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Preliminary Trials for Using Capsaicin to Aid Intestinal Epithelial Passage of Betanodavirus Vaccine in Goldfish *Crassius aurata*

[1]A.Y. Gaafar, [2]Y. Kawato, [3]H. Yamashita, [2]K. Mori, [1]A. Younes and [4]T. Nakai

[1]Veterinary Research Division, National Research Centre, Cairo, Egypt
[2]National Research Institute of Fisheries Science,
Fisheries Research Agency, Yokohama, Kanagawa, Japan
[3]Ehime Research Institute of Agriculture, Forestry and Fisheries, Ehime 798-0104, Japan
[4]Graduate School of Biosphere Science, Hiroshima University, Higashihiroshima 739-8528, Japan

Abstract: As a worldwide fish disease, piscine nodavirus (Betanodaviruses) infections have emerged as major constraints on the culture of marine fish all over the world causing severe economic losses. No live attenuated vaccine is produced against this disease and the effective vaccine is inactivated injectable vaccine, which need large sized fish to be applicable, while most epizootics and losses occur in fish at juvenile or larval stage. Oral vaccination trials against this virus are still not successful so far. So any method that facilitates the intestinal passage of the vaccine particles would be feasible. According to the fact that, some inflammatory substances which cause reversible pathology to the intestinal epithelium may be employed for the epithelial passage of vaccine particles. Capsaicin (Methyl vanillylnonenamide) which is naturally produced from hot chili pepper was investigated as a potential candidate in this study. Goldfish (*Crassius aurata*) and inactivated striped jack nervous necrosis virus (SJNNV) were used as the model fish and the tested vaccine, respectively. Both inflammatory substances and vaccine were introduced via anal intubation to fish. Capsaicin proved to be effective to aid the transepithelial passage of vaccine particles.

Key words: Betanodavirus · Capsaicin · Goldfish · *Crassius aurata* · Vaccine

INTRODUCTION

Mari-culture has always faced a risk of disease due to spreading of infectious pathogens. Vaccination is a main way for protection of fish against viral diseases. Due to lake of safe live attenuated viral vaccines, application of inactivated vaccines for fish needs more research because most of them are used parentally, which restricts its application to specific age or size of the fish rendering fry and fingerlings in severe risk of infection.

This fact appears clearly in case of Piscine Nodaviruses (Betanodaviruses) infections which are worldwide finfish diseases causing severe economic losses and have mostly been associated with juvenile stages in several marine fish species [1]. Nodaviruses have been always regarded as pathogens of marine fish, but there are few reports of Nodavirus-associated mortality episodes in fish species from freshwater [1, 2] and recently tilapia larvae reared in freshwater reported to be naturally susceptible to infection with a Nodavirus belonging to the RGNNV genotype [3].

The Nodaviridae are small (20-34 nm), nonenveloped isometric RNA viruses [4, 5] divided in two genera: the Alphanodavirus and the Betanodavirus, members of which infect insects and fish, respectively [6].

Pathogenesis and clinical signs of VNN are related to the neuroinvasive nature of the virus and the resulting effect on tissues such as brain and retina [2, 7-9]. In general, The most common histopathological changes in diseased fish are necrosis and vacuolation of the brain and the retina [5, 10, 11].

Encouraging results for preliminary vaccination trials using recombinant nodavirus coat protein as the immunogen have been reported [12-14]. However, DNA vaccines, constructed by cloning the encoding region of RNA2 from AHNV and SJNNV, have not proven efficacious [15].

Corresponding Author: Alkhateib Y. Gaafar, Veterinary Research Division, National Research Centre, Cairo, Egypt.
E-mail: alkhateibyg@yahoo.com.

As mentioned before most successful vaccination trials were injectable vaccines, which need suitable fish size 'large sized fish' to be applicable. While most epizootics and losses occur in young age, small sized fish.

So another route of vaccination rather than parenteral route should be investigated. Bath immunization with nano-encapsulated formalin-inactivated or binary ethylenimine-inactivated betanodavirus vaccines have been used to protect grouper larvae against VNN [16]. Vaccination studies are already being undertaken by some researchers and need to be fostered.

Recently, during studying the pathogenesis of some diseases in animals, associated with a primary dysfunction of intestinal intercellular tight junctions due to production of some compounds, for example, zonula occludens toxin (Zot), an enterotoxin expressed by *Vibrio cholerae* that reversibly opens tight junctions, [17], It was interesting that (Zot) was used to modulate the intestinal barrier permeability in a reversible manner allowing the passage of macromolecules in terrestrial animals, this was via the interaction between the toxin and zonula occludens (Paracellular tight junction). These same compounds however were used to develop innovative strategies for the delivery of macromolecules normally not absorbed through the intestine like drugs currently engineered by recombinant DNA techniques or even vaccines.

Following the same steps if a case of severe intestinal hyperemia could be produced safely in fish by natural chilly materials like capsaicin, dysfunction of intestinal intercellular tight junctions may occur, resulting in leakage of macromolecules like vaccine particles. This could give some hope that soon viral vaccines can be introduced to larvae and fingerlings through oral route or even incorporated with its food.

MATERIALS AND METHODS

Fish: Goldfish (*Crassius aurata*) with average weight about 12.4 g and average body length 75.0 mm were reared in flow through freshwater glass aquaria in the laboratory of fish Pathobiology, Graduate school of Biosphere Sciences, Hiroshima University. Before the experiments they were apparently healthy and were acclimatized for the laboratory conditions and fed daily 1% of their total body weight. Number of fish used for each treatment is mentioned in Tables (1 and 2). One day before anal intubation all fish stop feeding and each group was transferred to still water, 15 liters aerated aquaria, anal intubation was performed after anaesthetizing fish by 0.04 % eugenol solution.

The Tested Chemical: Capsaicin (Methyl vanillylnonenamide) was purchased from (Wako chemicals, Japan) and dissolved separately in 50 µl ethanol then diluted by 50 µl PBS (For each fish).

The Vaccine: Inactivated seven band grouper nervous necrosis virus (RGNNV; strain SGEhi00) was used in this study in a dose (10^{10} $TCID_{50}$/fish).

Determination of Capsaicin Effective Dose: Before starting the intestinal passage experiment, the needed effective nonlethal capsaicin dose was determined by a simple rapid test of the intestinal inflammatory reactivity against different doses of capsaicin. Capsaicin was serially diluted into different doses and anally intubated into the tested fish

According to (Table 1), after 1 hour fish were dissected for the intestine which was fixed rapidly with Davidson's fixative for 24 hours then transferred to 70% ethanol till histopathological examination.

Table 1: The experimental design for capsaicin dose determination for goldfish

No. fish/treatment	Dose/fish	Route	Duration of treatment	Comments
2 fish	5 mg	Anal intubation	1 hour	Firstly capsaicin was dissolved in 50 µl
2 fish	0.5 mg		Then fish were dissected	ethanol then diluted by 50 µl
2 fish	0.05 mg		for intestine	PBS for each fish
2 fish	0.005 mg			
2 fish control-ve	PBS			

Table 2: The experimental design for anal intubation of capsaicin and VNN vaccine for goldfish

No. fish/treatment	Dose/fish	VNN vaccine	Route	Duration of treatment
3 fish control -ve	PBS	-	Anal intubation	2 hours after vaccine intubation fish
3 fish	1 mg	-		were dissected for intestine***.
3 fish	PBS	10^{10} $TCID_{50}$/fish after 1h.*		
3 fish	1 mg	10^{10} $TCID_{50}$/fish after 1h.**		
3 fish	1 mg	10^{10} $TCID_{50}$/fish after 2h.**		
3 fish	1 mg	10^{10} $TCID_{50}$/fish after 3h.**		

* After PBS intubation, **after capsaicin intubation, * **dissection of control fish was after 5 hours of capsaicin or PBS intubation.

Determination of Best Timing of Vaccine Anal Intubation after Capsaicin: To determine the best timing for vaccine introduction after capsaicin administration according to the developing inflammatory reaction, three fish per group were used (Table 2). In the 1st group (Control -ve) fish were intubated with PBS. In the 2nd group fish were intubated with capsaicin only 1mg/fish. In the 3rdgroup fish were intubated with vaccine only. In the 4thgroup fish were intubated with vaccine after 1 hour of capsaicin administration. In the 5th group fish were intubated with vaccine after 2 hours of capsaicin administration. In the 6th group fish were intubated with vaccine after 3 hours of capsaicin administration. Two hours after vaccine intubation fish were dissected for intestine, which was fixed rapidly with Davidson's fixative for 24 hours then transferred to 70% ethanol then underwent histopathological, Immunohistochemical and Immunoflourescent examinations.

Histopathological Examination: The fixed specimens were processed through the conventional paraffin embedding technique (Dehydration through ascending grades of ethanol, clearing in chloroform and embedding in paraffin wax at 60°C). Paraffin blocks were prepared and cutting 4 µm-thick tissue sections by using microtome. Then 5 replicates from the same section were mounted on silane-coated slides, which were subjected to histological (H&E) and immunohistological (IFT, IHC) examination.

Immunohistological Examination: In all Immunohisto-detection methods in this study heat-induced antigen retrieval was needed for ideal staining. The paraffin sections mounted on slides were heated to 60° C for 30 minutes to retrieve the viral antigen inside the tissue. Anti-RGNNV Rabbit antiserum was used as primary antibody.

Immunohistochemistry: After deparaffinization, tissue sections were kept in methanol with 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase. The sections were incubated overnight with primary Anti-RGNNV rabbit antiserum (1:100 dilution) at 4°C. For negative controls, normal rabbit serum was used at equivalent concentrations. Also, endogenous nonspecific antigenicity was blocked by placing sections in 2.5% skimmed milk for 20 minutes. Then sections were incubated with secondary antibody Polyclonal Swine Anti-Rabbit Immunoglobulins/HRP (Dako); for 30 minutes at room temperature. The bound antibody was visualized using 0.016% diaminobenzidinetetrahydrochloride (DAB) substrate, 0.24% H_2O_2 in PBS (Wako, Japan) revealing brown precipitate. Sections were counterstained with Gill's hematoxylin and "Blued" with 0.1% ammonia water.

Immunoflourescent Technique: The technique was done according to Hayat [18]. As after deparaffinization, tissue sections were incubated overnight with primary Anti-RGNNV rabbit antiserum (1:100 dilution) at 4°C. For negative controls, normal rabbit serum was used at equivalent concentrations. After that, endogenous nonspecific antigens were blocked by placing sections in 2.5% skimmed milk for 20 minutes. Then sections were incubated with secondary antibody-FITC Conjugated Swine Anti-Rabbit polyclonal Immunoglobulin (Dako, Denmark) for 1 hour at 4°C in dark place. Sections were mounted by antifading water based mountant (Glycerol-PVD 1:1) then cover-slipped. Positive antigenic signals appear as green fluoresce under FITC-specific UV wavelength when viewed by Fluorescent microscope (Nikon Eclipse E200 + Hamamatsu CCD Camera).

RESULTS AND DISCUSSION

Determination of Capsaicin Effective Dose: Examination of histopathological samples (Figure 1) revealed that severe hemorrhages were obvious 1 hour after anal intubation of 5 mg capsaicin, moderate hyperemia was detected in intestinal mucosa with 0.5 mg, while no detectable tissue changes were noticed in lesser doses and PBS. So as a result, in the next experiment 1 mg/fish was the candidate dose.

The gross appearance and the histopathological picture of goldfish intestine showed noticeable inflammatory reaction due to its contact with capsaicin. which means that goldfish enterocytes contain vanilloid (TRPV1/VR1) capsaicin receptors as described by Zimov and Yazulla [19], which confirms goldfish as a reasonable model fish for studying capsaicin effects. The inflammatory effect resamples what occurs in rats as the vanilloid (Capsaicin) receptor VR1 when stimulated it mediates substance P release which is potent mediator for intestinal inflammation [20].

The microscopical picture of intestine showed graded degrees of inflammation and villar destruction after its contact with capsaicin. In this study the intensity of inflammation was dose/ exposure time dependent, so these two parameters should be carefully adjusted before using capsaicin with specific type and size of fish. In goldfish with the tested size, reasonable inflammatory reaction was obtained using 1 mg capsaicin/fish, capable

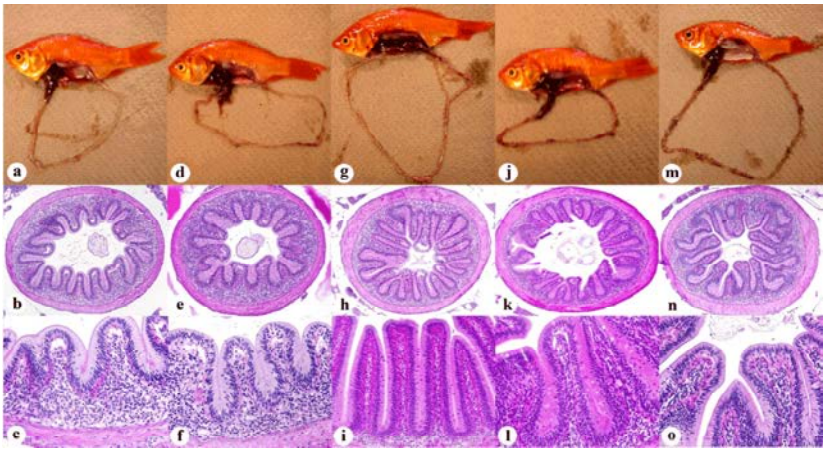

Fig. 1: Control goldfish dissected intestine showing normal coloration (a), with normal histological structure* (b,c). Goldfish anally intubated with 0.005 mg showing apparently non inflamed appearance (e), with normal histological structure (e,f). Goldfish anally intubated with 0.05 mg also showing faint reddening (g), with very mild subepithelial inflammation (h,i). Goldfish anally intubated with 0.5 mg showing reddening (j), with mild subepithelial inflammation (k, l). Goldfish anally intubated with 5 mg showing red intestine (m), with subepithelial inflammation (n,o).

*Microscopic pictures (b,e,h,k,n) 40X, (c,f,I,l,o) 200X, H&E stain.

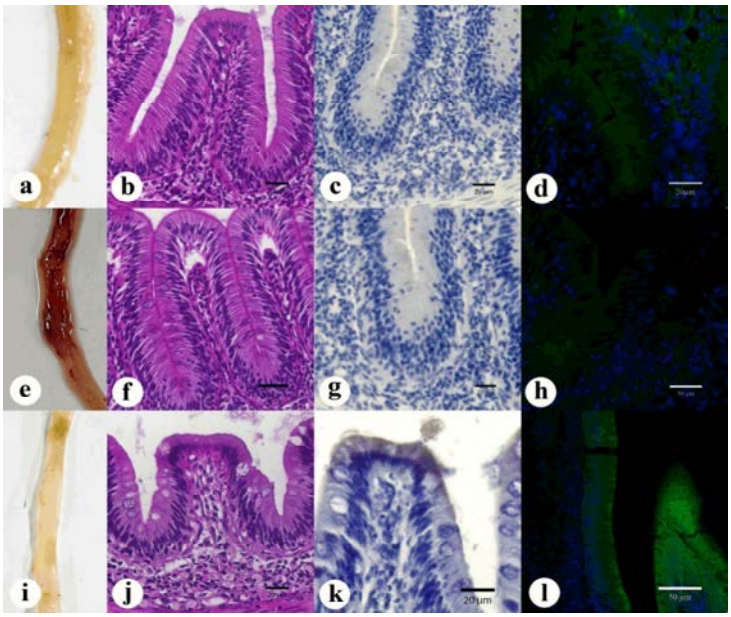

Fig. 2: Intestine of goldfish belonging to control -ve group showing no congestion (a) normal structure with no congestion or apparent abnormalities in H&E staining (b), In IHC staining* (c) and IFT** there is no evidence of VNN antigenic presence in intestinal tissue sections (d). Intestine of goldfish treated with 1mg capsaicin only showing congestion (e), moderate congestion in H&E staining (f), In IHC staining (g) and IFT there is no evidence of VNN antigenic presence in intestinal tissue sections (h). Intestine of goldfish intubated with VNN vaccine only after 1hour showing no congestion (i), normal structure with no congestion or apparent abnormalities in H&E staining (j), In IHC staining (k) and IFT (l) there is no evidence of VNN antigenic presence in intestinal tissue sections except some staining inside intestinal lumen in IHC and IFT sections.

* Immunoperoxidase staining with DAB as chromagen and Gill's hematoxylin as counterstain.

** Immunofluorescence using FITC conjugated antibody with DABI as nuclear stain.

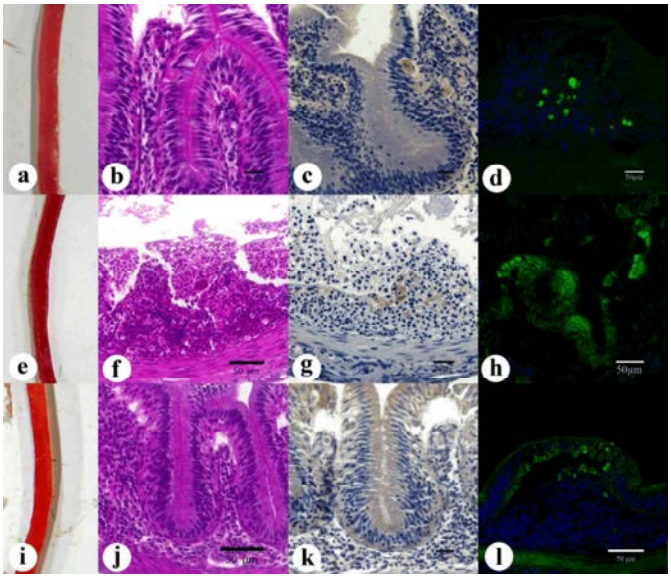

Fig. 3: Intestine of goldfish intubated with capsaicin then VNN vaccine after 1 hour showing reddening (a) moderate congestion, increased mucous cell secretions and frequent villar destruction in H&E staining (b), In IHC* staining (c) and IFT** there is few signals denoting VNN antigenic sub-epithelial presence in intestinal tissue sections (d). Intestine of goldfish intubated with capsaicin then VNN vaccine after 2 hours showing reddening (e) congestion, villar destruction in H&E staining (f), In IHC staining (g) and IFT there is some signals denoting VNN antigenic sub-epithelial presence in intestinal tissue sections and in-between tissue debris (h). Intestine of goldfish intubated with capsaicin then VNN vaccine after 3 hours showing also reddening (i) congestion, villar destruction in H&E staining (j), In IHC staining (k) and IFT there is some signals denoting VNN antigenic sub-epithelial presence in intestinal tissue sections and in-between tissue debris (l).
* Immunoperoxidase staining with DAB as chromagen and Gill's hematoxylin as counterstain.
** Immunofluorescence using FITC conjugated antibody with DABI as nuclear stain.

to promote the passage of vaccine particles through inflamed or intentionally damaged intestinal epithelium, after at least 1 hour of capsaicin intubation. Prolonged exposure of fish to 1 mg capsaicin; though was nonlethal; produced noticeable damage to the exposed intestinal wall. So it's recommended for succeeding experiments with prolonged exposures to use lower doses of capsaicin.

Determination of Best Timing of Vaccine Anal Intubation after Capsaicin: In the 1st group (Control -ve), intestine grossly and microscopically showed intact normal structure, with no antigenic staining or fluorescence (Fig. 2a-d).

In the 2nd group (Fish were intubated with capsaicin only), grossly intestine was severely inflamed and microscopically was congested, with no antigenic staining or fluorescence (Fig. 2e-h). In the 3rd group (Fish were intubated with vaccine only), intestine was intact with normal structure, while antigenic staining and fluorescence were localized on the tips of intestinal villi in the luminal side (Fig. 2i-l). In the 4thgroup (Fish were intubated with vaccine after 1 hour of capsaicin administration), grossly intestine was severely inflamed and microscopically was congested, with the antigenic staining and fluorescence localized on the tips of intestinal villi in the luminal side and in the interstitial tissue, especially in some resident mucosal macrophages (Fig. 3a-d). In the 5th group (Fish were intubated with vaccine after 2 hours of capsaicin administration), grossly intestine was also severely inflamed, by H&E staining, interstitial congestion, villous necrosis in some intestinal areas and intraluminal hemorrhage supervenes, also antigenic staining and fluorescence were localized on the tips of intestinal villi in the luminal side and in interstitial tissue and appearance of antigenic staining and fluorescence inside intestinal mucosal phagocytic cells (Fig. 3e-h). In the 6th group (Fish were intubated with vaccine after 3 hours of capsaicin administration), clearly the intestine was severely inflamed and microscopically there were interstitial congestion with apparent villous

necrosis in some intestinal areas with intraluminal hemorrhages, also with the antigenic staining and fluorescence were localized on the tips of intestinal villi in the luminal side and in interstitial tissue, appearance of antigenic staining and fluorescence inside intestinal mucosal phagocytic cells (Fig. 3i-l).

The presence of antigenic staining and Immunoflourescence inside the intestinal mucosa and inside the intestinal phagocytic cells denoted the subepithelial translocation of the vaccinal particles during the inflammatory process.

The quantity of the translocated vaccine particles (May or may not) be necessarily enough to produce immune reaction against them, as immunity initiation by vaccine needs a minimum threshold dose to activate cellular and humeral responses, this step needs more detailed investigations to fulfill.

In this study, it can be concluded that, capsaicin can be used successfully to aid the intestinal passage of large molecules like vaccine particles as demonstrated here with VNN vaccine. This step would be very helpful in production of oral vaccine adjuvant in vulnerable fish species instead of injectable vaccine, which is the next step in our group research.

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