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First detection of Shiga toxin producing *Escherichia coli* O157:H7 (*HlyA* gene) and enumeration of most probable number (MPN) of fecal coliforms and *Escherichia coli* in cage cultured oysters (*Crassostrea iredalei*) and water from southern Malaysia

Tee Ka Hong[1,3], George Bobby[1,3], Nurshafiqah Norizan[1,3], Nurshuhada Ariff[1,3], Siti Nor Khadijah Addis[2,3], Najiah Musa[1,4] and Sandra Catherine Zainathan[1,3]*

[1]Faculty of Fisheries and Food Sciences, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia.
[2]Faculty of Marine and Environmental Science, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia.
[3] Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia.
[4] Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia.
Email: sandra@umt.edu.my

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ABSTRACT

Aims: *Escherichia coli* O157:H7 is known to be transmitted via fecal-oral route, where water plays a role in the transmission process. Oysters as bivalves, bio accumulate pathogens from the water through filter feeding and are suspected to play a role as disease transmission vector. In Malaysia, the data on oyster's microbiological quality are limited. Hence, it was vital to conduct oyster related studies in Malaysia. The main objectives of this study include the enumeration of most probable number (MPN) of fecal coliforms and *E. coli* and isolation of *E. coli* from oyster (*Crassostrea iredalei*) and water sample for the detection of 16S rRNA and *HlyA* (Hemolysin A) genes of *E. coli* O157:H7.
Methodology and results: A total of 120 oysters and water samples (n=6) were collected from a fisherman village located in southern Malaysia. Total fecal coliforms and *E. coli* were determined using the MPN procedure. Colonies of *E. coli* were identified based on Gram staining, biochemical test, and PCR detection for the presence of 16S rRNA and *HlyA* gene of *E. coli* O157:H7. The enumeration results showed that the MPN of the fecal coliforms and *E. coli* found in the collected oyster samples do not meet the standard to be directed for human consumption ($0.72 \pm 0.19 \times 10^4$ MPN/100 g and $0.13 \pm 0.03 \times 10^4$ MPN/100 g, respectively). The PCR assays showed that 16 out of the 104 (15.38%) of *E. coli* isolated from water and oysters showed the presence of *HlyA* gene. The phylogenetic tree analysis showed there were genetic relationships between the *HlyA* gene of the *E. coli* isolated in this study with the ones isolated from calf and human faeces.
Conclusion, significance and impact of study: The detection of Shiga toxin producing *E. coli* O157:H7 (*HlyA* gene) in cage cultured oysters (*C. iredalei*) and water from southern Malaysia was first time reported here. In the future, more study can be conducted to study the expression of the *HlyA* gene and confirm of its identity as *E. coli* O157:H7 using different target genes such as *eaeA* (encodes a 94 kD outer membrane protein called intimin) and *Stx1* (Shiga toxin, *Shigella dysenteriae* type 1).

Keywords: Escherichia coli O157:H7, *HlyA* gene, fecal coliforms, *Crassostrea iredalei*, Malaysia

INTRODUCTION

The genus *Escherichia* is a member of the Enterobacteriaceae family and *Escherichia coli* is the most common aerobic organism in the intestinal tract of man and warm-blooded animals (Koteswar *et al*., 2017). *Escherichia coli* is the main member in fecal coliforms and have been used as index of sewage contamination (Feng *et al*., 2002). This is because *E. coli* can be found abundantly in human intestine and other animals as predominant, facultative anaerobe which in nature assisted to suppress the growth of harmful bacteria and had been known to produce certain amounts of vitamin B in the intestine (Armstrong *et al.*, 1996). However, some pathogenic *E. coli* are responsible to cause several diarrhoeal diseases in human due to the presence of explicit colonisation features, virulence factors and pathogenicity associated genes which are absent in

***Corresponding author**

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normal *E. coli* (Armstrong *et al.*, 1996; O'Sullivan *et al*., 2007). The known groups of diarrheagenic *E. coli* includes Verocytotoxigenic *E. coli* (VTEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAggEC) and Diffusely Adherent *E. coli* (DAEC) (Nataro and Kaper, 1998; Parry and Palmer, 2002; O'Sullivan *et al*., 2007; Price and Tom, 2007; Jensen *et al*., 2014). The transmission of *E. coli* is through fecal-oral route, as feces containing communicable agents such as *E. coli* can be shed by sick/infected host and acquired by the vulnerable/immune-compressed host through absorption of contaminated materials (Campbell and Reece, 2002; Katouli, 2010; Farlex Partner Medical Dictionary, 2012; WHO, 2016a). The shedding of *E. coli* into the water is highly possible to be bioaccumulated in shellfish particularly bivalves, as their feeding behaviour is to filter feed the food/any particles off large volume of water (Iwamoto *et al*., 2010).

Among the pathogenic strains of *E. coli*, *E. coli* serotype O157:H7 is known to have high impacts on human health status globally due to its low infection dose and related to diseases such as hemolytic uremic syndrome (Armstrong *et al.*, 1996; Motarjemi, 2014; WHO, 2016a). *E. coli* O157:H7 is the main serotype in a subset group known as Enterohemorrhagic *E. coli* (EHEC) under the Shiga toxin-producing *E. coli* (STEC). *E. coli* O157:H7 are known to produce toxin similar to *Shigella dysenteriae* (hence the name STEC), which causes severe damage to the lining of human intestine and other organs (Armstrong *et al.*, 1996). The diseases known to be caused by *E. coli* O157:H7 are grouped into four, including hemorrhagic colitis, hemolytic uremic syndrome (HUS), non-bloody diarrhea and asymptomatic infection. *HlyA* gene, also known as EHEC-*hlyA* gene, is one of the most important gene found in *E. coli* O157 that is associated with production of hemolysin toxin protein. It has been found in patients with hemolytic uremic syndrome (HUS) and can cause cell function failure and eventually lysis (Hughes *et al*., 1992; Schmidt *et al*., 1995; Mortajemi, 2014). The main reservoirs of these bacteria are ruminants such as cattle, however, it has been detected in bodies of water and waterborne transmission has been confirmed (WHO, 2016b). The possibility of *E. coli* O157:H7 being transmitted through shellfish in contaminated water had become a great concern due to the bioaccumulation characteristics of shellfish via filter feeding of particles in the water (Stewart *et al*., 2008; Bennani *et al.*, 2011; Walker *et al*., 2013).

Oysters are important cultured species in the commercial aquaculture system worldwide. In 2006, aquaculture industry which includes both inland and marine produced a total of 47.3 million tonnes (MT) of products worth of US$78 billion and by 2014, both inland and marine aquaculture produced 73.8 MT of products worth of US$160.2 billion (FAO, 2016). Asia produced about 65 MT (US$141.1 billion) in the aquaculture section and the production of molluscs such as oysters, mussels and others stand about 14 MT which worth around US$17 billion in 2014 (FAO, 2016). Malaysia had been reported to produce 42.6 thousand tonnes (around US$50 million) of molluscs in 2014 (FAO, 2016) and it was reported that commercial-scale production of oysters, of more than 100 tonnes per annum occurs in Malaysia (Nowland *et al.*, 2020). Oysters are filter feeder organisms where they filter the food particles from the surrounding environment including plankton, virus, bacteria and others (FAO, 2006; Elston *et al*., 2008; Wang *et al.*, 2008; Ueki *et al.*, 2010). Hence, oysters can be easily infested with pathogens as host or as carrier, such as *E. coli*. *E. coli* O157:H7 was reported to be found in collected oysters from France and in mussels and cockles from the Mediterranean coast of Morocco in 2006 and 2011, respectively (Gourmelon *et al*., 2006; Bennani *et al*., 2011).

There is insufficient research has been conducted in Malaysia and hence the knowledge on oyster's microbiological quality is scarce (Tan *et al*., 2014). In Malaysia, the monitoring of the microbiological quality of shellfish and shellfish waters have been carried out through enumeration of fecal coliforms and *E. coli* according to European Committee regulation (Directives 91/492/EC) and United States National Shellfish Sanitation Program (US NSSP) shellfish water classification in Selangor (Wan Norhana and Nor Ainy, 2004; Wan Norhana *et al*., 2016). In this study, the enumeration of *E. coli* and detection of *E. coli* O157:H7 in oysters and surrounding waters was conducted to gain more insight on the status of oysters reared in southern Malaysia.

MATERIALS AND METHODS

Sample collection

A total of 120 oysters (*C. iredalei*) and water samples (n=6) were collected from a fisherman village located in southern Malaysia (N01°28.904', E103°48.985') on 10 December 2016 and 31 December 2016. A number of activities were conducted in the fisherman village including jetty and big seaport, restaurants, boat repairing site, aquaculture activities including fish cage cultures, mussels' culture and its processing facilities (Yap *et al.*, 2010). The water quality parameters were measured using YSI Multiparameter Water Quality Meter (YSI Incorporated, USA) and API® Freshwater Aquarium Master Test Kit (Mars Fishcare North America, Inc). Oyster samples were randomly collected (n=60) from the same sampling site along with water sample (n=3) using sterile water collection bottle (500 mL) during each sampling time. The samples were transported back to Aquatic Organism Health Laboratory, Universiti Malaysia Terengganu in an ice box to maintain the temperature at approximately 4 °C and processed within 24 h at 4 °C. The oyster samples (n>120) were examined as followed; oyster samples which were with opened shell, empty contents and dead were eliminated and only live oyster sample (n=120) were used in this study.

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Enumeration of fecal coliforms and *E. coli*

The enumeration of fecal coliforms and *E. coli* in sample was determined by the values of most probable number (MPN) according to the methods by Feng *et al.* (2002), where it is statistical and consists of three phases which includes the presumptive, confirmed and completed phases.

Most Probable Number: Presumptive test for fecal coliforms and *E. coli*

The water sample was mixed vigorously for 30 sec manually. A total of 10 mL of water sample was diluted with 90 mL of Butterfield's phosphate-buffered dilution water (Feng *et al.*, 2002) and mixed well to reach 10× dilution. The water sample was then serially diluted to 1,000×, 10,000× and 100,000× respectively. For each water sample, 5 tubes of lactose broth (Lab M, UK) (10 mL) with the presence of inverted Durham tube were inoculated with 10 mL of each selected consecutive dilutions water sample (1,000×, 10,000× and 100,000×). The inoculated tubes were then incubated at 35 °C and examined at 24 h for the presence of gas production in the inverted Durham tube or appearance of fizz when gently agitated and the reactions were recorded. Gas-negative tubes were continued to be incubated for an additional 24 h, examined and the reactions were recorded again at 48 h.

For each sampling time, 6 pools of 10 oyster samples were homogenized separately according to each pool using BagMixer® lab blenders (Interscience, France). Two conditions were applied to the processed oyster samples according to the protocols by Bacteriological Analytical Manual of the Food and Drug Administration (Feng *et al.*, 2002). For pool of oysters that weighted more than 50 g, approximately 50 g of the homogenized sample was added with 450 mL of Butterfield's phosphate-buffered water and mixed well to make a solution of 1:10 dilution. For pool of oysters that weighted less than 50 g in a pool, percentage that is equivalent to half of the sample was weighted and adequate volume of Butterfield's phosphate-buffered water was added to make a solution of 1:10 dilution.

Different aliquots of 1:10 homogenate was used to inoculate 5 tubes of lactose broth (10 mL) to achieve 1 g, 0.1 g, 0.01 g and 0.001 g of original sample material, respectively. Confirmed test were performed on all presumptive positive (gas) tubes using methods listed in the next section.

Most Probable Number: Confirmation test for fecal coliforms and *E. coli*

A loopful of suspension was transferred from each gassing lactose broth tube from the presumptive test to a tube of *Escherichia coli* (EC) broth (Himedia, India) with the presence of inverted Durham tube. The inoculated EC broth tubes were then incubated for 24 h at 44.5 °C and examined for gas production. The EC broth tubes that were negative for gas production after 24 h were further incubated and examined again at 48 h. The results of this test were used to calculate the fecal coliforms' MPN according to Most Probable Number from Serial Dilutions in Bacteriological Analytical Manual (Blodgett, 2010) using three successive dilutions of gassing EC broth tubes. The combination of gas positive results was then used to refer to a statistical table for estimation of number of organism present. The EC broth tubes which are show positive gassing were further used for complete test for *E. coli*.

Most Probable Number: Complete test for *E. coli*

After gently agitating each gassing EC broth tube, a loopful of broth was streaked on Eosin Methylene Blue (EMB) (Himedia, India) agar plate and was incubated for 18 to 24 h at 35 °C. Suspicious *E. coli* colonies on EMB were selected with the characteristic of dark centered and flat, with or without metallic sheen. Up to three suspicious colonies were selected from each gassing EC broth tube. The selected colonies were then transferred to 1.5% NaCl Trypticase Soy Agar (TSA) agar plate (Conda, Canada) and were incubated for 18 to 24 h at 35 °C. Since identification of any one of the three colonies as *E. coli* was adequate to regard that EC broth tube as positive, hence not all three selected isolates were tested (Feng *et al.*, 2002). Gram stain was carried out according to Whitman (2004) using suspicious *E. coli* colonies. The colonies that appeared as Gram-negative, short rods were tested for indole, methyl red, Voges-Proskauer and citrate (IMViC) reactions and re-inoculated into lactose broth tube for confirmation of gas production.

All isolates that appeared as Gram-negative, short rods; showed IMViC patterns ++-- (biotype 1) (indole positive, methyl red positive, Voges-Proskauer negative, citrate negative) or -+-- (biotype 2) (indole negative, methyl red positive, Voges-Proskauer negative, citrate negative); fermented lactose with gas production within 48 h at 35 °C were recognized as *E. coli*. MPN of *E. coli* was calculated based on proportion of EC broth tubes in three successive dilutions that were *E. coli* positive according to Most Probable Number from Serial Dilutions in Bacteriological Analytical Manual (Blodgett, 2010).

Detection of 16S rRNA and *HlyA* genes

The isolates (n=104) that were recognized as *E. coli* after examination of Gram stain, IMViC reactions (biotype 1 and 2) and gas production from lactose were used to detect the 16S rRNA gene for identification purpose and *HlyA* gene for the detection of presence of *E. coli* O157:H7. *Escherichia coli* DSMZ 17076 was used as positive control and water as negative control at this stage.

Genomic DNA extraction of *Escherichia coli* samples

The genomic DNA extraction was conducted using NucleoSpin® Tissue kit (MachereyNagel, Germany)

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according to the protocol provided by the manufacturer (Macherey-Nagel, Germany). For the re-subcultured sample and positive control, 250 µL of the bacterial culture was transferred to 1.5 mL microcentrifuge tube prior to extraction. The tube was then centrifuged for 5 min at 8,000× *g*. The supernatant was removed, and the extraction was conducted accordingly. The eluted DNA was then stored at –20 °C until further analyses.

PCR amplification of 16S rRNA gene of extracted samples

Once pure genomic DNA was extracted, it was subjected to PCR assay to detect the target gene. The primers used for 16S rRNA gene PCR amplification were 8F: 5'-GTTTACCTTGTTACGACTT-3' and 149R: 5'-AGAGTTTGATCCTGGATGCTCAG-3' yielding a product at 1500 bp (Galkiewicz and Kellogg, 2008). The amplification was programmed as followed: 5 min at 95 °C, followed by 26 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 60 sec and extension at 72 °C for 2 min. A final extension of 72 °C for 10 min terminated the thermal cycling reaction. The nested PCR was carried out with a total of 25 µL PCR mixture containing: 12.5 µL 2× MyTaq™ Mix (BIOLINE), 8.0 µL RNase-free water, 1 µL (10 µM) 8F and 1 µL (10 µM) 149R were added to 2.5 µL extracted genomic DNA. The assay was conducted using T100™ thermal cycler (Bio-Rad, USA). For each PCR run, extracted genomic DNA from *E. coli* DSMZ 17076 were used as positive control and non-template mixture was used as negative control. After the assay, the amplicons were subjected to gel electrophoresis with a negative control (nucleic acid free), a positive control (amplified PCR products of *E. coli* DSMZ 17076) and a 100 bp DNA ladder (TrackIt™) in each electrophoresed gel. The gel electrophoresis was carried out for 45 min at 70 V. The electrophorized gel were visualized using Aplegen® Omega Lum™ G Imaging System (Gel company, USA). The presence of target gene was determined to be positive when amplicons were detected at 1,500 bp.

PCR amplification of *HlyA* gene of extracted samples

The primers used for amplification of *HlyA* gene were O157-3: 5'-GTAGGGAAGCGAACAGAG-3' and O157-4: 5'-AAGCTCCGTGTGCCTGAA-3' yielding a product at 361 bp (Wang *et al.*, 1997). The amplification was programmed as followed: 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 3 sec, annealing at 50 °C for 10 sec and extension at 74 °C for 35 sec. A final extension of 45 °C for 2 min terminated the thermal cycling reaction. The nested PCR was carried out with a total of 25 µL PCR mixture containing: 12.5 µL 2× MyTaq™ Mix (BIOLINE), 8.0 µL Rnase-free water, 1 µL (10 µM) O157-3 and 1 µL (10 µM) O157-4 were added to 2.5 µL extracted genomic DNA. The assay was conducted using T100™ thermal cycler (Bio-Rad, USA). For each PCR run, extracted genomic DNA from *E. coli* DSMZ 17076 were used as positive control and non-template mixture was used as negative control. The gel electrophoresis and visualisation of PCR product was carried out as mentioned above.

Gel extraction and DNA sequencing

The positive bands from PCR amplification were excised/cut and sent to First Base Laboratories Sdn. Bhd. for sequencing analysis. The obtained results were then analysed and compared with sequences in GenBank using BLAST NCBI (http://blast.ncbi.nlm.nih.gov). Based on the sequenced data from samples and matched sequences from GenBank, the sequences were aligned using BioEdit Sequence Alignment Editor software and a phylogenetic tree were generated using aligned sequences with MEGA 7.0.26 software, bootstrap value is 100.

RESULTS

Water quality parameter

Multiple water parameters were recorded at sampling site using YSI® Multiparameter Water Quality Meter (YSI Incorporated, USA) and API® Freshwater Aquarium Master Test Kit (Mars Fishcare, USA) (Table 1). Temperature (°C), conductivity as specific conductance (µS/cm), total dissolved solids (mg/L), salinity (ppt) and pH were in the range of "Water Quality Criteria and Standards for Freshwater and Marine Aquaculture" and "Site Selection and Water Quality in Mariculture" guidelines (PHILMINAQ, 2008; Prema, 2013), except dissolved oxygen (mg/L), ammonia (ppm) and nitrite (ppm) contents. For dissolved oxygen, it was found to be lower than the guideline range (3.0-7.0 mg/L). Ammonia content in water were found to be higher than guideline for both sampling times. Whereas, nitrate content in water were lower than the guideline for both sampling times. The nitrite content was lower for the first sampling but higher than the guideline for the second sampling.

Enumeration of fecal coliforms and *E. coli* in samples

Overall, the mean MPN of fecal coliforms and *E. coli* in collected water samples were $0.93 \times 10^4 \pm 0.36 \times 10^4$ MPN/100 mL and $0.68 \times 10^4 \pm 0.34 \times 10^4$ MPN/100 mL, respectively) (Table 2). The fecal coliforms count exceeded 43 MPN/100 mL according to the shellfish water classification criteria by US NSSP (National Shellfish Sanitation Program) for approved areas. The water sample with the highest MPN of fecal coliforms and *E. coli* for both sampling was W1 (2.30×10^4 MPN/100 mL), while the water sample with the lowest fecal coliforms and *E. coli* MPN was W2 (0.45×10^4 MPN/100 mL and 0.20×10^4 MPN/100 mL, respectively). Whereas, water samples W5 and W6 had similar MPN of fecal coliforms and *E. coli* (1.8×10^3 MPN/100 mL, respectively).

Eleven isolates consisting of biotype I and biotype II *E. coli* were isolated from the water samples after the

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Table 1: Average water quality parameter (triplicates) except nitrate, nitrite and ammonia by API® Freshwater Aquarium Master Test Kit during the first (10 December 2016) and second sampling (31 December 2016) time.

Equipment/kit	Parameters	1st sampling	2nd sampling	Guidelines (Based on "Water Quality Criteria and Standards for Freshwater and Marine Aquaculture" and "Site Selection and Water Quality in Mariculture" guidelines)
YSI Multiparameter Water Quality Meter	Temperature, °C	30.70 ± 0.06	29.70 ± 0.03	21.0-31.0 (Prema, 2013)
	Dissolved oxygen, mg/L	1.30 ± 0.14	2.40 ± 0.07	3.0-7.0 (PHILMINAQ, 2008), (>6 mg/L, Prema, 2013)
	Specific conductance µs/cm	45573.30 ± 35.27	43583.00 ± 20.82	No range provided
	Total Dissolved Solids, mg/L	29618.30 ± 21.67	28259.80 ± 30.33	<400 mg/L (Prema, 2013)
	Salinity, ppt	29.40 ± 0.03	27.90 ± 0.02	27.0-35.0
	pH	7.70 ± 0.06	7.80 ± 0.05	7.8-8.4
	Ammonium nitrogen, NH_4-N, mg/L	7.60 ± 0.16	2.50 ± 0.31	<0.1 (Prema, 2013)
	Ammonia nitrogen, NH_3-N, mg/L	0.30 ± 0.05	0.10 ± 0.01	<0.03 (Prema 2013)
API® Freshwater Aquarium Master Test Kit	Ammonia, ppm	2.00	1.00	<1.0
	Nitrite, ppm	0.00	0.25	<0.021
	Nitrate, ppm	0.00	5.00	<100

Table 2: Enumeration of fecal coliform and *E. coli* in water samples collected during 1st sampling and 2nd sampling (MPN/100 mL).

Sampling	Sample	Fecal coliform (MPN/100 mL)	*E. coli* (MPN/100 mL)
1st sampling	W1	2.30×10^4	2.30×10^4
	W2	0.45×10^4	0.20×10^4
	W3	1.70×10^4	0.78×10^4
2nd sampling	W4	0.78×10^4	0.45×10^4
	W5	0.18×10^4	0.18×10^4
	W6	0.18×10^4	0.18×10^4
Average		$0.93 \pm 0.36 \times 10^4$	$0.68 \pm 0.34 \times 10^4$

W1-6: Water samples 1-6

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Table 3: Number of *E. coli* identified in water samples collected according to biotype I and II.

Sampling time	Sample	*E. coli*		
		Biotype I	Biotype II	Total
1st sampling	W1	4	1	5
	W2	1	0	1
	W3	3	0	3
	Total (A)	8	1	9
2nd sampling	W4	2	0	2
	W5	0	0	0
	W6	0	0	0
	Total (B)	2	0	2
	A + B	10	1	11

W1-W6: Water samples 1-6

confirmation tests (Table 3). Only one isolate was identified as *E. coli* biotype II in the water sample W1 and there were 8 isolates have the *E. coli* biotype I from the collected water samples during the first sampling. As for the second sampling, only two isolates were identified as *E. coli* biotype I in water sample, W4.

In the meanwhile, the mean MPN of fecal coliforms and *E. coli* in the collected oyster samples were $0.72 \times 10^4 \pm 0.19 \times 10^4$ MPN/100 g and $0.13 \times 10^4 \pm 0.03 \times 10^4$ MPN/100 g, respectively) (Table 4). As for the first sampling, the pooled oyster samples with the highest fecal coliforms MPN was S5 (2.20×10^4 MPN/100 g) and *E. coli* was S3 (0.33×10^4 MPN/100 g), while the pooled oyster sample with the lowest MPN of fecal coliforms and *E. coli* was S2 (0.17×10^4 MPN/100 g and 0.04×10^4 MPN/100 g, respectively). In 2nd sampling, S8 had the highest MPN of fecal coliforms (0.70×10^4 MPN/100 g) and S10 had the highest MPN of *E. coli* (0.13×10^4 MPN/100 g). Lastly, S12 had the lowest fecal coliforms and *E. coli* MPN (0.13×10^4 MPN/100 g and 0.02×10^4 MPN/100 g, respectively).

A total of 93 isolates of *E. coli* consisting of biotype I and biotype II were isolated from the pooled oyster samples after the confirmation tests for the enumeration of *E. coli* (Table 5). In the first sampling, 49 isolates were identified as *E. coli* biotype I and 6 isolates as *E. coli* biotype II out of a total of 55 isolates of *E. coli* harvested from pooled oyster samples. Pooled oyster sample, S5 had the highest sum (biotype I and II) of *E. coli* (n=14), while pooled oyster sample, S2 had the lowest sum of *E. coli* (n=4). As for the second sampling, 37 isolates were identified as *E. coli* biotype I and one isolate as *E. coli* biotype II out of a total of 38 isolates of *E. coli* harvested from pooled oyster samples. Pooled oyster sample S8 and S9 had the highest sum of *E. coli* (n=8), while pooled oyster samples, S11 and S12 had the lowest sum of *E. coli* (n=5).

Detection of 16S rRNA and *HlyA* gene in pooled oyster samples and water samples

A total of 104 isolates of *E. coli* (both biotype I and biotype II *E. coli* isolated from pooled oyster samples and water samples) were subjected to PCR amplification according to 8F and 149R primers and amplicons with the expected size of 1500 bp was produced (Figure 1). Several bands were selected randomly (M8, M41, M69) for sequencing analysis and the result was analysed using NCBI BLAST. The randomly selected isolates of *E. coli* (M8, M41, M69) was identified as *Escherichia coli* O157:H7 strain 8368, complete genome (GenBank accession number: CP017444.1), with query cover and identities percentage of 96% to 99% (Table 6).

Table 4: Enumeration of fecal coliform and *E. coli* in oyster sample collected during 1st sampling and 2nd sampling (MPN/100 g).

Sampling	Sample	Fecal coliform (MPN/100 g)	*E. coli* (MPN/100 g)
1st sampling	S1	1.30×10^4	0.14×10^4
	S2	0.17×10^4	0.04×10^4
	S3	1.70×10^4	0.33×10^4
	S4	0.22×10^4	0.14×10^4
	S5	2.20×10^4	0.32×10^4
	S6	0.23×10^4	0.13×10^4
2nd sampling	S7	0.49×10^4	0.08×10^4
	S8	0.70×10^4	0.06×10^4
	S9	0.46×10^4	0.08×10^4
	S10	0.49×10^4	0.13×10^4
	S11	0.49×10^4	0.05×10^4
	S12	0.13×10^4	0.02×10^4
Average		$0.72 \pm 0.19 \times 10^4$	$0.13 \pm 0.03 \times 10^4$

S1-S12: Sample 1-12

Table 5: Number of *E. coli* identified in oyster samples collected according to biotype I and II.

Sampling time	Sample	*E. coli*		
		Biotype I	Biotype II	Total
1st sampling	S1	8	0	8
	S2	3	1	4
	S3	10	1	11
	S4	6	3	9
	S5	13	1	14
	S6	9	0	9
	Total (A)	49	6	55
2nd sampling	S7	6	0	6
	S8	8	0	8
	S9	8	0	8
	S10	5	1	6
	S11	5	0	5
	S12	5	0	5
	Total (B)	37	1	38
	A + B	86	7	93

S1-S12: Sample 1-12

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Table 6: Identification of *E. coli* isolated from oyster and water samples based on 16S rRNA sequencing.

Sample	Description	Query cover	Expected value	Identities	Accession no.
M8 (pooled oyster sample S2/ 1st sampling)	*Escherichia coli* O157:H7 strain 8368, complete genome	99%	0.0	96%	CP017444.1
M41 (water sample W1/ 1st sampling)	*Escherichia coli* O157:H7 strain 8368, complete genome	98%	0.0	97%	CP017444.1
M69 (pooled oyster sample S9/ 2nd sampling)	*Escherichia coli* O157:H7 strain 8368, complete genome	98%	0.0	98%	CP017444.1

Besides, a total of 104 isolates of *E. coli* (both biotype I and biotype II *E. coli* harvested from pooled oyster sample and water sample) also subjected to PCR amplification according to O157-3 and O157-4 primers to detect *HlyA* gene with the expected size of 361 bp (Figure 2). From the results, 16 isolates of *E. coli* showed amplified bands at 361 bp, which indicated the presence of *HlyA* gene in *E. coli* O157:H7 (Table 7). Three bands (M14, M41 and M90) were randomly selected for sequencing analysis and the result was analysed using NCBI BLAST. The randomly selected isolates of *E. coli* (M14, M41 and M90) was identified as *Escherichia coli* O157:H7 strain 8368, complete genome (GenBank accession number: CP017444.1), with query cover and identities percentage of 97% (Table 8).

Phylogenetic tree analysis

Based on the phylogenetic tree analysis (Figure 3), *HlyA* gene of *E. coli* isolated from oyster samples, M14 and M90 shared closer genetic relationship compared to the *E. coli* isolated from water sample M41, but all of the *E. coli* sampled in this study are grouped together (Figure 3). The phylogenetic analysis showed that *E. coli* isolated from this study (M14, M90 and M41) was found to be more related to complete sequence of *Escherichia coli* O157:H7 strain CP017444.1 and CP015832.1. Similarly, sequences found in this study were more related to CP016625.1 *Escherichia coli* O157:H7 strain FRIK944 (sequence found in cattle feces, USA) compared to BA000007.2 *Escherichia coli* O157:H7 str. Sakai DNA (sequence found in human feces, Japan and USA). This indicated that the *E. coli* isolated in this study most probably originated from cattle/goat farms. Lastly, in this phylogenetic tree, it was observed that CP017249.1 *Escherichia coli* strain NADC 5570/86-24/6565 had the weakest relationship with the *HlyA* gene of *E. coli* isolated from the samples in this study.

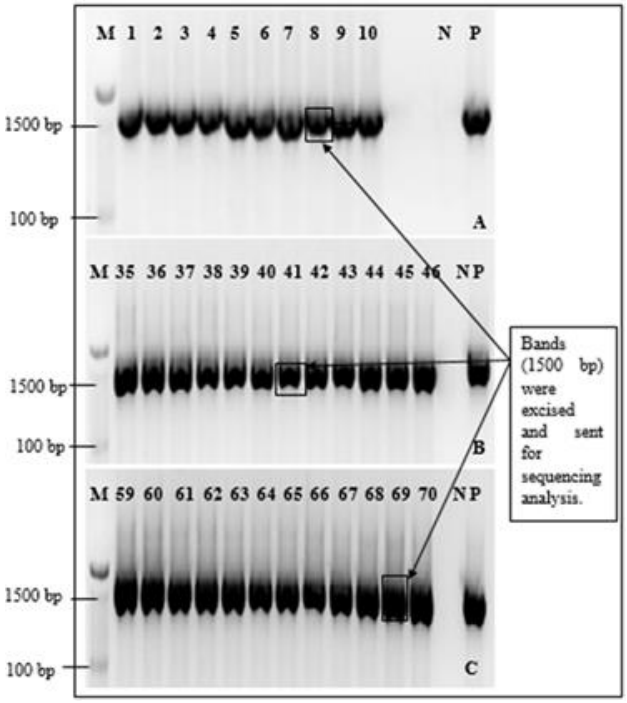

Figure 1: Representative electrophoresis gel of 16S rRNA amplicon of *E. coli* isolated from oyster and water samples. M: 100 bp DNA ladder, N: Water as a negative control, P: *Escherichia coli* DSMZ 17076 as positive control. (A) Lanes 1-7: *E. coli* isolated from pooled oyster sample S1, lanes 8-10: *E. coli* isolated from pooled oyster sample S2; (B) Lanes 35-37: *E. coli* isolated from pooled oyster sample S5, lanes 38 and 41: *E. coli* isolated from water sample W1, lane 39: *E. coli* isolated from water sample W2, lanes 40 and 42: *E. coli* isolated from water sample W3, lanes 43-46: *E. coli* isolated from pooled oyster S6. (C) Lane 59: *E. coli* isolated from water sample W1, lane 60: *E. coli* isolated from water sample W3, lanes 61 and 62: *E. coli* isolated from water sample W4, lanes 63 and 64: *E. coli* isolated from pooled oyster sample S7, lanes 65 and 66: *E. coli* isolated from pooled oyster sample S8, lanes 67-70: *E. coli* isolated from pooled oyster sample S9.

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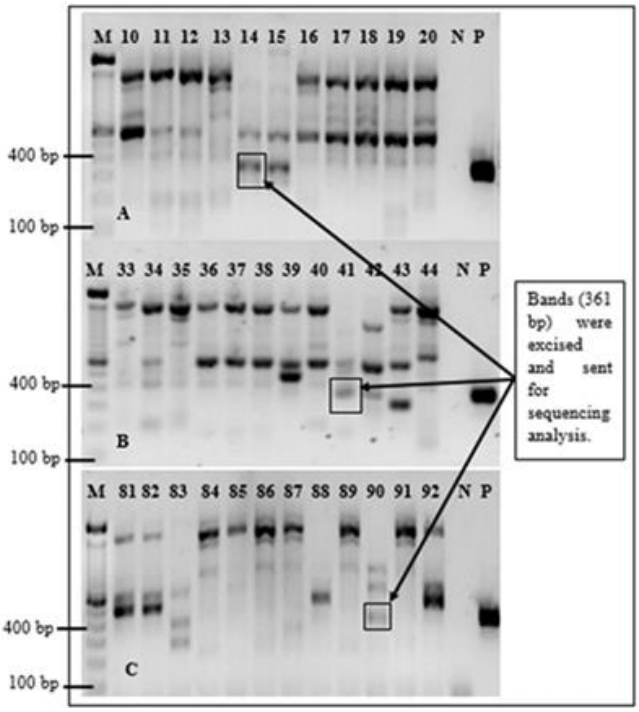

Figure 2: Representative of electrophoresis gel of *HlyA* gene amplicon of *E. coli* isolated from oyster and water samples. M: 100 bp DNA ladder, N: Water as a negative control, P: *Escherichia coli* DSMZ 17076 as positive control. (A) Lanes 10 and 11: *E. coli* isolated from pooled oyster sample S2, Lanes 12-20: *E. coli* isolated from pooled oyster sample S3, (B) Lanes 33-37: *E. coli* isolated from pooled oyster sample S5, lanes 38 and 41: *E. coli* isolated from water sample W1, lane 39: *E. coli* isolated from water sample W2, lanes 40 and 42: *E. coli* isolated from water sample W3, lanes 43 and 44: *E. coli* isolated from pooled oyster S6. (C) Lanes 81 and 82: *E. coli* isolated from pooled oyster sample S7, lanes 83 and 84: *E. coli* isolated from pooled oyster sample S8, lanes 85-87: *E. coli* isolated from pooled oyster sample S9, lanes 88 and 89: *E. coli* isolated from pooled oyster sample S10, lanes 90-92: *E. coli* isolated from pooled oyster sample S11.

Table 7: PCR amplification of *HlyA* gene in *E. coli* isolated from pooled oyster sample and water sample collected during 1st and 2nd sampling.

Sampling time	Sample	Code name of *E. coli* (Presence of *HlyA* gene)	Number of *E. coli* colonies with presence of *HlyA* gene
1st sampling	S1	M52	1
	S2	M8	1
	S3	M14, M15	2
	S4	M29, M30	2
	S5	M32, M56, M98, M100	4
	S6	M48	1
	W1	M41	1
	W3	M42	1
	Total (A)		13
2nd sampling	S8	M83	1
	S9	M69	1
	S11	M90	1
	Total (B)		3
	A + B		16

Table 8: Identification of *E. coli* isolated from oyster and water samples based on *HlyA* gene sequencing.

Code name	Description	Query cover	Identities	Accession no.
M14 (pooled oyster sample S3/ 1st sampling)	*Escherichia coli* O157:H7 strain 8368, complete genome	97%	98%	CP017444.1
M41 (water sample W1/ 1st sampling)	*Escherichia coli* O157:H7 strain 8368, complete genome	97%	97%	CP017444.1
M90 (pooled oyster sample S11/ 2nd sampling)	*Escherichia coli* O157:H7 strain 8368, complete genome	97%	98%	CP017444.1

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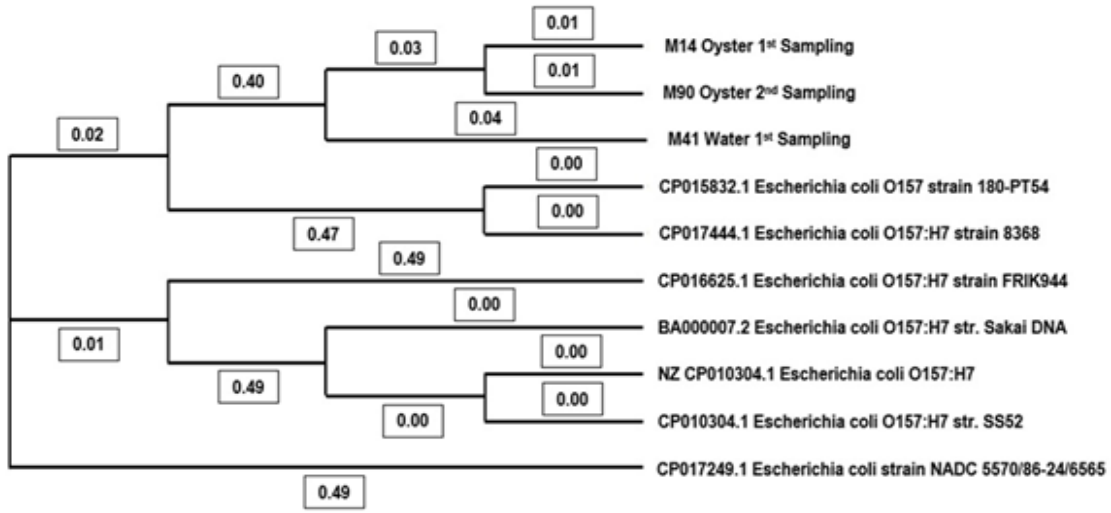

Figure 3: Phylogenetic tree analysis of *HlyA* gene of *E. coli* isolated from this study and selected sequences from Genbank.

DISCUSSION

Based on the results obtained in this study, the mean MPN of fecal coliform for oysters collected in December 2016, fell under the C category, where the MPN was between 6,000 to 60,000 MPN/100 g; while for *E. coli*, the mean MPN fell under the B category, where the MPN was between 230 to 4,600 MPN/100 g, according to the shellfish classification listed in Council Directive 91/492/EEC (Council of the European Union, 1991). Interestingly, the results found in this study was similar to the findings conducted by Vilariño *et al*. (2009) in Spain and Wan Norhana *et al.* (2016) in Malaysia, where the collected shellfish were categorized between class B and C. For oysters that fall into class B and C, it was advised that the harvested oysters to be subjected to depuration, heat treatment or relayed for a period of at least two months to reach class A, where the MPN of fecal coliforms to be less than 300 per 100 g and MPN of *E. coli* to be less than 230 per 100 g (Council of the European Union, 1991). The enumeration results showed that the MPN of fecal coliforms and *E. coli* found in the collected oyster samples did not meet the standard to be direct for human consumption. Similarly, Sorio and Peralta (2018) found high level of *E. coli* (460-940 MPN/100 g) in cage cultured oysters (*Crassostrea iredalei*) from selected production areas in Iloilo, Philippines (Sorio and Peralta, 2018). Based on Jeamsripong *et al.* (2018), the average concentrations of indicator bacteria observed in oysters from Phang Nga Bay, southern Thailand, ranged from 10^3-10^4 CFU/g oyster meat. These levels of bacterial contamination were reported to be against the regulations for maximum bacterial levels in shellfish for human consumption.

According to classification criteria as described by the US National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish: 2015 Revision (2015), the MPN of fecal coliforms in the water samples from this study exceeded the criteria of approved areas (<43 MPN/100 mL), which suggested that oysters harvested from these waters need to be relayed or treated before released to the market. Similar result was observed by Wan Norhana *et al*. (2016), where 90% of the collected seawater sample from coastal area near West Malaysia also exceeded the criteria of approved areas (<43 MPN/100 mL).

A report on the status of marine water quality at coastal areas in Malaysia (151 monitoring stations) according to Marine Water Quality Index (MWQI) (DOE, 2016), two monitoring stations in Penang, northern Malaysia were rated as poor or polluted (0-49) in 2015. At the same time, 12 marine stations in Johor, southern Malaysia and Penang were rated as moderate (50-79) according to MWQI. As MWQI was used as a method to reflect the marine water quality status and its categories are based on seven main parameters including dissolved oxygen (DO), nitrate (NO_3), phosphate (PO_4), unionized ammonia (NH_3), fecal coliforms, oil and grease (O&G) and total suspended solids (TSS). This indicated that the fecal coliforms and *E. coli* in water in this study is valid as most southern marine coastal areas were ranked as moderate according to MWQI. Other than that, Johor was also ranked as the third highest in production of water pollution loads, where 103,441 tonnes of pollution loads consisted of biochemical oxygen demand (BOD), suspended solids (SS) loads and ammoniacal nitrogen (AN) loads were produced in 2015 (DOE, 2016). Surprisingly, the main contribution source of BOD and AN was sewage, which were 51% and 84%, respectively; while sewage also contributed to 41% after piggery (49%) in the contribution of SS. Water pollution load is the quantity of contaminating substances that a waterbody is

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carrying at a specified time (DOE, 2016). The presence of fecal coliforms and *E. coli* in water and bivalves have been associated with sewage water contamination (human faeces) previously (Lipp and Rose, 1997; Feldhusen, 2000; Najiah *et al*., 2008; Adzitey *et al.*, 2010; Mortarjemi, 2014). In this study, the MPN of fecal coliforms and *E. coli* in water was higher compared to the oyster samples. Similarly, in a controlled experiment conducted by Ottaviani *et al.* (2017), the concentration of *E. coli* O157:H7 in the artificial seawater was higher than the concentration of *E. coli* O157:H7 in the mussel when examined at 24 and 48 h.

In this study, eight isolates were identified as *E. coli* biotype II and 96 isolates were identified as *E. coli* biotype I. *Escherichia coli* biotype II are variants of *E. coli* that showed negative reaction in indole production, while *E. coli* biotype I was positive reaction in indole production (Feng *et al.*, 2002). The detection of *E. coli* biotype II was usually minor upon detection, for instance, two isolates of *E. coli* biotype II out of 250 tested isolates isolated from human faeces (Papavassiliou, 1958) and 22 isolates of *E. coli* were identified as biotype II out of 622 tested isolates in a study on Enterobacteriaceae isolated from poultry meats (Stiles and Ng, 1981). Besides that, *E. coli* biotype II was also isolated from oysters in Canada (12 out of 50 isolates) and from rabbits in Belgium (19 out of 45 isolates) (Bernard, 1973; Okerman and Devriese, 1985).

The BLAST analysis result for 16S rRNA gene and *HlyA* gene of the selected *E. coli* showed that they matched with a region of the *E. coli* O157:H7 strain 8368, complete genome (GenBank accession number: CP017444.1) with a query cover and sequence identity of 97% to 99%. This is the first detection of *E. coli* O157:H7 isolated from oyster and water samples from Malaysia. In Malaysia, the presence of *E. coli* O157:H7 had been discovered only in marketed raw beef, raw cow, goat and buffalo milk previously (Radu *et al.*, 1998; Lye *et al.*, 2013, Dewanti-Hariyadi and Gitapratiwi, 2014). Positive detection of *E. coli* O157:H7 in oysters and other shellfish had been reported by previous studies in other countries. For instance, strains of *E. coli* O157 was isolated out of 150 oyster samples in France and were positive for three pathogenicity genes: *eae*, EHEC-*hlyA*, and *stx* (Guyon *et al.*, 2000). A strain of *E. coli* O157 which was *stx* negative, but *eae* and *ehxA* positive, was isolated from oyster sample collected near sewage treatment plant in France (Gourmelon *et al*., 2006). Three strains of *E. coli* O157:H7 were isolated from shellfish samples including mussels and cockles in Mediterranean Moroccan coast with positive detection of *stx1* and *stx2* virulence genes (Bennani *et al.*, 2011). The possibility for the presence of *E. coli* O157:H7 to be found in water and oyster samples in this study was most probably due to the location of oyster culture site near to dense human population and bioaccumulation by filter feeding ability of oyster. The oyster culture site was located near a fishermen village, shops and houses along the shorelines and high possibility of purging of human excretion directly to the water. *Escherichia coli* O157:H7 was once isolated in shellfish collected near an outlet of sewage water treatment in France (Gourmelon *et al*., 2006). A large outbreak of *E. coli* O157:H7 was associated with drinking municipal water contaminated with deer and elk feces in 1998 at Alpine, Wyoming (Olsen *et al.*, 2002). Similarly, high number of *E. coli* was found in cage cultured oysters (*Crassostrea iredalei*) from selected production areas in Iloilo, Philippines (Sorio and Peralta, 2018). The bioaccumulation of *E. coli* O157:H7 in shellfish was also confirmed through an experiment conducted by Ottaviani *et al.* (2017) where the presence of *E. coli* O157:H7 was confirmed in mussels that were put in contaminated artificial seawater after 96 h under experimental conditions. In Phang Nga, Thailand, the prevalence of total coliforms (TC), fecal coliforms (FC), *E. coli* were 99.3%, 94.4% and 93.1% in oyster meat and 94.8%, 79.2% and 78.1% in the estuarine water, respectively (Jeamsripong *et al.*, 2018). Whereas, the prevalence of *Shigella* in the pooled oysters were 7.6%, but *E. coli* O157:H7 were not detected (Jeamsripong *et al.*, 2018).

Based on the phylogenetic tree analysis, there is a higher possibility for the *E. coli* to be originated from calf faeces as there were multiple goat farms and residential areas in southern Malaysia near the sampling site. Hence, further study can be carried out by isolating *E. coli* from goat faeces and water samples at the multiple goat farms and investigating the relationship between them. The most possible reason for CP017249.1 *Escherichia coli* strain NADC 5570/86-24/6565 having the largest genetic changes compared to the ones isolated in this study is most probably due to the fact that they were characterized in 1988, as the line represent the amount of change of the evolutionary ancestry as time passed (Baum, 2008).

In this study, high ammonia and low dissolved oxygen were found in water. High level of ammonia has been reported to be accompanied with low dissolved oxygen due to the activity of the nitrifying and denitrifying bacteria (Kutty, 1987). Apart from that, low dissolved oxygen in water also may be due to limitation of water flow to the intertidal zone due to narrow and shallow geographic structure of Johore Strait with additional presence of Johore Causeway at the sampling location (Yap *et al.*, 2010). Besides that, the abnormal level of ammonia in the water can be the indicator of high load contamination of fecal matter, sewage and industrial waste.

Although the presence of *E. coli* O157:H7 in this study was low (15.35%), the impact of this finding should not be overlooked. Based on previous studies, the infectious dose for disease caused by *E. coli* O157:H7 was found to be very low. For instance, there was an outbreak associated with dry-cured salami with an estimation of fewer than five organisms of *E. coli* O157:H7 were consumed (Armstrong *et al.*, 1996) and that suggested possible infectious dose as low as ten organisms. Apart from that, the infectious dose of *E. coli* O157:H7 was also estimated by Paton and Paton (1998), which were 1 to 100 CFU in normal cases while for a vulnerable child, it might be in the range of 1 to 10 organisms. Since the infectious dose of *E. coli* O157:H7 is low, the oyster reared in southern Malaysia could signify a route of

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transmission with low level contamination in the marine environment.

More studies can be employed on the effect of the expression of the *HlyA* gene of the *E. coli* isolated in this study. For instance, the level of expression of the *HlyA* gene of the *E. coli* can be investigated through culture on blood agar at different stress level including temperature, humidity and introduction of antibiotics of different level. Besides, further study can be carried out to determine the identity of the other *E. coli* isolated from this study using other target genes such as *stx1*, *stx2*, *eae* and others to determine the presence or absence of other diarrhagenic *E. coli*. Besides that, strategies to control the high fecal coliforms and *E. coli* counts in the water and oyster samples can be developed in the future to improve the quality of water and oyster in the aspects of seafood safety.

CONCLUSION

The is the first detection of Shiga toxin producing *Escherichia coli* O157:H7 (*HlyA* gene) in cage cultured oysters (*C. iredalei*) and water from southern Malaysia. Out of the 104 isolates of *E. coli* isolated from the collected water and oyster samples, 16 isolates (15.38%) identified as *E. coli* biotype I showed the presence of *HlyA* gene. *HlyA* gene of *E. coli* isolated from collected samples in this study showed closer genetic relationship with CP016625.1 *Escherichia coli* O157:H7 strain FRIK944. The enumeration results showed that the MPN of fecal coliforms and *E. coli* found in the collected oyster samples do not meet the standard to be directed for human consumption.

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