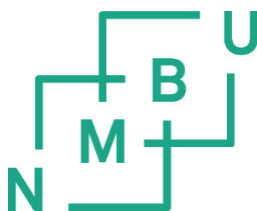# Nanoparticle based vaccine delivery of recombinant outer membrane proteins and characterization of *Aeromonas hydrophila* and *Edwardsiella* species in Asian Aquaculture

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Nanoparticle based vaccine delivery of recombinant outer membrane proteins and characterization of *Aeromonas hydrophila* and *Edwardsiella* species in Asian Aquaculture

Philosophiae Doctor (PhD) Thesis

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Acknowledgements

Summary

Asia is home to aquaculture and the region contributes more than two third of total global aquaculture production. The sustainability of aquaculture in this region is challenged because of diseases, mainly due to bacterial pathogens. Bacterial infections caused by *Aeromonas hydrophila* and *Edwardsiella tarda* result in large scale mortality of farmed fish. The outer surface of theses bacterium is made of different antigenic compound such as fimbriae and pili that define different serovars and serotypes. The major challenge in vaccine development has been to find antigens that are broadly protective against these bacterial pathogens. Definite identification of *Edwardsiella* spp. and *A. hydrophila* from different fish species is a prerequisite to identifying antigens with cross protective abilities, even across different serotypes. Species identification is also a challenge and recent developments have shown that *gyrB* is a useful molecular gene for the identification of different bacterial species and as a phylogenetic marker.

By comparing *gyrB* sequences obtained in this study, our findings show a significant genetic intraspecies and inter-geographical variation for *E. tarda* and *A. hydrophila* isolates from different aquatic organisms from different parts of Asia. Furthermore, our finding shows that combining 16S rRNA and *gyrB* sequence analysis and antigenic OMP protein characteristics for different bacterial strains could serve as an effective strategy for identifying the best suited antigenic proteins that are cross protective against *A. hydrophila* and *Edwardsiella* species. Recombinant DNA technology is useful to produce large amounts of OMP antigens. The small size and low antigenicity of these proteins require use of adjuvants to enhance the immune response after vaccination. Nanoparticles (NPs) are useful for encapsulation of small peptides and protein antigens and the cellular uptake of NPs is very high due to high surface-to-volume ratio. Further, oral vaccination is the least stressful immunization method of all age groups of fish. Rohu (*Labeo* species) is a popular farmed fish species in India and was used in this study for testing of vaccine efficacy. The method used to evaluate the host immune responses to bacterial antigens has been circulating antibody (IgM) levels and survival post challenge.

In the present study (Paper I & II) we have screened *Edwardsiella* spp. and *A. hydrophila* strains using biochemical test, 16S rRNA, *gyrB* and OMPs from different parts of Asia. *E. tarda* OmpA and *A. hydrophila* OmpW were delivered orally in Rohu encapsulated with NPs. We find that NP-rOMPs give better RPS and higher antibody titer compared with empty NP after post infection against *E. tarda* and *A. hydrophila* pathogenic strains (Paper III and IV).

Sammendrag

Akvakultur startet i Asia og i dag produseres mer enn to tredjedeler av den globale produksjonen i denne regionen. Bærekraftig utvikling av intensiv akvakultur utfordres av sykdom som hovedsakelig skyldes bakterielle infeksjoner, og spesielt infeksjoner forårsaket av *Aeromonas hydrophila* og *Edwardsiella tarda* resulterer i stor dødelighet. Den ytre overflaten av bakterien er laget av forskjellige antigener som fimbrier og pili som definerer forskjellige serovarer og serotyper. Den største utfordringen i vaksineproduksjon har vært å finne antigener som er kryssbeskyttende mot disse bakterielle patogenene og en nøyaktig identifisering av *Edwardsiella* arter og *A. hydrophila* isolert fra ulike fiskearter er viktig for å definere de antigener som er kryssbeskyttende på tvers av serotyper. Senere tids studier har vist at *gyrB* er et velegnet housekeeping-gen for identifisering av forskjellige bakteriearter. I denne studien (Paper I & II) har vi karakterisert *Edwardsiella* spp. og *A. hydrophila* ved hjelp av biokjemisk tester, 16S rRNA, *gyrB* og OMPs primere fra flere land i Asia.

Ved å sammenligne *gyrB*-sekvenser viser denne studien signifikante genetiske variasjoner mellom bakteriearter og inter-geografiske variasjoner for *E. tarda* og *A. hydrophila* isolater fra forskjellige fiskearter fra forskjellige deler av Asia. Videre viser vår undersøkelse at kombinasjon 16S rRNA, *gyrB* sekvensering og sekvensanalyse av OMP-protein fra forskjellige bakteriestammer er en måte å identifisere kandidat-antigener (proteiner) med potensielt kryssbeskyttende egenskaper for *A. hydrophila* og *Edwardsiella*-arter. Rekombinant DNA-teknologi er videre velegnet til å produsere store mengder OMP-antigen, men proteinenes størrelse og immunogenisitet gjør at det er nødvendig å bruke adjuvans for å oppnå god immunrespons/immunitet. Nanopartikler er velegnet for innkapsling av peptider/proteiner, og det cellulære opptaket av NP er høyt blant annet på grunn av forholdet mellom overflate og volum. Oral vaksinering er en lite stressende immuniseringsmetode for alle aldersgruppe av fisk. I denne studien (papir I og II) har vi benyttet antistoff-responser og overlevelse etter eksperimentell smitte for å vurdere vaksinenes effekt. Studiene er gjort i indisk karpe ved bruk av NP-rOMP formuleringer og disse gir høy RPS og høyt antistofftiter sammenlignet med kontrollgruppene testet mot *E. tarda* og *A. hydrophila*. OmpA antigener basert på *E. tarda* og OmpW for *A. hydrophila* ble administrert oralt i Rohu (Labeo-arter) innkapslet med NP (papir III og IV).

Abbreviations

APC	Antigen presenting cell
API 20 NE	Analytical profile index 20 non Enterobacteriaceae
CD	Cluster of differentiation
CFU	Colony forming unit
DCs	Dendritic cells
DPV	Days post vaccination
ELISA	Enzyme-linked immunosorbent assay
ERM	Enteric red mouth disease
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
gyrB	DNA gyrase subunit B
H2S	Hydrogen sulfide
IFN-γ	Interferon gamma
IgM	Immunoglobulin M
IM	Intramuscular injection
IP	Intraperitoneal injection
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IWC-ET	Inactivated whole cell *Edwardsiella tarda*
kDa	Kilo Daltons
LPS	Lipopolysaccharides
MAI	Motile aeromonad infection
MAS	Motile aeromonads septicemia
MHC	Major histocompatibility complex
MT	Metric ton
NK-cells	Natural killer cells
NPs	Nanoparticle
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PLGA	Poly D,L-lactide-co-glycolic acid
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rOmpA	Recombinant outer membrane protein A
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOFIA	The State of World Fisheries and Aquaculture
Tg	Transition temperature
TLR	Toll-like receptors
TSA	Tryptic soy agar
USD$	United States dollar
16S-rRNA	16 S ribosomal RNA

List of Publications

1) Dubey S, Maiti B, Kim SH, Sivadasan SM, Kannimuthu D, Pandey PK, Girisha SK, Mutoloki S, Chen SC, Evensen Ø, Karunasagar I, Munang'andu HM. Genotypic and phenotypic characterization of Edwardsiella isolates from different fish species and geographical areas in Asia: Implications for vaccine development. J Fish Dis. 2019 Mar 8. doi: 10.1111/jfd.12984

2) Dubey S, Maiti B, Sivadasan SM, Kannimuthu D, Girisha SK, Mutoloki S, Chen S, Evensen Ø, Karunasagar I, Munang'andu HM. *Aeromonas hydrophila* isolates obtained from different farmed aquatic species in India and Taiwan show high genotypic and phenotypic relatedness. Submitted to BMC Vet. Res.

3) Dubey S, Avadhani K, Mutalik S, Sivadasan SM, Maiti B, Girisha SK, Venugopal MN, Mutoloki S, Evensen Ø, Karunasagar I, Munang'andu HM. *Edwardsiella tarda* OmpA Encapsulated in Chitosan Nanoparticles Shows Superior Protection over Inactivated Whole Cell Vaccine in Orally Vaccinated Fringed-Lipped Peninsula Carp (*Labeo fimbriatus*). Vaccines (Basel). 2016 Nov 7;4(4).

4) Dubey S, Avadhani K, Mutalik S, Sivadasan SM, Maiti B, Paul J, Girisha SK, Venugopal MN, Mutoloki S, Evensen Ø, Karunasagar I, Munang'andu HM. *Aeromonas hydrophila* OmpW PLGA Nanoparticle Oral Vaccine Shows a Dose-Dependent Protective Immunity in Rohu (*Labeo rohita*). Vaccines (Basel). 2016 Jun 1;4(2). pii: E21. doi: 10.3390/vaccines4020021.

1.0 Introduction

1.1 Fisheries and Aquaculture

The world population is expected to reach 9.7 billion by 2050, which is going to put a significant pressure on food supply, particularly high value protein sources. Given that animal and plants proteins sources are reaching a ceiling, fish protein is considered as an important alternative, and aquaculture is and will be an important source of high value protein for a growing world population. Global fish production reached about 171 million tonnes (mt) by 2016 (Figure 1), with aquaculture accounting for 47%. The value of fisheries and aquaculture production in 2016 was estimated at USD\$ 362 billion, of which USD\$ 232 billion came from aquaculture. Global aquaculture production (including aquatic plants) in 2016 was 110.2 mt of an estimated value of USD\$ 243.5 billion. Farmed finfish production in the same year was 54.1 mt, equivalent to USD\$ 138.5 billion. Among the various sources of protein including fish, milk, meat and eggs, fish is of high nutritional value. Further, fisheries and aquaculture are important sources of food, nutrition, income and livelihoods for millions of people globally, and the annual per capita fish consumption reached a new record high of 20.3 kg in 2016[1].
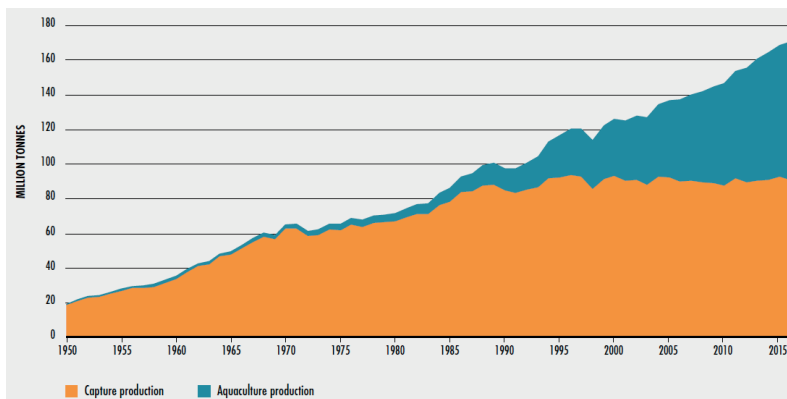
Figure 1. World capture fisheries and aquaculture production [1] SOFIA 2018, FAO

1.2 Asia-Pacific aquaculture production

Asia has been the home of aquaculture for thousands of years of which the Asia-Pacific region is the world leader in aquaculture production. Aquaculture in Asia accounts for 89.4% of total world production[2] (Table1 and Figure 2). Asian countries mainly China, India, Indonesia, Vietnam and

Bangladesh are the largest producers of farmed fish [3]. The per capita fish supply in this region is around 29 kg, which is 9 kg higher than the world per capita consumption [3]. There is need to produce an additional 30 to 40 mt fish/year in order to meet the demand of the growing human population by 2050 [3]. The five most important countries in Asia are China, India, Indonesia, Vietnam and Bangladesh with percentage of 61.5%, 7.1%, 6.2%, 4.5% and 2.8% respectively, while the rest of Asia contribute about 7.3%[1]. More than 60% of the world human population lives in the Asia-Pacific region where the two most populous countries China and India, contribute 1.4 and 1.2 billion people, respectively[4].

Table 1. Shows the share of fish production per continent (1,000 mt)

Continents	1995	2005	2015	2016
Africa	110	646	1772	1982
Americas	920	2177	3274	3348
Asia	*21678*	*39188*	*67881*	*71546*
Europe	1581	2135	2941	2945
Oceania	94	152	186	210
World	**24383**	**44298**	**76054**	**80031**
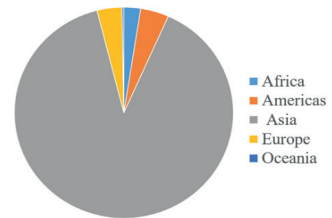
Figure 2. Global fish production by continent (FAO, Sofia 2018)

China is the largest exporter of fish in the region with an estimated value of US$19.8 billion followed by Thailand and Vietnam exporting US$7.1 billion and US$6.9 billion in 2013, respectively. The contribution of India was 4.6 billion in the same year[5]. In 2012, the region produced 58.5 mt of aquaculture products, accounting for 89% of the global production of 66.7 mt. To understand the factors influencing aquaculture development in the region, it is important to know the species cultured and the countries involved [6]. This region grows 129 finfish species which shows the diversity of aquaculture. Table 2 shows that the important cultured fish species are carp, catfish and tilapia contributing around 63% of total fish produced in 2016. Most of the production comes from Asian countries which are major contributors of global fish production.

1.3 Predisposing factors to disease prevalence in Asia-Pacific aquaculture

Aquaculture in Asian countries is still developing. There is lack of advanced technology in breeding, poor supply of quality seed stock, lack of basic infrastructure, insufficient knowledge of fish diseases, poor health management practices and lack of good quality feed. This region mainly produces fish in ponds that are not well managed and work is mostly done by untrained local people, making it a challenge for sustainable aquaculture growth [7]. Studies have shown that almost 50% of production losses are caused by diseases that are more severe in developing

countries where 90% of aquaculture production is done [8]. Some of the predisposing factors that play vital roles to disease transmission include poor water quality used for fish farming, which is mostly unchecked to determine dissolved oxygen levels (DO), pH, temperature, salinity, ammonia and nitrate content. Poor water quality favor the survival of bacteria species such as *A. hydrophila* and *Edwardsiella* spp. that are ubiquitous in aquatic environments.

Table 2. Major fish species produced in global aquaculture [1]

Finfish	Scientific name	2010	2012	2014	2016	% 2016
Grass carp	*Ctenopharyngodon idellus*	4362	5018	5539	6068	11
Silver carp	*Hypophthalmichthys molitrix*	4100	4193	4968	5301	10
Common carp	*Cyprinus carpio*	3421	3753	4161	4557	8
Nile tilapia	*Oreochromis niloticus*	2537	3260	3677	4200	8
Bighead carp	*Hypophthalmichthys nobilis*	2587	2901	3255	3527	7
Carassius	Carassius carassius	2216	2451	2769	3006	6
Catla	*Catla catla*	2977	2761	2770	2961	6
Freshwater fishes	*Osteichthyes* spp.	1378	1942	2063	2362	4
Atlantic salmon	*Salmo salar*	1437	2074	2348	2248	4
Rohu labeo	*Labeo rohita*	1133	1566	1670	1843	3
Pangas catfishes	*Pangasius hypophthalmus*	1307	1575	1616	1741	3
Milkfish	*Chanos chanos*	809	943	1041	1188	2
Tilapias	*Oreochromis* spp.	628	876	1163	1177	2
Torpedo-shaped catfishes	*Clarias batrachus*	353	554	809	979	2
Marine fishes	*Osteichthyes* spp.	477	585	684	844	2
Wuchang bream	*Megalobrama amblycephala*	652	706	783	826	2
Rainbow trout	*Oncorhynchus mykiss*	752	883	796	814	2
Cyprinids nei	Cyprinidae spp.	719	620	724	670	1
Black carp	*Mylopharyngodon piceus*	424	495	557	632	1
Snakehead	*Channa argus*	377	481	511	518	1
Other finfishes		5849	6815	7774	8629	16
Finfish total		38494	44453	49679	54091	100%

Other factors include overstocking commonly done with the view to increase output production but conversely leading to poor fish growth rate, inducing stress on fish due to increased competition of resources, sometimes leads to cannibalism leading to open wounds that serve as entry points for pathogens, and is often linked to increase in disease transmission index.

1.4 Major diseases in Asian Aquaculture

Diseases not only cause economic losses due to mortality, but they also reduce the fish quality, growth performance and feed conversion ratio. Fifteen Asian countries with aquaculture products valued at over USD$ $22.7 billion in 1990, lost USD$ $1.36 billion due to disease outbreaks [7]. In China, 15% of losses of total fish production are due to diseases. Diseases cause serious economic losses globally estimated around USD$ $1.05 to $9.58 billion /year [9]. Important viral disease in Asia aquaculture include spring viremia of carp virus (SVCV), Koi herpes virus (KHV),

viral nervous necrosis (VNN), Cyprinid herpesvirus-1 (CyHV-1) and the recently discovered tilapia lake virus (TiLV) [10]. Most of these viral diseases have limited tropism for a single or a few selected fish species. For example, SVCV is a disease for carp, TiLV for tilapia and CyHV-1 for cyprinid fish species. However, bacteria diseases caused by pathogens such as *Aeromonas hydrophila* and *Edwardsiella* spp. as well as the fungus *Aphanomyces invadans* with a tropism for multiple fish species tend to have a higher devastating impact on aquaculture than pathogens having a tropism for a few species [11, 12]. And as such, bacteria species causing diseases in multiple fish species are the major causes of mortality linked to high economic losses. Another factor prone to exacerbate disease transmission is polyculture involving the culture of different fish species together in one pond, which is commonly practiced in Asian aquaculture. For pathogens having tropism for multiple host species, they are bound to cause more losses in a polyculture system. As such, more effort is directed at developing vaccines and other control measures against bacteria diseases that infect the top farmed fish species in Asian aquaculture (Table 2) of which this study aims to contribute on vaccine development. To effectively combat the disease problem in Asian aquaculture there is need to prioritize by starting with vaccine development for diseases having the highest economic impact.

1.4.1 Major bacterial diseases in Asian aquaculture

There are several bacterial pathogens reported to cause diseases linked to high economic losses in Asian aquaculture[13]. The most prominent bacteria species with multi-host-species tropism include *Streptococcus* spp., *Vibrio* spp., *Edwardsiella* spp., and Aeromonads. Streptococci infections have largely been associated outbreaks in tilapia and less in carp, which accounts for the largest proportions of cultured fish species in Asia. On the other hand, diseases caused by *Vibrio* spp. are more in crustaceans and few in fish. However, infections caused by *A. hydrophila* and *Edwardsiella* spp. cause diseases in carp, tilapia and catfish of which these fish species account for 62 % of total global aquaculture production (Table 2). And as such, infections caused by *A. hydrophila* and *Edwardsiella* spp. account for some of the largest losses seen in Asian aquaculture, which raises the urgent need for rational mitigation measures against these pathogens. Their wide tropism for various fish species including the top- farmed fish species (Table 2) that account for >62% of global production puts these two bacteria species at the apex of the most devastating pathogens in aquaculture. Moreover, high antibiotics use against these pathogens linked to drug

resistance raises serious environmental concerns that urgently require an alternative approach for their control. Studies done in India, Bangladesh, Indonesia and Thailand show that antibiotics such as bacitracin, chloramphenicol, erythromycin, tetracycline and fluoroquinolones are widely used to treat these diseases [14]. Thayumanavan et al [15] reported of *A. hydrophila* strains resistance to bacitracin. Another concern is that these pathogens carry transferable drug resistance plasmids to other microbes and that they possess haemolyzing properties that could become a human health hazard [16]. Vaccination is considered to be a better option given that it eliminates the risk of drug resistance, prevents antibiotics deposition in the environment and protects consumers from excessive exposure to antibiotics through consumption of aquaculture products[17]. However, to develop effective vaccines, pathogens must be correctly characterized and additional studies are needed to determine if isolates obtained from different fish species share antigenic properties, particularly outer surface antigens would have to be characterized in detail. It is unknown whether different fish species influence or alter the genotypic and phenotypic properties of pathogenic bacteria. It is also unknown whether isolates obtained from different geographical areas have different phenotypic and genotypic properties associated with differences in virulence and antigenic properties. These knowledge gaps raise fundamental questions that warrant detailed investigations some of which create the basis for the present study.

1.3.2 Taxonomy and economic impact of *Edwardsiella* spp. in aquaculture

The genus Edwardsiella was first reported by Sakazaki in Japan in 1962 [18] and later described in detail by Ewing et al [19] to be a Gram-negative, short rod-shaped 1 µm diameter and 2-3 µm long, motile, facultative anaerobic member of the family Enterobacteriaceae. This organism is mesophilic, oxidase negative, indole positive, produces strong H_2S and infects a wide range of hosts including both fresh water and marine fish[20]. In 1980, the second member of the genus Edwardsiella was identified and characterized as *E. hoshinae* by Grimont et al[21]. Since then, it has been isolated from birds, reptiles, human feces and water sample[22]. The third member was classified as *E. ictaluri* by Hwake et al[23] in 1981 mainly causing disease in catfish [24-26]. In 2013, genotyping done by Abayneh et al[27] reclassified fish isolates previously classified as *E. tarda* to be *E. piscicida* while isolates from eel (*Anguilla anguilla*) were reclassified as *E. anguillarum* by Shao et al[28] in 2015. To date, five Edwardsiella species have been identified namely; *E. tarda*[19, 29], *E. ictaluri*[23, 30] *E. hoshinae*[31], *E piscicida*[27] and *E. anguillarum*[28]. As shown in Table 3, *E. tarda* has a wide host tropism including several farmed fish species in Asian aquaculture such as

carp, catfish, catla and tilapia (shown in Table 2) with most reports coming from the top ranked fish producing countries such as China, Vietnam and India. More than 25 farmed fish species are susceptible to Edwardsiellosis in which the disease causes high mortality [32].

Table 3. Fish species infected by *Edwardsiella tarda* in different countries

Fish/Aquatic species	Scientific Name	Country	Ref.
Grass carp	*Ctenopharyngodon idella*	Taiwan	[33]
Silver Carp	*Hypophthalmichthys molitrix*	China	[32]
Common carp	*Cyprinus carpio*	Japan	[34]
Nile Tilapia	*Tilapia nilotica*	China and Malaysia	[35, 36]
Catla	*Catla catla*	India	[37]
Rohu labeo	*Labeo rohita*	India	[38]
Pangasius catfish	*Pangasianodon hypophthalmus*	Vietnam, India	[39]
Torpedo-shaped catfishes	*Clarias batrachus*	India, Malaysia	[40]
Japanese eel	*Anguilla japonica*	China, Japan	[41]
Japanese flounder	*Paralichthys olivaceus*	South Korea, China and Japan	[42]
European eel	*Anguilla anguilla*	China, Spain	[43]
Bloch	*Anabas testudineus*	India	[44]
Snakeheads	*Ophiocephalus punctatus*	India, Malaysia	[45]
River catfish	*Pangasius pangasius*	Malaysia	[36]
Red sea bream	*Pagrus major*	Japan	[46]
Asian clam	*Corbicula fluminea*	Malaysia	[47]
Mullet	*Mugil cephalus*	Japan	[48]

Note that most of the fish species infected by *E. tarda* in Asian countries in Table 3 include many of the major fish species produced in aquaculture shown in Table 2.

Piscine *Edwardsiella* spp. are mainly pathogens of fresh and brackish water aquatic organisms [39, 49, 50] although they also cause sporadic outbreaks in cold-water species [51, 52]. In carp, catfish and other fish species, Edwardsiellosis causes septicemia, extensive skin lesions, fluid accumulation and pathology in various internal organs resulting in high mortalities and sporadic epidemics [53]. Mortality and morbidity range from 5% - 30% and 5% - 70% in cultured fish, respectively [54]. High morbidity and mortality in several fish species have been linked to increased production costs, reduced feed intake and delayed harvest [49]. Disease severity varies between moderate to high resulting in reduced market value culminating in loss of revenue for fish farmers. The disease mainly affects market size fish although it is not confined to age and size group. In terms of economic impact, it has been associated with serious losses in countries like South Korea in Olive flounder whose production value is estimated at 489.7 billion Korean Won (40 922 MT) of which mortalities due to Edwardsiellosis accounted for 56.5% of total fish loss in 2010 [55]. It has been reported to cause >50% mortality in 450g channel catfish (*Ictalurus punctatus*) [56] as well as revenue loss amounting to USD$600 million in largemouth bass (*Micropterus salmoides*) and striped bass in USA [49]. It is also causes high losses in species such Japanese eel (*Anguilla*

japonica)[29], Japanese flounder and red sea bream (*Pagrus major*) in countries like India, China, Japan, USA [57] and different European countries [22, 58].
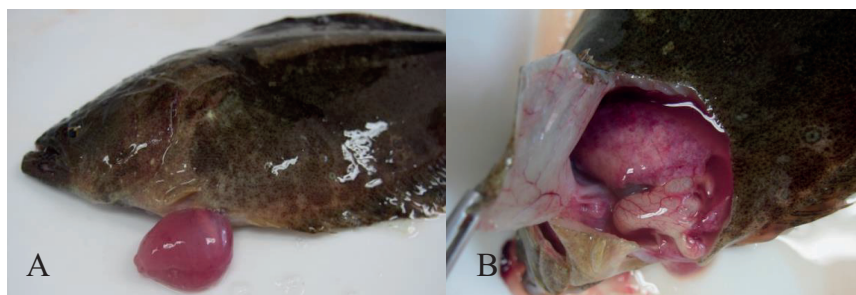
Figure 3. External sign of Olive flounder infected with *E. tarda*. (A) Abdominal distension (B) infection in Olive flounder in Jeju island, South Korea (source: Sung-Hyun Kim)

1.3.4 Taxonomy and economic impact of *Aeromonas hydrophila* in aquaculture

Aeromonads were first isolated more than 100 years ago from water and diseased animals[59]. They naturally inhabit aquatic environments such as freshwater, marine and estuarine waters. The taxonomic classification of Aeromonads has been revised several times of which initially it used to be in the Vibrionaceae family [60] but phylogenetic analysis showed that the genus *Aeromonas* was not closely related to *Vibrios* [61, 62] because it forms a monophyletic unit in the γ-3 subgroup of class Protobacteria. Therefore, in 1986, it was shifted to a new family called Aeromonadaceae [63]. The first complete genome sequence of *A. hydrophila* was carried out 20 years later in 2006 [64]. *A. hydrophila* is a Gram negative, facultative anaerobic, oxidase and catalase positive, rod shaped bacteria [59]. It has long been recognized as a pathogen of fish, bird, amphibians, and reptiles [65, 66]. In humans [67], it has been associated with a wide range of enteric and non-enteric diseases [68]. In fish, it causes motile aeromonad septicemia (MAS), motile aeromonad infection (MAI), hemorrhagic, ulcerative, septicemia, furunculosis and red sore disease [69-71]. It is one of the economically important fish pathogens in Asian aquaculture in which it has been reported to cause disease in >20 farmed fish species (Table 4).

Its economic impact is mostly devastating among commercial fish species such as carp, catfishes and tilapia that contribute >62% of global aquaculture production. In China MAS caused by *A. hydrophila* has been a prominent problem over the past 30 years. It caused significance economic losses amounting to 2200 tons of fish per year during the period 1989 to 1993 mostly affecting

Carassius carassius, *Megalobrama amblycephala* and *Hypophthalmichthys molitrix* in Zhejian Province in China [72]. In Indonesia, it caused mortality in common carp and goldfish amounting to losses of 820 tons, equivalent to $37.5 million in losses in 2001 and 2002. Major losses in catfish in Minnesota and North Dakota were reported resulting in high mortalities and economic losses in 2007 [73]. Since 2009 MAS outbreaks have occurred in Alabama (USA) leading loss of >3 million pounds per year [74]. In 2009, mortalities reached 2,200 tons and 10,500 tons in 2016 caused by the hypervirulent *A. hydrophila* (vAh) strains accounting for 35% MAS outbreaks in the USA [75, 76]. High mortalities with annual losses of millions of tons in species such as channel catfish and grass carp have continued to increase annually in China and USA [75-79]. In 2016, *A. hydrophila* caused mass mortalities in *O. niloticus* and *Sparus aurata* that resulted in high losses in Egypt [80].

Table 4. Fish species affected by *A. hydrophila* with geographic location

Common name	Scientific name	Country	Rf.
Grass carp	*Ctenopharyngodon idella*	China, India	[81]
Silver carp	*Hypophthalmichthys molitrix*	China, India	[82]
Common carp	*Cyprinus carpio*	India ,China	[83]
Nile tilapia	*Oreochromis niloticus*	India, China, Taiwan	[84]
Bighead carp	*Hypophthalmichthys nobilis*	China, Taiwan	[85]
Carassius	*Carassius carassius*	China	[82]
Catla	*Catla catla*	India	[86]
Rohu labeo	*Labeo rohita*	India, Bangladesh, Pakistan	[87]
Pangas catfishes	*Pangasius hypophthalmus*	Bangladesh	[88]
Torpedo-shaped catfish	*Clarias batrachus*	India	[89]
Spotted snakehead	*Channa punctatus*	India, Bangladesh	[90]
Soft shell turtle	*Amyda cartilaginea*	China, Taiwan, Vietnam	[82]
Eel	*Anguilla anguilla*	China, India	[82]
Blunt-snont bream	*Megalobrama amblycephala*	China	[82]
Marsh prawn	*Palaemonetes vulgaris*	China	[82]
Goldfish	*Carassius auratus*	India, Singapore	[91]
Chinese perch	*Siniperca chuatsi*	China	[92]
Crucian carp	*Carassius carassius*	China	[93]
Mud loach	*Misgurnus mizolepis*	South Korea	[94]
Chinese mitten crab	*Eriocheir sinensis*	China	[95]
Striped catfish	*Pangasius hypophthalmus*	Vietnam, India	[96]
Mrigal carp	*Cirrhunas mrigala*	India	[86]

Put together, these observation show that *A. hydrophila* together with *Edwardsiella* spp. have a wide tropism inclusive of the top-farmed fish species in aquaculture (Tables 2, 3 and 4) in which they cause high mortality and high economic losses putting the two bacteria species to be among the most important pathogens that urgently require development of protective vaccines to reduce their devastating impact in aquaculture.

1.6 Vaccines and vaccination

There has been a lot of research using different approaches aimed at developing protective vaccines against these pathogens but there has been no major breakthrough in the licensure of protective vaccines. There are several steps involved in development of protective vaccines; (i) identification of protective antigens, (ii) choice of antigen delivery systems, (iii) selecting the mode of immunization - injection, immersion or oral, and (iv) finally, defining measures of efficacy.

A: IDENTIFYING IMMUNOGENIC PROTEINS
• Broadly protective epitopes
• Evoke adaptive immune response

B: CHOICE OF ANTIGEN DELIVERY SYSTEM
• Intracellular antigen delivery (Replicative vaccines live/DNA)
• Extracellualr antigen delivery (Non-replicative vaccines IWC/subunit)

C: MODE OF VACCINE DELIVERY
• Injectable
• Immersion
• Oral vaccination

D: MEASURES OF EFFICACY & IMMUNE PROTECTION
• Relative percent survival/Kaplan Meyer's survival analysis
• Antibody response/serum inhibition tests *in vitro*

Figure 4. Schematic workflow of vaccine development

1.6.1 Identifying antigenic proteins

One of the fundamental requirements in vaccine design is to identify immunogenic proteins able to produce protective immunity in vaccinated individuals [97]. In the case of bacteria, this task can be difficult given that potentially bacteria have several immunogenic proteins that can be used for vaccine design. In the case of *A. hydrophila* and *Edwardsiella* spp., there have been several studies carried out aimed at identifying the protective antigens. For example, Hou et al [98] and Sakai et al [99] examined various proteins including GAPDH, OmpA and filament proteins (Fils) as vaccine candidate against *E. tarda*. Other proteins identified as potential vaccine antigens against *E. tarda* include flagellar protein D (FlgD) [100], flagelin protein C (FiC)[101], EseD[102], T3SS[102] and several OMPs [103, 104]. For *A. hydrophila* various antigens have been tested in different fish species with variable success and these include T3SS [102], GAPDH [105], DnaJ [106], major fimbrial protein A (fimA) [107], FlgD [100], OMPs [103] and several other genes [108]. Among these, the most widely explored both for *Edwardsiella* spp. and *A. hydrophila* are OMPs. Immunogenic OMP vaccines have been shown to have broad cross reactivity against variant strains of the same pathogen [109], but it is unknown if bacterial isolates from different fish species share common antigenic properties.

Figure 5. (A) Schematic layout of OmpX[110] showing surface loops (gray) and β-barrels (blue). (B) shows the topology plot of OmpX [111] β-barrels in the lipid bilayer (yellow) while surface loops are located in the extracellular layer designated as L1-L4.

1.6.1.1 Outer Membrane Proteins as vaccine candidates

Different studies have shown the potential role of OMPs as vaccine antigens in fish. OMPs are porin structures made of β-barrels that cut across the bacteria cell wall and membrane whose main function is transportation of various molecules into and outside bacteria. Their outer layer is made of surface loops and represent antigenic sites. Figure 5 shows the topology of *E. coli* OmpX [111] having eight β-barrel sheets cutting across the cell lipid bilayer thereby exposing the surface loops. Surface binding sites are highly conserved within bacterial species [112-114] and are recognized as none-self pathogen associated molecular patterns (PAMPS) by the host pattern recognition receptors (PRRs) such as toll like receptors (TLR) 2 and 4. In humans, OmpA has been shown to interact with APCs as a PAMP for *Klebsiella pneumoniae* and *Salmonella typhimurium* through TLR2 [115]. Similarly, the binding for *Shigella flexneri* and *Klebsiella pneumoniae* onto macrophages, natural killer (NK) cells and dendritic cells (DCs) was mediated through TLR2 [116, 117]. After OMP attachment on TLRs, the APCs are activated to produce various cytokines. For example, upregulation of IFN-γ secretion was observed in mice immunized with an OmpA vaccine [118] while OmpA was shown to render DCs and macrophages responsive to the cytokine CCL21 triggering their migration to secondary lymphoid organs [119]. *Shigella flexneri* OmpA has been shown to induce secretion of various pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, IFN-γ and IL-12p70 that coordinate type-1 adaptive immune responses [117]. Pore at al [117] showed that OmpA-stimulated macrophages production of MHC-II, CD80 and CD40 critical for activation of adaptive immune responses. Pore et al [120] also showed that OmpA-activated CD4+ cells to secrete IFN-γ and IL-2.

Table 5. List of OMPs of *A. hydrophila* and *E. tarda* used in fish vaccination

Bacteria	OMPS	Size of B-barrel	Fish species	Ref
Aeromonas hydrophila	OmpW	8 (Monomer)	*Labeo rohita*	[121]
	OmpTS	10 (Monomer)	*Labeo rohita*	[122]
	Omp48	16 (Trimer)	*Labeo rohita*	[123]
	OmpG	10 (Monomer)	*Anguilla anguilla*	[124]
	Aha1		*Cyprinus carpio*	[125]
	42 kDa OMPs		*Carassius auratus*	[126]
	43 kDa		*Trichopodus trichopterus*	[127]
Edwardsiella tarda	37 kDa		*Paralichthys olivaceus*	[128]
	OmpA	8 (Monomer)	*Cyprinus carpio*	[129]
	37 kDa OMP		*Paralichthys olivaceus*	[130]
	Omp85		*Labeo rohita*	[131]
	OmpA	8 (Monomer)	*Labeo rohita*	[132]
	Major OMP		*Anguilla japonica*	[133]
	44kDa OMP		*Oryctolagus cuniculus*	[45]
	OmpA	8 (Monomer)	*Paralichthys olivaceus*	[134]

In addition, OMPs induce production of specific antibodies in vaccinated individuals. Studies in humans have shown production of OmpA-specific IgA in mucosal secretions such as nasal wash, lung wash, and intestinal lavage [118] while in fish, OMPs evoke IgM responses [126, 135]. Table 5 shows examples in which various OMPs have been cloned and tested in fish in which they produced antibody responses associated with varying degrees of protection. Overall, these studies show that OMPs are recognized as PAMPs by the host PRRs and that they evoke various cytokine responses essential for modulating both the innate and adaptive immune responses in fish and that they are potent antigens able to induce protective antibodies against various pathogens including *A. hydrophila* and *Edwardsiella* spp.

1.6.2 Vaccine design and choice of antigen delivery system

There are two major antigen delivery systems used in vaccine design namely; (i) intracellular and (ii) extracellular antigen delivery systems.

1.6.2.1 Intracellular antigen delivery systems; delivery of antigens in intracellular compartments is mostly attained using replicative vaccines such as live attenuated and DNA vaccines that evoke cell-mediated (CMI) and humoral responses. Live attenuated vaccines have been used for both *Edwardsiella* spp. and *A. hydrophila* vaccine development. Live attenuated vaccine against *E. tarda* have attempted in zebrafish model [136] while Takano et al[137] tested five avirulent strains of *E. tarda* in flounder where they obtained a maximum RPS of 45%. Xioa et al [138] and Wang et al [139] showed that disruption of T3SS and T6SS led to attenuation of *E tarda* resulting in production of live vaccines while Sun et al [140] attenuated *E. tarda* into avirulent strain by serial passage

coupled with rifampicin treatment for use as a vaccine. In the case of *A. hydrophila*, Moral et al [141] disrupted the aroA gene to produce an auxotrophic aroA mutants while Liu et al [142] showed that use of the transposon Tn916-mutant strain J-1 *A. hydrophila* could serve as a live attenuated vaccine in fish. Sun et al [143] compared a subunit and DNA vaccine expressing the *E. tarda* antigen designated as rEta2 and showed high protection of 83% and 78% in flounder, respectively. In another study Sun et al [144] tested DNA vaccine encoding D15-like surface protein and obtained 57% protection in Japanese flounder. As for *A. hydrophila*, Vazquez-Juarez et al [145] used Omp38 and Omp48 to produce DNA vaccines against *A. veronii* in sea bass. Put together, these studies show that there have been several attempts aimed at developing live attenuated and DNA vaccines against *A. hydrophila* and *E. tarda* infection for use in aquaculture. Despite so, there are no licensed live attenuated and DNA vaccines against these diseases in aquaculture.

1.6.2.2 Extracellular antigen delivery systems; antigen delivery in extracellular compartments mainly comprise of whole cell inactivated (WCI) and subunit vaccines. As for subunit vaccines, various proteins have been used to vaccinate against *A. hydrophila* infection, such as the extracellular protease (epr) [146], GAPDH [105], DnaJ [106], fimA [107], FlgD [100] and OMPs [103]. Similarly, various immunogenic proteins tested as subunit vaccines against *E. tarda* infection include FIgD [100], FiC [101], EseD[102], T3SS[102] and OMPs [103, 104]. In general, both WCI and subunit vaccines require the support of adjuvants to enhance their immunogenicity, which in some cases have been associated with severe side effects.

Suffice to point out that the use of live attenuated vaccines raises the fear of reversion to virulence especially when vaccines are to be administered in multiple species. It is feared that for some host species, live vaccines might be protective while in other species the same strains could have attained residual virulence sufficient to cause clinical disease and mortality post vaccination. On the other hand, fish vaccinated with DNA vaccines have been viewed as being genetically modified organisms (GMOs) and the uptake in many countries/markets have been slow. The use of subunit and IWC-vaccines calls for use of adjuvants to enhance their immunogenicity which in some cases cause injection site side effects. Moreover, both subunit and IWC-vaccines do not evoke CMI rendering these antigens delivery system less effective for intracellular replicating pathogens. However, an ideal vaccine should induce both CMI and humoral immune responses, especially for intracellular replicating bacteria such as *A. hydrophila* and *Edwardsiella* spp. As shown in Table 6, live attenuated and DNA vaccines have low safety and yet they both evoke CMI and humoral

immune responses while subunit and IWC-vaccines that have high safety do not evoke CMI response, but only induce humoral responses. Hence there is need to develop other antigen delivery systems that are safe and able to evoke protective immune responses, typically balanced CMI and humoral immune responses.

Table 6. Comparison of antigen delivery systems

Parameter(s)	Intracellular delivery		Extracellular delivery	
	Live attenuated	DNA plasmid	Inactivated whole cell	Subunit protein
Production	Medium	Easy	Difficult	Easy
Difficulty of vaccine design	Medium/High	High	Low	High
Humoral responses	Yes	Yes	Yes	Yes
Cellular responses	Yes	Yes	No	No
Safety	Low	Low	High	High

1.6.3 Nano- and microparticles vaccine carrier

Advances in bioengineering show that nanoparticles (NPs) can be designed to deliver antigens intracellularly having the potential to evoke both CMI and humoral immune responses [147]. Many natural or synthetic polymers are used in nanomedicine for vaccine delivery where antigens are either encapsulated inside or covalently linked to NPs [148]. There are various biodegradable materials used in nanomedicine such as alginates, chitosan and polylactide co-glycolic acid (PLGA) NPs suitable for vaccine delivery that are safe with minor adverse effects in fish. They are also safe to consumers. Among these, the most widely explored are chitosan and PLGA NPs.

1.6.3.1 Chitosan nanoparticles

Chitosan is a non-toxic biodegradable natural polysaccharide isolated from crustaceans with great potential for gene delivery having variety of applications in nanomedicine [149] [150] [151]. It has been widely used for vaccine delivery in different fish species. Ramos et al. [152] showed that chitosan encapsulation of a ß-galactosidase plasmid coated on feed effectively delivered the DNA into Nile tilapia. Tian et al [153] used chitosan NPs to deliver plasmids encoding the major capsid protein of lymphocytic virus (LCDV) as an oral vaccine in Japanese flounder. RNA expression of the major capsid protein was subsequently detected in gills, kidney, spleen and intestine while the plasmid green fluorescence protein (GFP) was detected in tissues 90 days post vaccination. Chitosan NPs have also been used to deliver plasmid DNA encoding the *V. anguillarum* Omp38 in Asian sea bass (*Lates calcifer*) [154].

1.6.3.2 PLGA nanoparticles vaccine delivery and cellular uptake

PLGA NPs are made of hydrophobic polyester, biodegradable non-toxic material and have been used as a mode of vaccine delivery in fish and mammals. [155, 156]. They are attractive for oral vaccination because they easily adsorb on epithelial cells and penetrate the mucosal barrier where they are taken up by APCs. Cellular uptake of PLGA NPs is well documented in epithelial cells rendering PLGA NPs ideal for mucosal vaccine delivery [157-162]. They have the advantage that they are easy to produce and are relatively affordable. PLGA NPs can be bioengineered to improve cellular permeability so that they can easily attach on cell membranes and penetrate into cells mainly by endocytosis [163]. There are various forms of endocytosis in which NPs are engulfed and this can be through phagocytosis, clathrin-mediated endocytosis and micropinocytosis [164]. Apart from uptake by epithelial cells, NPs are engulfed by phagocytic cell such as macrophages, monocytes, neutrophils and dendritic cells in which phagocytosis is usually initiated by opsonisation. Opsonized NPs are recognized and attach to phagocytes via specific ligand-receptors that facilitate subsequent engulfment leading to formation of phagosomes. Cellular uptake of PLGA NPs is well documented in macrophages and DCs rendering PLGA NPs to be ideal for vaccine delivery [157-162].

Uptake of NPs by phagocytosis is governed by NP-physicochemical parameters that include size, shape and surface properties [164]. Depending on their size, NPs may aggregate in APCs via phagocytosis (0.5-10 μm), fluid phase (macro-) pinocytosis (0.5-5μm) or clathrin-coated pits (< 200 nm) [165, 166]. The upper limit for particle phagocytosis by APCs is in the range of 5-10 μm in diameter. As the size of particles changes from nanometer to micrometer size within the range for cellular uptake, a drastic reduction in uptake of particles is observed [162, 167, 168]. In addition to particle size, surface potential is important for particle uptake. NPs with zeta potential values > +25 mV or < -25 mV have high degrees of stability. Encapsulation carriers should include membrane penetrating peptides and polymers that disrupt the membrane when the pH declines in the endosome. Standley et al 2004 [169] made acid degradable nanoparticles that were stable at 7.4 pH but degrade at pH 5.0 in the acidic endosomal environment enabling the release of antigen into the cytosol, resulting in upregulation of MHC-I. Amphiphilic polymers have been shown to increase $CD8^+$ response and improve the vaccine potency [170]. These studies show that NP can be designed to deliver antigens via the intra- or extracellular route to evoke immune response linked to MHC-I or MHC-II pathways[171]. Figure 6 shows scanning transmission electron microscope

(TEM) of PLGA NP uptake of diameter around 1-2 μm in Atlantic salmon headkidney macrophages[172]. Apart from oral administration [173] PLGA NPs have also been tested for their efficacy in OMP vaccine delivery by intraperitoneal injection in various fish species such Rohu (*Labeo rohita*) against *A. hydrophila*.
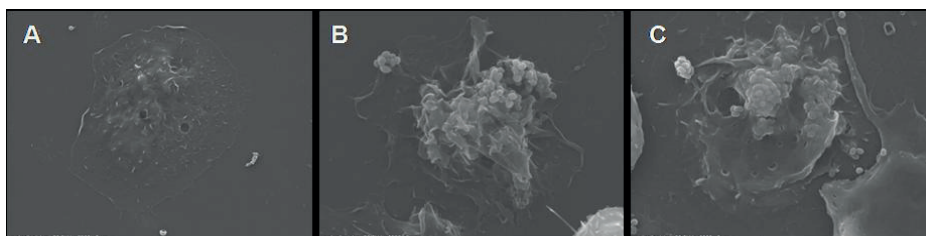
Figure 6. Uptake of PLGA microparticles in head kidney leukocytes from Atlantic salmon viewed by scanning electron microscopy (SEM, 20.0 kV, magnitude 6000x). Scale bar 2 μm. (A) Untreated adherent head kidney cell. (B) PLGA MPs outside/attached to adherent cell. (C) Internalized PLGA MPs in adherent cell [172]. Permission from Fredriksen 2008.

1.6.4 Mode of vaccine delivery and measures of efficacy

Table 7 shows a comparison of vaccination methods used in fish of which the most common is intraperitoneal injection followed by immersion and finally oral vaccination [174]. Vaccination by injection reliably delivers a predetermined antigen quantity directly into the peritoneal cavity of fish. Using this approach antigen doses have been correlated with long duration of immunity [175]. This is supported by observations made by Castro et al [176] who compared different routes of vaccine delivery and obtained different protection levels in which they recommended intraperitoneal injection as more effective than the other mode of vaccination. Disadvantages include handling stress, labor intensive and high cost given that fish have to be individually injected.

Immersion immunization has insignificantly low handling stress but has the disadvantage that the quantity of vaccine required could be large, the dose taken in by fish is difficult to determine and cannot be used in open water [177]. On the other hand, oral vaccines through feed has several advantages of being stress free and easy to administer the vaccine to large numbers of fish at one time. It is cost effective, reduces fish handling stress and is the easiest method for mass vaccination for all sizes of fish. However, it is difficult to determine the quantity of vaccine administered per fish through feed. Moreover, the challenge is to ensure that the antigen delivered remains intact and is able to pass through the acidic environment in the stomach in order to reach the intestines

for absorption [178]. Hence, the use of NPs designed to bypass the acidic environment in the stomach/foregut coupled with their ability to adhere to epithelial cells in order to penetrate the intestinal mucosa for antigen uptake by APCs render NPs to be ideal delivery vehicles of oral vaccines in fish.

Table 7. Comparison of the modes of vaccine delivery used for fish immunization

Parameter(s)	Injection	Immersion	Oral delivery
Dose control	Easy	Difficult	Difficult
Stress	High	Low	Very low
Natural infectious route	No	Similar	Similar
Labor cost	High	Medium	Low
Fish size	Limited	Unlimited	Unlimited
Use in open water	No	No	Yes

1.6.5 Measures of efficacy and immune protection

The most common approach for evaluating vaccine efficacy in vaccinated fish is by relative percent survival (RPS) as described by Amend [179]. In general, antibody responses are widely used as a way of measuring the immune response to vaccination and in recent years, circulating antibodies have been used as correlates of protective immunity in vaccinated fish [180]. In addition, serum inhibition test (SIT) is used in some studies to demonstrate the ability of serum obtained from vaccinated fish to inhibit bacterial growth *in vitro* the ability to inhibit bacteria *in vitro* correspond the antibody titer in vaccinated fish. These studies shown that humoral responses can be used as a measure of immune protection in vaccinated fish. However, growing evidence shows that CMI response play a vital role in providing protective immunity in vaccination fish. The major limiting factor for evaluating CMI responses is that fish species such as carp, catfishes and tilapia, cultured in developing countries, is the limited information available when it comes to gene sequences of immune cells and cytokines, *e.g.* genomic markers such as CD4 and CD8 T-cell, which are essential for evaluating CMI responses have not been characterized for some of these fish species. Hence, it is difficult to determine the role of CMI in these fish species, and as such, evaluation of host immune response to vaccination is mostly dependent on antibody responses. These factors to some extent, halt the development of protective vaccines against intracellular pathogens, like *Edwardsiella* spp. and *A. hydrophila*. Nevertheless, the search for protective vaccines continues given the devastating impact of these pathogens in Asian Aquaculture.

2.0 Main Objective

The overall objective of this study is to carry out genotypic and phenotypic characterization of *Edwardsiella* spp. and *A. hydrophila* originating from diseased farmed fish species in Asia, and to test the efficacy of OMP vaccines encapsulated in biodegradable polymeric chitosan and PLGA NPs in target fish species

2.1 Subgoals

a) Carry out phenotypic and genotypic characterization of *Edwardsiella* spp. and *A. hydrophila* from different farmed Asian aquatic organisms and geographical areas in Asia

b) Carry out phylogenetic comparison of OmpW and OmpA from different *E. tarda* and *A. hydrophila*, respectively, in order to determine their cross reactivity as broadly protective vaccine candidates against variant strains

c) Carry out cloning and expression of *E. tarda* OmpA and *A. hydrophila* OmpW followed by encapsulation in chitosan and PLGA-NPs and evaluate their efficacy as vaccine antigens encapsulated in chitosan and PLGA-NPs orally administered in *L. fimbriatus* and *L. rohita*.

3.0 Methodology

3.1 Principle outline of workflow

The layout workflow of this study program was divided into four phases (Figures 7 and 8). Phase-I (Figure 7) focused on determining the phenotypic and genotypic properties of *Edwardsiella* spp. and *A. hydrophila*. Phase-II (Figure 8) focused on comparing the phylogenetic properties of *Edwardsiella* spp. and *A. hydrophila* OMPs from different host species
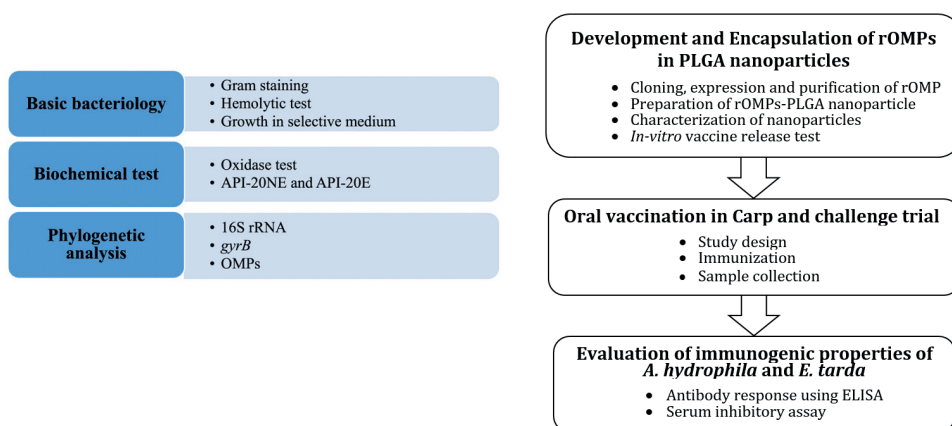
Figure 7. Shows the phenotype and genotype characterization steps of *Edwardsiella* spp. and *A. hydrophila* as well as phylogenetic comparison of their OMP-proteins (Phase I and II).

Figure 8. Shows workflow in expression of OMP proteins, encapsulation in chitosan and PLGA-NPs followed by vaccination and evaluation of immune responses (Phase III and IV)

and geographical areas with the view to determine their potential vaccine antigens. Phase-III focused on encapsulation of OMPs in chitosan and PLGA NPs for oral vaccine development. Finally, Phase-IV was focused on oral vaccination and challenge studies in rohu and carp.

3.2 Phenotype characterization of *Edwardsiella* spp. and *Aeromonas hydrophila* isolates

Phenotypic characteristics of *Edwardsiella* spp. and *A. hydrophila* isolates from 14 farmed fish and other aquatic organisms (Table 8) in India, South Korea and Taiwan was carried out using morphological and biochemical tests. All isolates were cultured on basic media such as Tryptone soy agar (TSA). In addition, all isolates were cultured on selective media specific for *E. tarda* and *A. hydrophila* propagation. Culture on 5% sheep blood agar (5% SBA) was done to determine their ability to lyse sheep red blood cells (rbcs). All isolates that replicated on selective

medium were preserved in glycerol broth at -80^{0}C of which *Salmonella–Shigella* agar (SSA) (Sigma-Aldrich, USA) was used for *E. tarda* and Rimler Shotts (RS) agar for *A. hydrophila* propagation. Morphological examination was done by Gram staining in order to determine whether isolates were rod or cocci shaped. In addition, all isolates were examined for motility on wet smears.

Table 8. Fish species sampled for and *A. hydrophila* and *Edwardsiella* spp. isolation

Fish species	Scientific name	Bacteria species	
		A. hydrophila	*Edwardsiella* spp.
Mrigal carp	*Cirrhinus mrigala*	√	(-)
Carassius spp.	*Carassius carassius*	√	(-)
Common carp	*Cyprinus carpio*	√	(-)
Nile tilapia	*Oreochromis niloticus*	√	√
Walking catfish	*Clarias batrachus*	√	√
Olive flounder	*Paralichthys olivaceus*	(-)	√
Catla	*Catla catla*	√	√
Rohu labeo,	*Labeo rohita*	√	√
Pangasius	*Pangasius hypophthalmus*	(-)	√
Chinese softshell turtle	*Pelodiscus sinensis*	√	(-)
Silver surfperch	*Hyperprosopon ellipticum*	√	√
Taiwanese worm eel	*Sympenchelys taiwanensis*	(-)	√
Spotted snakehead	*Ophiocephalus punctatus*	(-)	√
Climbing perch	*Anabas testudineus*	(-)	√

√=shows fish species sampled for bacterial isolation, (-) = not sampled

For biochemical characterization, all isolates that replicated on basic and selective media were examined using the analytical profiling index (API)-20E and API-20NE for enterobacteriaceae and non-enterobacteriaceae, respectively, according to manufacturer's instruction (BioMérieux, Marcy l'Etoile, France). Bacterial strain identification was carried out using the API catalogue Web version based on manufacturer's instruction (BioMérieux, Marcy l'Etoile, France) in comparison with *E. tarda* and *A. hydrophila* reference strains (papers I and II).

3.3 Genotype characterization of *Edwardsiella* spp. and *Aeromonas hydrophila* isolates

All isolates obtained from different farmed aquatic organisms and geographical regions in Asia showing phenotypic properties of *Edwardsiella* spp. and *A. hydrophila* were subjected to genotype characterization as described in detail in papers I and II. In both studies (papers I and II), genotype characterization was done by comparison of 16S rRNA, representing ribosomal RNA, and *gyrB* representing housekeeping genes. Primer sequences were designed from conserved regions of *Edwardsiella* spp. and *A. hydrophila* 16S rRNA and *gyrB* genes using CLC

6.9 version (papers I and II). PCR reactions were performed using Taq polymerase using the protocol provided by the supplier (Qiagen, Germany) followed by gel electrophoresis analysis. Positive bands were purified using the Qiagen PCR purification kit and commercially sequenced by Sanger Sequencing at GATC, Germany. After retrieval, all sequences were subjected to blast analysis using the National Center of Biotechnology Information (NCBI) database to identify sequences that were homologous with our isolates in MEGA v7. Genotype classification was carried out by blast analysis on 16r RNA and *gyrB* genes using phylogenetic analysis in MEGA v7. All phylogenetic trees were generated in MEGA v7 as described in detail in paper I and II.

3.4 Phylogenetic comparison of bacterial OMPs from different host species

Once all bacteria had been correctly characterized, the next step was to compare the genetic properties of their OMPs using phylogenetic analysis in order to determine antigenic similarities as an overture to vaccine development. DNA extracted for genotype characterization in Section 3.2 above was used for *A. hydrophila* OmpA and *E. tarda* OmpW sequencing. Primer sequences were designed from conserved regions of *Edwardsiella* OmpW and *A. hydrophila* OmpA using CLC 6.9 version. Sequencing was done commercially by MWG, Germany. After sequence retrieval, blast analysis was used to generate phylogenetic trees using MEGA v7 (detailed in papers I and II). Data generated from these studies was used to evaluate the similarity among OMPs from different host species and geographical areas in Asia.

3.5 Cloning, expression and purification of OMPs protein used for vaccine production

The *E. tarda* isolate used for rOmpA amplification was obtained from catfish (paper-III) while the *A. hydrophila* isolate used for OmpW cloning and expression was from rohu (paper-IV), both from India. Sequences used for cloning of *E. tarda* OmpA (FJ751236.2) and *A. hydrophila* OmpW (EU672512) have both been deposited in NCBI database. Both OmpA and OmpW were cloned and expressed in *Escherichia coli* M15 using IPTG. Purification of recombinant OMPs was done by denaturation and confirmation by SDS-PAGE as described in papers III and IV. Purified OmpA and OmpW proteins were used for vaccine development in the next stage.

3.6 Encapsulation and characterization of nanoparticles carrying OmpA and OmpW

Encapsulation of purified *E. tarda* OmpA and *A. hydrophila* OmpW was done in chitosan and PLGA NPs, respectively. Both chitosan and PLGA NP size determination was done using the Malvern ZetaSizer (NanoZS, Malvern Instruments, Worcester, UK) while protein encapsulation

efficiency was evaluated as described in papers III and IV. *In vitro* release tests were carried out in phosphate buffered saline (PBS) as previously described by Hori et al [181]. Both OmpW and OmpA antigens encapsulated in PLGA and chitosan NPs were subjected to similar *in vitro* release tests in which 500 uL supernatant was collected for release tests and replaced with an equal volume of PBS. Samples were collected at similar intervals of 1, 2, 8, 16, 24 and 48 hours followed by quantification of the released antigens as previously described by Lowry et[182] al in both OmpW and OmpA antigen release tests.

3.7 Vaccination and Challenge design

Preparation of *E. tarda* OmpA and *A. hydrophila* OmpW NP-vaccines for oral immunization was done by mixing commercial feed with NP-vaccines using a grinder followed by sieving. The vaccine-feed mixture was thoroughly mixed into dough followed by pressing through 2 mm diameter hand extruders. Pellets made from extruders were dried at room temperature for 24 hrs before storage until use. In both immunization studies, IWC-vaccines were prepared and used as positive controls while commercial feed without vaccines was used as negative control. Two fish species (Table-1) were used for immunization studies. For the *E. tarda* OmpA vaccine trial, *L. fimbriatus* was used while *L. rohita* was used for the OmpW vaccine efficacy trial. All vaccines were administered orally through feed. The duration of oral vaccine delivery and quantity of vaccine administered per fish are explained in papers III and IV. In both studies, challenge was done by intramuscular injection using highly pathogenic bacteria strains homologous with strains used for OMP and IWC-vaccine production at a dose of 0.1 ml/fish. Post challenge mortalities was recorded daily, and protection was determined using the Kaplan Meyer's survival analysis.

3.8 Serum inhibitory and ELISA assay

Blood samples collected prior to challenge in the OmpA and OmpW NP immunization studies, were analyzed by the enzyme linked immunosorbent assay (ELISA) to determine levels of specific, circulating antibodies post vaccination. In addition, the serum inhibition test (SIT) was carried out to determine the ability of sera from immunized fish to inhibit bacterial growth *in vitro* as previously described by Hamod et al[183] (papers III and IV).

Summary of Papers

Paper I

Edwardsiella spp. is one of the most important fish pathogens causing high economic losses in aquaculture. Herein, 37 isolates from ten different fish species from India, South Korea and Taiwan were examined to determine their phenotypic and genotypic properties. Morphological examination based on Gram stain, hemolysis tests and biochemical characterization using the API-20E system classified 30 of the 37 isolates as *E. tarda* of which the phenotypic homology based on API-20E classification was estimated at 85.71%. Phylogenetic analysis of 16S rRNA did not separate the isolates into different Edwardsiella phylogroups, but instead all 30 isolates were put together with *E. anguillarum*, *E. hoshinae*, *E. tarda*, *E. piscicida* and *E. ictaluri* reference strains as a single monophyletic group. On the contrary, the *gyrB* phylogenetic tree clearly separated our isolates into two groups of which group I, comprising 14 isolates was homologous with the *E. anguillarum* reference strain while group II that comprised of 14 isolates was homologous with the *E. piscicida* reference strain. Moreover, our isolates were separated from *E. tarda*, *E. ictaluri* and *E. hoshinae* reference strains clearly indicating that the 30 isolates were *E. piscicida* and *E. anguillarum* species infecting different fish species in Asia. Homology of the OmpW protein suggested that strains with broad protective coverage could be identified as vaccine candidates. This study underscores the importance of combining genotype with phenotype characterization for correct taxonomical classification of bacterial species.

Paper II

This study focused on comparing 28 *A. hydrophila* isolates from nine different aquatic organisms from India and Taiwan in order to determine their phenotypic and genotypic properties. The high phenotypic similarity (>90%) determined by the API-20NE biochemical characterization coupled with the high genotypic homology among isolates based on 16S rRNA (>98%) and *gyrB* (>96%) phylogenetic characterization suggests that a homogenous *A. hydrophila* taxon could be infecting different fish species in Asian aquaculture. The study also showed a high OmpA similarity among the 28 *A. hydrophila* isolates (>88%) suggesting that variant strains from different aquatic organisms and geographical areas share the same antigenic properties and that a single vaccine strain with a broad protective ability can be developed against these strains. Similar to observations made in paper-I, this study accentuate the importance of combining

genotyping with phenotyping for bacteria species identification and characterization, it also highlights the application of phylogenetic comparison of key bacteria antigens with the view to finding vaccine strain with broad protective properties against variant strains infecting different aquatic organisms.

Paper III

This study focused on encapsulation of recombinant *E. tarda* OmpA in chitosan NPs (rOmpA-NP) to produce an oral vaccine against *E. tarda*. The rOmpA-NP vaccine was administered in *Labeo fimbriatus* together with empty NPs as negative control and inactivated whole cell *E. tarda* (IWC-ET) vaccine as a positive control. The rOmpA-NP vaccine produced superior protection (PSCP=73.3%) over the IWC-ET vaccine (PSCP=48.28%) that corresponded with antibody responses of OD_{450}=2.430 and OD_{450}=1.735 for the rOmpA and IWC-ET vaccinated fish, respectively. Similarly, serum specific for rOmpA showed higher inhibition capacity of *E. tarda* on Tryptone soy agar (TSA) than serum from IWC-ET vaccinated fish. In summary, data from this study showed that the rOmpA-NP vaccine produced higher antibody levels that corresponded with superior protection over the IWC-ET vaccine clearly demonstrating that rOmpA encapsulated in chitosan NPs confer protective immunity in *L. fimbriatus* vaccinated against *E. tarda*.

Paper IV

A. hydrophila is a Gram-negative bacterium that causes high mortality in different fish species and at different growth stages. Although vaccination has significantly contributed to the decline of various diseases in aquaculture, the use of oral vaccines has lagged behind the injectable vaccines due to low efficacy, that being from primary immunization or by use of boost protocols. In this study, the OmpW of *A. hydrophila* was cloned, purified and encapsulated in PLGA nanoparticles for oral vaccination of *L. rohita*. To evaluate the efficacy of the NP-rOmpW oral vaccine, two antigen doses were orally administered in rohu with a high antigen dose that had twice the quantity of antigen compared to the low antigen dose. Data presented herein shows that OmpW orally administered using PLGA-NPs is protective against *A. hydrophila* infection and that the level of protective immunity is antigen dose-dependent.

4.0 Results and General discussion

The findings in this study were divided into four major groups as discussed below.

4.1 Phenotypic characterization *E. tarda* and *A. hydrophila*

Phenotypic characterization based on morphology and biochemical properties has been the main stay of bacteriology taxonomical classification since the 1930s when the Bergey's manual of systematic bacteriology was established. Paper-I shows that 30 of 37 isolates examined from 10 different fish species were characterized as *E. tarda* while paper II shows that 28 of 33 isolates from nine different aquatic organisms were classified as *A. hydrophila*. It is interesting to note that none hemolytic isolates both in papers I and II failed to propagate on selective media. In the case of *Edwardsiella* spp. isolates, only isolates from *P. hypophthalmus* and *C. batrachus* form India were non hemolytic and did not show characteristic properties on black centered colonies on SSA. As for biochemical tests, the difference seen is that isolates from North India were SAC negative and H_2S positive while isolates from South India were SAC positive and H_2S negative could be suggestive that adaption in different geographical areas could play a role in inducing biochemical differences among isolates of the same bacterial species. In addition, observations made in paper I that *P. hypophthalmus* from South India was the only fish species that had Edwardsiella isolates positive for SAC and negative for H_2S contrary to other fish species that were all SAC negative and H_2S positive suggests that some host species in different geographical areas can alter the biochemical properties of some bacteria species. However, there is a need for further studies to consolidate these observations.

However, the inability of the API-20E system to differentiate Edwardsiella isolates into different phylogroups in paper I points to lack of distinct phenotypic patterns in the API-20E biochemical elements having a confirmative profile that distinguishes *E. tarda* isolates from *E. piscicida* and *E. anguillarum*, which makes it unreliable for Edwardsiella intraspecies differentiation. This observation is consistent with Griffins et al [184] who failed to identify a discriminatory metabolic fingerprint able to differentiate Edwardsiella phylogroups into humans and piscine species. As mentioned by Reichley et al [185], these findings imply that phenotypic classification of *Edwardsiella* spp. regardless of the identification system used requires supplemental confirmation tests for correct taxonomical classification. There is need to establish

discriminatory phenotypic markers based on metabolic profiles if biochemical characterization has to be used for correct taxonomical classification of bacteria.

4.2 Genotypic characterization *E. tarda* and *A. hydrophila*

In 2015, using multilocus sequence typing (MLST) Abaynet et al [27] showed that *Edwardsiella* spp. previously classified as *E. tarda* infecting fish were distinctly different from those infecting humans and animals of which fish isolates were classified as *E. piscicida*. Reichley et al [185] used a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) to differentiate *E. tarda* isolates from *E. piscicida* and showed that isolates previously classified as *E. tarda* were *E. piscicida*. Similarly, Griffin et al [186] showed that archived isolates previously classified as *E. tarda* by the Aquatic Research and Diagnostic Laboratory, Stoneville, MS, USA prior to 2012 were identified as *E. piscicida* based on the repetitive sequence mediated PCR analysis using *gyrB* gene. Since 2015, *E. piscicida* has been isolated from several fish species [186]. These findings are supported by complete genome sequencing of *E. piscicida* from different fish species such as tilapia [187], channel catfish [188], grouper [189], turbot [190], which clearly show that fish isolates are distinct and homogenously related, and they are not close to *E. tarda* isolates from humans and animals. These findings are in line with observation made in paper-I in which isolates characterized as *E. tarda* using basic phenotypic characterization methods based on morphology, hemolysis and API-20E biochemical tests were classified as *E. piscicida* and *E. anguillarum* based on *gyrB* phylogenetic analysis.

An eminent observation made in papers I and II is the differences in discriminatory capacity at inter- and intraspecies levels observed between 16S rRNA and *gyrB*. Growing evidence shows that the discrimination power of 16S rRNA diminishes significantly at intraspecies level, especially for closely related taxons. As a result, its role in differentiation for *Edwardsiella* spp. has in recent years become questionable [184, 186, 191-193]. Moreover, some bacterial species possess multiple heterogenous rRNA copies further complicating differentiation of closely related species especially in cases where intragenomic heterogeneity exceeds interspecific variability [194, 195]. Consequently, high 16S rRNA sequence identity >99% does not imply high accuracy in microbial identification, especially for genera with closely related taxon [195, 196]. In paper I, 16S rRNA could not delineate *E. tarda* isolates from *E. piscicida* and *E. anguillarum* consolidating

previous observations of low discriminatory capacity. The intra-genomic heterogeneity varied from 0.0 - 1.10% with an overall sequence similarity of 98.9% in paper I.

As an alternative to 16S rRNA, single copy genes such as *gyrB,* and other housekeeping genes, are used for taxonomical classification of bacteria. *gyrB* encodes the ATPase domain of the DNA gyrase found in all prokaryote cells in which it contains conserved motifs that are species-specific making it is more resolute for intraspecies classification than 16S rRNA [197]. *gyrB* sequence alignment done by Griffin et al [186] averaged 99.8% (range 99.6% - 100.0%) homology among *E. piscicida* sequences while homology with *E. tarda gyrB* was <87%. Bujan et al [198] found 99.7% similarity among *E. piscicida* isolates using *gyrB* gene while the difference between *E. tarda* and *E. piscicida* was put at 82% [198]. Similarly, in paper I *gyrB* clearly separated the *E. tarda* reference strain from Asian fish isolates with a disparity of 82%. Isolates in paper I were put in two groups with a disparity of 2.9% between *E. piscicida* and *E. anguillarum* groups. The homology among all 14 *E. anguillarum* isolates was 100% while similarity among all 16 *E. piscicida* isolates was estimated at 99.8% using *gyrB* being similar to observations made by Griffin et al [186] and Bujan et al [198]. Similarly, paper II shows that *gyrB* had a higher resolution in differentiating *A. hydrophila* from *Vibrio harveyi* used as outgroup with maximal interspecies divergence, which was 10 times higher than 16S rRNA. Put together, the findings in papers I and II show that *gyrB* has a higher intra- and interspecies differentiation resolution than 16S rRNA, being in line with previous observations [195, 196].

4.3 Phylogenetic analysis of outer membrane proteins and vaccine development

The wide range of farmed fish species susceptible to *Edwardsiella* spp. *and A. hydrophila* make vaccine development a challenge. The main predicament is whether variant strains infecting different fish species can be protected by a single (or few) vaccine strain(s). Finding a single broadly protective vaccine strain against variant strains infecting different host species would be cost effective for use in various fish species and in different countries. This can be highly beneficial especially in countries where polyculture of different aquatic organisms is done. Phylogenetic analysis can be one approach to identify broadly protective antigens against variant strains. Phylogenetic analyses of OmpW and OmpA in papers I and II suggest that common OMP vaccines can be developed for use against *Edwardsiella* spp. and *A. hydrophila* strains from different host species and geographical areas in Asia. Although these findings require

further investigations involving immunization and challenge studies, developing broadly protective vaccines against variant strains infecting different farmed aquatic organisms would help reduce economical losses and promote expansion sustainable aquaculture.

An important challenge in fish vaccine development is designing vaccine delivery methods that are less strenuous on fish, promote easy handling of fish, easy to administer and less expensive by reducing workforce required for vaccination. Oral vaccination is perceived to be a better option than injectable and immersion vaccination because it is not strenuous on fish, easy to administer, does not require handling of individual fish and it can be applied throughout the production cycle. Although OmpA and OmpW have previously been shown to produce protection against *Edwardsiella* spp. and *A. hydrophila*, most previous studies focused on vaccine delivery by injection. For example, Maiti et al [199] showed that OmpW administered by injection produced 72% RPS in common carp vaccinated against *A. hydrophila* while Guan et al [200] obtained 70% RPS by injecting OmpG against *A. hydrophila*. Similarly, Tang et al [201] showed high protection in Japanese flounder using OMP against *E. tarda* while Maiti et al [202] showed protection in common carp using OmpA, and Liu used OmpC in Japanese flounder [203]. However, the ability of OMPs to induce protection against *A. hydrophila* and *Edwardsiella* spp. when administered as oral vaccines using NPs has not been widely explored. Data in papers III and IV show that OMP antigens encapsulated in both chitosan and PLGA NPs produced protective immunity in vaccinated fish suggesting that both chitosan and PLGA NPs are effective antigen carriers for oral vaccine delivery.

4.4 Evaluation of vaccine efficacy and immune protection

Various methods of evaluating immune response induced by OMP vaccines have been reported by different scientists [204-206]. These include; (ii) evaluating antibody response induced by vaccination, (ii) serum inhibition test (SIT) to test the ability of sera from vaccinated fish to inhibit bacterial growth *in vitro*, and (iii) passive immunization to determine the protection from antibodies generated from vaccinated fish when passively transferred in naïve unvaccinated fish. Luo et al [207] tested SIT and showed high inhibition of *Flavobacterium columnare* in vaccinated fish using OMP-vaccine while Hirts and Ellis [208] passively immunized Atlantic salmon using iron regulated outer membrane protein and showed high protection in passively immunized fish. In Papers III and IV high antibody levels were detected by ELISA and sera from OmpA and

OmpW vaccinated fish that had high inhibiting capacity of *Edwardsiella* spp. and *A. hydrophila* growth *in vitro*. However, passive immunization was not done.

Similar to observation made in paper III in which OmpW-NP produced higher protection than the IWC-vaccine in *L. fimbriatus*, Rahman et al [209] produced superior protection using OMF-vaccine that correlated with protection in rainbow trout and ayu against *F. psychrophilum,* than the IWC-vaccine. Similarly, Bricknell et al [204] showed a high correlation of antibody response with duration of protection in Atlantic salmon vaccinated against *A. salmonicida* using iron-regulated OMPs, which is in line with observation in paper-IV that the OmpW antigen dose corresponded with increase in antibody responses as well as the level of vaccine protection in rohu vaccinated against *A. hydrophila* infection. The corresponding increase in antibody response with vaccine protection suggest antibodies can be used as a measure of protective immunity for OmpA and OmpW NP vaccines. Moreover, the serum inhibition test done in papers III and IV show inhibition of bacterial growth *in vitro* corresponded with antibody levels in vaccinated fish using OmpA and OmpW NP-vaccines.

5.0 Main conclusions

- This study has shown that bacterial isolates from fish classified as *E. tarda* based on phenotypic characterization using selective medium, hemolysis test and API-20E were reclassified as *E. piscicida* and *E. anguillarum* using *gyrB* phylogenetic analysis.

- This has shown that *Edwardsiella* spp. infecting farmed fish in Asia belong to *E. piscicida* and *E. anguillarum* and that they are closely related homogenous taxons. Similarly, *A. hydrophila* isolates from different fish species and aquatic organisms in Asia form a homogenous taxon.

- *gyrB* has a higher inter- and intraspecies differentiation resolution than 16S rRNA in differentiating Edwardsiella isolates and

- The homology of the OmpA and OmpW proteins among bacterial isolates from various host species and geographical regions in Asia suggests that strains with broad protective coverage can be identified for use as vaccines against variant strains from several host fish species.

- The ability of OmpA and OmpW encapsulated in chitosan and PLGA NPs to produce high antibody levels corresponding with post challenge survival suggests that NPs can be used as delivery systems for oral vaccination of fish.

6.0 Future perspectives

- Future studies should seek to develop biochemical markers able to effectively differentiate Edwardsiella isolates into *E. tarda*, *E. anguillarum*, and *E. piscicida* in order to expedite the process of correct taxonomical classification of bacteria based on standardized phenotypic markers.

- Carry out immunization using OmpA and OmpW NP-vaccines followed by challenge studies in different fish species to verify the cross-protective ability of OMP vaccines against variant isolates from different host species and geographical areas in Aquaculture.

- Given that *Edwardsiella* spp. and *A. hydrophila* infection have been associated with intracellular replication, future studies should seek to elucidate the role of OMP-NP based vaccines in evoking CMI responses and to elucidate the protective mechanisms of CMI responses induced by OMP-NP vaccines.

- In order to improve the efficacy of OMP vaccines delivered by NPs, future studies should focus on increasing encapsulation efficiency, elucidate the mechanisms of antigen uptake and bio-distribution followed by the ultimate release of antigens intracellularly in order to provide feedback on optimal design and efficacy of NP-formulations.

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ORIGINAL MANUSCRIPT

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Genotypic and phenotypic characterization of *Edwardsiella* isolates from different fish species and geographical areas in Asia: Implications for vaccine development

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Abstract

The genus *Edwardsiella* is one of the major causes of fish diseases globally. Herein, we examined 37 isolates from ten different fish species from India, South Korea and Taiwan to gain insight into their phenotypic and genotypic properties, of which 30 were characterized as *E. tarda* with phenotypic homology estimated at 85.71% based on API-20E biochemical tests. Genotyping using 16S rRNA put all isolates together with *E. anguillarum*, *E. hoshinae*, *E. tarda*, *E. piscicida* and *E. ictaluri* reference strains in a monophyletic group. In contrast, the gyrB phylogenetic tree clearly separated *E. ictaluri*, *E. tarda* and *E. hoshinae* reference strains from our isolates and put our isolates into two groups with group I being homologous with the *E. anguillarum* reference strain while group II was homologous with the *E. piscicida* reference strain. Hence, our findings point to *E. piscicida* and *E. anguillarum* as species infecting different fish species in Asia. Homology of the *ompW* protein suggested that strains with broad protective coverage could be identified as vaccine candidates. This study underscores the importance of combining genotyping with phenotyping for valid species classification. In addition, it accentuates the importance of phylogenetic comparison of bacterial antigens for identification of potential vaccine candidates.

KEYWORDS
16S rRNA, *Edwardsiella tarda*, Genotype, gyrB, ompW

1 | INTRODUCTION

The genus Edwardsiella was first described by Ewing et al in 1965 (Abbott & Janda, 2006) as a distinct genus in the family Enterobacteriaceae. However, the first member in the genus, namely *Edwardsiella tarda*, was first characterized by Trabulsi and Ewing (1962), and it causes disease in humans, reptiles, animals and fish. In aquaculture, it is mainly a pathogen of fresh and brackish water aquatic organisms (Plumb, 1999; Shetty, Maiti, Venugopal, Karunasagar, & Karunasagar, 2014; Ullah & Arai, 1983) although it also causes sporadic outbreaks in cold-water species (Amandi, Hiu, Rohovec, & Fryer, 1982; Padrós, Zarza, Dopazo, Cuadrado, & Crespo, 2006). It causes septicaemia, extensive skin lesions and pathology in various internal organs linked to high mortalities and sporadic epidemics in different fish species (Plumb, 1999; Shetty et al., 2014; Ullah & Arai, 1983). In 1980, the second member of the genus *Edwardsiella* was characterized by Grimont, Grimont, Richard, and Sakazaki (1980) as *E. hoshinae*, which has been isolated from birds, reptiles, human faeces and water samples (Castro, Toranzo, Nunez, & Magariños, 2008; Grimont et al., 1980), while the third member was classified as *E. ictaluri* by Hawke, McWhorter, Steigerwalt, and Brenner (1981) in 1981 mainly causing disease in catfish (Kelly et al., 2018; Kim & Lovell, 1995; Reichley, Waldbieser, Soto, Lawrence, & Griffin, 2017). Recent advances in genotyping have led to the classification of fish isolates previously classified as *E. tarda* to be *E. piscicida* by Abayneh, Colquhoun, and Sørum (2013) in 2013, while isolates from eel (*Anguilla anguilla*) were classified as *E. anguillarum* by Shao et al (Ucko, Colorni, Dubytska, & Thune, 2016) in 2015. As the number of species in the genus *Edwardsiella* continues to increase, it is becoming evident that phenotypic characterization should preferably include culture characteristics, biochemical tests, serotyping and genotyping for correct taxonomic classification (Austin & Austin, 1989; Baird, Chikarmane, Smolowitz, & Uhlinger, 2003; Chen & Lai, 1998; Park, Wakabayashi, & Watanabe, 1983; Rashid, Nakai, & Muroga, 1994; Savan, Igarashi, Matsuoka, & Sakai, 2004). Moreover, combining phenotyping with genotyping allows for assessment of bacteria adaptation changes in the environment, which has particular relevance when it comes to determining whether universal vaccines containing antigenic determinants that confer protection across different species in different ecosystems can be developed.

Several studies have shown that the genus *Edwardsiella* constitutes a biochemically homogeneous taxon that exhibits typical characteristics of an enteric bacterium rendering biochemical tests to be a gold standard for phenotypic characterization (Kumar et al., 2007; Odds, 1981; Shetty et al., 2014). Despite this, studies have shown that biochemical tests produce conflicting results in extreme diverse strains (Stiles & Ng, 1981; Xie et al., 2015). Factors leading to extreme diversity within species include adaption to new hosts, mutations induced by environmental changes, and the presence of plasmids that control bacterial metabolism in the environment (Xie et al., 2015). Apart from biochemical phenotyping, genotyping based on sequence alignments is widely used for identification and taxonomic classification of bacteria. Among the genes widely used for genotyping is 16S rRNA whose presence in almost all bacteria is used to determine distant (e.g., intergenic) or close (e.g., intragenic) genealogical relationships (Figueras, Soler, Chacón, Guarro, & Martínez-Murcia, 2000; Shetty et al., 2014; Woese, 1987; Xiao et al., 2008). Despite this, several studies show that it has a poor discriminatory capacity and low phylogenetic resolution at species level (Bosshard et al., 2006; Janda & Abbott, 2007; Mignard & Flandrois, 2006). For example, Janda and Abbott (2007) showed 99.4%–99.8% similarity for *E. tarda*, *E. hoshinae* and *E. ictaluri* 16S rRNA, and yet all three species were distinguishable biochemically and by DNA homology. Moreover, rRNA sequence analysis is often at variant with DNA re-association, which is considered to be the absolute measure of relatedness (Fox, Wisotzkey, & Jurtshuk, 1992; Rainey, Lang, & Stackebrandt, 1994; Stackebrandt & Goebel, 1994). The major reason for this discrepancy is the low base substitution rate between rRNAs of closely related species resulting in large statistical errors in comparison of closely related genes (Yamamoto & Harayama, 1996). Therefore, protein-coding genes known to evolve faster than rRNAs are better phylogenetic markers of closely related species (Yamamoto & Harayama, 1996), which renders *gyrB* to be a more reliable phylogenetic marker given that it has four times higher mean synonymous substitution rate than 16S rRNA (Watanabe, Nelson, Harayama, & Kasai, 2001; Yamamoto & Harayama, 1996). This is supported by several studies showing that *gyrB* has a higher phylogenetic resolution for both intra- and interspecies relatedness than 16S rRNA (Jiao, Cheng, Hu, & Sun, 2010; Lan, Zhang, Wang, Chen, & Han, 2008; Yamamoto & Harayama, 1995, 1996).

Although bacterial phenotyping and genotyping are vital for the timely and correct identification of disease aetiological agents, development of protective vaccines calls for a good understanding of the immunogenic and protective antigens. Among the numerous antigens able to evoke protective immunity against bacterial infections are outer membrane proteins (OMPs) (Dubey, Mugimba, Mutoloki, Evensen, & Munang'andu, 2016; Dubey, Mutoloki, Evensen, & Munang'andu, 2016; Munang'andu, Paul, &

TABLE 1 Primers used in this study

Primer name	Primer (5'–3')	Reference
Edwardsiella tarda ompW	Forward: CATCAGGCCGGTGACGTGAT	This study
	Reverse: TCAGAAGCGGTAGCCGGCGC	
Edwardsiella tarda gyrB	Forward: GCATGGAGACCTTCAGCAAT	Lan et al. (2008)
	Reverse: GCGGAGATTTTGCTCTTCTT	
Edwardsiella tarda 16S rRNA	Forward: TAGGGAGGAAAGGTGTGAA	This study
	Reverse: CTCTAGCTTGCCAGTCTT	

TABLE 2 Morphology and growth scores for Indian, South Korean and Taiwanese isolates

Source/Fish species/ID	Country	ID	Mot.	Cat.	O/F test	Growth Media				
						TSA	BHI broth	SSA	5% SBA	β-H/C
1. India_*P. hypophthalmus*_EtI1	South India	EtI1	+	+	+, +	+	+	+	+	+
2. India_*P. hypophthalmus*_EtI2	South India	EtI2	+	+	+, +	+	+	+	+	+
3. India_*P. hypophthalmus*_EtI3	South India	EtI3	+	+	+, +	+	+	+	+	+
4. India_*P. hypophthalmus*_EtI4	South India	EtI4	+	+	+, +	+	+	+	+	+
5. India_*P. hypophthalmus*_EtI5	South India	EtI5	+	+	+, +	+	+	+	+	+
6. Taiwan_*H. ellipticum*_EtT6	Taiwan	EtT6	+	+	+, +	+	+	+	+	+
7. Taiwan_*S. taiwanensis*_EtT7	Taiwan	EtT7	+	+	+, +	+	+	+	+	+
8. Taiwan_*S. taiwanensis*_EtT8	Taiwan	EtT8	+	+	+, +	+	+	+	+	+
9. Taiwan_*S. taiwanensis*_EtT9	Taiwan	EtT9	+	+	+, +	+	+	+	+	+
10. S. Korea_*P. olivaceus*_EtSK10	South Korea	EtSK10	+	+	+, +	+	+	+	+	+
11. S. Korea_*P. olivaceus*_EtSK11	South Korea	EtSK11	+	+	+, +	+	+	+	+	+
12. S. Korea_*P. olivaceus*_EtSK12	South Korea	EtSK12	+	+	+, +	+	+	+	+	+
13- S. Korea_*P. olivaceus*_EtSK13	South Korea	EtSK13	+	+	+, +	+	+	+	+	+
14. S. Korea_*P. olivaceus*_EtSK14	South Korea	EtSK14	+	+	+, +	+	+	+	+	+
15. S. Korea_*P. olivaceus*_EtSK15	South Korea	EtSK15	+	+	+, +	+	+	+	+	+
16. S. Korea_*P. olivaceus*_EtSK16	South Korea	EtSK16	+	+	+, +	+	+	+	+	+
17. S. Korea_*P. olivaceus*_EtSK17	South Korea	EtSK17	+	+	+, +	+	+	+	+	+
18. S. Korea_*P. olivaceus*_EtSK18	South Korea	EtSK18	+	+	+, +	+	+	+	+	+
19. S. Korea_*P. olivaceus*_EtSK19	South Korea	EtSK19	+	+	+, +	+	+	+	+	+
20. India_*O. punctatus*_EtI20	North India	EtI20	+	+	+, +	+	+	+	+	+
21. India_*O. punctatus*_EtI21	North India	EtI21	+	+	+, +	+	+	+	+	+
22. India_*L. rohita*_EtI22	South India	EtI22	+	+	+, +	+	+	+	+	+
23. India_*L. rohita*_EtI23	South India	EtI23	+	+	+, +	+	+	+	+	+
24. India_*C. batrachus*_EtI24	North India	EtI24	+	+	+, +	+	+	+	+	+
25. India_*C. batrachus*_EtI25	North India	EtI25	+	+	+, +	+	+	+	+	+
26. India_*C. batrachus*_EtI26	North India	EtI26	+	+	+, +	+	+	+	+	+
27. India_*A. testudineus*_EtI27	North India	EtI27	+	+	+, +	+	+	+	+	+
28. India_*O. punctatus*_EtI28	North India	EtI28	+	+	+, +	+	+	+	+	+
29. India_*C. batrachus*_EtI29	North India	EtI29	+	+	+, +	+	+	+	+	+
30. India_*C. batrachus*_EtI30	North India	EtI30	+	+	+, +	+	+	+	+	+
31. India_*P. hypophthalmus*_EtI31	South India	EtI31	+	+	+, +	+	+	−	+	−
32. India_*P. hypophthalmus*_EtI32	South India	EtI32	+	+	+, +	+	+	−	+	−
33. India_*P. hypophthalmus*_EtI33	South India	EtI33	+	+	+, +	+	+	−	+	−
34. India_*P. hypophthalmus*_EtI34	South India	EtI34	+	+	+, +	+	+	−	+	−
35. India_*P. hypophthalmus*_EtI35	South India	EtI35	+	+	+, +	+	+	−	+	−
36. India_*C. batrachus*_EtI36	North India	EtI36	+	+	+, +	+	+	−	+	−
37. India_*C. batrachus*_EtI37	North India	EtI37	+	+	+, +	+	+	−	+	−

Notes. +: positive; −: negative; EtI: Edwardsiella *tarda* India; EtT: Edwardsiella *tarda* Taiwan; EtSK: Edwardsiella *tarda* South Korea; SSA: Salmonella–Shigella agar; TSA: tryptic soy agar; Mot: motility; Cat: catalase; O/F: oxidation and fermentation; BHI: brain–heart infusion; SBA: sheep blood agar; β-H/C: beta-haemolysis.
Only the first 30 isolates (1–30) that were positive for all phenotypic tests above were subjected to subsequent tests given in Tables 3 and 4 as well as in phylogenetic analyses.

Evensen, 2016; Munang'andu, Mutoloki, & Evensen, 2014). Several studies have shown the ability of OMPs to induce protective immunity against various fish diseases (Khushiramani et al., 2012; LiHua et al., 2019; Maiti, Shetty, Shekar, Karunasagar, & Karunasagar, 2011, 2012; Munang'andu, Mutoloki, & Evensen, 2015; Munang'andu & Evensen, 2015; Xing, Li,

Tang, & Zhan, 2018). The phylogenetic relatedness of *E. tarda* OMPs from different strains infecting various fish species is unknown, making it difficult to pinpoint which bacterial strains are best suited as vaccine antigens. Therefore, the objectives of the present study were threefold: (a) to examine *Edwardsiella* isolates from different fish species from India, South

TABLE 3 API 20E biochemical test results of *Edwardsiella tarda* isolates from India, South Korea and Taiwan

Source/Fish species/ID	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
1. India_P. hypophthalmus_Etl1	–	–	+	+	+	–	–	+	+	–	+	+	+	–	–	–	+	–	–	+	–
2. India_P. hypophthalmus_Etl2	–	–	+	+	–	–	–	+	+	–	+	+	+	–	–	–	+	–	–	+	–
3. India_P. hypophthalmus_Etl3	–	–	+	+	+	–	–	+	+	–	+	+	+	–	–	–	+	–	–	+	–
4. India_P. hypophthalmus_Etl4	–	–	+	+	+	–	–	+	+	–	+	+	+	–	–	–	+	–	–	+	–
5. India_P. hypophthalmus_Etl5	–	–	+	+	–	–	–	+	+	–	+	+	+	–	–	–	+	–	–	+	–
6. Taiwan_H. ellipticum_EtT6	–	–	+	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–	–	+	–
7. Taiwan_S. taiwanensis_EtT7	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
8. Taiwan_S. taiwanensis_EtT8	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
9. Taiwan_S. taiwanensis_EtT9	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
10. S. Korea_P. olivaceus_EtSK10	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
11. S. Korea_P. olivaceus_EtSK11	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
12. S. Korea_P. olivaceus_EtSK12	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
13. S. Korea_P. olivaceus_EtSK13	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
14. S. Korea_P. olivaceus_EtSK14	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
15. S. Korea_P. olivaceus_EtSK15	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
16. S. Korea_P. olivaceus_EtSK16	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
17. S. Korea_P. olivaceus_EtSK17	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
18. S. Korea_P. olivaceus_EtSK18	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
19. S. Korea_P. olivaceus_EtSK19	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
20. India_O. punctatus_Etl20	–	–	+	+	–	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
21. India_O. punctatus_Etl21	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
22. India_L. rohita_Etl22	–	–	+	+	–	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
23. India_L. rohita_Etl23	–	–	+	+	–	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
24. India_C. batrachus_Etl24	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
25. India_C. batrachus_Etl25	–	–	+	+	–	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
26. India_C. batrachus_Etl26	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
27. India_A. testudineus_Etl27	–	–	+	+	–	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
28. India_O. punctatus_Etl28	–	–	+	+	–	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
29. India_C. batrachus_Etl29	–	–	+	+	–	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
30. India_C. batrachus_Etl30	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–
DSMZ 30052	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	+	–

Notes. +: positive; –: negative; ONPG: ß-galactosidase (ortho-nitrophenyl-ß-D-galactopyranoside); ADH: arginine dihydrolase; LDC: lysine decarboxylase; ODC: ornithine decarboxylase; CIT: citrate utilization; H2S: H2S production; URE: urease; TDA: tryptophan deaminase; IND: indole production; VP: Voges–Proskauer; GEL: gelatinase; GLU: fermentation/oxidation of glucose; MAN: F/O of mannitol; INO: F/O of inositol; SOR: F/O of sorbitol; RHA: F/O of rhamnose; SAC: F/O of saccharose; MEL: F/O of melibiose; AMY: F/O of amygdalin; ARA: F/O of arabinose and OX: cytochrome oxidase. DSMZ 30052: *Edwardsiella tarda* reference strain (ATCC 23685).

Korea and Taiwan in order to determine their correct classification in view of recent changes in the *Edwardsiella* taxonomic classification; (b) to gain insight into the phenotypic and genotypic variability of the strains infecting different fish species in Asian aquaculture; and (c) to determine the variability of *ompW* as a potential antigenic determinant to be included in vaccines with broad coverage across species and environments.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolation

A total of 37 isolates obtained from different fish species that died of edwardsiellosis-suspected outbreaks in India, Taiwan and South Korea were used for this study (Table 2). All isolates used in this study were exempted from ethics approval given that they were obtained from fish that died of edwardsiellosis-suspected outbreaks. Ethics regulations in all three countries (India, South Korea and Taiwan) that provided samples for this study indicate that only experimental studies involving live animals require ethics approval while isolates from fish that died of disease outbreaks do not require ethics approval. All isolates were cultured in *Salmonella–Shigella* agar (SSA) (Sigma-Aldrich, USA) at 37°C overnight. Thereafter, purified single colonies from SSA were streaked on tryptic soy agar (TSA) after purification. All isolates were preserved in 30% glycerol broth and stored at −80°C until use. For retrieval, they were cultured in brain–heart infusion (BHI) broth.

2.2 | Morphology, catalase, haemolysis and motility tests

To test for haemolysis, all colonies were streaked on 5% sheep blood agar (SBA), and the plates were incubated at 37°C overnight.

TABLE 4 GenBank accession numbers for 16S rRNA, *gyrB* and *ompW*

Source/Fish species/ID	Host	Country	16 S rRNA	gyrB	ompW
1. India_*P. hypophthalmus*_EtI1	*Pangasionodon hypophthalmus*	India	MF973055	MG597291	MG458287
2. India_*P. hypophthalmus*_EtI2	*Pangasionodon hypophthalmus*	India	MF973056	MG597292	MG458288
3. India_*P. hypophthalmus*_EtI3	*Pangasionodon hypophthalmus*	India	MF973068	MG597293	MG458289
4. India_*P. hypophthalmus*_EtI4	*Pangasionodon hypophthalmus*	India	MF973069	MG597294	MG458290
5. India_*P. hypophthalmus*_EtI5	*Pangasionodon hypophthalmus*	India	MF973070	MG597295	MG458291
6. Taiwan_*H. ellipticum*_EtT6	*Hyperprosopon ellipticum*	Taiwan	MF967407	MG553555	MG208117
7. Taiwan_*S. taiwanensis*_EtT7	*Sympenchelys taiwanensis*	Taiwan	MF967408	MG553556	MG208118
8. Taiwan_*S. taiwanensis*_EtT8	*Sympenchelys taiwanensis*	Taiwan	MF967409	MG553557	MG208119
9. Taiwan_*S. taiwanensis*_EtT9	*Sympenchelys taiwanensis*	Taiwan	MF967410	MG553558	MG208120
10. S. Korea_*P. olivaceus*_EtSK10	*Paralichthys olivaceus*	South Korea	MF967414	MG553545	MG208107
11. S. Korea_*P. olivaceus*_EtSK11	*Paralichthys olivaceus*	South Korea	MF967415	MG553546	MG208108
12. S. Korea_*P. olivaceus*_EtSK12	*Paralichthys olivaceus*	South Korea	MF967416	MG553547	MG208109
13. S. Korea_*P. olivaceus*_EtSK13	*Paralichthys olivaceus*	South Korea	MF967417	MG553548	MG208110
14. S. Korea_*P. olivaceus*_EtSK14	*Paralichthys olivaceus*	South Korea	MF967418	MG553549	MG208111
15. S. Korea_*P. olivaceus*_EtSK15	*Paralichthys olivaceus*	South Korea	MF967433	MG553550	MG208112
16. S. Korea_*P. olivaceus*_EtSK16	*Paralichthys olivaceus*	South Korea	MF967434	MG553551	MG208113
17. S. Korea_*P. olivaceus*_EtSK17	*Paralichthys olivaceus*	South Korea	MF967435	MG553552	MG208114
18. S. Korea_*P. olivaceus*_EtSK18	*Paralichthys olivaceus*	South Korea	MF967436	MG553553	MG208115
19. S. Korea_*P. olivaceus*_EtSK19	*Paralichthys olivaceus*	South Korea	MF967437	MG553554	MG208116
20. India_*O. punctatus*_EtI20	*Ophiocephalus punctatus*	India	MF973071	MG662182	MG458292
21. India_*O. punctatus*_EtI21	*Ophiocephalus punctatus*	India	MF977454	MG662183	MG458293
22. India_*L. rohita*_EtI22	*Labeo rohita*	India	MF977455	MG662184	MG458294
23. India_*L. rohita*_EtI23	*Labeo rohita*	India	MF977456	MG662185	MG458295
24. India_*C. batrachus*_EtI24	*Clarias batrachus*	India	MF973072	MG662186	MG458296
25. India_*C. batrachus*_EtI25	*Clarias batrachus*	India	MF973073	MG662187	MG458297
26. India_*C. batrachus*_EtI26	*Clarias batrachus*	India	MF973074	MG597296	MG458298
27. India_*A. testudineus*_EtI27	*Anabas testudineus*	India	MF977457	MG458399	MG458399
28. India_*O. punctatus*_EtI28	*Ophiocephalus punctatus*	India	MF977458	MG458300	MG458300
29. India_*C. batrachus*_EtI29	*Clarias batrachus*	India	MF973075	MG458301	MG458301
30. India_*C. batrachus*_EtI30	*Clarias batrachus*	India	MF977459	MG489896	MG489896

Note. EtI: Edwardsiella *tarda* India; EtT: Edwardsiella *tarda* Taiwan; EtSK: Edwardsiella *tarda* South Korea.

Morphological examination of the colonies was performed by Gram stain followed by microscopic examination, while wet smear preparations from the cultured colonies were used for motility observation under a light microscope. The isolates were also examined for catalase reaction as well as growth in BHI broth, TSA and O/F test.

2.3 | Biochemical characterization using API 20E system

The analytical profile index (API) 20E, which constitutes a biochemical panel of 21 reactions, was used for the identification and phenotypic characterization of members of the family *Enterobacteriaceae* according to the manufacturer's guidelines (bioMérieux, Marcy l'Etoile, France). Briefly, streaks of single colonies from the overnight culture on TSA were used to make bacterial suspensions standardized to the McFarland 2 in sterile distilled water. The suspensions were put on API 20E biochemical test strips containing dehydrated media/biochemical reagents in 21 separate compartments. Each well in the compartment was filled to the brim with bacterial suspension. Thereafter, the strips were closed and put in a moist chamber for incubation at 37°C for 24 hr. Substrate assimilations were read after 24 and 48 hr. The final interpretation of the results was done after the second reading, which was 48 hr after the onset of incubation at 37°C. Bacterial strain identification was done using the Web version of API catalogue (Bosshard et al., 2006).

2.4 | Genomic DNA isolation

Genomic DNA from all isolates was extracted as previously described by Ausubel et al. (1995) with slight modifications. Briefly, all isolates were inoculated in 5 ml Luria–Bertani (LB) broth and incubated at 37°C overnight in a shaker incubator. For each sample, 1.5 ml of the culture was put in a microcentrifuge tube and centrifuged at 9,300 *g* for 10 min. Thereafter, the supernatant was removed, and the resultant pellet was resuspended in 567 µl of 1× TE buffer. To this, 30 µl of 10% SDS and 3 µl of proteinase K were added, followed by incubation at 37°C for 1 hr. Then, 100 µl of 5 M NaCl and 80 µl of CTAB-NaCl were added to each tube, mixed by vortexing and incubated at 65°C for 10 min. This was followed by adding an equal volume of chloroform–isoamyl alcohol (24:1), mixing gently and centrifugation at 10,000 rpm for 10 min. The aqueous layer was carefully removed and transferred into another sterile microcentrifuge tube to which an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) was added, mixed gently and centrifuged at 9,300 *g* for 10 min. The supernatant was transferred into another sterile microcentrifuge tube, and the DNA in the aqueous phase was precipitated using 600 µl isopropanol and, centrifuged at 12,000 rpm for 10 min in order to pellet the DNA. The supernatant was gently discarded, and the pellet was washed in 70% ethanol by centrifugation at 13,400 *g* for 10 min. Finally, after washing, the supernatant was gently discarded and the pellet was dried in a vacuum drier and dissolved in 50 µl 1× TE buffer and stored at −20°C. The DNA concentration and purity were determined using a NanoDrop® spectrophotometer (ND-1000, V3.3.0; Thermo Fisher Scientific, USA).

2.5 | Polymerase chain reaction amplification of 16S rRNA, *gyrB* and *ompW* genes

Primers for 16S rRNA, *gyrB* and *ompW* genes were designed from the conserved regions of *E. tarda* reference strain (NZ_QDAC00000000.1, DSMZ 30052). All primers were purchased
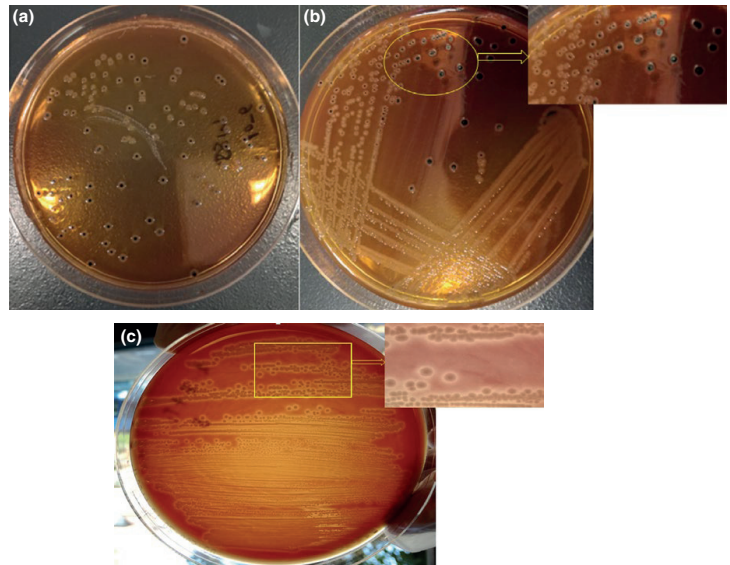
FIGURE 1 *Edwardsiella tarda* colonies on *Salmonella–Shigella* agar (SSA) and 5% sheep blood agar (5% SBA). (a, b) *E. tarda* colonies showing characteristic yellow appearance and black centred dots on the surface. (c) *E. tarda* colonies on 5% SBA exhibiting β-haemolysis zones around the colonies

from MWG-Biotech and used according to the manufacturer's recommendations (Eurofins, Germany) (Table 1). PCRs were carried out using AccuStart *Taq* DNA Polymerase HiFi (Quanta BioSciences) in 5 U/μL. Reactions comprised 50% glycerol, 20 mM Tris-HCl, 40 mM NaCl, 0.1 mM EDTA, and stabilizers, HiFi PCR Buffer (10X), 600 mM Tris-SO$_4$ (pH 8.9), 180 mM (NH4)$_2$SO$_4$, 50 mM MgSO$_4$ each of the four deoxynucleotide triphosphates (dNTP) at a concentration of 200 μM, 100–00 nM of each primer and 1 U of Taq polymerase (Quanta BioSciences). All PCRs were carried out in a programmable thermocycler (Bio-Rad, USA) at an initial delay of 94°C for 5 min followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final delay at 72°C for 5 min. PCR products were analysed by electrophoresis on 1.5% agarose–TAE gel stained with SYBR® Safe DNA Gel Stain (0.5 μg/ml) and visualized on a UV transilluminator. PCR products were purified using the Concert QIAquick PCR Purification Kit following the manufacturer's guidelines (Qiagen, USA). Purified products were commercially sequenced by GATC Biotech, Germany.

2.6 | Primer specificity test

The specificity of 16S rRNA, *gyrB*, and *ompW* primers was determined by PCR amplification of DNA extracted from the reference *E. tarda* isolate (NZ_QDAC00000001.1, DSMZ 30052) as a positive control and *Yersinia ruckeri*, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella enterica*, *Citrobacter* spp. and *Pseudomonas* spp. All PCR products were analysed by electrophoresis on 1.5% agarose–TAE gel, and PCR products were purified as described above.

2.7 | Sequencing and phylogenetic analysis

All DNA sequences were retrieved from GATC Biotech. Phylogenetic trees were made using the Molecular Evolutionary Genetics Analysis 7 (MEGA7) bioinformatics software (Kumar, Stecher, & Tamura, 2016). The evolutionary history for each tree was inferred using the Maximum Composite Likelihood method (Kimura, 1980) and is expressed as number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed. The genetic distances were computed using the Kimura 2-parameter method with 1,000 replicates of bootstrap value (Kimura, 1980). All positions containing gaps and missing data were eliminated. There were a total of 816, 326 and 499 positions in the final data set for the 16S rRNA, *gyrB* and *ompW* sequences, respectively. Estimates of evolutionary divergence between sequences were determined using the Jukes–Cantor model (Jukes & Cantor, 1969). Apart from sequences retrieved from GATC Biotech generated in this study, we included five sequences of reference strains from NCBI including *E. piscicida* recently characterized to designate isolates from fish,

and *E. anguillarum* reported to infect eels in the phylogenetic analysis as well as reference strains for *E. ictaluri* and *E. tarda* known to infected fish and other organisms.
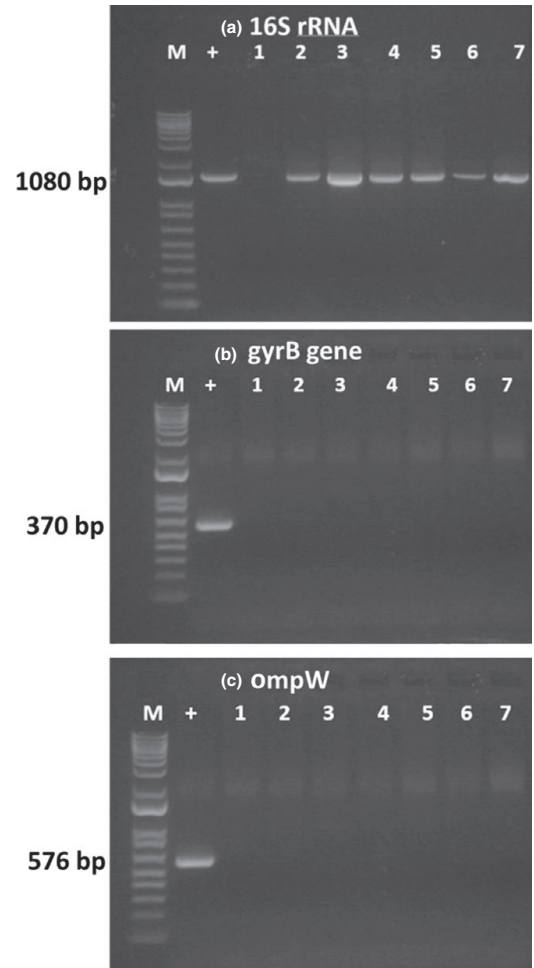
FIGURE 2 Results of the primer specificity test for 16S rRNA, *gyrB* and *ompW*. (a) A 1,080-bp PCR product was detected for *E. tarda* 16S rRNA reference strain as positive control (lane +), and no PCR product was detected for the negative control sterile water (lane 1), while PCR products were detected for *Salmonella enterica* (lane 2), *Escherichia coli* (lane 3), *Yersinia ruckeri* (lane 4), *Klebsiella pneumoniae* (lane 5), *Citrobacter* (lane 6) and *Pseudomonas* (lane 7). (b) A 376-bp PCR product was detected for the *E. tarda gyrB* reference strain (lane +), and no PCR product was detected for the negative control sterile water (lane 1), *S. enterica* (lane 2), *E. coli* (lane 3), *Y. ruckeri* (lane 4), *K. pneumoniae* (lane 5), *Citrobacter* (lane 6) and *Pseudomonas* (lane 7). (c) A 576-bp PCR product was detected for the *E. tarda ompW* reference strain (lane +), and no PCR product was detected for the negative control sterile water (lane 1), *S. enterica* (lane 2), *E. coli* (lane 3), *Y. ruckeri* (lane 4), *K. pneumoniae* (lane 5), *Citrobacter* (lane 6) and *Pseudomonas* (lane 7)

3 | RESULTS

The Results section is divided into phenotypic and genotypic characterization as shown below.

3.1 | Phenotypic characterization

3.1.1 | Growth and morphological properties

Table 2 shows the results for motility and haemolysis and growth scores for all isolates examined. All isolates were Gram-negative short rods, motile, O/F test positive and catalase positive (Table 2). All isolates proliferated on TSA, BHI broth and 5% SBA while only 30 showed black centred colonies on SSA after incubation at 37°C (Figure 1). Seven isolates proliferated without showing characteristic black centred colour on SSA and were non-haemolytic on 5% SBA, of which five were from *P. hypophthalmus* from South India and two from *C. batrachus* from North India.

3.1.2 | API 20E biochemical characterization

Of the 37 isolates examined using the API 20E system, only 30 showed biochemical traits characteristic of *E. tarda* based on the API catalogue score of 4,766,102 (Bosshard et al., 2006) (Table 3). The seven isolates that proliferated without characteristic black centred colour on SSA and were non-haemolytic on 5% SBA produced biochemical reactions not characteristic of *E. tarda* on the API 20E score system. All isolates classified as *E. tarda* were positive for nine biochemical reactions including LDC (lysine decarboxylase), ODC (ornithine decarboxylase), TDA (tryptophan deaminase), IND (indole production), MAN (F/O of mannitol), GEL (gelatinase), GLU (fermentation/oxidation of glucose), ARA (F/O of arabinose) and OX (cytochrome oxidase) irrespective of their origin and host species (Table 3). In addition, they were all negative to nine reactions that included ONPG (ß-galactosidase (ortho-ni-trophenyl-ß-D-galactopyranoside)), ADH (arginine dihydrolase), URE (urease), VP (Voges–Proskauer), INO (F/O of inositol), SOR (F/O of sorbitol), RHA (F/O of rhamnose), MEL (F/O of melibiose) and AMY (F/O of amygdalin) (Table 3). Major differences were observed in CIT (citrate), hydrogen sulphide (H_2S) and SAC (F/O of saccharose) reactions. Put together, all isolates had a similarity of 85.71% and 14.29% differences out of the 21 biochemical reactions tested using the API 20E system.

In terms of geographical variability, Indian isolates had some slight differences in biochemical reactions unlike Taiwanese and South Korean isolates that had similar reactions (Table 3). Variability among Indian isolates was observed in CIT, H_2S and SAC reactions. Isolates from South India were SAC positive and

H_2S negative, while all isolates from North India, South Korea and Taiwan were SAC negative and H_2S positive. Utilization of CIT accounted for the largest variability in which isolates from South and North India were either positive or negative suggesting that differences in CIT utilization were not influenced by the geographical origin of the isolates.

In terms of inter-host species variations, *P. hypophthalmus* from South India was the only fish species with isolates positive for SAC and negative for H_2S contrary to isolates from other fish species that were negative for SAC and positive for H_2S irrespective of the geographical origin. As for intra-host species variability, isolates from *P. hypophthalmus*, *C. batrachus*, *O. punctatus* and *P. hypophthalmus* from the same geographical locations were either positive or negative for CIT utilization (Table 3).

3.2 | Genotypic characterization

3.2.1 | Primer specificity test

Figure 2 shows the specificity test results for primers targeting 16S rRNA, *gyrB* and *ompW*. Figure 2a shows that primers targeting 16S rRNA produced a 1,080-bp PCR product from *E. tarda* (GenBank Acc. DSMZ 30052) (lane +) and no PCR product from sterile water (lane 1). Also, 16S rRNA primers produced PCR products from *S. enterica*, *E. coli*, *Y. ruckeri*, *K. pneumoniae*, *Citrobacter* spp. and *Pseudomonas* spp., as shown in lanes 2 to 7, respectively. Primers targeting the *gyrB* gene produced a 370-bp PCR product from *E. tarda* (GenBank Acc. DSMZ 30052) as shown in lane + in Figure 2b and no PCR products from sterile water and other bacterial species tested as shown in lanes 2 to 7, respectively. Similarly, primers targeting *ompW* produced a 576-bp PCR product from *E. tarda* (GenBank Acc. DSMZ 30052) as shown lane + in Figure 2c and no PCR product from sterile water and other bacterial species as shown in lanes 2 to 7, respectively.

3.2.2 | Phylogenetic analysis of 16S rRNA of *E. tarda*

Of the 37 samples examined, 16S rRNA primers produced PCR products from 33 isolates that were commercially sequenced by GATC Biotech. After retrieval, three sequences were characterized as *Citrobacter* spp., while 30 showed 97%–100% similarity (0.0 E-value) with the five *Edwardsiella* reference strains used in this study (Figure 3a). The 30 isolates classified as *Edwardsiella* spp. by 16S rRNA sequence alignment produced characteristic dark centred colonies on SSA and were haemolytic on 5% SBA. In addition, they were also classified as *E. tarda* using the API 20E biochemical tests. Only sequences classified as *Edwardsiella spp.* were

FIGURE 3 *Edwardsiella* 16S rRNA phylogenetic tree and sequence distance divergence relationship. (a) The tree clusters the isolates into one large monophyletic group comprising all 30 isolates from South Korea, India and Taiwan together with the *E. ictaluri*, *E. tarda*, *E. piscicida*, *E. hoshinae* and *E. anguillarum* reference strains while using the *A. hydrophila* reference strain as an outgroup. (b) The distance divergence of fish isolates together with the *E. ictaluri*, *E. tarda*, *E. piscicida*, *E. hoshinae* and *E. anguillarum* reference strains while using the *A. hydrophila* reference strain as an outgroup. The overall divergence for all 30 isolates varied between 0.000 and 0.009 (99.1%–100% similarity), while divergence of *Edwardsiella* isolates and the *A. hydrophila* reference strains was estimated at 0.13

(a)

21-India *O. punctatus* EtI21
20-India *O. punctatus* EtI20
27-India *A. testudineus* EtI27
1-India *P. hypophthalmus* EtI1
2-India *P. hypophthalmus* EtI2
26-India *C. batrachus* EtI26
25-India *C. batrachus* EtI25
NR 118487.1 *E. tarda*
24-India *C. batrachus* EtI24
29-India *C. batrachus* EtI29
5-India *P. hypophthalmus* EtI5
4-India *P. hypophthalmus* EtI4
KM676416.1 *E. hoshinae*
3-India *P. hypophthalmus* EtI3
NR 024769.1 *E. ictaluri*
FJ405308.1 K19 16S ribosomal RNA
8-Taiwan *S. taiwanensis* EtT8
7-Taiwan *S. taiwanensis* EtT7
6-Taiwan *H. ellipticum* EtT6
9-Taiwan *S. taiwanensis* EtT9
12- S. Korea *P. olivaceus* EtSK12
CP006664.1 1921633-1923140 *E. anguillarum*
10-S. Korea *P. olivaceus* EtSK10
22-India *L. rohita* EtI22
30-India *C. batrachus* EtI30
28-India *O. punctatus* EtI28
23-India *L. rohita* EtI23
18- S. Korea *P. olivaceus* EtSK18
19 S. Korea *P. olivaceus* EtSK19
14- S. Korea *P. olivaceus* EtSK14
13- S. Korea *P. olivaceus* EtSK13
17- S. Korea *P. olivaceus* EtSK17
16- S. Korea *P. olivaceus* EtSK16
11- S. Korea *P. olivaceus* EtSK11
15- S. Korea *P. olivaceus* EtSK15
CP016044.1 148583-149876 *E. piscicida*
MG984625.1 ATCC *A. hydrophila*

(91, 84, 65, 82, 44, 82)

(b)

Standard deviation / Divergence matrix (37 × 37)

#	Sequence
1	27-India_A._testudineus_EtI27
2	21-India_O._punctatus_EtI21
3	20-India_O._punctatus_EtI20
4	2-India_P._hypophthalmus_EtI2
5	1-India_P._hypophthalmus_EtI1
6	FJ405308.1_K19_16S_ribosomal_RNA
7	30-India_C._batrachus_EtI30
8	28-India_O._punctatus_EtI28
9	23-India_L._rohita_EtI23
10	22-India_L._rohita_EtI22
11	24-India_C._batrachus_EtI24
12	5-India_P._hypophthalmus_EtI5
13	4-India_P._hypophthalmus_EtI4
14	29-India_C._batrachus_EtI29
15	12-_S._Korea__P._olivaceus__EtSK12
16	26-India_C._batrachus_EtI26
17	25-India_C._batrachus_EtI25
18	19_S._Korea__P._olivaceus__EtSK19
19	18-_S._Korea__P._olivaceus__EtSK18
20	10-S._Korea__P._olivaceus__EtSK10
21	17-_S._Korea__P._olivaceus__EtSK17
22	16-_S._Korea__P._olivaceus__EtSK16
23	11-_S._Korea__P._olivaceus__EtSK11
24	14-_S._Korea__P._olivaceus__EtSK14
25	13-_S._Korea__P._olivaceus__EtSK13
26	15-_S._Korea__P._olivaceus__EtSK15
27	3-India__P._hypophthalmus__EtI3
28	8-Taiwan_S._taiwanensis_EtT8
29	7-Taiwan_S._taiwanensis_EtT7
30	6-Taiwan_H._ellipticum_EtT6
31	9-Taiwan_S._taiwanensis_EtT9
32	CP006664.1_1921633-1923140_E._anguillarum
33	CP016044.1_148583-149876_E_piscicida
34	KM676416.1_E._hoshinae
35	NR_024769.1_E._ictaluri
36	NR_118487.1_E._tarda
37	MG984625.1_ATCC_A._hydrophila

deposited in the National Center for Biotechnology Information (NCBI) databank (Table 4). Phylogenetic analysis grouped the 30 *E. tarda* isolates into one monophyletic group together with *E. tarda* (NR_118487.1), *E. ictaluri* (NR_024769.1), *E. anguillarum* (CP006664.1_1921633-1923140), *E. hoshinae* (KM676416.1) and *E. piscicida* (CP016044.1_148583-149876) reference strains (Figure 3a). As shown in Figure 3a, the phylogenetic tree does not delineate the reference strains into separate groups. Moreover, the disparity among isolates was estimated at 1.10% indicating that all isolates had close similarity of 98.9% together with all reference strains (Figure 3b). All isolates were distantly related to the *Aeromonas hydrophila* reference strain (MG984625.1 ATCC) used as an outgroup with a disparity of 13.0% pointing to a similarity of 87.0% between *Edwardsiella* isolates and *A. hydrophila* 16S rRNA sequences.

3.2.3 | Phylogenetic analysis of *gyrB* gene

Of the 37 isolates examined, only 30 produced PCR products using *gyrB* primers from the same isolates classified as *Edwardsiella spp.* by 16S rRNA primers (Table 4). Note that the *gyrB* phylogenetic tree clearly separated the *E. ictaluri* (CP001600.23948-5807), *E. tarda* (JX866988.2 ATCC) and *E. hoshinae* (KM660678.1 ATCC) reference strains from our isolates (Figure 4a). The *E. tarda* (JX866988.2 ATCC) and *E. hoshinae* (KM660678.1 ATCC) reference strains were placed next to each with sequence divergences of 18.0% and 16.7%, respectively, from our isolates (Figure 4b). On the other hand, *E. ictaluri* (CP001600.23948-5807) was placed close to group I and II isolates supported with a bootstrap value of 100% (Figure 4a) and had a low sequence divergence of 3.2% with our isolates (Figure 4b). It is noteworthy that the reference strain *E. anguillarum* (CP00664.11772788 1774696) was put together with 14 isolates from India and all isolates from Taiwan, which is supported by the distance matrix analysis that showed 100% homology of reference strain *E. anguillarum* (CP00664.11772788 1774696) with all isolates in group I (Figure 4b). On the other hand, all isolates from South Korea and three isolates from India were homologous with the *E. piscicida* (CP004141.13788-5696) reference strain. The sequence disparity between isolates in groups I and II was estimated at 2.9%. In addition, the distance matrix tree shows that all isolates from India, Taiwan and South Korea together with *E. anguillarum* (CP00664.11772788 1774696) and *E. piscicida* (CP004141.13788-5696) reference strains were distantly related

to the *A. hydrophila* reference strain (AF417622.1 ATCC) used as an outgroup with a sequence disparity of 31.9%.

3.2.4 | Phylogenetic analysis of outer membrane protein W (*ompW*) gene

Figure 5 shows the *ompW* tree for 30 isolates characterized as *Edwardsiella spp.* using the 16S rRNA and *gyrB* phylogenetic analysis. The *ompW* tree separates the isolates into two closely related groups (I and II). Group I comprised all Indian and Taiwanese isolates together with the *E. anguillarum* (CP006664.1 3552374-3552949) and *E. ictaluri* (CP020466.1 324388-32463) reference strains, while group II comprised South Korean isolates together with the *E. piscicida* (CP106044.1 1656679-1657254) and *E. tarda* (LC127084.1 1030742-1031317) reference strains (Figure 5a). All isolates were distantly related to the *A. hydrophila ompW* strain (HM063446) used as an outgroup with a sequence disparity of 58.8%. Put together, all 30 *ompW* sequences from groups I and II put together with all reference strains had a low sequence divergence of 5.0% pointing to a high similarity of 95% among the 30 isolates obtained from different aquatic organisms in Asia together with all reference strains used in this study.

4 | DISCUSSION

Bacterial cells are multistate automata that continuously sense changes in the environment to which they adapt (Bochner, 2008), and the phenotypic and genotypic variations observed here potentially reflect the environmental conditions in which bacteria proliferate by selecting for better-adapted strains. In aquatic environments in the wild, multiple host species coexist and bacterial phenotypic and genotypic changes can be caused by intra- and inter-host species adaptation.

4.1 | Phenotypic characterization of *E. tarda* isolates

The dark centred colonies seen on SSA in this study are consistent with those reported by other scientists (Wyatt, Nickelson, & Vanderzant, 1979). Concerning phenotypic traits based on biochemical properties, 30 isolates characterized as *E. tarda* had a similarity of 85.71% where all were positive for nine and negative for nine reactions giving a total of 18 identical reactions out of the 21 reactions tested on the API 20E strips. Wyatt et al. (1979) reported positive reactions for indole motility, ODC, LCD, maltose and glucose and

FIGURE 4 *Edwardsiella gyrB* phylogenetic tree and sequence distance divergence relationship. (a) The *gyrB* tree is divided into groups I and II. Group I consists of 17 homologous sequences from different fish species in India and Taiwan grouped with the *E. anguillarum* reference strain. Note that the *E. ictaluri* is placed close to group I and II isolates while *E. tarda* and *E. hoshinae* are placed further close to the *A. hydrophila* outgroup. Group II consists of all isolates from South Korea and three isolates from India that clustered together with the *E. piscicida* reference strain. (b) Nucleotide distance divergence of the 30 isolates used to generate the *gyrB* tree in A together with the *E. tarda*, *E. ictaluri*, *E. anguillarum*, *E. hoshinae* and *E. piscicida* reference strains. Rows 1–15 and 31 in column 1 show no nucleotide variability (0.000 divergence, 100% homology) for 16 isolates (orange) put in group I of the *gyrB* tree (a). Similarly, there was no nucleotide difference among sequences clustered in group II as shown in rows 17–26 (green) and in rows 27–30 (blue). The furthest divergence among all isolates was estimated at 0.032, while variability of *Edwardsiella* sequences and the *A. hydrophila* (AF417622.1) reference strain was estimated at 31.9%

(a)

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21-India O. punctatus EtI21
CP006664.11772788 1774696 E. anguillarum
8-Taiwan S. taiwanensis EtT8
27-India A. testudineus EtT27
30-India C. batrachus EtI30
9-Taiwan S. taiwanensis EtT9
7-Taiwan S. taiwanensis EtT7
6-Taiwan H. ellipticum EtT6
23-India L. rohita EtI23
22-India L. rohita EtI22
26-India C. batrachus EtI26
4-India P. hypophthalmus EtI4
25-India C. batrachus EtI25
20-India O. punctatus EtI20
28-India O. punctatus EtI28
29-India C. batrachus EtI29
2-India P. hypophthalmus EtI2
1-India P. hypophthalmus EtI1
5-India P. hypophthalmus EtI5
3-India P. hypophthalmus EtI3
24-India C. batrachus EtI24
GQ387361.1 E. tarda gyrB
CP004141.13788-5696 E. piscicida
17- S. Korea P. olivaceus EtSK17
18- S. Korea P. olivaceus EtSK18
19 S. Korea P. olivaceus EtSK19
16- S. Korea P. olivaceus EtSK16
15- S. Korea P. olivaceus EtSK15
13- S. Korea P. olivaceus EtSK13
12- S. Korea P. olivaceus EtSK12
11- S. Korea P. olivaceus EtSK11
14- S. Korea P. olivaceus EtSK14
10-S. Korea P. olivaceus EtSK10
CP001600.23948-5807 E. ictaluri
JX866988.2 ATCC E. tarda
KM660678.1 ATCC E. hoshinae
AF417622.1 A. hydrophila ATCC
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Group I, Group II (bracket labels)

(b)

Divergence / Standard deviation matrix (37 × 37)

Legend:

No.	Isolate
1	7-Taiwan_S._taiwanensis_EtT7
2	9-Taiwan_S._taiwanensis__EtT9
3	6-Taiwan_H._ellipticum_EtT6
4	28-India_O._punctatus__EtI28
5	29-India_C._batrachus_EtI29
6	21-India_O._punctatus__EtI21
7	30-India_C._batrachus_EtI30
8	23-India_L._rohita_EtI23
9	22-India_L._rohita_EtI22
10	27-India_A._testudineus_EtT27
11	8-Taiwan_S._taiwanensis__EtT8
12	26-India__C._batrachus__EtI26
13	4-India__P._hypophthalmus_EtI4
14	2-India__P._hypophthalmus__EtI2
15	25-India__C._batrachus_EtI25
16	1-India_P._hypophthalmus_EtI1
17	17-_S._Korea__P._olivaceus_EtSK17
18	18-_S._Korea__P._olivaceus_EtSK18
19	19_S._Korea__P._olivaceus_EtSK19
20	16-_S._Korea__P._olivaceus__EtSK16
21	15-_S._Korea__P._olivaceus__EtSK15
22	14-_S._Korea__P._olivaceus__EtSK14
23	13-_S._Korea__P._olivaceus__EtSK13
24	12-_S._Korea__P._olivaceus__EtSK12
25	11-_S._Korea__P._olivaceus__EtSK11
26	10-S._Korea__P._olivaceus_EtSK10
27	GQ387361.1_E.tarda_gyrB
28	24-India_C._batrachus_EtI24
29	5-India__P._hypophthalmus__EtI5
30	3-India__P._hypophthalmus__EtI3
31	20-India_O._punctatus_EtI20
32	CP001600.23948-5807_E._ictaluri
33	CP004141.13788-5696_E._piscicida
34	CP006664.11772788_1774696_E._anguillarum
35	JX866988.2_ATCC_E._tarda
36	KM660678.1_ATCC_E._hoshinae
37	AF417622.1_A._hydrophila_ATCC

negative reactions for VP, mannitol, KCN and citrate for 74 *E. tarda* isolates from catfish (Wyatt et al., 1979), which corroborates our findings. Amandi et al. (1982) found positive reactions from glucose, maltose, H_2S, ODC, LDC, indole, catalase and nitrate reduction and negative reactions for RHA, salicin, urease, mannitol, salicin, sorbitol and melibiose from chinook salmon (*Oncorhynchus tshawytscha*) isolates, which is also in agreement with our findings. Further, several other scientists have reported similar biochemical reactions from

(a)

6-Taiwan *H. ellipticum* EtT6
20-India *O. punctatus* EtI20
7-Taiwan *S. taiwanensis* EtT7
29-India *C. batrachus* EtI29
28-India *O. punctatus* EtI28
27-India *A. testudineus* EtI27
26-India *C. batrachus* EtI26
25-India *C. batrachus* EtI25
24-India *C. batrachus* EtI24
23-India *L. rohita* EtI23
22-India *L. rohita* EtI22
5-India *P. hypophthalmus* EtI5
4-India *P. hypophthalmus* EtI4
3-India *P. hypophthalmus* EtI3
2-India *P. hypophthalmus* EtI2
8-Taiwan *S. taiwanensis* EtT8
9-Taiwan *S. taiwanensis* EtT9
30-India *C. batrachus* EtI30
21-India *O. punctatus* EtI21
1-India *P. hypophthalmus* EtI1
CP006664.1 3552374-3552949 *E. anguillarum*
CP020466.1 324388-324963 *E. ictaluri*

Group I

CP016044.1 1656679-1657254 *E. piscicida*
LC127084.1 1030742-1031317 *E. tarda*
JQ923432.1 *E. tarda* OmpW
14- S. Korea *P. olivaceus* EtSK14
13- S. Korea *P. olivaceus* EtSK13
12- S. Korea *P. olivaceus* EtSK12
18- S. Korea *P. olivaceus* EtSK18
19 S. Korea *P. olivaceus* EtSK19
15- S. Korea *P. olivaceus* EtSK15
10-S. Korea *P. olivaceus* EtSK10
17- S. Korea *P. olivaceus* EtSK17
16- S. Korea *P. olivaceus* EtSK16
11- S. Korea *P. olivaceus* EtSK11

Group II

HM063446.1 *A. hydrophila* OmpW

(b)

Standard deviation / Divergence

[Large 36×36 divergence/standard deviation matrix]

Legend:

#	Label
1	29-India_C._batrachus_EtI29
2	28-India_O._punctatus__EtI28
3	27-India_A._testudineus_EtI27
4	26-India_C._batrachus__EtI26
5	25-India_C._batrachus__EtI25
6	24-India_C._batrachus_EtI24
7	23-India_L._rohita_EtI23
8	22-India_L._rohita__EtI22
9	5-India_P._hypophthalmus__EtI5
10	4-India_P._hypophthalmus__EtI4
11	3-India_P._hypophthalmus__EtI3
12	2-India_P._hypophthalmus__EtI2
13	8-Taiwan_S._taiwanensis__EtT8
14	7-Taiwan_S._taiwanensis_EtT7
15	6-Taiwan_H._ellipticum_EtT6
16	30-India_C._batrachus_EtI30
17	21-India_O._punctatus_EtI21
18	1-India_P._hypophthalmus_EtI1
19	9-Taiwan__S._taiwanensis__EtT9
20	20-India_O._punctatus_EtI20
21	19_S_Korea__P._olivaceus__EtSK19
22	18-_S._Korea__P._olivaceus__EtSK18
23	17-_S._Korea__P._olivaceus__EtSK17
24	16-_S._Korea__P._olivaceus__EtSK16
25	15-_S_Korea__P._olivaceus__EtSK15
26	12-_S._Korea__P._olivaceus__EtSK12
27	14-_S._Korea__P._olivaceus__EtSK14
28	13-_S._olivaceus___P_olivaceus__EtSK13
29	11-_S._Korea__P._olivaceus___EtSK11
30	10-S_Korea__P._olivaceus_EtSK10
31	JQ923432.1_E._tarda_OmpW
32	CP006664.1_3552374-3552949_E._anguillarum
33	CP016044.1_1656679-1657254_E._piscicida
34	CP020466.1_324388-324963_E._ictaluri
35	LC127084.1_1030742-1031317_E._tarda
36	HM063446.1_A._hydrophila_OmpW

FIGURE 5 *Edwardsiella ompW* phylogenetic tree and sequence distance divergence relationship. (a) The *ompW* phylogenetic tree separates the isolates together with the *E. tarda*, *E. anguillarum*, *E. ictaluri* and *E. piscicida* reference strains into two closely related groups (groups I and II). Group I consists of all isolates from India and Taiwan together with the *E. anguillarum* and *E. ictaluri* reference strains, while group II consists of all South Korean isolates together with *E. tarda* and *E. piscicida*. (b) Nucleotide divergence of fish isolates used to generate the *ompW* tree in A. Column 1 shows a summary of the *ompW* nucleotide divergence with the furthest divergence among isolates being estimated at 5.0% while variability with the *A. hydrophila* (AF417622.1) reference strain used as an outgroup from other isolates was estimated at 0.558

various fish species (Alcaide, Herraiz, & Esteve, 2006; Griffin et al., 2013; Padros, Zarza, Dopazo, Cuadrado, & Crespo, 2006) suggesting that common phenotypic traits do exist among *E. tarda* isolates from different fish species. That said, the number of studies contrasting biochemical reactions of isolates intra- and inter-host species in aquaculture is limited. We found positive SAC reactions and negative H_2S reactions in *P. hypophthalmus* isolates from South India, while the opposite profile was found in North Indian isolates from the same fish species. On this basis, different ecosystems might influence *E. tarda* adaptation altering its phenotype. In contrast, for CIT utilization, we found that Indian isolates from *P. hypophthalmus*, *O. punctatus* and *C. batrachus* were positive and negative for CIT within the same species, but not within the same individual, and thus were possibly influenced by host microenvironments. These findings show that *E. tarda* isolates from different fish species and geographical locations share a large proportion of phenotypic traits (85.71%), but that adaptation to host environments influences bacterial phenotypes. However, there is a need for more studies to consolidate these observations in different fish species cultured in different ecosystems.

4.2 | Genotypic characterization of *E. tarda* isolates

The 16S rRNA phylogenetic tree shows that all 30 isolates classified as *E. tarda* using the API 20E system together with the *E. tarda* (NR_118487.1), *E. ictaluri* (NR_024769.1), *E. anguillarum* (CP006664.1_1921633-1923140), *E. hoshinae* (KM676416.1) and *E. piscicida* (CP016044.1_148583-149876) reference strains form a monophyletic group indicating that the 16S rRNA phylogenetic marker could not delineate the reference strains and our isolates into different species. Moreover, the 16S rRNA distance matrix analysis showed a low differentiation capacity with a sequence disparity of 13.0% between the *A. hydrophila* (MG984625.1 ATCC) reference strain used as an outgroup and our Asian isolates, unlike the gyrB distance matrix analysis that had a higher sequence differentiation capacity of 31.9% between the *A. hydrophila* outgroup and our isolates. These observations are supported by the primer specificity test in which we showed that 16S rRNA primers produced PCR products from other genera in the family *Enterobacteriaceae* apart from *E. tarda* while *gyrB* primers only produced PCR products from *E. tarda*. Put together, these observations are in line with several other studies that have shown that 16S rRNA has a lower inter- and intraspecies phylogenetic differentiation capacity compared to *gyrB* (Dauga, 2002; Mun Huang, 1996; Wang, Lee, Tai, & Kasai, 2007; Yanez, Catalán, Apraiz, Figueras, & Martinez-Murcia, 2003).

Caro-Quintero and Ochman (2015) pointed out that 16S rRNA has a low rate of evolution that hinders its resolution and specificity of closely related bacterial species unlike protein-coding genes such as *gyrB* that evolve quickly culminating in high resolution and specificity. It is likely that these inherent factors could have influenced the low 16S rRNA intra- and interspecies differentiation capacity observed in this study.

Contrary to the 16S rRNA phylogenetic tree, the *gyrB* tree clustered our isolates into two groups with the majority of Indian and all Taiwanese isolates grouped together with the *E. anguillarum* reference strain while all South Korean isolates and three Indian isolates were grouped together with the *E. piscicida* reference strain, indicating that our isolates belong to *E. anguillarum* and *E. piscicida* species. In addition, the *gyrB* tree clearly separated the *E. ictaluri*, *E. tarda* and *E. hoshinae* reference strains from our isolates as shown that *E. ictaluri*, which is mostly isolated from catfish, was placed close to our isolates resulting in a low sequence disparity of 3.2% with group I and II isolates. On the contrary, *E. hoshinae*, which has mainly been isolated from birds and reptiles (Grimont et al., 1980), had a high sequence disparity of 16.7% with our isolates. These findings are in agreement with observations made by Abayneh et al. (2013), who showed that fish isolates were distinct from mammalian isolates and that they belong to either *E. piscicida* or *E. anguillarum*. Moreover, our findings show that all isolates classified as *E. tarda* using the API 20E system were classified as either *E. anguillarum* or *E. piscicida*, which is in agreement with Buján et al. (2018) who showed that fish isolates classified as *E. tarda* isolates using biochemical tests were re-affiliated to *E. piscicida* or *E. anguillarum* using genetic classification. Overall, these observations show that the gyrB phylogenetic marker had a higher interspecies differentiation capacity between our isolates and the *A. hydrophila* outgroup with a sequence disparity of 31.9%, unlike the 16S rRNA phylogenetic marker that had a low sequence disparity of 13.0% between our isolates and the *A. hydrophila* outgroup. In addition, our findings also show that the *gyrB* marker had a high intraspecies differentiation capacity to effectively classify all *Edwardsiella* reference strains used in this study into individual species unlike the 16S rRNA marker that could not differentiate the reference strains into individual species but instead grouped them in a single monophyletic group.

4.3 | Implications for vaccine development

Phylogenetic analysis has been widely used for the identification of vaccine antigens with broad protective coverage against variant strains. Peng et al. (2016) used phylogenetic analysis to identify

four highly conserved OMPs with broad protective ability against various *Vibrio parahaemolyticus* strains and showed that protection in vaccinated fish was highly dependent on homology of the vaccine strain with the challenge strain. Sequence alignments of *V. parahaemolyticus* OMPs showed close similarity with *A. hydrophila* and *P. fluorescens* OMPs of which protection in vaccinated fish corresponded with the level of OMP homology among the three bacterial species. Similarly, Ningqui et al (Li et al., 2010) used sequence alignments to show that *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* had 71.7%–99.2% OmpK homology of which cross-protection in orange-spotted grouper (*Epinephelus coioides*) correlated with the degree of similarity of their deduced OmpK amino acid sequences. In the present study, the *ompW* phylogenetic tree clustered the different isolates from various fish species into two closely related groups with the furthest disparity between isolates estimated at 5.0% pointing to a homology of 95% across all isolates, suggesting that a common strain can be used against isolates from different fish species in Asian aquaculture. Obviously, there is a need for experimental vaccination and challenge studies to explore and demonstrate cross-protective ability of *ompW* vaccines.

5 | CONCLUSIONS

This study underlines the importance of combining phenotyping with genotyping in the identification and classification of bacterial species. The high biochemical similarity of the 30 isolates classified as *E. tarda* from different Asian fish species corroborates previous studies (Odds, 1981) showing that the genus *Edwardsiella* constitutes a biochemically homogeneous taxon with minor intraspecies variations. Our findings show that *gyrB* is a better phylogenetic marker for inter- and intraspecies characterization of Edwardsiella isolates than 16S rRNA because it has a high differentiation capacity between species. In summary, our findings show that all isolates characterized as *E. tarda* using the API 20E biochemical test were classified as *E. anguillarum* or *E. piscicida* based on *gyrB* phylogenetic characterization. Finally, homology of the *OmpW* protein among our isolates points at the possibility of identifying vaccine candidates with broad protective coverage for use in different countries in Asia, pending experimental studies corroborating these findings.

AUTHOR CONTRIBUTION

SD conceived and designed the study, carried out laboratory and data analyses and prepared the manuscript. BM, SHK, SMS, DK, PKP, SKG and SCC collected the samples and carried out characterization of bacteria. SM, ØE and IK conceived and designed the study, supervised the study and prepared and finalized the manuscript. HMM conceived and designed the study, carried out data analysis and resource mobilization, supervised the study and prepared the manuscript.

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Paper-II

1 ***Aeromonas hydrophila* isolates obtained from different farmed aquatic species in India and**

2 **Taiwan show high genotypic and phenotypic relatedness**

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20

21 **Abstract**

22 **Background:** In aquaculture, interspecies transmission could play a vital role in transforming

23 non-pathogenic bacteria strains into virulent strains. For *Aeromonas hydrophila* having a tropism

24 for a broad host range, interspecies transmission can lead to emergence of hypervirulent strains.

25 Therefore, comparison of properties of *A. hydrophila* strains from different aquatic organisms

26 can shed light on host influence in altering bacteria properties. Herein, we compared 28 isolates

27 from nine different aquatic organisms from India (n=24) and Taiwan (n=4) to gain insight of the

28 genotypic and phenotypic properties of *A. hydrophila* isolates different host species in two Asian

29 countries.

30 **Results:** The high similarity (>90%) of the API 20 NE biochemical properties as well as the high

31 *gyrB* (>96%) and 16S rRNA (>98%) sequence similarity suggests that a closely related *A.*

32 *hydrophila* taxon could be infecting different aquatic organisms. Moreover, the high *ompA*

33 similarity (>88%) suggests that strains from different host species and geographic regions share

34 antigenic determinants.

35 **Conclusions:** Overall, this study accentuates the importance of combining genotyping and

36 phenotyping for accurate species identification and characterization. Moreover, it underlines the

37 potential of phylogenetic comparison of key bacterial antigens with an aim to identify potential

38 vaccine strains protective against strains from different host species in different geographic

39 regions.

40 **Keywords:** *Aeromonas hydrophila*/genotype/phenotype/16S rRNA/*gyrB*/*ompA*/vaccine

1.0 Introduction

41

42 *Aeromonas hydrophila* is a ubiquitous bacterium that infects amphibians, reptiles, fish and

43 mammals found in warm water environments (Yáñez et al., 2003). In fish, it causes a disease

44 known by various names such as motile aeromonad septicemia (MAS), motile aeromonad

45 infection (MAI) and hemorrhagic septicemia (HS) (Dash et al., 2014, Gong et al., 2015). In

46 infected fish, it causes internal and external hemorrhages as well as hypertrophy, dropsy, red

47 sores, necrosis, ulceration, and exophthalmia (Chu and Lu, 2008, Shen et al., 2013). Recently,

48 there has been an upswing of hypervirulent *A. hydrophila* (vAh) strains that cause high

49 mortalities with annual losses of millions of tons of farmed fish, such as channel catfish

50 (*Ictalurus punctatus*) and grass carp (*Ctenopharyngodon idella)* in China and USA (Rasmussen-

51 Ivey et al., 2016, Nielsen et al., 2001, Hemstreet, 2010, Zhang et al., 2002, Pang et al., 2015). In

52 2009, mortalities reached 2,200 tons and 10,500 tons in 2016 with vAh strains accounting for

53 35% of MAS outbreaks in the USA (Hemstreet, 2010, Rasmussen-Ivey et al., 2016). This trend

54 has also been reported in countries such as China where vAh strains cause high economic losses.

55 The emergence of vAh strains calls for characterization of field isolates in order to develop

56 effective vaccines against these highly pathogenic *A. hydrophila* strains.

57 Transfer of bacteria between species can result in diversification of bacterial properties

58 particularly during proliferation within a new host species. This applies to pathogens such as *A.*

59 *hydrophila* that infect a wide range of aquatic organisms, but little is known if intra- and inter-

60 host species transmission alter bacterial virulence and/or antigenic properties which could

61 potentially facilitate emergence of vAh strains or escape variants. Therefore, there is a need to

62 compare isolates from different aquatic organisms in order to determine to what extent host

63 species transfer influences bacterial genotypic and phenotypic traits, including variation in

3

64   antigenic structures of importance for vaccine protection. Biochemical characterization is a

65   standard method used for phenotyping of bacterial species although it sometimes produces

66   conflicting results due to extreme phenotypic diversity between and within species (Odds, 1981,

67   Abbott et al., 2003). However, biochemical tests are often time-consuming and expensive

68   rendering genotyping using rapid molecular biology tools a better option. The most widely used

69   molecular marker for bacterial genotyping is 16S rRNA because it is considered to be the most

70   reliable gene for determining distant (intergenic) and close (intragenic) genealogical

71   interrelationships between species (Figueras et al., 2000, Woese, 1987). Its presence in almost all

72   bacteria species as a multigene family makes it an ideal molecular marker for bacterial

73   genotyping (Patel, 2001). Despite so, 16S rRNA sequences vary in size and organization in their

74   rRNA variable regions which result in poor intra-species resolution. Housekeeping genes like

75   *gyrB* that encode the DNA gyrase B-subunit are used as an alternative suitable phylogenetic

76   marker for bacterial systematics (Watanabe et al., 2001). The *gyrB* genomic sequence has a

77   mean synonymous substitution rate four times faster 16S rDNA making it more reliable than 16S

78   rRNA for intra-species genotyping of some bacteria species (Yamamoto, 1996). Despite so,

79   concurrent application of *gyrB* and 16S rRNA has gained prominence in genotyping of different

80   bacteria species (Yamamoto and Harayama, 1998, Bavykin et al., 2004, Holmes et al., 2004).

81   While genotype and phenotype characterization are important for correct bacterial identification,

82   selection of protective vaccine strains against variant strains infecting different aquatic

83   organisms should be based on a detailed understanding of antigenic properties of the strains used

84   for vaccine production. Phylogenetic analyses of antigenic proteins from different isolates is

85   useful in identifying large neutralizing immunogenic proteins able to confer protection against

86   variant strains. Among the proteins shown to induce protective immunity in vaccinated fish is the

87 outer membrane proteins (OMPs) (Dubey et al., 2016a, Dubey et al., 2016b, Hamod et al., 2012,

88 Munang'andu et al., 2014, Munang'andu et al., 2016). OMPs are vital components of the

89 bacterial surface membranes recognized as pathogen-associated molecular patterns (PAMPs) by

90 pattern recognition receptors (PRRs) found on the surface of antigen presenting cells (APCs).

91 They play a vital role in activating cells of the adaptive immune system in producing long-term

92 protective immunity. Different OMPs have been characterized in *A. hydrophila* (Khushiramani et

93 al., 2007, Maiti et al., 2009, Khushiramani et al., 2012).

94 On this basis, we have compared genotypic and phenotypic properties of *A. hydrophila* isolates

95 from different fish species and farmed turtles, originating from different aquaculture

96 environments in India and Taiwan. Methods included morphological and biochemical tests, 16S

97 rRNA and *gyrB* (house-keeping gene) phylogenetic analyses. In addition, we compared the

98 *ompA* of *A. hydrophila* isolates with an aim to understand if host species and environments

99 would impact on outer membrane structures known to be important for immune protection.

100 **2.0 Material and methods**

101 **2.1 Bacterial isolation**

102 A total of 33 isolates obtained from nine different diseased aquatic organisms (see below) in

103 India and Taiwan were included in this study. Indian isolates were from eight host species

104 comprising of *Carassius auratus* (Gold fish), *Cirrhinus mrigala* (Mrigal), *Cyprinus carpio*

105 (Common carp), *Clarias batrachus* (Mangur), *Catla catla* (catla), *Labeo rohita* (Rohu) and

106 *Oreochromis niloticus* (Nile tilapia). The Taiwanese isolates were from three host species

107 comprising of *Hyperprosopon ellipticum* (California perch), *Pelodiscus sinensis* (soft-shelled

108 turtle) and *Oreochromis niloticus* as shown in Table 1. All isolates used in this study were

109 exempted from ethics approval given that they were obtained from fish that died of *A. hydrophila*

110 suspected outbreaks. Ethics regulations in all three countries (India and Taiwan) that provided

111 samples for this study indicate that only experimental studies involving live animals require

112 ethics approval while isolates from dead fish from disease outbreaks do not require ethics

113 approval. All isolates were initially cultured on tryptose soy agar (TSA) and tryptose soy broth

114 (TSB) for bacteria isolation from infected hosts before culture on Aeromonas isolation agar

115 (AIA) and Rimler Shotts (RS) medium, selective for *Aeromonas* isolation (Sigma-Aldrich,

116 USA). Characteristic single green colonies from AIA and yellow colonies from RS medium were

117 streaked on TSA for production of pure cultures. Thereafter, all isolates were preserved in 30 %

118 glycerol TSB and stored at -80^{0}C until use. For retrieval after -80 ^{0}C storage, brain heart infusion

119 (BHI) broth was used.

120 **2.2 Phenotypic characterization**

121 Phenotypic characterization was carried out by morphological, hemolysis and biochemical tests

122 as shown below.

123 **2.2.1 Morphological characterization and hemolysis examination**

124 The morphological examination was carried out after Gram staining by microscopic examination

125 of isolates cultured on TSA. For hemolysis test, all isolated were cultured on 5% sheep blood

126 agar (SBA), and the cultures were examined for lysis of red blood cells around the bacteria

127 colonies.

128 **2.2.2 Biochemical characterization using the analytical profile index (API) 20 NE system**

129 Biochemical tests were carried out using the analytical profile index (API) 20 systems for non-

130 Enterobacteriaceae (NE). Briefly, bacterial suspensions obtained from overnight culture on TSA

131 were standardized to MacFarland 2 and inoculated on API 20NE strips based on manufacturer's

132 guidelines (BioMérieux, Marcy l'Etoile, France). The strips were incubated at 37 ^{0}C for 48 h.

133   Substrate assimilations were read after 24 and 48 h. Interpretation of results was done after 48 h

134   using the VITEK 2 ID-GNB card identification software version 6.0 based on manufacturer

135   instructions (Bosshard et al., 2006).

136   **2.3 Genotype characterization**

137   Genotype characterization was carried out by extracting genomic DNA followed by sequence

138   comparison of different isolates using 16S rRNA and *gyrB* phylogenetic analyses as shown

139   below.

140   **2.3.1 Genomic DNA Extraction**

141   Genomic DNA from different isolates shown in Table 1 was extracted as previously described by

142   Ausubel et al. (Ausubel, 1995). The DNA pellet were suspended in 1 X Tris-EDTA buffer (pH

143   8.0). Concentration and purity were determined using the NanoDrop® spectrophotometer (ND-

144   1000, Thermo Fisher Scientific, USA).

145   **2.3.2 Genotyping of 16S rRNA, *gyrB* and *ompA* gene**

146   Primers for 16S rRNA, *gyrB* and *OmpA* genes were designed from the most conserved region of

147   *A. hydrophila* and the primers used are shown in Table 2. PCR was performed using AccuStart

148   *Taq* DNA Polymerase HiFi (Quanta, Biosciences), in a 5 U/μL in 50% glycerol, 20 mM Tris-

149   HCl, 40 mM NaCl, 0.1 mM EDTA, and stabilizers, HiFi PCR Buffer (10X) 600 mM Tris-SO4

150   (pH 8.9), 180 mM $(NH_4)_2SO_4$, 50 mM magnesium sulfate 50 mM $MgSO_4$ each of the four

151   deoxynucleotide triphosphates (dNTP) at a concentration of 200 μM, 100-500 nM for each

152   primer and 1 U *Taq* polymerase (Quanta, Biosciences). The PCR reactions were carried out at

153   94^0C for 5 min followed by 30 cycles of denaturation at 95^0C for 20 s, annealing at 56^0C for 1

154   min, extension at 72^0C for 1 min and a final delay at 72^0C for 5 min. Amplified PCR products

7

155 were analyzed by electrophoresis (ND-1000, Thermo Fisher Scientific, USA) and PCR products

156 were purified using the Concert QIAquick PCR Purification Kit based on manufacturer's

157 instructions (Qiagen, USA). Purified products were sequenced on a commercial basis by GATC-

158 Biotech (GATC-Biotech, Germany).

159 All retrieved DNA sequences were used for BLAST analysis in NCBI. The 16S rRNA, *gyrB* and

160 *ompA* phylogenetic trees were made using molecular evolutionary genetics analysis 7.0.26

161 (MEGA7) bioinformatics software. The phylogenetic construct was made with maximum

162 likelihood phylogeny using the neighbor-joining method (Saitou and Nei, 1987). The evolution

163 history for each tree is inferred using the maximum composite likelihood method in the unit of

164 the number of base pairs per site. The percentage of replicate trees in which the associated taxa

165 clustered in bootstrap test (1000 replicates) are shown next to the branches. The trees were drawn

166 to scale having branch length in the same units as those of the evolutionary distance used to infer

167 the phylogenetic trees. Genetic distances were computed Kimura's two-parameter with 1000

168 replicate of bootstrap value (Kimura, 1980). Codon positions included were

169 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There

170 was a total of 1381, 2610 and 1064 positions in the final datasets for the 16S rRNA, *gyrB* and

171 *ompA*, respectively. Evolutionary analyses were conducted in MEGA7 [2] showing distance

172 matrix and analyses were conducted using the Jukes-Cantor model [1]. Standard error estimate(s)

173 are shown above the diagonal.

174 **3.0 Results**

175 **3.1 Phenotypic characterization of Indian and Taiwanese isolates**

176 **3.1.1 Morphological examination and hemolysis on sheep blood agar**

177   All 33 isolates formed colonies on TSA and 5% SBA while on selective media, only 28 isolates

178   (84.84%, n=33) grew on AIA and RS was exhibiting and yellow colonies, respectively (Table 2,

179   Figure 1). In addition, the 28 isolates (84.84%, n=33) produced colonies that showed β-

180   hemolysis on 5% SBA while five isolates (15.15%, n=33) had no hemolytic zones around their

181   colonies on 5% SBA (Figure 1). Gram staining showed Gram-negative vibrio shaped bacteria

182   characteristic of *A. hydrophila* on microscopic examination.

183   **3.1.2 Biochemical characterization using the API 20 NE system**

184   Of 33 isolates examined for biochemical properties using the API-20 NE system, only 28 isolates

185   showed characteristic properties of *A. hydrophila* with an overall score of 6566654 on the

186   VITEK 2 ID-GNB card identification system (Bosshard et al., 2006). Five isolates that did not

187   grow on AIA and RS produced biochemical reactions that were not characteristic of *A.*

188   *hydrophila* based on the API 20NE characterization system (Bosshard et al., 2006). Phenotypic

189   similarities and differences were observed among the 28 isolates based on the 21-biochemical

190   tests carried out on the API-20 NE strips. All 28 isolates were positive for 15 and negative for

191   four biochemical tests carried out on the API-20 NE strips (Table 3) giving a similarity of

192   90.48% (n=21 biochemical tests). Major differences between isolates were based on the D-

193   arabinose (ARA) and malic acid (MLT). Table 5 summarizes the results of the ARA and MLT

194   tests obtained from the API-20 NE strips. In general, the differences between isolates were

195   classified into four major categories based on the ARA/MLT (-/+, +/-, +/+, -/-) utilization. These

196   results are summarized in Table 5, and show that isolates from two species *L. rohita* and *C.*

197   *carpio* (India) had a -/+ ARA/MLT utilization, with only one isolate from India being positive

198   (+/+) for both sugars. All isolates from *C. catla*, *C. batrachus,* and *C. mrigala* had +/-

199   ARA/MLT utilization (Table 5). The Taiwanese *H. ellipticum* and most of the *O. niloticus*

200 isolates from India were positive for both ARA/MLT (+/+). On the other hand, *P. sinensis* and *C.*

201 *auratus* isolates were ARA/MLT negative (-/-). In summary, these observations suggest that

202 utilization of these sugars could be influenced by host species adaption.

3.2 Genotype characterization based on 16S rRNA and *gyrB* genes

3.2.1 Polymerase chain reaction (PCR) amplification

205 The PCR products generated using primers targeting 16S rRNA yielded 840 bp amplicons while

206 primers targeting *gyrB* gene produced 580 bp PCR products for all isolates characterized to be *A.*

207 *hydrophila* using the API 20NE biochemical test above. The full-length *ompA* amplicon

208 generated by PCR produced a single band of 895 bp (Fig. 1C). The accession numbers for 16S

209 rRNA, *gyrB* and *ompA* sequences deposited in NCBI are shown in Table 4. Phylogenetic

210 analyses of sequences generated from 16S rRNA, *gyrB* and *ompA* sequences are shown in

211 Figures 2a, 3a, and 4a.

3.2.2 Phylogenetic analysis of 16S rRNA

213 Of the 33 isolates detected by PCR amplification, three isolates were characterized as

214 *Enterobacter cloacae* while two were characterized as *Acinobacter* spp. The remaining 28

215 isolates had 95-98% sequence similarities (E-value=0.0) with the *A. hydrophila* reference strain

216 (GenBank Acc. No. MG984625.1 ATCC 7966). The 16S rRNA phylogenetic tree put all isolates

217 in one clade although 11 isolates were in a subclade descending from the main clade (Figure

218 2A). Regarding geographical distribution, all Taiwanese isolates clustered together in subclade I

219 with two *P. sinensis* isolates that were identical. Isolates from *O. niloticus* and *H. ellipticum* were

220 also identical. Regarding host species distribution, isolates from *O. niloticus*, *H. ellipticum* and

221 *P. sinensis* clustered together in one subclade while isolates from *L. rohita* were in the main

222 clade. There was a general trend that isolates from the same host species and geographical origin

10

223 clustered together (100% homologous). For example, Indian isolates from *C. auratus* AhI17 and

224 AhI20, *O. niloticus* AhI16 and AhI19, *C. mirgala* AhI14 and AhI22 as well as the Taiwanese *P.*

225 *sinensis* isolates were 100% homologous as shown both in the phylogenetic tree (Figure 2A) and

226 distance matrix (Figure 2B). Figure 2B shows the distance matrix with the furthest disparity

227 between isolates of 0.020 indicating that all isolates had a similarity of >98.0%. Note that *Vibrio*

228 *harveyi* used as an outgroup, distantly related with all isolates examined in this study with a

229 disparity of 0.105 despite being in the Enterobacteriaceae family together with *A. hydrophila*.

230 **3.2.3 Phylogenetic analysis of *gyrB* gene**

231 Of the 33 isolates examined for *gyrB*, only 28 isolates produced PCR products of correct size.

232 All of these isolates were positive for AIA and RS growth on selective media. All 28 sequences

233 showed similarities of 95-98% (E-value=0.0) with the reference *gyrB* sequences (GenBank Acc.

234 No. ATCC_49140 *gyrB* gene). Figure 3A shows the *gyrB* phylogenetic analysis tree in which all

235 isolates clustered together in the same clade with the Taiwanese isolates being evenly distributed

236 among Indian isolates. Overall, the distance matrix output showed a closely related group with a

237 similarity >94.0% and the furthest disparity between isolates estimated at 6% (Figure 3B). It is

238 noteworthy that the disparity between *A. hydrophila* isolates and *V. harveyi* used as an out-group

239 determined by the *gyrB* distance matrix (1.116) (Figure 3B) was 10 times higher than the

240 disparity estimated by the 16S rRNA distance matrix (0.105) (Figure 2B).

241 **3.2.4 Phylogenetic analysis of *ompA***

242 The amplification of *ompA* using specific primers only detected 28 isolates as positive for *A.*

243 *hydrophila* out of 33 samples examined, similar to those detected by AIA and RS selective

244 media. The 28 isolates showed 97-99% similarities (E-value=0.0) with sequences from the

245 reference *ompA* of *A. hydrophila* strain (GenBank Acc. No. KP082946.1). Figure 4A shows the

246   *ompA* phylogenetic tree separating the 28 isolates into two closely related clades (I and II). Clade

247   II was less divergent than clade I, which was subdivided into four subgroups (IA, IB, IC and ID).

248   All Taiwanese isolates clustered in clade I. As for host species variability, *C. catla* isolates were

249   only found in clade I while *L. rohita* isolates were only found in clade II. Figure 4B shows the

250   distance matrix between isolates with the widest variability estimated at 11.7% indicating that all

251   isolates had >88.30% similarity. The isolates from the Taiwanese *O. niloticus* and the Indian *C.*

252   *carp* and *C. mrigala* were 100% homologous indicating that isolates from different geographical

253   regions and host species could have a similar *ompA* protein. The disparity between *A. hydrophila*

254   isolates and *V. harveyi* (GenBank Acc. No. KP026417.1) used as an out-group was estimated at

255   0.998 being >8 times higher than the furthest intra-species disparity among the 28 *A. hydrophila*

256   isolates (0.117) examined in this study.

257

258   **4.0 Discussion**

259   The main findings from this study are that *Aeromonas hydrophila* strains across host species and

260   geographic origin, are homogenous genetically and phenotypically, including surface antigens

261   that have been shown to be important for immune protection in vaccinated animals (Dash et al.,

262   2014).

263   All 33 isolates examined in this study grew on basic growth media such as TSA, TSB and 5%

264   SBA. On selective AIA and RS media, only 28 isolates grew with characteristic green opaque

265   and yellow colonies with black spots, respectively (Andělová et al., 2006, Altaf Ahmed, 1997).

266   Overall phenotypic characterization based on selective growth on AIA and RS media, motility,

267   β-hemolysis, colony, and morphological properties showed that only 28 out of 33 isolates

268   examined produced phenotypic traits characteristic of *A. hydrophila* (Coykendall, 1989,

269   Illanchezian et al., 2010, Santos et al., 1999). To validate these findings, all isolates were

270   subjected to biochemical tests using the API 20 NE system.

271   Biochemical tests have been the mainstay of bacterial identification and taxonomic classification

272   since the 1920s when the first Bergey's systematic bacteriology manual was introduced

273   (Buchanan and Gibbons, 1974, Bergey, Cowan, 1965). Of the 33 isolates examined, only 28

274   isolates that grew on selective media were characterized as *A. hydrophila* by API-20 NE. All *A.*

275   *hydrophila* isolates were positive for 15 reactions that included oxidase and indole; glucose,

276   maltose, and mannose fermentation; nitrate reduction; arginine, D-mannitol, N-acetyl-

277   glucosamine and D-maltose hydrolysis; gas and acetoin production from glucose; and, lysine

278   decarboxylation being in line with observations made by other scientists who found similar

279   biochemical properties in isolates from fish samples (Belgin ERDEM, 2011, Altwegg et al.,

280   1990). In addition, all isolates were negative for urea, esculin ferric citrate, trisodium citrate and

281   phenylacetic acid being in line with Martin et al, (Altwegg et al., 1990) who reported similar

282   biochemical properties from fish isolates. However, differences in arabinose and malic acid

283   reactions group the 28 isolates into four major groups as shown in Table 5. Within the same

284   geographic region, the bacterial strains are in different subgroups based on species of origin. This

285   could indicate that there is an adaption by host species, but the numbers are limited. So, while it

286   is possible to group the isolates into 4 based on arabinose and malic acid fermentation,

287   biochemical analysis show that the strains have an overall high phenotypic similarity (90%, n=21

288   biochemical reactions), and less than 10% phenotypic diversity.

289   Genotyping is vital for bacteria identification as well as determining the strain diversity within

290   species (Emerson et al., 2008). The fact that only isolates that grew on AIA and RS selective

291   media were characterized as *A. hydrophila* by the 16S rRNA and *gyrB* phylogenetic analyses

13

292   validates the use of selective media as part of phenotype characterization. Moreover, these

293   findings consolidate the biochemical characterization given that the API-20 NE classification

294   was in agreement with the 16 S rRNA and *gyrB* classification. *Aeromonas* is one of the most

295   tightly defined genera because of the high 16S rRNA intra-species similarities (96.7 – 100%)

296   (Martínez-Murcia et al., 2007, Martinez-Murcia et al., 1992). In concert with these observations,

297   our findings show a high intra-species similarity >98% for the 28 isolates from nine different

298   aquatic organisms from India and Taiwan suggesting that despite the wide range of host species

299   and different geographical areas from which the isolates originated, the genotypic similarity

300   among isolates was high. This is supported by the *gyrB* phylogenetic tree that showed a high

301   (>94%) homology among the 28 isolates consolidating observations that *A. hydrophila* isolates

302   from different aquatic organisms in India and Taiwan were closely related. That said, our

303   findings show that inter-species variability between *V. harveyi* used as an outgroup with *A.*

304   *hydrophila* isolates was >10 times higher for the *gyrB* phylogenetic tree than the 16S rRNA tree

305   suggesting that *gyrB* has a higher capacity in differentiating *A. hydrophila* isolates from other

306   *Vibrio* species than 16S rRNA. These observations are in line with several other studies that have

307   shown that housekeeping genes such as *gyrB* have a high capacity for inter-species

308   differentiation than 16S rRNA (Yamamoto and Harayama, 1995, Yamamoto, 1996, Soler et al.,

309   2003, Yáñez et al., 2003). This is also seen from the phylogenetic analysis of *gyrB* (Fig. 3A)

310   where it is shown that strains are subdivided to a greater extent that what was found for 16S

311   rRNA analysis (Fig. 2A). Put together, observations from the 16S rRNA and *gyrB* phylogenetic

312   analyses suggest that host adaptation and environment influence genotypic and phenotypic

313   properties of *A. hydrophila* to a lesser extent and high degree of similarity between strains is

314   seen.

14

315 The dilemma of selecting highly protective vaccine strains against diverse isolates infecting

316 different host species in different geographical areas can be a challenge in vaccine development.

317 To contend the diversity, growing evidence suggests that phylogenetic analyses of bacteria is a

318 reliable tool for identifying broadly protective antigens against variant bacterial strains (Reeve et

319 al., 2010, Paessler and Veljkovic, 2017, Chan et al., 2018). For example, Pizza et al (Pizza et al.,

320 2000) used whole genome sequencing and phylogenetic analysis while Maiden et al (Maiden et

321 al., 1998) used MLST dendrograms to identify conserved segments of OMPs of *Neisseria*

322 *meningitides* strains for use as vaccine candidates. Antibodies from 100% homologous conserved

323 proteins were broadly neutralizing against variant *N. meningitides* strains.

324 In fish, Ningqiu et al (Li et al., 2010) showed 71.7 – 99.2 % amino acid homology of *ompK* for

325 *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* and that protection in vaccinated orange

326 spotted grouper (*Epinephelus coioides*) correlated with the degree of identity of the deduced

327 amino acids. In the present study, the 100% homology of *H. ellipticum*, *O. niloticus* and *P.*

328 *sinensis* from Taiwan suggests that a common vaccine against *A. hydrophila* can be used against

329 isolates from different host species and geographical regions. Moreover, the 100% homology of

330 *C. mrigala* and *C. carpio* from India with the Taiwanese isolates in clade I support the notion

331 that a vaccine produced against *A. hydrophila* using *ompA* could be used in different countries

332 across different host species. Finally, the high similarity (>88%) of the *ompA* protein among all

333 the 28 isolates examined in this study further consolidate the notion that these bacteria are likely

334 to share common immunogenic properties, which could facilitate the design of vaccines that can

335 be used against *A. hydrophila* isolates infecting different host species in different geographical

336 areas. However, there is a need for further investigations to support these observations with

337   vaccine efficacy trials in order to determine the broadly protective ability of *ompA* proteins in

338   different host species.

339   In this study, we have shown a high phenotypic, and genotypic similarity among *A. hydrophila*

340   isolates from different aquatic organisms in India and Taiwan. There is >90% similarity in the

341   API 20NE biochemical properties, and >96% *gyrB* and >98% 16S rRNA sequence homologies.

342   Thus, isolates from different host species and geographical areas have a high similarity which

343   consolidates earlier observation that *Aeromonas* is a tightly defined genera with low intra-species

344   variability (Martínez-Murcia et al., 2007, Martinez-Murcia et al., 1992). These observations

345   suggest that environmental and host adaptational factors have low influence on phenotypic and

346   genotypic properties of *A. hydrophila*. In addition, we have also shown that isolates from

347   different host species and geographical areas have a high *ompA* protein homology suggesting

348   that common vaccines can be used against *A. hydrophila* induced disease in different aquatic

349   organisms and in different geographical areas. However, further studies are needed to expand on

350   these findings through *in vivo* studies, which can lay the ground for developing broadly

351   protective vaccines against *A. hydrophila* infections.

352   **5.0 Competing interests**

353   Authors declare no competing interests

354   **6.0 Authors contribution**

355   SD=Conception of study design, laboratory and data analysis, manuscript preparation; SMS,

356   BM, KD, SKG=Sample collection and bacteria characterization; SM, IK= conception of study

357   design, supervision and manuscript preparation; ØE= supervision, resource mobilization, data

358   analysis, and manuscript preparation; HMM=Conception of study design, data analysis,

359   manuscript preparation and overall supervision of the study.

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525

9.0 Figure legends

9.1 Figure 1. *Aeromonas hydrophila* colonies on Aeromonas isolation agar (AIA), Rimler Shotts (RS) agar and 5% sheep blood agar (5% SBA). **Figure 1A(1).** *A. hydrophila* colonies showing characteristic green color on AIA agar. **Figure 1B(2).** *A. hydrophila* colonies showing yellow colonies on RS agar while **Figure 1C(3)**. *A. hydrophila* on 5% sheep blood agar (5% BSA) exhibiting β-hemolysis zones around the colonies.

9.2 Figure 2. *A. hydrophila* 16S rRNA phylogenetic tree and sequence distance divergence matrix. **Figure 2A** shows that the tree clusters the isolates into one group. Twelve isolates including all Taiwanese isolates form a subclade that stretches out of the main clade. Note that two isolates from Taiwanese *Pelodiscuss sinensis* were placed next to each other. Similarly, two Indian *Oreochromis niloticus* isolates were also placed next to each other. All *O. niloticus* were put in the subclade while *Labeo rohita* and *Catla catla* were only found in the main. **Figure 2B.** Distance divergence of fish isolates used to generate the 16S rRNA tree in Figure 2A. Column-1 shows the divergence for all isolates in which rows 1-4, 9-15 and 24-30 shows 0.000-0.004 divergence forming the main clade (blue). Rows 5-8, 16, 17 and 19-24 show isolates with a divergence of 0.012 – 0.020 that form the subclade (brown). The overall divergence for all 28 isolates varied between 0.000-0.020 (98.0 - 100% similarity) while divergence of *A. hydrophila* with *Vibro harveyi* used as an out group was estimated at 0.105.

9.3 Figure 3. *A. hydrophila* gyrB phylogenetic tree and sequence distance divergence matrix. **Figure 3A.** All isolates were placed in one clade in which the Taiwanese isolates were randomly distributed among Indian isolates. The *A. hydrophila* reference strain was placed close the Indian *Cyprinus carpio* (ATCC_49140). **Figure 3B.** Nucleotide distance divergence of the 28 isolates

548   used to generate the *gyrB* tree in Figure 3A. Rows 2, 3 and 29 show nucleotide variability 0.000 –

549   0.0016. The overall divergence for all fish isolates varied between 0.000-0.047 (95.7-100%

550   similarity). The variability of *V. harveyi* (EU672845.1) used as an outgroup and *A. hydrophila*

551   (ATCC_49140) reference strains from other isolates were estimated at 1.116 and 0.024,

552   respectively.

553   **9.4 Figure 4.** *A. hydrophila ompA* phylogenetic tree and sequence distance divergence

554   relationship. **Figure 4A.** The *ompA* phylogenetic tree separates the isolates into two clades (I and

555   II). Clade I was further subdivided into four subgroups (clades IA-ID) separated by high bootstrap

556   values. Note that all Taiwanese isolates are clustered in IA and IB while IC and ID only comprised

557   of India isolates. Group II consist of 11 Indian homologous sequences supported by 99% bootstrap

558   score placed close to the *A. hydrophila ompA* reference strain (GenBank Acc. KF082946.1).

559   **Figure 4B.** The nucleotide divergence of fish isolates used to generate the *ompA* tree in Figure

560   4A. Column 1 shows a summary of the *ompA* nucleotide divergence. Fish isolates in subgroup 1A

561   in row 2-4 show no sequence variability (0.000 divergence, 100% homology) that form clade IA

562   (blue). Rows 5-7 shows closely related sequences with 0.038-0.040 divergence for clade IB

563   isolates (yellow), rows 9-14 with 0.068-0.071 divergence form clade C (green) and rows 28-29

564   with a disparity of 0.104 form clade ID. Similarly, rows 15-26 also show closely related sequences

565   with 0.112-0.117 divergence clustered in clade II of the *ompA* tree (brown). The overall divergence

566   for all fish isolates varied between 0.000-0.117 (88.3-100% similarity) while distances of

567   variability for the *V. harveyi* (KF026417.1) and *A. hydrophila* (KF082946.1) reference strains from

568   other isolates were estimated at 0.988 and 0.098, respectively.

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570 **Table 1.** Primer sequences for 16S rRNA, *gyrB*, and *ompA* genes

Primer name	Direction	Primer	GenBank Acc. No
Ah OmpA	Forward:	ATGATGAAAATGGCTCCTTCCC	KP082946.1
	Reverse:	TTACTTCTGAACTTCTTGTACGCC	
Ah gyrB	Forward:	TCCGGCGGTCTGCACGGCGT	ATCC_49140
	Reverse:	TTGTCCGGGTTGTACTCGTC	
Ah 16S rRNA	Forward:	AGGGGGATAACAGTTGGA	MG984625.1
	Reverse:	AACGTATTCACCGCAACA	ATCC 7966

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573 **Table 2.** Characterization of isolates based on growth media, catalase test, hemolysis and motility

No	Source	ID code	Origin	Gram stain	Motility	Growth Media				
						TSA	AIA	RS	5% SBA	β-H/C
1	*Labeo rohita*	AhI1	India	Gram –ve	+	+	+	+	+	+
2	*Labeo rohita*	AhI2	India	Gram –ve	+	+	+	+	+	+
3	*Catla catla*	AhI3	India	Gram –ve	+	+	+	+	+	+
4	*Catla catla*	AhI4	India	Gram –ve	+	+	+	+	+	+
5	*Pelodiscus sinensis*	AhT5	Taiwan	Gram –ve	+	+	+	+	+	+
6	*Pelodiscus sinensis*	AhT6	Taiwan	Gram –ve	+	+	+	+	+	+
7	*Hyperprosopon ellipticum*	AhT7	Taiwan	Gram –ve	+	+	+	+	+	+
8	*Oreochromis niloticus*	AhT8	Taiwan	Gram –ve	+	+	+	+	+	+
9	*Oreochromis niloticus*	AhT9	Taiwan	Gram –ve	+	+	-	-	+	-
10	*Hyperprosopon ellipticum*	AhT10	Taiwan	Gram –ve	+	+	-	+	+	-
11	*Labeo rohita*	AhI11	India	Gram –ve	+	+	+	+	+	+
12	*Labeo rohita*	AhI12	India	Gram –ve	+	+	+	+	+	+
13	*Clarias batrachus*	AhI13	India	Gram –ve	+	+	+	+	+	+
14	*Cyprinus carpio*	AhI14	India	Gram –ve	+	+	+	+	+	+
15	*Cyprinus carpio*	AhI15	India	Gram –ve	+	+	+	+	+	+
16	*Cirrhinus mrigala*	AhI16	India	Gram –ve	+	+	+	+	+	+
17	*Cirrhinus mrigala*	AhI17	India	Gram –ve	+	+	+	+	+	+
18	*Oreochromis niloticus*	AhI18	India	Gram –ve	+	+	+	+	+	+
19	*Carassius auratus*	AhI19	India	Gram –ve	+	+	+	+	+	-
20	*Catla catla*	AhI20	India	Gram –ve	+	+	+	+	+	+
21	*Oreochromis niloticus*	AhI21	India	Gram –ve	+	+	+	+	+	-
22	*Carassius auratus*	AhI22	India	Gram –ve	+	+	+	+	+	+
23	*Cirrhinus mrigala*	AhI23	India	Gram –ve	+	+	+	+	+	+
24	*Cirrhinus mrigala*	AhI24	India	Gram –ve	+	+	+	+	+	+
25	*Cyprinus carpio*	AhI25	India	Gram –ve	+	+	+	+	+	+
26	*Oreochromis niloticus*	AhI26	India	Gram –ve	+	+	+	+	+	+
27	*Cyprinus carpio*	AhI27	India	Gram –ve	+	+	+	+	+	+
28	*Carassius auratus*	AhI28	India	Gram –ve	+	+	+	+	+	+
29	*Catla catla*	AhI29	India	Gram –ve	+	+	-	+	+	-
30	*Carassius auratus*	AhI30	India	Gram –ve	+	+	-	-	+	-
31	*Catla catla*	AhI31	India	Gram –ve	+	+	-	+	+	-
32	*Labeo rohita*	AhI32	India	Gram –ve	+	+	-	+	+	-
33	*Carassius auratus*	AhI33	India	Gram –ve	+	+	-	-	+	-

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Table 3. API-20 NE characterization of *Aeromonas hydrophila* isolates from India and Taiwan

| Test | Sample number |||||||||||||||||||||||||||| |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
| NO3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TRP | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| GLU | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| ADH | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| URE | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ESC | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| GEL | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| PNPG | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| GLU | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| ARA | - | - | + | + | - | - | + | + | - | - | + | - | - | + | + | + | - | + | + | - | + | + | - | - | + | - | + | - |
| MNE | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MAN | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NAG | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MAL | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| GNT | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| CAP | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| ADI | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| MLT | + | + | - | - | - | - | + | + | + | + | - | + | + | - | - | + | - | - | + | - | - | - | + | + | + | - | - | - |
| CIT | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| PAC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **OX** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

API 20 NE result: Identification: (+) positive; (-) negative, full form of all the test are NO3 (potassium nitrate), TRP (L-tryptophane), GLU (D-glucose), ADH (L-arginine), URE (urea), ESC (Esculin ferric citrate), GEL (Gelatin), PNPG (4-nitrophenyl-β-Dgalactopyranoside), GLU (D-glucose), ARA (L-arabinose), MNE (D-mannose), MAN (D-mannitol), NAG (N-acetyl-glucosamine), MAL (D-maltose), GNT (Potassium gluconate), CAP (Capric acid), ADI (Adipic acid), MLT (Malic acid), CIT (Trisodium citrate), PAC (Phenylacetic acid) and OX (oxidase test).

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Table 4. Host species, country of origin and GenBank accession numbers of *Aeromonas hydrophila*

No	Host	Country	OmpA	gyrB	16 S NCBI	ID
1	*Labeo rohita*	India	MG019729	MG193618	MF806600	1-India *Labeo rohita* AhI1
2	*Labeo rohita*	India	MG019730	MG050699	MF806601	2-India *Labeo rohita* AhI2
3	*Catla catla*	India	MF988347	MG050700	MF806602	3-India *Catla catla* AhI3
4	*Catla catla*	India	MF988348	MG193619	MF806603	4-India *Catla catla* AhI4
5	*Pelodiscus sinensis*	Taiwan	MG019741	MG050709	MF948884	5-Taiwan *Pelodiscus sinensis* AhT5
6	*Pelodiscus sinensis*	Taiwan	MG019742	MG050710	MF948885	6- Taiwan *Pelodiscus sinensis* AhT6
7	*Hyperprosopon ellipticum*	Taiwan	MF988351	MG050707	MF967406	7- Taiwan *Hyperprosopon ellipticum* AhT7
8	*Oreochromis niloticus*	Taiwan	MG019740	MG050708	MF767520	8- Taiwan *Oreochromis niloticus* AhT8
9	*Labeo rohita*	India	MG010124	MG050701	MF806604	9-India *Labeo rohita* AhI9
10	*Labeo rohita*	India	MG010125	MG050702	MF927579	10-India *Labeo rohita* AhI10
11	*Clarias batrachus*	India	MG010126	MG193620	MF807157	11-India *Clarias batrachus* AhI11
12	*Cyprinus carpio*	India	MG010127	MG050703	MF927580	12-India *Cyprinus carpio* AhI12
13	*Cyprinus carpio*	India	MG010128	MG050704	MF927581	13-India *Cyprinus carpio* AhI13
14	*Cirrhinus mrigala*	India	MG010129	MG193621	MF927582	14-India *Cirrhinus mrigala* AhI14
15	*Cirrhinus mrigala*	India	MG010130	MG193622	MF927583	15-India *Cirrhinus mrigala* AhI15
16	*Oreochromis niloticus*	India	MG010131	MG193623	MF927584	16-India *Oreochromis niloticus* AhI16
17	*Carassius auratus*	India	MG010132	MG193624	MF928405	17-India *Carassius auratus* AhI17
18	*Catla catla*	India	MF988349	MG193625	MF927585	18-India *Catla catla* AhI18
19	*Oreochromis niloticus*	India	MG019731	MG193626	MF928406	19-India *Oreochromis niloticus* AhI19
20	*Carassius auratus*	India	MG019732	MG193627	MF962583	20-India *Carassius auratus* AhI20
21	*Cirrhinus mrigala*	India	MG019733	MG193628	MF948883	21-India *Cirrhinus mrigala* AhI21
22	*Cirrhinus mrigala*	India	MG019734	MG193629	MF942356	22-India *Cirrhinus mrigala* AhI22
23	*Cyprinus carpio*	India	MG019735	MG050730	MF942355	23-India *Cyprinus carpio* AhI23
24	*Oreochromis niloticus*	India	MG019736	MG193631	MF948882	24-India *Oreochromis niloticus* AhI24
25	*Cyprinus carpio*	India	MG019737	MG050706	MF942355	25-India *Cyprinus carpio* AhI25
26	*Carassius auratus*	India	MG019738	MG193632	MF942129	26-India *Carassius auratus* AhI26
27	*Catla catla*	India	MF988350	MG193633	MF943216	27-India *Catla catla* AhI27
28	*Carassius auratus*	India	MG019739	MG050705	MF942128	28-India *Carassius auratus* AhI28

Table 5. API 20 NE characterization of *Aeromonas hydrophila* isolates based on L-arabinose and malic acid utilization.

No	Host	Country	ID	ARA	MLT
1	*Labeo rohita*	India	1-India_Labeo rohita_AhI1	-	+
2	*Labeo rohita*	India	2-India_Labeo rohita_AhI2	-	+
9	*Labeo rohita*	India	9-India_Labeo rohita_AhI9	-	+
10	*Labeo rohita*	India	10-India_Labeo rohita_AhI10	-	+
12	*Cyprinus carpio*	India	12-India_Cyprinus carpio_AhI12	-	+
13	*Cyprinus carpio*	India	13-India_Cyprinus carpio_AhI13	-	+
23	*Cyprinus carpio*	India	23-India_Cyprinus carpio_AhI23	-	+
25	*Cyprinus carpio*	India	25-India_Cyprinus carpio_AhI25	+	+
3	*Catla catla*	India	3-India_Catla catla_AhI3	+	-
4	*Catla catla*	India	4-India_Catla catla_AhI4	+	-
18	*Catla catla*	India	18-India_Catla catla_AhI18	+	-
27	*Catla catla*	India	27-India_Catla catla_AhI27	+	-
11	*Clarias batrachus*	India	11-India_Clarias batrachus_AhI11	+	-
14	*Cirrhinus mrigala*	India	14-India_Cirrhinus mrigala_AhI14	+	-
15	*Cirrhinus mrigala*	India	15-India_Cirrhinus mrigala_AhI15	+	-
21	*Cirrhinus mrigala*	India	21-India_Cirrhinus mrigala_AhI21	+	-
22	*Cirrhinus mrigala*	India	22-India_Cirrhinus mrigala_AhI22	+	-
7	*Hyperprosopon ellipticum*	Taiwan	7-Taiwan_Hyperprosopon ellipticum_AhT7	+	+
8	*Oreochromis niloticus*	Taiwan	8-Taiwan_Oreochromis niloticus_AhT8	+	+
16	*Oreochromis niloticus*	India	16-India_Oreochromis niloticus_AhI16	+	+
19	*Oreochromis niloticus*	India	19-India_Oreochromis niloticus_AhI19	+	+
24	*Oreochromis niloticus*	India	24-India_Oreochromis niloticus_AhI24	-	+
5	*Pelodiscus sinensis*	Taiwan	5-Taiwan_Pelodiscus sinensis_AhT5	-	-
6	*Pelodiscus sinensis*	Taiwan	6-Taiwan_Pelodiscus sinensis_AhT6	-	-
17	*Carassius auratus*	India	17-India_Carassius auratus_AhI17	-	-
20	*Carassius auratus*	India	20-India_Carassius auratus_AhI20	-	-
26	*Carassius auratus*	India	26-India_Carassius auratus_AhI26	-	-
28	*Carassius auratus*	India	28-India_Carassius auratus_AhI28	-	-
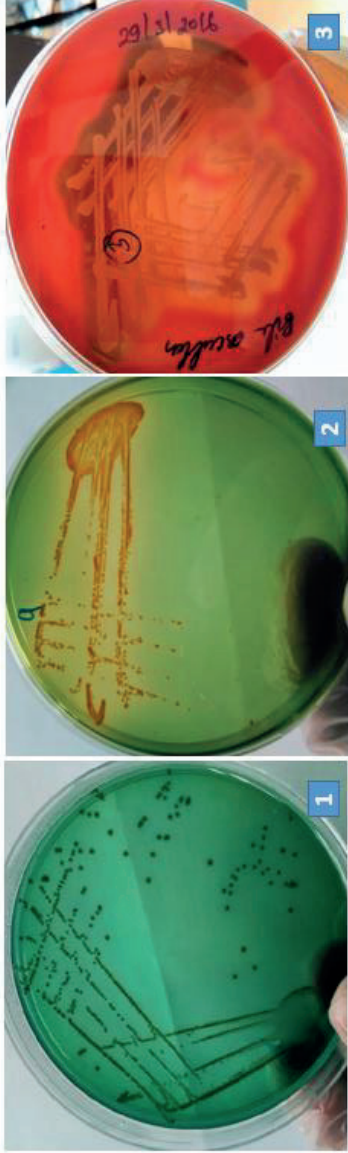
Figure 1. *A. hydrophila* on 1. Aeromonas isolation agar 2. Rimler Shotts (RS) medium 3. Blood agar
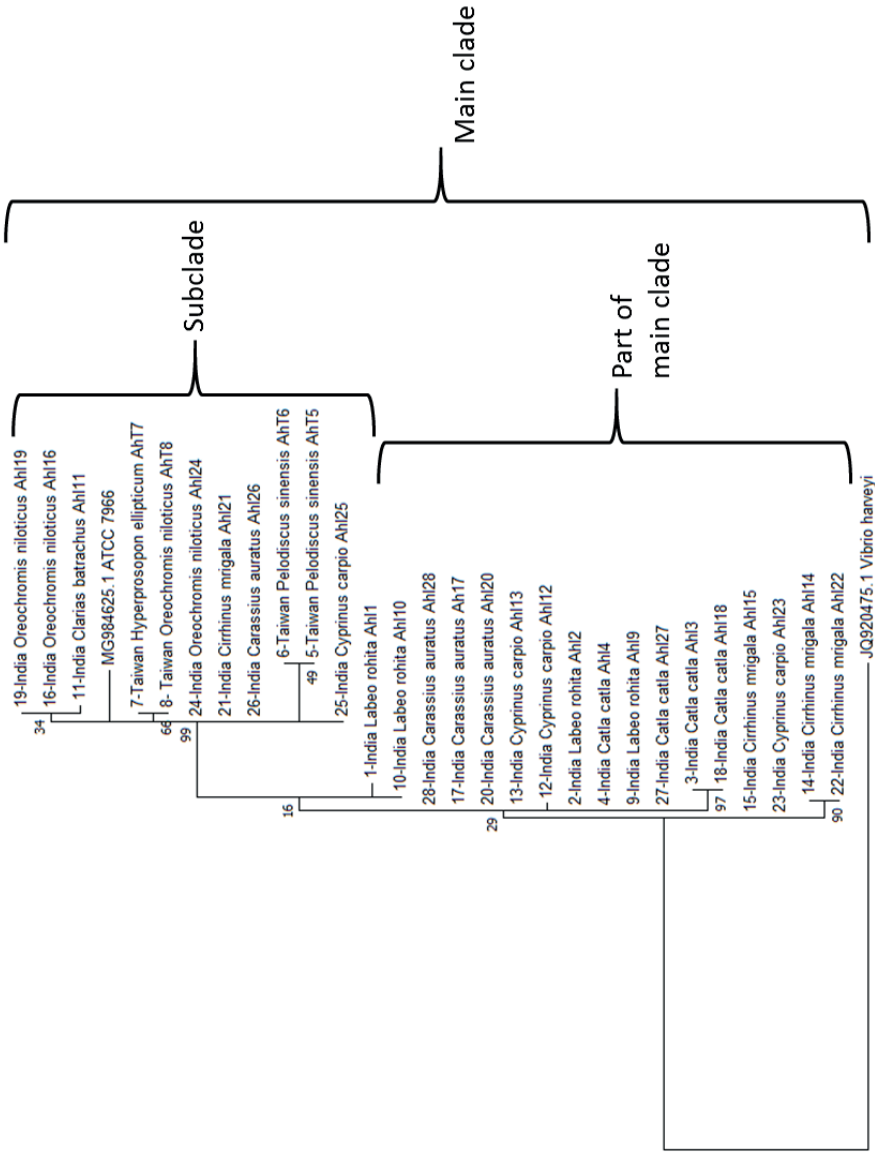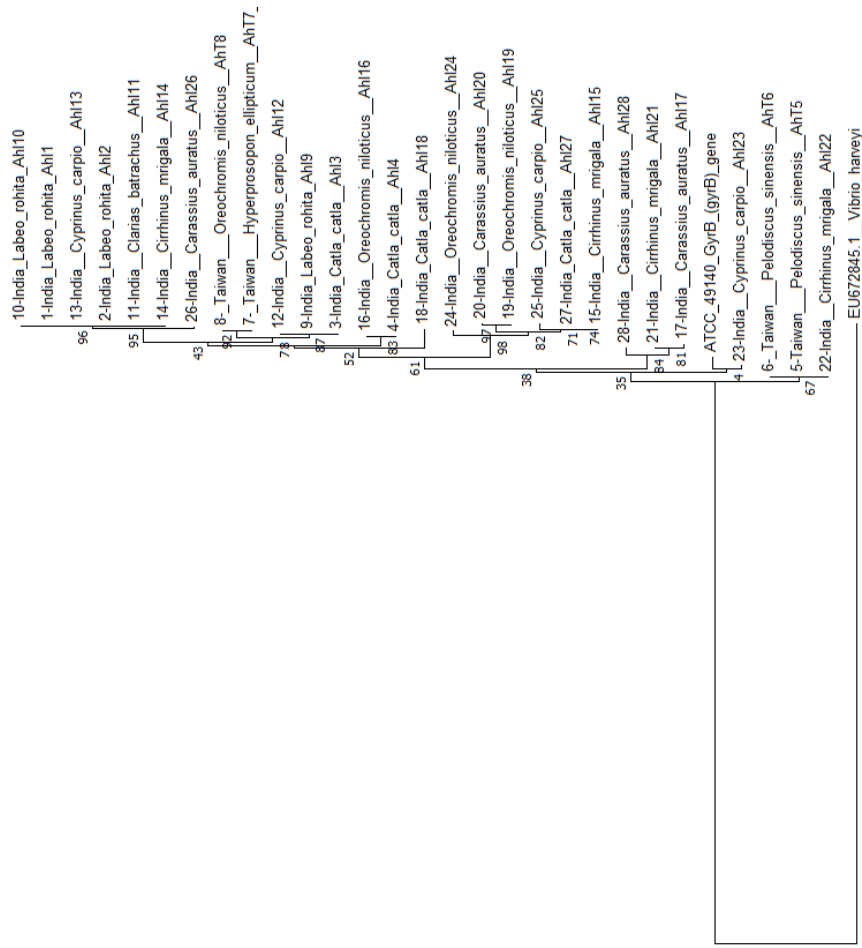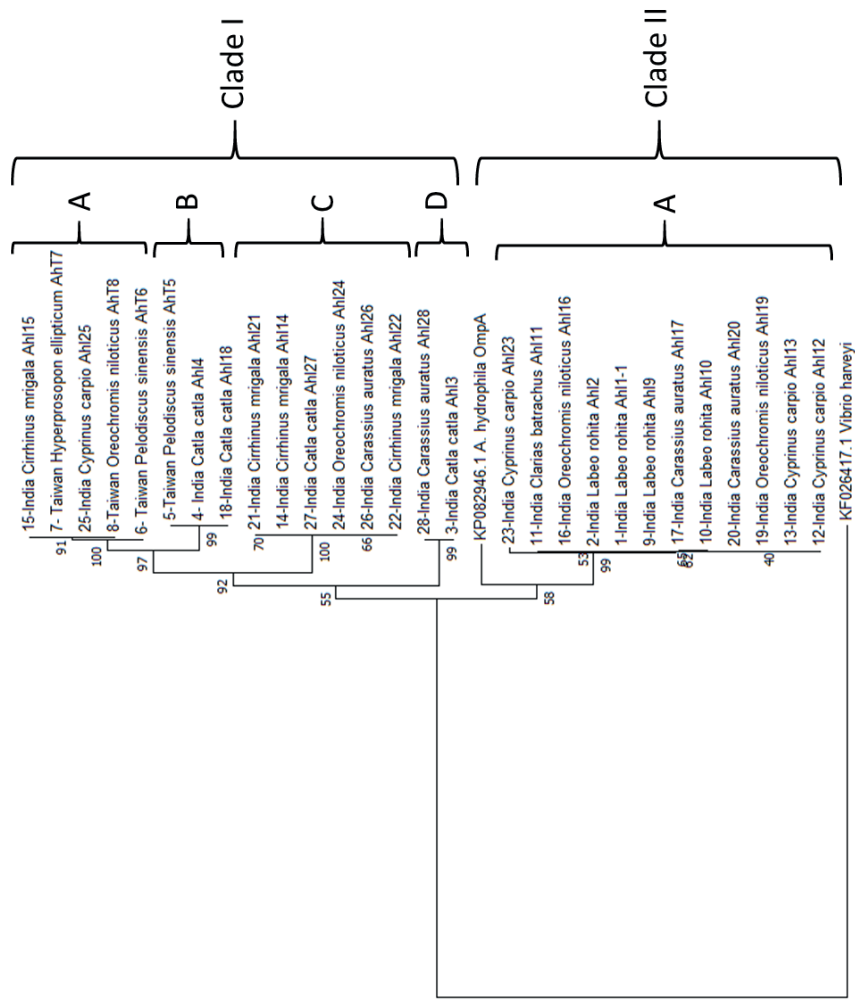
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29

Main clade

Subclade

Part of
main clade

19-India Oreochromis niloticus Ahl19
16-India Oreochromis niloticus Ahl16
11-India Clarias batrachus Ahl11
MG984625.1 ATCC 7966
7-Taiwan Hyperprosopon ellipticum AhT7
8- Taiwan Oreochromis niloticus AhT8
24-India Oreochromis niloticus Ahl24
21-India Cirrhinus mrigala Ahl21
26-India Carassius auratus Ahl26
6-Taiwan Pelodiscus sinensis AhT6
5-Taiwan Pelodiscus sinensis AhT5
25-India Cyprinus carpio Ahl25
1-India Labeo rohita Ahl1
10-India Labeo rohita Ahl10
28-India Carassius auratus Ahl28
17-India Carassius auratus Ahl17
20-India Carassius auratus Ahl20
13-India Cyprinus carpio Ahl13
12-India Cyprinus carpio Ahl12
2-India Labeo rohita Ahl2
4-India Catla catla Ahl4
9-India Labeo rohita Ahl9
27-India Catla catla Ahl27
3-India Catla catla Ahl3
18-India Catla catla Ahl18
15-India Cirrhinus mrigala Ahl15
23-India Cyprinus carpio Ahl23
14-India Cirrhinus mrigala Ahl14
22-India Cirrhinus mrigala Ahl22
JQ920475.1 Vibrio harveyi

34
66
99
16
29
97
90
49

0.0100

Figure 2A. Ah 16S rRNA phylogenetic tree

592
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Standard deviation

Divergence

Legend 1–15:

#	Label
1	17-India_Carassius_auratus_Ah17
2	20-India_Carassius_auratus_Ah20
3	28-India_Carassius_auratus_Ah28
4	13-India_Cyprinus_carpio_Ah13
5	6-Taiwan_Pelodiscus_sinensis_AhT6
6	5-Taiwan_Pelodiscus_sinensis_AhT5
7	25-India_Cyprinus_carpio_Ah25
8	26-India_Carassius_auratus_Ah26
9	12-India_Cyprinus_carpio_Ah12
10	2-India_Labeo_rohita_Ah2
11	1-India_Labeo_rohita_Ah1
12	3-India_Catla_catla_Ah3
13	18-India_Catla_catla_Ah18
14	15-India_Cirrhinus_mrigala_Ah15
15	23-India_Cyprinus_carpio_Ah23

Legend 16–30:

#	Label
16	24-India_Oreochromis_niloticus_Ah24
17	MG984625.1_ATCC_7966
18	JCQ920475.1_Vibrio_harveyi
19	11-India_Clarias_batrachus_Ah11
20	19-India_Oreochromis_niloticus_Ah19
21	16-India_Oreochromis_niloticus_Ah16
22	21-India_Cirrhinus_mrigala_Ah21
23	7-Taiwan_Hyperprosopon_ellipticum_AhT7
24	8-Taiwan_Oreochromis_niloticus_AhT8
25	14-India_Cirrhinus_mrigala_Ah14
26	22-India_Cirrhinus_mrigala_Ah22
27	4-India_Catla_catla_Ah4
28	9-India_Labeo_rohita_Ah9
29	27-India_Catla_catla_Ah27
30	10-India_Labeo_rohita_Ah10

Figure 2B. Ah 16S rRNA distance matrix

Figure 3A. *gyrB* gene phylogenetic analysis

0.1

32

597

598

Divergence

599

#	Species
1	6-Taiwan__Pelodiscus_sinensis__AhT6
2	5-Taiwan__Pelodiscus_sinensis__AhT5
3	ATCC_49140_GyrB_(gyrB)_gene
4	22-India_Cirrhinus_mrigala_Ahl22
5	8-Taiwan__Oreochromis_niloticus__AhT8
6	7-Taiwan__Hyperprosopon_ellipticum__AhT7
7	16-India_Oreochromis_niloticus__Ahl16
8	4-India_Catla_catla_Ahl4
9	21-India_Cirrhinus_mrigala_Ahl21
10	17-India_Carassius_auratus_Ahl17
11	28-India_Carassius_auratus_Ahl28
12	12-India_Cyprinus_carpio_Ahl12
13	9-India_Labeo_rohita_Ahl9
14	3-India_Catla_catla_Ahl3
15	14-India_Cirrhinus_mrigala_Ahl14
16	11-India_Clarias_batrachus__Ahl11
17	2-India_Labeo_rohita_Ahl2
18	13-India_Cyprinus_carpio_Ahl13
19	10-India_Labeo_rohita_Ahl10
20	1-India_Labeo_rohita_Ahl1
21	26-India_Carassius_auratus_Ahl26
22	27-India_Catla_catla_Ahl27
23	15-India_Cirrhinus_mrigala_Ahl15
24	20-India_Carassius_auratus_Ahl20
25	19-India_Oreochromis_niloticus_Ahl19
26	24-India_Oreochromis_niloticus_Ahl24
27	25-India_Cyprinus_carpio_Ahl25
28	18-India_Catla_catla_Ahl18
29	23-India_Cyprinus_carpio_Ahl23
30	EU672845.1_Vibrio_harveyi

600

601

Figure 3B. *gyrB* distance matrix

33

Figure 4A. Ah *ompA* phylogenetic tree.

Clade I

A
- 15-India Cirrhinus mrigala Ahl15
- 7- Taiwan Hyperprosopon ellipticum AhT7
- 25-India Cyprinus carpio Ahl25
- 8-Taiwan Oreochromis niloticus AhT8
- 6- Taiwan Pelodiscus sinensis AhT6
- 5-Taiwan Pelodiscus sinensis AhT5

B
- 4- India Catla catla Ahl4
- 18-India Catla catla Ahl18

C
- 21-India Cirrhinus mrigala Ahl21
- 14-India Cirrhinus mrigala Ahl14
- 27-India Catla catla Ahl27
- 24-India Oreochromis niloticus Ahl24
- 26-India Carassius auratus Ahl26
- 22-India Cirrhinus mrigala Ahl22
- 28-India Carassius auratus Ahl28

D
- 3-India Catla catla Ahl3

- KP082946.1 A. hydrophila OmpA

Clade II

A
- 23-India Cyprinus carpio Ahl23
- 11-India Clarias batrachus Ahl11
- 16-India Oreochromis niloticus Ahl16
- 2-India Labeo rohita Ahl2
- 1-India Labeo rohita Ahl1-1
- 9-India Labeo rohita Ahl9
- 17-India Carassius auratus Ahl17
- 10-India Labeo rohita Ahl10
- 20-India Carassius auratus Ahl20
- 19-India Oreochromis niloticus Ahl19
- 13-India Cyprinus carpio Ahl13
- 12-India Cyprinus carpio Ahl12

- KF026417.1 Vibrio harveyi

91
100
97
99
70
100
66
92
55
99
58
53
99
65
82
40

0.10

34

602

603

Divergence

604

1 | 8-Taiwan_Oreochromis_niloticus_AhT8
2 | 25-India_Cyprinus_carpio_Ahl25
3 | 15-India_Cirhinus_mrigala_Ahl15
4 | 7-_Taiwan_Hyperprosopon_ellipticum_AhT7
5 | 5-Taiwan_Pelodiscus_sinensis_AhT5
6 | 4-_India_Catla_catla_Ahl4
7 | 18-India_Catla_catla_Ahl18
8 | 6-_Taiwan_Pelodiscus_sinensis_AhT6
9 | 27-India_Catla_catla_Ahl27
10 | 24-India_Oreochromis_niloticus_Ahl24
11 | 26-India_Carassius_auratus_Ahl26
12 | 22-India_Cirhinus_mrigala_Ahl22
13 | 21-India_Cirhinus_mrigala_Ahl21
14 | 14-India_Cirhinus_mrigala_Ahl14
15 | 20-India_Carassius_auratus_Ahl20

16 | 19-India_Oreochromis_niloticus_Ahl19
17 | 13-India_Cyprinus_carpio_Ahl13
18 | 12-India_Cyprinus_carpio_Ahl12
19 | 17-India_Carassius_auratus_Ahl17
20 | 10-India_Labeo_rohita_Ahl10
21 | 1-India_Labeo_rohita_Ahl1-1
22 | 2-India_Labeo_rohita_Ahl2
23 | 11-India_Clarias_batrachus_Ahl11
24 | 16-India_Oreochromis_niloticus_Ahl16
25 | 9-India_Labeo_rohita_Ahl9
26 | 23-India_Cyprinus_carpio_Ahl23
27 | KP082946.1_A._hydrophila_OmpA
28 | 28-India_Carassius_auratus_Ahl28
29 | 3-India_Catla_catla_Ahl3
30 | KF026417.1_Vibrio_harveyi

605

606 **Figure 4B. Ah *ompA* distance matrix**

35

Paper-III

Edwardsiella tarda OmpA Encapsulated in Chitosan Nanoparticles Shows Superior Protection over Inactivated Whole Cell Vaccine in Orally Vaccinated Fringed-Lipped Peninsula Carp (*Labeo fimbriatus*)

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Abstract: The use of oral vaccination in finfish has lagged behind injectable vaccines for a long time as oral vaccines fall short of injection vaccines in conferring protective immunity. Biodegradable polymeric nanoparticles (NPs) have shown potential to serve as antigen delivery systems for oral vaccines. In this study the recombinant outer membrane protein A (rOmpA) of *Edwardsiella tarda* was encapsulated in chitosan NPs (NP-rOmpA) and used for oral vaccination of *Labeo fimbriatus*. The rOmpA purity was 85%, nanodiameter <500 nm, encapsulation efficiency 60.6%, zeta potential +19.05 mV, and there was an in vitro release of 49% of encapsulated antigen within 48 h post incubation in phosphate-buffered saline. Empty NPs and a non-formulated, inactivated whole cell *E. tarda* (IWC-ET) vaccine were used as controls. Post-vaccination antibody levels were significantly ($p = 0.0458$) higher in the NP-rOmpA vaccinated fish (Mean OD_{450} = 2.430) than in fish vaccinated with inactivated whole cell *E. tarda* (IWC-ET) vaccine (Mean OD_{450} = 1.735), which corresponded with post-challenge survival proportions (PCSP) of 73.3% and 48.28% for the NP-rOmpA and IWC-ET groups, respectively. Serum samples from NP-rOmpA-vaccinated fish had a higher inhibition rate for *E. tarda* growth on tryptic soy agar (TSA) than the IWC-ET group. There was no significant difference ($p = 0.989$) in PCSPs between fish vaccinated with empty NPs and the unvaccinated control fish, while serum from both groups showed no detectable antibodies against *E. tarda*. Overall, these data show that the NP-rOmpA vaccine produced higher antibody levels and had superior protection over the IWC-ET vaccine, showing that encapsulating OmpA in chitosan NPs confer improved protection against *E. tarda* mortality in *L. fimbriatus*. There is a need to elucidate the possible adjuvant effects of chitosan NPs and the immunological mechanisms of protective immunity induced by OMPs administered orally to fish.

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1. Introduction

Edwardsiella tarda is a member of the *Enterobacteriaceae* family that infects different fish species and mammals. In Channel catfish (*Ictalurus punctatus*), eels (*Anguilla anguilla*), and Japanese flounder (*Paralichthys olivaceus*), it causes emphysematous putrefaction, gangrene, and red disease [1]. It has been isolated from Red sea bream (*Pagellus bogaraveo*), Yellowtail (*Seriola lalandi*), Turbot (*Scophthalmus maximus*), Tilapia (*Oreochromis niloticus*), Mullet (*Mullus barbatus*), eels, Channel catfish, and Japanese flounder [1,2]. It is a zoonotic agent causing gastrointestinal and extra-intestinal infection in humans [3,4]. There are 61 different serovars of *E. tarda* identified based on somatic (O) and flagellar (H) antigens, infecting a wide range of hosts from different parts of the world [5]. In fish, there are no commercial vaccines available and, hence, there is a need for a conserved universal antigen for use in vaccine design. Bacterial outer membrane proteins (OMPs) are highly immunogenic and recognized as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on host cells. They are conserved among different serovars [6,7] and have attracted a lot of interest in vaccine design. They serve as antigenic sites because of their exposed epitopes on the outer surfaces of bacterial cell membranes [8–11] and are suitable molecules for genetic engineering since they are made of simple structures that can be produced in inclusion bodies and easily recovered in the exact native conformation (12).

Although injectable inactivated bacterial vaccines bring about a significant decrease in disease outbreaks in aquaculture [12], the use of oral vaccines, which would be more practical, has been hampered by a general lack of efficacy [13]. Adjuvants have the advantage of enhancing the immunogenicity of non-replicative antigens; by reducing the quantity of antigens required per dose and forming depots at injection sites, they reduce the number of boosters required to induce long-term protective immunity [12,14]. Moreover, current advances in fish immunology show that the fish gut is endowed with antigen-presenting cells (APCs) and processing mechanisms comparable to those seen in lymphoid organisms [15–17]. However, the challenge in the design of oral vaccines for finfish has been developing formulations that protect the antigens from the harsh environment of the stomach and/or the foregut, thereby facilitating antigen uptake in the hindgut. An alternative that has attracted a lot of interest in recent years is the use of biodegradable polymeric nanoparticles that permit a sustained or pulsed release of encapsulated antigens. Among the polymers used in vaccine delivery are Poly(D,L-lactic-co-glycolic) acid (PLGA) [18,19] and chitosan [20,21]. Chitosan is a natural biodegradable polysaccharide obtained from crustacean shells and has been used for targeted drug [22] and DNA vaccine delivery [23–25]. In the present study an oral vaccine based on the recombinant OmpA (rOmpA) antigen was encapsulated in chitosan nanoparticles and tested for protective ability against *E. tarda* infection in *L. fimbriatus*. Hence, we wanted to determine whether oral vaccination using the rOmpA antigen encapsulated in chitosan nanoparticles would afford higher protection than the levels obtained in our previous studies, in which the rOmpA protein was intraperitoneally injected in fish without nanoparticle encapsulation. We used *L. fimbriatus* not only because it is a food fish but also because of its importance as an endangered species on the International Union of Conservation for Nature (IUCN) red list of threatened fish species [26]. The wild population of *L. fimbriatus* has significantly declined, becoming nearly extinct in areas of its original distribution due to overharvesting and river pollution. In order to prevent its further decline, current efforts are aimed at rearing *L. fimbriatus* in aquaculture, but these are hampered by disease outbreaks due to infectious agents such as *E. tarda*. Hence, there is a need to develop highly protective vaccines with the capacity to induce long-term protection in vaccinated fish in order to increase the population of this species for food and nutritional security.

2. Materials and Methods

2.1. Expression and Purification of OmpA of E. tarda

A recombinant OmpA (rOmpA) cloned in our lab at the UNESCO MIRCEN for Marine Biotechnology, Mangalore was expressed in *Escherichia coli* M15 cells [11]. The *E. tarda* isolate (Strain PCF01, GeneBank Acc. No. FJ751236.2) used for amplification of the rOmpA gene in the present study was obtained from catfish (*Pangasius hypohthalmus*) from east coast of India [11]. Briefly, characterization of the rOmpA was carried out by initially extracting the genomic DNA from the bacteria. Extraction of the OMP was carried out as previously described by Filip et al. [27] and the steps of amplification, cloning and expression followed by characterization has been detailed in our previous study [11]. Briefly, PCR amplification of the extracted OmpA was carried out using three different primers based on the OmpA of *E. tarda* strain CK41 (GenBank Acc. EF528483). The PCR reaction was done in a thermal cycler (Applied Biosystems, Carlsbad, CA, USA) using a master mix consisting of 5 μL of $10\times$ buffer (100 mM Tris-HCl pH 8.3, 20 mM MgCl$_2$, 500 mM KCl, 0.1% gelatin), 50 μM deoxynucleotide triphosphates (dNTPs), 2 U Taq polymerase, and 20 pmol of each primer. PCR conditions included an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 60 °C , extension for 1 min at 72 °C , and a final delay at 72 °C for 5 min. PCR products were analyzed by gel electrophoresis using ethidium bromide and amplicons were purified for sequencing using the Qiagen kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Sequence alignments for the amplicons purified in this study with strain CK41 used in the primer design were used to determine the sequence similarity of the gene product generated in this study.

Cloning of the OmpA gene was carried out by excluding the region coding for the signal peptide using PCR conditions described above, except for the annealing temperature, which was set at 51 °C. The purified products were ligated into the 30-UA commercial vector (Qiagen) set at 16 °C for 2 h. Thereafter, the plasmids were transformed into the competent M15 *E. coli* cells followed by heat shock and were later grown in LB broth containing kanamycin (100 μg/mL) and ampicillin (25 μg/mL). The bacteria cultures were induced by 1 mM isopropyl thiogalactoside (IPTG) and were grown until the turbidity reached 0.5–0.7 OD$_{600}$. Analysis of recombinant expression of the OmpA protein was carried out using 12% SDS-PAGE as previously described [28]. For purification, IPTG induced recombinant cells were disrupted using the lysis buffer (6 M GuHCl; 0.1 M NaH$_2$PO$_4$; 0.01 M Tris-Cl; pH 8.0) while the cell debris was separated by centrifugation at $10,000\times g$ for 30 min. The supernatant was mixed with 50% Ni–NTA slurry at a ratio of 4:1and added to columns. Purification was achieved by washing using a wash buffer (8 M urea; 0.1 M NaH$_2$PO$_4$; 0.01 Tris-Cl; pH 6.3 and 5.9) followed by eluting the purified protein using an elution buffer (8 M urea; 0.1 M NaH$_2$PO$_4$; 0.01 M Tris-Cl; pH 4.5). The purity of the protein was analyzed using 12% SDS-PAGE while the concentration was determined using the method previously described by Lowry et al. [28]. The immunogenicity of the cloned rOmpA was tested using a polyclonal antibody produced from immunized rabbits in our previous study [11].

2.2. Preparation of Chitosan Nanoparticles

Chitosan nanoparticles were prepared by an ionic gelation process, as described by Gan et al. [23], with minor modifications. Briefly, purified low molecular weight (75%–85% deacetylated) chitosan (Sigma-Aldrich®, St. Louis, MO, USA), was put in 1% (w/v) acetic acid to obtain a 0.15% (w/v) chitosan concentration. Thereafter, 10 mg rOmpA was put in the chitosan solution, adjusted to pH 5.5, and maintained at 20 ± 2 °C up to the time of making the chitosan nanoparticles. The chitosan-rOmpA solution was flush mixed with sodium TPP (sodium tripolyphosphate, Sigma-Aldrich®). The nanoparticles were formed spontaneously via the TPP initiated ionic crosslink and coacervation mechanism at a chitosan:TPP weight ratio of 3:1. Nanoparticles (NPs) encapsulated with rOmpA were separated by centrifuging at 14,000 rpm for 30 min at 14 °C, freeze-dried, and stored at 4 °C [23].

2.3. Characterization and Encapsulation Efficiency of Chitosan Nanoparticles

Chitosan particle size was determined using the Malvern ZetaSizer (NanoZS, Malvern Instruments, Worcester, UK) particle estimator while the binding capacity of rOmpA was determined by completely dissolving the nanoparticle in 0.1 M NaOH containing 0.5% w/v SDS. After lyophilization, the size and zeta potential of chitosan NPs was measured using a zeta analyzer. One milliliter of the supernatant obtained during chitosan preparation was kept to check the loading efficiency. Released antigen was quantified using the method of Lowry et al. [28]. The protein encapsulation efficiency was determined using the following equation: Encapsulation Efficiency = [(Total amount of Protein − free amount Protein in supernatant)/total amount Protein] × 100.

2.4. In Vitro Release Test

The in vitro release test was performed to determine the timing of antigen release from the chitosan nanoparticles according to the method of Hori et al. [29] with minor modifications. Briefly, 10 mg of chitosan nanoparticles encapsulating rOmpA were suspended in 500 μL phosphate buffer saline (PBS). The vial was shaken horizontally in a water bath at 37 °C for up to 48 h. In vitro protein release was determined by drawing 500 μL supernatant, which was replaced with an equal volume of PBS, after the centrifugation at $10,000 \times$ g for 10 min. Samples were collected after 1, 2, 8, 16, 24, and 48 h. Released antigen was quantified using the method of Lowry et al. [28].

2.5. Vaccine Preparation for Oral Immunization

The chitosan NPs oral vaccine was prepared by mixing the rOmpA antigen with commercial feed for carp by powdering the feed using a grinder followed by sieving. The vaccine–feed mixture was thoroughly mixed and made into a dough followed by pressing through 2 mm diameter hand extruder. Thereafter, the pellets were dried at room temperature (29 ± 1 °C) for 24 h and stored at 4 °C till use. The inactivated whole cell (IWC) *E. tarda* vaccine was made according to Caipang et al. [30], with minor modifications. An overnight broth culture of *E. tarda* was adjusted to a concentration of 10^6 CFU/mL in PBS, inactivated for 1 h at 60 °C, and kept at 4 °C until use. The efficiency of inactivation was determined by plating 100 μL of the above bacterial suspension onto tryptic soya agar (TSA, Hi-Media, Mumbai, India) and the sterility monitored for three days. The oral vaccine was prepared at a proportion of 600 μL of IWC–*E. tarda* antigen thoroughly mixed with 120 g commercial feed followed by making a dough and pressing it through a 2 mm diameter hand extruder in the same pattern as preparation of the nanoparticle vaccines.

2.6. Vaccination and Challenge Study

Healthy *L. fimbriatus* weighing approximately 12 g on average were obtained from the Bhadra Reservoir Carp Centre, Karnataka, India and transported to the wet lab of the department in oxygenated bags. The health status of the fish used in this study was determined by collecting a representative random sample of 12 fish that were used for bacteriology, pathology, and histopathology examination. Bacterial pathogens examined were *Aeromonas hydrophila* and *E. tarda*, shown to infect different fish species in India [16,17,31] and the organs examined included the head kidney, spleen, pancreas, liver, gill, heart, and muscle. No bacterial infections, pathology, or histopathological changes were detected in any organs examined from the sampled fish. Fish used for the study were maintained in recirculating water at 28 °C with uniform aeration at a water flow rate of 3.0 L·min⁻¹ during acclimatization for a period of one month before performing the immunization experiments. They were fed ad libitum and anesthetized using tricaine methanesulfonate (80 μg/mL) before handling. The 160 fish included in this trial were equally distributed (Figure 1), with 40 in each of the four tanks. Fish in Tank 1 were vaccinated using the NP-rOmpA vaccine while those in Tank 2 were vaccinated with the IWC-ET vaccine. Fish in Tank 3 were vaccinated using empty NPs, designated as NP-Empty without rOmpA antigen, while those in Tank 4 served as an unvaccinated

control group. All vaccines were orally administered in feed at 6 µg/g of fish body weight for 21 days. Blood samples were collected from 10 fish from each group at 51 days post-vaccination (dpv), followed by challenge experiment with a pathogenic strain of *E. tarda* (PCF01, 2.4×10^8 CFU/mL) [11] at 51 dpv by intramuscular injection. Mortality was recorded daily until fish stopped dying. Protection was estimated using the Kaplan Meyer's survival analysis.
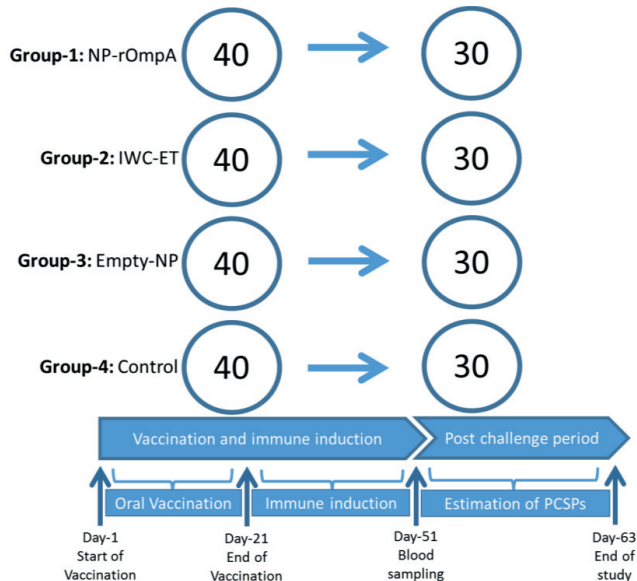
Figure 1. The study design for the oral vaccination trial of carp against *E. tarda* using different vaccines. Four vaccine groups were each allocated 40 fish. Group 1 was allocated the chitosan NP rOmpA vaccine, designated NP-rOmpA. Group 2 was vaccinated with an inactivated whole cell (IWC) *E. tarda* vaccine designated, IWC-ET. Group 3 was vaccinated with empty nanoparticles, without the rOmpA antigen, designated NP-Empty; Group 4 was left unvaccinated and served as the control group. The study timeline for the vaccination trial was segmented into (i) the oral vaccination period of 21 days; (ii) an immune induction period of 51 days post vaccination (dpv); and (iii) the post-challenge period. Blood samples were collected from 10 fish in each group and challenged at 51 dpv. The vaccination trial ended at 63 dpv when fish stopped dying after challenge.

2.7. Antibody Responses Detected by Enzyme Linked Immunosorbent Assay

Immune response to rOmpA vaccination was determined by measuring the serum antibody titer by ELISA. Briefly, ELISA plates (Greiner Bio-One, Frickenhausen, Germany) were coated with rOmpA (2 µg/well) diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Thereafter, the plates were washed in PBS and blocked with 350 µL of 3% BSA at 37 °C for 2 h. After washing, 100 µL fish sera (1:200 dilution) was added to each well and incubated at 37 °C for 2 h. Rabbit anti-rohu HRPO (horseradish peroxidase, DAKO, Sweden) conjugate was added to each well after washing and the plates were incubated at 37 °C for 35 min. The final reaction was obtained by adding tetramethylbenzidine (TMB) hydrogen peroxide substrate to each well and the plates were read at 450 nm absorbance using an EL_{X800} Universal microplate reader (BioTek, Winooski, VT, USA). Serum used in this study was from 10 fish sampled per group that were individually tested in duplicate and the mean OD-value determined was used to compare antibody responses between the vaccinated and control groups.

2.8. Serum Inhibitory Assay

The serum-mediated antibacterial activity was measured as described by Hamod et al. [32] with minor modifications. Briefly, 10 μL of overnight grown broth culture of *E. tarda* was adjusted to a density of 10^3 CFU/mL in PBS, to which 90 μL serum was added in a microtube. The serum plus bacteria suspension was mixed thoroughly, followed by incubation for 24 h at 30 °C. Thereafter, a 10-fold serial dilution of the serum bacteria mix was prepared from each sample and 100 μL of each was spread plated on TSA plates followed by incubation for 24 h at 30 °C. Bacterial colonies were counted and the results expressed as $\log_{10}$ CFU/mL. Reduction in bacterial growth was determined by subtracting counts of vaccinated fish from the control group. Serum samples used in this study were from fish from each group sampled at 51 dpv.

2.9. Statistical Analysis

All statistical analyses and plotting of graphs were carried out GraphPad version 6. Post-challenge survival analysis were carried out using the Kaplan Meyer's survival analysis. Differences were considered significant different at *p*-value = 0.05% and confidence limits of 95%.

3. Results

3.1. Expression, Purification and Concentration of Recombinant OmpA

The rOmpA protein was expressed after 4 h induction with 1 mM IPTG. The molecular weight (MW) was estimated to be 38 kDa (Figure 2) by 12% SDS-PAGE with 85% purity and at 1.0 mg/mL concentration.
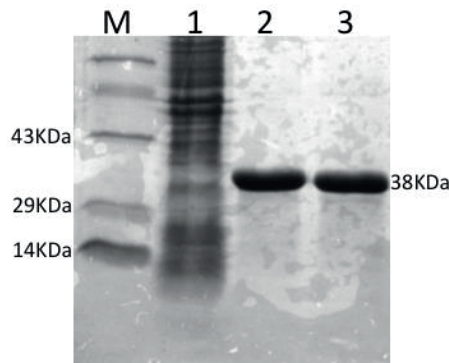
Figure 2. Expression of rOmpA protein. Lane M: Molecular protein marker; Lanes 1: Un-induced recombinant *E. coli* M-15 cell; Lane 2 and 3: Purified rOmpA.

3.2. Physiochemical Properties of Chitosan Nanoparticles Encapsulating rOmpA

The encapsulation efficiency of rOmpA antigen by chitosan NPs was estimated to be 60.06%, with the particles having an average diameter of 468.9 nm (Figure 3). Figure 4 shows the size and surface morphology of the freeze-dried chitosan nanoparticles, as determined by transmission electron microscope (Morgagni TEM, FEI, Eindhoven, The Netherlands). The charge of rOmpA encapsulated chitosan NPs was +19.05 mV.
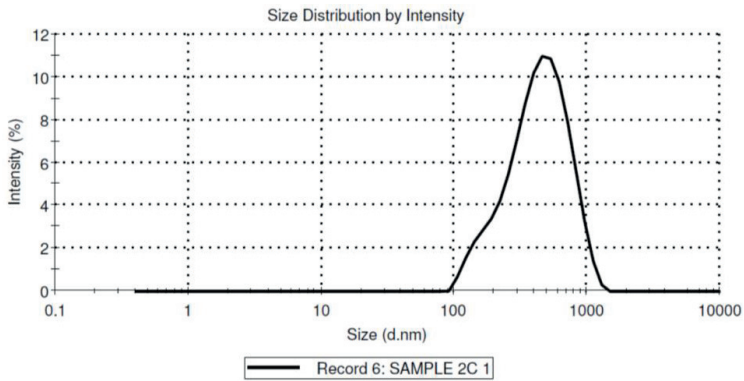
Figure 3. Chitosan nanoparticle size distribution intensity determined using Malvern ZetaSizer (NanoZS; Malvern Instruments, UK; www.malvern.com) at temperature of 25 °C, count rate (kcps) of 407, duration of 60 s and measurement position of 5.50 mm.
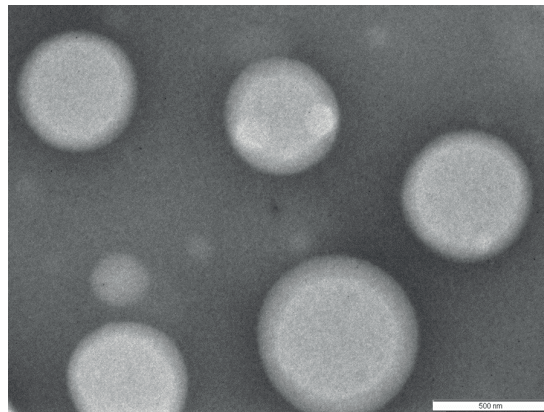
Figure 4. Size and surface morphology of freeze-dried chitosan nanoparticles, as determined by transmission electron microscope (Morgagni TEM, FEI, Eindhoven, The Netherlands).

3.3. In Vitro Release Assay

Figure 5 shows the quantification of rOmpA antigens released from encapsulated chitosan nanoparticles. The recombinant OmpA released from the NPs increased exponentially from less than 10% in 1 h to 49% by 48 h after the start of the in vitro release assay.
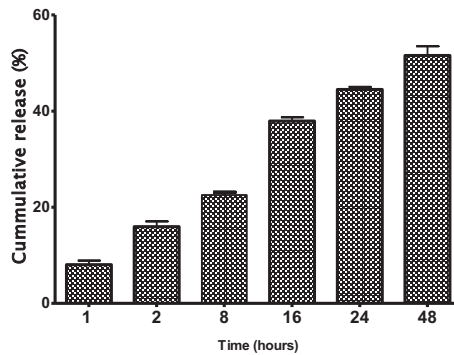
Figure 5. In vitro release of rOmpA protein from encapsulated NPs. Note the exponential increase in rOmpA release within 24 h of the start of the in vitro test.

3.4. Antibody Responses

Circulating antibody levels at 51 dpv in the different vaccine groups are shown in Figure 4. The highest levels were recorded in fish vaccinated with NP-rOmpA vaccine (Mean OD_{450} = 2.430), followed by the IWC-ET (Mean OD_{450} = 1.736). There was a significant difference (p = 0.0458) between antibody levels detected in the NP-rOmpA and IWC-ET group. Figure 6 shows that there was no antibody response detected in fish vaccinated using the NP-Empty vaccine and the unvaccinated control group.
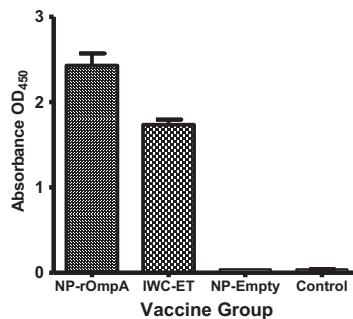
Figure 6. Antibody levels detected against *E. tarda* from fish vaccinated with the NP-rOmpA, IWC-ET, NP-Empty, and control groups at 51 dpv. Antibody levels from fish vaccinated with the NP-rOmpA were higher than antibody levels from those vaccinated with the IWC-ET vaccine. A significant difference (p < 0.0003) in antibody levels was detected between the NP-rOmpA and IWC-ET vaccinated groups. The NP-Empty and control groups did not show detectable antibodies against *E. tarda* at 51 dpv.

3.5. Serum Inhibition Test

E. tarda colony counts obtained on TSA plates after spread plate technique and incubation with a bacteria serum mixture from vaccinated and control fish are shown in Figure 7. Subtracting the colony counts of the vaccinated from control fish shows that the largest inhibition of bacterial growth was from the NP-rOmpA group with a reduction of 10×10^7 CFU/mL, followed by the IWC-ET group, which showed a reduction of 10×10^5 CFU/mL. This shows that fish vaccinated with the NP-rOmpA vaccine had 1.7 times higher inhibitory capacity than the group vaccinated with the IWC-ET vaccine. The group vaccinated with the NP without any antigen added, moderately inhibited *E. tarda* growth compared to the control group but to a lesser extent than the NP-rOmpA and IWC-ET.
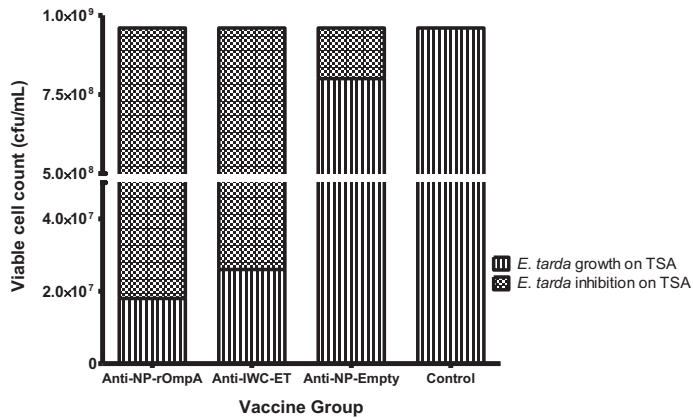
Figure 7. Shows *E. tarda* growth and inhibition on TSA after treatment of sera from vaccinated fish with cultured bacteria. The concentration of the bacteria used was 10^3 CFU/mL, to which 90 μL serially diluted sera were added in microfuge tubes followed by spread plate technique and incubation on TSA for 24 h at 30 °C. Bacterial growth for each group was determined by counting individual colonies on TSA while inhibition was calculated by subtracting the bacterial colony counts of vaccinated fish from the control group. Note that serum samples from the NP-rOmpA, IWC-ET, and NP-Empty vaccines were designated as anti-NP-rOmpA, anti-IWC-ET, and anti-NP-Empty, respectively. The serum used in this study was pooled from blood samples of 10 fish collected at 51 dpv.

3.6. Kaplan Meyer's Survival Analysis

Figure 8 shows the Kaplan Meyer's survival analysis of the vaccinated and control groups. Mortality in the IWC-ET, NP-Empty, and control groups started at 2 dpc while in the NP-rOmpA it started at 3 dpc. The highest protection was in the NP-rOmpA group, with post-challenge survival proportions (PCSP) of 73.33%, followed by the IWC-ET (PCSP = 48.28%), NP-Empty (23.33%), and control (PCSP = 27.59) groups. Comparative analysis of the vaccinated groups showed that there was a significant difference ($p = 0.0396$) between the NP-rOmpA and IWC-ET groups. For the NP-Empty group, there was no difference ($p = 0.989$) compared to the unvaccinated control group.
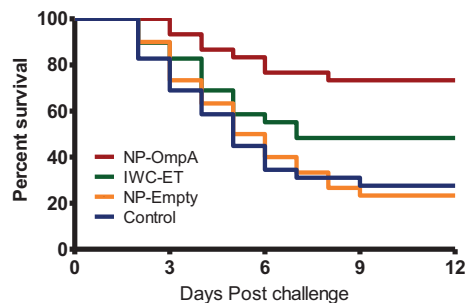
Figure 8. Kaplan Meyer's survival analysis of fish vaccinated against *E. tarda* using the NP-rOmpA, IWC-ET, and NP-Empty vaccine together with control fish. Mortality in the IWC-ET, NP-Empty, and control groups started two days post challenge (dpc), while in the NP-rOmpA group it started 3 dpc. The highest post-challenge survival proportion (PCSPs) was from the NP-rOmpA group (PCSP = 73.33%), followed by the IWC-ET group (PCSP = 48.28%), while the NP-Empty (PCSP = 23.33%) and control groups (PCSP = 27.59%) had the lowest PCSPs. There was a significant difference ($p = 0.0396$) in PCSP between the NP-rOmpA and IWC-ET groups. No significant difference ($p = 0.989$) was detected in PCSPs between the NP-Empty and control groups.

4. Discussion

Vaccination is one of the most effective disease control strategies and is the single most important factor for reduction of disease outbreaks in aquaculture [33]. Development of highly efficacious vaccines, particularly for many bacterial infections, is accredited to the use of adjuvants in vaccine formulations that boost the immunogenicity of non-replicative antigens [13] and prolong protection [34]. Koppolu and Zaharoff [35] showed that NPs have the capacity to efficiently deliver encapsulated antigens to activated macrophages and dendritic cells. Moreover, Zaharoff et al. [36] have shown that chitosan NPs enhance humoral and cellular mediated immune responses to vaccination in the absence of adjuvants. In the present study, the efficacy of chitosan NPs as an antigen delivery system for rOmpA and their ability to evoke protective antibody response in carp was evaluated. In our previous study [19], we showed that the infectious pancreatic necrosis virus (IPNV) variable protein 2 (VP2) that had a molecular weight (MW) of 36 KDa was encapsulated in 332 nm PLGA NPs at an encapsulation efficiency of 83%, suggesting that the 468.9 nm diameter chitosan NPs generated in this study were large enough to encapsulate the 38KDa rOmpA protein at a high encapsulation efficiency. However, the 60% encapsulation efficiency obtained in this study is in the range that has been shown to efficiently deliver encapsulated antigens in vivo by chitosan NPs [37,38]. However, at encapsulation efficiency >70% this has been shown to increase drug delivery in vivo [37], implying that the increased encapsulation (of rOmpA) might contribute to even higher protection in vaccinated fish. Given that nanodiameters <500 nm have been shown to be successfully endocytosed by antigen-presenting cells (APCs) [39–41], it is likely that the NPs produced in this study had the potential to be endocytosed by APCs in vaccinated fish. Furthermore, the anionic zeta potential of +19.04 mV would enhance uptake by APCs. In the referred study [42], it was surmised that phagocytic cells preferentially take up anionic NPs while cationic NPs are mostly ingested by nonphagocytic cells. It is not known if the same principles apply for fish but delivering NP-rOmpA orally to rohu elicited a systemic immune response (humoral antibodies). To what extent this involves both antigen uptake (over epithelium) and delivery to antigen-presenting cells remains to understood.

The OmpA protein is among the immunogenic proteins expressed on the outer surface of bacterial membranes [43]. Its potential as a vaccine candidate includes its cross-reactivity, surface exposure of antigenic epitopes, and conservation among different strains [43–45]. It stimulates macrophages and upregulates MHC-II, CD80, and CD86, expression resulting in activation of CD4+ T-cells and an adaptive immune response [45]. It has been shown to induce IgG and IgA both in systemic and mucosal components in higher vertebrates [46] and hence is likely to induce mucosal and systemic immune responses in fish vaccinated by the oral route, using mechanisms similar to those seen in higher vertebrates. In fish, it has been shown to induce protective immunity against *Vibrio harveyi* in Senegalese sole (*Solea senegalensis*, Kaup) [47], *E. tarda* in Japanese flounder (*Paralichthys olivaceus*) [48], *Vibrio anguillarum* in Asian sea bass (*Lates calcarifer*) [49], and *A. hydrophila* in carp [11]. As pointed out by Meenakshi et al. [50], OMPs are only protective in the presence of adjuvants and, hence, it is likely that the higher protection induced by the NP-rOmpA vaccine was due to the adjuvant effect of the chitosan NPs used to deliver the rOmpA antigens in this study [51]. On the contrary, the lower protection levels observed in the IWC-ET group could be due to the lack of an adjuvant that would be able to boost the immunogenicity of IWC antigens and the fact that there was possibly no depot formation for the slow release of the IWC-ET antigen. These factors could account for the low antibody levels detected in the IWC-ET group, resulting in low PCSP in vaccinated fish. On the contrary, the NP-rOmpA group that had an inherent adjuvant effect and a pulsed slow antigen release had high antibody levels that corresponded with high PCSPs in vaccinated fish. Taken together, these findings show that oral vaccination using the OmpA antigens encapsulated in chitosan NP induces higher protection than IWC-ET vaccines without adjuvants in vaccinated fish.

Although different NP vaccines have been used in aquaculture [18,19,52–54], few studies have evaluated their ability to release the antigens in vitro [55,56]. It is important that the ability of the NPs to release antigens in vitro be evaluated as this would give insight into their ability to induce

a protective immune response in vaccinated fish. In this study, at least 49% of the rOmpA antigen was released within 48 h after onset of the in vitro release test. This observation is in line with that of Ranjan and Nayak [56] who showed an in vitro release of 52%–55.6% of *A. hydrophila* OMPs within 48 h of incubation in PBS. To evaluate the in vivo release of rOmpA, we used the serum inhibition test to evaluate the ability of antibodies induced by rOmpA released in vaccinated fish to inhibit the growth of *E. tarda* on TSA. The serum inhibition test is an in vitro vaccine efficacy measure used to determine the ability of antibodies generated by vaccination to inhibit bacterial growth in vitro [11,57]. Given that in some cases the bacterial strain used for vaccine production is also used as the challenge strain for measuring the RPS of vaccinated fish, in such cases the serum inhibition test serves as an in vitro measure of vaccine efficacy, used to determine the protective ability of antibodies generated by the vaccine strain challenged using its homologous strain [11,57]. As seen from our findings, the NP-rOmpA group had a higher inhibitory capacity than the IWC-ET vaccinated fish, suggesting that the NP-OmpA produced higher levels of protective antibodies than the IWC-ET vaccine. This was supported by ELISA data that showed high antibody levels against *E. tarda*, which corresponded with higher PCSPs for fish vaccinated with the NP-rOmpA vaccine than the IWC-ET vaccine. The importance of serum inhibition tests to demonstrate the in vitro protective ability of antibodies generated by oral vaccination as a measure of in vivo antigen release and vaccine efficacy was found to be significant in this study. However, in order to improve the efficacy of OMPs delivered by NP vaccines, future studies should seek to increase the encapsulation efficiency, elucidate antigen uptake and bio-distribution, and determine the protective mechanism of OMPs and adjuvant effect of NP vaccines, as has been done for other oral vaccines for fish. Nevertheless, in this study we have shown that NP vaccines could serve as an effective oral immunization strategy in fish and would be a better alternative to inactivated whole cell oral vaccines without adjuvants.

5. Conclusions

This study has shown that *E. tarda* OmpA encapsulated in chitosan nanoparticles is protective when administered orally in Fringed-Lipped Penisula carp. The study also shows that the protection induced by the OmpA encapsulated in the chitosan nanoparticles was superior to inactivated whole cell vaccine without adjuvants. Hence, there is need to investigated the adjuvant effect of chitosan nanoparticles in fish.

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Paper-IV

Article

Aeromonas hydrophila OmpW PLGA Nanoparticle Oral Vaccine Shows a Dose-Dependent Protective Immunity in Rohu (*Labeo rohita*)

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Abstract: *Aeromonas hydrophila* is a Gram-negative bacterium that causes high mortality in different fish species and at different growth stages. Although vaccination has significantly contributed to the decline of disease outbreaks in aquaculture, the use of oral vaccines has lagged behind the injectable vaccines due to lack of proven efficacy, that being from primary immunization or by use of boost protocols. In this study, the outer membrane protein W (OmpW) of *A. hydrophila* was cloned, purified, and encapsulated in poly D,L-lactide-*co*-glycolic acid (PLGA) nanoparticles (NPs) for oral vaccination of rohu (*Labeo rohita* Hamilton). The physical properties of PLGA NPs encapsulating the recombinant OmpW (rOmpW) was characterized as having a diameter of 370–375 nm, encapsulation efficiency of 53% and −19.3 mV zeta potential. *In vitro* release of rOmpW was estimated at 34% within 48 h of incubation in phosphate-buffered saline. To evaluate the efficacy of the NP-rOmpW oral vaccine, two antigen doses were orally administered in rohu with a high antigen (HiAg) dose that had twice the amount of antigens compared to the low antigen (LoAg) dose. Antibody levels obtained after vaccination showed an antigen dose dependency in which fish from the HiAg group had higher antibody levels than those from the LoAg group. The antibody levels corresponded with post challenge survival proportions (PCSPs) and relative percent survival (RPS) in which the HiAg group had a higher PCSP and RPS than the LoAg group. Likewise, the ability to inhibit *A. hydrophila* growth on trypticase soy agar (TSA) by sera obtained from the HiAg group was higher than that from the LoAg group. Overall, data presented here shows that OmpW orally administered using PLGA NPs is protective against *A. hydrophila* infection with the level of protective immunity induced by oral vaccination being antigen dose-dependent. Future studies should seek to optimize the antigen dose and duration of oral immunization in rohu in order to induce the highest protection in vaccinated fish.

Keywords: *Aeromonas hydrophila*; rohu; PLGA; nanoparticle; oral; outer membrane protein

1. Introduction

Aeromonas hydrophila is a Gram-negative bacteria that causes hemorrhagic septicemia, dropsy, and mortality in different fish species at different growth stages [1,2]. Vaccination has proved to be an effective disease preventive strategy with ability to reduce disease outbreaks [3,4]. Although injectable vaccines that offer protective immunity have been developed for *A. hydrophila* [5,6], the development of oral vaccines has lagged behind the injectable vaccines due to lack of efficacy and antigen formulations that maintain antigen integrity and immunogenicity [7]. An immunologic adjuvant is any substance that is able to accelerate, prolong, or enhance antigen-specific immune response when used in combination with specific antigens [8]. Adjuvants enhance immunogenicity, reduce the amount of antigen required per dose and also reduce the number of boosters needed for long-term protective immunity [8–11]. As pointed out by Munang'andu and Evensen [9], adjuvants are designed to serve as antigen delivery vehicles and as immunostimulants that would be able to enhance antigen uptake. The search for oral adjuvants has attracted a lot of interest in biodegradable polymeric nanoparticles (NP) because of their dual ability to serve as antigen delivery vehicles and to permit a sustained release of antigens and a consequent reduction of booster vaccinations [9,12–15]. Among the polymeric systems, poly D,L-lactide-*co*-glycolic acid (PLGA) NPs have been widely used for the controlled delivery of peptides, synthetic proteins, and nucleic acids in humans [16]. Hence, this immunization strategy is being widely explored for the delivery of oral vaccines in finfish [12–15].

Given their large size, encapsulation of whole cell bacteria (0.5–5.0 μm diameter) in PLGA NPs (<500 nm), is not practical. Therefore, the practical approach is to identify immunogenic proteins found on bacterial surfaces that are able to induce protective immunity for use as vaccine candidates. These can then be used for encapsulation in PLGA NPs. Bacterial outer membrane proteins (OMPs) are among the potential candidates shown to evoke protective immune responses in vaccinated fish because of their exposed epitopes on cell surfaces [17]. The β-barrel architecture of OMPs is easily recognized by host pattern recognition receptors (PRRs) as pathogen associated molecular patterns (PAMP) [18]. OMPs have been widely studied as vaccine candidates for most of the *Enterobacteriaceae* spp. where their structural layout has been shown to play an important role in inducing protective immune responses in vaccinated fish. The objective of the present study was to assess the effect of recombinant *A. hydrophila* OmpW encapsulated in PLGA NP in inducing protection against mortality after oral delivery in rohu (*Labeo rohita*) and whether this effect is dose dependent.

2. Materials and Methods

2.1. Expression and Purification of A. hydrophila Recombinant OmpW Protein

A recombinant OmpW (rOmpW) clone generated in our lab at Mangalore, India, was expressed in an *Escherichia coli* M15 clone, as previously described [18]. The *A. hydrophila* isolate used for cloning and expression of the OmpW protein was isolated from rohu expressing clinical signs of epizootic ulcerative syndrome (EUS). Morphological, biochemical, and molecular characterization of the isolate have previously been described by Maiti *et al.* [18]. The sequence for the OmpW retrieved from this isolate has been deposited in the National Center for Biotechnology Information (NCBI) databank (accession no. HM063443.1), while its structural properties have also been described previously [18]. For large-scale production, *E. coli* containing the rOmpW clone was inoculated in 200 mL Luria Bertani (LB) broth and induced with 1 mM isopropyl thiogalactoside (IPTG) and purified using affinity chromatography. Expression and purity analysis of the rOmpW protein was done by 15% SDS-PAGE and the concentration measured as described by Lowry *et al.* [19].

2.2. Encapsulation of rOmpW in PLGA Nanoparticles

Encapsulation of rOmpW in PLGA nanoparticles (PLGA; 50:50 ratio; inherent viscosity: 0.45–0.6 dL/g; molecular weight (MW): 38,000–54,000 Purac Biomaterials, Montville, NJ, USA) was done using the $W_1/O/W_2$ double emulsion solvent evaporation method described previously [20–26]

with some minor modifications. Briefly, 10 mg of the peptide was dissolved in 2 mL (5 mg/mL) milli-Q water (pH 7.4) and emulsified in 20 mL dichloromethane (DCM) containing 100 mg PLGA using a high-speed homogenizer (Polytron, Kinematica AG, Littau-Luzem, Switzerland) at 16,500 rpm for 5 min on ice. Thereafter, 8 mL of 1% *w/v* poly vinyl alcohol solution (PVA; Average MW: 30,000–70,000; 87%–90% hydrolysed; Sigma, St. Louis, MO, USA) was added. Homogenization was carried out for 10 min and the emulsion formed was sonicated at 60 amplitude and 4 s pulse (Vibra Cell, VC 130, Sonics and Materials, Newton, CT, USA) on ice for 30 min. Thereafter, 90 mL 1% *w/v* PVA was added to the double emulsion solution formed after sonication. The double emulsion solution was allowed to evaporate at room temperature by keeping the dispersion overnight with stirring using a mechanical stirrer. The particles were separated by centrifugation at 22,000 rpm (4 °C) for 45 min. Finally, the pellet obtained was dispersed in 5% *w/v* trehalose solution and subjected to lyophilization. Empty PLGA nanoparticles without rOmpW protein were prepared using the same protocol.

2.3. Characterization of PLGA Nanoparticles and In Vitro Release Test

Encapsulation efficiency of rOmpW was determined by dissolving the particles in 0.1 M NaOH containing 0.5% *w/v* SDS followed by adjusting the pH to 7.4 [19]. The size and zeta potential of the PLGA nanoparticles after lyophilization was determined using the particle size analyzer (NanoZS, Malvern instruments, Malvern, Worcestershire, UK). An *in vitro* release test of rOmpW was carried out to determine the timing of antigen release from the PLGA nanoparticles by putting 10 mg PLGA nanoparticles encapsulating rOmpW in 500 mL phosphate buffered saline (PBS) in a water bath shaker at 37 °C. *In vitro* release of rOmpW was evaluated by drawing 500 μL supernatant, which was replaced with an equal volume of PBS after centrifugation at $10,000 \times g$ for 10 min. Samples for *in vitro* release were collected at 1, 2, 8, 16, 24, and 48 h intervals. The released rOmpW protein was quantified using the method of Lowry *et al.* [19].

2.4. Vaccine Preparations for Oral Delivery

To prepare the PLGA NPs rOmpW (NP-rOmpW) vaccine for oral immunization, commercial feed for rohu was ground and sieved. The NP-rOmpW vaccine was thoroughly mixed with feed and made into a dough. This was followed by pelletizing the vaccine-feed mixture by pressing through a hand extruder having a diameter of 2 mm. The pellets were dried at room temperature and stored at 4 °C until use.

2.5. Vaccination and Challenge

Healthy *Labeo rohita* with an average weight of 10 g were brought to the wet lab in oxygenated bags from the college farm, Mangalore, India. Examination of the health status of fish was based on clinical observations by checking for any abnormal appearances and swimming behavior. In addition, six were sacrificed for pathological examination. Both gross pathology and histopathology examination did not show pathological changes. Fish were kept in recirculating water at 28 °C with uniform aeration during acclimatization for a month and fed *ad libitum*. For vaccination, 160 fish were taken and distributed at equal numbers into eight tubs as shown in Figure 1. Group 1 was allocated 40 fish distributed in duplicate tubs, with 20 in each, and was vaccinated with a high-antigen dose of 8 μg/g of fish body weight designated the HiAg group. Likewise, Group 2 was also allocated 40 fish distributed to two tubs and was vaccinated with a low-antigen dose of 4 μg/g of fish body weight designated the LoAg group. Group 3 was fed with empty NPs without the rOmpW antigens, while group 4 was only given feed and left unvaccinated as a control group. The vaccine-coated feed was given to rohu twice daily for 21 days. There was no significant difference observed in feed intake and overall growth in all groups. Blood samples were collected from 10 fish in each group after 30 days post vaccination (dpv). Serum was separated from the blood and stored at −20 °C until use. After 30 dpv, fish were challenged using a pathogenic strain of *A. hydrophila* (Ah40, 2.7×10^7 cfu/mL) [27] by intramuscular injection at 0.1 mL/fish. The *A. hydrophila* isolate used for challenge is similar to the isolate that was used to

produce the NP-rOmpW vaccines [18]. Its pathogenicity in fish has been documented in previous challenges studies [27]. Mortalities were recorded and protection was estimated using the Kaplan Meyer's survival analysis.
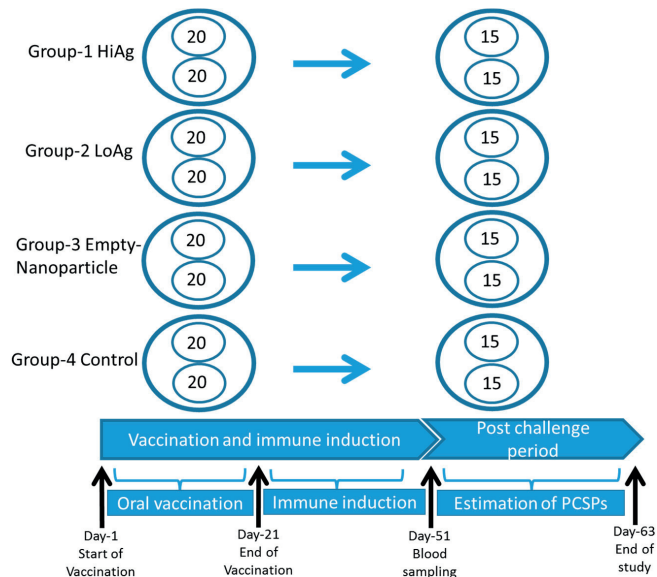

Figure 1. The study design for oral vaccination of rohu using different vaccine against *A. hydrophila*. Four vaccine groups were each allocated 40 fish. Group 1 was allocated a high-antigen dose of 8 µg/g of fish body weight of the rOmpW vaccine designated as the HiAg dose. Group 2 was vaccinated with a low-antigen dose of 4 µg/g of fish body weight of the rOmpW designated as the LoAg dose. Group 3 was vaccinated with empty nanoparticles, without the rOmpW antigen, designated as NP-Empty while Group 4 was left unvaccinated as a control group. The study time-line was segmented into three parts, namely, (i) the oral vaccination period of 21 days; (ii) immune induction period of 50 days post vaccination (dpv); and (iii) post challenge period. Blood samples were collected from 10 fish per group at 50 dpv after which fish were challenged with a virulent strain *A. hydrophila* at a concentration of 2.7×10^7 CFU/mL injected intramuscularly at 0.1 mL/fish. The vaccination trial ended at 83 dpv when fish stopped dying.

2.6. Serum Inhibitory Assay

The serum mediated antibacterial activity was measured as described by Hamod *et al*. [17]. Briefly, 10 µL of *A. hydrophila* grown overnight in broth culture was adjusted to 10^3 cfu/mL in PBS to which 90 µL serum was added in a microtube. The solution was mixed thoroughly and placed at 30 °C for 24 h. After incubation, a 10-fold serial dilution of serum containing bacteria was prepared for each mixture and 100 µL aliquots of each dilution was plated onto tryptone soya agar plates and incubated at 30 °C for 24 h. Bacterial colonies were counted and results were expressed as $\log_{10}$ cfu/mL. The serum used for this study was pooled from the 10 fish sampled at 30 dpv from each group described in Section 2.6 above. Reduction in bacterial count was obtained by subtracting the bacterial count of vaccinated fish from counts of PBS control fish.

2.7. Antibody Response to the rOmpW Protein

Antibody responses to rOmpW were analyzed using the enzyme linked immunosorbent assay (ELISA). Briefly, ELISA plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 2 µg/well

rOmpW diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were blocked with 350 μL 3% BSA at 37 °C for 2 h after washing using PBS. Thereafter, 100 μL fish sera (1:10 dilution) was added to each well and incubated at 37 °C for 2 h. The plates were washed three times with PBS, followed by adding rabbit anti-rohu HRPO at 1:200 dilution per well and incubated at 37 °C for 35 min. After washing, tetramethylbenzidine hydrogen peroxide (TMB) substrate was added to each well and the absorbance was read at 450 nm using an EL_{X800} Universal microplate reader (BioTek, Winooski, VT, USA).

3. Results

3.1. Antigen Preparation

3.1.1. Expression, Purification, Concentration, and Encapsulation Efficiency of OmpW

The rOmpW protein was expressed after 4 h induction with IPTG. The MW was determined to be 22 kDa using 15% SDS-PAGE. The purity of rOmpW was about 90% and the concentration was estimated at 1.2 mg/mL. The encapsulation efficiency of rOmpW was estimated at 53.56% and average particle size diameter was 370–375 nm. The zeta potential of rOmpW encapsulated PLGA nanoparticles was −19.3 mV.

3.1.2. *In Vitro* Release of the rOmpW Protein from PLGA Nanoparticles

In vitro release of rOmpW showed an exponential increase in the first 24 h of incubation (Figure 2), which progressed to the plateau phase after 24–48 h of incubation in PBS.
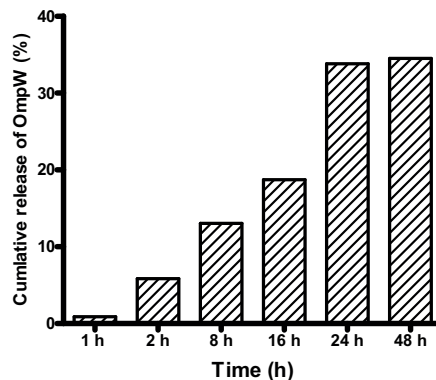
Figure 2. Shows *in vitro* release of rOmpW protein from encapsulated PLGA NPs observed within 48 h of incubation in phosphate buffered saline (PBS). Note that the release of rOmpW increased exponentially within 24 h after the start of the *in vitro* test.

3.2. Vaccination and Challenge of Rohu

3.2.1. Kaplan Meyer's Survival Analysis

A total mortality of 75% of the fish was achieved in the control group following challenge. In this group, mortality started one day earlier than the vaccinated groups (Figure 3). There was a significant difference in post challenge survival proportions (PCSPs) between the vaccinated and control groups. Figure 3 shows that there was a dose dependent effect on PCSPs with the HiAg group (8 μg/g of fish body weight), had a significantly higher PCSP ($p = 0.0435$) than the LoAg group. In terms of relative percent survival (RPS), the HiAg group had the highest RPS (79.99%), followed by the LoAg group (RPS = 37.33%) and the NP-Empty (RPS = 3.96%).
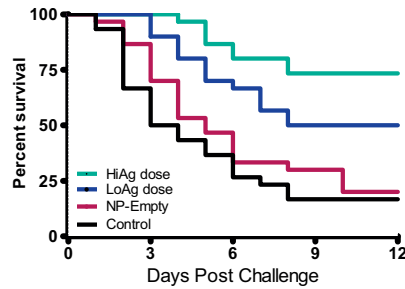
Figure 3. The Kaplan Meyer's survival analysis of fish vaccinated against *A. hydrophila* using with the NP-rOmpW vaccine administered at high- and low-antigen doses. The high-antigen (HiAg) dose was administered at a concentration of 8 μg/g of fish body weight, while the low-antigen (LoAg) dose was administered at a dose of 4 μg/g of fish body weight. Mortality in the control group started at two days post challenge (dpc), 3 dpc in the LoAg group and 4 dpc in the HiAg group. The highest post challenge survival proportion (PCSPs) were from the HiAg group (PCSP = 73.33%), followed by the LoAg group (PCSP = 48.28%), while the NP-Empty (PCSP = 20.00%) and control (PCSP = 16.67%) groups had the lowest PCSPs. There was a significant difference ($P = 0.0435$) between the HiAg and LoAg groups, while no significant difference ($P = 0.3104$) was observed between the NP-Empty and control groups.

3.2.2. Serum Inhibition of *A. hydrophila* Growth on Trypticase Soy Agar (TSA) Agar

Figure 4 shows results of *A. hydrophila* counts. There was a significant reduction in bacterial counts when bacteria were incubated with serum from vaccinated fish, compared to unvaccinated control fish, with a reduction to less than 2.0×10^7 for HiAg and around 3.0×10^7 for the LoAg group, compared to 1.0×10^9 for the control group. The unvaccinated control fish had higher bacterial growth than fish fed with empty PLGA NPs (Figure 4).
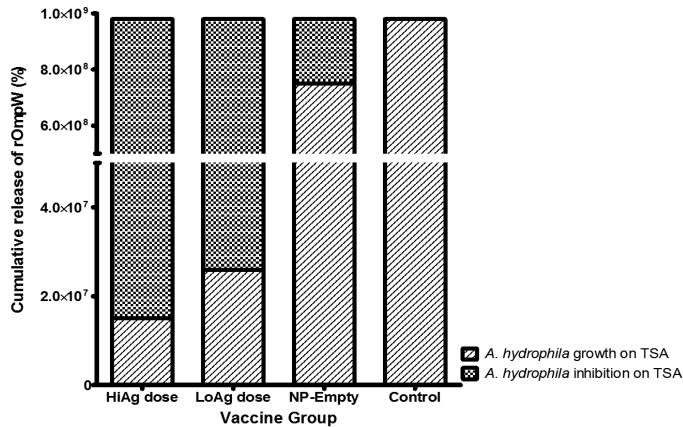
Figure 4. *A. hydrophila* growth and inhibition on trypticase soy agar (TSA) after treatment of sera from fish vaccinated with overnight culture broth of *A. hydrophila*. The *A. hydrophila* culture was used at a concentration of 10^3 cfu/mL to which 90 μL serially-diluted sera was added in microtubes followed by incubation on TSA for 24 h at 30 °C. *A. hydrophila* growth for each group was determined by counting individual colonies, as described by Lowry *et al.*, while inhibition was calculated by subtracting bacteria colony counts of vaccinated fish from the unvaccinated control group. The serum samples used in this study were pooled from 10 blood samples collected from each group at 50 dpv.

3.2.3. Antibody Responses

Figure 5 shows that antibody levels in the control groups (empty-NP and PBS groups) were significantly ($p < 0.00001$) lower than the vaccinated groups (HiAg and LoAg groups). The LoAg group (mean OD_{450} = 0.4160, SD = 0.0017, N = 10) had significantly lower antibody levels ($p < 0.001$) than for the HiAg group (mean OD_{450} = 0.4631, SD = 0.0025, N = 10) indicating that there was an antigen dose-dependency effect on the induction of antibody responses. Both the empty-NP and PBS control group showed no presence of circulating antibodies (Figure 5).
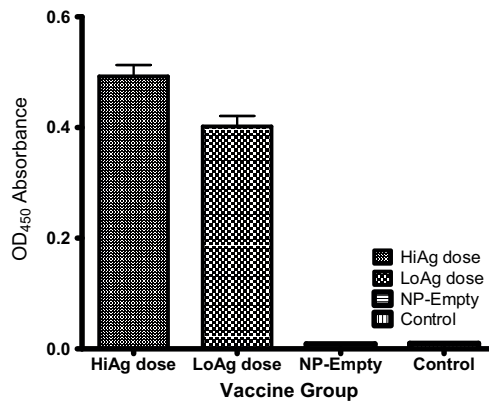
Figure 5. Antibody levels detected against *A. hydrophila* from fish vaccinated with the NP-rOmpW vaccine at HiAg dose, LoAg dose, NP-Empty, and control groups at 51 dpv. Note that antibody levels from fish vaccinated with the HiAg dose were higher than the LoAg dose. There was a significant difference ($p < 0.0003$) in antibody levels detected between the HiAg and LoAg dose groups, while the NP-Empty and control groups did not show presence of antibodies against *A. hydrophila* detected against at 50 dpv.

4. Discussion

Our findings show that PLGA NP-formulated OmpW used in this study was protective against lethal challenge with *A. hydrophila* in rohu. Further protection against mortality was antigen dose-dependent, *i.e.*, the HiAg group had the highest PCSP, and the LoAg group had a correspondingly low PCSP. This is the first study to document that PLGA NP-formulated OmpWs of *A. hydrophila* confer protective immunity in rohu. The encapsulation efficiency obtained was, however, lower than what has been reported by others [25] and it is, therefore, likely that the quantity of antigen released after oral vaccination *in vivo* was also low, resulting in lower protection than what was obtained from injectable vaccines [27–30]. Therefore, future studies should seek to increase the content of OmpW encapsulated in PLGA NPs in order to increase the protective ability of the OmpW antigen in vaccinated fish.

Biodegradable PLGA NPs have attracted a lot of interest as an antigen delivery system for oral vaccines because of their ability to enhance antigen uptake and ability to allow the slow release of antigens *in vivo*, an adjuvant depot effect [31–33]. The size of the NPs (nanodiameter < 400 nm) generated in this study suggests that these particles are of an adequate size for inducing systemic antibody responses. For systemic responses to occur it is anticipated that uptake and processing of the rOmpW antigens by cells of adaptive immune system for induction of protective immunity is required [31–33]. However, the encapsulation efficiency of 53.6% obtained in this study was lower than the mean 60%–70% encapsulation efficiency for PLGA NPs as shown by Kumara *et al.* and Danhier *et al.* [34,35]. Danhier *et al.* [34] observed that high encapsulation efficiencies increase the ability of NPs to deliver antigen *in vivo*; therefore, future studies should seek to increase the

encapsulation efficiency of rOmpW in PLGA NPs in order to induce higher protection levels in vaccinated fish.

The OmpW protein belongs to a family of small OMPs that are highly conserved among the Gram-negative bacteria species [36,37] and, being a highly immunogenic protein, able to induce protective immunity in vaccinated fish [27–30]. Qian *et al.* [30] obtained high protection with a relative percent survival (RPS) of 78 in large yellow croaker (*Pseudosciaena crocea*) when injected intraperitoneally with OmpW against *V. alginolyticus,* while Cai *et al.* [28] showed high protection (RPS = 92) in Crimson snapper (*Lutjanus erythropterus*) following intramuscular injection with OmpW against *V. alginolyticus.* Similarly, Mao *et al.* [30] showed high protection (RPS > 80) in large yellow croaker injected intraperitoneally with OmpW against *V. parahaemolyticus.* Maiti *et al.* [29] obtained high protection (RPS = 80) in carp on intraperitoneal injection with rOmpW against *A. hydrophila.* Results of these studies show that OmpW is protective against different bacteria species when administered by the intramuscular or intraperitoneal route [27–30]. The results obtained from parenteral delivery of rOmpW of *A. hydrophila* surpass those from oral delivery routes indicating that there is still room for improvement of oral PLGA NP-formulations Determining the ability of PLGA NPs to release the encapsulated antigens in vaccinated fish calls for *in vitro* tests that reflect their ability to release the antigens *in vivo.* In this study, *in vitro* release of rOmpW reached 34% within 48 h after incubation in PBS but was lower than the 50% *in vitro* release obtained by Rauta and Nayak [25] for the same antigen encapsulated in PLGA NPs. The kinetics of rOmpW *in vitro* release obtained in this study follow the common trend that starts with a rapid initial release of antigens within 24 h [25,38], followed by a continuous slow release over a long period. Hence, it is likely that a follow up on the *in vitro* release test carried out in this study after 48 h would have shown a prolonged continuous release of the rOmpW antigen from encapsulated NPs. To evaluate the functional characteristics of elicited antibodies post vaccination we performed a serum inhibition test using serum from vaccinated fish. Our findings show that there was an antigen dose dependency on inhibition of *A. hydrophila* growth on TSA, corresponding to *in vivo* challenge, where serum from the HiAg group had the highest inhibition capacity, lower in the LoAg group. These findings correspond with antibody levels obtained from the vaccinated fish where the HiAg group had the highest antibody levels with the LoAg group having relatively low levels. The NP-Empty and control groups had no circulating antibodies. Thus, antibody levels induced by rOmpW vaccines correspond with vaccine antigen quantity delivered by oral vaccination. These findings show that the serum inhibition test used in this study can be used as an *in vitro* measure of vaccine efficacy, in line with our previous findings in which we showed that antigen dose corresponded with protection in Atlantic salmon vaccinated against IPN [39,40]. Similar observations have been shown for furunculosis vaccines in salmon [41], and also in higher vertebrates in which antigen dose was seen to correlate with the induction of protective immunity. As pointed out in our previous studies [39–41], establishing an optimal antigen dose that correlates with protective immunity could serve as a measure of vaccine efficacy for fish vaccines. Hence, future studies should seek to determine the antigen dose and optimal duration of oral vaccination for OmpW that correlate with protective immunity in rohu. In addition, there is a need for detailed investigations to determine the protective mechanism of OmpW oral vaccination, as done for other vaccines [42,43]. There is also a need to elucidate the impact of PLGA NPs in fish vaccinated by the oral route. Nevertheless, this study has shown that OmpW is protective against *A. hydrophila* infection in rohu and that NP-based vaccines could serve as an effective oral immunization strategy for finfish.

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study design, data analysis and manuscript preparation. Øystein Evensen and Indrani Karunasagar participated in mobilizing resources. Øystein Evensen, Indrani Karunasagar and Hetron Mweemba Munang'andu = overall supervision of the project. All authors read and approved publication of the manuscript.

Conflicts of Interest: The authors declare no competing interests.

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