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Molecular Characterization of Antibiotic Resistance Genes in Staphylococcus Isolated from Cell Phone Users' and Non- Users' Ears

Tayabe Avazzadeh [1], Abbas Ali Rezaeian [1], Shafie Ghorbani Tazhandarreh [2], Roohollah Zarei Koosha [1], Davood Ghorbani Tazhandarreh [3], Abdolhassan Doulah [*4]

1. Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran
2. Department of Microbiology, Zanjan Branch, Islamic Azad University, Zanjan, Iran
3. Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran
4. Department of Cellular and Molecular Biology, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran

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***Corresponding author:**
Abdolhassan Doulah. Department of Cellular and Molecular Biology, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran
Phone: +989173723087
Fax: +986136742323

Email: h_doulah@yahoo.com

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Abstract

Introduction: Resistance to macrolide can be created by *erm* genes in *Staphylococcus*. The aim of the current study was to determine whether or not cell phone use can result in the antibiotic resistance of *16S rDNA*, *Coa*, *ermA, ermB* and *ermC* genes in *Staphylococci* isolated from cell phone users' and non- users' ears.

Methods: A total of 150 isolates of *Staphylococci* were tested by the disk diffusion method. The isolates were examined by PCR for *16S rDNA*, *Coa*, *ermA, ermB* and *ermC* genes.

Results: According to PCR results, in two statistical societies, 65.33% cell phone users with positive *Coa* had only one *erm*, 33.33% cell phone non-users with negative *Coa* had only one *erm* and 1.34% had a minority of genes, whereas 24% cell phone non-users with positive *Coa* had only one *erm*, 44% cell phone non-users with negative *Coa* had only one *erm* and 32% had a minority of genes. Results showed that *16S rDNA*, *Coa*, *ermA, ermB*, and *ermC* genes in the cell phone users group were more prevalent than the other group in *Staphylococci* isolated from ears.

Conclusion: It is revealed that the presence of *16S rDNA*, *Coa*, and *erms* genes had a significant relation to erythromycin and methicillin. Detection of *ermA, ermB* and *ermC* plays crucial roles in the molecular mechanisms, epidemiology of the efflux pump and methylase erythromycin ribosome. Since antibiotic resistant *Staphylococci* isolates may mutate and prompt constitutive resistances it is suggested that inducible resistance test should be implemented on erythromycin resistant sensitive isolates to prevent treatment failures.

Keywords: Cell Phone, Ear, *erm* Genes, Staphylococci

Introduction

Cell phones are becoming important in our lives, though they can be tremendously harmful for our health. They have become a source of infectious pathogens (1). The genus *staphylococcus* is composed of several species, many of which may be encountered in human clinical specimens. *Staphylococcus* is a major pathogen that poses a significant practical and theoretical clinical problem due to the high degree of carriage in the nasopharyngeal cavity. It causes a wide variety of infections in addition to Multidrug resistances (2- 4). Among the genus *staphylococcus*, the three main species which are clinically important are *S. aureus*, *S. epidermidis*, and *S. saprophyticus* (5). *S. aureus* is coagulase positive (5, 6). It can be found in the external environments and the anterior nares of 20- 40 % of healthy adults. Other sites of colonization include intertriginous skin folds, perineum and vagina.

Although this organism is a skin microflora, it can result in significant opportunistic infections under appropriate conditions (6). Coagulase-negative *Staphylococci* (CONS) are becoming increasingly important as a result of hospital-acquired infections, particularly nosocomial bacteremia (7), and neonatal sepsis (8). It has been revealed that increased nosocomial infections can be considered as a result of high rate of contamination of ears and cell phones with MRSA strains (1). The National Nosocomial Infection Survey (NNIS) reported that the incidence of CONS as a cause of nosocomial bacteremia has increased from 9% to 27% during the period from 1980 to 1989, to become the most common single cause of these infections (7). It was reported that there is an association between the dramatic increase of CONS, as a cause of nosocomial bacteremia and the increased rate of resistance of these pathogens to antimicrobial agents (9). According to Cinar *et al.* 52.63% of the isolates from 40 nursing students' mobile phones were identified as Coagulase-negative Staphylococci in addition to contaminating of mobile phones of 31.58% with *S. aureus* strain (10). In another study by Osman *et al.* found that the frequency of positive culture between cell phone and ear were 95% and 100%, respectively (1). *erm* genes are widely dispensed among many species of bacteria. In addition, a dozen resistance determinants have been explained (11). In *S. aureus*, erythromycin resistance is generally due to either ribosomal transformation by 23S rRNA methylases mediated firstly by *ermA*, *ermB* and *ermC* or active efflux pump of the antimicrobial doors by an ATP-dependent pump inserted by *msrA* (12, 13). *ermA* is very often harbored and mediated on the transposonTn554, which encodes spectinomycin resistance (14, 15), whereas *ermB* is usually associated with the penicillinase plasmid and transposon Tn551, pI258. The *ermC* gene which is obviously rare in microbial strains isolated from specimens prior to 1970, is often integrated on plasmids average in size from 2.4 to 5 kb (16, 17). All of the *erm* significations confer cross-resistance to macrolides, lincosamides, and streptogramin B agents (MLSB phenotype) (18).

Methods

In this study conducted for a six-month period from May to October in 2016, 150 clinical isolates of *Staphylococci* were collected from ears of two statistical populations of cell phone users and cell phone non-users at a rehabilitation center affiliated to Bushehr and Shiraz cities. The bacteria isolated from ears, were transported to the Microbiology Laboratory of Islamic Azad University of Jahrom, Iran and were confirmed by standard microbiological tests such as Gram staining, catalase, tube coagulase, slide and mannitol fermentation, oxidation fermentation and production of DNase enzyme (19). Antimicrobial susceptibility of the isolates was determined using Kirby-Bauer disk diffusion method corresponding to clinical and Laboratory Standard Institute (CLSI). Initially, 0.5 McFarland suspension of bacteria was primed and inoculated on Mueller-Hinton agar plates (Merck, Germany). The tested antimicrobial agents were penicillin (10U), tetracycline (1µg), erythromycin (15µg), vancomycin (10µg), methicillin (10µg), amikacin (10µg), ciprofloxacin (cp) (5µg) and cephalothin (cf) (5µg) (Table 1). The minimal inhibitory concentrations (MICs) of erythromycin were determined by E-Test (Bio Merieux) corresponding to CLSI (20). In this study, DNA was extracted from *Staphylococcal* (*S. aureus*, *S. epidermidis*) isolates by boiling. All *Staphylococcal* isolates were inoculated on blood and Mueller-Hinton agar plates at 37°C. After 24 hours, five colonies were suspended in 100 µl of TE buffer (1 mM EDTA, 10 mMTris, pH=7.8) and boiled at 100 C° for 10 minutes (21). Therefore, centrifuged bacterial suspensions diluted at 9000 rpm ×g for 30second at 4°C. After centrifugation, the supernatant solution

was collected and used as the DNA template for polymerase chain reaction (PCR). Primers were designed based on the *16S rDNA* (KX611101.1), *Coa* (AJ311979.1), *ermA* (KT803896.1), *ermB* (AF239772.1) and *ermC* (AF466402.1) genes sequences of *Staphylococci* obtained from Gene Bank utilizing the Gene Runner program. To account the specificity of the designed primers, they were analyzed in BLAST software of the desired primers. The primers were synthesized by Cinna Clon Co, Iran (Table 1). For further confirmation, the PCR product of each gene was analyzed by sequencing (Macrogen Research, Seoul, Korea). PCR amplification for *16S rDNA*, *Coa*, *ermA, ermB and ermC* genes detection PCR method was used to investigate the distribution of the *16S rDNA*, *Coa*, *ermA, ermB* and *ermC* genes among the isolates. The DNA was precipitated through boiling. The quantity and quality of the purified DNA were evaluated using gel electrophoresis and Nano Drop spectrophotometer (Nano Drop 8000 UV-Vis Spectrophotometer), respectively. Then, 4µl of each DNA was amplified in 25 µl of the reaction mixture, which consisted of 2.5 µl 10× the reaction buffer, 0.5 mM deoxynucleoside triphosphates (dNTPs), 0.5 mM $MgCl_2$, 1 µl of each primer and 0.5 U of Taq DNA Polymerase (Sinaclon, Iran). PCR was performed using the Eppendorf asterCycker (Hamburg, Germany) with an initial denaturation step of 94ºC for 4 minutes, followed by 35 cycles of 94ºC for 30 seconds, 58ºC for 30 seconds and the annealing temperature of 2ºC for one minute for the *Coa* and *16S rDNA* genes with the final extension of 72ºC for 5 minutes. But for *ermA, ermB* and *ermC* genes with an initial denaturation step of 94ºC for 4 minutes, followed by 35 cycles of 94ºC for one minute, 53ºC for 5 minutes and the annealing temperature of 72 ºC for 2 minutes, with the final extension of 72ºC for 10 minutes. To determine the presence of the desired amplicon, electrophoresis was performed on 1% gel agarose staining and subsequently visualized the PCR products using a UV transilluminator (UVT-20.S and UVT-20.SL, Iran). The sizes of the PCR products of the amplified *Coa*, *16S rDNA* and *ermA* genes were 821bp, 229bp and 139bp, respectively. DNA amplification was performed using strain-specific primers to detect *16S rDNA*, *Coa*, *ermA, ermB* and *ermC* genes. The results of the research were analyzed using the SPSS (version 18). Fisher's exact test or chi-square test, by way of analysis, was used to compare frequencies. P-value of ≤ 0.017 was narrated as statistically significant.

Results

In this study, the prevalence of *Staphylococcus* was evaluated, which contained five genes of *16S rDNA*, *Coa*, *ermA, ermB* and *ermC*. Frequency and abundance of each gene addressed, *Coa* +, 49 (65.3%),18 (24%), *Coa* -, 26 (37.4%), 57 (76%), *ermA*+, 54 (72%), 18 (24%), *ermA*-, 21 (28%), 57 (76%), *ermB*+, 50 (66.7%), 33 (44%), *ermB*-,25 (33.3%), 42 (56%) and *ermC*+, 62 (82.7%), 32 (42.7%), *ermC*-, 13 (17.3%), 43 (57.3%). These genes were isolated from ear samples, respectively. In addition, *16S rDNA* gene detected positive in all isolates (100%) (Figure 1). Six kinds of antibiotic resistant genotypes were obtained in coagulase-negative and coagulase -positive Staphylococcal isolates in both groups, with high-frequent genotypes of *coa+ /ermA+ /ermB+ /ermC+* (19), *coa− /ermA− /ermB− /ermC−* (14), *coa−/ ermA−/ ermB+/ ermC+* (10), and *coa−/ ermA−/ ermB−/ ermC+* (10) (Table 2). All individuals were over 18 years using cell phone more than 5 hours per day. Distribution and frequency of genes by sex (62% females vs 38% males) and age are shown in Figur 2. The frequency of *16S rDNA*, *Coa*, *ermA, ermB* and *ermC* genes was identified using PCR. In addition, the relationship of this frequency with sex, age and antibiotic type was investigated. A representative example of a multiplex PCR and uniplex PCR reaction for

the identification of *16S rDNA*, *Coa* and *ermA, ermB* and *ermC* genes is shown in Fig. 4 and 5. In addition, the specificity of the designed primers in this research was tested via DNA purification from *Staphylococcus* saprophyticus, *Staphylococcus epidermidis* was obtained from these strains, although No PCR product was found. Furthermore, the lowest concentration for the mentioned genes detected by our designed primers was 2 ng. In this study, a total of 150 isolates of *Staphylococci* isolated from ear in the statistical population of cell phone users at the rehabilitation center affiliated with Bushehr and Shiraz city, were examined. The results were 75 (50%), 49 (36.33%), 54 (36%), 50(33.33%) and 62 (41.33%), expressing the *16S rDNA*, *Coa*, *ermA*, *ermB* and *ermC* genes, respectively. In addition, the comparison between cell phone users and non-users showed significant differences among all genes (p = 0.0) (Figure 1). The antibiotic-resistant properties of each bacterial strains isolated from samples were determined using antimicrobial susceptibility analysis. Penicillin had the highest resistance among the isolates, whereas none of the isolates were resistant to vancomycin. The frequency of resistant isolates to each antibiotic is shown in Table 3. The results of antimicrobial sensitivity test showed that all isolates (related to both cell phone users and cell phone non-users) were susceptible to vancomycin (100% susceptible) and the majority of them were resistant to penicillin (98.8%). The results of antibiotic susceptibility test for other antibiotics are shown in the Table 3. The electrophoresis results of PCR products revealed that 48.4 % and 51.6 % of the isolates were positive and negative for *erm* genes, respectively. Data on the multidrug resistance (resistant to more than 2 antibiotics) of the isolates is summarized in Table 3. Statistical analyses showed no significant difference on the prevalence of the multidrug resistance properties (p > 0.05) regardless of sex, age and sample types. Prevalence of *erm* genes and the antibiotic resistance status of the isolates are provided in Table 3. In addition to penicillin and erythromycin, resistance to other antibiotics increased significantly in the presence of the *erm* genes in the cell phone users' isolates (Table 3). Moreover, it was observed that the presence of the *erm* genes was significantly higher among the multidrug resistant isolates (p = 0.042, chi value = 3.363) (Table 3). Moreover, 58 (33.7%) of the MRSA isolates were resistant to all antibiotics, excluding vancomycin (Table 3). Considering the results of statistical analyses, no significant correlation was found between the resistance to the methicillin, erythromycin and ciprofloxacin antibiotics and the presence of the *ermC* gene (Fisher test p=0.9, P=0.912, P=0.554) respectively. Moreover, there was no significant correlation between the distribution of the *ermC* gene and the Amikacin and Tetracycline resistance pattern, (chi test p= 0.226, p= 0.581) respectively. But resistance to all of the antibiotics tested in the strains harboring the *Coa*, *ermA* and *ermB* genes were significant. Regarding the relationship between erythromycin and methicillin resistance levels in the isolates of both groups, significant correlation was found between the resistance to erythromycin and methicillin antibiotics and the presence of the *erm* genes of the *Staphylococcal* strains. In this comparison, in samples of cell phone users (75unit), erythromycin resistance level in all isolates indicator is equal to the resistance methicillin level (fisher test and Pv = 0.000) (Table 4). Results of Statistical analysis shewed a significant correlation between *ermA* and *ermB* genes and erythromycin and methicillin antibiotics, in comparison with the fact that there was no significant correlation between *ermC* and erythromycin and methicillin antibiotics (Fisher test, P=0.912, p = 0.9), respectively.

Table 1. Primer sequences

Genes	Forward	Reverse
Coa	5accacaaggtactgaatcaacg3	5tgctttcgattgttcgatgc3
ermA	5tatcttatcgttgagaaggga3	5ctacacttggcttaggattgaaa3
ermB	5gtttactcttggtttaggatgaaa3	5gtttactcttggtttaggatgaaa3
ermC	5cttgttgatcacgataatttcc3	5atcttttagcaaacccgtattc3

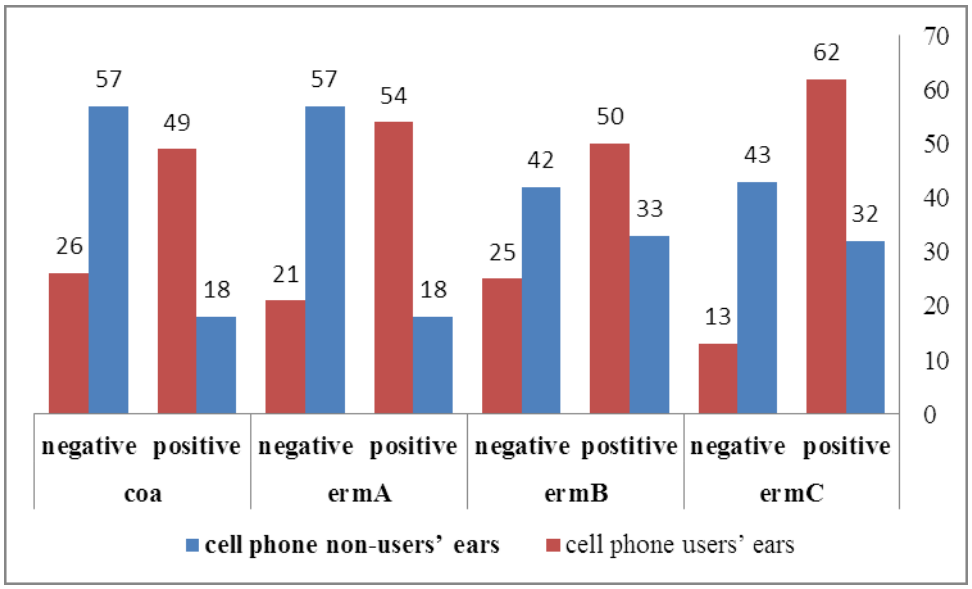

Figure 1. Distribution and frequency of the occurrence of *erm* genes in the studied populations

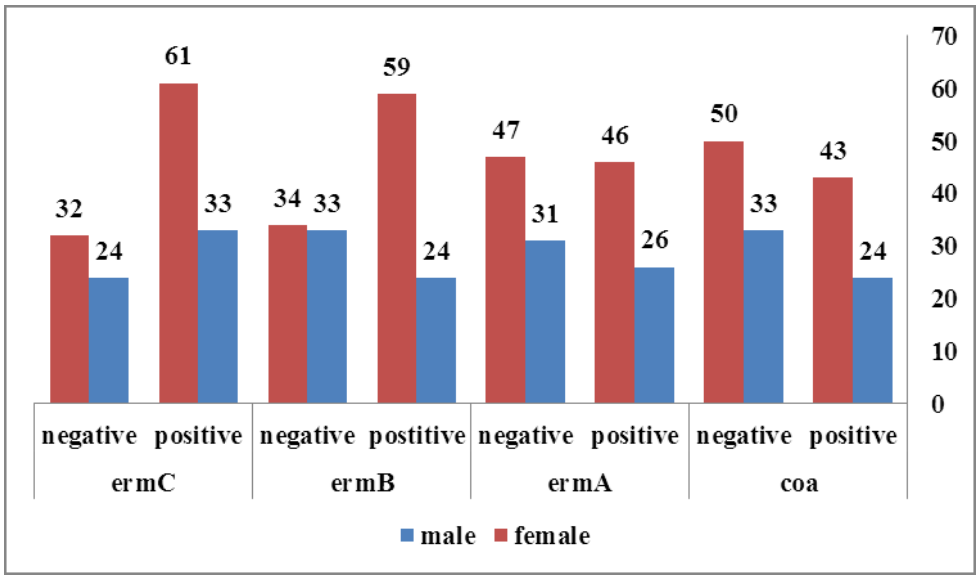

Figure 2. Distribution and frequency of genes by sex and age in the studied populations.

Table 2. Distribution and frequency of the genotypes of *coa* and *erm* genes in cell phone users and non-users by sex

sample	sex	*coa*		Genotypes							
				ermA+ ermB+ ermC+	***ermA+ ermB+ ermC-***	***ermA+ ermB- ermC-***	***ermA+ ermC- ermB+***	***ermA- ermB+ ermC+***	***ermA- ermB- ermC+***	***ermA- ermB+ ermC-***	***ermA- ermB- ermC-***
cell phone non-users' ears	Male	*coa*	Positive	1	0	5	2	0	0	0	0
			Negative	0	0	1	0	9	4	3	10
			Total	1	0	6	2	9	4	3	10
	Female	*coa*	Positive	5	2	2	0	0	0	1	0
			Negative	0	0	0	1	4	6	5	14
			Total	5	2	2	1	4	6	6	14
cell phone users' ears	Male	*coa*	Positive	1	2	2	9	1	0	1	0
			Negative	4	0	0	0	1	1	0	0
			Total	5	2	2	9	2	1	1	0
	Female	*coa*	Positive	19	4	2	6	2	0	0	0
			Negative	3	0	0	2	10	2	2	1
			Total	22	4	2	8	12	2	2	1

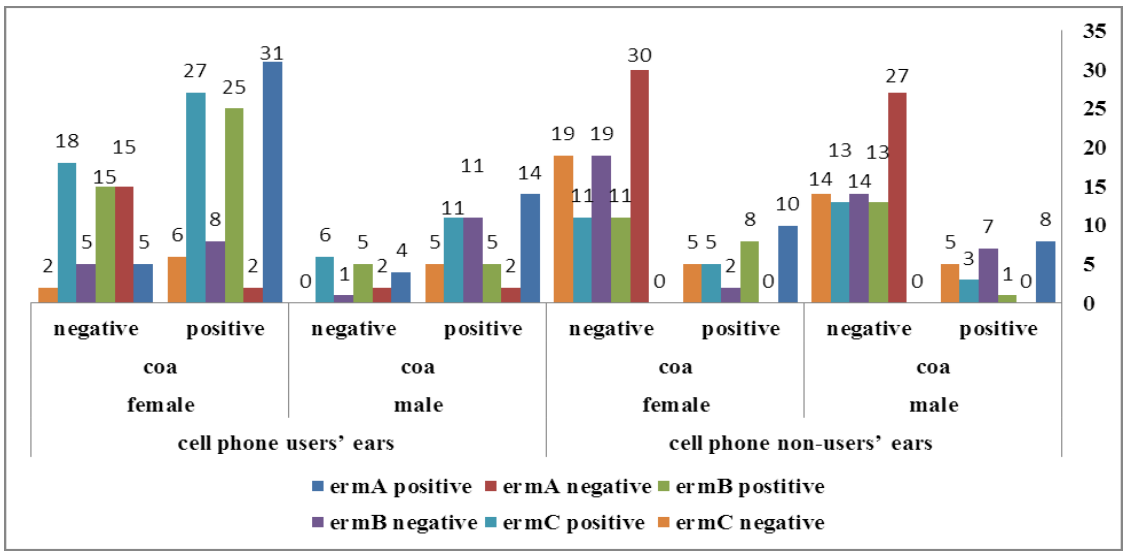

Figure 3. Distribution of the macrolide resistance genes *ermA*, *ermB* and *ermC* in Coagulase-negative and Coagulase-positive *Staphylococci* in tow statistical populations of cell phone users and non-users by sex (female and male)

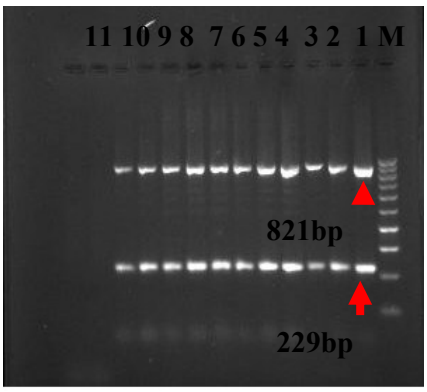

Figure 4. Gel electrophoresis of multiplex PCR products from the *16S rDNA* and *Coa* genes. The sizes of the amplicon of *16S rDNA* and *Coa* genes are 229 bp and 821 bp, respectively. M indicates the 100 bp DNA ladder. Lane 1 is the positive control. Lanes 2-11 show the results obtained from the ear isolates.

Table 3. Anti-microbial susceptibility analysis among the isolates

	Antibiotic	Disc contains(ng)	Drug resistance templet(%)		
			resistance	Intermediate	sensitive
cell phone users' ears	Tetracycline	30	21	0	54
	Erythromycin	15	38	4	33
	Amikacin	30	8	14	53
	Vancomycin	30	0	0	75
	Ciprofloxacin	5	0	0	75
	Cefalotin	30	6	4	64
	Penicillin	10	40	0	35
	Methicillin	10	31	3	41
cell phone non-users'ears	Tetracycline	30	39	0	36
	Erythromycin	15	38	2	44
	Amikacin	30	6	10	59
	Vancomycin	30	0	0	75
	Ciprofloxacin	5	3	0	72
	Cefalotin	30	27	9	39
	Penicillin	10	7	0	68
	Methicillin	10	30	1	44

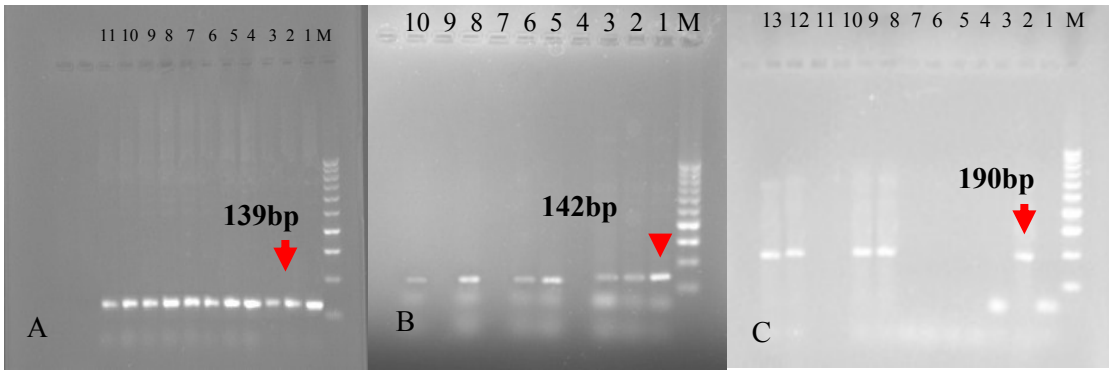

Figure 5. Gel electrophoresis of the PCR products from the *ermA* (A), *ermB* (B) and *ermC* (C) genes. Note that M indicates 100 bp DNA ladder in all the pictures. (A) The size of the amplicon of the *ermA* gene is 139 bp. Lane 1 is the positive control (*S. aureus* ATCC 43300). Lane 1 is the positive control. Lanes 2-11 show the results obtained from isolates of ear. (B) The PCR product of the *ermB* gene is 142 to 146 bp. lane 1 is the positive control. Lane 1 is the positive control. Lanes 2, 3, 5, 6, 8 and 10 show the results obtained from isolates of ear. (C) The size of the amplicon of the *ermC* gene is 190 bp. Lane 14 is the positive control and lanes 3, 9, 10 and 13 show the results obtained from isolates of ear.

Table 4. Relationship between the erythromycin, methicillin resistance and isolates

Sample			Erythromycin			total	test	PV
			Sensitive	Intermediate	Resistance			
cell phone non-users'ears	Methicillin	Sensitive	42	1	1	44	Fisher	0.00
		Intermediate	0	1	0	1		
		Resistance	2	0	28	30		
		Total	44	2	29	75		
cell phone users'ears	Methicillin	Sensitive	33	1	7	41	Fisher	0.00
		Intermediate	0	3	0	3		
		Resistance	0	0	31	31		
		Total	33	4	38	75		

Discussion

Staphylococcus strains cause to happen a high rate of morbidity and mortality due to severe nosocomial infections. Major issues in the emergence of MRSA strains are the difficulties in their treatment and the rising number of reported outbreaks in some parts of the world. Therefore, the multi-drug resistance properties of MRSA strains and the production of variety types of virulence factors cause the prevalence of infections. It has been directed that resistance to antibiotics can alter the expression of genes involved in illness agent (22). In this study, the frequency of antimicrobial resistance was very high. Our results showed a high prevalence of resistance toward many antibiotics that are routinely administered for the treatment of staphylococcal infection. Similar to most regions around the world (23, 24), resistance to penicillin was high in our studied populations (98.8 %). Despite the high frequency of MRSA among our specimens, our isolates at two statistical populations of cell phone users and cell phone non-users were resistant to erythromycin 6.50 % tetracycline 52 %, ciprofloxacin 0% (cell phone users) and to erythromycin 6.38 %, tetracycline 28 %, ciprofloxacin 4% (cell phone non-users). All isolates were susceptible to vancomycin (100% susceptible) which there were no statistically significant differences with the amount reported in Leski's *et al.* findings (99%) (12). Consistent with the results of some Asian and African countries, in which the percentage of resistance to erythromycin was lower than (0%) (24, 25) or was 30% (26, 27), erythromycin-resistant strains were more abundant in the current study (51.3%). However, higher prevalence of erythromycin-resistant strains have been observed in China (97.8%) (28), United Kingdom (90%) (29), and Australia (98%) (19). We also observed enhanced prevalence of tetracycline-resistant strains (61.3%) compared to previous reports from Lebanon (48% and 44%) (30, 31) and USA (5%) (32). However, Zhang et al. and Nimmo et al. reported a prevalence of 97.8% and 80% resistant isolates from China (28) and Australia (19), respectively. Moreover, the prevalence of gentamicin-resistant strains (47.7%) was more than reports from Nigeria (14.7%) (29), China (28.1%) (33) and Russia (19%) (25). In addition, the rates of resistance to clindamycin and ciprofloxacin were higher compared to Lebanon (30), Libya (22) Nigeria (24, 34) and Russia (25). Similar to the results obtained from several other studies, the sensitivity to vancomycin (27, 29- 31, 35) was observed among our isolates. In this study, we found high distribution of *ermA, ermB and ermC* genes among our isolates. However, the occurrence of *16S rDNA, Coa* and *erm* genes in cell phone users' and cell phone non-users' isolates was 75 (50 %), 49 (36.66%), 54 (36%), 50 (33.33%), 62 (41.33%) respectively. In addition, strains harboring the *ermA* and *B* genes were more abundant in the MRSA drugs. The high prevalence of *ermA* and *ermC* genes are reported in many countries and scholars have previously observed a high frequency of the genes (36, 37). In a Tunisian study, the *ermA* and *ermB* genes were detected in high frequency (38). All of the isolates of the two studies were collected from the ears. The results obtained from our study, similar to the reports from Netherlands, and findings about Turkey (39), found a high prevalence of genes encoding *ermA* and *ermB* isoforms resistant to erythromycin. Another study in Iran investigated the distribution of the *ermA* and *ermC* genes among pediatric patients, only 4.48% of the isolates carried both genes, but no detection of *erm* genes for 3.33% our isolates (40). Our results demonstrate three important findings: First, the percentage of MRSA strains and consequent multidrug resistance has increased in our population. Second, high remarkable rates of *ermA* and *ermB* were found. In addition, their distribution was the same as methicillin-resistance (MRSA) and MSSA S. aureus groups. Third, drug resistance was abundant in the analyzed populations. Despite the

geographical variations, information obtained from these regions worldwide level indicated a higher distribution of *ermA* and *ermB* in comparison with *ermC*. Importantly, the presence of *erms* genes mechanisms does demonstrate the level of their dependence on protein expression. However, There are many resistance genes and virulence factors in *Staphylococcus* strains that are encoded on cell phone genetic elements, which have been illustrated recently (41). Furthermore, it has been made of conjugative transposons among our population. This is most likely owing to their clinical importance in terms of conferred resistance is over abstruse by the SCCmec elements that confer methicillin resistance. To carry out the research, a survey of 37 tet (M) containing of *staphylococcal* strains was demonstrated by dot blot hybridization, two of which contained intTn (42). Sequencing of the methicillin-resistant strain Mu50 defined and explained the presence of a putative conjugative transposon (43). This observation suggested that the *ermA* and *ermB genes* could have caused the resistance to the erythromycin by conjugative transposons conferring resistance to the methicillin (41). In other words, resistance to the erythromycin strains integrated in transposons chromosomal copies conferring resistance to the methicillin (Figure 2). It is believed that more antibiotic resistance is acquired, and lower virulence factor was secreted (22, 44). Thus, as a consequence of high significant rate of methicillin and erythromycin drug resistance among our isolates, we can reduce the expression of *erms* genes. Moreover, it is suggested that the presence of the *ermA* and *ermB* genes and high resistance to erythromycin in samples of cell phone users and cell phone non-users is transferred by the SCCmec methicillin resistances, an indicator of increased resistance in population of cell phone users, but the expression of *ermC* genes is reduced by plasmids.

Conclusion

It is revealed that the presence of *16S rDNA*, *Coa*, and *erms* genes had a significant relation to erythromycin and methicillin. Detection of *ermA, ermB* and *ermC* plays crucial roles in the molecular mechanisms, epidemiology of the efflux pump and methylase erythromycin ribosome. Since antibiotic resistant *Staphylococci* isolates may mutate and prompt constitutive resistances it is suggested that inducible resistance test should be implemented on erythromycin resistant sensitive isolates to prevent treatment failures.

Ethical issues

Not applicable.

Authors' contributions

All authors equally contributed to the writing and revision of this paper.

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