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Detection of tetracycline resistance genes in bacteria isolated from fish farms using polymerase chain reaction

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Abstract

Five common tetracycline resistance genes *tet*(A), *tet*(B), *tet*(M), *tet*(O) and *tet*(S) were studied by polymerase chain reaction in 100 bacteria isolated from Iranian fish farms. In the antibiogram test most of the bacteria were either intermediately or completely resistant to tetracycline. Nine isolates out of 46 *Aeromonas* spp. contained either *tet*(A/M/S) resistant genes as follows: *tet*(A) in *A. veronii/sobria* (n = 1), *A. media* (n = 2), *A. aquariorum* (n = 1), and *A. veronii* (n = 3); *tet*(M) in one isolate of *A. sobria* and *tet*(S) in 1 isolate of *A. jandaei*. In other bacteria, *tet*(A) gene was detected in *Citrobacter freundi* (n = 1), *Pseudomonas putida* (n = 1); *tet*(S) was also identified in *Yersinia ruckeri* (n = 1), *Arthrobacter arilaitensis* (n = 1) and *P. putida* (n = 1). In total, 31 isolates (31.00%) contained the tetracycline resistance genes in which 21 bacteria (21.00%) showed the *tet*(S), nine bacteria (9.00%) contained the *tet*(A) and 1 bacteria (1.00%) was positive for *tet*(M). All of the *L. garvieae* isolates contained *tet*(S) in this study. The most widely distributed resistance gene was gene *tet*(A) and the least known resistance genes was *tet*(M) among the studied bacteria of the genus *Aeromonas* in this study.

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شناسایی ژنهای مقاومت به تتراسیکلین در باکتریهای جدا شده از مزارع پرورش ماهی با استفاده از واکنش زنجیره‌ای پلیمراز

چکیده

پنج ژن مقاومت به تتراسیکلین شامل *tet*(S) *tet*(A), *tet*(B), *tet*(M), *tet*(O) با استفاده از واکنش زنجیره‌ای پلیمراز در یکصد باکتری جدا شده از مزارع پرورش ماهی ایران مورد بررسی قرار گرفت. در آزمایش آنتی بیوگرام بیشتر این باکتریها دارای مقاومت متوسط یا مقاومت کامل به تتراسیکلین بودند. نُه جدایه از ۴۶ گونه *آئروموناس* دارای ژنهای مقاومت *tet*(S) *tet*(A), *tet*(M) بترتیب زیر: *tet*(A) در *آئروموناس ورونئی* بیووار *سوبریا* (یک مورد)، *آئروموناس مدیا* (۲ مورد)، *آئروموناس اکواریوروم* (یک مورد) *آئروموناس ورونئی* (۳ مورد) و *tet*(M) در یک جدایه *آئروموناس سوبریا* و *tet*(S) در یک جدایه *آئروموناس جاندائی* مشاهده شد. در سایر باکتری ها *tet*(A) در *سیتروباکتر فروندی* (یک مورد)، *سودوموناس پوتیدا* (یک مورد) و *tet*(S) در *یرسینیا روکری* (یک مورد)، *آرتروباکتر آریلائیتنسیس* (یک مورد) و *سودوموناس پوتیدا* (یک مورد) مشاهده گردید. بطور کلی ۳۱ جدایه (۳۱/۰۰ درصد) از باکتریها دارای ژنهای مقاومت به تتراسیکلین بودند که از این تعداد ۲۱ باکتری (۲۱/۰۰ درصد) *tet*(S)، ۹ باکتری (۹/۰۰ درصد) *tet*(A) و یک باکتری (۱/۰۰ درصد) *tet*(M) را دارا بودند. تمام ۱۷ جدایه باکتری *لاکتوکوکوس گارویه آ* مورد مطالعه در این تحقیق دارای ژن مقاومت *tet*(S) بودند. ژن *tet*(A) بیشترین ژن مقاومت به تتراسیکلین و ژن *tet*(M) کمترین ژن مقاومت در باکتریهای جنس آئروموناس مورد مطالعه در این تحقیق بودند.

واژه های کلیدی: باکتریها، تتراسیکلین، ژن مقاومت، مزارع پرورش ماهی، واکنش زنجیره ای پلیمراز

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Introduction

Aquaculture has been a growing activity for the last 30 years worldwide and this impressive development has been attended by some practices potentially damaging human and animal health.[1] Synthetic antibiotics have been widely used for human and animals, and misuse of antibiotics in many countries potentially contributes to the emerging and spread of antibiotic resistant bacteria and antibiotics resistance genes in the environment.[2]

Tetracycline is a broad-spectrum antibiotic used in human and animal medicine as well as aquaculture industry and at least 40 different tetracycline resistance genes (*tet*) have been characterized to date.[3] Resistance to tetracycline is governed by *tet* genes, which are involved in either active efflux of the drug, ribosomal protection or enzymatic drug modification.[4] Among the various *tet* genes, the *tet*(A), *tet*(B), *tet*(D), *tet*(E) and *tet*(G) are reported in gram-negative bacteria.[5] Whereas, the *tet*(K), *tet*(L), *tet*(M), *tet*(O), and *tet*(S) are significantly found in the gram-positive bacteria.[6]

Tetracyclines have been greatly used in aquaculture particularly to control furunculosis in salmonids and oxytetracycline is frequently used in most fish farming industries as a prophylactic agent.[7] They are broad-spectrum agents including tetracycline, chlortetracycline, doxycycline and minocycline which exhibit activity against a wide range of gram-positive and -negative bacteria.[8]

In a study conducted in Japan and Korea, 34 isolates including *Vibrio* spp., *Lactococcus garvieae*, *Photobacterium damsela* subsp. *piscicida*, and unidentified gram-positive bacteria out of 151 isolates from fish and aquaculture sites contained *tet*(M) gene. The majority of these bacterial isolates displayed high-level resistance with a minimum inhibitory concentrations (MICs) equal to or greater than 250 μg mL^{-1} of oxytetracycline and only four isolates had MICs less than 31.3 μg mL^{-1}. The *tet*(S) gene was also detected in *L. garvieae* from yellowtail in Japan and in *Vibrio* sp. from seawater in Korea.[9]

In Korea, 54 isolates of tetracycline-resistant *S. parauberis* contained the *tet*(M/O/S) genes, out of which 39 and 12 isolates contained the *tet*(M) and *tet*(S) genes, respectively, whereas three isolates contained both the *tet*(M) and *tet*(S) genes.[10] These studies suggest that the *tet*(S), *tet*(M), *tet*(O) genes of fish and aquaculture from Asia are involved in the acquisition of high-level resistance to tetracycline. In a recent study, 63.3% of *L. garvieae* isolated from diseased rainbow trout in Iran were resistant to oxytetracycline.[11] However, no molecular genetic studies were performed to investigate tetracycline resistance genes in the bacteria from fish farms and fish pathogenic bacteria in Iran. Therefore, the aim of this study was to detect the tetracycline resistant genes in bacterial isolates with aquaculture origin found to be resistant to tetracycline.

Materials and Methods

Bacteria and culture conditions. A total number of 100 bacterial isolates from diseased fish with apparent symptoms of fish diseases and from water samples where the fish were collected from Iranian fish farms were studied in this research. The bacteria were previously identified (unpublished data) based on their phenotypic, biochemical,[12] and 16S rDNA sequencing characteristics. Some 16S rDNA sequencing data were already registered in the GenBank database. *Aeromonas* spp. as a main gram-negative bacteria were accounted for approximately 46.00%, *Lactococcus garvieae* as the main gram-positive bacteria for 17.00% and the other bacteria for 37.00% (Table 1). The isolates were stored at –70 ˚C in tryptic soy broth (TSB; Scharlau Chemie, Barcelona, Spain) containing 10% glycerol (Caldic Deutschland Chemie, Düsseldorf, Germany).

In preparation for antibiotic susceptibility tests, isolates were cultured for 48 hr on brain heart infusion agar (BHI; Quelab Labratories, Montreal, Quebec, Canada). Individual colonies were re-cultured on BHI for ensuring purity of the isolates. Colonies were then suspended in 3 mL TSB and adjusted to 0.5 McFarland (Becton Dickinson, Franklin Lakes, USA) using a colorimeter for use in the antimicrobial susceptibility test.

Antimicrobial susceptibility test. Antimicrobial susceptibility tests were performed according to the Muller Hinton agar methods described by the Clinical and Laboratory Standards Institute as previously reported.[13] One hundred microliter of the 0.5 McFarland of each bacterial suspension were placed on Muller Hinton agar (Scharlau Chemie, Barcelona, Spain) and spread all over the plate. Antibiotic discs, each containing 30 μg tetracycline per disc (Padtan Teb Co., Tehran, Iran), were placed aseptically onto the surface of the seeded plates. Plates were incubated at 25 ˚C until 48 hr and the diameter of the inhibition zone for each bacterium was recorded. The breakpoint of inhibition zone was interpreted as follows: susceptible (S) ≥ 20 mm, intermediate (I) ≥ 8 mm, and resistant (R) ≥ 0 mm.

Polymerase chain reaction (PCR) and sequence analysis. Antimicrobial resistant genes were investigated using a single PCR assay. The stored isolates were cultured in TSB at 28 ˚C for 24 hr and DNA was extracted using boiling method.[14] The DNA extracted by this method was visualized by gel electrophoresis on a 0.9% agarose gel before being stored at –20 ˚C. The oligonucleotide primer sets were used to detect tetracycline resistance genes including *tet*(A), *tet*(B), *tet*(M), *tet*(O), *tet*(S), (Table 2).[15,16]

The primers were commercially synthesized by the CinnaGen Company (Tehran, Iran) (Table 2). Following PCR conditions were applied to each assay: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 μM dNTPs, 20 pmol of each primer, and 2 U *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany) per 50 μL reaction using

4 µL of DNA were extracted as the template. A gradient thermocycler (MG 5331; Eppendorf, Hamburg, Germany) was used to determine an optimal annealing temperature for the specific binding of the primer set to the template DNA. The optimal thermal parameters were as follows: initial denaturation at 94 ˚C for 5 min followed by 30 cycles of denaturation at 94 ˚C for 45 sec, annealing at 55 ˚C for 1 min and extension at 72 ˚C for 1 min. A final extension at 72 ˚C for 5 min at the end of the amplification cycles was included. Each sample was tested at least in duplicate and sterile water was used as a negative control.

The PCR products obtained from different tet genes were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide (1 µg mL^{-1}). The amplification products were visualized under a UV trans-illuminator (Armin Teb Novin Co., Tehran, Iran) and photographed.

The PCR products were directly sequenced using capillary DNA analyzer (ABI 3730; Applied Biosystems, Foster City, USA) after sequencing reactions with a cycle sequencing kit (BigDye Terminator V3.1; Applied Biosystems, Foster City, USA). Sequence generated from different *tet* genes were analyzed to assess the diversity of each gene compared to the GenBank data base using the BLAST program maintained by the National Center for Biotechnology Information (NCBI).[17] Nucleotide sequences of each *tet* gene were aligned using the multiple sequence alignment program CLUSTALW provided by MEGA 4.0 software.[18]

Table 1. Characteristics of the bacterial isolates.

No.	Bacterial isolation codes (No. of isolates)	Bacteria	Source/Tissue	Accession No.
1	1- 21 (21)	*A. veronei bv. sobria*	Fish/Kidney*, Aquarium/Water	JF313389-98 JF313414-15 JF313399
2	141-142 (2)	*A. veronei*	Fish/Kidney	-
3	23-31 (8)	*A. hydrophila*	Fish/Kidney	JF313400-03
4	143-150 (7)	*A. media*	Fish/Kidney	JF313404-07
5	43-44 (2)	*A. sobria*	Fish/Kidney	-
6	58 (1)	*A. caviae*	Aquarium/Water	-
7	59 (1)	*A. caviae/ A. media*	Fish/Kidney	-
8	78 (1)	*A. aquariorum*	Aquarium/Water	JF313412
9	112-113 (2)	*A. bestarium/ A. piscicola*	Fish/Kidney	-
10	73 (1)	*A. jandaei*	Fish/Kidney	JF313413
11	82 (1)	*Plesiomonas shigelloides*	Fish/Kidney	-
12	28-35 (7)	*C. freundi*	Fish/Kidney	-
13	36 (1)	*C. brakii*	Fish/Kidney	-
14	117-122 (6)	*Y. ruckeri*	Fish/Kidney	FJ870985
15	89-90 (2)	*P. putida*	Fish/Skin**	-
16	91-94 (6)	*Pseudomonas spp.*	Fish/Skin	-
17	301-306 (5)	*Microbacterium spp.*	Fish/Skin	-
18	331 (1)	*Vibrio anguillarum*	Fish/Kidney	-
19	311 (1)	*Chryseobacterium aquaticum*	Fish/Skin	FJ514480
20	312 (1)	*Paenibacillus sp.*	Aquarium/Water	FJ666319
21	512 (1)	*Acinetobacter sp.*	Fish/Skin	-
22	513-514 (2)	*Delftio acidovorans*	Fish/Skin	-
23	210-241 (17)	*L. garvieae*	Fish/Kidney	EU727199
24	245 (1)	*Enterococcus faecium*	Fish/Kidney	FJ870986
25	422 (1)	*A. arilaitensis*	Fish/Kidney	-
26	77 (1)	*Arthrobacter sp.*	Fish/Kidney	-

* Bacteria were isolated from fish kidney of the diseased fish (with hemorrhagic septicemia).
** Bacteria were isolated from fish skin of the diseased fish (with skin erosion and fin rot).

Table 2. Oligonucleotide sequences used as primers for polymerase chain reaction.

Gene	Primer name	Primer sequence	Product Size (bp)	References
***tet*(M)**	*Tet*(M)-F *Tet*(M)-R	5'-GTG GAC AAA GGT ACA ACG AG-3' 5'-CGG TAA AGT TCG TCA CAC AC-3'	406	15
***tet*(O)**	*Tet*(O)-F *Tet*(O)-R	5'-AAC TTA GGC ATT CTG GCT CAC-3' 5'-TCC CAC TGT TCC ATA TCG TCA-3'	515	15
***tet*(S)**	*Tet*(S)-F *Tet*(S)-R	5'-CAT AGA CAA GCC GTT GAC C-3' 5'-ATG TTT TTG GAA CGC CAG AG-3'	667	15
***tet*(A)**	*Tet*(A)-F *Tet*(A)-R	5'- GTGAAACCCAACATACCCC-3' 5'-GAAGGCAAGCAGGATGTAG-3'	888	16
***tet*(B)**	*Tet*(B)-F *Tet*(B)-R	5'-CCTTATCATGCCAGTCTTGC-3' 5'-ACTGCCGTTTTTTCGCC-3'	774	16

Results

Resistance to tetracycline. Antimicrobial susceptibility test of the bacterial isolates using tetracycline discs demonstrated strong resistance to tetracycline in several isolates, i.e., *Aeromonas* spp. (5/46), *Citrobacter freundi* (3/7), *Yersinia ruckeri* (3/6), *Pseudomonas putida* (1/2), *Penibacillus* spp. (1/1), *Acinetobacter sp*. (1/1) and *Lactococcus garvieae* (9/17), (Table 3).

Resistant genes. Antibiotic resistant genes detection on 100 bacterial isolates in this study showed 31 isolates (31.00%) contained the tetracycline resistant genes in which 21(21.00%) bacteria showed the *tet*(S), 9 (9.00%) bacteria contained the *tet*(A) and 1 (1.00%) bacteria was positive for *tet*(M). Nine isolates out of 46 *Aeromonas* spp. contained either *tet*(A/M/S) resistant genes as follows: *tet*(A) was detected in four isolate of *A. veronii bv. sobria* (n = 4), *A. media* (n = 2), *A. aquariorum* (n = 1); *tet*(M) in *A. sobria* (n = 1) and *tet*(S) in *A. jandaei* (n = 1). In other bacteria, *tet*(A) gene was identified in *C. freundi* (n = 1), *P. putida* (n = 1); *tet*(S) in *Y. ruckeri* (n = 1), *P. putida* (n = 1), *Arthrobacter arilaitensis* (n = 1) and *L. garvieae* (n = 17). The *tet*(B) and *tet*(O) genes were not detected in any of the examined isolates in our study (Table 3 and Fig. 1).

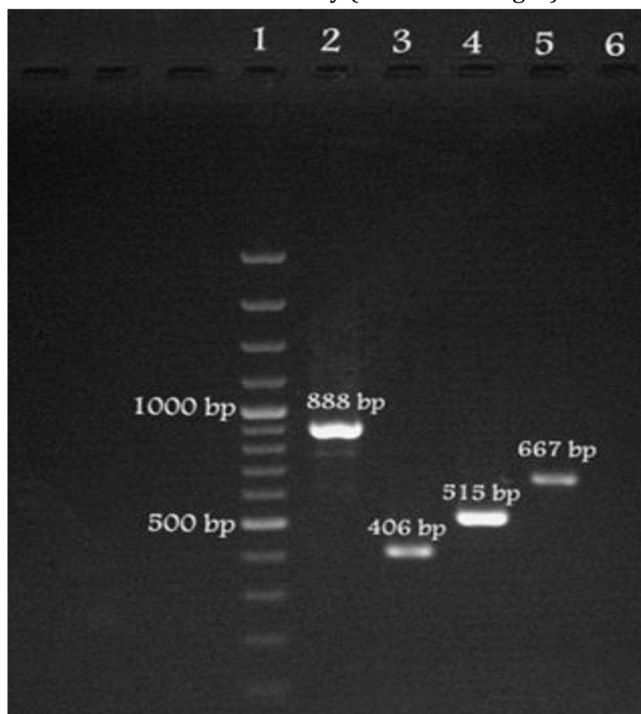

Fig. 1. Electrophoretic analysis of PCR amplified products for tetracycline resistant genes (A, M, O and S). Lane 1, DNA ladder molecular markers; lane 2, *tet*(A) PCR product-888 bp produced from *Pseudomonas putida* strain Fars-110 (JN937120); lane 3, *tet*(M) PCR product-406 bp obtained from *Aeromonas sobria* strain CW4 (JN806155); lane 4, *tet*(O), PCR product-515 bp obtained from *Campylobacter jejuni* strain Shiraz2 (JX853722); lane 5, *tet*(S) PCR product-667 bp produced from *Lactococcus garvieae* strain Iran.1S (JN998084).

Our results showed that the bacterial isolates contained *tet*(A) were completely resistant to tetracycline while those contained *tet*(S) were mostly resistant to tetracycline (Table 4).

Sequence analysis. BLAST search of the Iranian *tet*(A) and *tet*(S) sequences in GenBank showed sequence identities ranging from 99.00% to 100% for these two genes. Whereas, sequence analysis of the obtained *tet*(M) gene from the *A. sobria* (NCBI accession no. JN806155) revealed sequence similarity ranging from 98.00% to 99.00%. Partial sequence obtained for *tet*(M) in this study (349 bp) had a high identity (99.00%) to the known *tet*(M) genes detected in *Streptococcus pneumoniae* (NCBI accession no. FM201786). Our data showed a new genotype for *tet*(M) in Iran that has not been previously described elsewhere in the world.

The sequences of *tet*(A), *tet*(S) and *tet*(M) genes obtained in this study have been deposited in GenBank under accession numbers JN937120, JN998084 and JN806155, respectively.

Discussion

Many mechanisms are involved in tetracycline resistance and three different specific mechanisms have been identified so far: Antibiotic efflux pumps, including *tet*(A), *tet*(B), *tet*(C), *tet*(E) and *te*t(L) target modification with ribosomal protection protein (RPP) including *tet*(M), *tet*(O), *tet*(Q), *tet*(S) and *tet*(W), and antibiotic inactivation *tet*(X).[19] In this study, we examined 100 bacterial isolates from diseased fish and their aquatic environment in Iran for the presence of five tetracycline resistance genes (i.e., *tet*(A, B) as efflux pumps and *tet*(M, O, S) as RPPs class). One type of the efflux pump genes, *tet*(A) and two types of the RPP genes *tet*(M) and *tet*(S) were detected among the isolates. We found no genetic evidence for the presence of *tet*(B) and *tet*(O) in our experiments.

In the present study, the most frequent tetracycline-resistant gene was *tet*(S) (21.00%), followed by *tet*(A) (9.00%) and *tet*(M) (1.00%), (Table 3). In addition, the most widespread determinant in gram-positive and gram-negative bacteria were *tet*(S) and *te*t(A), respectively. Similarly, the *tet*(A) has a broad host range and is often carried by various environmental genera.[20] Some authors previously believed that efflux pump genes (e.g., *tet*(A), *tet*(D), *tet*(E)) are often carried by *Aeromonas* spp. in fish farm ponds,[21] and *Vibrio* spp. in marine environment,[22] and suggested that *tet* genes might have host specificity in different environments. However, recent studies have also demonstrated that *tet* genes are often located on the plasmids and can be horizontally transferred among bacterial strains.[23]

Only one of the isolates, *A. sobria* contained *tet*(M) gene in this study. The limited occurrence of *tet*(M) in the isolates was surprising because this class of tetracycline

Table 3. Resistance pattern of the bacterial isolates by antimicrobial susceptibility test and antibiotic resistance genes.

No.	Bacteria	Total No. of strains	Tetracycline			*tet* genes
			Sensitive (≥ 20 mm)	Intermediate (≥ 8 mm)	Resistant (0 mm < a < 8mm)	
1	***A. veronei bv. sobria***	21	5	13	3	4 *tet*(A)
2	***A. veronei***	2	1	-	1	-
3	***A. hydrophila***	8	5	3	-	-
4	***A. media***	7	1	6	-	2 *tet*(A)
5	***A. sobria***	2	-	1	1	1 *tet*(M)
6	***A. caviae***	1	1		-	-
7	***A. caviae/ A. media***	1	-	1	-	-
8	***A. aquariorum***	1	-	1	-	1 *tet*(A)
9	***A. bestarium/ A. piscicola***	2	1	1	-	-
10	***A. jandaei***	1	-	1	-	1 *tet*(S)
11	***Plesiomonas shigelloides***	1	-	1	-	-
12	***C. freundi***	7	-	4	3	1 *tet*(A)
13	***C. brakii***	1	-	1	-	-
14	***Y. ruckeri***	6	3		3	1 *tet*(S)
15	***P. putida***	2	-	1	1	1 *tet*(S) 1 *tet*(A)
16	***Pseudomonas spp.***	6	2	4	-	-
17	***Microbacterium spp.***	5	1	4	-	-
18	***Vibrio anguillarum***	1		1	-	-
19	***Chryseobacterium aquaticum***	1	-	1	-	-
20	***Paenibacillus sp.***	1	-	-	1	-
21	***Acinetobacter sp.***	1	-	-	1	-
22	***Delftio acidovorans***	2	-	2	-	-
23	***L. garvieae***	17	1	7	9	17 *tet*(S)
24	***Enterococcus faecium***	1	1	-	-	-
25	***A. arilaitensis***	1	1	-	-	1 *tet*(S)
26	***Arthrobacter sp.***	1	1	-	-	-

Table 4. Comparison of active efflux pump pattern *tet*(A) with ribosomal protective protein pattern *tet*(S) in 30 bacterial isolates containing *tet*(A) and *tet*(S) genes.

	***tet*(A, S)**	**Sensitive**	**Intermediate**	**Resistant**	**Total**
1	*tet*(A)	0	0	9	9
2	*tet*(S)	2	1	18	21
Total	-	2	1	27	30

resistant determinant has broadly been described in aquaculture,[9,13] river and channel sediments.[24] Nevertheless, this study reports the occurrence of *tet*(M) in the *A. sobria* for the first time. The occurrence of *tet*(M) in *Enterococcus* spp. isolated from integrated and traditional freshwater fish farm pond in Thailand is also reported.[25] According to current insights, *tet*(M) is the most widely distributed *tet* gene, being detected in at least eight gram-negative and 18 gram-positive genera, including the lactic acid bacterial genera *Enterococcus*, *Streptococcus*, and *Bifidobacterium*.[8] Some studies suggested that the origin of *tet*(M) is most probably the tetracycline-producing species of *Streptomyces* and that its integration into mobile genetic elements (plasmids and transposons) has led to its widespread distribution.[8] The presence of this gene has also been described in a range of bacteria including *Vibrio* spp., *L. garvieae*, *P. damselae* subsp. *piscicida* and some gram-positive bacteria isolated from healthy and diseased fish as well as from seawater in Japan and Korea.[9] These results support the fact that increasing numbers of gram-negative bacteria carry what has been previously considered as gram-positive specific tet genes, such as *tet*(M). Therefore, further epidemiological and molecular investigations are needed to evaluate the presence of genetically mobile antibiotic resistant genes in human and animal food chain.

Recent Iranian genotype of *tet*(M) was placed in genetically distant group, suggesting different origins for dissemination of resistance in this region. According to the published data on bacterial species in marine sediments, the majority of *tet*(M) possessing isolates were belonged to Bacillales (121 strains) Actinomycetales (three strains), Flavobacteriales (one strain) and Pseudomonadales (one strain).[26] This indicates that *tet*(M) is present in various bacterial species in marine sediments, which is the natural reservoir of the *tet*(M) gene. Moreover, our results supported that *tet*(M) might be derived from different origins in environment. Recently, Wu *et al*. carried out a phylogenetic analysis of *tet*(M) in soil and found that all the five *tet*(M) types obtained matched known genes in GenBank,

with sequence identities ranging from 98.00% to 100%.[26]

In our study, the *tet*(A) gene was found in 7 isolates of *Aeromonas* spp. one isolate of *C. freundi* and one isolate of *P. putida* in which all of the strains were resistant to tetracycline. Conversely, the *tet*(S) was also detected in tetracycline-sensitive isolates (n = 2) as well as tetracycline-intermediate isolates (n = 1). These results suggested that there was no direct correlation between the presence of *tet*(S) gene and simultaneous antibiotic resistance. Hence, it was likely that further genetic elements played an important role in antibiotic resistance.

The isolates of *L. garvieae* used in this study were either susceptible (6.00%), intermediate resistance (41.00%) or resistant (52.00%) to tetracycline. In a recent study conducted on *L. garvieae* isolated from diseased rainbow trout culture in 10 fish farms in Chaharmahal and Bakhtyari province of Iran, which is a major trout producing area, it was exhibited that 65.30% and 76.90% of the isolates were resistant to oxy-tetracycline and doxycycline, respectively.[11] Also in a previous study, it was demonstrated a high level of resistance to tetracycline among *L. garvieae* isolated from rainbow trout farms in the south and southwest of Iran during summer 2002 to winter 2008.[27] Our molecular investigations indicated that the continuous rise in the rate of tetracycline resistance in *L. garvieae* in Iran might contribute to the high frequency of *tet*(S) in 100% of our *L. garvieae* isolates in this investigation.

Tian *et al*. showed that Streptococci use two major mechanisms of antibiotic resistance, including efflux by proton antiporters, encoded by the *tet*(L) gene, and ribosomal protection; mediated by the *tet*(M), *tet*(O) and *tet*(S) genes.[28] In the present study, *tet*(S) incidence was only described in *L. garvieae* isolates among ribosome protection types of resistance genes.[28] Historically, the tet(S) gene has predominantly been found in gram-positive bacteria such as *Listeria monocytogenes* and *Enterococcus faecalis* isolated from humans and *L. lactis* isolated from cheese.[9] In gram-negative bacteria, only *Veillonella* spp., an oral bacterium, is known to possess *tet*(S).[29] Now this range is extended and includes a marine Vibrio species as well.[9] In the present study we also found *tet*(S) in additional gram-negative bacteria, i.e., *Y. ruckeri*, *P. putida* and *A. jandaei*.

In conclusion, our findings showed the importance of reducing antibiotic use and the need for appropriate vaccines to prevent the widespread emergence of resistance *L. garvieae*. Fortunately the development of fish vaccines and the use of selected disease resistant stocks nowadays have greatly limited the utilization of antibiotics. This study further emphasized the potential for aquaculture sources to act as a reservoir of antibiotic resistance genes, which could contaminate the environment and water sources as well as aquaculture-produced food products.

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