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Application of Horseshoe Crab-Turbidity Basis in the Development of a Sensitive Detection Assay for Gram Negative

[1]Nagi A. Al-Haj, [1]Mariana N. Shamsudin, [2]Raha A. Rahim and [3]Zamri Ishak
[1]Department of Microbiology and Parasitology, Faculty of Medicine and Heath Sciences,
[2]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular,
University Putra Malaysia, 43400 Serdang Selangor DE, Malaysia
[3]Biotechnology Center, Mardi, P.O. Box 12301, Malaysia

Abstract: Quantitation assay of *Escherichia coli*, *Samonell* sp. and *Vibrio cholerae* cells investigated by exploiting the component consistently present on the outer surface of Gram-negative bacteria. In this study, a simple marine biolysate-based method for simultaneous detection of the gram negative pathogenic bacteria based on lipopolysaccharide component was optimized. The detection technique focused on the surface of these bacterial species, which is covered by polysaccharides and has high affinity to marine biolysate. *E. coli*, *Samonell* sp. and *V. cholerae* with similar initial cell count per mL have different but consistent absorbance readings by using the spectrophotometer and turbidity meter. This revealed that different genera of Gram Negative bacteria can be directly differentiated through standard curve that is plotted from the carbohydrate and marine biolysate assays absorbance readings. Both the assays elucidated a qualitative and quantitative detection of the pure culture pathogens.

Key words: Lipopolysaccharide, biolysate horseshoe crab, turbidity assay, carbohydrate assay, food-borne bacteria, absorbance

INTRODUCTION

Turbidity methods offer possibilities for generating data required for addressing microorganism variability in risk modeling given that the results of these methods correspond to those of viable count methods The rapid detection of microbial contaminants in food is critical for ensuring the safety of consumers. Traditional methods for detection of foodborne bacteria as gold standard in the microbiology environment eventhe disadvantages of this technique like time-consuming growth in culture media, followed by isolation, biochemical identification and in some instances serology. Based on structural information regarding various unique molecules including the lipopolysaccharide is continuously applied in the development of faster, more convenient biochemical assays. Lipopolysaccharides (LPS) are major constituents of the cell wall of Gram-negative bacteria (Raetz, 1990). LPS molecules consist of a lipid core (lipid A) attached to a hydrophilic polysaccharide moiety (O-antigen). The lipid A core is believed to be responsible for most of the biological properties characteristic of bacterial endotoxins (Holst *et al*., 1993). High levels of similarity are eminent amongst the lipid A molecules of various bacterial species. A 2-keto-3-deoxyoctonate (KDO) links the lipid A moiety with the O-antigen. This O-antigen is the only variable region in the LPS molecule and is responsible for the diverse response of different bacterial strains to strain specific antibodies (Ni Eidhin and Mouton, 1993). LPS from gram-negative bacteria induce the amoebocytes of horseshoe crabs to aggregate and degranulate. Presumably, LPS-induced coagulation cascade represents an important defense mechanism used by horseshoe crabs against invasion of gram-negative bacteria (Armstrong and Rickles, 1982). The amoebocyte lysate has been used for decades as a tool to detect trace concentrations of LPS in solution: *Limulus* amoebocyte lysate (LAL) test (Levin *et al*., 1970; Novitsky, 1994). The molecular mechanism of coagulation in horseshoe crab has been established and involves a protease cascade. The initial activator of the clotting cascade, factor C functions as a biosensor that responds to LPS. Since factor C can be activated by femtograms of LPS, it is conceivable that it has an LPS binding region that exhibits exceptional high affinity for LPS. Consequently, this LPS binding domain can be used to detect gram negative

Corresponding Author: Nagi A. Al-Haj, Department of Microbiology and Parasitology, Faculty of Medicine and Heath Sciences, Universiti Putra Malaysia 43400 Serdang Selangor DE, Malaysia

bacteria like *Salmonella*, *Vibrio cholera* and *E. coli*. Considering the problems associated with food born disease associated LPS-grame netaive bacteria we developed a turbidity and colorimetric methods for the detection of gram negative LPS. The overall objectives of this study were two fold: to identify the best approach, in terms of accuracy and convenience, for determining growth parameters using turbidity data and to apply this approach to characterize the variation in growth parameters of *Escherichia coli*, *Salmonella* and *Vibrio cholerae*.

MATERIALS AND METHODS

Source of bacteria: The bacteria used in this study, were *5* reference strains of *Escherichia coli*, *5 V. cholera* strains were provided by (Department of Clinical Laboratory Sciences) and the *5 Salmonella* sp. were collected from the 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 Fig. 1.

Preparation of suspension in 1 mL tube with different cell concentrations: To run the bioassay, pure bacteria isolates (*Escherichia coli*, *Salmonella* sp. and *Vibrio cholerae*) were grown overnight in Erlenmeyer flasks containing LB broth (Merck, Germany) and transferred to Petri dishes plate containing chromocult coliform agar (Merck, Germany). The inoculating loop was sterilized by heating red hot in the gas burner flame. The lid of the LB broth (Merck, Germany) was lifted slightly. A small amount of the broth was taken with the inoculating loop and the lid closed. The lid of the chromocult plate was lifted just high enough to enable streaking of inoculum onto the agar surface. Lopes of cells was placed on agar and then streaked back and forth. The streak was repeated 2-3 times. The loop was flamed before proceeding to the next steps. All the plates were incubated upside down, with the agar-containing portion up. For each isolates, 3-5 plates were prepared.

Turbidity assay: To carry out the turbidity assay, 20 mL of marine biolysate put in a 20 mL pre-prepared anti-coagulant reagent (3% NaCl + 0.125% NEM + 150 μL absolute alcohol) which contain in an autoclaved flask. The process was proceeded the same preparation of isolates as mentioned above using suspension containing different number of bacteria colonies per mL. After the

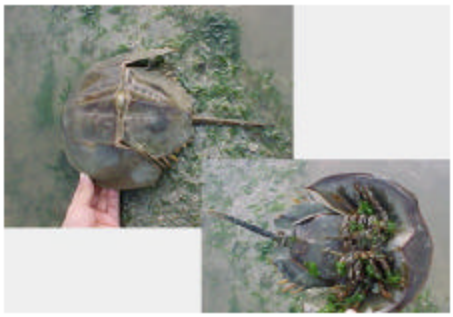

Fig. 1: Marine animal horseshoe crab

tubes are ready, 100 μL of marine biolysate blood was put into each tube and mixed well. The absorbance readings of the mixture were taken at 580 nm of turbidity meter (SECOMAM. Germany) after 30 min. To make sure the marine biolysate blood was functioned; absorbance reading of the blank bacterial tubes without reagent was taken before carrying on the carbohydrate assay and turbidity assay. Beside that, spread plate was needed to confirm the population of cell in each tube which had multiplied after 6 h incubation. Spread plate technique was carried out using a bend glass rod (Hockey stick in shape) dipped into a beaker containing alcohol. The alcohol wetted glass rod was passed through the flame. The flame was allowed to go instantaneously. The glass rod was cooled by touching it on a clean agar surface. The inoculum was spread out by moving the glass rod sideways. The agar plate was rotated so that the inoculum was well spread out. When the plate had dried out, the plate was inverted and incubated. Therefore, a serial dilution of each bacterial suspension was prepared. An aliquot of 0.1 mL from each diluted suspension was plated out, incubated, followed by colony count after 24 h. Initial bacterial population was determined by multiply colony forming unit to the dilution factor. Average count of 3 plates used to calculate the bacterial population.

Carbohydrate assay: Carbohydrate assay was used to determine the relative amounts of Lipopolysaccharide (LPS) carbohydrates present in each tube of bacterial suspension initially with different number of bacterial colony per tube for each given bacterial genera. The tubes were first cooled in an ice bath. After that 900 μL reagents A (4 mL dH_20 + 24 mL conc. H_2SO_4) was added. The tubes were shaken carefully and warmed to room temperature 32°C. The tubes were later boiled in water bath for exactly 20 min and immediately cooled to room temperature under cold water tap. About 20 μL reagents B (0.3 g cysteine Hcl in 10 mL dH_2O (fresh)) was added and mixed. Then

the tubes were let to stand for 30 min in the dark at room temperature. The step of reading absorbance at 450 nm in a spectrophotometer (UV1601, SHIMAOZU) was carried on after 30 min.

RESULTS

Biolysate-based turbidity assay: The marine biolysate it able to detect many gram negative bacteria *E. coli*, *Sal* sp. and *V. cholera*. The Turbidmeter (Germany) at 580 nm was used to verify a preparation of 5×10^{1} CFU mL^{-1} by comparison of the optical density to known standards, as shown in the (Fig. 2) the absorbance reading of respective serially diluted test bacteria upon coagulation with marine biolysate. Absorbance of turbidity of bacterial colony-forming unit/mL of different Genera with marine biolysate readings for three bacterial genera in different initial numbers of bacterial colony. Based on the figure, absorbance readings increased consistently from high bacterial cell numbers to low cell numbers.

Carbohydrate based assay: Carbohydrates in the form of capsular polysaccharides (PSs) are the major components on the surface of bacteria. These molecules are important virulence factors in many bacteria isolated from infected persons. Detection of these components confers protection against the disease. Based on carbohydrate assay the result show the absorbance reading at 415 nm for different bacterial genera studied, namely, *E.coli*, *Salmonella* sp. and *V. cholera*. Different numbers of bacterial colony per tube for each bacterial genus gave different absorbance readings. Bacterial populations per mL were based on the average count of the plates. Figure 3 shows the high concentrate of color activity of *Salmonella* sp. in compare to *E.coli* and *Vibrio cholera*

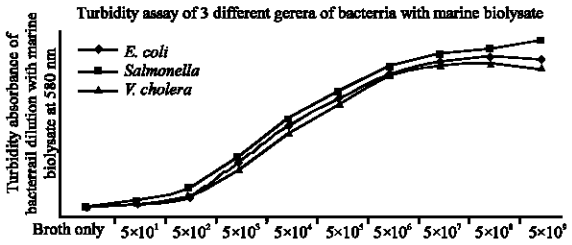

Fig. 2: Reactivity of marine biolysate (100 μL) towards different bacterial colony-forming unit/mL turbidity assay indicated that the high turbidity notice with the *Salmoenalla* sp. in compare to *E. coli* and *Vibrio cholera* when we added the marine biolysate lysate at 580 nm

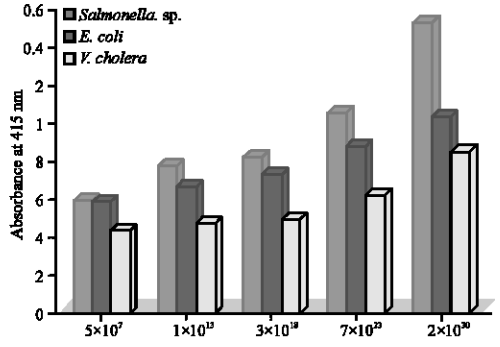

Fig. 3: The color activity of *Salmonella* sp., *E. coli* and *V. cholera* when carbohydrates assay measure at 415 nm

when the carbohydrate assay measure at 415 nm show that it can be differentiate between different genera of gram negative bacteria.

DISCUSSION

Our study revealed that this new approaches that can aid in the detection and identification of living microorganisms and that may play a role in planetary protection, particularly in the analysis and reduction of food borne complications. On the other hand, the use of this advanced detection methods to identify the potential wide range of gram negative microorganism may lead to reduction techniques that are targeted at groups of organisms of specific concern for planetary protection and the present study is a platform study to improve detection method of gram negative pathogens by exploiting surface polysaccharide structure of gram negative bacteria genera. The present study showed that both carbohydrate and turbidity assay, absorbance reading decrease accordingly upon serial dilutions. Serial diluting the bacteria reduced the cell count of bacteria per mL and thus corresponds to the reduction in absorbance reading. The absorbance reading also revealed that different bacterial genera gave absorbance reading at different ranges even when the initial cell count per mL for all 3 genera was similar. The group of serially diluted *Salmonella* sp. suspension recorded highest range of absorbance, followed by *E. coli* and *V. cholera*. Similarly for the marine biolyssate assay, the group of serially diluted *Salmonella* sp. suspensions recorded highest range of absorbance reading. However, the readings were higher for *E. coli* suspension than the *V. cholera* suspension and for both assay the highest cell count per mL for three genera didn't show any overlap in the absorbance reading and the reading for *Salmonella* sp.

are consistently high for the highest cell count. The present study optimized a simple method based on marine biolysate blood to detect gram negative pathogens since the marine biolysate only coagulate upon addition of gram negative bacteria. The method was specific and sensitive to detect the gram-negative pathogens. Unlike the conventional methods, current methods, the marine biolysate-based method was easy, less expensive and time saving since a single step of adding marine biolysate to the bacterial culture can detect gram negative bacteria. No cultivation or lysing of cells is required. The turbidity assay graph using marine biolysate blood illustrated different turbidity values among the 3 pathogens at 580 nm. *Salmonella* sp. still had the highest color activity among the three bacterial genera similar to the carbohydrate assay figure. The turbidity values could be corresponding to the coagulogen that formed when the biolysate blood mixed with the cell wall (lipopolysaccharide) of the pathogens. *Salmonella* sp. showed the highest absorbance reading which could be attributed to a longer polymer numbers in O-specific chain than the other 2 bacterial genera.

More coagulogens were formed and more amount of light was absorbed when *Salmonella* sp. was exposed to the biolysate blood. The turbidity meter measured the light that had been blocked by the coagulogen so the reading was high. The graph showed the highest color activity for *Salmonella* sp. followed by *Escherichia coli* and *V. cholerae* at 415nm. Differences in color activity and absorbance readings could be attributed to the type of carbohydrate in the lipopolysaccharide layer of gram-negative bacteria. Lipopolysaccharide is an extensive polymer of carbohydrate monomers at the cell wall of these pathogens (Seydel *et al*., 1999). Through, the carbohydrate assay, the relative amounts of lipopolysaccharide carbohydrates could be speculated. The carbohydrate assay complex as it could only scan for reasonable data on 3 types of sugars (hexose, 6-deoxyhexose and heptose) at various wavelengths. The sugars were found in the outer core and inner core separately. The trends of data for both assays were similar implicating the roles of the carbohydrate component of the lipopolysaccharide. *Salmonella* sp. recorded the highest activity in various numbers of cells at different bacterial cell suspensions, *V. cholerae* recorded the lowest activity and *E. coli* always showed second low activity. The main factor attributed to different absorbance readings could be the length of polymer chains in the three bacterial genera. The three bacterial genera had different length of polymer chain, which built up the O-antigen (Arnold *et al*., 1985 and Dubois *et al*., 1956). Thus, different levels of coagulogens affected the amount of light absorbing. Both assay recording for different genera but all genera had similar initial cell count per mL and all three genera were subjected to similar fold dilution as Fig. 2 and 3.

The trend in absorbance readings revealed that the different genera of gram-negative bacteria could be differentiated based on absorbance reading of both marine biolysate and carbohydrate assays. Both assays are being optimized to enable not only qualitative detection but also quantitative detection for the pathogens direct from the samples. Turbidity is proportional to the cell concentration (Begot *et al*., 1996; Metris *et al*., 2003, 2005). The range of proportionality depends on the size and shape of the bacteria, which can in turn be affected by environmental conditions. Turbidity is the ratio of intensities of the incident light intensity and the light scattered by the culture. The range of proportionality depends on the size and shape of the bacteria, which can in turn be affected by environmental conditions. Turbidity measurements are used to estimate the growth parameters of bacteria as an alternative to traditional plate counts (Metris *et al*., 2003, 2005). Estimation of microbial turbidity and carbohydrate measurement from absorbance measurements have the advantages of being rapid, non-destructive, inexpensive and relatively easy to automate as compared to many other techniques and particularly when compared to classical viable counts methods. On the other hand, absorbance detection of serially diluted cultures allowed accurate estimation of absorbance values and a method for estimation of lag time from such data was recently suggested (Cuppers and Smelt, 1993; Baranyi and Pin, 1999). Absorbance detection of serially diluted cultures of turbidity and carbohydrate estimation allowed accurate estimation of absorbance values and a method for estimation of turbidity and difference in color from such data was recently suggested (Cuppers and Smelt, 1993; Baranyi and Pin, 1999). Their use has increased as new trends in predictive microbiology have started to focus on the quantification of the variability of bacterial responses to food environments.

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