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Detection the Toxigenic Genes of *Vibrio cholerae* and *Staphylococcus aureus*

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Abstract: *Vibrio cholerae* has caused severe outbreaks of cholera worldwide with thousands of recorded deaths annually while the *Staphylococcus aureus* is one of the most significant pathogens causing nosocomial and community-acquired infections. Conventional detection methods for diagnosis of clinical samples, water and food based on culture, microscopy and biochemical testing are limited by the speed of detection, sensitivity and specificity, so it is necessary to develop innovative molecular methods for the rapid detect the presence genes, expression levels of the toxigenic and drug target genes in *S. aureus* and *V. cholerae* using PCR, sequencing and membrane array. The genes studied are SEA-SEJ (genes encoding *S. aureus* enterotoxins) *ace*, *zot*, *ctxA*, *ctxB*, *toxR* (toxigenic genes of *V. cholerae*) *Sav1017* and *AdaB* (protein synthesis and DNA synthesis genes in *S. aureus*. These techniques were carried out step by step with primers designing, PCR amplification, sequencing and detection of expression by membrane array. These assays are extremely robust, sensitive, specific and economical and can be adapted to different throughputs. Thus, a rapid, sensitive and reliable technique for detecting toxigenic genes of *S. aureus* and *V. cholerae* was suc-cessfully developed.

Key words: *Vibrio cholerae*, *Staphylococcus aureus*, enterotoxin, polymerase chain reaction, dot blot, sequencing

INTRODUCTION

Enterotoxins producing by food-borne pathogens, such as *V. cholerae* and *Staphylococcus aureus* are very diverse in their nature and keep causing major public health problems worldwide. Many high-risk pathogens that cause diseases in humans are transmitted through various food items. Due to increased morbidity and mortality leading to time lost in the work place and reduced productivity, food-borne disease across the world costs billions of dollars annually (Naravaneni and Jamil, 2005). Researchers are continuously searching for sensitive tools for detecting the enterotoxins of *S. aureus* and *V. cholerae*, therefore the diagnosis of contamination food has become an important concern for health committee. The conventional detection methods for diagnosis of clinical samples, water and food based on culture, microscopy and biochemical testing are limited by the speed of detection and sensitivity and specificity levels, so the appropriate monitoring and effective preventive measures can be undertaken to prevent an outbreak and nosocomial infection. Molecular methods, especially PCR have become more important in the diagnosis of *V. cholera* and *S. aureus* has greatly improved the speed, sensitivity and specificity of diagnostic tests which in turn facilitates early and informed decision-making related to patient management, infection control, treatment and prevention (Yang and Rothman, 2004). Staphylococcal Enterotoxins (SEs) are family of major serological types of heat stable enterotoxins (SEA through SEE and SEG through SEJ) (Becker *et al*., 2001) SEs functions both as potent gastrointestinal toxins, as well as superantigens that stimulate non-specific T-cell proliferation. Toxins production from *S. aureus* can cause toxic shock-like syndromes and have been implicated in food poisoning and several allergic and autoimmune diseases (Archer *et al*., 1998). *V. cholerae* enterotoxin genes including: accessory cholera enterotoxin (*ace*) cholera toxin (*ctxA* and *ctxB*), *toxR* (gene encoding the central regulatory protein) and also increasing intestinal permeability might cause diarrhea by leakage of water and

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electrolytes into the lumen under the force of hydrostatic pressure zonula occludens toxin (*zot*), toxin-coregulated pilus (*tcp*) repeats in toxin (*rtx*) and heat-stable enterotoxin (*sto*) virulence genes have also been targeted for amplification in both monoplex and multiplex PCR tests (Fields *et al*., 1992; Koch *et al*., 1993; Rivera *et al*., 1995; Singh *et al*., 2001). However, these tests cannot detect environmental non-O1/non-O139 serogroups of *V. cholerae* which do not possess the toxigenic or other virulence genes but are still responsible for sporadic and localized outbreaks of cholera-like diarrhoea (Kaper *et al*., 1995). A PCR test designed based on an outer-membrane protein (*ompW*) gene was able to detect non-O1/non-O139 strains of *V. cholerae* (Nandi *et al*., 2000) but showed some cross-reaction with *Vibrio mimicus*. However, another PCR test developed to detect all serogroups including non-O1/non-O139 serogroups of *V. cholerae* based on the outer membrane lipoprotein (*lolB*) gene was highly specific and could discriminate other *Vibrio* species (Lalitha *et al*., 2008). In this study, a PCR, dot blot hybridization and sequencing were developed to detect the presence of toxigenic and drug target genes in *S. aureus* and *V. cholerae*. Polymerase Chain Reaction (PCR) method has been used as compare in this study to study the presence of virulence gene of *V. cholerae*. In the study described, here a specific, rapid and easy technique, membrane assay was developed to identify the genes of *S. aureus* and *V. cholera*.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Bacterial strains, the bacterial isolates used in this study were collected from two different sources which were clinical (*S. aureus* and *V. cholera*) and environmental (*S. aureus*). Bacterial strains were cultured in Luria-Bertani medium (LB).

DNA isolation: Genomic DNA from each isolates was extracted from an overnight culture by using the pure gene bacterial genomic DNA extraction (Biosyntech Inc.) protocol for clinical isolates, the purity and quality of the DNA were determined by UV absorption with a UV spectrophotometer (Shimadzu UV-1601). An aliquot containing 100 ng of genomic DNA was used for Polymerase Chain Reaction (PCR).

Primers design: The generated forward and reverse primers were and for *Sav1017* gene were synthesized by QIAGEN Operon while the generated forward and reverse primers for *AdaB* gene and *V. cholera* were synthesized by first base laboratory.

Genes amplification: Using the thermal block cycler (T-personal) provided by Biometra, drug targets and toxigenic genes were detected by amplification using Polymerase Chain Reaction assay (PCR). A total of 25 μL reaction mixture contained 0.3 μL $MgCl_2$, 0.4 μL deoxynucleotide triphosphate or dNTP (dATP, dCTP, dGTP and dTTP) 2.5 μL. Buffer, 0.1 μL each forward and reverse primers of each isolates, 1 μL DNA template, 0.2 μL of Taq DNA polymerase and the balance is the sterile distilled water were prepared. DNA amplification gene for *S. aureus* was carried out according to the following thermal cycling profile: Initial denaturation at 96°C for 5 min, denaturation, annealing and extension reactions at 95°C for 25 sec, 59°C for 30 sec and 55°C for 60 sec for 35 cycles, respectively followed by the final extension reaction at 72°C for 30 sec. For *V. cholerae* virulence genes, initial denaturation at 94°C for 5 min, denaturation at 95°C for 1 min, annealing 59°C for 1 min and extension reactions at 75°C 1 min for 35 cycles. Then, PCR products were analyzed by electrophoresis in a 1.2% agarose gel at 75 V. After electrophoresis, the gel was stained with ethidium bromide and photographed under Alpha Innotech 2200. A 100 bp and 1 Kb DNA molecular weight ladder was used as the molecular size marker.

Sequencing: The PCR products yielding the bands at the expected sizes genes were then purified with PCR Purification Kit (Qiagen) and sent for commercial sequencing to confirm the identity of both genes at Institute of Biosciences, University Putra Malaysia.

Dot blot hybridization: After PCR-gel analysis, 6 μL of PCR product was denatured with 0.6 μL of denaturing. From this mix, 2 μL was spotted on cellulose membranes which were cross-linked on a UV irradiator. Prehybridization was done in 0.8 M NaCl, 0.2 M Tris-Cl (pH 8.0) 0.05 M EDTA-Na_2 and 1% sodium dodecyl sulfate. Specific PCR probe then labeled. Hybridization was performed for 2 h at 30°C in prehybridization buffer with 0.1-0.3 ρM of specific labeled probe. Filters were washed 3 times for 10 min each in 0.1 X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0) with 1% sodium dodecyl sulfate at 34°C and exposed to Kodak XAR-5 film. Finally, the film was developed.

RESULTS AND DISCUSSION

The generated forward and reverse primers were 5'-C TTGACCAGGTGCAGGAT-3'and5'-TATTACAGCCGC

CCAGA-3' for *Sav1017* gene while the generated forward and reverse primers for *AdaB* gene were 5'-CAA CCGCTTGGGCTGACA-3' and 5'-CGCCTGTAGGACGAT TGG-3'as shown in Table 1. Amplification of various genes through Polymerase Chain Reaction (PCR) with respective specific primers were sensitive at the DNA template concentration of 100 ng μL^{-1} for *V. cholerae* and *S. aureus* genomic DNA. This study demonstrates that the *adaB 321* and *sav 1017691* bp genes (Fig. 1 and 2). Optimization of the PCR parameters carried out involving variations in DNA template, primers, dNTP and $MgCl_2$ concentrations, as well as annealing temperature reaction. A set of optimized reaction mixture and PCR cycling condition successfully amplified *Sav1017* and *AdaB* gene's with band position at 691 and 321 bp in 5 clinical isolates of *S. aureus*. This result was obtained after several optimizations were done. In the first optimization with MgCl volume was 0.4 μL, multiple bands were been presented. This is may be due to the MgCl that can cause unspecific binding to the PCR reaction. The result was only obtained when the volume of MgCl was reduced to 0.3 μL. *V. cholerae* 01 isolates exhibited positive PCR results for *ctxA* gene at 564 bp and *toxR* gene at 779 bp as shown in Fig. 3, *ace* gene at 314 bp and *ctxB* gene at 460 bp (Fig. 4) and for *zot* gene at 1083 bp (Fig. 5). The membrane-based assay screenings of *S. aureus* and *V. cholerae* were performed by a gene probe method using the dot blot technique to detect toxin gene of PCR product that amplified by specific primer and genomic DNAs as target (Fig. 5). The strains that exhibited negative results by PCR which may explained by the deletion of the gene cluster were not used in membrane assay study, the boiled colony lysate was spot on each grid and probed probes individually. The presence of *S. aureus* and *V. cholerae* specific fragment was confirmed in the dot blot technique when a dot signal was seen on the spot area in the x-ray film. On the basis of the detection of specific gene in *V. cholerae* and *S. aureus*, first researchers screen the presence of the gene by using PCR method. The sequencing results showed both *sea* 544 bp and *seg* 400 bp genes were successfully amplified at the expected sizes (Fig. 6) then were sent for commercial sequencing to confirm identity of the genes, raveled sharp signals with minimum underlying peaks in the electrophorogram with most of the isolates showed >90% (95-99% homology) homology to both of genes sequences. *S. aureus* isolates of environmental.

Vibrio cholerae and *Staphylococcus aureus* can potentially cause a wide range of infections from relatively minor to life-threatening systemic illnesses, among healthy and ill people. However, the ability of both *S. aureus* and *V. cholerae* strains to produce disease in both clinical and food settings, depends, among other determinants, on its ability to produce its toxins. A

Table 1: PCR primers used for the detection of the genes in this study were listed

Target genes	Primers	Size of PCR product (bp)
sav1017	F 5'-CTTGACCAGGTGCAGGAT-3'	
(S. aureus)	R 5'-TATTACAGCCGCCCAGA-3'	691
adaB	F 5'-CAACCGCTTGGGCTGACA-3'	
(S. aureus)	R 5'-CGCCTGTAGGACGATTGG-3'	321
Sea	F 5'-ACGATCAATTTTTACAGC-3'	
(S. aureus)	R 5'-TGCATGTTTTCAGAGTTAATC-3'	544
Seg	F 5'-ACGTCTCCACCTGTTGAAGG-3'	
(S. aureus)	R 3'-TGAGCCAGTGTCTTGCTTTG-3'	400
Seh	F 5'-TCACATCATATGCGAAAGCAG-3'	
(S. aureus)	R 5'-TAGCACCAATCACCCTTTCC-3'	357
Ace	F 5'-TAAGGATGTGCTTATGATGGACACCC-3'	
(V. holerae)	R 5'-CGTGATGAATAAAGATACTCATAG-3'	314
ctxB	F 5'-GATACACATAATAGAATTAAGGATG-3'	
(V. holerae)	R 5'-GGTTGCTTCTCATCATCGAACCAC-3'	460
ctxA	F 5'-CGGGCAGATTCTAGACCTCCTG-3'	
(V. holerae)	R 5'-CGATGATCTTGGAGCATTCCCAC-3'	564
toxR	F 5'-CCTTCGATCCCCAAAGCAATAC-3'	
(V. holerae)	R 5'-AGGGTTAGCAACGATGCGTAAG-3'	779
Zot	F 5'-TGGCTTCGTCTGCTGCCGGCGATT-3'	
(V. cholerae)	R 5'-CACTTCTACCCACAGCGCTTGCGC-3'	1083

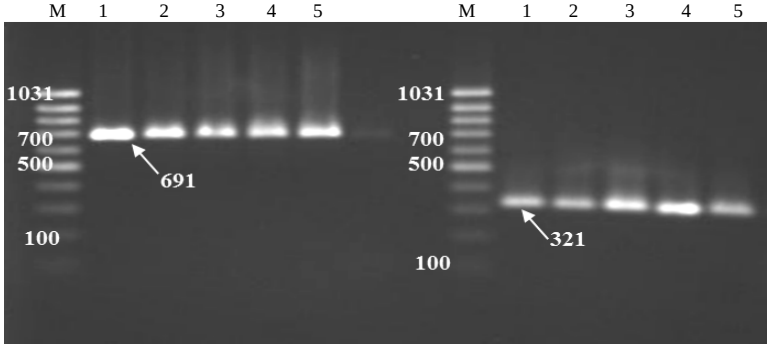

Fig. 1: Amplification of *S. aurues* enterotoxin genes by PCR. *Sav1017* gene lanes 1-5 a single band of size 691 and *AdaB* gene lanes 1-5, a single band of size 321 bp. M is a 100 bp DNA ladder (Fermentas)

number of Staphylococcal Enterotoxins (Ses) classified as A, B, C1, C2, C3, D or E can be produced by some strains (Pimbley and Patel, 1998). Most *S. aureus* strains isolated from patients with Toxic Shock Syndrome (TSS) a severe acute illness that rapidly leads to multiorgan system failure, produce a toxin known as Toxic Shock Syndrome Toxin-1 (TSST-1). While the *V. cholerae* is responsible for cholera, the clinical disease is characterized mainly by rapid onset of severe watery diarrhoea, vomiting and rapid dehydration which can lead to death if left, untreated (Chua *et al*., 2011). Early PCR tests were developed to target the cholera enterotoxin operon, since cholera outbreaks are associated with enterotoxin-producing *V. cholerae* (Fields *et al*., 1992; Koch *et al*., 1993; Shirai *et al*., 1991). A multiplexed PCR was later developed to identify biotypes of *V. cholerae* O1 based on the haemolysin toxin gene (*hylA*) gene and also the enterotoxin (Chua *et al*., 2011; Shangkuan *et al*., 1995). With the emergence of serogroup O139 of *V. cholerae*, PCR tests have also been developed to detect this serogroup (Albert *et al*., 1997; Rivera *et al*., 1995). Other *V. cholerae* 01 isolates that used in this study are caused outbreak in 1996 and 1998. All isolates exhibited positive PCR results for 5 genes, *ace*, *zot*, *ctxA*, *ctxB* and *toxR*. Although, the *V. cholerae* that studied exhibit virulence gene, Nonepidemic *V. cholerae* non-O1 serogroup strains which cause only sporadic, milder cases

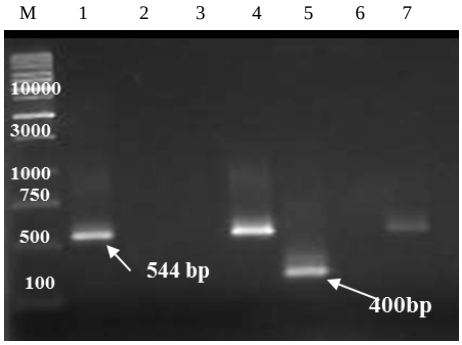

Fig. 2: Amplification of *S. aureus* isolates of environmental exhibited positive PCR results for both genes *sea* 544 bp lane 1, 4 and 7 and *seg* 400 bp lane 5. M is a 1 kb DNA ladder (Fermentas)

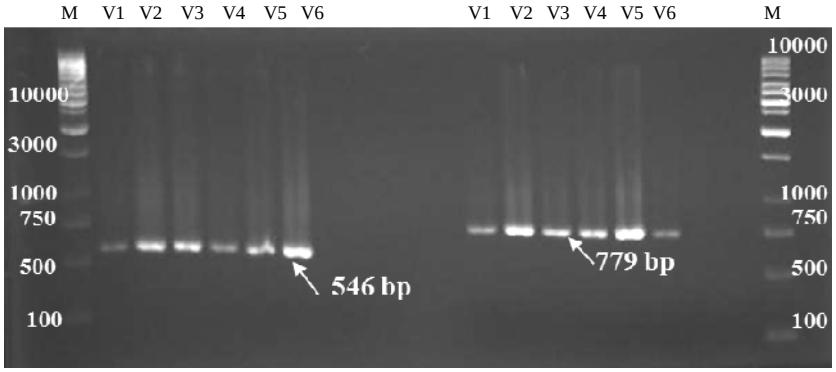

Fig. 3: Amplification of *V. cholerae* isolates exhibited positive PCR results for both *ctxA* gene at 564 bp and *toxR* gene at 779 bp. M is a 1 kb DNA ladder (Fermentas)

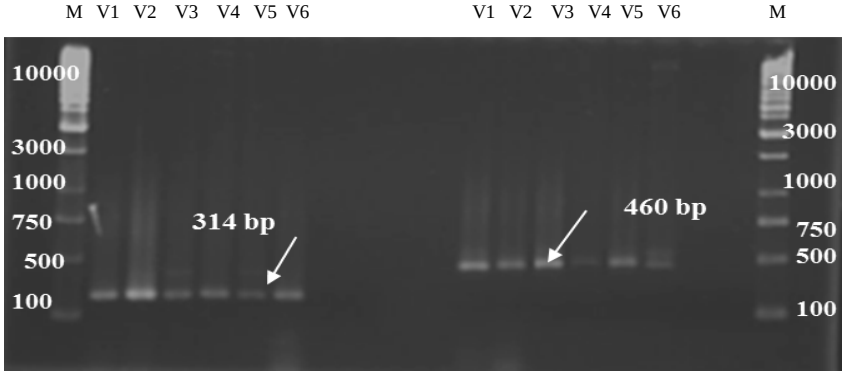

Fig. 4: Amplification of *V. cholerae* isolates exhibited positive PCR results for both *ace* gene at 314 bp and *ctxB* gene at 460 bp. M is a 1 kb DNA ladder (Fermentas)

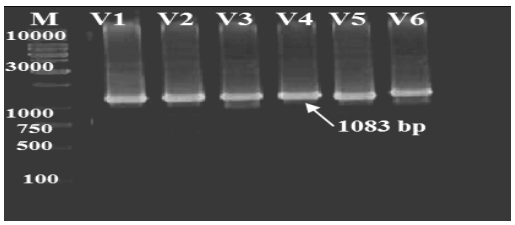

Fig. 5: Amplification of *V. cholerae* isolates exhibited positive PCR results for *zot* gene at 1083 bp. M is a 1 kb DNA ladder (Fermentas)

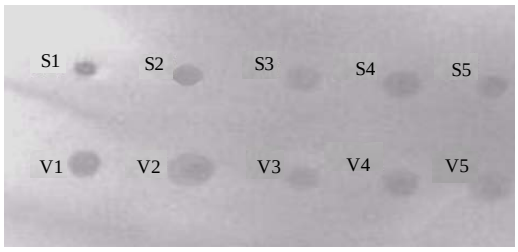

Fig. 6: Photograph of the x-ray film confirming the presence of enterotoxin genes in *S. aureus* and *V. cholerae* isolates, indicated by a dot: S1-S5 *S. aurues* isolates and dots V1-V5

of diarrhea, do secrete the RTX cytotoxins but do not secrete CT. Several groups proposed that virulence factors account for the clinical manifestations of diarrhea caused by non-O1 serogroup. On the basis of the detection of specific gene in *V. cholerae* and *S. aureus*, first researchers screen the presence of the gene by using PCR method. However, multiple sequence alignments of the nucleotide sequences showed that different pathogens have highly homologous sequences and it was difficult to design probes for identifying food-borne pathogens, especially among the Enterobacteriaceae. In this case, the detection of specific gene in *V. cholerae* and *S. aureus*, first researchers screen the presence of the gene by using PCR method. The strains that exhibited negative results by PCR which may explained by the deletion of the gene cluster were not used in membrane assay study. The membrane assay screening of *S. aureus* and *V. cholerae* were performed. The probes to detect toxin gene are PCR product that amplified by specific primer and genomic DNAs as target. The probes were manually prepared. The *S. aureus* gene specific probe hybridized only with *S. aureus* that carry the gene, as well as *V. cholerae*. His protocol can be used to screen and detection for virulence gene and drug target gene genomic DNA which obtain either by utilize commercial kit or by boiling method.

CONCLUSION

Therefore in this study, all primers and probes for identifying enterotoxin genes of *S. aureus* and *V. cholerae* were designed against genes specifically found in the respective pathogens, so as to prevent false-positive or false-negative results. Samples (genomic DNA) that fail to hybridize with the PCR probe do not give signal on film. The samples that give positive signal indicated that the specific gene exhibit in the sample isolates. This membrane assay utilize hybridization theory is non-radioactive. Thus, this technique can be used in many laboratories. Besides, it is also rapid and reliable method. Moreover, the simple equipment requirements of this method make it possible to be widely used by diverse laboratories. Taken together, the method is rapid, simple, specific and sensitive and is suitable for the detection of food-borne pathogens

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