# MOLECULAR IDENTIFICATION AND CLONAL RELATION OF ATYPICAL ISOLATE *Aeromonas salmonicida* USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

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**ABSTRACT**

*Aeromonas salmonicida* is responsible in many cases of furunculosis outbreaks resulting in economic loss of freshwater aquaculture. Four isolates of *A. salmonicida* have been isolated from fish collected in four different regions in Indonesia and its clonal relation has yet to be determined. In the research, *A. salmonicida* isolates and ATCC atypical isolate as the control had been checked for their clonal relation using Restriction Fragment Length Polymorphism (RFLP) method in which restriction enzyme of *Alu*I, *Hae*III, *Mbo*I and *Ehe*I were used. PCR test results using the primers 16S rDNA amplicon gave a positive response to the 1300 bp band. The result of RFLP analysis showed that *A. salmonicida* atypical isolates from Indonesia represent subspecies *smithia* except isolates from *C. macropomum* in Yogyakarta in MS and 16S rDNA regions. Isolates from Jambi, Pontianak and Semarang showed a slight variation on enzyme restriction sites. Isolates number 2, 3, 4 and 5 had the same restriction sites using *Alu*I enzyme with MS primer. The restriction enzymes that could give the best result for RFLP method of *A. salmonicida* were *Hae*III, *Mbo*I and *Ehe*I.

**KEYWORDS: *A. salmonicida,* furunculosis, PCR, RFLP, restriction enzyme**

## INTRODUCTION

*Aeromonas salmonicida* is the agent of etiological furunculosis disease which strikes many fish farmings and has caused a significant economic loss in freshwater aquaculture (McCarthy & Roberts, 1980; O'Brien *et al.,* 1994). Typical and atypical terms of *A. salmonicida* are used to differentiate among strains causing furunculosis on salmon and other strains causing furunculosis on both Salmon and other fishes (Austin & Austin, 1999; Inglis *et al.,* 1993).

Clinical signs of furunculosis include septicemia, losing of appetite, weakness and necroses, and hemorrhagic of gill, muscle and intestine (Hiney & Olivier, 1999). This bacterium has also been attributed to disease of ulcer on carp and flat fish and also erythrodermatitis on carp fish (Austin & Austin, 1999).

*A. salmonicida* is Gram-negative bipolar, having the character of optional anaerobic, motion less, no spore, no capsule, in form of short rod, fairish 1,3–2,0 µ best growth at temperature 18°C-25°C (Sakazaki & Ballows, 1981; Untergasser, 1989; Inglis *et al.,* 1993; Austin & Austin, 1999). *Aeromonas salmonicida* can grow in various media for example

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*Trypticase Soy Agar* (TSA) and *Brain Heart Infussion Agar* (BHIA) (Inglish *et al.*, 1993; Austin *et al.*, 1998).

Using RFLP of 16S rRNA gene, specific pattern-type for all strains of species *A. salmonicida* can be determined (Borrel *et al.,* 1997; Figueras *et al*., 2000). This method used two endonucleus simultan *Alu*I (AGCT), *Hae*III, *Nar*I and *Mbo*I (GATC) (Borrell *et al.,* 1997). The addition of two enzymes type *Nar*I (GGCGCC) and *Hae*III (GGCC) were needed to differentiate gene 16S rDNA *A. salmonicida, A. encheleia* of HG 11 (group hybridization 11) of *Aeromonas* spp. (Figueras *et al.,* 2000).

The research was aimed to study clonal relationship of all atypical isolates of *A. salmonicida* using PCR and Restriction Fragment Length Polymorphism (RFLP) from several areas in Indonesia .

## MATERIALS AND METHODS

### Fish

Fish samples used in this research was 470 individuals of goldfish (*Carpio cyprinus*) sized ± 10 cm in average weight. The fish was collected from several freshwater areas in Indonesia characterized by the occurrences of *A. salmonicida.*

### Isolate

*Aeromonas salmonicida* used were isolates from ATCC (isolate 1), *C. macropomum* in Yogyakarta (isolate 2), *O. niloticus* in Jambi (isolate 3), *C. carpio* in Pontianak (isolate 4), and *O.gouramy* in Semarang (isolate 5).

As the control was atypical isolate of *A. salmonicida* subsp. *smithia* (Austin *et al.* Product of Americans Type Culture Collection (ATCC) Number : 49393 by RFLP).

### PCR

Materials for DNA Amplification and Electrophoresis: easy DNA mini column (Qiagen), PCR Kit (Promega) for PCR Amplification of 16S rDNA using the primers 1A (5'-CGTTGGATATGGCTCTTCCT-3') and 16S rDNA 2A (5'-CTCAAAACGGCTGCGTACCA-3') (Hiney *et al*., 1992); primer MS1 (5'-AGGATGGCCTGCTCGTCACC-3') and MS2 (5'-GTGACG CCCAGGCCA TCCTC-3 ') (Fehr *et al*., 2007).

### RFLP

Materials used during the RFLP examination were : enzyme of *Alu*I (5'AGCT), *Hae*III (5'GGCC), *Mbo*I (5'NGATCN) and *Ehe*I (5'GGCGCC), acetic acid glacial, buffer loading (consisted of 0,25% blue bromphenol, 0,25% cyanol xylene and 30 % glyserol), 4% gel agarose metaphor (PMC Bioproduct) in 0,5 TBEx (Tris-Borate-EDTA) Buffer (Metaphor Agarose Gel stained using bromide ethidium 0,5 µg/ml), $dH_2O$, sol I for 100 ml ($AgNO_3$ 0,15 gr + 37%), sol II for 100 ml ($Na_2CO_3$ : 3gr + Formaldehyde 151 ml + sodium thiosulfat 5 µg/261 mg/ml where is endconcentration 2 mg/ml) and 8 % polycrylamide gel.

### Characterization

Characterization of bacterium was done through identification of morphology, such as Gram test, motility and ability of fermentation, IMVIC, and biochemical examination using biochemistry test.

## RESULTS AND DISCUSSION

### Results

The results of 16S rDNA amplification with a primer on all isolates of *A. salmonicida* gave a positive response to the 1300 bp band. The results also showed that isolates from Yogyakarta, Semarang, Pontianak and Jambi has similarities to isolates of *A. salmonicida.* This ATCC isolates amplified with the characterization of isolates from Indonesia indicated *A. salmonicida* (Figure 1).

The results of the test using RFLP on four isolates of atypical *A. salmonicida* from several regions in Indonesia and isolates from the ATCC with MS primary did not show any band with a restriction enzyme *Alu*I (Figure 2 and 3). Enzymes *Alu*I did not give a positive response to the five isolates because this enzyme has no restriction sites of DNA on all isolates. This can occur because of several factors that affect the action of the enzyme, namely: temperature (0°C: no activity; 38°C-40°C: increased enzyme activity, >38°C: decreased enzyme activity; 60°C: enzyme activity is discontinued), water, pH, concentration of enzyme (speed the process of formation or decomposition of the molecule following the concentration of enzyme substrate) and inhibitors (Brown, 2002).

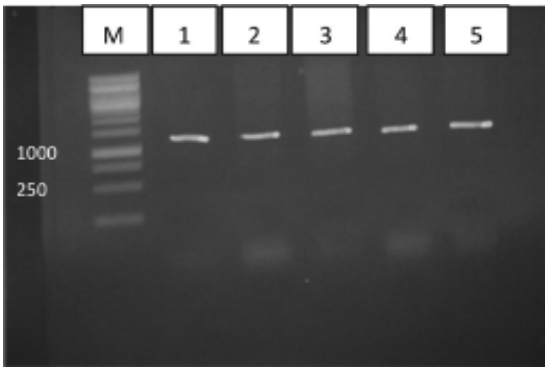


Figure 1. Results amplification *A.salmonicida* 4 isolates from Indonesia and from ATCC in the area of 16S rDNA bands appear at 1300 bp. (M: marker; 1: isolate ATCC; 2: Yogyakarta isolates; 3: Semarang isolates; 4: isolates Pontianak; 5: isolates Jambi

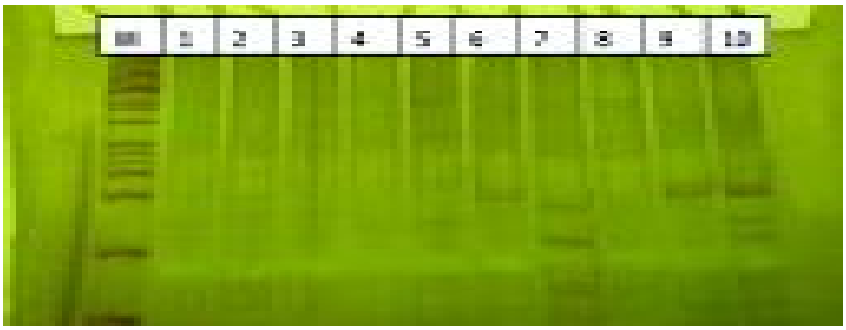

Figure 2. Result of RFLP-PCR method with MS primer of *A.salmonicida*, using *Alu*I enzyme (lanes 1-5) and using *Hae*III enzyme (lanes 6-10) , M= marker; 1 dan 6 : ATCC isolate, 2 and 7 : Yogyakarta isolate, 3 and 8 : Semarang isolate, 4 and 9 : Pontianak isolate, 5 and 10 : Jambi isolate

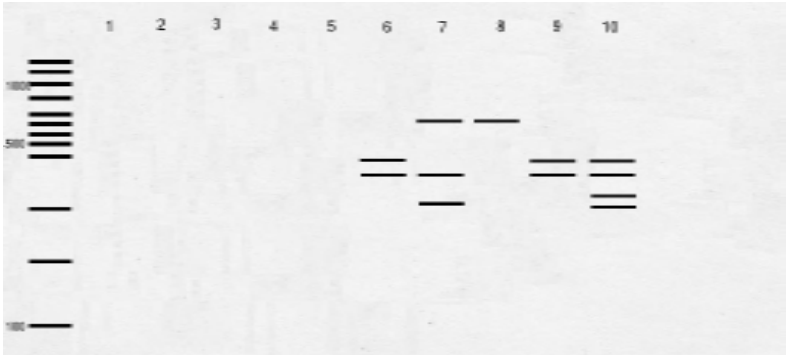

Figure 3. Scheme of RFLP-PCR method using the primary MS *A.salmonicida*, using enzymes *Alu*I (lanes 1-5) and enzymes *Hae*III (lanes 6-10), M = marker; 1 dan 6 : ATCC isolate, 2 and 7 : Yogyakarta isolate, 3 and 8 : Semarang isolate, 4 and 9 : Pontianak isolate, 5 and 10 : Jambi isolate

*Hae*III restriction enzyme showed similarity with isolates no. 1 and 4. Isolates 2, 3, 5 had very different places of restrictions on each other (Figure 4 and 5). Side-cutting enzymes used is shown in Table 1.

*Hae*III enzyme was used for PCR-RFLP along with the MS and 16S rDNA primers. With the MS primer, ATCC isolates showed the same restriction sites in isolates 4, while the isolates 2, 3, and 5 respectively show different restriction

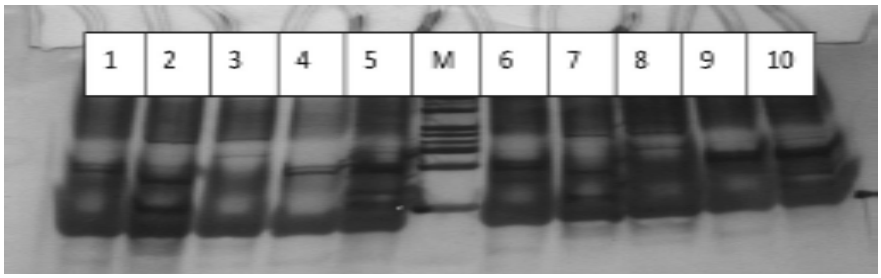


Figure 4. RFLP-PCR method of *A. salmonicida* atypical isolate using *Hae*III enzyme with MS primer (lanes 1-5) and PCR in 16S region (lanes 6-10). M= marker; 1 and 6 : ATCC isolate, 2 and 7 : Yogyakarta isolate, 3 and 8 : Semarang isolate, 4 and 9 : Pontianak isolate, 5 and 10 : Jambi isolate

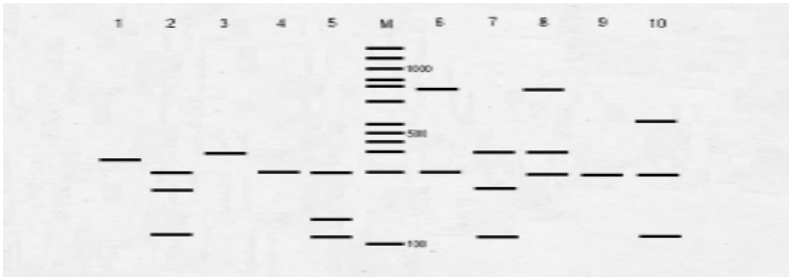


Figure 5. Scheme of RFLP-PCR method to isolate atypical *A. salmonicida* using the enzyme *Hae*III with MS primer (lanes 1-5) and the PCR in 16S region (lanes 6-10). M = marker; 1 and 6 : ATCC isolate, 2 and 7 : Yogyakarta isolate, 3 and 8 : Semarang isolate, 4 and 9 : Pontianak isolate, 5 and 10 : Jambi isolate

Table 1. Recognition results of the various restriction enzyme sites of the samples

| Restriction enzyme | Recognition site | Cutting side | Cut result |
|---|---|---|---|
| *Hae* III | 5'GGCC | 5'...GG ↓ CC... 3' | Positive |
| | 3'CCGG | 3'...CC ↑ GG... 5' | |
| *Alu* I | 5'AGCT | 5'...AG ↓ CT... 3' | Negative |
| | 3'TCGA | 3'...TC ↑ GA... 5' | |
| *Mbo* I | 5'NGATCN | 5'... N ↓ GATCN... 3' | Positive |
| | 3'NCTAGN | 3'... NCTAG ↑ N... 5' | |
| *Ehe* I | 5'GGCGCC | 5'...GGC ↓ GCC... 3' | Positive |
| | 3'CCGCGG | 3'...CCG ↑ CGG... 3' | |

sites. The amplification with 16S rDNA primer showed that 5 isolates of *A. salmonicida* have mutually different restriction sites. So, it can be concluded that the five isolates had a variety of strains in the area of the 16S rDNA gene (Figure 4 and 5).

In RFLP testing using *Mbo*I and *Ehe*I enzyme on 5 isolates of atypical *A. salmonicida,* isolates 3, 4, and 5 showed similar results compared to ATCC isolate (1). There was only one isolate which was isolate number 2 (isolates from Yogyakarta) that had different restriction sites compared to four isolates (Figure 6 and 7). The same result was also found in the use of enzymes *Ehe*I. 16S rDNA nucleotide sequences at 118-207 bp cut on *Mbo*I enzyme and the enzyme *Ehe*I 452-1050 bp were consistent with the results of the study by Martinez-Murcia *et al*. (1992) and Borell *et al.* (1997) (Table 2).

**Characterization**

Isolation and identification of *A. salmonicida* from several areas in Indonesia showed the same result.

Biochemical test against all isolates of atypical *A. salmonicida* suggests that there are similarities between isolates of atypical *A. salmonicida* subsp. *smithia* and ATCC by looking on the test results of ornithin dexarboxylase (-), the gas produced TSI (-), Voges-Proskauer test (-), acid from arabinose (+), sacrose (+), acid from D-sorbitol (SOR) (- ), acid from lactose (-), catalase (+), its ability to grow at 37° C (-), indole (-), methyl red (-), O / F (F), motility (-) and utilization of citrate (-) (Table 3).

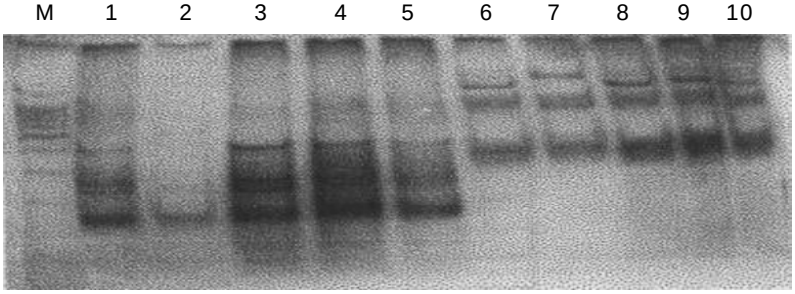


Figure 6. RFLP-PCR method of *A. salmonicida* atypical isolate with MS primer using *Mbo*I enzyme (lanes 1-5) and using *Ehe*I enzyme (lanes 6-10). M= marker; 1 and 6 : ATCC isolate, 2 and 7 : Yogyakarta isolate, 3 and 8 : Semarang isolate, 4 and 9 : Pontianak isolate, 5 and 10 : Jambi isolate

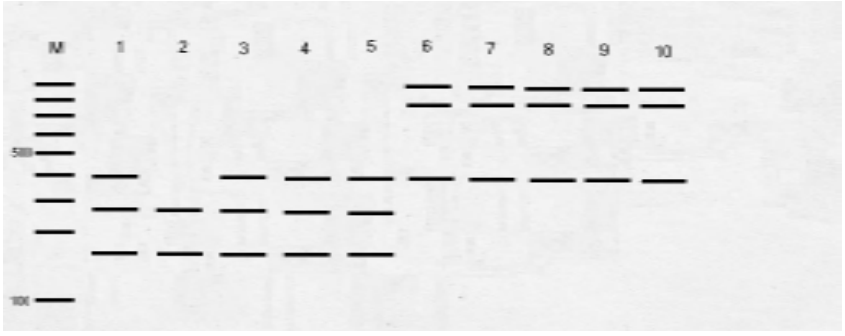


Figure 7. Scheme of RFLP-PCR method to isolate atypical *A. salmonicida* with primary MS using enzyme *Mbo*I (lanes1-5) and using the enzyme *Ehe*I (lanes 6-10). M = marker; 1 and 6 : ATCC isolate, 2 and 7 : Yogyakarta isolate, 3 and 8 : Semarang isolate, 4 and 9 : Pontianak isolate, 5 and 10 : Jambi isolate

Table 2. Comparison with the pattern of restriction enzyme endonukleus of *A. salmonicida* isolates

| Isolate | Enzime | | | |
|---|---|---|---|---|
| | ***Alu* I** | **Hae III** | **Mbo I** | **Ehe I** |
| ATCC | - | 360bp, 380 bp | 180 bp, 380 bp, 400 bp | 750 bp, 850 bp |
| Yogyakarta | - | 300 bp, 360 bp, 700 bp | 180 bp, 280 bp | 750 bp, 850 bp |
| Semarang | - | 300 bp, 320 bp, 360 bp, 380 bp | 180, bp 380 bp, 400 bp | 750 bp, 850 bp |
| Pontianak | - | 360 bp, 380 bp | 180 bp, 380 bp, 400 bp | 750 bp, 850 bp |
| Jambi | - | 700 bp | 180 bp, 380 bp, 400 bp | 750 bp, 850 bp |

Table 3. Result of biochemical characterization of *A. salmonicida* isolated from goldfish collected from Pontianak, Semarang, Yogyakarta, and Jambi

| Test Media | ***A. Salmonicida* atypical isolotes** | | | | |
|---|---|---|---|---|---|
| | **Pontianak** | **Semarang** | **Yogyakarta** | **Jambi** | **ATCC** |
| Brown Pigmen | + | + | + | + | + |
| Blood Agar | *β haemolysis* | *β haemolysis* | *β haemolysis* | *β haemolysis* | *β haemolysis* |
| Mc. Conkey | + | + | + | + | + |
| Agar | (colored) | (colored) | (colored) | (colored) | (colored) |
| Morfologi | Rod | Rod | Rod | Rod | Rod |
| | Gram - | Gram - | Gram - | Gram - | Gram - |
| Voges-Proskauer | - | - | - | - | - |
| Motility | - | - | - | - | - |
| Katalase | + | + | + | + | + |
| Fermentativ | + | + | + | + | + |
| Metabolisme | | | | | |
| Oxidase | + | + | + | + | + |
| TSI Agar : | | | | | |
| -Buff | As | As | As | As | As |
| -Slant | Alk | Alk | Alk | Alk | Alk |
| -H2S | - | - | - | - | - |
| Indol | - | - | - | - | - |
| MR | - | - | - | - | - |
| Sodium citrat | - | - | - | - | - |
| Urea | - | - | - | - | - |
| O/F | F | F | F | F | F |
| Agar 2% NaCl | + | + | + | + | + |
| Agar 4% NaCl | - | - | - | - | - |
| Gelatin | + | + | + | + | + |

Table 3. (Continued)

| Test Media | *A. Salmonicida* atypical isolotes | | | | |
|---|---|---|---|---|---|
| | Pontianak | Semarang | Yogyakarta | Jambi | ATCC |
| DNase | + | + | + | + | + |
| Grown at 37ºC | - | - | - | - | - |
| Glukosa | + | + | + | + | + |
| Laktosa | - | - | - | - | - |
| Sukrosa | + | + | + | + | + |
| Raffinosa | - | - | - | - | - |
| Sorbitol | - | - | - | - | - |
| Maltosa | + | + | + | + | + |
| Arabinosa | + | + | + | + | + |
| Dulcitol | - | - | - | - | - |
| Ornithin | - | - | - | - | - |

**Discussion**

*Hae*III enzyme provided a better result on DNA testing of MS gene RFLP and 16S rRNA of *A. salmonicida*. RFLP testing using enzyme *Mbo*I and *Ehe*I on 5 isolates of atypical *A. salmonicida* showed similar results for isolates 3, 4, and 5 with ATCC isolates (1). Isolates from Yogyakarta (2) has a different restriction site compared to the other four isolates. The same result was also found in the use of enzymes *Ehe*I. The result of 16S rRNA nucleotide sequence cutting in the enzyme *Mbo*I 118-207 bp and 452-1050 bp in *Ehe*I enzymes were consistent with the results of the study by Martinez-Murcia *et al*. (1992) and Borrell *et al*. (1997). Based on these results, it showed a specific pattern of relationships which are very close in all isolates of atypical *A. salmonicida* collection from several regions in Indonesia with atypical isolates of *A. salmonicida* subsp. *smithia* from ATCC. Application of RFLP method does not only uncover patterns that can be grouped in a single subspecies (Huys *et al*., 1997), but also can clarify phenotype pattern of 16S rRNA gene variation within *A. salmonicida* subsp. *smithia* (Graf, 1999).

In this regard, determination of endonucleosa enzyme to distinguish genes using the 16S rDNA RFLP method is basically not easy. Enzyme *Alu*I (5'AGCT) is not effective to be used in the primary RFLP testing of MS, but the enzyme *Hae*III (5'GGCC) can provide a clear difference between the five isolates of *A. salmonicida* either by using the primers MS or 16S. The RFLP test on isolates of atypical *A. salmonicida* on MS rDNA showed that the isolate number 2 (from Yogyakarta) is very different from the other 4 isolates based on the use of *Mbo*I (5'NGATCN) and *Ehe*I (5'GGCGCC) enzymes.

Restriction enzyme that can provide the best result for RFLP method of *A.salmonicida* is *Hae*III, followed in order of accuracy by *Mbo*I and *Ehe*I enzymes.

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