SHORT COMMUNICATION

Simultaneous virological and bacteriological screening of microbial isolates in *Perna viridis*

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Abstract:

The global consumption of shellfish has increased over the past few years concurrent with reports of viral and bacterial gastroenteritis outbreaks worldwide. This study was conducted to screen both viral and bacterial agents in *Perna viridis* using Transmission Electron Microscope (TEM) and molecular based detection methods. Direct genomic extraction methods were tested on mussels, which were purchased from Shah Alam wet market. Samples were subjected to Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for viral screening and conventional PCR for bacterial detection. In this study, no viral agent was discovered but PCR amplification of the *lamB* gene yielded a single band at 309 bp, identifying *E. coli* as being present in *Perna viridis* tissues. Continuous monitoring of shellfish for pathogens will help ensure its safety for human consumption. Further studies focusing on the relationship and quantitative measurement of fecal indicator bacteria in coastal areas/aquaculture settings should be addressed.

Keywords: *E. coli,* PCR, *Perna viridis*, RT-PCR, TEM

1. INTRODUCTION

Acute gastroenteritis is one of the main causes of morbidity and mortality and worldwide [1]. Factors influencing infectious diarrhoea vary among different geographical region, co-morbidities and host immune status [2]. In general, acute gastroenteritis can be caused by a wide array of pathogens, including bacteria, viruses, and parasites [3]. The mechanisms often incluse (i) excessive secretion of fluids induced by luminal toxins expressed by enteropathogens, (ii) inflammatory or cytotoxic damage of the ileal or colonic mucosa or (iii) penetration of the bacterium through the mucosa to the reticuloendothelial system [4].

The filter feeding nature of shellfish and probable unhealthy farming and handling practices may occasionally entail health risks due to the possible presence of hazards [5]. In particular, shellfish accumulate viral and bacterial pathogens in their tissues and can therefore act as bio-monitors to assess the quality of water. An example of a filter-feeding bivalve shellfish is *Perna viridis* or mussels, which are often consumed among the local population. Common pathogens found in shellfish that is associated with food poisoning include Norovirus (NoV) and *E.coli.* [6]. Consumption of undercooked or contaminated shellfish can lead to detrimental effects [7], especially when it is consumed raw. Microbial contamination in *Perna viridis* is undervalued yet poses significant health threats and economic risks in the shellfish and aquaculture industry. The aim of this study was to screen both viral and bacterial isolates in *Perna viridis* using TEM and molecular based detection methods for the purpose of raising public awareness.

2. MATERIALS AND METHODS

2.1 Sample collection and processing

Perna viridis purchased from Shah Alam market were transported to the laboratory and processed immediately according to procedures described by [8] with minor modifications. Briefly, *Perna viridis* were cleaned and whole tissues were removed by dissection, cut into small portions, mixed and homogenized. 6 mL of 0.05 mol/L glycine and 0.15 mol/L sodium chloride (NaCl) were added to 2 g of tissues and placed on shaker for 20 min at 4°C. The homogenate was centrifuged at 10,000 x g, 4°C for 15 min, and the pH adjusted to 7.2 – 7.4.

2.2 Viral concentration

Microbial particles were precipitated from the supernatant using 3 g of 6% (w/v) PEG with 0.87 g of 0.3 mol/L NaCl. The mixtures were rocked overnight and centrifuged at 10,000 x g, 4°C for 30 min [9].

2.3 Viral purification

Sample was placed on the top of a 20% (w/v) and 40% (w/v) sucrose gradient and centrifuged at 40,000 x g for 3 h at 4ºC using an ultracentrifuge (Himac CS120GXII Series, Hitachi). The opalescent band was removed and re-centrifuged for 1 h at 4ºC. Supernatant was discarded and pellet dissolved in 100 µL PBS.

2.4 Negative staining-electron microscopy

Samples were placed onto a copper-coated 300-mesh grid and stained with 3% (w/v) uranyl acetate for 5 min before being viewed using a scanning transmission electron microscopy (STEM) (INCA 350, Oxford Instruments, UK).

2.5 Viral extraction

Two mL of TriZol® Reagent was added to the pellet before homogenization. RNA was extracted from 140 µL of viral concentrates using QiaAmp Viral RNA Mini kit (Qiagen, Hilden, Germany). Viral nucleic acid was stored at -70ºC.

2.6 NoV screening of the *RdRp* gene by RT-PCR

Viral nucleic acid was analyzed for NoV using a one-step RT-PCR assay. *RdRp* primers used were as follows; NVF5046: 5' CGT GGG AGG GCG ATC GCA AT 3' and NVR5245: 5' TTT CTR ATC CAI GGR TCI AT 3'. Briefly, 2.5 µL of viral nucleic acid was added to 22.5 µL mixture of MyTaq™ One-Step RT-PCR (Bioline, USA) containing 12.5 µL My Taq One-Step Mix, 0.5 µL RNase Inhibitor, 0.25 µL Reverse Transcriptase, 7.25 µL of DEPC-Treated water, 1.0 µL of 10 µM forward and reverse primer. Cycling conditions consisted of reverse transcription at 44°C for 20 min, activation of Taq polymerase at 95°C for 1 min, 40 cycles of 95°C for 10 sec (denaturation), and 60°C for 10 sec (annealing) followed by a final extension at 72°C for 30 sec. Electrophoresis of samples was performed using a 2.0% (w/v) agarose gel at 60 V for 90 min.

2.7 Bacterial isolation and identification

Samples were cultured in nutrient broth at 37ºC for 24 h, then sub-cultured onto MacConkey (MAC) and Eosin Methylene Blue (EMB) agar to observe the colony morphology. Gram's staining was performed to determine the size, shape and arrangement of bacteria. Motility, Triple Sugar Iron Tests (TSI) and indole biochemical tests were performed using standard microbiological procedures.

2.8 Bacterial extraction

Colonies were incubated overnight in a 5 mL Luria Burtani (LB) broth at 37ºC. One mL of LB stock culture was taken and centrifuged at 2,500 x g, 4ºC for 4 min. The pellet was washed with 1 mL of Tris EDTA (TE) buffer and re-centrifuged. The pellet was re-suspended in 100 µL of TE buffer and boiled at 100ºC for 10 min for protein lysis. The suspension was centrifuged at 9,000 x g, 4ºC for 30 sec. The supernatant was collected and stored at -20ºC.

2.9 *E. coli* screening of the *lamB* gene by PCR

PCR amplification for *E. coli* was performed using 2X Taq Master Mix (PhileKorea Technology, Korea) using the *lamB* primer [10]. Briefly, 2 µL of DNA was added to 23 µL of the cocktail containing Taq Master Mix 12.5 µL, 8.5 µL of distilled water, 1.0 µL of 0.2 µM forward and reverse primer. The cycle conditions were as follows: denaturation at 94°C for 3 min, annealing at 60°C for 30 sec and extension at 72°C for 2 min. This cycle was repeated 30 times. Electrophoresis of samples was run using a 1.5% (w/v) agarose gel at 60 V for 90 min. *E. coli* ATCC 4157 was used as a positive control.

3. RESULTS

3.1 Negative staining-electron microscopy

Electron microscopic observations revealed bacteria-like particles following staining with 3% (w/v) uranyl acetate (Figure 1).

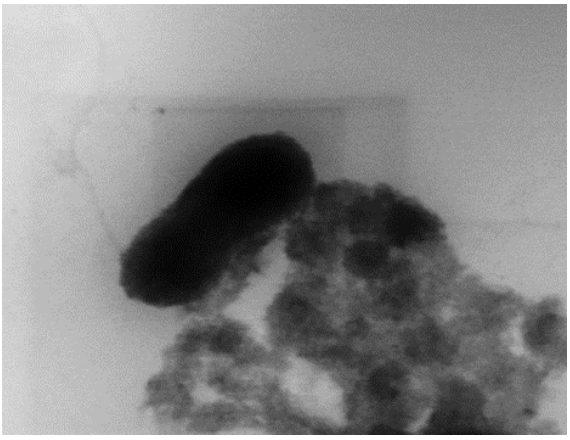

Figure 1: Presence of bacteria like particles were observed following staining and viewing at 160,000 x magnification.

3.2 NoV screening of the *RdRp* gene by RT-PCR

Although the positive control of NoV revealed a PCR product with a band size of 200 bp following the amplification of the *RdRp* gene, no bands were detected for the samples tested. This implies that the samples were not of NoV origin.

3.3 Bacterial isolation and identification

Initially, organisms revealed colony morphology resembling of *E. coli* and were sub-cultured onto MAC and EMB agar until pure culture with homogenous colonies were obtained. Gram stain revealed uniformed gram negative, rod shaped organisms arranged singly. All isolates produced bright pink colonies on MAC and green characteristic metallic sheen colonies on EMB agar.

Hanging drop slide prepared by broth culture and examined under 100X objective demonstrated motile organisms. The following TSI reaction was observed: (A/A) with gas and no precipitate indicative of *E. coli.* Indole test was positive due to the development of red coloured rings upon testing with Kovak's reagent.

3.4 *E. coli* screening of the *lamB* gene by PCR

Agarose gel electrophoresis of the PCR products of the *lamB* gene revealed bands at 309 bp (Figure 2).

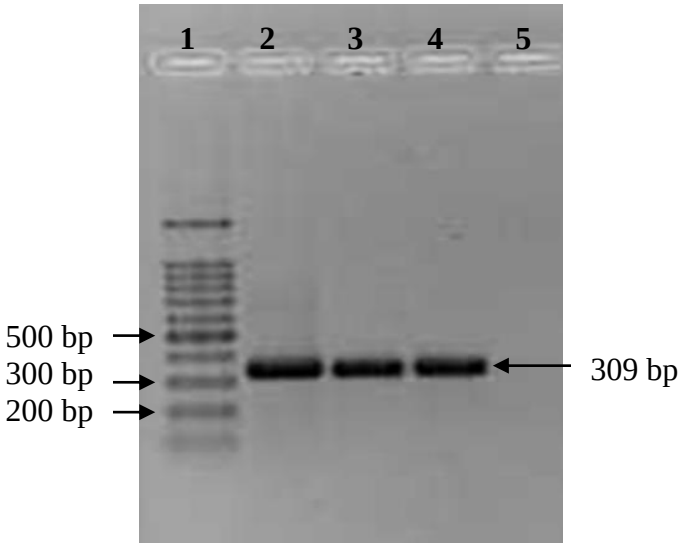

Figure 2: 1.5% (w/v) agarose gel. Lane 1: 100 bp DNA Ladder; lane 2: positive control; lane 3: inoculated sample from NA agar; lane 4: inoculated sample from EMB agar; lane 5: negative control.

4. DISCUSSION

The purpose of this study was to evaluate the simultaneous diagnosis of viral and bacterial contamination in *Perna viridis.* Samples used in this study were purchased from Shah Alam, but were originally harvested from Sungai Kurau, Perak. In order to maintain the viability of the microorganism, all mussels were kept at 4^0C prior to processing. Previous studies have only selected digestive glands as predilected sites for viral pathogens [11]. However, in this study, all tissues were salvaged in order to expedite and maximize the recovery of viral entities if any.

Recent study showed that in a gastroenteritis outbreak, both *E.coli* and NoV were detected in shellfish samples [12, 13], hence justifying the selection of organisms as probable causative agents of interest in this study. The presence of viral agents could be detected using electron microscopy [14] which necessitates the process of viral concentration and purification beforehand. The latter is a technique involving different gradients of sucrose used to separate viruses according to its molecular weight. In this study, 20 and 40 % (w/v) of sucrose were selected for viral separation purposes. Opalescent layers were initially observed at both gradient interfaces indicating the presence of particles in the processed tissues. Nevertheless, no viral agents were detected including NoV following screening using TEM, including NoV. To confirm this, RT-PCR was conducted using a set of NoV universal primers (NVF5046 and NVR5245) for the amplification of the *RdRp* gene. Molecular methods including RT-PCR are sensitive methods that can be used to detect the presence of viruses in shellfish [15]. The RT-PCR results corroborated the TEM findings, in which no bands were observed following the amplification of the *RdRp* gene, thus confirming the absence of NoV in the samples. This finding should be repeated with more time aimed towards the optimization process of the cycling conditions or the use of different primers targeting different genes of interest. Other than NoV, several other viruses including rotavirus, adenovirus, astroviruses and enterovirus account for cases of acute viral gastroenteritis [16, 17]. Like NoV, these agents are transmitted via the fecal-oral route through contaminated food and water, thus could be targeted in forthcoming studies.

Despite not having to detect viral agents, EM screening of *Perna viridis* did successfully disclose the presence of bacilli bacteria with filamentous organelles. Clinical microbiology screening using standard bacterial culture and biochemical tests methods were positive for the presence of *E. coli* in the samples tested. Furthermore, samples revealed positive amplification of the *lamB* gene with a single band at 309 bp upon gel electrophoresis, confirming the aetiology of the bacterial in sample of *Perna viridis.*

5. CONCLUSION

Shellfish accumulate bacterial and viral pathogens as they filter feed and can therefore act as bio monitors to assess the quality of water. The presence of pathogens in shellfish is a public health hazard. This study was conducted randomly as a screening purpose in the absence of any gastroenteritis outbreak. In this study, no viral agent was discovered but *E. coli* was successfully identified in *Perna viridis* tissues through microbiological methods and PCR amplification of the *lamB* gene. This study was undertaken for the purpose of raising public awareness addressing problems of hygiene and waste management related issues. Further studies focusing on the relationship and quantitative measurement of fecal indicator bacteria in coastal areas/aquaculture settings should be addressed.

ACKNOWLEDGEMENT

The authors would like to thank the Faculty of Health Sciences for the laboratory facilities and technical assistance in this study.

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