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Linezolid-resistant *Enterococcus casseliflavus* and *Enterococcus gallinarum* isolated from poultry farms in Kelantan, Malaysia

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ABSTRACT

Aims: Linezolid has become a decisive therapy in treating infections with vancomycin-resistant *Enterococcus* (VRE). Currently, the emergence of linezolid-resistant *Enterococcus* further complicates the therapeutic options and leads to global health threat not only in hospital setting but in the community. The study aimed at antimicrobial pattern of *Enterococcus* isolated from 6 poultry farms in Kelantan, Malaysia.
Methodology and results: Between February and December 2019, 300 broiler cloacal swab sample (*Gallus gallus domesticus*) were collected and screened for linezolid-resistant enterococci (LRE) using a standard biochemical and antimicrobial susceptibility tests. Among all the samples, 32.3% (n=97/300) grew *Enterococcus*, 71.1% (n=69/97) of it were identified *Enterococcus casseliflavus* by molecular identification, whilst remaining isolates 28.9% (n=28/97) were further identified as *Enterococcus gallinarum* by 16S rRNA sequencing. None of the isolates were found to exhibit high-level resistance to vancomycin. However, 3/97 (3.1%) were exhibit resistance to high-level gentamicin based on Kirby-Bauer disk diffusion test. Whereas 48/97 (49.5%) of isolates were observed to be resistant to ampicillin, 28/97 (28.9%) were resistant to penicillin. Surprisingly, among the two strains isolated, 18.6% (n=18/97) of it were resistant to linezolid. Isolates showed resistance to linezolid by disk diffusion test were verified by VITEK-2 automated system (bioMérieux, USA) with MIC ≥8 µg/mL. All antimicrobial susceptibility test and minimal inhibitory concentration (MIC) results were interpreted according to Clinical and Laboratory Standard Institute (CLSI).
Conclusion, significance and impact of study: In conclusion, this study has reported the prevalence of linezolid resistant *Enterococcus* (LRE) in highly intrinsic antibiotic resistant of *E. casseliflavus* and *E. gallinarum* in Malaysia poultry farms, alongside with the truancy of *van*A strains. The emergence of LRE strains is an alarming problem to the animal husbandry and healthcare setting worldwide. This could lead to potentially untreatable and life-threatening enterococcal infections. Even more worrying is the spread of LRE to geographical regions where these strains were previously unreported, which may pose a global health threat. Antimicrobial surveillance in poultry husbandry is thus, dimly necessary to prevent wide spread of multidrug-resistant bacteria.

Keywords: Linezolid-resistance, *Enterococcus*, poultry farm, Kelantan, Malaysia

INTRODUCTION

Enterococci are normal flora of gastrointestinal tract of human and animals. They are known to cause healthcare-associated infections (HCAIs) worldwide and become a major concern to the public health. In Malaysia, the first case of hospital acquired vancomycin-resistant enterococci (VRE) occurred in 1996 at University of Malaya Medical Centre (Riley *et al*., 1996). Even though VRE has been reported in Europe beforehand, the spread of VRE in Southeast Asia was scattered. Later on, it was reported in one of the chronic renal failure patient from Kuala Lumpur Hospital in 2006 (Zubaidah *et al*., 2006). The origin of VRE is unknown, but the emergence of VRE in human was believed to affiliate from the overuse of avoparcin as a growth promoter in the animal husbandry.

Furthermore, the rampant and misuse of glycopeptide antimicrobials have resulted in the accelerated of

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vancomycin-resistant *Enterococcus* strains in human and livestock (Hayes *et al.*, 2004). With the emergence of high vancomycin resistance, linezolid has become the last resort of antimicrobial used to treat the infections cause by Gram-positive bacteria, in particular involving strains harbouring resistances, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and VRE. Therefore, emergence of these resistance pathogens has prevented the treatment of such infections and this causes a major warrants worldwide surveillance (Arias and Murray, 2008; Cavaco *et al.*, 2017).

Linezolid-resistant enterococci (LRE) has been reported in 2001 (Auckland *et al.*, 2002), not less than a year after the approval of linezolid used by US Food and Drug Administration (FDA) for treating VRE infections in 2000 (Wang and Hsueh, 2009). Linezolid resistant has been reported in referring to the three most common mechanism; mutations in the V domain of 23S rRNA (Marshall *et al.*, 2002); mutations in the sequence of genes encoding the riboproteins *L3*, *L4* and *L22* (Bi *et al.*, 2018); and acquisition of linezolid-resistance genes, including *cfr*, *cfr(B)*, *optr*A and *poxt*A which associated with the mobile genetic elements (MGE) (Sadowy, 2018). Different studies have documented a range of non-clinical sources as reservoirs of multidrug-resistant (MDR) enterococci often carrying genes encoding to significant robust features (Wang *et al.*, 2015; Rushton-Green *et al.*, 2019). Thus, the study was conducted to determine the antibiotics susceptibility patterns among enterococci isolated from poultry, which has close association with human as a food source.

MATERIALS AND METHODS

Study design

A single proportion formula was used to determine the sample size. Previous study by Chan *et al.* (2008) reported of VRE prevalence in poultry was 5.3% at the confidence level of 95% and margin error of 5%.

Poultry cloacal swab samples

Six commercial poultry farms were identified in the six different districts in Kelantan state on the east-coast Malaysia. Estimated, there were 5000 to 8000 broilers per farm and approximately, 50 samples from different broiler flocks were collected within each farm. The chicken's cloaca swabs were taken after consented from the owners. The samples were taken from the chicken (*Gallus gallus domesticus*) using a sterile cotton swabs and inoculated immediately into brain heart infusion (BHI) broth (Oxoid, England) supplemented with 6.5% sodium chloride (NaCl). The samples were then incubated for 24 h at 37 °C. Samples with positive growth were further sub-cultured onto Slanezt and Bartley Medium (Oxoid, England) with antibiotic selection, 6 µg/mL of vancomycin (Oxoid, England). All red, maroon or pink colonies were taken from each sample for further genus identification.

Bacterial identification

Biochemical tests (preliminary identification)

The isolates recovered from the Slanezt and Bartley media (Oxoid, England) were presumptively identified as enterococci by colonial morphology, Gram's stain, the absence of catalase production, the presence of pyrrolidonylarylamidase by hydrolysis of L-pyrrolidonyl-β-naphthylamide (PYR; Oxoid, United Kingdom, England) and the ability to hydrolyse esculine and resistance to bile (Bile-Esculine azide test; Oxoid, United Kingdom, England). Further identification of species was carried out with a test scheme as based on carbohydrate fermentation, motility and colony pigmentation described by Facklam and Collins (1989) and Raeisi *et al.* (2017).

Molecular identification by PCR and 16S rRNA sequencing for species identification of Enterococcus

Bacterial lysates were prepared to obtain DNA by suspending a few colonies of *Enterococcus* in PCR tube containing 100 µL of DNase-free distilled water. The suspensions were boiled in water bath for 10 min and centrifuged at 10000× *g* for 3 min. Then, 5 µL supernatants were directly used as a template in a PCR reaction for further confirmation of *E. casseliflavus* species. A specific forward (5'-TCC TGA ATT AGG TGA AAA AAC -3') and reverse (5'-GCT AGT TTA CCG TCT TTA ACG -3') primers described previously by Jackson *et al.* (2004) were used to target the small region of superoxide dismutase (*sod*A) gene sequence. Other unidentified *Enterococcus* species were proceeded with 16S rRNA sequencing.

The 16S rRNA was amplified using the universal primer pair BakII-F (5'-AGT TTG ATC MTG GCT CAG-3') and BakII-R (5' GGA CTA CHA GGG TAT CTA AT 3') described previously by Goldenberger *et al.* (1997). Both DNA amplification assays for detecting *sod*A *E. casseliflavus* and 16S rRNA were done in 25 µL reaction volume. The PCR was performed using a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with initial cycle of denaturation at 95 °C for 5 min, 30 cycles consisting of denaturation at 95 °C for 30 sec, annealing for 30 sec at 58 °C and extension at 68 °C for 1 min, followed by final extension at 72 °C for 5 min. The PCR products were electrophoresed through 1.5% agarose gels (Promega, Madison, USA) at 90 V for 60 min. The impurified PCR products were sent to a commercial sequencing service (MATRIOUX, Singapore) according to the procedures and requirements delineated by the service providers. The sequence results were analysed using BLAST at the NCBI database to confirm the *Enterococcus* species (Bosshard *et al.*, 2004).

Antibiotic susceptibility test

All enterococci isolates were tested for susceptibility to seven antibiotics (Oxoid, England) by Kirby-Bauer disk diffusion method and interpreted according to Clinical

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Laboratory and Standards Institute (CLSI) guidelines and European Society of Clinical Microbiology and Infectious Disease (EUCAST) (www.eucast.org; version 10.0, June 2020). The results were analysed and stated as sensitive (S), intermediate (I) and resistance (R). The antibiotics tested are considered the most important drugs by World Health Organization (WHO) in treating Gram-positive bacteria and VRE infections. These include penicillin (10 µg), ampicillin (10 µg), high level gentamicin (120 µg), vancomycin (30 µg), teicoplanin (30 µg), linezolid (30 µg) and tigecycline (15 µg). Isolates showing resistance to linezolid by disk diffusion method were verified using VITEK-2 automated system (bioMérieux, USA) to determine for their minimal inhibitory concentration (MIC).

RESULTS

Prevalence of *Enterococcus* spp.

A total of 300 broiler's cloacal swab samples were obtained from the selected farms. Based on biochemical tests and molecular identification by PCR and 16S rRNA sequencing, the results showed that 32.3% (n=97/300) of the samples were confirmed as *Enterococcus* species. Among the 97 enterococcal strains isolated, majority (n=69/97; 71.1%) were identified as *E. casseliflavus* (Figure 1), whilst the remaining isolates were identified as *E. gallinarum* (n=28/97; 28.9%) by DNA sequencing of the 16S rRNA gene (Table 1). All the 28 isolates of 16S rRNA DNA sequence showed significant alignments with >99% homology identities to *E. gallinarum* strains in Genebank databases (Table 2).

Table 1: Distribution of *Enterococcus* species recovered from the poultry cloacal swabs in Malaysia.

Enterococcus species	Poultry cloacal swab (n)	Total (%)
E. casseiliflavus	69	71.1
E. gallinarum	28	28.9

Table 2: Identification of bacterial strains by 16S rRNA analyses.

Isolates number	Nearest match	GenBank access no.	Identity (%)
F3/C2/3(A)	*Enterococcus gallinarum*	MN915062	99.76
F3/C2/3(B)	*Enterococcus gallinarum*	CP046307	100.0
F3/C24	*Enterococcus gallinarum*	CP046307	99.45
F3/C2/7(A)	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/7(B)	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/8	*Enterococcus gallinarum*	CP046307	99.86
F3/C2/9	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/10	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/11	*Enterococcus gallinarum*	MN880439	100.0
F3/C2/12	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/13	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/14	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/15	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/16	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/17	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/18	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/19(A)	*Enterococcus gallinarum*	CP046307	100.0
F3/C2/20(A)	*Enterococcus gallinarum*	MH111475	99.61
F4/C1/16	*Enterococcus gallinarum*	CP046307	99.58
F4/C1/21	*Enterococcus gallinarum*	CP046307	100.0
F5/C1/9	*Enterococcus gallinarum*	CP046307	100.0
F5/C1/19	*Enterococcus gallinarum*	MN208191	99.48
F5/C2/01	*Enterococcus gallinarum*	CP046307	100.0
F5/C2/11	*Enterococcus gallinarum*	MN208191	99.61
F6/C1/23	*Enterococcus gallinarum*	CP046307	100.0
F6/C1/24	*Enterococcus gallinarum*	MH111475	99.35
F6/C2/08	*Enterococcus gallinarum*	CP046307	100.0
F6/C2/22	*Enterococcus gallinarum*	CP046307	100.0

F3- farm three, F4- farm four, F5- farm five, F6- farm six, C1- cage one, C2- cage two

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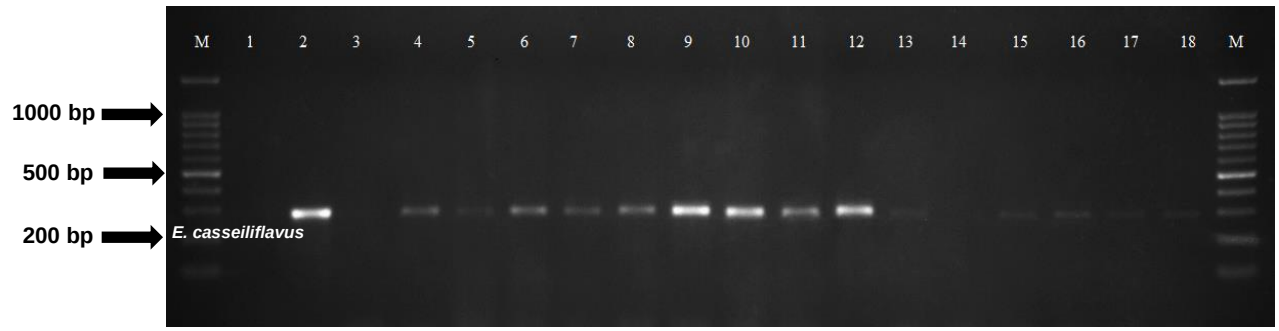

Figure 1: Conventional PCR analysis of a short region of *sod*A gene (288 bp) from *Enterococcus* spp. to identify *E. casseliflavus*. M, 100 bp plus marker; Lane 1, negative control; Lane 2, positive control; Lanes 3-18, *Enterococcus* spp. isolates (Lanes 3 and 14 are negative result; Lanes 4-13 and Lanes 15-18 are positive result).

Phenotypic antimicrobial susceptibility

Among the 97 enterococcal isolates, none of them showed resistant to vancomycin based on disk diffusion test (Table 3). Interestingly, there were 18/97 (18.6%) were resistant to linezolid. All isolates showed resistant to linezolid were further verified by VITEK-2 automated system (bioMérieux, USA) which MIC ≥8 µg/mL. The 18 isolates of linezolid-resistant *Enterococcus* (LRE) were identified as *E. casseliflavus* (n=15) and *E. gallinarum* (n=3) respectively. These LRE were isolated from three different farms which are from farm 3, farm 5 and farm 6 as stated in the (Table 4). All the 97 *Enterococcus* spp. have demonstrated 100% sensitivity to teicoplanin and tigecycline. Of all, 3/97 (3.1%) were identified to have resistant to high-level gentamicin whilst, 48/97 (49.5%) were resistant to ampicillin and 28/97 (28.9%) were resistant to penicillin. Based on the existing knowledge and findings, this is the first linezolid-resistant *Enterococcus* (LRE) isolated from intrinsic resistant strain of *E. casseliflavus* and *E. gallinarum*in poultry farms Kelantan, Malaysia.

Table 3: Frequency of antibiotic resistance of the ninety-seven *Enterococcus* spp. isolated from poultry farms in Kelantan, Malaysia.

Antimicrobial class	Antimicrobial agent	Number of resistant strains (%)		
		E. casseiliflavus (n=84)	*E. gallinarum* (n=13)	Total (n=97)
Beta-lactams	Penicillin	26 (30.9)	2 (15.4)	28 (28.9)
	Ampicillin	40 (47.6)	8 (61.5)	48 (49.5)
Aminoglycosides	High-level Gentamicin	3 (3.6)	0 (0.0)	3 (3.1)
Glycopeptides	Vancomycin	0 (0.0)	0 (0.0)	0 (0.0)
	Teicoplanin	0 (0.0)	0 (0.0)	0 (0.0)
Oxazolidinones	Linezolid	15 (17.9)	3 (23.1)	18 (18.6)
Glycylcycline	Tigecycline	0 (0.0)	0 (0.0)	0 (0.0)

DISCUSSION

Enterococci is one of the predominant genera inhabit in animal intestinal microbiome. *Enterococcus faecium* and *E. faecalis* are known to present the most in the cloacal swab of chickens, but still there was a higher prevalence of undifferentiated *Enterococcus* spp. (Pillay *et al.*, 2018). *Enterococcus casseliflavus*, *E. gallinarum* and *E. mundtii* are infrequently isolated from chickens (Simjee *et al.*, 2002). However, *E. casseliflavus* is more prominent in this study whilst, none of *E. faecium* was isolated. A rare cluster of infections with *E. gallinarum* and *E. casseliflavus* were also reported making an *Enterococcus* a foreseen nosocomial pathogen that can be challenging to treat especially those resistant to antibiotics. These two species have been associated with a wide variety of invasive infections such as endocarditis, bacteraemia, septicaemia, and meningitis (Dargere *et al.*, 2002; Iaria *et al.*, 2005; Verma and Baroco, 2017). With the increasing cases of infections and hospital outbreak (Britt and Potter, 2016), these two species should not be neglected. *E. casseliflavus* and *E. gallinarum* were the only species isolated in this study whilst, no *E. faecium* and *E. faecalis* was isolated. Disappearance of *E. faecium* and *E. faecalis* in this study remains unclear, but similar finding has been reported by Ayeni *et al.* (2016).

Even though, *E. faecium* and *E. faecalis* rank as the second and third most important nosocomial pathogens worldwide (Zhang *et al.*, 2017; Ramos *et al.*, 2020), other species of *Enterococcus* should not be neglected. With the ability to acquire and transmit antibiotic resistant genes to other species, it poses a significant treatment challenge which leads to increment of treatment failure, relapse, and higher rates of mortality. Estimated, 25-50%

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Table 4: Antibiotic resistance profiles of the eighteen linezolid-resistant *Enterococcus* (LRE) by minimum inhibitory concentration (MIC) assays by VITEK-2 automated system.

Isolates	MIC (µg/mL)
	Linezolid
E. casseilflavus F3/C1/09	≥8[R]
E. casseilflavus F3/C1/10	≥8[R]
E. casseilflavus F3/C1/13	≥8[R]
E. casseilflavus F3/C1/14	≥8[R]
E. casseilflavus F3/C1/18	≥8[R]
E. gallinarum F3/C1/19	≥8[R]
E. gallinarum F3/C1/20	≥8[R]
E. casseilflavus F3/C2/15	≥8[R]
E. casseilflavus F5/C1/15	≥8[R]
E. casseilflavus F5/C1/19	≥8[R]
E. casseilflavus F5/C1/26	≥8[R]
E. casseilflavus F5/C2/22	≥8[R]
E. casseilflavus F6/C1/11	≥8[R]
E. gallinarum F6/C1/23	≥8[R]
E. casseilflavus F6/C1/24	≥8[R]
E. casseilflavus F6/C2/08	≥8[R]
E. casseilflavus F6/C2/14	≥8[R]
E. casseilflavus F6/C2/23	≥8[R]

[R] -resistant
F3- farm three, F5- farm five, F6- farm six, C1- cage one, C2- cage 2

mortality occur from enterococcal bacteraemia (Bar *et al.*, 2006). However, question arises whether the associated high mortality is directly caused by the *Enterococcus*. It has been demonstrated that *Enterococcus* spp. possessed a peptide sex pheromone conjugal machinery conferring a different mobile elements resulted in a diverse co-transferred resistance phenotype (Conwell *et al.*, 2017).

We present the first known report of linezolid-resistant *Enterococcus* along with the truancy of *van*A VRE in the poultry farms, Malaysia. The prevalence of LRE in this study is 18.6% (18/97) in both *E. casseliflavus* and *E. gallinarum*. Studies by Cavaco *et al.* (2017) and Yoon *et al.* (2020) had reported the prevalence of LRE in poultry at 7.5% and 8.0% , which lower than our estimate. In contrast to our findings, where linezolid resistance was identified in *E. casseliflavus* and *E. gallinarum*, both Cavaco *et al.* (2017) and Yoon *et al.* (2020) studies reported *E. faecalis*. However, linezolid resistant has been reported in *Enterococcus* species *casseliflavus* and *gallinarum* of swine origin in China (Liu *et al.*, 2014). Linezolid-resistant isolates in animals are considered as a human health hazard and could disrupt the activities of commercial poultry farmers, leading to loss of revenue (Mehdi *et al.*, 2018). In a broiler operation system for example, antimicrobial resistance can be transferred to the environment and commercial broiler via the faecal-oral route.

Even though a high prevalence of vancomycin-resistant *Enterococcus* (VRE) carried a *van*A gene among poultry in Malaysia has been reported (Toosa *et al.*, 2001; Tan *et al.*, 2006), the absence of this strain in this study is not surprising. Subsequently, decreasing pattern of VRE in poultry husbandry has been notified with detection rate was only 2.0% and lower (Ong *et al.*, 2002; Chan *et al.*, 2008). Later, infrequent detection of human VRE clone in poultry samples may suggest a reverse transmission of VRE from humans to animals (Getachew *et al.*, 2013). The use of glycopeptide antibiotic avoparcin was indeed contributed to the emergence and spread of VRE. But, decreasing trend of VRE in poultry sample has been revealed by a world-wide ban on the use of avoparcin in livestock feed including Malaysia (Aarestrup *et al.*, 2001; Hauer *et al.*, 2002; Chan *et al.*, 2008).

Furthermore, there is none clinically significant *van*A-type resistance VRE isolated in this study. However, the species of *E. gallinarum* and *E. casseliflavus* carried mostly *van*C-type resistance VRE phenotype (intrinsic resistant) was predominantly detected. The intrinsic *van*C gene are commonly present in *E. gallinarum*, *E. casseliflavus* and *E. flavescens*. These species however encode only low levels of resistance to vancomycin antimicrobials (MIC ≤8 µg/mL) (Ahmed and Baptiste, 2018). Even though the *van*C genotype has been described for both *E. casseliflavus* and *E. gallinarum*, Radu *et al.* (2001), Ong *et al.* (2002) and Chan *et al.* (2008) reported that the isolates of these species from wet market chicken meat and from poultry farm showed no *van*C gene. This is similar to the finding of our study which *E. gallinarum* and *E. casseliflavus* strain isolated lacked the *van*C gene. With the majority of enterococci identified as *E. casseliflavus* and *E. gallinarum* in this study, it can be concluded that these isolates were conferring intrinsic low-level resistance to vancomycin (*van*C phenotype) without harbouring a vancomycin resistance gene, *van*C.

Species identification was done by PCR and 16S rRNA sequencing. All isolates resistant to linezolid has been identified with antibiotic susceptibility test by disk diffusion method and were further tested with Vitek-2 automated susceptibility testing (bioMérieux, USA) which MICs for linezolid is ≥8 µg/mL. Even though resistance of *Enterococcus* to linezolid has been relatively related to many molecular factors and genes, oxazolidinone resistance gene, *optr*A was the most reported element that contribute to increasing case of LRE in humans and animals worldwide (Cavaco *et al.*, 2017; Freitas *et al.*, 2017; Cai *et al.*, 2019; Elghaieb *et al.*, 2019). This gene are likely to be more easily widespread and could eventually be transferred to other strains or species because of the mobile elements that harboured them (Cavaco *et al.*, 2017). Linezolid resistant in *E. casseliflavus* resulted in a variety of co-transferred resistance phenotypes suggesting the presence of different mobile elements in natural isolates and supported that the potential for extensive horizontal gene transfer in previously neglected reservoir for enterococci (Conwell *et al.*, 2017).

In the other hand, transferable chloramphenicol-florfenicol resistance (*cfr*) gene was also identified to be the source of linezolid, lincosamide and streptogramin A

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compounds resistance. It was first identified in *Staphylococcus aureus* in 2006 that encodes for the rRNA methyltransferase. It is responsible to modify adenosine in the linezolid-binding region on the 23S rRNA, thus preventing antibiotic binding (Long *et al.*, 2006; Toh *et al.*, 2007) . It has been reported that *cfr* gene in *E. faecalis* strain isolated from cattle farm in China (Liu *et al.*, 2012). Moreover, multi-resistance gene *cfr* in *E. casseliflavus* and E. *gallinarum* of swine origin were also reported by Liu *et al.* (2014). Thus, the existence of linezolid resistance *E. casseliflavus* in this study may supported the conjectured of *cfr* gene emerged from animal strains of bacteria that exposed to natural compounds with an rRNA binding site similar to linezolid (Toh *et al.*, 2007). Intrinsic resistance of enterococci undoubtedly positions them well to acquire additional resistances on mobile genetics elements (Selleck *et al.*, 2019).

CONCLUSION

To summarise, we have first reported linezolid resistant *Enterococcus* (LRE) in highly intrinsic antibiotic resistant of *E. casseliflavus* and *E. gallinarum* in Malaysia poultry farm alongside with the truancy of *van*A gene. As no clinically important species of *Enterococcus* was isolated from this study, enhanced surveillance effort is dimly necessary to monitor the emergence and spread of multidrug resistant organism in *Enterococcus* and other pathogens. This is to prevent the spread of resistance genes from animals to humans and vice versa. Further genotyping study of *E. cassseliflavus* and *E. gallinarum* linezolid resistance are needed to elaborate the source of their resistant.

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