Research Article

Assessment of the outer membrane protein profile of *Vibrio parahaemolyticus* using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis method

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ABSTRACT

Aims: The bacterium *Vibrio parahaemolyticus* causes gastroenteritis in humans after the consumption of raw seafood. Therefore, a rapid detection system that can identify the presence of *V. parahaemolyticus* accurately is necessary. One potential detection system under development is based on antibodies. This study aimed to map the outer membrane protein profile of *V. parahaemolyticus*. **Methods and Results:** These outer membrane proteins (OMP) may be antigenic and can be used to produce antibodies. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was used to map the outer membrane protein profile of *V. parahaemolyticus*. The results of the analysis using SDS-PAGE to assess the OMP of *V. parahaemolyticus* show that the molecular weight (MW) of the major polypeptide band was in the range of 10–100 kDa. Overall, there were 31 bands observed, of which 11 were major bands with MW of 72, 67, 58, 44, 42, 40, 38, 37, and 10 kDa. **Conclusion, Significance, and Impact of Study:** The identified proteins have antigenic properties and can be used to produce antibodies for a rapid detection system (rapid test) to detect the presence of *V. parahaemolyticus*.

KEY WORDS: Antibody, Odium dodecyl sulfate-polyacrylamide gel electrophoresis, Outer membrane protein profile, *Vibrio parahaemolyticus*

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative bacterium, which is halophilic and thrives on marine ecosystems and the surrounding estuaries. Since it was discovered in 1950, *V. parahaemolyticus* bacterium has been known as one of the pathogens found in humans that cause acute gastroenteritis.[1,2] These bacteria are known to often contaminate the marine organisms that humans mostly consume. The problems caused by the *V. parahaemolyticus* bacteria commonly occur in summer.[3] The increase in the seawater temperature is one factor of the occurrence of gastroenteritis cases that are caused by *V. parahaemolyticus* bacteria.[4] *V. parahaemolyticus* bacteria grow in seawater with the optimum levels of NaCl 3% (grow well in NaCl level of 0.5–8%) in the temperature range of 5–43°C, pH 4.8–11, and water activity (a_w) 0.94–0.99. There are 34 species and one-third of these species are known to be pathogenic to humans.[5] There are 30 species of marine organisms that can be infected by *V. parahaemolyticus* bacteria,[6] but the most often contaminated species is shellfish.[7] The shellfish obtained from the market in several countries in Southeast Asia (Thailand, Vietnam, Malaysia, and Indonesia) was very susceptible to the contamination of *V. parahaemolyticus* bacteria.[8] Food ingredients which are contaminated with *V. parahaemolyticus* bacteria will cause diseases to consumers if the food is consumed raw.[9,10]

Raw fish sold in Spain was the source of pathogenic bacteria.[11] The research managed to detect the presence of *V. parahaemolyticus*, *Vibrio cholerae*, *Aeromonas,* and *Staphylococcus aureus*. In Japan, Honda *et al.*[12] reported that 40–60% *V. parahaemolyticus* causes food poisoning. Marlina[13] in her research also found that the bivalvia *Corbicula moltkiana* isolated from Singkarak Padang River, West Sumatra also contains *V. parahaemolyticus* bacteria. *V. parahaemolyticus* bacteria can also cause serious disease in shrimp and mollusks, causing mass death and economic losses.[14] According to Nair *et al.*[15] and Matsumoto *et al.,* Indonesia and Taiwan are included in one of

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the region spreads of pandemic cases caused by[16] *V. parahaemolyticus* bacteria.

Historically, the outer membrane proteins (OMP) of Gram-negative bacteria have been considered to be a part of the cell wall associated with virulence properties, with a significant role in virulence and pathogenicity in the host.[17,18] The outer membrane molecules of bacteria are composed of proteins, fats, and sugars, which can be easily recognized as a foreign substance by the immune system of the host. The outer membrane has a double layer consisting of a layer of phospholipids and lipopolysaccharide (LPS). LPS layer is also known as endotoxin and is considered a major virulence factor.

The outer membrane (outer membranes) of Gram-negative bacteria contains proteins such as Porin, receptors, and pores as well as lipids.[19] The proteins on the outer membrane are known to have high immunogenic properties because they have a role as epitopes on the cell surface.[20] The outer membrane protein has a significant role in virulence;[21] the outer membrane proteins of pathogenic bacteria are antigen protective.[22,23] The OMPs of *Aeromonas*, *V. parahaemolyticus,* and *V. alginolyticus* bacteria have virulence and immunogenic properties.[24,25] The OMPs are vaccine components which are potential to control fish disease.[26-31] The outer membrane protein of *V. alginolyticus* strain can be used as a vaccine candidate 9.[32] The results of research conducted by Desrina *et al*.[33] found that the OMPs of *V. alginolyticus* bacteria 74 kDa are capable of providing protection against *Vibrio* infection on tiger grouper fish. These properties enable the OMPs to be used in developing antibody-based rapid detection systems.

There have been many studies on the outer membrane profile of *V. parahaemolyticus*, but the protein profile of this bacterium is subjected to change due to many factors such as the environment, habitat, and niche condition in the host and temperature, such that gene expression subsequently affects protein synthesis.[34,35] Moreover, the methods and materials used for bacterial culture also have an effect. This is consistent with several research findings showing that there are differences in the molecular weight (MW) of the OMP of *V. parahaemolyticus* bacteria following different treatments. A study conducted by Maniyankode *et al*.[25] identified six outer membrane protein bands of *V. parahaemolyticus* ATCC 17802® with MWs of 116, 66, 45, 35, 25, and 18 kDa (Maniyankode *et al*., 2013). However, in a study by Abdallah *et al*.[21] on the same strain of bacteria in the context of UVC radiation treatment (240 J/m^2), it was found that, before radiation treatment, *V. parahaemolyticus* had 15 bands on the protein profile with six major OMP at 17, 18, 20, 21, 22, and 23 kDa, UVC radiation-induced changes to the OMP. These changes were manifested by the appearance or disappearance of proteins. The results of this study indicated that the sustainable characterization of protein bands is required, thus enriching the knowledge of the outer membrane protein profile of *V. parahaemolyticus* ATCC 17802®.

One effort that can be made to determine the effectiveness and characterization of proteins is to map proteins on the outer membrane (OMPs) of bacterial cells using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The aim of this study was to analyze the outer membrane protein profile of *V. parahaemolyticus* ATCC 17802® to aid in the development of antigenic candidates to detect *V. parahaemolyticus* in food efficiently and quickly.

MATERIALS AND METHODS

Tools and Materials

The materials used in this study were a pure culture of *V. parahaemolyticus* ATCC 17802®, obtained from AHM Biotech Indonesia (Bogor, West Java), TCG agar-BHI broth, TCA, sterile PBS, SDS, reducing sample buffer (RBS), Coomassie blue, methanol (Merck), glacial acetic acid, aquabidest, deionize water (SWFI), acrylamide (Sigma), Gibbus, APS (Sigma), TEMED (Merck), TBS base and buffer TBE, handscoon (Suntouch), nitrile gloves, Tris-HCl pH 8.8 and Tris-HCl pH 6.8 (Biomed), glycine (Bioworld), face masker, Sigma marker protein (MW 10–260 kDa) (GeneOn), Tris base (Sigma), sterile water, and yellow and blue tips (Biologix). The tools used in this study were test tubes, beaker glass, Petri dishes, micropipette, a laminar air flow hood, incubators, test tube rack, autoclave, glass plate, vortex, freezer, clamp Eppendorf tool incubator, pili cutter, centrifuge, yellow and blue tips (Biologix), inoculation needles, centrifuges, an electrophoresis apparatus (Bio-Rad), and a Gel Doc apparatus (BioRad).

Isolation of outer membrane protein

Before the OMPs were isolated, the pili of *V. parahaemolyticus* bacteria were cut. The isolation of OMP was carried out according to Sumarno, which was a modification of Evan's method.[36] The pellet was resuspended with PBS pH 7.4 until its volume was increased 10 times, then 0.05% SDS was added to 10 ml of PBS 0.005 ml and homogenized until dissolved. The clamp Eppendorf was stored in the freezer for 1 min. After that, it was vortexed for 1 min and then centrifuged at 12,000 rpm for 15 min isolated until a solution was saturated in the centrifuge again until it was translucent. The supernatant was taken and analyzed. The fluid was analyzed in the first 24 hours using H_2O and in the second 24 hours using PBS pH 7.4.

SDS-PAGE

The OMPs were detected using SDS-PAGE. This MW measurement was in accordance with the protocol proposed by BioRad. First, scanning electron microscopy (SEM) imaging was performed to ensure that the OMPs were free of bacterial pili, as shown in Figure 1. SDS-PAGE used a 12% (weight/vol) running gel and a 4% (weight/vol) stacking gel. The sample of OMP was diluted in RBS in a 1:1 ratio with a total volume of 20 ml and heated at 95°C for 10 min. SDS-PAGE began after assembling the apparatus; then, 10 µl of the marker was put into the first well and 20 µl of OMP was put in the other wells. Electrophoresis was carried out at 200 V for 50 min. Then, the gel was taken and stained with 0.1% Coomassie blue for 18 h and stained with a mixture of methanol 50%, acetic acid 40%, and aquabidest 40% (vol/vol) until the background was clear. The gel was stored in 10% acetic acid solution (referred to as crude protein).

OMP weight calculation of SDS-PAGE results

The OMP MW calculation was performed by Gel Doc (BioRad). The preparation of the gel. (1) Preparation of separating gel: Dissolved in a beaker glass 3.4 ml deionize water (SWFI), 1.3 ml acrylamide, 2.5 ml Gibbus 8.8, 100 µl SDS 10%, 100 µl APS 10%, and 10 µl TEMED and (2) the preparation of stacking gel: 3050 ml Devon water, 650 µl 10% acrylamide, 2.5 ml TBS base 8.8, 50 µl 10% SDS, 50 µl APS 10%, and 5 µl TEMED.

The separation process of the OMP of *V. parahaemolyticus* bacteria was done by seeding bacteria in biphasic TCG agar-BHI broth and incubating at 37°C for 24 h. After that, ultrasonication and centrifugation were carried out. The supernatant was discarded and the sediment was resuspended using PBS 10 times the volume of pellets. After that, the pili were cut and the OMPs were imaged using SEM with a magnification of ×10 to show that the OMP was free of pili [Figure 1]. Pili cutting were performed to obtain pure OMP because this would affect the results.

RESULTS

SDS-PAGE profile of OMP

The OMPs were then analyzed using SDS-PAGE to determine the types of proteins found in the outer membrane of *V. parahaemolyticus*. The advantages of this method are that the mechanism of protein classification is based on MW. The electrophoresis of the outer membrane protein in this study used a 12% separating gel. The protein bands are the results of SDS-PAGE electrophoresis [Figure 2]. The calculation of the MW of the OMP of *V. parahaemolyticus* was performed by Gel Doc (BioRad) [Figure 2] and compared with marker protein bands with an MW between 10 and 260 kDa.

Based on the SDS-PAGE results [Table 1] using 0.1% Coomassie blue staining, the OMP profile showed 31 bands. However, there were only about 10 prominent bands, which had MWs of 72, 67, 58, 52, 44, 42, 40, 38, 37, and 10 kDa. The MW of these proteins was determined based on interpolation using the marker (low molecular marker protein ladder).

DISCUSSION

The results of a study by Honda *et al*. showed that pili are widely distributed in *V. parahaemolyticus* and can trigger antibody responses.[37] Besides, pili can stimulate a protective response that targets antigen O, which can be used as a basis for the development of various protective bioconjugate vaccines against Gram-negative bacteria. Therefore, it is very important to separate the pili from the bacterial membrane.

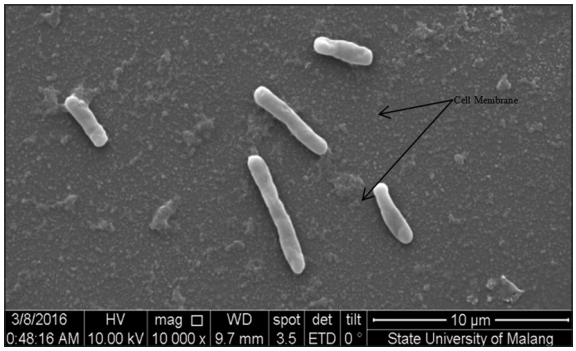

Figure 1: Outer membrane proteins *Vibrio parahaemolyticus* bacteria using scanning electron microscopy magnification of 10 kx (2) (Source: Private collection 2016)

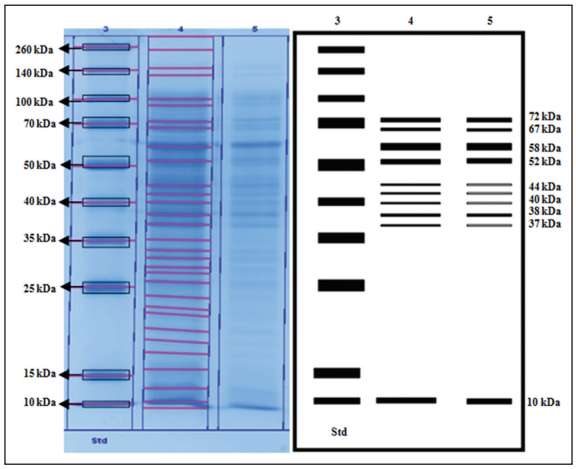

Figure 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane protein profiles of *Vibrio parahaemolyticus* ATCC 17802®, with Coomassie blue staining. Lane M marker, lane 1 and 2 are outer membrane protein of *V. parahaemolyticus*

Table 1: Molecular weight of outer membrane protein bacteria *V. parahaemolyticus*

Protein marker	Weight molecular of MPO
260	72
140	67
100	58
70	52
50	44
40	42
35	40
25	38
15	37
10	10

V. parahaemolyticus: Vibrio parahaemolyticus

In contrast to the results of this research, the research conducted Maniyankode *et al*. using the same strain of *V. parahaemolyticus* bacteria found four bands with an MW of about 52, 36, 22, and 21 kDa (Maniyankode *et al*., 2013). The research conducted by Koga and Kawata[38] also found six major bands on the outer membrane of *V. parahaemolyticus* bacteria strain 3283-61 (serotype O2: K3): 44, 36, 33, 26, and 22 kDa. The different types of proteins identified in the outer membranes of *V. parahaemolyticus* bacteria were due to the different methods, materials, and environment of the bacterial culture. These factors are known to affect the expression of genes that encode proteins in the outer membrane 9.[39] In general, environmental change affects the expression of OMP, and the increase in temperature may cause significant changes in the expression of OMP *Escherichia coli*.[40,41] The results of the research conducted by Abdallah *et al*.[21] on *V. alginolyticus* and *V. parahaemolyticus* also found that environmental stress can modify the synthesis speed of certain proteins that affect the outer membrane protein profiles.

Moreover, there are also some other factors such as serogroup differences, habitat variations, host range, and virulence[35] also known to influence the composition of the OMP *V. parahaemolyticus* bacteria. The strain of *Aeromonas bacteria* belonging to similar or different serogroups showed a difference in the patterns of protein (Sachan *et al.,* 2012).[41] The proteins on the outer membrane of *V. parahaemolyticus* have an important role during the cellular, and physiological processes, feel and respond to changes in the external environments effectively, and play an important role during the infection and induction of the host immune responses.[42,43] In an environment or food chain, cells of *V. parahaemolyticus* usually are forced into the stationary phase, the general phase to survive in the environment of various stress conditions including starvation, extreme temperatures, low pH, oxidative stress, shock osmotic, and exposure to UV light (DNA damage).[44] The proteins on the outer membrane of bacteria play an important role in the process of adaptation to environmental stress that will determine the pathogenicity of bacteria.[34]

The SDS-PAGE results on the OMP of *V. parahaemolyticus* show that the MW of the major polypeptide bands was in the range of 10–100 kDa. These results were obtained by comparing the band obtained with the standard protein MW. Large bands were observed in the range of 10.37 MW–72 kDa. In addition to the major band, there were also some minor bands with MWs in the range of 36–12 kDa.

The existence of two groups of proteins was an indication that there were differences in the level of the expression of each protein of the OMP of *V. parahaemolyticus*. Normally, the proteins on the outer membrane of bacteria are expressed in accordance with the objectives of the bacteria. The proteins on the outer membrane are expressed by cells with the aim of maintaining cell integrity and physiological purposes such as pore formation and mediated transport of molecules; OMP also acts as receptors.

This study found several types of OMP that has the potential to be used as an antigen and could trigger immune reactions. These proteins had MWs ranging from 10 kDa to 72 kDa. This is in line with the work of Maniyankode *et al.* showing that proteins with an MW of 18 kDa–54 kDa in the outer membrane of *V. parahaemolyticus* bacteria are potentially antigenic. The proteins on the outer membrane of *V. parahaemolyticus* have the potential to modulate the immune reaction. A study by Marlina[13] found that the crude extract of OMP *Aeromonas sobria* can trigger an immunogenic reaction, which is helpful for developing an antibody-based detection system. The proteins on the outer membrane are the primary antigens of bacteria. A study conducted by Nehlah *et al*.[20] found that some proteins with MWs of 23, 31, and 34 kDa isolated from the OMP of *V. alginolyticus* could be used for an effective vaccine because they can trigger an antibody response.

CONCLUSION

The SDS-PAGE analysis of the OMP of *V. parahaemolyticus* showed that the MWs of the major polypeptide bands were in the range of 10–100 kDa. Overall, there were 31 bands observed, of which 11 were major bands with MWs of 72, 67, 58, 44, 42, 40, 38, 37, and 10 kDa. Further, the analysis showed that the identified proteins have antigenic properties and could be used to produce antibodies for the development of a rapid detection system to detect the presence of *V. parahaemolyticus*.

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