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Isolation and Identification of *Enterococcus* spp. Resistant to Macrolides Antibiotics

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Abstract

Gram-positive enterococciare opportunistic and resistant to many antibiotics. This study aimed to investigate the presence of *Enterococcus* spp. in our community and whether these isolates are resistant to the macrolides class of antibiotics. Fifty isolates from 112 clinical samples were recognized as *Enterococcus* spp. and confirmed using Vitek-2 system. The current study found that 50/112 (44.6%) represented the total isolates, 38/50 (76%) of which were *Enterococcus faecalis*, while 12/50 (24%) were *Enterococcus faecium*, twenty (40%) isolates from root canals and 30 (60%) isolates from urine were isolated. The sensitivity of the enterococcal isolates to various macrolides (erythromycin, azithromycin and clarithromycin) antibiotics was determined by using the disk diffusion approach. Later, the minimum inhibitory concentrations (MICs) for erythromycin and the most resistant drug among this group were investigated using the agar dilution technique. And then molecular detection for *mef* gene was done using two specific primers via PCR technique. The current findings revealed high resistance rates to macrolide antibiotics which were reported in 21/50 (42%) of total isolates and at significant levels of MIC values for most isolates (57%). And as for the PCR results, it was negative for *mef* gene in all tested isolates.

Keywords: *Enterococcus* spp., Isolation, Identification, Macrolides antibiotics, Resistant

عزل وتشخيص المكورات المعوية المقاومة للمضادات الحيوية الماكروليدات

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الخلاصة

تعتبر المكورات المعوية إيجابية الغرام انتهازية ومقاومة للعديد من المضادات الحيوية. بحثت هذه الدراسة في انتشار المكورات المعوية في المجتمع وإذا كانت مقاومة لمجموعة الماكروليدات من المضادات الحيوية. تم تشخيص خمسين عزلة من 112 عينة سريرية على انها بكتريا المكورات المعوية وقد تم تأكيد ذلك باستخدام نظام التشخيص Vitek 2. وجدت الدراسة الحالية أن 112/50 (44.6٪) تمثل مجموع العزلات, 50/38 (76٪) عبارة عن بكتيريا *Enterococcus faecalis* بينما 50/12 (24٪) عبارة عن *Enterococcus faecium*، 20 (40٪) من العزلات أعلاه كانت من قناة جذر الاسنان و30 (60٪) من العزلات من الادرار. باستخدام طريقة انتشار القرص، تم تحديد حساسية عزلات المكورات المعوية لمضادات حيوية مختلفة من

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الماكروليدات (الاريثروميسين، أزيثروميسين وكلاريثروميسين). بعد ذلك، تم ايجاد الحد الأدنى من التركيزات المثبطة (MICs) للإريثروميسين، الدواء الأكثر مقاومة بين هذه المجموعة، باستخدام تقنية تخفيف الآغار، وبعدها تم التحري الجزيئي عن جين *mef* باستخدام بوادىء محددة وتقنية تفاعل البلمرة المتسلسل. كشفت النتائج الحالية عن معدلات مقاومة عالية للمضادات الحيوية الماكروليدات، والتي تم الكشف عنها في 50/21 (42٪) من مجموع العزلات ومستويات معنوية من قيم MIC لمعظم العزلات بنسبة (57٪). اما بالنسبة لنتائج تفاعل البلمرة المتسلسل فقد كانت سالبة لجين *mef* في كل العزلات المختبرة.

1. Introduction

Enterococciare is a gram-positive bacterium that can be found alone, in short chains or in pairs, under light microscope. They are frequently non-haemolytic, non-motile, facultative anaerobes and have colonies on blood agar that are 1-2 mm in distance [1]. Over the past decade, enterococci have developed as significant nosocomial microorganisms. They frequently exhibit several characteristics that allow them to thrive in the hospital setting, colonize in individuals, and spread illnesses such bacteraemia, endocarditis, peritonitis and infections of the urinary system, wounds, and medical devices. [2]. Antimicrobial drugs are commonly used in food animal production to treat and prevent bacterial illnesses, as well as in feed to promote growth [3]. Antibiotics use in animal breeding has been related to a rise in their resistance. Food animal resistance is remarkably analogous to nosocomial disease resistance including resistance to macrolides, nitrofurans, lincosamides, aminoglycosides, penicillin, streptogramins, quinolones, tetracycline and in rare cases, vancomycin [4]. Enterococci contain a number of mechanisms for acquired plus innate resistance to the main antibiotic groups used in medical practice, as well as effective genetic exchange pathways that aid in the spreading resistance genes of antibiotic [5]. Human infections are treated with macrolide antibiotics, with erythromycin being the antibiotic of choice for those who are sensitive to penicillin [6]. Various research has demonstrated that macrolide resistance spreads in streptococci, staphylococci and enterococci. Target modification via methylation of the 23S rRNA component to prevent binding of macrolide is mediated by *erm* genes (*ermTR, ermA, ermB* and *ermC,*), decomposition of the antibiotic molecule lactone ring, and efflux pumps are mechanisms that take antibiotic molecules out of the bacteria, i.e., genes *msrA, msrC, mreA, mefA* and *mefE* [7].

Aim of the Study

This study aimed to look into the presence of *Enterococcus* spp. in our community and whether these isolates are resistant to the macrolides class of antibiotics.

Materials and Methods

Study Sample:

For the duration of October 2022 to March 2023, 62 urine and 50 root canal specimens were collected from the Medical City Hospital, dentists' offices and labs to shed light on *Enterococcus* spp. resistance to macrolides antibiotics.

1. Bacterial Isolation:

- **Root Canal Samples**

In the infected root canals of 50 patients (15 males and 35 females) a sterile paper point and specific files were introduced for at least 5 to 15 seconds. The files and paper points were placed in sterilized Eppendorf tubes containing 1 ml of sterilized brain heart infusion broth which were then straight away transported to the microbiology lab for additional tests. Eppendorf material was vortexed for five seconds before being cultured on Pfizer special media [8].

- **Urine Samples**

Urine samples, collected in sterilized plastic cup containers from 62 persons with urinary tract infection, were then transferred to the laboratory. Each urine sample was centrifuged, the supernatant removed, and the pellet then went through a bacterial growth process on Pfizer medium for further identification of bacteria.

2. Bacterial Identification

- **Microscopic Examination:** The isolates were first Gram stained and then examined under a light microscope to determine their reactivity to stain, their shape and arrangement.
- **Culture Examination and Colony Selection:** Pfizer selected enterococci plates were streaked with a pure colony of the tested bacterium and cultured anaerobically for 24 hours at 37°C.
- **Detection by Biochemical Tests:** Bacterial isolates can be detected manually by biochemical tests like catalase test [9], growth in 6.5% NaCl [10], growth at 10°C and 45°C and growth at 9.6 pH value [11].
- **Detection by Vitek2 System:** The Vitek2 system was used to verify the results. The isolates were grown on Pfizer selective *Enterococcus* agar, then purified and created colonies by incubating at 37°C for 24 hr. anaerobically. The samples (suspension cells in 5 mL normal saline) were then placed into the Vitek kit which were checked after 5-7 hours.

3. Susceptibility Test

Disk diffusion technique was done by preparing plates containing sterile Mueller-Hinton medium, and the bacteria inoculum was used after comparing it with McFarland typical tube No. 0.5 (CFU/ml). The diluted bacteria were inoculated by swabbing method. Then, under sterile conditions, antibiotic disks from macrolides group (erythromycin, azithromycin and clarithromycin) were placed on the surface of culture media and incubated at 37°C for 18-24 hrs. Later on the width of the inhibition area for each disk was measured and compared to the standard inhibition area [12]. The diameters of the inhibition areas were determined in mm, and were explained into sensitive (S), resistant (R) and intermediate (I) groupings (Table **1**).

Table 1: Standard diameters of inhibition area (CLSI, 2021).

Antimicrobial Drug	Symbol	Disk Concentrations (µg)	Diameter of Resistance (mm) to Bacteria		
			Resistant	Intermediate	Sensitive
Erythromycin	E	15	≤13	14-22	≥23
Azithromycin	AZM	15	≤13	14-17	≥18
Clarithromycin	CLR	15	≤13	14-17	≥18

4. MIC Determination by Agar Dilution Method

Determination of the minimum inhibitory concentrations (MICs) for the most resistant antibiotic from Macrolides group in resistant isolates was done by using agar dilution method [13]. The antibiotic concentrations (2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 µg/mL) used for *Enterococcus* spp. were prepared by dissolving the antibiotic powder (Erythromycin) using 99.9% ethanol making stock solution and then diluted with distilled water to the chosen antibiotic concentrations.

The required dilution ranges solutions were prepared from the stock solution by using sterile cup container:

a. Twenty ml of cooled molten Mueller-Hinton agar (cooled to 50ºC before antibiotic adding) was added to each container, in addition to the antibiotic-free control.

b. Mixed well before pouring into Petri dishes. The antibiotic was then added pouring each concentration in turn. The dishes were stored at 4-8ºC until inoculation.
c. Organism suspension was made to match the turbidity of a 0.5 McFarland standard tube.
d. One to two µL of suspension was transferred by micropipette on to the surface of the agar. The inoculums must be allowed to be absorbed into the agar before incubation at 35-37ºC for 18-20 hr.

5. Molecular Detection of Macrolides Resistance Gene (*mef*).

DNA was extracted from pure bacterial colonies using a genomic DNA micro extraction kit from Norgen (Canada) following the manufacturer's instructions. Qubit 4 was used to measure the concentration of the extracted DNA in order to assess the quality of samples for subsequent uses. Accurately followed by the genotyping detection to *mef* gene using PCR technique as follows:

1. Primers Selection

Two primers were used for the detection of *mef* gene in this study. First primer was manufactured by a program, while the second primer was used from previous research (Table 2).

Table 2: The primers and their sequences used in conventional PCR

Gene	Sequence 5′ ➡ 3′	Size bp	References
mef	First primer **F:** TAACCCTAATAATAGACCCCC **R:** GGCAGGCAGTATCATTAATC	782	Newly designed by Nabu Scientific Foundation
	Second primer **F:** AGTATCATTAATCACTAGTGC **R:** TTCTTCTGGTACAAAAGTGG	367	[14]

2. PCR Amplification

The extracted DNA, primers and PCR premix were thawed at 4°C. The contents were temporarily vortexed to ensure they reached the bottom of their tubes. A 25-µl PCR mixture was made up of 15 µl of sterile deionized distilled water, 5 µl of PCR premix, 1 µl of each primer (forward and reverse), 3 µl of DNA template, and 5 µl of PCR premix.

After quickly mixing the PCR reaction tubes, the DNA was amplified using the thermocycler PCR instrument in line with the PCR protocol (Table 3).

Table 3: Program PCR amplification of *mef* gene

Stage	Temperature °C	Time	
Initial denaturation	94	4 min	30 Cycle
Denaturation	94	40 sec	
Annealing	47 (First primer) 50 (Second primer)	40 sec	
Extension	72	40 sec	
Final Extension	72	5 min	

3. Agarose Gel Electrophoresis

While stained with RedSafe dye to identify DNA, the samples were subjected to electrophoresis in 2% agarose gel for 50 minutes at 75 volts. Additionally, an ultraviolet transilluminator was used to obtain the agarose gel image.

Results

Isolation and Identification of *Enterococcus* spp.

Isolating and identifying *Enterococcus* spp. was the primary goal of sample collection. Each isolated bacterial species was identified using the Gram stain method. Under a light microscope, gram-positive cocci with spherical or ovoid shapes were arranged singly, in pairs, or in short chains. The colonies were grey, round and 2 mm in distance, with black areas around the colony and a black point in the middle on Pfizer Selective Enterococci medium (Figure **1**).

Figure 1: *Enterococcus* spp. on Pfizer Selective medium

All *Enterococcus*-related bacterial isolates tested negative for the catalase test, and that they can grow in 6.5% NaCl, pH 9.6, incubation at 10 and 45°C. The results of biochemical tests for isolates were established via Vitek-2 system (Figure **2**).

bioMérieux Customer: Microbiology Chart Report Printed November 26, 2022 9:16:07 AM AST

Patient Name: 1, . Patient ID: DFJUDR
Location: Physician:
Lab ID: 111 Isolate Number: 1

Organism Quantity:
Selected Organism : Enterococcus faecium

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	5.87 hours	**Status:**	Final
Selected Organism	93% Probability **Bionumber:**	**Enterococcus faecium** 1360034657737S1		
ID Analysis Messages				

Biochemical Details

2	AMY	+	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	+	15	AspA	+	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURu	-	25	AGAL	+	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	+	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	-	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	+	59	SAL	+	60	SAC	+	62	dTRE	-	63	ADH2s	+
64	OPTO	+															

Figure 2: Identification of *Enterococcus* spp. by Vitek 2 system.

The current study obtained 50/112 (44.6%) isolation distributed as: 38/50 (76%) of isolates were *Enterococcus faecalis*, while 12/50 (24%) of isolates were *Enterococcus faecium.* In accordance with source of isolation, the percent of isolation distributed as: 20 (40%) isolates from root canals samples and 30 (60%) isolates from urine samples.

Antibiotic Sensitivity Test and MIC Determination

The macrolides antibiotics, previously mentioned in Table **1**, were tested against *Enterococcus* spp. using the disk diffusion method (Figure **3**).

Figure 3: *Enterococcus* spp. sensitivity for macrolides antibiotics by disk diffusion technique.

After approving the diameter measurements of inhibition zones for *Enterococcus* spp., 21 of the fifty isolates (42%) were found to be resistant to macrolides group of antibiotics which were distributed as: 13/21 (38%) of isolates were *E. faecalis* and 8/21 (62 %) of isolates were *E. faecium*.

The minimum inhibitory concentrations (MICs) for erythromycin were established using the agar dilution method. The results of antibiotics susceptibility and MICs are shown in Table 4.

Table 4: Diameter measurements of inhibition zones and MIC for *Enterococcus* spp.

Isolate No.	Diameter Measurements of Inhibition Zones			MIC for Erythromycin (µg/mL)
	Erythromycin (mm)	Azithromycin (mm)	Clarithromycin (mm)	
1	11	12	13	32
2	10	9	13	8
3	10	9	13	8
4	9	9	14	16
5	10	9	14	8
6	R	R	10	16
7	7	8	11	16
8	8	8	13	32
9	8	9	10	1024
10	R	R	R	1024
11	10	10	13	8
12	7	6	9	1024
13	R	R	R	1024
14	R	R	R	1024
15	7	8	9	1024
16	R	R	R	1024
17	R	R	R	1024
18	R	R	R	1024
19	R	R	R	1024
20	R	R	R	1024
21	5	6	6	1024

Current study revealed that the MIC for 12 isolates was 1024 µg/mL meaning that these isolates were highly resistant to erythromycin. Two of the resistant isolates inhibited at 32 µg/mL of the antibiotics, 3 of the resistant isolates inhibited at 16 µg/mL, and the MIC for 4 isolates was 8 µg/mL.

Molecular Documentation of *mef* Gene

The results of PCR revealed that the *mef* gene was not found in any enterococcal isolate for both primers that were used (Figures 4 and 5).

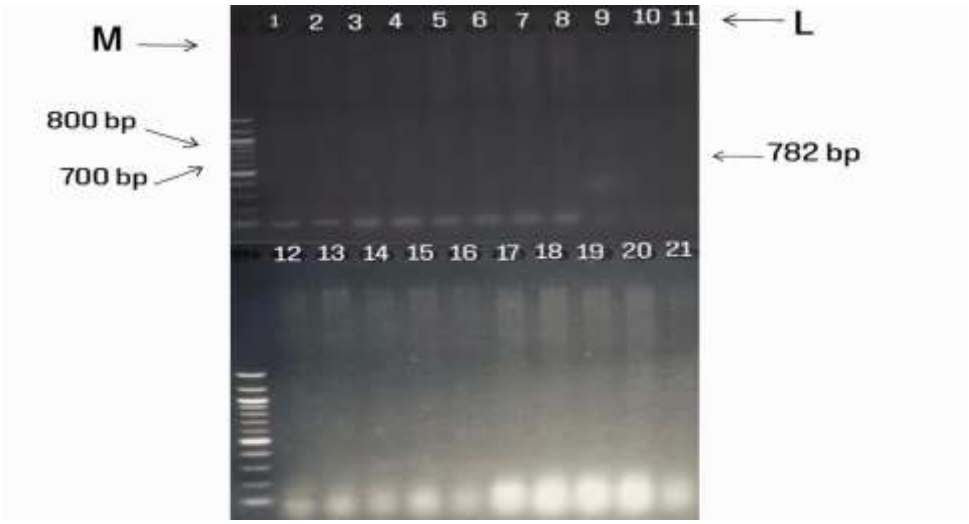

Figure 4: Agarose gel 2% electrophoresis of PCR amplified products for *mef* gene stained with RedSafe dye using first primer (75volt/cm for 50min). Lane M: l500bp Ladder marker. Lane L showed negative bands of 782bp of *mef* gene.

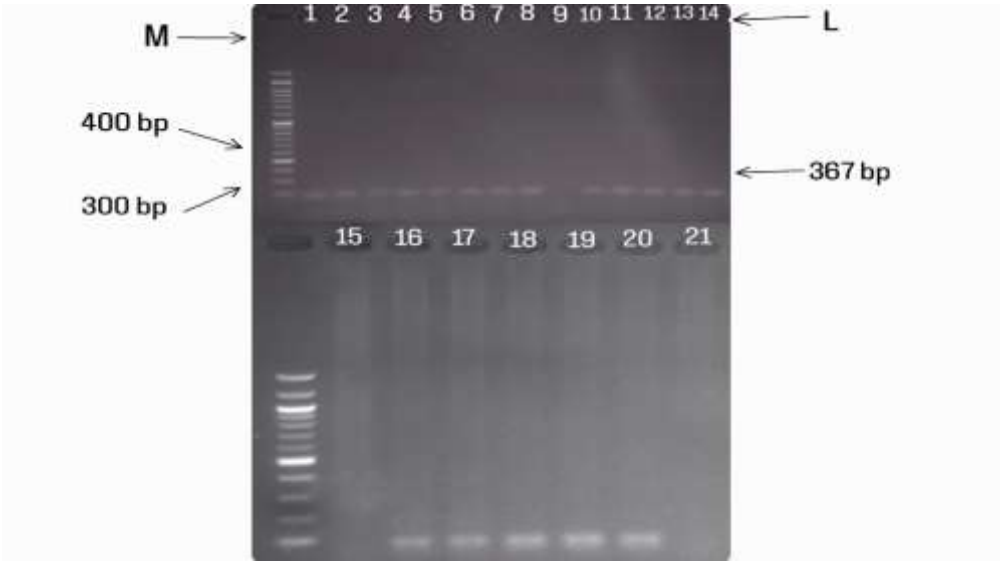

Figure 5: Agarose gel 2% electrophoresis of PCR amplified products for *mef* gene stained with RedSafe dye using second primer (75volt/cm for 50min). Lane M: l500bp Ladder marker. Lane L showed negative bands of 367bp of *mef* gene.

Discussion

Of 50 enterococcal isolates, 38 (76%) identified as *E. faecalis* and 12 (24%) were identified as *E. faecium*. A study by Abu Lila *et al*., [15] showed that among 131 enterococci

isolates, 67 (51.1%) identified as *E. faecalis* and 52 (39.7%) identified as *E. faecium.* Also, a local study by Jassim and Alash [16] showed that the percentage of *Enterococcus* spp. was 33.3%.

Sixty percent *Enterococcus* spp. isolates were from urine which is consistent with a local study carried out by Haider [17] who found 46.6% isolates of *Enterococcus* spp.in urine samples. This percentage was higher than the percentage (40.32%) of *Enterococcus* spp. isolated in the urine samples in another local study carried out by Salih.

The percentage of *Enterococcus* spp. isolates from root canal was 40%. This outcome is similar to the outcome of studies carried out by Al-shawi and Al-Quraishi [19] and Preethee *et al*. [20] who revealed that the ratio of *Enterococcus* spp. isolated from root canal was 55% and 46.87% respectively. Whereas previous studies published by Mahmoudpour *et al*. [21] and Haider [17] revealed that the percentages of *Enterococcus* spp. isolated from root canal were 10% and 14 % respectively. Also, a local study by Mustafa *et al.* [22] reported that the ratio of isolates that belonged to the genus *Enterococcus* was 83%.

The number of specimens, the origin of the isolates, hospitals consulted for each study, the sites geographically and the identification methods may all play a role in the variations in isolation percentages [23, 24].

Enterococci have increased resistance to several widely used antibiotics, including macrolides. which were found to be useful as an alternative for patients with penicillin allergy. However, the emergence of macrolide resistance restricted their use under certain conditions [25]. Henceforth, it was selected to study the latest changes in the resistance of bacteria to these antibiotics. Disk diffusion method was employed to assess *Enterococcus* spp. resistance to macrolides antibiotics, and the outcome revealed that 42% of *Enterococcus* spp. isolates were resistant toward macrolide antibiotics. This result well-matched with results of the study by Abu Lila *et al*. [15] who showed that the ratio of resistance of isolates of this bacteria isolated from different clinical cases to macrolides amounted to 50.4 %.

The entirely resistant isolates (n = 21, 42) showed substantial levels of resistance to the tested macrolide antibiotics. The most resistant antibiotic erythromycin that was chosen to find the MIC ranges used were between 2-1024 µg/mL. A latest study by Abu Lila *et al.*, [15] recorded that the MIC ranges used were between 0.125-1024 µg/mL. Lowest MIC value of 8µg/mL was recorded for few isolates (n = 4, 19%) which was compatible to the standard MIC in CLSI. [13]. However, the highest MIC value of 1024 µg/mL was recorded for most of the isolates (n = 12, 57%). The rest represented MIC=32 µg/mL (n = 2) and MIC=16 µg/mL (n = 3). Since most isolates resisted antibiotic disks that were used, and that most of the isolates had a high MIC value, meant that the results were identical and one complementary to the other in terms of their resistance to bacteria. This result is similar to the results reported by Ahmadpoor *et al* [26]. Without a doubt, the widespread usage of these antibiotics has facilitated the emergence of microorganism resistant to them. [27]. Macrolide resistance typically results from one of the following three mechanisms: (a) modification of the target site through methylation or mutation, which prevents the antibiotic from binding to its ribosomal target; (b) efflux of the antibiotic; and (c) drug-inactivated macrolides have low levels of activity against Enterobacteriaceae due to poor membrane invasion of these antimicrobials thus preventing their use in Enterobacteriaceae treatment [28].

This study detected the *mef* gene to update the knowledge on mechanistic and epidemiological traits of resistance. *Mef* gene was not found in any enterococcal isolate. The

results of study by Quiñones *et al.* [29] were similar to ours that no *mef* gene was detected among enterococcal isolates. This result is, however, in contrast to the report from Iran which found the *mef* gene in 8.3% of enterococcal isolates [30].

Also, Liang *et al.* [31] detected the *mef* gene only in 9 of 53 clinical isolates of *Enterococcus* species which may be due to the regional variation in the occurrence of the *mef* gene in enterococci. Another study by Ahmadpoor *et al.*, [26] reported that the gene-mediating efflux pump (*mef)* was detected in 70.8% of all enterococcal isolates. This finding might not be related to the efflux pump mechanism that this study detected., Rather to other mechanisms that bacteria use to develop resistance to this class of antibiotics.

Conclusion

Our community was contaminated in a percentage of 44.6% with *Enterococcus* spp., which was isolated in a higher percentage from urine samples (60%) than from tooth root canal samples (40%). Forty-two percent of the isolates were reported to have high levels of resistance to macrolide antibiotics with high levels of MIC values for most isolates (57%). In the molecular detection, the *mef* gene was not detected in any enterococcal isolate that were phenotypically resistant to the macrolides group of antibiotics.

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