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Genotypic profiles of virulent genes detected among the *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* isolated from swiftlets in Borneo

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ABSTRACT

Aims: The occurrence of multiple pathogenic *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* are important nosocomial and hazardous infection clinically challenge worldwide. Thus, the aim of this study was to screen for the virulent genes profiles to ascertain their prevalence in swiftlets in Borneo.
Methodology and results: The *Enterococci*, *E. coli* and *P. aeruginosa* bacteria were isolated from the swiftlets' faeces and air inside swiftlet houses, which located in the Southern, Central and Northern regions of Borneo. The isolates were identified to the species level by 16S rRNA sequencing assay. Specific primers were designed for detection of the potential virulence genes in *E. faecalis* (*ace*, *AS*, *efa*A and *gel*E), *E. coli* (*stx*) and *P. aeruginosa* (*opr*L) by PCR assay. A total of 38 *Enterococci*, 26 of *E. coli* and 2 of *P. aeruginosa* fecal and airborne bacteria were identified. Sixty-seven percent of *E. faecalis* isolates were detected positive for four virulence genes, 27% possessed three (*AS*, *efa*A, *gel*E) genes and 6% possessed two (*ace*, *AS*) genes. There were no *stx* genes detected among all the *E. coli* isolates. The *opr*L gene was detected in all the *P. aeruginosa* isolates.
Conclusion, significance and impact of study: Virulence genes are important in the pathogenesis of both clinical and avian infections which considered to be a serious public health threat. The high incidence of virulence genes detection in *E. faecalis* and *P. aeruginosa* indicates these genes were widely disseminated among the bacteria found in swiftlet houses, suggesting the important issues in the pathogenesis of infections and diseases which may cause potential health risks to humans.

Keywords: Virulent genes, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, swiftlets

INTRODUCTION

Pathogenic bacteria are defined as bacteria which caused infection and diseases. Most pathogenic bacteria are useful in various industries and harmless to humans, but some bacteria may cause diseases under certain conditions. Pathogens commonly found in wild birds that caused an outbreak were *Enterococcus* spp., *Escherichia coli*, *Klebsiella* spp. (Radimersky *et al*., 2010), *Pseudomonas aeruginosa* and *Enterobacter* spp. (Benskin *et al*., 2009).

Enterococci are facultative anaerobic Gram-positive diplococci bacteria. *Enterococcus faecalis* and *Enterococcus faecium* are the common commensal bacteria normally found inside the mammal's gastrointestinal tracts (Lebreton *et al*., 2014) and they are widely distributed in soil, water, plants, and even food products (Frazzon *et al*., 2009). *Enterococci* are opportunistic pathogens found in wild birds which may cause septicemic disease in a patient with the low immune system (Al-Talib *et al*., 2015). Multiple antibiotic resistance cases of *E. faecalis* have been reported in most 80-90% infections related to nosocomial (Fisher and Phillips, 2009), surgical wounds, blood and urinary tract which led to a high mortality rate. Research did previously discover that the pathogenicity of *E. faecalis* is closely linked to epithelial cells with the four main virulence genes, namely: aggregation substance (*AS*), adhesion of collagen (*ace*), gelatinase (*gel*E), endocarditis antigen (*efa*A) which were commonly investigated although the mechanism was not well known. *E. faecalis* are able to adhere to the host cell films and ecological surfaces in order to acquire nutrients needed and to evade the host immune response (Medeiros *et al*., 2014). The *AS* gene expressed is responsible in the sex pheromone-responsive plasmid (Tremblay and Archambault, 2013) in

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the aggregation and conjugation process between donors and recipients' bacteria. The *ace* gene is reported to act as a mediator in collagen and laminin adherence (Medeiros *et al*., 2014), contributing to the pathogenesis of endocarditis disease (Lebreton *et al*., 2011). In addition, the *gel*E is another zinc metalloprotease encoded genes in the chromosome which involves various hydrolysis processes. Preethee *et al*. (2012) have proved that *efa*A gene in *E. faecalis* is responsible for causing the failure of therapy in treating the resistant endodontic infections.

Escherichia coli as a Gram-negative rod-shaped bacterium under *Enterobacteriaceae* family is a normal flora found in the intestinal tract of mammalians and mostly harmless to the hosts. However, small numbers of *E. coli* namely Shiga toxin-producing *E. coli* (STEC 0157: H7) pose hazardous diseases called hemorrhagic colitis (HC) with the symptom of bloody diarrhea, acute abdominal cramps, vomiting, and large bowel inflammation (Andrew and Growther, 2011). These pathogenic *E. coli* are worldwide zoonotic pathogens that caused extra-intestinal diseases in birds especially chickens, turkeys (Schouler *et al*., 2012), cattle and ruminant hosts (Ferens and Hovde, 2011). *Escherichia coli* are classified based on their virulence factors. The STEC which produces harmful toxin named Shiga toxin 1 and Shiga toxin 2, causing infection by disrupting the host protein synthesis process (Chai, 2013). STEC affected mostly children, inducing HC followed by hemolytic uremic syndrome. Transmission of disease to people occurs via the ingestion of under-processed contaminated food and improper hygienic farm management.

Pseudomonas aeruginosa is another opportunistic pathogen, mostly isolated from soil, aquaculture environment, skin and even man-made environments for the purpose of development. *Pseudomonas aeruginosa* commonly caused diseases to animals and humans (Klockgether and Tümmler, 2017). However, *P. aeruginosa* is a common avian pathogen isolated mostly in birds and may infect the damaged avian tissues or low immunity patients with symptoms of inflammation and sepsis (Kalle *et al*., 2012). The bacteria colonized and multiplied in critical human organs such as urinary tract, kidneys, lungs, leading to high mortality. *Pseudomonas aeruginosa* poses virulence factor which is able to degrade the cell wall membrane of eukaryotic (Prithiviraj *et al*., 2005). *Pseudomonas aeruginosa* was the main pathogen causing high mortality and spoilage in fish and fish products (Abdullahi *et al*., 2013). Apart from that, *P. aeruginosa* is considered a high-risk pathogen because of their presence of outstanding intrinsic antibiotic resistance ability thus further decreased the clinical effectiveness (Cabot *et al*., 2016). Most of the *P. aeruginosa* are harmful and the *opr*L gene target is used precisely in the detection of bacteria. The *opr*L gene, peptidoglycan-associated outer-membrane lipoprotein is involved in protein synthesis and regulation in *P. aeruginosa* growth (Panmanee *et al*., 2008). Thus, the detection of *opr*L gene is vital in detection of its pathogenicity.

Nowadays, most of the swiftlet houses are built in the urban area, thus the unhygienic mismanaged waste and pollution may have cultivated more potential pathogens over the years. A study regarding the pathogenicity caused by these bacteria from the swiftlet industries is yet to be discovered. In this study, the virulent genes profiles of bacteria species including *E. facealis*, *E. coli*, and *P. aeruginosa* were studied in order to ascertain their prevalence in swiftlets in Borneo.

MATERIALS AND METHODS

Location of study areas

Sampling of the swiftlet bird fecal and airborne bacteria was carried out from March 2015 till September 2016 from the ten swiftlet houses located in the Southern, Central and Northern regions of Borneo (Figure 1). The sampling sites that were chosen for the Southern Sarawak were S1: Kota Samarahan (01°27'34.2''N 110°27'25.9''E), S2: Kuching (01°32'56.6''N 110°22'27.5''E), S3: Semarang (01°40'40.0''N 111°6'5.92''E), S4: Maludam (01°39'14.17''N 111°1'53.9''E), S5: Sepinang (01°40'11.8''N 111°7'5.9''E) and S6: Betong (01°24'0''N 111°31'0''E). The sampling sites chosen for the Central Sarawak were S7: Saratok (01°44'10.32''N 111°21'10.22''E), S8: Sarikei (02°6'3.75''N 111°30'39''E) and S9: Sibu (02°19'11.3''N 111°49'50.5''E). The sampling site chosen for the Northern Sarawak was S10: Miri (04°23'39.2''N 113°59'12.2''E).

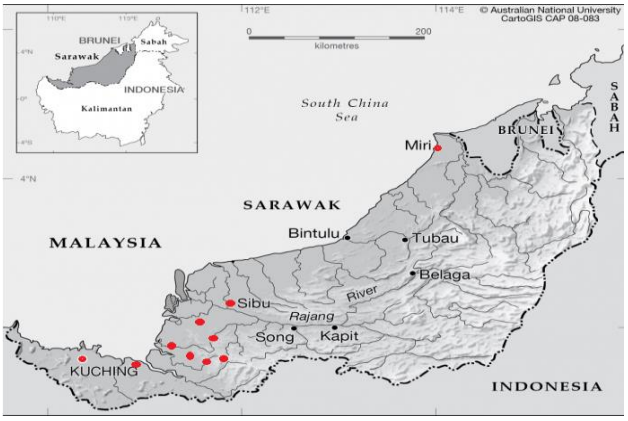

Figure 1: Location of study areas: Sarawak, Borneo (shown in red dots).

Isolation of feacal and airborne *Enterococci*, *E. coli* and *P. aeruginosa*

Five faeces samples were collected randomly from the floor of each swiftlet house of the sampling site as described by Nyakundi and Mwangi (2011). Each collected fecal sample was diluted in ratio 1:9 in sterile 0.85% saline solution. The diluted sample was then cultured on bile esculin agar, MacConkey agar and Pseudomonas agar (Merck, Germany), plates in duplicate

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using the spread plate method and incubated at 37 ± 1 °C for 24 h.

The collection of the indoor airborne samples procedures was carried out according to Malaysia Veterinary Health. The airborne bacteria were obtained using exposed plate count agar (Scharlau, Spain) in duplicate. The lid of the plates was lifted and exposed in the air for 15 sec inside the swiftlet house. The plates were incubated at 37 ± 1 °C for 24 h in the laboratory. The bacteria colonies were randomly selected and cultured on selective agar (Merck, Germany) and incubated at 37± 1 °C for 24 h.

Identification of airborne *Enterococci*, *E. coli* and *P. aeruginosa*

Ten colonies were randomly selected from each culture plate. The pure isolates were obtained for further identified by using biochemical tests. Isolates that showed Gram positive cocci, catalase-negative and Voges-Proskauer positive were presumed as *Enterococcus* spp. Isolates that showed Gram negative rod, lactose-positive, oxidase-negative and indole positive were presumed as *E. coli*. Isolates that showed Gram negative rod, Voges Proskauer-negative, indole-negative, methyl red-negative and catalase-positive were presumed as *Pseudomonas* spp. Species identification was confirmed by 16S rRNA sequencing.

DNA extraction

The bacteria DNA was extracted using the boiling method described by Sien *et al*. (2013) with minor modification. A volume of 1.5 mL of 24 h bacteria culture was then transferred into a 2.0 mL microcentrifuge tube and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded. After that, 500 µL of sterile distilled water was added and vortexed to suspend the cell pellet. The microcentrifuge tube was then boiled for 10 min and immediately transferred into ice for 5 min. Lastly, the microcentrifuge tube was centrifuged at 10,000 rpm for 10 min and the supernatant was collected. A total of 2 µL DNA extracted from each sample were then placed directly on the spectrophotometer (NanoDrop, Thermo Scientific) and measures in 230, 260 and 280 nm in order to acquire the DNA purity.

PCR for molecular detection of virulence genes (*ace*, *AS*, *efaA*, *gelE*) in *E. faecalis*

The PCR was performed according to the methods described by Dupre (2003). The *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 were used as positive and negative control respectively for the detection of virulence genes. The primers and reaction condition are given in Table 1. The PCR reactions were performed in reaction mixtures containing 2.5 µL of DNA, 1.0 µL each 20 pmol primers (First Base, Malaysia), 0.5 µL of 10 mM of deoxynucleoside triphosphate mix (Promega, USA), 1.5 µL of 25 mM $MgCl_2$ (Promega, USA), 5 µL of 5× Buffer solution (Promega, USA), 12.75 µL of distilled water and 0.75 µL of Taq polymerase (Promega, USA). PCR was performed with 30 cycles as follows: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 1 min, annealing for 1 min at the temperature shown in Table 1, primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were visualized with ethidium bromide staining after 1% agarose gel electrophoresis for 35 min at 80 volts.

Multiplex PCR for molecular detection of Shiga toxin (*stx* gene) in *E. coli*

Multiplex-PCR method was adopted (Son *et al*., 2015) with minor modification. The *E. coli* O157:H7 (EDL933 strain) used as positive control and *E. coli* ATCC 25922 used as negative control were included. The primer sequences (*stx* 1, *stx* 2) and reaction condition is given in Table 2. The PCR reactions were performed in reaction mixtures containing 5.0 µL of DNA, 0.5 µL each 20 pmol primers (First Base, Malaysia), 1.0 µL of 10mM of deoxynucleoside triphosphate mix (Promega, USA), 1.5 µL of 25 mM $MgCl_2$ (Promega, USA), 2.5 µL of 5X Buffer solution (Promega, USA), 12.5 µL of distilled water and 0.5 µL of Taq polymerase (Promega, USA). PCR was performed with 30 cycles as follows: initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 1 min, annealing at 95 °C for 1 min, primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were visualized with ethidium bromide staining after 1% agarose gel electrophoresis for 35 min at 80 volts.

PCR for molecular detection of *oprL* gene in *P. aeruginosa*

PCR analysis was performed for the detection of pathogenic *P. aeruginosa* as described by Xu *et al.* (2004) with minor modification. *Pseudomonas aeruginosa* ATCC 27853 was used as positive control and *E. coli* ATCC 25922 was used as negative control. The primer sequences for *opr*L gene are shown in Table 3. The PCR reactions were performed in reaction mixtures containing 5.0 µL of DNA, 1.0 µL each 20 pmol primers (First Base, Malaysia), 1.0 µL of 10mM of deoxynucleoside triphosphate mix (Promega, USA), 2.0 µL of 25 mM $MgCl_2$ (Promega, USA), 3.5 µL of 5X Buffer solution (Promega, USA), 11.0 µL of distilled water and 0.5 µL of Taq polymerase (Promega, USA). PCR was performed with 40 cycles as follows: initial denaturation at 96 °C for 5 min, denaturation at 96 °C for 1 min, annealing for 1 min at the temperature shown in Table 3, primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were visualized with ethidium bromide staining after 1% agarose gel electrophoresis for 35 min at 80 volts.

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Table 1: Primers sequence for adhesion of collagen (*ACE*), aggregation substance (*AS*), endocarditis antigen (*efa*A), gelatinase (*gel*E).

Primers name	Primer sequences (5'-3')	Annealing temperature (°C)
ACE-F	AAAGTAGAATTAGATCCACAC	56
ACE-R	TCTATCACATTCGGTTGCG	
AS-F	CCAGTAATCAGTCCAGAAACAACC	54
AS-R	TAGCTTTTTTCATTCTTGTGTTTGTT	
*efa*A-F	CGTGAGAAAGAAATGGAGGA	56
*efa*A-R	CTACTAACACGTCACGAATG	
*gel*E-F	AGTTCATGTCTATTTTCTTCAC	56
*gel*E-R	CTTCATTCTTTACACGTTTG	

Table 2: Primer sequence for Shiga toxin 1 (*stx*1) and Shiga toxin 2 (*stx*2).

Primer name	Primer sequence (5'-3')	Annealing temperature (°C)
*stx*1-F	ATAAATCGCCATTCGTTGACTAC	95
*stx*1-R	AGAACGCCCACTGAGATCATC	
*stx*2-F	TTAACCACACCCCACCGGGCAGT	95
*stx*2-R	GGATATTCTCCCCACTCTGACACC	

Table 3: Primer sequence for peptidoglycan-associated outer-membrane lipoprotein (*opr*L gene).

Primer name	Primer sequence (5'-3')	Annealing temperature (°C)
*opr*L- F	ATGGAAATGCTGAAATTCGGC	55
*opr*L -R	CTTCTTCAGCTCGACGCGACG	

RESULTS AND DISCUSSION

Prevalence of *Enterococcus, E. coli* and *P. aeruginosa*

A total of approximately 50 *Enterococci*, 30 *E. coli* and 5 *P. aeruginosa* were isolated biochemically from the ten swiftlet houses throughout the Borneo. Only 38 *Enterococci*, 26 *E. coli* and 2 *P. aeruginosa* were successfully identified through molecular sequencing analysis (Table 4). There were more fecal bacteria isolated mainly due to the differences in nutrient content available in the samples. The bacterial isolates from the swiftlet faeces were identified as *E. qallinacum* (51%, n=19), *E. faecalis* (35%, n=13), *E. harae* (8%, n=3), *E. coli* strain 1 (31%, n=8), *E. coli* strain 2 (35%, n=9), *E. coli* strain 3 (15%, n=4), *E. coli* strain 4 (3.8%, n=1), *E. coli* strain 5 (3.8%, n=1), *E. coli* 083:H1 (3.8 %, n=1) and *P. aeruginosa* (100%, n=2) . The airborne bacteria isolates from swiftlet houses were identified as *E. faecalis* (5%, n=2), *E. qallinacum* (1%, n=1) and *E. coli* strain 1 (7.6 %, n=2).

The detection of *Enterococci* and *E. coli* bacteria was widespread among the wild birds because birds can be the reservoirs for bacterial pathogens growth. No *E. faecalis* were being isolated from S6 (Betong) and no *E. coli* being isolated from S2 (Kuching). The absence of these pathogens may due to their geographical location, acidity (pH), moisture content, hygienic condition inside the swiftlet houses and temperature effects during different sampling at the different times of the year which may affect bacterial growth. Woo *et al*. (2017) has explained that environmental impacts especially geographical location can alter microbial community growth. Similar results were obtained with identification of *Enterococcus* spp. and *E. coli* in the Marabou Stock (*Leptoptilos crumeniferus*) and rooks droppings samples (Literák *et al*., 2007; Nyakundi and Mwangi, 2011). Besides, fecal *E. faecalis* was also found from the fecal samples obtained from dogs at veterinary hospitals (Ghosh *et al*., 2012), poultry (Kwon *et al.*, 2012), ducks and wild geese (Han *et al*., 2011). Our result of this study showed a low prevalence frequency for both *E. coli* and *Enterococcus* spp. as pathogen reservoirs in swiftlets. *Escherichia coli* was widely found in the polluted river water (Tambekar *et al*., 2008) and pig fecal samples (Meng *et al*., 2014). Research had proved that *E. faecalis* and *E. coli* are naturally present in the animal gastrointestinal tract and able to survive in a harsh bile content environment (Saitoa *et al*., 2018). Thus, we can assume that *Enterococcus* spp. and *E. coli* are common pathogens found in most avian. Only 2 isolates of *P. aeruginosa* were isolated from S8 (Sarikei). The study showed that *P. aeruginosa* is not a common avian pathogen found. Most *P. aeruginosa* occurs as second invader of skin or nasal infection. Thus, the detection of *P. aeruginosa* at S8 showing that the pathogens were originated from the skin debris of the birds not from the fecal sample itself.

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Table 4: The 16S rRNA sequencing homology search (BLAST) results of the *Enterococci*, *E. coli*, and *P. aeruginosa* isolates.

Sites	Source	16S rRNA sequencing			
		Bacterial species (Number of isolates)	Size(s) bp	Data base	Homology (%)
S2, S3, S5, S8, S7, S10	Faeces	*Enterococcus qallinacum* (19)	534	GenBank	99
S2, S4, S5, S7, S9, S10	Faeces	*Enterococcus faecalis* (13)	1445	GenBank	100
S1, S4	Faeces	*Enterococcus harae* (3)	1493	GenBank	99
S1, S4	Air	*Enterococcus faecalis* (2)	1445	GenBank	100
S5	Air	*Enterococcus qallinacum* (1)	534	GenBank	99
S1, S3, S4	Faeces	*Escherichia coli* strain 1 (8)	4887515	GenBank	99
S3, S5, S7, S8, S9	Faeces	*Escherichia coli* strain 2 (9)	5038386	GenBank	99
S6	Faeces	*Escherichia coli* strain 3 (4)	1537	GenBank	99
S3	Faeces	*Escherichia coli* strain 4 (1)	5386223	GenBank	99
S3	Faeces	*Escherichia coli* strain 5 (1)	4971461	GenBank	99
S3	Faeces	*Escherichia coli* 083:H1 (1)	4747819	GenBank	99
S8	Air	*Escherichia coli* strain 1 (2)	5038386	GenBank	99
S6, S9	Faeces	*Pseudomonas aeruginosa* (2)	1057	GenBank	100

S1-10: Sampling sites

Table 5: Distribution of genotypic virulence determinants among the *E. faecalis.*

Isolates (n=15)	Sampling sites	Sources	Detection by PCR[a]			
			ace	*AS*	*efa*A	*gel*E
SWF-SAM-B6	S1	Air	+	+	+	+
SWF-OPP-1D4	S7	Faeces	+	+	+	+
SWF-OPP-2A3	S7	Faeces	+	+	+	+
SWF-OPP-2C10	S7	Faeces	+	+	+	+
SWF-OPP-2D4	S7	Faeces	+	+	+	+
SWF-OPP-2D6	S7	Faeces	+	+	+	+
SWF-NEX-1E8	S6	Faeces	+	+	-	-
SWF-MAL-1B2	S4	Faeces	+	+	+	+
SWF-MAL-1C3	S4	Faeces	+	+	+	+
SWF-MAL-A3	S4	Air	+	+	+	+
SWF-MIRI-2A6	S10	Faeces	+	+	+	+
SWF-MIRI-2A10	S10	Faeces	-	+	+	+
SWF-KCH-2A1	S2	Faeces	-	+	+	+
SWF-SIBU-2C2	S9	Faeces	-	+	+	+
SWF-SPN-2A1	S5	Faeces	-	+	+	+

[a]Bands detected in PCR + : Present; - : absent.
S1-10: Sampling sites

Virulence genes (*ace, AS, efaA, gelE*) detection in *E. faecalis*

The virulence genes presented in the 15 *E. faecalis* isolates were successfully detected by PCR analysis using the specific primer sequences (Table 1). The distribution of genotypic virulence determinants among the *E. faecalis* is shown in Table 5 and the agarose gel electrophoresis banding patterns illustrated in Figure 2. There were 67 % isolates of *E. faecalis* detected positive for the four virulence genes. About 27% isolates of *E. faecalis* possessed three of the virulence genes and 6 % isolates of *E. faecalis* possessed two of the virulence genes. The *E. faecalis* isolates from S1, S4, S7, S10 confirmed the presence of the above four virulence genes. The result showed that the prevalence of virulence gene was not significantly correlated with the sampling sites. Besides, *E. faecalis* isolated from S6 (Betong) only revealed the presence of *ace* and *AS* genes. Lastly, *ace* gene was not detected from the *E. faecalis* isolated from S2, S5, S9, S10.

Most of the *E. faecalis* isolated carried most virulence genes. The high percentage of virulence genes detection indicated that they were high risk vector in transmission of diseases particularly through the swiftlet birds' fecal contamination. *E. faecalis* was also reported as the most

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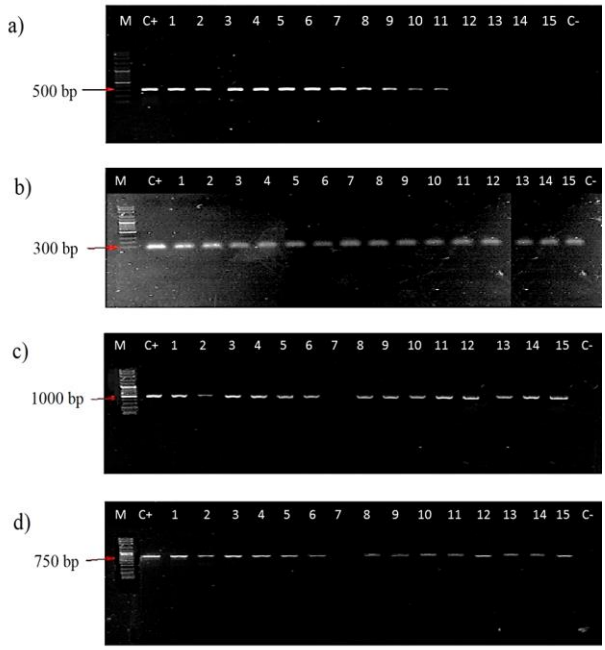

Figure 2: Agarose gel electrophoresis of gene amplification products from *E. faecalis* isolates. (a) *ace*; (b) *AS*; (c) *efa*A; (d) *gel*E. M: 1Kb ladder; C+: Positive control (*E. faecalis* ATCC 29212); 1-15: *Enterococcus* isolates; C-: Negative control (*E. coli* ATCC 25922).

common pathogens in nosocomial infection, causing fatal outcomes in patients. Cases of *E. faecalis* causing nosocomial infections were increasing especially in Italy thus causing threats to human health. The virulence genes which were expressed on the surface of the membrane (*ace*, *AS*, *efa*A, *gel*E) played a vital role in critical prerequisite for *E*. *faecalis*-induced diseases and their pathogenicity (Ocvirk *et al*., 2015). The virulence factors of *E. faecalis* enable them to evade the host immune response (Medeiros *et al*., 2014). The most widespread prevalence gene was *AS* gene (Table 5). A similar result was obtained revealed *Enterococcus* virulence genes especially *AS* gene together with cytolysin activator was found among *E. faecalis* and mostly isolated from beach water, dogs, humans, and birds in Australia (Ferguson *et al*., 2016). Similar result was also obtained stated that 87.8 % of *ace* gene and 69.4% of *gel*E gene were detected clinically. Similar result was proved by Ahmed *et al*. (2012) that *gel*E was the most abundant gene detected among *E. faecalis* from multiple environmental sources which enhance the survivability of *Enterococcus* especially during extra intestinal surroundings (Coque, 2002). The *efaA* gene of *E. faecalis* was detected and associated mainly with infective endocarditis. Similar result was obtained who had isolated *E. faecalis* strains from clinical and food samples that possess *efa*A genes in Brazil (Medeiros *et al*., 2014). Pathogenic *E. faecalis* had been proved by research findings as a culprit in therapy-resistant endodontic infections. The presence of the virulent strains among *E. faecalis* alone cannot confirm infection occurrence because there may be other mediators of pathogenicity (Kim and Marco, 2013). Pathogenicity of *Enterococcus* is mainly due to the ability of these virulent strains to overgrow in the infection sites and further multiple spreads all over the body. Host factors play a vital role in the ability of *Enterococci* to establish infection (Arias and Murray, 2012). However, the presence of these genes among *E. faecalis* strains from swiftlet warrants further studies to assess potential human health risks.

Shiga toxin (*stx*1 *and stx*2) gene detection in *E. coli*

When the *E. coli* isolates were analysed, the primers targeting the Shiga toxin gene generated bands of *stx*1 at size 500 bp and *stx*2 at size 250 bp for positive isolates. The agarose gel electrophoresis banding patterns of Shiga toxin detection is shown in Figure 3 and the distribution of genotypic virulence determinants among *E. coli* is shown in Table 6.

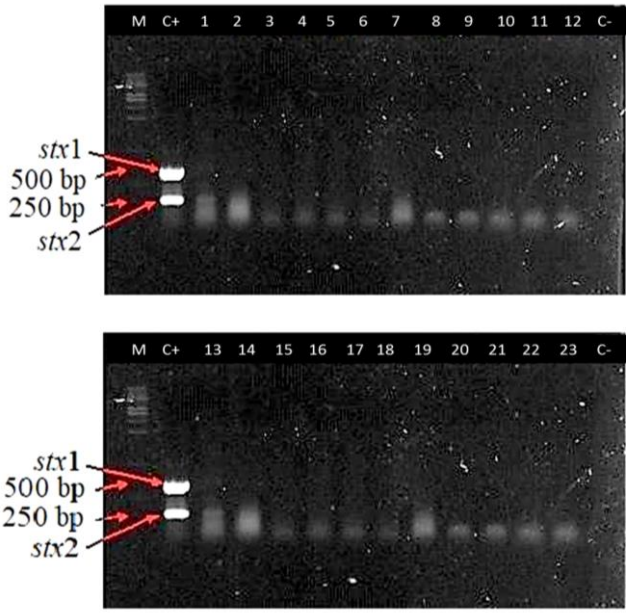

Figure 3: Agarose gel electrophoresis of *stx*1 and *stx*2 amplification products from *E. coli* isolates. M: 1 kb ladder; C+: Positive control (*E. coli* O157:H7 (EDL933 strain); 1-23: *E. coli* isolates; C-: Negative control (*E. coli* ATCC 25922).

In this study, there was no Shiga toxin (*stx*1 and *stx*2) detected among all the *E. coli* isolates. This may imply the absence of detectable Shiga toxin DNA in the specimen. Most of the *E. coli* is not hazardous but some portion of *E. coli* may cause diarrhea diseases such as STEC O157:H7 *E. coli*. Therefore, the pathogenicity of *E. coli* can be determined by characterizing their serotypes and virulence factor. Detection of *stx* genes are crucial in

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virulence study because the genes are responsible in the toxin secretion to disrupt the protein synthesis in their host. The present study was targeted the Shiga toxin genes mainly because all the STEC O157:H7 *E. coli* consists *Stx2* (*stx2*) and intimin (*eae*) genes which may link with severe disease (Adamu *et al*., 2014). Detection of STEC is vital to prevent any STEC infection outbreaks and treatment purposes to decrease further renal damage. The pathogen may spread through reservoir birds to human. Similar result was reported by Chai (2013), none of the *E. coli* isolated from aquaculture environment showed a positive in Shiga toxin gene detection. On the other hand, Meng *et al*. (2014) showed that 25% isolates of *E. coli* isolates from the pig fecal samples were detected with Shiga toxin genes. Based on the results of this study alone, we cannot prove that the *E. coli* isolate was harmless because there is non-O157 STEC available which cannot be cultured by selective and differential media in the laboratory (Gould, 2012). Apart from that, Grys *et al*. (2009) explained that false-negative results may occur during the Shiga toxin detection mainly due to the inhibition of the PCR, sequence variability for the primers and probes, the presence of the toxin gene in quantities less than the detection limit of the assay. Shiga toxins are encoded on a mobile genetic element that can be lost by the bacterial host (Foxman, 2011).

Table 6: Distribution of genotypic virulence determinants among the *E. coli*.

Isolates (n=23)	Sampling sites	Sources	Detection by PCR[a]	
			stx 1	*stx* 2
SWF-SAM-1E4	S1	Faeces	-	-
SWF-SAM-1E6	S1	Faeces	-	-
SWF-OPP-2D8	S7	Faeces	-	-
SWF-NEX-1D2	S6	Faeces	-	-
SWF-NEX-1D7	S6	Faeces	-	-
SWF-NEX-1E2	S6	Faeces	-	-
SWF-MAL-1A9	S4	Faeces	-	-
SWF-SIBU-2A5	S9	Faeces	-	-
SWF-SIBU-2D2	S9	Faeces	-	-
SWF-SPN-2A10	S5	Faeces	-	-
SWF-RK-2A4	S8	Faeces	-	-
SWF-RK-2A6	S8	Faeces	-	-
SWF-RK-2A10	S8	Faeces	-	-
SWF-RK-B3	S8	Air	-	-
SWF-RK-B10	S8	Air	-	-
SWF-SMR-1A1	S3	Faeces	-	-
SWF-SMR-1A3	S3	Faeces	-	-
SWF-SMR-1A6	S3	Faeces	-	-
SWF-SMR-1A10	S3	Faeces	-	-
SWF-SMR-1C7	S3	Faeces	-	-
SWF-SMR-1D1	S3	Faeces	-	-
SWF-SMR-1D6	S3	Faeces	-	-
SWF-SMR-1D8	S3	Faeces	-	-

[a]Bands detected in PCR + : Present; - : absent.
S1-10: Sampling sites

OprL gene detection in *P. aeruginosa*

The results showed that the *opr*L gene locus for the *P. aeruginosa* isolates tested was successfully detected using PCR targeting the specific primer (Table 3). The agarose gel electrophoresis banding patterns of *opr*L gene detection for *P. aeruginosa* is shown in Figure 4 and the distribution of genotypic virulence determinants is shown in Table 7.

The result of this study confirmed the presence of *opr*L gene in both isolates (Table 7) with the expected size of 504 bp is agreed with the research finding reported (Abdullahi *et al*., 2013). Similar result was reported that *op*rL gene was successfully detected in most of the *P. aeruginosa* isolates from the water (Abdullahi *et al*., 2013). The result showed that pathogenic *P. aeruginosa* was present in low frequency in the swiftlet houses especially at S6 and S9 is probably due to contamination in the feed or water in the surrounding area and bird skin infection outbreak. Apart from that, the birds flying in and out of the bird houses may introduce more bacterial pathogens from the outside environment into the houses. Current result showed that *P. aeruginosa* is not a common avian pathogen isolated mostly in birds because they were isolated in low frequency. Our study targeted *opr*L gene mainly because

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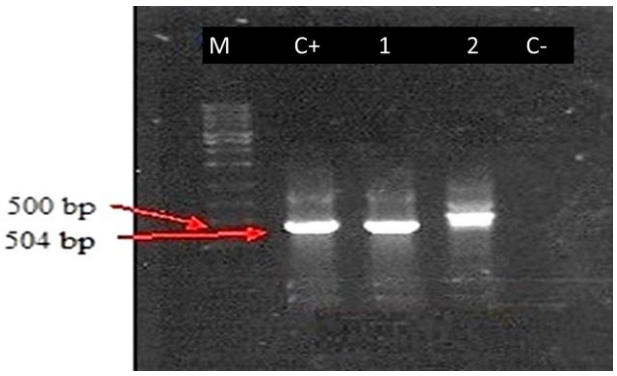

Figure 4: Agarose gel electrophoresis of *opr*L amplification products from *P. aeruginosa* isolates. M: 1 kb ladder; C+: Positive control (*P. aeruginosa* ATCC 27853); 1-2: *P. aeruginosa* isolates; C-: Negative control (*E. coli* ATCC 25922).

Table 7: Distribution of genotypic virulence determinants among the *P. aeruginosa*.

Isolates (n=2)	Sampling sites	Sources	Detection by PCR[a]
			*opr*L gene
SWF-SIBU-1B9	S9	Faeces	+
SWF-NEX-1C2	S6	Faeces	+

[a]Bands detected in PCR + : Present
S6, S9: Sampling sites

it is a peptidoglycan associated outer membrane lipoprotein which is sensitive and reliable for detection (Han *et al*., 2011). Most of the *P. aeruginosa* causes life-threatening infections and difficult to control clinically. The outer membrane proteins of *P. aeruginosa* encoded with *opr*L gene play the main role in the interaction between bacterium with the environment (Jami Al-Ahmadi and Zahmatkesh Roodsari, 2016). Thus, the *opr*L gene target was used precisely in the detection of them. *Psedomonas aeruginosa* produced pyocyanin as their virulence factor which degraded the cell wall membrane of eukaryotic cells, causing lysis and death in patients (Gellatly and Hancock, 2013) and mainly infected the damaged tissues or infected the low immunity patients with symptoms of inflammation and sepsis. Even though there was only a low frequency of *P. aeruginosa* isolated
from swiftlet, precautions should be taken. This bacterium remains a problematic culprit in hospitals, causing virulent and persistent infections despite antibiotic treatment.

CONCLUSION

Potential pathogens (*E. faecalis*, *E. coli*, and *P. aeruginosa*) showed a high prevalence in fecal samples. There were only pathogenic *E. facealis* detected in airborne samples. The high incidence of virulence genes detection in *E. faecalis* and *P. aeruginosa* indicates these genes were widely disseminated among the bacteria found in swiftlet houses which may transmit through the flying swiftlet inside the houses. The prevalence of pathogenic airborne bacteria was depended mainly on the frequency of the birds entering the house, air circulation and the hygiene condition inside the bird houses. Interaction human with swiftlet waste materials may spread pathogenic bacteria potentially creating health hazards in the community. Thus, rules and guidelines on swiftlet farming in Malaysia must be enforced. Swiftlet farming is recommended to build far from the city, away from the people.

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REFERENCES

Abdullahi, R., Lihan, S., Carlos, B. S., Bilung, M. L., Mikal, M. K. and Collick, F. (2013). Detection of *opr*L gene and antibiotic resistance of *Pseudomonas aeruginosa* from aquaculture environment. *European Journal of Experimental Biology* **3, 148-152.**

Adamu, M. T., Shamsul, B. M. T., Desa, M. N. and Khairani, B. (2014). A review on *Escherichia coli* O157:H7-the super pathogen. *Health and the Environment Journal* **5, 78-93.**

Ahmed, W., Sidhu, J. P. S. and Toze, S. (2012). Speciation and frequency of virulence genes of *Enterococcus* spp. isolated from rainwater tank samples in Southeast Queensland, Australia. *Environmental Science and Technology* **46, 6843-6850.**

Al-Talib, H., Zuraina, N., Kamarudin, B. and Yean, C. (2015). Genotypic variations of virulent genes in *Enterococcus faecium* and *Enterococcus faecalis* isolated from three hospitals in Malaysia. *Advances in Clinical and Experimental Medicine* **24, 121-127.**

Andrew, N. and Growther, L. (2011). Shiga toxin producing *E. coli*- an emerging pathogen a review. *World Journal of Science and Technology* **1, 2231-2583.**

Arias, C. A. and Murray, B. E. (2012). The rise of the *Enterococcus*: Beyond vancomycin resistance. *Nature Reviews Microbiology* **10, 266-278.**

Benskin, C. M. H., Wilson, K., Jones, K. and Hartley, I. R. (2009). Bacterial pathogens in wild birds: A review of the frequency and effects of infection. *Biological Reviews* **84, 349-373.**

Cabot, G., Zamorano, L., Moyà, B., Juan, C., Navas, A., Blázquez, J. and Oliver, A. (2016). Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. *Antimicrobial Agents and Chemotherapy* **60, 1767-1778.**

Chai, S. K. (2013). The use of loop-Amp in detection of Shiga toxin gene among the *Escherichia coli* from

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

aquaculture and other environments. B. Thesis. Universiti Malaysia Sarawak, Malaysia.

Coque, T. M. (2002). High occurrence of *esp* among ampicillin-resistant and vancomycin-susceptible *Enterococcus faecium* clones from hospitalized patients. *Journal of Antimicrobial Chemotherapy* **50, 1035-1038.**

Dupre, I. (2003). Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *Journal of Medical Microbiology* **52, 491-498.**

Fisher, K. and Phillips, C. (2009). The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology* **155, 1749-1757.**

Ferens, W. A. and Hovde, C. J. (2011). *Escherichia coli* O157:H7: Animal reservoir and sources of human infection. *Foodborne Pathogens and Disease* **8, 465-487.**

Ferguson, D. M., Talavera, G. N., Hernández, L. A. R., Weisberg, S. B., Ambrose, R. F. and Jay, J. A. (2016). Virulence genes among *Enterococcus faecalis* and *Enterococcus faecium* isolated from coastal beaches and human and nonhuman sources in Southern California and Puerto Rico. *Journal of Pathogens* **2016, 1-7.**

Foxman, B. (2011). Applications of molecular tools to infectious disease epidemiology. *In:* Molecular Tools and Infectious Disease Epidemiology. Elsevier Inc. Academic Press, United State. **pp. 23-39.**

Frazzon, A. P. G., Gama, B. A., Hermes, V., Bierhals, C. G., Pereira, R. I., Guedes, A. G. , d'Azevedo, P. A. and Frazzon, J. (2009). Prevalence of antimicrobial resistance and molecular characterization of tetracycline resistance mediated by tet(M) and tet(L) genes in *Enterococcus* spp. isolated from food in Southern Brazil. *World Journal of Microbiology and Biotechnology* **26, 365-370.**

Gellatly, S. L. and Hancock, R. E. W. (2013). *Pseudomonas aeruginosa*: New insights into pathogenesis and host defenses. *Pathogens and Disease* **67, 159-173.**

Ghosh, A., KuKanich, K., Brown, C. E. and Zurek, L. (2012). Resident cats in small animal veterinary hospitals carry multi-drug resistant *Enterococci* and are likely involved in cross-contamination of the hospital environment. *Frontiers in Microbiology* **3, 1-14.**

Gould, L. H. (2012). Update: Recommendations for diagnosis of Shiga toxin-producing *Escherichia coli* infections by clinical laboratories. *Clinical Microbiology Newsletter* **34, 75-83.**

Grys, T. E., Sloan, L. M., Rosenblatt, J. E. and Patel, R. (2009). Rapid and sensitive detection of Shiga toxin-producing *Escherichia coli* from nonenriched stool specimens by real-time PCR in comparison to enzyme immunoassay and culture. *Journal of Clinical Microbiology* **47**, **2008-2012.**

Han, D., Unno, T., Jang, J., Lim, K., Lee, S. -N., Ko, G., Sadowsky, M. J. and Hur H. G. (2011). The occurrence of virulence traits among high-level aminoglycosides resistant *Enterococcus* isolates obtained from feces of humans, animals, and birds in South Korea. *International Journal of Food Microbiology* **144, 387-392.**

Jami Al-Ahmadi, G. and Zahmatkesh Roodsari, R. (2016). Fast and specific detection of *Pseudomonas aeruginosa* from other *Pseudomonas* species by PCR. *Annals of Burns and Fire Disasters* **29, 264-267.**

Kalle, M., Papareddy, P., Kasetty, G., Mörgelin, M., van der Plas, M. J. A., Rydengård, V., Malmsten, M., Albiger, B. and Schmidtchen, A. (2012). Host defense peptides of thrombin modulate inflammation and coagulation in endotoxin-mediated shock and *Pseudomonas aeruginosa* sepsis. *PLoS ONE* **7, e51313.**

Kim, E. B. and Marco, M. L. (2013). Nonclinical and clinical *Enterococcus faecium* strains, but not *Enterococcus faecalis* strains, have distinct structural and functional genomic features. *Applied and Environmental Microbiology* **80, 154-165.**

Klockgether, J. and Tümmler, B. (2017). Recent advances in understanding *Pseudomonas aeruginosa* as a pathogen. *F1000Research* **6, 1261.**

Kwon, K. H., Hwang, S. Y., Moon, B. Y., Park, Y. K., Shin, S., Hwang, C. -Y. and Park, Y. H. (2012). Occurrence of antimicrobial resistance and virulence genes, and distribution of *enterococcal* clonal complex 17 from animals and human beings in Korea. *Journal of Veterinary Diagnostic Investigation* **24, 924-931.**

Lebreton, F., Depardieu, F., Bourdon, N., Fines-Guyon, M., Berger, P., Camiade, S., Leclercq, R., Courvalin, P. and Cattoir, V. (2011). d-Ala-d-Ser Van N-type transferable vancomycin resistance in *Enterococcus faecium*. *Antimicrobial Agents and Chemotherapy* **55, 4606-4612.**

Lebreton, F., Willems, R. J. L. and Gilmore, M. S. (2014). *Enterococcus* diversity, origins in nature, and gut colonization. *In:* Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Gilmore, M. S., Clewell, D. B., Ike, Y. and Shankar, N. (eds.). Massachusetts Eye and Ear Infirmary Press, Boston, Massachusetts. **pp. 205.**

Literák, I., Vanko, R., Dolejská, M., Čížek, A. and Karpíšková, R. (2007). Antibiotic resistant *Escherichia coli* and *Salmonella* in Russian rooks (*Corvus frugilegus*) wintering in the Czech Republic. *Letters in Applied Microbiology* **45, 616-621.**

Medeiros, A. W., Pereira, R. I., Oliveira, D. V., Martins, P. D., d' Azevedo, P. A., Van der Sand, S., Frazzon, J. and Frazzon A. P. (2014). Molecular detection of virulence factors among food and clinical *Enterococcus faecalis* strains in South Brazil. *Brazilian Journal of Microbiology* **45, 327-332.**

Meng, Q., Bai, X., Zhao, A., Lan, R., Du, H., Wang, T., Shi, C., Yuan, X., Bai, X., Ji, S., Jin, D., Yu, B., Wang, Y., Sun, H., Liu, K., Xu, J. and Xiong, Y. (2014). Characterization of Shiga toxin-producing *Escherichia coli* isolated from healthy pigs in China. *BMC Microbiology* **14, 5.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Nyakundi, W. O. and Mwangi, W. (2011). Isolation and characterization of pathogenic bacteria and fungi from *Leptoptilos crumeriferu* (Marabau Strok) dropping. *Journal of Applied Sciences and Environmental Management* **1, 93-103.**

Ocvirk, S., Sava, I. G., Lengfelder, I., Lagkouvardos, I., Steck, N., Roh, J. H., Tchaptchet, S., Bao, Y., Hansen, J. J., Huebner, J., Carroll, I. M., Murray, B. E., Sartor, R. B. and Haller, D. (2015). Surface-associated lipoproteins link *Enterococcus faecalis* virulence to colitogenic activity in IL-10-deficient mice independent of their expression levels. *PLoS Pathogens* **11, e1004911.**

Panmanee, W., Gomez, F., Witte, D., Pancholi, V., Britigan, B. E. and Hassett, D. J. (2008). The peptidoglycan-associated lipoprotein *Opr*L helps protect a *Pseudomonas aeruginosa* mutant devoid of the transactivator *Oxy*R from hydrogen peroxide-mediated killing during planktonic and biofilm culture. *Journal of Bacteriology* **190, 3658-3669.**

Preethee, T., Kandaswamy, D. and Hannah, R. (2012). Molecular identification of an *Enterococcus faecalis* endocarditis antigen *efa*A in root canals of therapy-resistant endodontic infections. *Journal of Conservative Dentistry* **15, 319.**

Prithiviraj, B., Bais, H. P., Weir, T., Suresh, B., Najarro, E. H., Dayakar, B. V., Schweizer, H. P. and Vivancoet, J. M. (2005). Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. *Infection and Immunity* **73, 5319-5328.**

Radimersky, T., Frolkova, P., Janoszowska, D., Dolejska, M., Svec, P., Roubalova, E., Cikova, P., Cizek, A. and Literak, I. (2010). Antibiotic resistance in faecal bacteria (*Escherichia coli*, *Enterococcus* spp.) in feral pigeons. *Journal of Applied Microbiology* **109, 1687e95.**

Saitoa, S. E., Harpa, J. R. and Fozoa, E. M. (2018). *Enterococcus faecalis* responds to individual exogenous fatty acids independently of their degree of saturation or chain length. *Applied and Environmental Microbiology* **84, e01633-17.**

Schouler, C., Schaeffer, B., Bree, A., Mora, A., Dahbi, G., Biet, F., Oswald, E., Mainil, J., Blanco, J. and Moulin-Schouleur, M. (2012). Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. *Journal of Clinical Microbiology* **50, 1673-1678.**

Sien, L. S., Lihan, S., Yee, L. T., HwaChuan, C. and ChanKoon, L. (2013). Isolation and characterization of antibiotic resistant bacteria from swiftlet feces in swiftlet farm houses in Sarawak, Malaysia. *Microbiology Indonesia* **7, 137-143.**

Son, I., Binet, R., Lin, A., Hammack, T. S. and Kase, J. A. (2015). Identification of five Shiga toxin-producing *Escherichia coli* genes by Luminex microbead-based suspension array. *Journal of Microbiological Methods* **111, 108-110.**

Tambekar, D., Jaiswal, V., Dhanorkar, D., Gulhane, P. and Dudhane, M. (2008). Identification of microbiological hazards and safety of ready-to eat food vended streets of Amravati City, India. *Journal of Applied Biosciences* **7, 195-201.**

Tremblay, C. -L. and Archambault, M. (2013). Interference in pheromone-responsive conjugation of a high-level bacitracin resistant *Enterococcus faecalis* plasmid of poultry origin. *International Journal of Environmental Research and Public Health* **10, 4245-4260.**

Woo, S., Yang, S.-H., Chen, H.-J., Tseng, Y.-F., Hwang, S.-J., De Palmas, S., Denis, V., Imahara, Y., Iwase, F., Yum, S. and Tang, S. L. (2017). Geographical variations in bacterial communities associated with soft coral *Scleronephthya gracillimum*. *PLoS ONE* **12, e0183663.**

Xu, J., Moore, J. E., Murphy, P. G., Millar, B. C., Redmond, A. O. B. and Elborn, J. S. (2004). Molecular (PCR) detection of *Pseudomonas* spp. other than *P. aeruginosa* directly from the sputum of adults and children with cystic fibrosis. *British Journal of Biomedical Science* **61, 147-149.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538