High-Level Resistance to Erythromycin and Tetracycline and Dissemination of Resistance Determinants among Clinical Enterococci in Iran

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Highlights of the study

- Distribution pattern of *erm* (A-C) was different among enterococci and staphylococci from the same geographic region.
- Alterations at the ribosomal level was more frequently detected in erythromycin and tetracycline resistance than efflux systems.
- Concurrent resistance mechanisms were more involved in resistance to erythromycin than tetracycline.

Keywords

Enterococcus · Erythromycin · Tetracycline · Antibiotic resistance determinants

Abstract

Objectives: The purpose of this study was to investigate the distribution pattern of genes responsible for erythromycin and tetracycline resistance and their association with resistance phenotypes in enterococcus isolates. **Materials and Methods:** Eighty-six *Enterococcus faecalis* and 26 *E. faecium* isolates were collected from 2 hospitals in Kerman, Iran. Minimum inhibitory concentration of erythromycin and tetracycline was determined and then genes encoding resistance to erythromycin – *erm* (A-C), *mef*, and *msr* – and tetracycline – *tet* (M), *tet* (O), *tet* (S), *tet* (K), and *tet* (L) – were

investigated. **Results:** In all resistant isolates ($n = 72$, 64%), high-level resistance to both tested antibiotics was found. The most prevalent *erm* gene was *erm* (B) (77.7%), followed by *erm* (A) (15.2%) and *erm* (C) (8.3%). Genes mediating erythromycin efflux were detected in 70.8% (*mef*) and 9.7% (*msr*) of resistant isolates. Regarding tetracycline, *tet* (M) was detected at the highest rate (50%), followed by *tet* (O) (31%) and *tet* (S) (11%). Export of tetracycline was found in 31% (*tet* (K)) and 12% (*tet* (L)) of isolates. **Conclusion:** A high prevalence of high-level resistance to both erythromycin and tetracycline was documented. Alterations at the ribosomal level was more frequently detected in erythromycin and tetracycline resistance than efflux systems. Concurrent resistance mechanisms were more involved in resistance to erythromycin than tetracycline.

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Introduction

Enterococci are considered as important nosocomial pathogens which are resistant to a wide range of antimicrobial agents through intrinsic and acquired mechanisms. Erythromycin and tetracycline are therapeutic agents used for treatment of enterococcal infections. Undoubtedly, the appearance of resistant strains is the result of the extensive use of these antibiotics [1, 2]. Methylation of 23S rRNA encoded by *erm* genes which reduces macrolide ability for ribosome binding is one of the most often associated erythromycin resistance mechanisms. Also, export of antibiotics mediated by genes encoding efflux pumps (*mef* and *msr*) is of the other mechanisms involved in macrolide resistance [3, 4].

As far as tetracycline resistance is concerned, 2 major mechanisms have been recognized in enterococci. The first, encoded by *tet* (M), *tet* (O), and *tet* (S) confers resistance through ribosomal protection (RP). The second group mediates efflux of tetracycline from cells and is represented by *tet* (K) and *tet* (L) genes [5].

In spite of the high frequency of enterococcal infections in our region, studies addressing erythromycin and tetracycline resistance in these organisms are few. The present study tested clinical isolates of enterococci by phenotypic and genotypic means to determine the presence and mechanism(s) of resistance to the mentioned antibiotics.

Methods

Isolates

In total, 112 isolates (*E. faecalis*, *n* = 86; *E. faecium*, *n* = 26) were collected from 2 hospitals in Kerman, Iran, during 2016–2017. All isolates were considered to be clinically relevant and mostly originated from urine (*n* = 67) and blood (*n* = 11) (Table 1). Species identification was carried out based on biochemical tests and molecular detection of the *ddl* gene [6].

Susceptibility Testing

The agar dilution method was performed in Mueller-Hinton agar (Merck, Germany) by the standard methods according to recommendations of the Clinical and Laboratory Standard Institute. *E. faecalis* ATCCC 29212 was used as the standard strain [7]. The antibiotics tested were erythromycin and tetracycline (Sigma Chemical Co., St. Louis, MO, USA).

PCR Analysis of Resistance Genes

The presence of genes encoding erythromycin resistance was investigated by amplification of *erm* genes using specific primers for *ermA*, *ermB*, and *ermC*. In addition, the genes involved in the erythromycin efflux system were determined by detection of *mef* and *msr* genes [8]. Duplex PCR assays were performed using 2

Table 1. Distribution of erythromycin- and tetracycline-resistance genes among clinical enterococcus isolates

Resistance determinant	<i>E. faecalis</i> (<i>n</i> = 62)	<i>E. faecium</i> (<i>n</i> = 10)	Total (<i>n</i> = 72)
<i>erm B</i>	51 (82.2)	5 (50)	56 (77.7)
<i>erm C</i>	4 (6.4)	2 (20)	6 (8.3)
<i>erm A</i>	9 (14.5)	2 (20)	11 (15.2)
<i>mef</i>	44 (70.9)	7 (70)	51 (70.8)
<i>msr</i>	6 (9.6)	1 (10)	7 (9.7)
<i>erm B</i> and <i>erm C</i>	2 (3.2)	0	2 (2.7)
<i>erm B</i> and <i>erm A</i>	3 (4.8)	0	3 (4.1)
<i>erm B</i> and <i>msr</i>	4 (6.4)	0	4 (5.5)
<i>erm B</i> and <i>mef</i>	35 (56.4)	3 (30)	38 (52.7)
<i>erm A</i> and <i>mef</i>	4 (6.4)	0	4 (5.5)
<i>erm C</i> and <i>msr</i>	1 (1.6)	0	1 (1.3)
<i>mef</i> and <i>msr</i>	2 (3.2)	1 (10)	3 (4.1)
<i>tet M</i>	31 (50)	5 (50)	36 (50)
<i>tet O</i>	18 (29)	5 (50)	23 (31)
<i>tet S</i>	5 (8)	3 (30)	8 (11)
<i>tet K</i>	19 (30)	4 (40)	23 (31)
<i>tet L</i>	8 (12)	1 (10)	9 (12)
<i>tet M</i> and <i>tet O</i>	5 (8)	3 (30)	8 (11.1)
<i>tet M</i> and <i>tet K</i>	10 (16.1)	2 (20)	12 (16.6)
<i>tet M</i> and <i>tet S</i>	2 (3.2)	0	2 (2.7)
<i>tet K</i> and <i>tet L</i>	2 (3.2)	1 (10)	3 (4.1)
<i>tet K</i> and <i>tet O</i>	1 (1.6)	1 (10)	2 (2.7)
<i>tet L</i> and <i>tet O</i>	4 (6.4)	0	4 (5.5)
<i>tet L</i> and <i>tet S</i> and <i>tet K</i>	0	1 (10)	1 (1.3)

primer pairs: *mef/ermA* and *msr/ermC*. Regarding tetracycline resistance, 5 *tet* genes using specific primers were investigated including those that confer resistance by RP: *tet* (M), *tet* (O), and *tet* (S) and efflux genes: *tet* (K) and *tet* (L) [9]. Multiplex PCR was optimized for multiplex primers of *tet* (K, L, M, S). PCR amplicons were checked by electrophoresis on 1% agarose gel. Strains harboring understudied resistance genes, kindly provided by Dr. Kalantar D, were used as positive controls [10, 11].

Results

The majority of studied isolates were resistant to both erythromycin and tetracycline (*n* = 72, 64%), including *E. faecalis* (*n* = 62) and *E. faecium* (*n* = 10). All resistant isolates expressed a high level of erythromycin resistance (MIC >128 µg/mL). Regarding tetracycline, all *E. faecium* and 90% of *E. faecalis* (*n* = 56) isolates showed high-level resistance (MIC >128 µg/mL), and the remaining ones represented MIC > 64 µg/mL (*n* = 4) and MIC >32 µg/mL (*n* = 1).

The *ermB* gene was the most common (*n* = 56, 77.7%), followed by *mef* (*n* = 51, 70.8%), *ermA* (*n* = 11, 15%), *msr*

Table 2. Frequency of enterococcus isolates in terms of the presence of only one resistant mechanism

Isolate	16SrRNA methylation ¹	Erythromycin efflux ²	RP mechanism ³	Tetracycline efflux ⁴
<i>E. faecalis</i> (n = 62)	14 (22.5)	5 (8)	31 (50)	9 (14.5)
<i>E. faecium</i> (n = 10)	3 (30)	1 (10)	4 (40)	0
Total	17 (23.6)	6 (8.3)	35 (48.6)	9 (12.5)

RP:ribosomal protection. ¹ Encoded by at least one of the *erm* (A–C) genes. ² Encoded by *mef* and/or *msr*. ³ RP encoded by at least one of the *tet* M, *tet* O, or *tet* S. ⁴ Encoded by *tet* K and/or *tet* L.

(n = 7, 9.7%), and *ermC* (n = 6, 8.3%). Comparing two enterococcus species, no significant difference in the distribution of erythromycin resistance determinants was found, although there were some differences between them, as shown in Table 1.

The *tet* (M) gene was found in half of the studied isolates. *Tet* (M) was the most prevalent RP gene (n = 36, 50%), whereas *tet* (S) was the least frequent one (n = 8, 11%). Among efflux genes, *tet* (K) was the most dominant gene (n = 23, 31%). All resistant isolates carried at least 1 erythromycin resistance gene, while among tetracycline-resistant isolates, 9 *E. faecalis* and 1 *E. faecium* did not harbor any resistance gene.

In total, in 32% of erythromycin-resistant isolates (23 of 72), one of the resistance mechanisms (*erm* genes or efflux system) was detected, whereas in 68% (49 of 72) of them, both mechanisms were involved. Also, the role of *erm* genes in erythromycin resistance (23.6%, 17/72) was more common than *mef/msr* genes (8.3%, 6/72) (Table 2). Conversely, in 71% of tetracycline-resistant isolates which contained one of the studied resistance genes (44 of 62), one mechanism and in 29% (18 of 62), both mechanisms (RP and efflux system) were detected. Similarly, the RP mechanism was more involved in tetracycline resistance than the efflux system (48.6 vs. 25%, respectively) (Table 2). No significant difference was found between resistance levels and the presence of one or more resistance genes conferring resistance to a particular antibiotic.

Discussion

The occurrence of macrolide and tetracycline resistance genes in 2 major enterococcal species was investigated in this study. Erythromycin resistance was more common (64%) than was previously described in our region or other countries [12–14] but less than that reported in China [1]. Similarly, as reported previously [1, 12, 14],

erythromycin resistance was expressed most often by *erm* B. Horizontal transfer of *erm* (B) may be involved which has been shown previously in the isolates from the same clone and even between animals and humans in genetically unrelated isolates [14, 15]. Following *erm* (B), *erm* (A) was dominant which is similar to other studies from Iran and China [1, 10] but in contrast to a report from Cuba [14]. In the present study, *erm* C was detected in 8% of isolates (n = 6). This is in contrast to other reports which did not describe any *erm* (C) [12, 14]. Also, the distribution pattern of *erm* (A–C) genes among enterococci from nonhuman specimens [16–18] was similar to our results. In comparison, the results of this study were in contrast to the situation in *Staphylococcus aureus* in which erythromycin resistance encoded by *erm* genes tends to be caused by *ermC*, followed by *ermA* and *ermB* [10].

As for genes responsible for erythromycin efflux, the *mef* gene was the most abundant determinant. This is in contrast to the report from Cuba which did not find any *mef* gene among enterococcal isolates [14].

The prevalence of tetracycline resistance in our isolates was lower than that reported in studies from Japan and Cuba [14, 19] but was higher than that in a report from China [1]. Among tetracycline-resistance genes, *tet* (M) was the most prevalent, however in less frequencies than other reports from China, Cuba, and Japan [1, 14, 19]. The prevalence of *tet* (L) in the present study was higher than that reported from China and Cuba, which reported 6.7 and 0% of *tet* (L) among *E. faecalis* isolates, respectively [1, 14]. Conversely, the prevalence of *tet* (L) in our study was lower than that reported among *E. faecium* isolates in Japan [19]. Also, a report from Japan did not find any *tet* (K) and low frequencies of *tet* (O) and *tet* (S); however, in our study, *tet* (K) and *tet* (L) were the second most dominant genes, and 11% of resistant isolates carried the *tet* (S).

In the present study, some disagreements were observed between the predicted and the actual tetracycline-resistant phenotype. This suggests that other mechanisms

could be involved in the development of resistance against tetracycline. As expected, 2 studied species showed the same distribution pattern of erythromycin- and tetracycline-resistance genes, and no significant differences were found between these species. This could be due to the fact that our isolates were collected from the same geographic area and at the same time with probably the same antibiotic treatment regimen. Furthermore, no differences were found in the MICs of tetracycline and erythromycin with related resistance genes. One possible explanation is that the actual level of resistance may be dependent on the copy number of resistance gene(s) within the bacterial cell as well as their level of expression. Interestingly, the alteration at the ribosomal level (methylation of 16srRNA and RP) were more involved in erythromycin and tetracycline resistance than did efflux systems. Conversely, in erythromycin-resistant isolates, the presence of both concurrent mechanisms was more evident than tetracycline resistance in which dominant involvement of only one resistance mechanism was observed.

Conclusion

The high prevalence of high-level resistance to both erythromycin and tetracycline was documented. The distribution pattern of *erm* (A-C) genes was unexpectedly different between enterococci and staphylococci from the same geographic region. Concurrent resistance mechanisms were more involved in resistance to erythromycin versus tetracycline.

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Statement of Ethics

The present study was approved by the Ethics Committee of the Research Council of Kerman University of Medical Sciences, Kerman, Iran (IR.KMU.REC.1397.329).

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author Contributions

Nikta Ahmadpoor: conceptualization, investigation, and writing of the original draft. Roya Ahmadrajab: conceptualization, investigation, review, and editing. Sarvenaz Esfahani: investigation and writing of the original draft. Zoya Hojabri: statistical analysis and writing of the original draft. Mohammad Hassan Moshafi: conceptualization, review, and editing. Fereshteh Saffari: conceptualization, writing of the original draft, and supervision.

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