyclines are in result of geographic regions, a difference in the program of infections control and the pattern of tetracyclines usage in clinical medicine and veterinary. Continued prescribing of tetracyclines in clinical setting and veterinary is one of the reasons in the increased rate of tetracyclines resistant isolates.

Tetracycline resistance can be mediated by efflux, ribosomal protection or chemical modification, but the first two mechanisms are the most clinically significant. A variety of resistance determinants may encode these mechanisms. Depending on the species, the tet (A) to tet (E) determinants are generally responsible for tetracycline resistance in *Enterobacteriaceae* (Fluit et al., 2005).

In the present study, among the eight detected tetracycline resistance genes, *tetA, tetB, tetC* and *tetD* were detected in 14.4%, 18.4%, 2%, and 4.4% of resistant isolates, respectively and *tetE, tetG, tetJ* and *tetY* genes not detected. Another study from Iran reported *tetA*, *tetB* and tetG in 28%, 14% and 6% of the tetracycline resistant *Salmonella* spp, respectively (Tajbakhsh et al., 2012). Both *tetA* and *tetB* genes generally were reported as the most common *tet* genes among the tetracycline resistant *Enterobacteriaceae* (Miranda et al., 2003; Tuckman et al., 2007; Tao et al., 2010; Momtaz et al., 2012). Our findings show a remarkable increasing rate in *tetD* gene distribution. In the current study, some of *E. coli* and *K. pneumoniae* resistant isolates have all four *tetA*, *tetB*, *tetC* and *tetD* genes simultaneously; however, the lack of some *tet* genes in a number of resistant isolates might be due to that, these resistant isolates follow other resistance mechanisms. Another similar investigation in South America studied the distribution of 20 members of *tet* family genes and found 24% of *tetA*, 8% of *tetB* and 4% of *tetE* genes; however, *tetC, tetD, tetG, tetJ* and *tetY* genes that coincided with our genes were not found in this study (Miranda et al., 2003). The results of this study indicate that *tet* genes in animal isolates have a similar dispersion rate with human isolates, partly.

In the present study, tigecycline was the most effective antimicrobial agents. Tigecycline is a chemically derivative of minocycline has a bacteriostatic mode of action against broad spectrum organisms (Kelesidis et al., 2008). Tigecycline is a therapeutic option for infections caused by carbapenem-resistant *Enterobacteriaceae* (Pournaras et al., 2011). Tetracycline-resistant *Enterobacteriaceae* isolates were reported by some studies (Fluit et al., 2005; Kumar, 2016). In the present study, resistance to tigecycline was observed in one isolate (0.4%) which was carbapenems resistant but fosfomycin susceptible. Resistance to tigecycline might be predictable to happen through the similar mechanisms that contribute to tetracycline resistance; but, tigecycline is principally unalterable by the commonly occurring resistance mechanisms (Fritsche et al., 2005). According to the PCR results, this tigecycline-resistant *E. coli* had the *tetD* gene. However, there is not reported the clear association between the presence of tetracycline resistance determinants and the resistance to tigecycline in *Enterobacteriaceae* (Fluit et al., 2005). AcrAB efflux pump is an RND-type efflux system that it`s over-expression may be the responsible of tigecycline resistance (Ruzin et al., 2005).

Resistance to antibiotics has become a serious problem for the medical community and has an increased effect on patients, doctors and even the community. Studies at clinical centers largely show that antibiotics are taking longer to be effective and cause the extended hospitalization of the patients, contribute to the increase in mortality rates and heavy financial burden. By recognizing a trend in the increase of resistance rates and by working to prevent it, we can fight against ultimate treatment failure. Availability and understanding of sensitivity and resistance statistics is the main bet to preventing these concurrencies. So in order to solve this problem, more cooperation between the doctors, hospital staff, insurance companies and pharmaceutical companies is of utmost importance. While this study worked with a large number of isolates that were collected from various sources, it would be highly advisable for further research to be undertaken with an even larger sample size and more isolated from other organisms. It seems especially important considering the danger that antibiotic resistance in the laboratory environment and modern microbiology at large poses. Reaching for the correct amount of antibiotics, like tetracyclines, to be prescribed would be crucial in preventing overdoses by the patients or the public.

Due to the distribution of *Enterobacteriaceae* isolates in an environment and even in foods, and tetracycline usage in animals, a bacterial transfer from one place to another easily achieve. On the other hand,

because of having the mobile genetic elements, resistance genes have been transferred rapidly between these organisms and even other species. One probable reason is that some studies selection criteria is diverse and hybridization methods are used as an alternative to PCR. Considering 29 *tet* genes are identified from the *tet* family, means that the screening of other genes from this group can determine their status in *Enterobacteriaceae* and other organisms everywhere in the world.

Conclusion

There is a moderate to high resistance rate to tetracyclines in Azerbaijan, Iran. The most effective tetracyclines against *Enterobacteriaceae* is tigecycline. The *tetA*, *tetB*, *tetC* and *tetD* genes, which act through the efflux pump, are identified as the main cause of tetracycline resistance in *Enterobacteriaceae* isolates; however, resistance to tigecycline occurs through the same resistance mechanisms to tetracycline.

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Conflict of interest

There is no conflict of interest to declare.

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