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

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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)

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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.