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Detection of cholera toxin (*ctxA* and *ctxAB*) genes in *Vibrio cholerae* isolated from clinical and environmental samples in Limbang Sarawak by multiplex polymerase chain reaction (PCR)

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ABSTRACT

Aims: Cholera epidemics have been occurred in Malaysia since 1991 till 2003 which can be proved from the records by the Infectious Diseases Division of the Ministry of Health. Moreover, there were also course of cholera epidemics from the year 1994 to 2003 which had been happened in Sarawak. Cholera outbreaks in Malaysia mostly caused by the El Tor O1 *Vibrio cholerae* serogroup. The aims of this study were to detect the presence of *V. cholerae* in clinical and environmental samples (n=28) from Limbang, Sarawak by collaboration with Sarawak Government Hospital and to detect the toxin genes from the isolates.
Methodology and results: All the isolates were sub-cultured in alkaline peptone water (APW). The boiled-cell method was used for DNA extraction. The total DNA extracted was amplified by polymerase chain reaction (PCR). Two types of PCR were used in this study which are 16S rRNA PCR and multiplex PCR. The results obtained from the study found out that 16 out of 28 (57.14%) samples were confirmed to be *V. cholerae* species. Four primers specific for *V. cholerae* were used in multiplex PCR (O1 type, O139 type, *ctxA* and *ctxAB*) to confirm the species type and the toxin genes. All samples shown positive for *V. cholerae* O1 serotype and 100% positive to all genes for the identification of *ctxA* and *ctxAB* genes.
Conclusion, significance and impact of study: From this study, it showed that multiplex PCR can be used for research purposes in molecular genetics field involving cholera outbreak.

Keywords: Vibrio cholerae, O1 serogroup, *ctxAB* genes, polymerase chain reaction

INTRODUCTION

Vibrio cholerae is a Gram-negative bacterium with non-spore rods that caused cholera disease. Vibrio species which found in marine environment most likely to prefer conditions such as salty, warm and alkaline. There are more than 200 serogroups of *V. cholerae* that successfully identified, but two biotypes of *V. cholerae* serogroup O1 are mostly pathogenic which are the "classical" and the El Tor (Mala *et al.*, 2014). Each O1 biotype has three serotypes which are Ogawa, Inaba and Hikojima. *V. cholerae* O1 strains are capable of interchanging serotypes between Ogawa and Inaba. The most prevalent causative agents of cholera epidemics are serogroups O1 and O139 (Hajia *et al.*, 2016). *V. cholerae* O139 Bengal is a new serogroup which has appeared in Bangladesh in year 1992. This type of serogroup has been isolated in Pakistan and India, while it is originally limited to areas of Southeast Asia. Some people believed that the O139 serogroup could derive the eighth pandemic. However, only small proportion of the total cases of cholera has been caused by this type of serogroup. Furthermore, other types of *V. cholerae* serogroup possibly causes human illnesses, yet they do not develop into an epidemic form (Zuckerman *et al.*, 2007).

According to Teh *et al.* (2012), cholera outbreaks in Malaysia mostly caused by the El Tor O1 *V. cholerae* serogroup. The O139 serogroup occasionally causes most cases compare to the non-O1/non-O139 because the non-O1/non-O139 *V. cholerae* serogroup has not been incriminated in major outbreak. The vehicles of transmission of cholera outbreak are mainly from contaminated drinking water, unwashed contaminated food and undercooked seafood that presence from *V. cholerae* endemic estuaries (Teh *et al.*, 2012; Griffith *et*

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al., 2006). According to Reimer *et al.* (2011), in Asia and Africa, the endemic and epidemic cases that recently occurred are increasingly associated with the genetically atypical El Tor variants which share characteristics of classical and El Tor strains. Cholera epidemics have been occurred in Malaysia since 1991 till 2003 which can be proved from the records by the Infectious Diseases Division of the Ministry of Health (Patrick *et al.*, 2012). Moreover, there were also course of cholera epidemics from the year 1994 to 2003 had been described recently in Sarawak (Patrick *et al.*, 2012). Many factors could contribute to the spread of this cholera disease. Lack of proper treated water supply and poor sanitation could lead and facilitated the spread of the diseases in Sarawak, especially in rural area which confronted by the toxigenic *V. cholerae* (Patrick *et al.*, 2012).

Furthermore, Rivera *et al.* (2001) in their research has been focusing on the presence of virulence-associated factors in *V. cholerae* populations. The virulence-associated factors studied include cholera toxin (*ctxA*). The polymerase chain reaction (PCR) is experimental method that can be used to detect the *V. cholerae* regulator and toxin genes (Teh *et al.*, 2012). Recent studies have shown that some vibrio genes are activated by the human body setting, therefore the species in the human body take a more virulent form. The cholera toxin is Vibrio's most significant virulence factor (Mala *et al.*, 2014). Overall, by applying molecular methods, there has been a rapidly growing trend towards microbial characterization as it prevents some of the limiting factors of culture-based bacterial detection. PCR is a proper method for detecting small amounts of microbial DNA, especially the direct PCR amplification and sequencing of bacterial genes that encode the small subunit rRNA. The 16S rRNA genes are extremely conserved and extremely variable (Schabereiter-Gurtner *et al.*, 2002). The rRNA (*rrn*) operon's genetic information offers useful taxonomic data (Chun *et al.*, 1999). Other molecular approach that can be used is a multiplex PCR. Multiplex PCR detecting multiple pathogens simultaneously in a single tube reaction has the potential to save time and effort, reducing the laboratory costs associated with testing (Chen *et al.*, 2012). Hence, PCR is an extremely particular, fast detection and delicate technique for detecting *V. cholerae* toxigenic strains (Blackstone *et al.*, 2007).

In the effort to solve the illnesses regarding human foodborne, many different methods have been evolved in these recent years. Some of the methods include microbiological, epidemiological and expert solicitation approaches and also intervention studies. One of the methods is the approach of epidemiological which involves the analysis of data from the outbreak investigations (Pires *et al.*, 2012). Therefore, the aim of this study is to identify bacterial species from clinical and environmental samples through molecular approach by 16S rRNA PCR and to detect the toxin genes of the bacteria (*V. cholerae*) using multiplex PCR.

MATERIALS AND METHODS

Bacterial strains

In collaboration with Sarawak Government Hospital (SGH), a total of twenty-eight (n = 28) clinical and environmental samples from Limbang, Sarawak were collected from SGH and analyzed in the laboratory of Faculty of Resource Science and Technology (FRST), UNIMAS. According to SGH, all samples were identified through passive case detection (PCD) and active case detection (ACD). The PCD approach gave more results compared to the ACD. ACD method was done by visiting houses in the villages that were infected based on secondary data. More active detection attempts are needed to control the cases by approaching populations with risk factors or those near infected area or people (Siahaan *et al.*, 2018), while PCD was done on samples that come directly to the health services. Detection by passive case method is normally done when patients come for examination and treatment.

From this study, the clinical and environmental samples were tested for bacterial analysis which include 15 from rectal swabs, three from stools, three from water samples, and seven from Moore swabs as stated in Table 1. All bacterial strains were isolated from these 28 samples. Then, the strains were subjected to enrichment and streaked on selective agar of *V. cholerae* species. Thiosulfate-citrate-bile-sucrose (TCBS) agar and CHROMagar Vibrio were used to select colony for culturing. After streaked and incubated overnight at 37 °C, the specific colony (TCBS = yellow; CHROMagar Vibrio = turquoise blue) were selected for sub-culturing in alkaline peptone water (APW) broth. The broth then incubated at 37 °C for 24 h and stored at –20 °C until future analysis (Madhusudana and Surendran, 2013).

DNA extraction for PCR amplification

The boiling-cell method was used to extract DNA in this study, according to the method by Kathleen *et al.* (2014) with modifications. Briefly, 300 µL of the culture was centrifuged at 10,000 rpm for 5 min. The supernatant was then discarded and the pellet was re-suspended in 200 µL of sterile double-distilled water and boiled at 95 °C for 10 min. After boiling the DNA, the boiled suspensions were immediately cooled in ice at 4 °C for 5 min. Next, the DNA were centrifuged at 10,000 rpm for 10 min and the supernatant was used for PCR analysis or stored at –20 °C for future use.

Amplification of 16S rRNA PCR

The PCR amplification of 16S rRNA PCR was carried out using two universal primers, 27F and 1492R (Hasegawa *et al.*, 2014) and primer sequences are presented in Table 2. The positive control used was ATCC 14035. According to the method by Kathleen *et al.* (2014), the cycling conditions used to amplify DNA were as followed: 95 °C for 10 min (initial denaturation); 26 cycles of 94 °C

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Table 1: Bacterial samples collected from Limbang, Sarawak in collaboration with Sarawak Government Hospital (SGH).

Sample	Sources	Case	Location	Coordinates (approximate)
VC001	Water sample 1	NA	Kampung Limpaku Pinang	4.879674, 115.019880
VC002	Water sample 2	NA	Kampung Limpaku Pinang	4.879674, 115.019880
VC003	Water sample 4	NA	Kampung Limpaku Pinang	4.879674, 115.019880
VC004	Moore swab 1	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC005	Moore swab 2	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC006	Moore swab 3	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC007	Moore swab 4	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC008	Moore swab 5	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC009	Moore swab 6	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC010	Moore swab 7	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC011	Rectal swab	PCD	Kampung Pahlawan	4.687784, 115.025586
VC012	Rectal swab	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC013	Rectal swab	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC014	Rectal swab	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC015	Rectal swab	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC016	Rectal swab	PCD	RH John Kelati	4.569667, 114.835611
VC017	Rectal swab	PCD	Kampung Bukit Luba	4.664801, 114.943439
VC018	Rectal swab	PCD	Kampung Lubok Tuan	4.603333, 114.888611
VC019	Rectal swab	PCD	Kampung Bukit Luba	4.664801, 114.943439
VC020	Rectal swab	PCD	RPR Rangau	4.756868, 115.007478
VC021	Rectal swab	PCD	Kampung Limpaong	4.767524, 115.001710
VC022	Rectal swab	PCD	Kampung Batu Danau	4.652792, 114.826880
VC023	Rectal swab	PCD	Kampung Pemukat	4.627540, 114.951687
VC024	Rectal swab	PCD	RH Imau	4.653031, 115.004280
VC025	Rectal swab	ACD	Kampung Merasam Parit	4.731611, 114.832736
VC026	Stool	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC027	Stool	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC028	Stool	ACD	Kampung Limpaku Pinang	4.880785, 115.019258

PCD: Passive Case Detection, ACD: Active Case Detection, NA: Not applicable.

Table 2: Primers used for 16S rRNA and multiplex PCR in the molecular study of *V. cholerae* strains isolated from Limbang, Sarawak.

Molecular techniques	Primers	Primer sequences (5'-3')	Amplicons (bp)	References
16S rRNA PCR	27F 1492R	F: AGAGTTTGATCMTGGCTCAG R: TACGGYTACCTTGTTACGACTT	1500	Hasegawa *et al.* (2014)
Multiplex PCR	O139F2 O139R2	F: AGCCTCTTTATTACGGGTGG R: GTCAAACCCGATCGTAAAGG	449	Hoshino *et al.* (1998)
	O1F2-1 O1R2-2	F: GTTTCACTGAACAGATGGG R: GGTCATCTGTAAGTACAAC	192	
	VCT1 VCT2	F: ACAGAGTGAGTACTTTGACC R: ATACCATCCATATATTTGGGAG	308	
	CTXAB-F CTXAB-R	F: GCCGGGTTGTGGGAATGCTCCAAG R: GCCATACTAATTGCGGCAATCGCATG	536	Goel *et al.* (2007)

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for 30 sec (denaturation), 55 °C for 1 min (annealing), 72 °C for 1 min 30 sec (extension); and 72 °C for 10 min (final extension) (Eppendorf Mastercycler Gradient). Each PCR reaction was optimized and performed in 30 µL reaction mixture consists of 15 µL of exTEN 2× PCR Mastermix (Base Asia), 1.2 µL of 10 µM of each primer, 9 µL of sterile double-distilled water, and 3.6 µL of each DNA template. The PCR product then electrophoresed by gel electrophoresis on 1.0% (w/v) agarose gel pre-stained with 1 µL of 10 mg/mL ethidium bromide (EtBr) in 1× TBE buffer at 90 V for 1 h. The stained gel was visualized under UV transilluminator. The positive DNA product then purified using Thermo Scientific GeneJET PCR Purification Kit (United States) and the sequencing part was then performed by First Base DNA Sequencing Services, Base Asia (Singapore). The DNA sequencing result was then compared using the Basic Local Alignment Search Tool (BLAST) server by the National Center for Biotechnology Information (NCBI).

Amplification of multiplex PCR

Four primers were used to perform multiplex PCR amplification. The primers used and the primer sequences were shown in Table 2. ATCC 14035 (*V. cholerae* O1 type) and ATCC 51394 (*V. cholerae* O139 type) were used as positive controls. According to the protocol by Hoshino *et al.* (1998), amplification was proceeded using a PCR Eppendorf Mastercycler Gradient with cycling conditions as followed: 94 °C for 5 min (initial denaturation); 35 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), 72 °C for 1 min (extension); and 72 °C for 7 min (final extension). A total of 30 µL reaction mixture which consists of 15 µL of exTEN 2× PCR Mastermix (Base Asia), 1.2 µL of 10 µM of each primer, 6.6 µL of sterile double-distilled water, and 3.6 µL DNA template was performed in PCR reaction for multiplex PCR. Each reaction mixture then separated by electrophoresis on a 1.5% agarose gel (90 V; 300 amp; 1 h), stained with EtBr and the DNA products were visualized under UV light.

RESULTS AND DISCUSSION

In this study, a total of 28 clinical and environmental samples collected in Limbang, Sarawak were isolated as represented by SGH in Table 1. However, there were only 16 out of 28 samples (57.14%) were identified as *V. cholerae* (Table 3). The positive strains of *V. cholerae* include nine from rectal swabs, two from stools, two from Moore swabs and three from water samples.

The PCR assay with universal primers for 16S rRNA used in this project, which are 27F and 1492R primers (Table 2) to confirm the identity of the isolates. As refer to Figure 1, 16 isolate samples showed positive result for bacteria identification using 16S rRNA PCR which there was a band seen at 1500 bp. As for the 12 samples that showed negative for the 16S rRNA PCR, it was found that they belonged to other bacterial species. For the 16 samples that showed positive results in 16S rRNA, their

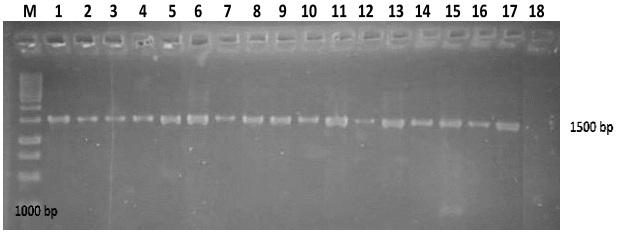

Figure 1: 16S rRNA PCR profile of 16 strains of *V. cholerae* isolates obtained using universal primers 27F and 1492R (1500 bp). Lane M = 1 kB DNA ladder; Lane 1: Positive control (ATCC 14035); Lane 2–17 = VC001, VC002, VC003, VC005, VC006, VC012, VC015, VC016, VC017, VC018, VC019, VC020, VC021, VC023, VC026, VC028; Lane 18 = Negative control (sterile double-distilled water).

Table 3: Positive *V. cholerae* isolates from Limbang, Sarawak using 16S rRNA PCR and multiplex PCR.

Samples	16S rRNA PCR	Multiplex PCR			
		O139-*rfb*	O1-*rfb*	*ctxA*	*ctxAB*
VC001	+	-	+	+	+
VC002	+	-	+	+	+
VC003	+	-	+	+	+
VC004	-	-	-	-	-
VC005	+	-	+	+	+
VC006	+	-	+	+	+
VC007	-	-	-	-	-
VC008	-	-	-	-	-
VC009	-	-	-	-	-
VC010	-	-	-	-	-
VC011	-	-	-	-	-
VC012	+	-	+	+	+
VC013	-	-	-	-	-
VC014	-	-	-	-	-
VC015	+	-	+	+	+
VC016	+	-	+	+	+
VC017	+	-	+	+	+
VC018	+	-	+	+	+
VC019	+	-	+	+	+
VC020	+	-	+	+	+
VC021	+	-	+	+	+
VC022	-	-	-	-	-
VC023	+	-	+	+	+
VC024	-	-	-	-	-
VC025	-	-	-	-	-
VC026	+	-	+	+	+
VC027	-	-	-	-	-
VC028	+	-	+	+	+

(+): positive result for the detection of *V. cholerae* through PCR;
(-): negative result for the detection of *V. cholerae* through PCR.

DNA were extracted and sent for sequencing after purified.

Based on the results from BLAST, it shows the 16 positive isolates were 83–99% similarity to *V. cholerae* strains, which include *V. cholerae* strain for O1 type.

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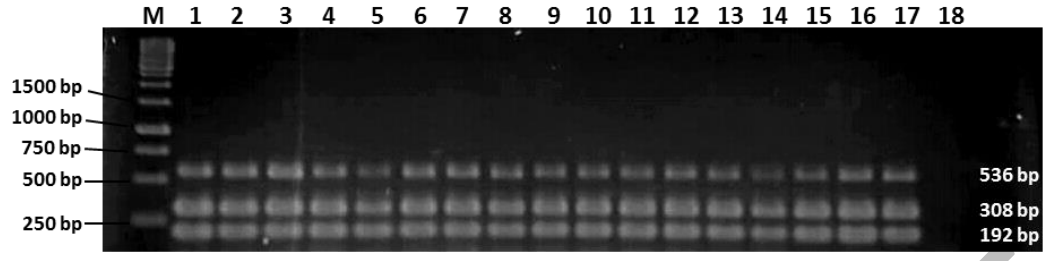

Figure 2: Multiplex PCR profile of 16 strains of *V. cholerae* isolates obtained using *V. cholerae* specific primers, O1-*rfb* (192 bp), *ctxA* (308 bp) and *ctxAB* (536 bp). Lane M = 1 kB DNA ladder; Lane 1 = Positive control (ATCC 14035); Lane 2–17 = VC001, VC002, VC003, VC005, VC006, VC012, VC015, VC016, VC017, VC018, VC019, VC020, VC021, VC023, VC026, VC028; Lane 18 = Negative control (Sterile double-distilled water).

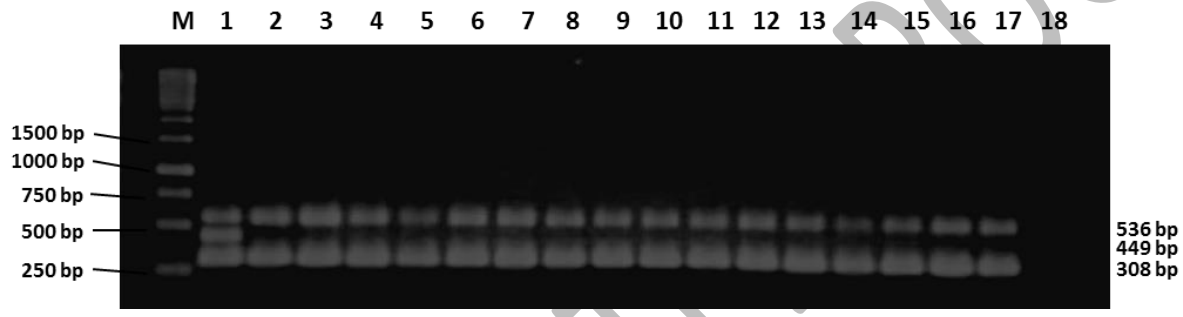

Figure 3: Multiplex PCR profile of 16 strains of *V. cholerae* isolates obtained using *V. cholerae* specific primers, O139-*rfb* (449 bp), *ctxA* (308 bp) and *ctxAB* (536 bp). Lane M = 1 kB DNA ladder; Lane 1 = Positive control (ATCC 51394); Lane 2 -17 = VC001, VC002, VC003, VC005, VC006, VC012, VC015, VC016, VC017, VC018, VC019, VC020, VC021, VC023, VC026, VC028; Lane 18 = Negative control (Sterile double-distilled water).

Other information from the BLAST included the taxonomy and alignments of the specific strains. From these results, it can be concluded that, *V. cholerae* species was a common bacterium found in clinical and environmental samples from patients or sewage/water samples, especially in rural area where safe water and food are rarely found.

According to Kathleen *et al.* (2014), 16S rRNA was chosen as the gene to sequence which the gene is adequate with 16S rRNA interspecific polymorphisms that are essential to contribute to a measurement that is discriminatively and statistically authentic. Universal primers are usually chosen as supplementary to the preserved areas and the variable region sequence is used for comparison taxonomy (Kathleen *et al.*, 2014). Moreover, the 16S rRNA gene sequence has been commonly used to determine a big number of bacterial strains and when there are many samples deposited, to compare the unknown strain sequence. The 16S rRNA gene also enables to measure the interactions between all bacteria as it is a universal gene in all bacteria. In general, the comparison of 16S rRNA gene sequences enables certain unknown bacteria to be discriminated to genus level and the classifying of strains at various and multiple levels (Kathleen *et al.*, 2014). Previous study showed that targeting 16S rRNA will not only able to detect culturable vibrio, but also the detection of vibrio cells in viable but non-cultural conditions (Da-Silva *et al.*, 2017). Most of the findings suggested that 16S rRNA-based phylogenetic relationships support species distinction between eubacteria, archaeobacteria, and eucaryotes. Furthermore, since they are essential components in all living organisms, 16S rRNA genes are present in high copy numbers (Kim and Jeong, 2001). Generally, the PCR amplification of 16S rRNA gene sequences is a helpful and promising technique to investigate clinical samples as it is a highly delicate technique of identification that overcomes the cultivation issues of fastidious or unknown bacteria (Schabereiter-Gurtner *et al.*, 2002).

The multiplex PCR assay was used to detect the *V. cholerae* species. It was performed by using four different specific primers for *V. cholerae* species. Two of the primers were specifically detect the biotype of *V. cholerae*, O139-*rfb* and O1-*rfb* (Hoshino *et al.*, 1998), while the other two were detecting the cholera toxin, *ctxA* (Hoshino *et al.*, 1998) and *ctxAB* (Goel *et al.*, 2007). From Figure 2, a total of 16 positive isolates showed positive bands for O1-*rfb*, *ctxA* and *ctxAB* genes, and none samples showed band for O139-*rfb* gene (Figure 3). The O1 specific primers, *ctxA* and *ctxAB* genes yielded a 192, 308 and 536 bp fragment, respectively (Hoshino *et al.*,

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1998; Goel *et al.*, 2007). The negative control used in this multiplex PCR was sterile double-distilled water and produced negative results. Therefore, it can be concluded that all 16 isolates possessed toxin genes and suspected to be the *V. cholerae* O1 type. *V. cholerae* O1 consist of two biotypes and each has two major serotypes depending on antigen factors. The two serotypes are Inaba, which expressing the A and C antigens, and Ogawa which expressing more A and B antigens and less C antigens (Pinto *et al.*, 2005). Further molecular study can be made to confirm the serotype of *V. cholerae* by detecting the *wbeT* gene which has a product of transferase activity (Ogawa phenotype requires its integrity) (Bakhshi *et al.*, 2015).

Moreover, *V. cholerae* O1 type has the genetic potential to produce cholera toxin. Epidemic cholera strains cause human disease by acquiring a CTX phage that allows *V. cholerae* strains to attach and generate cholera toxin in the intestinal lumen (Bakhshi *et al.*, 2015). The *ctxA* gene has been targeted as its activated product is liable for cholera toxin intracellular toxicity (Blackstone *et al.*, 2007) while, the *ctxAB* gene is known to play a key role in keeping virulence in *V. cholerae* and thought to be associated solely with O1 and O139 serogroup clinical strains (Pinto *et al.*, 2005). Clinical samples via PCR assessment showed that cholera toxin genes were present in 100% of the isolates, thus further emphasizing their key function in the virulence and pathogenicity of the *V. cholerae*. According to Goel *et al.* (2007), the cholera toxin gene (*ctx*) is crucial for causing cholera disease. The *ctxAB* genes encode cholera toxin, which is accountable for life-threatening cholera triggered by epidemic cholera diarrheal disease (Blackstone *et al.*, 2007). Some *V. cholerae* strains that lack *ctx* may also cause human disease, but these diseases are far less serious and rarely life-threatening. To differentiate toxigenic cholera from most non-toxigenic *V. cholerae* strains, the presence of *ctxAB* gene can be used (Blackstone *et al.*, 2007). Chakraborty *et al.* (2000) also added that, more than 95% of non-O1 and non-O139 strains absence in producing cholera toxin.

Furthermore, Piarroux *et al.* (2011) added that, the environmental could transmit bacterial strain to humans. This can be shown from the research of cholera epidemiology in South Asia where they hypothesized the condition of weather, for instance the La Nina phenomenon which might have induced the growth of *V. cholerae* in their environment. In this study, all clinical and environmental samples possessed cholera toxin. The samples might correlate with each other. Toxigenic *V. cholerae* O1 strains are present in the environment and may result from fecal contamination of the environment by people infected with *V. cholerae* (Alam *et al.*, 2014). Jutla *et al.* (2013) also added that, in epidemic cholera regions, high air temperatures create favorable environmental conditions for bacterial growth and result in a cholera epidemic following above-normal rainfall in combination with appropriate transmission mechanisms such as poor availability of safe water and the destruction of sanitation infrastructures that help to mix overflowing sewers with flood waters. Rainfall provides the most suitable mechanism for cholera spread through water cross-contamination, with the population having to rely on surface water for everyday use, especially in rural areas. The rains will lead to water runoff and sewage overflow. Such factors lead to increase risks of *V. cholerae* contamination (Mishra *et al.*, 2011). Moreover, there will be an increase in salinity when the water level decreases. High salinity provides ideal environmental conditions for the growth of cholera bacteria and increases the likelihood of a cholera epidemic, together with high precipitation and poor sanitation (Jutla *et al.*, 2013). Thus, it results in clinical cholera or sporadic diseases when a host is in contact with *V. cholerae* in the environment (Mishra *et al.*, 2011). As mentioned in the previous study regarding the *V. cholerae* bacteria in Malaysia by Mahapatra *et al.* (2014), outbreak of *V. cholerae* in Bintulu, Sarawak (2012) was caused by contaminated water and food. The research also mentioned that the cause of the outbreak was handling contaminated seafood and washing clothes with contaminated river water. Therefore, further molecular genetic relationship can be done in this study to identify the correlation between *V. cholerae*.

In molecular biology, PCR method is known to have high sensitivity level. There are several types of PCR tool with their specific functions including multiplex PCR. The study done by Chen *et al.* (2012) showed the sensitivity analysis between multiplex PCR and simplex PCR. From the study, it was found out that the simplex PCR has relatively lower detection limit compared to the multiplex PCR. Other than that, their study also compared the traditional detection method with that of multiplex PCR. These traditional or conventional methods are based on selective growing techniques which combined with the standard biochemical identifications. Their results showed that these traditional methods were time-consuming and challenging, easily getting errors during sampling and enumeration, and caused these pathogenic bacteria to be low in numbers. In addition, fast screening of large numbers of food samples to detect the presence of one or more pathogens cannot be achieved using low performance of these traditional methods. While, multiplex PCR has the capability to save time and effort as well as reducing laboratory costs which related to the test involved by detecting several pathogens in a single-tube reaction at once. Even though there was higher consistency achieved between multiplex PCR results and traditional culture techniques, multiplex PCR was a relatively reliable and helpful tool for fast testing of these pathogens. In conclusion, the multiplex PCR method is capable to be used in regular diagnostic laboratories and could also be used as a quick screening instrument.

Rivera *et al.* (2001) also added to their research that by using a multiplex PCR assay, *V. cholerae* O1 and O139 can be detected rapidly. Therefore, in this study, multiplex PCR was suitable to use to detect four specific primers of *V. cholerae* at one time.

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CONCLUSION

Vibrio cholerae is a common bacterium that usually found in rural area with poor sanitation and low hygiene practices. From this research, the bacterial isolates were successfully identified and the toxin genes (*ctxA* and *ctxAB*) of *V. cholerae* have also been detected. In this study, 16 out of 28 positive strains were confirmed to be *V. cholerae* serotype O1 species and carry toxigenic genes which is infectious and can cause diseases to human being. Besides, 16S rRNA PCR was a suitable approach to identify the species of the *V. cholerae* strains using universal primer and further confirmed by multiplex PCR for detection the serotype of the *V. cholerae* species. Moreover, multiplex PCR was suitable to use to detect multiple specific primers of *V. cholerae* at once. It was a sensitive, cost effective and time saving molecular method used in this study. Other virulence-associated factors involving *V. cholerae* could be analyzed as well for future study using multiplex PCR which could help in human health risk assessment such as haemolysin (*hlyA*), non-01 heat-stable enterotoxin (*stn*/*sto*), outer membrane protein (*ompU*), toxin-coregulated-pilus (*tcpA* and *tcpI*), ToxR regulatory protein (*toxR*), and zonula occludens toxin (*zot*).

In short, molecular characterization of bacterial strains provides useful information about the ecology of the specific bacteria, include *V. cholerae*. Phenotyping or fingerprinting techniques can be used to further characterize the *V. cholerae* strains such as random amplified polymorphic DNA (RAPD)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR. These fingerprinting techniques can be specifically determine the genetic relatedness between the strains where the correlation between *V. cholerae* strains can be identified.

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