INVESTIGATION OF THE BACTERIAL DISEASE OUTBREAKS IN FARM TILAPIA AND CATFISH IN INDONESIA

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by

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Supervisors' certificate

This is to certify that this thesis entitled "Investigation of the Bacterial Disease Outbreaks in Farm Tilapia and Catfish in Indonesia" submitted to the University of Stirling, UK by Heri Kurniawan, has been carried out under our supervision. This is further to certify that it is an original work carried out by the candidate and suitable for the degree of Philosophy.

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DECLARATION

I, the undersigned, hereby declare that this thesis has been composed entirely by me and has not been submitted for any other degree. The work presented in this thesis, except where specifically acknowledged, is the result of my own investigations which have been done by me independently.

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ABSTRACT

Infectious diseases remain the major challenge affecting the sustainability of aquaculture production due to mortalities and morbidities which caused significant economic losses. A comprehensive approach has not been widely adopted to understand the disease status in Indonesia. Therefore, a field study was conducted in 41 farms producing tilapia and catfish which were representative of the range of production systems identified in East Nusa Tenggara Indonesia. A semi structured questionnaire was designed to describe the current status of the farming systems, level of disease outbreaks and health management strategies adopted in this area. A disease diagnostic approach was adopted to understand the role of infectious diseases related to the outbreaks. Bacterial isolates and tissue samples were collected for bacterial identification and histopathology to confirm the aetiology. A combination of traditional identification methods and molecular methods were performed for bacterial identification and characterization. The results from the field study identified that the farming systems in the area of study were categorized as in the early stage of intensification, where good health and disease management and biosecurity practice were not in place within the visited farms. From the questionnaire provided, a total of 73% (n=30) of participating farms reported that they experienced high mortality where, 34% (n=13) of farmers identified that the diseases were the main cause of the mortality, where the farmers recognised fish disease from the presentation of external clinical signs. The gross presentation of moribund fish samples showed a minimum either one or a combined clinical sign of the disease such as loss of appetite, sluggish movement, and swimming near the water surface, with the complete absence of the reflex, redness, and other external signs. The abnormalities of internal clinical signs such as the enlargement and change colour of fish organs. The examination of histopathological from the tissue samples from fish with clinical signs presented several changes of the tissue including vacuolation, degenerative changed in glomerular, necrosis, infiltration of inflammatory cells, and the presence of excessive number of MMC. The Gram stain of the tissue samples was also showing the figure of rod shapes bacterial colonization. The vast majority of isolates were Gram-negative bacteria and dominated by motile *Aeromonas* species including A. *veronii, A. hydrophila,* and *A. caviae,* which then 40 representative samples were confirmed by 16s rRNA and the two housekeeping genes *rpoD* and *gyrB*. A total of 12 virulence

genes were detected with 95% (n=38) of all *Aeromonas* species positive for cytotoxic enterotoxin (*act)* gene. Whereas the aerolysin *(aerA)* gene was detected in 55% (n=22) of the strains. The *aerA* and a*ct* genes which is considered as one of the most important genes related to MAS disease, in the present study 53% (n=21) of the tested strains contained of the combination *act+aerA* genes. For the AMR, 39% (n=16) of the strains were resistant to a minimum 1 of antibiotics tested where oxytetracycline (30µg) was the most prevalent with 35% (n=14) of the *Aeromonas* strains where 13/14 of the same strains were also had *tetE* gene. All of the 6 *Aeromonas* strains selected for experimental challenge *in vivo* caused infection which led to the significant mortality of wax moth larvae compared with the unexposed controls. The strains of *Aeromonas* with different virulence profiles were administered to the larvae at the same concentration and incubation temperature but produced varied survival to the infected larvae for both tested *A. veronii and A, hydrophila* strains ($1x10^5$ CFU/ml). the strains categorized as high virulence caused all larvae dead since the second day post infection whereas the strains with low virulence caused 47% of larvae dead at the end of the experiment. These findings provide critical information on the role of motile *Aeromonas* in the occurrence of the disease outbreaks and pathogenic capacity of the motile *Aeromonas* species associated with infectious disease outbreaks in fish farming systems in Indonesia.

Keywords: *production system, tilapia, catfish, disease outbreaks, Aeromonas, virulence, pathogenicity, Indonesia*

List of Attendance and Presenting Conference

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3. Fisheries and Aquaculture Summit. 27 February 2021. United Kingdom
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List of abbreviations

act	cytotoxic heat-labile enterotoxin
aerA	aerolysin
H_2O_2	Hydrogen Peroxide
AH	Apparently Healthy
alt	cytotoxic heat-labile
AML	Amoxicillin
AMR	Antimicrobial Resistance
ast	cytotoxic heat-stabile
ATCC	American Type Culture Collection
AWERB	animal welfare and Ethical Review Body
CFU	Colony Forming Unit
DO	dissolved oxygen
ela	elastase
eno	Enolase
ENR	Enrofloxacin
ENT	East Nusa Tenggara
FAO	Food and Agriculture Organization
FCR	Food Conversion Ratio
FFC	Florfenicol
FQIA	Fish Quarantine and Inspection Agency
g	Gram
gcat	glycerophospholipid cholesterol acyltransferase
GSP	Pseudomonas *Aeromonas* Selective Agar
GUEP	General University Ethics Panel
gyrB	Gyrase subunit B
H&E	Haematoxylin and Eosin
hylA	haemolysin
ISO	International Organization for Standardization
KM	Kaplan Meier
KOH	Potassium Hydroxide
lafA	lateral flagella
lip	lipase
M	Moribund
MAS	Motile *Aeromonas* Septicaemia
MLST	Multilocus Sequence Typing
MMAF	Ministry of Marine Affairs and Fisheries
MMC	Melanomacrophage Centers
MR-VP	Methyl Red-Voges Proskauer
NAD	No Abnormalities Detected
NBF	Neutral Buffered Formalin

NCIMB	National Collection of Industrial Food and Marine Bacteria
NH_3	Ammonia
NO_2	Nitrite
OA	oxalinic acid
OD	Optical Density
OF	Oxidative-Fermentative
OR	Odds Ratio
OTC	Oxytetracycline
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Ribonucleic Acid
rpoD	RNA Polymerase Sigma (Sigma D) Factor
rRNA	Ribosomal Deoxyribonucleic Acid
RS	Rimler-Shotts
SEA	Southeast Asia
ser	serine protease
SPSS	Statistical Package for Social Sciences
SXT	sulfamethoxazole
T2SS	The type II secretion system
T3SS	The type III secretion system
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
vAH	Hypervirulent *A. hydrophila*
WSSV	White spot syndrome virus
µg	microgram
µl	microliter

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CHAPTER I. LITERATURE REVIEW

CURRENT STATUS OF TILAPIA AND CATFISH FARMING IN EAST NUSA TENGGARA, INDONESIA

1.1. Current Status of Aquaculture in Indonesia.

Indonesia is an archipelago country consisting of approximately 17.5 thousand individual islands with a complete coastline measuring 81 thousand km (Lakip, 2018). The country has immense capacity in its water resources for aquaculture including 16.9 million ha for marine, brackish water, and freshwater area reaching 2.96 and 2.8 million ha respectively (MMAF, 2019). Whilst there is indeed large resource capacity for aquatic farming, currently only 2.7% of the freshwater available area has been used for aquaculture (MMAF, 2019). Indonesia's fish production was generated mostly from the capture fisheries, providing approximately 96.5 million tonnes compared with 82.1 million tonnes from aquaculture (FAO, 2018). However, capture fisheries production was reported as having low potential in the future to meet the increasing demand of fish products from consumers. Furthermore, the efforts to fulfil the demands of fish product for human consumption through capture fisheries could promote overexploitation and exceed the maximum sustainable yield of the capture fisheries (Henriksson *et al.*, 2019; Napitupulu *et al.*, 2022). Therefore, the development of sustainable aquaculture in Indonesia became the major focus of Indonesian government to meet the increasing demand for fish products locally and export (MMAF, 2020). Indonesia has an ambitious target where aquaculture is expected to be the leading source of fisheries products by 2030 (Napitupulu *et al.*, 2022). It is not only the food production, but aquaculture sectors also play important roles in reducing unemployment rates in Indonesia (MMAF, 2022). In particular, tilapia farming which provides the greatest contribution of edible finfish from farming in Indonesia and provides significant employment (Phillips *et al.*, 2016; MMAF, 2022). Freshwater aquaculture plays an important role fulfilling the demand for edible fish products locally and if included as part of the normal diet, can address human health issues e.g. stunting of growth in children, which remains a significant issue in several parts of Indonesia including in the remote areas in East Nusa Tenggara (ENT) (MMAF, 2022). Recent studies have demonstrated uptake how important nutrient uptake of high-quality protein consumed through the inclusion of fish in the diet of children has reduced the rates of retarded growth from 436 thousand to 77 thousand

children in ENT (BPS, 2022). Therefore, the programs established to enhance fish production by the national and local governments have a broader aspiration than simply increasing income and will promote improved human health overall, if well managed. Government reports have shown there is an increased uptake of households participating in aquaculture, and the Government have provided resources including infrastructure, technology, training for the farmers as well as implementing regular monitoring and surveillance programmes (MMAF, 2022 & 2022).

The dominant aquaculture regions in 2017 were found in Java, South Sulawesi, and East Nusa Tenggara (ENT). In these regions of Indonesia seaweed remains one of the highest aquaculture production sectors, particularly in South Sulawesi and ENT, providing 2.3 and 1.9 million tonnes of seaweed, respectively. An overview of the annual aquaculture production within Indonesia is provided in Figure 1.1.

Source of the data: Ministry of Marine Affairs and Fisheries of Indonesia, 2018

Figure 1.1. Annual production of aquaculture in the regions of Indonesia per area in 2017 (source: KKP in number 2020).

A steady increase in production of all aquaculture commodities increased from 2015 to 2019, with a minor 2% reduction observed in 2018 and a 5.4% reduction in total production was observed in 2020 (Table 1.1). It is normal that the total production may fluctuate as there are several fish, shrimp and plants that contribute to the total aquatic food production in Indonesia, but the overall trend shows that seaweed is the

dominant plant and tilapia, followed closely by Clarias catfish species are the dominant finfish produced (Table 1.1).

Table 1.1. Indonesia aquaculture production from 2016 to 2020 (tonnes).

Commodity	2016	2017	2018	2019*	2020**
Tilapia	1,114,156	1,280,126	1,171,681	1,474,742	1,235,514
Clarias catfish	764,797	1,125,526	1,027,195	1,224,360	1,017,313
Shrimp	692,568	919,988	911,857	1,053,206	911,216
Milkfish	747,445	701,427	875,594	810,867	695,801
Carp	497,208	316,649	534,076	584,497	514,643
Pangasius	392,918	319,967	373,263	476,209	426,475
Gourami	132,334	234,084	173,345	227,468	212,139
Grouper	11,504	13,294	16,414	18,490	16,461
Snapper	7,890	8,432	9,835	7,686	7,250
Seaweed	11,050,301	10,547,553	10,320,297	9,918,455	9,923,259
other	591,107	590,945	358,249	534,543	496,036
Total	16,002,228	16,114,991	15,771,806	16,330,523	15,456,107

*Source of the data: MMAF, 2020

1.2. The Status of Aquaculture in the Area of Study (ENT).

East Nusa Tenggara consists of 5 large islands with the total land area of 48 thousand km^2. Timor Island is the largest land mass in ENT with 14.8 thousand km^2, and in 2022 had a population of 5.4 million as the data reported by Indonesia Central Bureau of Statistics (BPS, 2022). Aquaculture in ENT contributed 2.2 million tonnes to the national production of this sector (BPS, 2022). The Kupang region contributed the highest level of aquatic production with 1.9 M tonnes, followed by East Flores at 113.2 thousand tonnes (BPS, 2022). Intensive production in the ENT area occurs in marine, brackish water, and freshwater farming sites throughout the island. Excluding the seaweed production, greater production volumes were generated in the marine and brackish water systems compared with freshwater farming. The brackish water area is dominated by shrimp (*Penaeus sp.*) and milkfish (*Chanos chanos*) covering an area of 35.5 thousand ha with annual production of 36 thousand tonnes, whereas the marine production dominated by seaweed (*Eucheuma sp.*), pearls (*Pinctada sp.*), and grouper (C*romileptes sp.*) covers 5.9 thousand ha generated 51 thousand tonnes production per year (MMAF, 2020).

The capacity of freshwater aquaculture in ENT has not been as widely recognized in the region, particularly as there is more marine fish which are affordable to the local families. However, similar to other areas in Indonesia, marine fish

production has become stagnant and in 2017, decreased by approximately 20% from 173.296 tonnes in 2016 to 138.268 tonnes (BPS, 2018). To counteract this loss in dietary fish, the Indonesian government promoted and supported the development of freshwater farming by establishing the number of programs to support the development in this food production sector e.g. distribution of biofloc program in ENT to promote the environmental water quality in intensive farming systems and more efficient land use (KKP, 2020). The biofloc system maintains the water quality by promoting the nitrification and reduces the ammonium level in the water whilst supporting better dissolved oxygen in the freshwater intensive farms. This system allows the farmers to increase their stocking density with a minimum water change. This system has been promoted by the government as a part of the intensification development in the freshwater aquaculture systems (MMAF, 2022). The Government initiatives to promote aquaculture included provision of fish fry, improved accessibility to fish feed and medication, and regular farm-level monitoring. This approach was established by the government to help the farmers in developing the fish farming in the region and whilst these are positive, there remain significant challenges in these systems, particularly with disease outbreaks as they intensify.

1.3. Aquaculture Species in Indonesia.

Amongst the fish species cultured, tilapia and catfish are the two main freshwater species widely farmed in Indonesia where the total production of those fish species remains in the top five of the highest freshwater culture production in Indonesia (MMAF, 2018). Several characteristics that enhanced the popularity of tilapia include their ability to survive in wide range of water environments including in shallow and turbid waters, high marketability, and they are often considered to have higher resistance to the diseases compared with other freshwater farmed fish (Ng and Romano, 2013; Alam *et al.*, 2016). Tilapia is also able to tolerate the wide range of environmental conditions such as low dissolved oxygen (<2 mg/l) and high ammonia level for longer periods then most (Papuc *et al.*, 2019). The culture of both species is also considered suitable for medium and low-income societies in the region of Indonesia because the investment is relatively small compared with the other species e.g. shrimp (Sunarma, 2004; MMAF, 2020). The production of tilapia in the study area of ENT, Indonesia has intensified and increased in number of farming systems. Tilapia production increased by 74% and catfish by 77% from 2016 to 2018 in ENT (MMAF,

2018). In the national level Indonesian tilapia production was the second highest production behind China with 250 thousand metric tonnes (MMAF, 2022; El-Sayed and Fitzsimmons, 2023).

MMAF reported that tilapia production in 2017 in Indonesia was primarily found in the western area of Indonesia. West Java was the main producer with the highest number of tilapias generated at 343.4 thousand tonnes, followed by South Sumatra, Central Java and West Sumatra with 160.5, 120.7, and 114.4 thousand tonnes respectively (MMAF, 2018). Meanwhile, ENT contributed a smaller production level (Figure 1.2).

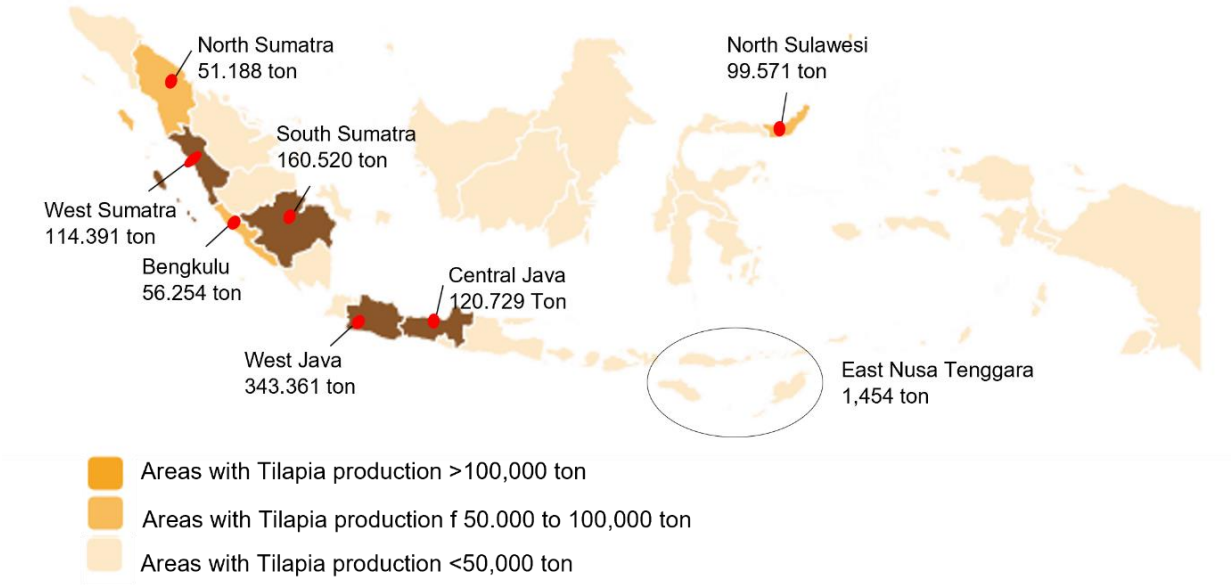

Figure 1.2. Annual production of tilapia farming in Indonesia (2017).
Source: MMAF in number, 2018.

The area of catfish production is primarily located in the western area of the country, similar with tilapia production. The top catfish production was West Java with 308.7 thousand tonnes, followed by East Java, South Sumatra, and Central Java with 248.5, 136.5, and 105.9 thousand tonnes respectively with ENT providing a lower production level (Figure 1.3).

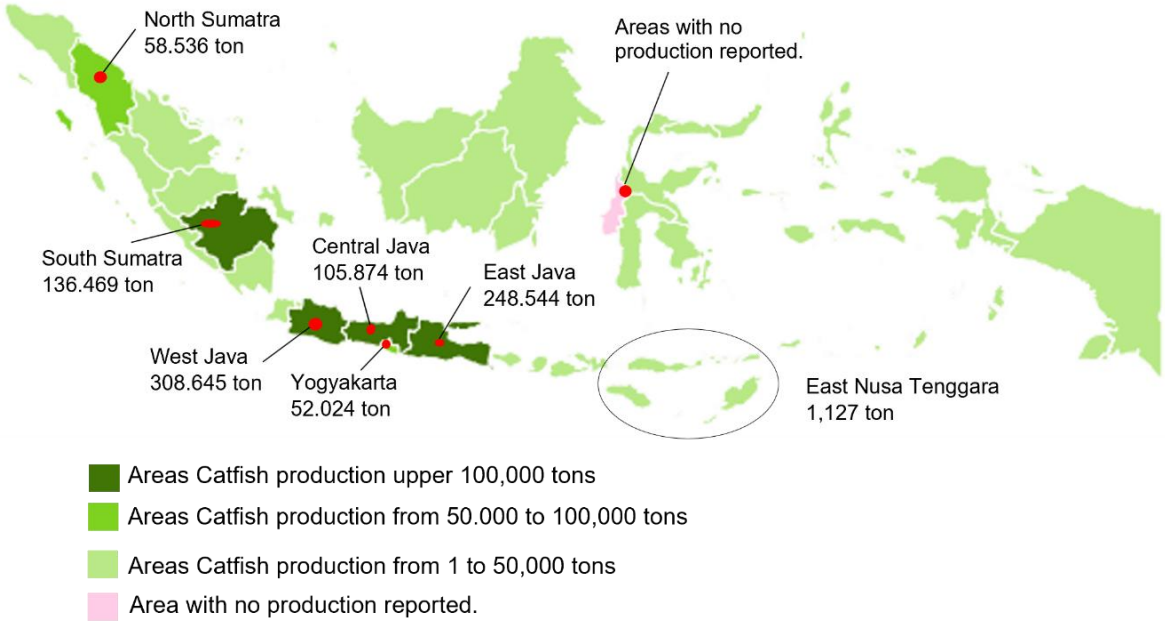

Figure 1.3. Production levels (ton) of catfish farming in Indonesia (2017). Source: MMAF in number, 2018.

1.4. Challenges Affecting Sustainable Aquaculture in Indonesia.

Disease outbreaks remain one of the major challenges affecting the aquaculture systems causing significant economic and animal losses in Indonesia. Several studies have identified bacterial disease outbreaks in Indonesia freshwater tilapia and catfish farms, where outbreaks due to the bacterial infection motile *Aeromonas* septicaemia (MAS) was reported in early 1980 costing economic loss of USD 1.3 million per year. More recently, MAS has been identified as the main disease in Indonesian tilapia and catfish farms, resulting in economic losses of USD 26.5 million per year (Wibowo, 2021). Other bacterial infections have also been reported e.g. outbreaks of *Streptococcosis* was reported to cost around USD 1 million of economic loss per year (NSAAH, 2015) with an overall economic cost of USD 400 million per year due to infectious disease in these aquaculture systems (Lusiastuti *et al.*, 2020).

In the study area of ENT, as this is considered a developing area of aquaculture the biosecurity practises applied are fragmented and often lacking in some farming systems. Anecdotal data has illustrated that the fish farmers in ENT region did not fully understand or apply a comprehensive health management practice or biosecurity approaches in their farms. Little, if any data, was available to support the

understanding on actual cause of the fish mortalities although most farmers describe these fish losses as a consequence of “disease” without the diagnostic or laboratory backup data to support an infectious outbreak, the actual cause of mortality is unknown. It is well recognised that as aquatic farming intensifies, the risk of disease also increases and appropriate biosecurity practises which include correct transportation of broodstock, supply of healthy fish fry are needed to reduce the emergence and re-emergence of infectious diseases (FAO, 2022). By not adopting a risk management strategy to avoid the disease transmission within, out of, and around the farm, all farmers run the risk of infectious disease outbreaks (Henriksson *et al.*, 2019; (Kayansamruaj *et al*., 2020). It is not simply the fish stocks that must be considered in a biosecurity plan but also the aquatic environment, both the water coming into the farm and the water leaving the farming system.

Environmental impacts can occur from intensification of the aquatic farming systems as without correct management applied, increasing water pollution can occur caused by improper application including excess feed, chemicals use, and medication which can all negatively affect the surrounding ecosystem (De, 2019). The freshwater fish farming systems in ENT are predominantly open resources, where they are exposed to the natural weather conditions as the majority of production systems are earthen based ponds or river/marine-based cages which are exposed to the natural environmental conditions. This can promote the risk and frequency host-pathogen interactions, and in some cases may be exacerbated by the extreme local weather in Indonesia which can affect the water quality and the behaviour of the farmed fish, which under certain conditions lead to disease (Henriksson *et al.*, 2019). The weather fluctuation in Indonesia ranges from dry to rainy seasons or vice-versa and are accompanied by high rainfall intensities and long dry season, which can result in unfavourable conditions for the fish promoting chronic stress and immunocompromised status and more susceptible to diseases (Lusiastuti *et al.*, 2020).

The Indonesian government has already established regulations related to the appropriate farm management practices relevant to the fish species being produced, however, uptake and implementation of the regulations had not been equally acknowledged by all of the farmers practising different stages of aquaculture. Implementing and policing best practises can be challenging in Indonesia systems due to the large variation in environmental conditions, farming systems, and social characteristics of local societies within the areas. The implementation of the policy

related to the farm management practices and biosecurity remained a concern to the sustainable development of fish farming sectors in Indonesia (MMAF, 2022).

A variety of prevention strategies and treatments have been established to control disease outbreaks in farmed fish species both locally and globally (Austin, 2019a). These are usually formulated based on a risk assessment of the infectious diseases and the susceptibility of the fish species. Clarity is required to provide these biosecurity practises and strategies, yet unfortunately such measures are rarely practised within many parts of SEA country including in Indonesia, unless there is a critical driver e.g. intensive farm or company policy (Kayansamruaj *et al.,* 2020). A systematic approach to support the disease prevention and appropriate control strategies relevant to Indonesian aquaculture system has not been fully developed. This remains critical given the trend in intensification of production and ensuring commercial viability of aquatic production and support the government strategic goal in aquaculture food production. The application of antibiotics and other chemical substances are a concern in the continuous development of fish farming in Indonesia. Excessive application without a therapeutic regimen remains the major concern in the use of antibiotics as this can promote antibiotic resistance, resulting in treatment failure. Antibiotics remain one of the most common methods of treatment during disease outbreaks globally in aquaculture including Indonesia (Sapkota *et al.*, 2008; Chitmanat *et al.*, 2016). Although, the Indonesian government has already established regulations regarding the use and spread antibiotics, the farmers are still able to obtain antibiotics in the private sectors (MMAF, 2020). The challenge of farmers adopting a more considered therapeutic approach in their farms is hampered by the fact that most of the farmers in the area of study have limited knowledge regarding the appropriate application of any treatments including antibiotics. Globally, the issue of antibiotic resistance is a major challenge where the excessive misuse of antibiotics can promote disease resistance and threaten the sustainability of the fish farming systems (Noga, 2010; Manyi-Loh *et al.*, 2018; Samreen *et al.*, 2021).

1.5. Bacterial infections impacting farmed tilapia and catfish, Indonesia.

Several reports have identified *Aeromonas* bacterial infections in Indonesian freshwater aquaculture systems, and the bacteria were recovered and identified from both apparently healthy and diseased tilapia samples in Indonesia (Amanu, Kurniasih and Indaryulianto, 2014; Rahayu, 2019; Azhari *et al.,* 2014; Manurung and Susantie,

2017; Hardi *et al.*, 2018). Similar reports have documented this MAS disease globally, in both catfish and tilapia farms (Cai *et al.*, 2012; De Jagoda *et al.*, 2014; Korni and Ahmed, 2020). Several *Aeromonas* species including *A. veronii, A. hydrophila, A. sobria, and A. caviae* were reported as fish pathogens causing outbreaks of MAS in multiple fish species (Cai *et al.*, 2012; Austin, 2019a) and *A, hydrophila* was commonly recovered and identified during fish MAS infections (Austin, 2019; Talagrand-Reboul *et al.*, (2020). The development of several molecular methods has contributed to the improved identification and characterization of motile *Aeromonas* species including *A. hydrophila* and *A. veronii* which have both been reported as aetiological agents of MAS infectious disease. It is the uptake of genomic molecular based assays that has provided better discrimination between closely related bacterial species has shown a wider range of *Aeromonas* species responsible for MAS infections (Janda and Abbott, 2010; Cai *et al.*, 2012; Rasmussen-Ivey *et al.*, 2016). A very wide range of genomic based identification tools have been developed including Multilocus Sequence Typing (MLST), which is a genetic typing-based assay that using DNA sequences from multiple bacterial samples and compares internal fragments of DNA using housekeeping genes which can then be used to distinguish between closely related bacterial species that traditional test-tube based identification may not have the sensitivity.

In the majority of aquatic diagnostic laboratories, a combination of traditional microbial identification methods with DNA based methods e.g. PCR are used for the identification and characterisation of the bacteria recovered. Traditional identification methods have a value and can be reliable in differentiating the viable and recovered bacterial to at least genus level but are not always able to provide species level identification with confidence. The reason being that most of the biochemical-based kits lack discriminatory power to distinguish closely related bacterial species. Depending on the identification level required, a combination of phenotypic and genotypic identification assays provide better diagnostic details and promote understanding related to the pathogenic diseases (Austin, 2019b). In cases of MAS, traditional identification methods were often hampered by inconsistent results which may be a consequence of the technical performance of the assay or the individual performing of the test as these are colorimetric assays can be rather subjective, and experience is required to interpret the results correctly. Even then, it is impossible in most closely related motile Aeromonads to rely on biochemical profiles to confirm

species-level identification due to the heterogenous nature of the strains within a single bacterial species. This leads to an unfortunate level of complexity in the biochemical profiles recovered as a result of strain variation from clinical and environmental isolates (Janda and Abbott, 2002; Beaz-Hidalgo *et al.*, 2015). Many diagnostic labs use the 16SrDNA PCR assay to identify their bacteria (Janda and Abbott, 2007; Burr *et al.,* 2012). However, identification of motile *Aeromonas* by 16S rRNA PCR gene analysis is limited as it cannot differentiate closely related bacterial species (Morandi *et al.*, 2005). Therefore, additional assays have been developed and applied including the use of the PCR assay using the housekeeping genes *gyrB* and *rpoD* that have better resolution for identification of motile aeromonad species, where both of these housekeeping genes were provided valuable molecular markers for the phylogenetic and taxonomic relationships study of *Aeromonas* into species level (Beaz-Hidalgo *et al.*, 2010). Identification of the bacteria is obviously the first step in being able to help support the right diagnostic approach and additional assays can be useful to characterise the bacteria more fully. In this case, using the molecular typing methods will help to identify the microbial population and the core virulence or antibiotic resistance factors they have. This can then elucidate not only the biosecurity practises but provide essential information to develop novel hygiene, sanitation, disease prevention and appropriate treatments.

1.6. Disease Monitoring and Surveillance, Indonesia.

Regular monitoring is important aspect in supporting the fish farming sector and can provide essential information on health and disease status within the sector. Adoption of routine disease monitoring combined with effective disease diagnosis can be a very effective early warning system and enable appropriate measures taken before the disease becomes established leading to high economic losses (Adams and Thompson, 2011; Austin, 2019b). Health and disease monitoring activities are provided by the Indonesian government as part of a national program through the Ministry of Marine Affairs and Fisheries. (MMAF). Most of the farms in the ENT study area are included in the government monitoring, however, at this stage, the purpose is detection of particular pathogen/disease rather than a comprehensive health strategy (MMAF, 2020). Whilst this is useful it is limited during diagnostic investigations as it does not take into account the farm history, disease outbreak history nor the histopathology or pathogens recovery methods required to confirm the diagnoses.

Instead, it provides a presence or absence of a particular infection or pathogen, which may or may not cause disease in the stocks. This is considered a high-level epidemiological monitoring activity.

An aquatic health plan or any policy developed for aquatic animal health is not possible without good quality of data relating to the overall aquatic health. These data should include the animal and the environmental health of the farm. These data then can be used for multiple purposes in support of sustainable aquaculture development e.g. improved disease control strategies, requirement for quarantine in transportation of live animal movement, and of course can provide critical information relevant to health certification which can all be achieved by conducting aquatic animal surveillance (Dvorak, 2009). Surveillance to monitor and avoid the introduction of disease is an important element of any biosecurity strategy, as it can identify the possible route of disease introduction into the site, spread of the disease within the site and to detect the emergence of a new disease. This approach will also ensure that control strategies can be implemented before the pathogen severity is increasing and wide spreading (Marcos-López *et al.*, 2010; Oidtmann *et al.*, 2013). Disease surveillance should be an integral and key part of all Government aquatic animal health services but requires a more holistic approach than that currently practised.

The high traffic of live fisheries commodities including fish fry and live fish products increases the likelihood of the introduction and spread of fish diseases from one country to another and from one area to another within the territory of Indonesia. This issue can be economically harmful and threaten the sustainability of the fisheries resources, including aquaculture in Indonesia. Efforts to protect and prevent the entry and spread of the diseases between areas, especially from abroad into the territory of Indonesia is carried out by Fish Quarantine and Inspection Agency (FQIA), Ministry of Marine Affairs and Fisheries. Based on Law No. 16 of 1992, covering animal, fish, and plant quarantine, and Government Regulation No. 15 of 2002 regarding Fish Quarantine. In the monitoring and surveillance activity conducted by MMAF through FQIA, the surveillance approach was therefore more focused on the detection and reporting of specific pathogens affecting the farmed animals of high economic value (e.g. shrimp) and is performed throughout several regions of Indonesia. These diseases or pathogens include specific viruses, parasites, fungi and or bacteria but are restricted to those that are known disease issues might affect the intensive farming systems. The monitoring and surveillance activities are conducted twice to

four times in a year in different area of Indonesia and include several farms. The farms visited were not necessarily associated to the occurrence of the disease outbreaks. The figure 1.4 provides details of the areas in Indonesia visited and the diseases included in the surveillance programmes in 2018, where the bacterial species recovery target from this program in 2018 included three bacterial species (*P. anguilliseptica, E. ictaluri, a*nd *Aeromonas spp.).* The traditional method was used to identify the bacterial isolates recovered from freshwater fish samples collected through the programs conducted by the Indonesian government. The bacterial isolates recovered from the regions of Indonesia were then sent to the central FQIA laboratory in Jakarta Indonesia to confirm the bacterial identity from previous results provided by the local FQIA laboratory. The identification results including three those bacteria targeted in 2018 from the surveillance conducted by FQIA were described in the Figure 1.4.

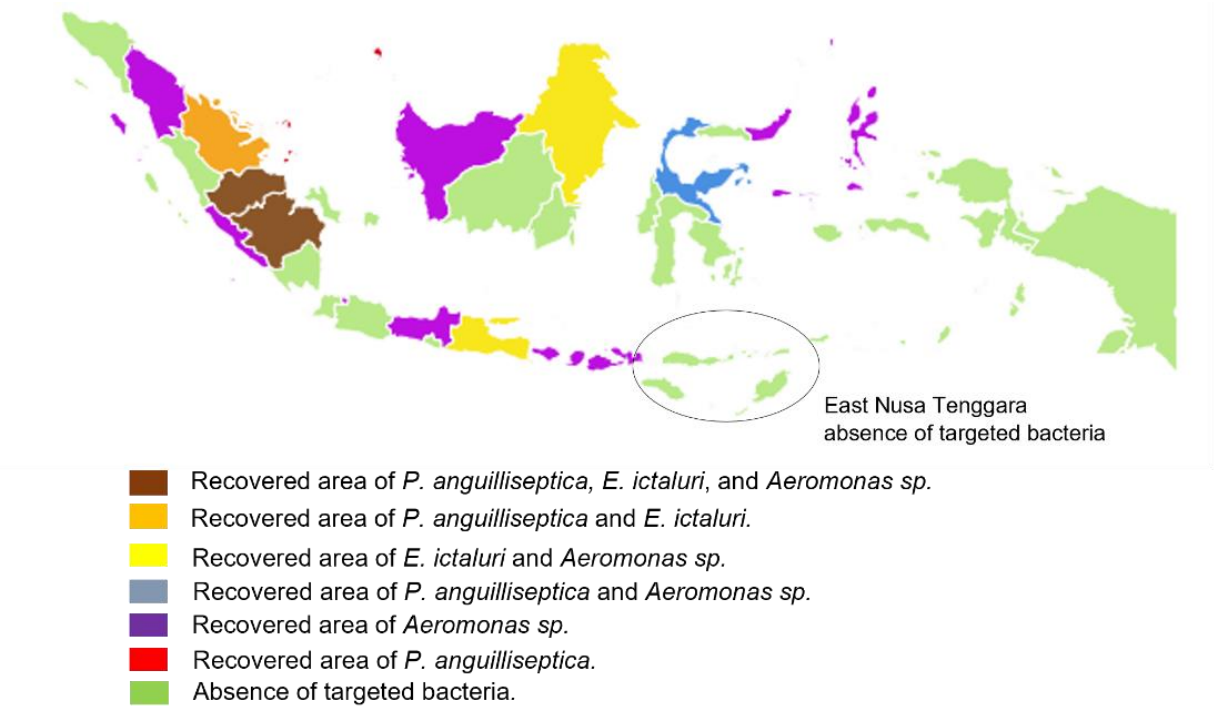

Figure 1.4. The area of the three targeted bacterial species (*P. anguilliseptica, E. ictaluri, a*nd *Aeromonas spp.)* recovered. Source of the data: Kepmen KP No. 58/2019 FQIA.

The interaction between maintaining fish health through high level disease management practices and provision of optimal environmental conditions are critical

to mitigate and control disease outbreaks. Whilst aquaculture policies in Indonesia incorporate all of these aspects (Cpib *et al.*, 2021), regulations provide a more general management approach and more specific details are required for the individual farming systems than those currently available. The lack of good management practises and appropriate biosecurity being practised on the fish farming sites is not that surprising given the diversity of the farming systems. In Indonesia farming system, the issue of antibiotic resistance was identified by the government. Only experienced farmers or companies have access to the antibiotics, which was a strategy applied within the “zero antibiotics” campaign by the government since 2012. Nevertheless, without adequate knowledge, management and efficacious alternatives to antibiotics farmers continue to apply the unproper application of antibiotics which can be found in the farms.

Understanding the level and challenge affecting the farming system in Indonesia is critical to identify the disease status and the current management approaches adopted in the farming system including tilapia (*Oreochromis spp*.) and Clarias catfish *(C. gariepinus)* farming in ENT, Indonesia.

1.7. The aims of study.

The ultimate aim of this study was to identify and characterise the bacterial diseases affecting farmed Clarias and tilapia species in ENT, Indonesia. This was achieved using several methods and approaches, including epidemiology, traditional disease diagnosis, water quality and genomic and phenotypic profiling of the bacterial pathogens recovered from the moribund fish. The overall results from the work performed will provide the most comprehensive data from which, improved biosecurity strategies and management practises can be developed to support the sustainable development of the existing and developing aquaculture systems in ENT, Indonesia. A series of activities were performed to address the study aim:

- Description of the current tilapia and catfish farming systems and challenges encountered in these farms (Chapter 2).
- Identification, recovery, and diagnosis of the key bacterial infections affecting productivity within the selected farms in ENT, Indonesia (Chapter 3).

- Microbial profiling of the motile *Aeromonas* species recovered from the farm sites during active infections to cluster the bacteria into pathogenic status using robust and reliable laboratory-based methods (Chapter 4).
- To investigate the infectivity and pathogenicity of the strains recovered from the affected fish using an insect-based infectivity assay and correlate these findings with the in vitro virulence and AMR profiles (Chapter 4).
- Provide a comprehensive and curated culture collection, specific to ENT, Indonesian freshwater aquaculture systems, from which improved biosecurity and novel treatments can be produced.

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CHAPTER 2. IDENTIFICATION OF FARMING PRACTICE AND CHALLENGES AFFECTING FARMED TILAPIA *(Oreochromis niloticus)* AND CATFISH *(Clarias gariepinus)* IN EAST NUSA TENGGARA, INDONESIA

2.1. Abstract.

Infectious diseases remain the significant challenge affecting the sustainability of aquaculture production due to animal losses from mortalities and morbidities as well as additional costs incurred. A comprehensive approach has not been widely adopted to confirm the disease status in Indonesia and so a field study was conducted in 41 farms producing tilapia and catfish which were representative of the range of production systems found in East Nusa Tenggara Indonesia. A semi structured questionnaire was designed to describe the current status of the farming systems, level of disease outbreaks and health management strategies adopted within this region. A total of 73% (n=29) of participating farms reported that they experienced high mortality that usually occurred during the seasonal change, where from those, 14 farmers identified that the diseases were the main cause of the mortality, where the farmers recognised the fish disease from the presentation of external clinical signs. The type of intensification system with lacking farmers sufficient knowledge applied by the farms was also identified as contributing factors affecting the production, where the farmers increased the density without sufficient information regarding the optimal density, the feeding intake including the FCR, and the unregulated and excessive application of antibiotics can cause the detrimental effect leading to the development of antibiotic resistance in bacteria and environments which compromise the fish welfare. The poor management practice was doubled by lack of knowledge regarding the production system and biosecurity practices where 78% (n=32) of farms used series water sharing system and 88% (n=36) used the same equipment without the disinfectant applied, these methods would increase the risk of infectious diseases transmission and harmful substances between ponds within the farms. Farmers reported using a range of antibiotics, where 44% (n=18) of the farmers who applied antibiotics did not understand the type or the application dose during the treatment within their farms. The limitation of fish fry was also potentially affecting the sustainability of the fish farms, where 76% (n=31) of fish fry were obtained from outside of the study area. The current study provided valuable

information regarding the current status of tilapia and catfish farming system and the fish health management applied by the farmers within this aquaculture sector, including the intensification system applied by the farmers that was not supported by the sufficient knowledge regarding the management practices and disease and health management.
Keywords: *production system, tilapia, catfish, disease outbreaks, Indonesia*

2.2. Introduction.

Infectious diseases remain the major challenge affecting the development of the global aquaculture sector, where several factors are associated with the emergence and re-emergence of infectious diseases (Lafferty *et al.*, 2015; Vouga and Greub, 2016; Bouwmeester *et al.*, 2021). Several issues are often described within the individual sectors, however, the global trend of intensification of aquaculture farming systems contribute towards the emergence and re-emergence of infectious diseases leading to outbreaks and economic losses (FAO, 2022). The contributing factors that exacerbated the disease situation can include poor farm or fish management practices and lack of appropriate biosecurity practises within the farming systems (Kayansamruaj *et al.,* 2020). Therefore, it is critical that the disease risks to these aquatic food production sectors are recognised and understood by those working in the farm to develop and implement efficacious biosecurity practises to mitigate the risk of production level losses from infectious disease outbreaks. At present, a systematic approach to disease prevention and control strategies relevant to Indonesian aquaculture has not yet been fully developed or widely implemented. This remains critical given the increasing trend towards intensification and the promising commercial viability of Indonesian aquaculture. There are several incentives and strategies applied by the Indonesian to promote sustainable aquatic food productivity within the country. An example is the government provision of national aquaculture support programs which included 371 packages of biofloc to be applied in the intensive tilapia and catfish culture systems, production of 222.4 million fish fry, 194.5 thousand of fish broodstocks, 300 ton of fish feed, and contributions towards development of aquaculture facilities (MMAF, 2020). However, these incentives are attractive but will fail to deliver sustainable growth if a comprehensive and cost-effective biosecurity strategy is not developed and applied.

Risk management is a large part of any biosecurity plan and can be complex with multiple influences affecting the level of risk identified (The World Bank, 2014). It is recognised that mis-managed intensification can increase the risk of pathogenic disease resulting in outbreaks, often observed as low productivity. This issue was also experienced by the farmers in the aquatic farming systems in Indonesia (Cao *et al.,* 2007; Henriksson *et al.*, 2019). Infectious diseases in aquaculture systems are complex by nature and the host-pathogen susceptibility/infectivity can be exacerbated due to changes in the local weather patterns. This is a known biological risk for the Indonesian sector, particularly as the majority of production systems are earthen based ponds or river/marine-based cages which are exposed to the natural environmental conditions. The weather fluctuation in Indonesia from dry to rainy seasons or vice-versa are accompanied by high rainfall intensities and long dry season, which is counterproductive leading to stress and immunocompromised stocks, more susceptible to diseases (Lukistyowati and Kurniasih, 2012).

In Indonesia, the issue of pathogenic diseases remains the main challenge affecting this sector, including in the area of study and in several parts of Indonesia. the disease outbreaks were often reported as the major limiting factors affecting the development of aquaculture systems which resulted significant production and economic losses. More recently, economic losses due to the impact of bacterial infections in Indonesia's farming system were estimated reached approximately USD 400 million per year (Lusiastuti *et al.*, 2020). Bacterial pathogens were often reported as the main cause of the disease outbreaks with significant losses incurred, where motile *Aeromonas* Septicaemia (MAS) outbreaks as the most reported caused of the outbreaks that led to the economic losses estimated at USD 26.5 million per year (Wibowo, 2021). MAS disease was reported since the early of 1980 in Indonesia, where natural disease outbreaks caused by MAS infection resulted economic loss reached USD 1.3 million in a year (Angka, 2001). Although the significant impact of the disease outbreaks, the information of aetiological agent of the disease outbreaks was still limited/fragmented. Therefore, the comprehensive study to investigate the aetiological agent of the disease outbreaks is considered important.

A variety of prevention strategies and treatments are applied to control the disease outbreaks globally (Austin, 2019a). However, the appropriate farm level health management and biosecurity practises required to reduce the occurrence and/or severity of disease outbreaks were not well acknowledged or implemented within

many parts of South East Asia (SEA) countries including in Indonesia (Kayansamruaj *et al.,* 2020). As a result, the aquaculture systems in this region commonly experienced natural disease outbreaks with varied levels of severity and economic impact. The lack of appropriate biosecurity practises can be more commonly seen in the early stages of establishing the fish farming system which results in poor farm and fish health management. Similarly, changes in intensification of the farming systems needs a revision of the biosecurity risks and adoption of different biosecurity practises. In ENT the fish farming system is still in the early stages of intensification and at risk of increasing numbers of natural disease outbreaks if the sufficient management practises are not adopted universally. Anecdotal evidence has shown that farmers in ENT reported to the government authority (FQIA Kupang) that their farms were often experiencing fish mortality which led to the economic losses. However, little if any data was available to support the actual cause of the fish mortalities although most farmers describe this as “disease” without the diagnostic or laboratory backup to support an infectious outbreak.

To develop a biosecurity plan, data on the farm, production purpose, species and infectious disease risks relevant to the farmed species must be identified. This can be done at a large scale (e.g. generic risks at national level) and then applied at a farm specific level which will encompass additional mitigation factors unique to the individual farming system. Part of this process is regular data collection which can be achieved using surveillance and monitoring methods, either passively or actively (Cameron, 2002). Indonesian Government has developed a disease surveillance programme as part of their regulatory activities which focused on the detection of specific diseases or pathogens. (MMAF, 2020). This is active surveillance and can be used to identify the specific disease risks at a broader level. This information can be applied to support decisions by the farmers into their stocks section, farm locations, and any preventative measures required e.g. vaccinations prior to purchasing and stocking the farm. However, this needs to be included as part of a wider surveillance strategy which investigates production level losses from all causes including those disease. Combined approach can provide robust knowledge on the actual disease status to promote improved biosecurity and reduce the risk of disease within the farms. The data obtained from comprehensive study also contribute to the effort in establishing national strategic planning on the health management and biosecurity practice in Indonesia, where those information is critical to reduce the vulnerability of

the sector from the pathogenic diseases causing significant losses due to mortality and morbidity (Dvorak, 2009; Subasinghe *et al.*, 2023).

2.3. The aim of study.

The overall aim of this study was to provide an improved understanding of the disease status and the current management approaches adopted in tilapia (*Oreochromis spp*.) and Clarias catfish *(C. gariepinus)* farming systems practised in East Nusa Tenggara, Indonesia. This was performed by implementing a semi-structured questionnaire to secure current information on the farm background, production system, husbandry, disease and fish health management strategies. For diagnostic purposes, biological samples were taken at the time of the questionnaire and laboratory investigations confirmed the disease status and aetiological agent.

2.4. Materials and Methods.

2.4.1. Data Collection and Selection of Farms.

Data collection. A total of 41 farms located in East Nusa Tenggara participated in the questionnaire performed between January to April 2020. These farms included both tilapia and catfish culture situated in the two large islands, Flores and Timor Island. A survey framework was developed following the methods in Cameron (2002) to help identify the farms and ensure that the farms included in the survey were representative of those across the sampling locations. First, the distribution of the farms was grouped into the regions in the two selected islands and the total number of farms was derived from the annual monitoring report (2019) which was conducted by the Fish Quarantine and Inspection Agency Regional Office of Kupang (FQIA Kupang) and from the local Department of Marines and Fisheries. These data are collected as part of the competent authorities' activities regarding the policies, monitoring, and surveillance in the local level of Eats Nusa Tenggara (ENT) who have responsibility for collecting and compiling the aquaculture data in ENT. This was considered the best course of farms at the time of developing the sampling framework. In addition to the survey questionnaire, fish samples were collected for diagnostic purposes in confirming the health/disease status and the data included gross presentation of both external and internal clinical signs which were recorded at the time of sampling. Water parameters were also checked and recorded during the farm visit. The distribution of the farms was then grouped into the regions in the two selected island (Table 2.1).

Table 2.1. The location and number of fish farms per regions included in the study.

Island	Regions	Number of Farms
Timor	Kupang City	14
	Kupang Regency	5
	Timur Tengah Selatan (TTS)	1
	Timur Tengah Utara (TTU)	3
	Belu	6
Flores	Manggarai	3
	Ngada	3
	Ende	2
	Sikka	4
Total		41

Farms selection. The two islands Timor and Flores were chosen because both of the islands had the highest number of farms and also the highest production among the islands located in East Nusa Tenggara (MMAF, 2018). Therefore, these were considered as representative of the farming systems within the study area. The sample size of fish farm was determined by random sampling techniques from the method developed by Cameron, (2002). The members of the farm population were selected randomly where each farm sample had the similar chances to be selected, the selected farm samples were then coded with unique ID. In detail, there were 120 freshwater fish farms recorded in East Nusa Tenggara, where 102 farms located in the two main islands (Timor and Flores Island), including 72 farms located in Timor Island and 30 farms in Flores Island. From those population 40% were randomly selected for the sampling, 29 farms (40%) of the total 72 farms in the Timor Island) and 12 (40%) of the total 30 farms and in the Flores Island) which then randomly selected by using random sample calculator to determine which farms will be visited, the selected farm were then coded using the unique number. The number of farms selected represented the variety and the total population of tilapia and catfish farming systems practiced in the East Nusa Tenggara, Indonesia. The selected farms were then contacted by the author's colleagues in the FQIA Kupang in order to confirm their availability and production activity prior to the survey and sampling. The location of 41 farms visited during the field study were shown in the Figure 2.1.

Figure 2.1. The area of study in East Nusa Tenggara Indonesia. The number shown in the map represented the total number of recorded farms within each island (Source: maps.google.com).

2.4.2. Questionnaire Design.

The questionnaire was designed to gather information on the current status and management practises of farms level located in ENT, Indonesia. The questionnaire was broken down into the following sections: farm background, husbandry and water management, stocking and production information, and health and disease management practises including disease prevention and control strategies applied. The questionnaire was pilot tested by the author's colleagues in the Ministry of Marine Affairs and Fisheries (MMAF), amendments were made accordingly prior to implementing the survey. Those included in the pilot testing were not included in the final survey. The questionnaires were delivered face-to-face to each participant per farm visited by author. A copy of the questionnaire is provided in Appendix I.

2.4.3. Questionnaire Data analysis.

All the data obtained from the questionnaires were recorded in the Microsoft excel 2018 spreadsheets which contained categorical and numerical data types. The

calculation of mean, median, percentages, and frequencies were performed to provide descriptive statistics. Fisher exact test was performed to examine the association between the potential related factors including the gender of owner, farm location, type of farms, operation duration, type of culture systems, type of ponds, fry area suppliers, water sources, farm management practice, measure taken during the disease outbreaks, and pond preparation with the occurrence of mortality within the farms (Table 2.2).

Table 2.2. Description of associated factors assessed through the questionnaire in correlation with the occurrence of mortality.

Related factors	Categories
Farm Description	Farm's location, size, source of water, operation duration, and gender of owner.
Farming system	Farms production system, culture types (monoculture, polyculture, mix culture system, pond types, number of ponds, and farm records.
Stocking information	source of fish fry, fish density, feeding intake, and production.
Biosecurity, and water management	source of water, water quality parameters, acclimatization, ponds fertilization, water and equipment sharing system, probiotic, vaccine, antibiotics used, and disinfectants applied.
Fish disease and health management	mortality reports, cause of mortality, time of mortality occur, clinical signs of the disease fish, relevant stressors related to the mortality (Temperature, water parameters), measures taken during the disease and mortality, treatments, and monitoring.

The categorical (TRUE/FALSE) data as the potential associated factors to the mortality within the farm were evaluated by using Fisher exact test where 95% confidence intervals (p-value < 0.05) and Odd Ratio (OR) were considered in this test. All the analyses were conducted by using R programs version 3.5.1.

2.4.4. Biological Sampling.

Biological samples were collected at the same time as the questionnaire was performed with maximum of 10 fish were sampled at each farm visited, which always included a minimum of 1 apparently healthy fish. Apparently healthy fish were considered as those that were active and feeding and had no gross external clinical signs of the disease. Fish selected for biological sampling were removed by net with

150 cm long and 50 cm diameter. Fish samples from the farms located near to the Fish Quarantine and Inspection Agency (FQIA) Kupang Regional Office Laboratory (Accredited: ISO 17025;2015) were transported to the laboratory and oxygen was added to the plastic bags containing fish samples and placed into the fibreglass container and transported to the laboratory which took between 1-2h. All fish samples located in Flores Island were directly sampled onsite.

All fish samples were killed by overdose of anaesthetic tricaine methane sulfonate (MS222, Finquel) with the dose of 100 mg/l by immersion and external and internal gross clinical signs of disease were examined, as described in Austin & Austin, (2012) and Jia *et al.*, (2022). In brief, immersion preparation for fish samples by dissolving the anaesthetic compound with sterile water in the tank, and pH was measured at 7, once the anaesthetic solution ready then the fish samples were added slowly into the tank to minimize the stress due to handling. To ensure the death confirmation of fish samples, concussion of the brain by striking the cranium with destruction of the brain before the fish return to the consciousness (Animal Procedures Comittee, 2009; Owen and Kelsh, 2021). The body weight and length were measured and recorded. Samples for viable bacterial recovery were aseptically taken from each fish from the liver, kidney, and spleen inoculated onto the TSA media, incubated at 28^0C and observed for bacterial growth for a maximum of 48h. For histopathology, liver, kidney, spleen, brain, and gills were collected, all tissue samples were fixed in 10% Neutral Buffer Formalin (NBF) for approximately 24h before processing into the wax block (Miranti, 2010). All the wax embedded tissue blocks were transported to the Institute of Aquaculture, Stirling University and 5um thick tissue sections cut and stained for Haematoxylin and Eosin (H&E) and Gram stain for histopathology examination. The biological samples were subsequently investigated and described chapter 3.

2.4.5. Water Quality Assessment.

At each farm water quality was measured using a HI9829 portable meter (HANNA Instruments, Woonsocket, U.S.A) following the manufacture protocol. Briefly, each pond where the fish samples were collected, the water quality was measured directly by dip/immersing the sensor of the portable meter into the water and adjusted based on the water parameters. The parameters included temperature, nitrite, nitrate,

NH_3, dissolved oxygen (DO), while pH was also checked from each sampled pond using pH test kit (Merck).

2.4.6. Ethics.

Ethical approval was obtained from the General University Ethics Panel (GUEP) at the University of Stirling (Number 745) and approved on 11 December 2019. Fish sampling and dissection methods have been carried out in accordance with the animal welfare and Ethical Review Body (AWERB) the University of Stirling Panel, based on the UK Animals (Scientific Procedures) Act, 1986 revised 2013). AWERB log in number 009 and has been approved on 5 October 2019. Ethical approval was granted for the survey.

2.5. Results

2.5.1. Questionnaire Information.

Overview. All selected farms within the area of study participated during the field study. Although all participants engaged with the study, 71% (n=29) of these were farm owners and were happy to engage, whereas 39% (n=14) needed more persuasion to engage with the study. Their hesitancy was primarily due to their lack of knowledge in engaging with these types of studies but once explained, they were happy to participate. For example, when farmers were asked about the use of antibiotics including the type and the antibiotic suppliers, they were more hesitant in their responses. All tilapia farms were in active production during the time of visit, it enabled the biological sampling process of tilapia for further study (Chapter 3 and 4). Meanwhile, catfish samples were able to be collected from 17/41 farms that in active production circle.

Although this was not a gender-based study, 83% (n=34) of the project participants identified as male and 17% (n=7) were female. The age of participants was between 31 to 65 years old. A total of 15% (n=6) participants with the age ≥ 60 years old were retired public servant who also previously engaged with the fish farming. The participants who experienced the disease problems claimed to recognise the disease by observing the clinical presentation of their fish. They also reported that they used a range of antibiotics as intervention to the fish during the disease outbreaks, although some of them did not aware regarding the type and the dose of antibiotics applied at those time. Based on the participant's responses, none of them sold the diseased fish for human consumption.

The distribution of 41 visited farms during the questionnaire were in the two main islands, where Timor Island contributed 29 farms located in 5 regions and 12 farms were in the four regions in Flores Island as shown in Figure 2.2.

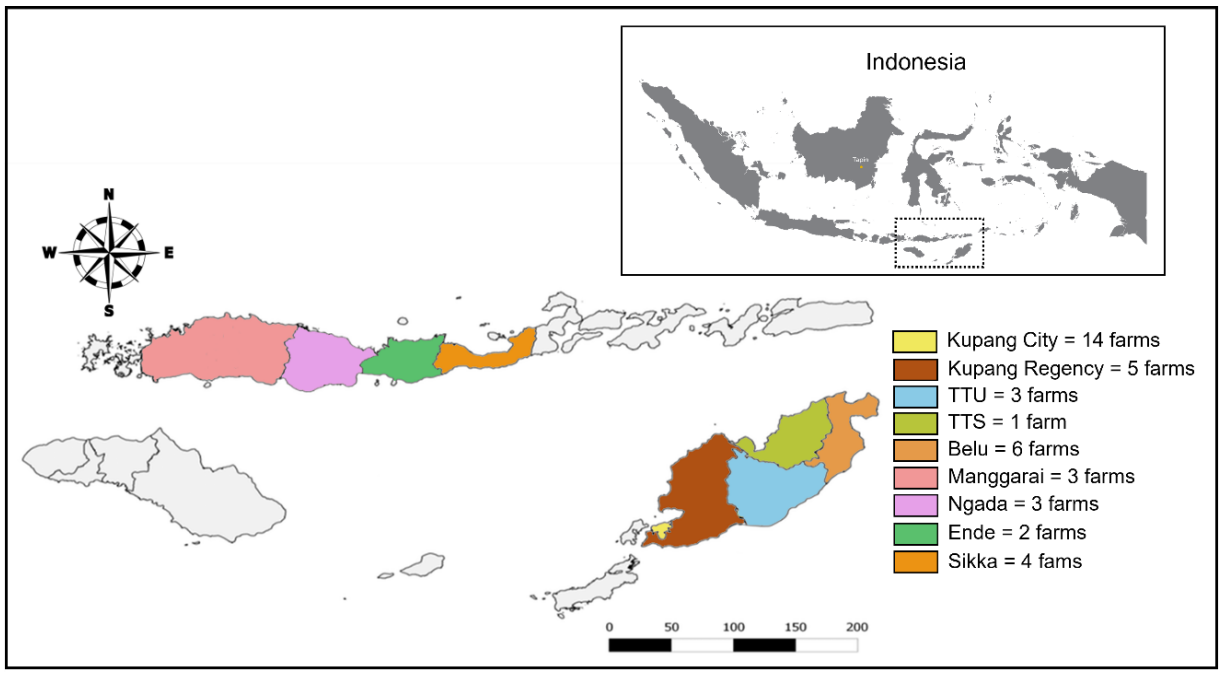

Figure 2.2. The visited area of field study conducted in East Nusa Tenggara, Indonesia.

The roles of participants interviewed. The roles of participants varied with 71% (n=29) farms owners, where 2 of them were also engaged as farms worker. Only 2 male participants were involved as a manager only in the visited farm. All the female engaged were only working in the farming practice as the main job. Of the female participants, 10% (n=4) of farmers identified as the farms owner and 1 of them as a worker in the farms located in Kupang. In Flores Island 1 female identified as an owner in the small-scale farm (Table 2.3).

Table 2.3. The number of gender description and role of the questionnaire participants within the visited farms.

Gender	All	Owner only (n)	Manager only (n)	Worker only (n)	Owner + worker (n)
Female	7	5	0	1	1
Male	34	22	2	9	1
Total	41	27	2	10	2
% Female	17	12	0	2	2

Fish Species. All of the farms with tilapia farming during the time visited stocked Nile tilapia *(O. niloticus)* species, where different strains of *O. niloticus* were

stocked including *O. niloticus bleeker*, called locally as “local tilapia” which was the most widely culture tilapia in Indonesia. The second strain was Bogor Enhanced Strain Tilapia or BEST strain and then Genetically Supermale Indonesia Tilapia or GESIT strain (Figure 2.3).

Figure 2.3. Image of *O. niloticus* species most widely farmed tilapia species in Indonesia.

The walking catfish, *Clarias gariepenus* (Fig. 2.4) was the most commonly cultured catfish species in the area of study and comprised of 2 strains - sangkuriang catfish (*C. gariepinus var*) and also dumbo (*C.gariepinus strains dumbo*). The fry from both catfish strains were purchased and transported from the island of Java which is approximately 1 thousand miles from the fish farms in ENT.

Figure 2.4. Image of catfish (*C. gariepinus*) species.

Farm Production System.

The data on the farming systems was compiled and presented in Table 2.4**.** The size of a total 41 visited fish farms ranged from the smallest with 600 m^2 to the largest with 26,000 m^2 where the average size at 3,256±5 m^2. Meanwhile, the number of ponds per farm ranged from 2 to 10 ponds with average 5±2 ponds/farm. From all ponds within the visited farms in total, although farms were all in active production, only 84% of the total ponds were stocked and 16% having some ponds empty. The most predominant the range number of ponds within the farms was 3 to 5 ponds per farm with 49% (n=20) followed by 34% (n=14) farms that had more than 5 ponds and only 17% (n=7) of the production sites included had only two ponds. The range of the farming practice duration were from 1 to 12 years at the visited time, with the average duration of 5±3 years. Based on the range criteria of the farming duration, 71% of the farms visited had been operated between 2 to 5 years at the time visited, this number was followed by the range of 6 – 10 years and the range of more than 10 years with 21 and 7 % respectively. The total production was obtained by recording the total weight (kg) when the fish were sold. The production cycle time varied and was dependant on the harvested time which was influenced by the market demand and farms conditions. Based on the information provided by the respondents, the production volume ranged from 50 to 5,500 kg/farm/year with average total production 531.8±898 kg/farm/year.

Table 2.4. Farm information and production data collected during the field study in 2020.

Unique ID	Island	Regency	Farm size (m^2)	Experience (year)	Number of pond/farms	Volume production/year (kg/farm)
58	Timor	Kupang City	10,000	4	6	100
59	Timor	Kupang City	1,200	5	4	100
62	Timor	Kupang City	800	5	4	100
65	Timor	Kupang City	11,000	7	7	500
74	Timor	Kupang City	14,000	3	6	80
77	Timor	Kupang City	8,000	5	7	100
78	Timor	Kupang City	1,500	5	5	400
82	Timor	Kupang City	800	6	2	250
83	Timor	Kupang City	1,200	4	4	100
85	Timor	Kupang City	1,000	8	2	60
89	Timor	Kupang City	600	4	2	90
90	Timor	Kupang City	650	4	4	100
91	Timor	Kupang City	12,000	5	6	100
93	Timor	Kupang City	1,000	1	5	100
24	Timor	Kupang	1,000	3	2	100
28	Timor	Kupang	3,400	4	8	400
29	Timor	Kupang	1,600	4	4	300
31	Timor	Kupang	26,000	15	10	5,500
36	Timor	Kupang	1,400	4	4	80
37	Timor	TTS	800	2	3	100
41	Timor	TTU	850	5	4	100
42	Timor	TTU	8,000	3	10	100
45	Timor	TTU	700	4	2	80
48	Timor	Belu	2,000	10	4	450
49	Timor	Belu	3,000	4	8	100
55	Timor	Belu	1,200	4	5	2,200
56	Timor	Belu	1,600	5	4	80
114	Timor	Malaka	900	12	4	500
117	Timor	Malaka	750	5	2	50
05	Flores	Manggarai	1,000	5	4	90
06	Flores	Manggarai	2,000	5	6	300
07	Flores	Manggarai	1,000	2	4	100
10	Flores	Ngada	650	4	2	100
13	Flores	Ngada	2,000	4	3	100
14	Flores	Ngada	1,600	9	4	250
15	Flores	Ende	2,000	5	6	1,000
16	Flores	Ende	1,200	5	6	100
17	Flores	Sikka	900	5	3	80
21	Flores	Sikka	1,000	6	6	450
98	Flores	Sikka	1,000	10	4	500
113	Flores	Sikka	2,200	3	6	100
Total			133,500	213	192	15,490
Average			3,256	5	4.68	378
Standard Deviation (SD)			5,007	3	2	898

The scale of the visited farms. An attempt was made to scale the farms based on the regulation established by MMAF of Indonesia (PER. 13/MEN/2018). In

the present study, small-scale farm was classified as those that measured < 0.5 ha were the most prevalence farm with 83% (n=34) of the total farms, this number was followed by large scale farms with 10% (n=4), where all the large-scale farms visited within the area of study were located in Kupang regions as shown by Table 2.5.

Table 2.5. The percentages of farms by scale per region in the study area.

Island	Regions	Farm Scale %(n)		
		Small	Medium	Large
Timor	Kupang City (n = 14)	22 (9)	5 (2)	7 (3)
	Kupang Regency (n=5)	7 (3)	2 (1)	2 (1)
	TTS (n=1)	2 (1)	0	0
	TTU (n=3)	7 (2)	0	0
	Belu (n=6)	15 (6)	0	0
Flores	Manggarai (n=3)	7 (3)	0	0
	Ngada (n=3)	7 (3)	0	0
	Ende (n=2)	5 (2)	0	0
	Sikka (n=4)	10 (4)	0	0
Total (%)		83 (34)	7 (3)	10 (4)

There were 6 types of materials used to produce the ponds with the greatest percentage of all farms visited having a concrete only pond base material (n=17, Figure 2.6).

Table 2.6. The variation in pond construction and type within the study site.

Island	Regions	Type of pond %(n)					
		Earthen only	Concrete only	Tarpaulin only	Earthen +concrete	Concrete +tarpaulin	Mix type ponds
Timor	Kupang City (n = 14)	10 (4)	20 (8)	0	0	0	5 (2)
	Kupang Regency (n=5)	0	2 (1)	2 (1)	2 (1)	5 (2)	0
	TTU (n=1)	0	0	0	0	0	2 (1)
	TTS (n=3)	2 (1)	2 (1)	0	2 (1)	0	0
	Belu (n=6)	5 (2)	5 (2)	0	0	5 (2)	0
Flores	Manggarai (n=3)	5 (2)	2 (1)	0	0	0	0
	Ngada (n=3)	5 (2)	2 (1)	0	0	0	0
	Ende (n=2)	2 (1)	2 (1)	0	0	0	0
	Sikka (n=4)	2 (1)	5 (2)	0	0	0	2 (1)
Total (%)		32/13	42/17	2 (1)	5 (2)	10 (4)	10 (4)

Concrete pond was the most common pond type-based materials among the visited farms, and then followed by earthen and tarpaulin pond. The figure below was

showing the type of pond based on constructed materials that located in different region as shown in the Figure 2.5 to 2.7.

Figure 2.5. Image of concrete pond type where 29 farms located in Kupang region used the concrete ponds type.

Figure 2.6. Image of tarpaulin ponds type with intensive farming system located in Sikka, Flores Island.

Figure 2.7. Image of earthen pond located in Belu regions.

The farming system of visited farms. An attempt was made to describe the production intensity of the farms using the following criteria regulated by the government based on the technology applied, where semi-intensive already performed better management practice, the density between 25-50 fish/m^2, commercial feed, and basic treatment. Whereas intensive system applied more routine biosecurity and health management practice supported by various treatment including the use of antibiotics and probiotics, aeration, use commercial feed with additional treatment such as probiotics., and higher fish density also applied. The survey results showed that 68% (n=28) of the farmers interviewed described themselves as practising semi-intensive production compared with 29% (n=12) intensive, and only 2% (n=1) extensive farming systems. The farming system per regions in area of study were shown in Table 2.7.

Table 2.7. The percentages of the farming system type per region in area of study.

Island	Regions	Type of farming system %(n)		
		Intensive	Semi-intensive	Extensive
Timor	Kupang City (n = 14)	5 (2)	27 (11)	2 (1)
	Kupang Regency (n=5)	2 (1)	10 (4)	0
	TTS (n=1)	0	2 (1)	0
	TTU (n=3)	0	7 (3)	0
	Belu (n=6)	10 (4)	5 (2)	0
Flores	Manggarai (n=3)	0	7 (3)	0
	Ngada (n=3)	2 (1)	5 (2)	0
	Ende (n=2)	0	5 (2)	0
	Sikka (n=4)	10 (4)	0	0
Total (%)		29 (12)	68 (28)	2 (1)

The culture system of visited farms. A total of 95% (n=39) of all farms interviewed and included in the study reported they were practising monoculture, compared with on 5% (n=2) farms using polyculture systems and was similar throughout the farming regions (Table 2.8). The 2 polyculture farms included one that was described as extensive and one that was considered as semi-intensive, where the semi-intensive farm stocked tilapia mixed with carp and gourami.

Table 2.8. The percentages of the pond systems per region in the area of study.

Island	Regions	Type of pond system % (n)	
		Monoculture	Polyculture
Timor	Kupang City (n = 14)	32 (13)	2 (1)
	Kupang Regency (n=5)	10 (4)	2 (1)
	TTS (n=1)	2 (1)	0
	TTU (n=3)	7 (3)	0
	Belu (n=6)	15 (6)	0
Flores	Manggarai (n=3)	7 (3)	0
	Ngada (n=3)	7 (3)	0
	Ende (n=2)	5 (2)	0
	Sikka (n=4)	10 (4)	0
Total (%)		95 (39)	5 (2)

Water sources supplied to the visited farms. A combination of water sources was provided which included springs, wells, and river water, with a higher number 10% (n=5) of farms in the Kupang region used springs and river only water in their farm. The water sources used, depended on the abundance of water within the region and 24% (n=10) farms used both spring and well water for their farms (Table 2.9).

Table 2.9. The range of the sources of water supply in the study site.

Island	Regions	Sources of Water Supplied to the Farms %(n)						
		Springs only	River only	wells only	springs +wells	springs +river	River +wells	Mix type ponds
Timor	Kupang City (n = 14)	12 (5)	2 (1)	5 (2)	5 (2)	0	5 (2)	5 (2)
	Kupang Regency (n=5)	5 (2)	7 (3)	0	0	0	0	0
	TTU (n=1)	0	0	0	0	2 (1)	0	0
	TTS (n=3)	0	0	0	2 (1)	5 (2)	0	0
	Belu (n=6)	0	0	2 (1)	5 (2)	7 (3)	0	0
Flores	Manggarai (n=3)	0	2 (1)	0	2 (1)	2 (1)	0	2 (1)
	Ngada (n=3)	0	0	0	5 (2)	0	0	2 (1)
	Ende (n=2)	0	0	0	0	0	5 (2)	0
	Sikka (n=4)	0	0	0	5 (2)	5 (2)	0	0
Total (%)		17 (7)	11 (5)	7 (3)	24 (10)	21 (8)	10 (4)	10 (4)

Farms record keeping. Although most farms reported that they kept the farm records, however, only 29% (n=12) of farms included in this study were able to demonstrate their record keeping. The remaining 71% (n=29) claimed to keep the record but no evidence was provided. There were of course, several reasons for this and it may not be accurate to assume that they did not keep farm records, but instead it may be that they were not comfortable in sharing these with the author. Irrespective of whether the farmer shared evidence of the records kept or not, stocking information and mortality appeared to be the data kept by most of the farmers. This could not be validated for all of the farms visited but was reported during the interview, the most data recorded by the farmer in this study, where only 7 % (n=3) of farms recorded detailed which included stocking production, mortality, feeding intake, fish disease, and water quality (Figure 2.8).

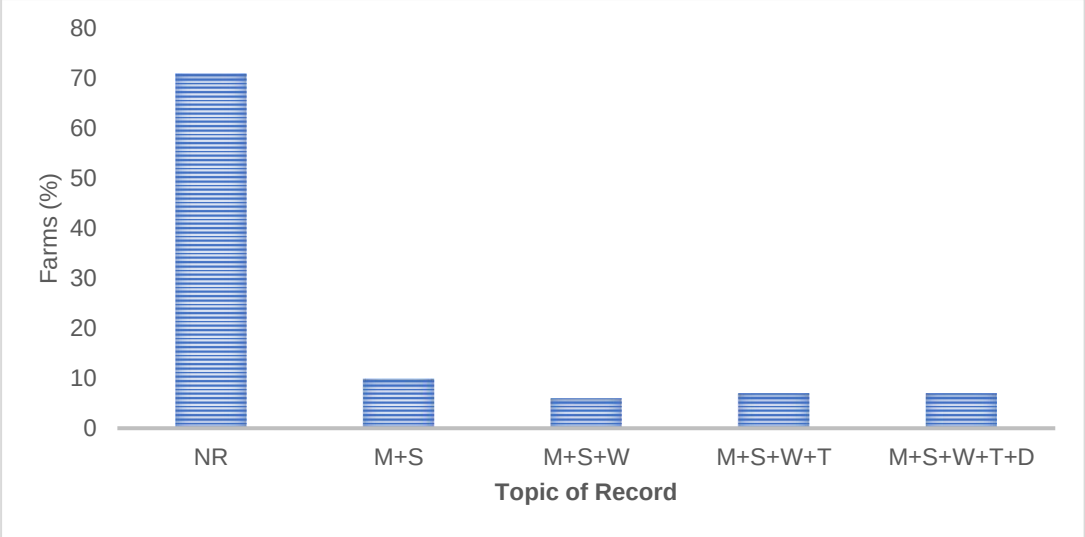

Figure 2.8. The type of record keeping within the farms in the area of study.

*NR: No Record, M: mortality, S; stocking, W: water quality, F: feed, D: disease.

Stocking and production information.

The source of fish fry. The fish supplied to the farms were predominantly from outside of the regions in ENT, where a total of 73% (n=30) of all farms obtained their fry from East Java and only 15% (n=6) of the farms used local sources. A total of 7% (n=3) of farms interviewed used multiple fry suppliers including Java and other areas. Based on the information provided by the farmers, they were more likely to purchase the fish fry from Java even though the price was higher than the local supply, this choice was associated with their individual experience, where they considered that fry from Java had better quality compared with fry produced locally. Irregular availability of fish fry from the local producers in ENT was also an important consideration in their choice of fry sources. Fish fry from local production were mostly obtained from government hatchery, and small number from own breeding. The area of fish fry supplier was shown in the Figure 2.9.

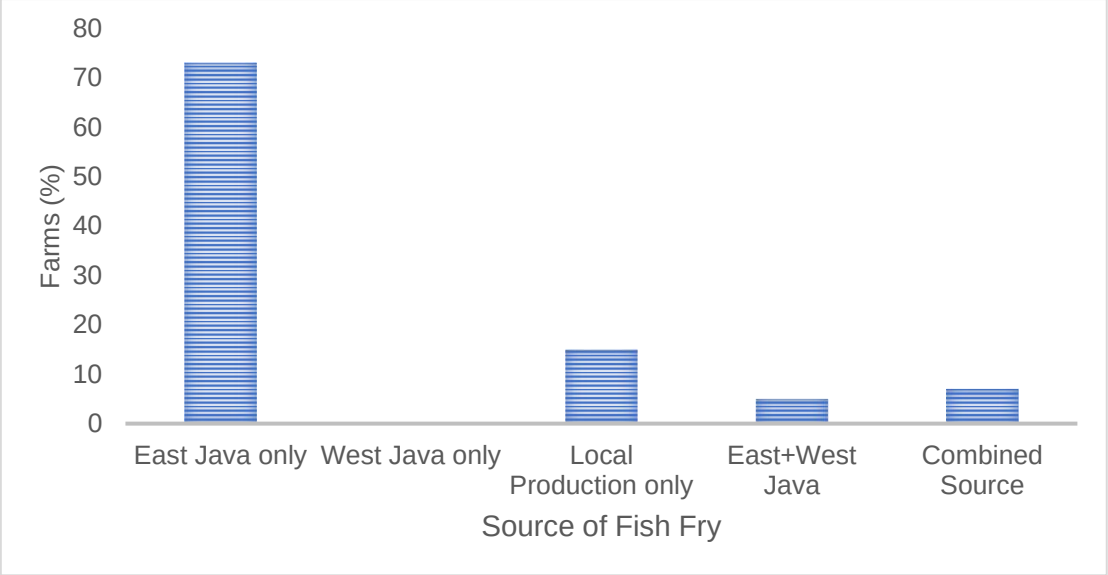

Figure 2.9. The percentages of fish fry supplier and its combination within the study area.

Stocking densities. Data on stocking density was irregular as farmers were often not sure or did not want to say, however % (n=29 tilapia farmers in this study reported their stocking density ranged from 20 – 100 fish/m^2, and 17% (n=7) tilapia farms applied more than 100 fish/m^2. Tilapia farms using biofloc system reported a higher stocking density compared with non-biofloc ponds, with the stocking density ranged from 100 to 500 fish/m^2. The catfish farmer included in the study reported higher densities of 20% (n=8) farms stocked more than 100 fish/m^2 (Figure 2.10).

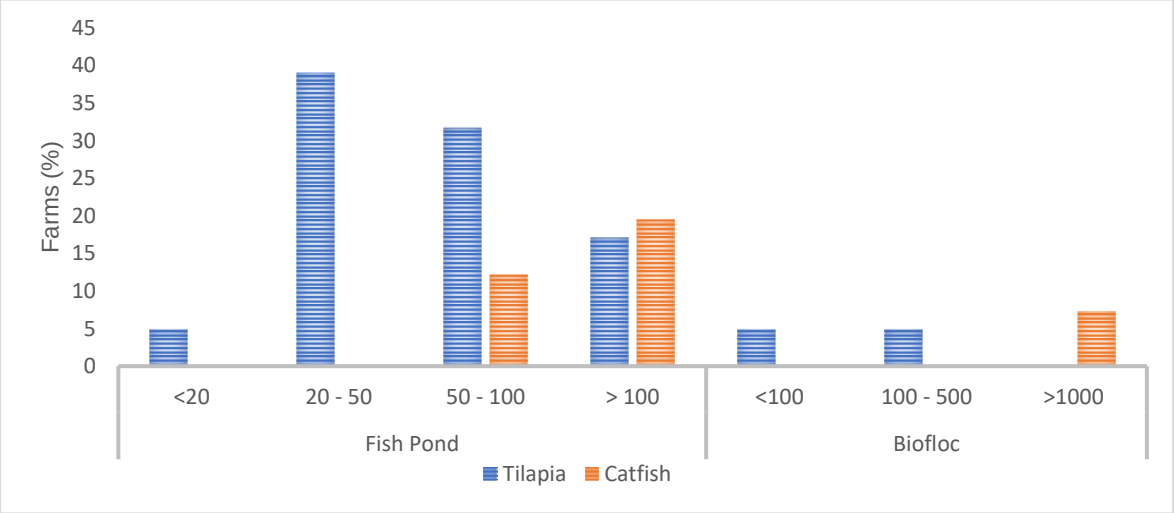

Figure 2.10. The percentages of fish density applied into the ponds within the visited farms.

Feeding intake. The frequency of feeding applied in the visited farm were majority twice a day that usually given in the morning and afternoon, where a total of 76% (n=32) of all fish farmers in this study fed their fish twice daily and 20% (n=8) with 3 times daily. The remaining 5% (n=2) of fish farms reported that they fed their fish stocks based on the fish appetite where they noticed while observing within the ponds. All farms used commercial feed purchased from private/agents and small number were obtained from government. Only 1 farm used homemade feed which was also supported by commercial feed in the tilapia farm. Although, more intensification method applied within the visited farms, only 7% (n=3) fish farms were able to provide Food Conversion Ratio (FCR) in determining the feeding intake. It was clear that FCR was not widely applied in the visited farms (Figure 2.11).

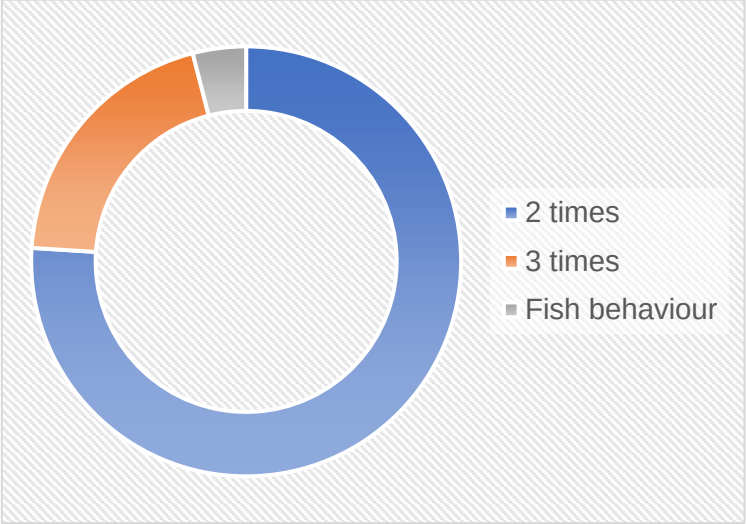

Figure 2.11. The percentages of the daily feeding intake applied by the farms in the area of study.

Husbandry and water management. The range of husbandry practises and water management strategies applied within the farming population included are presented in Table or Figure 2.12). In general, all farmers (100%, n=41) practised some form of acclimation of their fish stocks prior to stocking the farm and 63% of both tilapia (n=26) and catfish farms(n=17) applied fertilisation to their ponds prior to stocking. The number of 78% (n=32) of farms used in series system to transfer the water between ponds within their farm. A high percentage 87% (n=36) of all farmers interviewed did not practise disinfection or sanitation with their equipment between ponds.

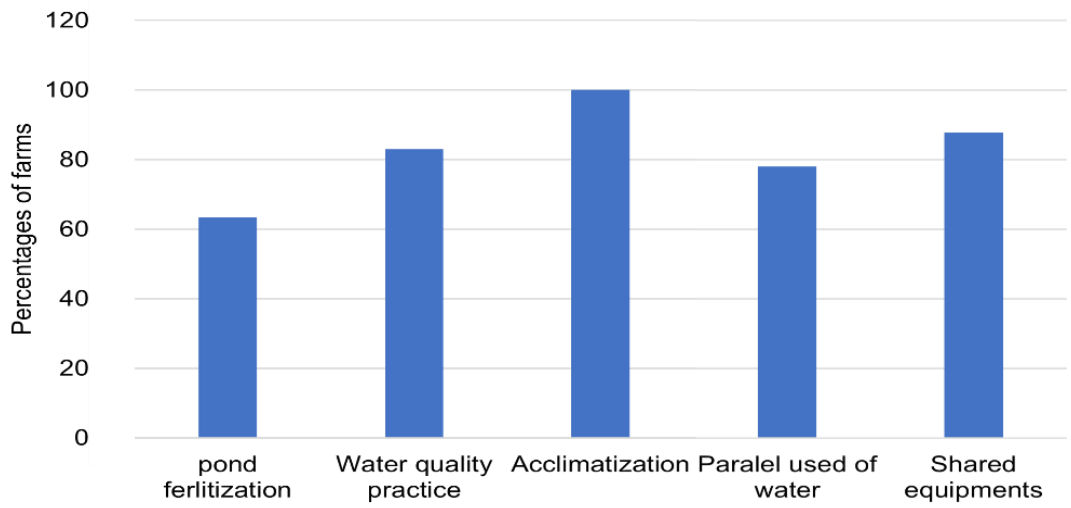

Figure 2.12. The percentages of husbandry and water management applied by visited farms.

Pond fertilization and probiotics applied by the visited farms. Ponds fertilization during the production system were also applied, where 44% (n=18) used organic fertilizer such as soybean and waste from animals, where 20% (n=8) treated with artificial (commercial) fertilizers to their farms. Probiotics were also used by 39% (n=16) of farmers where 15% (n=6) of them applied the probiotics mixed with fish feed and the remaining 24% (n=10) of the farms applied directly to the water to maintain the water quality based on the information provided by the farmers (Figure 2.13).

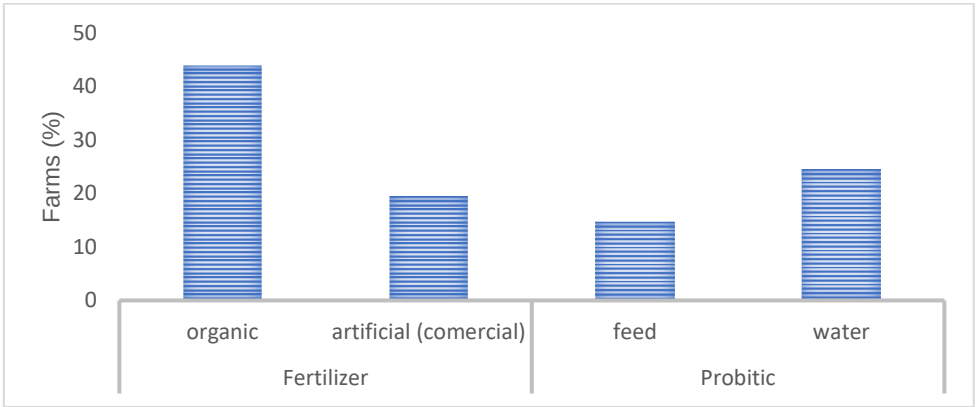

Figure 2.13. The percentages of pond fertilizer and probiotics applied by the farmers per farm.

Water quality monitoring. Only 22% (n=9) of all farmers interviewed claimed to perform no water quality checks at all and 10% (n=4) of farmers performed an

impressive range of water quality checks including pH, temperature, turbidity, NO_2, and NH_3. The majority of farmers 78% (n=32) did practise some form of water quality check but this was varied and irregular. For those that measures pH the method used included strips provided by the Government and was only included in 17% (n=7) of all farms included in the study (Figure 2.14).

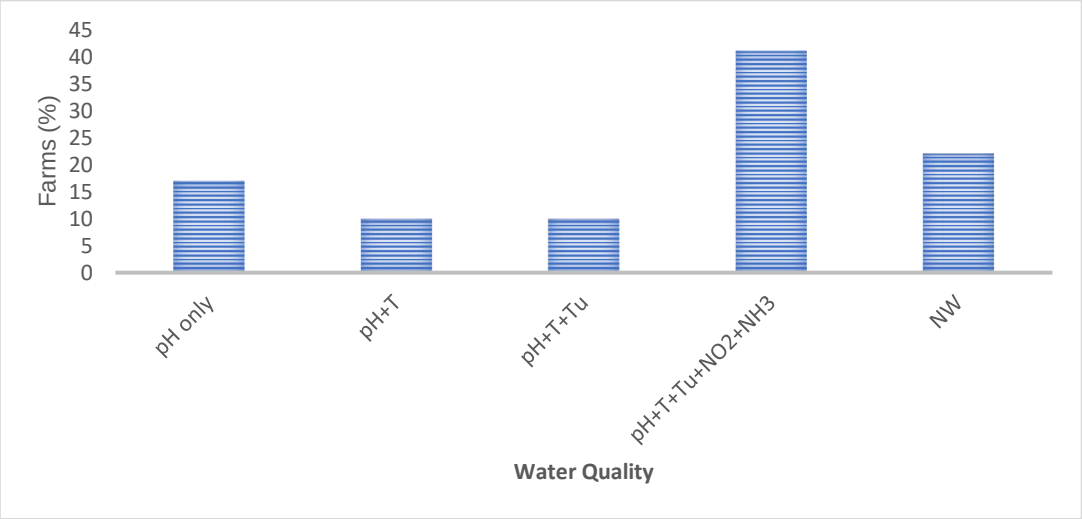

Figure 2.14. The percentages of water parameters that checked by the farmers and its combination within the visited farms. T: temperature, Tu: turbidity, NW: No Water Check.

Fish disease and health management strategies.

Farmers included in the study claimed to recognise fish disease by visual presentation of their stocks, including irregular fish behaviour and appearance, which followed by mortalities. At the time of sampling, 61% (n=25) of farms exhibited fish presenting with these clinical signs which were described as disease problems as reported by the farmers (Figure 2.17). The fish farmers interviewed in this study reported that they were able to recognise their diseased fish by observing the external appearance of the fish without the need for laboratory diagnosis or confirmation. A total of 34% (n=14) of the fish farmers reported that fish disease was the most common cause of mortalities on their farms, where this was reported by 22% (n=9) farms located in the Timor Island and the remaining farms located in the Flores Island. Whilst fish disease was considered the primary cause of mortalities in the farms, other issues were reported, where farmers claimed contributed to the disease and/or mortalities. These

included, poor water quality, weather where the rapid changes of weather from heavy rains to long dry weather, overcrowding because of the high densities and poor fry quality (Figure 2.15).

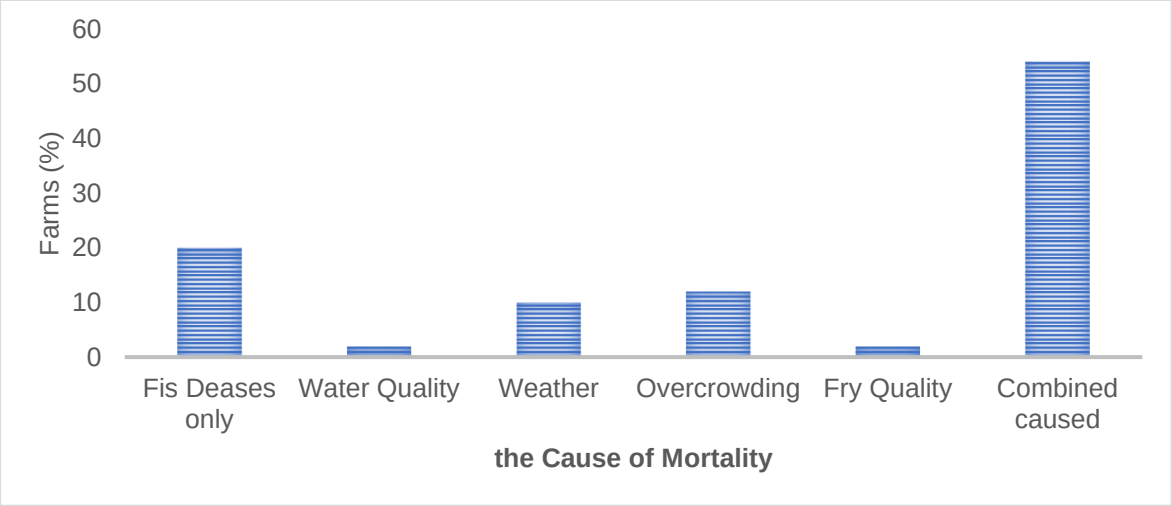

Figure 2.15. The percentages of fish mortality cause and its combination reported by the farms during the field study.

An attempt to identify what the clinical signs identified and to correlate these with the biological data acquired during the sampling was performed. From the questionnaire data. A range of changes were observed by the farmers during the outbreaks based on the questionnaire provided, this attempt was performed to understand the knowledge level in detecting the fish abnormality identified within their farms during the outbreaks, where 46% (n=18) of tilapia and 20% (n=8) of catfish farmers reported no abnormalities in their diseased/dead fish. More clinical signs were recognised and reported by the intensive tilapia farms compared with the catfish farms. However, more of the catfish farmers reported abnormal swimming in the catfish compared with the tilapia farmers (Fig. 2.16).

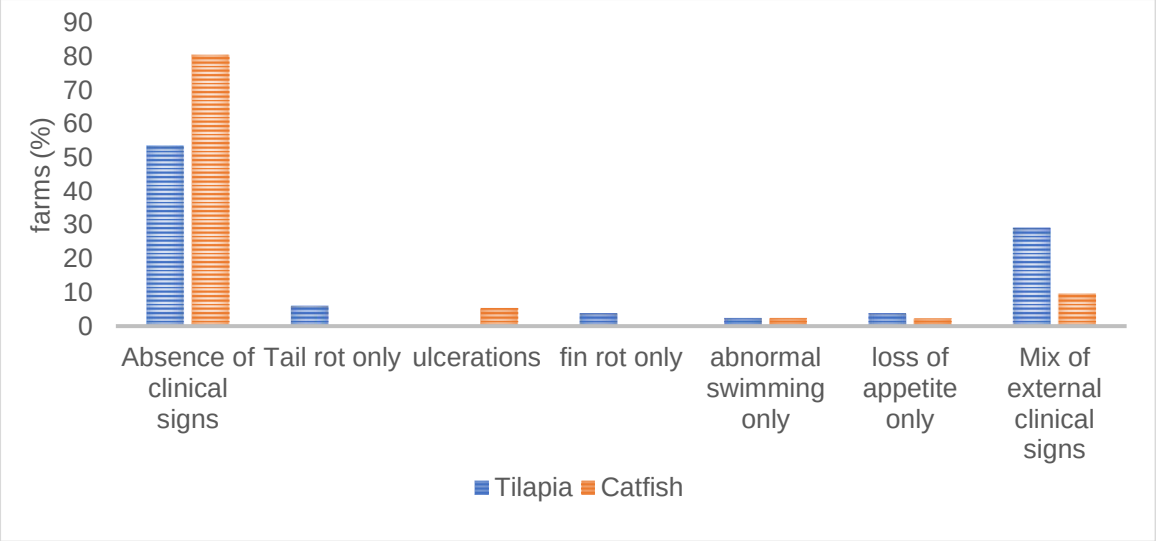

Figure 2.16. The percentages of clinical signs of the disease based on the data reported by the farmers during the field study.

The time of mortality occurred. Farmers reported a higher risk of mortalities occurring during seasonal changes, particularly between dry to rainy season with March, April, and October being described a month with high mortalities on the farms. Higher levels of mortalities were reported in the farmed tilapia, but a similar trend was observed between both the catfish and tilapia farmers (Figure 2.17).

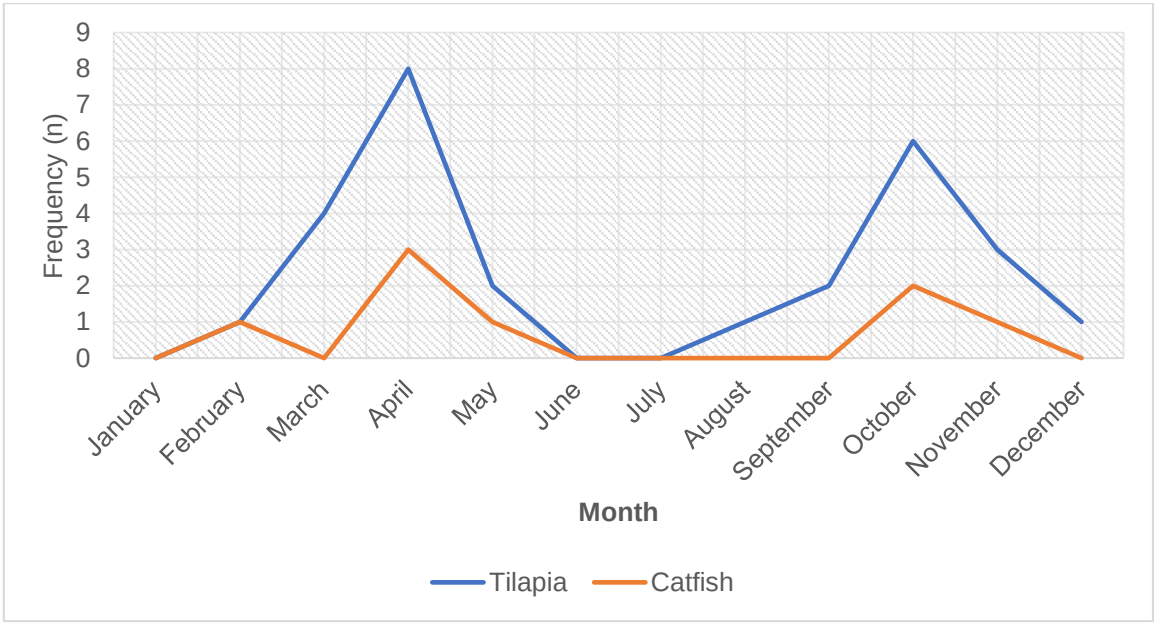

Figure 2.17. The frequency of the outbreaks during the period of time as reported by tilapia and catfish farmers.

The intervention applied during the disease outbreaks. As expected with such a varied farm population a range of intervention practises were identified from the survey questionnaire, with water exchange being the most commonly reported method applied by farmers during disease outbreaks on their farms (39% n=16, Figure 2.18). Farmers also reported the use of antibiotics and combined treatments using antibiotics mixed with traditional herbal ingredients were the most common treatments administrated by the farmers when the disease occurred (44% (n=18). Based on the farmers who applied antibiotics, most recognised that the antibiotic treatment reduced the severity of the infection, as observed by lower levels of fish losses on their farms compared with no antibiotics being used. All the farmers who applied the antibiotics reported that they acknowledged the benefit and impact of antibiotics application, however, only half of the farmers who used antibiotics had sufficient understanding regarding the action and dose which they had acquired from private/agents and government through training and monitoring activities. Measure taken during the disease outbreaks were summarized in the Figure 2.18.

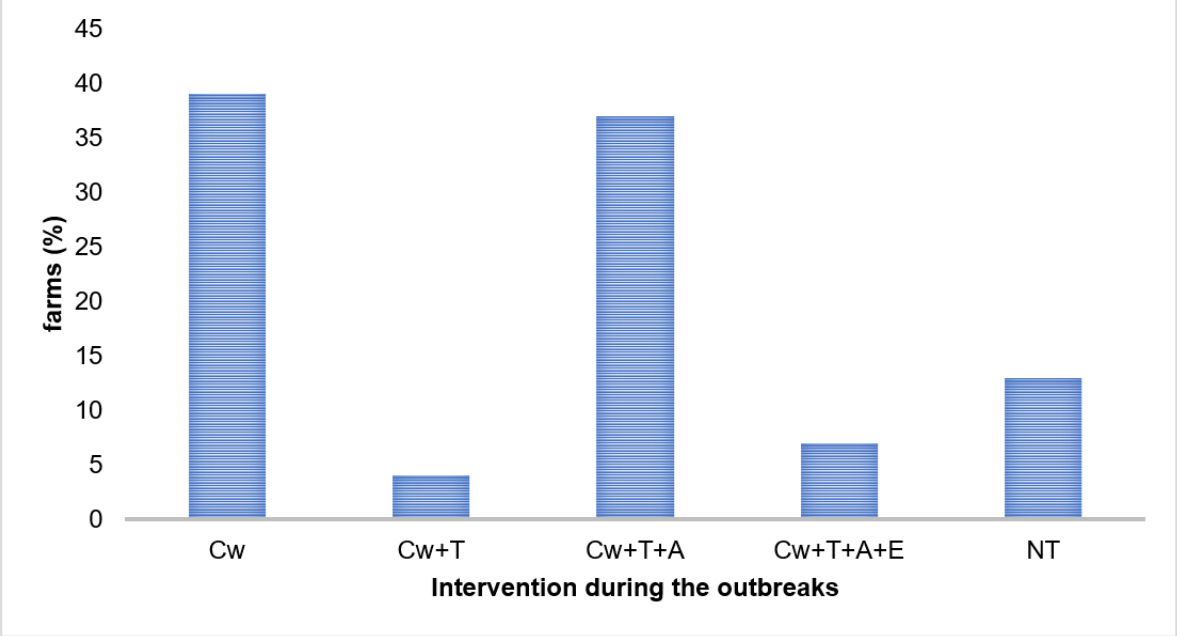

Figure 2.18. The percentages of intervention during the disease outbreaks and its combination applied by the visited farms. CW: change water only, T: traditional treatment, A: antibiotics, E: emergency harvest, NT: no treatment.

Disposal of the dead fish. In this study, farmers considered approximately between 1 to 2 fish mortalities per day as a usual or acceptable mortality, however during outbreaks, they reported having 10 or more fish dead per day which was higher than acceptable levels of mortalities described. Most of the farmers (61%, n=25) discarded the dead fish but 17% (n=8) either discarded or used the dead fish as broodstocks feed. Only 5% (n=2) of farms used the dead fish only for broodstock feed. No farms reported that the used the dead fish for either human consumption or selling to market (Figure 2.19).

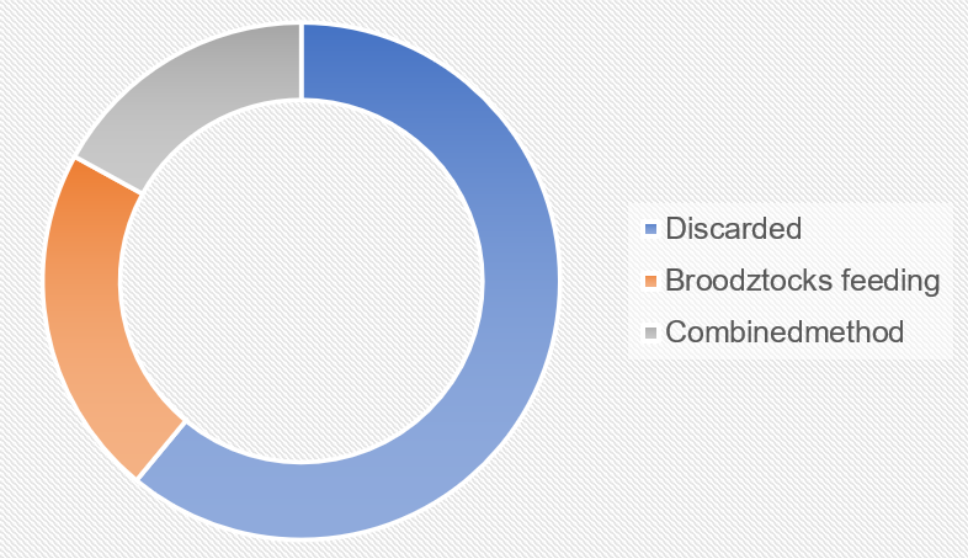

Figure 2.19. The percentages of the disposal of the dead fish applied by the farmers.

Antibiotics application within the visited farms. Forty four percent (n=18) of the farmers interviewed claimed to use antibiotics and of these, nearly all of them (88% or 16/18) used the antibiotic oxytetracycline. Other antibiotics were described by the participants however these were in much smaller number of farms (Figure 2.22). Of the 62% (11/18) of farmers who used antibiotics, the antibiotics were supplied from both government and private companies depending on who supplied the fish fry to their farms. The antibiotics were given by most of farmers through immersion to the fish that identified by the farmers presenting the abnormalities, where the suspected disease fish were collected and immersed into the prepared water, and few farmers applied the antibiotics mixed with feed. However, little information available regarding the dose of antibiotics, only 30% (5/18) of farmers who used the antibiotics were able

to provide the dose of application with the range between 5 -10gr/1000L water. The type of antibiotics used within the visited farms was shown in the Figure 2.20.

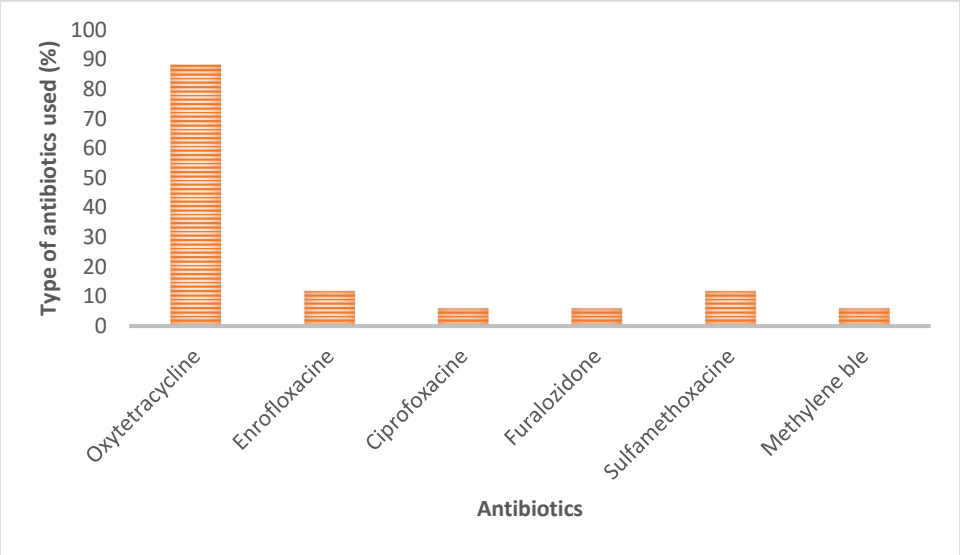

Figure 2.20. The percentages of antibiotics used by the farmers as a treatment during the disease outbreaks.

Regular monitoring activities in the area of study.

Regular monitoring for specific pathogens was provided by the government as part of a national surveillance program. The purpose of this activity was to identify the particular pathogenic diseases that might emerge or re-emerge in several region in Indonesia including in ENT. The other purpose was to assess the production cycle of farms among the programs that had been established by government.

Monitoring activities conducted by the Indonesian Government were performed with the fish farms to check for specific pathogens/diseases and to assess the productivity of the farms that were established by the Government. Based on the feedback from the farmers 83% (n=34) of farms had been visited by either national or local government institutions within last 5 years, where this was a part of national programs. The Government also supplied the antibiotics as a treatment during the disease outbreaks to 17% (3/18) farmers (from government only) and 27% (5/18) of farms obtained the antibiotics from combined of government and private sectors (Figure 2.21).

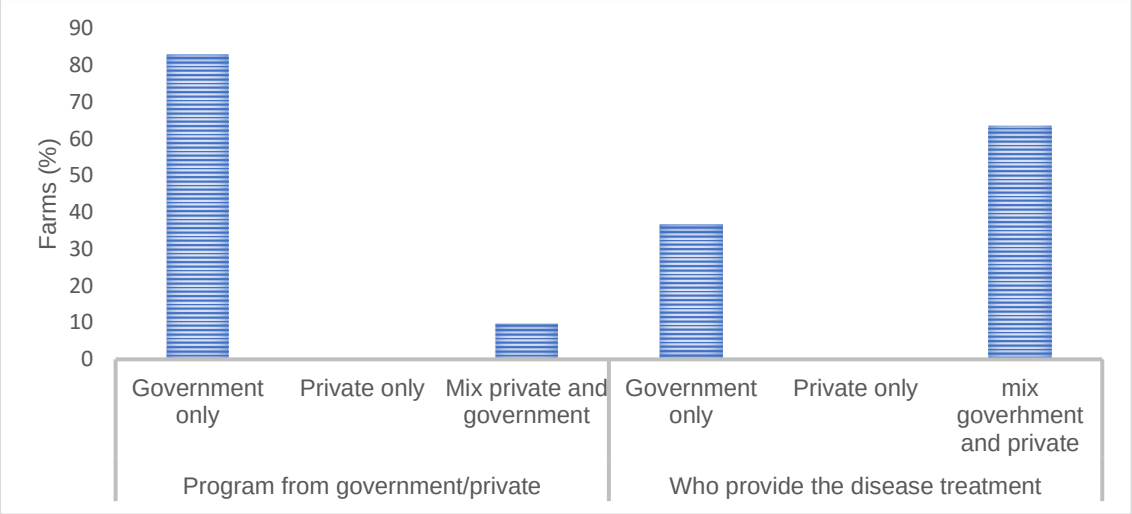

Figure 2.21. The monitoring program conducted in the area of study and antibiotics supplier to the visited farms.

2.5.2. Assessment of Potential Association Between Variables Associated with the Mortality.

An attempt was made to explore the strength of an association between the farm level variables and mortality (as reported by the fish farmers). The aim of this analysis was to correlate the relative risk of a farm-level activity (variables) with the outcome of mortality. In this case, if the Odds Ratio (OR) is greater than 1 then the variable and outcome are associated and correlated, and the risk of this variable being associated with the outcome can be determined. If the OR value is less than 1, then the relative risk of this activity is less likely to be associated with the outcome mortality and may even be protective (Fay, 2023).

From the OR data presented in Table 2.10, there was a greater risk of the farmer being male and have disease or mortality on the farm however, this was not statistically significant (OR = 2.37, p-value = 0.36). It is unlikely that this is a true risk factor as most of the participants were male. The production system was also analysed where the semi-intensive was less likely associated with the mortality compared with the intensive system (OR = 0.15 and p-value = 0.12). The duration of farming practices was also analysed, where the longer production time was less likely to be associated with the mortality (OR = 0.35 and p-value = 0.4). Sources of fry which were also not significantly associated to the mortality.

The measure taken during the disease outbreaks were also analysed, where the changing water and combined of water changing and treatment were not significantly associated to the occurrence of the mortality with p-value=0.08 and 0.09 respectively. However, there was a significant association between the use of the combination of antibiotics and traditional treatment to reduce the occurrence of mortality within the farms (p-value = 0.03) and based on the Odds Ratio indicated that this application was more likely reduced the risk of mortality (OR = 0,19). The water sources were also not significantly associated with the mortality. However, the water sharing system within the visited farm can become a problem, where most of the farms used the series system to share the water between ponds within the farm. Series sharing system was applied by 78% of the total farms increase the transferable of the disease or harmful substants from one pond to the other within the farm, as shown from the test where there was significant relation between the series water system with the occurrence of the disease outbreaks with p-value = 0.04. One potential problem was also identified where most of the farmers used the same equipment in their farms, based on the fisher exact test, the shared equipment provided the significant association to the disease outbreaks by p-value = 0.01. Shared equipment used could transfer either of the disease and/or harmful substants within the farm which correlated with the mortality. The statistic data of the potential association of several factors with the mortality were shown in Table 2.10.

Table 2.10. The assessment of the variables in association to the mortality by using Fisher Exact test.

Category	Frequency (n=41)	Percentages (%)	Disease outbreaks (n=30)	p-value	Odds Ratio (OR)
Gender of owner					
Male	34	82.93	26	0.36	2.37
Female	7	17.07	4	0.36	0.42
Farming location					
Timor island	32	78.05	25	0.21	2.77
Flores	9	21.95	5	0.21	0.36
Type of farm		0			
Semi-intensive	28	68.29	18	0.12	0.15
Intensive	12	29.27	7	0.23	4.16
Time of operation		0			
> 5 years	20	48.78	15	1	1.19
6 - 10 years	19	46.34	14	1	1.04
>10 years	2	4.88	1	0.4	0.35
Type of pond culture		0			
Monoculture	39	95.12	29	0.46	2.81
Polyculture	2	4.88	1	0.46	0.35
Type of pond					
Earthen	14	34.15	10	1	0.91
Cement	18	43.90	15	0.72	1.42
Tarpaulin	1	2.44	0	0.3	0.3
Mix	8	19.51	5		
Fry supplier					
Java	31	75.61	22	0.7	0.61
Non-Java	8	19.51	7	0.41	2.97
Mix of Java + non-Java	2	4.88	1	0.46	0.35
Water sources					
wells only	3	7.3	2	1	0.7
river only	7	17.1		0.65	2.45
springs only	5	12.2	3	0.59	0.5
ground water only	4	9.8	3	1	1.1
Mix source water	22	53.7	16	1	0.93
Series system of water	32	78.0	26	0.04	5.14
Shared equipments	36	87.8	29	0.01	15.1
Fish disease	25	60.98	17	0.48	0.49
Cause of mortality					
NA	16	39.0	13	0.48	2.01
Fish disease only	9	22.0	6	0,68	0.67
weather	5	12.2	4	1	1.52
overcrowding only	5	12.2	4	1	1.52
Mix	5	12.2	3	0,32	0.3
Measure taken					
Change water	17	41.46	15	0.08	4.34
Treatment		0.00			
Emergency harvest		0.00			
Mix change water treatment)	24	58.54	15	0.08	0.23
Treatment					
Traditional	1	2.44		0.26	0
Vaccines		0.00			
Antibiotics		0.00		1	0.72
Others		0.00			
mix traditional antibiotics	18	43.90	10	0.03	0.19
Pond preparation					
pond fertilization	26	63.41	18	0.71	0.57
Probiotics	16	39.02	10	0.28	0.42

2.5.3. Fish Samples.

The sampled of fish in the current study was presented in the Figure 2.24. A total of 264 fish were sampled from the 41 farms at the time of questionnaire and the number of fish selected per farm ranged from 5 to 10 samples. From the total tilapia and catfish samples collected as shown in Figure 2.23, 49% (n=129) of fish samples showed at least one or more internal/external clinical signs as observed at the time of sampling, A wide range of clinical signs were observed, both externally and internally and are provided in Figure 2. 22. The external clinical signs identified with the range of loss of appetite, sluggish movement, and swimming near the water surface, with the complete absence of the reflex. 72% (n=102) of the moribund tilapia presented the external clinical signs, where 35% (n=55) had a combined clinical sign. Meanwhile, 24% (n=20) of catfish samples presented a combined clinical sign. The details of the data were described in the Chapter 3, section 3.5.1.

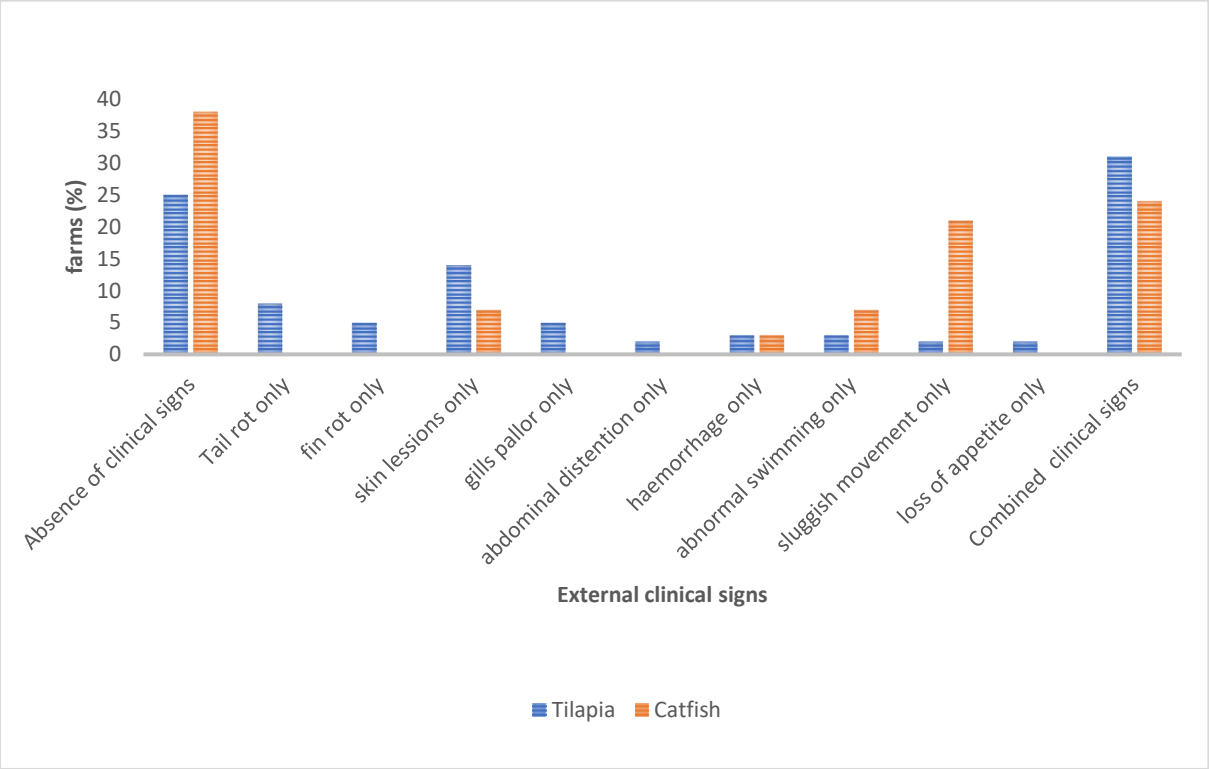

Figure 2.22. The percentages of the external clinical signs of fish (tilapia and catfish) samples collected in this study.

2.5.4. Water Quality

The physicochemical characteristics of water samples from the study area including temperature, pH, Nitrite (NO_2), Nitrate (NO_3), Dissolved oxygen (DO) and Ammonia (NH_3). The range of water qualities level checked during the field study were among the optimum level for tilapia and catfish growth, except in 1 farm that applied the biofloc system where the pH beyond the optimum range with 9.1..The field study also identified the water quality level of farms with the biofloc system on the maximum level of optimum ranges for freshwater farming such as NH_3, where the highest NH_3 level at 0.01 was identified in the two farms with the biofoc system as presented in Table 2.11.

Table 2.11. The range of water quality in the study area checked during the field study.

Farms water quality	**Range**	**Mean**	**Unit**	**Optimum range**
Temperature	27 – 32	28.41	^{0}C	25 – 32
pH	7 – 9.1	7.73		6.5 – 8.5
Nitrite (NO_2)	0 - 0.04	0.02	ppm	≤ 0,05 ppm
Nitrate (NO_3)	0.01 - 0.22	0.13	ppm	≤ 2 ppm
Dissolved oxygen (DO)	04 - 06	4.37	ppm	≥ 3 ppm
NH3	0 - 0.01	0.009	ppm	< 0,02 ppm

***** SNI7550:2009, Maimunah and Kilawati (2020), and Zhang *et al.*, (2023)

2.6. Discussion.

The aim of this study was to provide information to confirm the cause of fish mortalities in the farms and provide a more comprehensive understanding on the disease status and the current management approaches adopted in tilapia (*Oreochromis spp*.) and Clarias catfish *(C. gariepinus)* farming systems practised in ENT, Indonesia. In the area of study, freshwater farming particularly farmed tilapia and catfish developed widely and more recently, has started to intensify production. One of the drivers supporting intensification in this sector is the range of programs provided by the government to promote aquaculture in Indonesia including biofloc program in ENT (KKP, 2020). Freshwater farming production in ENT remains lower compared with other area such as West Java and East Java because traditionally, ENT relied more on marine fisheries, where the area of study is one of the highest marine production areas in Indonesia (MMAF, 2018). However, marine production recently has decreased, e.g. 2017 marine fish production decreased by approximately 20% from 173.296 tonnes in 2016 to 138.268 tonnes in 2017 (BPS, 2018), primarily through a decline in tuna (KKP, 2019). Furthermore, the Government considered overexploitation of capture fisheries will affect the sustainability of Indonesian fish supply in the future (Wiadnya *et al.*, 2018), hence the investment in freshwater aquaculture systems. Therefore, intensification of fish farming was considered necessarily to fulfil the increasing demand of the fish products locally and export and provided resilience to the Indonesian aquaculture sector.

The farms included in the study were located in the two main islands Timor and Flores Island which are the top 2 freshwater fish farming production areas in ENT, Indonesia (MMAF, 2018). The highest number of farms were identified in Timor Island and followed by Flores Island and reflected the higher levels of production of tilapia and catfish in ENT (BPS, 2018; MMAF, 2020). In Timor Island, the freshwater farming systems were concentrated in the Kupang regions, most likely due to the abundant supply of freshwater sources in this region. Although different number of farms located in ENT, the homogenous system applied was identified where the majority of farms were categorized as in the early stage of intensification. The intensification of aquatic farming system applied in the last couple of year in ENT, has raised the level of reported mortalities, and in this study, it was identified that the majority of farmers experienced mortality in their farms which caused significant losses in their stocks. Several factors identified from the work performed in this study could exacerbate

disease outbreaks, transmission and higher levels of fish mortalities. The rapid intensification without sufficient knowledge regarding the best management practice and production system, combined with increasing stocking densities would certainly contribute towards the infectious disease spread within the farm and in the systems. From the survey data provided it was clear that the farmers were not confident using FCR to to optimise growth in their stocks and over or under feeding could impacting the water quality, particularly in undigested organic matter but also compromise the health of the fish. The area of study was categorized as being in the early stages of intensification. This was evidenced by the use of aerators, pumps, water quality measurement, and consumables including probiotics, antibiotics, and vitamins (Henry, 2021). Thus, showing investment in the farm themselves and moving away from existence or extensive farming practises. However, intensification has to be managed effectively to control disease and appropriate health management practises must be developed and implemented to obtain the best fish growth performance.

In the present study, from the statistical analysis there were no significant relationships between the type of farming system with the occurrence of mortality within the farms. However, from the Odds Ratio (OR) the intensive production system applied by farms with OR more than 1 (OR=4.16) in the area of study were more likely associated to the mortality compared with the semi–intensive system (OR=0.15) which was closer to zero indicated that semi-intensive system applied was less likely cause mortality. However, given the small sample sizes additional work would be needed to confirm. Follow on studies, perhaps including longitudinal studies within individual fish farms over 2-3 production cycles would help clarify the actual risks from confounders. This was not possible in the existing study but might be possible to include additional sample or testing within the government surveillance and monitoring programmes in the future. In the current study, several issues related to the water management were identified, which would influence the water quality and growth of the animals, resulting in low productivity and exacerbate the occurrence of fish mortality within the visited farms. Of these, the water treatment where only a few farms allocated pond for water precipitation and treatment from water source before sharing to each pond within the farm. Pond for water treatment and sedimentation is required to prevent the harmful sediments and toxic substances, since the source of water were also supplied from surface water such as river which might already been contaminated, since the surface water were usually used for irrigation and other activities that in consequence of

pollution of the surface water. This approach is also important to maintain the optimal water condition before sharing to each pond, since the extreme weather in the of study might compromise the physical and biochemical characteristic of water in the optimal environment for fish such as water temperature, DO, pH, ammonia, etc. Therefore, it required pond for water treatment and filtration to maintain the optimal level of water from the water sources before sharing to the stocking ponds as an effort to optimize the production (Drózdz *et al.*, 2020). The allocation of pond for sedimentation and filtration can precipitate such substances that might harmful to the environment and also fish pra and post production (Boyd and Massaut, 1999). The fish farming in the area of study needs to be allocated and constructed for water filtration, treatment, and also as water storage to supply the demand during the production cycle. This approach also required since the limitation of water sources in some part of area in the Timor Island, ENT. In the area of study, the farms were more concentrated in the regions with more abundance source of water, where the main water supply were from the ground water and from river. Ground water is considered more consistent in quality and quantity (Summerfelt, 2015). Water sources are important part in the farming system, although in this study there were no significant association between the water sources and the occurrence of the mortality, where the water source was not significantly associated with the mortality. However, the issue was identified where the water sharing system between ponds with the farms was mostly constructed in a series system, this construction system increase the risk of the transmission of the disease and/or harmful substances, as shown from the statistical analysis that there was significant association between the series water sharing system with the mortality, where in the current study, most of the farmers were used the series system in sharing the water between each pond. This method also enhanced the spread of the pathogenic diseases between the disease fish to the healthy fish within the farms (Chitmanat *et al.*, 2016).

Providing fish with optimal water quality is critical to their health and wellbeing and in this study, most of the farmers performed basic water quality checks, ranging from temperature and pH, with approximately 50% of the farms included in the study also measuring turbidity. Monitoring the more comprehensive water parameters including temperature, DO, NH3, nitrite, nitrate, and pH are required because it is integrated and associated between the water parameters (Levinton, 1982). Water temperature was also strongly associated to the water productivity. The correlation

between the parameters was described previously where the temperature as an example, water with low temperatures is higher nutrients then the water with higher temperature (Fujaya *et al.*, 2022). Therefore, the temperature is associated to the other parameter including DO level. During the current study, although DO is one of the most important factors, only a small proportion of farms were also monitoring the DO. In the farming system, DO is the main limiting factor for the growth and welfare of fish, when the oxygen level is exceeded the optimum range, the fish tend to stop feeding which then slower the growth rate and the emerging of the infectious diseases which related to the stress (Summerfelt, 2015). Although, the DO level in the present study still in the tolerate range for fish including tilapia and catfish, the temperature instability affected by the rapid change caused significant fluctuation in the DO level. NH_3 is also important water parameter because it relates to the fish well-being. High level of NH_3 will cause fish stress or even fish mortality (Summerfelt, 2015). Furthermore, in a small number, NH3 can cause fish stress, damages gills and other tissues of fish (Shoko *et al.*, 2014). Measuring the NH_3 is also important because it in correlation to the feeding rate, excessive feeding can increase the NH_3 level in the water in the pond. In the current study, higher NH_3 level were found in the farms that applied biofloc system with much higher fish density, more excessive feeding intake, and also with a minimum water changing during the culture compared with the pond that used concrete or earthen pond. Probiotics were used in the biofloc system to maintain the water condition including reduce the NH_3 level. The heterotrophic process of bacterial biosynthesis that produce bacterial biomass to enabling the microbial process (Brune *et al.*, 2012). The high NH_3 level was also associated to the low oxygen that hindering the transferring process from NH_3 into nitrite, and low oxygen level also caused incomplete nitrification process. Therefore, aeration was also applied to increase the oxygen level to enhance the oxidation process in the water pond. This approach was taken to support the nitrification process from nitrite into nitrate even though the aeration was not always sufficient in promoting the nitrification process (Brune *et al.*, 2012). The nitrification process was also related to the pH level, the nitrification will be affected and even stopped by the low pH level (Boyd and Massaut, 1999). Considering the important of maintaining the water quality during the production cycle including support the fish performance, health, survival, and fish growth. However, in the current study the water quality was not checked regularly where only small number of farms were able to provide the water quality record. The farmers need

to monitor the water quality as a regular basis including during the seasonal changes where the weather fluctuation occur rapidly. This approach is also necessary for further action applied by the farmer if the water parameters beyond the optimum range, so the farmers can practice the intervention to treat the water condition such as filtration, water treatment from the preparation prior to the stocking by using probiotics, water changing, and another treatment to the water that can promote the optimal environment for fish. Good water management practices is strongly associated with the success of fish production (Chitmanat *et al.*, 2016).

Understanding the tools used by the farmers to help promote the optimal condition of the farming system and the stocks welfare. The efforts including improvements in any biosecurity practises. In the present study, farmers claimed to use both fertilizer to the ponds and probiotics. This technology aims at maintaining and improving the water quality in the farming system by using probiotics and specific microorganisms to form microbial protein from organic waste in the water including fish waste (Brune *et al.*, 2012). In the current study, probiotics were predominantly used by the farms together with biofloc system. Those farms adopting this technology applied higher stocking density with the support of aeration system and probiotics mixed directly to the water. This approach was considered necessarily to maintain the water conditions that favourable to the fish. The farms with biofloc system also had advantages where the environment relatively small and isolated which reduced the exposure to the extreme weather which is one of the challenges in the farming system in Indonesia. However, since this method was categorized as a new system in the study area, the farmers tended to face the issues such as the diseases still occurred and doubled by the limit knowledge regarding the disease fish treatment, the low growth even with the higher feeding intake, and the cause of fish mortality that they difficult to acknowledge within their farms. Some farmers suggested that they required further assistant and training regarding the management practice to support the productivity. The development of fish farming especially the farms related to government programs were regularly monitored by the government through MMAF. However, this activity was still inefficient since the large number of fish farming and located in the different island of Indonesia including in the area of study (MMAF, 2020).

The management practice also identified as the constrains, where most of the farmers used the same equipment in their farms. Shared equipment could transfer either of the disease, or harmful substants between ponds within the farm. Based on

the statistical analysis, shared equipment within the farm provided significant association with the occurrence of mortality, where the p-value = 0.01. The equipment should not be shared between each pond or farms because large number of the disease causing agents are still survive within a range amount of time and can be transferred through the shared used of equipment (Dvorak, 2009). This practice doubled by lack of disinfection applied by the farmers. Only few farmers were able to provide the evidence regarding the disinfection in the visited farms, although, this is important practice in the farming system to prevent the contamination including pathogens contamination (Kasai et al., 2002). Most of the dead fishes were discarded during the outbreaks, although during the field study, the dead fish were still can be found in the pond. The uncollected dead fish can contribute to the biosecurity risk which can become the source of the infection that can be spread in the water system (Dvorak, 2009). Based on the farmers feedback, no dead fish were sold for human consumption but some of them utilized the dead fish as feed for other animal broodstocks e.g. pigs.

Water exchange was the most common intervention taken when the disease occurred. Half of the farms applied several interventions including the use of herbal ingredients such as turmeric (*C. domestica*) and Ketapang (*T. catappa)* and also antibiotics. The use of mixed traditional and antibiotics application methods significantly reduced the occurrence of mortality based on the statistical analysis (p-value = 0.004). This also supported by the analysis of the risk factors related to the mortality (OR=0.15) which was closer to zero indicated that these methods potentially decreased the risk of mortality within the farms. Several reports also suggested that the application of antibiotics was the common measure applied during the outbreaks (Sapkota *et al.*, 2008; Chitmanat *et al.*, 2016). This approach was also true in Indonesia where antibiotics application was the most method used to address the disease in the farming system. Although most of the farmers in the area of study still have limited knowledge regarding the appropriated application of the antibiotics. The farmers who used antibiotics in this study, did not aware regarding the correct type and the dose of the antibiotics applied during the disease, although most of them understand in terms of the negative effects of the antibiotics, including residual effect of antibiotics and the risk of antibiotics resistance. The farmers reported that most of the antibiotics were obtained from private/companies who mostly the fry suppliers. The excessive use and misuse of antibiotics can lead to the antibiotic resistance which

become global challenges and detrimental effect to the environment (Noga, 2010). In Indonesia, the distribution and access of antibiotics were limited for aquaculture, only experienced farmers or companies have access to the medication including antibiotics as the programs that had been established by the government to limit the spread and reduce the use of antibiotics in the farming system since 2012 (Ministry Regulation PER.04/MEN/2021). In terms of the antibiotics type in the current study, oxytetracycline was the most prevalent used by the farmer. This finding was in agreement with the global reports where the class of tetracyclines including oxytetracycline were widely used in the aquaculture industry, animal husbandry, and human therapy because of low cost and high efficacy against a broad spectrum of bacteria, parasites, and fungi (Mo *et al.*, 2017). The farmers in the study were convinced that this type of antibiotic strongly helped them in addressing the disease problems, however, no evidence was provided to support their perception. Tetracycline reported had strong efficacy in tackling the pathogenic bacteria including Gram-positive and Gram-negative by stressing protein and linked with 305 rRibosome to limit the aminosil on the ribosome side which compromising the peptide linkage (Chopra and Roberts, 2001). Antibiotic was also reported In aquaculture where tetracycline and their compounds were used in 60% of the antibiotics application (Sekkin and Kum, 2011; Suzuki, 2021). This antibiotics were also use not only to treat the diseases but also as a prevention and in the freshwater farming system and environment (Yuningsih, 2005; Cañada *et al.,* 2009; Mostafa Shamsuzzaman and Kumar Biswas, 2012; Skwor *et al.*, 2020). Although the use of tetracycline compounds was banned in some country including EU in 2016 (Castanon, 2007), and also in Indonesia where the used of tetracycline were limited, however, tetracycline class remains the most commonly used in animal production and aquaculture in different countries including in Indonesia (Suzuki and Hoa, 2012), in Malaysia (Thiang *et al.*, 2021), Thailand (Lulijwa, Rupia and Alfaro, 2020), and other Asian countries (ASEAN, 2013).

The other issue in the intensive system mostly applied by the farmers in the area of study was the lack of information regarding the appropriate approach to increase the production including by increasing the density. The farmers tended to increase the density in each pond within their farms, without sufficient data regarding the optimum density to obtain the optimum productivity. This can be seen during the field study where farmers were hesitated to provide the feedback on the optimum

density of both tilapia and catfish. This condition doubled by lack of information provided to the farmers regarding the effect of increasing the density and feed conversion for the stocks. Hence, the feeding intake might either not sufficient to the fish growth or even excessive feeding occur which then resulted the negative effects to the fish, reduce the water quality, and negatively affecting the environment. The high density also cause negative effects including physical damaged, increased stress level, and also affected the culture condition which often led to the disease problems (Føre *et al.*, 2018).

The extreme weather also remains the limiting factor in Indonesia farming system. As shown in the current study where the outbreaks commonly occur in the season change. During these stages the daily temperature changed rapidly, this was followed by the other water parameters including the DO level, pH level, etc also fluctuated. This condition was unfavourable to the fish and caused the stress which than compromising the immune system and then caused the fish more susceptible to the infectious diseases. More specifically to the time range of the outbreaks, the farms experienced high mortalities in March - April. This result has in agreement with the previous outbreaks reported in Indonesia (Manumpil *et al.,* 2015; Hernawaty, 2018) and also globally (Ibrahem *et al*., 2008; Li *et al.*, 2020). In this period of time, the temperature in particular was change significantly, the rapid changes of environment condition can lead to the stress of fish and increase the potential pathogenicity of infectious disease which then causing the fish mortality. A few farmers were identified during the current study applied more isolated farming system to address the weather issue including the rapid changes of weather by using the more isolated farms and also monitored the water parameters regularly including temperature, DO, pH, and also NH_3. This approach might reduce the mortality level during the extreme weather in Indonesia (Tarnadi *et al.,* 2015). Whilst a gender-based study was not performed, data from this project clearly showed a male dominance in the farming systems included with female participants being more involved in the small-scale family business farm. This is not unique to Indonesia and has been reported in fish farming system globally (Frangoudes and Pascual-fernández, 2005). The involvement of women in the aquaculture sectors was reported in almost all of the phases from fish production, processing and distribution, and contribute to the family income to the preserving the ecosystem (Gopal *et al.*, 2020). Furthermore, the engagement of women generated additional income to the household in the area of study and this

also true to the national level in Indonesia (Sari et al., 2017; MMAF, 2020). In 2015, 12% of women were reported involved in the fish farming (Pettersen and Alsos, 2007). In the current study, the female involvement was slightly higher than global report where 17% of female engaged with the farming system in the visited farms. The increasing number of female engagement was also related to the several programs establish by government through MMAF to enhance the women participation and the capability by establishing training and several programs (Sari et al., 2017; MMAF, 2020). The women participation playing important roles in the farming practice cycles within the visited farms where female involvement was identified. Women participation in this sector contributed significantly to the family income, where previously was mostly as a supporting system with the farming system (MMAF, 2020; Puteri et al. 2021). The statistical analysis in correlation to the mortality within the farm identified that there was no significant relationship between gender involvement with the occurrence of mortality in the freshwater farming system in East Nusa Tenggara. The current study also identified that he women involvement in the farming system resulted the productivity that can be compared with the male. As shown in the data where the fish farming with women involvement distributed slightly lower risk of mortality occur than the farming system without women engagement in the farm based on the statistical analysis. This positive finding on female involvement would support the effort of greater women participation by providing more opportunities for women to engage in the fish farming in the study area. Although, this might associate with the other relating factor such as the knowledge and/or experience of participants, based on the data from the questionnaire, 5/7 female who involved in the farming system of visited farms with the operation duration more than 5 years. Therefore, the statistical results identified in association with the gender might be influenced by experience of the female who engaged within the visited farms. The farmers experience contributed positively significant to the success of the fish farming (Goswami *et al.*, 2020).

Experience is considered as important factor related to the success of the farming system, the farmers tend to use their experience to interpret the information and the current condition in their farm in making decision including the farming practice and management practice applied in their farm. In the present study, in general the farmers experiences were categorized as early stages. Although, there was no statistically significant relationship between the farm experiences with the relative risk of fish mortality, from the statistical analysis showing that the farms were less likely to

experience the disease outbreaks with more experience within the farms. This is in agreement with previous studies in Indonesia where the more experience within the farms the higher probability of the success of production (Hermawan *et al.*, 2017). The farmers experience also related to the consideration in making decisions regarding the issue arise in their farms more rapidly and confidence (Føre *et al.*, 2018). The limitation of fish fry quality and quantity produced in East Nusa Tenggara could become a constraint to the success and sustainability of the farming system in the study area. The fish fry that grown in the farming area were obtained from the hatchery where most of them from the outside region of the study area, only a small number of fish fry was produced in the hatchery located in the study area. Different environment and long transportation from the fish fry producer to the farms will be a limiting factor, although all of the farmers practiced acclimatization prior to the stocking and ponds preparation prior to the stocking, they often experienced the number of mortalities in the early stages of stocking. The quality of stocking fry also significantly affected the productivity (YIDH, 2018). The issue associated to the success of the farming in the area of study (MMAF, 2020). However, the production of fish fry to continuously meet the demand of the fish farming in the study area remains the challenges hampering the production, because the hatchery sector in the area of study was still low in productivity and not able to meet the required production to supply the fry demand in the area of study. Government has already acknowledged the issue of fish fry limitation in term of quantity and also quality. Therefore, several programs were also established by Indonesia government through MMAF included providing the farms with the training from the hatchery level to marketing level, supported with the infrastructure, fry, feed, and also regular monitoring (Nenobais, 2021). The hatchery belong to the government and private sectors were also supported to increase the productivity in quantity and quality with sustainable production for long term and able to supply the increasing demand of fish products (Darwisoto *et al.*, 2015; MMAF, 2022).

The ability to keep regular and high-quality farm records is essential to support the daily and longer-term management of any farming practise (Prajapati et al*., 2015)*. Farm record keeping is important contributing to the sustainability of the farming practice, it can be used as an early recognition of the problems within the farm and to monitor the production, health, and disease problems. Farm records can also help as an early warning in the acknowledging the disease problems and their impact that might occur in the farming system which can lead to the disease outbreaks (Dvorak,

2009). Most of farmers reported that they kept farm records, however, when asked to provide their record. Only a small number of farms were able to provide their farm's record during the field study and most of them by the paper-based record. Stocking information and production were the most common reports recorded by the farmers and followed by the record of mortality number. This is understandable because the stocking, production information, feeding intake, and mortality provided them directly to the financial aspects (Prajapati et al*., 2015)*. Only small number recorded the clinical signs and potential cause of the disease outbreaks, this probably because the lack knowledge of the important in recording the time of the disease occur, potential cause of mortality, and the gross clinical presentation of the disease or dead fish. This type of record can be used as an alarm and further treatment. Lack of awareness regarding the farm record keeping resulted the non-maintaining the farm records (Prajapati et al*., 2015)*. Furthermore, the record keeping including the mortality record and the clinical signs as this information will be beneficial as an early-stage information for the farmers (Dvorak, 2009).

Regular monitoring is important as a control and to understand the status within the farms which included the management and husbandry practice, biosecurity, disease problem, and mitigation approach within the farming system. In the study area the monitoring was provided by the government as a national program through the Ministry of Marine Affairs and Fisheries. Most of the farm in the area of study were also visited. However, this program was not employing comprehensive diagnostic of fish disease, management practice, and or biosecurity but the purpose was focussing on the particular pathogen and also particular fish species (MMAF, 2020). Therefore, the problems of the outbreaks were remained unsolved and even the farmers reported the outbreaks were often occur in their farms. The current study is considered essential in understanding the aetiological agents of the disease associated with the outbreaks in the study area by employing comprehensive study from the field study level into the laboratory level. The biological samples were also collected in the current study, where the fish samples from moribund fish showed the gross presentation ranging from loss of appetite, dullness, a sluggish movement, and swimming near the water surface, with the complete absence of the reflex. The clinical signs presented in the current study might associate with pathogenic disease as the clinical manifestation shown from the fish samples. This presentation of the gross clinical signs were in agreement of the previous report in the freshwater farming in Indonesia described that

the Gram-negative bacteria caused the infected fish showing the clinical signs shown in the current study (Hardi *et al.*, 2018; Rahayu, 2019). The neighbour country, such as Vietnam also reported that the main problem of fish farming production was affected by the pathogenic diseases (Steinbronn, 2009).

2.7. Conclusion.

The majority of farms in the area of study were categorized as at the early stages of intensification system where the lack of farmers knowledges and experiences on the best biosecurity and health and disease management practice were identified in the current study. In consequence, the system was inevitable from the increased of fish disease risk, where infectious diseases remain the main issues effecting the production system in the area of study. As reported during the field study where most of the farmers experienced the disease outbreaks that caused significant losses, where the farmers were able to observe the disease fish stocks through the gross clinical signs of the diseases. Therefore, require further approach in addressing the issue hampering the production along with the proper management practice applied, where until now it was still not fully acknowledged and addressed in the area of study.

Further identification of the bacterial isolates collected in the field study will be studied in the Chapter 3.

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CHAPTER 3. DIAGNOSTIC INVESTIGATION INTO BACTERIAL DISEASE WITHIN FARMED TILAPIA AND CATFISH, INDONESIA.

3.1. Abstract

Disease outbreaks are commonly reported by the farmers in Indonesia including in the area of study which caused significant economic losses. However, a comprehensive approach including the disease diagnosis has not been widely adopted to confirm the disease status in Indonesia's aquaculture systems. Therefore, a disease diagnostic approach was adopted where a total of 246 fish samples from tilapia and catfish, were included in this study. Bacterial isolates and tissue samples were collected for bacterial identification and histopathology assessment to confirm the aetiology. A combination of traditional identification methods and molecular methods were performed for bacterial identification. The gross presentation of moribund fish samples showed a minimum one of clinical signs of the disease such as loss of appetite, sluggish movement, and abnormal swimming with the complete absence of the reflexive response, and haemorrhages. The abnormalities of internal clinical signs such as the enlargement and the colour alteration of fish internal organs. The examination of histopathological from the tissue samples of fish with clinical signs presented several deviations of the tissue including large number of vacuolation, degenerative changed in glomerular, necrosis, infiltration of inflammatory cells, and the aggregations of MMC. The Gram stained of the tissue samples was also showing the figure of rod shapes bacterial colonization. Most of bacterial isolates were Gram-negative bacteria and dominated by *Aeromonas* species including *A. veronii, A. hydrophila,* and *A. caviae,* which then 40 representative samples were confirmed by 16s rDNA and the two housekeeping genes *rpoD* and *gyrB*. The bacterial species in the present study from the molecular methods were evolutionary illustrated had close relationships with the isolates recovered from the farmed fish in the neighbouring countries. Therefore, this study was able to establish the clinical figures of the observed fish samples and the involvement of motile *Aeromonas* in causing infection which may led to the occurrence of the disease outbreaks.

Keywords*: tilapia, catfish, disease, clinical signs, mortality, bacteria, disease*

3.2. Introduction.

Aeromonas species are found in a wide range of aquatic and terrestrial environments as well as the gut microbiome of vertebrate species including fish and human (Janda and Abbott, 2010; Rasmussen-Ivey *et al.*, 2016). Several members of the motile *Aeromonas* species including *A. veronii, A. hydrophila, A. sobria and A. caviae* are reported as pathogenic with resulting infections of Motile *Aeromonas* Septicaemia (MAS) in multiple fish species (Cai *et al.*, 2012; Austin, 2019a). Care must be taken on initial recovery of these motile *Aeromonas* species, particularly in association with infectious disease and diagnoses, given their ubiquitous nature and rapid growth *in vitro*. In Indonesia, *Aeromonas* was reported as the most commonly recovered bacteria identified from farmed tilapia species isolated from both apparently healthy and diseased samples (Amanu *et al.,* 2014; Rahayu, 2019; Azhari *et al.,* 2014; Manurung and Susantie, 2017; Angraeni *et al.,* 2018). A wide range of clinical signs of disease are described from MAS infected freshwater fish, which are not pathognomonic as other bacterial species can produce these clinical signs (Hanson *et al.,* 2021, Pękala-Safińska, 2018). Thus, making it difficult to identify if the MAS strains recovered from the affected fish species are the primary causative agent or a passenger. This will hinder the production of efficacious biosecurity practises including vaccine development.

The development of molecular tools e.g. PCR have contributed to the improved identification and characterization of many motile *Aeromonas* species including *A. hydrophila* and *A. veronii* associated with infectious disease. Although vigilance is required as not all MAS infections are due to a single bacterium and without a comprehensive approach this can be a point of failure within a diagnostic investigation. For a long time, the most dominant MAS species recovered and identified was *A. hydrophila.* However, with the uptake of molecular tools which provide a more rapid and sensitive identification method, better discrimination between closely related motile *Aeromonas* species has clarified the range of bacteria recovered from aquatic MAS infections to species-level (Janda and Abbott, 2010; Cai *et al.*, 2012; Rasmussen-Ivey *et al.*, 2016). Recent studies have improved identification of the bacteria and confirmed other motile *Aeromonas*, including *A. veronii* as a primary cause of MAS infection in freshwater fish species (Chen *et al.*, 2019).

The development of effective disease control strategies is essential to promote food security and requires understanding and knowledge on the disease status,

health, and welfare of the farmed stocks as well as optimal biosecurity strategies. Adoption of routine disease monitoring combined with disease diagnosis (not pathogen confirmation) is the gold standard approach to support disease control and reduce mortalities due to bacterial infections from MAS (Adams and Thompson, 2011; Austin, 2019b). Diagnostic practises rely on a combined approach to ascertain the problem, i.e. is the mortality or morbidity infectious or environmental, husbandry, nutritional etc. Thus, supporting informed decisions on the treatment strategy, is a cost-effective manner. Farm and production level data are essential information to support a diagnosis and when combined with gross clinical signs of the disease and high-quality biological samples, will ensure the best health or disease management strategy is adopted at the time to promote welfare of the stock. The strength of a diagnostic approach is the combination of information, which includes detection and identification of pathogens and distinction between the apparently unhealthy and healthy host (Austin, 2016). The term apparently healthy is often used in diagnoses until the histopathology samples have confirm the health or disease status of all animals sampled and is applied to those animals that do not show any behavioural abnormalities nor evidence of external or internal clinical signs of disease.

The diagnostic approaches in aquaculture are similar to those applied in terrestrial animal and medical fields, but the methods employed are often tailored to the production system, current knowledge, and laboratory resources available to support the sample identification. It is more common nowadays that both traditional and molecular and/or immunological methods are performed within most aquatic diagnostic facilities (Adams and Thompson, 2011), however, this will be dependent on the laboratory resources and status. All diagnostic methods rely on the recovery of viable cultures from diseased fish, with only a very limited number of exceptions. Viable bacterial recovery is required for the antibiogram assay which informs on the most efficacious antibiotic for treatment. Most diagnostic approaches include both phenotypic and genotypic assays to identify the bacteria, with the genotypic work providing identification in closely related bacterial species or detection of pathogen specific genes associated with infectivity. In Indonesia, like many countries in Southeast Asia (SEA) there remains reliance on more traditional bacterial assays which will provide identification to genus but may be more limited to confirm species-level identification for some bacterial species. The traditional methods often lack the level of discrimination required to differentiate to species level with closely related

pathogens and often provide inconsistent results due to the variability of the biochemical profiles obtained particularly relevant to the motile *Aeromonas* group as these are very heterogeneous within species. Furthermore, the absence of clinical inequity of sufficient training staff may also impair accuracy in the results for the biochemical profiling as these are colorimetric assays, where distinction of the varied colours can be more subjective compared with a single band on a PCR gel (Janda and Abbott, 2002; Beaz-Hidalgo *et al.*, 2015). This scenario is relevant to the aquatic laboratory in East Nusa Tenggara where there are a small number of experienced staff processing a high number of samples through varied laboratory tests from customers including fish farmers. Therefore, adopting molecular assays will support a more cost-effective and improve quality of the bacterial identification results, beneficial to the fish farmers (Janda and Abbott, 2007; Burr *et al.,* 2012). The cost of these assays has significantly reduced over the years making this an attractive option and there are now several assays available which can be used within the ENT fish diagnosis laboratories to differentiate between the motile *Aeromonas* species and identify the core virulence and antimicrobial resistance genes known at the bacterial species level. Such tools are not yet in place within the laboratories and would require a quality control system to ensure that they remain fit-for-purpose within the diagnostic unit but also can provide essential evidence on the pathogenicity and antimicrobial genetic data to improve future microbial epidemiology studies, monitoring and surveillance strategies.

Histopathology remains the only true diagnostic tool available in aquaculture and any other diagnostic method supports pathogen detection/identification or host response to the health issue under investigation. Therefore, pathology samples are invaluable at the onset of any disease investigation because it can provide a robust description of the status of the animals at the cellular level. Tissue alteration in fish maybe due to genetic, poor husbandry conditions or sub-optimal environment or induced by pathogen, and often in aquaculture a combination of factors. Ultimately all factors can compromise the growth and survival of the farmed animals, but the health management decisions and economic costs incurred will be vastly different depending on the cause (Brum et al., 2014; Santos et., 2012).

3.3. The study aims.

The overall aim of this study was to provide a comprehensive description of the disease status and the identification of bacterial isolates recovered from farmed fish presenting with clinical signs of disease to species level. A diagnostic approach was taken to confirm the aetiology and bacterial identification performed using a suite of traditional and molecular assays.

3.4. Material and Methods

3.4.1. Fish biological samples.

The collection of biological samples for bacterial recovery and tissue collection were described in the Chapter 2 (section 2.4.1). The biological samples were collected from 41 farms which included tissue samples from apparently healthy and diseased tilapia and catfish and bacterial recovery from the same animals.

3.4.2. Bacterial Isolation and Identification.

Bacterial Recovery and Isolation.

Samples for bacterial recovery were aseptically taken using a streak plate method according to the Austin & Austin (2012). Basically, a sterile bacteriology loop was inserted into the kidney, liver, and/or spleen of the fish and inoculated directly onto tryptone soya agar (TSA, Oxoid) plates, and incubated for 24 to 48 hours at 28^{o}C as described by Legario *et al.*, (2020). The bacterial growth on the agar was observed and a single colony subculture performed, where the dominant colony (based on the colony shape, size, colour) was aseptically removed from the original agar plate and subcultured onto a new TSA plate, incubated at 28^{0}C and used for the subsequent assays described Each single colony was also subcultured onto selective agar including GSP and RS medium, where the first to help in identifying the *Aeromonas* genus and the later to distinguish the *A. hydrophila* species. The specific mediums that were also performed during the present study to help in the identification of *Aeromonas* species. *Pseudomonas Aeromonas* Selective Arar Base (GSP) was used to preliminary identify the *Aeromonas* genus, the positive results were indicated by the colour change of colony into yellow from green colour. Furthermore, Rimler-Shotts (RS) agar medium was also performed to specifically identify *A. hydrophila* species, where the positive results were shown by colour change from red to yellow colour.

Identification of Bacterial Isolates.

A combination of traditional and molecular bacterial identification and characterisation methods were used. The primary bacterial recovery and preliminary bacterial identification was performed in the Fish Quarantine Regional Office Laboratory (FQIA) of Kupang, East Nusa Tenggara, Indonesia within the Government laboratories. Traditional bacterial identification assays were performed to provide a presumptive identification to species but more commonly genus level. Traditional

Gram stain was not performed in Indonesia and instead the potassium hydroxide test (3% of KOH) was employed with additional catalase and oxidase tests performed according to Barrow and Feltham (2003). The KOH test was based on the difference in the chemical reactions, KOH was able to dissolve the thin layer of peptidoglycan of the cell walls of Gram-negative bacteria (Suslow. *et al.,* 1982). Bacteria were considered Gram-negative when the result of the test showed a cloudy/thick reaction on the test slide and Gram-positive where no reaction occurred. Motility test was performed by inoculating the bacterial colony into sulphur indole motility (SIM) medium and incubating at 28^0C for 24 hours, the bacteria were considered motile when it was growing and spreading on the media (Sudarsono, 2008). The catalase test was used to detect the production of catalase enzyme of bacteria following the method of Hadioetomo (1993). Oxidase test was conducted to determine the bacteria in the production of oxidase strips (Oxoid), the pure colony was directly placed onto the filter paper area of the strip and spread over the strip and read after 1 minute. A positive result was when the oxidase indicator turned blue or purple colour where no reaction indicating oxidase negative. Additional conventional biochemical methods were performed in Indonesia as described in Table 3.1.

Table 3.1. Culture tube method performed in the identification of *Aeromonas* species performed in Indonesia.

Biochemical Test		Positive Result	Negative Result
Indole		red ring	No red ring
MR		red	yellow
VP		pink/red	yellow
Citrate		blue	green
Urea		pink/red	yellow
Gelatine		liquid at 4^0C	solid at 4^0C
Lysine decarboxylase		purple	yellow
Ornithine decarboxylase		purple	yellow
Glucose		yellow	red
Sucrose		yellow	red
Lactose		yellow	red
Arabinose		yellow	red
Maltose		yellow	red
Mannitol		yellow	red
Inositol		yellow	red
Dulcitol		yellow	red
Sorbitol		yellow	red
Growth on the specific medium	GSP	yellow	red
	RS	yellow	green

Biochemical identification was performed following the methods as described by Indonesian National Standard (SNI) Number: 7303.1:2015, and SNI No, 8096.1:2015 which referred to the (Bergeys. *et al*.,1923; Cowan and Steel, 1993; Austin & Austin, 2007) detail of the methods as described in the protocol of FQIA Microbiology laboratory of Indonesia.

All isolates from bacterial identification were stored into semisolid agar medium (Merck), where the composition of the semisolid medium followed the manufacture instruction, with 100 ml sterile water with 0.3 g of beef extract, 0.35 g agar, and 0.5 g peptone (FQIA protocol of Microbiology lab.). The bacterial samples were transported to the Institute of Aquaculture, Stirling University for further identification and analysis.

Bacterial Recovery for the Identification of the Bacterial Strains.

A total of 40 bacterial samples preliminary identified as members of the motile *Aeromonas* species by the conventional methods described above, were used in this study and confirmation of the primary bacterial identification results were performed at IoA laboratory prior to conducting the molecular assays performed at University of Stirling. At this stage the bacterial strains were identified as *A. veronii (n= 22), A. hydrophila (n= 14)* and un-speciated *Aeromonas sp* (n=3), and *A. caviae (n= 1)*. On arrival at the University of Stirling, all bacterial samples were grown from pure cultures, in the semisolid's agar onto TSA. Primary identification tests performed to ensure purity and no changes post-transportation prior to being stored in 15% glycerol stocks at -20^{0}C. These included Gram-stain, cytochrome-oxidase activity using oxidase strips (Oxoid-UK). Motility with hanging drop method, catalase production by 3% of hydrogen peroxide (H_2O_2), and oxidation/fermentation – OF test as described in Frerichs & Millar (1993). As well as the primary identification tests, biochemical profiles of the 40 strains were performed using the API 20E test (BioMerieux, France) following the manufacturer's protocol except the inoculated strips were incubated at 28^{0}C and the results were read after 48h (Crumlish, 2011). All tests were conducted in parallel with two reference strains *A. hydrophila* National Collection of Industrial and Marine Bacteria (NCIMB) 9240 and *A. veronii* the American Type Culture Collection (ATCC) 36524. The biochemical reaction of API 20E performed in the current study was described in the Table 3.2.

Table 3.2. The biochemical reaction of API 20E performed in the current study**.**

API 20E abbreviation	Biochemical Test	Reaction	
		Positive	Negative
ONPG	o-Nytrophenyl-B-D-galactosidase	Yellow	Colourless
ADH	Arginine dehydrolase	Red/Orange	Yellow
LDC	Lysine decarboxylase	Orange	Yellow
ODC	Ornithine decarboxylase	Red	Yellow
CIT	Citrate utilization	Deep Blue	Yellow
H2S	H2S production	Black Deposit	Colourless
UREA	Urease	Red/Orange	Yellow
TDA	Tryptophan deaminase	Dark Brown	Yellow
IND	Indole production	Red Ring	Yellow Ring
VP	Acetoin production	Pink/Red	Colourless
GEL	Gelatinase	Black Diffusion	No Black Diffusion
GLU	Glucose fermentation	Yellow	Blue/Green
MAN	mannitol fermentation	Yellow	Blue/Green
INO	Inositol fermentation	Yellow	Blue/Green
SOR	Sorbitol fermentation	Yellow	Blue/Green
RHA	Rhamnose fermentation	Yellow	Blue/Green
SAC	Sucrose fermentation	Yellow	Blue/Green
MEL	Melibiose fermentation	Yellow	Blue/Green
AMY	Amylase fermentation	Yellow	Blue/Green
ARA	Arabinose fermentation	Yellow	Blue/Green
OXY	Oxidase	purple	No colour change

PCR Amplification and Sequencing of 16S rDNA, *gyrB* and *rpoD.*

Bacterial DNA was extracted from single purified colonies following SSTNE/salt precipitation DNA extraction method (Dwiyitno *et al.*, 2018), with minor modification. The bacterial cells were lysed by adding lysis solution and proteinase K into the DNA pellet, RNAse treatment, protein precipitation by mixing 5M NaCl solution, DNA precipitation using isopropanol and washed with 70% ethanol, and DNA hydration by adding Tris-EDTA (TE) buffer which composed of 10mM Tris-HCl containing 1mM EDTA-Na2 into the DNA pellet. As the dilution buffer, TE buffer primary function was to solubilize nucleic acids while protecting them from enzymatic lysis. The concentration of the extracted DNA was measured by Nanodrop (Thermo) spectrophotometer. The DNA samples were stored in the -20^{0}C until required.

PCR amplification was performed to produce specific DNA fragments *in vitro* by using DNA templates. The 16S rDNA gene was PCR amplified using universal primer set 20F (5'-AGAGTTTGATCATGGCTCAG-3') and 1500R (5'-CGGTTACCTTACGACTT-3') which amplifies approximately 1501-bp region of the gene. The method followed that as described in Weisburg *et al.,* (1991). PCR

amplification of DNA gyrase subunit B (*gyrB)*) gene was performed as described by (Martino *et al.*, 2011). The sequencing primers used in the amplification were purchased from Eurofins Genomic with the forward primer *gyrB_F* (5'-GGGGTCTACTGCTTCACCAA-3') and reverse primer *gyrB_R* (5-CTTGTCCGGGTTGTACTCGT-3'). RNA Polymerase subunit D (*rpoD*) gene was performed as described by Yamamoto *et al.,* (2000). The sequencing primers used in the amplification was purchased from Eurofins Genomic with the forward primer 70Fs (5'-ACGACTGACCCGGTACGCATGTA-3') and reverse primer 70Rs (5-ATAGAAATAACCACGTAAGTT-3'). The amplification of 16S rDNA, *gyrB* and *rpoD* were performed in a 25 µl reaction mixture consisting of 12.5 µl of 2X HS MyTaq MasterMix (Bioline, UK), 1.0 µl of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 µl of DNA template and 8.5 µl of Milli-Q water to volume. The PCR amplification of 16S rDNA was performed with the initial denaturation at 95^0C for 1 min, and then followed by 30 cycles of denaturation at 94^0C for 15 s, the next steps was annealing at 56^0C for 20 s, and then extension at 72^0C for 1 min, with the last steps was final extension of 72 ^{0}C for 2 min. Meanwhile, the PCR amplification of both housekeeping genes, the initial denaturation was performed at 95^0C for 2 min, and then followed by 35 cycles of denaturation at 95^0C for 10 s, where annealing temperature at different degree for both *gyrB* and *rpoD* with 59^0C and 56^0C respectively for 30 s, and then the next steps was extension at 72^0C for 30 seconds, with the final extension of 72 ^{0}C for 2 min for both housekeeping genes.

The PCR products of 16S rDNA and both housekeeping genes were obtained by electrophoresis in a 1% (w/v). Agarose gel prepared on 0.5X TAE (Tris-acetate-EDTA) buffer with ethidium bromide (0.5 µg/ml), The 50X TAE buffer working stock was prepared with the composition of 242 g tris base in double distilled H2O, 57.1 ml glacial acetic acid in 100ml 0.5 M EDTA solution (pH 8.0) where the volume was adjusted into 1 L. the 0.5X TAE buffer was then prepared by mixing 10 ml of 990 ml of prepared 50X TAE buffer. Agarose gel was then visualized on UV transilluminator. A positive control was used which include type strain *A, hydrophila* NCIMB 9240 and milli Q water as a negative control. Gel electrophoresis of the motile *Aeromonas* strains showing the target size of a positive band at approximately 669bp for *gyrB* and 820 bp for the *rpoD* housekeeping gene were then selected for DNA purification prior to sequencing, in brief, the DNA from the PCR products were then purified by using the Qiagen purification kit followed the manufactures protocol. The purified DNA

samples were then sent to the Eurofins Genomic, Germany. Sequences were aligned with ClustalW algorithm against phylogenetically related organism available in GenBank in the National Center for Biotechnology information website (http;//www.ncbi.nlm.nih.gov)in order to determine the species of bacteria with the highest homology to the queried bacterial isolates. The query length of more than 1400 and with the query coverage of equal or greater than 98% were then selected for further blasted and pulled into the phylogenetic tree. All strains were pooled together with the respective references. The phylogenetic tree was constructed by the neighbour-joining method by using Molecular Evolutionary Genetic Analysis (Mega) X from the results of sequence blasts. In brief, the FASTA sequences from the current study and references from GenBank were imported into Mega X and then the new alignment was created from the DNA sequences data of both data from the present study and the references DNA by using ClustalW, the phylogenetic trees were then constructed by using Neighbour-Joining to evolutionary estimated the relationships between different DNA sequences.

3.4.3. Histopathology

Haematoxylin and Eosin (H&E) and Gram Staining were used for histopathology examination following the methods described in Del-Pozo *et al.*, (2010). Tissues samples including kidney, spleen, and liver were taken from both moribund fish presenting with clinical signs of disease and apparently healthy fish that were judge as those with no gross clinical signs of the disease evident. Tissues were placed into 10% neutral buffered formalin (NBF), fixed, and then processed to produce wax embedded tissue blocks. The fixation and blocking were performed in Indonesia and transported to the University of Stirling. From each sample, 5um thick sections were cut (Leica RM 2035 microtome, Leica Macrosystems Ltd. Milton Kynes, UK), 2 slides were made: the first stained with H&E and the second Gram stained to identify the presence of the bacteria, in order to make Gram-negative (decolorized) cells visible, safranin as red acidic dye was used, Gram-negative bacteria appeared red/pink, whereas Gram-positive bacteria cells remained purple. Tissue sections were examined under the light microscope from the lower magnification from 40X to 100X magnification and with the digital slide scanner (ZEISS axioScan.Z1, ZEISS Germany).

3.5. Results

3.5.1. Fish Biological Samples

A total of 264 fishes from both farmed tilapia and catfish were sampled from 41 farms, where from each farm a minimum of 5 to 7 fish samples were collected including a minimum of 1 apparently healthy fish (Chapter 2 section 2.5.3). The tilapia samples consisted of 129 moribund samples and 50 of apparently healthy samples. The average weight of the tilapia was 166.4g±48.5g with a minimum of 86g and a maximum of 256g. Most of the tilapia (65% of the total tilapia sampled) were between 100 to 200 g and only 8% of the fish samples were less than 100g (Table 3.3).

Table 3.3. The number of tilapias sampled collected including moribund and apparently healthy samples from the farms visited.

ID of farm	Geographical Area	Total number of Fish sampled/farm (n)	Average samples weight (g)	Moribund (n)	Apparently Healthy (n)
58	Timor	5	126	1	4
59	Timor	5	128	4	1
62	Timor	7	204	5	2
65	Timor	5	224	4	1
74	Timor	5	134	4	1
77	Timor	6	148	5	1
82	Timor	5	154	4	1
83	Timor	5	96	4	1
89	Timor	5	124	4	1
90	Timor	6	110	4	2
91	Timor	5	256	4	1
24	Timor	5	223	6	1
28	Timor	5	144	4	1
29	Timor	7	89	4	1
31	Timor	5	110	4	1
36	Timor	5	128	4	1
37	Timor	5	214	4	2
41	Timor	6	210	4	1
42	Timor	5	162	4	1
48	Timor	5	167	4	1
49	Timor	5	202	2	3
55	Timor	5	232	3	2
114	Timor	6	198	4	1
117	Timor	5	245	4	1
5	Flores	5	133	4	1
6	Flores	5	157	4	1
7	Flores	5	126	4	1
10	Flores	5	188	4	1
14	Flores	5	154	4	1
15	Flores	5	193	2	3
16	Flores	6	86	5	2
21	Flores	5	141	4	1
17	Flores	5	244	3	2
113	Flores	5	206	1	4
Total		179	5656	129	50
Average		4.4	138.0	3.1	1.2
Standard Deviation (SD)		0.6	48.7	1.0	0.9

The catfish samples consisted of 54 moribund and 31 apparently healthy samples with the weight ranges from the smallest with 124g into the highest with 294g from 85 catfish samples collected in total. The average weight was 214±50g with 65% of farms had the average weight of catfish sample more than 200g (Table 3.4).

Table 3.4. The number of fish sampled collected including moribund and apparently healthy samples from the visited farms.

ID of farm	Geographical Area	Total number of Fish sampled/farm (n)	Average samples weight (g)	Moribund (n)	Apparently Healthy (n)
58	Timor	5	184	4	1
59	Timor	5	124	1	4
62	Timor	5	226	2	3
74	Timor	5	214	4	1
77	Timor	5	247	4	1
82	Timor	5	204	2	3
90	Timor	5	168	1	4
91	Timor	5	276	4	1
24	Timor	5	242	4	1
29	Timor	5	187	3	1
31	Timor	5	290	3	2
37	Timor	5	168	2	3
41	Timor	5	220	4	1
114	Timor	5	148	4	1
5	Flores	5	264	4	1
6	Flores	5	294	3	2
7	Flores	5	182	4	1
Total		85	3638	54	31
Average		5.0	214.0	3.2	1.8
Standard Deviation (SD)		0.0	50.0	1.1	1.1

Clinical signs of the fish samples.

The clinical signs of the moribund fish collected were assessed by naked eye (gross) and recorded. From the total number of fish sampled in the study (tilapia and catfish samples), 70% of the fish showed at least one or more of internal/external clinical signs of disease, as judged grossly by the naked eye.

External clinical signs of tilapia samples,

Further examination of the external clinical signs observed in the tilapia only fish showed that 72% (n=129) of the tilapia sampled at the time of the study showed a minimum of one clinical sign including a sluggish movement and swimming near the water surface with the complete absence of reflex, tail/fin rots, gills pallor, and peritoneal cavity. The fish samples combined clinical signs were presented by 31% (n=55) of collected tilapia samples. Tail/fin rots only were also shown by the fish samples with the same proportion with 22%. This number was followed by abnormal swimming only including sluggish movement with 16% of the total fish samples. The combined clinical signs were the most frequently presented where 31% of fish samples had a combined external clinical sign (Figure 3.1).

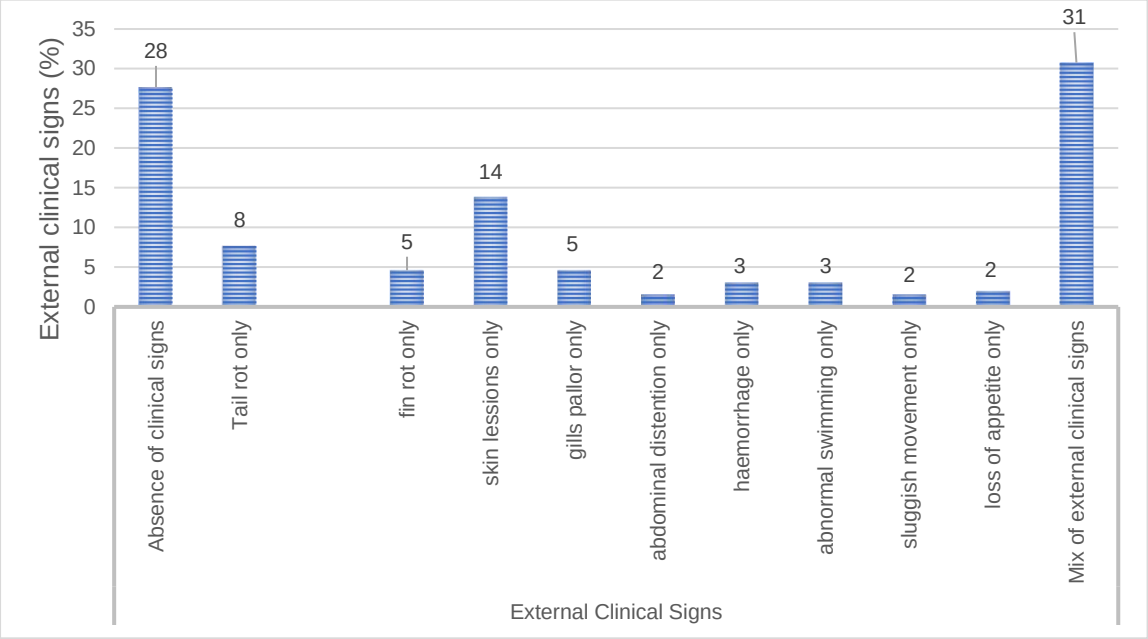

Figure 3.1. The percentages of external clinical signs of tilapia samples collected in present study.

Apparently healthy tilapia samples showed no apparent sign of abnormalities from external observation. Meanwhile, the moribund tilapia samples demonstrated a combined external clinical sign of disease as described in the Figure 3.3 to 3.5, where the apparently healthy tilapia was shown in the Figure 3.2.

Figure 3.2. Apparently healthy tilapia with no obvious external clinical signs of disease.

A moribund fish tilapia showed a minimum of one clinical sign, and these ranged from haemorrhages on the body surface, tail/fin rot, detached scales, corneal opacity, and exophthalmia. (Figure 3.3),

Figure 3.3. Typical gross presentation of moribund tilapia where red circles highlighted areas of haemorrhages.

The other clinical signs that were observed from the moribund samples from as exophthalmia, detached scales, tail/fin rots (Figure 3.4).

Figure 3.4. The tilapia sample with external clinical sign including detached scales (red circle) and exophthalmia (red arrow), and fin rots (blue circle).

Exophthalmia was also observed from the moribund fish from different farms as shown in the Figure 3.5.

Figure 3.5. The moribund tilapia samples with sign of more severe exophthalmia and corneal opacity.

Figure 3.5 showed the diversity in internal clinical signs of disease observed in the moribund farmed tilapia sampled where 58% (n=105). More of the moribund fish (12%, n=17) presented with combined clinical signs which is expected compared with lower number of fish displaying only single clinical signs of disease internally (Figure 3.5). A higher percentage of the moribund fish had extra fluid in the peritoneal cavity and 17% (n=30) of the apparently healthy fish samples with absence of clinical signs Figure 3.6).

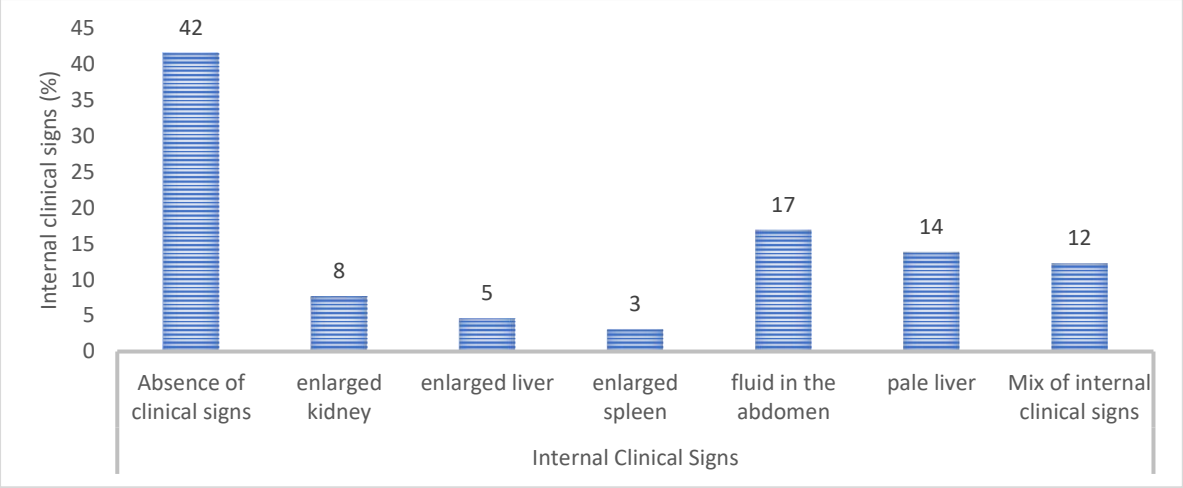

Figure 3.6. The percentages internal clinical signs of tilapia samples collected in this study.

The internal clinical signs of moribund tilapia samples were showing several clinical abnormalities included peritoneal cavity build with extra fluid, enlargement of the internal organs, and pale liver as shown in the Figure 3.7.

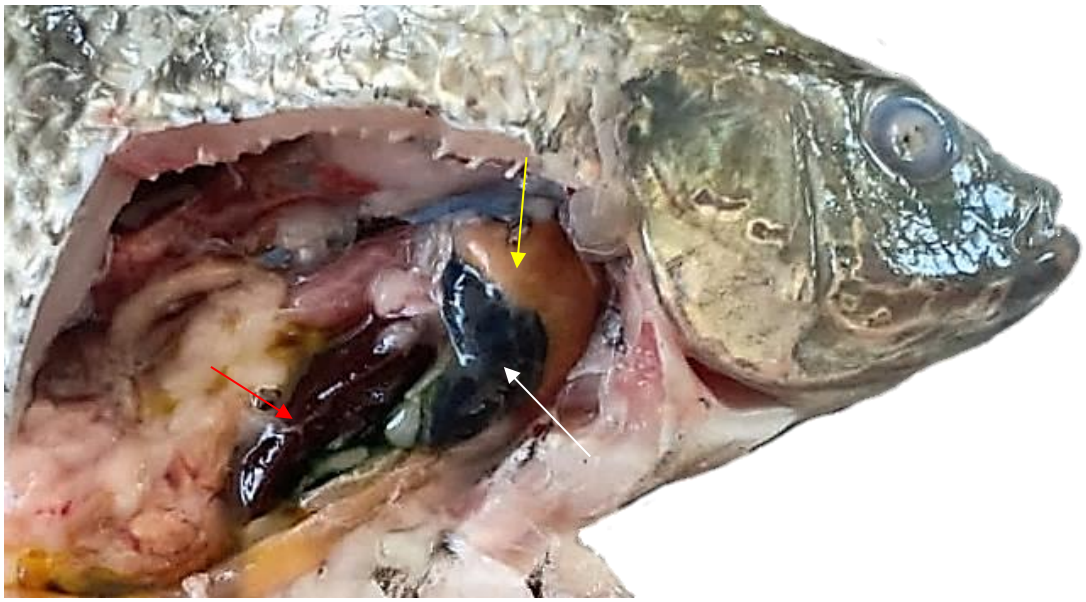

Figure 3.7. Fish samples with discoloration and white spot of liver (yellow arrow), distended of gall bladder (white arrow), spleen enlargement (red arrow).

Clinical signs of catfish samples from the visited farms.

A total of 623% (n=53) of catfish samples showed a minimum of one clinical sign included ulcerations, fin/tail rots, haemorrhages, where the fish samples with

combined clinical sign were presented by 24% (n=20) of total samples and followed by unusual swimming only with 16% (Figure 3.8).

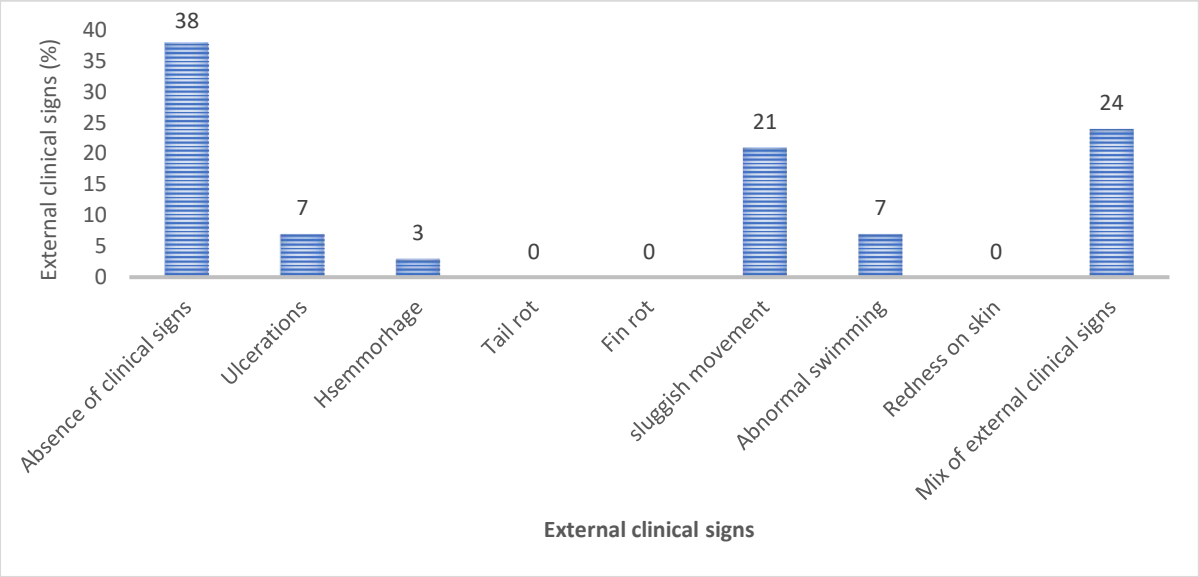

Figure 3.8. The percentages external clinical signs of catfish samples collected in this study.

The gross clinical presentation of the apparently unhealthy catfish samples was showing a minimum of 1 clinical sign of abnormalities, this included abnormal body shape, tail/fin rots, redness on the body surface, and ulcerations (Figure 3.9).

Figure 3.9. The lesions of catfish samples were shown on the body surface.

Catfish samples with no apparent clinical signs of the diseases were shown in the 57% (n=48) of the samples. From all samples collected, the catfish samples with combine clinical signs with 14% (n=12) whereas the highest internal clinical signs presented was liver enlargement with 21% (n=18) (Figure 3.10).

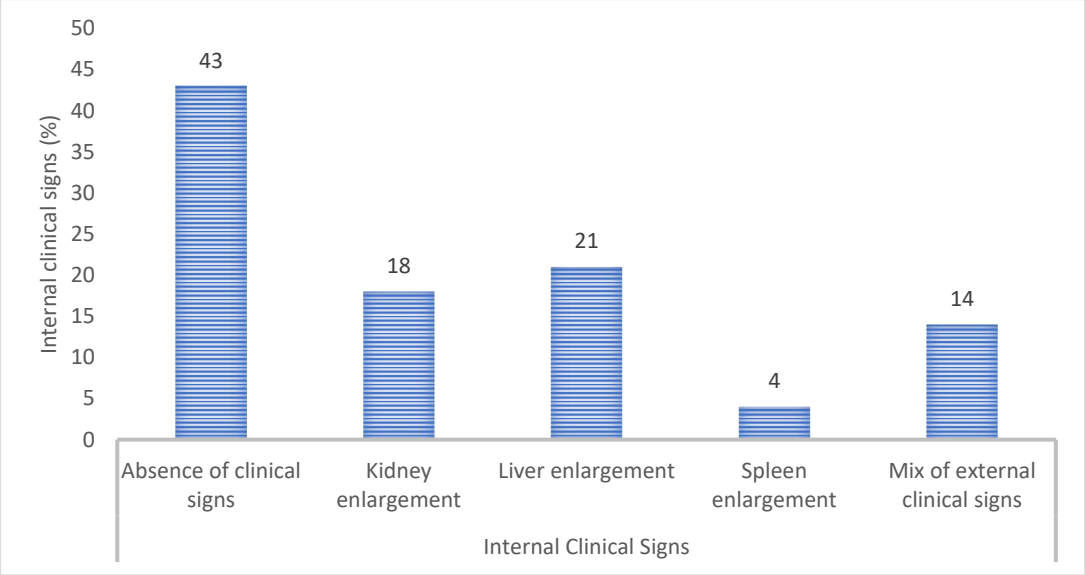

Figure 3.10. The percentages internal clinical signs of catfish samples collected in this study.

The internal clinical signs of apparently unhealthy catfish samples were showing several clinical abnormalities including the pale and friable mottled liver and enlargement of the internal organs as shown in the Figure 3.11.

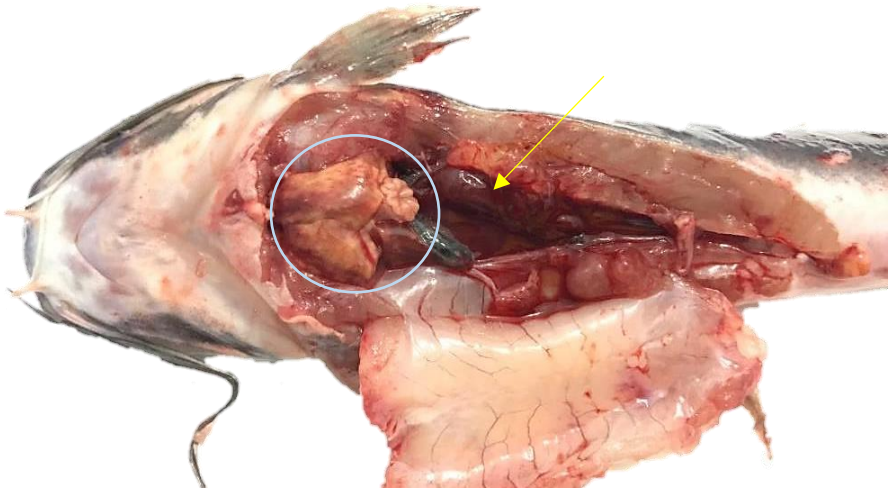

Figure 3.11. Internal organs of catfish sample with kidney congestion (yellow arrow) and friable mottled liver (blue circle).

Histopathology

The examination of histopathological from the tissue samples from tilapia with clinical signs showed several changes of the tissue including vacuolation, degenerative changes in the glomerular tissue, necrosis, infiltration of inflammatory cells, and the aggregations of Melano Macrophages Center (MMC). A range of the most common pathology observed in the fish tissues is provided below.

Kidneys from tilapia samples with clinical signs showing varying degenerative changes in glomerular epithelium, infiltration of inflammatory cells which compromised the renal tissue architecture, congestion of the glomerulus, necrosis, karyolitic and cytoplasmic eosinophilia of the tubular epithelial cells, and vacuolation of renal tissue (Figure 3.12).

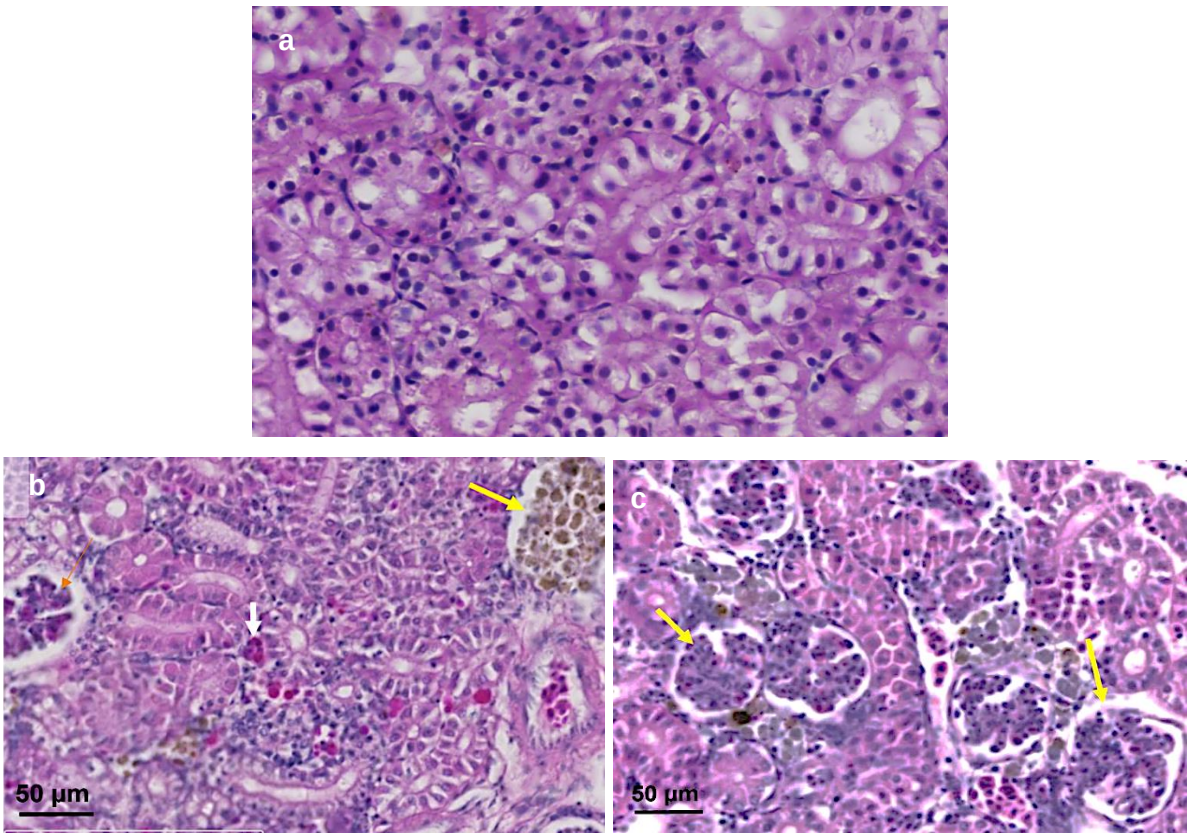

Figure 3.12. The kidney tissue of tilapia samples a. Kidney tissue from apparently healthy tilapia where no sign of abnormalities presented including normal renal tubule both proximal and distal tubule, b. Showing the degenerative changed in glomerular (yellow arrow), and mild haemorrhages (white arrow), c. abnormal tissue including distanced tubular and elements.

Liver tissue from the moribund tilapia sample showed the severe vacuolation in the hepatocytes, macrovascular steatosis (Figure 3.13).

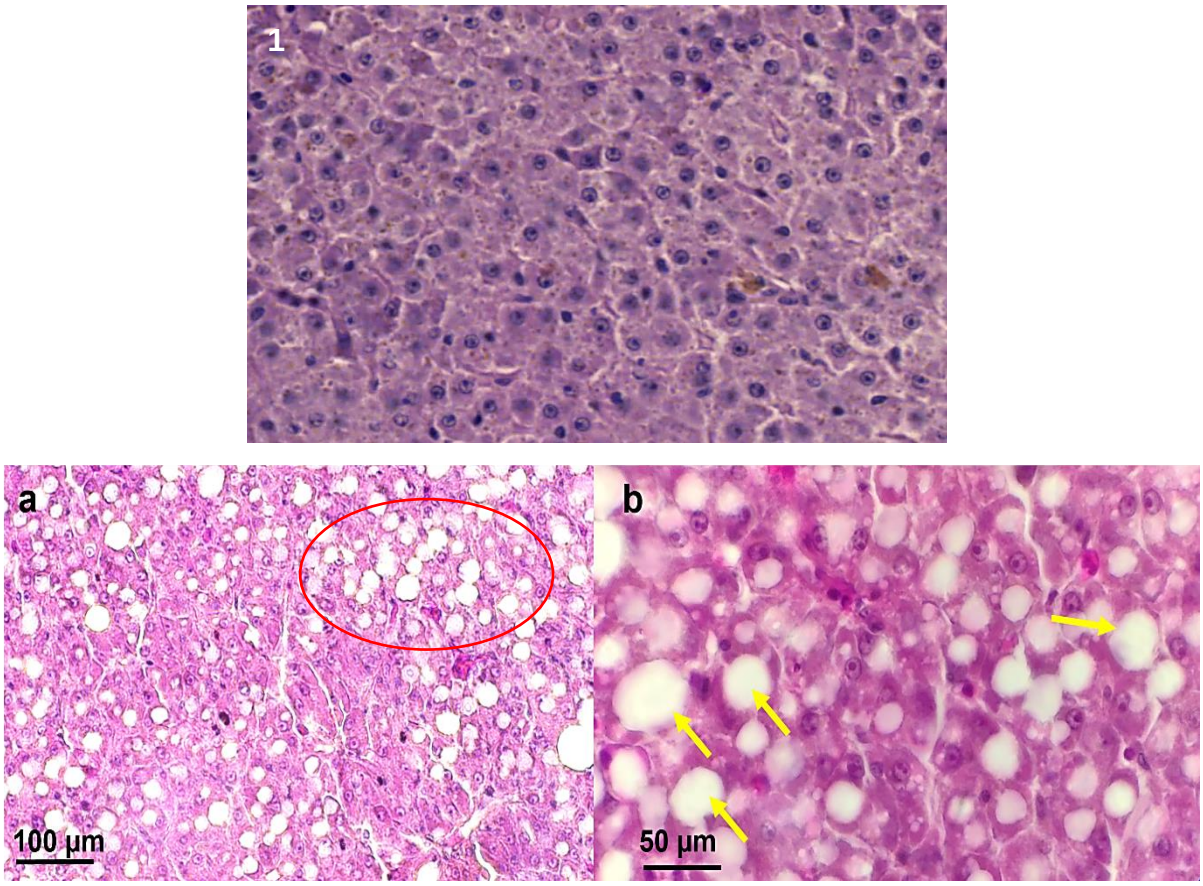

Figure 3.13. Liver tissue of apparently healthy tilapia where no sign of abnormalities observed (1), liver tissue showing the increased of vacuolation area (red circle) (a, X40) and showing severe vacuolation in hepatocytes (yellow arrows) (b, X100).

Splenic tissue of tilapia samples with clinical signs showing the increased number of Melan Macrophage Centers (MMC) presentation of phagocytes and encapsulation in the spleen organs (Figure 3.14).

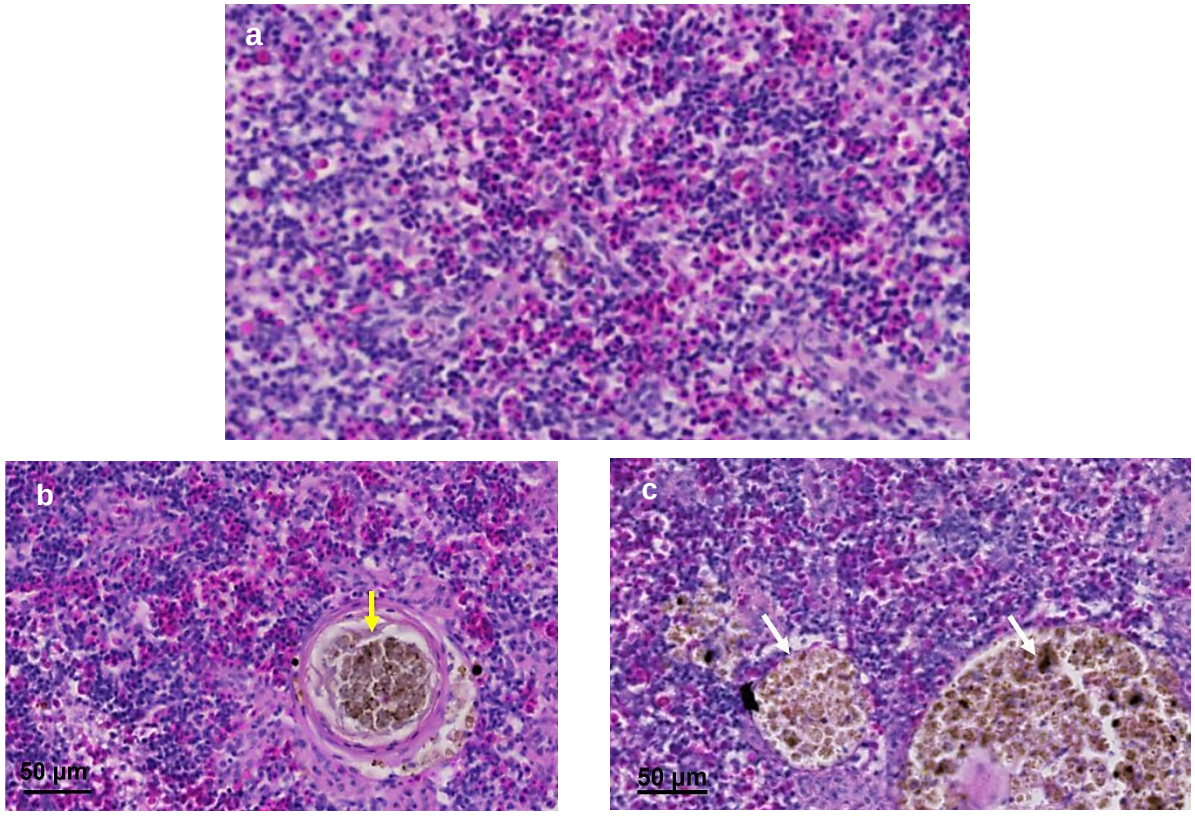

Figure 3.14. The tilapia spleen tissue. a. Spleen tissue from apparently healthy tilapia where no sign of abnormalities observed b. the encapsulation of the spleen (yellow arrow), c. increased number of MMC (hemosiderin) presentation (white arrows) (b, X100).

Gram-negative rod shapes bacteria with the size approximately 0.5 – 1µm with and 2 - 4µm length, were shown in the spleen tissue of moribund fish tilapia sample with the external clinical signs including haemorrhages, fin and tail rots, and also internal clinical sign including kidneys enlargement and fluid in the peritoneal cavity (Figure 3.15).

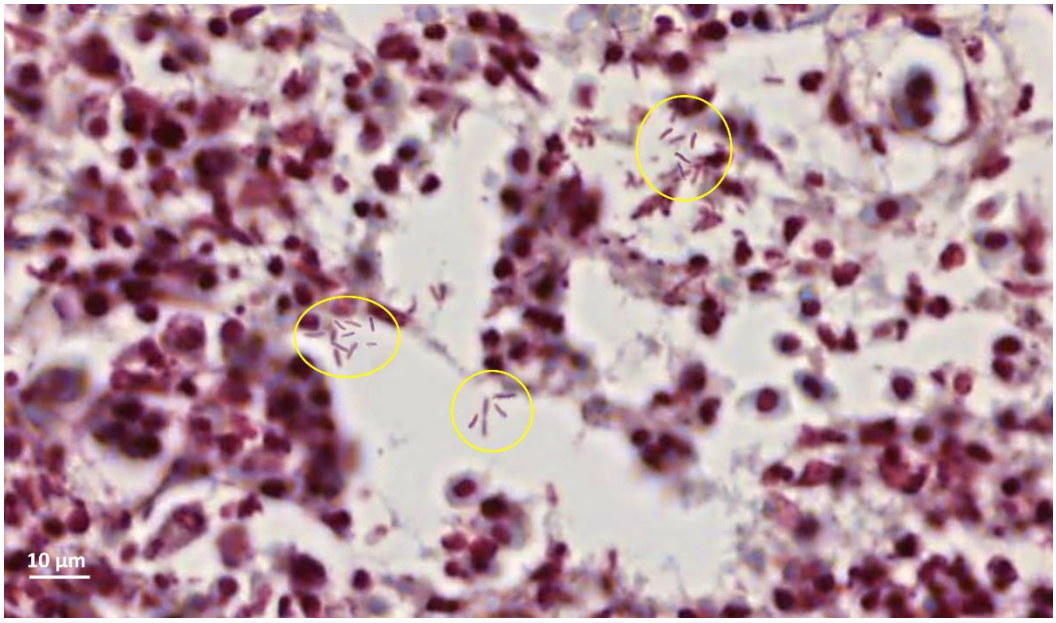

Figure 3.15. Gram-negative rod shape bacteria observed in the spleen tissue were highlighted with yellow circle from moribund tilapia sample (fish ID: 77T3S) presented with haemorrhages on the body surface, fin and tail rots, and also kidneys enlargement (10µm).

3.5.2. Bacterial Isolation and Identification.

Bacterial identification from tilapia and catfish samples.

The bacterial identification results performed in this chapter identified a range of bacterial genus with some isolates identified to species level (Fig 3.16). From the identification results performed using both primary and biochemical tests, the isolates with similar patterns were grouped together and the mostly likely match to species level applied. This identified most of the isolates as *Aeromonas* species where 57% (n=134) of them were recovered from moribund fish samples. *A. veronii* was the most frequently identified which was recovered from both apparently healthy and moribund fish samples with 15% (n=36) and 26% (n=62) respectively, whereas *A. hydrophila* as the second highest number of recovered isolates, were predominantly identified from moribund fish samples with 24% (n=56) much higher than from apparently healthy fish samples with only 3% (n=6). These numbers were followed by *A. caviae* and *Aeromonas sp* with smaller proportion. The most common identification profiles per species identified are shown in Table 3.5, where 3 isolates could not be identified to species level but were instead allocated to *Aeromonas sp*. The primary identification performed in Indonesia with additional selective medium in the test were able to

presumptively identify the *Aeromonads* species, where the primary test of all *Aeromonas* species identified in the current study were all Gram-negative, Oxidase +, Catalase +, motile, and Fermentative. Additional secondary biochemical test also performed, where all *Aeromonas* species were glucose positive and also growth on GSP medium. Meanwhile, *A. hydophila* species was the only *Aeromonas* produced gas reaction as indicated during the glucose test and the only *Aeromonas* species growth positively on specific RS medium. Meanwhile, only *Aeromonas* categorized as *Aeromonas sp*. was presented catalyst reaction on TSIA agar, *A. veronii* and *Aeromonas sp* in the present study were positive citrate and ornithine decarboxylase, both of them were also negative to arabinose fermentation, and only *A. veronii* was negative to mannitol fermentation as shown in the Table 3.5.

Table 3.5. The biochemical profiles of bacterial samples recovered from the present study from the conventional method.

Bacteria isolate			*A. veronii (n=)*	*A. hydrophila*	*A. caviae*	*Aeromonas sp.*
Primary ID Results	Gram		-	-	-	-
	Motility		+	+	+	+
	OF		F	F	F	F
	Oxidase		+	+	+	+
	Catalase		+	+	+	+
Biochemical Results	Triple Sugar Iron Agar (TSIA)	Butt	A	A	A	A
		Slant	A	A	A	C
		H2S	-	-	-	-
		Gas	-	+	-	-
	Indole		+	+	+	+
	MR		+	+	+	+
	VP		+	+	-	+
	Citrate		+	-	-	+
	Urea		-	-	-	-
	Gelatine		+	+	+	+
	Lysine decarboxylase		+	+	-	+
	Ornithine decarboxylase		+	-	-	+
	Glucose		+	+, gas +	+	+
	Sucrose		+	+	+	+
	Lactose		-	-	-	-
	Arabinose		-	+	+	-
	Maltose		+	+	+	+
	Mannitol		-	+	+	+
	Inositol		-	-	-	-
	Dulcitol		-	-	-	-
	Sorbitol		-	-	-	-
Growth on the specific medium		GSP	yellow	yellow	yellow	yellow
		RS	green	yellow	green	green

*A: Alkaline reaction, C: Catalyst reaction

The traditional biochemical identification performed in Indonesia were able to identify the predominant from 154 bacterial isolates recovered from tilapia samples in the current study. Including 46 isolates from apparently healthy and 108 from moribund tilapia samples. Aeromonads species was the most predominant bacteria identified with a total of 81% (n=124), where 64% (n=98) of Aeromonads were isolated from moribund tilapia samples (Figure 3.16). A higher number of bacteria were identified as *A. hydrophila* with 34% (n=52) from the moribund fish, but 3% (n=4) of strains identified as *A. hydrophila* were also recovered from apparently healthy fish. Likewise, higher number of *A. veronii* recovered from moribund tilapia samples, however, the *A. veronii* strains were also identified from apparently healthy tilapia samples with 12% (n=18) as shown in the Figure 3.16.

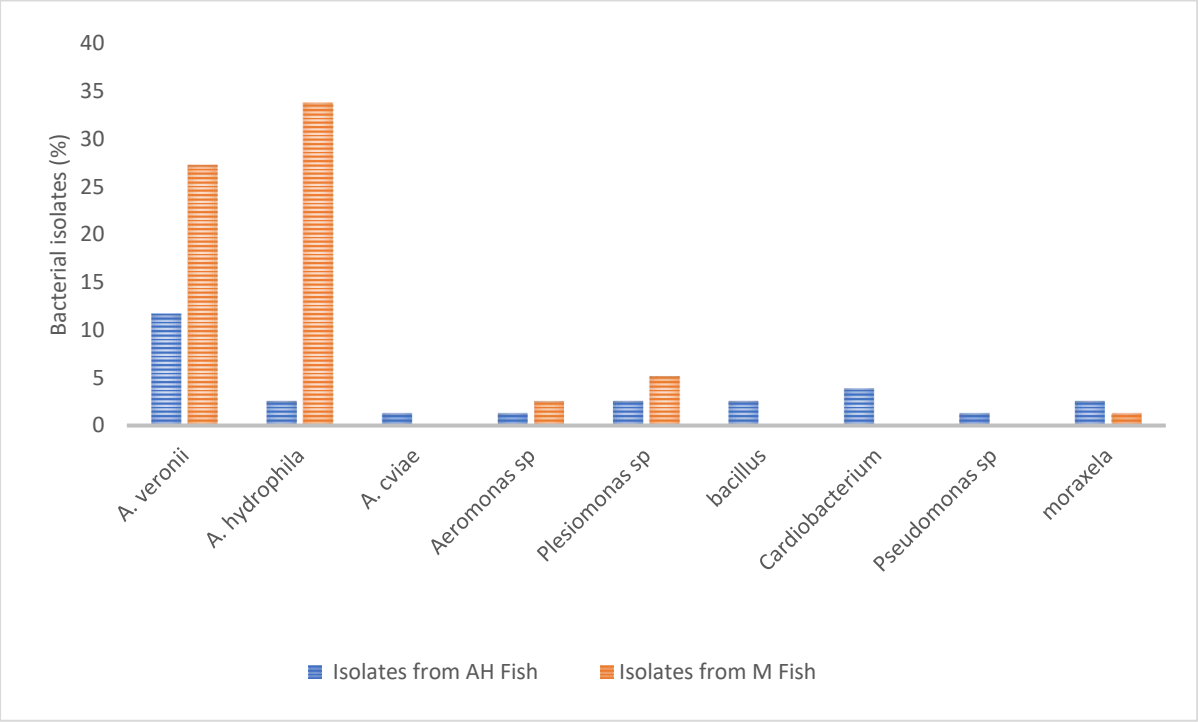

Figure 3.16. The percentages of bacterial isolates recovered from moribund and apparently healthy tilapia samples. *AH: Apparently Healthy fish sample, M: Moribund fish.

The bacterial isolates recovered from catfish samples were also identified *Aeromonas* as the vast majority bacterial isolates with 78% (n=76) from the total of 82 bacterial isolates recovered from catfish samples in the current stud. However. The Aeromonads were recovered from apparently healthy catfish with 41% (n=40), higher

than the Aeromonads isolates recovered from moribund catfish samples with 37% (n=36) as shown in the Figure. 3.17.

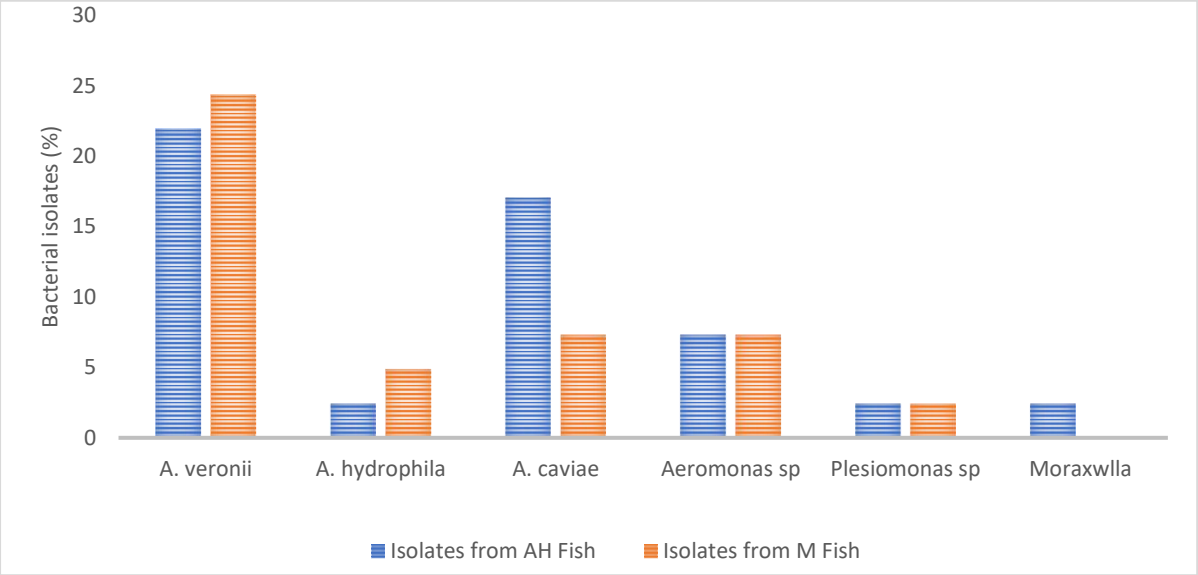

Figure 3.17. The percentages of bacterial isolates recovered from moribund and apparently unhealthy catfish samples. *AH: Apparently Healthy fish sample, M: Moribund fish.

API 20E. The commercially available kits called API 20E (bioimeriuex) were used to provide the biochemical profile of the bacteria identified in section of bacterial identification from tilapia and catfish samples (3.5.2) These kits are widely used in a large number of facilities in Indonesia and other SEA countries. Given the frequency of motile aeromonads recovered, the API 20E biochemical profiles were performed using the *Aeromonas* strains recovered in the Table 3.6. The API 20E performed in the current study were not able to identify each *Aeromonas* into the species level due to the highly varied biochemical reaction resulted by using API 20E. Therefore, the biochemical results were analysed combined with the primary test performed prior to the API 20E test. There were 7 different groups identified based on the biochemical reaction which were highlighted with different colour as shown in the Table 3.6. The first group consisted of the highest number of isolates (n=19) which also shared similar characteristic with positive control (*A. hydrophila* NCIMB 9240). These including the isolates 64HK, 92HK, 5HK, 115HK, 1HK, 4HK, 62HK, 70HK, 33HK, 69HK, 38HK, 75HK, 86HK, 36HK, 67HK, 127HK, 145HK, 68HK, and 95HK. The second largest

number of isolates (n=6) included the isolates number 97HK, 63HK, 164HK, 24HK, 73HK, and 28HK. The third largest isolates (n=5) included 162HK, 13HK, 192HK, 61HK, and 71HK, and followed by the group with 4 isolates including 130HK, 65HK, 166HK, and 105HK. The remaining three groups were consisted of two isolates of each group.

In correlation to the primary test results, the biochemical reaction of API 20E provided additional information to help the identification of bacterial isolates recovered from the current study. The API 20E biochemical reaction from the present study were able to identify the most common reaction related to the *Aeromonas* species, where the bacterial samples and positive control (*A. hydrophila* NCIMB 9240) always resulted positive reaction to the fermentation of GEL, GLU, MAN, and SAC, and always positive to ADH and TDA. whereas the API 20E results always generated negative reaction to ODC, H2S, and UREA, and also negative fermentation to INO, SOR, and RHA as finding in the present study. Those similarity of the biochemical reaction between the isolates in the present study and positive control provided valuable information in identification of *Aeromonas*, where all the tested isolates based on primary and API 20E test were categorized as *Aeromonas sp.* However, the variable's reactions were identified to the remaining biochemical reaction, this compromised the identification into the species level, which was still difficult to determine. This issue also doubled by lack of discrimination of the test, where the API 20E system gave false positive or false negative reaction to LDC, VP, GEL, and fermentation of GLU, SOR, and RHA. Although in the current study, GEL and GLU were identified as always positive and the reaction of SOR and RHA always negative but still could not provide adequate information. Consequently, the identification of *Aeromonas* into the species level using API 20E still could not be established in the current study. Therefore, further effort to use molecular test considered critical in confirming the identification into the species level.

Table 3.6. The API 20E biochemical characteristic of motile *Aeromonas* species recovered from the current study.

Biochemical Test		A. hydrophila NCIMB 9240	64HK	21HK	104HK	130H	162HK	144HK	97HK
Primary Test	Growth on TSA	+	+	+	+	+	+	+	+
	Gram Stain	Negative/Rod	Negative/ Rod	Negative/ Rod	Negative/ Rod	Negative/ Rod	Negative/ Rod	Negative/ Rod	Negative/ Rod
	Motility	+	+	+	+	+	+	+	+
	Oxidase	+	+	+	+	+	+	+	+
	Catalase	+	+	+	+	+	+	+	+
	OF	F	F	F	F	F	F	F	F
API 20E	ONPG	+	+	-	+	-	+	+	+
	ADH	+	+	+	+	+	+	+	+
	LDC	+	+	-	-	-	+	+	+
	ODC	-	-	-	-	-	-	-	-
	CITE	+	+	+	+	+	+	+	+
	H2S	-	-	-	-	-	-	-	-
	UREA	-	-	-	-	-	-	-	-
	TDA	+	+	+	+	+	+	+	+
	IND	+	-	+	+	+	+	+	+
	VP	+	+	+	+	+	+	+	+
	GEL	+	+	+	+	+	+	+	+
	GLU	+	+	+	+	+	+	+	+
	MAN	+	+	+	+	+	+	+	+
	INO	-	-	-	-	-	-	-	-
	SOR	-	-	-	-	-	-	-	-
	RHA	-	-	-	-	-	-	-	-
	SAC	+	+	+	+	+	+	+	+
	MEL	-	-	-	-	-	+	-	-
	AMY	-	+	+	+	+	+	+	+
	ARA	+	-	-	+	+	+	-	-
	OXY	+	+	+	+	+	+	+	+
API 20E Code		7267126	7227125	2267125	3267127	2267127	7267127	3267125	7267125
API20E ID Result		*Aeromonas* sp.	*Aeromonas* sp.	*Aeromonas* sp.	*Aeromonas* sp.	*Aeromonas* sp.	*Aeromonas* sp.	*Aeromonas* sp.	*Aeromonas* sp.

similar biochemical characteristic with 92HK, 5HK, 115HK, 1HK,4HK, 62HK, 70HK, 33HK, 69, 38HK, 75HK, 86HK, 36HK, 67HK, 127HK145HK, 68HK, 95HK, NCIMB9240

similar biochemical characteristic with 141HK

similar biochemical characteristic with 311HK

similar biochemical characteristic with 65HK, 166HK, 105HK

similar biochemical characteristic with 13HK, 102HK, 61HK, 71HK

similar biochemical characteristic with 93HK

similar biochemical characteristic with 63HK, 164HK, 24HK, 73HK, 28HK

Given the recovery of bacteria from the apparently healthy fish, an attempt was made to cluster the clinical signs from the tilapia and catfish with the presumptive identification of the bacteria at species-level. Aeromonads species was the primary bacteria identified with a total of 81% (n=124), where 80% (n=98) of them were isolated from moribund tilapia samples (Table 3.7). The isolates identified as belonging to the

motile *Aeromonas* species were then selected for full identification and characterisation as performed in chapter 4.

Table 3.7. The motile *Aeromonas* species selected for further identification and characterization recovered from tilapia samples.

Bact. ID	Farm Unique ID	Fish sample number (n)	M/AH	Clinical Signs		Organ's bacteria recovered	API 20E	Conventional methods
				External	Internal			
64HK	58	Timor	AH	absence of clinical signs	absence of clinical signs	Liver	*Aeromonas sp*	*A. veronii*
21HK	59	Timor	M	tail rot	flids on the abdomen	Spleen	*Aeromonas sp*	*A. veronii*
104HK	62	Timor	M	abnormal swimming	absence of clinical signs	Kidney	*Aeromonas sp*	*A. veronii*
28 HK	65	Timor	M	tail rot, ulcerative lesions	kidneys congestion, red pallor	Kidney	*Aeromonas sp*	*A. hydrophila*
93HK			M	*peritoneal cavity*	fluid in the abdominal	Kidney	*Aeromonas sp*	*A. hydrophila*
130HK	74	Timor	M	fin and tail rot	absence of clinical signs	Spleen	*Aeromonas sp*	*A. hydrophila*
65HK	77	Timor	M	tail rot, redness on skin, loss of appetite	fluid in the abdominal, kidneys congestion	Kidney	*Aeromonas sp*	*A. hydrophila*
92HK			M	Tail and fin rot, ulcerations	Fluid in the abdominal, haemorrhages of the liver	Kidney	*Aeromonas sp*	*A. hydrophila*
5HK	82	Timor	M	fin and tail rot	absence of clinical signs	Kidney	*Aeromonas sp*	*A. hydrophila*
115HK	83	Timor	M	*peritoneal cavity*	fluid in the abdominal	Kidney	*Aeromonas sp*	*A. hydrophila*
13HK	85	Timor	M	ulcerations	fluid in the abdominal, kidneys congestion	Kidney	*Aeromonas sp*	*A. hydrophila*
102HK	89	Timor	M	abnormal swimming	pale of the liver	Kidney	*Aeromonas sp*	*A. hydrophila*
141HK	90	Timor	AH	abnormal swimming	absence of clinical signs	Kidney	*Aeromonas sp*	*A. veronii*
144HK	91	Timor	M	abnormal swimming	pale of the liver, fluid in the in the abdomen	Kidney	*Aeromonas sp*	*A. veronii*
61HK	24	Timor	M	fin and tail rot	pale of the liver	Kidney	*Aeromonas sp*	*A. hydrophila*
97HK	28	Timor	M	tail and fin rot, ulcerations	fluid in the abdominal, redness of the liver	Kidney	*Aeromonas sp*	*A. hydrophila*
145HK			M	abnormal swimming	absence of clinical signs		*Aeromonas sp*	*A. veronii*
1HK	29	Timor	M	skin lesions	absence of clinical signs	Liver	*Aeromonas sp*	*A. hydrophila*
166HK	36	Timor	M	haemorrhage	absence of clinical signs	Liver	*Aeromonas sp*	*A. hydrophila*
4HK	41	Timor	M	loss of appetite	liver and spleen enlargement	Kidney	*Aeromonas sp*	*A. veronii*
63HK	42	Timor	M	loss of appetite	kidney enlargement	Spleen	*Aeromonas sp*	*A. veronii*
62HK	48	Timor	M	tail rot	enlargement of liver and spleen	Spleen	*Aeromonas sp*	*A. veronii*
162HK	5	Flores	M	fin rot	pale of the liver	Kidney	*Aeromonas sp*	*A. veronii*
95HK	7	Flores	M	fin and tail rot	kidneys congestion, red pallor	Spleen	*Aeromonas sp*	*A. veronii*
71HK	10	Flores	M	tail rot, loss of appetite	pale liver	Spleen	*Aeromonas sp*	*A. veronii*
164HK	14	Flores	M	tail rot, loss of appetite	pale liver	Kidney	*Aeromonas sp*	*A. veronii*
24HK	16	Flores	M	abnormal swimming, loss of appetite	kidney enlargement	Kidney	*Aeromonas sp*	*A. veronii*
105HK	21	Flores	M	abnormal swimming	absence of clinical signs	Kidney	*Aeromonas sp*	*A. veronii*

*M: moribund, AH: apparently healthy

The *Aeromonas* species from moribund catfish samples representing a minimum of one external clinical sign, The Aeromonads were recovered from apparently healthy catfish with 41% (n=40), which was higher than the Aeromonads isolates recovered from moribund catfish samples with 37% (n=36) of the total bacteria recovered (Table 3.8).

Table 3.8. The motile *Aeromonas* species selected for further identification and characterization recovered from catfish samples.

IOA Bact. ID	Farm Unique ID	Fish sample number (n)	M/AH	Clinical Signs		Organ bacteria recov-ered	API 20E	Conventional methods
				External	**Internal**			
33HK	58	3	M	ulcerations	kidneys enlargement	Kidney	*Aeromonas sp.*	*A. veronii*
36HK	91	3	M	sluggish movement	enlargement of liver and spleen	Kidney	*Aeromonas sp.*	*A. hydrophila*
69HK	74	7	M	abnormal swimming	absence of clinical signs	Kidney	*Aeromonas sp.*	*A. veronii*
38HK	59	5	M	sluggish movement	kidneys enlargement	Spleen	*Aeromonas sp.*	*A. veronii*
73HK	62	2	M	abnormal swimming	absence of clinical signs	Spleen	*Aeromonas sp.*	*A. veronii*
75HK	77	5	M	lesions	yellowish liver	Spleen	*Aeromonas sp.*	*A. veronii*
86HK	90	1	H	absence of clinical signs	absence of clinical signs	Spleen	*Aeromonas sp.*	*A. veronii*
67HK	24	1	M	sluggish movement	absence of clinical signs	Spleen	*Aeromonas sp.*	*Aeromonas sp.*
127HK	41	6	H	sluggish movement	liver congestion	Kidney	*Aeromonas sp.*	*A. caviae*
145HK	5	3	M	sluggish movement	absence of clinical signs	Liver	*Aeromonas sp.*	*A. veronii*
31HK	29	4	H	abnormal swimming	kidneys enlargement	Kidney	*Aeromonas sp.*	*Aeromonas sp.*
68HK	7	1	M	lesions	absence of clinical signs	Kidney	*Aeromonas sp.*	*Aeromonas sp.*

*M: moribund, AH: apparently healthy

Molecular identification methods

Molecular identification performed in the current study including 16S rDNA sequencing and two housekeeping genes (*gyrB and rpoD*) were able to confirm the identify into the species level of the tested *motile Aeromonas* recovered from the current study.

16s rDNA genes sequencing.

A series of molecular assays were performed to identify the strains to species level. The first assay was the 16S rDNA PCR and the gel electrophoresis of the motile *Aeromonas* strains showed the target size of a positive band at 1501bp was achieved for all 40 strains using the Eubacterial primer set. The 16S rDNA sequence results were cleaned and compared against other sequence data held within the public domain of National Center for Biotechnology Information (NCBI) data. All of the isolates sent away for 16S rDNA sequence had a query length of more than 1400bp and with the query coverage of equal or greater than 98%, this was performed using comparison with sequences held in the GenBank database showed that the

Indonesian strains were identified as either *A. veronii, A. hydrophila,* or *A. caviae*. The phylogenetic information was obtained by blasting the DNA sequences with the closely related species and producing the phylogenetic tree with 1 species as an outlier. The genetic relatedness of the motile *Aeromonas* isolates identified from the 16S rDNA analyses was illustrated in figure 3.18.

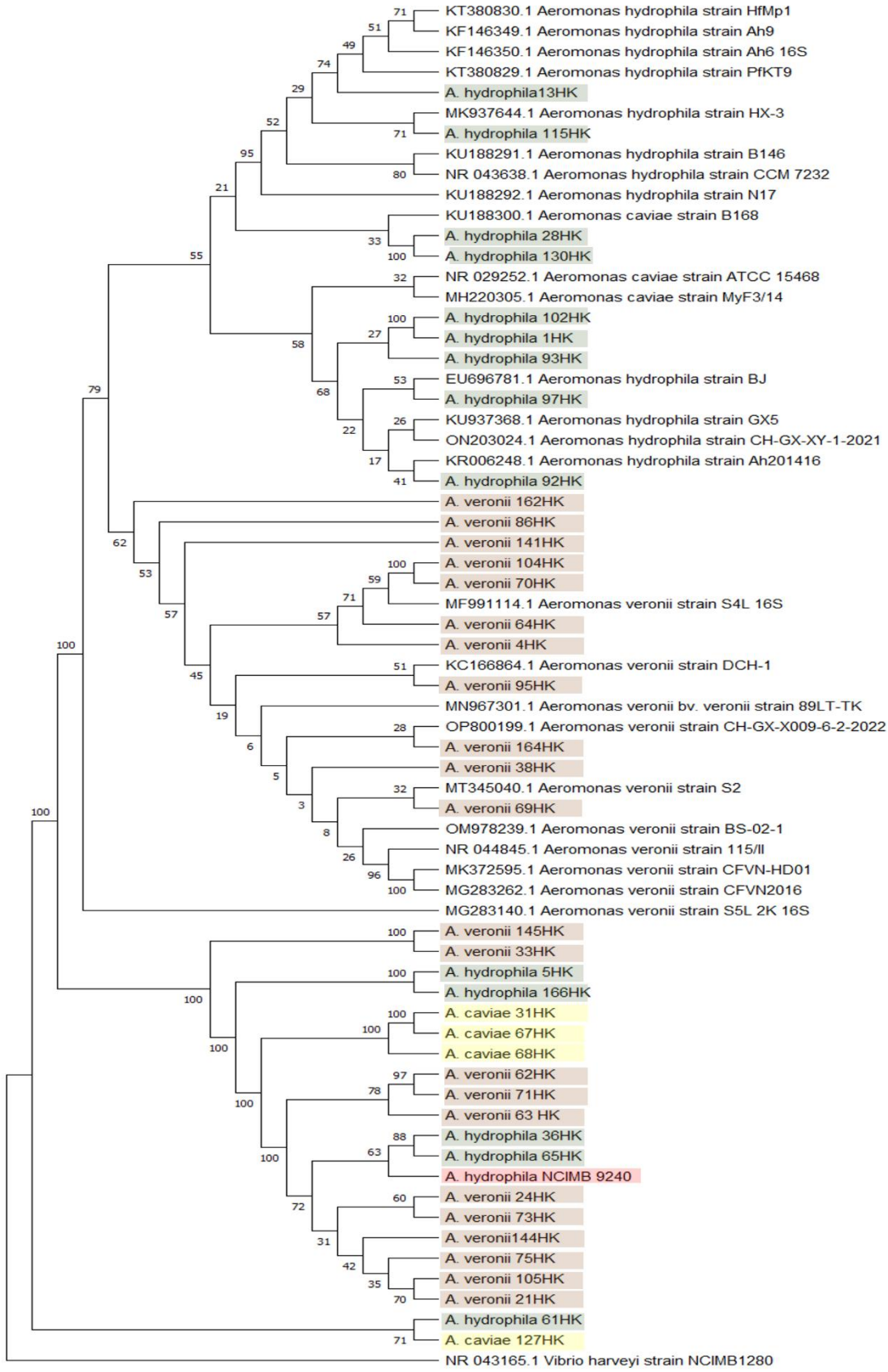

Figure 3.18. Neighbours Joining of phylogenetic tree was constructed based on the 16s rDNA gene of motile *Aeromonas* in the present study and the closely

related species. *vibrio harveyi* NCIMB1280 was used as an outlier in constructing the phylogenetic tree. Percentages of the bootstrap value (1000 replicates) were shown at each branch. The strains from the current study and the positive strain were highlighted with different colours.

The phylogenetic tree constructed from the strains recovered from the moribund fish in the area of study illustrated that strains had evolutionary relationships with the *Aeromonas* strains from NCBI. *A. hydrophila* species were illustrated as 3 main cluster groups (Figure 3.18), where the majority of *A. hydrophila* strains (9 strains) including *A. hydrophila* 13HK, 115HK, 28HK, 130HK, 102HK, 1HK, 93HK, 97HK, and 92HK were among the first group. The remaining *Aeromonas* strains recovered from the current study were grouped in 1 cluster group with *A. hydrophila* NCIMB 9240 All of the 9 strains from first cluster group were recovered from moribund tilapia samples. The 9 strains were group at the same cluster with the strains acquired from the GenBank database whit the query coverage of greater than 98%, where 24 sequences of *A. hydrophila* and *A. veronii* with the highest query coverage and percent identity (99-100%) were pulled together to construct the phylogenetic tree (Figure 3.18). This including the strains recovered from neighbouring country such as Malaysia and Vietnam. In details, *A. hydrophila* 13HK which was recovered from tilapia samples with the clinical signs including ulcerations and kidney enlargement shared a common ancestor with strains recovered from diseases of tilapia (KF146350, figure 3.18) which was recovered from a ulcer skin of red hybrid tilapia (*O. niloticus)* from river cage culture in Malaysia. The strains were also demonstrated as a close relation to the characterization of pathogenic *A. hydrophila* in Vietnam (KU188292). *A. hydrophila* NCIMB 9240 (positive control) shared the closest relationships with the strains 36HK and 65HK.

The other group where *A. veronii* strains from the phylogenetic tree was divided into two groups comprised with similar number of strains in each group, 11 *A. veronii* strains each group. The first shared the common similarity to the *A. veronii* strains recovered from both catfish and tilapia, including the strains recovered from the diseased catfish in Indonesia and from tilapia in Malaysia with assession number MG283140. More specifically, *A. veronii* 38HK and 141HK along with 9 A. veroni recovered from the current study shared more common ancestor to the *A. veronii*

strains BS-02-1 with assession number OM978239 which was recovered from the disease catfish in Indonesia.

Analysis of *gyrB* and *rpoD* sequence alignments of motile *Aeromonas* strains.

***gyrB* housekeeping gene.** The phylogenetic tree constructed by performing the *gyrB* gene sequences from the current study and from NCBI references where *V. harveyi* strain ATCC 33842 as an outlier was showing that the motile *Aeromonas* strains from the present study (*A. hydrophila* 115HK, 92HK, 166HK, 5HK, 130HK, 36HK, 13HK, 61HK, and 28HK) were illustrated evolutionary close related to the *Aeromonas* strains from the references. In detail, 9 of *A. hydrophila* strains from this study were illustrated located in a group with the bootstep value 66 with four strains of *A. hydrophila* from NCBI references including *A. hydrophila* strains CECT5744 with assession number JN711805.1, *A. hydrophila* strains BMP1 with assion number MT935699.1, *A. hydrophila* strains 295 (assession number DQ519366.1, and A*. hydrophila* strains D14 (assession number MT967985.1. The remaining *A. hydrophila* strains from this study were also located at the same group with the strains mentioned above with the bootsteps value 73 including *A. hydrophila* 102 HK, 97HK, 1HK, and 93HK (Figure 3.19).

A. veronii strains recovered from the present study were illustrated divided into 2 cluster groups. The first group comprised of 11 *A. veronii* strains form this study (*A. veronii* 162HK, 105HK, 38HK, 141HK, 104HK, 21HK, 145HK, 33HK, 164HK, 69HK, and 86HK) were among the group consisted of the strains from GenBank including A. *veronii* strains CECT 44864 (assession number EF465527.1), *A. veronii* strains DK-*A.veroni*-42 (assession number KJ747142.1), *A. veronii* strains HNZZ-3 (assession number KR537458.1), and *A. veronii* strains GYC2 (assession number KU543617.1). the remaining strains were group in 1 cluster group including (*A. veronii* 75HK, 73HK, 63HK, 24hk, 64HK, 70HK, 71HK, 95HK, 62HK, 144HK, and 4HK). An outlier *vibrio harveyi* strain ATCC 33842 (assession number: EU672845.1) were located outside of the cluster group (Figure 3.19).

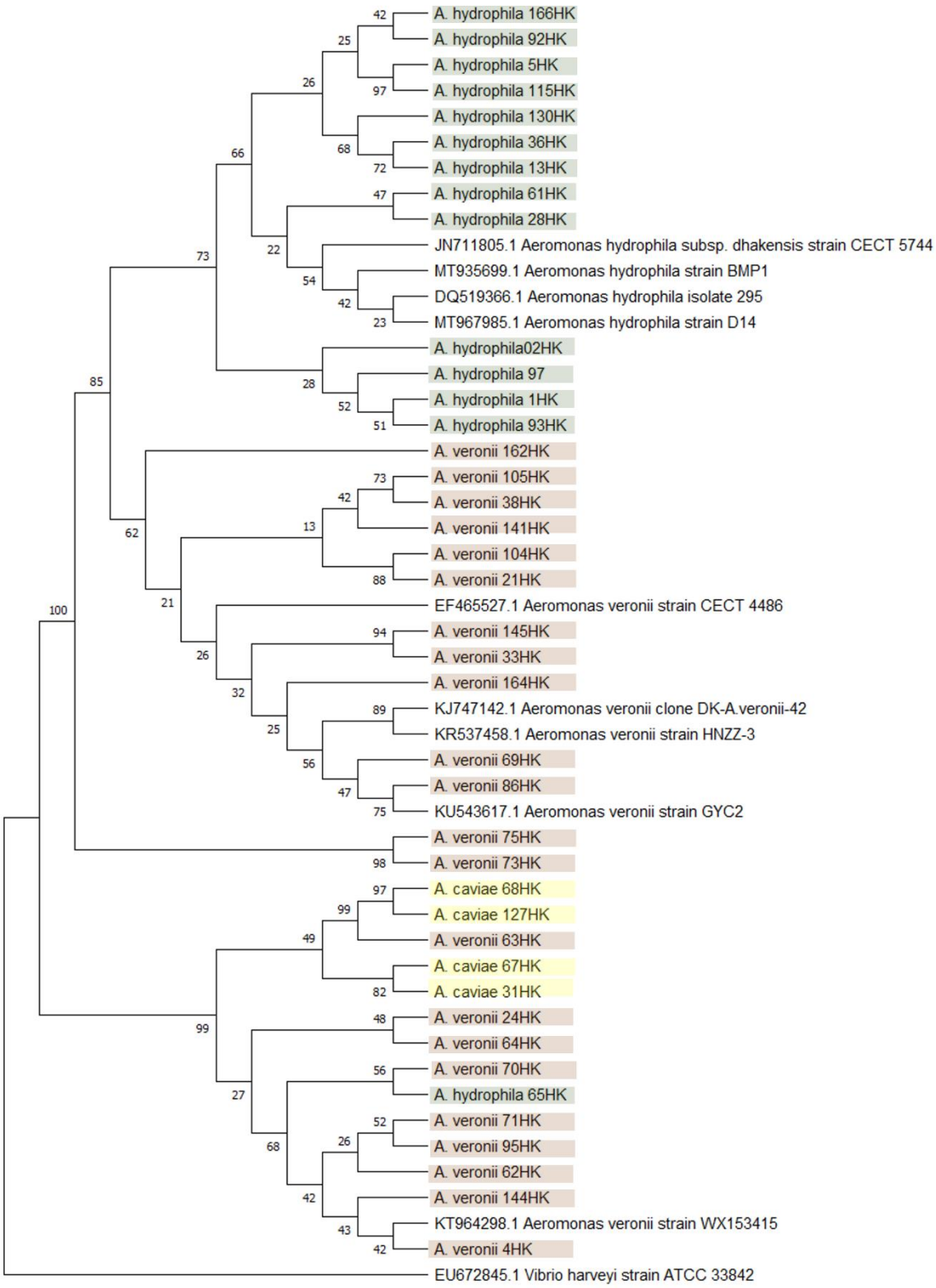

Figure 3.19. Neighbours Joining of phylogenetic tree was constructed based on the *gyrB* gene sequences of the motile *Aeromonas* randomly selected in the present study and the closely related species from GeneBank database. Percentages of the bootstrap value (1000 replicates) were shown at

each branch. The strains from the current study were highlighted with different colours.

***rpoD* housekeeping gene.** The phylogenetic tree constructed by *rpoD* gene sequences was showing that the motile *Aeromonas* strains recovered in the current study were closely related to *A. hydrophila* and *A. veronii* from GenBank database. *A veronii* strains from the current study were divided into two groups. In detail, 11 strains of *A. hydrophila* recovered from the current study (*A. hydrophil* 28HK, 92HK, 102HK, 61HK, 130HK, 13HK, 115HK, 5HK, 1HK, and, 65HK) were located at the same group with *A.hydrophila* from references strains. The group was consisted of the reference strains including *A. hydrophila* strain ZSWL-31 with assession number KU230411.1, *A, hydrophila* strain AE-57 with assession number AY987671.1, and *A, hydrophila* strain A14 with assession number KC601678.1. the remaining group consisted of *A. hydrophila* 93HK, 36HK, and 166HK.

The first group consisted of 8 strains (A. veronii 105HK, 141HK, 21HK, 38HK, 64HK, 145HK, 62HK, and 63HK) were illustrated close related to four reference strains including *A. veronii bv. Sobria* UGH129 (assession number: LC547061.1), *A. veronii strain Ae53* (assession number: AB828779.1), *A. veronii strain TCMB1-2018* (assession number: MK396842.1), and *A. veronii bv. Veronii* strain ATCC 35624 with assession number KC601667.1. The remaining strains recovered in the present study were grouped in the cluster with bootstrap value 100. *V. vulnificus* strain JY1305 with assession number EF647821.1 was used as an outlier (Figure 3.20).

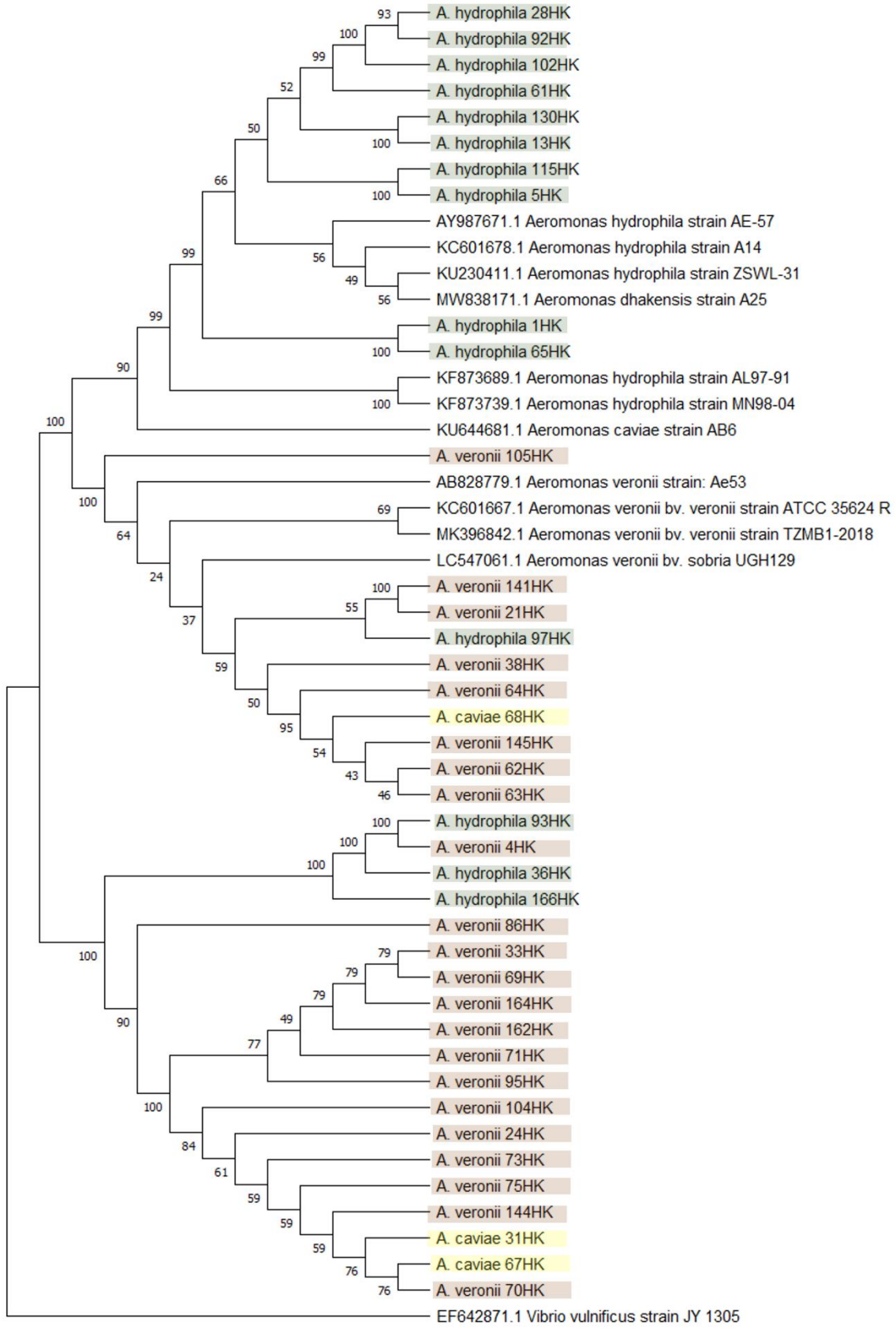

Figure 3.20. Neighbours Joining of phylogenetic tree was constructed based on the *rpoD* gene sequences of motile *Aeromonas* selected in the present study and the closely related species from NCBI references. Percentages of

the bootstrap value (1000 replicates) were shown at each branch. The strains from the current study were highlighted with different colours.

The Comparative Identification of phenotypic profiles and molecular method. The comparison of conventional biochemical test tube methods, commercial miniaturised API 20E, and the 3 molecular identification methods performed in this study demonstrated a very high level of agreement with 37 out of 40 isolates confirmed to species level (Table 3.9). Only 3 strains were not identified to species level using the conventional test tube methods and these were identified to species level as *A. caviae* with 100% agreement between the 3 molecular tests performed (isolate 67HK, 31HK, and 68HK, Table 3.9). The least sensitive method was the biochemical profile using the API 20E kit, which was unable to confirm the identification to species level but did provide a complex of *hydrophila/caviae/veronii* (Table 3.9).

Table 3.9. Comparative identification results of phenotypic profiles and molecular method.

IoA Bact. ID	Conventional methods	API 20E	16S rDNA	*rpoD*	gyrB
64HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
21HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
104HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
28 HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
93HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
130HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
65HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
92HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
5HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
115HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
13HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
102HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
141HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
144HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
61HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
97HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
145HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
1HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
166HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
4HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
63HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
62HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
162HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
95HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
71HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
164HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
24HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
105HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
33HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
36HK	*A. hydrophila*	*Aeromonas sp.*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
69HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
38HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
73HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
75HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
86HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
67HK	*Aeromonas sp.*	*Aeromonas sp.*	*A. caviae*	*A. caviae*	*A. caviae*
127HK	*A. caviae*	*Aeromonas sp.*	*A. caviae*	*A. caviae*	*A. caviae*
145HK	*A. veronii*	*Aeromonas sp.*	*A. veronii*	*A. veronii*	*A. veronii*
31HK	*Aeromonas sp.*	*Aeromonas sp.*	*A. caviae*	*A. caviae*	*A. caviae*
68HK	*Aeromonas sp.*	*Aeromonas sp.*	*A. caviae*	*A. caviae*	*A. caviae*

3.6. Discussion.

In this study an attempt was made to identify the bacteria recovered from moribund catfish and tilapia and to compared conventional v's molecular methods to confirm the identification to species-level. The most common bacteria recovered from the moribund fish belonged to the motile *Aeromonas* absence of clinical signs group and were all associated with a wide range of clinical presentations in the fish including ulceration, swelling, skin and fin erosion, and haemorrhagic septicaemia. As none of these clinical signs are considered to be pathonogmic for the bacterial species, investigation of the gross clinical signs in the farmed tilapia sampled in this study, ranged from loss of appetite, a sluggish movement, and swimming near the water surface, with the complete absence a reflexive response. Likewise, from the catfish samples fin/tail rot, haemorrhages. Internally the moribund fish had clinical signs of pale liver, spleen congestion, and kidney haemorrhages. Similar clinical descriptions in both fish species have been provided previously (Hassan *et al.*, 2017; Legario *et al.*, 2023). The finding in this study were in agreement to the previous report in Indonesia from the tilapia and catfish farms (Hardi *et al.*, 2018; Rahayu, 2019). Global reports showed similar manifestation associated to MAS symptoms as shown in the current study including abnormal swimming, loss of appetite, pale gills ulcerations, and haemorrhages caused by *Aeromonas* species from fish, including tilapia (M. Randy White, 1991; Chen *et al.*, 2019; Korni and Ahmed, 2020; Adah *et al.*, 2021). Meanwhile, apparently healthy fish samples collected in the present study of both tilapia and catfish were showing no clinical sign of the diseases. The apparently healthy tilapia and catfish with normal behaviour and active feeding, and also no signs of morphological abnormality with no excessive mucus produced, clear and no detached scales for tilapia, clear eye colour, clear red gills (Lathifah, 2015; Aich *et al.*, 2022). Normal signs of fish were observed in the apparently healthy fish samples collected in the current study during the field study.

Assessing the factors associated to the abnormalities presented in the tissue samples is difficult to determine and probably due to the numerous variables affecting the changes of the tissue. In terms of fish internal organs, the liver, kidney, and spleen are known associated to the diseases because their ability to trap the circulating pathogens. Therefore, those organs are often targeted in determining the diseases (Agius and Roberts, 2003; Humprey, 2007). The histological of kidney section showed several manifestations including tubular generation, glomerular dilation of the

Bowman's capsule and necrosis. These abnormalities were also identified in the previous reports (Shrimp *et al.*, 2016; El Latif *et al.*, 2019; Abdel-Latif and Khafaga, 2020). The tissue kidney degeneration including the distal kidney luminal epithelium might associate to the toxins observed as hyaline droplets, where the toxins and associated might be produced by bacteria. As previous report where the same abnormalities associated with the bacterial infection (Abdel-Latif and Khafaga, 2020; Korni and Ahmed, 2020). The inflammatory cells of kidney tissue might be associated with the presence of pathogens including bacteria and or virus (Ferguson, 2016). As evidence in the present study where the presence of gram-negative rod shapes bacteria in the internal organs of the fish samples, with the range size of approximately 0.5 - 1 µm with and 2 – 4 µm length, where the range size of Aeromonas spp. Are between 0.4 - 1 µm with and 1 – 4 µm length (Cutter *et al.*, 2012). Kidneys from fish samples with clinical signs showing varying degenerative changes in glomerular epithelium, infiltration of inflammatory cells which compromised the renal tissue architecture, congestion of the glomerulus, and necrosis. Necrosis of the kidney was reported associated with the release of toxins and extracellular products such as haemolysin, protease, elastase by *A. hydrophila* (Donta and Hadow, 1978, Asao et al, 1984, Lallier et al, 1984; AlYahya *et al.*, 2018; Afifi et al, 2000).

Splenic tissue of fish samples with clinical signs showing the high number of the Melano macrophage canters presentation (MMC) of phagocytes. MMC is a collection of macrophages that contain hemosiderin, lipofuscin, ceroids, and melanin pigment. Agrarians of MMC mostly caused by inflammation (Steinel and Bolnick, 2017; Aliza *et al.*, 2021). These changes might be associated to the stress as a result of dramatic changes in the environment conditions such as temperature, where during the time survey there was seasonal changes from dry to rainy session. The environment variations enhanced the pathogenicity of various pathogens including *Aeromonas* species, as evidence some of rod shapes bacteria were found in the spleen. The presentation of MMC may indicated of the environmental stress such us variations of water temperature (Balamurugan et al. 2012), bioindicator of pollution (authiman *et al*., 2012), toxic agents (pulsford et al, 1992), and may also related to the development of immune response to the bacterial antigens that might as a response to the environment conditions and also pathogens (Agius and Roberrt, 2003). Overall, the cellular pathology identified and described for the moribund fish examined in this study were in agreement with other studies and the presence of Gram-negative rod

shape bacteria were observed in the spleen tissue of the moribund tilapia. There is a need to identify the presence of the bacteria with the presence of the pathology and in this study the bacteria observed in the organs were observed with pathology identified as hydropic degeneration. This finding was in agreement with a previous study in catfish challenged with the bacterium *A. hydrophila* (Laith and Najiah, 2014). To clarify the role of the bacteria, further studies are necessary to confirm the pathogenicity of the bacteria recovered from the fish and this work is described in chapter 4.

The abnormalities were also identified from the liver tissue. the vacuolation as shown in the current study might be associated to the several factors and also sometime observed in the tissue liver of the apparently healthy tilapia. However, the vacuolation may cause hepatomegaly and causing the disturbing of the liver function. Therefore, this manifestation needs to be assessed carefully because it may also relate to the nutrition response contributed to the intracellular accumulation (Ferguson, 2006). This was also associated with the feeding intake where most of the farming practiced unproper feeding intake that might lead to the excessive feeding practices applied with the commercial feed that caused the fat accumulation. Degeneration of the liver, and also vacuolation of hepatocytes as identified in the current study indicated that the liver functions have been compromised. The damage of the liver with different level of infection including hepatocyte degeneration and necrosis were also previously reported previously (Bilen and Bilen, 2013; Abdel-Latif and Khafaga, 2020; Korni and Ahmed, 2020; Abdel-Latif and Khafaga, 2020).

Most bacterial isolates identified from this study from both tilapia and catfish samples were Gram-negative rod-shaped bacteria. Internal organs targeted for bacterial isolation in the current study were the kidney, spleen, and liver. The liver and kidney are two important organs in the fish metabolism systems (Cao et al., 2016). Liver and kidney are the most predominant affected organs during acute septicaemia (Laith and Najiah, 2014). Furthermore, liver and kidney have a high potential as the organs where bacteriological alteration frequently occurs (Kron, 2012). In the current study, bacterial isolates including *Aeromonas* species were primarily isolated from the fish internal organs presented the internal clinical sign of the diseases. This finding indicated that the *Aeromonas* species playing important roles in causing the diseases which led to the disease outbreaks in the area of study, where the finding was supported by the presentation of rod shapes Gram-negative bacteria within the affected tissue organs of moribund fish. During the infection of pathogenic diseases,

kidney, spleen, and liver were among the most severely organs affected when bacterial infection (Plumb & Hanson, 2011; Beaz and Jos, 2012).(Beaz and Jos, 2012).

In the present study, *Aeromonas* species were confirmed as the common species recovered and identified in both tilapia and catfish samples, which was similar to previously published reports for tilapia in Indonesia (Amanu *et al.,* 2014; Rahayu, 2019; Azhari *et al.,* 2014; Manurung and Susantie, 2017; Angraeni et al., 2018), and also an agreement with the previous global reported (Cai *et al.*, 2012; De Jagoda *et al.*, 2014; Korni and Ahmed, 2020). The ubiquity nature of *Aeromonas* species allow the inevitable contact between *Aeromonas* species and animal including fish in the natural habitat in the freshwater farming ecosystem (Hu *et al.*, 2012). The motile *Aeromonas* absence of clinical signs group are complex and often associated with MAS infections in a wide range of farmed freshwater fish species. It is the combination of their presence, sub-optimal environmental or increased stress within the fish that are considered exacerbators of the growth of these bacteria enabling establishment and infection (Beaz-Hidalgo *et al.*, 2010; Tavares-Dias and Martins, 2017). Hence, they are often described as secondary pathogens or opportunistic pathogens and there is a need to clarify their pathogenic capacity within the different strains recovered from the environment, host species microbiome from those that are truly pathogenic in the tilapia and catfish.

The most common *Aeromonas* species identified in the present study, were *A. veronii, A. hydrophila* and *A. caviae* which were confirmed using a combination of laboratory-based methods. These 3 bacterial species are commonly described as the global cause of MAS infections in freshwater farming systems (Vega-Sánchez *et al.*, 2014; Mzula *et al.*, 2019). These bacteria species were reported as responsible for causing haemorrhages, erosions, and other pathological abnormalities in tambaqui fish (*Collosoma macropomum*) from Brazil (Ariede *et al.*, 2018). Furthermore, the wide range of clinical presentation observed in this study has previously been reported in fish including abnormal swimming and loss of appetite (Hamid *et al.*, 2016), abdominal swelling, increased of ascetic fluid, and other clinical symptoms (Zhou *et al.*, 2013; Hassan *et al.*, 2017). *Aeromonas* species have been associated with MAS in farmed fish with the clinical sign of the disease including external and internal haemorrhages, fin/tail rot, ulcerative disease(Latif-Eugenín et al., 2016). These reports were also displayed by the clinical signs of the fish samples in this study.

Conventional bacterial identification methods are widely applied, particularly in SEA country laboratories and can in some cases, be valuable. This is more often related to the lack of species-specific treatment or preventative measures. However, conventional identification methods are unable to differentiate closely related bacterial species which can potentially affecting the identification results, since there is a level of variation within the species which means that the level of variation in the biochemical reactions cannot predict the *Aeromonas* species due to the heterogeneity. Therefore, ambiguous information regarding the strains in association to the disease outbreaks can occur resulting in misidentification (Austin, 2019a). The primary identification tests e.g, Gram, motility, oxidase, catalase, etc as also performed in the current study are valuable to confirm the bacterial species to genus level but biochemical profiling can add advantages and should, in most cases, provide a species-level identification. In this study the biochemical profiles obtained from the API 20E test was not sensitive or specific enough to identify the *Aeromonas* species, and this lack of discrimination from the biochemical profiles of motile aeromonads is well recognised (El Latif *et al.*, 2019; Abdel-Latif and Khafaga, 2020).

API 20E in the current study was not able to identify a single *Aeromonas* species, the method only able to distinguish the *Aeromonas* into the genus level supported by primary biochemical test results which included Gram-stain, O/F fermentation, catalase, oxidase, and motility. In the present study, API 20E testing system found difficult to distinguish the identity of Aeromonads. Previous report also identified the challenge of API 20E system in the identification of Aeromonads (Hassan *et al.*, 2017; Legario *et al.*, 2023). Due to the challenges of analysing the API 20E results, each biochemical profile from API 20E were analysed individually in comparison to the control strains and the references. Based on the API 20E results, some of the biochemical profiles from API 20E and the traditional methods (tubes methods) which previously conducted in Indonesia had some agreement, for example both have the same results of lysine decarboxylase, citrate, hydrogen sulphide, urea, indole, voges-proskuer, gelatine, glucose, mannose, sorbitol, rhamnose, and arabinose, where these reaction are considered in the identification of *Aeromonas.* In the *Aeromonas* identification the biochemical reaction individually from API 20E were also provided valuable information to support the primary biochemical test, therefore, by matching the reaction results with conventional method will help the identification process. As finding in the current study, where the agreement was identified in several

biochemical reaction including indole. VP, glucose, and gelatinase mannitol and gas production which the *Aeromonas* species tended to produce those reaction. Gas production also associated to *A. hydrophila* where the species often produced this.

The use of molecular identification methods will be essential and gain more attention in confirming the species level of the bacterial species, this is also because complexity of the conventional method and the limitation of API 20E in accurately identifying *Aeromonas* species and the other challenge was the biochemical identification often provide inconsistent results in both clinical and environmental isolates (Janda and Abbott, 2002; Beaz-Hidalgo *et al.*, 2015). The identification of bacterial phylogeny and taxonomy using 16S rDNA has become more common because it can be used for the confirmation of the identity of bacteria in general and provided valuable information in the identification over time (Janda and abbot, 2007). In this study, the sequence results showed that the bacterial isolates were closely related to *A. veronii* and *A. hydrophila* with the range of 98-100% similarity to the published gene sequencing in the GenBank database. This suggested that 16S rDNA method was useful in confirming the *Aeromonas* into the species level. This is in agreement to the reported regarding effectivity of 16S rDNA (Janda and Abbott, 2007; Burr et al., 2012). However, more recently, the lack of discrimination between very closely related *Aeromonas* species (e.g. the taxonomy of *Aeromonas* has been experiencing the update and change to this end) has illustrated the need for a more specific PCR sequence assay. In this study comparative sequence results from the 16s rDNA sequencing were then analysed using the additional *gyrB* and *rpoD gene.* The 16s rDNA gene is widely used and generally acceptable as stable and specific biomarker due to its highly conserved nature with micro heterogeneous segments of polymorphism able to separate bacterial species (Alperi *et al.,* 2008). However, the identification of the *Aeromonas* genus remains challenging and often cause miss identification because there are high interspecies similarity of 16S rDNA sequence which ranges between 97 to 100% (Navarro and Martínez-Murcia, 2018). These issues are also aggravated by the overlapping biochemical profiles and limit data on the association between phenotypic and genotypic identification contributes to the complexity of its taxonomy (Ormen et al., 2005). Considering the limitation of 16s rDNA gene sequencing, the housekeeping genes were also often used as confirmatory level into the identification into the species lever of *Aeromonas* (Hassan *et al.*, 2017). In the current study, the housekeeping genes including *gyrB* and *rpoD* confirmed the

identification results into the species level of motile *Aeromonas* recovered in this study. Both methods provided a good agreement with 16S rDNA sequencing, demonstrating that all 3 can be used and further investigations are required to compare the variability between the 3 DNA sequence results as it is not intended to use all 3 assays. This would not be cost effective in resources and may delay the identification process. Instead, a more comprehensive study could be performed to identify the specificity and sensitivity thresholds between the 3 molecular sequence methods applied in this study.

The housekeeping genes *gyrB* and *rpoD* have many advantages in the phylogenetic studies (Gonçalves Pessoa *et al.*, 2019). As well as in the current study where both housekeeping genes were able to confirm the identity of the tested *Aeromonas* strains. Therefore, the use of the two housekeeping genes is widely applied in taxonomic study and the method provides higher reliability in the phylogenetic classification of *Aeromonas* (Soler *et al.*, 2004). Similar reports also suggested that the housekeeping gene provided the valuable information on the taxa level and beneficial in identification of close related taxa including *Aeromonas* strains (Khor *et al.*, 2015; Vega-Sánchez *et al.*, 2014; Fernández-Bravo and Figueras, 2020).

The present study provided valuable information regarding the involvement of Gram-negative bacteria in the occurrence of the disease outbreaks in the study area, an identified *Aeromonas* species as the most common bacterial group recovered from the clinically affected fish. This finding is in agreement with the previous report that identified the important roles played by *Aeromonas* species in the occurrence of the disease outbreaks in freshwater farming systems (Vega-Sánchez *et al.*, 2014; Mzula *et al.*, 2019). The combination of clinical presentation with bacterial recovery and histopathology results demonstrated an infectious disease with the identification of the motile Aeromonads supporting that the key disease at the time of sampling was MAS. The comparative laboratory identification methods have shown the need for a combination of identification approaches and can provide a useful strategy for future epidemiological and/or disease diagnostic investigations relative to the ENT fish farming sector.

3.7. Conclusion.

Gross presentation of the disease fish samples in the current study showed that the involvement of bacterial pathogens in establishing the diseases. This result was also supported by the presentation of the rod shapes bacteria in the tissue organs of the fish samples with clinical sign of the diseases, where Gram-negative bacteria including *Aeromonas* species were the most predominant bacteria identified from the fish samples with the clinical signs of the diseases. This finding manifested the critical roles played by *Aeromonas* species in the occurrence of the disease outbreaks in the area of study. Further characterization of the *Aeromonas* species and potential pathogenicity test will be assessed in the Chapter 4.

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CHAPTER 4. CHARACTERIZATION OF *AEROMONAS* SPECIES ASSOCIATED WITH CLINICAL OUTBREAKS OF MOTILE *AEROMONAS* SEPTICAEMIA (MAS) IN INDONESIA.

4.1. Abstract.

Indonesia is one of the largest aquaculture producers worldwide, and like most intensive aquaculture systems, suffers from animal losses due to infectious disease outbreaks. The present study aimed to characterize the motile *Aeromonas* strains recovered from natural disease outbreaks occurring in tilapia and catfish *(Clarias spp).* A combination of phenotypic and genomic methods was applied, where a total of 40 bacterial isolates recovered from moribund and apparently healthy fish belonged to motile *Aeromonas* species identified as *A. veronii* (n=22), *A. hydrophila* (n=14), and *A. caviae* (n=4) which previously identified by conventional and 16S rDNA sequencing and further confirmed by *rpoD* and *gyrB* housekeeping genes in current study. Six selected strains were tested for pathogenicity using the *Galleria mellonella* wax moth larvae model to determine the effect of bacterial virulence profiles on larvae survival post inoculation. A total of 12 virulence genes were detected, and 95% (n=38) of the *Aeromonas* species were positive for cytotoxic enterotoxin (*act)* gene. Whereas the aerolysin *(aerA)* gene was detected in only 55% (n=22) of the motile *Aeromonas* strains. The *aerA* and a*ct* genes are considered as one of the most important genes related to MAS disease, and 53% (n=21) of the Indonesian *Aeromonas* strains contained both *act+aerA* genes. Ninety eight percent of the bacteria were resistant to the antibiotic amoxicillin and 40% (n=16) of the strains were resistant to a minimum 1 antibiotic tested. Resistance to oxytetracycline (30µg) was found in 35% (n=14) of the *Aeromonas* strains where 13/14 of the same strains had the tetracycline resistant *tetE* gene. Infectivity trials were performed using the wax moth larvae model where all 6 *Aeromonas* strains selected caused infection resulting in mortality of wax moth larvae compared with the unexposed control animals. Isolates were chosen based on their different virulence profiles, administered to the larvae by injection at the same concentration and incubation temperature, resulting in varied infectivity/mortality. The *A. veronii* strain 4HK categorized as high virulence in the current study which had gave no larvae survived in the exposed larvae within 96h post exposure to the bacteria, whereas isolates *A. veronii* 105HK with low virulence profile gave 37% (n=14) in the

exposed animals by day 4 which was the end of the experiment. A similar trend was observed with the *A. hydrophila* strains and for all animal experiments, infectivity rate was correlated with incubation temperatures. *A. hydrophila* strains 93HK with low virulence caused 53% (n=16) survival, whereas *A. hydrophila* strains 92HK categorized as high virulence caused only 13% (n=4) larva survived. These findings provide critical information on the pathogenic capacity of the motile *Aeromonas* species associated with infectious disease outbreaks in fish systems in Indonesia.

Key words: *fish disease*, *bacterial infection, Aeromonas, virulence profiles, antibiotics resistance.*

4.2. Introduction.

Motile *Aeromonas* strains were often recovered from moribund fish presenting with a wide range of clinical signs of disease including ulcerative, haemorrhagic, and septicaemic infections in freshwater and ornamental fish (Beaz-Hidalgo *et al.*, 2015; Bebak *et al*., 2015; Eisa *et al*., 2015; Austin, 2021). Several motile *Aeromonas* species have been described as associated with natural disease outbreaks in aquaculture systems, where *A. veronii, A. hydrophila, A. sobria and A. caviae* were more commonly reported as fish pathogens causing Motile *Aeromonas* Septicaemia (MAS) (Cai *et al.*, 2012). Various studies have described the prevalence of *Aeromonas* in the Indonesia fish farming system, recovered and identified from farmed moribund and apparently healthy tilapia (Amanu *et al.,* 2014; Rahayu, 2019; Azhari *et al.,* 2014; Manurung and Susantie, 2017; Angraeni *et al.,* 2018). *A. hydrophila* strains in particular were reported as the aetiological agent causing catfish fry mortality over 1 to 2 weeks, in Indonesia (Lukistyowati and Kurniasih, 2012). Previously, researchers considered the motile *Aeromonas* to only be “secondary” or opportunistic pathogens usually associated with stress induced disease outbreaks in fish and other animals (Janda and Abbott, 2010). However, *A. hydrophila* has been described as a primary bacterial pathogen in catfish (*clarias sp.)* (Sarkar and Rashid, 2012). Relatively recently, studies have identified a hypervirulent (vAH) *A. hydrophila* with alternate secretory mechanism and virulence factors compared with non-hypervirulent *A. hydrophila* which are described as critical factors in pathogenesis, distinguishing these unique hyper-virulent strains (Bebak *et al.*, 2015; Rasmussen-Ivey *et al.*, 2016). The vAH strains of *A. hydrophila* belong to Sequence Type (ST) 251 identified using Multi Locus Sequence Typing (MLST) and

due to their hyper virulent nature, act more as primary pathogens in the fish farms producing high mortalities over acute periods of time (Bebak *et al.*, 2015 ; Pang *et al.*, 2015; Ngo *et al.*, 2022). vAH strain has been an increasingly important challenge affecting cyprinid fish industry throughout China since 1989 leading to huge economic losses (Zhang *et al.*, 2014; Wang *et al.*, 2011; Pang *et al.*, 2015; Rasmussen-Ivey *et al.*, 2016). In the USA, MAS outbreaks caused by vAH strains have been reported in several farms and affected the channel catfish industry in 2019, and economic losses incurred that reached 3 million dollars annually, equivalent to 10,500 tonnes of dead fish (Pridgeon and Klesius, 2011; Hossain *et al.*, 2014; Rasmussen-Ivey *et al.*, 2016).

The pathogenicity of the motile *Aeromonas* group is governed by virulence factors and their expression within a susceptible host resulting in the establishment of infection. *Aeromonas* virulence factors were often reported as contributing factors to the development of infection within the affected host including fish (Latif-Eugenín *et al.*, 2016; Tomás, 2012). The virulence factors of *Aeromonas* are described as multifactorial, often linked with the production/secretion of enzymes and toxins e.g. cytotoxins, haemolysin, lipases, and proteases. Their survival in aquatic environments is down to their ability to form biofilms by using specific metabolic pathways, and they are able to mediate virulence factor expression (Allan and Stevenson, 1982; Rasmussen-Ivey *et al.*, 2016). The more common virulence factors identified in many motile *Aeromonas* species associated with disease include protease and haemolysis play an active role in the pathogenesis (Janda and Abbott, 2010). Meanwhile, the enzyme lipase has a more diverse function within the numerous pathogenic infections and is considered variable in many of the *Aeromonas* species (Stehr *et al.*, 2003). Biofilm formations contribute to the colonisation of the bacteria, thus promoting adherence and establishment of disease (Chen *et al.*, 2014).

The diversity of virulence profiles detected within the *Aeromonas* complex and their strain-variation within species show the variety of pathogenic mechanisms employed in a wide range of environments (Roges *et al.*, 2020; Rasmussen-Ivey *et al.*, 2016). Most studies have combined the phenotypic expression of the virulence factor with the presence of the gene responsible for the virulence factor e.g. the type II secretion system (T2SS) is often reported present in most members of *A. hydrophila* recovered from diseased fish and is integral in the extracellular secretion of wide manifestation of virulence factors including aerolysin, DNases, and proteases (Tseng, Tyler and Setubal, 2009; Pang *et al.*, 2015; Ruhil Hayati *et al.*, 2015). Aerolysin is

considered as one of the most potent virulence factor related to the cytotoxic enterotoxin *act* contributing to establishment of disease (Heuzenroeder, *et al.,* 1999; Galindo *et al.*, 2004; Iacovache *et al.*, 2016). Aerolysin is described as a pore-forming toxin which damages host cell membrane absorbency, resulting in osmotic lysis and cell necrosis (Cirauqui *et al.*, 2017; Xu *et al.*, 1998). Vadivelu *et al.*, (1995) also described that aerolysin is generally observed in the *A. hydrophila* strains recovered from fish presenting with bacteraemia, which is a typical clinical sign of MAS infection. Furthermore, *Aeromonas spp.* have strong association with transfer of virulence factors that encode of the type III secretion system (T3SS) (Sha *et al.*, 2009; Beaz Hidalgo, 2013). T3SS function as the injecting effector into the host cells and has been identified in the motile *Aeromonas spp.* co-regulating a multitude of virulence factors e.g. cytotoxic enterotoxin, DNA adenine methyltransferase, flagella, lipopolysaccharides, and DNA methylation ((Sha *et al.,* 2002; Braun *et al.*, 2002; Sierra *et al.*, 2010; Beaz Hidalgo, 2013; Rasmussen-Ivey *et al.*, 2016). The *act* gene in particular has been described as critical in establishing the infectivity process as it can cause multiple cellular changes in the susceptible host e.g. haemolytic, cytotonic, and cytotoxic activities but unlike other virulence factors exported via T3SS and T6SS, *act* is exported through the T2SS (Sha *et al.*, 2002; Galindo *et al.*, 2004; Erova *et al.*, 2012). Based on comparative genomic analysis, other virulence genes such as *alt* and *ast* are also among the factors often associated with the pathogenicity of *A. hydrophila* (Rasmussen-Ivey *et al.*, 2016). The *ast* gene contributes in causing intestinal fluid accumulation (Sha et al., 2002). The *Aeromonas* species including *A. hydrophila* express diverse degradation enzymes that contribute to virulence including collagenase, elastase, enolase, lipase, metalloprotease, and serine protease (Rasmussen-Ivey *et al.*, 2016). It is clear that these motile *Aeromonas* species have a large number of virulence factors from which they can utilise during infectivity in a susceptible host.

Significant progress has been made in differentiating pathogenic from non-or weakly pathogenic strains of MAS complex, and it provides essential information on the disease aetiology and novel treatments. In Indonesia's isolates virulence profiling of *Aeromonas* species remains limited. Studies over the last 5 years have focused more on detection and description of the individual genes associated with a specific host susceptibility e.g. aerolysin gene was reported to be presented in *A. hydrophila* isolated from natural diseased catfish *(C. gariepinus)* (Indrawati *et al.*, 2020). However,

further work is required to profile these into related clusters of high, medium, and low virulence groups at the bacterial species level to ensure optimal diagnosis and treatments are provided.

During a bacterial disease outbreaks, antibiotics remain the key treatment strategy applied however, without due diligence antimicrobial resistance (AMR) can rapidly develop (Rhodes *et al.*, 2000). In Indonesia, AMR investigations are limited and are more frequently reported only in areas where the most intensive farming systems and the largest aquaculture production occurs in Indonesia, e.g. Java. Strict regulations in antibiotic use were established in Indonesia. Nonetheless, assessment and regular monitoring on the application of antibiotics including their efficacy and also the study on AMR resistance profiles more widely in the area of Indonesia remains important. Globally, several studies reported that *Aeromonas* species were already exposed to the indiscriminate practice of antibiotics supporting rapid AMR development to a wide range of antibiotics critical for animal and human health. These included tetracyclines, penicillin, cephalosporins, carbapenems, aminoglycosides, chloramphenicol, and trimethoprim (Janda and Abbott, 2010; Figueras *et al.,* 2011; Vega-Sánchez *et al.*, 2014; Odeyemi and Ahmad, 2017). The intrinsic microbial resistance in Aeromonads is chromosomally mediated resistance to β-lactamase (Stratev and Odeyemi, 2016). Therefore, treatment with this antibiotic and similar type of antibiotics would be futile. AMR development can be natural and acquired where antibiotic resistance genes detected in the bacteria can be acquired through mobile genetic elements including plasmids, transposons and integrons, that facilitate the rapid spread of resistance among bacteria including *Aeromonas* (Adeleye *et al.*, 2011; Stratev and Odeyemi, 2016). The presence of resistance genes in mobile genetic elements could be transmitted through three pathways including lateral DNA transfer, namely transformation, transduction, and conjugation (Romero *et al.,* 2012). Tetracycline resistance as an example reported in most bacteria is due to the acquisition of new genes that are often associated with the presence of mobile genetic elements (Chopra and Roberts, 2001; Roberts, 2003). Therefore, understanding the presence, role, and the mechanism of genes related to antibiotic resistance is considered important due to the raising concerns of antibiotics resistance globally including Indonesia.

The use of animal for research experiments is considered prominent in developing the knowledge and contribute in solving the biological and biomedical

issues affecting both human and animal (Andersen and Winter, 2019). The use of fish as an experimental model in assessing the pathogenicity of bacteria including *Aeromonas* remain the “gold standard” in aquatic disease research (Cengizler, 2022). The appropriate host for experimental challenges is critical however, the infrastructure and resources for in vivo experimental challenge studies are not always readily available and alternative models are required. These remain limited but some alternatives can be used as an intermediate step which expands knowledge from the in vitro laboratory based studies into the next phase of animal studies without needing the use of whole fish. The introduction of the greater wax moth (*Galleria mellonella)* is one alternative model which is reported as an effective intermediate stage between in vitro and full host-specific in vivo studies in vertebrate species. One of the attractive features is the simplicity and reliability of establishing infection in these animals (Desbois and Coote, 2011). Furthermore, the model is able to assess several strains clearly and quickly to provide a holistic understanding of virulence to complement genetic analysis study, where this model has been used to investigate the virulence of various human pathogens including Gram-positive and Gram-negative bacteria (Peleg *et al.*, 2009; Champion *et al.*, 2009). Several reports investigated the use of *G. mellonella* in the study of bacterial pathogens recovered from fish had been successfully achieved (Desbois and Coote, 2011; McMillan *et al.*, 2015; Djainal *et al.*, 2020; Six *et al.*, 2019).

4.3. Study Aim.

The aim of this study was to characterise the virulence and antibiotic resistance profiles of the range of strains at species-level recovered from farmed tilapia and catfish in East Nusa Tenggara, Indonesia and to investigate infectivity using the alternative wax moth larvae model.

4.4. Material and Methods.

4.4.1. Bacterial DNA Extraction.

In total 40 bacterial strains were included in this study: *A. veronii (n= 22), A. hydrophila (n= 14)* and *A. caviae (n= 4)*, with identification profiles described in chapter 3 (Chapter 3 Section 3.5.2). Bacterial DNA was extracted from single purified colonies following SSTNE/salt precipitation DNA extraction method (Dwiyitno *et al.*, 2018), with minor modification as described in the Chapter 3 (section 3.4.1). All bacterial DNA samples were stored as aliquots at -20C until required.

4.4.2. Antibiotic Susceptibility Test Using Kirby Bauer Disc Diffusion Method.

The antibiotic susceptibility test was carried out using Kirby-Bauer Antibiotic Disc Method according to Bauer *et al.*, (1966) and detected the viable antibiotic susceptibility of each bacterial strain. The antibiotic impregnated paper discs were purchased from Oxoid UK and stored at -20C prior to use, then at 4^0C during the study. The antibiotics selected included those most commonly used/available in the area of study. The antibiotic and concentration were Amoxicillin (AML, 10µg), Enrofloxacin (ENR, 5µg), oxalinic acid (OA, 2µg), florfenicol (FFC, 30µg), oxytetracycline (OT, 30µg), sulfamethoxazole (SXT, 25µg).

The method applied for the antibiotic sensitivity assay followed Crumlish. *et al*., (2002). In brief, pure culture was grown overnight and the bacterial suspension measured to give a turbidity at a McFarland standard of 1, which expected to provide the concentration of 2.5 x 10^8 CFU/ml. From this suspension in sterile saline, a bacterial lawn was produced by aseptically plating 100µl of the bacterial suspension onto the TSA plate and then spreading across the TSA agar, and antibiotic discs aseptically dispensed onto the agar plate, using disc dispenser (Oxoid, UK). The plates were incubated at 28^0C for 24h. The zone of inhibition (no bacterial growth) was measured using digital callipers and interpretation followed IoA bacteriology laboratory standards, where resistance ≥10 mm inhibition, partially sensitive 11-15 mm or sensitive ≥16 mm inhibition.

Detection of antibiotic resistance genes.

The DNA recovered from all bacterial strains was screened for the presence of putative genes encoding resistance to tetracyclines (*tet*A, *tet*B, *tet*C, *tet*D, *tetE*, *tet*G, *tetM*), and sulphonamides (*sul1*, *sul*2, *sul*3) by PCR (Table 4.1). These data were then

combined with the agar disc diffusion inhibition zone measurements to produce a comprehensive AMR/AST profile.

Table 4.1. The primers set to detect antimicrobial resistance genes.

Targeted Gene	Primer Pair	Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
Tetracycline					
tetA	tetA-F	GTAATTCTGAGCACTGTCGC	62	957	(Hossain *et al.*, 2018)
	tetA-R	CTGCCTGGACAACATTGCTT			
tetB	tetB-F	CTCAGTATTCCAAGCCTTTG	57	436	(Hossain *et al.*, 2018)
	tetB-R	CTAAGCACTTGTCTCCTGTT			
tetC	tetC-F	CTTGAGAGCCTTCAACCCAG	58	418	Syrova. *et al.,* 2018
	tetC-R	ATGGTCGTCATCTACCTGCC			
tetD	tetD-F	AAACCATTACGGCATTCTGC	54	787	Syrova. *et al.,* 2018
	tetD-R	GACCGGATACACCATCCATC			
tetE	*tetE*-F	GTGATGATGGCACTGGTCAT	62	1199	(Hossain *et al.*, 2018)
	tetA-R	CTGCCTGGACAACATTGCTT			
tetG	tetG-F	CAGCTTTCGGATTCTTACGG	56	844	Syrova. *et al.,* 2018
	tetG-R	GATTGGTGAGGCTCGTTAGC			
tetM	tetM-F	ACACGCCAGGACATATGGAT	54	536	(Skwor *et al.*, 2020)
	tetM-R	ATTTCCGCAAAGTTCAGACG			
Sulphonamide					
sul1	sul1-F	CTTCGATGAGAGCCGGCGGC	71	417	Syrova. *et al.,* 2018
	sul1-R	GCAAGGCGGAAACCCGCGCC			
sul2	sul2-F	AGGGGGCAGATGTGATCGAC	54	249	Syrova. *et al.,* 2018
	sul2-R	GCAGATGATTTCGCCAATTG			
sul3	sul3-F	GAGCAAGATTTTTGGAATCG	52	789	Syrova. *et al.,* 2018
	sul3-R	CATCTGCAGCTAACCTAGGG CTTTGGA			

4.4.3. Detection of Bacterial Virulence *(in vitro*).

A series of assays were performed to determine the expression of virulence factors *in vitro*. These methods followed protocols described in Legario *et al.*, (2020), with minor modifications. In detail, a pure colony of each bacterial strain was inoculated into 15 ml centrifuge tube containing 10 ml of Tryptic Soy Broth (TSB, Oxoid UK), incubated at 28^0C for 24h, centrifuged at 3500 x g for 15 minutes at 4^0C and the cell pellet resuspended in sterile 0.85% (w/v) saline solution. The cell density was measured using spectrophotometer and OD_{600nm} = 0.5 was obtained for each bacterial strain. A total of 20 µl of each strain at this concentration was inoculated into the selective agar representative of the individual virulence phenotypic assays (Table 4.2), and the inoculated agar was then incubated for 48h at 28^0C and the results were recorded. All tests were performed in duplicate per strain. The *A. hydrophila* strain

NCIMB 9240 was used as a positive control in all assays and was expected to give positive results as described in Table 4.2 for each assay performed.

Table 4.2. Phenotypic agar-based virulence detection assays.

Virulence activity	Media	Positive results	Reference
Haemolysis	Horse agar	Clear zone surrounding the inoculation	Brendan & Janda, 1987)
Protease	skimmed milk agar	Clear zone surrounding the inoculation	Vermelho et al., 1996
Lipase	Tween 80 agar	white halo around the inoculation	Plou et al., 1998
Gelatinase	Gelatine agar	Clear zone surrounding the inoculation	Goodner, 1958
DNAse	DNAse agar base + 0.01% toluidine blue	pink zone around the inoculation	(Weckman and Catlin, 1957; Steitfeld *et al.*, 1962)
Biofilm formation	Congo red agar	Black colonies with a dry crystalline consistency	Freeman et al., 1989

Detection of the virulence genes *in vitro*

All samples were screened for the presence of 12 common virulence genes identified for motile *Aeromonas* species (table 4.3). The methods for each assay were followed according to the publication with slight modifications in the annealing temperatures, denaturation time, and extension time.

Table 4.3. Virulence genes PCR screening for *Aeromonas.*

Virulence Factor	Gene	Primer	Nucleotide Sequence (5'- 3')	Expected product size (bp)	Annealing Temperature (°C)	Reference
Aerolysin	*aer*	aerA_F	CCTATGGCCTGAGCGAGAAG	431	69	Nawaz. *et al*., 2010
		aerA_R	CCAGTTCCAGTCCCACCACT			
Hemolysin A	*hylA*	hylA-F	ATGAGTTTTGCCGATAGTTTATTTTTCCTGA	1320	67	(Khor *et al.*, 2015)
		hylA-R	TTACGATTCCTGAGCGGGCTTGTCGGCCGGCGTG			
Cytotoxic enterotoxin	*act*	act_F	AGAAGGTGACCACCACCAAGAACA	232	65	Nawaz. *et al*., 2010
		act_R	AACTGACATCGGCCTTGAACTC			
Heat-labile cyototonic enterotoxin	*alt*	alt_F	TGACCCAGTCCTGGCACGGC	442	66	Nawaz. *et al*., 2010
		alt_R	GGTGATCGATCACCACCAGC			
Heat-stable cytotonic enterotoxin	*ast*	ast_R	TCTCCATGCTTCCCTTCCACT	331	65	Nawaz. *et al*., 2010
		ast_R	GTGTAGGGATTGAAGAAGCCG			
Cholesterol acyl transferase	*gcaT*	gcaT_F	CTCCTGGAATCCCAAGTATCAG	237	65	Nawaz. *et al*., 2010
		gcaT_R	GGCAGGTTGAACAGCAGTATCT			
Serine protease	*ser*	ser_F	CACCGAAGTATTGGGTCAGG	350	64	Nawaz. *et al*., 2010
		ser_R	GGCTCATGCGTAACTCTGGT			
Lipase	*lip*	lip_F	CAYCTGGTKCCGCTCAAG	247	63	Nawaz. *et al*., 2010
		lip_R	GTRCCGAACCAGTCGGAGAA			
DNAse	*exu*	exu_F	RGACATGCACAACCTCTTCC	323	61	Nawaz. *et al*., 2010
		exu_R	GATTGGTATTGCCYTGCAAS			
Lateral flagella	*lafA*	lafA_F	CCAACTTYGCYTCYMTGACC	738	62	Nawaz. *et al*., 2010
		lafA_R	TCTTGGTCATRTTGGTGCTY			
Enolase	*enolase*	enolase_F	ATGTCCAAGATCGTTAAAGTGAT	1302	60	(Khor *et al.*, 2015)
		enolase_R	TTAAGCCTGGTTCTTCACTTCTT			
Elastase	*ela*	ahyB_F	ACACGGTCAAGGAGATCAAC	513	62	Nawaz. *et al*., 2010
		ahyB_R	CGCTGGTGTTGGCCAGCAGG			

Each PCR reaction was performed in a 25 µl mixture consisting of 12.5 µl of 2X HS MyTaq mastermix (bioline UK), 1.5 µl of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 µl of DNA template, and Milli-Q water to volume. The PCR products were analysed by electrophoresis in a 1% (w/v) agarose gel prepared in 0.5X TAE buffer with ethidium bromide (0.5 µg ml^{-1}), visualized on a UV transilluminator and documented. A GeneRuler™ 1kb Plus DNA Ladder (Thermo

Scientific™, UK) was run together with the PCR products to enable estimation of the size of the amplified fragments.

4.4.4. Investigating the Pathogenicity of the Tested Strains Challenged with The Wax Moth Larvae.

Six bacterial strains were selected for the infectivity trials performed in the wax moth larvae. The strains selected were among the strains recovered in the current study and categorized as low virulence (LV), medium virulence (MV), and high virulence (HV) of both *A. veronii* and A. *hydrophila* strains as shown in the Table 4.4.

Table 4.4. The motile *Aeromonas* strains used in the challenge test.

***Aeromonas* species**	**Bact. ID**	**Phenotypic Virulence (n)**	**Virulence genes (n)**	**Virulence genes composition**	**Virulence categorization**
A. veronii	105HK	2	2	*act, ser*	LV
	62HK	5	6	*aerA, act, alt, ser,lip,ela*	MV
	4HK	6	7	*aerA, act, ast, gcat, ser, lip, eno*	HV
A, hudrophila	93HK	5	5	*act, gcat, ser, lip, eno*	LV
	115HK	6	7	*aerA, act, gcat, lafA, lip, eno, ela*	MV
	92HK	6	10	*aerA, hylA, act, alt, ast, gcat, exu, lip, eno, ela*	HV

*PV: LV: Low Virulence, MV: Medium Virulence, HV: High Virulence

The selected 3 strains from *A. veronii* and 3 strains represented the virulence genes from low (with 2 - 4 genes), medium (5 - 6 genes), and high (≥ 7 genes). The three selected *A. hydrophila* strains were also categorized as 3 virulence level, however, since the lowest virulence genes identified was 5 genes, therefore, the low group was 5-6 genes, medium (7-8 genes), and high (9-10 genes). The bacterial suspension was grown from (-20^{0}C) glycerol stocks in 10 ml of TSB, incubated for 18 h at 28^{0}C, then centrifuged at 3500xg for 15 minutes at 4^{0}C and the cell pellet resuspended in sterile 0.85% saline to achieve an Optical Density (OD_{600}) value of 1, which gave 2.5x10^8 CFU/ml. The viable colony counts at the OD value were confirmed using the Miles and Misra drop counts (Miles, Misra and Irwin, 1938). Ten-fold serial dilutions were performed aseptically with dilution factors of 10^{-1} to 10^{-7}. 20µl of each dilution were then inoculated into six divided area on TSA media, the number of colonies forming unit (CFU) was counted after 24h of incubation. A small selection of identification and characterisation assays were performed on each of the 6 isolates used to confirm that they had not changed or loss their virulence status post-storage.

These were performed using the methods described in section (Chapter 3, Section 3.4.13) and include the following tests (Gram-stain, cytochrome-oxidase activity. Motility, catalase and oxidation/fermentation – OF test). Any isolate that had a change in the identification or characterisation profiles were not used and replaced with an alternative strain as appropriate.

Wax moth larvae.

Wax moth larvae were purchased from UK waxworms Ltd, Sheffield, UK, stored in the dark at 4^0C and used within 14 days arrival at Stirling. The healthy criteria of larvae was described by Andrea *et al.,*, (2019). The larvae were selected upon arrival and stored in 4^0C fridge until required for testing. The unhealthy larvae were not included including the larvae with darker colour as effect of melanisation, dark spots at their body and leg, no clogged prolegs as this will compromise the injection process, and the larvae that not active/moving.

Experimental design.

A total of 4 experiments were performed with the wax moth larvae which had a similar experimental design, but each was investigating a different variable on the survival of the animals. The animals were randomly allocated to the treatment groups in each experiment with each treatment group having duplicate samples (n=15). In all experiments, the animals were injected using a 50-µl Hamilton syringe (Sigma-Aldrich Ltd) into the last left proleg and administered 10µl of a suspension (e.g. bacterial suspension, antibiotic solution, or Phosphate Buffer Saline (PBS) that contained of sodium chloride (NaCl) 4 g, potassium chloride (KCl) 0.1g, disodium phosphate (Na_2HPO_4) 0.72g, and monopotassium phosphate (KH_2PO_4) 0.123g in 500ml distilled water. The syringe was cleaned between experiments with consecutive washes of 1% (w/v) sodium hypochlorite, 70% ethanol, and sterile water. In each experiment there were 2 negative control groups: Negative control (PBS) and Negative control (No handling). The first control, the animals were not handled or injected and the second control the animals were injected with sterile PBS to confirm that injection did not cause any mortalities. The larvae were then assessed every 24h for survival which was observed as the live larvae by checking the larvae movement and the moribund larvae as the one that experienced the melanisations and with passive movement. The cumulative number of survivals in each group was calculated and analysed using

Kaplan-Meier plots. Relative virulence in the larva was calculated as the cumulative area under Kaplan-Meier plots and this approach can distinguish the virulence of each isolate (Mcmillan *et al.,* 2015).

Effect of temperature on the larvae survival after injection. In the first experiment, the effect of temperature was explored on the survival of the larvae exposed to bacteria. This experiment was conducted to investigate the effect of temperature on the testing larva survival and to find the suitable temperature for the challenge test of different strains with virulence profiles. In this experiment, the treatment was grouped into 4 group including TG1 (*A. veronii* 62HK) and TG2 (*A. hydrophila* 115HK) which were categorized as medium level (MV) virulence profiles. The concentration of the bacteria injected was 10µl of $1x10^5$ CFU/ml to each wax moth larva. Once all of the animals had been injected, they were then stored at 15, 22, 28, or 37^0C and monitored twice a day for four days where moribund or dead animals removed and counted. The survival data was then calculated to determine the influence of temperature on larval survival after inoculation with *Aeromonas* strains and to determine the suitable incubation temperature for further experiments infection performing the motile *Aeromonas* strains that had varied virulence profiles.

Effect of heat killed *Aeromonas* strains to the larvae survival. In the second experiment, a similar design was applied, however, the bacteria in TG1 and TG2 were heat killed prior to administration to the larvae. This experiment was designed to explore the effect of viable v's non-viable *Aeromonas* strains on survival of the wax moth larvae, as *Aeromonas* strains often secrete toxins, and these are considered important virulence factors. The same bacterial strains and species were used as above, however, they were grown to $1x10^7$ CFU/ml and then heat killed following the methods described in Djainal *et al.*, (2020). Heat-killing was confirmed by the absence of viable *Aeromonas* colonies when 100µl of heat killed bacterial suspension was plated across the TSA and then incubated at 28^0C for 48h. If the heat killing was successful, then no bacteria would grow, and the animals were then exposed to the bacteria. The treatment groups were incubated at 28^0C for four days and the survival of the animals monitored as described above for the temperature experiment.

Virulence of Motile *Aeromonas* in the Wax Moth Larvae. The third experiment was where larvae exposed to the bacterial strains as described above with the treatment groups as shown in Table 4.5. The treatment group in this experiment consisted of 6 treatment group (TG1 – TG6) with 6 motile *Aeromonas* strains with different virulence level for each *Aeromonas* species. Different concentration of each strain was also performed to assess the effect of different cell concentration to the larvae survival. For the control, 2 groups of negative control were used including PBS injection (TG7) and no handling (TG8). The viable number of bacterial colonies in the haemolymph of the *Aeromonas* Strains was performed by randomly selected the surviving larvae in each group post exposure and harvesting the larvae haemolymph. For this, the last abdominal segment of each larva was removed with sterile scissors and the haemolymph (approximately 5-20µl) were harvested. Of this, 10µl will be diluted in 90µl of PBS and plated into TSA media and then Ten-fold serial dilutions were performed aseptically until the viable number of colonies were achieved following Mile and Misra method (Table 4.5).

Table 4.5. The treatment group of different virulence profiles of motile *Aeromonas* strains.

Treatment Group (TG)	Bacterial Strains	Bact. ID	Virulence Level	Larvae used for each experiment group (n)		
				$1x10^3$CFU/ml	$1x10^5$CFU/ml	$1x10^7$CFU/ml
TG1	*A. veronii*	105HK	LV	30	30	30
TG2		62HK	MV	30	30	30
TG3		4HK	HV	30	30	30
TG4	*A. hydrophila*	93HK	LV	30	30	30
TG5		115HK	MV	30	30	30
TG6		92HK	HV	30	30	30
TG7	PBS injection			30		
TG8	No treatment			30		

*LV: Low Virulence, MV: Medium Virulence, HV: High Virulence

Exploring antibiotic treatment in wax moth larvae. The final experiment was Efficacy of antibiotics administered to the larvae post infection was determined following the methods of Desbois and Coote, (2011). This experiment was done to validate the wax moth larvae model to be good infection model i.e. infection can occur and also can be treated with the application of antibiotics. In this experiment, the

treatment was grouped into 6 group including TG1 (*A. veronii* 62HK) and TG2 (*A. hydrophila* 115HK) which were categorized as medium level (MV) virulence profiles, the concentration of the bacteria injected was 1 x 10^5 CFU/ml. In each experiment there were 2 negative control groups: negative control (PBS) and Negative control (no handling). Positive control groups were also performed in this experiment for each strain. each larva was exposed to1x10^5 CFU/ml by injection into each larva and then store in the incubator at 28^0C without antibiotics treatments. Once all larvae were confirmed to be alive after 2h post bacterial exposure, antibiotics in PBS were administered in the same route as bacteria. The antibiotic used as the therapy was oxytetracycline and erythromycin (Oxoid, UK). The survival was assessed at 24 and 48h post antibiotic exposure. As negative controls PBS only were injected and group of larvae without treatment. The dose of antibiotics each injection was 1 µl/g larvae in 10 µl PBS according to Desbois and Coote (2011).

Statistical Analysis.

The data were analysed descriptively by using the frequency of the larvae mortality during the experimental time. The SPSS Kaplan Meier method was used for the statistical analysis by plotting the survival data and differences between the variables were calculated using the log-rank test where p value ≤ 0.05 indicated the statistically significant. The negative controls were also plotted in the analysis and were used to analyse the comparison between all variables. All data analyses were performed by using IBM SPSS statistic 26.

4.5. Results

4.5.1. Antibiotic Susceptibility Test using Kirby Bauer Disc Diffusion Method

The antibiogram results of the 40 motile *Aeromonas* strains tested using the Kirby Bauer susceptibility disc diffusion methods is presented in Fig 4.1 to 4.3. Care must be taken as different numbers of strains were recovered for each species; therefore, the data was presented as percentages of each strain within the respective *Aeromonas* species including *A. veronii* (n=22), *A, veronii* (n=14), and *A. caviae* (n=4). The testing result of motile *Aeromonas* strains to the tested antibiotics were group into three different groups including sensitive, partially sensitive, resistant.

The sensitive proportion of motile *Aeromonas* to the tested antibiotics were shown in Figure 4.1. All the tested motile *Aeromonas* strains were sensitive to ENR. A higher proportion of sensitive were also shown by the tested strains to SXT, where all of *A. veronii* and *A, hydrophila* were sensitive to SXT and 50% (n=2) of *A. caviae* strains were sensitive to SXT. All *A. veronii* strains were also sensitive to FFC, whereas 93% (n=13) of *A. hydrophila* strains were also sensitive to. FFC sensitivity proportion of *A. hydrophila* strains to OA was the highest among the tested strains with 86% (n=12) strains. Meanwhile, the least sensitive was shown to OTC to all the tested strains (except AML), where 43% (n=6) *A. hydrophila* strains were sensitive to OTC was the lowest among the tested antibiotics. Likewise, *A. veronii* sensitivity to OTC was also the lowest with 64% (n=14) than the other tested antibiotics.

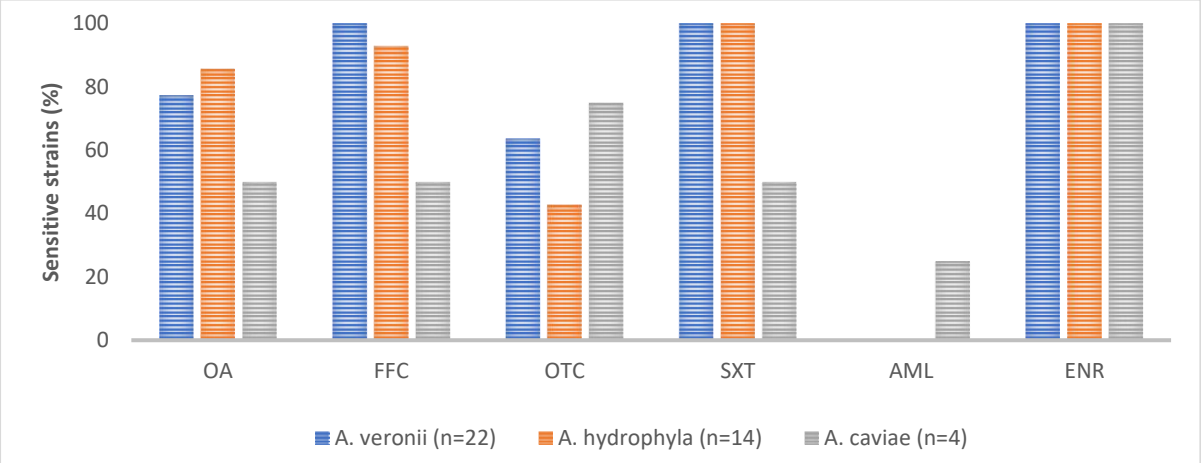

Figure 4.1. Percentages of sensitive motile *Aeromonas* strains to the tested antibiotics. Amoxicillin (AML, 10µg), Enrofloxacin (ENR, 5µg), oxalinic acid (OA, 2µg), florfenicol (FFC, 30µg), oxytetracycline (OT, 30µg), sulfamethoxazole (SXT, 25µg).

The partially sensitive proportion of motile *Aeromonas* to the tested antibiotics were shown in Figure 4.2. The lowest proportion was shown in the partially sensitive group compared to the other groups, where none of *A. hydrophila* strains categorized as partially sensitive to the tested antibiotics. Whereas *A. veronii* strains were partially sensitive to OA with 14% (n=3) and to OTC with 9% (n=2). Meanwhile, the proportion of partially sensitive also shown by *A. caviae* strains, where 25% (n=1) were partially sensitive to three different antibiotics including OA, FFC, and AML (Figure 4.2).

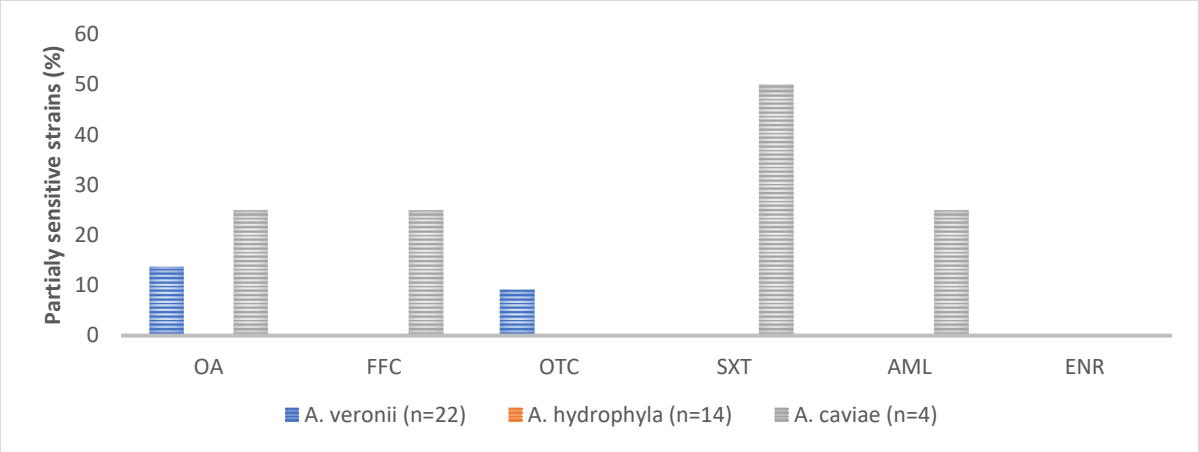

Figure 4.2. Percentages of partially sensitive motile *Aeromonas* strains to the tested antibiotics. Amoxicillin (AML, 10µg), Enrofloxacin (ENR, 5µg), oxalinic acid (OA, 2µg), florfenicol (FFC, 30µg), oxytetracycline (OT, 30µg), sulfamethoxazole (SXT, 25µg).

The resistant proportion of motile *Aeromonas* to the tested antibiotics were shown in Figure 4.3. Motile *Aeromonas* strains during the invitro assay showed primary resistant to AML, where 98% (n=39) of the tested strains had complete resistance as shown by the 0 mm zone of inhibition, whereas only 1 *A. caviae* strain was partially sensitive to AML. By excluding AML, the highest resistant was shown by motile *Aeromonas* strains to OTC, where 35% (n=14) of the tested strains were resistant to OTC, *A. hydrophila* strains categorized as the highest resistant proportion with 57% (n=8) of the tested *A. hydrophila* strains, this number was followed by *A. veronii* with 23% (n=5) of the tested *A. veronii* strains, and the lowest proportion was *A. caviae* with 25% (n=1). 14 strains categorized as resistance to OTC had diameter of inhibition

zone ≤ 10 including 8 of *A.hydrophila* strains with the Bact.ID 65HK, 92HK, 13HK, 102HK, 61HK, 97HK, 36HK, and 5HK. Whereas *A. veronii* with 5 strains with the ID 4HK, 145HK, 144HK, 62HK, and 38HK, and 1 HK. Only 1 of *A. caviae* strains showing resistant to OTC with the ID 127HK. Meanwhile, small proportion of the motile *Aeromonas* strains were also resistant to OA and FFC, where 9% (n=2) of *A. veronii* and 14% (n=2) of *A. hydrophila* strains were showing resistant to OA as combination with OTC, only 2 of the tested strains resistant to FFC only.

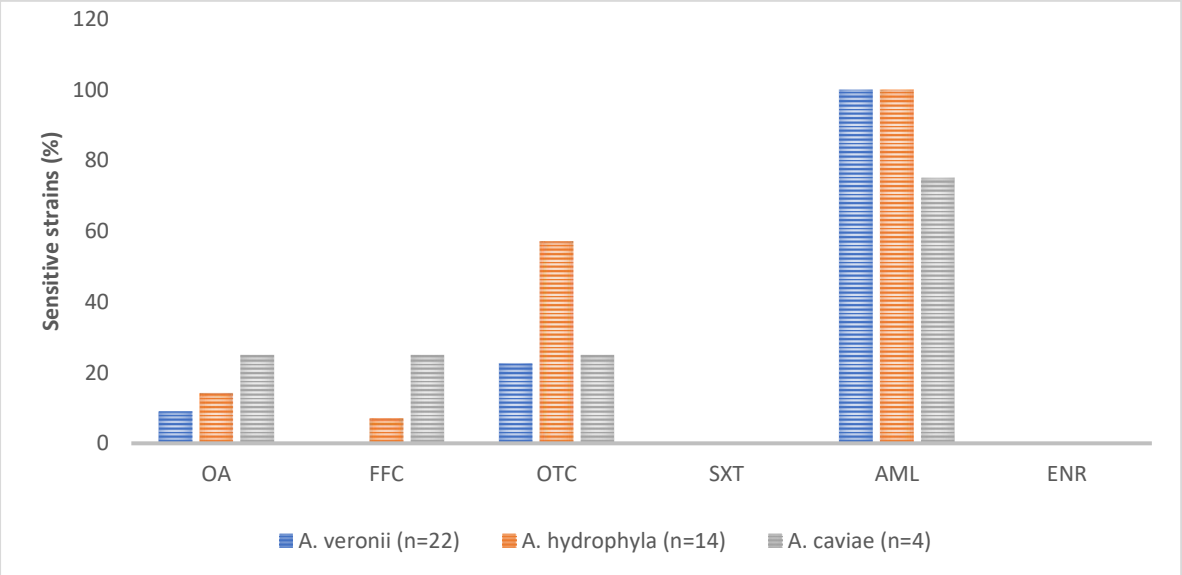

Figure 4.3. Percentages of resistant motile *Aeromonas* strains to the tested antibiotics. Amoxicillin (AML, 10µg), Enrofloxacin (ENR, 5µg), oxalinic acid (OA, 2 µg), florfenicol (FFC, 30µg), oxytetracycline (OT, 30µg), sulfamethoxazole (SXT, 25 µg).

The resistant strains to the tested antibiotics were shown in the Table 4.6. By excluding AML, 39% (n=16) of strains resistant to the tested antibiotics. OTC was the most frequently expressed where 14 strains categorized resistant to the OTC with either resistance to the OTC alone or the combination with the other antibiotics tested including OA. The *tetE* gene was the only encoding gene detected in 13 out of 14 strains that showed resistance to either OTC only or combined OTC+OA had the *tetE* gene. The correlation with the resistance to *OTC* as determined by the Kirby Bauer method and the same strains were also positive by PCR for tetE which encodes for *OTC* resistance. The resistance OTC per species showed that *A. hydrophila* distributed the highest number among the tested *Aeromonas* strains where 59% (n=8)

of *A. hydrophila* strains resistance to OTC and had the *tetE* gene. This number was followed by *A. veronii* and *A. caviae.* Only *A. caviae* strain that resistance to OTC was not presenting the *tetE*.

Table 4.6. The number of resistance profiles expressed by the tested motile *Aeromonas* strains.

Motile *Aeromonas* Strains	Bact.ID	Resistant to Antibiotics (n)				*TetE* gene (n)
		OTC only	OA only	OTC+OA	FFC only	
A. veronii (n=22)	144HK	0	0	1	0	1
	4HK	1	0	0	0	1
	62HK	1	0	0	0	1
	33HK	0	0	1	0	1
	145HK	1	0	0	0	1
A. hydrophila (n=14)	65HK	1	0	0	0	1
	92HK	0	0	1	0	1
	5HK	1	0	0	0	1
	13HK	1	0	0	0	1
	102HK	1	0	0	0	1
	61HK	1	0	0	0	1
	97HK	0	0	1	0	1
	36HK	1	0	0	0	1
	1HK	0	0	0	1	0
A. caviae (n=4)	127HK	1	0	0	0	0
	67HK	0	0	0	1	0
Total (n)		10	0	4	2	13
Percentages		24	0	10	5	32

The gel electrophoresis visualization from the PCR amplification of the specific genes were showing that of *tetE* targeted size was achieved at 1100 bp as shown in the Figure below (Figure 4.4).

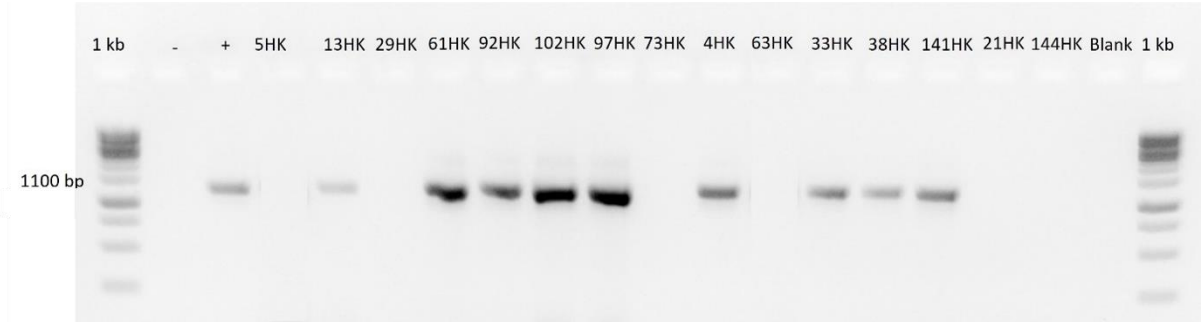

Figure 4.4. PCR amplification of the *tetE* gene, 1 kb ladder, Milli Q water as a negative control (-) and *A. hydrophila* NCIMB 9240 as a positive control (+),

from this figure 9 of motile *Aeromonas* samples presented in the gel with the product size 1100bp. *A. hydrophila* (13HK, 61HK, 92HK, 102HK, and 97HK) were positive to *tetE* gene, meanwhile *A. veronii* (4HK, 33HK, 38HK, and 141HK) were also positive to the *tetE.*

The amplified products of the *tetE* gene presented by motile *Aeromonas* strains in the current study were blasted and analysed with the GeneBank database. The amplified *tetE* products were selected for sequencing to confirm the identity of the gene. The BLAST analysis from the NCBI illustrated that the amplified products were closely related to the *tetE* presented by the reference strains with the query coverage ≥ 99%. The phylogenetic tree of the 4 strains selected for sequence with *tetE* gene described those strains had evolutionary relationships with the reference strains from NCBI where the *tetE* products from *A. hydrophila* 61HK, *A. hydrophila* 1HK, and *A. veronii* 145HK were closely related to the *tetE* from *E. coli* with assession number Y19229.1. The *A. veronii* 4HK was closely related to strains *Aeromonas sp strain A148* with assession number MK592881.1 (Figure 4.5).

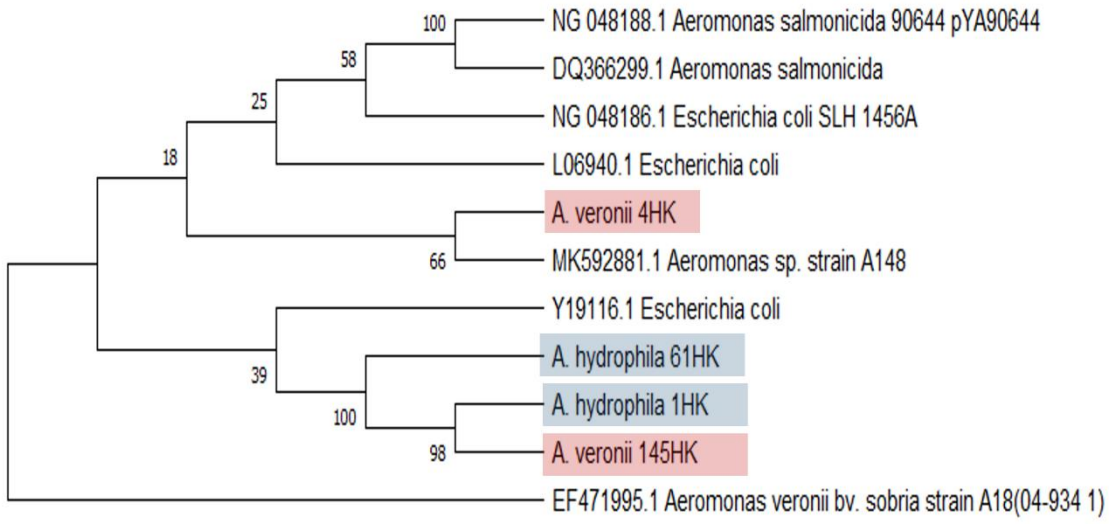

Figure 4.5. Neighbors Joining of phylogenetic tree was constructed based on the sequences of the *tetE* amplified products from motile *Aeromonas* in the present study and the closely related species presented the *tetE* gene. Percentages of the bootstrap value (1000 replicates) were shown at each branch.

4.5.2. Characterization of Virulence Factor from the Tested Motile *Aeromonas* Strains.

The Phenotypic Virulence of Motile *Aeromonas* strains.

The phenotypic virulence of the tested motile *Aeromonas* strains were presented as percentages of each strain within the individual *Aeromonas* species including *A. veronii* (n=22), *A, veronii* (n=14), and *A. caviae* (n=4). In the species level, all of the *A. hydrophila* strains displayed haemolysis, protease, lipase, gelatinase, and DNAse activity when tested on the selective agars. The most common phenotypic virulence factor expressed by all the tested strains was gelatinase, where all of *A. hydrophila* and *A. caviae* expressed gelatinase. The next most common virulence factor expressed was protease and lipase where only all *A. hydrophila* strains expressed both of the virulence factors. This number was followed by *A. veronii* with 91% (n=20) and *A. caviae* with75% (n=3). The less common expression was biofilm formation where was expressed by 57% (n=8) of the *A. hydrophila* strains and followed by *A. veronii* and *A. caviae* with 41% (n=9) and 25% (n=1) respectively. Haemolysis which is considered as one the most important virulence factor was also expressed by all of the *A. hydrophila* strains tested, greater than the number of expressions from both *A. veronii* and *A. caviae* (Figure 4.6).

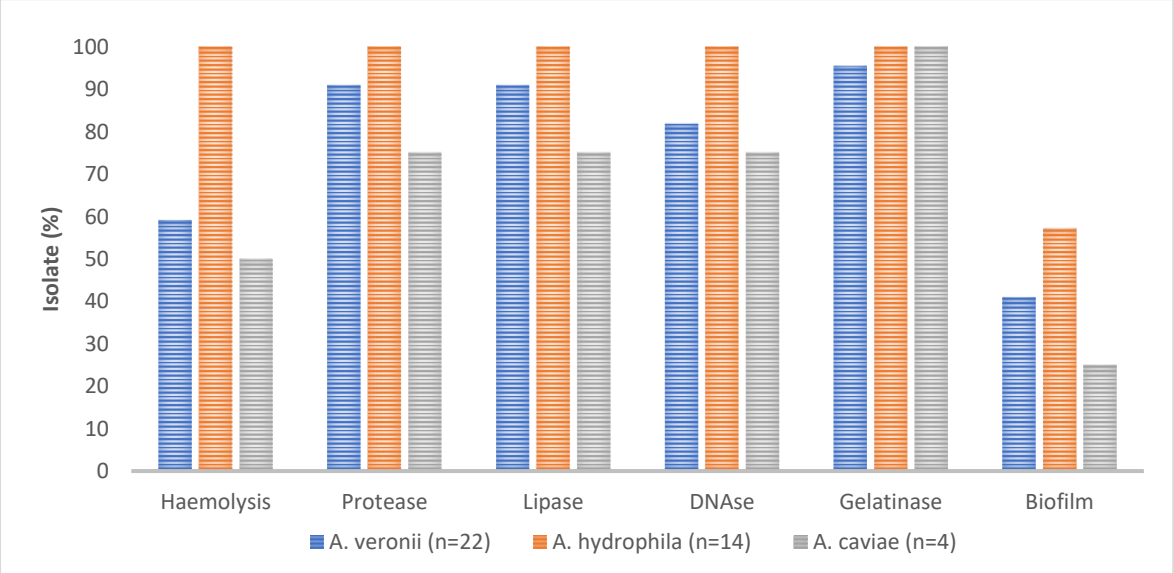

Figure 4.6. The percentages of phenotypic virulence factor of the tested motile *Aeromonas* strains phenotypically assayed.

Evaluating the patterns of phenotypic virulence, it was found that 40% (n=16) of the tested strains expressed all virulence phenotypes. Where, A. *hydrophila* strains distributed 57% (n=8) of the tested *A. hydropjila* strains, this proportion was followed by *A. veronii* with 32% (n=7) of the tested *A. veronii* strains. Likewise, with similar trend, 43% (n=6) of tested *A. hydrophila* strains had 5 combined phenotypic virulence and followed by *A. veronii* with 14% (n=3). Meanwhile the lowest combination consisted of two phenotypic virulence, where each 1 of *A. veronii* and *A. caviae* strain had these two phenotypic variances combined (Figure 4.7).

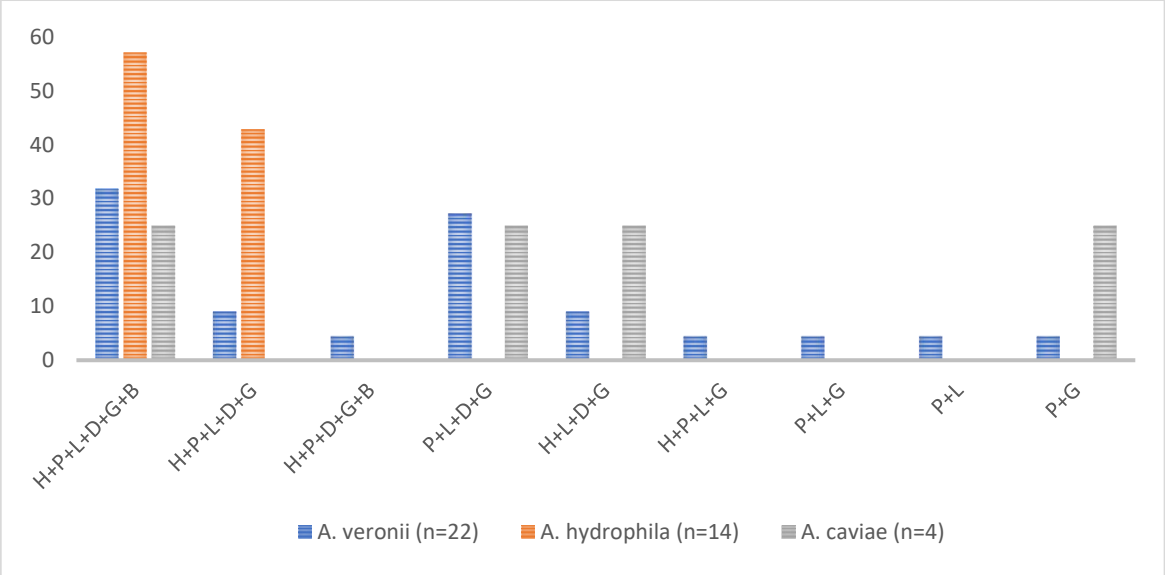

Figure 4.7. The pattern of the virulence factors phenotypically tested as a group of motile *Aeromonas*. H: hemolysis, P: protease, L: lipase, D; DNAse, G: gelatinase, B; Biofilm.

The positive expression was shown by the presentation of the area around the wells more specifically DNAse activity were showing pink colour surrounding the wells, except the biofilm formations where the positive results were shown as darkened of the strains colonies area. the positive results of the phenotypic virulence expression of the tested motile *Aeromonas* strains on the agar media of each test were shown in the Figure 4.8.

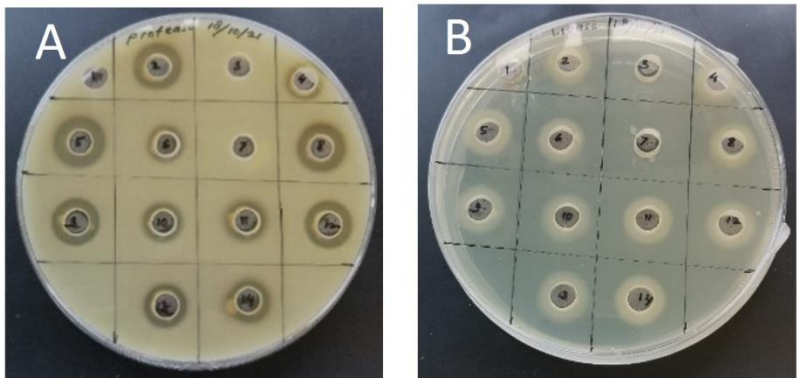

Figure 4.8. The reaction of phenotypic virulence profiles from motile *Aeromonas* strains of extracellular protease (A), lipase (B). Positive reactions were indicated by the zone around the wells and negative results were shown by no zones developed around the wells.

The presence of virulence genes from motile *Aeromonas* strains.

In the level of motile *Aeromonas* as a group, a higher number of strains from each of the 3 bacterial species had the act gene detected. This gene is associated with the affecting the fluid secretion. *act* gene was detected within bacterial strains tested genotypically with 95% (n=38) of the total strains. This figure was followed by *lip* with 70% (n=28) and both of *ser* and *aerA* were presented at the same number by 58% (n=3) of the total strains. Among the species level, the highest number of virulence genes detected was from the *A. hydrophila* strains. The *act* gene was the most frequently detected by the tested strains where, all *A. caviae* strains (n=4) had *act* gene and followed by *A. veronii* with 95% (n=20) of the tested A. veronii strains and *A. hydrophila* with 93% (n=13) of the tested *A. hydrophila* strains. The *aerA* gene which is responsible for producing the aerolysin toxin is often presented by the strains recovered from the diseased fish and considered as one of the most important gene contributing to the infection required for pathogenicity and was detected in 71% (n=10) of the *A. hydrophila* strains followed by *A. veronii* and *A. caviae* with the same proportion. The *hylA* was only presented by *A. hydrophila* with small proportion 14% (n=2) of the total strains. *A, hydrophila* strains were also presented by far the highest *gcat* and *lafA* gene where both with same proportion where 93% (n=13) of *A. hydrophila* strains had *gcat* and *lafA* genes. Virulence genes were detected in all of the *A. hydrophila* strains

tested, whereas none of the *A. veronii* and *A. caviae* strains presented *hylA* gene (Figure 4.9).

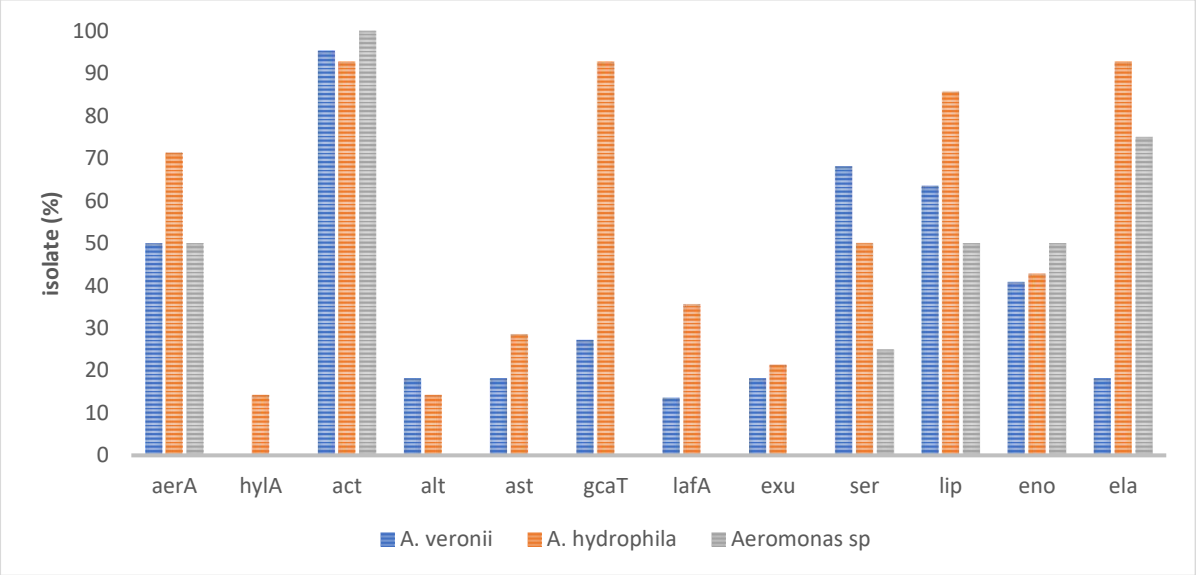

Figure 4.9. The percentages of phenotypic virulence factor of the tested motile *Aeromonas* strains phenotypically assayed.

The virulence genotypes of each motile *Aeromonas* strains recovered from the current study was described in the Table 4.7. The virulence genes presented were ranging from the lowest with two genes that presented by *A. veronii* 86HK and *A. caviae* 31HK, where both strains were recovered from Catfish samples. Whereas the highest number of genes were presented by *A. hydrophila* 92HK with 10 genes and followed by the species *A. hydrophila* 65HK. Meanwhile, the highest among *A. veronii* strain was *A. veronii* 4HK and 144HK with the proportion (7 genes).

Table 4.7. The distribution of virulence genes presented by motile *Aeromonas* strains.

Motile *Aeromonas*	Bact.ID	Origin	Virulence Genes
A. veronii	64HK	Tilapia	*act* *gcat* *lip* *ela*
A. veronii	21HK		*aerA* *act* *lip* *eno*
A. veronii	104HK		*act* *ast* *ser* *lip*
A. veronii	141HK		*aerA* *act* *gcat* *exu* *ser*
A. veronii	144HK		*aerA* *act* *ast* *gcat* *ser* *lip* *eno*
A. veronii	4HK		*aerA* *act* *ast* *gcat* *ser* *lip* *eno*
A. veronii	63HK		*aerA* *act* *lafA* *ser* *lip*
A. veronii	62HK		*aerA* *act* *alt* *exu* *ser*
A. veronii	70HK		*act* *alt* *ast* *ela* *ser*
A. veronii	162HK		*aerA* *act* *exu* *ser*
A. veronii	95HK		*aerA* *act* *alt* *lip*
A. veronii	71HK		*aerA* *act* *lafA* *exu* *ser* *eno*
A. veronii	164HK		*aerA* *act* *gcat* *lip*
A. veronii	24HK		*act* *lafA* *ser* *lip*
A. veronii	105HK		*act* *ser*
A. veronii	33HK	Catfish	*aerA* *act* *alt* *ser* *lip* *ela*
A. veronii	69HK		*act* *gcat* *lip* *eno*
A. veronii	38HK		*aerA* *ser* *lip* *eno*
A. veronii	73HK		*act* *eno*
A. veronii	75HK		*act* *gcat* *lip* *eno*
A. veronii	86HK		*act* *ser*
A. veronii	145HK		*act* *ser* *lip* *eno*
A. hydrophila	28 HK	Tilapia	*aerA* *act* *gcat* *lip* *ela*
A. hydrophila	93HK		*act* *gcat* *ser* *lip* *ela*
A. hydrophila	130HK		*aerA* *act* *gcat* *lip* *ela*
A. hydrophila	65HK		*aerA* *hylA* *act* *alt* *gcat* *lafA* *ser* *lip* *ela*
A. hydrophila	92HK		*aerA* *hylA* *act* *alt* *ast* *gcat* *exu* *lip* *eno* *ela*
A. hydrophila	5HK		*act* *gcat* *exu* *lip* *ela*
A. hydrophila	115HK		*aerA* *act* *gcat* *lafA* *lip* *eno* *ela*
A. hydrophila	13HK		*aerA* *act* *alt* *exu* *ser* *lip* *eno* *ela*
A. hydrophila	102HK		*act* *ast* *gcat* *lafA* *ser* *eno* *ela*
A. hydrophila	61HK		*aerA* *ast* *gcat* *lafA* *lip* *eno* *ela*
A. hydrophila	97HK		*aerA* *act* *ast* *gcat* *lafA* *ser* *eno* *ela*
A. hydrophila	1HK		*aerA* *act* *gcat* *lip* *ela*
A. hydrophila	166HK		*aerA* *act* *gcat* *lip* *ela*
A. hydrophila	36HK	Catfish	*aerA* *act* *gcat* *ser* *lip*
A. caviae	67HK	Catfish	*aerA* *act* *lip* *ela*
A. caviae	127HK		*aerA* *act* *eno* *ela*
A. caviae	31HK		*act* *lip*
A. caviae	68HK		*act* *ser* *eno* *ela*

Gel electrophoresis of the motile *Aeromonas* strains tested to the virulence genes were described on the Figure 4.10 to 4.13 which included *act, lip, eno,* and *aerA* genes.

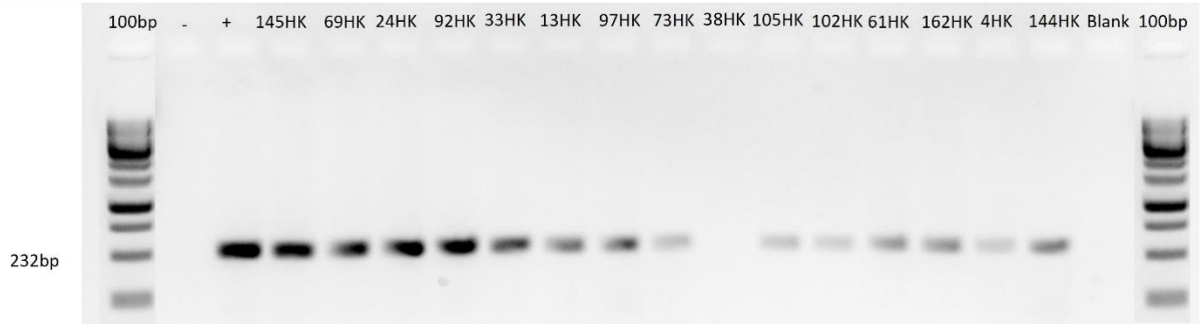

Figure 4.10. PCR amplification of the *act* gene, with 100bp ladder, Milli Q water as a negative control (-) and *A. hydrophila* NCIMB 9240 as a positive control (+). All tested samples presented in the gel with the product size 232bp except *A. veronii* 38HK.

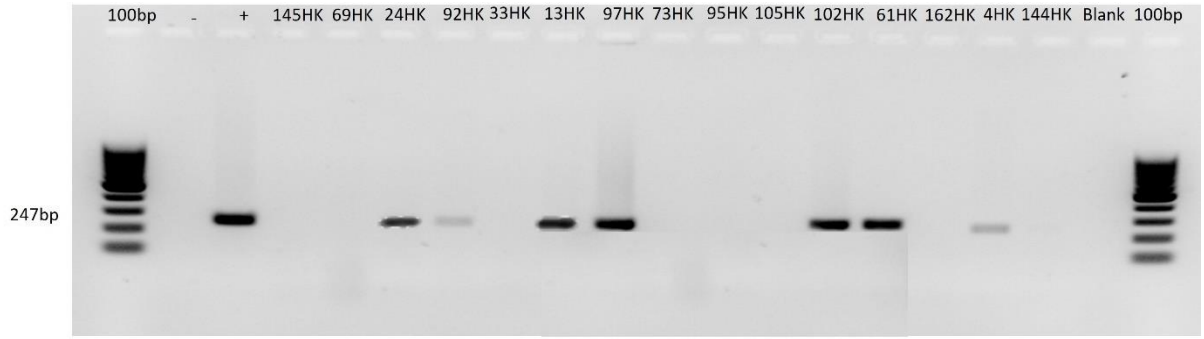

Figure 4.11. PCR amplification of the Lipase (lip) gene, with 100bp ladder, Milli Q water as a negative control (-) and *A. hydrophila* NCIMB 9240 as a positive control (+), 7 motile *Aeromonas* samples presence in the gel with the product size 247bp including *A. hydrophila* (24HK, 92 HK, 13HK, 61HK, and 61HK) and *A. veronii* (102HK and 4HK).

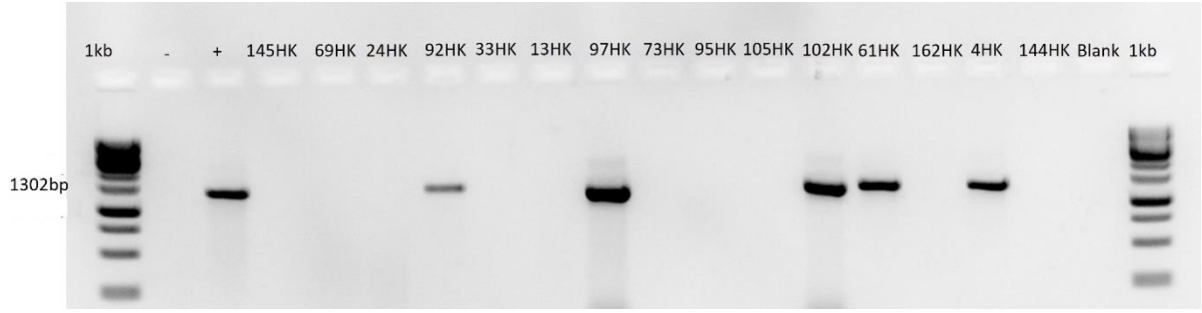

Figure 4.12. PCR amplification of the Enolase *(eno*) gene, with 1kbp ladder, Milli Q water as a negative control (-) and *A. hydrophila* NCIMB 9240 as a positive control (+), 5 motile *Aeromonas* samples presence in the gel with the product size 1302bp including *A. hydrophila* (92 HK and 61HK) and *A. veronii* (102HK and 4HK).

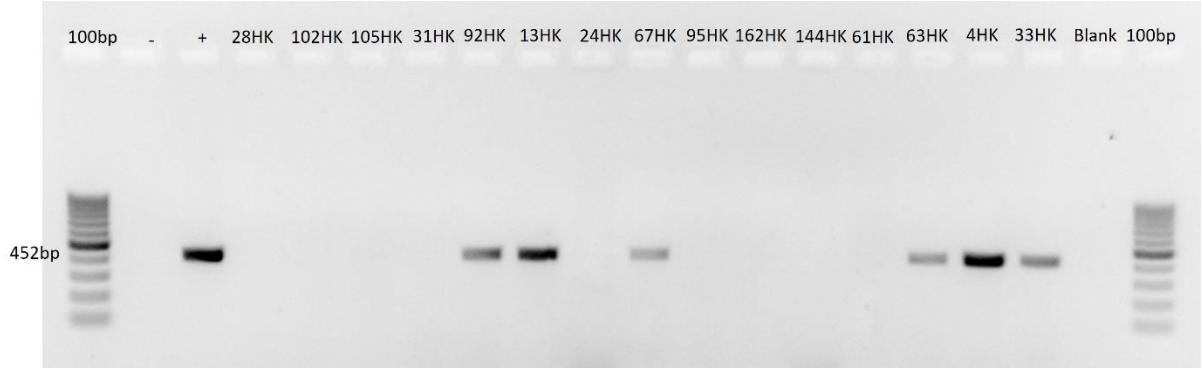

Figure 4.13. PCR amplification of the Aerolysin (*aerA*) gene, with 100bp ladder, Milli Q water as a negative control (-) and *A. hydrophila* NCIMB 9240 as a positive control (+), 6 motile *Aeromonas* samples presence in the gel with the product size 452bp including *A. hydrophila* (92 HK and 13HK), *A. caviae* (67HK), and *A. veronii* (63HK, 4HK, and 33HK).

The Pattern of Virulence Genes of Motile *Aeromonas* Strains.

An attempt was made in effort to find the most common virulence gene pattern or combination between all the strains screened in this study. The grouping of 2 gene combinations indicated that the strains contained *aerA+act* and this was by far the most common combination of virulence genes detected with 53% (n=21) of all strains tested. The combination of both genes was also considered as the most important genes related to pathogenicity as these genes encode for aerolysin and enterotoxin. Furthermore, the combination of three genes predominantly had *act+aerA+lip* genes

that were presented by 38% (n=15) strains from the samples showing the clinical signs of either external, internal or both (Table 4.8). This three genes combination also considered playing important role in correlation to the ability of the strains in causing the disease, where *lip* contribute to increasing the severity of infection. Interestingly, most of the strains with higher virulence genes detected had also higher clinical signs identified, i.e., the *A. hydrophila* with 9 and 10 strains detected (65HK and 92HK) were recovered from fish samples with several clinical signs including external clinical signs such as fin and tail rots, redness on the skins, loss of appetite, and ulcerations and internal clinical signs including kidneys congestion, fluid in the abdominal and haemorrhages. This also true in the *A. veronii*, where the higher virulence genes presented by both *A. veronii*144HK and 4HK with 7 genes were also recovered from fish samples with more frequently clinical signs. The strains with the combined *act*+*aerA*+*lip* was also grouped based on the number of virulence genes governed the strains categorized as low virulence (LV), medium virulence (MV), and High virulence (HV). The categorization based on the number of genes where *A. veronii* from low (with 2 - 4 genes), medium (5 - 6 genes), and high (≥ 7 genes. However, since the lowest virulence genes identified in *A. hydrophila* strains was 5 with the highest 10 genes, so *A. hydrophila* strains were categorized as low (5 – 6 genes), medium (7 - 8 genes), and high (9 - 10 genes). Where all of the strains categorized as HV were recovered from the internal organs of tilapia samples with combined clinical signs of the disease. This categorization was considered required in the current study to group the strains based on the virulence level and to help in selecting the strains for further challenge test in wax moth larvae model.

Table 4.8. The distribution of clinical signs of fish samples where the strains containing the *act+aerA+lip* genes combined.

Aeromonas strains	Bact. ID	Origin	Clinical signs		Organ's origin	Virulence genes combination	Vir. level
			External	Internal			
A. hydrophila	92HK	Tilapia	Tail and fin rot, ulcerations	Fluid in the abdominal, haemorrhages of the liver	kidney	*aerA+act+hylA+alt+ast+gcat+exu+lip+eno+ela*	HV
A. hydrophila	65HK	Tilapia	Tail rot, redness on skin	Fluid in the abdominal, kidneys congestion	Kidney	*aerA+hylA+act+alt+gcat+lafA+ser+lip+ela*	HV
A. hydrophila	13HK	Tilapia	Ulcerations	Fluid in the abdominal, kidneys congestion	kidney	*aerA+act+alt+exu+ser+lip+eno+ela*	HV
A. hydrophila	115HK	Tilapia	Abdominal distention	Fluid in the abdominal	Kidney	*aerA+act+gcat+lafA+lip+eno+ela*	MV
A. veronii	144HK	Tilapia	Abnormal swimming	Pale of the liver, fluid in the in the abdomen	Kidney	*aerA+act+ast+gcat+ser+lip+eno*	MV
A. veronii	4HK	Tilapia	Loss of appetite	Liver and spleen enlargement	Kidney	*aerA+act+ast+gcat+ser+lip+eno*	HV
A. veronii	21HK	Tilapia	Tail rot	Fluids on the abdomen	Spleen	*aerA+act+lip+eno*	LV
A. veronii	63HK	Tilapia	Gills pallor	Redness of spleen	Spleen	*aerA+act+lafA+ser+lip*	MV
A. veronii	62HK	Tilapia	tail rot	enlargement of liver and spleen	Spleen	*aerA+act+alt+lip+ser*	MV
A. hydrophila	28 HK	Tilapia	Tail rot, ulcerative lesions	Kidney's congestion, red pallor	Kidney	*aerA+act+gcat+lip+ela*	LV
A. hydrophila	130HK	Tilapia	fin and tail rot	absence of clinical signs	Spleen	*aerA+act+gcat+lip+ela*	LV
A. hydrophila	5HK	Tilapia	fin and tail rot	absence of clinical signs	Kidney	*aerA+act+gcat+ser+lip*	LV
A. veronii	144HK	Tilapia	loss of appetite	kidney enlargement	Spleen	*aerA+act+last+gcat+ser+lip++ela*	HV
A. veronii	95HK	Tilapia	fin and tail rot	kidneys congestion, red pallor	Spleen	*aerA+act+alt+lip*	LV
A. caviae	67HK	Catfish	sluggish movement	absence of clinical signs	Spleen	*aerA+act+lip+ela*	LV

4.5.3. Investigating the pathogenicity of the tested strains challenged with the wax moth larvae.

Effect of temperature on the larvae survival post infection with the tested strains. The survival of *A. veronii* strain 62HK which was considered to have medium virulence, was greater in the animals incubated at the lowest temperature (15^0C) compared with any other incubation temperature (Fig 4.14). A similar trend was observed in survival between all of the temperatures with no mortalities in the control (no experiment) and 1 mortality in the TG3 (injected PBS). A statistically significant difference was found between different temperature. In brief, there were 37% (n=11) of larvae survived at the end of experiment time periods (96h) at 15^0C incubation. This number was followed by the incubation at 22^0C where 3% (n=1) of larvae survived at

the end of the experimental time. No larvae survive at incubation temperatures 28 at the end of experiment time. whereas at 37^{0}C, no larvae survive since the third day post exposure.

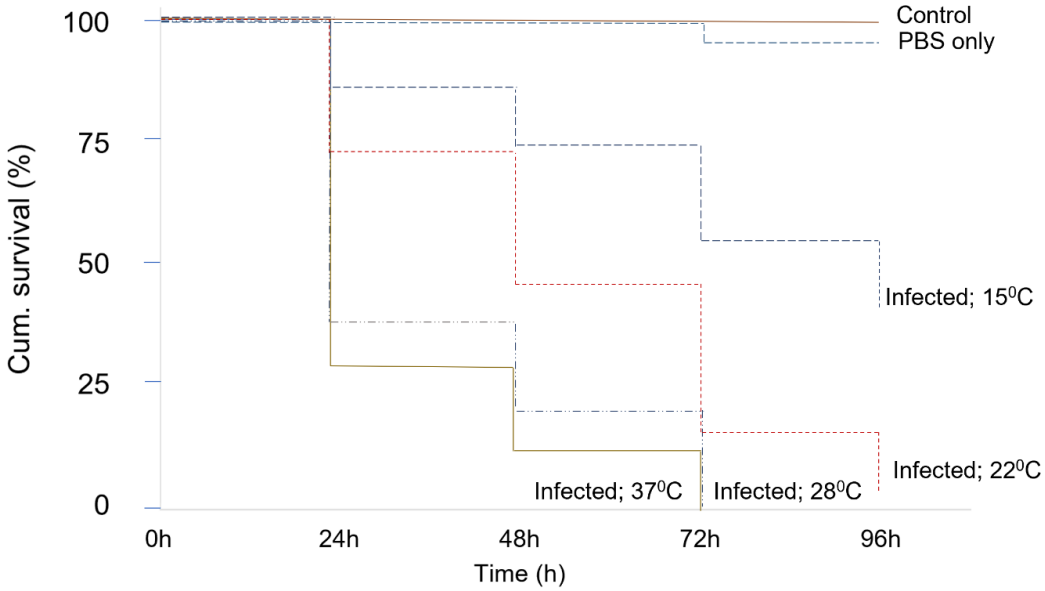

Figure 4.14. Cumulative survival of larvae infected with *A. veronii 62HK,* PBS only and no handling (control) and then stored at different temperatures.

The second treatment group of larvae exposed with *A. hydrophila* 115HK were also showing the identical survival trends with the group infected with *A. veronii 62HK.* The incubation at lowest temperature provided the highest survival rate to the postinoculation larvae with 37% (n=11) of the tested larvae survived at the incubation 15^{0}C during the experimental time period. This figure was followed by the incubation temperature at 22^{0}C where 13% (n=4) of larvae survived. The identical trend with the previous test above (infection with *A. veronii* 62HK) where the lowest survival rates were shown at the end of experiment were the incubation at 22^{0}C and 37^{0}C where all infected larvae dead. However, the incubation temperature with the fastest result provided where all larvae death was the incubation at 37^{0}C where no larvae survived since the third day of post infection (Figure 4.15).

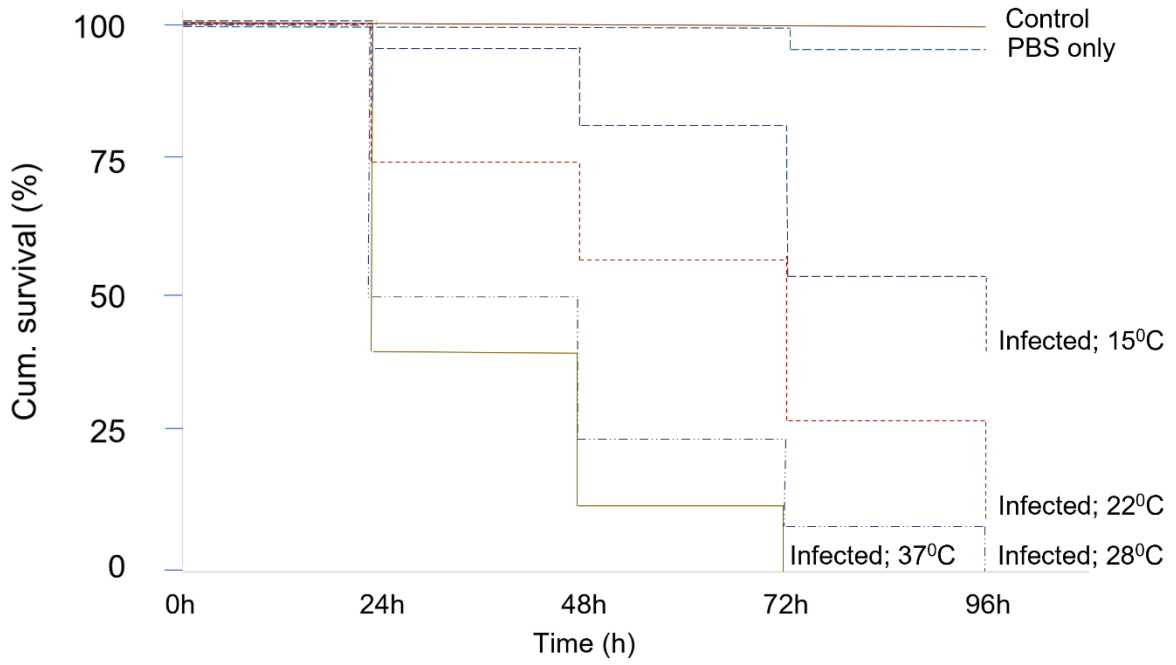

Figure 4.15. The cumulative survival of infected with *A. hydrophila* 115HK incubated at different temperatures, PBS only and no handling (control) as negative controls.

The morphology of infected larvae and stored at different temperatures changed gradually where the infected larvae the TG1 (infection with *A. hydrophilai* 115HK) showed melanisation post exposure since day 1 of experiment, where 65 - 70% larvae each strains treatment experiences the melanisation into light brown, light dark and dark black. The larvae with the incubation at 28 and 37^{0}C showed the melanization into dark brown and black since the second day of post infection (Figure 4.16).

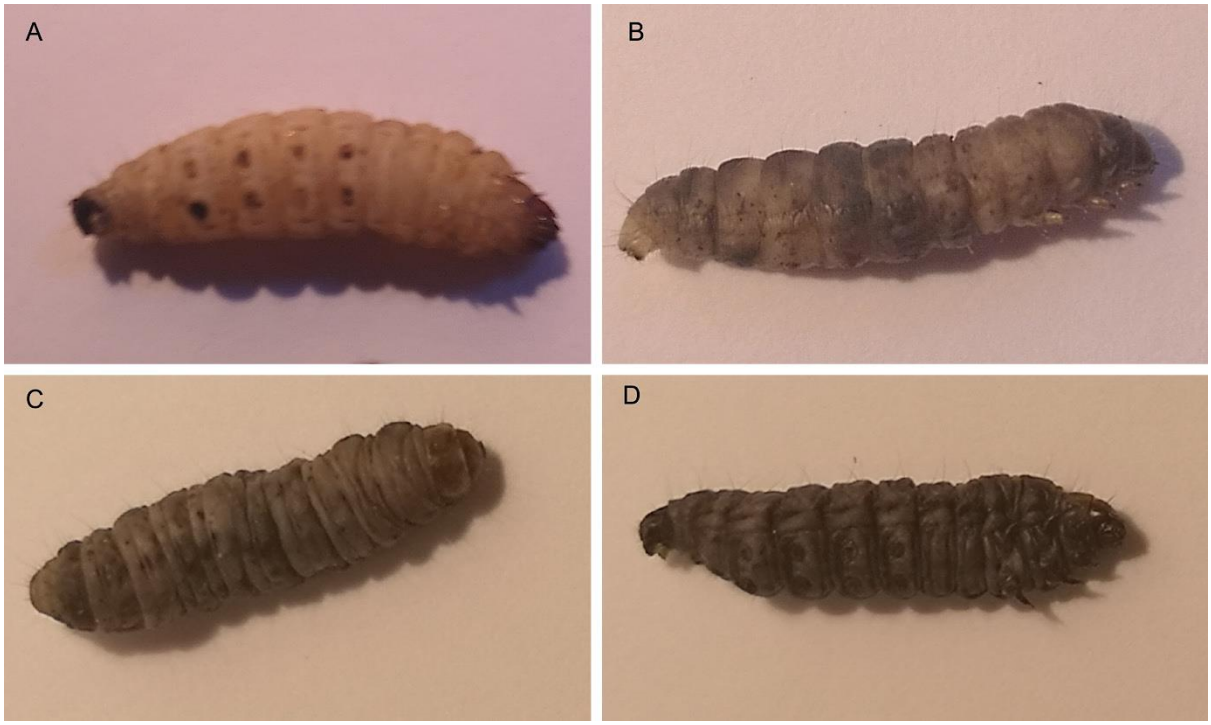

Figure 4.16. The infected larvae during the experiment, the larvae was exposed with *A. hydrophila* 115HK. A. 2h post exposure, most of larvae experienced which was started from the pro-leg area, B. the first 24h the larvae experience the melanization on the part or all body surface into light and dark brown, C. the 48h post exposure, most of the larvae turned into dark brown and dark black, D. 72h post exposure, most of the larvae changed colour into dark black.

Effect of heat-killed (HK) *Aeromonas* strains to the survival of tested larvae.

Heat-killed bacteria were also performed to the two strains to study the effect of the inherent toxic nature of bacterial material in causing the larvae mortality (Figure 4.17). The heat-killed strains were confirmed by a lack of colonies on TSA agar. there was significant effect of heat-killed strains to the larvae survival where 97% (n=29) of all larvae survived in the TG1 (heat-killed *A. veronii* 62HK) and TG2 (heat-killed *A. hydrophila* 115HK). Whereas the positive controls (infected with *A. veronii* 62HK and *A. hydrophila* 115HK) were causing no larvae survived since the third day of infection.

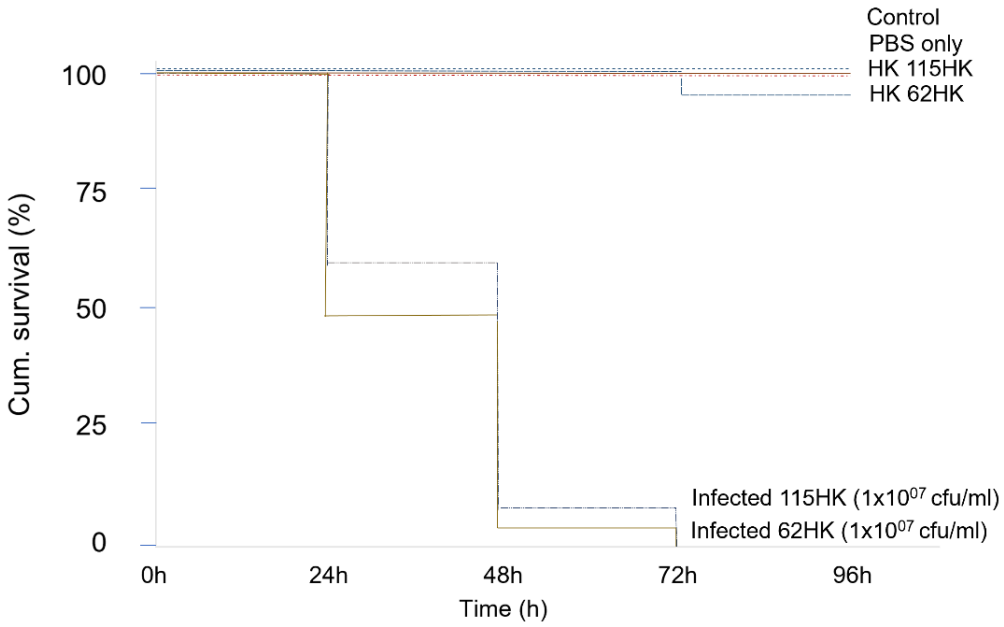

Figure 4.17. The effect of heat-killed larvae to the larvae survival. HK strains generated significant survival compared with the positive control (the strains without heat-killed).

The experiment of heat-killed strains to the larvae were showing significant difference where until the end time of infection only 1 larva dead. Meanwhile all larvae dead when infected with the strains without previously heat-killed. The change colours of larvae infected without heat-killed strains were started at the second day post infection into the dark black (Figure 4.18).

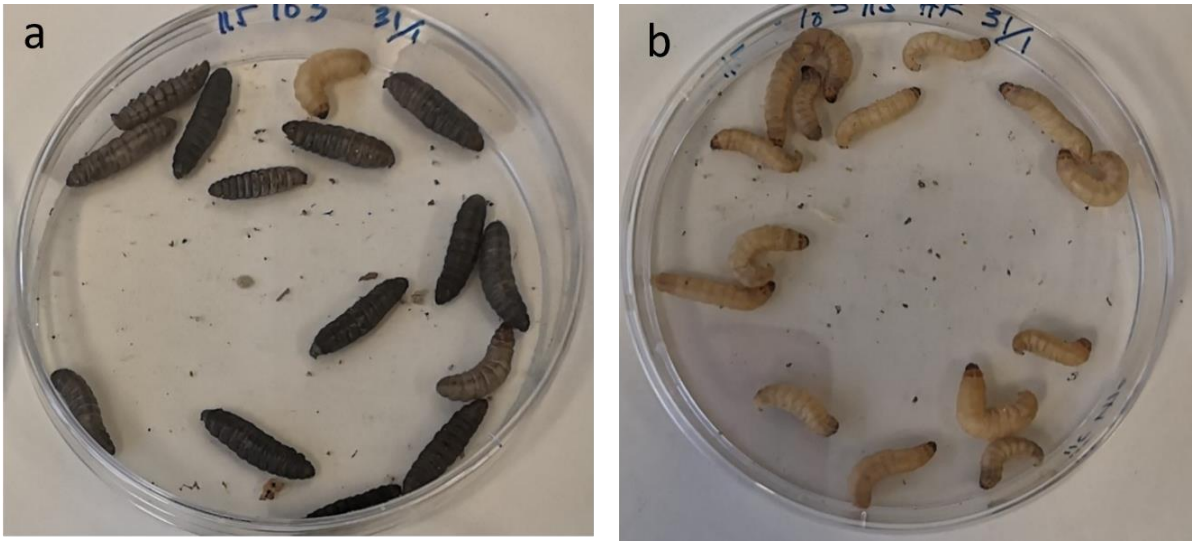

Figure 4.18. The effect of heat-killed strains to the larvae mortality, a. infected *with A. hydrophila 115HK*, and b. injected with heat-killed *A. hydrophila 115HK*

4.5.4. Effect of different motile *Aeromonas* concentration to the larvae survival.

The Kaplan Meier analysis was showing that he log rank of the overall comparisons were significant with p value < 0.01 to all treatment groupThe higher concentration of *A.veroniii* 105HK the lower survival rate of the infected larvae. the concentraion of $1x10^7$ CFU/ml resulted the lowest survival rate with 33% (n=10) at the end of experiments time. Negative control where the larvae injected with PBS only and no handling larvae (control) had the highest survival rate as shown in the Figure 4.19.

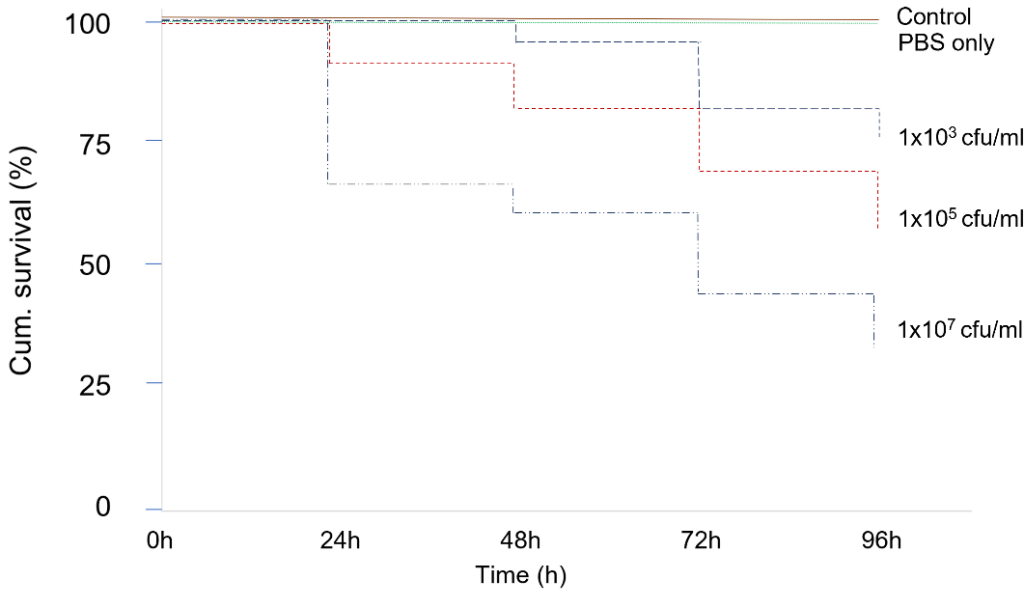

Figure 4.19. The Cumulative survival of infected with different concentration of *A. veronii 105HK*, PBS only and no handling (control) were used as a negative controls.

The two concentration $1x10^5$ CFU/ml and $1x10^7$ CFU/ml provided the lowest survival rate where 0% of larvae survive at the end of post infection. The highest dose of infected strain resulted the fastest of no larvae survived since the 72h post infection. Whereas the lowest concentration ($1x10^3$ CFU/ml) provided the highest survival among the doses of infection where 60% (n=18) of larvae survived at the end of experiment (Figure 4.20).

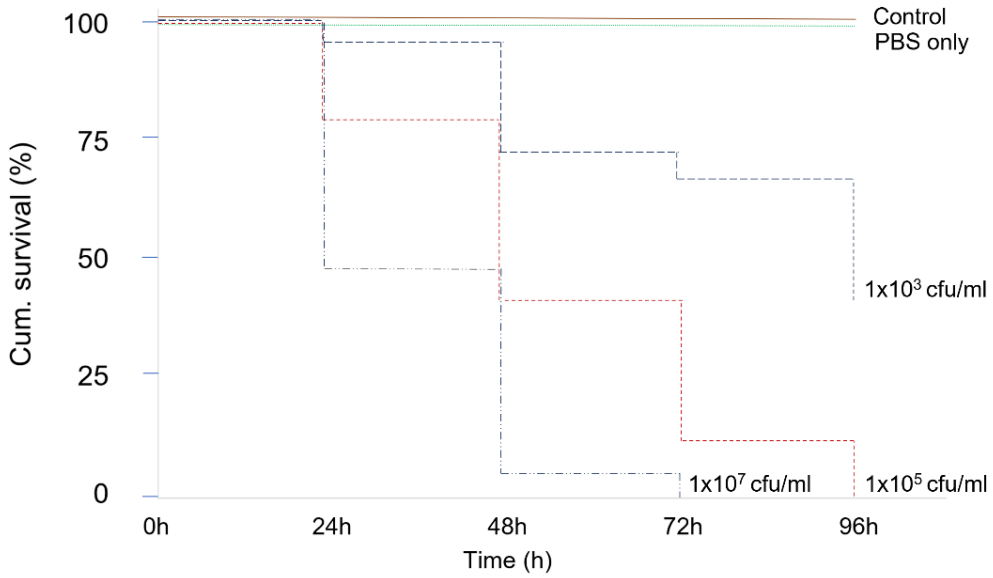

Figure 4.20. The Cumulative survival of infected with different concentration of *A. veronii 62HK*, PBS only and no handling (control) as the negative controls.

Larvae mortality infected with different concentration of *A.veroniii* 4HK.

The highest survival was found in the lowest concententration of infected larvae with 40% (n=12) at the end experiments time and highest concentration caused the no larvae survive since the second day post infection (Figure 4.21).

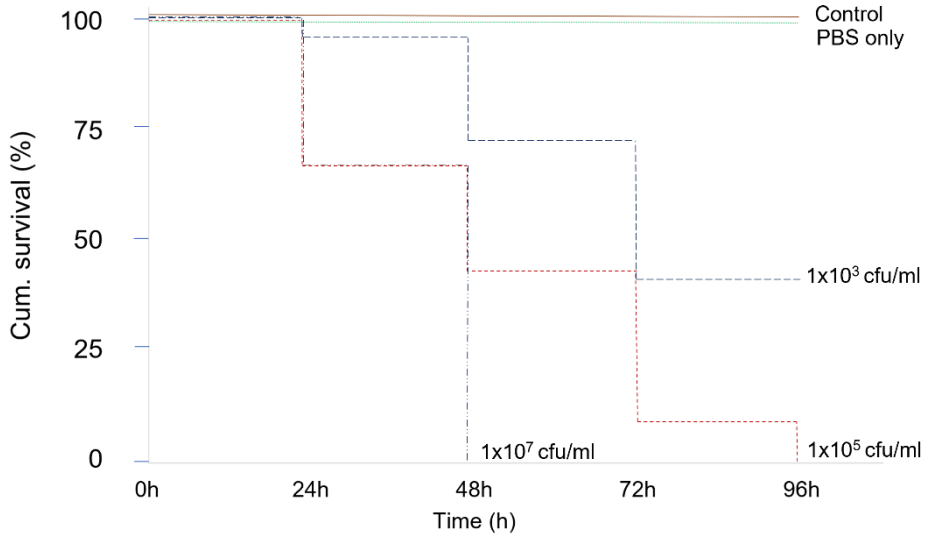

Figure 4.21. The Cumulative survival of larvae infected with different concentration of *A. veronii 4HK*, PBS only and no handling (control) as the negative controls.

Larvae survival infected with different concentration of *A.hydrophila 93HK*. the KM plot was showing that the higher concentration of *A.hydrophila 93*HK the lower survival rate of the infected larvae. The highest survival was found in the lowest concententration of infected larvae with 73% (n=22) at the end time peiods of the experiments (Figure 4.22).

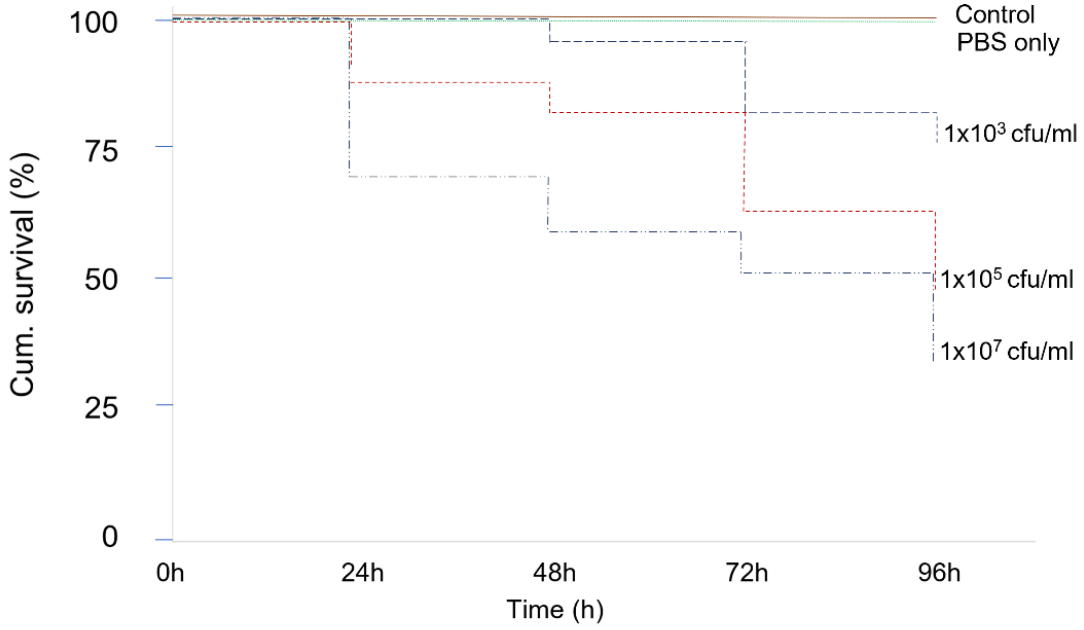

Figure 4.22. The Cumulative survival of larvae infected with different concentration of *A. hydrophila 93HK*, PBS only and no handling (control) as the negative controls.

The infected larvae survival with different concentration of *A.hydrophila 115HK*. KM plot was showing that the higher concentration of *A.hydrophila* 115HK the lower survival rate of the infected larvae. the highest survival was found in the lowest concententration of *A. hydrophila 115HK* with 60% (n=18) at the end time peiods of the experiments. The highest concentration cause no larvae survive since the third day post infection (Figure 4.23).

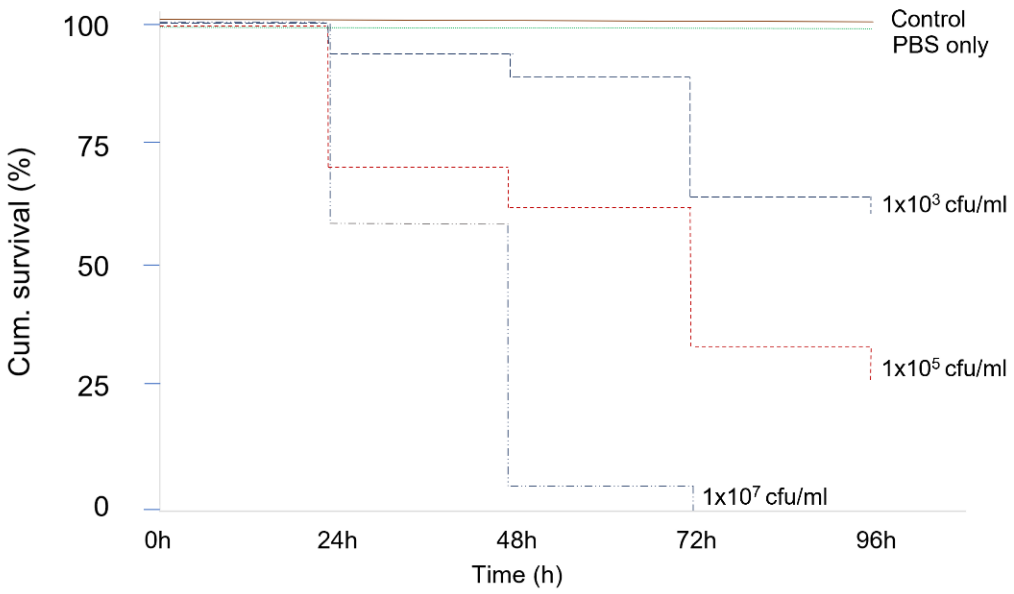

Figure 4.23. The Cumulative survival of infected with different concentration of *A. veronii 4HK*, PBS only and no handling (control) as the negative controls.

Larvae mortality infected with different concentration of *A.hydrophila 92HK*. The KM plot indicated that the highest strains concentration caused the lowest survival rate, and the lowest concentration provided the highest survival to the infected larvae. The lowest survival rate was resulted by the infection with the concentration $1x10^7$cfu/ml where no larvae survive at the second day post infection (Figure 4.24).

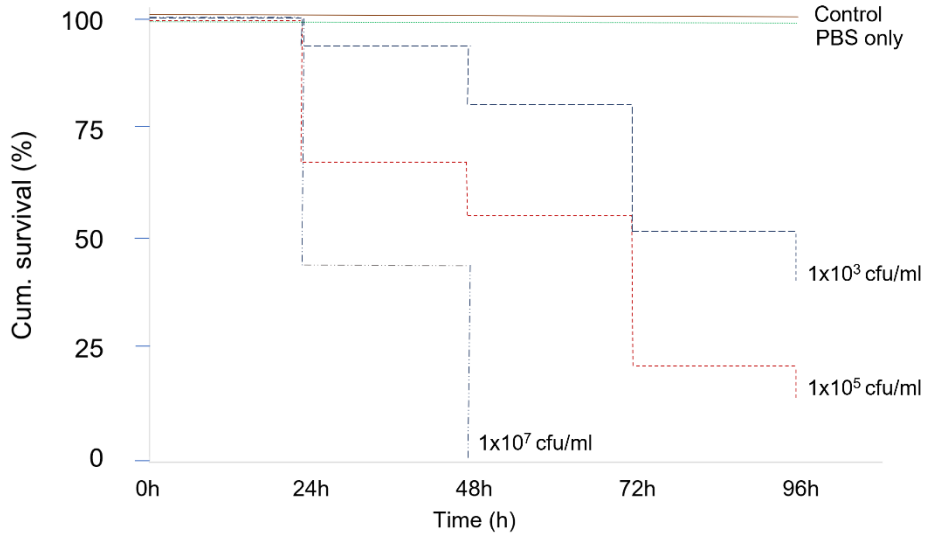

Figure 4.24. The Cumulative survival of infected with different concentration of *A.hydrophila 92HK*, PBS only and no handling as the negative controls.

The infection effect of strains with different concentration was clearly shown in the figure, where the higher concentration the greater number of larvae experienced the melanisation from both treatment with *A. veronii* 62HK and *A, hydrophila* 115HK (Figure 4.25).

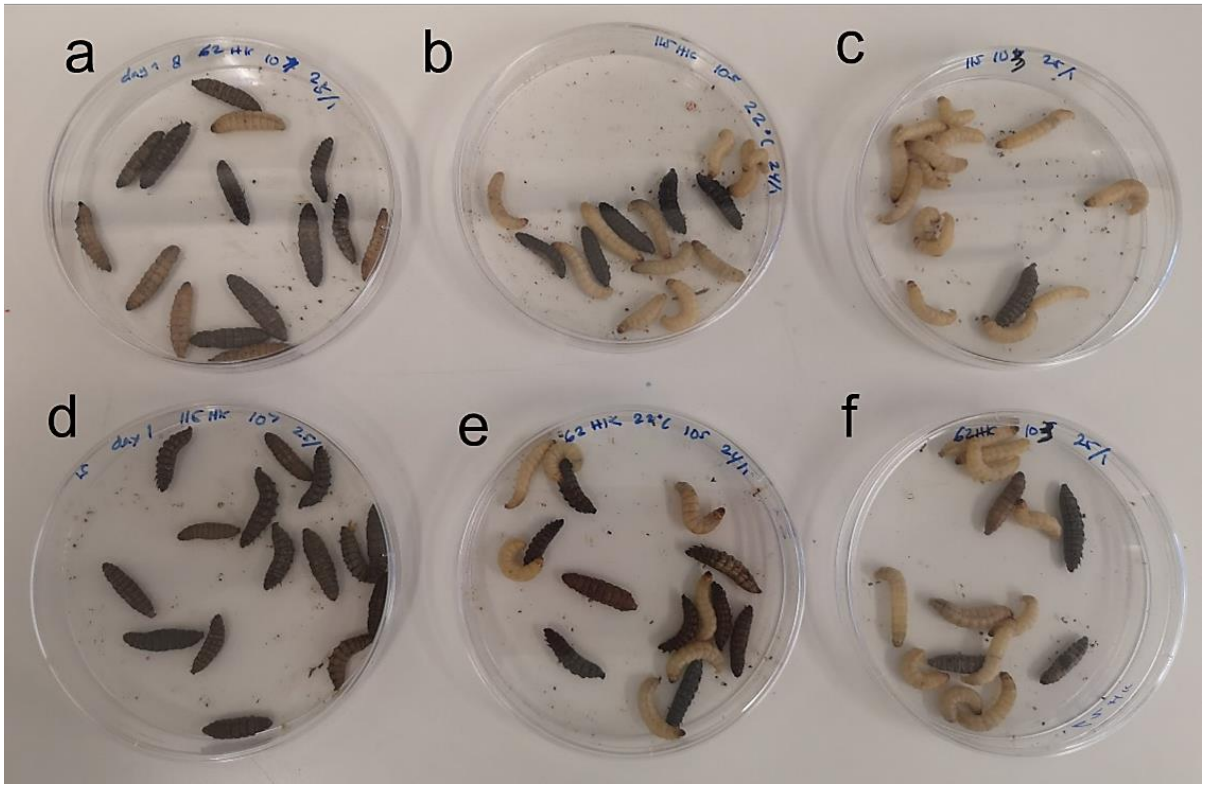

Figure 4.25. The effect of different concentrations of CFU on the infected larvae. Different concentrations resulted in different numbers of larvae with morphological changes during the experiments time exposed with *A. veronii* 62HK and *A. hydrophila* 115HK. a and d. infection with $1x10^7$ cfu/ml, b and e. infection with $1x10^5$ CFU/ml, c and f. infection with $1x10^3$ cfu/ml.

4.5.5. Effect of different *Aeromonas* strains with the same concentration.

The treatment of infection with the same strain's concentration were tested to 6 *Aeromonas* strains. Both negative controls were showing no mortality during the experiment time periods. The effect of infection of 6 different strains were significant to the larvae survival in each case.

The larvae were infected with the *A. veroniii* strains (1x10^5 CFUml). KM plot indicated that the lowest survival rates were found in the treatment infection with *A. veronii* 4HK and 62HK where no larvae survive at the end of experiments times. The highest survival rates between the tested strains was found in the infection with *A. veronii* 105HK with 53% (n=16) larvae survive (Figure 4.26).

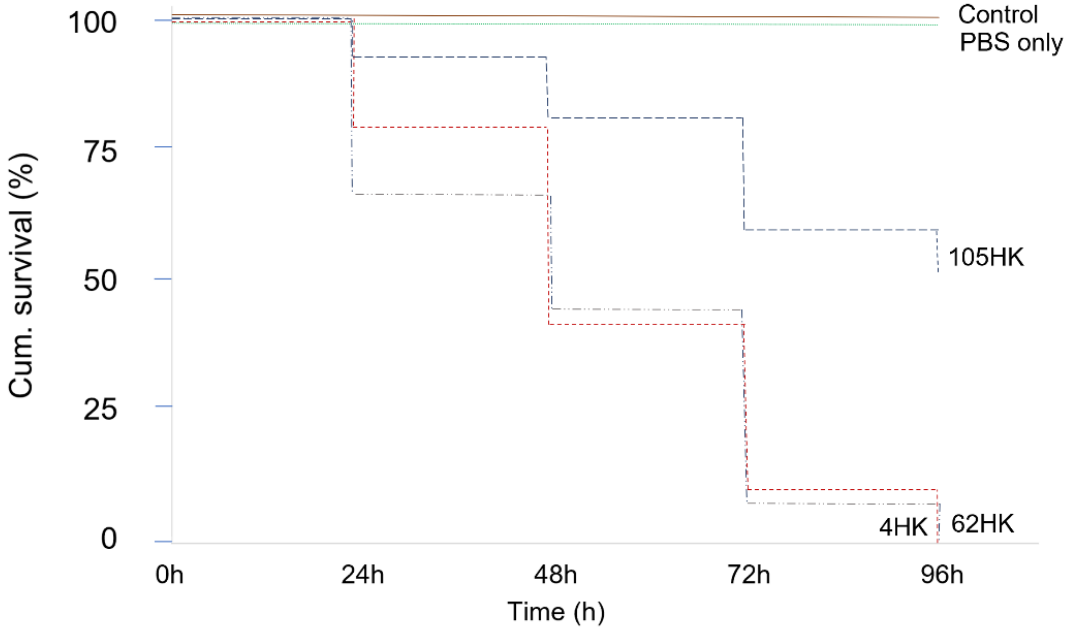

Figure 4.26. The Cumulative survival of infected larvae with motile *A. veronii* strains (1x10^5 cfu/ml)**.** PBS only and no handling (control) as the negative controls.

The larvae were infected with the *A.hydrophila* strains (1x10^5 CFU/ml).

KM plot was showing that the lowest survival rates were found in the treatment infection with *A. hydrophila* 92HK where only 13% (n=4) survival rate at the end of experiment. The highest survival rates between the tested strains was infection with 93HK with 43% (n=13) of larvae survive (Figure 4.27).

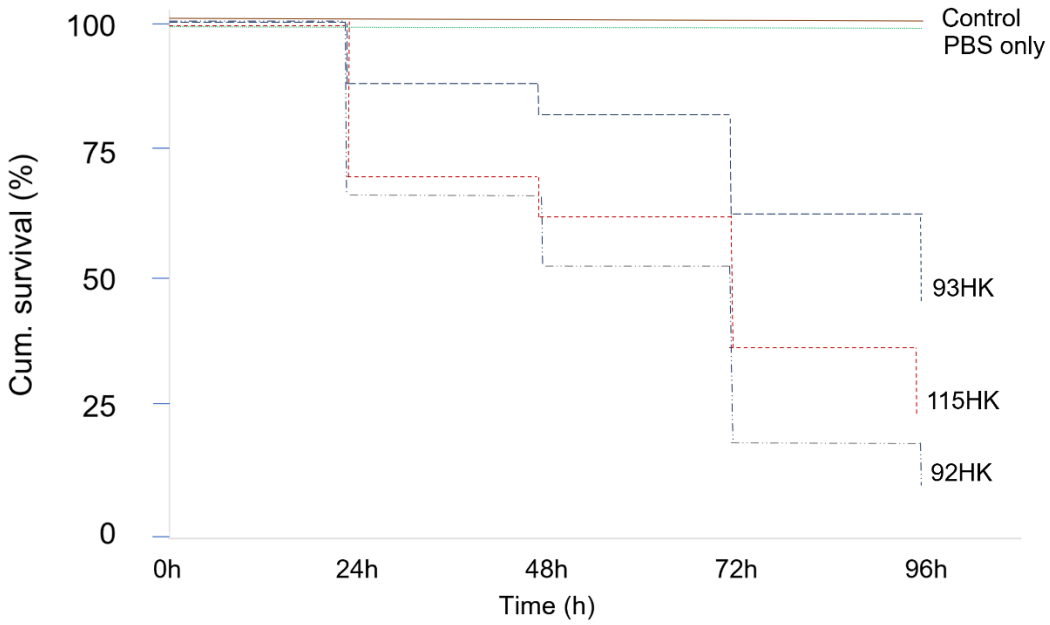

Figure 4.27. The Cumulative survival of infected larvae with motile *Aeromonas* strains ($1x10^5$ cfu/ml)**.** PBS only and no handling (control) as the negative controls.

In the present study, the treatment of infection with the same strain's concentration were tested to 6 motile *Aeromonas* strains ($1x10^5$ CFU/ml). The effect of infection was significant to the larvae mortality. The strain with the higher virulence gene resulted the greater mortality number for both *A. hydrophila* and *A. veronii* strains. *A. hydrophila* 92HK with the highest virulence genes (10 genes) generated the highest mortality rate at 72h among *A. hydrophila* with 24 larvae dead. Likewise, *A. veronii* 4HK as the highest virulence genes resulted the highest mortality with 28. Contrary, the strains with the lowest virulence genes generated the lowest mortality rates, where *A. hydrophila* 93HK and *A. veronii* 105HK resulted 11 and 10 larvae dead at the 72h post inoculation (Figure 4.28).

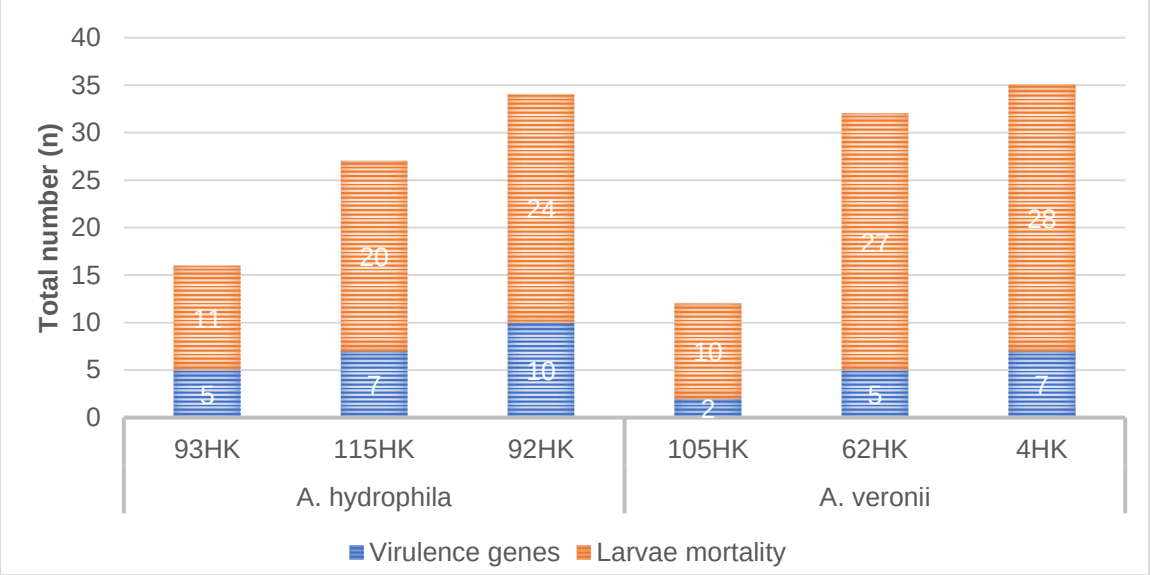

Figure 4.28. The total number of mortality in association with the different virulence genes governed motile *Aeromonas* strains ($1x10^5$ CFU/ml)**.** The graph was showing the total mortality of the 72h post inoculation because during this time the data provided obvious different mortality number, whereas at the end of the experiments two of the strains caused all the larvae mortile.

4.5.6. Antibiotics treatment to the infected larvae with the tested strains.

The treatment of antibiotics to two different group of larvae infected with the strains from *A. hydrophila* and *A. veronii* were showing significant survival rate to the infected larvae based on the log rank analysis with p value < 0.01. The positive control groups were all dead at the end of the experiments time and all no mortality was found in the negative controls. The KM plot was showing that the antibiotics treatment increase the survival rates of the infected larvae during the experiments time. However, the survival rate decreased at the 72 and 96h post infection and antibiotics treatment (Figure 4.29).

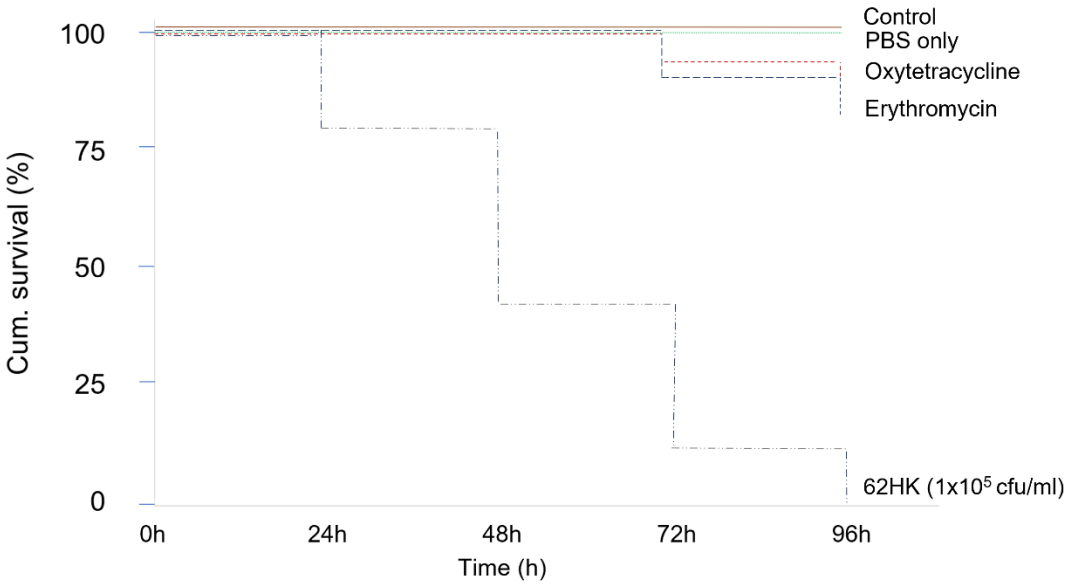

Figure 4.29. The cummulative percent survival of wax moth larvae exposed to A. veronii strain 62HK and treated with either oyxtetracycline or ethyromycin antibiotics.

The morphological changes between the infected larvae with *A. veronii* 62HK without further antibiotics treatment and the infected larvae which then treated with antibiotics affected significant morhological changes in terms of melanisation (Figure 4.30).

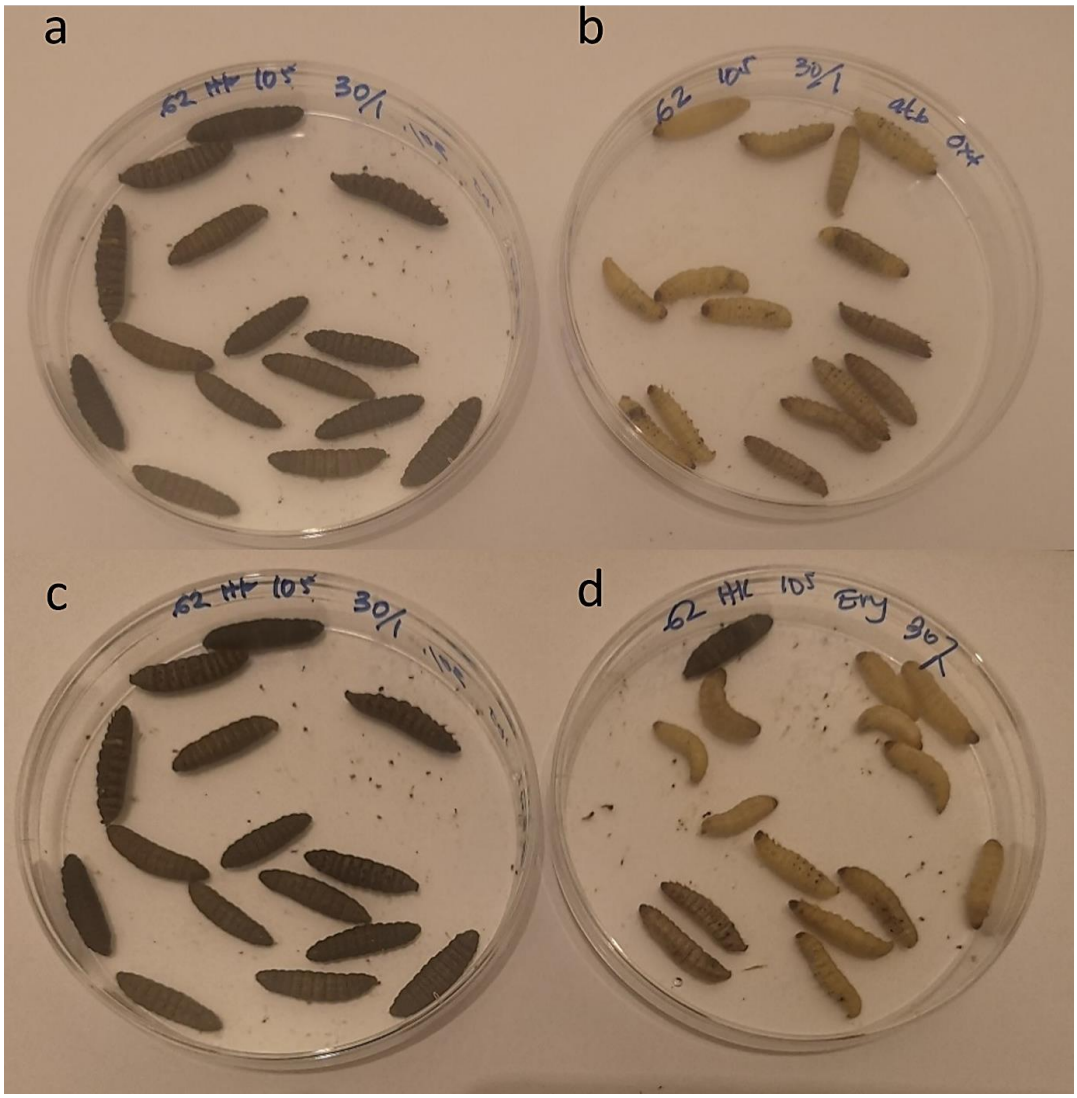

Figure 4.30. The effect of antibiotics to the infected larvae at the end of experiment time. a and c, the infected larvae, b and d, the infected larvae and then treated with antibiotics.

The larvae were infected with the *A. hydrophila 115HK.* The larvae infected with the *A. hydrophila 115HK* ($1x10^5$ CFU/ml) and then treated with antibiotics were showing significant increase the survival rates of the infected larvae with *A. hydrophila* strains and then treted with two different antibiotics. the highest survival rates was shown in the amoxiciline treatment altough the survival rate decreased sin the second and fourth day post infection (Figure 4.31).

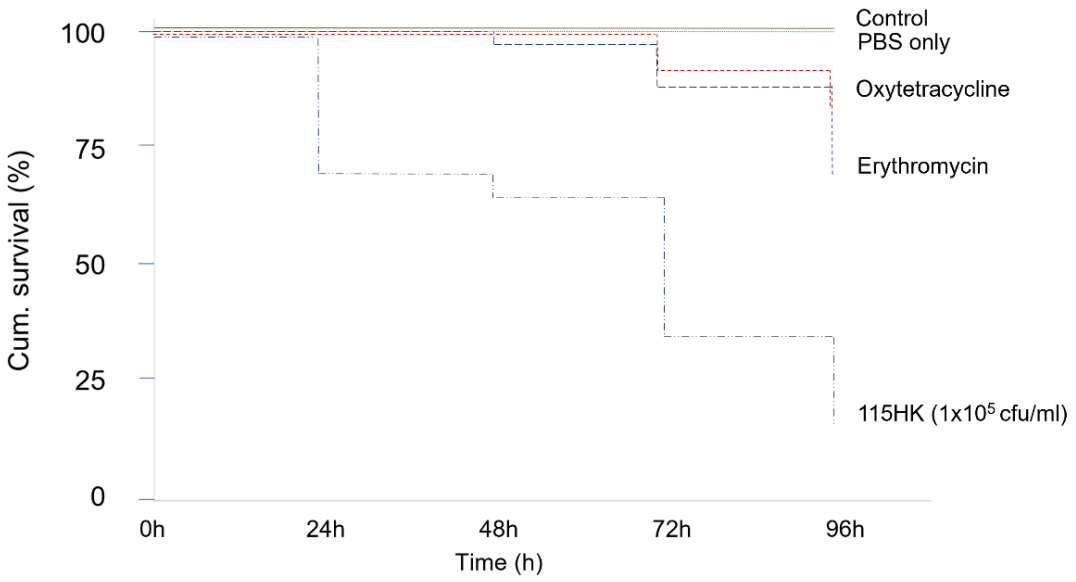

Figure 4.31. The Cumulative survival of infected larvae with with motile *Aeromonas* strains (1x10^5 CFU/ml) and treated with antibiotics**.** Positive control (PC) of *A. hydrophila 115HK* (1x10^5 CFU/ml), PBS only and no handling (control) as negative controls.

The morphological changes between the infected larvae with *A. hydrophila* 115HK without further antibiotics treatment and the infected larvae which then treated with antibiotics shown significant morhological presentation where the larvae without antibitocs treatment experiences severe melanization post infection (Figure 4.32).

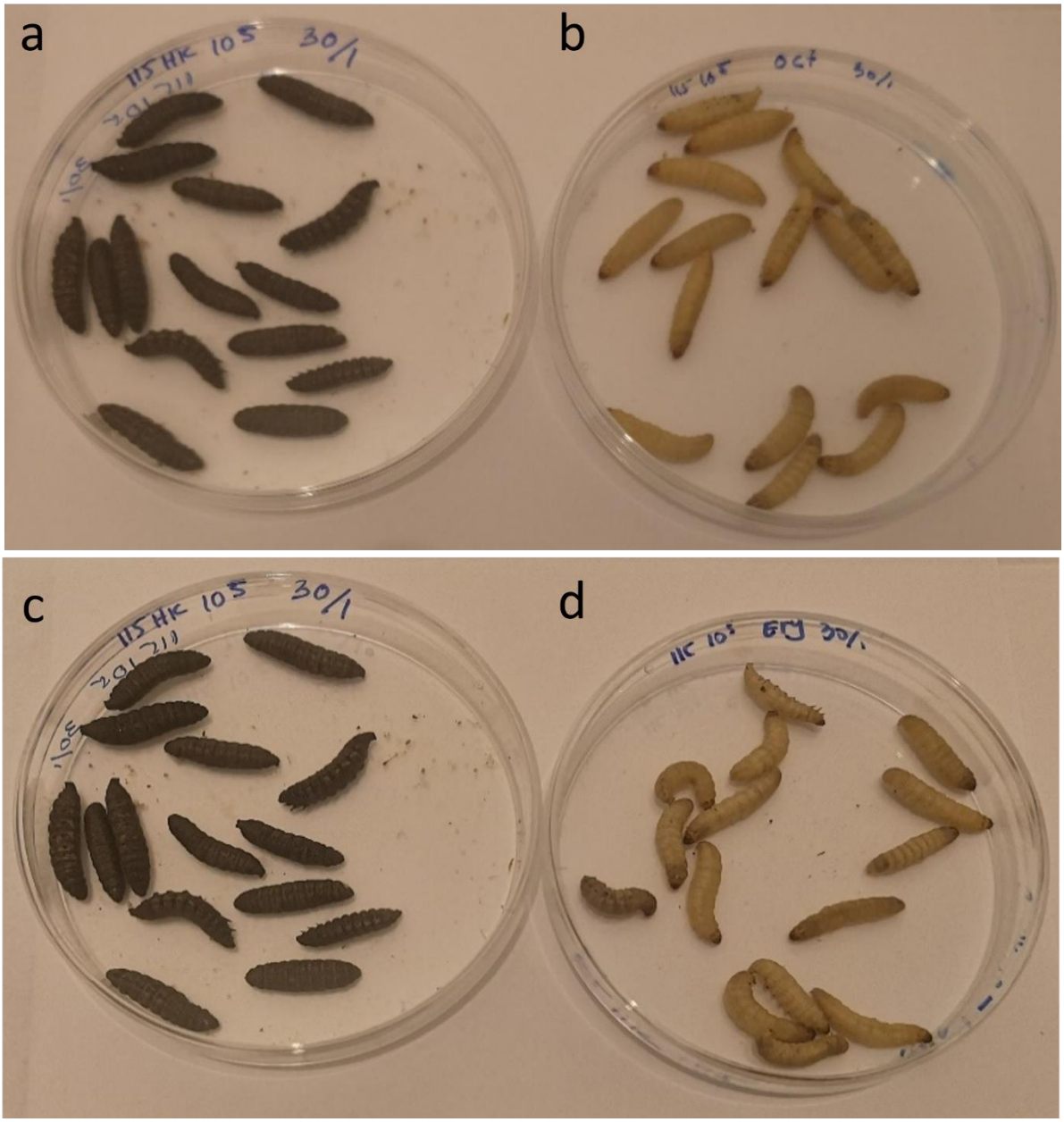

Figure 4.32. The effect of antibiotics to the infected larvae at the end of experiment time. a and c, the infected larvae, b and d, the infected larvae and then treated with antibiotics.

4.5.7. Viable Number of Bacterial Isolates Recovered from the Post Infection Larvae.

The viable number of bacterial colonies in the haemolymph of larvae post infection were showing the ability to grow and duplicate until 48h. The bacterial recovery at 72h onwards was not possible because most of the larvae were dead. In

the first 2h post inoculation the log10 CFU/ml were showing at the same log growth rate between 1.9 x10^5 CFU /ml as the lowest to 8.0x10^5 CFU /ml. The lowest growth was the strains 93HK which was the lowest virulence profiles previously tested in vitro, and the highest was the strains 115HK as medium level of virulence profiles from the previous invitro assay. The viable number of the bacterial colony was increasing as the longer duration of post infection until 48h. The number of viable colonies at ranged of 10^8-10^9 CFU/ml at 24h of post infection. Meanwhile, at 48h only 1 strain had the log phase at 10^8 CFU/ml and 5 strains with 10^9 CFU/ml with the range from 1.2 to 1.6x10^9 CFU /ml (Figure 4.33).

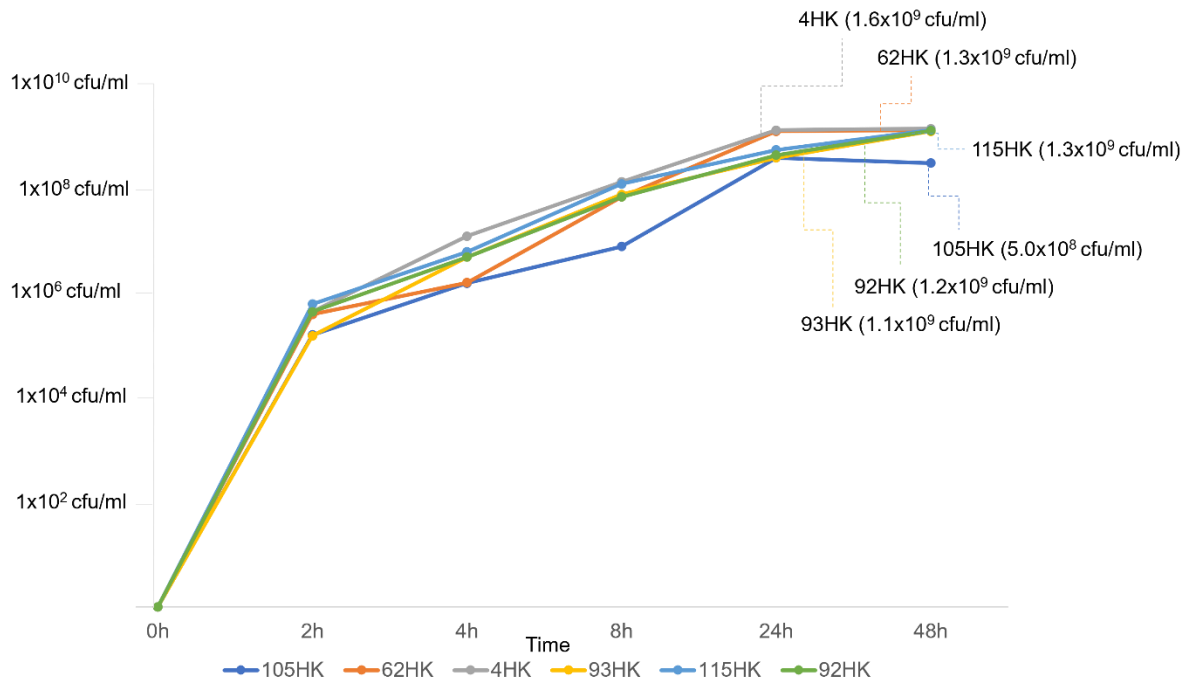

Figure 4.33. The log phase of the *Aeromonas* strains colonies viable number in the haemolymp of post infected larvae (log_{10} CFU/ml). data points indicated the mean.

4.6. Discussion

The purpose of this study was to characterize motile *Aeromonas* strains associated factor including virulence and antibiotics resistance profiles of the strains collection recovered from naturally infected farmed tilapia and catfish in Indonesia, to identify the pathogenic diversity of the species and strains variation. Where the present study was able to characterize the virulence and antibiotics resistant profiles of the tested motile *Aeromonas* strains. The clustering of the strains based on the virulence level were also performed, where the tested motile *Aeromonas* strains were categorized as high, medium, and low virulence. Clustered strains were then also linked with the clinical signs presented by fish samples collected, where all the strains categorized as high virulence were recovered from moribund fish samples presenting a minimum of one clinical sign of the diseases. The pathogenic status of the tested strains can be identified and characterized through robust and reliable laboratory-based methods, where in the current study the representative strains were then selected to determine the pathogenicity of the strains by using the wax moth larvae model *in vivo*. The challenge experiment results confirmed the role of virulence contributed to the infection. The challenge test was able to investigate the relationships between the virulence profiles governed the strains with the pathogenicity of the strains, as finding from the challenge test. Antibiotics resistance profiling also provided valuable information on the level of antibiotics practice in the study area. Therefore, these results will help the authority in establishing policy and further approach to be taken to support the productivity and the sustainability of fish farming to meet the increasing demand of fish production. The current study also able to provide information on the most effective method taken in effort to identity and characterize the aetiological agents of the disease. This would be significant in effort to address the issues including the disease outbreaks that often occur and remain as the major challenge affecting the farming system in Indonesia.

The level of antibiotics used in the area of study was also assessed by testing the representative motile *Aeromonas* strains recovered from the present study, where 98% (n=39) of the tested motile *Aeromonas* strains were resistant to amoxicillin. In other area of Indonesia, the resistant of *Aeromonas* species to amoxicillin were also reported with lower proportion, where 77,72% of *A. hydrophila* recovered from catfish farming were resistant to (Wulandari *et al.,* 2019). *Aeromonas spp.* recovered from diseased walking catfish in Java Indonesia were also identified resistant to amoxicillin

with 80% of the tested *Aeromonas spp.* In neighbouring Southeast Asia (SEA) country, higher proportion was reported where 99% of *Aeromonas* isolated from snakehead fish were resistant to amoxicillin (Pham Thi *et al.*, 2023). Globally, with almost similar proportion to the current study where 96–99% of *Aeromonas* recovered from carps pond in Poland were resistance to amoxicillin (Zdanowicz *et al.,* 2020). The resistance proportion identified in the current study was expected given the intrinsic chromosomal resistance to this antibiotic in the motile Aeromonads (Hassan *et al.*, 2017; Laith and Najiah, 2014; De Jagoda *et al.*, 2014). *Aeromonas* produce different β-lactamases which converse resistance to a broad spectrum of β-lactam antibiotics (Stratev and Odeyemi, 2016; Chen, Ko and Wu, 2012). β-lactamases is grouped into four different groups, where broad spectrum β-lactamases was classified as Class A, its deliberate resistance to penicillin including amoxicillin, cephamycin and monobactams (Chen, Ko and Wu, 2012).

Oxytetracycline continues to be used for disease fish treatment including caused by bacterial pathogens as well as for non-infectious condition and is a common antibiotics for prophylaxis and treatments either applied alone or the combination with other antibiotics (Chopra and Roberts, 2001; Roberts, 2003; Roberts, 2005). In the present study, the oxytetracycline was also identified resistant to the tested strains with the proportion 35% (n=14) of motile *Aeromonas* samples. The previous report in other area of Indonesia, the higher proportion of oxytetracycline resistance was identified, where 40% of *Aeromonas spp.* isolated from diseased catfish in Yogyakarta Indonesia (Sofiyanti *et al.*, 2021). Into species level, *A. hydrophila* was identified with the highest amount of resistant to oxytetracycline with 57% of the total *A. hydrophila* strains tested in the current study. Whereas, previous study was also reported that 54.5% of *A. hydrophila* isolated from West and Central Java Indonesia farming system were reported resistance to oxytetracycline (Mawardi *et al.*, 2023). In the neighbouring country, the lower proportion were identified where the 13% of *Aeromonas* samples isolated from the freshwater fish were resistant to the oxytetracycline (Fauzi *et al.*, 2021), Globally, higher resistant number was reported where 60% of *Aeromonas* isolates derived from market fish in Egypt were resistant to oxytetracycline (Hafez *et al.*, 2018). This data identified the association between the resistance profiles with unproper use of antibiotics as treatment in the study area, where during the field study the farmers reported that the antibiotics were applied as a treatment during the outbreaks. As evidence, the antibiotics still can be found during the field study. In

agreement with previous finding in more intensive farming system in Java Island where the frequently used of antibiotics positively associated with the prevalence of antibiotics resistance (Mawardi *et al.*, 2023). This also true globally, where the use of antibiotics is one the most important factor leading to the emergence of resistance in the bacterial pathogens (Stratev and Odeyemi, 2016; Robinson *et al.*, 2016). Tetracycline including oxytetracycline resistance are categorized as acquired resistance genes (Chopra and Roberts, 2001; Roberts, 2005). Oxytetracycline and enrofloxacin were categorized as have a wide spectrum of activity against Gram-positive and Gram-negative bacteria (Sofiyanti *et al.*, 2021). The oxytetracycline resistance in most bacteria including *Aeromonas* strains in the current study due to recovery that often associated to the mobile elements (Chopra and Roberts, 2001; Roberts, 2003). The unproper or excessive use of antibiotics associated to the increasing risk of the transmission of mobile genetic elements including plasmids, transposons, and integrons carrying the resistance profiles via horizontal transfer, where this issue were doubled by poor health management and biosecurity practice applied as finding within the visited farms. The mobile elements might also contribute to the development of the oxytetracycline resistance along with the genes related. Although the plasmid was not studied in the present study, the previous report identified that oxytetracycline resistance profiles emerged and also found to be plasmid encoded (Chaudhury *et al.*, 1996; Aoki, 2000; Gudmundsdottir, 1998; Roberts, 2003). As a mobile genetic element, plasmids carrying microbial resistance gene and are transmitted among bacteria of various species via horizontal gene transfer (Stratev and Odeyemi, 2016).

In relation to oxytetracycline resistance profiles, *tet* encoding genes were often studied, where there are currently 38 different *tet* and *otc* genes described include 23 genes as the code for ribosomal protection proteins, three genes codes for an inactivating enzyme, and one gene was categorized as unknown mechanism of resistance (Roberts, 2005). In the current study, the *tetE* gene was the primary gene identified in the strains with oxytetracycline resistance profiles. The previous reports suggested the involvement of *tetE* gene in the tetracycline resistance mechanism (Balassiano *et al.*, 2007). A good correlation was also found in the present study where 93% (n=13) of the tetracycline resistance strains were also had the *tetE* gene. Higher than the previous report in Indonesia, where only 6% (n=2) 0f the tested *Aeromonas spp*. recovered from walking catfish also had *tet* encoding gene (Sofiyanti *et al.*, 2021).

The proportion of *tetE* encoding genes identified in the current study were also higher than the previous report where only 30% of *Aeromonas* with resistance to *otc* were also presented *tet*A to *tetE* encoding genes (Schmidt *et al.*, 2001). In the species level, all of *A. hydrophila* and *A. veronii* strains with oxytetracycline resistance were also had *tetE* gene. Marginally higher than previous report where 90% *A. hydrophila* with oxytetracycline resistance also had *tetA* or *tetB* positive genes (DePaola *et al.*, 1988; Adams *et al.*, 1998; Aoki, 2000). Different proportion of *tet* genes presentation within the resistance to the tetracycline class among motile Aeromonads might associate to either difference environment along with different physical conditions or difference in local genetic exchange mechanism (Schmidt *et al.*, 2001). The present of *tet* genes might also associate with the ability to spread into the new genera, therefore the *tet* data is experience the ongoing change into this end (Roberts, 2005). The associated factors contributed to the oxytetracycline resistant profiles in the present study were also identified, where the unproper and or excessive use of antibiotics including oxytetracycline to treat the disease fish including during the occurrence of the disease outbreaks contributed to the increasing proportion of resistant profiles. Therefore, it required further and regular monitoring program to assess the status of antibiotics and to be able to provide information to the authority to establish the regulation and also to the stakeholders including fish farmers.

The majority of the tested *Aeromonas* samples in the present study were sensitive to the remaining tested antibiotics in the present study, where all of the strains were sensitive to enrofloxacin (ENR), whereas previous study reported that 26% *A. hydrophila* isolated from freshwater environment in West and Central Java were resistant to ENR (Mawardi *et al.*, 2023). Meanwhile, the other study identified that *Aeromonas* species including *A. hydrophila* and *A. veronii* were sensitive to the florfenicol, sulfamethoxazole, and enrofloxacin (De Jagoda *et al.*, 2014; Hassan *et al.*, 2017). Whereas, in the current study, small proportion 5% (n=2) of motile *Aeromonas* tested were resistant to florfenicol, where both strains were also resistant to oxytetracycline. The less resistance proportion of the tested antibiotics compared with oxytetracycline in the current study might associate with less frequently used of those antibiotics compared with oxytetracycline in the area of study. As evidence from the information provided by the visited farmers during the field study, where most of them were only used oxytetracycline to treat the disease fish within their farms. However, without policy/regulation supported by regular monitoring and proper knowledge

farming practice including the spread and the use of antibiotics, the issue of AMR profiles remains challenging. As finding in the present study where several types of antibiotics were used by the farmers without sufficient knowledge to support their approach. Unproper and excessive used of antimicrobial drugs as prophylaxis and treatment the last decade, strongly contributed to the increased number of resistant of *Aeromonas* strains along with oxytetracycline (Adebayo *et al.*, 2012). This issue of AMR also influenced by the presence of resistance genes in mobile elements such as plasmids, transposons, and integrons facilitate the rapid spread among bacteria including *Aeromonas* strains (Stratev and Odeyemi, 2016). Therefore, resistance genes through various mechanism can be transmitted from one bacterium to another which lead to the spreading of resistance strains (WHO, 2020). This finding was also provided valuable information to the competent authority locally and in the national level in establishing further regulation related to the antibiotics. Although several policies had been established, this approach would need continuous comprehensive study to support the programs and assess the level of the farming system including the antibiotics application. This would help the sector in addressing the potential issue that might occur, can be used as an early warning system, and the mitigation approach to be taken.

The present study was also able to analyse the diversity of virulence profiles by performing the phenotypic and genotypic assay *in vitro*, where 6 virulence were assayed phenotypically, and 12 virulence encoding genes were tested by PCR assay. From phenotypic virulence test results, haemolysis activities with the ability to obliterate the cell blood were possessed in the 65% (n=26) of bacterial strains tested, from the tested *Aeromonas* strains *A. hydrophila* expressed the highest number of haemolysis activity with 93% (n=13) of *A. hydrophila* strains tested on blood agar. This proportion was higher than previous study where 44% of *Aeromonas* species isolated from marine fish samples expressed the haemolytic activity (Reshma, 2015). Previous report also suggested that *A. hydrophila* and *A. veronii* are among the *Aeromonas* species that had the haemolytic activity (Pessoa *et al.*, 2020). The result indicated that the strains from the current study have the ability to produce virulence factor like haemolysin which degraded the red blood cells on red blood agar media (Yadav and Kumar, 2022). Several reports indicated that the expression of haemolysis on red blood agar indicated that the *Aeromonas* species were pathogenic with the ability to secrete the pore forming toxins (Hafez *et al.*, 2018; Chen *et al.*, 2022). Therefore,

haemolysin is considered as the most important virulence factors where this activity is often studied and characterized from pathogenic *Aeromonas* strains. Meanwhile, lipase and protease were presented in the 93% (n=37) of tested motile *Aeromonas* strains. Slightly lower than previous report where Lipase phenotype was reported expressed by all *A. hydrophila* (Abu-Elala *et al.*, 2015). High percentages of protease activity in *Aeromonas* were also reported by the previous studies (Abd-El-Malek, 2017; Pessoa *et al.*, 2020). Although, both virulence factors lipase and protease have diverse roles, but are often linked as contributing factor with the other virulence in numerous pathogens including bacterial pathogens (Stehr *et al.*, 2003). The extracellular lipase playing role in nutrient acquisition by digesting lipids, adherence to host cells and host tissues, and synergistic interaction with other enzymes (Gácser *et al.*, 2007). Furthermore, lipase activity showing the ability to hydrolyse the triglycerides, generating glycerol and fatty acids (Rasmussen-Ivey *et al.*, 2016). It was also reported that lipase contributed to causing pathogenic infection by harming the intestinal epithelium of fish (Beaz Hidalgo et al., 2016). Therefore, assessing lipase is considered important related to virulence profiles and the ability of bacteria in affecting the host, where in the present study, lipase with *lip* encoding gene was also identified as a combination with the other virulence genes (*aerA* and *act*) that contributed to the occurrence of the disease. As evidence, all of the tested motile *Aeromonas* strains with those genes' combination (*aerA*+*act*+*lip*) were recovered from moribund fish samples presenting clinical signs of the disease. Meanwhile, the other phenotypic virulence protease has the ability to degrade the protein within the host cells and tissues. It also reported contributed to promoting the expression of clinical signs of the diseases fish including discoloration of scales and the degradation of mucosa which then enhance the progression of the pathogens (Beaz Hidalgo et al., 2016). High prevalence of protease were identified from *Aeromonas* species including including in the present study, and with higher proportion where 100% of *A. hydrophila* isolated from farmed fish expressed protease activity (Abd-alla *et al.*, 2017). Different ability in producing protease of *Aeromonas* species were due to the genetic variations responsible for the protease production and might also associated to the different environment (Swift *et al.*, 1997). DNAse phenotypic virulence tested in the current study as extracellular endonucleases that responsible in cleaving the DNA and also producing a high concentration of oligonucleotides (Willis *et al.*, 2016), where in the current study DNAse was expressed by 88% (n=35) of the strains tested, lowere than

previous report in Indonesia where 100% of *A. hydrophila* recovered from catfish expressed DNAse (Kusdarwati et al., 2021). DNAse contributes in enhancing the spread of infectious pathogenic bacteria to the host and resulting the nucleotides from the rapid growth of the bacteria through DNA hydrolysis (Fox and Holtman, 1968). Haemolysis activity, DNAse, and gelatinase reported determine the pathogenicity of bacteria including *Aeromonas* species in fish (Chandrarathna *et al.*, 2018). Where in the current study, high gelatinase was also expressed by the tested motile *Aeromonas* strains. The gelatinase is used by bacteria in progressing to the host cell membrane and also used for metabolic developments by hydrolysing the gelatine compounds into polypeptides, peptides, and amino acids (Balan *et al.*, 2012). The lowest number of phenotypic expressions were both haemolysis activity and biofilm formation where biofilm was possessed by 45% (n=18) of the total samples. Biofilm formations reported potentially increase the colonisation of the bacterial pathogens (Chen *et al.*, 2014). The biofilm formation was also often reported correlated to the expression of resistance profiles where in the present study, 78% of the strains expressed biofilm on media agar also reported provided resistance profile to the tested antibiotics *in vitro*. Therefore, biofilm considered as a good indication of antibiotics resistance expressed by bacterial pathogens, where biofilm formation reported act as the antimicrobial agents and host defences (Costerton *et al.,* 1995; Lynch *et al.,* 2002).

Detection of virulence genes is useful indicator for assessing the pathogenicity of certain microorganisms as reported previously where virulence profiles contributed to the pathogenicity of bacterial pathogens (Beaz and Jos, 2012; Robertson *et al.*, 2014; ZG and C, 2016; Rasmussen-Ivey *et al.*, 2016). Among them, the type II secretion system (T2SS) was reported often presented in all known member of *A. hydrophila* related to the diseases. T2SS is integral in the extracellular secretion of wide array of virulence factors including aerolysin (Tseng *et al.,* 2009; Pang *et al.*, 2015; Ruhil Hayati *et al.*, 2015). Aerolysin is among the virulence factors in the T2SS that considered as the most important virulence factor and often associated to the enterotoxin *act* (Galindo *et al.*, 2004), where both *aerA* and *act* genes are also reported involved in the haemolysis activity causing degradation of red blood cells (Rasmussen-Ivey *et al.*, 2016). From the results in the present study, the *act* gene presented not only from the strains with positive haemolytic expression but also presented from the strains absence of haemolysis activity on the blood agar. In the current study, *act* gene was the most prevalence gene presented by 95% (n=38) of motile *Aeromonas* strains

tested. In agreement with the previous study where *act* gene was highly prevalent (Hoel, Vadstein and Jakobsen, 2017; Nhinh *et al.*, 2021). *act* gene is responsible in affecting the fluid secretion (Sha, Kozlova and Chopra, 2002; Austin, 2011). *act* gene was also contributes to the multiple effects including haemolytic (Rasmussen-Ivey *et al.*, 2016). *act* is also integrated with multifactorial activities aside from enterotoxin activity, it also has cytotoxic and haemolytic activities (Xu *et al.*, 1998; Sha *et al.,* 2002). Unlike the other virulence factors which are exported through the T3SS or T6SS, *act* is exported through T2SS (Chopra and Roberts, 2001; Sha *et al.*, 2002). Meanwhile, *aerA* as one of the most important virulence factors in pathogenic infection and even considered as the major contributor to the virulence of pathogenic *Aeromonas* (Heuzenroeder, *et al.,* 1999; Rieger and Barreda, 2011; Iacovache *et al.*, 2016). In the current study, aerolysin with encoding *aerA* was detected in 58% (n=23) of the total of *Aeromonas* strains tested, where in the species level 71% (n=10) of *A, hydrophila* strains presented aerolysin gene which was higher than previous study where 62% of *A. hydrophila* recovered from the milkfish samples in Gresik region Indonesia had aerolysin gene (Fikri *et al.*, 2022). The proportion was higher than previous report where aerolysin also identified in 35% of *A. hydrophila* in Indonesia (Indrawati *et al.*, 2020). Globally, *aerA* was reported presented by 76% of bacterial isolated recovered from fish (Oliveira *et al.*, 2012). Aerolysin is known increase the pathogenicity of *A. hydrophila*, where Aerolysin contributes in causing osmotic lysis and cell necrosis as effect of pore-forming toxin (Cirauqui *et al.*, 2017; Xu *et al.*, 1998). Aerolysin also contributes to causing haemolysis and lesion as identified in the current study where the strains with aerolysin gene were recovered from moribund fish with the clinical sign of the diseases including haemorrhages and lesion. Furthermore, Vadivelu *et al.*, (1995) also described that aerolysin was detected in the *A. hydrophila* which was causing bacteraemia. *aerA* as encoding of aerolysin and *hylA* encoding haemolysis gene detection is reliable approach for assessing the pathogenicity of the strains (Heuzenroeder *et al.,* 1999). Moreover, the absence of *aerA* and *hylA* genes within the strains significantly reduce the pathogenicity level of the *Aeromonas* strains (Chen *et al.*, 2022). Aerolysin with encoding *aerA* gene along with cytotoxic heat-labile enterotoxin (*act*), cytotoniic heat-labile enterotoxin *(alt*), and cytotonic heat-stabile enterotoxin (*ast*) are among the virulence factors which are likely corelate to the haemorrhagic septicaemia on the infected fish (Sha *et al.*, 2002). In the present study heat-stable cytotonic enterotoxin (*ast*) which is causing intestinal fluid accumulation

was identified in 20% (n=8) of the tested motile *Aeromonas* strains. It has been also reported that the other genes, *gcat* and *lip* genes are playing important role in affecting the overall pathogenicity of *Aeromonas* infections, as *gcat* and *lip* genes enhance the severity of infection (Pemberton *et al.,* 1997). The *gcat* gene was presented in both of *A. hydrophila* and *A. veronii* tested strains, however, *gcat* was predominantly presented by the majority of *A. hydrophila* with 87% (n=12) of *A. hydrophila* strains tested. As finding in the previous study where *gcat* gene was most frequently identified from 76% of *A. hydrophila* recovered from diseased fish farms (Taha *et al.*, 2021). Although *gcat* is not categorized as the main virulence factors, it however participates in altering infection including by digesting plasma membrane of host cells, and leading to lysis of host organs (Beaz-Hidalgo *et al.*, 2010; Rasmussen-Ivey *et al.*, 2016). Meanwhile, the *lip* gene which contributes to the increasing of aeromonads infection severity by participating in the alteration of the host plasma membrane (Pemberton *et al.,* 1997). In the current study, *lip* gene was presented by 70% (n=28) of the strains tested, lower than previous report where 87% of the tested *Aeromonas* isolated from tambaqui fish (*Collosoma macropomum*) had *lip* gene (Pessoa *et al.*, 2020). The other virulence genes assessed in the current study enolase with encoding gene *eno* has functions as a heat shock protein and regular transcription by binding host chromatin/cytoskeletal structure (Sha *et al.*, 2009). In the present study, *eno* gene was presented by 43% (n=17) of the strains. The remaining genes tested *was* lateral flagella (*lafA*) gene, where the tested *Aeromonas* strains including *A. hydrophila* produces this for surface movement/swarming and the support the ability to form the biofilm formation (Canals *et al.*, 2006; Rasmussen-Ivey *et al.*, 2016). In the current study, 20% (n=8) of the strains were presenting *lafA* gene, lower than previous report where approximately 50% of *Aeromonas* species had either *lafA* or *lafB* encoding gene of lateral flagella (Kirov *et al.*, 2002). Additionally, flagellar glycosylation was considered to be linked with the ability to form biofilms (Rasmussen-Ivey *et al.*, 2016). Therefore, *lafA* gene playing important role to the ability of the strains in performing the biofilm formation. The other tested virulence gene *exu* as a DNA encoding gene which also considered as contributing factor for the bacterial pathogenicity, in the current study was presented by 18% (n=7) of the strains tested. The *exu* gene was reported responsible in enabling the strains to prevent the antibacterial host defences, and enhance the ability of the strains to invade and sustain withing the host (Tomás, 2012, Pessoa *et al.*, 2020). The *exu* gene contributes to the DNAse activity, where it

playing role in enhancing the spread and the alteration of the pathogenic disease causing infection to the host (Fox and Holtman, 1968).

The current study was also identified the potential association between phenotypic and genotypic assayed of the related virulence profiles as individually or a combination of the virulence profiles. *aerA* gene is often associated with the haemolytic activity. The presentation of the *aerA* and *hylA* genes indicated the ability in degrading the red blood cells as shown in the haemolytic activity on red blood agar and pathogenicity level of the strains (Chen *et al.*, 2022). Where in the current study, from 29 strains expressed haemolysis activity, 76% (n=22) of them were also presenting *aerA.* Meanwhile*, hylA* gene as encoding gene of haemolysis was only presented by 7% (n=3) of the strains with haemolytic activity on blood agar. The expression of haemolysis is not always presenting the particular gene because the expression might also be influenced by another contributing factor (Heuzenroeder *et al.,* 1999). Analysing the haemolysis expression with the combination of the genes related was showing a promising result where all the strains expressed haemolysis were also presented either 1 of the gene or a combination of the genes related. This also indicated that the expression was not only associated with a single gene related but also the genes in combined. Haemolytic activity was reported influenced by several virulence genes that contributed to the haemolytic activity (Kanai and Wakabayashi, 1984). The other tested phenotypic virulence was extracellular protease, where it possess in the most of virulent pathotypes (Rasmussen-Ivey *et al.*, 2016). Protease activity was often reported associated to the production of *ser* gene as encoding gene for protease, that playing role in the caseinolytic activity (Esteve and Birkbeck, 2004). In association of extracellular protease, the genotypic virulence's related were also analysed where 53% (n=21) strains presented the *ser* gene were also expressed the protease activity on skimmed milk agar, the proportion was lower than previous report where around 59% of *Aeromonas* expressing protease activity were also had the ser gene (Pessoa *et al.*, 2020), No *ser* gene was identified from negative protease expression. In correlation to the protease activity, the elastase with the ability to support infection and colonization by damaging tissue and degrading immune proteins were presented by 50% (n=20) of the strains with positive expression to protease. When the two of gene related were combined, only 11% of the strains with protease activity were negative to each of the gene and the genes combined. This indicated that the protease expression in the protease agar media associated with the two tested

genes related (*ser* and *ela* genes), although the expression of protease activity was not only influenced by the single gene related, i.e, *ser* gene not only as a single contributing factor for the protease activity but also involving other gene which made them able to degrade the casein. Meanwhile, *lip* gene was presented in 76% of the strains expressed lipase on lipase agar media. This result was slightly lower than previous report where lipase phenotypically were also had a lip gene, where around 82% of the *Aeromonas* species isolated from tambaqui fish positive lipase activity (Pessoa *et al.*, 2020). This finding will help further study in efforts to understand the presentation of virulence profiles within the bacterial pathogens, this including Aeromonads by targeting the most potent virulence profiles associated with the diseases outbreaks, where this remain hampering the production and sustainability of farming system in Indonesia. DNAse phenotypic virulence tested in the current study as extracellular endonucleases that producing a high concentration of oligonucleotides and also reported responsible in degrading the DNA (Willis *et al.*, 2016), where only 18% (n=7) of the strains that had *exu* as encoding gene of DNAse also expressed DNAse activity on media agar from 88% (n=35) of the strains with the expression of DNAse. This might associate to the various contributing factors for DNAse expression on DNAse agar media which not only the presence of single gene including *exu* gene but associated with other factor, where this would need further study. The lowest number of phenotypic expressions was biofilm formation where biofilm was possessed by 45% (n=18) of the total samples. Where this expression reported potentially increase the colonisation of the bacterial pathogens (Chen *et al.*, 2014). Therefore, it often associated to the expression of resistance profiles, where in the present study, 78% of the strains expressed biofilm on media agar also reported provided resistance profile to the tested antibiotics *in vitro*. Therefore, biofilm considered as a good indication of antibiotics resistance expressed by bacterial pathogens, where biofilm formation reported act as the antimicrobial agents and host defences (Costerton *et al.,* 1995; Lynch *et al.,* 2002). Lateral flagella encoding genes including *lafA* gene is also often associated to the ability in performing biofilm, where in the current study showed the promising relation between the biofilm formation and *lafA* gene. As reported previously where lateral flagella are important contributing factor to form biofilm formation and adherence of epithelial cell of *Aeromonas* species (Gavín *et al.*, 2002).

The virulence factors detected and reported from the MAS strains are often corelated to the cause of the diseases that are complex and diverse (Janda and

Abbott, 2010). The ability of *Aeromonas* species in causing the disease is associated with the structure composition of *Aeromonas* cells that are supported by the specific characteristic including the specific secretion system. These specificity are often studied and reported initiating the infection process by adhering and invading the tissue of different host (Fernández-Bravo and Figueras, 2020). Where in the present study, a minimum of 4 virulence genes were presented by all the tested strains with *aerA*+*act* genes combination as the predominant gene's combination within the tested *Aeromonas* strains. Both genes were considered as the most important genes associated to the pathogenic infection of *Aeromonas* related to the disease outbreaks. Into the strains level, the virulence genes governed tested *A. hydrophila* strains were more prevalence than the other *Aeromonas* strains tested in the current study, with the ranged from 5 to 10 genes. The proportion of virulence profiles detected in the current study has an agreement with the previous study globally where Talagrand-Reboul *et al.*, (2020) reported that *A, hydrophila* was commonly considered as one of the most pathogenic species as supported by the high number of virulence profiles. Previous study also suggested that a positive correlation between the number of virulence profiles and the ability in causing the disease of Aeromonads (Sha *et al.*, 2002; Rasmussen-Ivey *et al.*, 2016). More specifically to the virulence genes, these factors are good indicator for assessing the pathogenicity of certain microorganism, where high number of virulence genes within the strains are manifestation of the pathogenicity level (Hoel, Vadstein and Jakobsen, 2017). In the present study, the combination of both virulence factors (*aerA*+*act*) was the most prevalence with 53% (n=21) of the tested strains had that combination. This finding described the potential association of this genes combined with the occurrence of the disease, where the two genes (*aerA* and *act*) are the most important virulence factors significantly contributes to the pathogenicity of motile *Aeromonas* (Chopra and Houston, 1999; (Sha *et al.,* 2002). Furthermore, the combination *aerA*+*act* was identified from the moribund fish samples with clinical sign of the disease in the present study. This strongly supported the previous report on the important role played by *aerA*+*act* gene combination contributed to the establishment of infection which led to the disease outbreaks within the area of study. The involvement of the two genes within the *A. hydrophila* were also often recorded causing the pathogenic diseases in the fish farming globally (Rasmussen-Ivey *et al.*, 2016; Sha *et al.,* 2002). Furthermore, the combination of *aerA*+*act*+*lip* in the current study was the highest among three genes combination

where 38% (n=15) of the tested motile *Aeromonas* strains had this combination. All strains presented the three genes combination were also recovered from moribund fish samples with clinical signs of the disease. This indicated that the establishment of infection caused by *aerA* and *act* was also enhanced by the present of *lip* gene as contributing factor that increase the severity of infection. The current study also able to identify the critical roles played by motile *Aeromonas* strains that governed by varied virulence profiles associated to the occurrence of the disease outbreaks within the area of study. As finding during the present study where Gram-negative bacteria screened in the abnormal organ tissue of moribund fish samples. Furthermore, the strains categorized as high virulence level (HV) were recovered from moribund fish samples with the most clinical signs of the disease. This strongly indicated that the ability of the strains in infecting the host associated to the virulence profiles governed the strains.

The current study showed that *G. mallonella* infection model to be potentially useful model for studying *Aeromonas strains* pathogenicity associated with the virulence profiles of the tested strains. In the infectious process, the structural composition of bacterial cell has a great influence on the ability in causing the disease, where in the present study, the virulence profiles associated with the ability of the tested strains in altering infection of to the tested larvae. This finding indicated that the virulence profiles were good evidence for assessing the pathogenicity of certain microorganism. The previous study were also reported that strong correlation between the virulence profiles and the pathogenicity of *Aeromonas* in fish and other host (Li *et al.*, 2011; Hoel, Vadstein and Jakobsen, 2017). In the present study, the effect of infection from 6 different strains were significant to the larvae survival in the challenge experiment using wax moth larvae model *in vivo*. Lowest survival rates were identified from both *A. veronii* and *A. hydrophila* strains with the high virulence level (HV). Whereas, the highest survival was found in the *A. veronii* and *A. hydrophila* strains with the lowest virulence profiles (LV). This finding identified that the virulence genes significantly associated to the ability the tested strains in causing infection/mortality of the tested larvae. Previous study reported that different strains with different virulence profiles level might associate to the different pathogenicity level of bacterial pathogen (ZG and C, 2016).

The effect of temperature of two motile *Aeromonas* strains to the larvae post infection generated different survival rate to the infected larvae. The similar studies on

the Gram-negative bacteria also reported the association between incubation and the survival of post infection larvae (Desbois and Coote, 2011; McMillan *et al.*, 2015; Djainal *et al.*, 2020). Although this test was performed to identify the suitable incubation temperature for the main challenge test, the results also provided valuable information where temperature as one of environment variables and stress condition associated with the pathogenicity of certain bacterial pathogen (ZG and C, 2016). The host pathogen interaction activates response as a result of antigen exposure (Fernández-Bravo and Figueras, 2020). The infected larvae in all different incubation temperatues were causing the different level of survival rate where the highest survival rate was found in the incubation of 15^0C which the lowest incubation temperature as shown in the current study. The highest incubation temperature (37^0C) caused the highest number of mortality where all larvae dead from the 24h of post infection and the fastest time periode in causing the mortality of all infected larvae. This trend might associated with the more effective conditions of the strains for growing and causing the infection, as this supported by thermoregulated virulence profiles governed by the *Aeromonas* strains. *G. malonella* were more susceptable to the infection in the higher temperature. This indicated that the temperature also contributed in compromising the immune system led to more susceptible host (Pirarat *et al.*, 2011; Oliveira *et al.*, 2012). As finding during the filed study in the Chapter 2 in the section 2.5.1.5 where the mortality of fish farmed occurred approximately during period of March/April where the temperature raised, and also around October when the temperature reached the highest during the year. The same result where the mortality increased during the range of the same time period reported in Indonesia (Manumpil *et al.,* 2015; Hernawaty, 2018).

The preliminary tests were also performed prior to the main challenge experiment, where injection with live *Aeromonas* cells is required in the infection of *G. mallonela. S*ignificant level of survival rates between the infection with live inoculum and heat killed *Aeromonas* strains. As identified in the present study where the heat-killed strains provided by far higher survival rate compared with the infection with live inoculum. This indicated that the live cell of *Aeromonas* was required in causing the infection which then led to the mortality. Similar observation were also reported where the infection was depended of the injected inoculum (Desbois and Coote, 2011; McMillan *et al.*, 2015; Djainal *et al.*, 2020). The requirement of live cell to allow the infection of the tested larvae was also tested by using antibiotics. The information

gathered from this test also provided the information on the suitability of wax moth larvae as an alternative model to identify the efficacy of certain antibiotics *in vivo*. In the present study, the treatment of antibiotics to two different group of larvae infected with the strains from *A. hydrophila* and *A. veronii* were showing significant survival rate to the post infection larvae. Similar observation were also suggested that *G. malonella* model was suitable in assessing the efficacy of antibiotics treatment of infected larvae (Desbois and Coote, 2011; McMillan *et al.*, 2015; Djainal *et al.*, 2020). As the global challenge, the antibiotics application promote another problems including the development of antibiotic resistance bacteria and also reducing the efficacy of antibiotics (Rhodes *et al.*, 2000; ZG and C, 2016). As shown in the current study where the larvae still experienced the mortality even with the application of antibiotics post infection. This might illustrated that the development of drug resistant strains associated to the physiological and molecular mechanism responsible for the genetic basis resistant in bacteria and host (ZG and C, 2016). The antibiotics resistance also indicated that the requirement and the ability of bacterial pathogen to survive in the presence of an antibiotic or chemical where it previously susceptible to certain antibiotics (Krkošek, 2010). Although, this would need further study on the mechanism related to the AMR resistant strains. The variety of antibiogram profiles of the *Aeromonas* species can be attributed to the selective environment pressures such as inappropriate use of antibiotics and strain-specific properties (Janda and Abbott, 2010) as a result *Aeromonas* species has been reported resistance to number of antibiotics (Janda and Abbott, 2010; Figueras *et al.,* 2011; Vega-Sánchez *et al.*, 2014).

The pathogenicity of the *Aeromonas* strains also associated to the concentration level of injected strains to the tested larvae. In the present study, the viable number of *Aeromonas* species has a strong relation to the survival of infected larvae where all the tested strains were showing similar trends. The higher concentration of infected *Aeromonas* the higher number of larvae mortality within the time periods of post infection. Similar studies were also reported where the infection was dependent on the concentration of the injected bacteria (Desbois and Coote, 2011; McMillan *et al.*, 2015; Djainal *et al.*, 2020). This also identified that the pathogenicity associated with cell concentration of bacterial pathogens and duration of post infection (ZG and C (2016). The viable number of the bacterial colony in the haemolymph of larvae post infection were showing the ability to grow and replicate until the 48h. The viable number of the bacterial colony was increasing as the longer

duration of post infection until 48h post infection. This finding showed that the strains had the ability to replicate within the period of times (Djainal *et al.*, 2020; Six *et al.*, 2019b). In the current study. Although, the growing viable number of *Aeromonas* strains were determined, the viable number trends of different strains replication were identical where at 48h the viable number were the same at 10^9 CFU/ml except 1 strain with the log phase of colonies at the viable number 10^8 CFU/ml. This suggested that different survival of the tested larvae as a results of post infection more likely associated with the virulence profiles which that related to the pathogenicity, as also identified in the previous study (ZG and C, 2016).

4.7. Conclusion

The virulence profiling of motile *Aeromonas* strains in the current study has been achieved and also was able to demonstrate the presence of virulence profiles that governed the motile *Aeromonas* strains. This specificity of motile *Aeromonas* strains supported the ability in causing infection which led to the diseases, as evidenced from the experimental infection *in vivo*, where significant infection caused by *Aeromonas* strains with high virulence profiles led to the significant mortality of the tested larvae. Meanwhile, the antibiotics resistance profiles identified in the current study were most likely associated with the ability of the strains in the acquisition of new genes that were often linked with the presence of mobile genetic elements, where unproper use of antibiotics in the study area enhanced the risk of the resistance profiles development as AMR profiles identified in the current study. This finding would help further research in the diagnosis and comprehensively understand the role of the virulence factor associated to the disease, and the approach to be taken in addressing the disease problems caused by bacterial pathogens.

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CHAPTER 5. GENERAL DISCUSSION

5.1. Principal Aim of the Study

The drive for increased aquaculture development within Indonesia is promoting intensification and has resulted in a fragmented approach to the freshwater production sector in East Nusa Tenggara (ENT). Intensification increases the risk of infectious diseases and requires a more robust biosecurity approach which must be developed and applied to the individual farms, based on the biological and ecological risks to the farmed species on site (Henriksson *et al.*, 2018; Henry, 2021). To support the aquaculture intensification more sustainably, the Indonesia farms need improved knowledge on best management practises within their farming systems to reduce the increased mortalities reported (Henriksson *et al.*, 2018; Kayansamruaj *et al.*, 2020). The causes of fish mortalities within these systems, are not well documented due to a lack of diagnostic capacity, both on the farm and within the laboratory setting where the focus of disease control currently is more focused on identification of specific diseases or pathogen surveillance rather than identification of the causes of morbidity and mortalities including a wider range of diseases. For the system to be correctly developed, more reliable information on the farming practises, challenges and disease outbreaks is critical. The approach taken in this study, was to combine biological samples with questionnaires to identify farmers knowledge and investigate the actual cause of the disease. Any bacterial samples recovered from the fish were identified and characterised using the combination of both traditional culture and molecular identification methods. This approach provided a greater level of knowledge in this area and provided evidence of the natural disease outbreaks occurring on the fish farms included in the study.

5.2. The Status of Farming Practice in the Area of Study.

Similar to other freshwater farming systems in SEA countries, there is a wide diversity in the range of production systems, even in those producing the same fish species (Kayansamruaj *et al.,* 2020). More intensive production is found in Java Island, which has been developing aquaculture longer than the ENT study. In this study, ENT was categorized as being in the early stages of intensification and considered to be an excellent case study to learn valuable lesson and support sustainable development (Henry, 2021; MMAF, 2020). Irrespective of the farming practise or system, infectious

diseases were identified as a major challenge in ENT with the severity of the fish losses having a significant impact on all the production systems included. This is similar to other studies which have identified infectious bacterial diseases as a major challenge affecting the development of the global aquaculture sector (Lafferty *et al.*, 2015; Vouga and Greub, 2016; Bouwmeester *et al.*, 2021). It is well recognised that infectious diseases are complex, particularly in the aquatic environment and several variables identified in this study were considered as contributing to the level of fish morbidity and mortalities. In the present study, poor farm or fish management practices and lack of both generic and more specific biosecurity practises were identified throughout the farming population as described in Chapter I, Table 2.10, Therefore, it is critical to comprehensively understand the disease risk both within the systems holistically but also at an individual farm level to develop effective biosecurity plans that can be shared and implemented to support the ambitious growth target of Indonesia aquaculture by 8.5% per annum up to 2030 (Henry, 2021; MMAF, 2022). If the farms in ENT are to positively contribute to this ambitious target, then the data generated from this study can support better biosecurity practises and more strategic approaches to the control and treatment of infectious bacterial diseases especially in the farmed tilapia and catfish species.

At the start of the study, limited or fragmented data was available on infectious disease outbreaks within these farming systems and this lack of critical base line data was identified as a knowledge gap. To address this a comprehensive diagnostic approach was taken and combined with questionnaire-based information on the farming practises and farmer knowledge to identify the diversity in animal husbandry and farm management practises. To confirm the data provided by the farmers, biological samples were taken at the time of interview to investigate the aetiology of the morbidity observed/reported. These types of data are essential to develop robust aquaculture health strategies, support appropriate regulation and develop comprehensive mitigation and disease prevention measures to address the fish losses experienced (Cameron, 2002; Weiss *et al.*, 2019; Austin, 2019b; Suzuki, 2021). This is particularly important as the freshwater aquaculture sector in ENT continues to thrive and develop as intensification and expansion of the sector can increase the risk of disease outbreaks (Suzuki, 2021; FAO, 2022). Although not completely unexpected, the lack of farmers knowledge on appropriate farm management practices, including biosecurity and treatments within the visited farms were identified as a major constraint

in the sustainable development of the sector. From the questionnaire data, it was clear that a single biosecurity practise was not in place (Chapter I, Table 2.10), which is not surprising given that the biosecurity plan for any farm must be situated in the requirements of the farm itself. The diversity of systems and production levels was typical of many freshwater aquaculture finfish producers found in SEA, and improvements were identified which would reduce the risk of disease entry and transmission in the fish farming system (Mary Opiyo *et al.*, 2020; Suzuki, 2021; Subasinghe *et al.*, 2023).

Biosecurity practice was identified as a limiting factor affecting the production and sustainability of the sector, where the practice to reduce the risk of pathogens emergence, reemergence, and spread was not appropriately applied. The lack of disinfection and using the same nets as applied by most of the visited farms enhanced the risk of pathogen transmission and associated disease outbreak, and improvement in this regard could be an easy "win" for the sector by developing these practises, which are very cost-effective in reducing the prevalence of the infectious pathogens into the farm, transmission within the farm, and of course treatment of any water prior to leaving the farm would benefit the surrounding aquatic environment and the farms downstream or sharing the water body. Simple biosecurity approaches are proven to be cost-effective risk management in tackling and preventing the issue of disease outbreaks (Dvorak, 2009; Subasinghe *et al.*, 2023). Moreover, effective biosecurity practice also contributes to the fish welfare within the farming system, whereas inadequate biosecurity practice is the major constraint compromising the development of aquaculture sector in the last three decades (FAO, 2019, 2020a).

In efforts to help fish farmers to address the issues affecting their farms in the area of study and wider area in Indonesia, knowledge and technology transfer is also critical and must be established by the government, and not only focus on the distributing aquaculture materials and infrastructures. This can be achieved through the farmers workshops, training packages, sharing information between the farms, and also regular monitoring and surveillance, because lack of knowledge contributes to the ineffectiveness of biosecurity as a tool to prevent or tackle the transmission and negative effects of pathogenic diseases. The comprehensive study at the farm level, including diagnosis on the disease and associated factors as applied in the current study, also needs to be performed regularly rather than only focusing on specific infectious diseases. This approach will be critical in supporting the success and

sustainability of the farming system, where the data collected produce a base line to support the establishment of regulations, including control strategies to be applied, and can also be used as an early warning on potential issues that may affect the production system and then measures taken to mitigate the issue. Aquatic health management and biosecurity practice is vital to reduce the vulnerability of the aquaculture sector to infectious diseases and long-term management of adequate biosecurity planning and health and disease play a critical role in the success and sustainability of this sector (Dvorak, 2009; Austin, 2019; Subasinghe *et al.*, 2023).

5.3. The Disease Status of Farmed Tilapia and Catfish.

In the study performed, data on the gross external and internal clinical signs, with the histopathology and the identification of the bacteria, demonstrated the involvement of bacterial pathogens as the causative agents of the infection. The clinical signs identified during the present study were in agreement with previous reports (Mohamed Fawzy *et al.*, 2014; Hardi *et al.*, 2018; Kristianingrum *et al.*, 2021) and are not pathognomic of specific diseases but consistent with the wider descriptions of similar diseases affecting catfish and tilapia farmers in the SEA region (Kristianingrum *et al.*, 2021; Legario *et al.*, 2023) This is also true for global reports where similar signs to MAS were identified associated in the current study including abnormal swimming, loss of appetite, pale gills, ulcerations, and haemorrhages from fish, including tilapia (M. Randy White, 1991; Chen *et al.*, 2019; Korni and Ahmed, 2020; Adah *et al.*, 2021). The predominant bacteria recovered from the presenting with clinical signs of disease were Gram-negative, rod-shaped bacteria identified as members of the motile aeromonads, and this was similar for both tilapia and catfish samples where more than 80% Aeromonads were preliminary identified (Chapter 3, section Bacterial Isolation and Identification). The ubiquitous nature of the motile aeromonads means that these bacteria are often recovered, particularly from moribund freshwater fish as they are part of the gut microflora. This finding itself was not unusual but additional study was required to clarify the species and the pathogenicity factors.

The identification of the *Aeromonas* species remains challenging as many of the assays used, particularly the traditional biochemical profiles, are not sufficiently sensitive enough to discriminate between closely related species, but more concerning is the degree of variability within a single species (Beaz-Hidalgo *et al.*, 2015;

Fernández-Bravo and Figueras, 2020; Legario *et al.*, 2023). Similar issues arose in this study where the identification profiles using both traditional test tube or the commercial miniaturised API 20E kit gave different biochemical profiles for isolates belonging to the same species. To add to the complexity, similar biochemical profiles were identified between different *Aeromonas* species in the current study. This in itself has been reported previously and the limited data on the association between phenotypic and genotypic identification results associated with specific motile *Aeromonas* species also contributed to the complexity (Navarro and Martínez-Murcia, 2018; Ormen et al., 2005). Differentiation to species level is critical to support the development of efficacious and novel prevention and treatment strategies. In the present study comparative analyses of the varied bacterial identification methods utilised showed good agreement between the primary (Gram, oxidase, O/F, catalase, and motility tests) and the *16S rDNA* sequence data. More recently, the use of the two housekeeping genes *gyrB* and *rpoD* have been investigated to support improved species-level identification and provide higher reliability in the phylogenetic classification of the motile *Aeromonas* complex (Soler *et al.*, 2004; Khor *et al.*, 2015; Vega-Sánchez *et al.*, 2014; Fernández-Bravo and Figueras, 2020). In the present study, a high level of agreement was found between the 3 molecular tests performed and so effectively any of the 3 tests could be used and it would be a rare situation where all 3 would be required. Hafez *et al.*, (2018) and Ador *et al.* (2021) reported that molecular methods provided sensitive, rapid, and reliable data for identifying specific pathogens. Molecular methods are also useful for simultaneously identifying various bacterial pathogens that are difficult to identify by biochemical techniques or challenging to culture or take longer period for growing *in vitro*. Therefore, during the disease outbreaks, the use of molecular methods is critical in the diagnostic laboratory where the need of accurate and rapid identification of pathogens in the fish population presenting the clinical signs of the disease in efforts to prevent and address the occurrence of pathogenic infection.

In many SEA laboratories, including those in Indonesia, there is a lack of resources and capacity to use a sufficient number of assays to confirm the identification of the bacteria. Therefore, from the work performed here, it is clear that the combination of the primary identification tests (traditional) and the *16S rDNA* or *rpoD* or *gyrB* could be recommended to obtain species level identification of the motile *Aeromonas* species associated with disease in the freshwater tilapia and catfish

farming systems in ENT. Previous reports also identified the benefit of the housekeeping genes including *gyrB* and *rpoD* in accurately recognising and characterizing the *Aeromonas* strains in farmed fish (Chen *et al.*, 2019; Hossain *et al.*, 2020; Fernández-Bravo and Figueras, 2020; Legario *et al.*, 2023). The uptake of these housekeeping genes is an important modernisation in microbial identification of a closely related group of bacteria and can help to improve the accuracy in detecting the diversity and phylogenetic relationships for intra genus species identification of the motile aeromonads (Rajwar and Sahgal, 2016).

5.4. Pathogenicity and Infectivity studies

The identification results from Chapter 3 clearly showed the best laboratory-based methods including the phenotypic and genotypic assay to distinguish the *A. hydrophila* and *A. veronii* species which were recovered from the affected fish ENT systems. This is a significant improvement but does not provide sufficient information on the pathogenic from the non-pathogenic strains. Without this additional level of detail, any surveillance or monitoring strategies or preventative treatments may not detect the appropriate strains or could be produced against the wrong bacterial strains. This would significantly impair the promise of vaccines or other non-antibiotic treatments as they would have the wrong microbial target, i.e. they may be developed to non-pathogenic or weakly pathogenic strains. To understand the pathogenicity of the motile aeromonad strains recovered in ENT, a series of phenotypic and genotypic assays was performed. The genotypic assays performed investigated the presence or absence of 12 genes which are all reported to be associated with pathogenicity in *A. hydrophila* or *A. veronii* (Latif-Eugenín *et al.,,* 2016; Fernández-Bravo and Figueras, 2020; Legario *et al.*, 2023).

Highest number of virulence genes were detected by PCR reactions from *A. hydrophila* strains where a minimum of 42% (n=5) and a maximum of 83% (n=10) genes were recovered from *A. hydrophila* strains. The profiling of the virulence genes detected the combination of *act*+*aerA* genes to be the most common from all the tested motile *Aeromonas* strains recovered from moribund fish samples that presented several clinical signs externally and internally, and similar findings have been reported for other *A. hydrophila* or *A. veronii* isolates associated with disease outbreaks globally (Hafez *et al.*, 2018; Abdel-Latif and Khafaga, 2020; Nhinh *et al.*, 2021). These genes are important in pathogenicity for these bacteria as they cause host cell damage of

absorbance membrane, osmotic lysis and cell necrosis and combine with enterotoxin gene to cause haemolysis and contribute to MAS infection (Galindo *et al.*, 2004; Rasmussen-Ivey *et al.*, 2016). As the majority of the virulence genes combinations identified were found within tested motile *Aeromonas* strains recovered from moribund fish with several clinal signs externally and internally. The cellular change of moribund fish samples could appear grossly as haemorrhages and internal bleeding of the organs and motile *Aeromonas* strains were also recovered that were later identified to cause haemolysis on blood agar media.

It would not currently be cost-effective to perform all of the virulence gene assays in this study for every bacterial strain recovered and so representatives of strains were selected, including the strains categorized as low, medium, and high virulence for *A. hydropila* and *A. veronii,* where the high virulence strains always had the combined of *act+aerA+lip* genes, corresponding to 58% (n=8) of the tested *A. hydrophila* strains and 27% (n=6) of A. *veronii.* Currently these assays are performed as single PCRs but a multiplex PCR could be developed to reduce the cost and time and could confirm the virulence of the bacteria. These assays could easily be developed and implemented for most diagnostic assays and the data collected and reported, similar to other competent authorities in other countries, to improve the disease surveillance and diagnostic capacity.

There is immense value to the *in vitro* laboratory-based work performed in this study, however, to confirm the pathogenicity it is common practise to perform *in vivo* infectivity studies in the target host species. The use of fish as an experimental model remains the “gold standard” in aquatic disease research (Cengizler, 2022). Unfortunately, this was not possible during the study and so anon-fish model of infection was used. The wax moth larvae model is one of an alternative models which is reported as an effective intermediate stage between *in vitro* and full host-specific *in vivo* studies in vertebrate species. Innate immune responses function similarly in the infection between insects and fish especially for pathogen recognition and gene expression and this supports the use of this model. One of the attractive features is the simplicity and reliability of establishing infection in these animals (Desbois and Coote, 2011). In the current study, the wax moth larvae model performed in the current study was able to assess the pathogenicity level associated to the virulence profiles of motile *Aeromonas* strains, where the different level of virulence profiles significantly caused different level of infection and survival rate of the tested larvae. The model

also able to demonstrate the effectiveness in detecting the efficacy and level of antibiotics and also proved that the infection was caused by live cells. Therefore, the wax moth larvae model can be used in the diagnostic method to address the issue of pathogenic diseases. The model has also been shown to be an excellent alternative model for infectivity studies in aquatic pathogens including *A. hydrophila* (Korany *et al.*, 2019; Six *et al.*, 2019). Furthermore, the finding in the current study will need further test to confirm the results in the present study with more refined fish infectivity trials with significant dataset outcome from which the protocols for pathogenicity can be developed, robust and animal models can also be developed from which the most effective vaccines produced.

5.5. Recommendations.

1. One of the key findings from this study was the lack of farmers knowledge and lack of cost-effective biosecurity practises. The data generated from this study can provide a range of simple and advanced biosecurity practises applicable to the diversity of farming systems and production levels in ENT. These strategies will include recommendations on hygiene and sanitation practises as well as development of polyvalent vaccines for the tilapia and catfish sector.
2. The range of laboratory-based methods used in this study highlighted the challenges using biochemical or colorimetric assays for identification of the motile aeromonads. Future recommendations for any diagnostic laboratory or to support the competent authorities in their disease monitoring practises would be to use both primary and molecular methods to accurately identify and characterize the bacterial pathogens including motile *Aeromonas*. The inclusion of both primary and one of the molecular identification assays would be cost effective and significantly improve capability within those responsible for the health monitoring and disease surveillance.
3. Detection of virulence profiles governing bacterial pathogens can help in addressing the issue of pathogenic infections and the development of specific and cost-effective vaccines. Still, the main driver of AMR emergence is the inappropriate use of antibiotics. Therefore, the simple approach to address this is to reduce the number and volume of antibiotics used and this is considered as the most effective and reliable approach that can be achieved by improving

knowledge and awareness, introducing regulation and regular monitoring, and producing standardized protocols.

4. The wax moth larvae model was shown to be a useful intermediate animal challenge model to confirm infectivity and identify the environmental variables influencing pathogenicity. This could be used to refine fish studies and reduce the number of vertebrate animal studies in line with the 3Rs (refinement, reduction, and replacement).
5. The baseline data produced from the current study can contribute significantly to addressing the issue of pathogenic diseases and support the sustainability of farming system as long-term programs with more improved and integrated farming practice and less environment impact, where the data can also inform policy makers as well as local farmers to support the development of sustainable aquaculture in ENT and more widely across Indonesia.

5.6. Future Perspective Research Work

The work performed in this study has produce a curated bacterial culture collection and a tissue biobank from which to develop novel treatment and intervention methods e.g. vaccines. Future work should continue to expand on the curated collection and could include the use of whole genome sequencing (WGS) to fully elucidate the virulence and pathogenicity factors associated with the specific diseases and pathogens. These kinds of databanks can become useful in the future development of fish disease monitoring programmes. Whilst the wax moth larvae study showed the infectivity potential of the bacterial species recovered from fish, further work is required to confirm this using whole fish studies. These kinds of infectivity study can now be refined based on the data provided in this study and may lead to the development of infectivity models which would be critical to confirm the efficacy of any disease prevention strategy adopted. The data from this study will be shared with the Indonesian competent authority.

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APPENDIX I

QUESTIONNAIRE:

Information on the Status and Prevalence of Bacterial Infections Affecting Intensive Farmed Tilapia in Indonesia

Introduction: The aim of this questionnaire is to gather quantitative and qualitative information on the current knowledge, practices and prevention of infectious aquatic diseases on these farms. This information will remain confidential, and when used in publications, the names and specific locations of the farms will not be disclosed but will be re-coded.

I. Farm Background Information

a. Gender farm owner: ☐ Male ☐ Female

b. GPS : Click here to enter text.

c. Farm Size?

☐ 0-1 ha	☐ 2-5 ha	☐ 6 or more ha

Exact size: Click here to enter text.

d. Type of farming system?

☐ Extensive (natural food only)

☐ Semi – intensive (natural food + artificial feeding)

☐ Intensive (artificial feeding only)

e. How long has the farm operated?

☐ 0-2 years	☐ 2-5 years	☐ 5-10 years	☐ > 10 years

f. Total number of ponds in farm and wow many ponds are currently in use?

☐ 0-4 ponds Used:	☐ 4-8 ponds Used:	☐ 8-10 ponds Used:	☐ >10 ponds Used:

i. Type of ponds culture?

☐ Monoculture

☐ Polyculture

☐ Mix culture

g. What type of ponds system?

☐ Earthen ponds

☐ Cement ponds

☐ Tarpaulin ponds

☐ Others, please specified:

Click here to enter text.

h. Volume of Production in 2018 and 2019?

☐ 1 – 100 tonnes	☐ 100 – 500 tonnes	☐ 500 – 1,000 tonnes	☐ 1.000 or more tonnes

Exact production: Click here to enter text.

i. Source of fry?

☐ Wild

☐ Hatchery

☐ Mix

If it was from hatchery are there:

☐ Private

☐ Government

☐ others, please specify:

: Click here to enter text.

j. Did you practice acclimatization of your fish stocks prior to stocking?

☐ No

☐ Yes

☐ Sometimes

If Yes, Please provide in more details what method of acclimatization did you use?

Click here to enter text.

k. Do you keep farm records?

☐ No

☐ Yes

☐ Sometimes

If Yes, what type of report do you keep?

☐ Electronic

☐ Papers

What information of farm records do you keep?

☐ Production

☐ Feeding Conservation Ratio

☐ Diseases/mortalities

☐ Water quality parameters

☐ Others, please mention below:

Click here to enter text.

II. Background of person interviewed

a. Please tick all that apply

☐ Owner	☐ Manager	☐ Worker	☐ Family member

b. Person interviewed name : Click here to enter text.

Gender : ☐ Male ☐ Female

Address : Click here to enter text.

c. How many years of experience do you have working in fish farm?

☐ < 1 year ☐ 1 – 2 years ☐ 3 - 5 years ☐ > 5 years

III. Fish Diseases and Health Management

a. Do you have any fish disease problems on your farm? If yes go to III.b, if no go to III,i.

☐ Yes	☐ No

If yes: can you please give details of how often the problems occur?

☐ No details

☐ Don’t know

☐ Give details:

Click here to enter text.

b. Do you know what caused of the disease outbreaks?

☐ No

☐ Yes

☐ Sometimes

If yes, please describe?

Click here to enter text.

c. Do you report mortality/disease outbreaks to government fisheries authorities?

☐ No

☐ Yes

☐ Sometimes

If yes, please describe?

Click here to enter text.

b. During disease outbreaks, what have been your largest losses from 2018-2019?

☐ Losses : Click here to enter text. %

☐ Economic losses : Rp. Click here to enter text.

c. If disease outbreak occurs, what measure had been taken (tick all that apply).

☐ Nothing	☐ Change water	☐ Treatment	☐ Emergency harvest	☐ Others Please specify: Click here to enter text.

d. Do you know about disease treatment?

☐ No

☐ Yes

If Yes go to III.e, if No go to III.g.

If yes, please tick who provided the disease treatment methods?

☐ Government

☐ Other farmers

☐ Self

☐ Others, please specified

Click here to enter text.

e. What treatment did you apply during recent disease outbreak?

☐ Traditional medicines	☐ vaccines	☐ Antibiotics	☐ Others
Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

f. How do you know if your fish is sick (tick all that apply)

☐ Fish stop feeding

☐ Fish change color

☐ Fish change behavior

☐ Abnormal shape or size

☐ Marks (lesions, tumor, ulcers) in body

☐ Damaged fins

☐ Swelling eyes or bulging stomach

☐ Fish dies

☐ Behaviour

☐ Appearance

☐ Don’t know

☐ Others, Please specify:

Click here to enter text.

g. In what culture/stages period diseases occur?

☐ First month of culture

☐ Middle of culture period

☐ Last month of culture period

☐ Others: Click here to enter text.

h. What do you do with disease fish/shrimp?

☐ Discard	☐ Burn	☐ Eat	☐ Sell, where do you sell? Click here to enter text.

Other use e.g. feed to livestock: Click here to enter text.

i. Is there any fish disease monitoring from government institution?

☐ Yes	☐ No

If yes: please describe what kind of fish disease monitoring concern on?

Institution	Activities
Click here to enter text.	Click here to enter text.

j. Is there any program from government/private companies in addressing fish disease?

☐ Yes	☐ No

If yes: please describe what program?

Institution/Private Companies	program
Click here to enter text.	Click here to enter text.

V. Husbandry, feed and water management

a. Do you fertilization of pond

☐ Yes	☐ No	☐ Sometimes

If yes, Please mention what fertilization:

Click here to enter text.

b. Do you practice any water quality management Y/N Water management (water change, water quality control and measurement)

☐ Yes	☐ No	☐ Sometimes

If yes, Please describe what management?

Click here to enter text.

c. Any chemical used for water quality?

☐ Yes	☐ No	☐ Sometimes

If Yes, Please mention what chemical and when?

Click here to enter text.

d. The frequent of feeding

☐ Once a day

☐ Twice a day

☐ Others, please specified: Click here to enter text.

FARM CONTACT INFORMATION

Name of farm	: Click here to enter text.
Owner of farm	: Click here to enter text.
Address	: Click here to enter text.
Contact telephone number	: Click here to enter text.
Email	: Click here to enter text.
Farm Id Number	: Click here to enter text.

APPENDIX II

KEY INFORMANTS LIST OF QUESTIONS:

Introduction: The aim of this interview is to gather quantitative and qualitative information on the activities engaging with fish farming in East Nusa Tenggara, Indonesia. This information will remain confidential, and when used in publications, the names and specific activities will not be disclosed but will be re-coded.

Please Identify and describe the activities that you are engaged in that are associated with Fish farming in East Nusa Tenggara. Please thick all that apply.

☐ feed suppliers	☐ seed suppliers	☐ hatcheries	☐ medicine suppliers	☐ others

1. **List of Question.**
   a. What types of products do you supply to farmers? *(examples provided, please tick all that apply)*
      ☐ vaccines
      ☐ probiotics
      ☐ antibiotics
      ☐ feed
      ☐ fish
      ☐ others, please specify.
      : Click here to enter text.

   b. What types of farmers do you sell your product?
      ☐ Intensive farmers
      ☐ Semi intensive farmers
      ☐ Conventional farmers.
   c. Does your product provided meet the need of farmers? (Y/N)

   d. Do you provide information to the farmer regarding your product associated with fish farming?
      ☐ No.
      ☐ Yes.

If yes, please specify what information do you provide

: Click here to enter text.

e. Do you contact the farmers regarding your product application? (Y/N/Sometimes)
f. Do the farmers contact you regarding your product? (Y/N/Sometimes)
g. Do the farmers provide any information to you regarding their fish farming?

☐ No.

☐ Yes.

If yes, please specify.

Click here to enter text.

KEY INFORMANTS (KI) CONTACT INFORMATION:

Name of KI	: Click here to enter text.
Company/Institution	: Click here to enter text.
Contact telephone number	: Click here to enter text.
Email	: Click here to enter text.
KI Id Number	: Click here to enter text.

APPENDIX III

The Protocol of Kirby-Bauer Antibiotic Disc Diffusion Test

Purpose

To measure inhibition of bacterial activity by impregnated antibiotic disk method (Kirby-Bauer Method)

Procedure

Materials

Pure bacterial culture on agar plate
Sterile saline solution (0.85% or 2%)
Antibiotic disks to be tested and disk dispenser
70% Ethanol
Glass spreader and beaker
Bunsen burner and lighter
Bacteriological loop
Agar plate of appropriate growth media
Bijoux container
Gilson pipette and sterile tips (100µl)
Ruler

Method

1. Ensure culture under test is pure by examining colony morphology. A culture is considered to be pure when the colony size, shape, colour etc are uniform in appearance.
2. Remove 1 colony using a sterilised bacteriological wire loop and inoculate into 3 mls of sterile saline solution. Note if cfu very small, will require more (aim to obtain turbidity equal to McFarland 3)
3. With the bottle cap replaced, suspend the culture in the saline solution by gently inverting.
4. Pipette 100µl bacterial suspension onto the surface of a pre-labelled agar plate.

5. Sterilise a glass spreader by immersing in 70% ethanol then passing the spreader through a Bunsen flame to burn off the excess ethanol.
6. Once slightly cooled, gently spread the suspension over the whole surface of the agar plate and replace the petri dish lid.
7. Replace the glass spreader to 70% ethanol beaker to sterilise. DO NOT PASS THROUGH FLAME AS THIS WILL IGNITE ETHANOL
8. Allow the plate to dry for approximately 1 minute then dispense the antibiotics onto the agar plate by placing the antibiotic disc dispenser over the plate and pressing firmly downwards. The six disks will be evenly distributed onto the agar surface.
9. Leave plate for 5-10 minutes to dry then invert the plate and incubate at a suitable temperature. Ensure antibiotic discs have not detached from agar surface.
10. Check plates after 24hrs and record after 48hrs. If incubating at a low temperature. Record results after 72hrs.

Results

Examine agar plate for bacterial growth. If the antibiotic(s) have effectively inhibited growth, there will be areas of clearing around each disk known as zones of inhibition. If present, this indicates that the organism under test is sensitive to that particular antibiotic(s). The absence of a clear area indicates that the organism under test is resistant to that particular antibiotic(s). Using a ruler, measure the inhibition zone, taking two readings and obtain the average measurement and record in lab book.

Sensitive (S)	= over 16mm
Partially sensitive (PS)	= 11-15mm
Resistant	= 0-10mm