Past, current, and future trends in fish vaccines development

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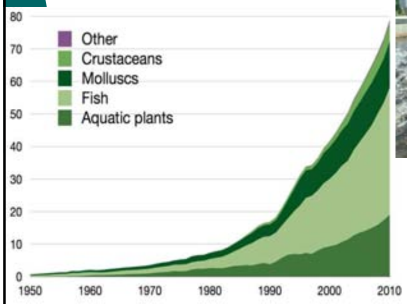
Trend in aquaculture production



Global aquaculture production in million tonnes, 1950-2010, FAO

http://en.wikipedia.org/wiki/File:Global_aquaculture_production.png

Intensification of fish production and consequences

- Maximize production
- Limited space
- High density
- Complete diet
- High water exchange

http://www.fao.org/docrep/007/y5728e/y5728e05.htm

Fish diseases

- Common aquatic diseases in Thailand (1999)

Catfish (hybrid catfish)	• Protozoan parasites • Columnaris disease • Epizootic ulcerative syndrome (EUS) • Mycobacteriosis • Aeromonas
Gourami fish	• Protozoan parasites • Psuedomonas spp. • Aeromonas • Crustacean parasites • E. tarda
Giant freshwater prawn	• Black-gill disease • Body and walking leg erosion
Frog	• Aeromonas • Iridovirus
Soft shell turtle	• Aeromonas • Protozoan parasites
Giant tiger prawn	• White spot disease • Yello head disease • Vibriosis
Grouper and seabass	• Vibriosis • Columnaris • Viral diseases

Aquatic Animal Health Research Institute (AAHRI) Department of Fisheries, Bangkok, Thailand

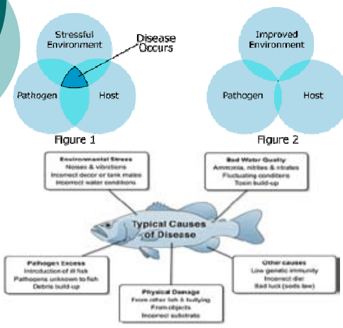
What causes fish disease?

1. Environmental disorders:
   - Temperature
   - Oxygen level
   - pH
   - Environmental toxins e.g. NH_3, NO_2^-, heavy metals, chlorine, chloramine
   - Fish farming condition
   - etc.

2. Heredity and individual defects

3. Pathogens: Bacteria, parasites, virus

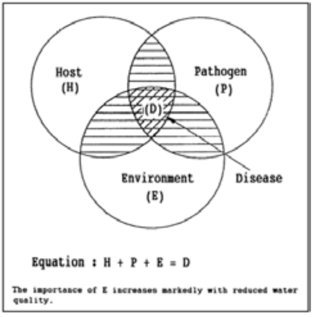
What causes fish disease?



Fish diseases are the result of interaction between a pathogen, a fish (host), and a stressful environment (Figure 1). Even if the pathogen is present, a disease outbreak will not occur unless the environment becomes too stressful for the fish (Figure 2).

http://www.seagrant.umn.edu/aquaculture/diagnosis_fish_disease

Control of fish diseases



Equation : H + P + E = D

The importance of E increases markedly with reduced water quality.

http://www.fao.org/docrep/009/t1623e/T1623E06.htm

1. Husbandry
2. Chemotherapy and chemoprophylaxis
3. Vaccination

Fish diseases and interventions

Examples of antibiotics used in aquaculture

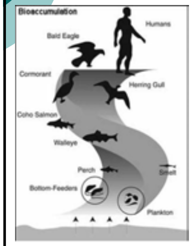
Antibiotics are used in aquaculture as prophylactics, as growth promoters and in the treatment of diseases.

Prophylactic use of antibiotics is defined as the administration of antibiotics in advance of disease occurrence and this is a common practice in shrimp hatcheries in Asia to reduce the incidence of diseases (GESAMP, 1997). A recent review (Graslund and Bengtsson, 2001) report the widespread prophylactic use of antibiotics in both shrimp hatcheries and in shrimp ponds in Southeast Asia. Antibiotics are usually administered in aquatic feeds and most commercial shrimp feeds contain antibiotics (Flaherty *et al*., 2000).

http://www.fao.org/docrep/006/y4743e/y4743e0e.htm

Possible consequences of fish diseases intervention by medications

The use of antibiotics in fish farming is associated with new hazards in fishery products that are not encountered in wild captured species. The main hazards are antibiotic residues and the development of antimicrobial resistance in bacteria that may be transferred to consumers of farmed fish.



Beetseh et al., 2013

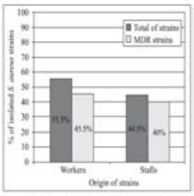
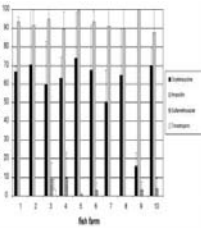


Figure 1. Percentage of multidrug resistant (MDR) and non multidrug resistant (NMDR) *Staphylococcus aureus* strains isolated from two fish market workers and fish stalls.

Albuquerque et al., 2007



Penders et al., 2008

Diseases prevention: vaccination vs chemoprophylaxis

Chemoprophylaxis

Advantage:

- can provide a useful means of helping to control some diseases e.g. bacteria and parasite (both curative and preventive measure, independent of physiological status)

Disadvantage:

- have no direct effect to viral infection
- residue problems and increasing consumer concerns
- development of drug resistance amongbacterial pathogens
- work only as long as they are present in appropriate concentration in the target organ

Vaccination:

Advantage:

- a preventative measure
- can act against bacterial, viral and, at least experimentally, parasitic infections and they will usually act specifically against only the targeted pathogens

Disadvantage:

- vaccines are not "cure all" remedies but are an integral part of comprehensive health management programs

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Vaccine development: from idea to licensed product

Identification

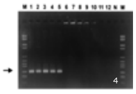
Process development

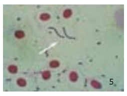
Production

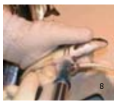
Validation

Documentation

License







ชาญณรงค์ รอดคำ, โรคติดเชื้อแบคทีเรียในปลา

http://www-csgc.ucsd.edu/NEWSROOM/NEWSRELEASES/NewsroomIndx.html
http://aem.asm.org/content/64/10/4065/F1.expansion.html
http://www.thefishsite.com/articles/817/practical-considerations-disease-diagnosis-and-treatment
Ben North, pharmaq UK

Vaccine development

Ideally, a vaccine should be:

1. Safe for both the fish, the administrator and the consumer
2. Have a broad strain or pathogen coverage
3. Provide 100% protection
4. Give a long-lasting protection, preferable as long as the production cycle
5. Be easy to apply
6. Be applicable in various species
7. Be cost effective and
8. Be readily licensed or registered.

Vaccine development

- Of principal importance in the entire vaccine development process is the precise identification of the causative organism, including the existence and significance of serotypes, and a full understanding of the epidemiology of the disease.
- Knowledge on the prevailing diseases, their economic significance and the pathogens associated are key information required to support a vaccination program.
- Unfortunately such information are still lacking for most pathogens involved.
- Far too often, disease outbreaks are described based on disease signs and not on the isolation and characterization of the pathogen.

Vaccine development

Fish diseases or their causative agents against which the development of vaccines has been investigated (Ellis,1988)

- <u>Bacterial diseases</u>
: Aeromonas hydrophila
: Bacterial kidney disease
(Renibacterium salmoninarum)
: Edwardsiella tarda
: Edwardsiella ictaluri
: Vibrio salmonicida (cold water vibriosis)

- <u>Viral diseases</u>
: Infectious pancreatic necrosis (IPN)
: Viral haemorrhagic septicemia (VHS)
: Infectious haematopoietic necrosis (IHN)
: Channel catfish virus
: Spring viraemia of carp (SVC)

- <u>Parasites</u>
: Ichthyopthiriasis
: Costiasis
: Trypanosomiasis
: Diplostosomiasis

- <u>Commercially available vaccines</u>
: Vibriosis
: Enteric redmount (ERM)
: Furunculosis

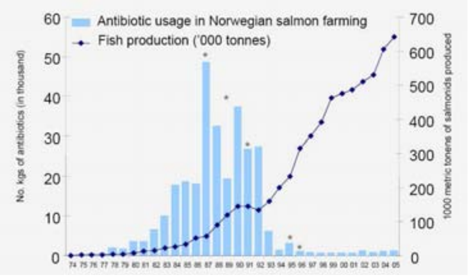
Fish vaccines used today (Mitchell, 2007)

- <u>Bacterial diseases</u>
: Yersinia ruckeri
: Aeromonas salmonicida
: Vibriosis (multiple strains and species)
: Edwardsiella ictaluri
(Enteric septicemia of catfish)
: Morantella viscosus
: Flavobacterium columnare
: Bacterial kidney disease
: Pasteurellosis
: Mycobacteriosis
: Streptococcosis

- <u>Viral diseases</u>
: Infectious pancreatic necrosis
: Infectious salmon anemia
: Infectious haematopoietic necrosis
: Koi herpes virus

- <u>Parasites</u>
: Piscirickettesiae

Successful of vaccination and the reduction of antibiotics usage



Fish vaccines development: in the past and current

- In the past and present, vaccines have relied on the isolation of fish whole pathogens
- Of the types currently in use, the most common are described below:
  - Killed or inactivated vaccines
  - Live, attenuated vaccines
  - Toxoids (inactivated toxins)

Fish vaccines development: in the past and current

Some methods of producing killed and attenuated vaccines

<u>Inactivation</u>

- Heat
- Formalin
- Other "micro-toxins"
  E.g. ß-propiolactone
- Pressure (French press)
- Irradiation

<u>Attenuation</u>

Non-specific:

- Mutagen culture passing
- Natural strains
- Aging cultures

Specific

- Genetically-engineered

Fish vaccines development: in the past and current

Killed vaccine

<u>Advantages</u>
- Safe and generally quick to develop
- Most common in human vaccines
- Usually excellent protection if humoral response is primary

<u>Disadvantages</u>
- Little residual effect
- Adjuvant generally needed
- Inactivation substance can be a concern
- May not be seen like live organism to immune system
- Not great for stimulation of cell-mediated response

Live, attenuated vaccine

<u>Advantages</u>
- Mimics natural infection and immune response

<u>Disadvantages</u>
- Danger to reversion to virulence
- Shelf-life, storage conditions and reviving can be critical to efficacy
- Adjuvant difficult

Fish vaccines development: trend in the future

Current new type of vaccine

1. Subunit vaccine
2. DNA vaccine

Subunit vaccine

- Subunit vaccines are defined as those containing one or more pure or semi-pure antigens. In order to develop subunit vaccines, it is critical to identify the individual components out of a myriad of proteins and glycoproteins of the pathogen that are involved in inducing protection.
- Indeed, some proteins, if included in the vaccine, may be immunosuppressive, whereas in other cases immune responses to some proteins may actually enhance disease.
- Thus, it is critical to identify those proteins that are important for inducing protection and eliminate the others.
- Combining genomics with our understanding of pathogenesis, it is possible to identify specific proteins from most pathogens that are critical in inducing the immune responses.

Subunit vaccine

- The potential advantages of using subunits as vaccines are the increased safety, less antigenic competition, since only a few components are included in the vaccine, ability to target the vaccines to the site where immunity is required, and the ability to differentiate vaccinated animals from infected animals (marker vaccines).
- One of the disadvantages of subunit vaccines is that they generally require strong adjuvants and these adjuvants often induce tissue reactions. Secondly, duration of immunity is generally shorter than with live vaccines.

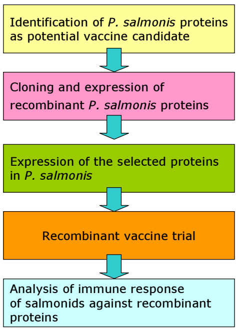
Subunit vaccine


Available online at www.sciencedirect.com

Vaccine

A vaccine against the salmonid pathogen *Piscirickettsia salmonis* based on recombinant proteins

Vivian Wilhelm, Alvaro Miquel, Luis O. Burzio, Mario Rosemblatt, Esteban Engel, Sebastián Valenzuela, Gustavo Parada, Pablo D.T. Valenzuela


Identification of *P. salmonis* proteins as potential vaccine candidate

Cloning and expression of recombinant *P. salmonis* proteins

Expression of the selected proteins in *P. salmonis*

Recombinant vaccine trial

Analysis of immune response of salmonids against recombinant proteins

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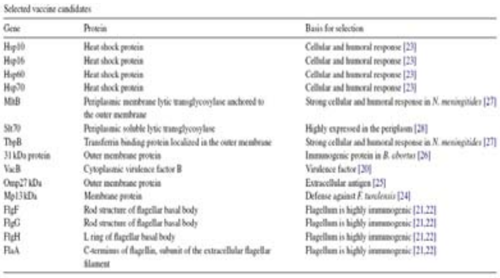
Subunit vaccine

1. Identification of *P. salmonis* proteins as potential vaccine candidate


Selected vaccine candidates

Gene	Protein	Basis for selection
Hsp10	Heat shock protein	Cellular and humoral response [23]
Hsp16	Heat shock protein	Cellular and humoral response [23]
Hsp60	Heat shock protein	Cellular and humoral response [23]
Hsp70	Heat shock protein	Cellular and humoral response [23]
MltB	Periplasmic membrane lytic transglycosylase anchored to the outer membrane	Strong cellular and humoral response in *N. meningitides* [27]
Slt70	Periplasmic soluble lytic transglycosylase	Highly expressed in the periplasm [28]
TbpB	Transferrin binding protein localized in the outer membrane	Strong cellular and humoral response in *N. meningitides* [27]
31 kDa protein	Outer membrane protein	Immunogenic protein in *B. abortus* [26]
VacB	Cytoplasmic virulence factor B	Virulence factor [20]
Omp27 kDa	Outer membrane protein	Extracellular antigen [25]
Mp13 kDa	Membrane protein	Defense against *F. tularensis* [24]
FlgF	Rod structure of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlgG	Rod structure of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlgH	L ring of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlaA	C-terminus of flagellin, subunit of the extracellular flagellar filament	Flagellum is highly immunogenic [21,22]

Subunit vaccine

2. Cloning and expression of recombinant *P. salmonis* proteins

Cloning of *P. salmonis* antigen coding region

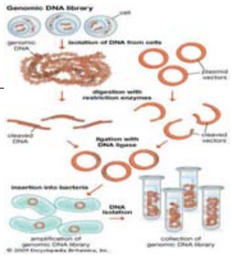
Isolation of genomic DNA from *P. salmonis*

Predicted coding regions of selected antigen were isolated by PCR amplification

PCR products were ligated to pGEMT and used to transform Novoblue competent cells

Positive clones were selected by blue/white screening using lacZ alpha-complementation

DNA analysis and sequencing



http://www.britannica.com/EBchecked/media/128149/A-genomic-DNA-library-is-a-collection-of-DNA-fragments


https://www.fishersci.ca/browseResults.do?cid=2643427

Subunit vaccine

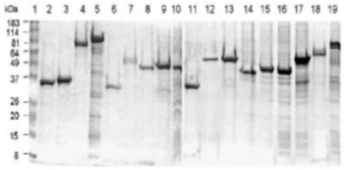
3. Expression of the selected proteins in *P. salmonis*

http://www.sciencedirect.com/science/article/pii/S1046592802000128

The amplified coding regions werecloned into the expression vector pET$_{32}$a in frame with the thioredoxin gene of *E. coli* and a histidine-tag domain

E. coli BL$_{21}$(DE$_{3}$) competent cells were transformed with the constructs and expression of recombinants was induced by incubation in LB-ampicillin supplemented with 1mM IPTG

Recombinant proteins used for immune analysis were purified by a Ni-agarose column (Qiagen)



SDS-PAGE analysis of purified *P. salmonis* recombinant proteins expressed in *E. coli*: (1) molecular weight markers; (2) Trx-Hsp$_{10}$; (3) Trx-Hsp$_{16}$; (4) Trx-Hsp$_{60}$; (5) Trx-Hsp$_{70}$; (6) Trx-Omp-C; (7) Trx-FlaA-C; (8) Trx-FlgF; (9) Trx-FlgG; (10) Trx-FlgH; (11) Trx-Mp$_{13}$; (12) Trx-$_{31}$ kDa protein; (13)Trx-TbpB-N; (14) Trx-MltB-N; (15) Trx-MltB-C; (16) Trx-Slt$_{70}$-N; (17) Trx-Slt$_{70}$-C; (18) Trx-VacB-N; (19) Trx-VacB-C. N and C refer to amino and carboxyl domains, respectively.

Subunit vaccine

3. Expression of the selected proteins in *P. salmonis*

It was of interest to study if the antigen candidates are expressed by the pathogen during infection. Therefore, extracts of *P. salmonis* growing in CHSE-214 cells were analyzed by Western blot for the presence of the native proteins. Specific monoclonal antibodies were used to detectthe presence of the selected proteins in these extracts.

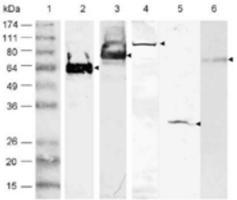


Fig. 2. Western blot analysis of antigens present in *P. salmonis*. Gel was loaded with 30–40 μg of total *P. salmonis* protein and the blotted membranes were analyzed with a 1:200 dilution of each monoclonal antibody: (1) molecular weight markers; (2–6) monoclonal antibody against Hsp60, Hsp70, VacB, 31 kDa protein and TbpB, respectively.

Subunit vaccine

4. Recombinant vaccine trial

Three different vaccine formulations:

Vaccine 1: Trx-Hsp70 (a)
Trx-Hsp60 (b)
Trx-FlgG (c)

Vaccine 2: Trx-TbpB-N (d)
Trx-MltB-C (e)
Trx-MltB-N (f)

Vaccine 3: Trx-FlaA-C (g)
Trx-Omp-C (h)

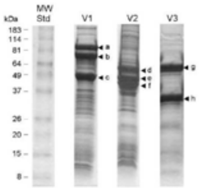


Fig. 3. SDS-PAGE analysis of recombinant vaccine formulations. Oil in water formulations containing 10 μg of each recombinant protein were used to immunize fish. MW Std: molecular weight markers; V1: formulation containing partially purified Trx-Hsp70 (a), Trx-Hsp60 (b) and Trx-FlgG (c). V2: formulation containing partially purified Trx-TbpB-N (d), Trx-MltB-C (e), Trx-MltB-N (f). V3: formulation containing partially purified Trx-FlaA-C (g) and Trx-Omp-C (h).

Subunit vaccine

5. Analysis of immune response of salmonids against recombinant proteins

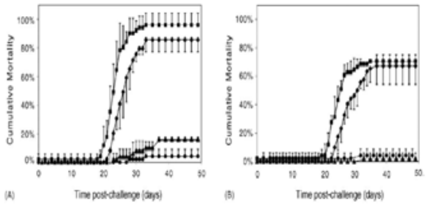


Fig. 4. Protection of recombinant vaccine against SRS in Atlantic salmon. (A) Cumulative mortality of control and immunized fish challenged with a dose of 8 × LD50 of *P. salmonis*. (B) Cumulative mortality of control and immunized fish challenged with a dose of 2 × LD50 of *P. salmonis*. (■) Control fish immunized with adjuvant; (●) fish immunized with formulation V1; (▲) fish immunized with formulation V2; (◆) fish immunized with formulation V3. The curves represent the average cumulative mortalities of four tanks of fish and the standard deviation of each value is indicated as a bar.

Subunit vaccine

5. Analysis of immune response of salmonids against recombinant proteins

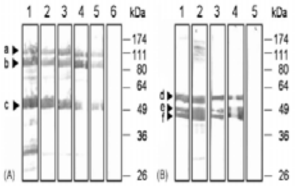
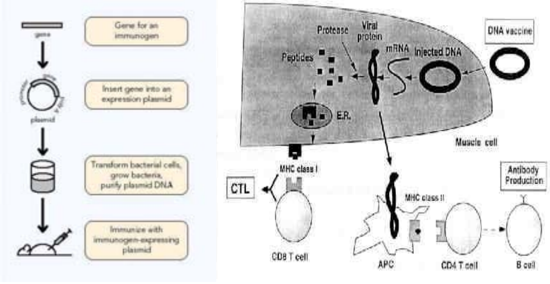


Fig. 5. Western blot analysis of serum obtained from salmon immunized with recombinant formulations V1 and V2. (A) Blots containing purified Trx-Hsp70 (a), Trx-Hsp60 (b) and Trx-FlgG (c) tested with serum of fish immunized with V1 formulation (lanes 1–5) and serum of non-immunized fish (lane 6). (B) Blots containing purified Trx-TbpB-N (d), Trx-MltB-C (e) and Trx-MltB-N (f) tested with serum of fish immunized with V2 formulation (lanes 1–4) and serum of non-immunized fish (lane 5).

DNA vaccine

- The most recent development in vaccinology is immunization with polynucleotides. This technology has been referred to as genetic immunization or DNA immunization.
- The basis for this approach to immunization is that cells can take up plasmid DNA and express the genes within the transfected cells.
- Thus, the animal acts as a bioreactor to produce the vaccine.
- This makes the vaccine relatively inexpensive to produce.
- Some of the advantages of polynucleotide immunization is that it is extremely safe, induces a broad range of immune responses (cellular and humoral responses), long-lived immunity, and, most importantly, can induce immune responses in the presence of maternal antibodies.
- Although this is one of the most attractive developments in vaccinology, there is a great need to develop better delivery systems to improve the transfection efficiency in vivo.

DNA vaccine



http://biology.kenyon.edu/slonc/bio38/scuderi/partii.html

DNA vaccine

Vaccine 27 (2009) 2150–2155

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Protection of Japanese flounder (*Paralichthys olivaceus*) against *Vibrio anguillarum* with a DNA vaccine containing the mutated zinc-metalloprotease gene

Hui Yang[a,b], Jixiang Chen[a,*], Guanpin Yang[a], Xiao-Hua Zhang[a], Rui Liu[a], Xiaoping Xue[b]

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[b] Department of Life Sciences, Northwestern Polytechnical University, 127 Youyi Road, Xi'an 710072, PR China

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DNA vaccine

Methodology

1. Cells and bacterial strains
2. Plasmid construction
3. *In vitro* transfection of recombinant plasmid (pEGFP-N1/m-empA7) in cell lines
4. Detection of m-EmpA7 expressed in transfected eukaryotic cells
5. Prechallenge of *V. anguillarum* W-1 in Japanese flounder
6. Immunization and experimental challenge
7. Detection of injected DNA in fish tissues
8. Detection of in vivo transcription of m-empA7 gene by RT-PCR
9. Detection of in vivo expression of m-EmpA7 by Western-blot
10. Analysis of antibody response
11. Statistical analysis

DNA vaccine: Methods and results

Mutated pET24d(+)/m-*empA7* (Glu^{374} ⟶ Lys mutation)

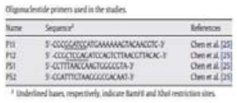
PCR

ORF of m-*empA7* was inserted in frame with EGFP reporter gene of pEGFP-N1

pEGFP-N1/m-*empA7* (recombinant plasmid)

Gene cloning, isolation of plasmid, DNA sequence

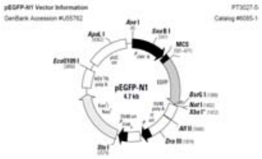
Plasmid construction



Oligonucleotide primers used in the studies.

Name	Sequence[a]	References
P11	5'-CGCGGATCCATGAAAAAACTACAACCTC-3'	Chen et al. [25]
P12	5'-CCGCTCGAGATCCAGTCTTAACCTTACAC-3'	Chen et al. [25]
PS1	5'-CCTTTTAACCAAGTGGGCGTA-3'	Chen et al. [25]
PS2	5'-CGATTTCTAAGGGCGACAAT-3'	Chen et al. [25]

[a] Underlined bases, respectively, indicate BamHI and XhoI restriction sites.



http://www.pkclab.org/PKC/vector/pEGFPN1.pdf

DNA vaccine: Methods and results

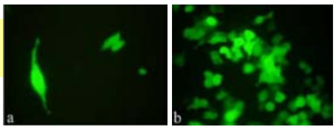
In vitro transfection of pEGFP-N1/m-empA7 in cell lines

chinese hamster ovary (CHO) and human embryonic kidney (HEK293T) cell lines were seeded in 6-well plates at 75-80% confluence

purified pEGFP-N1/m-*empA7* was transfected to cell lines, pEGFP-N1 was used as negative control (4 μl of plasmid in 100 μl transfection buffer

expression of m-*empA7* was determined by WB and observation of green fluorescence from the EGFP fusion protein was done under a fluorescent microscopy

Fig. 2. Western-blot analysis of m-EmpA7 expressed in transfected cells. The membrane was probed with a polyclonal antibody raised against EmpA. Lane 1, CHO cells; lane 2, CHO cells transfected with pEGFP-N1; lane 3, CHO cells transfected with pEGFP-N1-m-empA7; lane 4, HEK293T cells; lane 5, HEK293T cells transfected with pEGFP-N1; lane 6, HEK293T cells transfected with pEGFP-N1-m-empA7.



DNA vaccine: Methods and results

In vivo evaluation of m-empA7 as immunogent

Prechallenge of *V. anguillarum* in japanese flounder

18 japanese flounders (9 g BW) were randomly dividedto 3 groups

Bacterial suspension (0.1 ml) was injected (IP) in fish (3.5×10^7, 3.5×10^6, 3.5×10^5 cfu ml^{-1})

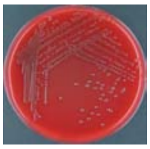
Disease progress was followed for 14 days

Randomly sampled dead fish were recorded in order to determine median lethal dose(LD50)

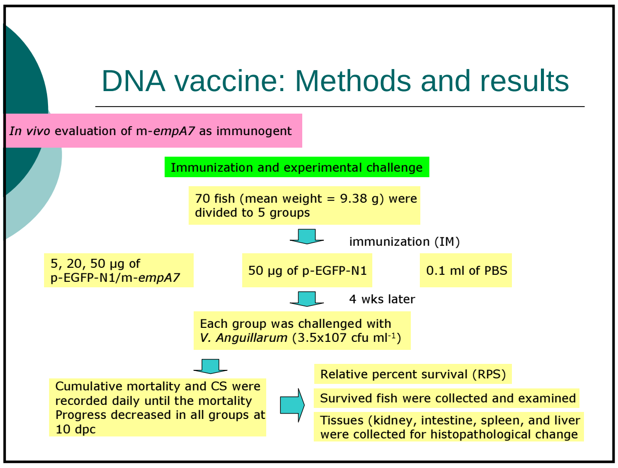
LD50= 3.5×10^7 cfu ml^{-1}

Japanese flounder (*Paralichthys olivaceus*), Hirame (JP)

http://toshichile.blogspot.com/2012/07/tokyo-fish-wholesale-market-price-from.html



http://atlas.life.ku.dk/microatlas/veterinary/bacteria/Vibrio_anguillarum/



DNA vaccine: Methods and results

In vivo evaluation of m-*empA7* as immunogent

Immunization and experimental challenge

70 fish (mean weight = 9.38 g) were divided to 5 groups

immunization (IM)

5, 20, 50 µg of p-EGFP-N1/m-*empA7*

50 µg of p-EGFP-N1

0.1 ml of PBS

4 wks later

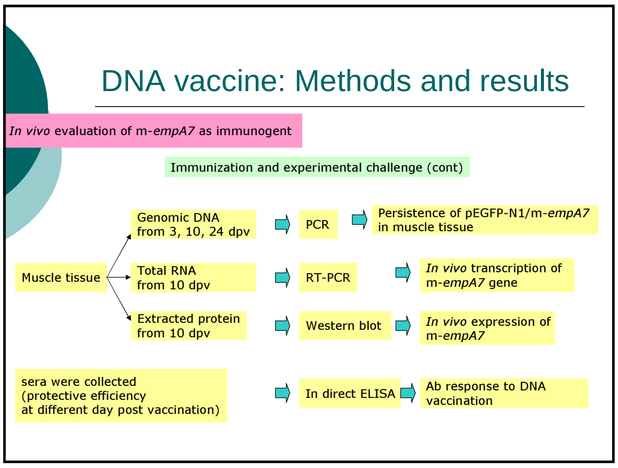
Each group was challenged with *V. Anguillarum* (3.5x107 cfu ml^{-1})

Cumulative mortality and CS were recorded daily until the mortality Progress decreased in all groups at 10 dpc

Relative percent survival (RPS)

Survived fish were collected and examined

Tissues (kidney, intestine, spleen, and liver were collected for histopathological change



DNA vaccine: Methods and results

In vivo evaluation of m-*empA7* as immunogent

Immunization and experimental challenge (cont)

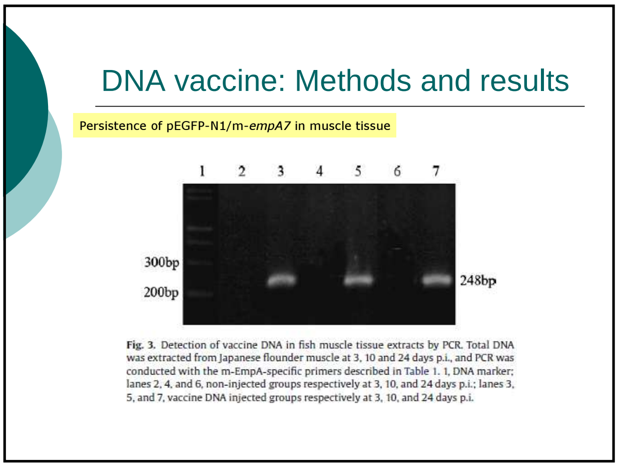
Muscle tissue

Genomic DNA from 3, 10, 24 dpv → PCR → Persistence of pEGFP-N1/m-*empA7* in muscle tissue

Total RNA from 10 dpv → RT-PCR → *In vivo* transcription of m-*empA7* gene

Extracted protein from 10 dpv → Western blot → *In vivo* expression of m-*empA7*

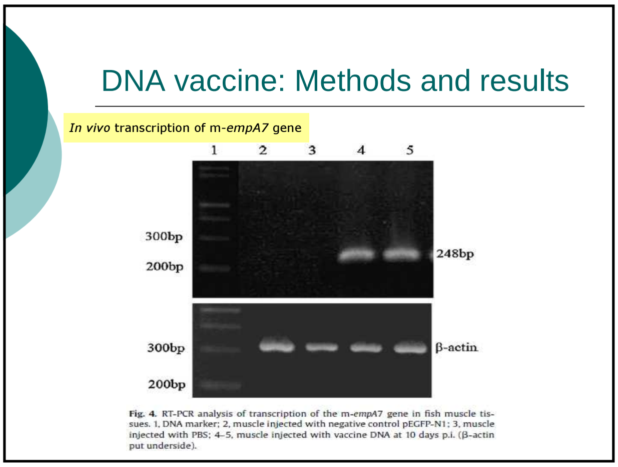
sera were collected (protective efficiency at different day post vaccination) → In direct ELISA → Ab response to DNA vaccination



DNA vaccine: Methods and results

Persistence of pEGFP-N1/m-*empA7* in muscle tissue

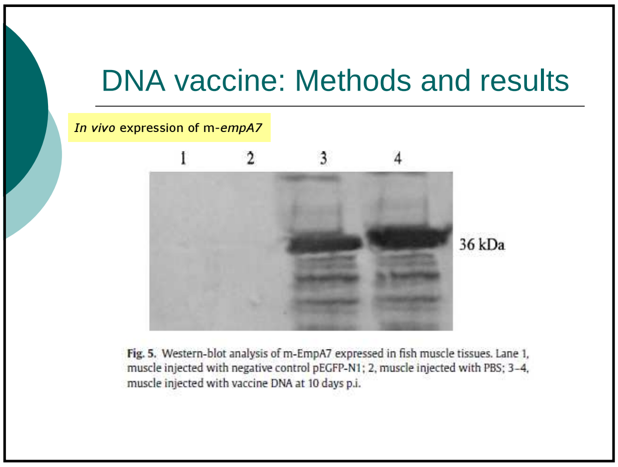
Fig. 3. Detection of vaccine DNA in fish muscle tissue extracts by PCR. Total DNA was extracted from Japanese flounder muscle at 3, 10 and 24 days p.i., and PCR was conducted with the m-EmpA-specific primers described in Table 1. 1, DNA marker; lanes 2, 4, and 6, non-injected groups respectively at 3, 10, and 24 days p.i.; lanes 3, 5, and 7, vaccine DNA injected groups respectively at 3, 10, and 24 days p.i.



DNA vaccine: Methods and results

In vivo transcription of m-*empA7* gene

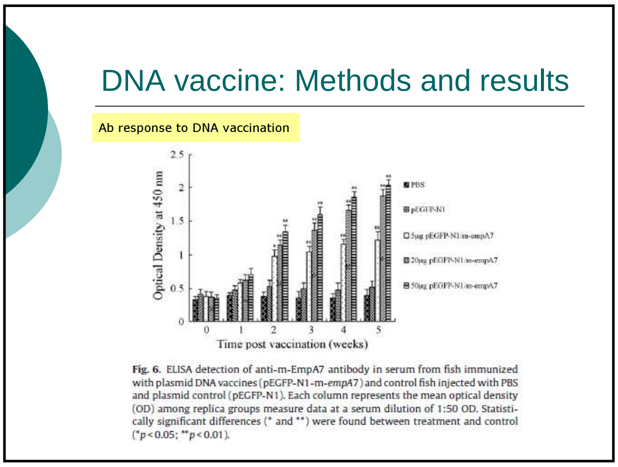
Fig. 4. RT-PCR analysis of transcription of the m-*empA7* gene in fish muscle tissues. 1, DNA marker; 2, muscle injected with negative control pEGFP-N1; 3, muscle injected with PBS; 4–5, muscle injected with vaccine DNA at 10 days p.i. (β-actin put underside).



DNA vaccine: Methods and results

In vivo expression of m-*empA7*

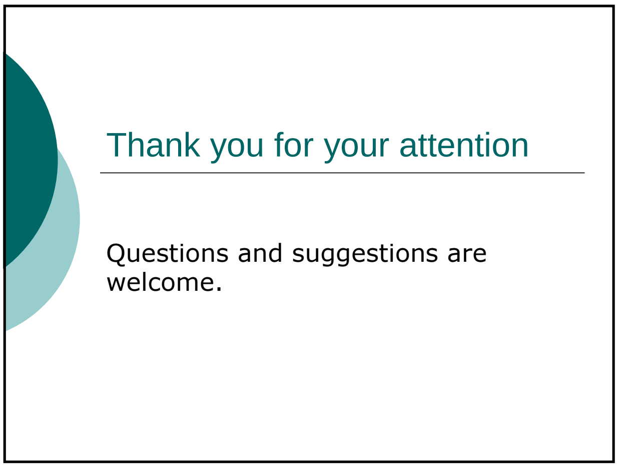
Fig. 5. Western-blot analysis of m-EmpA7 expressed in fish muscle tissues. Lane 1, muscle injected with negative control pEGFP-N1; 2, muscle injected with PBS; 3–4, muscle injected with vaccine DNA at 10 days p.i.



DNA vaccine: Methods and results

Ab response to DNA vaccination

Fig. 6. ELISA detection of anti-m-EmpA7 antibody in serum from fish immunized with plasmid DNA vaccines (pEGFP-N1-m-*empA7*) and control fish injected with PBS and plasmid control (pEGFP-N1). Each column represents the mean optical density (OD) among replica groups measure data at a serum dilution of 1:50 OD. Statistically significant differences (* and **) were found between treatment and control (*$p < 0.05$; **$p < 0.01$).



Thank you for your attention

Questions and suggestions are welcome.