EFFICACY OF LIVE ATTENUATED *Vibrio harveyi* VACCINE ACQUIRED THROUGH MATERNAL IMMUNITY AGAINST *Vibrio* SPP. IN ZEBRAFISH (*Danio rerio* F. Hamilton, 1822)

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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November 2022

Chair : Ina Salwany bin Md Yasin, PhD
Institute : Bioscience

Outbreaks of vibriosis in mariculture have caused major setbacks in the aquaculture industry. Vibriosis is a disease that could cause fatal hemorrhagic septicemia and exophthalmia in marine animals. Furthermore, the increase in demand for protein has pushed farmers to utilize a more complex system with higher density to increase output. The demand for fish fry has also pushed the hatchery industry to increase production. However, juvenile fish exposed to vibriosis would develop acute symptoms resulting in high mortality. The vaccination of broodstock could help in tackling the initial immunity delivered to the fish fry to counter this threat. Maternal transfer of immunity helps increase the survivability of fish fry when dealing with harmful pathogens. A live attenuated *V. harvey*i vaccine (LAVh) from a three-point knock-out on its serine endoprotease gene was established previously. This study aimed to determine the efficacy of three derivatives of the LAVh vaccine on the zebrafish (*Danio rerio*) model to provide immunological protection against *Vibrio alginolyticus, V. parahaemolyticus,* and *V. harveyi*. In the initial study phase, the median lethal dose (LD_{50}) for pathogenic *Vibrio* spp. in adult zebrafish and 21-days post-hatching (dph) zebrafish fry were determined. Subsequently, a vaccine safety study, the antibody level, and the effective dosage of LAVh vaccine to confer 80% survival (ED_{80}) in the adult zebrafish model was determined. As a result, the $LD_{50\text{-}144h}$ of *V. alginolyticus, V. parahaemolyticus,* and *V. harveyi* in adult zebrafish by intraperitoneal (i.p.) infection was 1 x 10^5, 10^6, and 10^6 CFU/mL respectively. The $LD_{50\text{-}144h}$ for the same pathogens in 21-dph juvenile zebrafish by immersion was 1 x 10^7 CFU/mL. In the second study phase, adult zebrafish were vaccinated by intraperitoneal (i.p.) injection with $ED_{80\text{-}144h}$ of LAVh vaccine. The specimens were sampled bi-weekly to plot an antibody profile. Subsequently, on week six (6), the relative percent survival (RPS) of vaccinated adult zebrafish was determined by challenge test. The remaining batches of vaccinated zebrafish were let to spawn and the antibody level of larvae were monitored for 4 weeks post hatching. Results of antibody profiling

in the adult zebrafish model indicated that freeze-dried LAVh delivers a longer immunological protective duration. Offspring's antibody profiling had determined that offspring of the formalin-killed *Vibrio harveyi* (FKVh) vaccination group had failed to provide immunological protection against *V. alginolyticus*. The freeze-dried LAVh vaccine was determined as a suitable candidate for further immunological studies. In the final study phase, juvenile zebrafish from freeze-dried LAVh and FKVh were vaccinated by immersion with vaccines formally vaccinated to their predecessor. The vaccination dose was set at 1 x 10^7 CFU/mL. A sampling of the vaccinated zebrafish juvenile was conducted weekly for four (4) weeks to determine their antibody profile and pro-inflammatory gene expression. At the end of week four (4), the vaccinated juveniles were challenged with pathogenic strains of *Vibrio* spp. As a result, both groups manages to confer antibody production against antigens *Vibrio* spp. However, gene expression of pro-inflammatory interleukin 1β (il1β) in the FKVh vaccinated group was elevated for 2 weeks as compared with that of the freeze-dried LAVh vaccinated group. The RPS of both vaccination groups against pathogenic *Vibrio* spp. displayed 100% immunity. Overall, the freeze-dried LAVh vaccine manage to confer maternal immune protection for its offspring, provides a long duration of immunological protection, cross-protection coverage against pathogenic *Vibrio* spp., and a longer shelf-life. It is proposed for the LAVh vaccine to be commercially available for the use of farmers to protect their products against Vibriosis outbreaks.

Keywords: Immunologial protection, Live attenuated *Vibrio harveyi* vaccine (LAVh), maternal immunity, vibriosis, zebrafish.

SDG: GOAL 14: Life Below Water

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KEBOLEHUPAYAAN VAKSIN *Vibrio harveyi* YANG DILEMAHKAN DIPEROLEHI MELALUI KEIMUNAN IBU MENENTANG *Vibrio* SPP. TERHADAP IKAN ZEBRAFISH (*Danio rerio* F. Hamilton, 1822)

Oleh

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November 2022

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Wabak vibriosis dalam marikultur telah menyebabkan kemerosotan besar dalam industri akuakultur. Vibriosis ialah penyakit yang boleh menyebabkan perdarahan septisemia yang membawa maut dan eksoftalmia dalam haiwan marin. Tambahan pula, peningkatan permintaan terhadap protein telah mendorong petani untuk menggunakan sistem yang lebih kompleks dengan kepadatan yang lebih tinggi untuk meningkatkan pengeluaran. Permintaan terhadap anak ikan juga telah mendorong industri penetasan meningkatkan pengeluaran. Walau bagaimanapun, ikan juvenil yang terdedah kepada vibriosis akan mengalami gejala akut yang mengakibatkan kematian yang tinggi. Pemvaksinan induk boleh membantu dalam meningkatkan imuniti awal pada anak ikan untuk menentang ancaman ini. Pemindahan imuniti ibu membantu meningkatkan kemandirian anak ikan apabila berhadapan dengan patogen berbahaya. Vaksin *V. harveyi* yang dilemahkan (LAVh) hasil daripada penghapusan tiga tapak yang berbeza pada gen serine endoproteasenya telah hasilkan sebelum ini. Kajian ini adalah bertujuan untuk menentukan keberkesanan tiga terbitan vaksin LAVh yang diuji pada model ikan zebrafish (*Danio rerio*) untuk memberikan perlindungan imunologi terhadap *Vibrio alginolyticus*, *V. parahaemolyticus*, dan *V. harveyi* . Fasa awal kajian ini, dos maut median (LD_{50}) untuk patogen *Vibrio* spp. akan ditentukan pada zebrafish dewasa dan anak ikan zebrafish berumur 21-hari selepas penetasan (hsp). Kemudiannya, kajian terhadap keselamatan vaksin, tahap antibodi, dan dos berkesan vaksin LAVh untuk memberikan 80% kemandirian (ED_{80}) pada model ikan zebrafish dewasa telah ditentukan. Hasilnya, $LD_{50\text{-}144j}$ *V. alginolyticus*, *V. parahaemolyticus* dan *V. harveyi* pada ikan zebrafish dewasa melalui jangkitan intraperitoneal (i.p.) adalah 1 x 10^5, 10^6, dan 10^6 CFU/mL. $LD_{50\text{-}144h}$ bagi patogen yang sama pada ikan zebrafish juvana 21 hsp secara rendaman adalah 1 x 10^7 CFU/mL. Dalam fasa kajian kedua, ikan zebrafish dewasa telah divaksinasi melalui suntikan intraperitoneal (i.p.) dengan vaksin LAVh $ED_{80\text{-}144h}$. Sampel spesimen telah diambil dua minggu sekali untuk pemplotan profil

antibodi. Seterusnya, pada minggu keenam (6), melalui ujian cabaran, peratusan kemandirian relatif (PKR) ikan zebrafish dewasa yang telah divaksin telah ditentukan. Manakala, baki kumpulan ikan zebrafish yang divaksin dibiarkan untuk mengeluarkan telur dan tahap antibodi larva dipantau selama 4 minggu selepas penetasan. Keputusan profil antibodi dalam model ikan zebrafish dewasa menunjukkan bahawa LAVh yang kering beku memberikan tempoh perlindungan imunologi yang lebih lama. Pemprofilan antibodi anak ikan juga telah menentukan bahawa anak ikan dari pemvaksinan induk menggunakan vaksin daripada *Vibrio harveyi* yang dimatikan menggunakan formalin (FKVh), gagal memberikan perlindungan imunologi terhadap *V. alginolyticus*. Vaksin LAVh kering beku telah ditentukan sebagai calon yang sesuai untuk kajian imunologi selanjutnya. Dalam fasa kajian akhir, ikan zebrafish juvana daripada kumpulkan vaksin LAVh kering beku dan FKVh telah divaksinkan secara rendaman dengan vaksin yang telah diberikan kepada induk masing-masing. Dos vaksinasi telah ditetapkan pada 1 x 10^7 CFU/mL. Seterusnya, pensampelan juvana ikan zebrafish yang divaksinkan telah dilaksanakan setiap minggu selama empat (4) minggu untuk menentukan profil antibodi dan ekspresi gen proinflamasi. Pada pengakhiran minggu keempat (4), ikan juvana yang divaksinkan telah dicabar dengan strain patogen *Vibrio* spp. Hasilnya, kedua-dua kumpulan berjaya menunjukkan penghasilan antibodi terhadap antigen *Vibrio* spp. Walau bagaimanapun, ekspresi gen interleukin 1β (il1β) proinflamasi daripada kumpulan vaksin FKVh berada pada tahap yang tinggi selama 2 minggu jika dibandingkan dengan kumpulan vaksin LAVh kering beku. PKR kedua-dua kumpulan vaksinasi terhadap patogen *Vibrio* spp. menunjukkan imuniti 100%. Secara keseluruhannya, vaksin LAVh kering beku berjaya memberikan perlindungan imunisasi ibu kepada anak-anaknya, memberikan perlindungan imunologi jangka panjang, perlindungan serangkai terhadap patogen *Vibrio* spp., dan jangka hayat vaksin yang lebih lama. Vaksin LAVh perlu dihasilkan secara komersil untuk kegunaan penternak bagi melindungi hasil ternakan dari wabak Vibriosis.

Keywords: Keimunan ibu, perlindungan imunologi, vaksin *Vibrio harveyi* yang dilemahkan (LAVh), vibriosis, zebrafish.

SDG: MATLAMAT 14: Kehidupan Dalam Air

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Date: 7 November 2024

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LIST OF ABBREVIATIONS

%	percentage
*	asterisk
±	plus minus
≈	approximately
©	Copyright
®	Registered
µ	micro
µL	microliter
½	half
Aeromonas sp.	*Aeromonas* species
Bhd.	Berhad
bp	base pair
cc	cubic centimeter
CD	cluster of differentiation
cDNA	Complementary DNA
CFU	colony forming unit
dH_2O	distilled water
ddH_2O	double distilled water
dph	days post hatching
DNA	deoxyribonucleic acid
ED_{80}	Effective dosage 80%
EU	European Union
FKVh	Formaline-kill *Vibrio harveyi* vaccine
g	gravitational force

g	gram
G	gauge of needle
gDNA	genomic DNA
hpf	Hours post fertilization
HRP	Horseradish Peroxidase
I.P.	Intraperitoneal
IgG	Immunoglobulin G
IgM	Immunoglobulin M
L	liter
LAVh	Live attenuated *Vibrio harveyi* vaccine
LD_{50}	Lethal dosage 50%
M	molar
mL	milliliter
mRNA	Messenger RNA
Na_2CO_3	Sodium Carbonate
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
nm	nanometre
^oC	degree Celsius
OD	optical density
p	Significant value
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
pH	power of hydrogen
ppt	part per thousand

qPCR	quantitative polymerase chain reaction
D. *rerio*	*Danio rerio*
RNA	ribonucleic acid
rpm	rotation per minute
RPS	relative percent survival
RT enzyme	reverse transcriptase enzyme
Sdn.	Sendirian
sp.	species
spp.	several species
TAE	Tris-acetate-EDTA buffer
TCBS	Thiosulfate-Citrate-Bile Salt-Sucrose Agar
™	Trademark
TMB	3,3',5,5'-tetramethylbenzidine
UK	United Kingdom
v	version
V. alginolyticus	*Vibrio alginolyticus*
V. anguillarum	*Vibrio anguillarum*
V. harveyi	*Vibrio harveyi*
V. parahaemolyticus	*Vibrio parahaemolyticus*
v/v	volume/volume percentage
w/v	weight/volume percentage
wph	week post hatching
α	alpha
β	beta
γ	gamma

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Vibriosis is a disease caused by pathogenic *Vibrio* spp. bacterium (Bullock, 1977; Novriadi, 2016). It is a Gram-negative luminous bacterium with a dependency on sodium chloride; due to this, its spread is relevant to the rapid development of marine aquaculture in Asia and South America (Austin & Zhang, 2006). It is commonly found in marine warm waters around the world and affects multiple species of farmed finfish, some of the most notable ones are grouper (*Epinephelus* spp.), snapper (*Lutjanus* spp.), Asian seabass (*Lates calcarifer*) Pacific salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) (Tendencia, 2002; Rodkhum et al., 2005; Chong et al., 2011; Aris et al., 2016; Chin et al., 2020; Mohamad et al., 2019). In Malaysia (Ransangan & Mustafa, 2009), the Philippines (Tendencia, 2002), and Vietnam (Dong et al., 2017); *Vibrio harveyi*, *Vibrio alginolyticus,* and *Vibrio parahaemolyticus* are the main culprits for the outbreaks of vibriosis in cage cultured Asian seabass (Dong et al., 2017) and multiple species of farmed grouper (Mohamad et al., 2022). High demands forced farmers to increase the stocking high density in cage culture environments, and also hatchery husbandry (Schipp et al., 2007). This condition promotes stress in hatcheries towards the fish fry and increases the chance of pathogenic infection. Furthermore, the high loss of fish larvae was attributed to Vibriosis during an outbreak (Silva et al., 2014). Dong et al. (2017) then further reiterated this notion, stating that Vibriosis is highly susceptible to juvenile fishes with a mortality rate of around 40% during outbreaks in juvenile Asian Seabass (*Lates calcarifer*) culture.

A method of reducing the chances of infection is via vaccination of the broodstock. This helps provide the fry with early protection against diseases and has been shown fruitful; offspring of fish from vaccinated broodstock has shown enhance immune cells development (Ye et al., 2016), an increase in innate (Zhang et al., 2013), and humoral (Hanif et al., 2004) immunity, with higher survival rate (Nisaa et al., 2017). Several attempts to deliver maternal immunity have been successful; Tilapia (*Oreochromis niloticus*) broodstock was vaccinated against *Streptococcus agalactiae* and confer higher relative percent survival (RPS) compared with non-vaccinated larvae (Sukenda & Rahman, 2018), live attenuated *Vibrio anguillarum* increases the lysozyme transferred and accelerated the development of adaptive immune-response (Ye et al., 2016) in Zebrafish (*Danio rerio*). Another success was the transfer of innate immunity from broodstock of marine sea bream (*Sparus aurata L.*) vaccinated with *Photobacterium damsela* subsp. p*iscicida* SK7 (*Phdp*) while also maintaining a level of humoral immunity to larvae until day 14 post-hatching (Hanif et al., 2004). Therefore, this study supports the investigation of maternal transfer from vaccinated broodstock to larvae after vaccination of live

attenuated *Vibrio harveyi* vaccine to provide multiple protection against pathogenic *V. harveyi, V. alginolyticus,* and *V. parahaemolyticus.* Worth mentioning; a study has been conducted, in which the transfer of maternal immunity in zebrafish has been recorded against *Aeromonas hydrophila* has shown to be remarkable at protecting their embryos (Wang et al., 2009), it is proposed to have booster vaccines by immersion for the larvae to raise the antibody level.

1.2 Problem statement

In adult teleost, different methods of vaccine administration have shown various effects on the increment of specific humoral responses when vaccinated against Vibriosis (Collado et al., 2000). Adult teleost vaccination can be administered orally in foods, this method limits the interaction between direct human contact, and reducing stress; the intraperitoneal injection will provide the best results relative to the duration of protection but has limitations in its application at a commercial level; bath vaccination via immersion is the easiest way to administer vaccines (Wang et al., 2020). Despite having multiple routes to administer vaccines, the immune efficacy depends heavily on the bacterial species, the concentration of vaccine, the species, and the size of the animal (Li et at., 2016). It is at the larval and juvenile development stage which has been a bottleneck for developing a sustainable and viable fish farming industry (Ye et al., 2016). A notable way of developing protection against disease is via the transfer of maternal immunity toward the larvae. The quality and quantity of immune factors transferred to the offspring are greatly affected during vitellogenesis. In regards to this, the maternal transfer is the ideal method for humoral immune development in larvae because they are very much vulnerable to the elements (Ye et al., 2016). The rise of demand for commercial fish fingerlings can be met by vaccinating berried broodstocks, this can provide fish larvae with early immuno-protectant against pathogenic bacteria and increases the production output of hatcheries. Previously, a study by Aslizah (2019) managed to produce a live attenuated *Vibrio harveyi* vaccine by nullifying the function of its serine protease with a three point knock-out gene technique. Continuation of that study, we present an investigation to further understand the transfer of maternal immunity towards their offspring and the period of immunological protection that the vaccine provides to both the broodstock and fish fry.

However on a laboratory scale, the use of some commercial marine fish to study the relationship between maternal broodstock immunity and their offspring is unsuitable, due to the amount of specimen and frequent spawning rate needed for this study. More over, marine commercial fish model requires a high degree of maintenance and skill to handle the fishes, they are also somewhat large and requires a spacious operational hatchery to spawn eggs, the use of seawater in an inland and urban research facilities also adds up to the problem. Furthermore, the high amount of feeding required is expensive coupled with the short spawning period, strengthens this problem.

Due to this, a preferred fish model would be of those that are able to be maintained in an urban area, able to be maintained in a small compartment with basic maintenance skill, has a high and frequent spawning rate and has a fast growing period. A potential alternative animal model would be the use of zebrafish (*Danio rerio*). There are many studies conducted on marine bacterium using zebrafish to justify the use of this fish species. This study has a novelty of using zebrafish as a broodstock for maternal immunity transfer after vaccinating with live attenuated *Vibrio harveyi* vaccine.

1.3 Significant of the study

The common practice for farmers to increase the resistance of fish larvae is the use of antibiotics (Gao et al., 2014). However, the rise of antibiotics has also encouraged the development of resistance in pathogens by selective pressuring them into a horizontal transfer of resistance genes to the different organisms (Aris et al., 2016; Zhu et al., 2006). Due to this, multiple alternatives were suggested, one of them was immunostimulation of immune cells by vaccination (Frans et al., 2011). Vaccination can be a principle disease management strategy and an indispensable method in the reduction of antibiotic usage in aquaculture production and promoting stimulant of immunity in teleost (Zhang et al., 2014). Vaccines were primarily constructed from killed or inactivated organisms of a particular disease, however, this leads them to become weak in their immunogenic composition and requires an adjuvant or immunomodulator and multiple doses to upkeep the protection level (Kenneth et al., 2005). Recent molecular advancement in the field of immunology has given rise to the development of live attenuated vaccines (Yang et al., 2015). These vaccines offer a significant advantage over their killed-vaccine predecessor; an increase in macromolecules delivery (Kenneth et al., 2005), high immune efficacy, mimic natural infection (Liu et al., 2018) and provoking a wide range of immune responses (Zhou et al., 2010). That being said, the best live attenuated vaccines are the ones that can provide replicating antigens, able to stimulate the mucosal, humoral, and cell-mediated immune response (Lin et al., 2015), while also providing cross-protection against different strains (Shoemaker et al., 2009, Gao et al., 2014; Yilmaz et al., 2022). As a reference, live attenuated *Vibrio harveyi* (T4D Mutant) vaccine candidate has shown effective cross-protection for Japanese flounder (*Paralichthys olivaceus*) against *Vibrio alginolyticus* (Hu et al., 2012).

Nonetheless, it can be associated with the fact that most vaccines are species-specific vaccines and are only effective at guarding against a specific strain of bacterium. In the note of this, the *Vibrio* spp. family has a variety of antigenic strains and serotypes that makes antibodies developed from those vaccines incompetent to bring forth enough protection against simultaneous infection of different *Vibrio sp*. (Ina-Salwany et al., 2019). The common ancestry of *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi* to be members of the Harveyi clade, a subset of Vibrios core gene group (Urbanczyk et al., 2013), being in the same ancestry route gives an advantage at producing multiple cross-species vaccines able at defending against these pathogens. In 2016,

Aris et al. developed a live attenuated *Vibrio harveyi* vaccine by deleting mutation of the serine protease gene (VHS); the serine protease acts as a chaperone or provides thermal resistant properties for proteolytic enzyme activities (Aris et al., 2016), this inhibits the virulence of the mutant *V. harveyi* and was successful in providing immuno-protectant for marine fish against pathogenic strains of *V. alginolyticus, V. parahaemolyticus,* and *V. harveyi.* Live attenuated vaccines do have an offset, in terms of their stability. This is highly dependent on temperature, handling, storage, and distribution (Schlegl & Hahn, 2012). This could be countered by capitalizing on freeze-drying techniques in their development (Yang et al., 2007).

1.4 Objective of the study

To our knowledge, there were no studies at present assessing the derivatives of live attenuated vaccines on teleost species and their effects on maternal immune transfer in regards to the protection capability of their offspring. The framework of the research study as stated in Figure 1.1.

The three (3) main objectives of this study are as stated below:

1. To establish the survival and immunological assessment of three (3) derivatives of live attenuated *Vibrio harveyi* vaccine on zebrafish (*Danio rerio*) model,

2. To evaluate the efficacy of vaccinated zebrafish to confer maternal immunity against pathogenic *Vibrio* spp. for their progeny,

3. To determine the efficacy of vaccinating zebrafish fry from vaccinated broodstock to confer immunity protection against pathogenic *Vibrio sp.,*

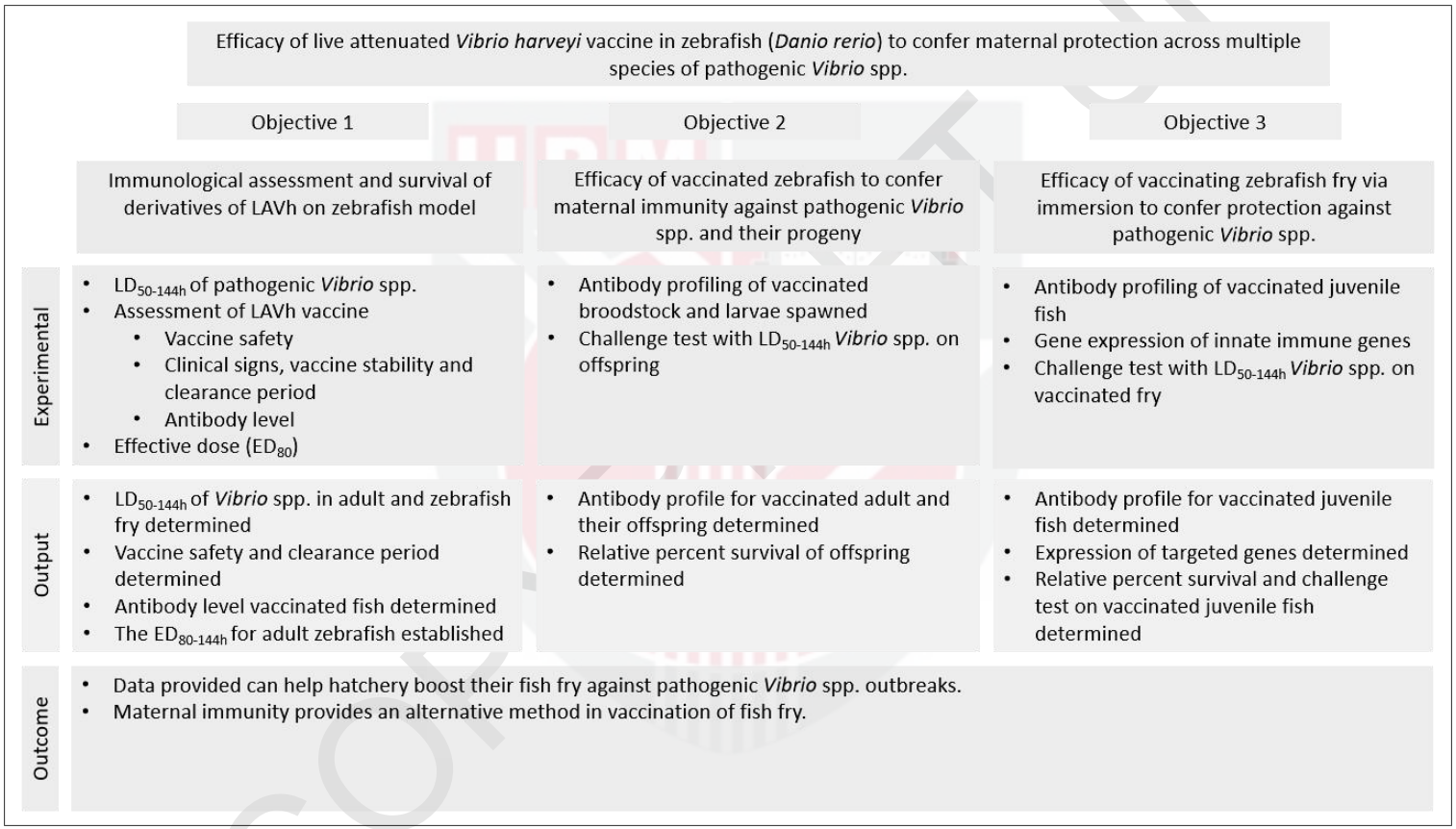

Figure 1.1 : Research framework of this study.

1.4 Hypothesis of the study

The hypothesis of this study is as stated below:

Hypothesis 1:

H_0: The live attenuated *Vibrio harveyi* (LAVh) vaccine causes high mortality and only invokes a small amount of antibody production response.

H_1: The live attenuated *Vibrio harveyi* (LAVh) vaccine cause minor mortality at the expense of invoking high antibody production in its response.

Hypothesis 2:

H_0: Adult zebrafish vaccinated with live attenuated *Vibrio harveyi* (LAVh) vaccine has low survival when challenged with pathogenic *Vibrio* spp. and their maternal immune transfer does not confer protection towards their progeny.

H_1: Adult zebrafish vaccinated with live attenuated *Vibrio harveyi* (LAVh) vaccine have high survival when challenged with pathogenic *Vibrio* spp. and confer maternal immune protection towards their progeny.

Hypothesis 3:

H_0: Juvenile zebrafish vaccinated with formalin-killed *Vibrio harveyi* (FKVh) have a higher immune response as compared with juvenile zebrafish vaccinated with live attenuated *Vibrio harveyi* (LAVh) vaccine.

H_1: Juvenile zebrafish vaccinated with live attenuated *Vibrio harveyi* (LAVh) vaccine has a higher immune response as compared with juvenile zebrafish vaccinated with formalin-killed *Vibrio harveyi* (FKVh) vaccine.

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