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Proteolytic Activity of *Vibrio parahaemolyticus* Isolated from *Epinephilus spp.* A Preliminary Report

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Vibrio infection is becoming more and more common worldwide. A Protease producing bacterium was isolated and identified from the eye of Epinephillus spp using phenotypic, morphological and 16S rRNA identification methods. Protease screening was carried out by the caseinolytic method. The unknown bacterium was found to have 98% maximum identity with Vibrio parahaemolyticus in NCBI database after BLAST. The bacterium produces protease enzyme on a nutrient agar medium containing 10% casein. The aim of this research was to identify the bacterium in question and its ability to produce extracellular proteases, which may be its virulence factor so that its biotechnological application can be explored in future.

Key words: Epinephillus spp, proteolytic, Vibrio parahaemolyticus, 16S rRNA.

Vibrio infections are becoming more and more common worldwide. The United States Centers for Disease Control and Prevention (CDC) estimates that 8,028 Vibrio infections and 57 deaths occur annually in the United States (Mead *et al.*, 1999). Of these infections, 5,218 are foodborne in origin (Mead *et al.*, 1999) . Three major syndromes, gastroenteritis, wound infection, and septicema, are caused by pathogenic *vibrios*. Within the genus *Vibrio, V. cholerae*, *V. Parahaemolyticus* and *V. vulnificus* have long been established as important human pathogens in various parts of the world. Generally, these organisms are contracted after the patient has consumed raw or undercooked seafood, such as oysters, shrimp, and fish (Thompson *et al.*, 2004). Hence, identification and characterization of *Vibrio* isolates are of significant importance to public health, safety of the human food supply and seafood industry.

Vibrio parahaemolyticus is one of the major *Vibrio species* associated with vibriosis in marine shellfish (Lee *et al.*, 2003). There are several reports on the pathogenecity of *V. parahaemolyticus* in marine finfish (Hatai *et al.*, 1981). *V. parahaemolyticus* has been isolated from skin ulcers in red seabream and other marine fishes in Japan (Hatai *et al.*, 1981, Kusuda *et al.*, 1979). Wong *et al.* (1986) reported *V. parahaemolyticus* as a predominant species associated with vibriosis in the kidney or spleen of Lates calcarifer. Liao *et al.* (2004) reported *V. parahaemolyticus* as one of the secondary invaders along with other *vibrios* in cobia culture in Taiwan. Virtually little or no report exist on the isolation of *V. Parahaemolyticus* from the eye of fish.

According to Bergey's manual of systematic bacteriology (Brenner *et al.*,

2005), members of the genus *Vibrio* (*Family:Vibrionaceae*) are Gram negative, usually motile rods having a facultative fermentative metabolism. They are generally able to grow on marine agar and on the selective medium thiosulfate-citrate bile salt-sucrose agar (TCBS), and are mostly oxidase positive. We made an attempt for identification and characterisation of a *V. parahaemolyticus* strains isolated from the eye of diseased groupers *(Epinephelus spp.)* and to further investigate its ability to produce an extracellular protease that may be the virulence factor of the bacterium.

Bacterial proteases are mainly involved in providing peptide nutrients for the microorganism. However, the production of bacterial proteases could contribute to the pathogenesis of infections, and therefore they could be considered virulence factors. In fact, some authors regard proteases as the main virulence factors present among the extracellular factors (Secades and Guijaro, 1999). Although direct evidence revealing the molecular mechanisms by which bacterial proteases contribute immensely in the development of the pathology is still lacking, it has been suggested that proteolytic enzymes of fish pathogens, such as *Aeromonas hydrophila, Vibrio anguillarum*, *Vibrio vulnificus*, *Aeromonas salmonicida*, *Flexibacter columnaris* and *Flexibacter psychrophilus*, play an important role in causing massive tissue damage in the host, which may aid the establishment of infection (Secades and Guijaro, 1999).

In this paper, we report the identification of protease producing *Vibrio parahaemolyticus* strain with ability to produce protease enzyme from the eye of diseased grouper (*Epinephilus spp*). To our knowledge, this is the first report.

MATERIALS AND METHODS

Bacterial strain: Grouper (*Epinephillus* spp) were obtained from an open fish farm located in Pusat Penyilidikan Ternakan air payau 81550 Gelang Patah Johor Bahru, Malaysia. The fish were kept in ice and transported to the research laboratory within 1 hour. The fish was subsequently washed with distilled water visceral organs were separated and gastrointestinal tract (GI) was removed. On the other hand the eye was dissected aseptically and kept separately.

A homogenate solution of the dissected eye and GI tract each were prepared by adding 0.9% NaCl solution (10:1; Volume: weight). Serial dilution was made by mixing this homogenate solution with sterilized distilled water. Diluted samples (0.3ml) were aseptically poured in nutrient agar and cultured plate were incubated at 37^0C overnight and examined for development of bacterial colonies after incubation period (Geethanjali, *et al.,* 2011). The strains were grown overnight with shaking (112 rpm) in Luria Bertani (LB; DIFCO Laboratories) medium at 37ºC. Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS; DIFCO Laboratories) Agar was used also as a selective agar to differentiate *V. vulnificus* and *V. Parahaemolyticus*, but the medium is supplemented with 2% NaCl. The isolates obtained on TCBS agar, after 24 h of incubation at 37°C resemble the sucrose non-fermenting isolates of *vibrio spp*. The pure colonies were subsequently cultured on chromagar, which is a medium design specially for the growth of *Vibrio spp*, growth were observed after 24h of incubation at 37°C. Preliminary phenotypic characterization was conducted for identification according previously described method by Brenner *et al.,* (2005). After presumptive colony morphology on TCBS and Chromagar, gram staining was conducted to deduce the gram reaction of the strain. Casein hydrolysis was conducted using 10% casein plate assay.

16S rRNA SEQUENCE HOMOLOGY

The genomic DNA extraction was performed using Promega Genomic DNA Purification Kit, according to manufacturer's protocol. Purified DNA was quantified spectrophotometrically using a Nano Drop-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington,

DE, USA) and diluted to a final concentration of 100 ng/µl using DNase/ RNase-free double-distilled water (ddH_2O). Oligonucleotide primers for amplification of the 16S rRNA gene and subsequent sequencing were designed using conserved sequences detected within a Clustal X nucleotide alignment of the *Vibrio* 16S nucleotide sequences obtained from the NCBI database. 16S rRNA gene sequences from 15 separate *Vibrio species* were used for the sequence alignment. Derived primer sequences were evaluated for predicted efficiency using the oligocalculator, online (Kibbe, 2007) and NetPrimer computer software (Premier Biosoft International, Palo Alto, CA, USA). The primers used for PCR amplification were: 16SF [5'-GTTTGATCATGGCTCAGATTG- 3'] and 16SR [5'-CTACCTTGTTACGACTTCACC-3'].These primers were synthesized by First BASE Laboratories sdn Bhd (Shah Alam, Selangor, Malaysia). The PCR was performed as described by (Hoffmann *etal.*, 2010). Briefly, in a 50 µl volume with Hot-StarTaq Master Mix (Qiagen, Valencia, CA, USA) containing 400 µM dNTP (each of dATP, dCTP, dGTP and dTTP), 5 U of HotStart Taq Polymerase (Qiagen), 1x Taq polymerase buffer (Qiagen), 2.5 mM $MgCl_2$ and a 300 nM concentration of each primer with ~100 ng of DNA template. The cycle conditions were 1 cycle 95^{o}C for 2 minutes, 40 cycles 0f 95^{o}C for 15 sec, 52^{o}C for 25 sec, 72^{o}C 35 sec followed by a final 7 minutes extension cycle at 72^{o}C. The PCR products were purified using QIAquick purification kit according to manufacturers' protocol. The PCR product was analysed by 1% agarose gel electrophoresis and visualized with UV irradiation after staining with ethidium bromide. Both strands of amplified PCR products were sequenced by First BASE Laboratories sdn Bhd (Shah Alam, Selangor, Malaysia). DNA sequences were edited and assembled using DNAStar, Inc. (Madison, WI) Lasergene SeqMan II 5.07 sequence analyses software. After analyzing and assembling the respective sequences, a consensus sequence was used to query the NCBI BLAST database at NCBI to confirm strain identity of the bacterium in question.

Screening for Proteolytic Activity

The purified bacterial isolates were seeded on 10% casein-agar plates and were incubated at 37^{o}C for 24 h. The microbes showing a clear zone of casein hydrolysis were identified as protease producers. Depending upon the maximum relative diameter of the zones, one strain was selected for further experimental studies.

Protease Production

The culture medium used in this work for the protease production as described by Almas *et al.* (2009) contained (wv^{-1}) 3% nutrient gelatin, 0.8% nutrient broth, 0.5% casein, 0.01% $MnCl_2$ and 1.2 ml of 20% glycerol. The culture was maintained at 37^{o}C for 24 - 72 h in a shaking incubator (150 rpm). After 72 h of growth, the cells were harvested at 10,000 rpm for 15 min and the supernatant thus obtained was used as crude enzyme

Protease Assay

The proteolytic activity was determined by caseinolytic method described previously (kuniz, 1947) with some modifications, using azocasein as a substrate. Crude enzyme (1ml) was mixed with 1 ml of 1% azocasein (sigma-Aldrich) solution, dissolved in 0.02M Tris-HCl (trihydroxy aminomethane hydrochloride) buffer (pH 8.5) and was incubated in a water bath at 55^{o}C for 10 min. The reaction was then terminated by the addition of 1 ml of 5% Trichloroacetic acid (TCA). The mixture was kept at 4^{o}C for 15 min followed by centrifugation at 4000 rpm for 20 min. Supernatant (1ml) was mixed with 1 ml of 0.4 N NaOH and the absorbance was read at 440 nm. The blank was prepared by adding TCA before incubation at 55^{o}C. One unit of protease activity is defined as the amount of enzyme that produces an increase in absorbance of 0.01 under assay conditions. The absorbance was measured using 100 VIS spectrophotometer (Buck scientific, USA).

RESULTS AND DISCUSSION

Morphological and physiological characteristics of the bacterial strain were investigated according to the methods described previously by Brenner *et al.,* 2005. It was identified as a member of *Vibrio* species. This is evident by the ability of the strain to grow on chromagar and TCBS. The bacterium produces colonies with blue to green centre on TCBS. The preliminary characterization of the isolate is summarized in Table 1. The bacterium was found to be gram negative short rods with ability to produce extracellular protease.

In addition to the preliminary identification of the bacterium using conventional morphological and physio-logical methods; further strain identification using 16S rRNA gene amplification and sequencing was performed to confirm its identity. This was carried out because sequence analyses of the 16S rRNA can be used to identify bacteria with ambiguous biochemical profile. The sequences generated after PCR and DNA sequencing were edited using DNAStar, Inc. (Madison, WI) Lasergene SeqMan II 5.07 sequence analyses software. After analyzing and assembling the designated sequences, a consensus sequence was used to query the NCBI database at NCBI to confirm strain identity. The homology of the unknown bacterium to *V. Parahaemolyticus* was 98 % with accession number of JF779831. We consider *V. Parahaemolyticus* as the bacterium isolated from the eye of *Epinephilus spp.* To our knowledge this is the first *V. Parahaemolyticus* isolated from the eye of grouper (*Epinephilus spp).* This bacterium has significant effect on public health as it grows at a body temperature of 37°C, suggesting that it may be a potential human pathogen.

Fish diseases of bacterial origin have been one of the most important factors of economic loss since the beginning of marine fish culture (Balebona *et al*., 1998). Bacteria of the genus *Vibrio* are ubiquitous in marine and estuarine aquatic ecosystems in which finfishes and shellfishes occur naturally and are farmed (Denner *et al*.,2002; Heidelberg *et al*.,2002). Several *Vibrio* spp. form part of the natural biota of fish and shellfish (Otta *et al*.,1999; Ruangpan *et al*., 1991) and these bacteria behave as opportunistic fish pathogens in marine environments. The principal bacteria identified in epidemic diseases of marine finfishes and shellfishes are *Vibrio anguillarum, V. alginolyticus, V. fluvialis, V. furnissii, V. harveyi, V. parahaemolyticus and V. Vulnificus* as reported by (Buck *et al*., 1990; Liao *et al*., 2004).this is in conformity with this preliminary report. *Vibrio parahaemolyticus* is one of the major *Vibrio* species associated with *vibriosis* in marine shellfish (Lee *et al*., 2003). These bacteria are also important in public health and many of them are capable of producing gastrointestinal disorders in humans who have ingested contaminated fish and shellfish (Nolan *et al*., 1984)

Furthermore, the isolate has positive proteolytic activity. Most extracellular protease produced by pathogenic bacteria are considered as virulence factor (Secades and Guijaro, 1999). This research aimed at identifying the bacterium isolated from the eye of *Epinephilus spp* and screening for protease production which may be its virulence factor. Further research will focus on characterization and purification of the protease enzyme so that its biotechnological application can be explored, such as selecting potent protease inhibitors to see the feasibility of developing new drugs.

Table 1: Characteristics of the Strain

Characteristics	Strain
Gram staining Reaction	Negative
Cellular Shape	Short rod
Colony colour on TCBS	Colonies with blue to green centres
Growth on Chromagar	Positive
Range of Growth Temperature	30°C, 37°C & 42°C
Casein Hydrolysis	positive

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