# DEVELOPMENT OF MUCOADHESIVE NANOVACCINE AGAINST COLUMNARIS DISEASE IN RED TILAPIA (*OREOCHROMIS* SP.)

Miss Sirikorn Kitiyodom



A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Veterinary Pathobiology
Department of Veterinary Pathology
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2019

# การพัฒนาวัคซีนอนุภาคนาโนแบบเกาะติดเยื่อเมือก เพื่อป้องกันโรคคอลัมนาริสในปลาทับทิม

น.ส.สิริกร กิติโยดม



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

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| Thesis Title | DEVELOPMENT OF MUCOADHESIVE NANOVACCINE AGAINST COLUMNARIS DISEASE IN RED TILAPIA (*OREOCHROMIS* SP.) |
| By | Miss Sirikorn Kitiyodom |
| Field of Study | Veterinary Pathobiology |
| Thesis Advisor | Associate Professor NAPADON PIRARAT, Ph.D., D.V.M. |
| Thesis Co Advisor | Assistant Professor CHANNARONG RODKHUM, Ph.D.,D.V.M. |
| | TEERAPONG YATA, Ph.D. |

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the FACULTY OF VETERINARY SCIENCE

(Professor ROONGROJE THANAWONGNUWECH, Ph.D.,D.V.M.)

DISSERTATION COMMITTEE

..... Chairman

(Associate Professor THEERAYUTH KAEWAMATAWONG, Ph.D.,D.V.M.)

..... Thesis Advisor

(Associate Professor NAPADON PIRARAT, Ph.D., D.V.M.)

..... Thesis Co-Advisor

(Assistant Professor CHANNARONG RODKHUM, Ph.D.,D.V.M.)

..... Thesis Co-Advisor

(TEERAPONG YATA, Ph.D.)

..... Examiner

(Assistant Professor YAOWALAK PANYASING, Ph.D.,D.V.M.)

..... Examiner

(Assistant Professor DACHRIT NILUBOL, Ph.D.,D.V.M.)

..... External Examiner

(Associate Professor Win Surachetpong, Ph.D.,D.V.M.)

สิริกร กิติโยดม : การพัฒนาวัคซีนอนุภาคนาโนแบบเกาะติดเยื่อเมือก เพื่อป้องกันโรคคอลัมนาริสในปลาทับทิม. ( DEVELOPMENT OF MUCOADHESIVE NANOVACCINE AGAINST COLUMNARIS DISEASE IN RED TILAPIA (*OREOCHROMIS* SP.)) อ.ที่ปรึกษาหลัก : รศ. น.สพ.ดร.นพดล พิษารัตน์, อ.ที่ปรึกษาร่วม : ผศ. น.สพ.ดร.ชาญณรงค์ รอดคำ,ดร.ธีรพงศ์ ยะทา

ปลานิล (*Oreochromis* sp.) เป็นปลาน้ำจืดที่มีความสำคัญและมีผลผลิตสูงมากในประเทศไทย ในการเลี้ยงปลานิลพบว่าปัญหาโรคติดเชื้อคอลัมนาริสเป็นโรคที่มีความสำคัญมีสาเหตุจากเชื้อแบคทีเรีย *Flavobacterium columnare* การป้องกันด้วยวัคซีนจัดเป็นวิธีที่มีความเหมาะสมที่สุดในการป้องกันและควบคุมโรค โดยพบว่าพยาธิกำเนิดของโรคมักเกิดบริเวณเยื่อเมือกของปลาเป็นส่วนใหญ่มักมีรอยโรคบริเวณเหงือกและผิวหนัง ดังนั้นการให้วัคซีนนาโนที่มีคุณสมบัติเข้าเกาะติดเยื่อเมือกจึงมีความเหมาะสมในการป้องกันโรคคอลัมนาริส โดยในการศึกษาครั้งนี้เราได้ทำการคัดเลือกเชื้อสายพันธ์ที่เหมาะสมในการนำมาพัฒนาวัคซีน เป็นเชื้อก่อโรคที่แยกได้จากพื้นที่การเลี้ยงจริง ทำการศึกษาลักษณะรูปร่างและลักษณะของเชื้อด้วยหลักทางชีวโมเลกุล พร้อมทั้งศึกษาความรุนแรงของเชื้อ การศึกษาวิจัยครั้งนี้เราได้พัฒนานาโนวัคซีนลูกผสมไคโตซาน (CS-NE) ผ่านวิธีอิมัลชิฟิเคชั่น และเทคนิคการทำให้เป็นเนื้อเดียวกัน ตามด้วยการห่อหุ้มอนุภาคด้วยพอลิเมอร์แบบเกาะติดเยื่อเมือก “ไคโตซาน” หลังจากนั้นทำการตรวจคุณสมบัติทางเคมีกายภาพของ CS-NE คุณสมบัติการเข้าเกาะติดเยื่อเมือก ประสิทธิภาพของวัคซีน และการประเมินการตอบสนองทางภูมิคุ้มกัน พบ CS-NE มีคุณสมบัติประจุบวก ขนาดเล็กระดับนาโน มีรูปร่างขนาดกลม และมีความสามารถในการเข้าเกาะติดเยื่อเมือกได้อย่างยอดเยี่ยม ดังแสดงผ่านกล้องจุลทรรศน์แบบอิเลคตรอน, ฟลูออเรสเซนต์ และเครื่องสเปคโตโฟโตมิเตอร์ หลังจากนั้นนำวัคซีนที่เตรียมได้ทำการให้วัคซีนด้วยการแช่นาน 30 นาที ทำการทดสอบด้วยการแช่เชื้อพิษในห้องปฏิบัติการที่ 30, 60, 90 และ 120 วันหลังการให้วัคซีน พบอัตราการตายในกลุ่มปลาที่ไม่ได้ให้วัคซีนคือ 89, 91, 71 และ 61% ตามลำดับ และปลาในกลุ่มที่ได้รับวัคซีน CS-NE มีค่าอัตรารอดสัมพัทธ์ (RPS) คือ 78, 61, 50 และ 36 ตามลำดับ ซึ่งจากผลการทดลองจะเห็นได้ว่าการออกแบบวัคซีนจำลองแบบคุณสมบัติเข้าเกาะติดเยื่อเมือกของเชื้อแบคทีเรียเชื้อเป็นมีส่วนช่วยอย่างมากในการเข้าเกาะติดเยื่อเมือกและดูดซึมของแอนติเจน ดังแสดงให้เห็นจากการศึกษาทางเนื้อเยื่อวิทยาของ MALT ในการศึกษานี้ได้ทำการประเมินการตอบสนองทางภูมิคุ้มกันของปลาที่ได้รับวัคซีน CS-NE ได้แก่ serum bactericidal activity: SBA , ELISA-IgM จำเพาะต่อเชื้อ *F. columnare* และการแสดงออกของยีน พบ SBA และ ELISA-IgM มีปริมาณสูงขึ้น มีลักษณะทางเนื้อเยื่อวิทยาของ MALT ที่มีการตอบสนองที่มากขึ้นของเซลล์เม็ดเลือดขาวและการเก็บกินแอนติเจน สอดคล้องกับผลการแสดงออกที่เพิ่มขึ้นของยีน *IgT*, *IgM*, *TNF* $\alpha$, *IL1*$\beta$ and *MHC-1* ในเหงือก ไตส่วนหน้าและม้าม จากผลการศึกษาแสดงให้เห็นว่าการแช่วัคซีนแบบเกาะติดเยื่อเมือกเป็นวิธีที่มีประสิทธิภาพในการป้องกันและควบคุม โรคคอลัมนาริสในปลานิลได้



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| สาขาวิชา | พยาธิชีววิทยาทางสัตวแพทย์ | ลายมือชื่อนิสิต ..... |
| ปีการศึกษา | 2562 | ลายมือชื่อ อ.ที่ปรึกษาหลัก ..... |
| | | ลายมือชื่อ อ.ที่ปรึกษาร่วม ..... |
| | | ลายมือชื่อ อ.ที่ปรึกษาร่วม ..... |

# # 5975523531 : MAJOR VETERINARY PATHOBIOLOGY

KEYWORD: Tilapia, Nanovaccine, chitosan, Mucosal immunity, columnaris disease

Sirikorn Kitiyodom : DEVELOPMENT OF MUCOADHESIVE NANOVACCINE AGAINST COLUMNARIS DISEASE IN RED TILAPIA (*OREOCHROMIS* SP.). Advisor: Assoc. Prof. NAPADON PIRARAT, Ph.D., D.V.M. Co-advisor: Asst. Prof. CHANNARONG RODKHUM, Ph.D.,D.V.M., TEERAPONG YATA, Ph.D.

Tilapia (*Oreochromis* sp.) is very important and high production of freshwater fish in Thailand. Columnaris disease has been now recognized as one of the most serious infectious diseases in farmed tilapia. The disease is caused by *Flavobacterium columnare*. Among the prevention and control strategies, vaccination is one of the most effective approach. According to the pathogenesis of this bacteria, the characteristic lesion is almost at mucosal area of skin and gill. Therefore, hypothesized that the mucosal nanovaccine with mucoadhesive characteristic could be suitable vaccination method to control columnaris disease. In this study, we determined vaccine strain candidate by clinical field isolation, morphology and molecular characterization and virulent ability test. We prepared chitosan-complexed nanovaccines (CS-NE) through emulsification and homogenization techniques followed by coating with mucoadhesive polymer chitosan. The physiochemical properties of CS-NE were analyzed. Their mucoadhesive characteristics, vaccine efficacy and immune responses were also evaluated. The analysis of hydrodynamic diameter and zeta-potential also indicated the successful modification of CS-NE that were positively charged, nano-sized and spherical. In vivo mucoadhesive study demonstrated the excellent affinity of the CS-NE toward fish gills as confirmed by TEM, bioluminescence imaging, fluorescent microscopy, and spectrophotometric quantitative measurement. Following vaccination with the prepared nanovaccines by immersion 30 mins, the challenge test was then carried out 30-60-90- and 120-days post-vaccination and resulted in 89,91,71 and 61 % mortalities, respectively in the control fish. The RPS of CS-NE vaccinated fish was calculated at 78,61,50 and 36, respectively. As a result, the formulated biomimetic nanovaccine mocking the mucoadhesive characteristic of live *F. columnare* can help achieve better adsorption on mucosal surfaces and more efficient vaccine efficacy that revealed in MALT histology. We evaluated immune response of CS-NE fish vaccinated include serum bactericidal activity, ELISA-IgM specific *F. columnare*, MALT histology and relative gene expression. Significantly higher serum bacterial activity and ELISA-specific IgM antibodies in CS-NE was also seen. The MALT histology revealed a significant higher leucocyte cell accumulation and antigen uptake, in accordance with our result of up-regulation of *IgT, IgM, TNF* $\alpha$*, IL1*$\beta$ and *MHC-1* genes in gill, kidney and spleen. Our study demonstrated the feasibility of mucoadhesive nanovaccine-immersion vaccination as an effective delivery method for prevention and control columnaris disease in tilapia.



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| Field of Study: | Veterinary Pathobiology | Student's Signature ..... |
| Academic Year: | 2019 | Advisor's Signature ..... |
| | | Co-advisor's Signature ..... |
| | | Co-advisor's Signature ..... |

# ACKNOWLEDGEMENTS

First, and most of all, I would like to deeply thank Associate Professor Dr. Nopadon Pirarat for his invaluable mentorship and guidance throughout the process of this study. I would like to extend my sincere gratitude to Assistant Professor Dr. Channarong Rodkhum for his assistance and suggestions. I would like to special thanks to Dr. Teerapong Yata for helping me in numerous ways and giving me many wonderful learning experiences.

I would like to thank my thesis committee members and researcher : Associate Professor Dr. Theerayuth Kaewamatawong, Assistant Professor Dr. Dachrit Nilubon, Associate Professor Dr. Win surachetpong, Assistant Professor Dr. Yaowalak Panyasing, Dr. Sasithon Temisak and Dr. Katawut namdee for their valuable suggestions and encouragement.

I would like to acknowledge with heartfelt thanks to all grant for supporting this work include the 90th Anniversary of Chulalongkorn University Scholarship; GCUGR1125622098D, National Nanotechnology Center; P1752586 and P1751449, Ratchadaphiseksomphot Endowment Fund; CU-GR_61_013_31_003 and Thailand Graduate Institute of Science and Technology Scholarship; SCA-CO-2560-4578-TH, National Science and Technology Development Agency (NSTDA, Thailand).

Last of all, I would like to thank my family, my colleague, my friends and everyone else who give encouragement and support to be able to finish this chapter.

Sirikorn Kitiyodom

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# CHAPTER I

# INTRODUCTION

## Importance and rationale

Aquaculture has become a great source of high-quality protein worldwide. Globally, fish currently represent approximately 17% of animal protein supply and 6.7% of all protein for human consumption. Tilapia (*Oreochromis* sp.) is very important freshwater fish in Thailand. It has high nutritional profile. The production of tilapia is a high value and a main culture species of freshwater aquaculture in Thailand that is about half of all production from freshwater aquaculture (DOF, 2019). The current trend in aquaculture is toward increased intensification of aquatic production. Many factors predispose for fish disease such as overcrowding, climate change and poor farm management. Fish disease problems caused by bacteria, virus, fungi, parasites and other undiagnosed (Bondad-Reantaso et al., 2005). Bacterial infection caused by *Flavobacterium columnare*, the causative agent of columnaris disease, has been now identified as one of the most serious infectious diseases in farmed tilapia. This bacterium distributes worldwide and one of the important bacterial diseases in fish (Shoemaker et al., 2011). This pathogen is a gram negative, thin, rod and filamentous bacterium with gliding motility and yellow rhizoid colony formation (LaFrentz and Klesius, 2009; Shoemaker et al., 2007). *F. columnare* infections may result in skin lesions, fin rot and gill necrosis, with a high degree of mortality 60 to 90%, leading to severe economic losses (Declercq et al., 2013). It is well established that vaccination is the most effective approach for prevention of infectious diseases in aquaculture (Austin, 2012). The immune system of fish is like higher vertebrates. It has the innate and adaptive immune response (Castro and Tafalla, 2015). Aquaculture vaccines are roughly administered through major three routes i.e. bath or immersion, in-feed or oral, and injection (Gudding et al., 2014). While immersion vaccination is the most applicable mode of delivery of these routes

of administration, that is easy of administration, lack of stress induction and suitability for mass vaccination at all ages (Austin, 2012; Soto et al., 2015). This method suffers from low potency as the efficiency of antigen uptakes through the gills and skin is limited (Gudding et al., 2014). Therefore, in this study, we will develop a mucoadhesive nanovaccine delivery system to circumvent this problem. Nanovaccine is used of nanoparticles as adjuvants and efficient delivery systems in fish vaccine development. This is used to improve administration and efficacy of vaccines that help for increase immunostimulatory properties, increase delivery efficiency (site specific delivery of antigens), reduction of the dose, reduction of the adverse effect, enhanced bioavailability, helped release and protection of antigen from degradation (Aklakur et al., 2016; Zhao et al., 2014). Nanotechnology is the administer of tiny particles varying in size, shape, composition, and surface properties. Nanoparticles can be facilitating the cellular uptake of antigens, increase the ability of antigen presentation and induce specific immune response against the antigen. The nanoparticles have many types such as polymeric nanoparticles, nanoliposomes, carbon nanotubes and ISCOMs. In this study, we are interested and used polymeric nanoparticles to develop mucoadhesive nanovaccine. Polymeric nanoparticles have the capacity to conjugate or encapsulate antigens within itself or on their surface (Aklakur et al., 2016; Ji et al., 2015; Zhao et al., 2014). Chitosan (CS), sometimes known as deacetylated chitin found in the exoskeletons of crustaceans, is a natural polycationic linear polysaccharide that exhibits mucoadhesive properties (Esmaeili et al., 2010; Saikia et al., 2015). Among polymers, chitosan has been exploited for the design of mucoadhesive dosage forms due to its excellent biocompatibility and biodegradability (M Ways et al., 2018; Najafi-Hajivar et al., 2016). It could stimulate good adaptive immune response, both cellular and humoral against the conjugated antigen (Arca et al., 2009).

Tilapia have four mucosal associated lymphoid tissue (MALTs) that nasopharynx-associated lymphoid tissue (NALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and gut-associated lymphoid tissue (GALT). As immunological sites, mucosae can increase a robust immune response after vaccination. MALTs are a disperse location of leukocytes. They have humoral and cellular immunity response in these mucosal lymphoid tissues (Castro and Tafalla, 2015; Rombout et al., 2014). The external constituent of skin, gills, and gut is a mucous gel secreted by various epidermal or epithelial mucus cells which forms a layer of a gel-like substance covering the epithelial cells (Koshio, 2016). More importantly, these organs are directly associated with the mucosal immunity of fish (Guardiola et al., 2014). The fish mucus is mainly composed of water and glycoproteins, containing vast number of mucins, high molecular weight negatively charged oligosaccharides (Grosell et al., 2010; Perez-Vilar, 2007). Since fish gills are considered a mucosal surface associated with the mucosal immunity, targeting mucoadhesive vaccines to the mucosal surface could be exploited as an effective method for immersion vaccination. It has been suggested that electrostatic force attraction is crucial for the mucoadhesive mechanism, which is affected by the complexation between positively charged polymer and negatively charged materials such as cell surface and mucin in a biomembrane environment (Silva et al., 2012). Therefore, we hypothesized that a mucoadhesive polymer could be exploited to deliver antigen preparation to mucosal membranes of tilapia. Immersion vaccination work on the MALTs that main target for mucosal vaccine development (Munang'andu et al., 2015). Also, we hypothesized that mucoadhesive polymer could be exploited to deliver antigen preparation to mucosal membrane of tilapia. In this study, we research to design, develop, produce, analyze and evaluate immersion mucoadhesive nanovaccine for protection against *F. columnare* infection in red tilapia.

**Objectives**

1. To physicochemically characterize and to design the mucoadhesive nanovaccine prepared from inactivated *F. columnare*.
2. To investigate the mucoadhesive properties of the prepared mucoadhesive nanovaccine.
3. To evaluate the immune responses and protective efficacy of the prepared nanovaccine against *F. columnare* immersion challenge.

**Hypotheses**

1. The mucoadhesive nanovaccine can develop and produce against columnaris disease in tilapia.
2. The mucoadhesive nanovaccine has mucoadhesive properties effect on mucosal area of tilapia.
3. The mucoadhesive nanovaccine can induce immune responses and modulate high protective efficacy against columnaris disease in tilapia.

**Advantages of Study**



1. This study will develop the mucoadhesive formalin killed nanovaccine prototype that can be used to against *F. columnare* infection in Tilapia.
2. This study showed Nano delivery system to improve efficacy of inactivated immersion vaccine.
3. This study might lead to understand about the mucoadhesive formalin killed nano vaccine can stimulate immune system and reduce mortality loss of *F. columnare* infection in Tilapia.

Conceptual Framework

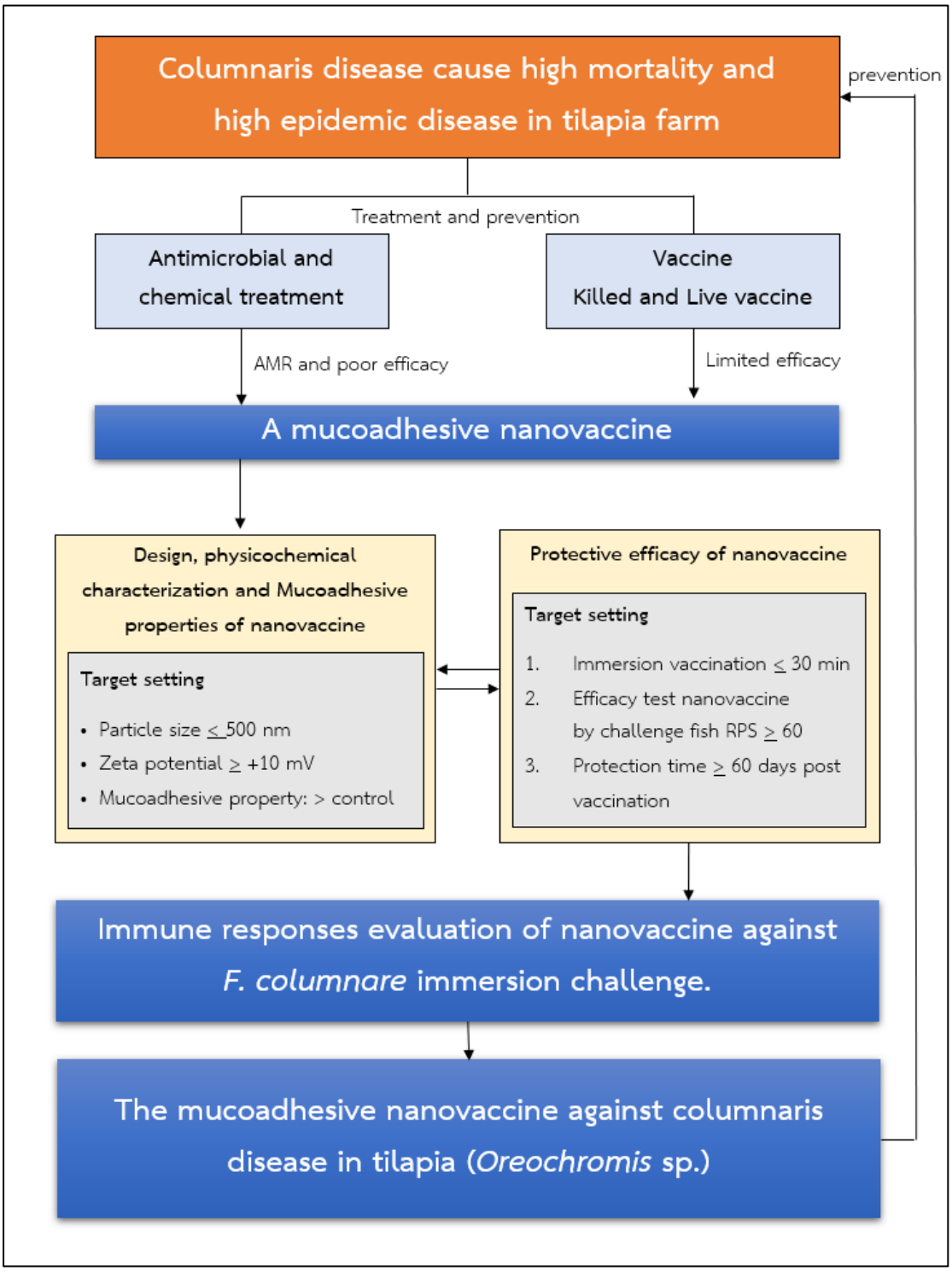


**Figure 1:** Conceptual framework

# CHAPTER II

# Literature Review

## 2.1 Tilapia (*Oreochromis* sp.)

Tilapia is classified in the cichlid family, genus Oreochromis and phylum Chordata. Tilapia is a very important freshwater fish in Thailand with a high nutritional profile, fast growth, tolerance to poor water quality and disease resistance. In 2017, freshwater production of tilapia in Thailand at 217,928 tonnes accounted for 53% of all production (413,263 tonnes) from freshwater aquaculture (Figure 2). Areas for tilapia production in Thailand include northeastern, central, northern and southern regions (DOF, 2019).

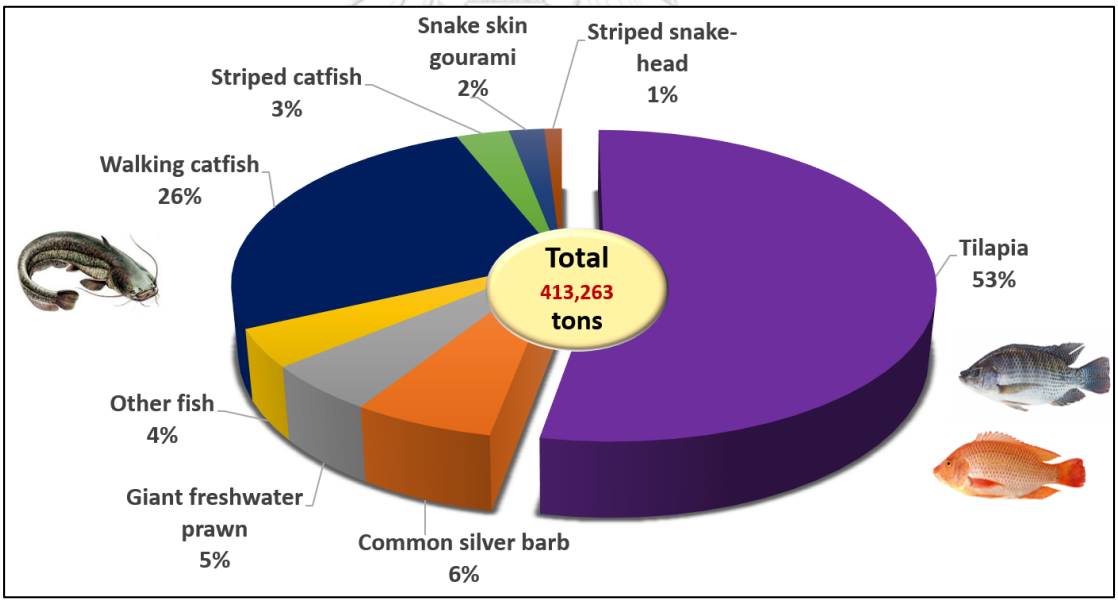


**Figure 2:** Production of freshwater aquaculture by species in Thailand, 2017 (DOF, 2019)

Culture of red tilapia has increased rapidly in Thailand because of its higher value relative to Nile tilapia. The principal cultured strain of Thai red tilapia is a hybrid comprising a gene pool of two main species: *Oreochromis niloticus* and *O. mossambicus* (Figure 3). The Thai red tilapia was developed by the Department of Fisheries from the red variant *O. mossambicus* that was originally found in Thailand in 1968 (Pongthana et al., 2010).

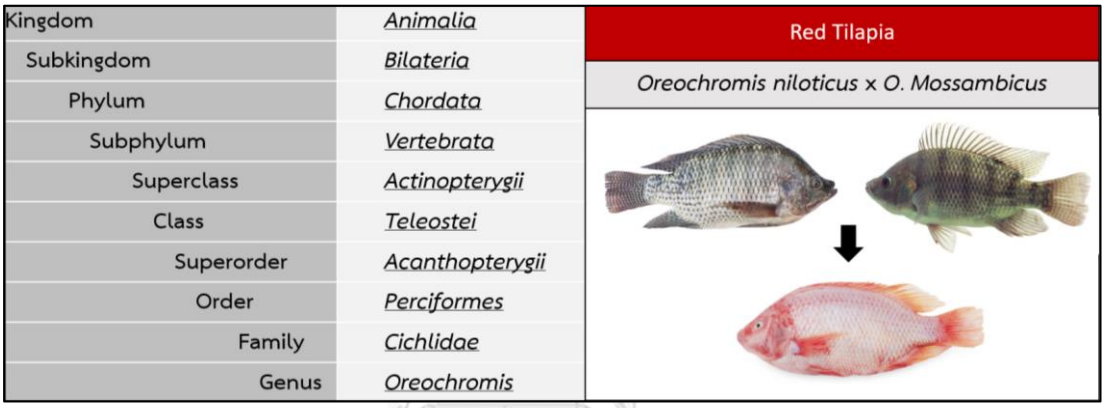


**Figure 3:** Taxonomy of the Thai red tilapia (*Oreochromis* sp.)

Tilapia culture has increased rapidly and trended to intensive farming. However, many predisposing factors such as high density, climate change and poor farm management encourage disease outbreaks. Causative agents include bacteria, viruses, fungi, parasites and other undiagnosed elements (Bondad-Reantaso et al., 2005). Bacterial infectious diseases are considered to be the main cause of economic losses in tilapia culture. Columnaris disease is a prominent bacterial infection caused by *Flavobacterium columnare* in freshwater fish, with outbreaks emerging and reemerging in Thai tilapia culture (Dong et al., 2015a; Grabowski et al., 2004).

### 2.2 Tilapia immune system and mucosal immunity

The immune system of fish is similar to higher vertebrates and can be divided into two components as the innate (non-specific) and the adaptive (specific) immune responses. Innate immunity is the first line of defense against infection and is

commonly divided into three compartments. First, physical barriers include the skin, gills, gut and mucus that contains lectins, lysozymes, pentraxins, complement proteins, antibacterial peptides and immunoglobulin. Second, humoral immune response contains antimicrobial enzymes, complement proteins, non-specific proteins and other cytokines/chemokines. Finally, cellular immune response contains nonspecific cytotoxic cells and phagocytosis by macrophage and dendritic cells. The adaptive immune response is composed of humoral and cellular responses that require specific antigen recognition and response. Adaptive immunity is generated via specific effector cells that contain B and T lymphocytes, immunoglobulin, helper T cells, memory T cells, cellular cytotoxicity and other cytokines (Castro and Tafalla, 2015; Koppang et al., 2010; Munang'andu et al., 2015).

An encounter with a pathogenic organism through mucosal tissues is initially blocked by physical barriers such as mucus, scales and the epithelium. Fish mucus contains different humoral components with antimicrobial activity such as complement factors, lysozyme or immunoglobulins (Igs). If the pathogen succeeds in penetrating the epithelium, it encounters innate cellular immunity, triggered as the first step by pattern recognition receptors (PRRs) that recognise common pathogen-associated molecular patterns (PAMPs) characteristic of many microbial agents. The uptake of the antigen primes and releases cytokine mediators that attract different cell types to release inflammatory processes and antigenic presentation through major histocompatibility complex (MHC) expression in the lymphoid tissues to activate the primary responses of antigen-specific lymphocytes. These variable receptors are able to specifically recognise the molecular characteristics of the pathogen, setting the basis for further secondary responses and memory (Castro and Tafalla, 2015; Munang'andu et al., 2015).

The lymphoid organs in fish (Figure 4) are the thymus, the head kidney and the spleen. Mucosal surfaces are the first physical barrier to protect the fish as active immune tissue. Four mucosal-associated lymphoid tissues (MALTs), including the gut (GALT: gut-associated lymphoid tissue), skin (SALT: skin-associated lymphoid tissue), gills (GIALT: gill-associated lymphoid tissue) and nostril (NALT: nasopharynx-

associated lymphoid tissue) are immune responses that function in fish (Parra et al., 2015; Salinas et al., 2011).

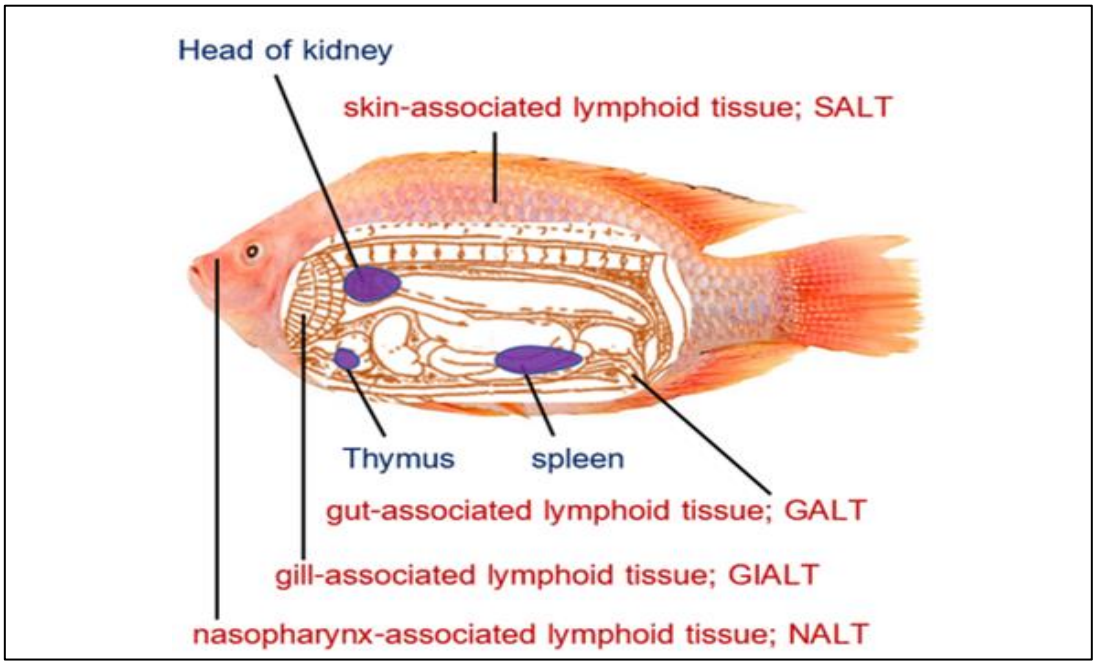


**Figure 4:** The lymphoid organs and MALTS in tilapia

Humoral and cellular immunity influence the response in these mucosal lymphoid tissues. Mucosal B-lymphocytes and immunoglobulins (Igs) are important players in the immune response. The main function of B cells is to produce immunoglobulin (Ig) on their surface and secrete antigen-specific antibodies in response to immune challenges. Teleost express three different Ig isotypes on the B cell surface including IgM, IgT/Z and IgD. IgT/Z reveals many functional similarities with mammalian IgA, while Ig plays a main role in adaptive immunity by recognizing and eliminating pathogens through various processes such as complement activation and phagocytosis. Functions of lymphocytes include innate and adaptive responses in teleost fish (Fillatreau et al., 2013; Mashoof and Criscitiello, 2016).

Cellular-mediated immunity is present in all mucosal organs, while production of T and B cells is specific for antigens. Teleost fish also have thymus-derived T cells that can be subdivided into distinct subpopulations as cytotoxic T cells, helper T cells, regulatory T cells and non-specific cytotoxic cells. Although

many fish T cell-specific antibodies are now available, those that recognize well-characterized T cell molecules are unavailable in many species. Previous studies have described genes encoding a number of cell marker molecules including *Cd3*, *Cd4*, *Cd8*, *MHC-I* and *MHC-II* in a variety of fish species. The increasing availability of relevant antibodies will improve our understanding of fish immune systems. T cells are abundant in mucosal tissues of teleosts and it is already known that teleost mucosal contains abundant numbers of T cells. The route of antigen delivery into antigen presenting cells (APCs) is deterministic of the type of cellular-mediated immune response induced by vaccination. Recognition of antigens is dependent on *MHC-I* or *MHC-II* molecules that bind and present antigens to the T cells (Castro and Tafalla, 2015; Koppang et al., 2010; Munang'andu et al., 2015; Parra et al., 2015; Salinas, 2015).

In fish, antigens enter through all four mucosal-associated lymphoid tissues (MALTs) during immersion/bath vaccination. Immersion vaccination is the best practical administration route to induce mucosal responses and MALTs are an important target for mucosal vaccine development and formulation. Analyses of vaccine efficacy have focused on relative percent survival (RPS), post challenge accumulative mortality, gene expression analysis, pathogen load measurement, histological analysis and levels of specific IgM antibodies against pathogens in both mucosal and systemic compartments (Munang'andu et al., 2015; Soto et al., 2015).

**2.3 Mucosal associated lymphoid tissue (MALT)**

MALT, as the mucosal areas of the respiratory, integument and digestive systems (gills, nasal, skin, intestine and hind gut) are in contact with the external environment and often exposed to pathogens. The immune system prevents pathogen entry or mounts a local immune response. Fish live in aquatic environments where microorganisms are more abundant than in terrestrial environments. The whole-body surface of fish is covered by mucus, which is one of the initial immune barriers inhibiting the attack of pathogens. Teleost fish lack some lymphoid structures such as germinal centers, B cell follicles and lymph nodes.

However, teleost fish have diffuse lymphoid cells, with the absence of organized lymphoid structures, as established characteristics of mucosal-associated lymphoid tissue (MALT) that plays a predominant role regarding immunoglobulin T-IgT antibodies which have specialized mucosal immunity and possess similar functions to mammalian IgA. Importantly, all four MALTs include gill-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT), gut-associated lymphoid tissue (GALT) and skin-associated lymphoid tissue (SALT) (Salinas, 2015; Zhang et al., 2010).

Mucosal delivery of vaccines as immersion or oral immunization is the chosen vaccination method against infectious diseases in aquaculture. These vaccination applications significantly decrease the working cost of vaccination in aquaculture and are suitable methods for mass vaccination. Vaccine antigens that are administered via the mucosal route are taken up by the MALTs of teleost fish as GiALT (Kato et al., 2013; Korbut et al., 2016; Ohtani et al., 2015), NALT (Tacchi et al., 2014); GALT (Adelmann et al., 2008; Korbut et al., 2016; Ohtani et al., 2015), SALT (Ototake et al., 1996). Most leukocyte types are present in fish MALTs, including leucocytes and phagocytes (neutrophils, macrophages, DCs, B cells, T cells and plasma cells (Rességuier et al., 2017; Salinas, 2015; Zhang et al., 2010).



**Gill associated lymphoid tissue: GIALT**

The gills are bilaterally located on either side of the pharynx and lie on cartilaginous bases which are called gill arches. The gills consist of four paired arches, each containing two rows of posterior laterally orientated filaments (primary lamellas) covered by respiratory epithelium. The filaments are supported along the proximal third of their length by an interbranchial septum of connective and muscle tissue. The position and fundamental structures of the gills of teleosts are explained in Figure 5. The gills form a similar arch-like arrangement with interfering branchial slits for the water to pass laterally from the buccopharyngeal cavity through the gills and out. Fish gill morphology of teleosts presents as an interbranchial septum,

parting the ends of the filaments and separated from the hind part opening of the operculum (Reece et al., 2012; Wilson and Laurent, 2002).

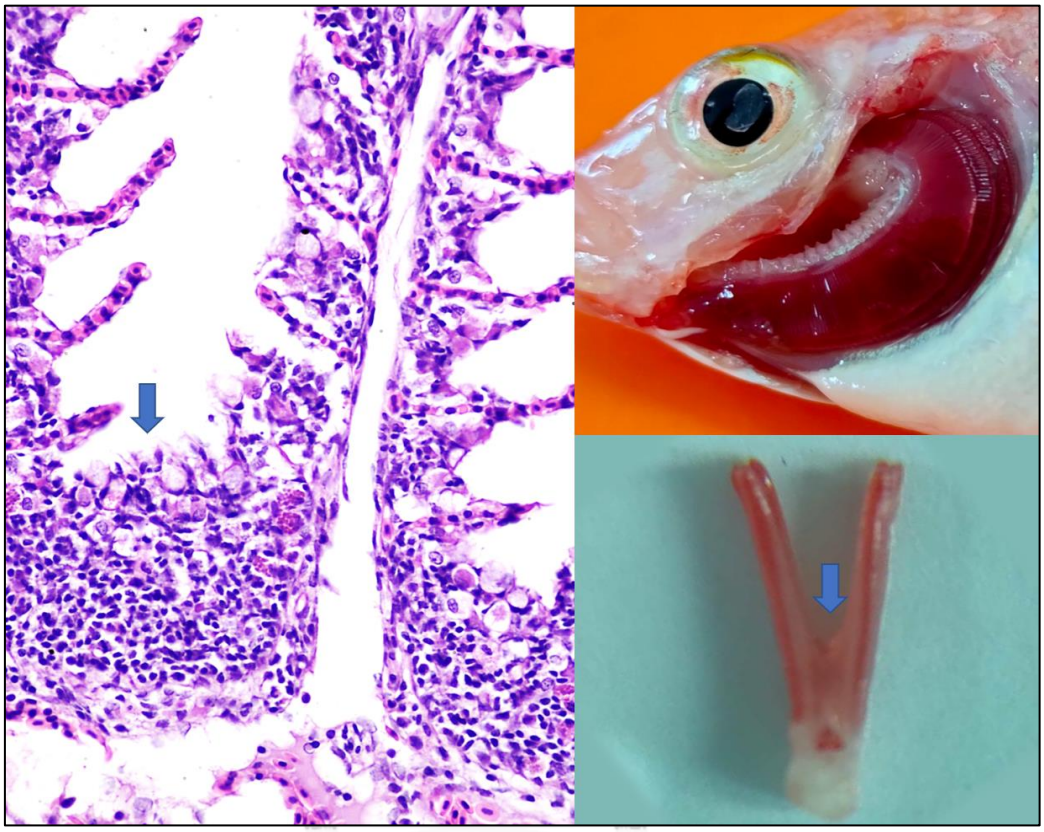

**Figure 5:** The presence of gill-associated lymphoid tissue (GIALT) in the inter-filament space in tilapia.



The basic functional unit of the gill is the filament, which supports rows of plate-like lamellae. The lamellae (secondary lamellae) are premeditated for gas exchange and consist of a hollowed flap-shaped structure surrounded by a thin epithelium. An intense blood flow runs in-between supportive cells called pillar cells. The lamellae are positioned for the blood flow to be counter-current to the water flow over the gills. Water flows over the filaments from the efferent aspect to the afferent aspect, in the opposite direction of the blood flow. The lamellar gas-exchange surface is covered by epithelium squamous cells (pavement cells), mitochondria rich cells (chloride cells), while ionocytes and mucous cells are found

frequently in the filament epithelium. Demands for ion regulation can often upset this balance (Evans et al., 2005; Reece et al., 2012; Wilson and Laurent, 2002; Zayed and Mohamed, 2004).

The respiratory epithelium has multifunctional purposes. These include gas and ion transport, nitrogenous waste excretion and hormone production (Evans et al., 2005). The mucosa forms a barrier between the pathogen and the external environment. Numerous reports have revealed the presence of a highly developed and active immune system. The gills form mucous surfaces created for selective transport and mechanisms to prevent infections. The physical barrier of the fish gills consists of the gill epithelium, a glycocalyx layer and the mucus layer (Powell et al., 1994). In addition, gill-associated lymphoid tissues (GIALT) adjust interactions with local microbiota and prevent pathogen infections. A GIALT structure has been found in teleosts in the interbranchial lymphoid tissue (ILT) shown in Figure 5. Modern teleosts have lymphoepithelium at the base of primary gill filaments, while the gills demonstrate the presence of T lymphocytes embedded in a meshwork of reticulated epithelial cells in ILT (Reece et al., 2012; Wilson and Laurent, 2002).

Innate and adaptive immune system components have been detected in the gills such as Mx, MHC I, MHC II and T cell receptor (TCR) transcripts, Immunoglobulin M and Immunoglobulin T (Haugarvoll et al., 2008; Rességuier et al., 2017; Takano et al., 2004). The level of major histocompatibility complex (MHC) transcripts in the gills was relatively high compared to the head kidney and spleen (Koppang et al., 1998). The ILT must be considered as a part of the gill-associated lymphoid tissue (GIALT), which is defined as one of the four main mucosal immune compartments found in teleost. The caudal edge of the interbranchial septum and intraepithelial tissue at the base of the gill filaments in teleosts reported here may be a suitable location for immune surveillance of gill infections, as well as a target site for new vaccine approaches. Research results of epithelial immunity showed leukocyte accumulations, lymphocyte cell aggregation and cell proliferation within a teleost gill epithelium network. In fish mucosal membranes, intraepithelial leukocytes mainly

consist of T cells and some macrophages (Haugarvoll et al., 2008; Kato et al., 2013; Rességuier et al., 2017; Takano et al., 2004; Zapata and Amemiya, 2000).

**Nasopharynx-associated lymphoid tissue: NALT**

The NALT is the first line of immune protection. The NALT in humans and terrestrial animals is considered as the first line of defense against airborne antigens and used for nasal delivery of vaccine. The NALT is present like other teleost MALTs. NALT contains diffuse lymphoid cells and lacks tonsils and adenoids. Teleost-NALT is capable of mounting strong innate and adaptive immune responses for nasal vaccine delivery. The NALT-olfactory system is also capable of raising strong innate and adaptive immune responses. Nasal vaccination is a very effective and new method for the control of both viral and bacterial aquatic infectious diseases in fish (Sepahi and Salinas, 2016; Tacchi et al., 2014).

**Gut associated lymphoid tissue: GALT**

The most significant GALT in tilapia is comprised of diffuse populations of leucocytes in both the epithelium and lamina propria. Lymphoid cells in both the epithelium and lamina propria of the intestine may also participate in processing particles/antigens, with presentation to other lymphoid cells in the gut that comprise macrophages and epithelial lymphocytes that are apparent in enterocyte vesicles and in the lamina propria (Doggett and Harris, 1991; Rombout et al., 1993; Salinas, 2015). The gut mucosal associated lymphoid tissue in fish is different from mammals because the former lacks Payer' s patch and antigen transporting M cells. It is normally composed of organized lymphoid cells, macrophages and granulocytes. Intraepithelial lymphocyte: IEL, the component of gut-associated lymphoid tissue, plays a major role in the mucosal defense mechanism response against intraluminal foreign antigens. Teleost-GALT is capable of raising local immune responses for vaccine delivery. These observations confirm that highly efficient antigen uptake

takes place in the hind-midgut region (Doggett and Harris, 1991; Kiristioglu et al., 2002; Rombout et al., 1993).

Based on present knowledge, immune cells in the teleost intestine as $CD8^+$/TCR$\alpha\beta^+$ T cells dominate the $CD4^+$ subset. Most TCR$\gamma\delta^+$ T cells are almost certainly CD8$\alpha^+$. Most B cells among intraepithelial lymphocyte: IEL are IgT/Z+, while IgM+ B cells are present in the connective tissue (lamina propria). A part of the intraepithelial tissue may contain non-specific cytotoxic cells (NCC), indicated as small granular lymphocytes. Antigen presenting cells (APC) are also shown. Commensal microbes are coated with Ig. Polymeric immunoglobulins are considered as the main players of mucosal defence, while the polymeric Immunoglobulin Receptor (pIgR) plays an important role in the transport of immunoglobulin molecules. The extracellular part of the receptor is divided by a proteinase and co-secreted with the IgT or IgM as a protective secretory component (SC). Transport of immunoglobulins by pIgR towards the lumen, cleavage of pIgR extracellular component and delivery to the mucus occurs as a pIg-SC complex or as SC alone. The presence of dendritic cells in fish gut is debatable (Doggett and Harris, 1991; Rombout et al., 1993; Rombout et al., 2014; Salinas, 2015).



**Skin associated lymphoid tissue: SALT**

The skin acts as a mucosal surface that conceals abundant mucus-producing cells, lacks keratinisation and retains living epithelial cells in direct contact with water. Teleost fish represent a skin-associated adaptive immune system containing immunoglobulins (Igs). Teleosts contain IgT (also called IgZ in some species) as a mucosal tissue. Teleost fish have been shown to contain a skin-associated lymphoid tissue (SALT), which was reported to contain secretory cells that produce mucus (i.e., goblet cells), lymphocytes, granulocytes, macrophages and Langerhans-like cells (Lovy et al., 2008; Lugo-Villarino et al., 2010; Salinas et al., 2011). The skin of fish lacks keratinization and is coated by a mucosal layer that lacks organized lymphoid structures. Interestingly, the structure of the teleost SALT resembles that of the gut-

associated lymphoid tissue (GALT). B cell and Ig responses occur in the skin of teleosts. The structural characteristics of IgT in skin mucus look like gut mucus IgT, while the IgT/IgM ratio in skin is much higher than that found in serum. However, skin mucus IgT amounts were significantly lower than found in gut mucus (Løken et al., 2020; Salinas et al., 2011; Xu et al., 2013).

**2.4 Immune related genes in tilapia**

Many researchers used the mRNA expression of cytokine genes as a tool to measure immune responses against pathogenic bacteria. These are important mediators of the immune system and represent an essential part of innate and adaptive immune responses in fish (Low et al., 2003; Panigrahi et al., 2004).

**Interleukin 1β: *IL1β***

Interleukin-1β is a potent pro-inflammatory cytokine that is essential for host-defence responses to infection and damage. IL-1β has been shown to be protective against several bacterial, viral and fungal infections. It is produced and secreted by a variety of cell types such as monocytes and macrophages. Interleukin 1β is secreted and circulated systemically, whereas IL-1α is generally associated with the plasma membrane of the producing cell and so acts locally. IL-1β is mainly produced by monocytes and macrophages, whereas IL-1α expression is more widespread. The two genes are regulated differentially during development and in response to environmental changes. This results in different functional contributions from these cytokines during immune responses (Mantovani et al., 2019). IL-1B is produced as an inactive 31 kDa precursor, termed pro-IL-1β, in response to molecular motifs carried by pathogens called 'pathogen associated molecular patterns' (PAMPs) that act through pattern recognition receptors (PRRs) on macrophages to regulate pathways that control gene expression. The primed cell should confront a further PAMP, or DAMP (danger associated molecular pattern as endogenous molecules released from

dead cells) to induce the processing and secretion of an active IL-1β molecule. IL-1β applies its protective action against infections by activating several responses including the rapid recruitment of neutrophils to inflammatory sites, activation of endothelial adhesion molecules, induction of cytokines/chemokines and the stimulation of specific adaptive immunity. The inflammatory response to infection consists of several protective effector mechanisms. Among the proinflammatory cytokines, IL-1β offers peak potential to cause damage to the host tissues. Several mechanisms are devoted to controlling its transcription and processing by inflammasome action (intracellular) and inhibition of its receptor signaling through IL-1ra and type II decoy receptors (extracellular) (Dinarello, 1996; Mantovani et al., 2019; Sahoo et al., 2011; Takeuchi and Akira, 2010).

In fish, many studies have confirmed the rapid induction of IL-1β expression in response to inflammatory stimulation including acute phase-response, activation of macrophages, subsequent secretion of cytokines such TNF and IL-6 and activation of T, B and NK cells. In fish leukocytes, IL-1β is transcriptionally upregulated during infection and proinflammatory stimuli. Moreover, teleost IL-1β show their ability to stimulate immune cell proliferation and proinflammatory factor expression (Balm et al., 1995; Uribe et al., 2011; Vallejo et al., 1992; Wang et al., 2009).



**Tumor necrosis factor-alpha: *TNFα***

The classical pro-inflammatory cytokines are IL-1β and TNF-α. TNF-α is a type II transmembrane glycoprotein, with an extracellular C-terminal domain and a cytoplasmic tail. It can be found both as a membrane-bound and a soluble molecule. TNF-α protein stimulates phagocytic activity (Zou and Secombes, 2016), thereby promoting leukocyte recruitment and activation to the inflammatory process. TNF-α is a cytokine that participates in systemic inflammation and regulation of immune cells. It is produced mainly and activated macrophages as a membrane or secreted form. TNF-α is mainly produced by activated monocytes and

macrophages, applies many immunological functions such as regulating inflammation and cellular immune responses, hemorrhagic necrosis of transformed tumours, enhancing neutrophil phagocytosis and cytotoxicity, and modulation of the expression of many cytokines including IL-1, IL-6 and chemokines The active form of TNF**α** binds to two distinct receptors of the cell surface, TNFR1 and TNFR2, which provoke different cellular responses including cellular differentiation, proliferation and apoptosis. No data are available on the bioactivity of TNF-**α** in sharks, whereas teleost fish show the presence of TNF-**α** and TNF-**α** receptors. TNF-**α** was shown to enhance teleost macrophage respiratory burst activity, neutrophil migration and lymphocyte proliferation (Scapigliati et al., 2006; Vassalli, 1992; Zou and Secombes, 2016).

**Major histochemistry class 1: *MHC I***

The function of MHC molecules is to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T cells. The results are almost always destructive to the pathogen. Virus infected cells are killed, macrophages are activated to kill bacteria living in their intracellular vesicles and B cells are stimulated to produce antibodies that eliminate or neutralize extracellular pathogens. Two independent properties of the MHC make it difficult for pathogens to evade immune responses. MHC is polygenic and contains several different MHC class I and MHC class II genes (Grimholt et al., 2015; Zou and Secombes, 2016).

Major histocompatibility complex (*MHC*) molecules are cell surface-expressed, highly polymorphic, heterodimeric glycoproteins. In teleost fish species, major histocompatibility complex MHC-I and MHC-II molecules play an essential role in the immune response to pathogens through their ability to present peptides to $CD8^+$ and $CD4^+$ T cells, respectively. Products of the MHC class I *(MHC-I)* and MHC class II *(MHC-II)* genes encode cell-surface glycoproteins involved in the binding and presentation of peptides to the T cell receptors (TCRs) of T lymphocytes. Major

histocompatibility complex (MHC)-I proteins present peptides from endogenous sources, such as those derived from viruses, to $CD8^+$ T cells, whereas MHC-II molecules mainly present peptides from exogenous sources, such as those derived from extracellular pathogens, to $CD4^+$ T cells. These tri-molecular interactions of MHC, peptide and TCR are central to the generation of antigen-specific immune responses (Grimholt et al., 2015; Okamura et al., 1993; Zou and Secombes, 2016).

**Immunoglobulin T: *IgT* and Immunoglobulin M: *IgM***

It is well known that the main components of the humoral adaptive immune response are immunoglobulins (Igs). The Ig isotypes that have been described in teleosts include IgM, IgD and IgT. IgM represents the key Ig in the plasma of teleosts and is the main player in systemic immune responses. IgT also appears in mucosal secretions and is involved in responses against several pathogens. IgT is reported to be involved in mucosal immunity of many teleosts. It is expressed as a monomer in serum but as a polymer in gill, gut and skin mucus. Ig plays a main role in adaptive immunity by recognising and eliminating pathogens through various processes such as complement activation and phagocytosis. The function of lymphocytes includes innate and adaptive responses in teleost fish (Fillatreau et al., 2013; Mashoof and Criscitiello, 2016).



**2.5 *Flavobacterium columnare***

*F. columnare* is the causative agent of columnaris disease, one of the principal bacterial diseases in freshwater fish species such as tilapia (Dong et al., 2015a; Grabowski et al., 2004), Catfish (Dong et al., 2015c; Shoemaker et al., 2011) and Grass carp (Zhu et al., 2012). This pathogen is a negative, long slender rod-shaped filamentous bacterium that shows characteristic rhizoid and non-rhizoid yellow colonies in specific culture medium as a result of gliding motility (Figure 6). *F. columnare* bacteria are typically 3 to 10 μm long and 0.3 to 0.7 μm wide (Declercq et al., 2013; LaFrentz and Klesius, 2009; Shoemaker et al., 2007).

| | |
|---|---|
| Kingdom | *Bacteria* |
| Subkingdom | *Negibacteria* |
| Phylum | *Bacteroidetes* |
| Class | *Flavobacteriia* |
| Order | *Flavobacteriales* |
| Family | *Flavobacteriaceae* |
| Genus | *Flavobacterium* |
| Species | *Flavobacterium columnare* |

| Biochemical test | *Flavobacterium columnare* |
|---|---|
| Flexirubin pigment | + |
| Congo red absorption | + |
| Cytochrome oxidase | + |
| Catalase | + |
| Nitrate reduction | – |
| Production of $H_2S$ | + |
| Starch hydrolysis | + |
| Esculin hydrolysis | – |
| Gelatin hydrolysis | + |
| Casein hydrolysis | + |
| Tyrosine hydrolysis | – |
| Arginine dihydrolase | – |
| Lysine decarboxylase | – |
| Ornithine decarboxylase | – |
| Tributyrin hydrolysis | + |
| Lecithin hydrolysis | + |
| Tween-20 hydrolysis | + |
| Chondroitin AC lyase | + |
| Acid from fructose | – |
| Acid from glucose | – |
| Acid from galactose | – |
| Acid from glycerol | – |

**Figure 6:** Taxonomy and Biochemical test of *Flavobacterium columnare*

The bacterium forms three colony morphotypes including rhizoid, rough and soft. *F. columnare* can be induced to form different colony morphotypes by exposure to infection, starvation and serial culture. The rhizoid type has been shown to be virulent in fish, whereas the derivative rough and soft types are non-virulent (Dong et al., 2016; Kunttu et al., 2009; Laanto et al., 2014).

Cells of the rhizoid and soft morphotypes were observed to display an organised structure within the colony, whereas in the rough type this internal organisation was absent. Planktonic cells of the rhizoid and rough morphotypes produced large membrane vesicles that were not seen on the cells of the soft morphotype. The vesicles were purified and determined. Two proteins, *OmpA* and *SprF*, with predicted functions were identified. *OmpA* is a virulence factor in several bacterial pathogens, mostly related with adhesion and invasion, while *SprF* is a protein connected with gliding motility and protein secretion of *F. columnare*. The rhizoid morphotype secreted protein absent in the rough and soft morphotypes, indicating an association with bacterial virulence. The virulence of *F. columnare* was associated with three factors as the coordinated organisation of cells, a secreted protein and outer membrane vesicles (Kunttu et al., 2009; Laanto et al., 2014).

Bacterial cells have structures that facilitate surface adhesion, biofilm formation and cell-cell interactions. The ability of bacteria to form biofilms can

influence virulence and promote persistent infections. Bacteria in the biofilm are covered by an extracellular polymeric substance (EPS) layer that protects the cells from environmental factors (Beveridge et al., 1997). This bacterium colonizes the skin and gills in the first steps of pathogenesis. Colonization of the fish tissue can be divided into stages of attraction, adhesion and aggregation. Mucus from the skin and gills of the fish promotes chemotaxis of *F. columnare*. This bacterium has gliding motility and the ability to adhere is a necessity for successful colonization of the host tissue. Adhesion of *F. columnare* to the gill tissue is the main step in the pathogenesis of columnaris disease. The surface of fish is coated with mucus made up of high molecular weight glycoprotein. Bacterial pathogens utilize fish mucus as a nutrient source and mucus promotes chemotaxis of *F. columnare* (Declercq et al., 2013; Decostere et al., 1999; Klesius et al., 2008; Shoemaker and LaFrentz, 2015). In addition, *F. columnare* produces an enzyme that degrades chondroitin sulphates A and C and hyaluronic acid, the complex polysaccharides of connective tissue, resulting in the destruction of skin, muscle and gill tissue (Declercq et al., 2013; Shoemaker and LaFrentz, 2015).

There is an extraordinary genetic diversity among isolates of *F. columnare*. High variations between isolates of *F. columnare* cultured from different fish hosts and geographical regions have been demonstrated in several studies (Dong et al., 2015b; Figueiredo et al., 2005; LaFrentz et al., 2018; LaFrentz and Klesius, 2009). Phylogenetic analyses based on 16S rRNA gene sequences have identified three phylogenetic clusters that correspond to the three originally described genomovars (Darwish and Ismaiel, 2005). The 16S-RFLP technique has been used for *F. columnare* genotypic classification. In restriction fragment length polymorphism (RFLP) assay, a portion of the 16S rRNA gene is amplified, digested with a single restriction enzyme and the resultant DNA fragments are resolved by electrophoresis (Darwish and Ismaiel, 2005; Dong et al., 2015a; LaFrentz et al., 2017; Olivares-Fuster et al., 2007). Four genetic groups and six genomovars (I, II, II-A, II-B, III and IV) have been organized. A multilocal phylogenetic analysis (MLPA) of the 16S rRNA and 6 housekeeping gene

sequences was conducted to decode the genetic diversity of four district *F. columnare* groups (LaFrentz et al., 2018).

An increased understanding of which genetic groups are most prevalent in different regions may help in the prevention and vaccination for columnaris disease. Genetic diversity can result in antigenic variations that may render vaccines ineffective against heterologous isolates. Most currently available successful vaccines induce host responses against antigens that are highly conserved in the targeted pathogens (Telford, 2008). In Thailand, the concurrence of three genetic groups 2, 3 and 4 was found in diseased red tilapia (*Oreochromis* sp.) (Dong et al., 2015a; Kayansamruaj et al., 2017; LaFrentz et al., 2018).

**2.6 Columnaris disease**

*F. columnare* infections may result in fin rot, gill necrosis and skin lesions that are often found as grey to white lesions (saddle back lesion). Mortality rates can be extremely high (Figure 7), with 60 to 90% common mortality, leading to severe economic losses (Declercq et al., 2013; LaFrentz and Klesius, 2009). Columnaris disease can affect fish of all ages but is more prevalent in young fish. Columnaris disease outbreaks are generally associated with overcrowding, handling and stressful rearing conditions such as low dissolved oxygen, high ammonia and organic load. Typically, outbreaks occur when the water temperature is warmer (25-32$^{o}$C) (Declercq et al., 2013; LaFrentz and Klesius, 2009).

**Figure 7:** High mortality red tilapia were infected *F. columnare* in Kanchanaburi province, Thailand.

Prevention of the disease includes farm management, chemical usage and vaccination. For chemical usage, potassium permanganate ($KMnO_4$) and antibiotics such as oxytetracycline or sulfonamide can be used to control the bacterial outbreak. Another method to suitably prevent and control columnaris disease is through vaccination (Darwish and Ismaiel, 2005; Shoemaker et al., 2005).

**2.7 Vaccination in fish**

Current vaccine administrations in fish farming are based on three major methods including injection, oral and immersion vaccination (Figure 8). Injection intraperitoneally is most effective and gives long protection. Disadvantages of vaccine administration by injection are increased stress from handling, high labor cost and unsuitability for small fish. Oral vaccine is cost-efficient and considered as the most practical method for vaccination. It can be mixed with feed for mass vaccination of all fish sizes, with lower labor cost and low stress. Disadvantages of oral vaccine administration include weak/short protection and the large amount of antigen required. The efficacy of oral vaccine depends on antigen protection in the feed against gastric degradation and antigen uptake in the gut. Immersion and bath vaccination are usually manipulated by dipping the fish in a diluted vaccine solution for short or long time. Advantages of this vaccination method are less stress, lower

labour cost and it is suitable for mass vaccination of all sizes of fish. Disadvantages of immersion vaccination include the large amount of vaccine required, lower efficacy and short protection time (Austin, 2012; Gudding et al., 2014).

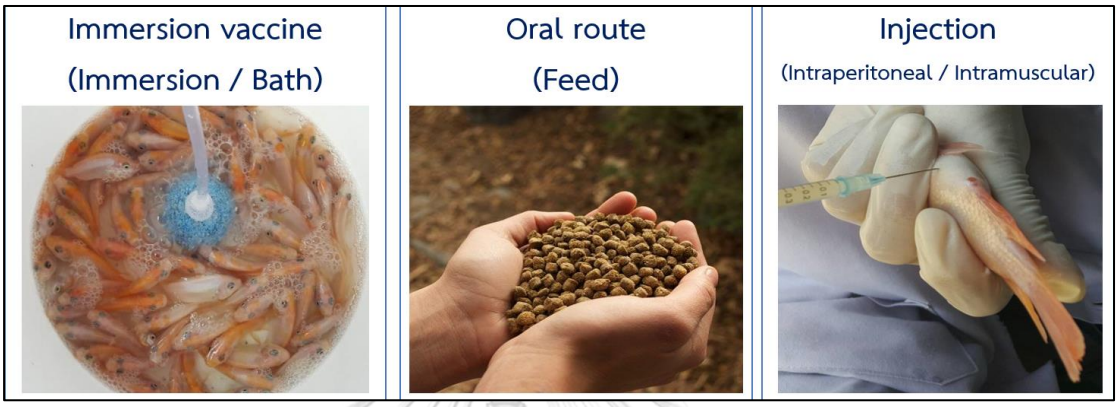


**Figure 8**: Fish vaccination

The ideal approach for development of an effective vaccine is identification of the key virulence factors. This component then stimulates an innate and/ or adaptive immune response of fish to counter the pathogen. Fish vaccines were developed with adjuvants, immunostimulants and delivery methods as alternative techniques for vaccine delivery (other than injection such as nano delivery system), and the traditional or new generation adjuvants. Current understanding of fish vaccinology is based on microbiology and immunology. Recent advances in molecular biology and enhanced knowledge of protective antigens signaled rapid developments in new generations of vaccines for use in fish (Adams, 2019; Plant and LaPatra, 2011; Tafalla et al., 2013).

Conventional aquaculture vaccines were inactivated and live attenuated that begin by culturing target pathogens (Ma et al., 2019; Plant and LaPatra, 2011). This range of vaccines has successfully protected fish against disease. Modern vaccine technology has targeted specific pathogen components. Vaccines developed using these methods may include subunit or recombinant DNA vaccines that contain novel antigens produced using various expression systems. Other technologies, such as

mRNA vaccines, have been developed globally and appear to induce greater levels of immunity than traditional vaccine technology (M Ways et al., 2018; Ma et al., 2019).

### 2.8 *Flavobacterium columnare* vaccine

Research studies on *F. columnare* have included killed and live vaccines. However, the efficacy of formalin killed vaccine with and without adjuvants by intraperitoneal (Grabowski et al., 2004), immersion (Grabowski et al., 2004; Leal et al., 2010) and oral administration (Leal et al., 2010) resulted in limited efficacy. A modified live vaccine was developed against columnaris disease by immersion. This vaccine has now been licensed by Intervet/Schering-Plough Animal Health (AQUAVAC-COL) for use in channel catfish (Shoemaker et al., 2011; Shoemaker et al., 2005) and largemouth bass (*Micropterus salmoides)* (Shoemaker et al., 2011). Attenuation of *F. columnare* resulted in a high efficacy vaccine(Shoemaker et al., 2011; Shoemaker et al., 2005); however, in Thailand there is only one licensed commercial vaccine for control of *S. agalactiae*. This vaccine is also available in Indonesia, Brazil and several Central American countries.

In tilapia culture, columnaris is considered a significant disease. However, immersion vaccination with formalin-killed bacterins resulted in limited effectiveness (Grabowski et al., 2004; Leal et al., 2010). The development and use of live attenuated immersion vaccines showed efficacy in catfish (Shoemaker et al., 2011) but several concerns were raised about their biosafety and permission in Thailand. To improve the efficacy of formalin-killed immersion vaccine, research and development are constantly needed.



### 2.9 Nanovaccine

Nanovaccines consist of nanoparticles attached or formulated with antigen components that induce immune response for disease prevention. Nanoparticles have diameters of 1 to 1,000 nm. They are designed with specific particle

characteristics such as size and surface properties including surface charge, surface modification and hydrophobicity to control delivery, improve targeting and stimulate the immune system. Particle sizes are important for the interaction of biomolecules and biodistribution *in vivo.* Nanoparticle uptake capabilities can be influenced by particle size and surface properties (Aklakur et al., 2016; Zaman et al., 2013; Zhao et al., 2014).

Nanoparticles have been increasingly used to design vaccine delivery systems. They offer efficient modes in targeted delivery, providing stability to antigens and adjuvant properties. They can enter the antigen presenting cells and induce appropriate immune responses. Many types of nanoparticles are applied in fish vaccine delivery such as polymeric nanoparticles, nanoliposomes, carbon nanotubes and immune stimulating complexes (ISCOMs). The induction of immune responses by various nanoparticles can be modulated via different mechanisms such as pattern recognition receptors (PRRs) activation, cytotoxic T-lymphocyte induction, T-helper (Th) activation, cytokine production in diverse ways, B cell activation and antibody production (Aklakur et al., 2016; Najafi-Hajivar et al., 2016). Currently, many materials have been tested in delivery systems for vaccination administration in fish. The important considerations are efficacy and the final cost of the nanovaccine for large-scale industrial production in fish farming. In this study, we are interested in polymeric nanoparticles to develop mucoadhesive nanovaccine. Polymeric nanoparticles have the capacity to conjugate or encapsulate antigens within their matrix or by adsorption or conjugation on the surface (Aklakur et al., 2016; Ji et al., 2015; Zhao et al., 2014). Chitosan is a naturally derived biodegradable and biocompatible polymer that can stimulate good adaptive immune response, both cellular and humoral against the conjugated antigen (Arca et al., 2009).

**2.10 Nano delivery system**

The nano-delivery system has been increasingly used to design vaccine delivery systems as efficient modes that focus on delivery, providing potent antigens and adjuvant properties.

Chitosan is a cationic polymer derived from chitin and N-acetyl-D-glucosamine (acetylated unit) that is naturally found in the exoskeletons of crustaceans. Chitosan is a low cost, non-toxic, biodegradable and biocompatible mucoadhesive polymer that is positively charged and has the ability to temporarily open intercellular tight junctions. Chitosan exhibits potential adjuvant properties that can be exploited in vaccine delivery. This polymer can encapsulate or adsorb antigens, giving rise to the formation of nanoparticles. In mucosal vaccination by immersion, chitosan has mucoadhesive properties, prolongs the time of the loaded antigen at mucosal sites and is suggested to increase antigenic uptake. Chitosan improves antigen uptake at mucosal epithelia by vaccine access to subepithelial antigen-presenting cells (APCs) and increases local immune responses. The mechanism of immune induction showed that the chitosan polymer specifically interacted with APC and CD4+ T cells to enhance antigen presentation, with activation of macrophages and the complement pathway. Chitosan promotes dendritic cell maturation by inducing type I interferons (IFNs) and enhances antigen-specific T helper 1 (Th1) cells. Chitosan-based vaccine delivery systems are increasing vaccine efficacy (Carroll et al., 2016; Esmaeili et al., 2010; Najafi-Hajivar et al., 2016). Complexation of antigen-coated chitosan particles with nanoemulsion is used to increase the stability of these carrier systems and slow the release of the adsorbed antigen. Therefore, this study will research nanoparticle systems using nanoemulsions and chitosan nanoparticles to increase vaccine efficacy.

# Chapter III

# The potential of mucoadhesive polymer in enhancing efficacy of direct immersion vaccination against *Flavobacterium columnare* infection in tilapia.

## Publication

**Kitiyodom S**[a], Kaewmalun S[b], Nittayasut N[b], Suktham K[b], Surassmo S[b], Namdee K[b], Rodkhum C[c], Pirarat N[a]*, Yata T[b]** (2019) The potential of mucoadhesive polymer in enhancing efficacy of direct immersion vaccination against *Flavobacterium columnare* infection in tilapia. Fish Shellfish Immunol 86: 635-640. (IF 3.370)

## List of authors

Sirikorn Kitiyodom and Nopadon Pirarat

Wildlife Exotic and Aquatic Pathology-Research Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

Somrudee Kaewmalun, Naiyaphat Nittayasut, Kunat Suktham, Suvimol Surassmo, Katawut Namdee and Teerapong Yata

National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani, 12120, Thailand

Channarong Rodkhum

Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

**Abstract**

Vaccination is the most effective approach for prevention of infectious diseases in aquaculture. Although immersion vaccination is more applicable compared to in-feed/oral administration and injection, this method suffers from low potency as the efficiency of uptake of antigens through mucosal membranes is limited. In this study, we have successfully developed a mucoadhesive vaccine delivery system to enhance the efficacy of direct immersion vaccination against *Flavobacterium columnare*, the causative agent of columnaris disease in red tilapia. A formalin-killed negatively charged, bacterial cell suspension was used to prepare a mucoadhesive vaccine by electrostatic coating with positively charged chitosan. Our results demonstrate that the chitosan-complexed vaccine greatly increases its mucoadhesiveness, thus increasing the chances of vaccine uptake by the gill mucosa and improving the protection obtained against columnaris infection. The surface charge of the chitosan-complexed vaccine was altered from anionic to cationic after chitosan modification. Tilapia were vaccinated with the prepared chitosan-complexed vaccine by immersion. The challenge test was then carried out 30- and 60-days post vaccination, which resulted in a high level of mortalities in the non-vaccinated and uncomplexed vaccine groups. A high relative percentage survival (RPS) of vaccinated fish was noted with the mucoadhesive vaccine. Our results indicated that the naked vaccine failed to protect the fish from columnaris infection, which is consistent with the mucoadhesive assays performed during the study showing that the naked vaccine was unable to bind to mucosal surfaces. This system is therefore an effective method for immersion vaccination in order to deliver the antigen preparation to the mucosal surface membrane of the fish.

**Keywords:** Bath vaccination Chitosan Columnaris disease Tilapia

**Introduction**

Tilapia (*Oreochromis sp.*) is one of the most important fish species produced in fish farming (Fitzsimmons et al., 2011). Bacterial infection caused by *Flavobacterium columnare*, the causative agent of columnaris disease, has been now identified as one of the most serious infectious diseases in farmed tilapia (Wonmongkol et al., 2018). *F. columnare* is a Gram-negative, rod and slender filamentous bacterium with gliding motility and yellow rhizoid colony formation (Dong et al., 2015b). *F. columnare* infections may result in skin lesions, fin rot and gill necrosis, with a high degree of mortality, leading to severe economic losses (Loch and Faisal, 2015). It is well established that vaccination is the most effective approach for prevention of infectious diseases in aquaculture (Assefa and Abunna, 2018). Aquaculture vaccines are roughly administered through major three routes, i.e. bath or immersion, in-feed or oral, and injection (Dadar et al., 2017). While immersion vaccination is the most applicable mode of delivery of these routes of administration, this method suffers from low potency as the efficiency of antigen uptakes through the gills and skin is limited (Huising et al., 2003). Chitosan (CS), sometimes known as deacetylated chitin found in the exoskeletons of crustaceans, is a natural polycationic linear polysaccharide that exhibits mucoadhesive properties (M Ways et al., 2018). Among polymers, chitosan has been exploited for the design of mucoadhesive dosage forms due to its excellent biocompatibility and biodegradability (Cheung et al., 2015). The external constituent of skin, gills, and gut is a mucous gel secreted by various epidermal or epithelial mucus cells which forms a layer of a gel-like substance covering the epithelial cells (Koshio, 2016). More importantly, these organs are directly associated with the mucosal immunity of fish (Guardiola et al., 2014). The fish mucus is mainly composed of water and glycoproteins, containing vast amount of mucins, high molecular weight negatively charged oligosaccharides (Grosell et al., 2010; Perez-Vilar, 2007). Since fish gills are considered a mucosal surface associated with the mucosal immunity, targeting

mucoadhesive vaccines to the mucosal surface could be exploited as an effective method for immersion vaccination. It has been suggested that electrostatic force attraction is crucial for the mucoadhesive mechanism, which is affected by the complexation between positively charged polymer and negatively charged materials such as cell surface and mucin in a biomembrane environment (Silva et al., 2012). Therefore, we hypothesized that a mucoadhesive polymer could be exploited to deliver antigen preparation to mucosal membranes of tilapia. The main overall aim of this study was to investigate the application of chitosan to facilitate efficient delivery of inactivated vaccines to fish mucosal surfaces. In this study, we prepared chitosan-complexed vaccines as schematically shown in Figure 1. The physiochemical property of chitosan-complexed vaccines was analyzed, and their mucoadhesive characteristics and protective effect against columnaris disease were evaluated. Throughout this paper, the abbreviation CS-vaccine will be used to refer to inactivated *F. columnare* vaccines complexed with mucoadhesive polymer chitosan.

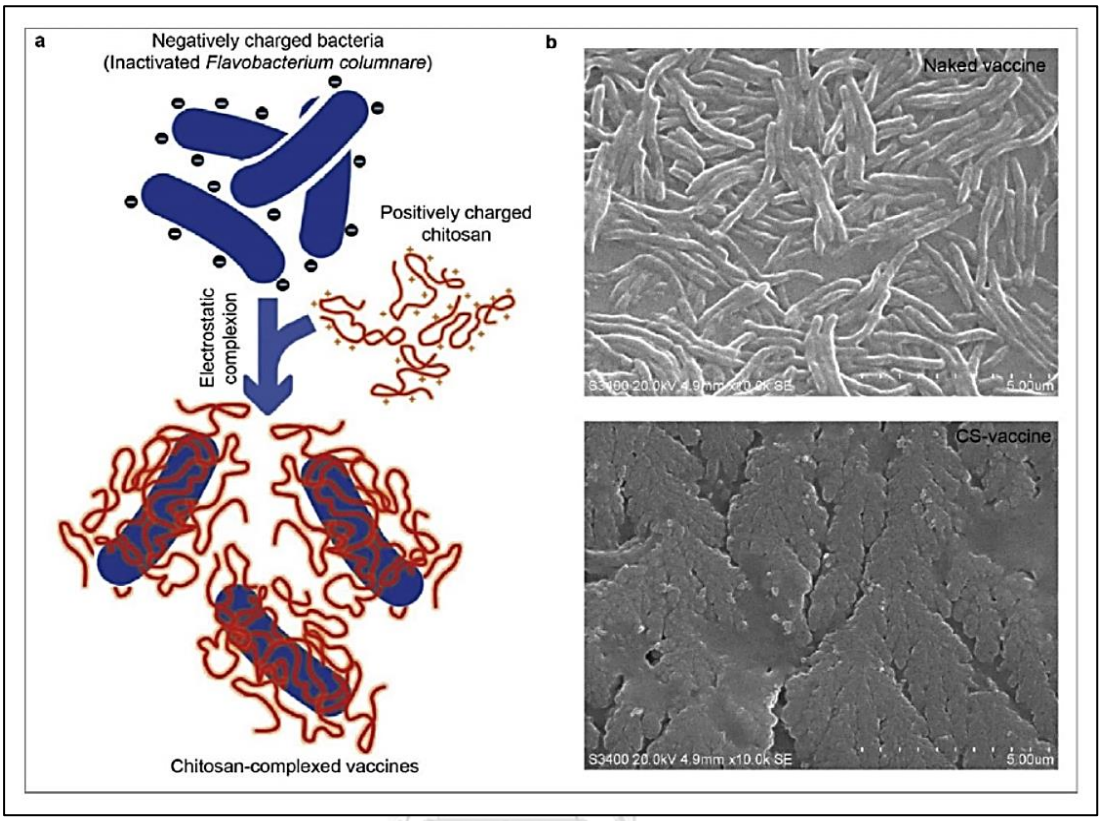


**Figure 1.** Surface characteristics of the chitosan complexed vaccines. a). Schematic diagram of the chitosan-complexed vaccines. Negatively charged vaccines prepared by formaldehyde inactivation were electrostatically assembled with cationic chitosan polymers to form chitosan-vaccine complexes. b) . SEM images of the surface morphology of the chitosan-complexed vaccines in comparison with naked vaccines.

## Materials and methods

Ethics Statement Care of laboratory animals and animal experimentation were conducted in accordance with animal ethics guidelines and approved protocols. All animal experiments were approved by the Animal Ethics Committee of Chulalongkorn University, 1831020. Upon termination of the study, all fish were euthanized according to appropriate guidelines.

*Fish and experimental conditions*

Healthy red tilapia (*Oreochromis sp.*) with an average weight of 10 g were purchased from a tilapia breeding farm, Thailand and used for the experiment. Fish were distributed into 800 L fiber tanks containing water under continuous aeration and with continuous water flow (80% water change per day). Air and water temperatures were measured daily and were 25–33 °C and 25–28 °C, respectively. Dissolved oxygen (DO) content and pH were measured weekly using a DO meter and pH meter, and values were within acceptable ranges of 5.24–5.98 mg L−1and 7.48–8.16.

*Bacteria and vaccine preparation*

Bacterial cultures (*F. columnare* isolated from red tilapia, *Oreochromis sp.*, in Thailand) (Dong et al., 2015b) used for vaccine preparation and challenge test were grown in Tryptone Yeast Extract Salt (TYES) broth medium (pH 7.2) and incubated at 25–28 °C for 48 h (Grabowski et al., 2004). In order to prepare inactivated vaccines, bacterial cells were harvested by centrifugation at 3,000 g at 4 °C for 40 min, resuspended in phosphate buffered saline (PBS) containing 0.2% formalin, and incubated at 4 °C for 20 h. Formalin-killed bacteria suspensions were washed three times by centrifugation and re-suspended in PBS (adjusted to $10^9$ colony forming units (cfu/mL)). Subsequently, an aliquot of bacterial cells was used to complex with chitosan (Sigma-Aldrich) by adding 1% w/v of chitosan (previously dissolved in a solution of 1% acetic acid) to the prepared formalin-killed vaccine at a ratio of 1: 1 (v/v). The mixture was stirred for 30 min at 25 °C.



*Surface characterization of the chitosan-complexed vaccines*

Zeta potential of vaccine preparations were determined using a Zetasizer Nano ZX (Malvern Instruments). The vaccine solution was diluted 1000 times in DI water before measurement. All measurements were performed at 25 °C. The data are given as mean ± SEM based on the measurements of three replicate samples.

The morphology of vaccine preparations was observed using an environmental scanning electron microscope (E-SEM, S-3400, Horiba). The samples were diluted by distilled water at ratio of 1:50 onto the surface of carbon tape. The samples were then observed at of 5000–20,000× magnifications with electron beam energy of about 20 kV.

***Mucoadhesive properties of the chitosan-complexed vaccines***

DAPI (4′,6-diamidino-2-phenylindole) (Sigma-aldrich) solution was added to the formalin-killed bacteria suspension (final concentration: 0.5 μg/ mL) and incubated in the dark at room temperature for 5 min. The suspension was washed three times by centrifugation and resuspended in PBS and the stained cells were used to prepare the chitosan- complexed vaccines. Fingerling tilapias (10 g) were divided into 3 groups; control, naked vaccine, and CS-vaccine groups (5 fishes each) in 3 replicate tanks. Fish were immersed in 107 cfu/mL of vaccine preparations for 30 min. Following direct immersion and euthanasia by rapid chilling (2°–4 °C) until loss of orientation and operculum movements and subsequent holding times in ice-chilled water, fish gills were harvested. Attachment of vaccines to mucosal surfaces was examined by determining the fluorescent signal of DAPI-stained vaccines using a Nikon Eclipse TE2000-U fluorescence microscope. Photographic images were obtained by using 4× magnification and fluorescent setting with an excitation max of 358 nm and emission max of 461 nm. Fish gills were also observed using a bioluminescence imaging instrument (Bruker). To quantity the attached bacteria, 100 μl of Glo® lysis buffer (Promega) was added to 1 g of gill tissue, incubated for 10 min at 37 °C, and homogenized with PYREX® 3 mL Glass Pestle Tissue Grinder. Homogenized tissues were then centrifuged for 5 min at 10,000 g to remove debris. One hundred microliters of the supernatants were transferred to an opaque 96-well plate for fluorescence measurement. Fluorescence was read with a fluorescence plate reader at 358 nm/ 461 nm.

*Vaccine efficacy*

Tilapia Fingerlings (10 g) were divided into 3 groups; control, naked vaccine, and CS-vaccine groups (15 fish each) in 2 replicates. Fish were immersed with $10^7$ CFU/mL of vaccine preparations for 30 min. At 30 and 60 days after immersion vaccination, fishes were challenged with $1\times10^6$ CFU/mL concentration of a virulent strain of *F. columnare* for 1 h the same isolate used to prepare the vaccine as previously described (Dong et al., 2015b). Cumulative mortality and survival rate were recorded for 10 days after immersion challenge. Columnaris disease caused by *F. columnare* was confirmed by clinical signs of necrotic gills, fin rot, skin erosion or necrotic muscle, followed by a characteristic rhizoid pattern of growth on a low nutrient agar medium (Dong et al., 2016).

*Statistical analysis*

GraphPad Prism software (version 5.0) was used to generate graphs and perform statistical analyses. One-way analysis of variance, or repeated measures analysis of variance, followed by Tukey post-hoc tests were used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant and denoted as follows: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Survival curves were generated for the vaccinated fish and unvaccinated fish. The numbers of fish which died after challenge test were recorded. Relative percentage survival (RPS) was calculated as 1 – (mortality rate of vaccinated fish/mortality rate of control fish) (Amend, 1981).



**Results**

*Surface characteristics of the chitosan-complexed vaccines*

Figure 1b shows the SEM image of naked vaccines and chitosan complexed vaccines. The morphology of the chitosan-complexed vaccines exhibited well-formed vaccines complexed with chitosan forming a rough surface. When we analyzed the zeta potential of the CS-complexed vaccines, we observed that the zeta potential shifted from a negative value of $-6.44 \pm 1.00$ mV for naked vaccines,

to a positive value of +11.62 ± 2.29 mV for the CS-complexed vaccines. These data proved the positive charge of the CS-complexed vaccines in contrast to the negatively charged surface of the naked vaccines.

***Mucoadhesive property of the chitosan-complexed vaccines***

The affinity of chitosan-complexed vaccines toward a mucosal surface of fish gill was studied using DAPI-stained *F. columnare.* Quantification of fluorescent signal in gill tissues after tissue lysis showed that a significant higher mean fluorescence intensity (MFI) were achieved with CS-vaccines compared to naked vaccines. As shown in Fig. 2a, treatment with CS-vaccines and naked vaccines resulted in ~2- and ~ 1.25-fold increase of fluorescent signal compared to the control (non-treated) group, respectively. Fluorescence microscopy revealed a large number of CS-vaccines attached to fish gills. In contrast, a few particles of naked vaccines were observed on gills of fish (Fig. 2b). Consistently, bioluminescence imaging revealed that incorporation of vaccines with chitosan polymer allowed efficient attachment to mucosal surfaces, as indicated by higher fluorescent intensity than that obtained with naked vaccines (Fig. 2c).

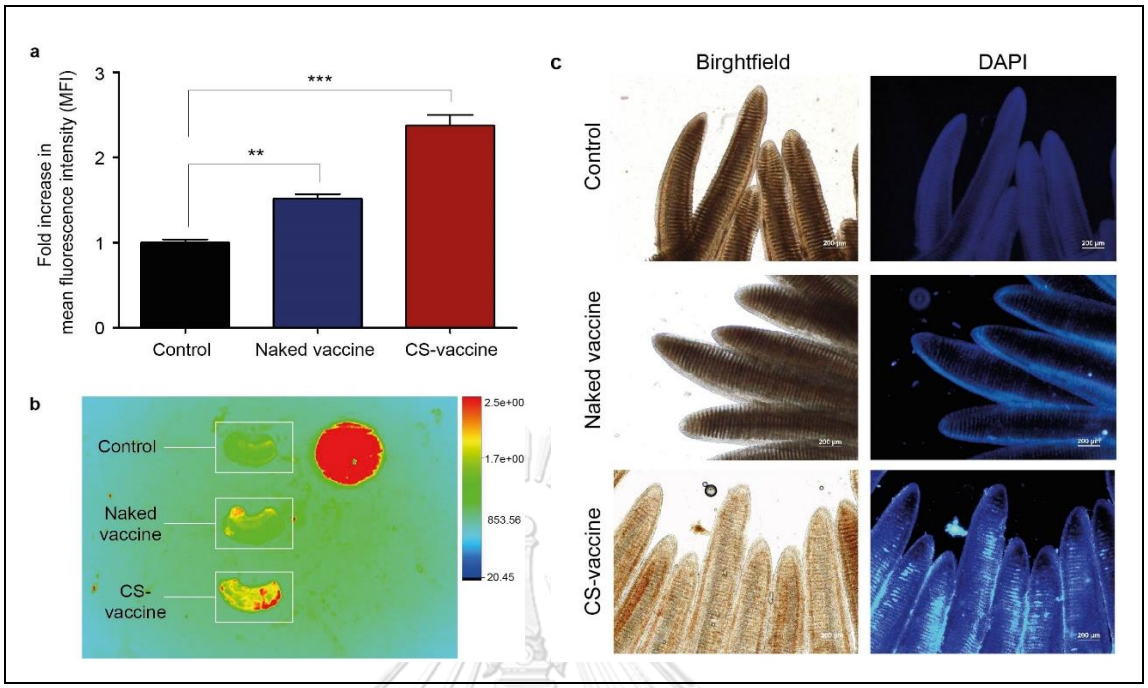


**Figure 2** Accumulation of mucoadhesive in gill tissue after direct immersion vaccination a) Quantitative analysis of DAPI-stained vaccines in fish gill tissues. Experiments were performed in triplicate and data presented as fold-increase in mean fluorescence intensity (MFI) compared with the control (non-vaccinated fishes). b) Bioluminescence imaging of fish gills following direct immersion of naked vaccines and CS-vaccines. c) Representative microscopic fluorescence images of vaccines complexed with chitosan in fish gill after direct immersion as examined by fluorescence microscopy. Scale bar = 200 μm



### *Protective effect of mucoadhesive vaccines against flavobacterium infection*

Vaccinated and control fish were held for 30- and 60-days following vaccination before they were challenged with virulent *F. columnare*. The time course for bath immunization and challenge is shown in Fig. 3a. The fish tolerated the vaccination procedure well. It has been suggested that a positive effect by vaccination is a relative percent survival (RPS) greater than 50% (Dodds and Schultz, 1998). At 30 days post vaccination with naked and CS vaccines, the RPS were 4 and 81%, respectively, as shown in Table 1. In this study, mortality in an equivalent group

of non-vaccinated and naked vaccine groups were 90% and 87%, respectively versus 17% mortality in the CS-vaccine group. The prolonged protective effect could be also observed in fishes vaccinated with CS-vaccines at 60 days post vaccination (Table 1). Percentage survival after bath challenge of vaccinated and control groups is shown in Fig. 3b. We observed at least one of the following clinical signs in all the moribund and dead fish; hemorrhage, splenomegaly, lesion on the trunk, and/ or eroded tail and mouth (Fig. 3c).

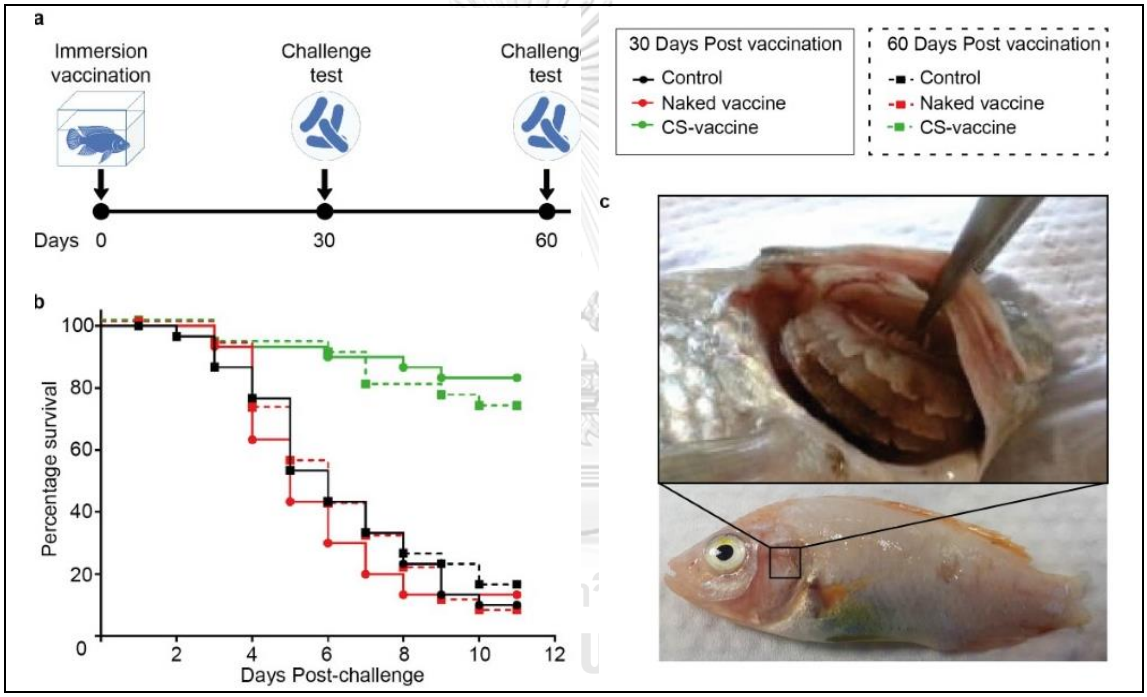


**Figure 3.** Vaccine efficacy. (a) Time course for bath immunization and challenge. (b) Percentage survival after bath challenge of vaccinated and control groups. The survival rates following challenge with 1 x$10^6$ CFU/mL *F. columnare* are presented. (c) Clinical signs of columnaris infection following bath challenge with *F. columnare*. Diseased Tilapia showing prominent yellowish deposits in the gills (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

**Table 1**

Average percent mortality of tilapia after bath challenge ( 30- or 60-days post vaccination) with *Flavobacterium columnare*.

| Group | Replicate tank | *n* | Average mortality (%) | Average survival (%) | RPS | Remark |
|---|---|---|---|---|---|---|
| Control (30 days) | 1 | 15 | 90 | 10 | – | – |
| | 2 | 15 | | | | |
| Naked vaccine (30 days) | 1 | 15 | 87 | 13 | 4 | ns |
| | 2 | 15 | | | | |
| CS-vaccine (30 days) | 1 | 15 | 17 | 83 | 81 | ** |
| | 2 | 15 | | | | |
| Control (60 days) | 1 | 15 | 87 | 13 | – | – |
| | 2 | 15 | | | | |
| Naked vaccine (60 days) | 1 | 15 | 97 | 3 | 0 | ns |
| | 2 | 15 | | | | |
| CS-vaccine (60 days) | 1 | 15 | 37 | 63 | 58 | * |
| | 2 | 15 | | | | |

Immersion vaccination for 30 min with $1\times10^6$ CFU/mL *F. columnare*. * and ** indicate significant difference compared with control group ($p < 0.05$ using Bonferroni test following one-way ANOVA).



## Discussion

Targeting the vaccine to the particular biological site, where action is needed is difficult. For most aquaculture fish species, immersion vaccines must be directly taken up and processed by appropriate cells of the immune system (Aklakur et al., 2016). Therefore, the use of safe and cheap carriers capable of efficient delivery of antigens to target cells is of importance. In this study, we have successfully developed a mucoadhesive vaccine delivery system to circumvent this problem. We chose *F. columnare*, the causative agent of columnaris disease, as a representative model antigen for this proof-of-concept study.

We hypothesized that positive charges of chitosan can enhance the adhesion of inactivated vaccines to negatively charged mucosal membranes, which increases

gill accessibility (Charlie-Silva et al., 2018). In our study, mucoadhesive vaccine was prepared by electrostatic coating of inactivated *F. columnare* with a cationic chitosan polymer. Our results showed that the complexation of vaccines with cationic polymers generates positively charged vaccine complexes allowing better adsorption on mucosal surfaces and enhanced protective effect against columnaris disease. A Naked vaccine was used as a control to confirm that they are unable to bind to the mucosal membrane in the absence of chitosan polymers and thus failed to protect fishes from columnaris infection. This enhanced protective effect against infectious diseases may result from the mucoadhesive property of the chitosan polymer. Mucoadhesive polymers increases the contact time with the mucosa (Shaikh et al., 2011) thereby increasing the potential of enhancing antigen uptake by the antigen presenting cell. The main mechanism of chitosan mucoadhesion appears to be electrostatic interaction between the positively charged polymer and negatively charged materials such as mucus and cell surface (Alexander et al., 2011; Ali and and Bakalis, 2011; Carvalho et al., 2010; Chaturvedi et al., 2011; Yadav et al., 2010). Another possible explanation could be the adjuvant ability of chitosan (Carroll et al., 2016; Xia et al., 2015; Zeng, 2016). Chitosan has been extensively investigated for its immunogenic activities, especially via the mucosal routes (Baudner et al., 2003; Li et al., 2013; Zeng, 2016). Therefore, this strategy could be used as an effective method in particular for direct immersion vaccination of fishes.



Despite these promising results, a large range of related clinical parameters need to be measured and monitored over a period of time. Further research should be undertaken in farmed tilapia in order to determine the effectiveness of Mucoadhesive vaccine against *F. columnare*. In addition to related clinical significances, such as Average Daily Gain (ADG), Feed Conversion Ratio (FCR) and survival/ mortality rate, adverse side-effects (pain and stress) and long-term safety should be evaluated in parallel. The immune response to the vaccine should also be assessed.

## Conclusion

The vaccine strategy, presented here, is an improved version of a killed vaccine that target the mucosal membrane of tilapia fish. Specifically, we reported on the preparation of mucoadhesive vaccines as well as their physicochemical and biological properties. The analysis of TEM image and zeta-potential also suggested the successful modification of vaccines by chitosan. In vivo mucoadhesive study demonstrated the excellent affinity of the chitosan-complexed vaccines toward fish gills as confirmed by bioluminescence imaging, fluorescent microscopy, and spectrophotometric quantitative measurement. Most interestingly, mucoadhesive polymer could increase the efficacy of killed vaccines. Taken together, our study demonstrated the feasibility of mucoadhesive particle as an effective delivery method for a vaccine against infectious *F. columnare* in tilapia by immersion vaccination.

## Acknowledgments

This work was supported by the grant from Ratchadaphiseksomphot Endowment Fund; CU-GR_61_013_31_003, National Nanotechnology Center; P1752586 and P1751449, and Thailand Graduate Institute of Science and Technology Scholarship; SCA-CO-2560-4578-TH, National Science and Technology Development Agency (NSTDA, Thailand). Faculty of Veterinary Science, Chulalongkorn University, is also acknowledged.

# Chapter IV

# Enhanced efficacy of immersion vaccination in tilapia against columnaris disease by chitosan-coated “pathogen-like” mucoadhesive nanovaccines.

**Publication**

**Kitiyodom S**, Yata T, Yostawornkul J, Kaewmalun S, Nittayasut N, Suktham K, Surassmo S, Namdee K, Rodkhum C*, Pirarat N* (2019) Enhanced efficacy of immersion vaccination in tilapia against columnaris disease by chitosan-coated “pathogen-like” mucoadhesive nanovaccines. Fish Shellfish Immunol 95 (2019): 213-219. (IF 3.370)

**List of authors**

Sirikorn Kitiyodom and Nopadon Pirarat
Wildlife Exotic and Aquatic Pathology-Research Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

Teerapong Yata
Biochemistry Unit, Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

Jakawan Yostawornkul, Somrudee Kaewmalun, Naiyaphat Nittayasut, Kunat Suktham, Suvimol Surassmo and Katawut Namdee
National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani, 12120, Thailand

Channarong Rodkhum
Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

**Abstract**

Red tilapia (*Oreochromis s*p.) has become one of the most important fish in aquaculture. Bacterial infection caused by *Flavobacterium columnare*, the causative agent of columnaris disease, has been now identified as one of the most serious infectious diseases in farmed red tilapia and cause major financial damage to the producers. Among the effective prevention and control strategies, vaccination is one of the most effective approach. As the surface of living fish is covered by mucus and directly associated with the mucosal immunity, we therefore hypothesized that better adsorption on mucosal surfaces and more efficient vaccine efficacy could be enhanced biomimetic nanoparticles mimicking the mucoadhesive characteristic of live *F. columnare.* In this work, we describe an effective approach to targeted antigen delivery by coating the surface of nanoparticles with mucoadhesive chitosan biopolymer to provide "pathogen-like" properties that ensure nanoparticles binding on fish mucosal membrane. The physiochemical properties of nanovaccines were analyzed, and their mucoadhesive characteristics and immune response against pathogens were also evaluated. The prepared vaccines were nanosized and spherical as confirmed by scanning electron microscope (SEM). The analysis of hydrodynamic diameter and zeta-potential also suggested the successful modification of nanovaccines by chitosan as indicated by positively charged and the overall increased diameter of chitosan-modified nanovaccines. In vivo mucoadhesive study demonstrated the excellent affinity of the chitosan-modified nanovaccines toward fish gills as confirmed by bioluminescence imaging, fluorescent microscopy, and spectrophotometric quantitative measurement. Following vaccination with the prepared nanovaccines by immersion 30 min, the challenge test was then carried out 30- and 60-days post-vaccination and resulted in high mortalities in the control. The relative percent survival ( RPS) of vaccinated fish was greater than 60% for mucoadhesive nanovaccine. Our results also suggested that whole-cell vaccines failed to protect fish from columnaris infection, which is consistent with the mucoadhesive assays showing that whole-cell bacteria were unable to bind to mucosal surfaces. In conclusion, we could use this system to deliver antigen

preparation to the mucosal membrane of tilapia and obtained a significant increase in survival compared to controls, suggesting that targeting mucoadhesive nanovaccines to the mucosal surface could be exploited as an effective method for immersion vaccination.

**Keywords:** Immersion vaccination, Chitosan, Columnaris disease, Tilapia

**Introduction**

According to 2015 UN Food and Agriculture Organization (GLOBEFISH- Analysis and information on world fish trade), Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis sp.*) have increasingly recognized as one of the most important freshwater fish in aquaculture. Inevitably, several bacterial diseases can cause major financial damage to the producers of tilapia. Bacterial infection caused by *Flavobacterium columnare*, the causative agent of columnaris disease, has been now identified as one of the most serious infectious diseases in farmed tilapia (Wonmongkol et al., 2018). *F. columnare* are gram negative, rod and slender filamentous bacterium with gliding motility and yellow rhizoid colony formation (Dong et al., 2015a) and colonizes the mucosal surfaces of fishes (gills and skin) in the initial steps of pathogenesis (Shoemaker and LaFrentz, 2015). *F. columnare* infections may result in skin lesions, fin rot and gill necrosis, with a high degree of mortality, leading to severe economic losses (Loch and Faisal, 2015). The development of effective and affordable prevention and control strategies for columnaris infection is therefore warranted. It is well established that vaccination is the most effective approach for prevention of infectious diseases in aquaculture. In fact, fish vaccines are mostly administered through major three routes of administration as bath or immersion, second through in-feed or oral and the third by injection. While immersion vaccination is more applicable, but this method suffers from low potency as the efficiency of uptake of antigens through the gills and skin are limited.

Nanoparticle platforms can be categorized as organic-based ( e. g. , lipid nanoparticles, biodegradable polymeric nanoparticles, and viral vectors), inorganic-based, or a hybrid combination of the two. The use of nanotechnology has been extensively exploited for controlled release and targeted delivery of drugs, vaccines, and biopharmaceuticals in order to improve their effectiveness for the prevention and treatment of human and animal diseases. It is well established that nanotechnology-based delivery system can play an important role in addressing the issue of inefficient targeting antigen to the action site which causes the administration of large doses of vaccine (Aklakur et al., 2016). A number of previous studies investigating the different aspects related to nanoparticle vaccine and demonstrated their advantages over conventional vaccines. The use of nanotechnology-based delivery system has provided a tremendous opportunity to design new formulations of nanovaccine in order to effectively and selectively deliver antigens to appropriate sites, provide stability to antigens, and act as efficient adjuvants (Vinay et al., 2018). One purpose of this study was to exploit nanoencapsulation technology to enhance the efficacy of inactivated *F. columnare*.

Another purpose of this study was to exploit a mucoadhesive polymer-based delivery system to circumvent the issue of inefficient targeting antigen to fish mucosal surfaces. Chitosan (CS), sometimes known as deacetylated chitin found in the exoskeletons of crustaceans, is a natural polycationic linear polysaccharide (M Ways et al., 2018). Among polymers, chitosan is one of the most studied form of bioadhesive polymers (Saikia et al., 2015) and has been extensively used in numerous applications in pharmaceutical and biomedical areas e.g. in drug delivery and tissue engineering due to its outstanding biological properties such as mucoadhesiveness, biocompatibility and biodegradability (Cheung et al., 2015). Taken together, we hypothesized that the efficacy of killed vaccines could be enhanced by nanoencapsulation in combination with incorporation of mucoadhesive characteristic. In this study, we prepared different formulations of nanovaccines as schematically shown in Fig. 1. The physiochemical properties of chitosan-complexed nanovaccine were analyzed, and their mucoadhesive characteristics and immune response against

model antigen were also evaluated. Throughout this paper, the abbreviation WC, CS, NE and CS-NE will be used to refer to inactivated whole-cell *F. columnare* vaccines, polyme (chitosan) nanovaccines, nanoemulsion vaccines and the hybrid nanoemulsion vaccines coated with mucoadhesive polymer chitosan, respectively.

**Materials and methods**

The procedures of animal experiments were approved by the Animal Ethics Committee of Chulalongkorn University, 1831020, and in accordance with animal ethics guidelines and approved protocols.

***Fish and experimental conditions***

Red tilapia (*Oreochromis sp.)* were used, with an average weight of 10 g. Fish were distributed into fiber tanks containing water under continuous aeration. Air and water temperatures were monitored daily and values were within acceptable ranges of 25–33 °C and 25–28 °C, respectively. Dissolved oxygen (DO) content and pH were examined weekly and were within ranges of 5.24–5.98 mg/ L and 7.48–8.16, respectively.



***Bacteria and nanovaccine preparation***

Bacterial cultures used for nanovaccine preparation were grown in Tryptone Yeast Extract Salt (TYES) broth medium (pH 7.2) and incubated at 25–28 °C for 48 h (Grabowski et al., 2004). In order to prepare inactivated vaccines, bacterial cells were collected by centrifugation at 3,000 g at 4 °C for 40 min, resuspended in phosphate-buffered saline (PBS) containing 0.2% formalin, and incubated at 4 °C for 20 h. Formalin-killed bacteria suspensions were washed three times by centrifugation and resuspended in PBS. Viable counts were determined by plating. After being adjusted to an identical number of bacterial cells based on an optical density-based approach (equivalent to $10^9$ colony forming units (cfu)/ mL predetermined by plating), an aliquot of bacterial cells was sonicated at 40% amplitude for 30 s and used to

prepare different formulations of nanovaccine. Chitosan solution of 0.5% w/v concentration in 1% aqueous acetic acid was also prepared. To prepare polymeric nanovaccines, an aliquot of sonicated bacterial cells (20% w/w) was mixed with 33% (w/w) of chitosan solution and 47% (w/w) of water. Stirring was continued for another 1 h at room temperature. To prepare nanoemulsion, an aliquot of sonicated bacterial cells (30% w/w) was mixed with 6% (w/w) of polyoxyethylene (20) sorbitan monolaurate, 2% (w/w) of medium chain triglycerides (Miglyol) and 62% (w/w) of water. The mixture was homogenized by sonicator probe at 40% amplitude for 5 min. To prepare chitosan complexed nanoemulsion (hybrid), complexation of preformed nanoemulsions with chitosan was performed by adding 1% of small molecular weight (50–200 kDa; Sigma) chitosan (previously dissolved in a solution of 1% acetic acid) to the prepared nanoemulsion at a ratio of 1: 1 (v/v). The mixture was stirred for 1 h at room temperature.

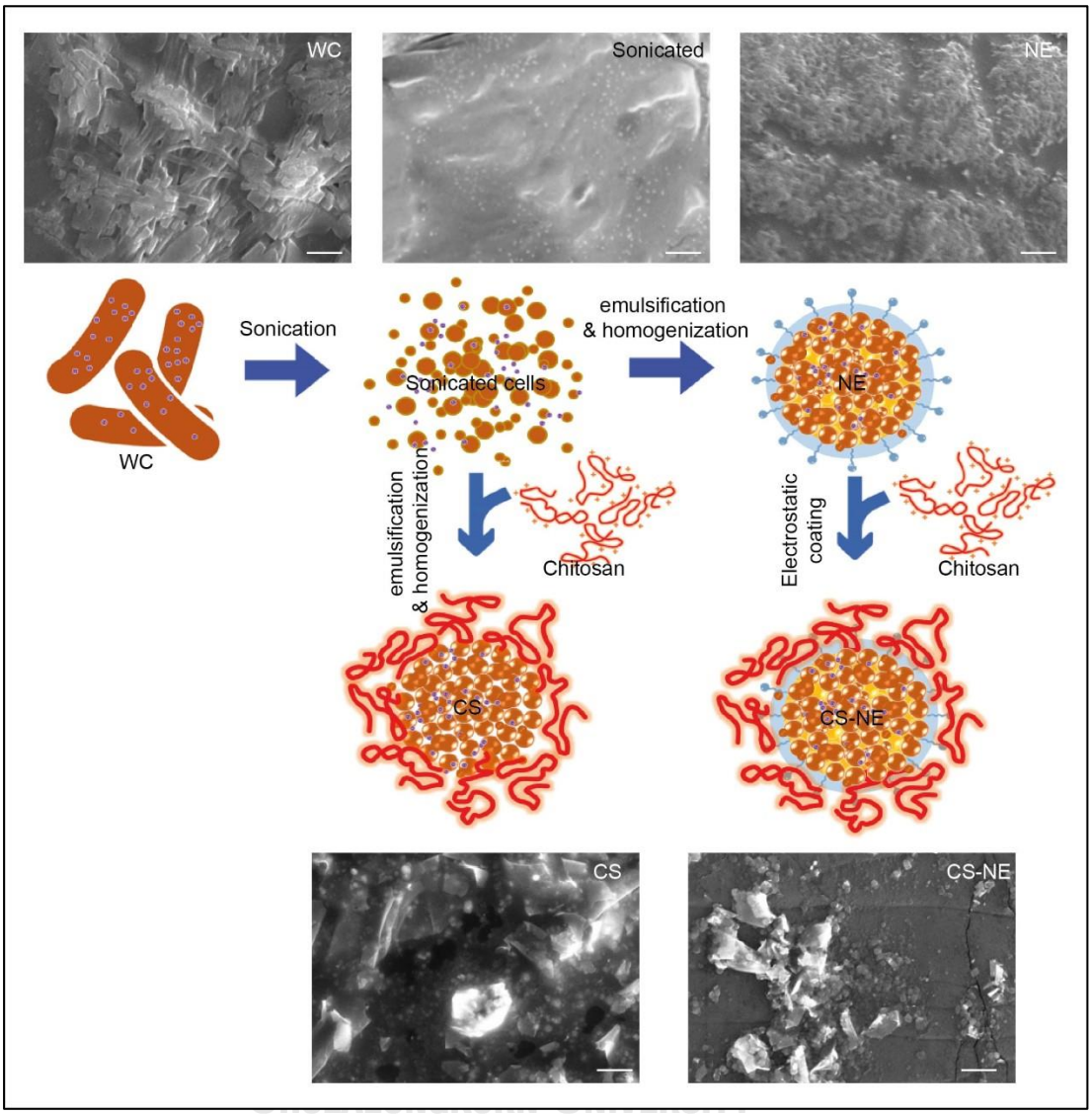


**Figure 1.** Preparation and physical characteristics of different vaccine formulations used in this study. Schematic diagram of the prepared vaccines. Negatively charged vaccines prepared by formaldehyde inactivation were physically broken down, followed by reformation of nanoparticles with/ without assembling with cationic chitosan polymers. SEM images of the surface morphology of different nanovaccine formulations in comparison with inactivated whole-cell vaccines are also shown. Scale bar=10 μm.

*Surface characterization of nanovaccines*

Zeta potential of nanovaccine preparations were measured using a Malvern Instruments Zetasizer Nano ZX. The nanovaccine suspension was diluted 1,000 times in Deionized water before measurement. All measurements were performed at 25 °C. The data are given as mean ± SD based on the measurements of the samples from three replicates. The morphology of nanovaccine preparations was observed using an environmental scanning electron microscope (E-SEM, S-3400, Horiba, Japan). The samples were diluted by distilled water at 1:50 ratio onto the carbon tape. The samples were then investigated at magnification of 5,000–20,000 times with electron beam energy of 20 kV.

*Mucoadhesive characterization of nanovaccines*

Fingerling tilapias (10 g) were divided into 4 groups; control, whole cell vaccine, polymeric (CS) nanovaccine, nanoemulsion vaccines and hybrid CS complexed nanoemulsion vaccine groups (5 fish each) with 3 replicates. Fish were immersed with $10^7$ cfu/mL of vaccine preparations for 30 min. Following direct immersion and euthanasia, fish gills were harvested. Attachment of vaccines to mucosal surfaces as determined by the fluorescent signal of DAPI-stained vaccines was examined using a Nikon Eclipse TE2000-U fluorescence microscope. Fluorescence images were obtained by using 4X magnification and fluorescent setting. A second experiment was carried out to determine whether vaccines could be detected in the mucosal membranes. Following immersion, euthanasia and gill dissection, accumulation of DAPI-stained vaccines was observed using a bioluminescence imaging instrument (Bruker). For quantitative measurement, 100 µl of Glo® lysis buffer (Promega) was added to 1 g of gill tissues, incubated for 10 min at 37 °C, and homogenized with PYREX® 3 mL Glass Pestle Tissue Grinder. Homogenized tissues were then centrifuged for 5 min at 10,000 g to remove cell debris. One hundred microliters of the supernatants were transferred to an opaque 96-well plate for fluorescence measurement. Fluorescence intensity was measured with a fluorescence plate reader at 358 nm/461 nm.

*Vaccine efficacy*

Fingerling tilapias (10 g) were divided into 5 groups; control, wholecell vaccine, polymeric (CS) nanovaccine, nanoemulsion vaccines and hybrid CS-complexed nanoemulsion vaccine groups (25 fish each) with 3 replicates. Fish were immersed in aerated 2 L bath solutions containing 20 mL (1:100 dilution) for 30 min. Control immersion baths was prepared by 2 L sterile water. After vaccination, fish were transferred to fiber tanks containing water under continuous aeration. At 30 and 60 days after immersion vaccination, Fish were challenged with $1\times10^6$ CFU/mL lethal concentration of a virulent strain of *F. columnare* for 1 h. Cumulative mortality and survival rate were recorded for 10 days after immersion challenge.

*Statistical analysis*

GraphPad Prism software (version 5.0) was used to generate graphs and perform statistical analyses. One-way analysis of variance, or repeated measures analysis of variance, followed by Tukey post-hoc tests were used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant and denoted as follows: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Survival curves were generated for the vaccinated fish and unvaccinated fish. The numbers of fish which died after challenge test were recorded. Relative percent survival (RPS) was calculated as 1 - (mortality rate of vaccinated fish/mortality rate of control fish) x100.



**Results**

*Physicochemical characteristics of different nanovaccine formulations*

We analyzed the zeta potential, size, and the appearance of the prepared nanovaccines. As shown in Table 1, we observed the zeta potential shifts from a negative value for whole-cell vaccines and uncomplexed NE, to a positive value for the CS vaccines and NE following complexation with CS polymers (CS-NE). These data proved the positive charge of the cationic nanovaccines in contrast to the negatively charged surface of the uncomplex vaccines. Formation of nanovaccines

was also confirmed by the measurement of particle size. Size measurement of different formulations of nanovaccines in Table 1 revealed that CS nanovaccine has an average diameter of 350 ± 50 nm. Our results also showed that CS-NE has an average diameter of 2-fold greater than the uncomplexed NE.

**Table 1**

Physicochemical properties of nanovaccines after formulations.

| Formulation | Average diameter (nm) | Zeta potential (mV) | PDI |
|---|---|---|---|
| WC | 1,900 ± 400 | −15 ± 5.0 | 0.42 |
| CS | 350 ± 50 | 27.5 ± 2.5 | 0.12 |
| NE | 175 ± 25 | −22.5 ± 2.5 | 0.14 |
| CS-NE | 350 ± 50 | 28.5 ± 6.5 | 0.19 |

Values were the means of three replicate samples. The data were presented as mean ± SD.

*Mucoadhesiveness of the prepared nanovaccines*

The affinity of different vaccine preparations toward mucosal surfaces of fish gills was studied using DAPI-stained *F. columnare.* Quantification of fluorescent signal in gill tissues after tissue lysis showed that a significant higher mean fluorescence intensity (MFI) were achieved with CS nanovaccines and CS-NE vaccines compared to whole-cell vaccine and nanoemulsion vaccines. As shown in Fig. 2a, treatment with CS nanovaccines and CS-NE vaccines resulted in 4 to 5- fold increase of fluorescent signal compared to control ( non-treated) group, respectively. Fluorescence microscopy revealed that a large number of CS nanovaccines and CS-NE nanovaccines can bind to fish gills, whereas a few particles were observed on gills of fish immerged in water containing whole-cell vaccines or NE vaccines ( Fig. 2b). Consistently, bioluminescence imaging revealed that incorporation of nanovaccines with chitosan biopolymer mediated efficient attachment to mucosal surfaces, as indicted by higher fluorescent intensity than of naked vaccines (Fig. 2c).

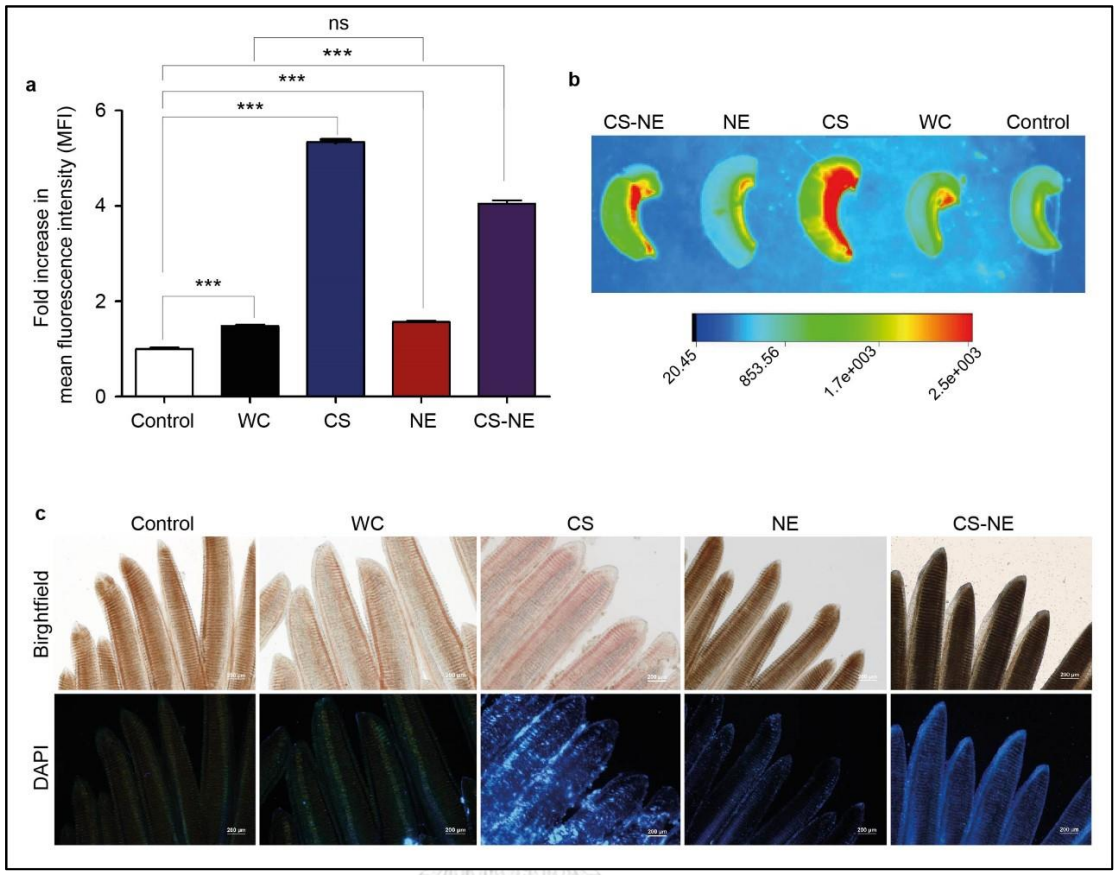


**Figure 2.** In vivo accumulation of mucoadhesive vaccines in gill tissues after direct immersion. a) Quantitative analysis of DAPI-stained vaccines in fish gill tissues. Experiments were performed in triplicate and data presented as fold-increase in mean fluorescence intensity (MFI) compared with the control (non-vaccinated fish). b) Fluorescence imaging of fish gills following direct immersion of different formulations of nanovaccines compared to whole-cell vaccines. (c) Representative microscopic fluorescence images of nanovaccines in fish gill slices after direct immersion as examined by fluorescence microscopy. Scale bar=200 μm.



### *Protective effect of mucoadhesive vaccines against flavobacterium infection*

Vaccinated and control fish were held for 30- and 60-days following bath vaccination before they were challenged with virulent *F. columnare.* Time course for bath immunization and challenge is shown in Fig. 3a. There was no fish died after the vaccination. A positive effect of vaccination is determined by a relative percent

survival (RPS) greater than 60%. At 30 days post vaccination with all vaccine formulations, the RPS were greater than 60%, as shown in Table 2. In this study, mortality in an equivalent group of non-vaccinated fish was 87%. The prolonged protective effect could be also observed in fish vaccinated with nanovaccines at 60 days post vaccination (Table 2). However, a loss of protection after initial effectiveness was observed in a group vaccinated with whole-cell bacteria as shown by mortality in this group was lower than 50%. Percentage survival after bath challenge of vaccinated and control groups is shown in Fig. 3b. We observed at least one of the following clinical signs in all the moribund and dead fish; hemorrhage, lesion on the trunk, and/or eroded tail (Fig. 3c). Moreover, chitosan polymer alone fails to protect tilapia from columnaris infection. Following immersion with the chitosan polymer by immersion 30 min, the challenge test was then carried out 30- and 60-days post immersion and resulted in high mortalities.

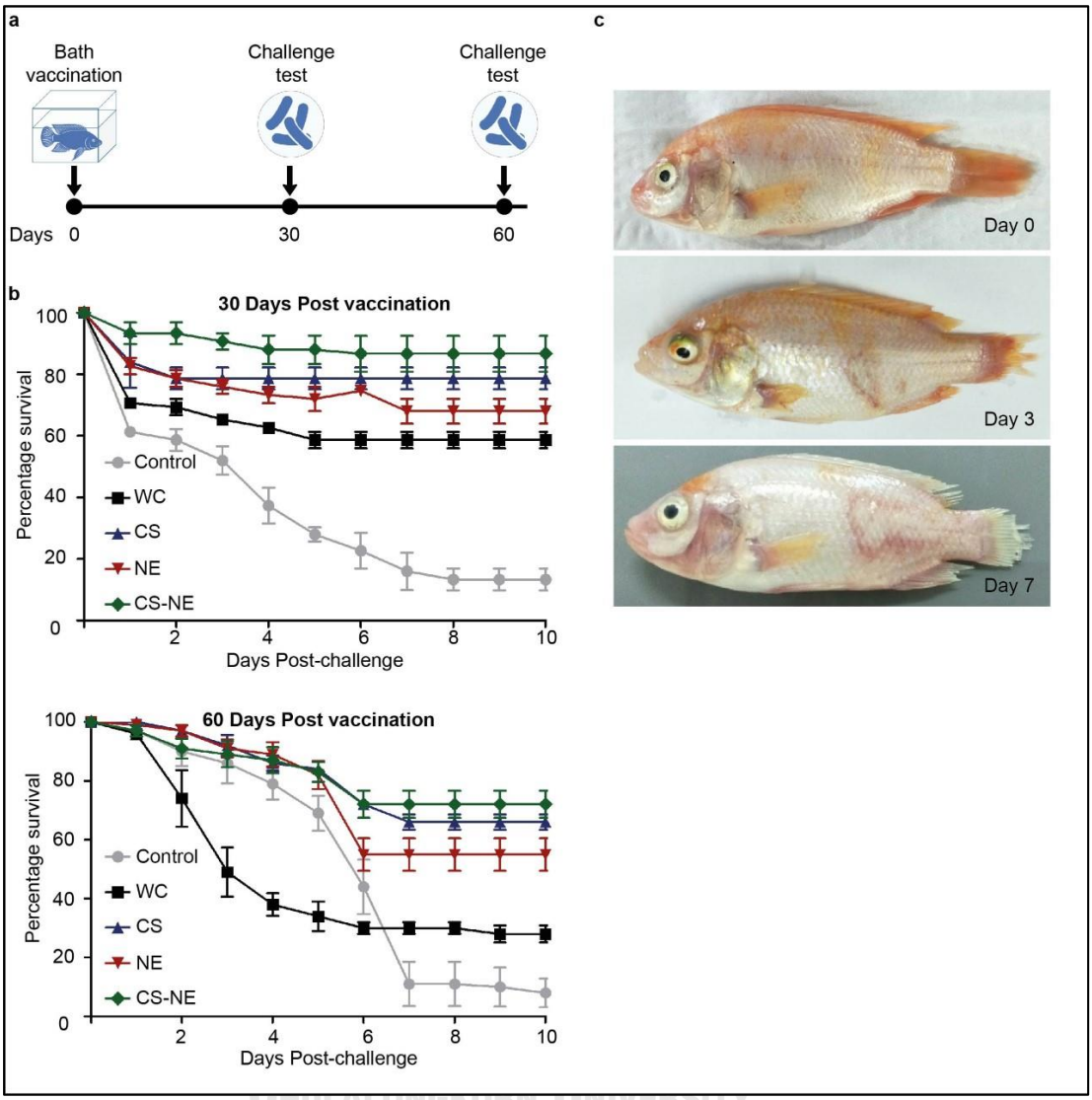




**Figure 3**. Vaccine efficacy. (a) Time course for immersion immunization and challenge test. (b) Percentage survival after bath challenge of vaccinated and control groups. The survival rates following challenge with $1\times10^{6}$ CFU/ mL *F. columnare* are presented. (c) Clinical signs of columnaris disease following bath challenge with *F. columnare*.

**Table 2**

Average percent mortality of tilapias after bath challenge ( 30- or 60-days post vaccination) with *F. columnare.*

| Group | Replicate tank | *n* | Average % mortality | Average % survival | RPS |
|---|---|---|---|---|---|
| Control (30 days) | 1 | 25 | 87 | 13 | – |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| WC vaccine (30 days) | 1 | 25 | 41 | 59 | 53 |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| CS vaccine (30 days) | 1 | 25 | 21 | 79 | 76 |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| NE vaccine (30 days) | 1 | 25 | 32 | 68 | 63 |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| CS-NE vaccine (30 days) | 1 | 25 | 13 | 87 | 85 |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| Control (60 days) | 1 | 25 | 92 | 8 | – |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| WC vaccine (60 days) | 1 | 25 | 72 | 28 | 22 |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| CS vaccine (60 days) | 1 | 25 | 34 | 66 | 63 |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| NE vaccine (60 days) | 1 | 25 | 45 | 55 | 51 |
| | 2 | 25 | | | |
| | 3 | 25 | | | |
| CS-NE vaccine (60 days) | 1 | 25 | 28 | 72 | 70 |
| | 2 | 25 | | | |
| | 3 | 25 | | | |

Immersion vaccination with $1\times10^{6}$ CFU/ mL *F. columnare* for 30 min immersion exposure.

**Discussion**

Among the effective prevention and control strategies, vaccination is one of the most effective approach. Vaccines for use in fish against infectious pathogens can

be broadly categorized as traditional and modern vaccines. The former includes killed vaccines and attenuated vaccines while the latter includes recombinant technology vaccines and synthetic peptide vaccines as well as DNA vaccines, all of which are in progress around the world (Correia et al., 2014; Dellagostin et al., 2011). In general, all of these strategies have shown advantages and disadvantages. Despite several efforts to develop effective vaccines against a large number of diseases using recombinant vaccines and recombinant DNA technology, the inherent limitation of these modern antigens is their low immunogenicity in comparison to the more traditional vaccines. The poor immunogenicity frequently observed in recombinant antigens is associated with a lack of exogenous immune activating components (Jorge and and Dellagostin, 2017). At the present, most of licensed aquaculture vaccines are in the form of live attenuated, killed/inactivated microorganisms (McVey and and Shi, 2010; Unnikrishnan et al., 2012). Traditional vaccines are generally more effective than modern vaccines. The explanation behind this fact is that protection is mediated by the combination of multiple antigens composed of lipopolysaccharides, lipoproteins, complex polysaccharides as well as proteins (LaFrentz and Klesius, 2009; Shoemaker et al., 2011; Shoemaker et al., 2009). Traditional vaccines that express these multiple antigens thus provide the most efficacious immunity superior to recombinant vaccines. As compared to inactivated vaccines, live attenuated vaccines can be highly effective (Da Costa et al., 2015; Rizzi et al., 2012). However, their potential risk of reversion of the microorganism for a more virulent phenotype can occur (Shimoji et al., 2002; Unnikrishnan et al., 2012). This major concern has become a limitation for their use in aquaculture including in Thailand. Although killed/ inactivated vaccines may be less effective than attenuated vaccines, they are typically safer.

Nanotechnology-based delivery system has been extensively used in vaccine development as it is effortless to deliver, protect the antigen from degeneration and is found to be efficient with a single dose resulted from slow release of the encapsulated antigen (Walters et al., 2015). As mentioned earlier, we hypothesized that the efficacy of killed vaccines could be enhanced by nanoencapsulation

technology. We evaluated the average size and zeta potential to characterize the nanovaccine prepared from formalin-killed and sonicated *F. columnare* via the emulsification and homogenization technique. Our results also showed that the prepared nano-sized vaccines are well-dispersed in water and provided excellent protective effect against columnaris disease following immersion vaccination as compared to inactivated whole-cell bacteria. This result could be explained by the finding that smaller nanoparticles ranging from 1 to 100 nm can be easily delivered to lymph nodes because they can be readily internalized by dendritic cells and retained for a longer period of time at the vaccine administration site (Reed et al., 2013). Moreover, the use of nano-sized vaccines improves immunogenicity in the absence of adjuvants such as alum, which are inflammatory mediators. Gills, skin, and gut are important organs that directly associated with the mucosal immunity of teleost fish and play a very important part of the fish immune defenses, protecting the body from the first encounter of infectious pathogens (Guardiola et al., 2014). The external constituent of skin, gills, and gut is a mucous gel which forms a layer of a gel-like substance covering the epithelial cells (Koshio, 2016). The fish mucus is mainly composed of water and glycoproteins, containing a vast majority of mucins, high molecular weight negatively charged oligosaccharides (Carroll et al., 2016; Perez-Vilar, 2007).



As colonization of the mucosal surfaces of fish skin and gills is the first step of *F. columnare* infection (Shoemaker and LaFrentz, 2015), we therefore hypothesized that better adsorption on mucosal surfaces and more efficient vaccine efficacy could be enhanced by biomimetic nanoparticles mimicking the mucoadhesive characteristic of live *F. columnare*. Our results also confirmed that the positively charged nanovaccines increased attachment to fish gill tissues mainly by an efficient binding of nanovaccines to the negatively charged mucosal membranes. Another possible explanation for enhanced protective effect could be the adjuvant ability of chitosan (Carroll et al., 2016; Chang et al., 2010; Li et al., 2013). Chitosan has been widely

studied for its immunogenic activities, especially via the mucosal routes (Baudner et al., 2003; Xia et al., 2015; Zeng, 2016).

Despite these promising results, a basis for the safety of novel vaccines must be established before regulatory agencies approve initiation of animal clinical trials. Further research should be undertaken in farmed tilapia in order to identify both intrinsic toxicity of the product and immunotoxicity arising from the host immune response to the new vaccine. Moreover, some related clinical parameters are required to be measured and monitored over a period of time, such as Average Daily Gain (ADG), Feed Conversion Ratio (FCR).

**Conclusion**

The strategy, as presented here, is an improved version of inactivated nanovaccine surfaced modified with chitosan biopolymers and targeted to the mucosal membrane of tilapia. Specifically, we reported here the preparation of mucoadhesive nanovaccines via the emulsification and homogenization method as well as their physicochemical and biological properties. The analysis of SEM image and zetapotential also suggested the successful modification of nanovaccines by chitosan. In vivo mucoadhesive studies demonstrated the excellent affinity of the chitosan-complexed nanovaccines toward fish gills as confirmed by bioluminescence imaging, fluorescent microscopy, and spectrophotometric quantitative measurement. By taking advantage of the unique characteristics of the fish mucus, the present study demonstrated that targeting mucoadhesive vaccines to the fish gill mucosal surface could be exploited as an effective method for immersion vaccination. Interestingly, our data confirmed that the complexation of nanovaccines with cationic chitosan polymers generates positively charged vaccine complexes. As a result, biomimetic nanoparticles mimicking the mucoadhesive characteristic of live F. columnare can help achieve better adsorption on mucosal surfaces and more efficient vaccine efficacy. Taken together, our study demonstrated the feasibility of

mucoadhesive nanoparticle as an effective delivery method for an inactivated vaccine against infectious *F. columnare* in Tilapia by immersion vaccination.

**Acknowledgments**

This work was supported by the grant from the 90th Anniversary of Chulalongkorn University Scholarship; GCUGR1125622098D, Ratchadaphiseksomphot Endowment Fund; CU-GR_61_013_31_003, National Nanotechnology Center; P1752586 and P1751449, and Thailand Graduate Institute of Science and Technology Scholarship; SCA-CO-2560-4578-TH, National Science and Technology Development Agency (NSTDA, Thailand). Faculty of Veterinary Science, Chulalongkorn University, is also acknowledged.

# Chapter V

# Modulation of the mucosal immune response of red tilapia (*Oreochromis sp.)* against columnaris disease using biomimetic-mucoadhesive nanovaccine.

**Publication**

**Kitiyodom S,** Tullus C, Rodkhum C, Thompson K, Katagiri T, Temisak S, Namdee K, Yata T*, Pirarat N* . Modulation of mucosal immune response against columnaris disease by biomimetic-mucoadhesive nanovaccine in red tilapia (Oreochromis sp.). Fish Shellfish Immunol (submitted)

**List of authors**

Sirikorn Kitiyodom, Clara Tullus and Nopadon Pirarat

Wildlife Exotic and Aquatic Pathology-Research Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

Channarong Rodkhum

Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

Kim Thompson

Moredun Research Institute, Pentlands Science Park, Penicuik, UK

Takayuki Katagiri

Laboratory of Fish Health Management, Course of Aquatic Biosciences, Tokyo University of Marine Science and Technology, Tokyo, Japan

Sasithon Temisak

Bio Analysis Group, Chemical Metrology and Biometry Department, National Institute of Metrology (NIMT), Pathum Thani, 12120, Thailand

Katawut Namdee
National Nanotechnology Center ( NANOTEC) , National Science and Technology Development Agency (NSTDA), Pathum Thani, 12120, Thailand

Teerapong Yata
Biochemistry Unit, Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, Thailand

**Abstract**

Columnaris, a highly contagious bacterial disease caused by *Flavobacterium columnare*, is recognized as one of the most important infectious diseases in farmed tilapia, especially during the fry and fingerling stages of production. The disease is associated with characteristic lesion in the mucosa of affected fish, particularly their skin and gills. Vaccines that can be delivered via the mucosa are therefore of great interest to scientists developing vaccines for this disease. In the present study, we characterized field isolates of *F. columnare* obtained from clinical columnaris outbreaks in red tilapia to select an isolate to use as a candidate for use in our vaccine, for which we characterized its colony morphology, genotype and virulence status. The isolate was incorporated into a mucoadhesive polymer chitosan-complexed nanovaccine (CS-NE), the efficacy of which was determined by experimentally infecting red tilapia that had been vaccinated with the nanoparticles by immersion. The experimental infection was performed 30-days post-vaccination (dpv), which resulted in 89 % of the unvaccinated control fish dying, while the relative percentage survival (RPS) of the CS-NE vaccinated group was 78 %. Histology of the mucosal associated lymphoid tissue (MALT) showed a significant higher presence of leucocytes and a greater antigen uptake by the mucosal epithelium in CS-NE vaccinated fish compared to control fish and whole cell vaccinated fish, respectively, and there was statistically significant up-regulation of *IgT*, *IgM*, *TNF**α***, *IL1-**β*** and *MHC-1* genes in the gill of the CS-NE vaccinated group. Overall, the results

of our study confirmed that the CS-NE particles achieved better adsorption onto the mucosal surfaces of the fish, elicited great vaccine efficacy and modulated the MALT immune response better than the conventional whole cell-killed vaccine, demonstrating the feasibility of the mucoadhesive nano-immersion vaccine as an effective delivery system for the induction of a mucosal immune response against columnaris disease in tilapia.

**Keywords**: red tilapia, mucosal immunity, MALT, columnaris disease, mucoadhesive nano-immersion vaccine

**Introduction**

Tilapia (*Oreochromis sp*.) is an important freshwater fish for global aquaculture. As tilapia aquaculture has expanded, there has been an increasing trend for intensification of the production system, resulting in overcrowding of stock. This, together with other factors, such as climate change and poor farm management, have increased the tilapias' susceptibility to bacterial infections. *Flavobacterium columnare*, the causative agent of columnaris disease, has been characterized as one of the most serious infectious bacterial diseases in farmed tilapia (Shoemaker et al., 2011). This pathogen is a gram negative, filamentous, thin-rod bacterium with gliding motility and yellow rhizoid colony formation (LaFrentz and Klesius, 2009). Studies on the genetic diversity of *F. columnare* have resulted in the bacterium being classified into 4 distinct genetic groups (I, II, III and IV). Genetic group II, III and IV have all been reported in tilapia (LaFrentz et al., 2018), with genetic group IV commonly associated with highly virulent stains affecting red tilapia (Dong et al., 2015a; Kayansamruaj et al., 2017; LaFrentz et al., 2018). Several attempts have been made to phenotype this bacterium and characterize its virulence factors, including one of its secreted proteins, outer membrane vesicles and the coordination of bacterial cell organization, all of which have been associated with its rhizoid morphotype (Kunttu

et al., 2009; Laanto et al., 2014). The relationship between genotypic variation, phenotypic characteristics and variations in virulence still remains to be resolved.

*Flavobacterium columnare* infections result in skin lesions, fin rot, gill necrosis and high levels of mortality, especially during the fry and fingerling stages of production, leading to severe economic losses (Declercq et al., 2013). The mucosal area of the fish, such as the skin and gills, tend to be the main sites affected by the bacterium.

The mucosal areas of the fish (i.e. gills, nares, skin, intestine and hind gut) are in contact with the external environment and therefore are potential routes of infection by bacteria present in the water. Immersion vaccination is an ideal route for delivering the vaccine to the mucosal tissues, it is ease to administer, less stressful than injection vaccination and is suitability for mass vaccination of fish. The method is associated with low efficacy, however, as antigen uptake through the gills and skin is limited (Soto et al., 2015). We previous demonstrated the use of a nanovaccine for *F. columnare*, with mucoadhesive characteristics that could enhance uptake of the vaccine at the mucosal surface, through the efficient binding of the positively charged vaccine nanoparticles to the negatively charged mucosal membranes, resulting in higher levels of protection when compared with a conventional killed vaccine (Kitiyodom et al., 2019). However, the induction of the immune response at the mucosal site and the interaction between the mucosal associated lymphoid tissues (MALT) and antigen after vaccination have not been clearly elucidated. MALT sites have been shown to elicit a robust immune response after immersion vaccination. Previous studies using mucosal vaccines in fish have examined the uptake of vaccine antigens administered to MALT sites i.e. nasopharynx-associated lymphoid tissue (NALT) (Tacchi et al., 2014); skin-associated lymphoid tissue (SALT) (Ototake et al., 1996); gill-associated lymphoid tissue (GiALT) (Kato et al., 2013; Korbut et al., 2016; Ohtani et al., 2015) and gut-associated lymphoid tissue (GALT)

(Adelmann et al., 2008; Korbut et al., 2016; Ohtani et al., 2015). Using a mucoadhesive nanovaccine to target the immune response at the mucosal surface, such as gills or skin, may be an effective method for mucosal immune modulation and protection against columnaris disease in tilapia. We, therefore, characterized a *F. columnare* isolate (genetically and phenotypically) as a potential vaccine candidate, and use this to develop a mucosal killed-nanovaccine mimicking the mucoadhesive characteristics of live *F. columnare*. We assessed the mucosal immune response induced in the MALT by the vaccine, examining MALT histology and the expression of immune related genes within the gill of vaccinated fish.

**Materials and methods**

All animal experiments were approved by the Animal Ethics Committee of Chulalongkorn University, IACUC1831020. Upon termination of the study, all fish were euthanized according to appropriate guidelines. This project has been reviewed and approved by the biosafety committee of the Faculty of Veterinary Science, Chulalongkorn University, in accordance with the faculty regulations and policies governing the biosafety, IBC1831052.



***Fish and experimental conditions***

Healthy red tilapia (*Oreochromis sp.),* with an average weight of 5 g (n= 600 fish) were acclimatized for 2 weeks and distributed into 200 L fiberglass tanks (4 tanks) containing water under continuous aeration. Air and water temperatures were monitored daily and were maintained within acceptable ranges of 25–33°C and 25–28°C, respectively. Dissolved oxygen (DO) content were observed daily and maintained at 5 mg/L. Total ammonia (TAN) and pH were measured weekly and were within range of 0.1 mg/L and 7.48–8.16, respectively. Experimental fish were fed twice per day, and water was changed by fifty percent every second day.

*Bacteria isolation and identification*

Thirty-two *F. columnare* isolates were collected from the gills and skin of moribund tilapia during 2016-2018 (Table 1). *F. columnare* isolates were confirmed by species-specific PCR (Table 2) (Welker et al., 2005).

**Table 1:** Information of *F. columnare* 32 isolates

| Isolates | Geographical | Organ | Year | Case mortality rate (%) | Colony morphology |
|---|---|---|---|---|---|
| **F-K16/2** | Kanchanaburi | Gill | 2016 | 80 | yellow rhizoid |
| **F-K16/4** | Kanchanaburi | Gill | 2016 | - | Yellow non-rhizoid |
| **F-K17/1*** | Kanchanaburi | Gill | 2017 | 95 | yellow rhizoid |
| **F-K17/2** | Kanchanaburi | Skin | 2017 | - | yellow non-rhizoid |
| **F-K18/1** | Kanchanaburi | Gill | 2018 | 50 | yellow rhizoid |
| **F-K18/2** | Kanchanaburi | Gill | 2018 | 50 | yellow non-rhizoid |
| **F-K18/6** | Kanchanaburi | Skin | 2018 | 50 | yellow rhizoid |
| **F-R16/1** | Ratchaburi | Gill | 2016 | 75 | yellow rhizoid |
| **F-R17/4** | Ratchaburi | Gill | 2017 | 90 | yellow rhizoid |
| **F-R18/1** | Ratchaburi | Gill | 2018 | 80 | yellow rhizoid |
| **F-A16/3** | Angthong | Gill | 2016 | 80 | yellow rhizoid |
| **F-A17/2** | Ayutthaya | Skin | 2017 | 20 | yellow non-rhizoid |
| **F-A17/3** | Ayutthaya | Skin | 2017 | 20 | yellow rhizoid |
| **F-A18/1** | Ayutthaya | Gill | 2017 | 50 | yellow rhizoid |
| **F-S16/1** | Samutsonkham | Gill | 2016 | - | yellow rhizoid |
| **F-S16/3** | Samutsonkham | Skin | 2016 | - | yellow non-rhizoid |
| **F-S17/1** | Samutsakhon | Gill | 2017 | - | yellow rhizoid |
| **F-S17/2** | Samutsakhon | Gill | 2017 | - | yellow rhizoid |
| **F-S17/4** | Samutsakhon | Skin | 2017 | - | yellow non-rhizoid |
| **F-P16/1** | Phetchaburi | Gill | 2016 | - | yellow non-rhizoid |
| **F-P18/1** | Phetchaburi | Gill | 2018 | - | yellow rhizoid |
| **F-N16/1** | Nakhonpathom | Skin | 2016 | - | yellow non-rhizoid |
| **F-N16/3** | Nakhonpathom | Gill | 2016 | - | yellow non-rhizoid |
| **F-N17/1** | Nakhonpathom | Gill | 2017 | 80 | yellow rhizoid |

| | | | | | |
|---|---|---|---|---|---|
| **F-N17/2** | Nongkhai | Gill | 2017 | 90 | yellow rhizoid |
| **F-N17/3** | Nongkhai | Gill | 2017 | 60 | yellow rhizoid |
| **F-N17/4** | Nongkhai | Gill | 2017 | 60 | yellow rhizoid |
| **F-N17/5** | Nongkhai | Gill | 2017 | 60 | yellow non-rhizoid |
| **F-N17/6** | Nakornratchasima | Gill | 2017 | 50 | yellow rhizoid |
| **F-N17/8** | Nakornratchasima | Skin | 2017 | 50 | yellow non-rhizoid |
| **F-M17/1** | Mukdahan | Skin | 2017 | 60 | yellow rhizoid |
| **F-M17/3** | Mukdahan | Gill | 2017 | 60 | yellow rhizoid |

(*: asterisk symbol mark on high virulent strain used in this study)

**Table 2:** Primers were used for species-specific PCR and 16S rRNA sequencing.

| Primer name | Sequences | Product | Product (bp) | Reference |
|---|---|---|---|---|
| FCISRFL | TGCGGCTGGATCACCTCCTTTCTAGAGACA | *F. columanare* identification | 500 | (Welker et al., 2005) |
| FCISRR1 | TAATYRCTAAAGATGTTCTTTCTACTTGTTTG | | | |
| UN-20/20F | AGAGTTTGATC(AC)TGGCTCAG | 16S rRNA gene amplification | 1450 | (Darwish and Ismaiel, 2005) |
| R1438 | GCCCTAGTTACCAGTTTTAC | | | |
| F582 | CAGTGGTGAAATCTGGT | 16S rRNA gene sequencing | | (Darwish and Ismaiel, 2005) |
| F1274 | AGTTCGGATCGGAGTCTGC | | | |
| R1117 | AACCATGCAGCACCTTGAA | | | |
| R584 | GAGCGACCAGATTTCACCAC | | | |



Bacteria were cultured in 5 mL Tryptone Yeast Extract Salt (TYES) broth at 28°C for 48 h, centrifuged at 6000 g for 5 min and the supernatant discarded. Cell pellets were suspended in 200 µl sterile water, boiled for 10 min, cooled immediately on ice and briefly centrifuged. The supernatant was used as DNA template or stored at -20°C until used. *F. columnare*-specific primers, FCISRFL and FCISRRI, were used to amplify the partial sequence of 16S-23S ISR as described by

Welker et al. (2005). Amplification was performed as 25 µl reactions containing 12.5 µl Master Mix (GoTaq Green; Promega), 0.2 µM of each PCR primer and 5 µl DNA template (100–400 ng genomic DNA). The following steps were performed for the PCR procedure: denaturation at 94 ℃ for 5 min and then 30 cycles of amplification at 94 ℃ for 30 s, annealing at 45℃ for 45 s; extension at 72℃ for 7 min and held at 4℃. PCR products were run on a 1% agarose in Tris-Borate-EDTA (TBE) buffer at 100 V, stained with Red Safe and viewed under UV light using a gel documentation system (Vilber Lourmat).

Only one *F. columnare* isolate (F-K17/1) was selected from the *F. columnare* collection for use in the vaccine. This isolate had the highest level of virulence in clinical outbreaks of the disease (Table 1). A nearly full-length sequence of the 16S rRNA gene of *F. columnare* F-K17/1 was amplified using universal primers UN-20/20F and R1438 as described by Darwish and Ismaiel (2005). PCR products were cleaned using a NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) and submitted for sanger sequencing (1st BASE Pte Ltd) using six primers (Table 2). The sequences from these reactions were assembled using CAP contig assembly program and used to perform phylogenic analysis as described by LaFrentz et al. (2018). A total of twenty-four 16s rRNA gene sequences of *F. columnare*, *F. psychrophilum* and *F. johnsoniae* were used for generating the phylogenetic tree. Twenty-two published 16s rRNA sequences of *F. columnare* were downloaded from NCBI and 2 sequences were extracted from *F. columnare* genomes of this study. All sequences were aligned and trimmed using the Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar et al., 2016). The phylogenetic tree was generate based on the maximum likelihood method based on the Kimura 2-parameter model (K2+G) with 1,000 replicates (Kimura, 1980), which is the best nucleotide substitution model tested using MEGA7, and the best model with the lowest Bayesian Information Criterion scores.

*Bacteria and vaccine preparation*

Bacterial cultures used for vaccine preparation were grown in TYES broth at 25–28°C for 48 h (Grabowski et al., 2004). Formalin-killed bacteria were used 0.2% formalin and incubated at 4°C for 20 h. Bacterial cells were collected by centrifuging at 3,000 g at 4°C for 30 min. Formalin-killed bacteria were washed three times by centrifugation as desribed above and resuspended in Phosphate Buffer Saline: PBS (bacterial concentration $10^{10}$ colony forming units (CFU) $ml^{-1}$). Bacterial concentration of vaccine preparations was 108 CFU $ml^{-1}$. Four groups of fish were including in the study: (1) whole cell killed bacteria vaccine (WC); (2) nanovaccine (CS-NE); (3) polymer blank (polymer) and (4) PBS (control). Formulation of the vaccine was carried out according to Kitiyodom et al. (2019) [10]. In brief, to prepare the WC vaccine, an aliquot of bacterial cells (15% w/w) was mixed with PBS (85% w/w). To prepare the CS-NE vaccine, an aliquot of sonicated bacterial cells by a sonicator probe at 40% amplitude for 10 min (30% w/w) was mixed with 6% (w/w) of polyoxyethylene (20) sorbitan monolaurate, 2% (w/w) of medium chain triglycerides (Miglyol) and 62% (w/w) of water. The mixture was homogenized using a sonicator probe at 40% amplitude for 5 min. The complexation of the nano-emulsion with chitosan was performed by adding 1% of chitosan (previously dissolved in 1% acetic acid) to the nano-emulsion at a ratio of 1:1 (v/v). The mixture was stirred for 1 h at room temperature.



*Vaccination and vaccine efficacy test*

Red tilapia (5 g) were divided into 4 groups; control, WC, CS-NE and polymer groups (150 fish each, 1 tank/group). Fish were immersed in vaccine solutions, diluted 1:100 dilution with tank water (i.e. $10^6$ CFU $ml^{-1}$) for 30 min with aeration. After vaccination, fish were transferred into fiberglass tanks containing 200 L of water. After 30 days post-vaccination (dpv), fish (30 from each group, 3 replicate tanks) were challenged with a lethal concentration 80 (LC80) of a virulent strain of F-K17/1 by immersion for 1 h. The time course for immunization and challenge is presented in Figure 2A, with fish held for 30 dpv before challenging them with *F. columnare*. The

cumulative mortality and survival rates were recorded for 14 days after challenge and the relative percent survival (RPS) calculated i.e. RPS = 1 - (mortality rate of vaccinated fish/mortality rate of control fish) x 100 (Austin, 2012).

*Mucoadhesive property*

To assess the mucoadhesive attachment of the vaccine to the gills, bacterial were stained with a fluorescent stain (DAPI: 4,6- diamidino-2-phenylindole) which had been added to the vaccine formulation. Red tilapia (5 g) were divided into 4 groups; control, WC, CS-NE and polymer groups (5 fish each). Fish were immersed with 106 CFU/ml of vaccine preparations in water (1: 100 dilution) for 30 min. Following immersion and euthanasia, the fish gills were sampled. Attachment of the vaccines to mucosal surfaces was assessed by observing the fluorescent signal of the DAPI-stained bacteria on the tissue under a fluorescence microscope.

*Mucosal immune response*

Red tilapia (5 g) were divided into 4 groups; PBS (control), whole cell killed bacteria vaccine (WC), hybrid chitosan-complexed nanoemulsion vaccine (CS-NE) and polymer blank (polymer) groups (150 fish each). Fish were immersed in vaccine solution (1:100 dilution) for 30 min as described above. At 3 and 21 dpv, 6 fish per group were collected for tissue samples include gill, nares, skin, spleen, head kidney and hind gut for histology. For real time quantitative reverse-transcription RT-PCR (qRT-PCR) gene expression, gill was collected at 1,3,14 and 21 dpv from 6 fish from each group.

*Histology*

Morphological changes of the MALT in the gill, nares, skin and hindgut of fish from the four groups were evaluated. Tissues were collected and fixed in 10% formalin, processed routinely, embedded in paraffin, sectioned at 5 µm, and stained

with hematoxylin and eosin (H&E). The tissue sections were examined by light microscopy for descriptive interpretation.

***Immunohistochemistry***

Gill samples of fish after vaccination were also used for immunohistochemistry staining (Slaoui and Fiette, 2011). The slides were deparaffinized, endogenous peroxidase activity blocked with 3% $H_2O_2$ solution in methanol at room temperature for 10 min. The slides were then blocked with 2% fetal bovine serum in PBS. A. monoclonal anti-*Flavobacterium columnare* antibody (Ango science SpA) was diluted to 1:1,000 in PBS and added to the sections on the slides and incubated for overnight. A secondary antibody conjugate with peroxidase (Nichirei) was added to the sections on the slides incubated 1 hours at 37°C. The slides were added 3,3'-Diaminobenzidine (DAB) substrate solution, counterstained with hematoxylin and visualized under a light microscope.

***Gene expression determined by qRT-PCR***

RNA isolation and cDNA synthesis: RNA was extracted from 30 to 40 mg of each gill tissue sample using the Rneasy Minikit (QIAGEN) following the manufacturer's instructions. RNA samples were stored at -80°C until used. RNA quantity and quality were determined using the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK).and adjusted to a final concentration of 1 µg µL -1. To convert mRNA to complementary DNA (cDNA), a Quantinova Reverse Transcription kit (QIAGEN) was employed using the manufacture's protocol. The cDNA sampled from the gill samples was analyzed by qRT-PCR for the expression of immune genes (table 3), including interleukin-1 (*IL-1*), tumor necrosis factor alpha (*TNF**α***), MHC class 1 (*MHC 1*), immunoglobulin M (*IgM*) and immunoglobulin T (*IgT*).

The qRT-PCR was performed in 96-well plates using Luna® Universal qPCR master mix (New England Biolab Inc., USA) according to manufacturer's instructions. Individual 20 µL reactions consisted of 10 µL Luna® Universal qPCR master mix and cDNA diluted at 1:10 as the template. The optimal annealing temperature for all primers was determined using the thermal gradient feature of the CFX96 Real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The cycling profile was as follows: enzyme activation was carried out at 95 °C for 1 min, followed by 45 cycles of denaturing at 95 °C for 15 s, and annealing and primer extension at 55 and 60 °C for 30 s. The *β-actin* served as an internal control for cDNA normalization. Gene expression was calculated as relative to the *β-actin* using the 2-ΔΔCt method (Pfaffl, 2001). The gene expression data were normalized to the reference genes *β-actin* and expressed as a comparison of vaccinated fish relative to control fish.

**Table 3:** Primers used in qRT- PCR

| Gene | Target | Sequence forward/reverse (5'-3') | Product (bp) | Reference |
|---|---|---|---|---|
| *β-actin F* | housekeeping gene | AAGGACCTGTACGCCAACAC | 196 | (Pirarat et al., 2011) |
| *β-actin R* | | ACATCTGCTGGAAGGTGGAC | | |
| *TNFα F* | inflammation related gene | CTCACAGATAGCGGCATCAA | 190 | (Pirarat et al., 2011) |
| *TNFα R* | | CCTGGGCTCTCTCTGTGTTC | | |
| *MHC IIβ F* | adaptive immune-related gene | TCAGCACAGCAGATGGATTC | 175 | This study |
| *MHC IIβ R* | | GCCTGCTTCACTCCAAACTC | | |
| *IL-1β F* | adaptive immune-related gene | AAGATGAATTGTGGAGCTGTGTT | 175 | This study |
| *IL-1 β R* | | AAAAGCATCGACAGTATGTGAAAT | | |
| *IgM-F* | adaptive immune-related gene | TGGTACTGGGGGTCAAACAT | 156 | (Pirarat et al., 2011) |
| *IgM-R* | | TAAGCGATCCATTCCAGTCC | | |
| *IgT-F* | adaptive immune-related gene | AGACACACCAGAGTGATTTCAT | 78 | This study |
| *IgT-R* | | AGACACACCAGAGTGATTTCATCAG | | |

### *Statistical analysis*

GraphPad Prism software (Version 8.0) was used to generate graphs and perform statistical analyses. One-way and Two-way analysis of variance, or repeated measures analysis of variance followed by Turkey's multiple comparison test were used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

## Results

### *Bacteria isolation and identification*

A total of thirty-two bacterial isolates of *F. columnare* were collected from moribund tilapia between 2016 and 2018 from tilapia farms all over Thailand, which had a history of high mortality and morbidity in their fish. The bacteria were confirmed as *F. columnare* by species-specific PCR, the results of which are shown in Table 3. The highest level of mortality observed in the farmed fish was with *F. columnare* isolate (F-K17/1) and was therefore selected for subsequent analyses i.e. near-complete sequencing of its 16S rRNA gene and phylogenetic analysis. The phylogenetic tree constructed with the twenty-two 16s rRNA gene sequences of *F. columnare*, and on each of *F. psychrophilum*, and *F. johnsoniae* is shown in Figure 1 (*: sequence used in this study). The 16s rRNA gene-based tree were constructed using the maximum likelihood method based on the Kimura 2-parameter model (K2+G) with 1,000 replicates [20]. F. columnare isolate F-K17/1 was displayed in genetic group 4 as shown in Figure 1.

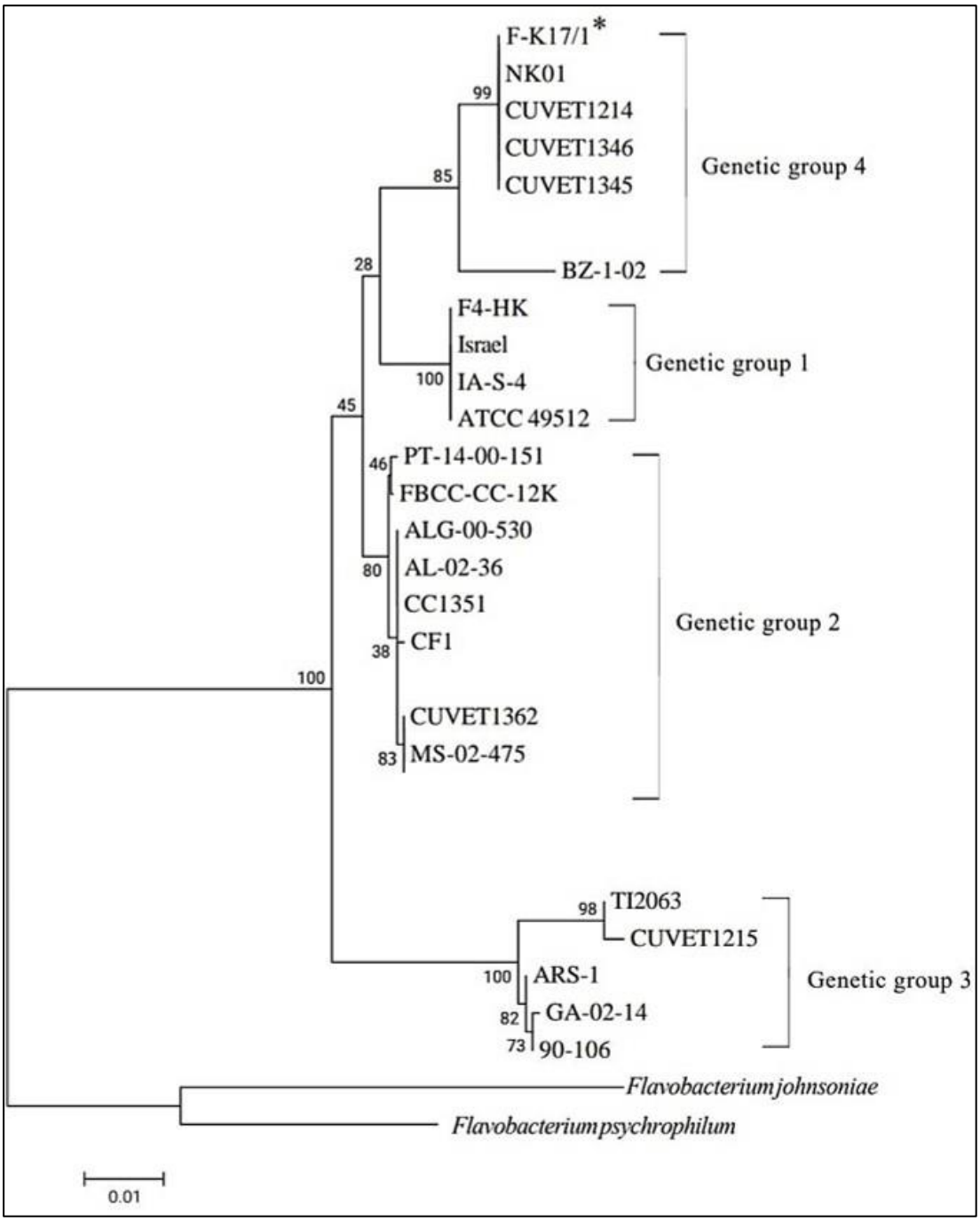


**Figure 1:** A total 25 16s rRNA gene sequences of *Flavobacterium columnare, F. psychrophilum* and *F. johnsoniae* were used for generating the phylogenetic tree (*: sequence used in this study).

*Vaccine efficacy*

Red tilapias (5 g) were divided into 4 groups; control, WC, CS-NE and polymer groups (150 fish each, 1 tank/group). No fish died after vaccination. The mortality of the non-vaccinated fish, WC, CS-NE and polymer were 89, 20, 42 and 87% mortalities, respectively. The RPS value of the vaccinated and control group after challenge is shown in Figure 2B, while the RPS value of CS-NE group was greater than 60%.

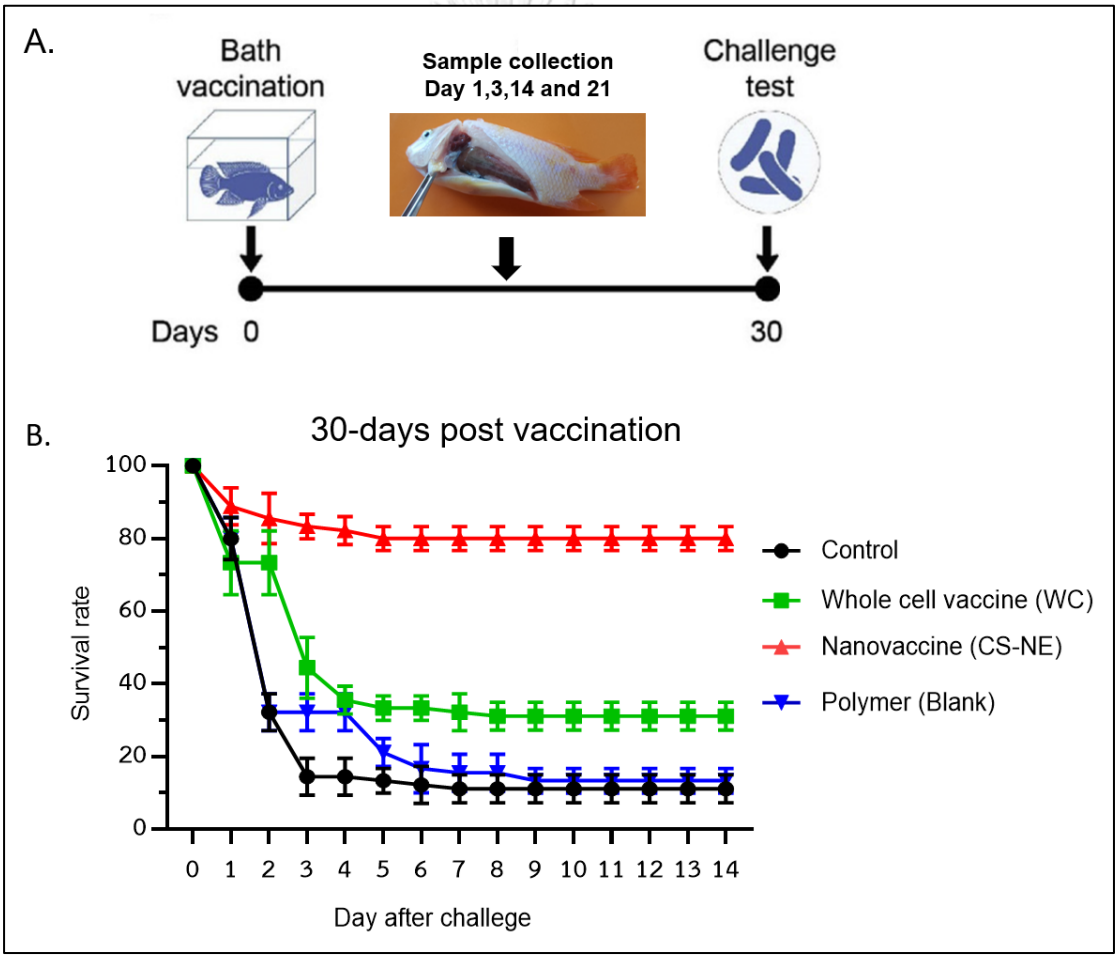


**Figure 2:** Vaccine trial. A) Time course for bath vaccination, sample collection and challenge test, fish (30 from each group, 3 replicate tanks). B) Percentage survival after bath challenge of vaccinated and control groups with $1\times10^{6}$ CFU/ mL *F. columnare* strain of F-K17/1 by immersion for 1 h.

*Mucoadhesive property*

The attachment of the bacteria in the vaccine preparations to the surface of gills was examined using DAPI-stained *F. columnare* (Figure 3). The fluorescence staining in the gills of the CS-NE vaccine group was much stronger (Figure 3G) than the other groups, while only a small amount of staining was observed on gills of fish immersed in the WC vaccine (Figure 3F).

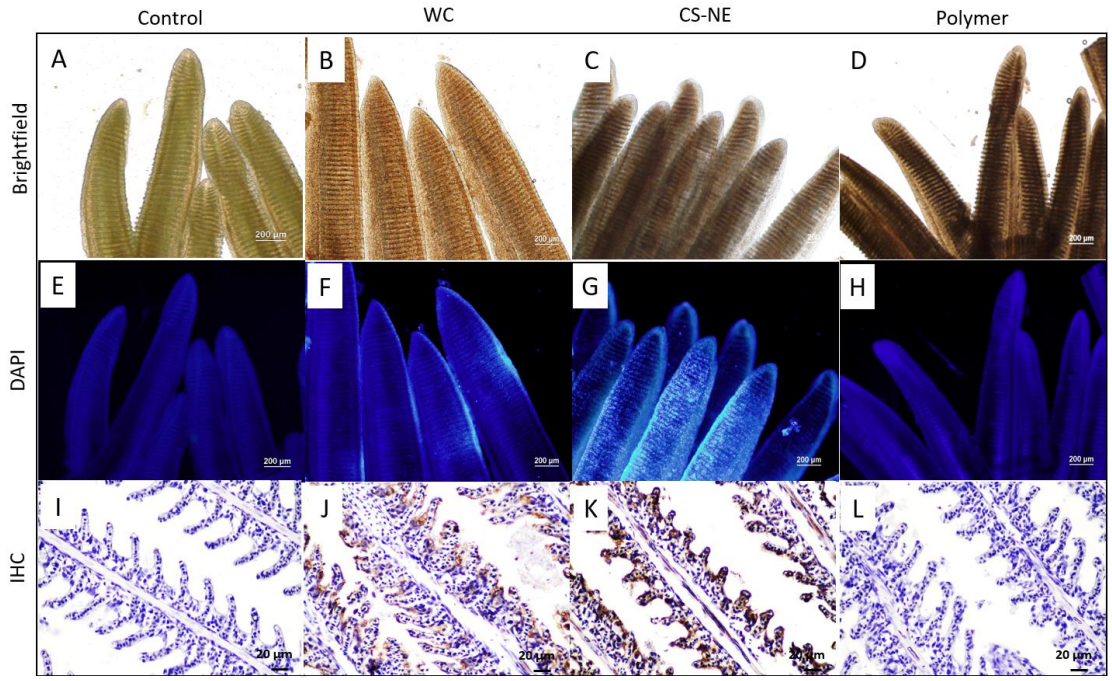




**Figure 3:** Microscopic fluorescence (DAPI) and immunohistochemistry (anti-*F. columnare* monoclonal antibody) of vaccine uptake by gills after immersion vaccination. A,E,I) Gill of fish in control group; B,F,J) Gill of fish in whole cell group, with limited particles observed on their gills; C,G,K) Gill of fish vaccinated with CS-NE showing strong binding of the particles to gills; D,H,L) Gill of fish in polymer group.

*Histology and Immunohistochemistry*

At 3 dpv, a higher lymphocyte aggregation was observed in the GiALT histology at the base of inter-branchial junction (arrowhead), forming a discrete nodular structure of GiALT. The presence of eosinophilic granular cell infiltration

(arrow) was frequently seen in the GiALT structure of CS-NE fish (Figure 4B). The WC and polymer vaccinated fish showed a moderate response of lymphocyte aggregation at the base of inter-branchial junction (Figure 4C&D). At 21 dpv, a greater accumulation of lymphocytes was seen in gill histology (Figure 4F) with thickening of primary gill lamellae evident (arrowhead) in CS-NE group compared with other groups. In NALT histology, at 3 dpv, CS-NE fish showed the increase number of mucous goblet cells and lymphocyte infiltration into olfactory epithelium (arrowhead) (Figure 5B). There was evidence of intra-epithelial lymphocytes and goblet cells hyperplasia in the olfactory of WC vaccinated fish (Figure 5C). At 21 dpv, the CS-NE vaccinated group had a marked increase in intraepithelial lymphocytes (arrowhead) and infiltration of lymphocyte into the lamina propria of NALT (Figure 5F), resulting in the thicker and broader in the width of lamina propria layer when compared with other groups. The olfactory structure also showed folding (circle) in its arrangement. The olfactory epithelium with many cytoplasmic vacuoles containing homogeneous pale pink substance frequently appeared to protrude into the lumen. The presence of intraepithelial lymphocytes could be moderately observed in the WC vaccinated fish (Figure 5G). At 3 dpv, a greater extent of mucous cells, hyperplastic epithelium and intraepithelial lymphocytes (arrowhead) were present in the SALT histology of CS-NE and WC vaccinated fish (Figure 6B & 6C). At 21-dpv, CS-NE and WC vaccinated fish had greater lymphocyte infiltration in the squamous epithelium (arrowhead) and goblet cell hyperplasia could also be observed. Many eosinophilic granular cells (arrow) were observed in the epidermal epithelium and sub-epidermal area in CS-NE fish at 21-DAV (Figure 6F). At 3 dpv, GALT histology of the hind gut or posterior intestine of CS-NE vaccinated fish revealed a greater extent of hyperplastic goblet cells containing homogeneous pale pink color in their cytoplasm, intraepithelial lymphocyte and lymphocyte infiltration in lamina propria (arrowhead) (Figure 7B). The GALT of the WC vaccinated fish also had an increase number of mucous goblet cells with homogeneous pale pink staining of cytoplasmic

vacuoles and intra-epithelium lymphocytes in the mucosal layer (Figure 7C). At 21 dpv, CS-NE and WC vaccine fish also displayed intra-epithelium lymphocytes and lymphocyte infiltration in intestinal epithelium and lamina propria (Figure 7F & 7G).

In the immunohistochemistry, strong brown staining was seen in the gill of CS-NE vaccinated fish, with staining especially seen in the mucous goblet cells and the epithelial lining of the secondary lamellae (Figure 3K). Positive staining was also seen in the gills of the WC vaccinated fish, but the extent of this staining was lower than the CS-NE vaccinated fish (Figure 3J). No positive reaction was evident in the gills of the control or polymer immersed fish (Figure 3I and 3L, respectively).

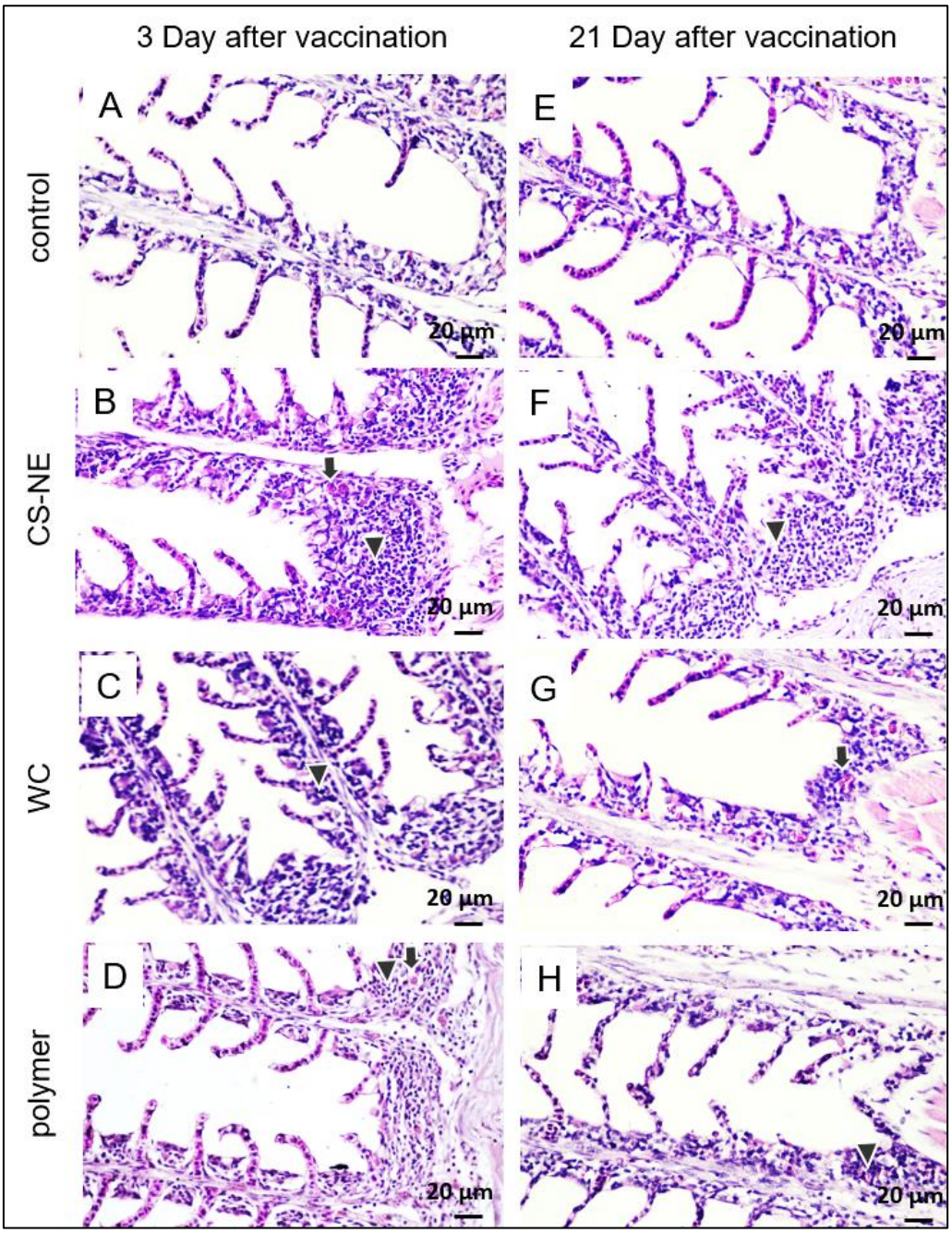


**Figure 4:** Gill histology (gill associated lymphoid tissue) of tilapia at 3-days post vaccination A) control, B) CS-NE, C) WC and D) polymer. Gill histology at 21 dpv of E) control, F) CS-NE, G) WC and H) polymer. Lymphocyte aggregation at the base of inter-branchial junction (arrowhead) and eosinophilic granular cell infiltration (arrow). Scale bar=20 µm

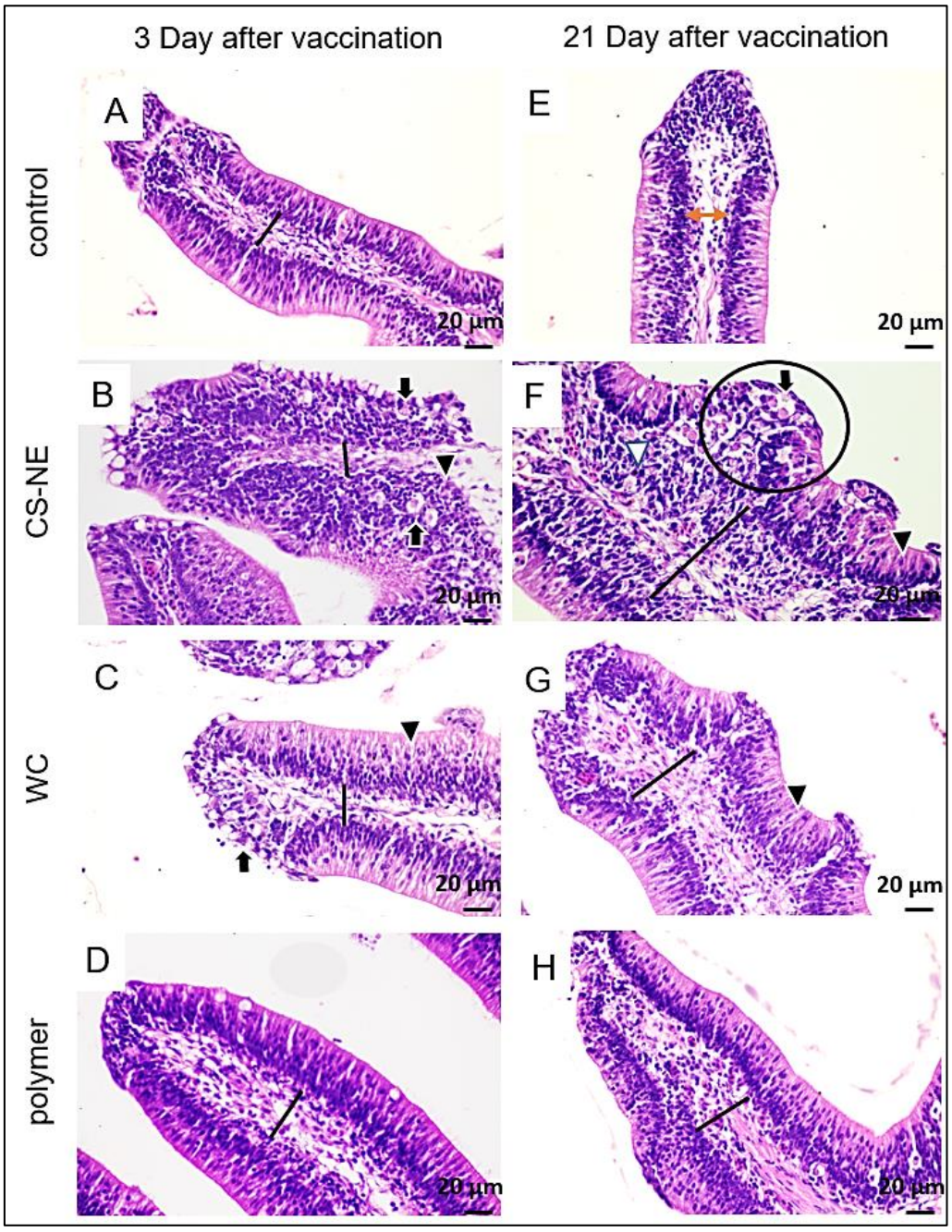


**Figure 5:** Nostril histology (Nostril associated lymphocyte tissue) of tilapia at 3 days post vaccination (dpv) of A) control, B) CS-NE, C) WC and D) polymer. Nostril histology at 21 dpv E) control, F) CS-NE, G) WC and H) polymer. Lymphocyte infiltration at intraepithelial and lamina propria (arrowhead), eosinophilic granular cell infiltration (arrow), the olfactory epithelium folding (circle). The lines indicate the width of the lamina propria. Scale bar=20 µm.

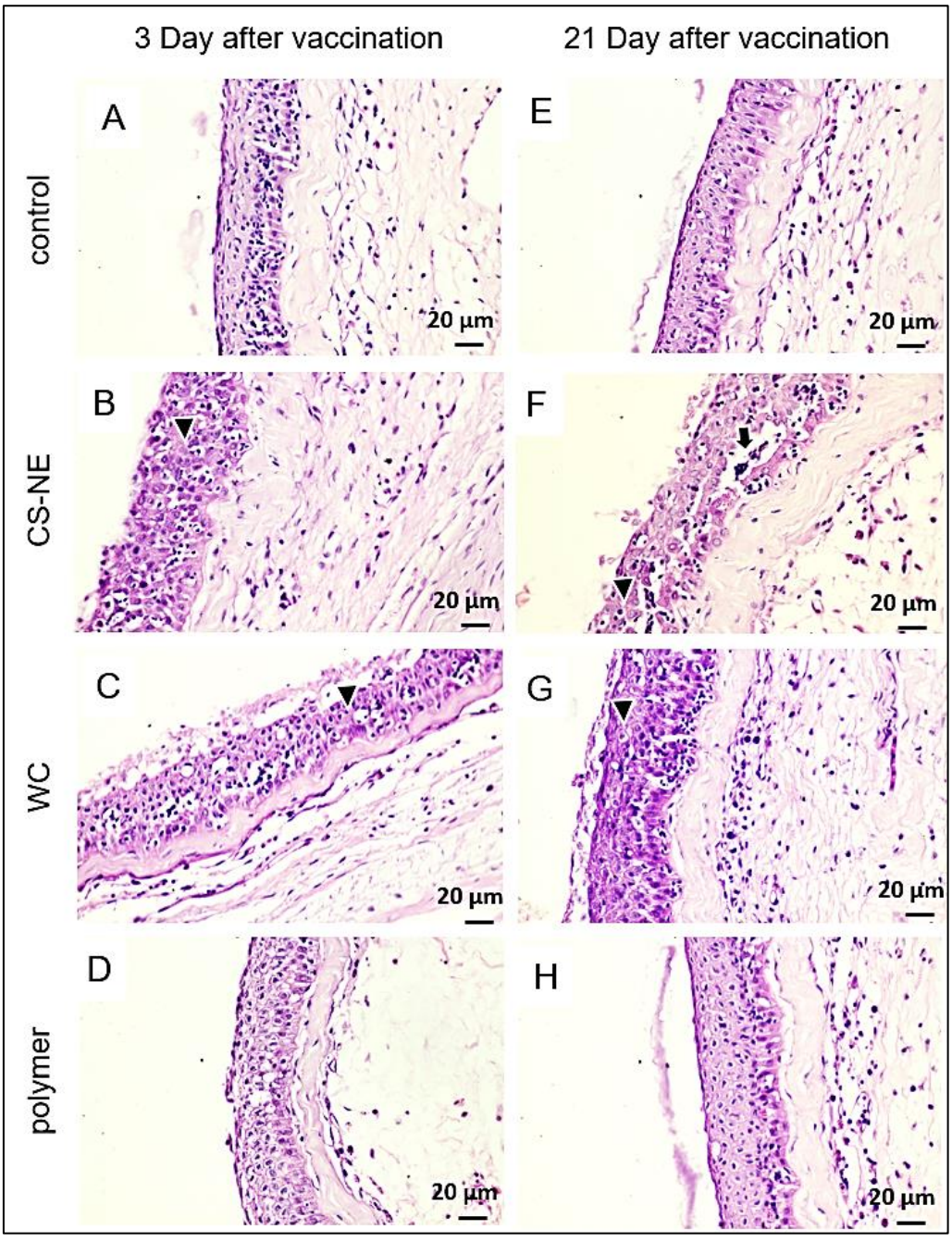


**Figure 6:** Skin histology (SALT) of tilapia at 3-DAV of A) control, B) CS-NE, C) WC and D) polymer. Skin histology at 21-DAV of E) control, F) CS-NE, G) WC and H) polymer that showed lymphocyte infiltration at intraepithelial and lamina propria (arrowhead). Scale bar=20 µm

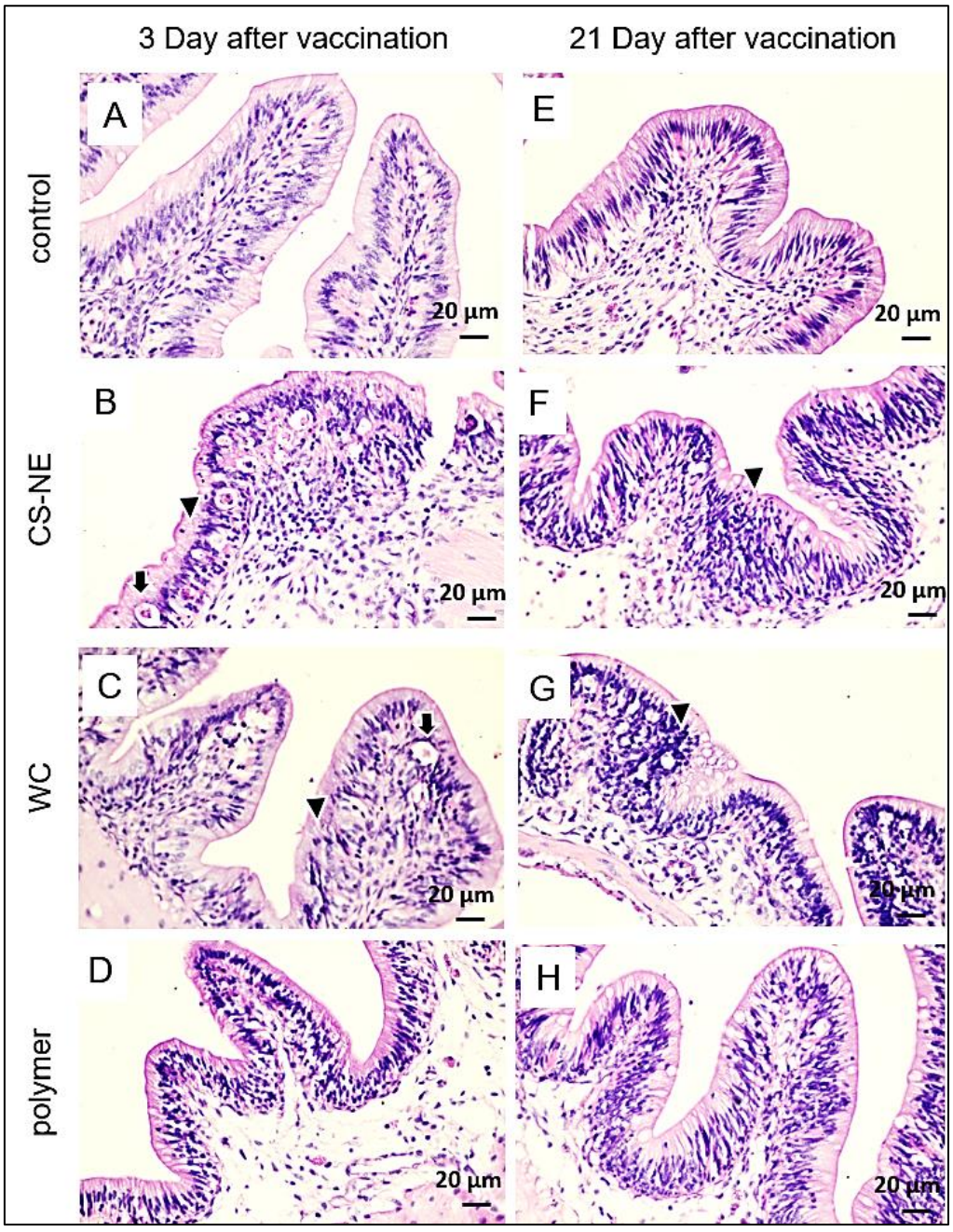


**Figure 7:** Hindgut histology (GALT) of tilapia at 3-DAV of A) control, B) CS-NE, C) WC and D) polymer. Hindgut histology at 21-DAV of E) control, F) CS-NE, G) WC and H) polymer that showed lymphocyte infiltration at intraepithelial and lamina propria (arrowhead). Scale bar=20 µm

***Gene expression with Real time PCR***

The expression of the 5 immune genes that were examined, *IgM, IgT, IL1β*, *TNF-α* and *MHC1* gene in the gills of each of the experimental groups at 1, 3, 14, 21 dpv. The expression of *IgM, IgT, IL1β, TNF-α* and *MHC 1* was higher significantly in the CS-NE compare with WC and polymer vaccinated groups (Figure 8). Significantly higher expression of *IgM* and *IgT* genes was measured in the gill of CS-NE vaccinated group at each time point measured post-vaccination compared to the other groups of fish. The genes encoding *IL1β, TNF-α* and *MHC1* genes were also up regulated at each time point, especially in the CS-NE vaccinated group. This is a first report confirming that immersion vaccination induces a mucosal IgT response in tilapia.

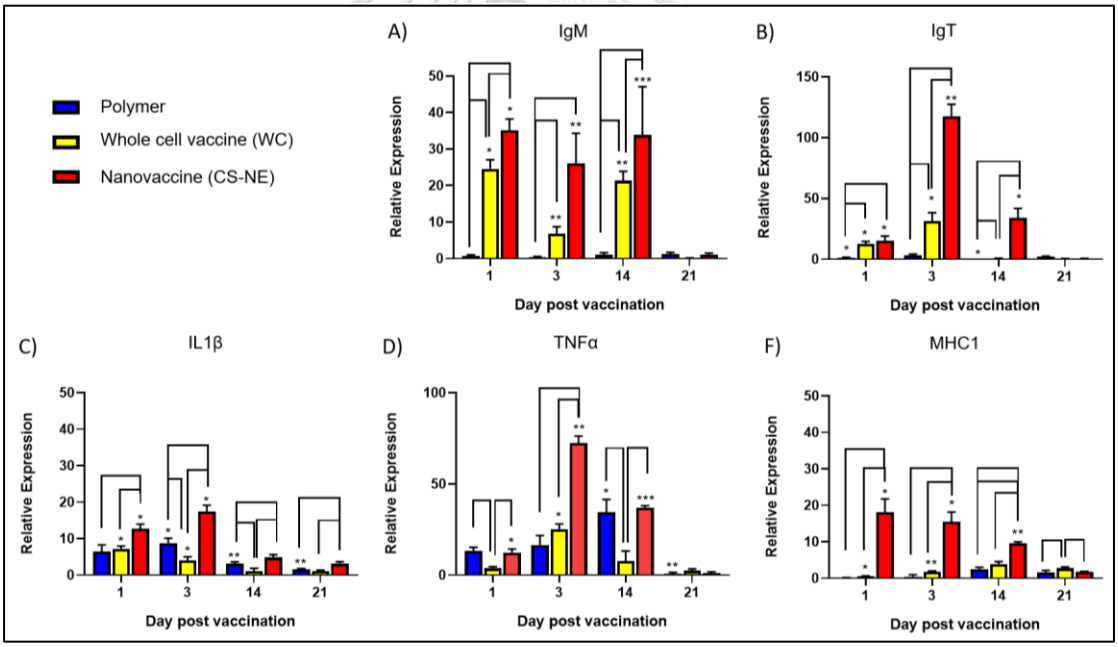


**Figure 8:** Gene expression in the gill of vaccinated fish relative to control fish. The expression of a set 5 immune genes- A) *IgM*, B) *IgT*, C) *IL1β*, D) *TNF-α* and E) *MHC1* in CS-NE, WC, Polymer vaccinated fish relative to control fish at 1,3,14,21 days post-vaccination.

**Discussion**

It is well recognized that vaccination is the most effective approach for the prevention of bacterial diseases in aquaculture. Generally, the first step to make a successful vaccine is selection of an appropriate bacterial isolate, based on its phenotypic and genetic characteristics. Since *F. columnare* colonizes the mucosal surfaces of the fish (skin and gills) during the early stages of the infection (Shoemaker and LaFrentz, 2015), it would seem appropriate that the muco-adhesive properties of the selected isolate be considered when developing an effective mucosal vaccine for columnaris disease. Several studies have suggested that the rhizoid morphotype of *F. columnare* is associated with its virulence. Factors such as its adherence ability, gliding motility, biofilm formation and capsule production, have all been related with the mortality and pathogenicity levels of the bacterium (Kunttu et al., 2009; Laanto et al., 2014; Telford, 2008). In the present study, the bacterial isolate selected as our vaccine candidate (*F. columnare* isolate F-K17/1) was chosen based on its rhizoid morphotype, virulence and genotype. It was a highly virulent isolate, belonging to genetic group IV and having a typical rhizoid morphotype. However, because of the lack of information on the role of the genetic diversity in pathogenicity of the columnaris disease, it is unclear if this isolate will provide cross protection against other genotypes (I, II, and III), and this needed to be further investigated.



Understanding that the clinical signs, disease pathogenesis and pathological changes associated with this disease are almost restricted to the external surfaces of the fish, such as skin damage, gill necrosis and fin erosion (Declercq et al., 2013), the major concerns of using an immersion vaccine consisting of conventional killed bacteria is that antigen adsorption and uptake through the gills and skin is limited, partly due to the short contact time at the mucosal site. To overcome this, we have successfully developed a mucoadhesive nanovaccine and delivery system using a nanotechnology-based platform (Kitiyodom et al., 2019). The results of the study

suggest that there is better adsorption of the vaccine onto the mucosal surfaces, and this results in greater vaccine efficacy compared to conventional formalin killed immersion vaccine. The enhanced vaccine efficacy by the biomimetic nanoparticles results from the particles mimicking physical and biological characteristics of live *F. columnare*. The uptake of the encapsulated bacterial antigen by the gill is higher and persists for longer in CS-NE vaccinated fish, as demonstrated from by the results of the fluorescence and immunohistochemistry. The positively charged CS-NE is presumably enters the MALT mainly by efficient binding to the negatively charged mucosal membranes. The enhanced protection obtained might result from the mucoadhesive property of the chitosan polymer. Mucoadhesive polymers increase the contact time of the vaccine with the mucosa, thereby increasing the potential of enhancing antigen uptake by the antigen presenting cells (Carroll et al., 2016). Another possible reason for the enhanced protective effect could be the adjuvant effect of the chitosan (Chang et al., 2010; Li et al., 2013).

Diffuse MALT (D-MALT) are considered to be a network of diffuse leukocytes that are disseminated along the mucosal surfaces of all vertebrates, including fish. Unlike higher vertebrates, the organized MALT (O-MALT) do not exist in fish except on the inter-brachial lymphoid tissue described in salmon (Salinas, 2015). The mucosal surface, armed with MALT, plays a very important role in the immune defenses of fish, protecting the animal from the first encounter of infectious pathogens (Guardiola et al., 2014). There a great deal of evidence confirming that vaccine antigens administered via the mucosal route are taken up by the MALT of teleost fish (e.g. GiALT (Kato et al., 2013; Korbut et al., 2016; Ohtani et al., 2015); NALT (Tacchi et al., 2014); SALT (Ototake et al., 1996) and GALT (Adelmann et al., 2008; Korbut et al., 2016; Ohtani et al., 2015). Our study showed that the GiALT in the CS-NE vaccinated fish showed a greater extent of lymphocyte aggregation at the gill-interbrachial lamellae, forming a rigid, discrete, well-organized nodular structure

of the associated lymphoid tissue and acting as an important cell mediated immune response at the mucosal surface. No O-MALT was observed in any of the other MALT (nostril, skin or gut) of the tilapia. To the best of our knowledge, this is the first report of the morphological characteristic of O-MALT within the inter-brachial lymphoid tissue of tilapia. However, the physiological and immunological basis for the maturation of antibody response need to be further elucidated as clearly described in Peyer's patches and tonsils of higher vertebrates. The MALT histology reflected the results of the immune gene expression (*IgM, IgT, IL1β, MHC-1* and *TNF-α*), which was significantly upregulated in the gills, promoting the transportation of bacterial antigens into the lymphoid structure and subsequent induction of systemic immune responses, such as the production of specific antibodies (Haugarvoll et al., 2008; Koppang et al., 2010; Rességuier et al., 2017). Up-regulation of *IgT* and *IgM* at the gill-mucosal site, strongly indicated an important role of these immunoglobulin in protecting the ill against *F. columnare* infection. Interestingly, *MHC-I* gene expression was highly upregulated in the gills of the CS-NE vaccinated fish. This might be explained that some elements of antigens delivered through nanoparticle (NPs) were transferred to cytoplasmic vacuoles of APCs and presented by MHC class I molecules (Amigorena and and Savina, 2010; Brode and and Macary, 2004; Pati et al., 2018), suggesting the potential intracellular antigen presentation by polymeric NPs (Hamdy et al., 2007; Tanaka et al., 2010). Many researchers mentioned that lipid NPs can induce CD8+ T cell expansion by efficient antigen cross presentation against infections (Brandtzaeg, 2007; Tanaka et al., 2010). Moreover, there are some reports which show effective induction of cell-mediated immunity, which would play a key role in protection against intracellular pathogens, by mucoadhesive CS-NE (Agnihotri et al., 2004; Khan et al., 2019; Pati et al., 2018).



In conclusion, we applied an innovative nanotechnology to develop a mucosal vaccine delivery system suitable for enhanced vaccination of tilapia by

immersion. The vaccine candidate was carefully selected based on its rhizoid morphotype, virulent property and genetic characteristics. The biomimetic nanoparticles mimicking the physical and biological characteristics of live *F. columnare*, achieving better adsorption onto the mucosal surfaces of the fish and inducing a strong mucosal immune response, resulting in a significant increase in RPS of tilapia against columnaris disease. The MALTs play an important role for initiating and potentiating mucosal immune responses against columnaris disease in tilapia. The results suggest that the charged-mucoadhesive nanovaccine modified by chitosan-based nanoemulsion is an effective platform for effect immersion vaccination against infectious diseases of aquatic animals.

**Author contributions**

N.P., T.Y., and C.R. were involved in the design and supervision of all experiments. N.P., S.K. were involved in conducting the biological experiments including in vivo studies. S.K., C.T., T.K., T.Y., K.T., S.T. and K.N. were performed the statistical analyses and wrote the manuscript text. All authors reviewed the manuscript. Declaration of competing interest. The authors report no conflicts of interest in this work.



**Acknowledgments**

This work was supported by the grant from the 90th Anniversary of Chulalongkorn University Scholarship; GCUGR1125622098D, Ratchadaphiseksomphot Endowment Fund; CU-GR_61_013_31_003 and Thailand Graduate Institute of Science and Technology Scholarship; SCA-CO-2560-4578-TH, National Science and Technology Development Agency (NSTDA, Thailand). Faculty of Veterinary Science, Chulalongkorn University is also acknowledged.

# CHAPTER VI

# Discussion and conclusion

## Discussion

The frequent occurrences of mass mortality and high morbidity were investigated in Tilapia farms in Thailand during 2016-2018. We collected fish sample in many provinces including Kanchanaburi, Ratchaburi, Nakhonpathom, Ayutthaya, Angthong, Phetchaburi, Samutsonkham, Samutsakhon, Nakornratchasima, Mukdahan and Nongkhai across Thailand. Thirty-two bacterial isolates of *F. columnare* were confirmed by species-specific PCR (Welker et al., 2005). The bacteria were isolated from moribund tilapia with clinical signs of necrotic gills, depigmented and necrotic lesions of the skin, and necrotic fins.

The eight bacterial isolates from high mortality cases were selected to research in this study. The two bacterial (FK17/1 of 550 bp and FN17/4 of 450bp) that showed high virulent selected to study of genetic group. The two bacterial strains were identified into genetic group 2 and 4, respectively. According to result of prior studies, the genetic group 2, 3 and 4 of *F. columnare* were reported in tilapia (LaFrentz et al., 2018). The genetic group 4 was mostly found in diseased red tilapia (Dong et al., 2015a; Kayansamruaj et al., 2017; LaFrentz et al., 2018). In this research, red tilapias were challenged with two representative morphotypes, rhizoid and non-rhizoid of *F. columnare* to determine their virulence properties. The results showed that the typical rhizoid isolates were the highly virulent which was responsible for 90% mortality of experimental fish. The non-rhizoid isolate was avirulent to red tilapia similar to previous studies (Dong et al., 2016; Kunttu et al., 2009; Laanto et al., 2014). The virulence factors such as the coordinated organization of cells, a secreted protein and outer membrane vesicles have been reported in *F. columnare* rhizoid morphotype (Kunttu et al., 2009; Laanto et al., 2014). As

mention by many researchers, bacterial strain for successful vaccine can be selected from rhizoid morphotype and the virulent property (Kunttu et al., 2009; Laanto et al., 2014; Telford, 2008). In this study, the F-K17/1 strain was selected to use as a vaccine strain candidate regarding to its high virulence, rhizoid morphotype and genetic group 4.

In this study, we have successfully developed a mucoadhesive vaccine and its delivery system using nanotechnology-based platform. Vaccine was improved by nanotechnology act as delivery system and adjuvant. Nanotechnology-based delivery system has been used in vaccine development as it is easier to deliver, the antigen protection from degeneration, slow release of the encapsulated antigen and induce immune response (Aklakur et al., 2016; Ji et al., 2015; Walters et al., 2015). We determined the physiochemical characteristics of vaccine by the average size, zeta potential and polydispersity. Our results confirmed that the prepared nano-sized vaccines are well-dispersed in water and provided excellent protective effect against columnaris disease following immersion vaccination as compared to inactivated whole-cell bacteria. This result could be explained by the finding that smaller nanoparticles can be easily delivered to lymphoid tissue and retained for a longer period at the vaccine administration site (Reed et al., 2013). Moreover, the use of nano-sized vaccine itself can improve the immunogenicity properties in the absence of adjuvants (Zaman et al., 2013; Zhao et al., 2014).

Mucosal associated lymphoid tissues are important organs that directly correlated with the mucosal immunity of teleost fish and play a very important part of the fish immune defenses, protecting the body from the first encounter of infectious pathogens (Guardiola et al., 2014). As colonization of the mucosal surfaces of fish is the first step of *F. columnare* infection (Shoemaker and LaFrentz, 2015). Our results confirm that better adsorption on mucosal surfaces and more efficient vaccine efficacy could be enhanced by biomimetic nanoparticles mimicking the

mucoadhesive characteristic of live *F. columnare*. The positively charged nanovaccines could be taken into MALT mainly by an efficient binding of nanovaccines to the negatively charged mucosal membranes. This enhanced protective effect against infectious diseases may result from the mucoadhesive property of the chitosan polymer. Mucoadhesive polymers increases the contact time with the mucosa thereby increasing the potential of enhancing antigen uptake by the antigen presenting cell (Carroll et al., 2016). Another possible reason for enhanced protective effect could be the adjuvant ability of chitosan (Chang et al., 2010; Li et al., 2013). Chitosan has been broadly studied for its immunogenic activities, especially via the mucosal routes (Baudner et al., 2003; Xia et al., 2015; Zeng, 2016). Therefore, this strategy could be used as an effective method for direct immersion vaccination of fishes.

Mucosal delivery of vaccine might be a suitable vaccination route against infectious diseases in aquaculture. Evidences confirmed that vaccine antigens administered via the mucosal route are taken up by the MALT of teleost fish: GiALT (Kato et al., 2013; Korbut et al., 2016; Ohtani et al., 2015), NALT (Tacchi et al., 2014), GALT (Adelmann et al., 2008; Korbut et al., 2016; Ohtani et al., 2015), SALT (Ototake et al., 1996). GiALT-fish in CS-NE vaccination showed higher significant lymphocyte aggregation at interbrachial lamellae, acting as an important cell mediated immune response at the mucosal surface. The result is relevant with the mRNA gene expression of *IgM*, *IgT*, *IL1*β, *MHC-1* and *TNF*-α which was higher significantly in the gills, promoting the transportation of vaccine antigens into the lymphoid organs and for the subsequent induction of systemic immune responses, such as the production of specific antibodies (Haugarvoll et al., 2008; Koppang et al., 2010; Rességuier et al., 2017). Interestingly, *MHC-I* mRNA gene expression was highly upregulated in the gills and head kidneys of CS-NE fish. This might be explained that some elements of antigens delivered through nanoparticle (NPs) were transferred to cytoplasmic

vacuoles of APCs and presented by MHC class I molecules (Amigorena and and Savina, 2010; Brode and and Macary, 2004; Pati et al., 2018), suggesting the potential intracellular antigen presentation by polymeric NPs (Hamdy et al., 2007; Tanaka et al., 2010). Many researchers mentioned that lipid NPs can induce $CD8^+$ T cell expansion by efficient antigen cross presentation against infections (Brandtzaeg, 2007; Pati et al., 2018). Moreover, there are some reports which show effective induction of cell-mediated immunity, which would play a key role in protection against intracellular pathogens, by mucoadhesive nanovaccine (CS-NE) (Agnihotri et al., 2004; Khan et al., 2019; Pati et al., 2018).

The ELISA is recognized as a sensitive and accurate method. It has been demonstrated to be able to detect the antibody against *F. columnare* (Shoemaker et al., 2011). In this study, the in-house ELISA development was average optical density values at 450 nm of the negative sera were 0.167 and the standard deviation was 0.027. Therefore, the cut-off point of the indirect ELISA was calculated with the mean of the negative control plus 3 standard deviations (Crowther, 2001; Tankaew et al., 2017) and the value was set to 0.2480. For the interpretation, tilapia serums that had an OD value higher than the cut-off value were determined as *F. columnare* IgM antibody positive. In this study, humoral immune responses by ELISA-specific IgM antibodies were significantly higher in CS-NE fish. Significantly higher serum bacterial activity in CS-NE was also seen. Our study proved that immersion via mucoadhesive nanovaccine could also induce higher systemic humoral immune response and long-lasting protection against *F. columnare* bath challenge in tilapia. The elevation of serum antibacterial activity is considered a nonspecific response to inhibit the growth of bacterial that contains several elements include antimicrobial enzymes, complement system, non-specific proteins and other cytokines/chemokines etc. (Munang'andu et al., 2015).

**Conclusion**

The mucoadhesive nanovaccine presented here is an improved version of a killed vaccine that target the mucosal membrane of tilapia fish. Specifically, we characterized the vaccine strain candidate by clinical field isolation, identification and virulent test. We explained the preparation of mucoadhesive vaccines as well as their physicochemical and biological properties. The analysis of TEM image and zeta-potential also suggested the successful modification of vaccines by chitosan. In vivo mucoadhesive study demonstrated the excellent affinity of the chitosan-complexed vaccines toward fish gills as confirmed by bioluminescence imaging, fluorescent microscopy, and spectrophotometric quantitative measurement. Our data confirmed that the complexation of nanovaccines with cationic chitosan polymers generates positively charged vaccine complexes. As a result, biomimetic nanoparticles mimicking the mucoadhesive characteristic of live *F. columnare* can help achieve better adsorption on mucosal surfaces and more efficient vaccine efficacy that revealed in MALT histology. We evaluated humoral immune responses of CS-NE fish vaccinated include high serum bactericidal activity and high immunoglobulin M specific *F. columnare.* Histology of immune organs revealed a significant increase cell mediated immune response by a higher induction of leucocyte cell infiltration and antigen uptake, in accordance with our result of gene expression in gill, kidney and spleen. Our study demonstrated the feasibility of mucoadhesive nanovaccine as an effective delivery method for a vaccine against infectious *F. columnare* in tilapia by immersion vaccination.

# CHAPTER VI

## Further Investigation and Future direction

In conclusion, we could use mucoadhesive nanotechnology to deliver antigen to the mucosal membrane and MALT of tilapia and induce appropriate immune responses, resulting in a significant increase in relative percent survival of tilapia against columnaris disease. Therefore, targeting mucoadhesive nanovaccines to the mucosal surface could be exploited as an effective method for immersion vaccination.

In future study, exact protection time of vaccine in 4-8 months should be further investigated. Study about an optimal schedule, dose and route for the booster vaccination for longer time protection should be also determined.

In addition, research about cross protection of mucoadhesive vaccine with high genetic diversity of *F. columnare* strains and field trial should be conducted. Moreover, some related clinical parameters are required to be measured and monitored over a period, such as Average Daily Gain (ADG) and Feed Conversion Ratio (FCR) and other immune parameters to determine the efficacy of vaccine should be established.



Finally, the mucoadhesive nanovaccine is probably a versatile platform polymeric nanocarrier with excellent mucoadhesive characteristic for immersion vaccine development. So, it would be better to apply this platform for another bacterial and viral immersion vaccine such as vaccines against *Streptococcus agalactiae, Aeromonas hydrophila* and Tilapia Lake Virus etc.

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# VITA

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|---|---|
| **NAME** | Sirikorn Kitiyodom |
| **DATE OF BIRTH** | 24 June 1982 |
| **PLACE OF BIRTH** | Bangkok |
| **INSTITUTIONS ATTENDED** | Chulalongkorn University |
| **HOME ADDRESS** | 64/30 Tharinporn villa Watchalor Bangkruy Nonthaburi 11130 |
| **PUBLICATION** | 1. Kitiyodom S, Kaewmalun S, Nittayasut N, Suktham K, Surassmo S, Namdee K, Rodkhum C, Pirarat N and Yata T 2019a. The potential of mucoadhesive polymer in enhancing efficacy of direct immersion vaccination against Flavobacterium columnare infection in tilapia. Fish & shellfish immunology. 86: 635-640.<br>2. Kitiyodom S, Yata T, Yostawornkul J, Kaewmalun S, Nittayasut N, Suktham K, Surassmo S, Namdee K, Rodkhum C and Pirarat N 2019b. Enhanced efficacy of immersion vaccination in tilapia against columnaris disease by chitosan-coated “pathogen-like” mucoadhesive nanovaccines. Fish & shellfish immunology. 95: 213-219.<br>3. Kitiyodom S, Khemtong S, Wongtavatchai J and Chuanchuen R 2010. Characterization of antibiotic resistance in Vibrio spp. isolated from farmed marine shrimps (Penaeus monodon). FEMS microbiology ecology. 72(2): 219-227.<br>4. Yostawonkul J, Kitiyodom S, Kaewmalun S, Suktham K, Nittayasut N, Khongkow M, Namdee K, Ruktanonchai UR, Rodkhum C and Pirarat N 2019. Bifunctional clove oil nanoparticles for anesthesia and anti-bacterial activity in |

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**AWARD RECEIVED**

1. TGIST scholarship Outstanding student award 2019
National Science and Technology Development Agency: NTSDA

2. The gold medal and excellent level of the 2020 higher education innovation award
The Thailand Research Expo 2020, National research council of Thailand