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Comparison of the efficiency of methods and selective agars for enumerating *Vibrio parahaemolyticus* in shrimps

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Abstract *Vibrio parahaemolyticus* is a major food borne pathogen that causes rejection of tiger shrimps (*Penaeus monodon*) consignments at the export market. It was artificially inoculated into tiger shrimps and recovery was evaluated by using nine standard procedures incorporating most probable number (MPN) method or direct plating and four selective agars. The enrichment step in alkaline salt peptone water (ASPW) and 20 h direct plating on thiosulfate citrate bile salts sucrose (TCBS) agar gave the highest recovery (76.9%) of *V. parahaemolyticus* in shrimps and followed by primary and secondary enrichment steps in alkaline peptone water (APW) using MPN method (71.5%). *V. parahaemolyticus* was not recovered in salt polymyxin broth (SPB) using either direct plating or MPN. Three tube MPN method using enrichment media ASPW and APW gave recoveries of 64.2% and 62.1% respectively. The enrichment in glucose salt teepol broth (GSTB) using MPN method resulted in 57.9% recovery whereas it was 24.7% in saline glucose sodium dodecyl sulphate peptone water (GST). The recovery in GST using direct plating was 50.4%. ASPW and APW with double enrichment steps are recommended as the most superior enrichments for enumeration of *V. parahaemolyticus* in tiger shrimps using direct plating and MPN method respectively. The differences observed among four selective media namely TCBS, triphenyl tetrazolium soya tryptone agar (TSAT), sodium dodecyl sulphate polymyxin sucrose agar (SDS) and CHROM agar Vibrio (CV) for enumerating *V. parahaemolyticus* were not significant ($p>0.05$).

Keywords Efficiency – enrichment – enumeration – *Vibrio parahaemolyticus* – shrimps

INTRODUCTION

Vibrio parahaemolyticus is a major food borne pathogen that causes rejection of consignments of tiger shrimps at the export market. Detection and enumeration of *V. parahaemolyticus* in shrimps has become important as some European Union countries have imposed zero tolerance for this organism in their importation of shrimps. In the last three and half years, 39 shrimp consignments produced in Malaysia were rejected in the countries of the European Union due to the presence of *V. parahaemolyticus* (personal communication, Ministry of Health Malaysia). To establish effective control measures to reduce the risk of prevalence of *V. parahaemolyticus* and to ensure the safety of shrimps, efficient analytical methods for the detection of *V. parahaemolyticus* in shrimps and the environment must be available.

There are various methods available for the detection of *V. parahaemolyticus* in food. Quantitative procedures either direct plating or most probable number (MPN) are occasionally used for the enumeration of *V. parahaemolyticus* [1]. It has

Journal of Science and Technology in the Tropics (2008) 4: 19-25

been noted that different methods to detect *V. parahaemolyticus* have different sensitivities [2]. Through observations and visits in various shrimp factories in Malaysia, it has been noticed that the laboratories for testing of this organism are using different methods and media. Therefore it is high time to compare these methods to determine the most reproducible method for testing of *V. parahaemolyticus*.

There are different methods using different enrichments such as alkaline peptone water (APW), alkaline salt peptone water (ASPW), salt polymyxin broth (SPB), glucose salt teepol broth (GSTB) etc for selective isolation of *V. parahaemolyticus*. Different culture media are also being used for the enumeration of *V. parahaemolyticus*. Thiosulphate citrate bile salts sucrose (TCBS) agar is a selective medium commonly used for the isolation of this organism and other members of the genus *Vibrio* from seafood. This medium supports good growth of most species while inhibiting most non-vibrios [3].

Vibrio mimicus and *Vibrio vulnificus* cannot easily be distinguished from *V. parahaemolyticus* on TCBS agar as they form similar type of green colonies on TCBS [4]. Therefore three other culture media namely: Triphenyl tetrazolium soya tryptone agar (TSAT)**,** Sodium dodecyl sulphate polymyxin sucrose agar (SDS) and CHROM agar Vibrio (CV) that can be used for enumerating *V. parahaemolyticus* were compared to find out the efficiency in recovering un-stressed cells of *V. parahaemolyticus* from artificially contaminated shrimps.

The aim of this study was to find the most reproducible method and culture media for quantitative detection of *V. parahaemolyticus* in shrimps and it was evaluated by comparing nine different standard methods, direct plating or MPN and four selective agars.

MATERIALS AND METHODS

Brackish water shrimps (*Penaeus monodon*) which are commonly known as black tiger shrimps were used in this study. Shrimps needed for the experiments were collected in sterile polythene bags from a shrimp processing plant in Kuala Selangor and transported to the microbiological laboratory at Universiti Kebangsaan Malaysia in an iced styrofoam box and kept in frozen storage (below -18^{0}C) until use. Frozen shrimps were kept in refrigerator (4-8^{0}C) for thawing a few hours before using for the experiment. The muscle portion of shrimp was cut aseptically into small pieces using sterile scalpel. 25 g or 50 g was weighed into a sterile stomacher bag and blended in a stomacher with 225 mL or 450 mL diluent described below under each method at low speed for 30 seconds.

Preparation of inoculum

Pure culture of *V. parahaemolyticus* (clinical O3:K6 strain which was tdh positive and trh negative) confirmed with polymerase chain reaction (PCR) and BIOLOG microlog system was used throughout the study. A loopful of *V. parahaemolyticus* which was stored in long term preservation medium at room temperature was streaked on nutrient agar (3%NA) containing 3% sodium chloride (NaCl) and incubated at 37^{0}C for 18 h. A loopful from this agar was inoculated into brain heart infusion broth containing 3% NaCl and incubated for 18 h at 37^{0}C. The culture was diluted decimally to the inoculum level with sterile 0.1% peptone containing 3% NaCl (pH 7.0) and the level of inoculum was determined by spread plating 0.1 mL portions of dilution on duplicate plates of TCBS agar.

Comparison of methods

The method for detection of *V. parahaemolyticus* by the International Standard Organization (ISO) recommends selective enrichment in SPB and ASPW or saline glucose sodium dodecyl sulphate peptone water (GST). The pH was adjusted at 7.4 in SPB and basal medium was sterilized by autoclaving. Polymyxin B sulphate (PB, CalBiochem) was prepared separately and sterilized by filtration. PB was added to the basal medium before used. 25 g of shrimps were stomached with 225 mL SPB, ASPW (pH 8.6) and GST (pH 8.6) separately and a known concentration (10^4 to 10^5

cfu/mL) of *V. parahaemolyticus* was inoculated. Decimal dilutions (from 10^{-2} to 10^{-6}) were prepared in 0.1% peptone containing 3% NaCl (pH 7.0). Dilutions from 10^{-1} to 10^{-4} were spread plated (0.1 mL) on TCBS (Oxoid) agar for direct enumeration. One mL from each dilution (from 10^{-1} to 10^{-6}) was transferred to 10 mL of SPB, ASPW and GST separately in 3-tube MPN method. Tubes and plates were incubated at 37^0C for 20 h. Tubes with visible growth were streaked on TCBS and TSAT agar and incubated at 37^0C for 20 h. Results were calculated in MPN/g and cfu/g based on number of tubes that yielded growth of organism on TCBS and TSAT and colonies on plates of TCBS agar [5].

The procedure for testing of *V. parahaemolyticus* recommended by the Ministry of Health (MOH) Malaysia is based on the Australian/New Zealand Standard. 25 g of shrimps was stomached with 225 mL of 0.1% peptone with 3% NaCl and a known concentration of *V. parahaemolyticus* was inoculated. Decimal dilutions were prepared in 0.1% peptone (3% NaCl). One mL from each dilution was transferred to 10 mL of APW (primary enrichment, pH 8.6). Tubes were incubated at 37^0C for 6 h and 1 mL from each tube was transferred to freshly prepared tubes of 10 mL APW (secondary enrichment). A loopful from each primary enrichment medium was streaked on TCBS agar. Secondary enrichments and plates were incubated at 37^0C for 18 h. A loopful from each secondary enrichment medium was streaked on TCBS agar and incubated at 37^0C for 18 h. The tubes from which *V. parahaemolyticus* cells were detected on plates were considered to be positive for this organism. Results were calculated in MPN/g [6].

The American Public Health Association (APHA) describes the method as follows: 50 g of shrimps was stomached with 450 mL of phosphate buffered saline (PBS, pH 7.2) and inoculated with *V. parahaemolyticus*. Dilutions were prepared (from 10^{-2} to 10^{-6}) in PBS and 10 mL from 1:10 homogenate was added into 3 tubes of double strength APW (10ml). Similarly 1 mL from all dilutions (from 10^{-1} to 10^{-6}) was inoculated into 10 mL of single strength APW and incubated at 37^0C for overnight. A loopful from APW showing growth was streaked on TCBS agar and incubated at 37^0C for overnight. The plates were examined for *V. parahaemolyticus* colonies, and the MPN values were calculated [7].

According to the method described by the Food and Agriculture Organization (FAO), 450 mL of 3% dilution water was added into 50 g of shrimps and blended. *V. parahaemolyticus* was inoculated and decimal dilutions were prepared. Ten mL from 1:10 dilution was added into 3 tubes of double strength GSTB (10ml, pH 7.4). One mL from all other dilutions was transferred into 10 mL of single strength (3 tubes) GSTB (pH 7.4). After overnight incubation at 37^0C tubes showing growth were streaked on TCBS agar and incubated at 37^0C. The plates were examined for *V. parahaemolyticus* colonies, and the MPN values were calculated [8].

Comparison of culture media

25 g of shrimps was stomached with 225 mL of 0.1% peptone containing 3% NaCl. Inoculum of *V. parahaemolyticus* was prepared as mentioned above and serial ten fold dilutions in 0.1% peptone (3%) were prepared. Shrimps were artificially inoculated with a final estimated *V. parahaemolyticus* concentration of 10^5-10^6 cfu/g. Decimal dilutions (10^{-2}-10^4) were prepared in 0.1% peptone (3%) and mixed well by vortex. Each dilution (from 10^1-10^4) was spread plated (0.1 mL) on TCBS agar, TSAT agar, SDS agar and CV agar in duplicate for direct enumeration. Plates were then incubated at 37^0C for 20 h and colonies of *V. parahaemolyticus* were counted in cfu/g.

Recovered organisms in shrimps were confirmed as *V. parahaemolyticus* using Analytical Profile Index (API 20E, bioMerieux) and minimal number of biochemical tests [7] in each method. API 20E identification was done according to the manufacture's instructions.

Un-inoculated samples of shrimp were tested in parallel using each method to confirm the absence of naturally occurring *V. parahaemolyticus* in shrimps used for the experiment. Percentage re-

covery of *V. parahaemolyticus* was calculated based on the approximate level of inoculum (cfu/mL) in each occasion and the number of organisms recovered in each method. All experiments were repeated usually three times and in each case three samples (triplicates) were analyzed independently.

Statistical analysis
In the first experiment, the number of organisms recovered was calculated as a percentage and in the second experiment the number of organisms recovered was converted to $\log_{10}$ cfu/g and analyzed statistically by ANOVA using the SPSS 13.1. Means of three replicates were reported. Differences among methods of testing and culture media were examined for level of significance ($p<0.05$) by Duncan's multiple range test.

RESULTS AND DISCUSSION

Data comparing methods of testing for *V. parahaemolyticus* by quantitative recoveries are presented in Figure 1. The performances of 20 h direct plating method in ASPW were superior to that of other methods and gave the highest recovery of 76.9%. The next highest recovery of 71.5% was obtained from primary and secondary enrichments

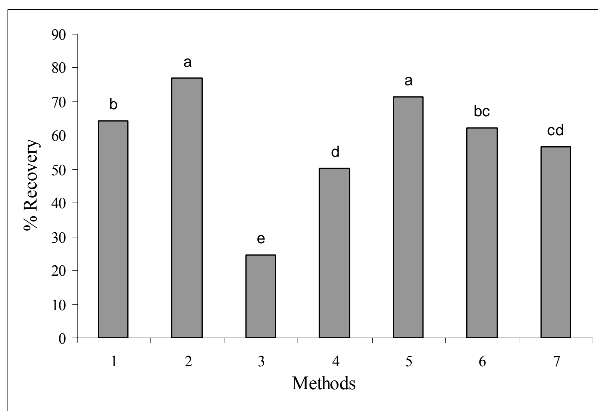

Figure 1. Percentage recovery of *V. parahaemolyticus* in shrimps (*Penaeus monodon*) using different methods. 1: ISO-ASPW MPN; 2: ISO-ASPW Direct plating; 3: ISO-GST MPN; 4: ISO-GST Direct plating; 5: AS/NZS-APW MPN; 6: APHA-APW MPN; 7: FAO- GSTB MPN. a-e: Different alphabets for different methods show significant differences ($P<0.05$). Each bar represents average of three independent experiments.

in APW using MPN method. The difference observed was not significant among these two methods. Two steps enrichment in APW using MPN method resulted in significantly higher recovery (71.5%) than any single step enrichment MPN method described in this text. It was noticed that *V. parahaemolyticus* colonies were well isolated in this method than any other methods described here.

Vibrio parahaemolyticus was not recovered in SPB using either direct plating or MPN. Concentration of PB in SPB was 12.34 µg/mL or 100 IU/mL taking 1 mg as equivalent to 8100 IU, as with PB according to manufacturer's instructions. Ottaviani *et al*. [9] also found that growth was inhibited in SPB for many microorganisms. Kampelmacher *et al*. [10 found that some strains of *V. parahaemolyticus* in pure culture appear to be inhibited by 70 IU per mL of PB. The unsuitability of SPB with 250 µg of polymyxin per mL was indicated by studies of Karunasagar *et al*. [11] on 27 samples of raw and processed shrimp, of which 10 samples showed the presence of *V. parahaemolyticus* when GSTB was the enrichment broth, whereas none were positive when SPB (250 µg of polymyxin per mL) was used. They compared recovery of *V. parahaemolyticus* from inoculated fish homogenates using various enrichment broths namely: salt broth, SPB (0.25µg/mL and 2.5µg/mL of Polymyxin), GSTB and direct plating on TCBS. They concluded that recovery by direct plating appeared to be good. This suggests that during the period of incubation of homogenates in broths, the *V. parahaemolyticus* isolates are overgrown by the other flora. This was further supported by the observation from sterile fish homogenates inoculated with *V. parahaemolyticus* cells reached the upper limit of detection by the MPN technique in salt broth and SPB with 0.25 µg of polymyxin per mL. Even in the absence of competing flora, SPB with 2.5 µg of polymyxin per mL did not yield good recovery, suggesting the toxicity of polymyxin to *V. parahaemolyticus*. The least recovery was observed in GSTB. Based on these results Karunasagar *et al*. [11] suggested that direct plating on TCBS might be more useful in obtaining a good estimate of

the *V. parahaemolyticus* counts in seafood. This technique is less laborious and results are obtained a day earlier as compared with the MPN technique. Their findings are also in agreement with our present findings. Hagen *et al*. [12] compared two enrichment broths (APW and SPB) for their ability to recover *V. parahaemolyticus* inoculated into each of five seafoods (crab legs, oysters, shrimps, lobsters and sharks). Recovery of *V. parahaemolyticus* from each seafood immediately after inoculation was significantly higher ($P<0.05$) with APW than with SPB. The concentration of PB in SPB was 2.5μg/mL and pH 7.4.

Three tube MPN method using enrichment media such as ASPW (ISO) and APW (APHA) gave recoveries of 64.2% and 62.1% respectively and there was no significant difference between these two methods. Eyles *et al*. [13] and Oscroft [14] also found that APW was most effective for the isolation of *V. parahaemolyticus* from oysters and prawns. The enrichment in GSTB using MPN method resulted in 56.7% recovery whereas it was 24.7% in GST. The recovery in GST using direct plating was 50.4%.

Figure 2 represents the recovery of *V. parahaemolyticus* in log cfu/g using four plating media. The differences observed among four media were not significant ($p=0.932$). Colonies of *V. parahaemolyticus* observed on TCBS was green in colour and the size was 2-5 mm. Selective agents in TCBS are sodium thiosulphate, sodium taurocholate, ferric citrate and pH of 8.6 while the diagnostic agent is sucrose [15]. *V. parahaemolyticus* produced dark red colonies with a diameter of more than 2 mm on TSAT. Bile salt is the selective agent in TSAT while sucrose and triphenyltetrazolium chloride (TTC) are diagnostic agents [16]. Green colour and 2-5 mm size colonies were observed on SDS agar. Selective agent in SDS is sodium dodecyl sulphate and the diagnostic agents are sucrose and sulphatase [17]. The colonies produced on CV were violet in colour. CV medium containing substrates for beta-galactosidase was developed specifically to differentiate *V. parahaemolyticus* from other bacteria by using a chromogenic substrate, instead of sugar fermentation, used in traditional growth media such as TCBS [4].

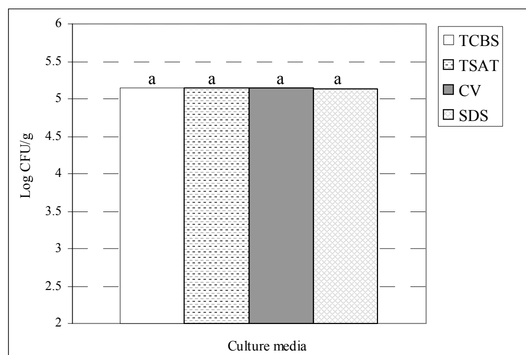

Figure 2. Recovery of *V. parahaemolyticus* on different culture media. a: same alphabet for different culture media indicates no significant differences ($P>0.05$).

There was no any advantage over the other in selective agents or diagnostic agents in four culture media compared for unstressed pure organisms of *V. parahaemolyticus.* It is required to conduct field studies to evaluate the behaviour of background microflora when isolating *V. parahaemolyticus* from culture environment. Estuarine waters generally contain a significant number of *Pseudomonas* species and to a lesser extent *Flavobacterium* and *Photobacterium* species [18]. Thus a medium intended for use in such environments must be able to inhibit or at least differentiate among such genera. It was noted by Villari *et al*. [19] that the choice of the culture medium may depend on the expected level of bacterial contamination of the sample. Samples with an expected high level of background microbial flora should be analyzed through more selective media [19].

In sum, ASPW with single enrichment step is recommended as the most superior method for enumeration of *V. parahaemolyticus* in shrimps using direct plating technique. There was no significant difference in the recovery of *V. parahaemolyticus* on TCBS, TSAT, SDS or CHROM agar *Vibrio*. The plating efficiency of TSAT agar, SDS agar and CHROM agar *Vibrio* appeared to be comparable to that of TCBS, which is the most commonly used media for enumeration of *V. parahaemolyticus*.

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