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Molecular Characterization of Horseshoe Crab Anti-Lipopolysaccharide Factor C-Peptide for Hybridization-Based Detection Method of Gram Negative Bacteria

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Abstract: Recent advances in molecular techniques have revolutionized the detection of microorganism. The development of a molecular-based technique for detection of the three different targets of Enterbacteriacae was undertaken. Primer and probe were designed based on specific pepted of novel hemolymph protein of horseshoe crabs (Factor C anti-LPS) *Tachypleus tridentatus* that is believed to be involved in the binds to the lipopolysaccharide of *Escherichia coli*, *Salmonella* and *Vibrio cholerae*. The aim of our study the exploit part of cell wall polysaccharide in the development of improved detection method based on molecular approaches. In the gene detection assay, Lipopolysaccharide gene of *Salmonella*, *V. cholera* and *E. coil* were hybridized to anti-LPS factor gene found in the biolysate of the marine animals. The wzm and wzt genes encoding O-polysaccharide genes were amplified in these pathogens and the LPS factor C were amplified from the marine lysate. Development of a PCR-based technique for detection of the food-borne pathogens particularly *Sa Salmonella*, *V. cholera* and *E. coil* were achieved. Thus rapid, sensitive and reliable techniques for the detection of food-borne pathogens developed.

Key words: Gram negative bacteria, lipopolysaccharide, horseshoe crab, factor C anti-LPS, polymerase chain reaction, sequencing, dot blot hybridization

INTRODUCTION

Traditional methods of detection food-borne pathogens, which cause disease in humans, are time-consuming and laborious, so there is a need for the development of innovative methods for the rapid detection of food-borne pathogens. Recent advances in molecular cloning and recombinant DNA techniques have revolutionized the detection of pathogens in foods. Many high-risk pathogens that cause disease 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). Because outbreaks of food-borne illnesses may be under-reported by as much as a factor of 30, the number of cases of gastroenteritis associated with food is estimated to be between 68 million and 275 million per year. Even at the lower end of this range, food-borne disease constitutes a major public health problem. Quality and safety of foods is important in human health. Common pathogenic bacteria that are the causes of food-borne diseases include strains of *Salmonella*, *Virio cholera* and *Escherichia coli* (Naravaneni and Jamil, 2005). Conventional microbio logical methods for detection of these bacteria, however, usually include multiple subcultures and biotype-or serotype-identification steps and thus are laborious and time-consuming (Swaminathan and Feng, 1994; Blackburn, 1993). One of the inherent difficulties in the detection of food pathogens is that they are generally present in very low numbers (<100 cfu g^{-1}) in the midst of up to a million or more other bacteria. These microbes may be lost among a background of indigenous microflora and substances in the foods themselves may hinder recovery. There is also the difficulty of demonstrating that the strains recovered from a food sample are, indeed, pathogenic to human beings (Sockett, 1991). Rapid and easy detection of pathogenic organisms will facilitate precautionary measures to maintain healthy food (Naravaneni and Jamil,

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2005). The advent of gene probe techniques has allowed the development of powerful tests by which particular bacterial strains can be rapidly identified without the need for isolating pure cultures (Rasmussen *et al*., 1994; Cohen *et al*., 1993). The polymerase chain reaction (PCR) is a technique for *in vitro* amplification of specific segments of DNA by using a pair of primers (Nguyen *et al*., 1994). The recently developed techniques for amplifying specific DNA sequences *in vitro* allow the detection of very small amounts of target DNA in various specimens. Theoretically these procedures can detect even one molecule of target DNA. By amplifying a sequence that is unique to the pathogenic micro-organism of interest, the *in vitro* amplification methods can be used to indirectly detect extremely low concentrations of microbes.

MATERIALS AND METHODS

Source of bacteria: Bacteria used in this study were 5 reference strains of *Escherichia coli* (ATCC 25922, 23519, 32520, 12799 and 12810), 5 *V. cholera* strains were provided by (Department of Clinical Laboratory Sciences, Faculty of Medicine and Health Sciences, UPM) and the 5 *Salmonella* sp. were collected from Bacteriology Department, Faculty of Veterinarian UPM.

Marine biolysate source: Live marine invertebrates were collected for the marine biolysate aseptically using hypodermic needle (18 G), A sterile container filled with anticoagulant was used for the collection container.

DNA extraction of factor C marine biolysate and LPS biosynthesis gene from *E. coli*, *V. cholera* and *Salmonella* sp.: The marine biolysate was further processed for amplification of Factor C anti-LPS gene through the PCR assay. DNA was extracted using QIAGEN QIAamp Tissue kit (Qiagen Inc, Germany) with minor modification. While, DNA of five isolate of each *E. coli*, *V. cholerae* and *Sal.* sp. were extracted DNA following QIAGEN Tissue kit instruction (Qiagen Inc.).

Amplification of factor C anti-LPS gene of marine biolysate: The amplification was carried out in a total volume of 25 μL containing 3 μL of DNA template, 1X reaction buffer (Research Biolabs), 1.8 mM $MgCl_2$ (Reserarch Biolabs), 200 μM dNTPs mix (Fermentas Life Sciences), 1 μL of 5 pmole of any one of the forward 5'ACTTTGGCAGAGCGGAGAT3' and reverse 5'CCACGAGGGACACCAGAAC3 primer 1 U μL^{-1} of taq polymerase (Reserarch Biolabs). The cycling profiles followed were initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 61°C for 1 min and elongation at 72°C for 2 min with final extension at 72°C for 5 min. The amplification was performed in a DNA thermal cycler (Biometra-2 Thermoblock). About 10 μL of the PCR products were mixed with 3 μL of loading dye and separated on a 1.4% agarose (Promega) gel electrophoresis and the gel was then stained in 1.0 mg mL^{-1} ethidium bromide.

Amplification of wzm, wzt from *E. coli*, *V. cholera* and *Salmonella* sp.: PCR was used to detect and amplify the presence of LPS Biosynthesis (wzm, wzt) genes in *E. coli*, *V. cholerae* and *Salmoenlla* sp. isolates. Two oligonucleotide primers that were utilized in this study were synthesized by (BioSynTech) Table 1 amplification of wzm gene was performed in a final volume of 25 μL containing 1X BST buffer (Fermentas Life Sciences); 1.8 mM $MgCl_2$ (Biosyntech Inc.), 200 um dNTPs (Finzymes), 5 IU taq polymerase (Research Biolabs), 5 pmoles of each primer (wzm F and R) and 300 ng μL^{-1} DNA template. The DNA amplification steps performed were initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification steps consisting of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 2 min. The amplification was stopped following with a single final extension at 72°C for 7 min. Whilst the amplification of wzt gene was performed in a final volume of 25 μL containing 1X BST buffer (BioSynTech), 1.8 mM $MgCl_2$ (BioSynTech), 200 μM dNTPs mix (Fermentas Life Sciences), 10 pmoles of each primer, 500 ng of genomic DNA and 1U of Taq polymerase (BioSynTech). The PCR amplification steps were initial denaturation (95°C, 2 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and elongation (72°C, 2 m in) with an extra extension (72°C, 7 min). The PCR for both genes were performed in a DNA thermal cycler (Biometra-TRIO Thermoblock). Gene sequencing of Factor C gene from marine biolysate gene and LPS genes of *E. coli*, *V. cholera* and *Salmonella* sp. Purified PCR product was sent for commercial sequencing. Both the strands of the purified PCR product were sequenced commercially (Reserarch Biolabs) with Forward and Reverse primers.

Table 1: The sequences of primers and biotin labeled probes used in detection and amplification of wzm and wzt genes

Gene	Sequence 5' 3'	Amlpicon size (bp)	Reference
wzm	5'-CTTCACGCGATGTCGATA-3'		
	3'GTCTTGGACGCTGATTGC-5'	869	Present study
wzt	F-TTGGTGGTGTACTGGGTT		
	R-GCATGATTGTGGTGTTCC	180	Present study

The DNA sequences were then analyzed by computer assisted BLASTN package (National Center for Biotechnology Information, Bethesda).

Dot blots hybridization: Dot blot assay was also used, chromosomal DNA was labeled with Hors Reddish Peroxidase (HRP) using the Direct Nucleic Acid Labeling Kit ECL (Amersham Pharmacia Biotech, UK) and then probe used was prepared by PCR amplification of the target gene using primer pair wzm-F, wzm-R and F-anti-LPS, R-antiLPS as described by Al-Haj *et al*. (2007) (Table 1). The amplified products were purified by using the PCR purification Kit (QIAquick PCR Purification Kit) and labeled with horseradish peroxidase by the gluteraldehyde conjugation method using the Direct Nucleic Acid Labeling Kit ECL (Amersham Lifesciences). For each isolates, 100 ng of chromosomal DNA was denatured by heating at 96°C for 10 min and spotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech, UK). DNA was then fixed on to the filter by UV cross-linking by incubating in a UV cross-linking chamber (UV-Cross linker UVC-500, USA) for 3 min at the room temperature. The prehybridization and hybridization temperature were both 42°C. All filters were pre-hybridized for 1 h in 5×SSC (1.5 M sodium chloride, 0.15 M sodium citrate). Hybridization was carried out overnight with heat-denatured probe. Detection was performed using the phototope-star detection kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, UK).

RESULTS

Detection and amplification of Factor C anti-LPS gene of marine limulus ameobocyte: Preliminary detection of Factor C anti-LPS gene of marine limulus ameobocyte demonstrated a single band at a position 110 bp (Fig. 1).

Sequence analysis of Factor C anti-LPS gene (*Tachypleus tridentatus* anti-lipopolysaccharide factor) anti-LPS: The factor C anti-LPS gene codes for anti-lipopolysaccharide. The factor c anti-LPS gene sequence of *Tachypleus tridentatus* when blasted against the Gene Bank sequences and gave 100% homology to factor c anti-LPS gene (e.g., gi|12655910|gb|AF227150.1).

Detection and amplification of wzm and wzt from *E. coli, V. cholera and Salmonella* sp.: The preliminary detection of wzm by PCR showed that all the isolates of *E.coli*, *Salmonella* sp. and *V. cholerae* used in this study showed presence of wzm gene isolates with wzm gene showed a single band at position 869 bp. The primers

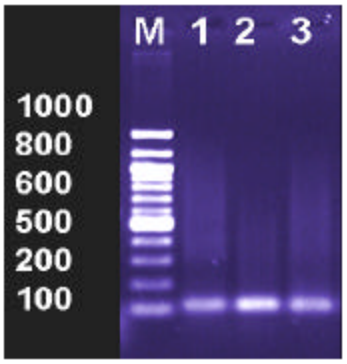

Fig. 1: The Factor C anti-LPS gene positive isolates have a single band present in the region between the ladders of 100 bp of the ladder to 200 bp (Lane M). The actual band position is at 110 bp

synthesized for wzm gene of *Burkhloderia pseudomallei* successfully amplified a single fragment in each of the Gram-negative bacteria- *Salmonella* sp., *E. coli* and *V. cholerae*. The single band had showed a strong signal when amplified using the primer sets designed for wzm gene of *B. pseudomallei* encoding for polysaccharide ABC transporter gene. The primers designed for wzt gene of *B. pseudomallei* encoding for lipopolysaccharide O-antigen transport gene wzt gene of *B. pseudomallei* also successfully amplified a single fragment in each of the Gram negative bacteria- *Salmonella* sp., *E. coli* and *V. cholerae*. The band showed a strong signal at a position in the region of 180 bp (Fig. 2). The sequence using the primer set for wzt gene of *B. pseudomallei* for each bacterial genera was found between 100-200 bp in length (Fig. 3).

Sequence analysis of wzm gene from *E. coli, Salmonella* sp. and *V. cholerae* (*Elongation factor Tu*): Wzm gene codes for Elongation factor Tu. The wzm gene sequence of *E. coli* and *S. typhimurium*, respectively when blasted against the Gene Bank gave homology to all the wzm genes of *S. typhimurium* 99% *V. cholera* 99% and *E. coli*, 100% for the protein called Elongation factor Tu while all isolates and determined a consensus sequence (AE014075.1, DQ773092.1 and X55116.1). The sequencing analysis showed in considerable variation in sequences among the isolates, which showed that although all the isolates produced bands at the same position, the nucleotide variations indicate strain differences. The DNA sequencing results of *E. coli* wzm gene analyzed using BLASTIN program function.

Sequence analysis of wzt gene from *E. coli, Salmonella* sp. and *V. cholera*: Wzt gene sequence of *V. cholera*, E. *coli* and *S. typhimurium*, respectively when blasted

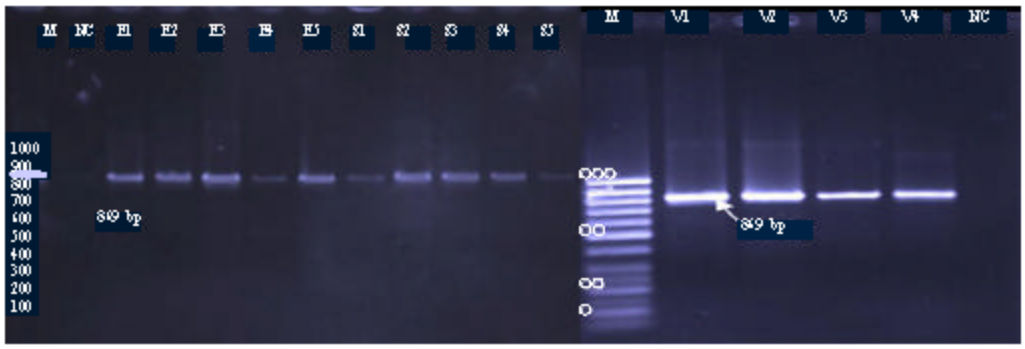

Fig. 2: Amplification of a single DNA band with a size at the region of 800-900 bp using primer set for wzm gene of *B. pseudomallei*. Lanes S1-5 = 5 *Salmonella* sp. isolates; Lane E1 =ATCC *E. coli* isolates and Lanes E2-5 = 4 *Escherichia coli* isolates; Lane V1-4 = 4 *V. cholerae* isolates Lane M indicated 100 bp DNA ladder; Lane NC = negative control

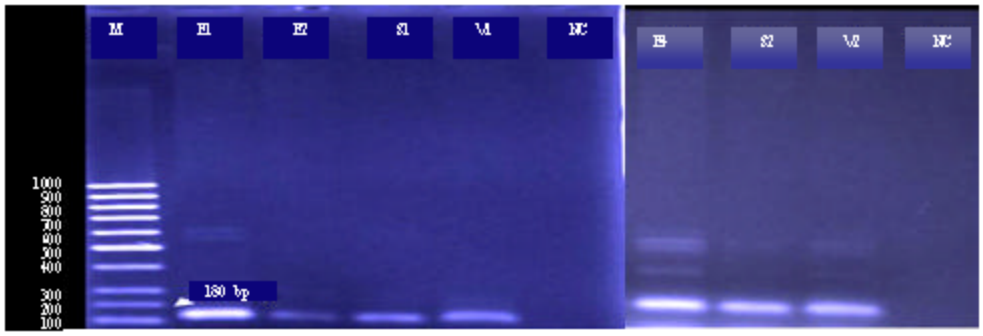

Fig. 3: Amplification of a single DNA band with a size at the region of 100-200 bpn using primer set for wzt gene of *B. pseudomallei*. Lane E1=ATCC *E. coli* isolates, Lane E2= *E. coli* isolate; Lane S1= *Salmonella* sp. isolate; Lane V1= *V. cholerae* isolate; E4= *E. coli* isolate; Lane S2= *Salmonella* sp. isolate; Lane V2= *V. cholerae* isolate; Lane M indicated 100bp DNA ladder; Lane NC = negative control

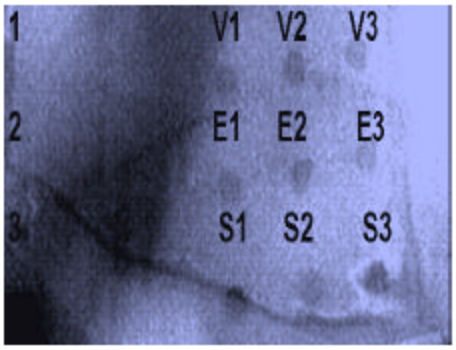

Fig. 4: Dot blot hybridization of peptide blood of marine biolysate anti-LPS and wzm gene of three different genera of *Enterobacteriacea*. 1-3 *S. aurues* isolates didn't show any signal of dot. Dots, V1-V3 *V. cholera*, dots E1-E3: *E. coli*, dot S1-S3 *Salmonella* sp.

against Gene Bank gave 97-100% homology to all the *wzt* genes of *V. cholera*, *E. coli* (100%) and *S. typhimurium* (97%) (AE004326.1, U00096.2 and AE008858.1), respectively as presented in the Gene Bank. Wzt gene produced a significant alignment corresponding to ribosomal subunit protein gene fractions after the Blast analysis.

Dot blot hybridization: A peptide probe method using the dot blot technique was used to confirm the presence of specific peptide based product of a different amplified gene from the marine biolysate Factor C anti-LPS and wzm gene amplified from *S. typhimurium*, *E. coli* and *V. cholerae* detected in PCR. The peptide hybridize probe product of wzm gene was confirmed in the dot blot technique when a positive signal for the spots were seen on the X-ray film (Fig. 4). The amplified product of wzm was labeled with Horse Radish peroxidase (HRP) and used as a probe in dot blot hybridization. Gram-positive *S. aurues* isolates was used as negative controls. The repeated technique failed to have the same product of Factor C anti_LPS when labeled with HRP and used wzt as a probe in dot blot hybridization.

DISCUSSION

In the gene detection assay, the LPS gene of *E. coli* was hybridized to the anti-LPS factor gene found in the biolysate of the marine animal. The wzm and wzt genes encoding O-polysaccharide genes were amplified from the pathogen and the LPS factor C were amplified from the marine biolysate. The hybridization results clearly demonstrated that wzm, hybridized with anti-LPS factor C peptide of marine biolysate, thereby showing hybridization ability to the different bacterial genes. Hence, it was suggested that the successful hybrdization of wzm gene to the anti-LPS genes can be further developed for a DNA probe assay providing another rapid detection of the epidemiologic *E. coli* and its related genera. The combination assay of polysaccharide detection using biolysate blood and the molecular probe application is feasible for the detection of gram negative bacteria in routine food testing in order to reduce the consumer exposure to contaminated food. The successful amplification of purification and sequencing of Factor C sensitive LPS from *Tachypleus tridentatus* (CrFC) enable the design of probe development of the dot blot assay. Similarity 100% showed that the correct target is amplified *Tachypleus tridentatus*. There was no variation in the sequencing band at the same size of 110 bp, suggesting that sequencing of *Tachypleus tridentatus* fragment could be an important tool and confirmation of this product. An intracellular clotting factor, factor C, found in the horseshoe crab biolysate is a LPS-sensitive serine-protease zymogen, which participates in the initiation of the hemolymph clotting system (Nakamura *et al*., 1986). The primers for wzm gene were designed to encode the polysaccharide ABC transporter gene in *B. pseudomallei*, which is also a Gram-Negative bacterium. Amplification of wzm and wzt genes from the Gram negative genera has potential application in the dot blot hybridization assay using the probe based on anti LPS-factor C gene of limulus ameobocyte. Further investigation need to be carried out after performing the dot blot hybridization. The value of the assay in diagnostics of Gram negative genera is not completely shown in the present study but the investigation thus far performed in the present study indicated binding of the probe designed from anti-LPS factor C gene to both wzm and wzt genes of different genera. The variation within the wzm and wzt genes among the Gram negative bacteria genera can be further exploited as Gram negative markers when coupled to anti-factor C gene probe. The gene probe is thought to differentiate Gram negative genera based on fragments of the wzm and wzt genes. The hypothesized has to be further investigated for verification. Through this molecular work, an important finding was discovered. The primer should be designed specifically (Smits *et al*., 1999) for the organisms or closely related and used particularly for the bacteria. Similar size fragments do not necessarily encode for similar functions the gene. In consequence, sequence analysis was necessary to confirm the correct amplification of the gene. Every PCR product that was successfully amplified must be subjected to nucleotide sequence analysis to confirm functional identity. The observation is meaningful to reinforce the trustworthy of research result. In this research, DNA-targeted peptide nucleic acid of marine biolysate (anti_LPS) and wzm gene of bacterial PCR product were used to achieve dot blot hybridizations Gram-positive *Staphylococcus aureus* was tested using the biotin labeled probe as a negative control. This result agreed with finding reported by Nguan *et al*. (2004) who were designed of an improved LPS binding and neutralizing peptide, charge balance of the peptide is a critical parameter in addition to its structure. It was clearly shown that the hybridization result when we used only *wzm* gene which was successfully amplified from the *E. coli*, *V. cholera* and *Salmonella* sp. against the factor C anti-LPS gene binds to gram-negative bacteria, such as *E. coli*, *V. cholera* and *Sal.* sp., but not other microbes, such as *S. aureus*. On the other hand, when we used the same procedure with the wzt gene, there was no signal of hybridization. This could be attributed to specificity of wzm gene which express (elongation factor protein) of the ABC transporter that had been found in both prokaryotic and eukaryotic systems and were responsible for the import and export of various proteins, peptides, polysaccharides and drugs (Fath and Kolter, 1993). The hybridization results demonstrate that wzm hybridized with the anti-LPS factor C peptide of marine biolysate, thereby showing to hybridization present in the different bacterial genes. Successful of wzm gene in these genera to the anti-LPS genes can be developed used further a DNA probe assay emphasis the fast detection of the epidemiologically *E. coli* and its related genera. Molecular aspect of the study revealed variation in gene function among Gram negative bacteria of similar DNA fragment size amplified using homologous primer. Peptide hybridization with a wide range of bacteria to emphasis the new technology that is needed to fully understand the interactions between factor C anti-LPS and surface bacterial gene for detection the water-food borne diseases. Epidemiological molecular typing and investigation will aid in developing more effective strategies in preventing and controlling, the sources of Human and water-foodborne pathogens by fast interception of the transmission when the source of pathogens identified in the food chain. Formulating of strategies for reducing the incidence of Human and water-foodborne illness in the population will be enhanced.

CONCLUSION

Optimization of molecular approach for detection foodborn pathogens which include gene probes and surface component based assay, are novel achievements, which will be of great value in the modern diagnostic era. These molecular systems appear promising and could be readily applied in the Human diagnosis as well as food and water analyses since molecular approach is specific, sensitive, relatively simple and rapid. Current study is especially a significant contribution to the human diagnosis and food industry research. Therefore, the routine application of the molecular systems optimized and the primers probes developed in this study will definitely contribute towards early diagnosis of foodborn pathogens in clinical microbiology laboratories.

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