The screening of pathogenic *Aeromonas hydrophila* based on its hemolytic and proteolytic properties

Pongsaton Juntarut¹,²*, Sommai Chiayvareesajja² and Damrongsak Faroongsarng³

**ABSTRACT:** We investigated the hemolytic and proteolytic activities of the 2015 pathogenic strains of *A. hydrophila* isolated from diseased freshwater fishes in the farms located in the central and northern regions of Thailand that the strains were kindly provided by the Inland Aquatic Animal Health Research Institute (AAHRI), Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand. The results clearly show that 5 strains namely: AH4C-SPT58AAHRI, AH8ONUD58AAHRI, AH10HNUD58AAHRI, AH11HNUD58AAHRI and AH13HNUD58AAHRI contained both hemolytic and proteolytic properties might have been caused by extracellular enzymatic products secreted from the isolates. Those exoenzymes may correspond to biotoxins which play an important role in the pathogenesis associated with motile aeromonas septicaemia in infected fishes. Our results suggested that those strains may have high lethality and potential yielding motile aeromonas septicaemia in commercial freshwater fish species in the Thai aquaculture industry.

**Keywords:** *Aeromonas hydrophila*, biotoxins, proteolytic activity, hemolytic activity

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**Introduction**

The pathogenic *Aeromonas hydrophila* has currently been reported its virulence causing high lethality in cultured freshwater fishes (Hossaina et al., 2014). Most importantly, its infection has caused serious problems associated with gross clinical signs of motile aeromonas septicaemia (Austin, 2011) such as surface or fin base haemorrhages, skin and gills lesions, abdominal distension, skin necrotic ulcers, fins erosion and exophthalmia (Zhang et al., 2016) in infected fish. This pathogenic bacterium has been one of the major pathogens in both scale and scaleless fish in Asian countries. It causes disease outbreaks which leads to economic losses in freshwater intensive culture systems (Nielsen et al., 2001). This has led to especial concern in Thailand where it has been reported that the bacterium could infect several aquatic animals such as cultured edible finfish (Boonyaratpalin, 1987), ornamental fish (Jongjareanjai et al., 2009), farmed frogs (Huys et al., 2003) and fairy shrimps (Saejung et al., 2011). Because of the role of exotoxic factors producing by the bacterium such as enterotoxins (Santos et al., 1988), hemolysins (Pollard et al., 1990), aerolysins (Singh et al., 2008), adhesins (Fang et al., 2004), protease (Esteve and Birbeck, 2004) and lipase (Cascón et al., 1996), the infection can cause serious...
pathogenicity in the fishes. The assay of hemolytic and proteolytic activities are simple techniques that have frequently been used to screen the pathogenic strains of *A. hydrophila* isolated from fishes (Esteve and Birkbeck, 2004; Pridgeon et al., 2013). Therefore this study was targeted on the hemolytic and proteolytic properties of pathogenic *A. hydrophila* strains that recently isolated from the important edible fish species in aquaculture of Thailand. This *in vitro* study aims to verify that 2015 strains of pathogenic *A. hydrophila* originally isolated from the farmed fishes are able to produce extracellular enzymatic products associated with their β-hemolytic and proteolytic properties.

**Materials and Methods**

**Bacterial strains**

Five strains of pathogenic *A. hydrophila* were isolated from the diseased fishes associated with motile aeromonas septicaemia in the farming around the central and north regions of Thailand in 2015. All pathogenic isolates, including AH4CSPT58AAHRI, AH8ONUD58AAHRI, AH10HNU58AAHRI, AH11HNU58AAHRI and AH13HNU58AAHRI were kindly provided by the Inland Aquatic Animal Health Research Institute (AAHRI), Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand (Table 1).

In addition, the reference strains of *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538, obtained from Assoc. Prof. Dr. Sanae Kaewnopparat, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University that had been previously purchased from the Department of Medical Sciences, Thailand.

**Table 1** The pathogenic *A. hydrophila* isolated in 2015 that provided by the AAHRI, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand.

<table>
<thead>
<tr>
<th>Strain codes</th>
<th>Isolation sources (Genus species)</th>
<th>Sampling area</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH4CSPT58AAHRI</td>
<td>Walking catfish <em>(Clarias sp.)</em></td>
<td>PathumThani</td>
</tr>
<tr>
<td>AH8ONUD58AAHRI</td>
<td>Nile tilapia <em>(Oreochromis niloticus)</em></td>
<td>Uttaradit</td>
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<tr>
<td>AH10HNU58AAHRI</td>
<td>Channel catfish <em>(Ictalurus punctatus)</em></td>
<td>Uttaradit</td>
</tr>
<tr>
<td>AH11HNU58AAHRI</td>
<td></td>
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<tr>
<td>AH13HNU58AAHRI</td>
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**Bacterial culture conditions**

All bacterial strains were cultured in tryptic soy agar or broth (Difco) and incubated at 37 °C for the reference strains, and 30 °C for pathogenic *A. hydrophila*. The growth rates were measured by the determination of the optical density at 600 nm (OD$_{600}$) of each inoculated bacteria at various time intervals. The OD$_{600}$-
values were plotted against time intervals to show exponential growth phase of each bacterial strain that modified from Perni et al. (2005). In addition, the bacterial densities were measured by standard drop plate technique that modified from Herigstad et al. (2001) in which the OD$_{600}$-values were corresponded to that of $\sim 10^8$ cfu/ml. The bacterial strains were stored long-term in microcentrifuge tubes containing 50% (v/v) glycerol in tryptic soy broth and were kept in -80 °C.

**β-hemolytic activity assay**

The determination of β-hemolysis was modified from Pollard et al. (1990). Briefly, the stocking aliquots of each of 2 reference strains and 5 strains of pathogenic *A. hydrophila* were overnight cultured until reaching the exponential phase. Then, bacterial cells were washed and re-suspended in sterile phosphate buffered saline (Difco), and adjusted to the OD$_{600}$-value corresponded to $\sim 10^8$ cfu/ml. Finally, 20 µl of bacterial suspension was dropped onto the Mueller-Hinton agar (HiMedia) plate containing ~20 ml of 5% defibrinated sheep blood (Salaya Pet Hospital, Thailand), while the drops of sterile phosphate buffered saline (Difco) was considered as a negative control. The plates were incubated at optimal temperature for 24 h before the determination of proteolysis activity. The protease activity was determined according to the appearance of clear zone forming around the bacterial colony. A positive result was interpreted as the appearance of clear zone around them while the negative one was as visible observed translucent zone. Each bacterial strain and control was performed in twice replications.

**Proteolytic activity assay**

The determination of protease was modified from Banerjee et al. (2007). Briefly, stocking aliquots of reference and pathogenic *A. hydrophila* strains were cultured until reaching the exponential phase. The bacterial cell suspension was washed by centrifuging and the neglected culture medium was replaced using sterile phosphate buffered saline. Each suspension was altered to a cell density of $\sim 10^8$ cfu/ml by OD$_{600}$-value adjustment. Finally, 20 µl of each bacterial suspension was dropped on to the skim-milk agar (Difco) plate whereas negative control were done by dropping 20 µl of sterile phosphate buffered saline. The plates were incubated at optimal temperature for 24 h before the determination of proteolysis activity. The protease activity was determined according to the appearance of clear zone forming around the bacterial colony. A positive result was interpreted as the appearance of clear zone around them while the negative one was as visible observed translucent zone. Each bacterial strain and control was performed in twice replications.

**Results**

The β-hemolytic activity was observed in pathogenic *A. hydrophila* strains and a reference of *S. aureus* ATCC 6538. While in case of *E. coli* ATCC 8739, a translucent ring covering the colony was observed resulting in non-β-hemolysis against sheep erythrocytes (Figure 1). However, our findings demonstrated 2 different degrees of hemolysis in 3 β-hemolytic strains: AH4C-SPT58AAHRI, AH8ONUD58AAHRI and *S. aureus* ATCC 6538 exhibited larger clear zone rings than the other 3 strains of AH10HNUD58AAHRI,
AH11HNUD58AAHRI and AH13HNUD58AAHRI. The strains with smaller clear zone rings also transmitted light at the central spot of the colony. While, non-β-hemolytic E. coli ATCC 8739 showed the zone of greenish ring around the colony with a translucent center indicating an unhemolyzed area. In addition, the study on the proteolysis based on lyses casein and gelatin in skim-milk agar showed that pathogenic A. hydrophila strains could breakdown the protein composition in agar causing clear zones surrounding their colonies which were clearly estimated as proteolytic strains. Unfortunately in cases of the reference strains, there was no clear zone ring around the colony (Figure 2). Also notice that no hemolysis and proteolysis was observed in the group of negative control when sterile phosphate buffered saline was dropped on the tested agars.

**Figure 1** The β-hemolytic of bacterial strains; A) control, B) E. coli ATCC 8739, C) S. aureus ATCC 6538, D) AH4CSPT58AAHRI, E) AH8ONUD58AAHRI, F) AH10HNUD58AAHRI, G) AH11HNUD58AAHRI and H) AH13HNUD58AAHRI.
The hemolysis and proteolysis of pathogenic A. hydrophila were considered as virulent strains based on their hemolytic and proteolytic properties. These isolates had the ability to breakdown defibrinated sheep blood agar plates showing a transparent zone around the colony (Figure 1). Combination of those strains may exhibit proteolytic properties that could breakdown casein and gelatin in skim-milk agar (Figure 2). The hemolysis and proteolysis of pathogenic A. hydrophila may be due to the virulent factors defined by the extracellular enzymes produced by the strains. These factors acted as the major exotoxins inducing pathogenesis in infected animals that are frequently been used to screen the pathogenic strains of A. hydrophila isolated from diseased fishes (Esteve and Birkbeck, 2004; Pridgeon et al., 2013). Therefore this study was targeted on the hemolytic and proteolytic properties of the strains that recently isolated from the important edible fish species in aquaculture of Thailand. To compare with other studies, the evidence has been suggested by the report of Pridgeon et al. (2013) that highly virulent isolates carrying hemolytic, protease and also nuclease activity owing to the exotoxins such as hemolysin, aerolysin, elastase (metalloprotease) and 5´-nucleotidase infected channel catfish. Likewise, our results clearly revealed that the strains of

**Figure 2** The proteolytic activity of bacterial strains; A) control, B) E. coli ATCC 8739, C) S. aureus ATCC 6538, D) AH4CSPT58AAHRI, E) AH8ONUD58AAHRI, F) AH10HNUD58AAHRI, G) AH11HNUD58AAHRI and H) AH13HNUD58AAHRI.

**Discussion**

This study clearly shows that recent 2015 fish isolates of pathogenic A. hydrophila were considered as virulent strains based on their hemolytic and proteolytic properties. These isolates had the ability to breakdown defibrinated sheep blood agar plates showing a transparent zone around the colony (Figure 1). Combination of those strains may exhibit proteolytic properties that could breakdown casein and gelatin in skim-milk agar (Figure 2). The hemolysis and proteolysis of pathogenic A. hydrophila may be due to the virulent factors defined by the extracellular enzymes produced by the strains. These factors acted as the major exotoxins inducing pathogenesis in infected animals that are frequently been used to screen the pathogenic strains of A. hydrophila isolated from diseased fishes (Esteve and Birkbeck, 2004; Pridgeon et al., 2013). Therefore this study was targeted on the hemolytic and proteolytic properties of the strains that recently isolated from the important edible fish species in aquaculture of Thailand. To compare with other studies, the evidence has been suggested by the report of Pridgeon et al. (2013) that highly virulent isolates carrying hemolytic, protease and also nuclease activity owing to the exotoxins such as hemolysin, aerolysin, elastase (metalloprotease) and 5´-nucleotidase infected channel catfish. Likewise, our results clearly revealed that the strains of
A. hydrophila had hemolytic and proteolytic properties. It might be because of the secreting of exotoxins, such as hemolysin, aerolysin and protease against erythrocytes and proteins in the agars. Similarly, Esteve and Birbeck (2004) found that pathogenic A. hydrophila strain EO63 from European eel (Anguilla anguilla) had both hemolytic and proteolytic properties. The authors suggested that hemolytic A. hydrophila produce toxins corresponding to aerolysin against human erythrocytes, while 2 types of proteases including serine protease against casein and metalloproteases against elastin and casein were observed. Furthermore, Singh et al. (2008) demonstrated that pathogenic A. hydrophila strains isolated from Channa punctatus and Labeo rohita, containing aerolysin genes could produce aerolysin that breakdown rabbit erythrocytes. In addition, enterotoxin, an extracellular enzyme related to β-hemolytic activity, may be released by some pathogenic A. hydrophila previously isolated from diseased fish (Singh and Sanyal, 1992). The β-hemolysin also considered as a virulent factor associated with hemolytic activity that has been investigated by the strains of clinical A. hydrophila against animal erythrocytes and especially mouse red blood cells (Brenden and Janda, 1987). Later, β-hemolysin gene could be used as a specific marker to detect the virulent strains of A. hydrophila isolated from diseased fish (Singh and Sanyal, 1992). On the other hand, the use of virulent genes involving with exotoxins such as cytolytic enterotoxin, hemolysin and bacterial outer membrane to detect the clinical isolates associated with motile aeromonas septicaemia in rainbow trout, Oncorhynchus mykiss (Cagatay and Şen, 2014) has been evident. Interestingly, multiple virulent factors could coordinate to such a degree of virulence causing lethality in infected fish. This was proved by Hu et al. (2012) who investigated the strains of A. hydrophila isolated from diseased fish containing more virulent genes of aerolysin, cytotoxic enterotoxin, cytotoxic enterotoxin, temperature-sensitive protease and serine protease, compared to isolates from healthy fish and water samples had high potential in causing fish disease. These genes definitely involve in producing exotoxins that contribute to the mechanisms effecting immune responses in infected fish. Reyes-Becerril et al. (2011) clearly demonstrated that gilthead seabream (Sparus aurata) infected by virulent A. hydrophila had decreased in cellular innate immunity of phagocytosis, respiratory burst activity and peroxidase leucocyte while, significant increases in hepcidin and cytokine interleukin-1β might be induced by the exotoxins that play an important role in the first line of defense of post-infection.

Our in vitro results clearly show that recent pathogenic A. hydrophila strains isolated from diseased fish in 2015 having both hemolytic and proteolytic properties might produce the exotoxins corresponding to the properties. This proof may be helpful to understand their ability to produce the toxins associated with motile aeromonas septicaemia in Thai aquaculture. The further study based on the molecular approach of the virulent genes related to extracellular enzymes of these isolates would be an issue of clarification that might be useful for the rapid and accurate diagnosis of the disease, and also reducing the incidence of disease outbreaks. In addition, the in vivo determination of the degrees of virulent among the 5 pathogenic A. hydrophila
strains should be very soon investigated in fish specimens, which are of commercial importance in cultured species in Thailand. Based on the virulence properties of these recent pathogenic strains, the further chemotherapeutic agents or other alternatives must be immediately prepared for strategic control of the diseases such as motile aeromonas septicaemia to reduce the risk of the outbreaks that would definitely increase the production costs and loss of revenue in Thai aquaculture industry.

Conclusion

Our study clearly demonstrated that in 2015 the 5 recent pathogenic A. hydrophila strains: AH4CSP58AAHRI, AH80NUD58AAHRI, AH10HNUD58AAHRI, AH11HNUD58AAHRI and AH13HNUD58AAHRI previously isolated from 3 species of farmed freshwater finfish: Nile tilapia (O. niloticus), walking catfish (Clarias sp.) and channel catfish (I. punctatus) had hemolytic and proteolytic properties based on in vitro investigation. It may be due to the role of extracellular enzymes defined as exotoxins corresponding to hemolysins and proteases produced by the isolates. Furthermore, the detection of virulence genes that are responsible for producing those extracellular enzymatic products in the isolates should be clearly revealed. This study might prove useful for controlling diseases associated with motile aeromonas septicaemia in Thai aquaculture.

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References


