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Incidence and virulence characteristics of *Aeromonas* spp. in Malaysian fish and shrimp

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Abstract

A total of 86 samples from five species of aquaculture products including tilapias (*Oreochromis mossambicus*), red hybrid tilapias (*Orechromis* sp. × *Orechromis* sp.), walking catfishes (*Clarias batrachus*), common snakeheads (*Channa striata*), and white-leg shrimps (*Litopenaeus vannamei*) were obtained from three local wet markets in Kuala Lumpur, Malaysia. Using the *Aeromonas* isolation agar, 72 *Aeromonas* isolates (83.7%) were identified; 43 *A. veronii* biovar *sobria* (50%), 21 *A. hydrophila* (24.4%), and eight *A. caviae* (9.3%). The 72 *Aeromonas* isolates were then subjected to haemolysis, proteolysis, and lipolysis tests to determine their virulence characteristics. All the *Aeromonas* isolates demonstrated haemolytic activity (100%); 57 isolates expressed beta-haemolytic activity (79.2%), while the remaining 15 expressed alpha-haemolytic activity (20.8%). Besides that, the *Aeromonas* isolates revealed proteolytic activity (100%), and only 57 of the isolates showed lipolytic activity (79.2%). The results demonstrated that *Aeromonas* spp. were present in various commercial aquaculture products in Kuala Lumpur, Malaysia. The results from the virulence tests also showed that *Aeromonas* spp. possessed a variety of different virulence factors that may have aided in their pathogenesis of *Aeromonas*-associated diseases. The present work highlighted the importance of proper food handling practices and audited processes from fish farms to consumers to prevent the spread of foodborne pathogens, and the occurrence of *Aeromonas*-associated diseases in humans.

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Introduction

The emergence and vast distribution of multidrug-resistant aeromonads has become a considerable threat to aquaculture farms and healthcare institutions worldwide (Bebak *et al*., 2015; Mzula *et al*., 2019). *Aeromonas* is a facultative anaerobic, Gram-negative rod species of the Aeromonadaceae family, and are common inhabitants isolated from bodies of water and different types of food such as frozen chicken and seafood (Daskalov, 2006; Abdelhamed *et al*., 2017). *Aeromonas* expresses a range of virulence factors including haemolysis, proteolysis, and lipolysis, all of which contribute to the development of various diseases in fish (Igbinosa *et al*., 2012; Abdelhamed *et al*., 2017), thus being responsible for huge economic losses in the aquaculture industry due to fish depreciation (Austin and Austin, 2016; Peterman and Posadas, 2019). In a study by Elgendy *et al*. (2024), motile aeromonads were identified in earthen-pond-farmed *Oreochromis niloticus* that suffered massive mortalities in Egypt during the summer of 2020. This study highlighted a strong association between poor water quality, *Aeromonas* infection, and tilapia mortalities. The findings provided information on virulence, antibiotic resistance, and potential treatment strategies for both fish and human health (Elgendy *et al*., 2024).

Besides *Aeromonas* being a future concern for increased economic burden due to a myriad of diseases inflicted upon fish in aquaculture farms, it is also responsible for a broad spectrum of gastrointestinal diseases among humans (Mohan *et al*., 2017). The common manifestations include a range of diarrhoeal diseases, severe wound infections, bacteraemia, and gastrointestinal symptoms such as abdominal pain, nausea, and vomiting (Batra *et al*., 2016). Of all the *Aeromonas* spp. discovered thus far, *A. hydrophila*, *A. veronii* biovar *sobria*, and *A. caviae* have been incriminated as the main pathogens in causing *Aeromonas*-associated human diseases

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(Janda and Abbott, 2010), and are usually transmitted *via* foodborne infections or direct contact with diseased fish (Zmysłowska *et al*., 2009).

Aeromonas infection is not a notifiable disease in Malaysia, and the prevalence of *Aeromonas* in multiple species of aquatic consumables in Malaysia is outdated (Radu *et al*., 2003). Several studies were conducted after Radu *et al*. (2003), but the sample sizes were too small to infer to the general population in Malaysia. In the present work, we thus aimed to isolate and identify clinically relevant strains of *Aeromonas* spp. from freshwater fish and shrimp samples in Kuala Lumpur and Selangor, Malaysia, and to characterise their virulence factors together with their haemolytic, proteolytic, and lipolytic activities.

Materials and methods

Isolation and identification

A total of 86 freshwater food samples of five different species were collected from three different market locations in Kuala Lumpur, Malaysia. The species of aquaculture samples were chosen based on the most eaten fish by the locals in this region of Malaysia. The five different species of aquaculture samples included 25 tilapias (*Oreochromis mossambicus*), 25 red hybrid tilapias (*Oreochromis* sp. × *Oreochromis* sp.), 27 walking catfishes (*Clarias batrachus*), four common snakeheads (*Channa striata*), and five white-leg shrimps (*Litopenaeus vannamei*). All samples were placed separately in the original plastic bags provided by the vendor, and transported within an hour back to the microbiology laboratory. After the samples in the tryptic soy broth were incubated at 37°C for 24 h, the samples were streaked on *Aeromonas* agar with an inoculation loop using the dilution streaking method. Suspected *Aeromonas* colonies were visually identified based on the colony morphology of dark green convex circular colonies (0.5 - 1.5 mm). A sterile inoculation loop was used to scrape up the suspected colony, and streak it onto nutrient agar. The nutrient agar plate was incubated upside down at 37°C for 24 h. *Aeromonas* isolation agar (Merck, Germany) was based on the formulation of Ryan in 1985, which supports the growth of *Aeromonas* spp. Analytical Profile Index (API) 20E kits were used to identify the bacterium suspected to be *Aeromonas* to species level.

Virulence tests

Haemolysis screening test

Samples from nutrient agar were streaked onto 5% sheep blood agar. The plates were incubated upside down at 37°C for 24 h. The results were recorded by visual identification either as beta-, alpha-, or gamma-haemolysis.

Proteolysis screening test

Samples from nutrient agar were streaked onto a pre-prepared skimmed milk agar (Merck, Germany). The plates were incubated upside down at 37°C for 24 h. The results were recorded by visual identification as either positive, indicating a clear halo around the colonies; or negative, agar remained cloudy with the growth of bacterium.

Lipolysis screening test

Samples from nutrient agar plate were streaked onto a phenol red agar with 1% substrate, prepared according to Ramnath *et al*. (2017). The plates were incubated upside down at 37°C for 24 h. The results were recorded by visual identification with a positive result noted as a change of colour from red to yellow surrounding the colonies on the agar.

Results

Pure cultures were obtained from a total of 86 aquaculture samples, among which 72 (83.7%) of them were found to be *Aeromonas* positive (Table 1). *Aeromonas* spp. were isolated from 19 (76%) of 25 tilapia (*Oreochromis mossambicus*) samples, 23 (92%) of 25 red hybrid tilapia (*Oreochromis* sp. × *Oreochromis* sp.) samples, 25 (92.6%) of 27 walking catfish (*Clarias batrachus*) samples, two (50%) of four common snakehead (*Channa striata*) samples, and three (60%) of five white-leg shrimp (*Litopenaeus vannamei*) samples.

All *Aeromonas* samples were biochemically identified using primarily APIWEB and Aerokey II as a cross-confirmation. The *Aeromonas*-positive samples were subsequently identified as *A. veronii* biovar *sobria* (n = 43) (50%), followed by *A. hydrophila* (n = 21) (24.4%) and *A. caviae* (n = 8) (9.3%).

All the *Aeromonas* isolates exhibited (n = 72) haemolytic properties, with 57 of them displaying beta-haemolytic properties, and the remaining 15 displaying alpha-haemolytic properties. A total of

Table 1. Prevalence of *Aeromonas* spp. in various aquaculture food samples.

Type of sample	Number of positive sample	Total number of sample	Frequency	Number of sample with *A. hydrophila*	Number of sample with *A. caviae*	Number of sample with *A. veronii* biovar *sobria*
Tilapia	19	25	76%	4	2	13
Red hybrid tilapia	23	25	92%	6	3	14
Walking catfish	25	27	92.6%	7	3	15
Common snakehead / Snakehead murrel	2	4	50%	2	nd	nd
White-leg shrimp	3	5	60%	2	nd	1
Total number of samples	**72**	**86**	**83.7%**	**21**	**8**	**43**

nd = not detected.

20 out of 21 (95.2%) *A. hydrophila* were beta-haemolytic, while one (4.8%) was found to be alpha-haemolytic. Exactly half of eight *A. caviae* isolates (50%) were beta-haemolytic, while the other half (50%) was alpha-haemolytic. A total of 33 out of 43 (76.7%) *A. veronii* biovar *sobria* isolates were beta-haemolytic, while the remaining 10 (23.3%) were alpha-haemolytic (Figure 1).

Additionally, all the *Aeromonas* isolates were positive for proteolytic activity (n = 72) (100%). A portion of the *Aeromonas* isolates (n = 57) were positive for lipolytic activity (79.2%), while the remaining did not exhibit any lipolytic properties (n = 15). A total of 19 (90.5%), 5 (62.5%), and 33 (76.7%) out of 21 *A. hydrophila*, 8 *A. caviae*, and 43 *A. veronii* biovar *sobria* were lipolytic positive, while the remaining were lipolytic negative (Figure 2).

The present work investigated the number of virulence factors for each specific isolate obtained. In *A. hydrophila*, 19 out of 21 (90.5%) isolates possessed three virulence characteristics, while the remaining two possessed only two virulence characteristics (9.5%) (Figure 3). In *A. caviae*, five out of eight (62.5%) isolates possessed three virulence characteristics, while the remaining three possessed only two virulence characteristics (37.5%) (Figure 4). In *A. veronii* biovar *sobria*, 33 out of 43 (76.7%) isolates possessed three virulence characteristics, while the remaining 10 possessed only two virulence characteristics (23.3%) (Figure 5). Overall, all the *Aeromonas* isolates possessed at least two virulence factors.

Discussion

The difference in *Aeromonas* spp. populations observed in the present work corroborated the findings from other researchers. The most relevant study by which the present work was inspired was the prevalence study of *Aeromonas* by Radu *et al*. (2003). Even though the methods and materials used by them were completely different from the present work, they reported 48 *A. veronii* biovar *sobria* isolates, ten *A. hydrophila* isolates, and two *A. caviae* isolates, in a total of 60 confirmed *Aeromonas* spp. samples. This suggested that the prevalence of different *Aeromonas* spp. in Malaysia may not have changed much in the last two decades. Abd-El-Malek (2017) also found a very similar proportion of *Aeromonas* spp., with *A. veronii* biovar *sobria* being the most frequent species found (n = 11) (22%), followed by *A. hydrophila* (n = 7) (14%) and *A. caviae* (n = 1) (2%).

Moreover, Khor *et al*. (2015) also reported very similar proportions of *A. veronii* biovar *sobria* (n = 44) (43%), *A. hydrophila* (n = 6) (6%), and *A. caviae* (n = 4) (4%) among 102 isolates from freshwater lakes. In another study conducted by Hafez *et al*. (2018), he found 34 *A. veronii* biovar *sobria* isolates (50%), 14 *A. sobria* (20.6%), and ten of each *A. hydrophila* and *A. caviae* (14.7% each) among 68 confirmed isolates from three different types of frozen fish (mackerel, herrings, and fish fillets).

Last but not least, in an experimental study conducted by Hu *et al*. (2012), a total of 25 out of 42

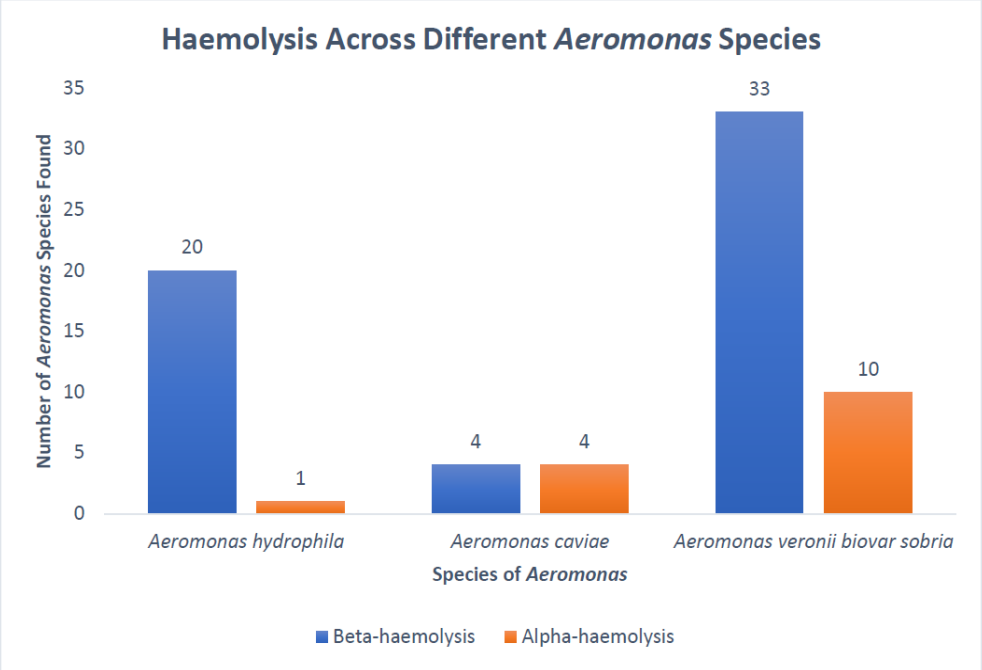

Figure 1. Number of *Aeromonas* spp. positive for haemolysis activity.

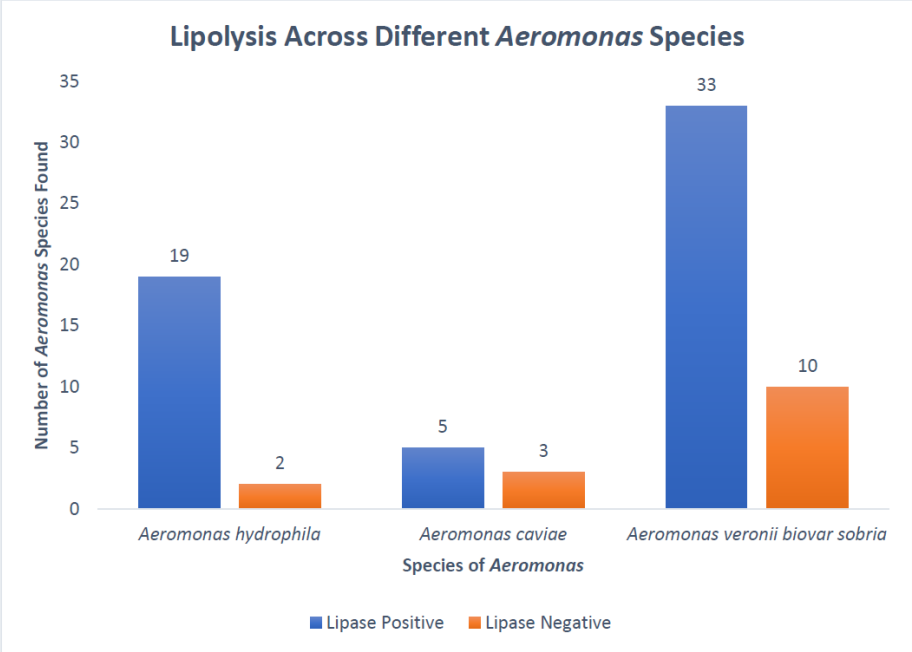

Figure 2. Number of *Aeromonas* spp. positive for lipase activity.

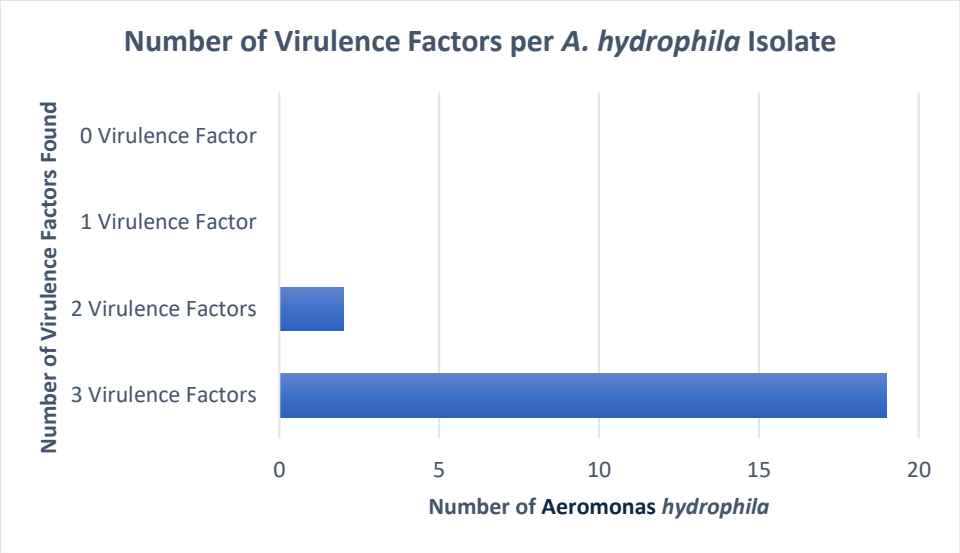

Figure 3. Number of virulence factors among *A. hydrophila* isolates.

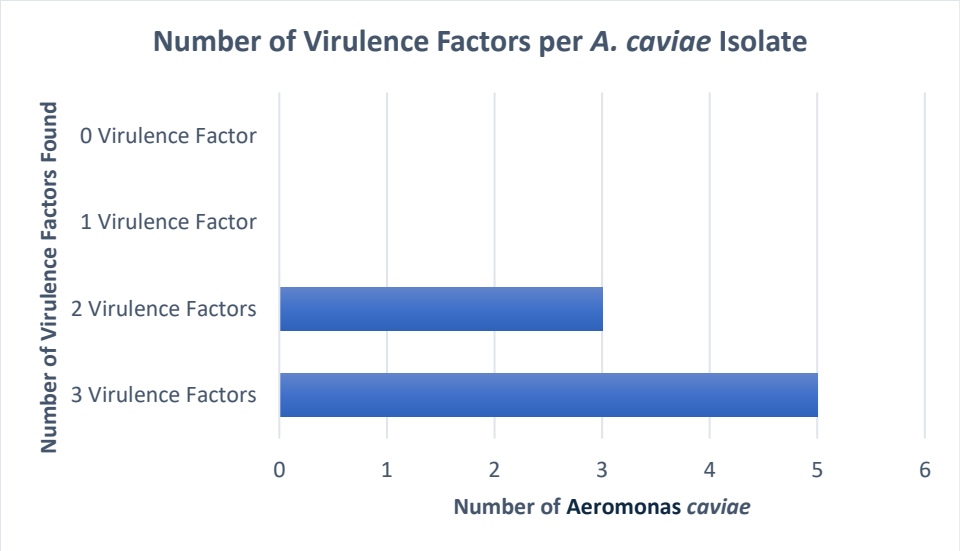

Figure 4. Number of virulence factors among *A. caviae* isolates.

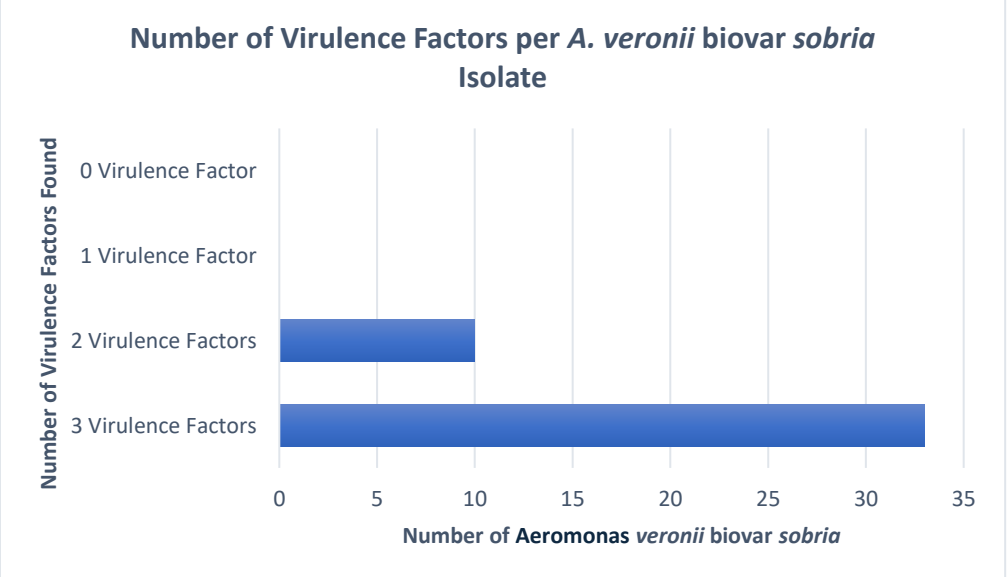

Figure 5. Number of virulence factors among *A. veronii* biovar *sobria* isolates.

isolates (60%) and 14 out of 42 isolates (33%) from diseased fish were confirmed to be *A. veronii* biovar *sobria* and *A. hydrophila*, respectively, by gyrB housekeeping genes, which is also one of the most accurate method for identification of *Aeromonas* spp. to date (Martínez-Murcia *et al*., 2008; Hu *et al*., 2012; Wu *et al*., 2015). The same study also reported that 90 out of 120 (75%) isolates found in healthy fish were confirmed to be *A. veronii* biovar *sobria* (Hu *et al*., 2012). All of these studies have a similar dominant species and proportion of *Aeromonas* isolates, as observed in the present work.

In contrast, Lau *et al*. (2020) reported that *A. caviae* was the most frequent species found (n = 17) among 30 isolates, while the other two *Aeromonas* spp. belonged to *A. rivuli* (n = 9) and *A. dhakensis* (n = 4). Although the results of Lau *et al*. (2020) were confirmed with PCR and rpoD gene sequencing, the small sample size may not be adequate to compare to the population observed in the present work as those aeromonads were only isolated from two different species of fish in a single aquaculture hatchery.

Moreover, Ghenghesh *et al*. (2014)'s study also showed that *A. hydrophila* (n = 44) was the most common species among 99 *Aeromonas* isolates obtained from a wide variety of different sources. Two other studies also concluded that *A. hydrophila* was the dominant species isolated (Nielsen *et al*., 2001; Guerra *et al*., 2007), while another study demonstrated that *A. sobria* was the most frequent species isolated instead (Beaz-Hidalgo *et al*., 2010). Puthucheary *et al*. (2012) also found that *A. aquariorum* was the dominant species among other species found in his study. However, a study has shown that *A. aquariorum* was previously misidentified as *A. hydrophila*, and this could also be the case in the present work due to the lack of accurate species differentiation methods like gene sequencing (Aravena-Román *et al*., 2011).

Since *Aeromonas* spp. have already been documented to be found in the environment and various aquatic species for more than two decades (Wu *et al*., 2019; Dudley, 2022), the presence of *Aeromonas* found in the present work was not surprising. The observed prevalence of *Aeromonas* in the present work may be attributed to contaminated water sources, and improper and polluted freshwater fish handling procedures from the fishmongers to the wet market stalls. It may also be attributed to the different geographical circumstances in which the present work was conducted. Differing sources to which *Aeromonas* spp. were extracted, such as differing species or water sources, may also contribute to the difference in prevalence of a particular species.

From the results of the haemolytic test (Figure 1), it can be concluded that *A. hydrophila* had the most beta-haemolytic strains, followed by *A. veronii* biovar *sobria*, with at least 76.7% of them exhibiting beta-haemolytic potential. Several reports corroborated these findings that both *Aeromonas* strains exhibit strong beta-haemolytic activity (Monfort and Baleux, 1991; Abd-El-Malek, 2017; Hoel *et al*., 2017).

However, these findings are in stark contrast with the study of Radu *et al*. (2003), where the majority of *Aeromonas* strains (43/60) were alpha-haemolytic. Additionally, haemolytic activity in the study of Radu *et al*. (2003) was assessed using 5% human defibrinated RBC, which suggested that haemolytic activity might differ based on the type of blood agar used; the present work used sheep's blood. Radu *et al*. (2003) also did not clearly categorise haemolytic activity based on the species, so it was difficult to properly make a detailed comparison with the present work.

The results for haemolytic activity among *A. caviae* isolates corroborated the findings of John and Abdulla (2013), where it was noted that beta-haemolytic strains were infrequent in *A. caviae*, but were within the range of 70 to 72% (between 175 and 182 isolates) from fish and water samples, respectively. Similarly, another study also found that 65% of 17 *A. caviae* isolates possessed haemolytic activity (Yadav *et al*., 2014). However, there were only eight *A. caviae* strains found in the present work. As such, the comparison to the studies mentioned earlier may not be accurate. In contrast, based on other studies, it was found that most *A. caviae* isolates did not exhibit any form of haemolytic activity (Monfort and Baleux, 1991; Hoel *et al*., 2017). To summarise, the results for haemolytic test clearly demonstrated *Aeromonas*' haemolytic potential, with the majority of them being able to exhibit beta-haemolytic properties. Therefore, they are very efficient in breaking down blood cells, and causing congestion in various internal organs of organisms. Their strong beta-haemolytic potential has been proven to be one of the key virulence factors in the pathogenesis of *Aeromonas*-associated diseases, such as in a few studies in which intraperitoneal challenged fishes with isolated *Aeromonas* spp. manifested with

enlarged livers, congested spleen, and damaged kidneys (Bidin *et al*., 2019; Abdel-Latif and Khafaga, 2020; Pauzi *et al*., 2020).

In the present work, all the *Aeromonas* isolates (n = 72) (100%) were positive for proteolytic activity. This indicated that the isolated *Aeromonas* spp. were very adept at breaking down simple protein structures. The frequency of occurrence of proteolytic-positive *Aeromonas* spp. were in broad agreement with those of other studies which found that most *Aeromonas* spp. were also protease-positive (Zmysłowska *et al*., 2009; Chu *et al*., 2013), and possessed protease genes (Takahashi *et al*., 2014; Skwor *et al*., 2014; De Silva *et al*., 2018). In another literature, Chakraborty *et al*. (2019) noted that *A. hydrophila* produced maximum protease enzymes after incubation at 37°C after 18 - 24 h. Moreover, Chakraborty *et al*. (2019) has also specifically mentioned in their study that *A. hydrophila* continued to exhibit protease activity even at high temperatures of 70°C, which further highlights the importance of proper food handling measures especially when cooking food products. From Chakraborty *et al*. (2019)'s study, it can be assumed that fish would need to be thoroughly processed and cooked or there can be a possible cross-contamination with other foods during the food preparation process. There were no other conflicting studies of *Aeromonas* concerning protease production.

A total of 19 (90.5%), five (62.5%), and 33 (76.7%) (Figure 2) out of 21 *A. hydrophila*, eight *A. caviae*, and 43 *A. veronii* biovar *sobria* were lipase positive, while the remaining isolates were lipase negative. The frequency of occurrence of lipase-positive strains of *Aeromonas* spp. agreed with PCR gene studies, where the prevalence of detected lipase genes ranged from 56 to 72% (Yang *et al*., 2017; Hossain *et al*., 2018), with differences between the aforementioned studies and the present work lying in their sample sizes and the species of the sample *Aeromonas* spp. were isolated from. However, the findings of the present work disagreed with Abd-El-Malek (2017) on *Aeromonas* spp. found in raw and ready-to-eat fish, where lipase was only detected in 17.1% of the total isolates, and Yano *et al*. (2015)'s study, in which only 24% of 87 *Aeromonas* isolates were detected to harbour the lipase gene.

Based on the analyses on all three different *Aeromonas* spp. obtained in the present work, it was noted that both haemolytic and proteolytic activities were observed in all the *Aeromonas* isolates (100%). Lipolytic activity was the only factor that stood out. Therefore, when we analysed our results based on the total number of virulence factors each *Aeromonas* isolate had in the present work, we obtained similar results as those under the detection of lipase activity. For *A. hydrophila*, 19 out of 21 (90.5%) isolates possessed three virulence characteristics, while the remaining two possessed only two virulence characteristics (9.5%) (Figure 3). Meanwhile, for *A. caviae*, five out of eight (62.5%) isolates possessed three virulence characteristics, while the remaining three possessed only two virulence characteristics (37.5%) (Figure 4). Finally, for *A. veronii* biovar *sobria*, 33 out of 43 (76.7%) isolates possessed three virulence characteristics, while the remaining ten possessed only two virulence characteristics (23.3%) (Figure 5). It is also important to note that all of the *Aeromonas* isolates possessed at least two virulence factors. Since *Aeromonas* isolates found in the present work were only investigated for three different virulence factors, the isolates could not be defined as highly pathogenic. Many studies in the past were conducted using targeted PCR gene sequencing, and found numerous other virulence factors (Di Pinto *et al*., 2011), among which was aerolysin (De Silva *et al*., 2018) and cytotoxic enterotoxins (Chacón *et al*., 2003). Consequently, this analysis was unable to cross-reference to other studies conducted previously since an equal comparison cannot be justified as the present work did not explore all the possible virulence genes using a gene sequencing method.

The findings that there was a high frequency of important virulence factors observed in the present work corroborated the notion that *Aeromonas* spp. isolated from the freshwater food samples possessed different types of virulence factors. The obtained results suggested that potentially virulent *Aeromonas* strains are common in commercial aquaculture fishes, which may be a cause of concern for public health (Igbinosa *et al*., 2012; FAO, 2020).

Conclusion

The present work demonstrated that *Aeromonas* spp. were indeed present in various species of aquaculture food samples in Kuala Lumpur, Malaysia, and shown to possess a different variety of virulence factors, such as haemolysis, proteolysis, and lipolysis. The prevalence of *Aeromonas* observed in the present work necessitates close monitoring of such species and future studies

into its pathogenesis of associated diseases. The results of the present work would provide preliminary data to establish appropriate management and biosecurity practices that are essential in the aquaculture and public health sectors.

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