Sultan & Gouda | J Pure Appl Microbiol | 16(2):1179-1186 | June 2022
Article 7665 | https://doi.org/10.22207/JPAM.16.2.46
Published Online May 31, 2022
Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

The Association of the *mazEF* Toxin-antitoxin System and Vancomycin Resistance in Clinical Isolates of Vancomycin Resistant *Enterococcus faecalis*

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Abstract

Vancomycin resistant enterococci are challenging bacteria as they are difficult to be eradicated. Toxin-antitoxin (TA) systems are genetic elements located in most prokaryotic genomes. The *mazEF* TA system is harbored by a plasmid among *Enterococcus faecalis* (*E. faecalis*). To explore the relation between the existence of *mazEF* TA system and vancomycin resistance among clinical isolates of *E. faecalis*. Samples were collected from patients showing clinical picture of infection. Isolates of *E. faecalis* were identified by standard microbiological methods and their antimicrobial susceptibility patterns were detected by disk diffusion method. In addition, the E-test was used to confirm vancomycin resistant isolates. All the *E. faecalis* isolates were screened for the *mazEF* TA system by PCR. A total of 180 *E. faecalis* strains were identified with a vancomycin resistance rate of 30.6%. Vancomycin resistance was significantly associated with prolonged hospital stay (P= 0.04) and ICU setting (P= 0.001). The *mazEF* TA system was detected among 100% of vancomycin resistant isolates, while only 33.6% of the vancomycin sensitive isolates carried the system with a significant difference (P= 0.002). In addition, there was a significant association between the *mazEF* TA system- positive strains and the ICU setting (P= 0.02). A significant association was found between vancomycin resistance and the presence of the *mazEF* TA system among *E. faecalis* isolates. This association supports the current efforts to utilize the *mazEF* TA system as a possible target for novel antibacterial agents; however, further studies on a wider scale are necessary.

Keywords: Enterococci, vancomycin resistance, TA systems, *mazEF* TA system, VRE

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(Received: March 8, 2022; accepted: April 24, 2022)

Abbreviations: GIT, gastrointestinal tract; E. faecalis, Enterococcus faecalis; HAIs, healthcare associated infections; VRE, vancomycin resistant enterococci; TA, Toxin-antitoxin; MHA, Mueller-Hinton agar; CLSI, Clinical and Laboratory Standard Institute; ICU, intensive care unit; PSK, post-segregation killing; MDR, multidrug resistant; SD, standard deviation.

Citation: Sultan AM, Gouda NS. The Association of the mazEF Toxin-antitoxin System and Vancomycin Resistance in Clinical Isolates of Vancomycin Resistant *Enterococcus faecalis*. *J Pure Appl Microbiol.* 2022;16(2):1179-1186. doi: 10.22207/JPAM.16.2.46

INTRODUCTION

Enterococci are facultative anaerobic Gram-positive cocci that belong to the commensal microbiota of human gastrointestinal tract (GIT). *Enterococcus faecalis* (*E. faecalis*) is considered to be the most predominant in the GIT followed by *E. faecium, E. durans* and *E. hirae.*[1] The enterococci are common human pathogens that can cause different community-acquired infections and healthcare associated infections (HAIs)[1,2] with *E. faecalis* responsible for about sixty percent of these nosocomial enterococcal infections.[3]

The continuous exposure to antibiotics has led to the selection of resistant enterococcal strains and increase in their prevalence.[4] Add to that, the enterococci are known by their genomic plasticity that can acquire and disseminate antibiotic resistance genes by horizontal gene transfer.[1,5] Among enterococcal resistant strains, vancomycin resistant enterococci (VRE) are the most concerning. Infections caused by VRE are challenging to clinicians as vancomycin has been traditionally considered as the last resort for eradication of enterococcal infections.[1,6] Furthermore, vancomycin resistance among enterococcal isolates is mediated by mobile genetic elements as plasmids which help the spread of the resistance.[7] Disturbingly, VRE have been increasingly reported as etiological agents of various HAIs,[7] where *E. faecalis* contributes to as high as 20% of VRE isolates.[8]

Toxin-antitoxin (TA) systems are genetic elements located in most prokaryotic genomes that were first identified in bacterial plasmids and later they were located on chromosomes.[9] The TA systems are organized as one operon consisting usually of two genes. A single TA module consists of a stable toxin protein and the unstable antitoxin, either protein or RNA, which counteract the toxin effect. With ideal growth conditions, the antitoxin binds to the toxin forming a TA complex, hence blocking the toxin's activity.[10] The antitoxin can also neutralize the toxin through inhibiting its synthesis or by protecting the cellular targets from the toxin.[11] In the presence of stress conditions, the antitoxin gene transcription is down-regulated or the antitoxin itself is degraded by cellular proteases, thus freeing the toxin from the antitoxin effect. Following that, the free toxin inhibits important cellular functions such as replication, gene expression and ATP synthesis resulting in cellular death or persistence.[10,12]

Bacterial TA systems contribute to various biological functions such as stress endurance, plasmid stabilization, bacteriophage resistance, programmed cell death, bacterial persistence and biofilm production.[9-11] The TA systems are classified into eight types according to antitoxin nature and its mechanism of counteracting the toxin.[9,10] The most common and distinct type of TA systems is the type II system in which the antitoxin is a protein that typically inhibits the toxin's activity through the formation of TA complex.[10]

The *mazEF* TA system is a type II TA system that has been well characterized among several bacteria including *E. faecalis* in which it was found to be harbored by a twenty kb plasmid.[13] The *mazEF* TA system consists of MazE antitoxin that binds to and inhibits the effect of MazF toxin. The MazF toxin is an endoribonuclease that can cleave mRNA at specific sites (ACA sequences) resulting in protein synthesis inhibition, while the MazE antitoxin is a labile protein that can be degraded by ClpAP serine protease.[14]

Objectives

The aim of this study was to explore the relation between the existence of *mazEF* TA system and vancomycin resistance among clinical isolates of *E. faecalis*.

METHODS

Samples collection

We have conducted a prospective study over a period of 15 months from August 2020 till October 2021. We have enrolled patients admitted to Mansoura University Hospitals and showing clinical picture of infection where different samples were collected from them aseptically. Labeled samples were transported immediately to the Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University-Egypt for further microbiological investigations. The following data for study subjects were collected; gender, age, type of sample, duration of hospital stay, the presence of chronic illness and stay in intensive care unit (ICU).

Identification of *E. faecalis* isolates

The collected samples were processed by standard microbiological techniques and the identification of enterococcal isolates was based

on colonial appearance, Gram-stained films and biochemical methods including optochin resistance, catalase test, growth in 6.5% sodium chloride and blackening of bile-esculin. Species-level identification was executed by the automated VITEK 2 as per manufacturer's instructions.

Antimicrobial susceptibility testing

The antimicrobial susceptibility patterns of the *E. faecalis* isolates were identified by the disk diffusion method using Mueller-Hinton agar (MHA) as per the guidelines of Clinical and Laboratory Standard Institute (CLSI).[15] The disk diffusion method was conducted using the following set of antibiotic disks (Oxoid, UK); ampicillin (10µg), ampicillin/sulbactam (10/10µg), chloramphenicol (30µg), erythromycin (15µg), ciprofloxacin (5µg), tetracycline (30µg), doxycycline (30µg) and vancomycin (30µg). After 24 hours incubation at 35°C, the diameter of inhibition zone produced by each antibiotic disk was measured and the results were interpreted based on the CLSI guidelines.[15] The identified vancomycin resistant *E. faecalis* isolates were confirmed by E-test strips according to manufacturer's instructions (bioMerieux, France) and the MIC values were interpreted following the CLSI breakpoints.[15] Vancomycin resistant *E. faecalis* ATCC 51299 strain was used as a positive control.[15] Isolates showing resistance to three or more different antibiotic classes were considered as multidrug resistant (MDR).[16,17]

Detection of the *mazEF* TA system

All identified *E. faecalis* isolates were subjected to plasmid DNA extraction by using GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA). Following plasmid DNA extraction, the *mazEF* TA system was detected by PCR using the following primers; Forward: 5-ATGATCCACAGTAGCGTAAAGCGT-3; Reverse: 5-TACCAGACTTCCTTATCTTTCGG-3. These *mazEF* TA loci-specific primers result in amplification products of 505 bp.[7]

The PCR test was performed in a final reaction volume of 50 µL that contained the template DNA, Taq PCR Master Mix (QIAGEN-UK) and specific primers. The PCR started with initial denaturation for 2 minutes at temperature of 95°C that was followed by thirty five cycles of denaturation (at 94°C for 1 minute), annealing (at 58°C for 45 seconds) and extension (at 72°C for 30 seconds), then a final extension at 72°C for 10 minutes.[7] Finally, agarose gel electrophoresis was used to analyze the amplification products.

Statistical analysis

The obtained data were processed and statistically analyzed using the computer program SPSS version 22.0. Descriptive data were presented as frequency (number-percentage) or means ± standard deviation (SD). Categorical nominal variables were compared using the Chi-square test such as gender (male, female), type of sample (urine, blood, and wound drainages), presence of chronic illnesses, ICU setting and presence of the *mazEF* TA system. Continuous numerical data were compared using Student T test such as age and duration of hospital stay. A P-value <0.05 was considered statistically significant.

RESULTS

During the study period, a total of 258 enterococcal strains were isolated from patients showing evidence suggestive of infection at Mansoura University Hospitals. Out of the enterococcal isolates, 180/258 (69.8%) were *E. faecalis*, 73/258 (28.3%) *E. faecium* and 5/258 (1.9%) *E. durans* as shown in Table 1. Out of the 180 *E. faecalis* strains, 111 (61.7%) were isolated from urine samples, 60 (33.3%) from blood samples and 9 (5.0%) from wound drainages (Table 2).

Based on the antibiotic susceptibility results, we found that 55 (30.6%) *E. faecalis* isolates were resistant and 125 (69.4%) were sensitive to vancomycin. The resistance rates of *E. faecalis*

Table 1. Isolated species of enterococci

Species	No.	%
E. faecalis	180	69.8
E. faecium	73	28.3
E. durans	5	1.9
Total	258	100

Table 2. Distribution of *E. faecalis* isolates in different clinical samples

Sample	No.	%
Urine	111	61.7
Blood	60	33.3
Wound drainages	9	5.0
Total	180	100

isolates to other studied antibacterials were as follows; ampicillin (45.6%), ampicillin/sulbactam (45.6%), ciprofloxacin (34.4%), erythromycin (68.9%), chloramphenicol (47.2%), tetracycline (72.2%) and doxycycline (72.2%) as demonstrated in Table 3. It should be noted that all vancomycin resistant isolates exhibited MDR profiles.

On comparing between vancomycin resistant and vancomycin sensitive *E. faecalis* patient groups, prolonged hospital stay and ICU setting were significantly associated with the vancomycin resistant group (P= 0.04 and P= 0.001, respectively). Nevertheless, no significant relation was found regarding gender, age, sample type or the presence of chronic illness as presented in Table 4.

Table 3. Antimicrobial resistance patterns of *E. faecalis* isolates

Antimicrobials	Disk content (µg)	No.	%
Ampicillin	10	82	45.6
Ampicillin/sulbactam	10/10	82	45.6
Ciprofloxacin	5	62	34.4
Erythromycin	15	124	68.9
Chloramphenicol	30	85	47.2
Tetracycline	30	130	72.2
Doxycycline	30	130	72.2
Vancomycin	30	55	30.6

The presence of *mazEF* TA system among all *E. faecalis* strains was examined by PCR. The *mazEF* TA system was detected among 55 (100%) vancomycin resistant *E. faecalis* isolates, while only 42 (33.6%) vancomycin sensitive isolates harbored the system and such difference was statistically significant (P= 0.002), Table 5.

Of the identified 97 *mazEF* TA system-positive *E. faecalis* strains, 49 (50.5%) were isolated from male patients and 48 (49.5%) from female patients. Fifty nine strains (60.8%) harboring the *mazEF* TA system were recovered from urine, 33 (34.0%) from blood and 5 (5.2%) from wound drainages. A significant association was found between the ICU setting and *mazEF* TA system- positive *E. faecalis* strains (P= 0.02). However, no significant relation was found with gender, age, sample type, the presence of chronic illness or the duration of hospital stay as shown in Table 6.

DISCUSSION

Treatment of enterococcal infections has became a big challenge because of the antibiotics misuse and dissemination of resistance genes.[4,5] In the present work, 69.8% of the isolated enterococci were *E. faecalis* which was in line with previous reports.[3,18] In a previous study conducted in Egypt, El Kholy and his colleagues reported a rate of 4% for vancomycin resistance among

Table 4. Comparison between patients infected with vancomycin sensitive and vancomycin resistant *E. faecalis* isolates

Characteristics	Vancomycin sensitive *E. faecalis* n=125	Vancomycin resistant *E. faecalis* n=55	*P*-value
Gender			
Male	63 (50.4)	29 (52.7)	0.87
Female	62 (49.6)	26 (47.3)	
Age (years)	48±15	46±12	0.88
Type of sample			
Urine	75 (60.0)	36 (65.5)	0.65
Blood	44 (35.2)	16 (29.1)	0.88
Wound drainages	6 (4.8)	3 (5.5)	0.79
Duration of hospital stay (days)	5±2	10±1	**0.04**
Presence of chronic illnesses	52 (41.6)	21 (38.2)	0.62
ICU setting (admission or transfer)	10 (8.0)	24 (43.6)	**0.001**

Values are given as mean ± SD, or number (percentage); Bold values indicate statistical significance.

Table 5. Distribution of the *mazEF* TA system among vancomycin sensitive and vancomycin resistant *E. faecalis* isolates

	Vancomycin sensitive *E. faecalis* n=125	Vancomycin resistant *E. faecalis* n=55	*P*-value
mazEF TA system			
Present	42 (33.6)	55 (100)	**0.002**
Abscent	83 (66.4)	0 (0.0)	

Values are given as number (percentage); Bold values indicate statistical significance.

Table 6. Comparison between patients infected with *mazEF* TA system- positive and *mazEF* TA system- negative *E. faecalis* isolates

Characteristics	*mazEF* TA system-positive *E. faecalis* n=97	*mazEF* TA system-negative *E. faecalis* n=83	*P*-value
Gender			
Male	49 (50.5)	43 (51.8)	0.89
Female	48 (49.5)	40 (48.2)	
Age (years)	47±14	50±13	0.77
Type of sample			
Urine	59 (60.8)	52 (62.7)	0.78
Blood	33 (34.0)	27 (32.5)	0.80
Wound drainages	5 (5.2)	4 (4.8)	0.69
Duration of hospital stay (days)	7±4	6±1	0.86
Presence of chronic illnesses	41(42.3)	32 (38.6)	0.69
ICU setting (admission or transfer)	28 (28.9)	6 (7.2)	**0.02**

Values are given as mean ± SD, or number (percentage); Bold values indicate statistical significance.

enterococcal isolates[19]; however, Ghonaim et al. few years later found that 20.9% of enterococcal isolates were resistant to vancomycin.[20] In our study, the rate of vancomycin resistance was 30.6% which is higher than the previously reported rates in earlier studies.[19,20] Such increment in vancomycin resistance suggested an escalation of the VRE problem in our healthcare settings that can be worrisome and adding an extra challenge for the clinicians.

Nevertheless, compared to our findings, higher rates of vancomycin resistance were reported in other localities as in Iran (39.5%),[7] and Ethiopia (41.7%).[16] Such higher vancomycin resistance rates could be explained by the improper implementation of antibiotic stewardship policies and inadequate infection control measures that enhance the acquisition, expression and the spread of resistance genetic determinants. On the other hand, in a recent epidemiological study in Europe, only 1.1% of *E. faecalis* isolates were found to be vancomycin resistant.[21] Similarly, lower rates of vancomycin resistant *E. faecalis*, ranging from 1.9% to 5.3%, were reported in the United States,[22] where a rate of less than 1% been reported in China.[23] Notably, all vancomycin resistant strains identified in our study exhibited MDR profile which was consistent with the findings of Yilema and his colleagues.[16] Such findings emphasized the current challenge to eliminate these isolates.

In the current work, a significant association was found between vancomycin resistant *E. faecalis* isolates and prolonged duration of hospital stay as well as ICU setting.

Prolonged hospital stay is associated with longer exposure to resistant strains that are endemic in the environment. Moreover, extended hospital stay is usually accompanied with antibacterial usage placing the nosocomial pathogens under more selective pressure. The ICU setting was also associated with the *mazEF* TA system- positive *E. faecalis* strains (P= 0.02). Patients admitted to the ICU settings are frequently exposed to antibiotics as well as invasive procedures or devices. In addition, the TA systems can spread between different bacteria by horizontal gene transfer.[9] Therefore, without proper implementation of infection control measures, the ICU can serve as an environment that enhance the spread of genetic determinants, including TA systems and resistance genes, which can explain our findings.

In our study, all vancomycin resistant *E. faecalis* isolates harbored the *mazEF* TA system as compared to only 33.6% of the vancomycin sensitive isolates. Our findings revealed a significant association between vancomycin resistance and the presence of the *mazEF* TA system among *E. faecalis* isolates (P= 0.002). In line with our results, Sadeghifard and his colleagues detected the *mazEF* TA system among 100% of vancomycin resistant *E. faecalis* and 31.4% of the vancomycin sensitive isolates.[7] Moreover, Moritz and Hergenrother have detected the *mazEF* TA system on plasmids extracted from all VRE which was consistent with our results.[24] Also, they reported that the *mazEF* TA system was located on the same plasmid with *vanA* resistance gene in more than ninety percent of the cases.[24] All these findings have suggested that the *mazEF* TA system plays a considerable role in vancomycin resistance among enterococcal isolates.

In addition to *E. faecalis*, the *mazEF* TA system was detected among *E. faecium* isolates. Moritz and Hergenrother studied 61 vancomycin resistant *E. faecium* isolates and detected the *mazEF* TA system on the plasmids extracted from all these isolates.[24] Similarly, Soheili and his colleagues studied 29 *E. faecium* isolates and detected this TA system on the plasmids from all isolates.[13] Also, Arredondo-Alonso et al. detected the *mazEF* TA system among *E. faecium* isolates.[25]

Bacterial TA systems play a major role in stabilization of plasmids[10] including those harboring vancomycin resistance genes.[7,26] Besides, TA systems mediate post-segregation killing (PSK) in which the plasmid-free daughter cell fails to express the antitoxin and killed by the toxin, while cells harboring the plasmids, encoding the TA system along with other resistance genes, survive,[11,27] which can explain the ubiquitous presence of *mazEF* TA system in vancomycin resistant isolates.[7] Furthermore, the PSK can lead to the selection of resistant cells which underlines the role of TA systems in antibiotic resistance.

Additionally, type II TA systems, such as the *mazEF* TA system, are commonly associated with the development of bacterial persistence.[11,27,28] The type II toxins reduce bacterial metabolism leading to a dormant state in which the cellular pathways commonly targeted by antibiotics are inactive.[11,27,29] Therefore, the *mazEF* TA system might induce bacteriostasis among the vancomycin resistant isolates which can explain our findings.

In our study, the *mazEF* TA system was detected in all vancomycin resistant *E. faecalis* isolates; therefore, it could be used as a potential target for novel antibacterial agents. Several strategies have utilized the TA systems as targets for new antibiotics.[10] In one strategy, in silico derived toxin and antitoxin-mimicking peptides have been used to disrupt the TA interaction.[30,31] Other studies have reported the usage of the acyldepsipeptide antibiotic (ADEP4) to activate ClpP protease with subsequent antitoxin degradation and bacterial death.[32,33] The antisense peptide nucleotide (PNA) targeting either HipB or MazE antitoxin mRNA in *Escherichia* coli inhibited the antitoxin translation and resulted in a lethal effect.[10,34] Although these approaches seem promising, further investigations are still needed before their clinical use.[10]

CONCLUSION

Our findings have pointed out the growing challenge of VRE in our healthcare settings. Almost one third of the isolated *E. faecalis* strains were resistant to vancomycin and all of these resistant isolates harbored the *mazEF* TA system. Notably, the ICU setting was significantly associated with the *mazEF* TA system- positive *E. faecalis* strains which highlighted the role played by such intense environment in disseminating mobile genetic elements between different bacterial strains. Our study revealed a significant association between vancomycin resistance and the presence of the

mazEF TA system among *E. faecalis* isolates. These findings support the current efforts to utilize the *mazEF* TA system as a possible target for novel antibacterial agents against resistant bacteria; however, further studies on a wider scale are required to test the effectiveness of these drugs.

ACKNOWLEDGMENTS

The authors would like to thank the members of Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University for providing the related support to compile this work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The study was approved by the institutional research board of Faculty of Medicine, Mansoura University, Egypt with protocol number R.22.01.1572.

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