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Evaluation of identification techniques for the fish pathogen, *Aeromonas hydrophila*, from Indonesia

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ABSTRACT

Aims: This study evaluated the accuracy of three methods used in the identification of *Aeromonas hydrophila*, a Gram-negative bacterium found in warm aquatic environments. *A. hydrophila* samples from Indonesia were tested using (a) SNI 7303, developed by the Indonesian government, (b) the method of Dorsch and (c) the method of Cascón. The results obtained were compared to that of the gold standard method, which used 16S rDNA sequences.
Methodology and results: Based on the Indonesian government standard identification method SNI7303, we identified 56 out of 95 samples as *A. hydrophila*. The samples were then screened using the PCR amplification approach developed by Dorsch and Cascón. Of the 56 samples, only 20 samples were found to be positive by either the Dorsch or Cascón methods. DNA from these 20 samples was amplified using common 16S rDNA primers and the sequences compared with available 16S rDNA sequences from the GenBank. Phylogenetic analyses on the data were performed using Clustal X and MEGA 5 software.
Conclusion, significance and impact of study: Of the 56 samples positively identified as *A. hydrophila* using the BSN method, identity in only five samples were positively confirmed using the16S rDNA method, giving an accuracy of only 8.9%. In this connection, the Dorsch method was 31.3% accurate while the Cascón method provided 45.5% correct identification. When all three methods were used in combination, 71.4% of the samples were correctly identified. The results of the study show that methods used to identify *A. hydrophila* cannot be used with confidence to identify *A. hydrophila* from Indonesia and probably from other tropical regions as well. The genetic diversity of *Aeromonas* bacteria in Indonesia appears to be considerably higher than that encountered by Dorsch or Cascón. Therefore, there is a need to develop a new simple method to identify *A. hydrophila* from tropical regions.

Keywords: Aeromonas hydrophila, identification, phylogenetic, 16S rDNA

INTRODUCTION

Aeromonas hydrophila is a pathogenic bacterium found in various aquatic environments, such as fish ponds, rivers, lakes, even in sparkling chlorinated drinking water reservoirs. It is pathogenic to shrimps, frogs, and fishes (Martin-Carnahan and Joseph, 2005; EPA, 2006). The infection caused by this bacterium can lead to significant mortality rates within a short period, causing losses to fish and shrimp breeders (Illanchezian *et al.*, 2010; Mangunwardoyo *et al.*, 2010). The disease symptoms vary, but infected fish generally show skin ulcers and haemorrhaging in the skin, gills, and oral cavity (Gardenia *et al.*, 2010).

High mortality in gourami fish occurring over short periods has caused great losses to fish breeders. For example, in Lubuk Pandan, a city in the West Sumatra Province of Indonesia, 47 tons of gourami worth USD235,000 and 2.7 million juvenile fish worth USD200,000 died in three days (Diraja, 2007 in Eka, 2010). Laboratory experiments have shown that 10^5–10^{10} CFU/mL *A. hydrophila* can kill the fish in only three days after infection (Triyaningsih *et al.*, 2014). Moreover, *A. hydrophila* is frequently associated with human diarrhea. Alberts *et al.* (1990) found that 12.2% of toddlers with acute diarrhea in Dhaka, Bangladesh, tested positive for *A. hydrophila*. Hence, a rapid method to determine *A. hydrophila* concentration in fish ponds and to ensure the supply of safe drinking water is urgently needed.

The difficulty in distinguishing between *A. hydrophila* and other species within the *Aeromonas* genus stems from the complexity of its identifying characters, sometimes varying even within the species (Soler *et al.*, 2004; Ottaviani *et al.*, 2011). Identification using classical

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phenospecific methods is hence error-prone as shown by Beaz-Hidalgo *et al.* (2010) in the recent *Bergey's Manual of Systematic Bacteriology*. For example, a positive ADH (arginine dihydrolase) test and hydrolysis of glutamine cannot differentiate between *A. hydrophila* and *A. sobria*. Moreover, conventional methods that do not employ genetic constitution tend to be tedious and complex (Abbott *et al.*, 2003).

The gold standard method in the identification of bacteria involves analysis of 16S rDNA. However, this method is dependent on DNA sequencing, facilities for which are not available in many laboratories. Therefore, there is a need to develop alternative practical and accurate identification methods for *A. hydrophila*. A standard biochemical method to identify *A. hydrophila* SNI 7303 (BSN, 2009) has been developed by Indonesian Marine and Fisheries Department. This official protocol is frequently used to detect *A. hydrophila* in Indonesia (Tanjung *et al.*, 2013; Hardi *et al.*, 2014; Tulung *et al.*, 2014).

Other DNA-based methods for the identification of *A. hydrophila* have been reported by Dorsch *et al.* (1994) and Cascón *et al.* (1996). Dorsch *et al.* (1994) designed primers to amplify specific regions of the 16S rDNA of *A. hydrophila*. The 685 bp amplicon that distinguishes *A. hydrophila* from other *Aeromonas* species has been used for identification of the former by Chu and Lu (2008) and Pandove *et al.* (2013). Adopting a different approach, Cascón *et al.* (1996) designed PCR primers from the lipase gene of *A. hydrophila* H3. This method is rapid and specific in identifying *A. hydrophila* isolated from aquatic environments (Hiney and Smith, 1998; Abdullah *et al.*, 2003; Salimi *et al.*, 2013; Afsari *et al.*, 2014). The PCR product, a 760 bp amplicon, has also been employed by Lee *et al.* (2000) to identify *A. hydrophila* in diseased fishes in Korea, while Swaminathan *et al.* (2004) successfully detected four out of nine *A. hydrophila* isolates from fish and water using the method of Cascón *et al.*

Nevertheless, both the methods of Dorsch *et al*. and Cascón *et al.* have not been validated against the gold standard established using complete sequence of 16S rDNA (Jiang *et al*., 2006). This is the first research, to our knowledge, that evaluates the accuracy (sensitivity) of the methods of Dorsch *et al.* (1994) and Cascón *et al.* (1996) for the identification of *A. hydrophila* from a population of *Aeromonas* in Indonesia**.**

MATERIALS AND METHODS

The isolation of *Aeromonas hydrophila*

In order to sample *Aeromonas* species from diverse environments, we collected bacteria from different sources. Bacteria samples were isolated from the intestines of healthy and diseased fish obtained from Balai Riset Perikanan Budidaya Air Tawar (Research Institute of Freshwater Aquaculture) Sempur Bogor and Sukabumi. Other bacteria samples were isolated from fish ponds in West Java (Indonesia) cities, including Tasikmalaya, Garut, Bandung, Indramayu, and Sukabumi. When initially cultured on Rimler-Shotts medium + novobiocin, the colonies of suspected *A. hydrophila* appeared white with a bright yellow zone in the middle of the colony. The isolates were sub-cultured on Trypticase Soy Agar (TSA) slants and incubated at 28 °C for 24 h before being subjected to an array of tests and analyses, including Gram staining, motility test, oxidase test, blood agar test, oxidative-fermentative test and Rimler-Shotts+novobiocin test (Rimler and Shotts, 1973; BSN, 2009). During this procedure, the morphology of the isolate under investigation was compared with that of the reference *A. hydrophila* ATCC 7966 obtained from Microbiologic Co.

Bacterial DNA isolation and analysis

To isolate bacterial DNA, the bacterial colony was re-suspended in one milliliter PBS (Phosphate Buffer Saline) and homogenized using a vortex mixer. The tube was centrifuged at 1680 rcf for 3 min. The supernatant was discarded and the pellet was mixed with 100 µL 1× TE pH 8.0, and homogenized using a vortex mixer. The sample was incubated in boiling water for 10 min, and then centrifuged for 1 min at 1680 rcf. A total of 100 µL of the lysate was transferred to a new 1.5 mL sterile tube and dissolved in 900 µL cold 1× TE. Several sample aliquots of 200 µL were stored at −20 °C for use as template DNA in conjunction with specific primers designed by Dorsch *et al.* (1994), Cascón *et al.* (1996), and Jiang *et al.* (2006) for PCR. Following electrophoresis on 1% agarose gel, DNA sequencing was performed at Macrogen Korea. The PCR solution mix and primers used are shown in Table 1. The sequencing results were subjected to BLAST analysis, with sequences aligned and compared with the other *Aeromonas* 16S rDNA from the GenBank database NCBI (National Center for Biotechnology Information;http://www.ncbi.nml.nih.gov/BLAST/Blast.cgi). Eleven sequences of *Aeromonas* 16S rDNA were chosen from BLAST analysis for comparison with 16S rDNA sequence from 20 samples which were identified as positive using the Cascón and Dorch methods. The comparison was made using phylogenetic analysis based on program of Clustal X and MEGA 5 software (Tamura *et al.*, 2011). To construct a phylogenetic tree, we used three 16S rDNA sequences from *A. hydrophila* as references*, viz.* (1) 16S rDNA generated from living *A. hydrophila* (ATCC 7966) from Microbiologic Co, (2) 16S rDNA sequence *of A. hydrophila* (ATCC 7966) available at GenBank NCBI, and (3) 16S rDNA sequence of *A. hydrophila* (ATCC 4910) available at GenBank NCBI. Other reference 16S rDNA sequences were later identified from the NCBI database based on the BLAST results. We also used the 16S rDNA sequence of *Tolumonas auensis* strain DSM 9187 from GenBank as an out-group. *Tolumonas auensis* is a member of the Aeromonad group, but is of a different genus from *Aeromonas*.

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Table 1: Primer sequences and amplification programs used.

	Dorsch *et al.*, 1994	Cascón *et al.*, 1996	Jiang *et al.*, 2006 (16S rDNA)
Size	685 bp	760 bp	±1450 bp
F 5'-3'	GAAAGGTTGATGCCTAATACGTA	AACCTGGTTCCGCTCAAGCCGTTG	AGAGTTTGATCCTGGCTCAG
R 5'-3'	CGTGCTGGCAACAAAGGACAG	TTGCCTCGCCTCGGCCCAGCAGCT	GGTTACCTTGTTACGACTT
Initial denaturation	95 °C, 5 min	95 °C, 5 min	95 °C, 5 min
Cycle	28	28	28
Denaturation	95 °C, 15 sec	95 °C, 15 sec	95 °C, 30 sec
Annealing	57 °C, 15 sec	65 °C, 15 sec	54 °C, 30 sec
Extension	72 °C, 30 sec	72 °C, 30 sec	72 °C, 90 sec
Final Extension	72 °C, 5 min	72 °C, 5 min	72 °C, 5 min
Enzyme	2× Green Kappa 2G fast ready mix	2× Green Kappa 2G fast ready mix	2× Green GoTaq ready mix, Promega

RESULTS AND DISCUSSION

Aeromonas hydrophila isolation

Using Rimler and Shotts (1973) selective medium, we obtained 95 bright yellow color isolates with white edges, consistent with the characteristics of *A. hydrophila*. Of the 95 isolates, further screening using the SNI 7303 method (BSN, 2009) brought the number down to 56. The isolates were further screened using the methods of Dorsch *et al.* (1994) and Cascón *et al.* (1996).

Bacterial DNA isolation and Identification

DNA amplification using the Dorsch method produced amplicons similar in size to those of the control sample *A. hydrophila* ATCC 7966. On the other hand, the Cascón method produced amplicons of varying sizes, some of them similar to the amplicon size produced by the control isolate. With respect to *A. hydrophila* identification, the Cascón and Dorsch methods produced inconsistent results. For example, in an examination of the control isolate (*A. hydrophila* ATCC 7966) and six test isolates, all of the test isolates (SfB, SfN, SfL, SfM, SfP, and FpT9) produced 685 bp amplicons using Dorsch method. However, using the Cascón method, only two isolates (SfB and FpT9) produced amplicons similar in size to the control amplicon. The other four samples (SfN, SfL, SfM, SfP) either generated amplicons of different sizes, or no relevant product was produced altogether (Figure 1).

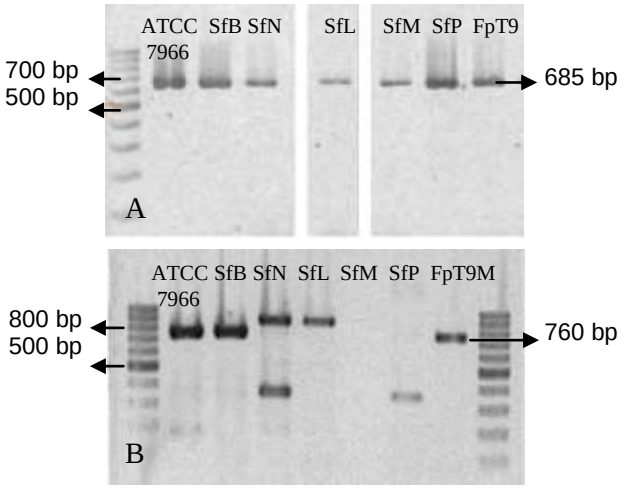

Figure 1: DNA amplification using specific primers for *A. hydrophila*. A, Method of Dorsch *et al.* (1994) B, Method of Cascón *et al.* (1996). Amplicons from the reference culture (*A. hydrophila* ATCC 7966) and six test isolates (SfB, SFN, SfL, Sf, SfP, FpT9). M, 100 bp ladder.

Of the 56 isolates selected using SNI 7303 (BSN, 2009), only 17 samples were positively identified as *A. hydrophila* using the Dorsch method, and only 11 samples were similarly identified based on the Cascón method. Seven samples were consistently positive for both methods and 20 samples were positive either for the Cascón or the Dorch method (Table 2).

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Table 2: Screening for *A. hydrophila* in 20 bacterial isolates based on the BSN method, the Dorsch method, the Cascón method, and the Jiang method 16S rDNA-based phylogenetic analysis.

Name of Isolate	SNI 7303 (2009)	Dorsch *et al.*, (1994)	Cascón *et al.*, (1996)	16S rDNA Jiang *et al.*, (2006)
ATCC 7966 (Control)	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
1.SfB	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
2.FpT_9	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
3.FpB_1	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
4.HfM_1	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
5.HfNp	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
6.SfP	*A. hydrophila*	*A. hydrophila*	unidentified	*A. media*
7.$FpIS_1$	*A. hydrophila*	unidentified	*A. hydrophila*	*Aeromonas* sp.
8.$FpIS_3$	*A. hydrophila*	unidentified	*A. hydrophila*	*Aeromonas* sp.
9.FpIA	*A. hydrophila*	*A. hydrophila*	unidentified	*A. taiwanensis*
10.FpC	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*Aeromonas* sp.
11.HfLp	*A. hydrophila*	*A. hydrophila*	unidentified	*Aeromonas* sp.
12.HfL_1	*A. hydrophila*	unidentified	*A. hydrophila*	*A. veronii*
13.HfL_2	*A. hydrophila*	*A. hydrophila*	unidentified	*Aeromonas* sp.
14.FpG	*A. hydrophila*	*A. hydrophila*	unidentified	*A. jandaei*
15.HfTp	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. jandaei*
16.SfM	*A. hydrophila*	*A. hydrophila*	unidentified	*A. veronii*
17.SfL	*A. hydrophila*	*A. hydrophila*	unidentified	*A. veronii*
18.SfN	*A. hydrophila*	*A. hydrophila*	unidentified	*A. veronii*
19.HfMn	*A. hydrophila*	*A. hydrophila*	unidentified	*A. veronii*
20.FpT_1	*A. hydrophila*	unidentified	*A. hydrophila*	*A. veronii*
21-56 not sequenced				

Note: Sf, Sick Fish; Hf, Healthy Fish; Fp, Fish Pond

Other than the control isolate *A. hydrophila* ATCC 7966, the isolates that were strongly identified as *A. hydrophila* were the isolates SfB, HfM_1, HfNp, FpT_1, FpC, FpT_9. and FpB_l. To assess both the Dorsch and Cascón methods, we made a comparison with 16S rDNA sequences from all of the 20 samples that were tested positive to either method. We also included isolates that did not produce amplicons using both the Dorsch and Cascón methods. The phylogenetic analysis also included 12 different *Aeromonas* species from GenBank and one species *T. auensis* strain DSM 9187 as an out-group. The phylogenetic tree was constructed based on multiple alignments of the 16S rDNA regions, using the Neighbor-Joining method. The result in the form of a phylogenetic tree is shown in Figure 2.

Based on this phylogenetic tree, the species of *Aeromonas* are very closely related, showing between 96-99% identity among the tested isolates (Figure 2). The result of this research is consistent with the work of Martinez-Murcia *et al.* (1992) where an analysis based on 16S rDNA reached 98-100% similarity in identity among species of *Aeromonas*. It was still difficult to distinguish between the minute differences in genotypes (Martinez-Murcia *et al*., 1992; Martinez-Murcia *et al.*, 2005).

Based on the 16S rDNA of 20 samples, there were five other samples that could not be identified as a distinct *Aeromonas* species. The phylogenetic analysis produced two major groups. However, five sub groups were discernible on closer examination. The first group consisted of three sub groups, where the first sub group included *A. hydrophila*, the second sub group included *A. media*, while the third sub group was very difficult to distinguish because it consisted of more than one species. One sample from the third sub group was very similar to *A. taiwanessis*. The second group consisted of two sub groups, while the fourth sub group comprised only *A. jandaei,* and the fifth sub group comprised only *A. veronii*. This phylogenetic division was very close to the

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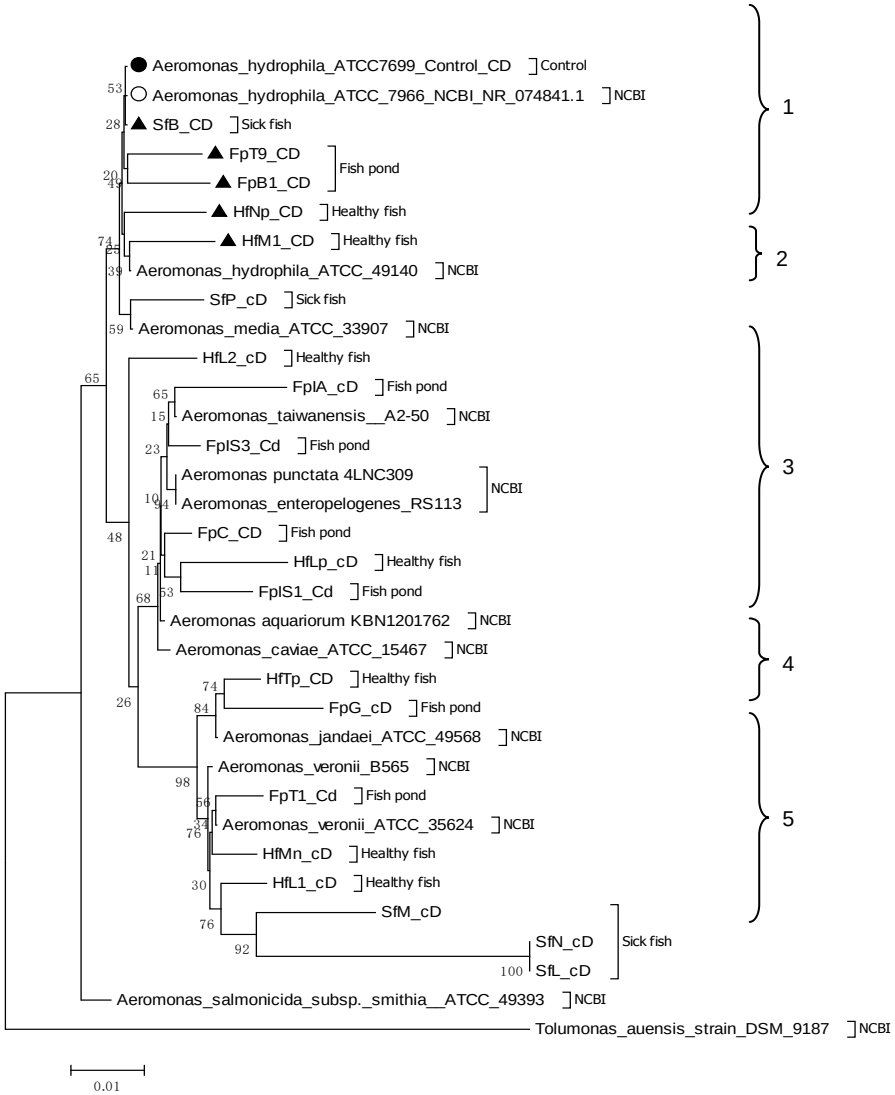

Figure 2: The Neighbor-Joining Phylogenetic Tree constructed using the 16S rDNA sequences from one control isolate, 20 test sample isolates, 12 sequences of *Aeromonas* sp. as references, and one non-*Aeromonas* sp. *T. auensis* strain ATCC DSM 9187 as the out-group. The values at the branches show the Bootstrap 5000 times; ●,16S rDNA generated from living *A. hydrophila* (ATCC 7966) from Microbiologic Co; ○, 16S rDNA sequence *of A. hydrophila* (ATCC 7966) available at GenBank NCBI; ▲,the sample isolates that belong to *A. hydrophila* group. CD, +Cascón/+Dorsch; cD, −Cascón/+Dorsch; Cd, +Cascón/−Dorsch and cd, −Cascón/−Dorsch.

phylogenetic structure of Martinez-Murcia *et al.* (1992). In this study, we identified at least five species of *Aeromonas* in our collection, viz. *A. hydrophila*, *A. media*, *A. taiwanensis*, *A. jandaei* and *A. veronii* (Table 2).

Based on Table 2 and the phylogenetic tree in Figure 2, five isolates which were positively identified using 16S rDNA method as *A. hydrophila* were also assigned the same identity using the BSN, Cascón and Dorsch methods. The isolates, SfB, FpT_9, FpB_1, HfNp and HfM_1 (Table 2), were in the same group as the three 16S rDNA sequences of *A. hydrophila,* and it could therefore be concluded that the five isolates were *A. hydrophila.*

There were 15 other isolates that were not confirmed as *A. hydrophila*. Of these, nine isolates that were identified as *A. hydrophila* using both the BSN and Dorcsh methods were designated by 16S rDNA analysis as *Aeromonas* sp., (HfLp, HfL_2), *A. veronii* (SfL, SfN, SfM, HfMn), A*. taiwanensis* (FpIA), *A. jandaei* (FpG), and *A. media* (SfP). Four isolates that tested positive for *A. hydrophila* using both BSN and Cascón methods were reassigned as *A. veronii* (FpT_1,HfL_1) and two isolates of *Aeromonas* sp. ($FpIS_1$, $FpIS_3$) by 16S rDNA analysis. Finally, two isolates that were positively identified using all three methods were re-classified as *A. jandaei* (HfTp) and *Aeromonas* sp. (FpC).

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The results from this study showed that there was no distinct division between species of *Aeromonas* found in healthy fish and diseased fish. For example, *A. hydrophila* and *A. veronii* were found in both healthy fish and diseased fish. *Aeromonas media* was only found in a diseased fish in this study, but this was the only time the species was encountered in the 95 original samples. Two other species, *A. taiwanensis* and *A. jandaei*, were found in healthy fish and in pond water. According to Kompanets *et al*. (1992) and Molinari *et al*. (2003), *A. hydrophila* can thrive in healthy fish. The virulence of this species is attributed to virulence genes such as cytotoxic aerolysin (*aerA*), enterotoxin (*act*), lipase (*lip*), cytotoxic enterotoxins (*ast,alt*), glycerol phospholipid cholesterol acyltransferase (*gcat*), DNases (*exu*), elastase (*ahyB*), serine protease (*ser*), ADP ribosyltransferase (*aexT*), and the structural gene flagellin (*fla*) (Nawaz *et al.*, 2010; Puthucheary *et al*., 2012).

From Table 2, a test using the SNI 7303 protocol (BSN, 2009) would yield 56 positive results, but with only five samples also testing positive with the 16S rDNA method, thereby giving an accuracy of only 8.9%. Of the 16 samples positively identified using the Dorsch method, only five samples matched positive readings in the 16S rDNA method (31.3% accuracy). Of the eleven samples positively identified using the Cascón method, only five samples were corroborated by test results using the 16S rDNA method (45.5% accuracy). Of the seven samples that were tested positive using all of the three methods, only five samples were validated as positive using the 16S rDNA method, giving an accuracy of 71.4%.

The Cascón method produces positive results in species other than *A. hydrophila*, and so in regard, it is lacking in test specificity. Ottaviani *et al*. (2011) reports that the Cascón method also tests positive for other *Aeromonas* species such as *A. popoffii*, and *A. bestarium*. Further analysis of the lipase DNA sequences might provide a clearer picture of the phylogeny of *Aeromonas* sp. The Dorsch method is similarly lacking in test specificity in that, from a BLAST analysis, the Dorsch primers would be expected to recognize 16S rDNA sequences of species such as *A. media*, *A. encheleia*, A. *veronii*, A. *salmonicida*, and *A. bestiarum*. This explains why test results using Dorsch primer are not always accurate. While the Cascón primers are very specific for the *A. hydrophila* lipase, there are approximately 100 *Aeromonas* lipase sequences in the Genebank database (accessed 30 October 2015), and hence the Cascón primers might amplify lipase genes from *Aeromonas* species other *A. hydrophila*. This could explain the observation of multiple band sizes of the PCR products in our study (Figure 1).

CONCLUSION

The results from this research show that identification of *Aeromonas* to the species level is very difficult because they are very closely related. Very definitive identification cannot be made using biochemical tests such as the BSN method, or PCR-based tests like the Cascón method or the Dorsch method only. Accuracy of the BSN methods was 8.9 %, and this figure refers only to positive SNI 7303 (BSN, 2009) test results that were confirmed by the 16S rDNA approach. It does not take into account the possibility that true *A. hydrophila* among the original 95 samples collected might have been wrongly tested negative in the first screening. The accuracy of the Dorsch and Cascón methods were 31.3% and 45.5% respectively when compared against the gold standard procedure based on 16S rDNA (Jiang *et al.*, 2006). Even when all the three methods were used together, the accuracy was only 71.4%. The three common methods used to identify *A. hydrophila* are not suitable for the identification of *A. hydrophila* with confidence in Indonesia and probably in other tropical countries too. Hence, there is a need to develop a new simple method for this purpose. On the other hand, *A. hydrophila* can be found in healthy and diseased fish. This indicates that the *A. hydrophila* consist of at least two strains, one strain can be pathogenic and the other may not pathogenic. Instead of developing identification method for *A. hydrophila* is probably more useful to identify pathogenic *Aeromonas* species.

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