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Evaluation of Prevalence of Trimethoprim Resistance Genes in Gram Negative Bacilli Isolated from Clinical Specimens of Patients Admitted to the Pars Hospital, Tehran

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ABSTRACT

Trimethoprim is a bacteriostatic and broad-spectrum antibiotic used to treat infections, particularly urinary tract infections. Among *dfrA* genes, five genes including *dfrA5*, *dfrA1*, *dfrA12*, *dfrA7* and *dfrA17* are more important for resistance to trimethoprim. The aim of this study was to evaluate the frequency of encoding trimethoprim resistance genes in clinical specimens isolated from patients hospitalized in the pars Hospital, Tehran, Iran. Two hundred fifty clinical specimens including sputum, blood, body fluid, wound, urine and secrations were cultured on blood agar and EMB. After incubation at 37°C for 24 hours, conventional biochemical tests were used to isolate and identify gram negative bacteria. Then, antibiotic susceptibility test was performed by disk diffusion agar method according to Clinical and Laboratory Standards Institute (CLSI 2020) guideline. Genomic DNA of trimethoprim resistant bacteria was extracted and polymerase chain reaction (PCR) was performed to detect the resistance genes. Hundred gram negative bacteria including *Escherichia coli* (42%), *Pseudomonas* (15%), *Klebsiella* (24%) and *Acinetobacter* (19%) isolated from clinical specimens. Based on the PCR results, *dfr1* gene followed by *dfr5* and *dfr17*, was the most frequent among trimethoprim resistant gram negative bacteria. The presence of dfr genes plays an important role in antibiotic resistance to trimethoprim

1. Introduction

Trimethoprim is a bacteriostatic and broad-spectrum antibiotic (Aggeliki et al., 2008) that interferes with function of dihydrofolate reductase enzyme and inhibits the production of tetrahydrofolic acid (Arabi, 2015). Tetrahydrofolic acid is a necessary cofactor in the production of thymidine and DNA (Bou et al., 2000). Trimethoprim very selectively act on prokaryotic bacterial cells, leaving mammalian cells unaffected. Sulfonamide cannot interact with mammalian cells because these cells do not synthesize folic acid, and thus have no dihydropteroate synthase target enzyme. Instead they take up folic acid from their environment, which most bacteria cannot do because they lack a transport system for this purpose (Ola Skold, 2001). Resistance to sulfonamides has been associated with five main mechanisms, including a permeability barrier, a naturally insensitive intrinsic DHFR, spontaneous chromosomal mutations in the intrinsic DHPS (folP) and DHFR (folA) genes involved in the

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folic acid pathways, increased production of the sensitive target enzyme by upregulation of gene expression or gene duplication, and the acquisition of alternative DHPS (*sul*) and DHFR (*dfr*) genes with integrons, plasmids, and transposons (Schrijver et al., 2018). Trimethoprim resistant gene is present on both plasmids and bacterial chromosomes (Braun-Fahrländer et al., 2002). Among several resistance mechanisms, the acquirement of DHFR variants encoded by dfr genes is the most common mechanism for TMP resistance, which results in high-level resistance in various bacteria (Seputiené et al., 2010). To date, more than 30 different dfr genes are known, which are usually found in gene cassettes within integrons (Cambray et al., 2010; Seputiené et al., 2010). *DFR* gene is divided into families A and B, including *dfrA* and *dfrB*, which is comprised of at least 20 *dfrAs*. Among *dfrA* genes, its five genes including $dfrA_5$, $dfrA_1$, $dfrA_{12}$, $dfrA_7$ and $dfrA_{17}$ are more important for resistance to trimethoprim (Grape et al., 2007). Our knowledge of resistance to sulphonamides and trimethoprim is few in developing countries. Some reports indicate that prevalence of resistance in Enterobacteriaceae from these countries is about 33%-96%, meanwhile in developed countries it is about 3.5%-7% (Jin et al., 2009). Despite antibiotics are widely used in treatment.; and, the likelihood of resistance to antibiotics is increasing. Assuming that the majority of clinical specimens possess trimethoprim resistant genes and there are few studies on prevalence of trimethoprim resistant genes in Iran, thus the aim of this study was to evaluate the presence of *dfr* genes in gram-negative bacilli isolated from clinical specimens from patients hospitalized in the Pars Hospital, Tehran, Iran.

2. Materials and Methods

2.1. Sampling

The studied population included all patients admitted to the intensive care unit of Pars Hospital, Tehran, Iran, from January to February 2016. Two hundred fifty clinical specimens were taken from patients including sputum, blood, body fluid, wound, urine and secretions. Then, specimens were immediately transported to the lab. All specimens were cultured on EMB and blood agar and incubated at 37°C for 24 h. To identify the isolated bacteria gram staining and biochemical tests (Varghese and Joy 2014).

2.2. Antibiotic Susceptibility test

Antibiotic susceptibility test of the isolates to trimethoprim (200 µg/ml; ROSCO Company) was determined by the Kirby Bauer disk diffusion method according to the CLSI 2020 guidelines by the Kirby Bauer disk diffusion method on Mueller-Hinton agar. Plates were incubated at 37℃ for 24 hours.

2.3. DNA extraction

Genomic DNA of the isolated gram- negative bacteria was extracted by using a DNA extraction kit, (Pooyagene Azma Company, Iran), according to the standard protocol contained in the kit.

2.4. Polymerase Chain Reaction

Dfr gene specific primers were designed by using the sequences in the NCBI Gene Bank and studies. Accuracy and specificity of primers was analysed and confirmed by using the Primer Express, Mega 7 and BLAST software in NCBI Gene Bank. Table 1 shows the sequences of the designed primers.

Table 1. primers used in this study

Tm	bp	primer	name
49	425	F: TGGTAGCTATATCGAAGAATGGAGT R: TATGTTAGAGGCGAAGTCTTGGGTA	dfr1
50	341	F: AGCTACTCTTTAAAGCCTTGACGTA R: GTGTTGCTCAAAAACAACTTCG	dfr5
52	155	F: GAGCTGAGATATACACTCTGGCACT R: GTACGGAATTACAGCTTGAATGGT	dfr12
50	289	F: ACATTTGACTCTATGGGTGTTCTTC R: AAACTGTTCAAAAACCAAATTGAA	dfr17

Amplification of genomic DNA was carried out in 50 µL volumes consisting 12.5 µl 2X Master Mix Amplicon, 10 pmol of each of the primers, 10 ng genomic DNA and 9.5 µl distilled water in 25 µl final volume. The PCR amplification was done with 35 cycles of initial denaturation at 94°C for 5 min, 30 cycles (each of 30s at 94°C, 49°C (annealing dfr1), 50°C (annealing dfr5), 52°C (annealing dfr12), 50°C (annealing dfr17) For 20 seconds, 72°C for 30 seconds), and a final extension at 72°C for 7 min. The PCR products were detected by electrophoresis on a

1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and staining with ethidium bromide (0.5 ug/ml). The results were analysed using SPSS 22 software.

3. Results

In a two-month period, 215 urines, sputum, wound and blood samples were collected from the patients admitted to Pars Hospital (Figure 1). Of all isolated specimens, 56 cases were isolated from women and 44 cases were isolated from men. A total of one hundred resistant trimethoprim gram -negative isolates were isolated.

The isolated gram negative bacteria belonged to 4 genus of gram negative bacteria including *Escherichia coli* (42%), *Pseudomonas* (15%), *Klebsiella* (24%) and *Acinetobacter* (19%).

3.1. PCR Results

In this study, hundred gram negative bacteria resistant to trimethoprim isolated of clinical specimens. Characterization of dfr has been performed by amplification of four parts of gene using in PCR.These four gene including dfr1, dfr5, dfr 12, dfr 17with approximate molecular weightsof 425, 341, and 155, 289 bp (Fig. 2).

Polymerase Chain reaction results of 42 *E. coli* strains isolated from clinical specimens revealed that 24, 28, 6 and 24 strains had dfr1, dfr5, dfr12 and dfr17 respectively. Among *E. coli* strains, 5 strains had none of the dfrA genes and one strain had all genes (Figure 3).

Of 19 *Acinetobacter* strains isolated, 11strains had *dfr1*, 6 stains had *dfr5*, 1 strain had *dfr12* and 6 strains had *dfr17* (Figure 4). Among the *acinetobacters*, 3 strains had none of *dfr* genes.

Out of 24 *Klebsiella* isolates, 16 strains with *dfr1*, 13 strains with *dfr5*, and 10 strains with *dfr17* detected, and dfr*A12* was not found in *Klebsiella* (Figure 5).

Of fifteen *Pseudomonas* strains, presence of *dfr1*gene in 7 strains, *dfr5* gene in 6 strains, and *dfr17* gene in 11 strains were detected (Figure 6). Among the *Pseudomonas*, 3 strains did not have any of *dfr* genes.

It was found that 11% of trimethoprim resistant bacteria lacked any of the *dfrA* genes; in other words, 89% of the trimethoprim-resistant bacteria had at least one gene of *dfrA*. There is a significant relationship between *dfrA* genes in bacteria (P <0.031) (Figure 6).

In this study, it was found that a number of bacteria only had a trimethoprim resistant gene; 14% of bacteria only had *dfrA1*, 12% of resistant bacteria only had *dfrA5*, and 7% of bacteria had only *dfrA17* and there was not *dfrA12* alone in any of the bacteria.

Of the isolated samples, only 18% simultaneously had *dfrA1*, *dfrA5* and *dfrA17* genes and this difference was significant and generalizable to the whole population (P <0.04).

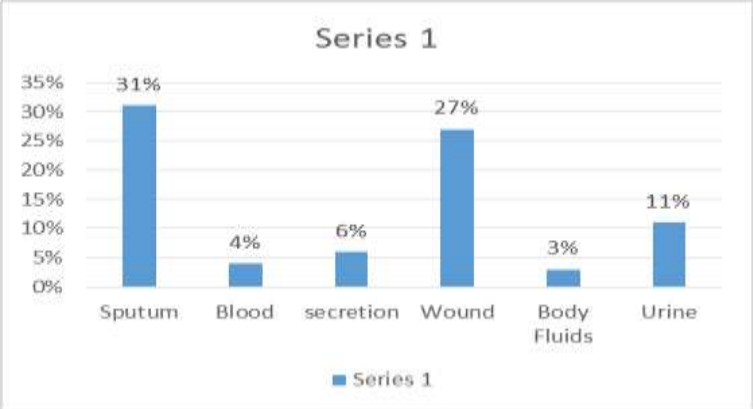

Figure 1: Percentage of samples taken from clinical specimens

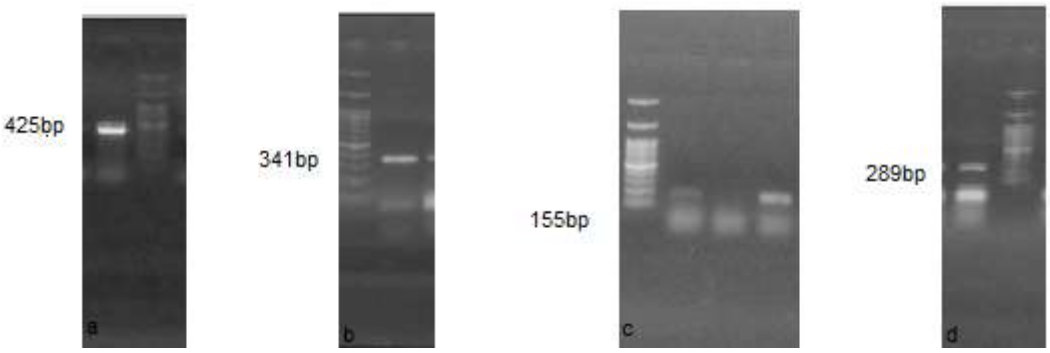

Figure 2: PCR product; a) dfr1; b) dfr5; c) dfr12; d) dfr17

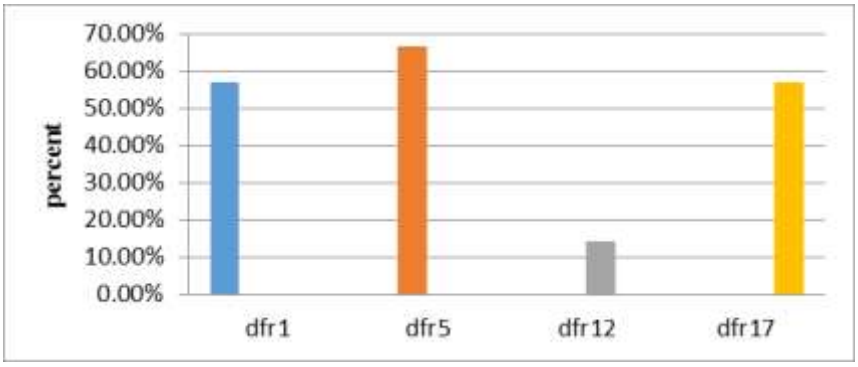

Figure 3: frequency of dfr gene in *E. coli*; numbers are in percentage

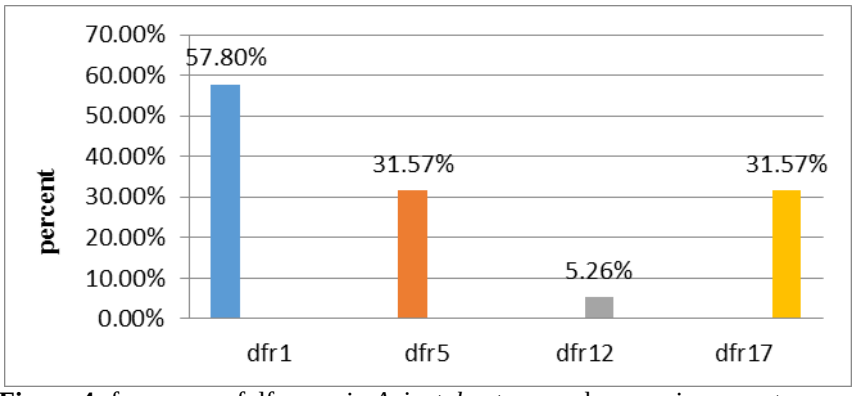

Figure 4: frequency of dfr gene in *Acinetobacter*; numbers are in percentage

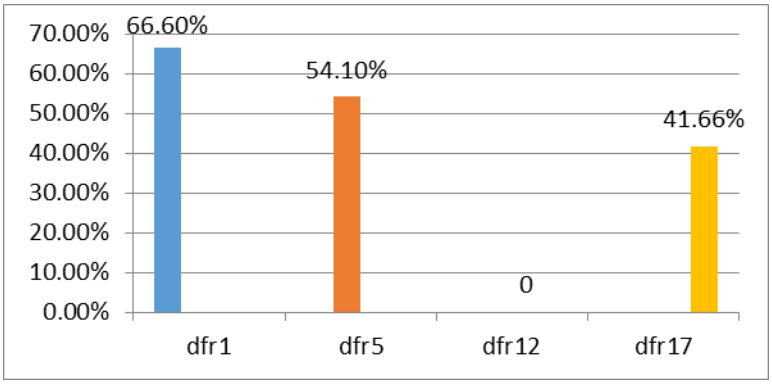

Figure 5: frequency of dfr gene in Klebsiella; numbers are in percentage

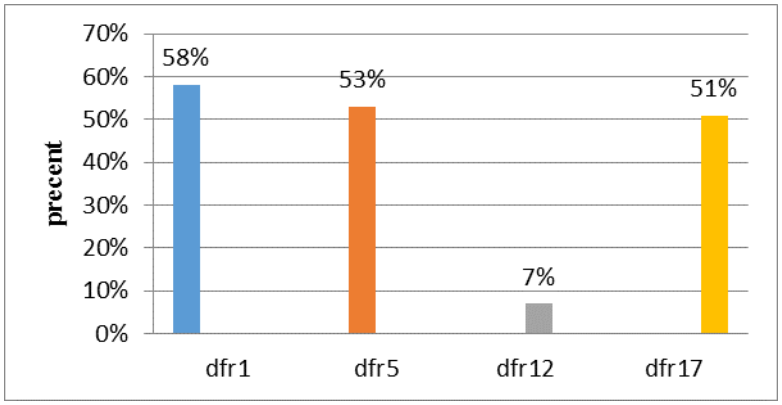

Figure 6: Frequency of dfrA genes in the isolated bacteria

4. Discussion

Infections caused by bacteria are considered as an important factor in threatening the health of people (Jin et al., 2009). Nosocomial infections appear in a patient under medical care in the hospital. With increasing infections, there is an increase in prolonged hospital stay, long term disability, increased antimicrobial resistance and increased mortality rate (Ahmed khan et al., 2017).Incidence of nosocomial infections depends on a number of factors, including the immunity in patients, the use of invasive instruments to tissue, such as catheters and sondage, as well as the resistance of bacteria causing infection to different types of antibiotics and various antimicrobial agents (Karah et al., 2008). The highest and the lowest frequency among gram-negative bacteria in this study were related *to Escherichia coli* (42%) and *Pseudomonas* (15%), respectively. In a study conducted by Didgar in Iran, 3321 samples were taken from blood, urine, ulcer, respiratory secretions and cerebrospinal fluid. Of these, the most common bacterium was *Escherichia coli* and the least common was *Acinetobacter* (Didgar et al., 2014), which is partially consistent with Our finding. In different studies ,among nosocomial pathogens *Escherichia coli* had the highest rates particularly among urinary tract infections (Pommier et al., 2019). Blahna examined the role of horizontal gene transfer in development of resistance to trimethoprim-sulfamethoxazole in Uropathogenic *E. coli* in Europe and Canada. He reported that 37.9% of the total population studied had dfr1 gene. They reported that horizontal gene transfer plays a very important role in transfer of resistance genes between bacteria (Blahna et al., 2006). Lee examined the prevalence of Trimotroperm-

resistant dihydrofolate reductase genes in *Escherichia coli* isolates in Korea; 77 strains of *Escherichia coli* were isolated. They indicated that 72 of strains contained *dfr17*, which had the highest frequency among the *dfr* genes, followed by *dfr12* and *dfr5*, respectively (Lee et al., 2001). In this study, the highest frequency was related to dfr1 (58%), followed by *dfr5* (53%) and *dfr17* (51%), which is consistent with previous studies. Yu examined the presence of integrons and genes existing on it in the *E. coli* isolated from urine specimens. Yu claimed that these strains had integron 1 and integron 2 but lacked integron 3. They reported that all specimens (100%) had *dfr17*, *dfr12* and *dfr5* genes (Yu et al 2003). Moreover, Brolund reported the frequency of dfrA1 gene at 96% in *E. coli* and 96% in *Klebsiella* (Brolund et al., 2010). In this study, the highest frequency of dfr gene in *Escherichia coli* was related to *dfr5* gene, which is completely consistent with other studies such as Lee and Blanha. In this study, the highest frequency of *dfr* gene was related to *dfr17* gene (73.3%) in *Pseudomonas* and *dfr1* (66.66%) in *Klebsiella* and *dfr1* (57.8%) in *Acinetobacter*. There are few reports of antibiotic resistant genes on integron and its transmission between bacterial strains; however, it is completely clear that two new dfr genes which cause antibiotic resistance in the bacterium are on the gene cassette located on integron. The *dfr17* gene is one of the genes found in the gene cassette (Chang et al., 2011). This gene is located precisely in the protected area and has a strong promoter with very high expression level. This gene, along with *dfr5* and *dfr12*, is located in class 1 integron, while the dfr1 gene is related to class 2 integrons. Through the studies, it has been found that class 2 integrons have been more prevalent than class 1 integrons in strains resistant to trimethoprim (Domínguez et al., 2018). There is still no solid evidence that why presence of *dfr12* gene is lower in trimethoprim resistant strains than the other *dfrA* genes (Al-Assil et al., 2013). The stability over time and throughout the intervention suggests that the epidemiological fitness cost of the most common dfr-genes or of plasmids carrying these genes is very low (Domínguez et al., 2018). The fact that *dfrA* and *dfrB* were indicates possible future shifts in the *dfr*-gene distribution. Multiple *dfr*-genes occur but are unusual. The PCR screening performedfor *dfrA1*, *dfrA5*, *dfrA7*, *dfrA8*, *dfrA1*, *dfrA14* and *dfrA17* detected coexistence of two genes in four out of 320 *E. coli* isolates and one out of 54 *K. pneumoniae* isolates (Faltyn et al., 2019). The prevalence of dfr-genes and integrons in *K. pneumoniae* has not been studied systematically before and was surprisingly different from *E. coli*. The low prevalence of integrons class I and II could suggest other mechanisms at play or that transfer of *dfr*-genes in *K. pneumoniae* does not occur (Bhosle et al 2020, Dandachi et al 2018). Recent studies in mice showed that plasmids carrying varying resistance genes were easily transferred from an *E. coli* donor to *K. pneumoniae* in a mouse model. Trimethoprim resistance is often associated with other resistance determinants. This gives the possibility for co-selection of trimethoprim resistant strains and plasmids carrying both *dfr*-genes as well as other resistance determinants by the use of other antibiotic classes (Brolund et al., 2010).

In this study, which was conducted on gram-negative bacteria resistant to trimethoprim, it was found that 89% of strains had *dfrA* genes responsible for resistance to trimethoprim, and 11% of the bacteria studied did not have any of these genes. Therefore, it was found that the presence of *dfrA* genes plays an important role in antibiotic resistance to trimethoprim. Other factors responsible for trimethoprim resistance should be studied to determine the strategies for preventing the transfer of resistance genes between bacteria.

Refereces

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