The Differences between the Expression Levels of axe-txe Genes in Chloramphenicol-Sensitive and Penicillin-Resistant Enterococcus faecium Isolates
(Perbezaan antara Tahap Ekspresi Gen axe-txe dalam Pencilan Enterococcus faecium yang Peka Kloramfenikol dan Tahan Penisilin)

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ABSTRACT
Toxin-antitoxin (TA) systems are important regulatory modules in bacterial physiological functions. In this study, Axe-Txe TA system of 20 Enterococcus faecium clinical isolates was investigated by polymerase chain reaction (PCR) using self-designed primers. The functionality of this TA system in two E. faecium isolates was evaluated by analysing the expression level of axe-txe genes using real-time quantitative PCR (RT-qPCR) in penicillin-resistant and chloramphenicol-sensitive environments at different points of time. Colony-forming units (CFU) of the bacteria were also measured at a similar point of time. The selection of these two isolates for TA functionality study was determined based on the susceptibility patterns of the two isolates to penicillin, the chloramphenicol via Kirby-Baur, and broth microdilution methods, which were then interpreted based on CLSI guidelines. Axe-Txe TA system was detected in both chromosomes and plasmids (100%, each) in all 20 isolates, while the selected two E. faecium isolates were sensitive to chloramphenicol (MIC = 4 µg/mL) and resistant to penicillin (MIC = 256 µg/mL). Although higher axe-txe genes expression was also observed in a chloramphenicol-sensitive environment at half an hour of the incubation period compared to the penicillin-resistant environment, higher expression of the axe-txe genes was found in the penicillin-resistant environment at 1 h incubation period compared to the chloramphenicol-sensitive environment. Nevertheless, E. faecium isolates in both environments exhibited higher expression of txe gene (toxin) at the 24 h incubation period. Provided that the functionality of TA systems of E. faecium isolates may vary in different antibiotic environments, various environmental conditions need to be considered in the role of TA systems as potential antimicrobial targets. Different expression of TA genes in different antibiotic environments and points of time may influence the discovery and development of drugs in the future.

Keywords: axe-txe genes; Enterococcus faecium; toxin-antitoxin system
INTRODUCTION

Clinical enterococci isolates have been identified as among the major nosocomial pathogens resulting in significantly high morbidity and mortality rates worldwide (Arias & Murray 2013). Among these pathogens, *Enterococcus faecium* was the most notorious species due to its intrinsic ability to develop antimicrobial resistance and acquire abundant mobile genetic elements (Hidron et al. 2008). This pathogen was involved in device-related infections in 40% of medical care units, exhibiting 80% and 90% resistance to vancomycin and ampicillin, respectively (Hidron et al. 2008).

Type II toxin-antitoxin (TA) systems are regulatory molecules involved in the maintenance of mobile genetic elements, which contain the genes for antibiotic resistance, virulence factors, and secretory components in bacteria (Palmer et al. 2010). With the TA systems, the genetic determinants are retained within a bacterial population to ensure its survivability under stressful environments, which consist of heat, nutrient deficiency, and selective pressure of antibiotics (Coussens & Daines 2016; Van Melderen & Saavedra De Bast 2009). Therefore, TA systems are important for the physiological function of bacteria, and the distribution of these systems varies from one bacterium to another. Furthermore, the TA system consists of stable toxin and an unstable antitoxin, with genes encoded in the operons located on plasmids and chromosomes. The unstable nature of the antitoxin leads to its vulnerability to the degradation of cellular enzymes, resulting in the killing of bacteria by the toxin, which is known as programmed cell death (PCD) (Hu et al. 2010). However, the harmful nature of toxin is halted when antitoxin is bound to it, forming a complex of proteins.

Axe (toxin)-Txe (antitoxin) system was first described as a plasmid of *E. faecium*, which was known as pRUM, a functional plasmid segregational stability cassette (Grady & Hayes 2003). It was proven in recent evidence that toxins were responsible for various cellular functions, such as inhibitory actions on protein synthesis, replication of DNA, and synthesis of the cell wall in response to various growth conditions (Lee & Lee 2016). Therefore, observation on the functionality of Axe-Txe system through gene expression was highly recommended, especially when *E. faecium* was challenged with different susceptibility conditions of antibiotics during its growth, namely sensitive and resistant. In this study, it was found that *E. faecium* was challenged with chloramphenicol (antibiotic-sensitive) due to this antibiotic function in hindering the protein synthesis in a bacterium, while penicillin (antibiotic-resistant) was used due to its function in hindering the cell wall synthesis.

MATERIALS AND METHODS

IDENTIFICATION OF ISOLATES

In this study, only 20 *E. faecium* clinical isolates were retrieved from the previous culture collection, in which the culture was then stored at -70 °C (Weng et al. 2013). The re-identification of the isolates was conducted using the Gram staining method, biochemical tests, and species-specific PCR (CLSI 2016; Kariyama et al. 2000).

ANTIBIOTIC SUSCEPTIBILITY TESTING

Selected antibiotic discs, such as penicillin (10 units, Oxoid) and chloramphenicol (30 μg, Oxoid), were used in the antibiotic susceptibility test through the Kirby Baur method, followed by interpretation of findings based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2016). In respect of the antibiotic challenge, penicillin-resistant and chloramphenicol-sensitive *E. faecium* strains obtained through disc-diffusion method were used to determine the minimum inhibitory concentration (MIC) of penicillin and chloramphenicol, while these two antibiotics were prepared for broth microdilution testing based on the CLSI guidelines (CLSI 2016). The absorbance reading for each microplate was measured at 625 nm wavelength using the microplate reader (Dynex, USA) before and after the plate was incubated at 37 °C for 16 to 20 h. This test was performed in triplicate. The MIC values were determined using the comparison between the absorbance readings in the pre-incubation and post-incubation periods of each plate. Notably, the lowest concentration of an antimicrobial agent with clear suspension was recorded, and the absence of an increase in the absorbance reading in post-incubation was considered as the MIC value (Chuah et al. 2014).

CHROMOSOMAL DNA EXTRACTION FOR axe-tee GENE DETECTION

Each *E. faecium* isolate was cultured into the nutrient broth (SIGMA, USA) and incubated overnight at 37 °C. The DNAs were extracted using the DNA extraction KIT (Real Biotech Corporation, Taiwan). The procedures of extraction were conducted according to the instructions from the manufacturer with slight modifications. The bacterial pellet was mixed with 200 μL of lysozyme (SIGMA, USA), and gently inverted for every two to three min, then incubated at room temperature for 10 min. Following that, 200 μL of Buffer GB was added to the mixture before being shaken for five seconds. The mixture tube was inverted for every three min and it was incubated at 60 °C for 10 min. In the case of the DNA elution, 200 μL of Elution Buffer was used,
followed by the addition of 200 µL of absolute ethanol, which was then gently vortexed. Then, the mixture was transferred to the GD column and centrifuged for two min at 14,000 rpm. This was followed by the addition of 400 µL of Buffer W1 into the column, which was centrifuged at 14,000 rpm for 30 s. Subsequently, 600 µL of Wash Buffer was added and then the mixture column was centrifuged at the same rpm for the same duration. Further centrifugation was then performed at 14,000 rpm for three min to remove any residues in the mixture. The GD column was transferred into a fresh 1.5 mL DNAse/RNase-Free tube, while 50 µL of pre-heated Elution Buffer was added into the centre of the column matrix. The column was allowed to stand for three min before being centrifuged at 14,000 rpm for 30 s. The DNA was then extracted and stored at -30 °C until further use.

PLASMID DNA EXTRACTION FOR axe-txe GENE DETECTION
Each *E. faecium* clinical isolate was cultured in nutrient broth (SIGMA, USA) at 37 °C overnight, while the plasmid was extracted using the plasmid extraction KIT (Analytik Jena, Germany). The processes of this extraction started with the centrifugation of 5 mL of overnight cultures to form cell pellets. The cell pellets were then added into 200 µL of Resuspension Buffer and pipetted several times. Then, 200 µL of Lysis Buffer was added to the mixture, which was gently inverted for five to 10 times. The mixture tube was added into 300 µL of Neutralisation Buffer and inverted for four to six times before being centrifuged further for two min at 12,000 rpm. Following that, the supernatant was loaded into the column and centrifuged at 12,000 rpm for one min before 500 µL Washing Solution A was added and centrifuged at the same rpm for the same duration. Another 700 µL of Washing Solution A was added and centrifuged again at the same rpm and duration before the empty column was centrifuged at the maximum speed for two min to remove the remaining buffer. Finally, 50 µL of Elution buffer was added into the column and stored for one min at the room temperature. After that, it was centrifuged at 8,000 rpm for one min, while the plasmid DNA was stored at -30 °C. Contaminants, including chromosomal DNAs, were removed through the treatment of plasmid with 0.2 µL of ethidium bromide (Promega, USA).

EVALUATION OF THE axe-txe TA SYSTEM
All isolates were subjected to a polymerase chain reaction (PCR) for the amplification of *axe-txe* genes through chromosomal or plasmid DNA. The primers were designed by utilising the sequences obtained from the gene bank (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The list of primers used is listed in Table 1. Generally, the PCR amplification was performed in DNA thermocycler (Bio-Rad, Singapore), and the uses of the amplification parameters consisted of initial denaturation at 95 °C for two min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 52°C for 10 s, extension at 72 °C for 20 s, and a final extension at 72 °C for five min. The PCR products were visualised on 1% of agarose gel electrophoresis, which was then analysed in the gel documentation system (BioRad, Singapore). Moreover, the purified PCR products were sequenced by MyTACG Bioscience Enterprise (Malaysia), while the aligned nucleotide sequences were searched in a BLAST® at (https://blast.ncbi.nlm.nih.gov/) to analyse the similarity of the sequenced gene in GenBank library.

| Primer | Sequence (5’-3’) | Size (bp) | Accession No |
|--------|-----------------|-----------|--------------|
| txe    | CCTAATCCAGCAAAAGGGGAA
         | GGCCTGGTCTGATGATGCTTG | 123 | NC_021170.1 |
| axe    | AGTGTTCATCAACGTCTTTGTAGCAT
         | GCAGTAGCTTATCAATTTTCCGC | 161 | NC_021170.1 |

ANTIBIOTIC CHALLENGE PREPARATION AND EVALUATION OF COLONY-FORMING UNIT OF CELLS
In the assessment of two different antibiotic conditions as environmental stressors in *axe-txe* gene expression studies, two *E. faecium* isolates were categorised as penicillin-resistant and chloramphenicol-sensitive based on the susceptibility patterns through the Kirby-Baur and broth microdilution methods. The standardised inoculums were prepared by inoculating three to five completely isolated colonies into 5 mL of nutrient broth (SIGMA, USA). The optical density was determined by BioPhotometer (Eppendorf, Germany) at 600 nm. This was followed by
the preparation of penicillin and chloramphenicol, each amounting to 4096 µg/mL (Oxoid, United Kingdom). Then, 100 µL of each antibiotic was added into 900 µL of culture, which was mixed thoroughly. Since at 0 min was considered as the first step of adding the antibiotic, the treated culture was incubated at 0 min, 30 min, 60 min, 3 h, 6 h, and 24 h. The culture was then centrifuged at 14,000 rpm for 1 min. Next, the supernatant was discarded, and 1000 µL of nutrient broth (SIGMA, USA) was added and centrifuged again at the same rpm and duration. Tenfold serial dilution-agar plate technique was performed, which was followed by the incubation of all plates overnight at 37 °C. It is important to note that the test was performed in triplicate. The colony growths were calculated using colony counter (Stuart, United Kingdom), while the cell survival was measured through the comparison between the colony-forming unit (CFU) of cells treated by antibiotics to the CFU of the untreated cells.

STRESS INDUCTION
The stress on E. faecium strains was the result of the antibiotics treatment of penicillin and chloramphenicol within a given time interval, which started at 0 min, 30 min, 60 min, 3 h, 6 h, and 24 h. The RNAs were extracted from the bacteria in two stress conditions at each time point, while the level expression of Axe-Txe TA system was evaluated by RT-qPCR.

REAL-TIME QUANTITATIVE PCR (RT-QPCR)
The primer sequences for the axe-txe genes and Ent-tuf primer (Halvorsen et al. 2011) were designed using the GenScript software, and all primers were synthesised by the Integrated DNA Technologies Company (Singapore). The appropriate amplicon length was amplified by all primers using 5 µL of complementary (c) DNA in a total volume of 20 µL per reaction in a Mastercycler® realplex 2 (Eppendorf, Hamburg, Germany) with an Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, USA). The procedures started with an initial denaturation at 95 ºC for two min, followed by 40 cycles of this process at 95 ºC for 10 s, 52 ºC for 15 s and 72 ºC for 20 s. Overall, the test was performed in triplicate. The RT-qPCR results were then analysed quantitatively through the estimation of the fold changes in the expression of axe-txe genes under certain time intervals in the treated and non-treated E. faecium isolates. This estimation was performed by calculating the CT values based on the established formula (Schmittgen & Livak 2008).

RESULTS AND DISCUSSION
A toxin-antitoxin (TA) system is prominent in most Gram-positive and negative bacteria. Significant attention has been brought to the TA system among scientists over the last few decades due to its new potential antimicrobial target discoveries (Chan et al. 2016; Yang & Walsh 2017). Furthermore, the functionality of TA systems has also been highlighted as important regulatory modules, which are involved in bacterial physiological adaptations and maintenance of mobile genetic elements (MGEs). Therefore, this study was conducted to investigate the distribution of Axe-Txe TA system and the functionality of the TA system in two different antibiotic conditions in the E. faecium clinical isolates.

![FIGURE 1. Distribution of axe-txe loci among E. faecium clinical isolates](image-url)
It was found that all *E. faecium* isolates possessed 100% of Axe-Txe TA system in their chromosomes and plasmids. Figure 1 illustrates the distribution of *axe-txe* loci in *E. faecium* clinical isolates, and the sequencing results demonstrated that the *txe* and *axe* genes possessed 98.0% and 100% of similarities with the GenBank, respectively. The Axe-Txe TA system was initially detected in a novel plasmid from a clinical isolate of *E. faecium*, which was involved in the plasmid segregational stability cassette. Recently, it has been detected in *Bacillus* sp, *Escherichia coli*, and several evolutionary-diverged species (Grady & Heyes 2003). It was found that the Axe-Txe TA system was prominent in the clinical *E. faecium* isolates. This phenomenon was indicated by the abundant Axe-Txe and other TA systems found in clinical enterococci, especially in antibiotic-resistant enterococci (Rosvoll et al. 2010). A study by Moritz and Hergenrother (2007) found lower prevalent rate (75%) of plasmid-borne Axe-Txe TA system among vancomycin-resistant enterococci (VRE) isolates. However, none of Axe-txe TA system was detected in 75 VRE chromosomal DNAs in their study.

Although the true prevalence of the Axe-Txe TA system among clinical enterococci is still unknown, several types of TA systems have been recently recognised (Lee & Lee 2016; Soheili et al. 2015). The presence of Axe-Txe TA system in both loci (chromosome and plasmid) in the *E. faecium* isolates in this study would contribute to an insight on the possibility of diverse biological functions of Axe-Txe TA system among the isolates and their successful evolutionary strategies. Furthermore, it was found that chromosomal TA systems would be incorporated into different types of bacterial responses, while the systems with plasmid-borne would be horizontally transmitted among inter- and intra-species of bacteria via mobile genetic elements (MGEs) (Coussens & Daines 2016; Van Melder & Saavedra De Bast 2009). Moreover, these MGEs also carried antibiotic and virulence genes, and their stability was maintained by TA systems. These characteristics led to high adaptability profiles and the increased resurgence of multidrug-resistant enterococci as observed in several hospital settings (de Kraker et al. 2013; Koch et al. 2004; Wisplinghoff et al. 2004). For instance, all vancomycin-resistant enterococci (VRE) was shown to confer the plasmids with the mazEF TA system, which was formerly identified in a Gram-negative bacterium, *E. coli*. Notably, *mazEF TA* gene co-existed with the *vanA* gene (vancomycin-resistant gene) in more than 90% of these plasmids (Moritz & Hergenrother 2007). Figure 2 illustrates the gel electrophoresis image of *axe-txe* genes detected in the chromosomes and plasmids in the representative *E. faecium* isolates.

![Gel electrophoresis image of axe-txe genes detected in the chromosomes and plasmids in the representative E. faecium isolates](image-url)
TABLE 2. Susceptibility results and minimum inhibitory concentration (MIC) of *E. faecium* towards penicillin and chloramphenicol

| Antibiotics      | Susceptibility (Kirby Baur) | MIC (µg/mL) |
|------------------|-----------------------------|-------------|
| Penicillin       | Resistant                   | 256*        |
| Chloramphenicol  | Sensitive                   | 4*          |

*MIC of penicillin values are interpreted according to the CLSI guidelines (2017) as sensitive (≤8 µg/mL) and resistant (≥16 µg/mL), *MIC of chloramphenicol values are interpreted according to the CLSI guidelines (2017) as sensitive (≤8 µg/mL) and resistant (≥32 µg/mL).

Despite the immense data on TA systems published in the literature, there is still insufficient information regarding the functionality of the TA systems among enterococci. It was reported in several works of research that few TA systems were shown to be functional due to the exposure of the bacteria to stressful environments (Coussens & Daines 2016; Van Melder & Saavedra De Bast 2009). For instance, *yoeB* toxin gene was activated when the degradation of *yefM* antitoxin was promoted by the heat stress in *E. coli* (Janssen et al. 2015). In a previous study, toxin genes (*mazF*, *relE*, and *higB*) were highly expressed upon the heat challenge in both *E. faecium* and *E. faecalis* clinical isolates (Soheili et al. 2015).

Stressful environments were considered upon the exposure of *E. faecium* isolates to two different antibiotics (penicillin and chloramphenicol) in a 24 h incubation period in our study. To the best of our knowledge, there is insufficient data on the evaluation of TA system in different antibiotic environments. Meanwhile, penicillin-resistant and chloramphenicol-sensitive isolates were confirmed by MIC test based on the CLSI guidelines outlined in this study (CLSI 2016). Table 2 illustrates the susceptibility results of *E. faecium* isolates by Kirby Baur and broth microdilution methods, the effect of these antibiotics was investigated by measuring the cell viability.

![Effect of Antibiotics on Cell Viability](image.png)

**FIGURE 3.** The percentage of survivors after treated with penicillin and chloramphenicol over 24 h
Figure 3 illustrates the percentage of viable cells after the treatment of penicillin and chloramphenicol for 24 h. Approximately 50% reduction in cell viability (as counted by CFU/mL) was observed in the 3 h post-incubation in a penicillin-resistant isolate compared to the 1 h incubation in a chloramphenicol-sensitive isolate in the study. Furthermore, the inability of penicillin to inhibit the peptidoglycan synthesis of the cell wall of enterococci was due to the presence of penicillin-binding protein 5 (pbp5), encoding a class B penicillin-binding protein (PBP) (Sifaoui et al. 2001), which possessed a low affinity for β-lactams antibiotics, including penicillin. Overall, this finding could support the survivability of the bacterium investigated in this study.

Antibiotics Treatment in *E. faecium*

The level of expression of *axe-txe* genes was evaluated by RT-qPCR. Each TA gene in different antibiotic environments was evaluated through the measurement of the level of its gene expression compared to the normal conditions evaluated using the comparative C\(_{\text{act}}\) method (ΔΔC\(_{\text{act}}\) Method). Figure 4 illustrates the differences between the expression levels of *axe-txe* genes in two different antibiotic environments with the colony-forming unit of *E. faecium*. Upon half an hour of the incubation, a higher level of *axe-txe* gene expression was observed among the chloramphenicol-sensitive *E. faecium* isolates compared to the penicillin-resistant *E. faecium* isolates. It was believed that a few TA systems were used to selectively kill or inhibit the growth of several bacterial populations in different environmental stressors (Aizenman et al. 1996). In this case, the growth inhibition by chloramphenicol might be assisted by the presence of Axe-Txe module, which actively functioned upon the transcription of more genes, as shown through the reduction of CFU counts. Notably, a study demonstrated that the presence of chloramphenicol would boost the transcription of *hp0968-hp0967* Type II TA genes in *Helicobacter pylori* isolates (Cárdenas-Mondragón et al. 2016).

It is noteworthy that the expression of the *axe* gene (antitoxin) was significantly high at almost eight folds compared to the *txe* (toxin) gene in this condition. It was inferred that constant inhibition by chloramphenicol on the bacteria cells might activate other regulatory modules to actively promote the production of antitoxin to neutralise the cognate toxic, which subsequently reduces the bacterial apoptosis in the antibiotic-sensitive environment. Moreover, bacterial apoptosis or programmed cell death was associated with the
accumulation of antitoxin in the cell (Christensen et al. 2004). Although axe-txe genes in *E. faecium* isolates were not fully activated in a penicillin-resistant environment at the initial stage, higher expression of axe-txe genes was observed among penicillin-resistant *E. faecium* isolates at 1 h incubation period, and the expression of txe gene (toxin) was higher in both environments starting from three to six hours of incubation periods. Notably, the CFU counts constantly decreased in chloramphenicol-sensitive strains compared to the penicillin-resistant strains.

It could be seen from another study that the activation of axe-txe genes might be delayed in antibiotic-resistant environments due to the involvement of unknown different regulatory mechanisms as proposed (Grady & Hayes 2003). As an effect from physiological stress conditions, it was found in this study that the expression of toxin genes was higher than antitoxin genes. In Type II TA system, the promoter was shared by both Ta genes, and the transcription of Ta proteins was higher in stressful environments. However, due to the instability of the antitoxin and its susceptibility to being degraded by environmental stressors could reduce the autolysis of bacteria cells, which was enhanced by *ε/ζ* systems in persistent environmental stimuli (Mutschler & Meinhart 2011). Provided that no intracellular targets for Ta complex released from the promoter site, preventing the uncontrolled production of antitoxin (Boss et al. 2013). Nonetheless, minimum increment in the fold changes of txe gene in a penicillin-resistant environment at the 6 h and 24 h incubation periods was recorded, compared to the chloramphenicol-sensitive environment. This finding indicated a lower degree of stressor in the former condition compared to the latter (*E. faecium* isolate was not inhibited by the penicillin). Moreover, the lack of environmental stressors could reduce the autolysis of bacteria cells, which was enhanced by *ε/ζ* systems in persistent environmental stimuli (Mutschler & Meinhart 2011).

**CONCLUSION**

This study has contributed to an insight into the functionality of the TA system in various environmental stresses to the antibiotics among *E. faecium* clinical isolates. It was found that the Axe-Txe Type II TA system was prominent in both chromosome and plasmids in the highly versatile bacterium of the hospital setting selected in this study. Furthermore, although the antibiotic-resistant *E. faecium* isolates exhibited a delayed expression of axe-txe genes compared to the antibiotic-sensitive isolates, further discussion on the functionality of other TA systems is crucial to determine whether only a certain type of Type II TA system or multiple different toxin-antitoxin modules are involved. Last but not least, the identification of intracellular proteins is equally important to identify any potential interactions between TA systems and host proteins.

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