

ORIGINAL ARTICLE

# Multi-Drug Resistance Patterns in Bacteria Isolated from Various Sources upon Common Related Virulence Factors by PCR in Iran

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## ARTICLE INFO

### Keywords:

Multi-drug resistance

Bacteria

PCR

Iran

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Received: February 5, 2018

Revised: November 12, 2018

Accepted: November 28, 2018

## ABSTRACT

**Background:** The current study was conducted to investigate the frequency of resistance in the bacteria isolated from various sources, in Shiraz, Iran. Acquisition of new resistance genes is an important factor in the increasing incidence of resistant strains. A critical feature of resistance gene transfer is their stability to adapt rapidly to a new host and make serious consequences.

**Methods:** A total of 520 samples were chosen from human and animal sources in order to investigate the frequency of antibiotics resistance mobile genes using PCR assay.

**Results:** The rates of 70%, 52%, 16.5%, 8.5%, 8%, 4%, 9.2% and 6.8% were confirmed for several genes including *tetO*, *tetA*, *tetB*, *tetM*, *tetR*, *gyrA*, *blaz*, and *blaSHV*, respectively. Our results have revealed a pool of mobile genetic elements in the bacteria isolated from various sources in Iran.

**Conclusion:** Our findings indicated un-regulated use of antibiotics in the food production chains which require more investigation.

Please cite this article as: Berizi E, Sayyadi M, Golkari H, Shahed A. Multi-Drug Resistance Patterns in Bacteria Isolated from Various Sources upon Common Related Virulence Factors by PCR in Iran. Int J Nutr Sci 2019;4(1):43-48. doi: 10.30476/IJNS.2019.81435.1003.

## Introduction

The widespread use of antibiotics in humans and animals has raised several concerns related to the public health issues. Additionally, using antimicrobial agents as growth promoters in animals resulted in considerable increase in the population of resistant bacteria in animals and humans (1). The prevalence of antimicrobial multi-drug resistance among various food borne pathogens has considerably increased during last decade's (2).

Transferring the multi-drug resistant in bacteria is a phenomenon depending basically on mobile genetic elements such as plasmids, transposons and gene determinants in the integrons. As such, the mechanisms of resistance acquisition in a bacterial cell are closely related to the origin of the isolates (3).

Mechanisms involved in the antibiotic resistance have developed to inactivation of enzymatic functions (such as beta lactamases, phosphotransferase).

Limitation of the bacterial entrance (caused likely by penicillin binding protein), antibiotic efflux (e.g. ATP-dependent efflux system), and genetically target modification (for instance due to the methylation of DNA) has eventually led to a crisis in antibiotic resistance (4). The theory of the dissemination of resistant bacteria and resistant genes via the food chain was previously addressed, where, the identification of antibiotic resistant mobile elements were shown in bacteria that colonize both in animals and humans (5).

In the current study, multiple antimicrobial-resistant genotypes were observed in various human and animal sources in Shiraz, Iran. Little is known about the phenotypic and genotypic characteristics of antimicrobial resistance in food borne pathogens, especially in developing countries. As the result of the inappropriate use of several antibiotics for different purposes such as growth promotion and prevention of infectious diseases which finally led to increase the rates of antimicrobial resistance (6). In human, the appearance of resistant strains of bacteria cause them more virulent and thus need longer periods of time for treatment. Eventually, as a major consequence, treatment failure is remained problematic in the developing countries (7).

In Iran, antibiotic resistance has been reported to occur in human, including a cross sectional study of *Staphylococcus aureus* (8), using a combined PCR and immune assay in the susceptible bacteria to beta-lactams and *Clostridium difficile*. The isolates were then characterized using ribotyping, PCR to detect *tcdA*, *tcdB* and *cdtB* genes (9). The aim of this study was to determine the prevalence of some resistance genes of public health hazard in different bacteria such as *S. aureus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Cl. difficile* and *Escherichia coli* isolated from various sources in Shiraz, Iran.

## Materials and Methods

From September to December 2017, two hundred fecal samples from poultry were collected before

being selectively sub-cultured for campylobacter. Additionally, 100 *S. aureus* isolated from different animals and human sources were used as follow, 120 poultry carcasses for *E. coli* and 100 fecal and carcasses for *C. difficile* and *Cl. perfringens*. The procedures for isolation of the microorganisms were based on the Food and Drug Administration (USA). The isolates were further identified using the species-specific primers (Table 1). Details of mobile genetic amplicons corresponding to each antibiotic were presented in Table 2.

For DNA extraction, the specimens were initially homogenized using a sterilized blender. One gram of the preparation was then homogenized in nine mL of normal saline. DNA extraction was carried out using the DNA extraction kit, as was recommended (Qiagen, Tehran, Iran). Briefly, the samples were centrifuged at 10,000 g for 10 min. The supernatants were discarded before adding 250 µL of buffer 1 (resuspension solution contained 100 µg/mL RNase) and 250 µL of buffer 2 (Lysis buffer). Totally, 550 µL saturated phenol was then added, mixed thoroughly and centrifuged at 8,000 g for 5 min. The supernatant was collected into a new micro-centrifuge tube; the same volume of the phenol was added and centrifuged at the same speed.

The clear phase was collected into a new tube, before adding sodium acetate (2M, pH~5.2). The aliquot was mixed with 1.5 mL 100% ethanol, kept at -20°C for 1 h, centrifuged at 12,000 g, the supernatant was then discarded and the DNA pellet was washed using 80% ethanol, before being dried and resuspended in 30 µL TAE until further use. The concentration of DNA was subsequently estimated by absorbance at 260 nm and the purity of DNA was checked by taking the ratio of OD reading at 260 nm and 280 nm using a UV visible spectrophotometer (Biophotometer 6131, Eppendorf AG, Germany).

The specificity of primers was confirmed by amplification of 100 ng purified DNA/µL as positive (Positive control for *C. jejuni*, *Cl. difficile*, *S. aureus*,

**Table 1:** Bacterial isolates confirmed by species- specific primers.

Bacterial Spp	Sequence (5'-3')		Target gene	Annealing	Amplicon size (bp)	Ref.
	Forward	Reverse				
<i>S. aureus</i>	ATAGAGATGCTGGTA-CAGG	GCTTCCGATTGTTC-GATGC	<i>coa</i>	57°C	720	(22)
<i>Cl. difficile</i>	GTGTACAATGAAAGTC-CAAGTTTACGCG	CACTTAGCTCTTTGATT-GCTGCACT	<i>tcdA</i>	55°C	1200	(23)
<i>C. jejuni</i>	CTA TTT TATTTT TGA GTG CTT GTG	GCT TTA TTT GCC ATT TGTTTT ATT	<i>mapA</i>	59°C	589	(24)
<i>Cl. perfringens</i> (type A)	GGAGATGGTTGGATATT-AGG	GGACCAGCAGTTGTA-GATA	<i>cpe</i>	55°C	233	(25)
<i>E. coli</i>	CTT CGG TAT CCT ATT CCC GG	GGA TGC ATC TCT GGT CAT TG	<i>stx2</i>	56°C	484	(26)

**Table 2:** Primers used for specification of various bacterial isolates.

Antimicrobial family, virulence genes, or genetic group	Genetic marker	Sequence (5'-3')		Annealing	Amplicon size (bp)	Ref.
		Forward	Reverse			
<i>Beta-lactams</i>	<i>Blaz</i>	CAAAGAT-GATATAGTTGCT-TATTCTCC	TGCTTGACCACTTT-TATCAGC	55°C	421	(10)
	<i>bla<sub>SHV</sub></i>	TCGCCTGTGTAT-TATCTCCC	CGCAGATAAATCAC-CACAATG	50°C	768	(27)
<i>Tetracycline</i>	<i>tetA</i>	GTGAAACCCAA-CATACCCC	GAAGGCAAGCAG-GATGTAG	50°C	888	(27)
	<i>tetB</i>	CCTTATCATGC-CAGTCTTGC	ACTGCCGTTTTTTC-GCC	50°C	774	(27)
	<i>tetR</i>	ACAACCCGTA-AACTCGCC	TTCCAATACG-CAACCTAAAG	52°C	366	(28)
	<i>tetM</i>	CTCGAACAAGAG-GAAAGC	GCATTC-CACTTCCCAAC	55°C	778	(29)
	<i>tetO</i>	AACTTAG-GCATTCTGGCTCAC	TCCCCTGTTC-CATATCGTCA	56°C	515	(19)
	<i>Enrofloxacin</i>	<i>gyrA</i>	ATG AGC GAA TTA GCC AAA GA	GCA ACC GTC CAA CAC TTC AT	62°C	150

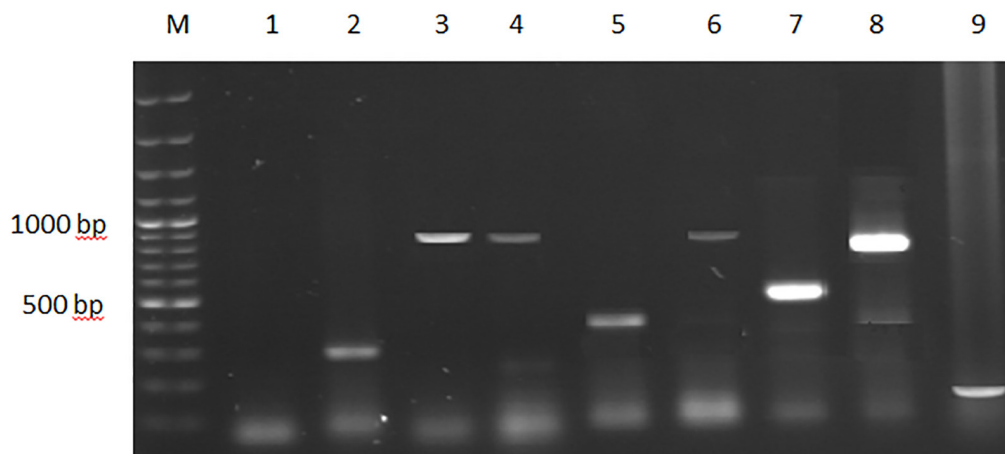
*Cl. perfringens* and *E. coli* were provided by Department of Food Hygiene and Quality Control, Shiraz University, Shiraz Iran) and DNA free water as negative control. PCR was carried out on 2 µL of DNA template in a final reaction mixture of 25 µL containing 2.5 µL of 10x PCR buffer, 3 mM MgCl<sub>2</sub>, 200 mM of each of dNTPs, 400 µM of each of forward and reverse primer, 2 units of Taq DNA polymerase (Cinagene, Iran). The amplified products were subsequently electrophoresed in agarose gel, stained with ethidium bromide, and photographed under UV light.

## Results

Twenty five percent of *E. coli* were confirmed from poultry carcasses that were carrying *tetA*

(18%), *tetM* (6%), *tetR* (8%) and *bla<sub>SHV</sub>* (6.8%) genes. However, in 4% of the isolates, *gyrA* gene corresponding enrofloxacin, were also confirmed. In 18 samples, *C. perfringens* strains were confirmed by *cpe* gene from carcasses of cattle, sheep and goats. The isolates were also screened by PCR (Figure 1) for the presence of *tetA*, *tetB* corresponding to the tetracycline, from which all the strains were carrying both genes. In 12 out of 18 tetracycline resistant strains, patterns of resistance were attributed to one or more of these genes. This study was approved by the Local Ethics Committee.

Of the 100 *S. aureus* species phenotypically and genetically (having thermonuclease and coagulase genes) confirmed from human and animal sources, the frequencies of *blaz* corresponding to the β-lactamase



**Figure 1:** A representative agarose gel analysis using specific primers to detect mobile resistant amplicons; Lane M, DNA ladder; Lane 1, negative control (no template); Lane 2, 360 bp corresponding to *tetR*; Lane 3, 768 bp corresponding to *bla<sub>SHV</sub>*; Lane 4, 778 bp corresponding to *tetM*; Lane 5, 420 bp corresponding to *blaz*; Lane 6, 888 bp corresponding to *tetA*; Lane 7, 515 bp corresponding to *tetO*; Lane 8, 774 bp corresponding to *tetB*; Lane 9, 150 bp corresponding to *gyrA*.

**Table 3:** Prevalence of different antibiotics resistant mobile genes in the bacteria

	<i>tetA</i>	<i>tetB</i>	<i>tetM</i>	<i>tetR</i>	<i>tetO</i>	<i>gyrA</i>	<i>bla<sub>Z</sub></i>	<i>bla<sub>SHV</sub></i>
<i>E.coli</i>	18%	-	6%	8%	-	4%	-	6.8%
<i>S. aureus</i>	-	-	-	-	-	-	9.2%	-
<i>C. jejuni</i>	23%	14%	-	-	70%	-	-	-
<i>Cl. difficile</i>	5%	-	2.5%	-	-	-	-	-
<i>Cl. perfringens</i>	6%	2.5%	-	-	-	-	-	-

gene was 9.2% of all the isolates. Out of 200 fecal samples collected from poultry, 48% of cases were confirmed to be contaminated at least with one of the species of *Campylobacter*, from which 23% and 14% possessed *tetA* and *tetB*, respectively. In broiler chicken, *tetO* was confirmed in 70% of the *C. jejuni* isolates. The *tetA* (5%) and *tetM* (2.5%) genes were detected in 38% of *Cl. difficile* isolated from fecal content of mice. Moreover, 6% and 2.5% of the *Cl. perfringens* isolated from cattle carcasses were respectively carrying *tetA* and *tetB* genes (Table 3).

### Discussion

Un-regulated use of different anti-bacterial agents for therapy and growth promotion has been considered as an emerging problem, worldwide. The study provided a useful insight into the level of resistance of various bacteria confirmed in food animals in Shiraz, southern Iran. In many previous studies, it was shown that the use of antibiotics is a risk factor for the selection of multi-resistant bacteria in both humans and animals (3). Previous studies suggested that conventional methods for monitoring antibiotic susceptibility were not reliable. Problems remained unsolved when it reported the overlapping inhibition zones for *bla<sub>Z</sub>* positive and negative strains of *Staphylococcus* using a disc diffusion technique (10).

Our results indicated alarming multi-resistance frequencies for the bacteria isolated from food animals, where the rates of 70%, 52%, 16.5%, 8.5%, 8%, 4%, 9.2% and 6.8% were confirmed for several resistance genes including *tetO*, *tetA*, *tetB*, *tetM*, *tetR*, *gyrA*, *bla<sub>Z</sub>*, and *bla<sub>SHV</sub>*, respectively. The frequency of various mobile resistance genes was formerly reported from elsewhere, for instances, 58.1% in Greece (11) and 100% in Spain (12). The role of class integrons, the mobile genetic elements that were well known for the efficient spread of antibiotic resistance genes were due to mobilization.

However, the presence of various gene cassettes in the clinically isolated bacteria is of great concern in the horizontal transmission of them (13). This condition was previously reported from Japan and China indicating that such mobile resistance cassette is widespread in Asia (14, 15). Earlier studies in Iran, using an antibiogram test on total of

food samples from animal origins in Sanandaj and Ahvaz, the resistance to tetracycline and ampicillin were respectively confirmed in 55% and 50% of the samples (16). According to reference 37, the distribution of antibiotic-resistance genes in the *E. coli* isolates included *tetA* and *tetB* (52.63%), *dfrA1*, *qnrA*, *catA1* and *cmlA* (36.84%) and *sull* and *ereA* (47.36%), respectively.

High prevalence of three corresponding tetracycline resistance genes, was indicated an extensive unregulated use of tetracyclines in poultry industry in Iran. Nine strains (15.78%) implying the presence of multi-resistant pattern in *E. coli* (17). Moreover, presence of *EAST1*, *STb* and *LT* genes were confirmed in *E. coli* isolated from raw milk and un-pasteurized cheese from Shahrekord, Central area of Iran. This study revealed that 21.66% of the *E. coli* strains isolated from raw milk and unpasteurized cheeses (18). The high frequencies of the main tetracycline resistant genes such as *tetO* and *tetA* in the bacteria isolated from poultry carcasses is of a major concern in the industry, in Iran (19).

The prevalence of antimicrobial resistance in normal microbiota gives a promising solution for understanding the process of antimicrobial-mediated selection in a population (20). Finally, our study demonstrated that the bacteria which investigated here, contained plasmids carrying multi-antibiotic resistance pattern in food producing animals, the fact that previously reported from other areas of the countries, as well which need closer investigations (21).

### Conclusion

Results of our study confirmed the high frequencies of the anti-bacterial resistance genes in the bacteria isolated from various sources, in Iran.

### Acknowledgement

We would like to thank the staff of the Department of Food Hygiene and Quality Control for their technical support.

### Conflict of Interest

None declared.

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