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# Detection of β-haemolysin Gene in *Aeromonas* Isolated from Retail Fish in Malaysia

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**Abstract:** A total of 29 samples of *Aeromonas veronii biovar sobria* and 8 samples of *Aeromonas hydrophila* were isolated from retail fish in Malaysia wet market. Haemolysin-specific polymerase chain reaction approach was used to screen the β-haemolysin gene in these samples. In this study, we found approximately 57% of the Aeromonas samples showed haemolytic activity on agar plate. Six out of the haemolytic *Aeromonas* spp. and 1 non-haemolytic sample were detected carrying the β-haemolysin gene by using PCR. This β-haemolysin gene was also detected in *Aeromonas veronii biovar sobria* in this study which is not being reported in other study.

**Key words:** Aeromonas, β-haemolysin gene, Polymerase chain reaction

## Introduction

Aeromonas species are gram-negative rod-shaped bacteria that can be found naturally in aquatic environment. Historically *Aeromonas* belongs to the family Vibrionaceae, but now it is grouped in its own family, Aeromonadaceae (Chopra and Houston, 1999). *Aeromonas* can be characterized into at least 14 genospecies which can be further subdivided into two large groups, the mesophilic, motile such as *A. hydrophila, A. veronii biovar sobria* and *A. caviae*, and the non-motile, psychrophilic group such as *A. salmonicida* (Merino *et al.*, 1995). The mesophilic *Aeromonas* has always been associated with human infections while *A. salmonicida,* the only non-motile *Aeromonas* has been considered as fish pathogen (Khalifa *et al.*, 2001 and Cipriano, 2001).

Aeromonas is widely distributed in both fresh and salt water and most of the food we consume such as meat, vegetables and seafood. They can also be found in soil, sewage and wastewater (Son *et al.*, 2003). Transmission was found largely due to ingestion of contaminated water, food or inoculation of bacteria through an opening in the skin. Thus, indicates why this organism is closely related to two major human diseases, gastroenteritis and septicemia (Merino *et al.*, 1995).

Generally, the mesophilic *Aeromonas* are considered as potential human pathogens. These *Aeromonas* species have been associated with a wide range of infections in humans, including septicemia, meningitis, pneumonia, gastroenteritis and respiratory tract infections such as epiglottis (Chopra and Houston, 1999). Systemic infections are also found due to contamination of lacerations and fractures with *Aeromonas*-rich waters. *Aeromonas* species that commonly associate with gasroenteritis are *A. caviae, A. hydrophila* and *A. veronii biovar sobria.* Therefore, these *Aeromonas* species are considered as enteropathogens and appear to be an important cause of acute diarrhoea in children. These *Aeromonas* species can also cause sporadic diarrhoea or dysentery in immuno-compromised patients, which can be severe or even life-threatening. An increase incidence of *Aeromonas* isolated from travellers with diarrhoea also showed that they may be an important cause for the infection (Janda, 1991; Wang *et al.*, 2003 and Vila *et al.*, 2003). It is also a concern in fish diseases as *Aeromonas* spp. live in aquatic environment (Baloda *et al.*, 1995; Thayumanavan *et al.*, 2003 and Castro-Escarpulli *et al.*, 2003).

A number of factors, such as immuno-competence, underlying illness and age of the patients, infection dose and expression of sufficient virulence factors by *Aeromonas* can affect the ability of this organism to cause disease. It was found that mesophilic *Aeromonas* have ability to express a range of extracellular biologically active substances, as possible virulence factors that play an important role in its pathogenicity. These main putative virulence factors are exotoxins, endotoxins, presence of S-layers, fimbriae or adhesins and its capability to form capsules (Merino *et al.*, 1995). One of the exotoxins, haemolysin is considered to play a vital role in its enterotoxigenicity (Asao *et al*, 1986). This has been demonstrated by fluid accumulation in the rabbit ileal loop test after several passages and the suckling mice, which showed *Aeromonas* as being a potential pathogen in gastrointestinal disease (Son et *al.*, 2003; Janda, 1991 and Asao *et al.*, 1986). The clear zone formation on blood agar due to haemolysis indicates the production of haemolysins by several *Aeromonas* strains. Haemolysins belong to a larger group of pore-forming bacterial cytolysins, can cause leakage of the cytoplasmic contents by disrupting of the cell membrane, followed by death of the cell (Kingombe *et al.*, 1999 and Wang *et al.*, 2003). Thus, it clearly shows how dangerous an *Aeromonas* infection could be.

There are several types of haemolysins produced by this organism including beta-haemolysin and alpha-haemolysin (Janda, 1991). It was known that the majority of *Aeromonas* species that isolated from infected human are haemolytic. Furthermore, a research carried out by Xia *et al.*, 2004 showed that all pathogenic strains in humans

contain the β-haemolysin gene.

## Materials and Methods

**Bacterial Samples and Culture Media:** Thirty seven samples of *Aeromonas* spp. isolated from retail fish were used in this study. The samples were given by Professor Son Radu from University Putra Malaysia. All the *Aeromonas* samples was characterized up to species level in previous study (Son *et al.*, 2003). Generally, the bacteria including *E.coli* culture (ATCC 25922) were grown on Brain Heart Infusion agar (0.78% w/v brain extract, 0.97% w/v heart extract, 1% w/v peptone, 0.5% w/v sodium chloride, 0.25% w/v disodium hydrogen phosphate, 0.2% w/v dextrose, 1.5% w/v agar) with 5% defibrinated human blood or brain heart infusion broth overnight at 37°C prior to any experiment.

**Determination of Haemolytic Activity:** The study of haemolytic activity was carried out by culturing the bacteria on a blood agar. Positive haemolytic activity was shown as a clear haemolysis zone formation surrounding the bacterial colony on the blood agar plates after overnight incubation at 37°C.

**Preparation of Bacterial Template DNA:** A method used for genomic DNA preparation in this study was adapted from Baloda *et al.*, 1995. Basically, a colony of the bacteria was suspended in 200 µl of sterile distilled water in a microfuge tube. The suspension was then heated to 100°C for 10 min followed by centrifugation at 10,000 xg for 5 min. The supernatant containing bacterial genomic DNA was transferred to a new microfuge tube and can be used for polymerase chain reaction.

**Polymerase Chain Reaction and Electrophoresis:** Polymerase chain reaction (PCR) was performed using the reagents purchased from Promega, U.S.A.The β-haemolysin specific oligonucleotide primers (Research Biolabs) were synthesized according to the sequence published by Xia *et al.*, 2004. The reaction mixture was prepared according to the manufacturer's guidelines. The 25 µl PCR mixture contained 200 ng of bacterial total genomic DNA, 1X reaction buffer, 0.3 mM dNTP mix, 2.0mM $MgCl_2$, 1µM of each β-haemolysin forward (5'-CAA GGA GGT CTG TGG CGA CA-3') and B-haemolysin reverse (5'-TTT CAC CGG TAG CAG GAT TG-3') primers, appropriate amount of deionized water and 2.5U of Taq DNA polymerase. The PCR was performed in a termal cycle (Techne) under the following optimised conditions: 94°C for 3 min (1 cycle), 94°C for 1 min, 50°C for 45 seconds, 72°C for 45 seconds (30 cycles) and 72°C for 10 min (1 cycle). Ten µl of the PCR products was then electrophoresed on 1% agarose gel. The PCR products were visualized under UV light after staining with 0.5 µl/ml ethidium bromide and photographed.

## Results

From a total of 37 *Aeromonas* spp. tested, 21 or ≈57% of the samples were found have positive haemolytic activity on blood agar plate. These included 14 out of 29 samples of *A. veronii biovar sobria* and 7 out of 8 *A. hydrophila* samples as shown in Table 1. The size of the amplified products obtained by the PCR was ≈208 bp as predicted. Of the 37 *Aeromonas* isolates tested, the target gene was detected in 7 isolates. These included 2 out of 8 *A. hydrophila* and 5 out of 29 *A. veronii biovar sobria*. All except 1 of the *A. veronii biovar sobria* were also performed positive haemolytic activity in blood agar plate. No PCR product was obtained when total genomic DNA of *E.coli* ATCC 25922 was used as the template. Part of the PCR results is shown in Fig. 1(A) and (B).
One hundred bp ladder (Fermentas) (lane 1, 14 and 15), water as the negative control (lane 2), *E. coli* ATCC 25922 as the negative control (lane 3). A 208 bp DNA fragment was detected in *A. veronii biovar sobria* (lane 7, 8, 9 and 11) and *A. hydrophila* (lane 13 and 19) when the samples were separated on 1% agarose gel followed by ethidium bromide staining.

## Discussion

In this study, PCR was carried out to detect the 208 bp β-haemolysin gene from *Aeromonas* isolates in Malaysia. Based on the results, most of the *Aeromonas* samples obtained from Malaysia wet market were found carrying the β-haemolysin gene. The size of the β-haemolysin gene detected is similar to what was reported in China (Xia *et al.*, 2004). However besides *A. hydrophila,* some of our *A. veronii biovar sobria* isolates were found carrying the β-haemolysin gene. This is different from what was previous reported by Xia *et al.*, 2004 where this gene was only found in *A. hydrophila.*
In this research, some of the *Aeromonas* isolates showed positive result in β-haemolytic activity but the β-haemolysin gene could not be detected in PCR study. This might be due to the particular *Aeromonas* carry other haemolysin gene such as *ahh*1 gene, *aer*A gene, *asa*1 gene that encodes haemolysin toxin as well (Wang *et al.*, 2003; Heuzenroeder *et al.*, 1999; Baloda *et al.*, 1995 and Pollard *et al.*, 1990).

Table 1: Summary of organisms, haemolysin production, and PCR probe results of the *Aeromonas* strains

| Strains | Organism | Heamolysin[a] | PCR amplification product (208 bp)[b] |
|---|---|---|---|
| AH1 | *A. hydrophila* | + | + |
| AH2 | *A. hydrophila* | + | - |
| AH3 | *A. hydrophila* | + | - |
| AH4 | *A. hydrophila* | + | - |
| AH5 | *A. hydrophila* | + | + |
| AH6 | *A. hydrophila* | + | - |
| AH7 | *A. hydrophila* | + | - |
| AH8 | *A. hydrophila* | - | - |
| AS1 | *A. sobria* | + | - |
| AS2 | *A. sobria* | + | - |
| AS3 | *A. sobria* | + | - |
| AS4 | *A. sobria* | + | - |
| AS5 | *A. sobria* | + | + |
| AS6 | *A. sobria* | + | + |
| AS7 | *A. sobria* | + | - |
| AS8 | *A. sobria* | + | + |
| AS9 | *A. sobria* | + | - |
| AS10 | *A. sobria* | + | - |
| AS11 | *A. sobria* | + | - |
| AS12 | *A. sobria* | + | - |
| AS13 | *A. sobria* | + | - |
| AS14 | *A. sobria* | + | + |
| AS15 | *A. sobria* | - | - |
| AS16 | *A. sobria* | - | - |
| AS17 | *A. sobria* | - | - |
| AS18 | *A. sobria* | - | - |
| AS19 | *A. sobria* | - | - |
| AS20 | *A. sobria* | - | - |
| AS21 | *A. sobria* | - | - |
| AS22 | *A. sobria* | - | - |
| AS23 | *A. sobria* | - | + |
| AS24 | *A. sobria* | - | - |
| AS25 | *A. sobria* | - | - |
| AS26 | *A. sobria* | - | - |
| AS27 | *A. sobria* | - | - |
| AS28 | *A. sobria* | - | - |
| AS29 | *A. sobria* | - | - |
| ATCC 25922 | *E.coli* | - | - |

[a] Presence or absence of haemolysin production of the strains.
[b] Presence or absence of β-haemolysin gene.

In this case, we cannot conclude the particular *Aeromonas* isolate possesses the β-haemolysin gene just based on its β-haemolytic activity on blood agar. Therefore, a more reliable method such as PCR can be used for further gene characterization, detection and confirmation.

There is an exception where β-haemolysin gene is detected in an *Aeromonas* isolate by PCR but haemolytic activity of the isolate was no shown on blood agar plate. This unusual phenomenon might be due to the β-haemolysin gene is suppressed or had been mutated due to some environmental factors and cause the bacteria lost its haemolytic activity (Wang *et al.*, 2003).

β-haemolysin has been reported as an important virulence factor in understanding the pathogenicity of *Aeromonas* spp. (Asao *et al.*, 1986; Pollard *et al.*, 1990 and Baloda *et al.*, 1995). It is also very important for us to take this matter seriously as *Aeromonas* is an emerging pathogen that lives in the aquatic environment, which has direct access to the fishes that we consume. Although the prevalence of food bone infections due to *Aeromonas* infection in Malaysia is low at this moment, the bacteria might become an emerging human pathogen one day since the trend of raw seafood consumption is getting popular in this country (Chopra and Houston, 1999 and Son *et al.*, 2003).

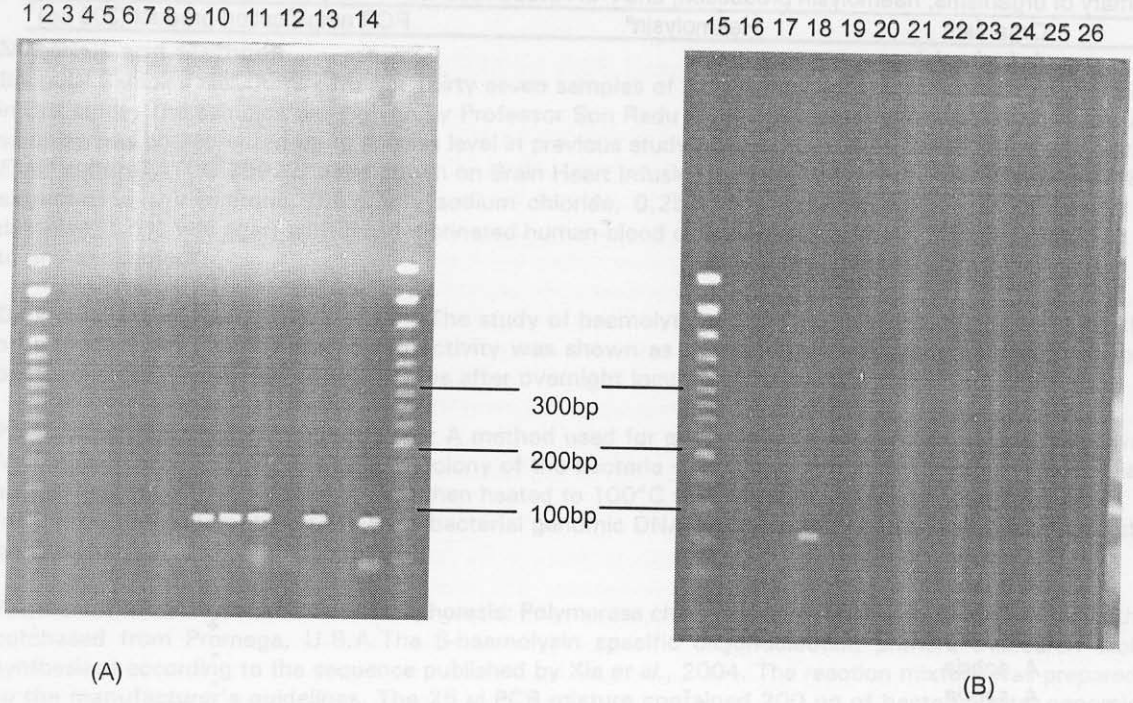


Fig. 1(A) and (B) : Detection of β-haemolysin gene in *Aeromonas* Species by PCR

Overall, a reliable detection method such as multiplex PCR can further be developed for rapid characterization and detection of the *Aeromonas* infection based on their haemolysin genes in the future.

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