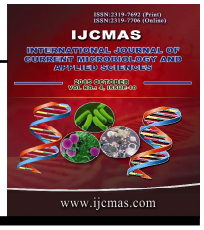
Int.J.Curr.Microbiol.App.Sci (2015) 4(10): 249-255

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 10 (2015) pp. 249-255**
http://www.ijcmas.com



Original Research Article

Mechanisms of Resistance to Erythromycin among Viridans Group Streptococci (VGS) Isolated from Blood Cultures

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Keywords

VGS, Blood cultures, Erythromycin resistance, MLS_B phenotype, M phenotype, *erm*B, *mef*A/E

A B S T R A C T

The aim of this work was to evaluate erythromycin resistance mechanisms of viridans group streptococci (VGS) isolates obtained from blood cultures. Fifty VGS isolates were obtained from patients suffering from infective endocarditis as suggested by their primary physicians. The macrolide resistance mechanisms of erythromycin resistant isolates were studied by double disc test and real time PCR. Fifteen (30%) VGS isolates resistant to erythromycin were distributed genetically as follows: *erm*B (53.3%), *erm*B+*mef*A/E (13.3%) (Those isolates containing *erm*B whether alone or combined with *mef*A/E account for MLS_B phenotype), *mef*A/E (26.6%) (account for M phenotype) and only phenotypically (6.8%) in rank of order. From our study, we concluded that constitutive MLS_B phenotype associated with *erm*B was the predominant mechanism of macrolide resistance among our erythromycin resistant VGS isolates and M phenotype was associated with *mef*A/E.

Introduction

Viridans group streptococci (VGS) are part of the oral flora and cause infections such as infective endocarditis, septicemia and neutropenia in immunocompetent and immunocompromised patients. VGS have accounted for 25 to 30% of bacteremic episodes among patients with malignancies and are the most common cause of early bloodstream infections among hematopoetic stem cell transplantation recipients (Ergin *et al.,* 2011).

Resistance to macrolides and other antibiotics among blood cultures of VGS is a major concern and could compromise currently available prophylactic and therapeutic regimens (Nandhakumar *et al.,* 2008). It has been shown that VGS can be important reservoirs of resistance genes for the more pathogenic streptococci like *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Brenciani *et al.,* 2014).

There are three different resistance mechanisms causing macrolide resistance in VGS isolates, the first is the target site modification, mediated by the erythromycin

resistance methylases, encoded by the *erm*A or *erm*B genes, conferring resistance to macrolide, lincosamide and streptogramin B antibiotics (MLS_B phenotype). Expression of MLS_B resistance can be constitutive ($cMLS_B$) or inducible ($iMLS_B$). The second is the active drug efflux mechanism, mediated by a membrane-bound efflux protein encoded by *mef*A gene conferring resistance to 14- and 15- membered macrolides only (M phenotype). Finally, the third mechanism of resistance is a mutation in the streptococcal 23S rRNA or ribosomal protein genes, leading to resistance to macrolide or streptogramin B antibiotics (MS phenotype) (Ergin *et al.*, 2006).

The aim of this work was to evaluate erythromycin resistance mechanisms of viridans group streptococci (VGS) isolates obtained from blood cultures.

Materials and Methods

The present study was conducted at the Central Microbiology Laboratory, Clinical Pathology Department, Ain Shams University, Cairo, Egypt during the period from January 2014 to October 2014.

Samples selection and susceptibility testing

The study was conducted on 50 blood culture specimens with positive signals obtained from patients suffering from infective endocarditis as suggested by their primary physicians. VGS blood culture isolates were identified by colony morphology, gram stain, catalase test and optochin test. Susceptibility to erythromycin was determined by disc diffusion method on Mueller- Hinton agar plates supplemented with 5% sheep blood. One isolate per patient was used. *S. pneumonia* ATCC 49619 was used as quality control. Isolates were determined using a double disc test with erythromycin (15μg) discs on Mueller-Hinton agar plates containing 5% sheep blood (CLSI, 2009).

Real – time PCR for detection of erythromycin resistance genes

DNA Extraction

Cell lysis for Gram-positive bacteria

One ml of cultured cells was transferred into a 1.5 ml microtube. The tubes were centrifuged at 15,000 g for 1 min to harvest the cells and the supernatant was discarded. The cell pellets were resuspended in 300 μl of cell resuspension solution. Two μl of lysozyme solution was added to each tube and mixed well by inverting. The tubes were incubated at 37°C for 60 minutes with occasional inverting then centrifuged at 15,000 g for 1 min and the supernatant discarded. Then the pellets were resuspended in 300 μl of cell lysis solution.

RNase treatment

1.5 μl of RNase Solution was added and mixed by inverting. The tubes were incubated at 37°C for 15–30 min and cooled on ice for 1 min.

Protein precipitation

100 μl of Protein Precipitation Solution was added and vortexed vigorously for 20-30 sec, then centrifuged at 15,000 g for 5 min.

DNA precipitation

The supernatants were transferred to clean 1.5 ml microtubes each containing 300 μl Isopropanol >99%. The samples were mixed by inverting gently for 1 min. then centrifuged at 15,000 g for 1 min (DNA

should be visible as a small white pellet). The supernatant was discarded and the tube was drained briefly on cleaned absorbent papers. 500 µl 80% Ethanol was added to each tube and inverted several times to wash the DNA pellets then were centrifuged at 15,000 g for 1 min. The ethanol carefully discarded. The tubes were left to dry in air at room temperature for 10–15 min.

DNA hydration

50-100 µl of DNA Hydration Solution was added to the dried DNA pellet in each tube. The DNA was hydrated by incubation at 65°C for 60 min. The samples were stored at -20°C till use.

DNA amplification and detection

The primers used for *erm*B and *mef*A/E genes and the thermal cycling conditions according to (Ergin *et al.*, 2011).

The amplification program was followed immediately by a melt program consisting of 1 minute at 95°C, 30 sec at 55°C then again to 95°C for 30 sec.

The greenish horizontal line in the graph is the threshold line at which the fluorescence begins to be detected (The point at which the amplification plot crosses the threshold is the cycle threshold=Ct) as shown in figure 1 & 2 (Muldrew, 2009). The T_m (temperature at which 50% of DNA is single stranded) of samples which were identical or close to that of positive control were considered the gene of target as shown in figure 3 & 4.

Results and Discussion

In our study, 35 (70%) were sensitive to erythromycin, while 15 (30%) were resistant to erythromycin as shown in table 1, 66.7% (n.10) constitutive MLS_B phenotype as shown in table 2 and 33.3% (n. 5) M phenotype as shown in table 3. Erythromycin resistant VGS isolates were distributed genetically as follows: *erm*B (53.3%), *mef*A/E (26.6%), *erm*B+*mef*A/E (13.3%) and only phenotypically (6.8%), one isolate did not carry any of macrolide resistance genes studied here as shown in table 4.

Our results agreed with Ergin *et al.* (2011) who studied 50 VGS blood cultures in which the percentage of erythromycin resistance was 36% (n. 18) and 7 were intermediate resistant (14%), 64% (n. 16) were $cMLS_B$ phenotype and 36% (n. 9) were M phenotype. The percentage genotypic carriage of each gene was: *erm*B, 56% (n. 14); *mef*A/E, 28% (n. 7); *erm*B+*mef*A/E, 8% (n. 2). Two of the isolates did not carry any of macrolide resistance genes studied there.

Uh *et al.* (2004) studied on 106 VGS blood cultures with rate of non-susceptibility to erythromycin 33.9%, 22 (61.1%) of 36 erythromycin non-susceptible isolates expressed constitutive resistance to macrolide–lincosamide–streptogramin B antibiotics (a constitutive MLS_B phenotype); 13 isolates (36.1%) expressed an M phenotype; and 1 isolate, a Streptococcus bovis isolate, had an inducible MLS_B resistance phenotype. *Erm*B was found in isolates with constitutive/inducible MLS_B phenotypes, and *mef*A in isolates with the M phenotype. In 3 isolates (2 isolates with a constitutive MLS_B phenotype and in 1 isolate with the M phenotype), none of *erm*A, *erm* B, *erm* C or *mef* A was detected by PCR.

Achour *et al.* (2004) studied on a total of 169 *S. mitis* isolates were recovered from 66 neutropenic patients at the Tunisian Bone Marrow Transplant Centre. Of these, 120 (70%) were resistant to erythromycin, 58

strains (48.5%) of isolates had an MLS_B phenotype and 57 (47.5%) displayed the M phenotype.

Rodriguez-Avial *et al.* (2005) reported that 45.6% (n=79) of VGS were resistant to erythromycin, of the 79 erythromycin-resistant strains, 61% displayed constitutive macrolide – lincosamide - streptogramin B ($cMLS_B$) resistance, 35% displayed the M phenotype, and 4% displayed the inducible MLSB ($iMLS_B$) phenotype.

Elia and colleagues (2009) stated that 18.7% (78 out of 416) of VGS were resistant to erythromycin and the leading resistance gene was *erm*B (74.3% of the isolates), but disagreed in the following order of these genes; *erm*B+*mef*A combination (17.9%) and *mef*A alone (7.7%).

In contrast to our study Zolezzi *et al.* (2004) from Spain reported that the M phenotype was predominant (59.38%) among their erythromycin-resistant VGS isolates from oropharynx samples, and they found the *mef*A/E gene in all the strains with M phenotype and *mef*E was the predominant subclass (95.36%).

From our study, we concluded that constitutive MLS_B phenotype associated with *erm*B was the predominant mechanism of macrolide resistance among our erythromycin resistant VGS isolates and M phenotype was associated with *mef*A/E.

Table.1 Distribution of erythromycin resistance among all isolates

Erythromycin Resistance		**Total**
Erythromycin Resistance	Count	15
	%	30.0%
Total	Count	50
	%	100.0%

Table.2 Distribution of MLS_B phenotype resistance among all isolates

MLS_B phenotype		**Total**
	Count	10
	%	66.7%
Total	Count	15
	%	100.0%

Table.3 Distribution of M phenotype resistance among erythromycin resistant VGS isolates

M phenotype		Total
	Count	5
	%	33.3%
Total	Count	15
	%	100.0%

Table.4 Distribution of patterns of resistance by PCR among erythromycin resistant VGS isolates

Resistance by PCR			Total
	*erm*B	Count	8
		%	53.3%
	*erm*B + *mef* A/E	Count	2
		%	13.3%
	mef A/E	Count	4
		%	26.6%
	No (*erm*B or *mef*A/E)	Count	1
		%	6.8%
Total		Count	15
		%	100.0%

Figure.1 Results of Syber Green real-time PCR in amplification plot with cycle's number on x axis and florescence on y axis (*erm*B gene)

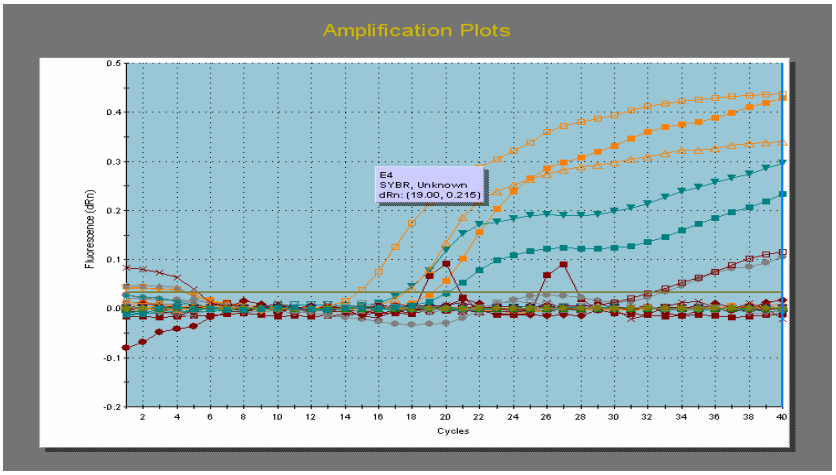


Figure.2 Results of Syber Green real-time PCR in amplification plot with cycle's number on x axis and florescence on y axis (*mef*A/E gene)

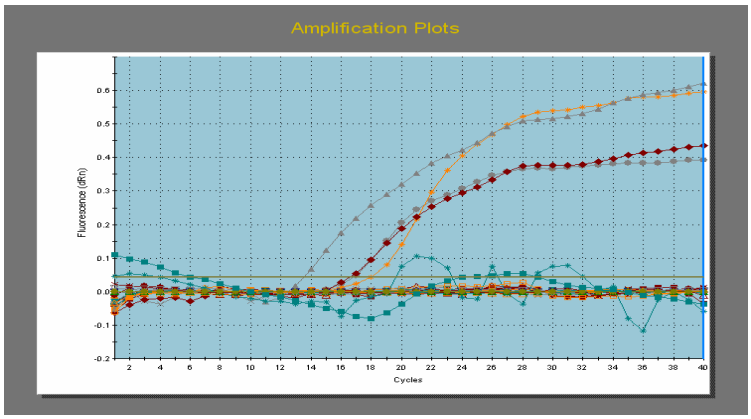


Figure.3 Results of melting curve, average T_m= 76.75–77.3°C (*erm*B)

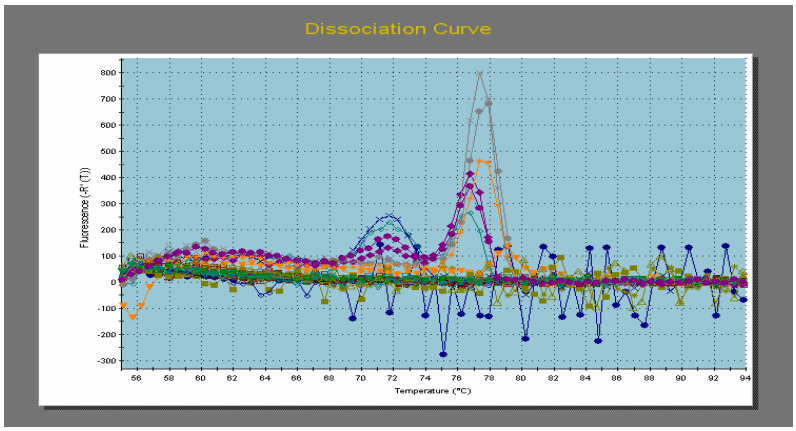
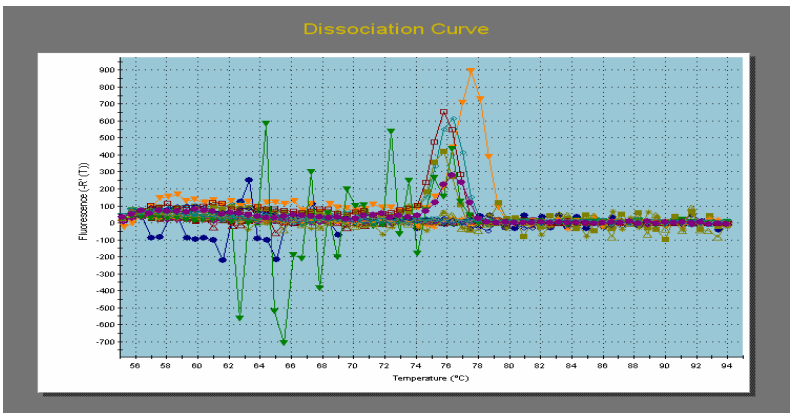


Figure.4 Results of melting curve, average T_m= 75.75–77.55°C (*mef*A/E)



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