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Reduced susceptibility of *Enterococcus* spp. isolates from Cairo University Hospital to tigecycline: Highlight on the influence of proton pump inhibitors

Reem Mostafa Hassan[a], Doaa Mohammad Ghaith[a,*], Dalia Kadry Ismail[a], Mai Mahmoud Zafer[b]

[a] Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, 1 Al-Saray Street, Al-Manial, Cairo 11559, Egypt
[b] Department of Microbiology and Immunology, Faculty of Pharmacy, Ahram Canadian University, 6th of October City, Egypt

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ABSTRACT

Objectives: The incidence of reduced susceptibility to tigecycline (TIG) is increasing. This study aimed to analyse the in vitro activity of TIG against *Enterococcus* spp. isolates recovered from hospitalised patients and to evaluate the effect of omeprazole on the in vitro antimicrobial activity of TIG against several enterococcal species.
Methods: A total of 67 *Enterococcus* clinical isolates were identified by MALDI-TOF/MS and multiplex PCR. Minimum inhibitory concentrations (MICs) of TIG alone and in combination with omeprazole (10, 30 and 60 mg/L) were determined by broth microdilution. Antibiotic susceptibility to other antibiotics was determined by disk diffusion. The presence of *van*, *tet*(X) and *tet*(X1) genes was tested by multiplex PCR.
Results: Of the 67 *Enterococcus* isolates, 2 (3.0%) were resistant to TIG and 13 (19.4%) were intermediate-resistant according to EUCAST. The frequencies of resistance to norfloxacin (80.6%), doxycycline (80.6%), levofloxacin (74.6%) and ciprofloxacin (71.6%) were highest, whilst that of vancomycin (25.4%) was lowest. The *vanA* gene was detected in 11 *Enterococcus* isolates (8 *Enterococcus faecalis*, 3 *Enterococcus faecium*), *vanB* in 3 *Enterococcus* isolates (2 *E. faecium*, 1 *E. faecalis*) and *vanC-2/3* in 3 *Enterococcus casseliflavus*. Nine isolates (13.4%) were positive for *tet*(X1). TIG resistance occurred both in patients receiving or not TIG and/or omeprazole. Omeprazole increased TIG MICs by 4–128-fold.
Conclusions: The possibility of selection of TIG-non-susceptible *Enterococcus* in the gut may occur with long-term use of omeprazole. Omeprazole influenced TIG activity in a concentration-dependent manner. To our knowledge; this is the first report of TIG-non-susceptible *Enterococcus* spp. in Egypt.

1. Introduction

Armed with multiple antibiotic resistance determinants, *Enterococcus* spp. isolates 'take advantage' of this opportunity and expand within their ecologic niche (i.e. the gastrointestinal tract of hospitalised patients) to gain the upper hand and to dominate the intestinal microbiota. From the gastrointestinal tract, multidrug-resistant (MDR) enterococci disseminate rapidly in the hospital environment. Indeed, *Enterococcus* spp. are a leading cause of nosocomial infections and are second only to *Staphylococcus* spp. as a cause of Gram-positive nosocomial infections [1].

Tigecycline (TIG) exhibits bacteriostatic activity against a large range both of Gram-positive, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (VRE), and Gram-negative bacteria (except *Pseudomonas aeruginosa* and *Proteus mirabilis*) [2]. Similar to all tetracyclines, TIG binds to the 16S rRNA of the 30S ribosomal subunit and inhibits the association of aminoacyl-tRNA. Interestingly, TIG interacts with the ribosomal target with a five-fold higher affinity, overcoming the main mechanisms of resistance to classical tetracyclines (i.e. ribosomal protection and active efflux) [3]. Resistance to tetracycline is mediated by multiple genes but follows two general strategies, namely efflux of the antibiotic and ribosomal protection, e.g. *tet*(M), *tet*(O), *tet*(S). Efflux pumps encoded by *tet*(K) and *tet*(L)

* Corresponding author.
E-mail addresses: doaa.ghaith@kasralainy.edu.eg, doaaghaith@gmail.com (D.M. Ghaith).

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are plasmid-borne determinants conferring resistance to tetracycline but not minocycline. The flavin-dependent monooxygenase Tet(X) is a resistance mechanism against TIG that was detected in *Bacteroides fragilis* strains. The Tet(X) protein can modify narrow- and expanded-spectrum tetracyclines and requires NADPH, Mg_2 and O_2 for its activity [4,5]. Tet(X) can also accept TIG as a substrate, therefore bacterial strains harbouring the *tet*(X) gene are highly resistant to TIG [6]. Increased expression of the *tet*(L)-encoded major facilitator superfamily (MFS) pump and the *tet*(M)-encoded ribosomal protection protein were hypothesised as being capable of conferring TIG resistance in clinical isolates of *Enterococcus* [7]. To date, there have been several published reports of TIG resistance in *Enterococcus*, some of them related to intra-abdominal procedures [8,9]. The mechanism of resistance remains unknown. However, TIG resistance has been increasingly reported, especially with prolonged use of omeprazole not only in enterococci-associated infections but also in *Acinetobacter baumannii* [10,11].

Omeprazole is a proton pump inhibitor (PPI) that is widely used in Egypt as an over-the-counter medication for the treatment of symptoms of gastroesophageal reflux disease and may also be given together with antibiotics to treat gastric ulcer caused by infection with *Helicobacter pylori*, which reaches rates of up to 90% in the Egyptian community [12,13].

Whether the concomitant use of omeprazole could influence the in vivo and in vitro activity of TIG is worthy of investigation. Therefore, the aim of this study was to analyse the in vitro activity of TIG against *Enterococcus* spp. isolates recovered from hospitalised patients and to evaluate the effect of omeprazole as an example of a PPI on the in vitro antimicrobial activity of TIG against several enterococcal species.

2. Materials and methods

2.1. Bacterial isolates

From October 2013 to February 2015, a total of 67 non-duplicate *Enterococcus* spp. isolates (one per patient) were randomly selected from different clinical specimens submitted for bacteriological testing. These samples were obtained from hospitalised inpatients admitted to Kasr Al-Ainy Hospital (Cairo, Egypt). The Kasr Al-Ainy School of Medicine is a tertiary care academic medical hospital belonging to Cairo University. Of the 67 patients, 39 (58.2%) were male and 28 (41.8%) were female; intensive care unit (ICU) patients represented 41 (61.2%) of the 67 patients, whilst 26 (38.8%) were from different departments (urology, chest, gastroenterology, etc.). The age of the patients ranged from 13–53 years. Nine patients (13.4%) were prescribed TIG for a concomitant respiratory or wound infection with a pandrug-resistant (resistant to carbapenems and aminoglycosides or quinolones) *Klebsiella pneumoniae* or *A. baumannii* organism for a duration of 7–10 days; moreover, omeprazole was administered to 38 (92.7%) of the 41 ICU patients as prophylaxis for stress ulcer and to 5 (19.2%) of the 26 patients in different departments for gastroesophageal reflux disease.

2.2. Bacterial species identification

All isolates were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI/TOF-MS) on a microflex LT instrument (Bruker Daltonik GmbH, Leipzig, Germany) with flexControl v.3.0 software (Bruker Daltonik GmbH) for the automatic acquisition of mass spectra in the linear positive mode within a range of 2–20 kDa according to the manufacturer's instructions [14]. All samples were prepared in duplicate to test the reproducibility of the system. Multiplex PCR was performed for *Enterococcus* spp. identification with primers specific for *Enterococcus faecalis*, *Enterococcus casseliflavus* and *Enterococcus faecium*. DNA amplification was performed as previously described [15]. Each PCR assay was performed in duplicate and blank samples were included in all PCR reactions.

2.3. Detection of resistance genes

Multiplex PCR for *van* genes, including *vanA*, *vanB*, *vanC-1* and *vanC-2/3*, was performed using the following strains as positive controls: *E. faecium* BM4147 (*vanA*); *E. faecalis* V583 (*vanB*); and *E. casseliflavus* ATCC 25788 (*vanC*) [15]. PCR was also performed on all of the isolates for the presence of resistance genes associated with TIG [*tet*(X) and *tet*(X1)] that could have been responsible for the observed antibiotic resistance [16].

2.4. Antimicrobial susceptibility testing

All isolates were tested for antimicrobial susceptibility by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. The antimicrobials tested included ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), doxycycline (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), norfloxacin (10 μg), linezolid (30 μg), vancomycin (30 μg), teicoplanin (30 μg) and nitrofurantoin (300 μg). In vitro antimicrobial susceptibility for TIG alone was determined by the disk diffusion method. Guidelines for performance and interpretation from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were followed for susceptibility determination of TIG as follows: disk diffusion (15 μg), susceptible, ≥18 mm, and resistant, ≤15 mm; minimum inhibitory concentrations (MICs) by broth microdilution method for enterococci, TIG MIC, susceptible, ≤0.25 mg/L, intermediate 0.5 mg/L, and resistant, >0.5 mg/L [17]. *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 were used as quality control reference strains for all antimicrobial susceptibility testing procedures.

The broth microdilution method was also used to determine the MIC of TIG in the presence of the PPI omeprazole. Briefly, 10^4 CFU in cation-adjusted Mueller–Hinton broth were inoculated into microplates containing a series of two-fold concentration increments of TIG in combination with omeprazole (10, 30 and 60 mg/L). Omeprazole concentrations were chosen based on the usual dosage of omeprazole and its pharmacokinetics. Inoculated microplates were incubated at 37 °C for 24 h in ambient air. Growth (bacterial cells only) and contamination (TIG and omeprazole only, to detect reagent contamination) controls were included through all testing steps. The MIC was defined as the lowest drug concentration that inhibited visible growth of the micro-organism [18].

2.5. Statistical methods

Data were coded and entered using IBM SPSS Statistics v.22.0 (IBM Corp., Armonk, NY). Data were summarised using frequency (count) and relative frequency (percentage).

3. Results

The most common source of the *Enterococcus* isolates was urine samples (44/67; 65.7%), followed by pus/wound swabs (12/67; 17.9%), blood cultures (6/67; 9.0%), and tissue sample, pleural fluid, cerebrospinal fluid, ascetic fluid and prostatic discharge (1/67; 1.5% each). Identification of the isolates classified them as *E. faecalis* ($n = 44$; 65.7%), *E. faecium* ($n = 20$; 29.9%) and *E. casseliflavus* ($n = 3$; 4.5%). Results of MALDI-TOF/MS analyses coincided with the results predicted by the multiplex PCR analysis used for isolate identification.

3.1. Antibiotic susceptibility testing

Of the 67 *Enterococcus* spp. isolates, 2 (3.0%) were resistant to TIG, including 1 *E. casseliflavus* and 1 *E. faecalis*, both of which were VRE; none of the *E. faecium* isolates were resistant to TIG. Moreover, 13 isolates (19.4%) showed intermediate TIG resistance, 12 of which were *E. faecalis* and 1 was *E. faecium*. There was 100% agreement in interpretation between TIG testing using broth microdilution and disk diffusion methods.

TIG consumption was recorded in nine patients (1.4%) from whom *Enterococcus* spp. were isolated; one patient had a TIG-resistant isolate (*E. faecalis*), six patients had intermediate-resistant isolates (5 *E. faecalis* and 1 *E. faecium*) and two patients had TIG-susceptible isolates. The relationship between TIG-non-susceptibility, location of patients, and TIG and omeprazole consumption is shown in Table 1.

High-level resistance to the antibiotics tested was observed among the isolates. Among the antimicrobial agents tested, the frequencies of resistance to norfloxacin (54/67; 80.6%), doxycycline (54/67; 80.6%), levofloxacin (50/67; 74.6%) and ciprofloxacin (48/67; 71.6%) were highest, whilst those of ampicillin (30/67; 44.8%), amoxicillin/clavulanic acid (30/67; 44.8%), vancomycin (17/67; 25.4%), teicoplanin (19/67; 28.4%) and nitrofurantoin (11/67; 16.4%) were lowest; all isolates were susceptible to linezolid.

The effect of adding omeprazole (as a representative PPI) at different concentrations on the MICs of TIG in *Enterococcus* spp. is shown in Table 2. There was no change in the MICs in all isolates with the addition of 10 mg/L omeprazole. However, at omeprazole concentrations of 30 mg/L and 60 mg/L, TIG MICs increased substantially (4–128-fold).

3.2. Detection of antimicrobial resistance genes

The *vanA* gene was detected in 11 *Enterococcus* isolates (8 *E. faecalis* and 3 *E. faecium*), the *vanB* gene in 3 *Enterococcus* isolates (2 *E. faecium* and 1 *E. faecalis*) and the *vanC-2/3* gene in 3 *Enterococcus* isolates (all *E. casseliflavus*). The presence of the resistance genes *tet*(X) and *tet*(X1) was also determined. The *tet*(X1) gene was observed in 9 *Enterococcus* isolates (13.4%), comprising 2 TIG-resistant isolates (1 patient administered TIG) and 7 TIG-intermediate-resistant isolates (6 patients administered TIG). None of the *Enterococcus* isolates was identified to carry the *tet*(X) gene.

4. Discussion

TIG has been newly introduced into clinical practice in Cairo University Hospital. It has been shown to exhibit an extended spectrum of activity against a variety of aerobic Gram-positive and Gram-negative pathogens. As clinical experience with TIG increases, it is important to investigate the mechanisms of bacterial resistance towards it. Therefore, the aim of this study was to investigate the in vitro activity of TIG against *Enterococcus* spp. isolates recovered from Cairo University Hospital inpatients and to evaluate the effect of omeprazole on the in vitro antibacterial activity of TIG against *Enterococcus* spp.

The prevalence of *E. faecalis* in this study (65.7%) was comparable with the distribution of *Enterococcus* spp. in different parts of the world [19–22]. The present study revealed that urinary tract infection was the most frequent infection (65.7%). There was a high prevalence of vancomycin resistance among *Enterococcus* spp. isolated from Cairo University Hospital. The rate of 25.4% VRE isolates reflects a threat limiting treatment options in our hospital. A similar high rate of resistance has been reported from other Egyptian studies where 25% VRE were isolated from paediatric patients [23] and 9.5% VRE in healthcare workers in ICUs [24]. Several studies have outlined that TIG resistance remains seldom reported in Gram-positive bacteria, including *Enterococcus* spp. [8,10,25,26]. Another study declared that *E. faecium* isolates are more TIG-resistant than *E. faecalis* isolates [27]. In the current study, 13 isolates (19.4%) having intermediate resistance to TIG were identified. A recent study from the Tigecycline Evaluation and Surveillance Trial (TEST) revealed that all *E. faecium* strains isolated in the Middle East and Africa recovered between 2004–2011 remained susceptible to TIG and linezolid, including VRE isolates [28].

In the present study, ICU patients represented 61.2% of patients from whom *Enterococcus* spp. was isolated. Most patients (38/41) admitted to ICU were prescribed prophylactic treatment for a stress ulcer. Although treatment options are debatable, PPIs are

Table 1
Enterococcus spp. isolates showing reduced susceptibility to tigecycline (TIG).

Species	ID-date of isolation	Sample type/ward	TIG resistance	*tet* (X1)	TIG consumption	Omeprazole consumption	VRE	Other antibiotic resistance phenotypes
E. faecalis	CU-EA-2013	Urine/ICU, surgical	R	+	Yes	Yes	*vanA*	AMP, AMC, CIP, LVX, DOX, VAN, TEC, NIT, NOR
E. casseliflavus	CU-FA-2013	Urine/ICU, gastroenterology	R	+	No	Yes	*vanC*	AMP, AMC, LVX, DOX, VAN, NIT, NOR
E. faecalis	CU-HR-2014	Blood/ICU, surgical	I	+	Yes	Yes		AMP, AMC, LVX, DOX, NIT, NOR
E. faecalis	CU-GF-2014	Blood/ICU, chest	I		No	No		LVX, DOX, NIT, NOR
E. faecalis	CU-WS-2014	Urine/ICU, surgical	I		No	Yes		AMP, AMC, LVX, DOX, NOR
E. faecalis	CU-OY-2014	Urine/ICU, neurosurgery	I		No	Yes		LVX, DOX, NIT, NOR
E. faecium	CU-MH-2014	Blood/ICU chest	I	+	Yes	No	*vanA*	AMP, AMC, LVX, DOX, VAN, TEC, NIT, NOR
E. faecalis	CU-HA-2014	Urine/ICU, gastroenterology	I	+	Yes	Yes		AMP, AMC, LVX, DOX, NIT, NOR
E. faecalis	CU-MM-2014	Urine/ICU, surgical	I		No	No		AMP, AMC, LVX, NIT, NOR
E. faecalis	CU-AG-2014	Urine/urology ward	I		No	No		LVX, DOX, NOR
E. faecalis	CU-ZF-2014	Blood/hepatology ward	I	+	Yes	Yes		AMP, AMC, LVX, NIT, NOR
E. faecalis	CU-FD-2014	Urine/gastroenterology ward	I	+	No	Yes		AMP, AMC, CIP, LVX, NIT, NOR
E. faecalis	CU-MW-2015	Urine/neurology ward	I		No	No		AMP, AMC, LVX, DOX, NOR
E. faecalis	CU-DS-2015	Pus/ICU, surgical/trauma	I	+	Yes	Yes		LVX, DOX, NIT, NOR
E. faecalis	CU-SR-2015	Blood/ICU, surgical/trauma	I	+	Yes	Yes		AMP, AMC, CIP, LVX, NIT, NOR

VRE, vancomycin-resistant enterococci; ICU, intensive care unit; R, resistant; I, intermediate; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CIP, ciprofloxacin; LVX, levofloxacin; DOX, doxycycline; VAN, vancomycin; TEC, teicoplanin; NIT, nitrofurantoin; NOR, norfloxacin.

Table 2
Effect of omeprazole at three different concentrations on the minimum inhibitory concentrations (MICs) of tigecycline (TIG) in clinical isolates of *Enterococcus* spp.

Number of isolates	MIC (mg/L)				
	Category	TIG alone	TIG + omeprazole at:		
			10 mg/L	30 mg/L	60 mg/L
30 (19 *E. faecalis*, 10 *E. faecium*, 1 *E. casseliflavus*)	S	<0.125	<0.125	0.5	16
22 (12 *E. faecalis*, 9 *E. faecium*, 1 *E. casseliflavus*)	S	0.25	0.25	8	16
13 (12 *E. faecalis*, 1 *E. faecium*)	I	0.5	0.5	8	16
2 (1 *E. faecalis*, 1 *E. casseliflavus*)	R	1	1	16	32

S, susceptible; I, intermediate; R, resistant.

offered in our hospital even for short intervals as recommended by different studies [29,30].

TIG has recently been introduced in the Egyptian market few years ago. It is mostly used for the treatment of infections caused by MDR Gram-negative micro-organisms [31], which have a high incidence among healthcare-associated infections in our hospital [32,33]. This ongoing use may lead to the development of resistance or reduced susceptibility to TIG in enterococci, which are normal residents of the human gastrointestinal tract. From a clinical perspective, treatment options for infections with TIG-resistant strains will be very limited, especially in cases associated with other mechanisms of resistance to other classes usually used in the treatment of *Enterococcus*, i.e. vancomycin, ampicillin or high-level gentamicin resistance.

Reports regarding TIG resistance have been published from different parts of Egypt, mainly in Gram-negative organisms. In a study conducted by Amer, one isolate of *Klebsiella* spp. (1/22) showed resistance to TIG whilst 40% (12/30) of *A. baumannii* were resistant to TIG [34]. Another study found 17.3% of *K. pneumoniae* isolates to be resistant to TIG from a total collection of 139 carbapenem-resistant clinical isolates [35]. Also Hassan et al. noted reduced susceptibility to TIG in 6 (9.5%) of 63 MDR *A. baumannii* isolates, with insertion sequence IS*Aba1* detected in 3 of them [36].

Presence of the *tet*(X1) gene has also been associated with TIG resistance [16]. The current results showed that nine isolates (13.4%) were positive for the *tet*(X1) resistance gene that may be implicated in TIG resistance. The *tet*(X1) variant was found in TIG-non-susceptible *A. baumannii* isolates in China, but not yet in *Enterococcus* [37,38].

Other mechanisms could be present in the TIG-intermediate-resistant isolates without *tet*(X1) and *tet*(X), such as overexpression of MepA [one of the multi-antimicrobial extrusion protein (MATE) family]. Another study revealed several mutations in the ribosomal protein gene *rpsJ*, encoding the S10 protein of the 30S ribosomal subunit, in four *E. faecium* strains (two clinical strains and two laboratory-generated mutants) showing reduced susceptibility to TIG (all TIG MICs between 0.25 mg/L and 0.5 mg/L) [39].

Using RT-qPCR and whole-genome sequence analysis, another study demonstrated a significant correlation between the *tet*(L)/*tet*(M) plasmid copy number and the TIG MIC in *Enterococcus* spp. and stated that TIG resistance may be produced by a complex interaction between various resistance mechanisms [7].

The data presented in this study indicate that in vitro susceptibility to TIG can be influenced by the addition of omeprazole in the test medium [8]. The effect of omeprazole as an example of a PPI appeared negligible for most clinical isolates when the concentration was low (10 mg/L), but with much higher doses (60 mg/L) susceptibility decreased markedly, indicating that this impact might be concentration-dependent. Ni et al. demonstrated the effect of PPIs, including omeprazole, lansoprazole and pantoprazole, on a collection of Gram-positive and Gram-negative micro-organisms, including *E. faecalis* [40]. In contrast to the current study, a concentration of 10 mg/L omeprazole could increase the TIG MIC of *E. faecalis* by two-fold. The mechanism by which PPIs influence the in vitro activity of TIG is still unclear and needs to be further explored.

In conclusion, as clinical experience with TIG increases, it is important to investigate the mechanisms of bacterial resistance towards it. The *tet*(X1) gene was observed in 9 *Enterococcus* isolates (13.4%), comprising 2 TIG-resistant and 7 TIG-intermediate-resistant isolates. Considering the use of TIG to treat intra-abdominal infections where there has also been long-term use of omeprazole, it becomes important to consider the possibility of selection of TIG-non-susceptible *Enterococcus*. Further studies are needed to specify the most important mechanism of TIG-non-susceptibility among *Enterococcus* spp. in Egypt.

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Competing interests

None declared.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2017.12.005.

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