



Genetic diversity of *Enterococcus faecalis* isolated from environmental, animal and clinical sources in Malaysia



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ABSTRACT

Enterococcus faecalis ranks as one of the leading causes of nosocomial infections. A strong epidemiological link has been reported between *E. faecalis* inhabiting animals and environmental sources. This study investigates the genetic diversity, antibiotic resistance and virulence determinants in *E. faecalis* from three sources in Malaysia. A total of 250 *E. faecalis* isolates were obtained consisting of 120 isolates from farm animals, 100 isolates from water sources and 30 isolates from hospitalized patients. Pulse-field gel electrophoresis-typing yielded 63 pulsotypes, with high diversity observed in all sources ($D = \geq 0.901$). No pulsotype was common to all the three sources. Each patient room had its own unique PFGE pattern which persisted after six months. Minimum inhibitory concentrations of Vancomycin, Gentamicin, Penicillin, Tetracycline, Nitrofurantoin, Levofloxacin, Ciprofloxacin and Fosfomycin were evaluated. Resistance to Tetracycline was most prevalent in isolates from farm animals (62%) and water sources (49%). Water isolates (86%) had a higher prevalence of the *asa1* gene, which encodes for aggregation substance, whereas clinical (78%) and farm animal isolates (87%) had a higher prevalence of the *esp* gene, encoding a surface exposed protein. This study generates knowledge on the genetic diversity of *E. faecalis* with antibiotic resistance and virulence characteristics from various sources in Malaysia.

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Introduction

Enterococcus faecalis is found in a variety of environments, such as soil, water, plants, and animals [1]. In humans, as well as in other mammals, these microbes are mainly found in the gastrointestinal tract as commensals. However, *E. faecalis* may become an opportunistic pathogen in individuals whose immune systems are compromised [2]. The virulence associated genes in human pathogenic *E. faecalis* encode among others a collagen-binding protein (*ace*) [3], an aggregation substance (*asa1*) [4], a haemolysin activator (*cylA*) [4], an endocarditis antigen (*efaA*) [5], a surface protein (*esp*) [4], gelatinase (*gelE*) [6] and two recently identified putative surface antigens, *EF0591* and *EF3314* [7]. *E. faecalis* has also been shown to acquire resistance to a wide range of antibiotics [8]. As a result, enterococci have emerged as one of the leading therapeutic challenges associated with enterococcal infections including urinary tract infections (UTI) [2]. Around the world

E. faecalis remains one of the most frequently recovered species from enterococcal infections in humans [9].

Due to the prevalence of *E. faecalis* in nosocomial infections, studies have suggested hospital settings as a source for antibiotic-resistant strains [10]. Additional studies suggest environmental sources including animals and water can serve as important sources for antibiotic resistant *E. faecalis* strains [1] as human populations, animal populations, and the environment are all interconnected [1]. Selection and persistence of antibiotic resistance might be attributed to a variety of factors including horizontal transfer of resistance genes among bacteria, the misuse or overuse of antibiotics in humans and animals, and environmental contamination through livestock slurry and plant wastewater. The rate of development of resistance appears to have accelerated in the past decade and today multiple antibiotic resistant bacteria constitute a global problem [11].

It is important to investigate the genetic relationships between microbes, such as *E. faecalis*, that are found in both the environment and hospitals, as a possible relationship between the different sources may be established. Although a number of studies have investigated the prevalence and characteristics of antibiotic resistance among enterococci in clinical and environmental settings in

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Malaysia [12–17], such studies are typically limited to vancomycin-resistant enterococci and/or studies of a limited geographical area. In this study, *E. faecalis* from the feces of farm animals, water sources and hospital patients in Malaysia were characterized. The genetic relationships, virulence determinants and antibiotic susceptibilities shared between human and environmental *E. faecalis* isolates from different sources were assessed. In addition, these same characteristics were assessed from the same location after a period of six months to assess the persistence of each type of isolate in each source.

Materials and methods

Study site and sample collection

Sampling was carried out in two states representing different geographical regions in Malaysia; Selangor (West Malaysia) and Sabah (East Malaysia). Study sites comprised of chicken and cattle farms, wastewater treatment plants, rivers and hospitals. All farms and water sources were located within a 15 km radius of the hospitals in Selangor and Sabah respectively. The sampling areas in Sabah comprised of small to medium residential communities surrounded by rural agricultural regions as opposed to Selangor which included sampling areas around semi-urban development constituting smallholder farms. Sampling was conducted at two different sampling times, June and December 2014. Details of the sampling procedure and the distribution of samples obtained in this study can be found in Supplementary material 1.

Isolation and identification of *E. faecalis*

Suspected *E. faecalis* appearing as typical black to brown colonies on BAA agar, indicating esculin hydrolysis, were transferred on Slanetz and Bartley (SlaBa) agar (Oxoid, UK) and identified by growth and biochemical reactions as described by Olutiola et al. [18].

Confirmation of *E. faecalis* identity by sequencing of 16S ribosomal DNA

All presumptive *E. faecalis* isolates, including the clinical *E. faecalis* isolates obtained from hospital patients, were further characterized by 16S rDNA sequencing as a confirmation from phenotypic testing as proposed by Marchesi et al. [19]. Total DNA was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia). Primers were obtained from First BASE Laboratories, Malaysia. Species identification was determined from the best-scoring reference sequence of the BLAST output and whether the best-scoring reference sequence in the database had a sequence identity of 98% with *e*-values 10^{-5} and at least 96% query coverage.

PFGE analysis

Pulse Field Gel Electrophoresis (PFGE) was performed (3 replicates per isolate) subsequent to DNA digestion with *Sma*I (Promega, USA) as described by Weng et al. [17]. The PFGE marker (Promega, USA) containing lambda concatemers and lambda-digested *Hind*III fragments was used as a size standard. Comparison of the PFGE fingerprints was analyzed with Cliqs 1D Pro software (Cliqs 1D Pro, USA).

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) was determined for all *E. faecalis* isolates against a range of antibiotics using the

broth microdilution technique according to standard recommendations [20]. The list of antibiotics tested in this study is provided in Supplementary material 2. These antibiotics were chosen because they are either used in both human medicine and animal husbandry or because previous studies have reported *E. faecalis* resistance to them [21]. All antibiotics were purchased from Oxoid (UK) and Nacalai Tesque (Japan). The results were interpreted according to the cut-off levels proposed by CLSI guidelines [20].

Screening for *vanA* and *vanB* genes

All isolates were subjected to PCR for *vanA* and *vanB* genes according to Dutka-Malen et al. [22]. Primers were obtained from First BASE Laboratories, Malaysia.

Putative virulence markers

All primers for testing the presence of putative virulence markers were selected according to Creti et al. [7]. Primers for all virulence markers tested in this study are listed in Supplementary material 3. Primers were obtained from First BASE Laboratories, Malaysia.

Statistical analysis

The prevalence of antibiotic resistance phenotype to each antibiotic among *E. faecalis* isolates from all sources was compared using the chi-squared test. A P-value of <0.05 was considered to be statistically significant. Simpson's index of diversity (D) was calculated [23] to assess the differentiation of *E. faecalis* pulsotypes by PFGE. PFGE analysis was based on Dice similarity coefficient and unweighted pair group method using arithmetic averages (UPGMA) clustering with position tolerance and optimization coefficient of 1.5%.

Results

Sample collection

In this study, one isolate per sample was haphazardly picked for analysis. A total of 250 *E. faecalis* isolates were obtained throughout this study; 120 from farm animal feces, 100 from water sources and 30 from hospital patients (Supplementary material 1).

Diversity of *Enterococcus faecalis* isolates by PFGE

The analysis based on the dendrogram generated from the PFGE profiles grouped the *E. faecalis* isolates into 63 pulsotypes (with $\geq 90\%$ similarity) with 44 clonal populations and 19 isolates that were treated as unique. The PFGE patterns of samples from Selangor and Sabah showed distinct differences. The complete dendrogram is shown in Fig. 1.

A total of 27 pulsotypes for isolates from farm animal feces, 47 for isolates from water sources and 8 for clinical isolates were obtained. Isolates from the same farm clustered together, with the exception of four isolates in pulsotypes XLII and XLVIII which displayed identical PFGE patterns between Farm A and Farm B, as shown in Fig. 1. There was no overlapping of PFGE patterns between isolates from chicken and cattle feces. All isolates from animal drinking water showed similar PFGE patterns to those from farm animals with respect to the farms sampled. Isolates from river water and wastewater showed large genetic variability. *E. faecalis* from wastewater did not cluster according to the two wastewater treatment plants that were sampled, although farm samples did cluster according to the source farm. In addition, this study found identical PFGE patterns between two pulsotypes consisting both

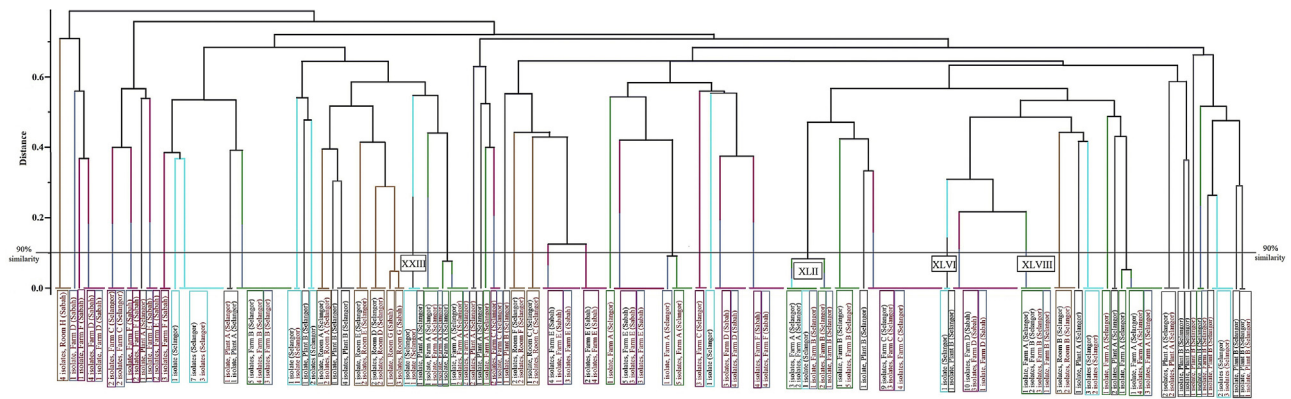


Fig. 1. Dendrogram of similarity among the observed PFGE macrorestriction patterns of *Sma*I-digested DNA from 250 *Enterococcus faecalis* isolates from farm animal feces, water sources and hospital patients at two different sampling times (July and December 2014). Dendrogram was generated using Dice similarity coefficient and UPGMA clustering with position tolerance and optimization coefficient of 1.5%. A total of 63 pulsotypes were identified at 90% similarity.

Note: Black text denotes samples collected in July 2014; Red text denotes samples collected in December 2014. ■ Cattle, ■ chicken, ■ water from troughs, ■ wastewater, ■ river water, ■ hospital patients.

wastewater and river water isolates as shown in clusters XXIII and XLVI in Fig. 1. Clinical strains isolated from patients occupying the same room had the same PFGE pattern, which differed from one room to another (Fig. 1). There was no overlapping of PFGE patterns between the three sources. The PFGE patterns obtained were highly variable for pooled isolates from each of the three sources (Simpson's diversity index; river and sewage wastewater $D = 0.975$, farm animals $D = 0.951$ and hospital patients $D = 0.901$).

Persistence of *Enterococcus faecalis* pulsotypes

All the pulsotypes obtained for the clinical strains from each of the rooms in Selangor persisted after six months (Fig. 1). Similarly, previously observed PFGE patterns were recovered in all farms after a follow-up period of at least six months (Fig. 1); in addition some variant pulsotypes were observed after the six month sampling period. In contrast, pulsotypes for samples from river water and wastewater after a period of six months showed considerable genetic transience and diversity among *E. faecalis* isolates. The complete dendrogram and correlation between the pulsotypes, antibiogram and virulence genes are presented in Supplementary material 4.

Antibiotic susceptibility test

Antibiotic resistance patterns of all *E. faecalis* isolates are presented in Figs. 2 and 3. Additional data on the antibiotic resistance profile of *E. faecalis* from all sources tested is available in Supplementary material 5. Of the total isolated *E. faecalis* in this study, 80% were resistant to at least one of the antibiotics tested. Comparison of the prevalence of antibiotic resistance of *E. faecalis* between Sabah and Selangor revealed variable differences in the proportion of antibiotic resistant *E. faecalis*, depending on the antibiotic tested (Table 1).

Isolates from farm animal feces and water sources were most commonly resistant to Tetracycline (Fig. 2). In contrast, 7 out of 30 clinical *E. faecalis* isolates were found to be resistant to Penicillin (2 isolates), Levofloxacin (2 isolates), Ciprofloxacin (1 isolate), Tetracycline (1 isolate) and Nitrofurantoin (1 isolate). The highest frequency of resistance in this study (except to Vancomycin and Nitrofurantoin), was found among isolates from farm animal feces. Multi-resistance (≥ 2 antibiotics) was common among isolates from water sources (74%) and farm animal feces (73%) (Fig. 3). River water held a higher percentage (83%) of multi-resistant *E. faecalis*

isolates compared to wastewater (60%). None of the clinical isolates in this study demonstrated multi-resistance.

Twenty four out of 250 isolates (9.6%) in the present study that possessed *vanA* were resistant to high levels of Vancomycin (MIC 32 $\mu\text{g/ml}$ to 128 $\mu\text{g/ml}$) with the exception of one isolate from river water that possessed the *vanA* gene but did not express Vancomycin resistance. There was no specific correlation observed between antibiogram patterns and the groupings obtained by PFGE (Supplementary material 4).

Prevalence of virulence markers

Distribution of nine virulence markers tested in the study varied between sources. All isolates carried at least one of the virulence genes tested, except for one isolate from cattle feces. Virulence gene *gelE* was found to be the most common factor (75.6%) in *E. faecalis* isolates in this study (Table 2). Water isolates had a statistically ($P < 0.05$) higher prevalence of the *asa1* gene than the other two sources as shown in Table 2. A high proportion of isolates from river water were found to have the *asa1* gene (93%), whereas isolates from wastewater had an equally high prevalence of both *asa1* (83%) and *ace* (83%) genes. Clinical isolates revealed high prevalence of the *esp* (87%) and *gelE* (83%) genes. However the *EF3314* gene was not present in any of the clinical isolates tested. Isolates with the same PFGE pattern showed different virulence profiles in a few cases in this study (Supplementary material 4).

Discussion

Genetic variability of *E. faecalis*

The genetic relationship between *E. faecalis* isolates from the different sources mentioned was analyzed by genotyping using PFGE which has previously been used to identify clonal relationships among isolates [24].

The clustering of PFGE patterns according to Selangor and Sabah suggests geographical localization. The high diversity observed in each of the 3 sources ($D = \geq 0.901$) is not particularly surprising. It is possible that the exposure to physical and chemical stresses may have resulted in evolution of wide diversity which is necessary for the adaptation of *E. faecalis*. The evolutionary process such as mutation, selection and recombination might have played a role in the evolution of environmental stress tolerance and resulted in observed high diversity. [25]. *E. faecalis* is also a ubiquitous colonizer in the gut of mammals and sauropods [1].

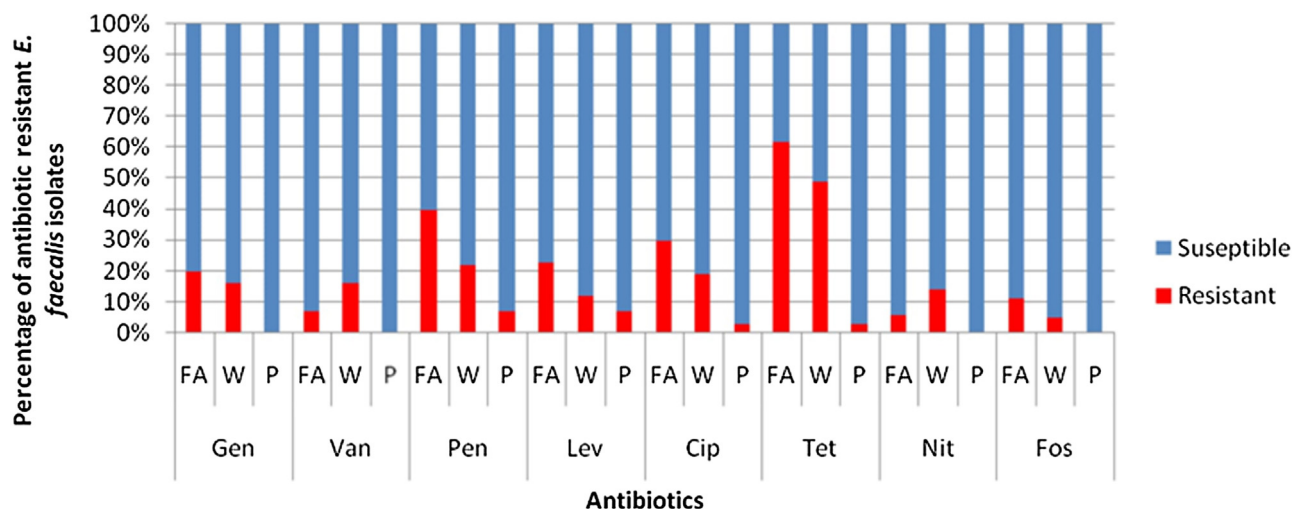


Fig. 2. Distribution of antibiotics for *Enterococcus faecalis* isolated from farm animal feces, water sources and hospital patients. Note: GEN = Gentamicin, VAN = Vancomycin, PEN = Penicillin, LEV = Levofloxacin, CIP = Ciprofloxacin, TET = Tetracycline, NIT = Nitrofurantoin, FOS = Fosfomycin, FA: farm animals, W: water, P: patients.

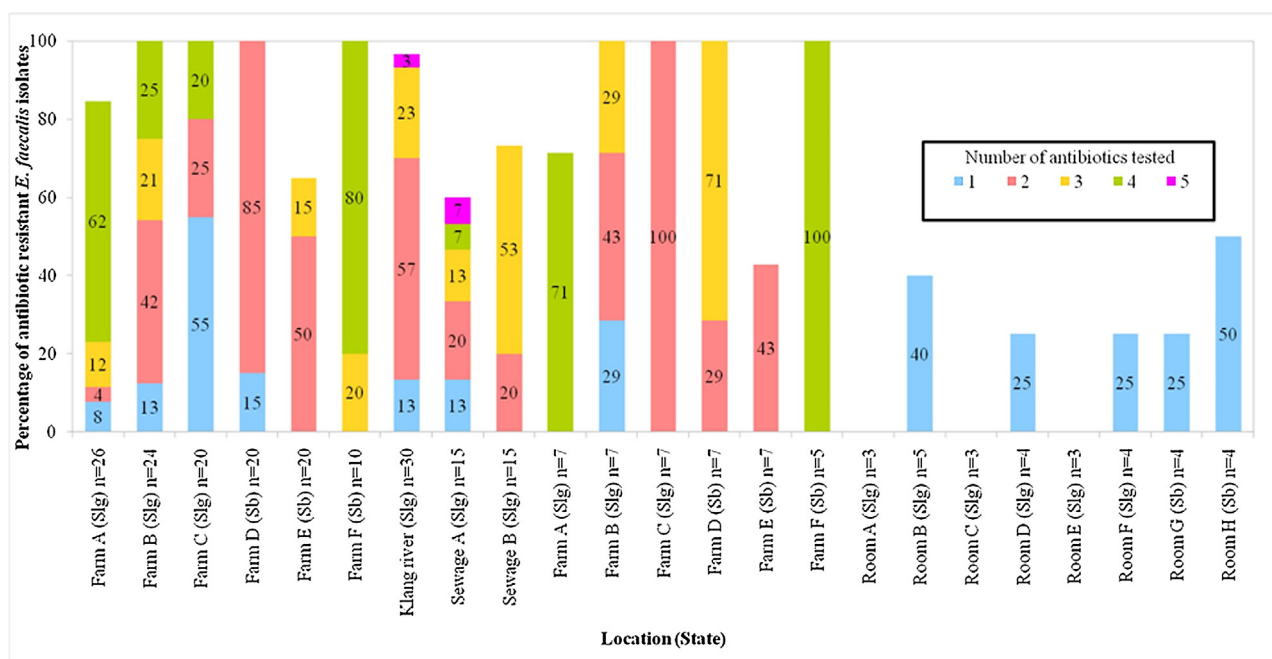


Fig. 3. Prevalence of multi resistant antibiotic (≥ 2 antibiotics) *Enterococcus faecalis* isolates from farm animal feces, water sources and hospital patients. Note: Slg: Selangor, Sb: Sabah.

Table 1
Prevalence of antibiotic resistant *Enterococcus faecalis* isolates in Sabah (n = 77) and Selangor (113) in percentage (number of isolates).

	VAN	HL-GEN	TET	PEN	CIP	LEV	NIT	FOS
Sabah	2.59% (2)	35.06% (27)	67.53% (52)	32.46% (25)	45.45% (35)	5.19% (4)	5.19% (4)	3.89% (3)
Selangor	9.73% (11)	24.78% (28)	49.56% (56)	44.25% (50)	23% (26)	28.31% (32)	7.08% (8)	11.50% (13)

Note: VAN = Vancomycin, HL-GEN = high-level Gentamicin, TET = Tetracycline, PEN = Penicillin, CIP = Ciprofloxacin, LEV = Levofloxacin, NIT = Nitrofurantoin, FOS = Fosfomycin.

This study reports overlapping pulsotypes between Farm A and Farm B (Fig. 1) which are both chicken farms. The two farms are approximately 5 km distance from each other. Farms traditionally do not operate in isolation and farm staff within a locality may well visit other farms with some regularity, as well as using shared resources such as delivery trucks [26].

All isolates from animal drinking water in this study showed identical PFGE patterns to those from farm animal feces with

respect to the farms sampled from. These results may indicate that *E. faecalis* is disseminated or maintained within a herd by contaminated water. This study also reports identical PFGE patterns of isolates from wastewater and river water that were approximately 8 km from each other (Fig. 1). Waste from hospitals and farms in the areas investigated are discharged into the sewer system. The treated sewage effluent from both treatment plants is discharged into the Klang river [16].

Table 2
Prevalence of virulence genes among *Enterococcus faecalis* isolates from all sources sampled.

Source	Location (State)	Number of isolates with virulence gene present								
		<i>esp</i>	<i>gelE</i>	<i>cylA</i>	<i>asa373</i>	<i>asa1</i>	<i>ace</i>	<i>efaA</i>	<i>EF0591</i>	<i>EF3314</i>
Chicken (n = 50)	Farm A (Selangor) (n = 26)	23	15	12	13	14	19	23	7	8
	Farm B (Selangor) (n = 24)	22	21	0	3	13	13	13	6	5
Cattle (n = 70)	Farm C (Selangor) (n = 20)	17	19	7	8	13	12	14	1	1
	Farm D (Sabah) (n = 20)	16	15	4	8	15	15	13	1	8
	Farm E (Sabah) (n = 20)	13	16	6	0	12	12	12	5	5
	Farm F (Sabah) (n = 10)	2	7	9	4	6	2	7	10	4
River (n = 30)	Klang river (Selangor) (n = 30)	9	18	2	9	28	14	16	1	3
Treated sewage wastewater (n = 30)	A (Selangor) (n = 15)	1	10	1	2	12	12	10	1	3
	B (Selangor) (n = 15)	0	9	2	7	13	13	12	2	2
Animal drinking water (n = 40)	Farm A (Selangor) (n = 7)	7	5	4	2	5	6	7	3	1
	Farm B (Selangor) (n = 7)	7	7	0	0	7	7	7	0	0
	Farm C (Selangor) (n = 7)	5	7	2	1	4	4	6	0	0
	Farm D (Sabah) (n = 7)	2	6	1	0	6	7	1	0	1
	Farm E (Sabah) (n = 7)	5	4	4	0	6	6	6	0	0
	Farm F (Sabah) (n = 5)	1	5	5	0	5	1	4	5	0
Hospital Serdang (n = 22)	Room A (Selangor) (n = 3)	3	3	0	0	3	0	3	3	0
	Room B (Selangor) (n = 5)	5	5	0	1	3	5	1	0	0
	Room C (Selangor) (n = 3)	0	3	0	3	3	3	0	0	0
	Room D (Selangor) (n = 4)	3	3	0	0	1	0	3	0	0
	Room E (Selangor) (n = 3)	3	0	3	0	0	0	0	0	0
	Room F (Selangor) (n = 4)	4	3	0	0	4	0	3	0	0
Hospital LahadDatu (n = 8)	Room G (Sabah) (n = 4)	4	4	0	0	0	4	0	4	0
	Room H (Sabah) (n = 4)	4	4	0	4	4	0	0	0	0
Total		156	189	62	65	177	155	161	49	41

For each patient room, clinical strains had the same PFGE pattern, which was different from other rooms. This suggests probable hospital to patient transfer, possibly via contact with fixed materials within the specific patient room.

Genomic persistence of *E. faecalis*

Mostly identical PFGE pattern was recovered in all farms after at least six months of follow-up although some variant pulsotypes were recovered as well (Fig. 1). Consequently, the farm animals examined appeared to be sources of *E. faecalis*, whose persistence over time may be a function of survival and proliferation of some resident population. In contrast, a great diversity was observed among *E. faecalis* isolated from water sources after a six month interval. Isolates from river and wastewater appear to be transient populations that fluctuate [26]. All clinical strains from Selangor persisted after 6 months in each of the rooms tested. This may suggest the patients picked up *E. faecalis* from the individual patient rooms, i.e. hospital bedding, shared bathroom within the room, as a result of infection; this confirms the nosocomial nature of *E. faecalis*. Similar results were seen by Papaparaskevas et al. [27] which found persisting clusters of *E. faecalis* PFGE patterns within a specific ward over a period of seven months.

Antibiotic susceptibility patterns of *E. faecalis*

A number of studies in Malaysia have reported antibiotic resistant *E. faecalis* from farm animals [12,13], water sources [14–16] and clinical sources [17]. So far in Malaysia, little emphasis has been given to the prevalence and diversity of MAR (multiple antibiotic resistant) *E. faecalis* and it was of interest to assess this. Most of the antibiotics used in this study were categorized by the World Health Organization as Rank I, i.e. critically important to human health (Supplementary material 5). Therefore, the high percentage of resistant isolates (80%) observed among *E. faecalis* isolates are

of concern for both clinical treatments as well as for the ecological implications for the transmission of this opportunistic pathogen.

Antibiotic resistant enterococci have been detected previously in livestock in Malaysia [12,13], and has led to suggestions of an epidemiological link between livestock and human infections [12,13]. A high level of Tetracycline resistance in *E. faecalis* isolated from farm animals (62%) in this study pose similar results to Butaye et al. [28] in Belgium, which reported Tetracycline resistant *E. faecalis* in almost all isolates (79%) from broilers. As intestinal inhabitants, enterococci are under selective pressure due to the routine supplement of antibiotics in livestock feed. In Malaysia, there are currently 97 antimicrobials registered for use according to the National Pharmaceutical Control Bureau (NPCB) of the Ministry of Health, Malaysia, unfortunately more than half of the antibiotics registered with the Ministry of Health for food animals in Malaysia are not recommended for veterinary use by the World Health Organization (WHO) [29]. A high percentage of multi-antibiotic resistant *E. faecalis* isolates was obtained from both chicken (82%) and cattle (70%). Tetracycline is one of the classes of antibiotics that are commonly and currently used in animal husbandry and human medicine in the Southeast Asian region [29].

In previous reports, core issues affecting the bacteriological quality of rivers available in Malaysia have been highlighted [14–16]. The current study, found comparable rates of antibiotic resistant *E. faecalis* isolates from river water (83%) as compared to sewage wastewater (60%). It is clear that a more integrated water management and monitoring system is vital for the community. While only 23% of clinical *E. faecalis* isolates were observed as antibiotic resistant in this study, reports in Turkey and Japan demonstrated that underlying urinary tract diseases predispose patients to repeated UTIs and exposure to antibiotics such as Fluroquinolones, leading to the selection of resistant *E. faecalis* isolates and the development of UTIs which may be caused by Quinolone resistant *E. faecalis* [30,31]. Although clinical strains of patients in the same room had the same PFGE pattern, the antibiotic resistant profiles were not identical in all the strains from the same patient

room (Supplementary material 4). No correlation was observed between antibiotic treatment and resistance of isolates for specific patients with antibiotic resistant *E. faecalis*. This suggests diversity and an exchange of antibiotic determinants among the population in a particular clinical setting.

vanA and *vanB* resistance have been linked with outbreaks of VRE and may be transferred to other organisms [32]. Studies have suggested that the occurrence of *vanA* in feces of animals may be of risk to humans through direct contact or ingestion of contaminated products [32]. The risk factors for VRE infection in humans are hospitalization and antibiotic treatment [33]. The *vanA* phenotype is related to a high level of inducible resistance to Vancomycin and cross-resistance to Teicoplanin, whereas the *vanB* phenotype has variable levels of inducible resistance only to Vancomycin [34]. The absence of resistant behavior even when the *vanA* gene is present, displayed by one of the isolates in this study, was also observed by Ribeiro et al. [35].

Prevalence of virulence markers

A number of genes suggested to play a role in the virulence properties of *E. faecalis* were assessed in this study. The *gelE* gene, which is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides, was found to be the most common marker (75.6%) in *E. faecalis* isolates in this study. Similar results were seen by other researchers in a number of countries [36,37].

A high frequency of the *esp* gene was found in both farm animals (87%) and clinical (78%) *E. faecalis* isolates in this study. The *esp* gene encodes a surface exposed protein and is important for the initial adherence during biofilm formation and urinary tract colonization.

The *asa1* gene, which encodes for aggregation substance, was found to be more common in river water (93%) and wastewater (83%) isolates as compared to the other sources. The *ace* gene (83%) was also a common virulence marker found in isolates from wastewater. A study by Sidhu et al. [38] of reported high prevalence of *ace* (74%) and *asa* gene was found in 47% of *E. faecalis* isolates in that study.

The *EF3314* gene was not present in any clinical isolates. No correlation was apparent between PFGE pulstypes and the virulence profiles of the strains. This observation is in agreement with the findings of Comerlato et al. [8] who observed no clonal relationship among *E. faecalis* isolates that influenced the distribution of virulence determinants.

To the best of our knowledge, this report remains to be the first to describe phenotypic and genotypic characteristics of *E. faecalis* isolates from farm animals, water and patients in East and West Malaysia. Although the study design of this experiment is insufficient to fully address the transmission of *E. faecalis* from farms and environmental sources to hospitals, due to low number of samples and great diversity of *E. faecalis* strains, the present investigation gives insight into the genetic diversity of *E. faecalis* isolates recovered from different sources in Sabah and Selangor, Malaysia. The high antibiotic resistance level with MAR pattern among the strains should be of concern for public health. A better knowledge of genotypic traits of *E. faecalis* might help in the design of strategies for the prevention and treatment of *E. faecalis* infections.

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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 <http://dx.doi.org/10.1016/j.jiph.2017.02.006>.

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