Faculty of Resource Science and Technology

Selection of Vaccine Candidates Against *Pseudomonas koreensis* Using Reverse Vaccinology and a Preliminary Efficacy Trial in *Tor tambroides* (Empurau)

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Master of Science
2024

Selection of Vaccine Candidates for *Pseudomonas koreensis* Using Reverse Vaccinology and a Preliminary Efficacy Trial in *Tor tamborides* (Empurau)

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A thesis submitted

In fulfilment of the requirements for the degree of Master of Science

(Biochemistry)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2024

DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

……

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude and appreciation to all those who have contributed to the completion of this master's thesis. First and foremost, I am deeply indebted to my supervisor, Associate Professor Dr. Chung Hung Hui, for his invaluable guidance, support, and expertise throughout the entire research process. His insightful feedback, constructive criticism, and continuous encouragement were instrumental in shaping this thesis and enhancing its quality. I am truly fortunate to have had his guidance throughout this journey. I would also like to acknowledge the financial support of Malaysian Ministry of Higher Education under Fundamental Research Grant Scheme (F07/FRGS/85428/2022).

I would also like to extend my heartfelt thanks to my Animal Biotechnology Lab mates: Melinda and Ivy. Their collaborative spirit, intellectual discussions, and constructive feedback have greatly enriched my research experience. I am grateful for the camaraderie, friendship, and shared knowledge we have fostered, which have undoubtedly contributed to the success of this thesis.

My gratitude extends to my family members: my father Kho Boo Teng, my mother Fam Say Tho, and my sister Wendy Kho Jia Min for their unwavering support and encouragement throughout this academic journey. Their understanding, patience, and belief in my abilities have been a constant source of motivation. Furthermore, I would like to extend my sincere appreciation to my close friends Eirene and Min Kim for their company.

ABSTRACT

The unregulated and/or incorrect use of antimicrobial agents against diseases of farmed aquatic species poses a considerable threat to the development and growth of a sustainable aquaculture industry. Hence, vaccination has emerged as a pivotal strategy to mitigate this threat. This study marks the first utilization of reverse vaccinology to develop subunit vaccines against *Pseudomonas koreensis* infection in Empurau (*Tor tambroides*). WGS analysis revealed that the genomic size of *P. koreensis* CM-01 is 6,171,880 bp and has a G+C content of 60.5%. The proteome (5,538 proteins) was screened against various filters to prioritize proteins based on features that are associated with virulence, subcellular localization, transmembrane helical structure, antigenicity, essentiality, non-homology with the host proteome, molecular weight, and stability, which led to the identification of eight potential vaccine candidates. These potential vaccine candidates were cloned and expressed, with six achieving successful expression and purification. The antigens were formulated into two distinct vaccine mixtures, Vac A and Vac B, and their protective efficacy was assessed through *in vivo* challenge experiments. Vac A and Vac B demonstrated high protective efficacies of 100% and 81.2%, respectively. Histological analyses revealed reduced tissue damage in vaccinated fish after experimental infection, with Vac A showing no adverse effects, whereas Vac B exhibited mild degenerative changes. Quantitative real-time PCR results showed a significant upregulation of *TNF-α* and downregulation of *IL-1β* in the kidneys, spleen, gills, and intestine in both Vac A- and Vac B immunized fish after challenged with *P. koreensis*. Additionally, *IL-8* exhibits tissue-specific differential expression, with significant upregulation in the kidney, gills, and intestine, and downregulation in the spleen, particularly notable in Vac A immunized fish. The research

underscores the effectiveness of the reverse vaccinology approach in fish and demonstrates the promising potential of Vac A and Vac B as recombinant subunit vaccines.

Keywords: *Pseudomonas. koreensis*; *Tor tambroides*; Reverse vaccinology; Subunit vaccines; Protective efficacy

Pemilihan Kandidat Vaksin untuk Pseudomonas koreensis Melalui Vaksinologi Terbalik dan Percubaan Keberkesanan Awal dalam Tor tambroides (Empurau)

ABSTRAK

Penggunaan agen antimikrobial yang tidak terkawal dan/atau tidak betul terhadap penyakit-penyakit spesies akuatik yang diternak membawa ancaman yang signifikan terhadap perkembangan dan pertumbuhan industri akuakultur yang mampan. Oleh itu, vaksinasi telah muncul sebagai strategi penting untuk mengurangkan ancaman ini. Kajian ini menandakan penggunaan pertama vaksinologi terbalik untuk membangunkan vaksin subunit terhadap jangkitan Pseudomonas koreensis *dalam empurau* (Tor tambroides). *Analisis penjujukan genom keseluruhan (WGS) mendedahkan bahawa saiz genom* P. koreensis *CM-01 adalah 6,171,880 bp dan mempunyai kandungan G+C sebanyak 60.5%. Proteom (5,538 protein) telah disaring terhadap pelbagai penapis untuk mengutamakan protein berdasarkan ciri-ciri yang dikaitkan dengan virulensi, lokalisasi subselular, struktur heliks transmembran, antigenisiti, keesensialan, ketiadaan homologi dengan proteom hos, berat molekul, dan kestabilan, yang membawa kepada pengenalpastian lapan kandidat vaksin berpotensi. Kandidat vaksin berpotensi ini diklon dan diekspresikan, dengan enam mencapai kejayaan ekspresi dan penulenan. Antigen tersebut dirumuskan kepada dua campuran vaksin yang berbeza, Vac A dan Vac B, dan keberkesanan perlindungannya dinilai melalui eksperimen cabaran in vivo. Vac A dan Vac B menunjukkan keberkesanan perlindungan yang tinggi masing-masing sebanyak 100% dan 81.2%. Analisis histologi mendedahkan pengurangan kerosakan tisu pada ikan yang divaksin selepas jangkitan percubaan, dengan Vac A tidak menunjukkan kesan buruk, manakala Vac B menunjukkan perubahan degeneratif yang ringan. Keputusan PCR real-time kuantitatif menunjukkan peningkatan yang signifikan dalam ekspresi TNF-α dan penurunan ekspresi IL-1β dalam*

buah pinggang, limpa, insang dan usus dalam kedua-dua ikan yang diimunisasi Vac A- dan Vac B selepas dicabar dengan P. koreensis. *Selain itu, IL-8 menunjukkan ekspresi pembezaan khusus tisu, dengan peningkatan yang signifikan di buah pinggang, insang, dan usus dan penurunan di limpa, terutamanya ketara dalam ikan yang diimunisasi dengan Vac A. Kajian ini menekankan keberkesanan pendekatan vaccinology terbalik dalam ikan dan menunjukkan potensi menjanjikan Vac A dan Vac B sebagai vaksin subunit rekombinan.*

Kata Kunci: Pseudomonas koreensis; Tor tambroides*; Vaksinologi terbalik*; *Vaksin subunit; Keberkesanan perlindungan*

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
AHL	N-acyl derivatives of homoserine lactone
APS	Ammonium persulfate
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic-resistance genes
B cells	B lymphocytes
BUSCO	Benchmarking Universal Single-Copy Orthologs
$CaCl_2$	Calcium chloride
CCW	Counterclockwise
CFU	Colony forming unit
$CheY^P$	Chemotaxis signaling molecule phospho-CheY
CpG	Cytosine-phosphorothioate-guanine
CW	Clockwise
cyclic-di-GMP	Cyclic dimeric guanosine monophosphate
DEG	Database of Essential Genes
DNA	Deoxyribonucleic acid
DOF	Department of Fisheries Malaysia
dpc	Days post challenge
dpi	Days post immunization
DVS	Department of Veterinary Services
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolysaccarides
FAO	Food and Agriculture Organization

fap	Flagellin-associated protein
FCA	Freund’s complete adjuvant
FIA	Freund’s incomplete adjuvant
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Gross domestic product
HCN	Hydrogen cyanide
HEPA	High-efficiency particulate absorbing
IAA	Indole-3-acetic acid
IFN	Interferon
Ig	Immunoglobulin
IL-1β	Interleukin-1β
IL-8	Interleukin-8
IPTG	Isopropyl β- d-1-thiogalactopyranoside
IUCN	International Union for the Conservation of Nature
Lao PDR	Lao People's Democratic Republic
LB	Luria-Bertani
LKIM	Fisheries Development Authority of Malaysia
LPNS	List of Prokaryotic Names with Standing
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAFI	Ministry of Agricultural and Food Industry
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MLSA	Multilocus sequence analysis
MT	Metric tonnes

NaH_2PO_4	Monosodium phosphate
NAP	National Agrofood Policy
NCBI	National Center for Biotechnology Information
NCCs	Non-specific cytotoxic cells
NETs	Neutrophil extracellular traps
NO	Nitric oxide
OD600	Optical density at 600 nm
PBL	Peripheral blood leukocytes
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGAP	Prokaryotic Genome Annotation Pipeline
PGPR	Plant growth-promoting rhizobacteria
PUFA	Polyunsaturated fatty acid
PVCs	Potential vaccine candidates
RND	Resistance-Nodulation-Division
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPS	Relative percentage of survival
rRNA	Ribosomal ribonucleic acid
S.O.C. Medium	Super optimal medium with catabolic repressor medium
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
T cells	T lymphocytes
Tc cells	Cytotoxic T-cells
TCS	Two-component signal

TGF-β	Transforming growth factor-β
Th cells	Helper T cells
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Tris-HCl	Tris Hydrochloride
USDA	United States Department of Agriculture
VFDB	Virulence Factor Database
WC-MALDI-TOF MS	Whole-cell matrix-assisted laser-desorption time-of-flight mass spectrometry

CHAPTER 1

INTRODUCTION

Aquaculture involves the cultivation of aquatic organisms such as fish, crustaceans, mollusks, and even aquatic plants in a regulated fashion to allow the independence of wild catches. It is emerging as an important economic agribusiness, worldwide. According to Food and Agriculture Organization (FAO), global aquaculture production hit a record-breaking 122.6 million metric tons (MT) in 2020, with China alone contributing 57% of the global aquaculture volume (FAO, 2022). The increasing demand for aquaculture production stems from the fact that the wild harvesting of numerous seafood species has already reached or surpassed the maximum sustainable yield (Dulvy et al., 2021). As a result, aquaculture offers a viable alternative, enabling farmers to cultivate these species in controlled environments and thus easing the burden on wild stocks. Consequently, this approach holds the potential to restore depleted wild stocks and aid in the recovery of endangered and threatened species in the future (Osathanunkul & Suwannapoom, 2023). However, the fish that are kept in captivity are more prone to infection as the fish are reared at higher densities than wild individuals, which enables pathogens to spread quickly (Bouwmeester et al., 2021). Consequently, disease outbreaks pose a significant challenge to productivity and lead to substantial economic losses in the aquaculture industry.

Tor tambroides, locally known as “kelah merah” in Peninsular Malaysia or empurau in Sarawak, is a promising freshwater fish species from the Cyprinidae family. The cultivation interest in this species arises from its significant cultural and socioeconomic importance, encompassing its roles as food, ornamental, and sporting fish (Lau et al., 2021). Due to the decline in natural populations and distribution of *T. tambroides*, there has been a

pronounced surge of interest in artificial propagation, for aquaculture production and conservation purposes (Ng & Andin, 2011).

The infectious diseases in fish are often caused either by bacteria, fungi, viruses, and parasites. Among them, bacteria pathogens account for the majority of diseases in fish farming (Dhar, 2014). Diverse *Pseudomonas* spp. are pathogenic to fish, resulting in significant economic losses in the aquaculture sector. Fish pathogenic pseudomonads include *Pseudomonas aeruginosa*, *P. putida*, *P. luteola*, *P. fluorescens*, *P. koreensis*, *P. anguilliseptica*, *P. baetica*, *P. chlororaphis*, *P. plecoglossicida*, and *P. pseudoalcaligenes* (Austin & Austin, 2016). The mortality of diseased cultured *Tor tambroides* was associated with *P. koreensis.* The diseased fish displayed scale loss, exophthalmia, abdominal swellings, and some haemorrhaging at the fins and gills (Kho et al., 2023). *P. koreensis* was also described as the causal agent of eye lesions in golden mahseer (*Tor putitora*) from India (Shahi & Mallik, 2014).

The common practice in treating bacterial infections involves the application of antibiotics. However, the long-term use and misuse of antibiotics in the aquaculture industry have reportedly caused many adverse effects on humans, fish, animals, and the environment. For instance, the unregulated use of antibiotics in aquaculture exerted a selective pressure on aquatic bacterial species, and thus generated reservoirs of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in the aquatic environment. From these reservoirs, ARB from the aquatic environment may reach humans directly or the ARGs may disseminate to other bacteria through horizontal gene transfer, eventually reaching human pathogens (Amarasiri et al., 2020).

Vaccination is one of the alternatives that have been recommended to be employed in the management of aquaculture as it has been successful in preventing or reducing the