Research Journal of Biological Sciences 4 (4): 509-513, 2009
ISSN: 1815-8846
© Medwell Journals, 2009

Evaluation of Safe Attenuated *Vibrio alginolyticus* for Oral Vaccination of *Lates calcarifer* Against Vibriosis

[1,2]Nurul H. Idris, [3]Nagi A. Al-Haj, [1,2]Mariana N. Shamsudin, [4]Raha A. Rahim
[1]Department of Marine Science and Aquaculture, Institute of Bioscience,
[2]Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences,
[3]Laboratory Immunotherapeutic and Vaccine, Institute of Bioscience,
[4]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular,
University Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Abstract: Safe attenuation has been done on marine pathogen *Vibrio alginolyticus* using naturally acidified fructose against vibriosis. Attenuation was confirmed by injecting the attenuated bacterium into fish where the survival rate was 100% compared to 50% survival in fish injected with non-attenuated bacteria. The attenuated bacterium was then evaluated for oral vaccination of *Lates calcarifer* (Asian seabass). Fish were fed with fish pellet incorporated with attenuated and non-attenuated bacterium of *V. alginolyticus* for 30 days. They were measured for serum antibody production by conventional agglutination titer and also monitored for the fish weight gain to observe the health improvement. Vaccinated fish showed comparable increased in weight gain, 90% survival after challenge and significantly high antibody titer compared to other treatment and control.

Key words: Vaccines, *Vibrio alginolyticus*, *Lates calcarifer*, minimal inhibitory concentration, polymerase chain reaction

INTRODUCTION

Infectious diseases are major problems affecting aquaculture causing heavy economic losses to fish farmers. Chemotherapy, vaccination and other prophylactic measures are generally adopted to control microbial infection (Vine *et al*., 2004) but development of drug resistant microbes (Kawakami *et al*., 1997) has prompted alternatives treatment of infection. In aquaculture, multiple setbacks from use of antibiotics are frequently documented (Miranda and Zemelman, 2002). Conventional immunostimulant agent employed chemical basis of antigen attenuation, rendering toxicity to host cells. The present study hypothesized that alteration of infective target can disrupt infection establishment and in turn induce positive immune response of host cells. A bacterial surface structure gene, especially those involve in infection establishment will affect virulence of pathogen when altered but the whole intact cell can still induce response of host cells. The attenuated bacteria will find use as an immunostimulant, improving relevant fish health parameters (Raetz *et al*., 1998; Sabine *et al*., 2000). The weakening of vital cellular structure is desirable since, growth can be inhibited and if target is part of bacterial outer wall, it is duly recognized by host immunity (Gladys *et al*., 2006). Target gene alteration through disruption by a naturally derived acidified fructose is used for pathogen attenuation. The health outcome of fish through feeding with fish feed incorporated with safely attenuated antigen was studied. Therefore, the efficacy of the modified fish feed in improving fish health through immune response and fish survival upon challenging with virulent bacterial agent were assessed.

MATERIALS AND METHODS

Bacterial isolate: Pure culture of a marine pathogen *V. alginolyticus* (ATCC17749) reference strain that was bought from American Type Culture Collection (ATCC) was maintained in sterile *Luria bertanii* (Merck) broth supplemented with 2% sodium chloride with 15% glycerol at -80°C. For use, the culture was revived on Mueller Hinton agar (Merck) supplemented with 2% sodium chloride and subsequently cultured on Thiosulphate Citrate Bile Salts (TCBS, Merck) agar supplemented with 2% sodium chloride. The *V. alginolyticus* isolates will show bright yellow colonies on TCBS agar.

Corresponding Author: Nagi AL-Haj, Laboratory of Immunotherapeutic and Vaccine, Institute of Bioscience, University Putra Malaysia, 43400, Serdang, Selangor, Malaysia

***In vitro* attenuation efficacy:** *V. alginolyticus* isolates treated with naturally derived acidified fructose were determined for growth inhibiton through the plate and bacterial reduction tube assay. The growth of *V. alginolyticus* is confirmed to be inhibited when no growth was seen in agar plates streaked with the treated bacteria. The Minimal Inhibitory Concentration (MIC) assay was conducted to obtain the MIC value of the natural substance on *V. alginolyticus*. The bacterial concentrations were adjusted to 0.5 McFarland and MIC values were then used in bacterial treatment to obtain mRNA.

Molecular assay: The effect of growth inhibition by the non-toxic natural agent on a selected infective gene of *V. alginolyticus* was determined using RT-PCR assay. Type strain (ATCC 17749) was treated with natural non-toxic substance based on the MIC value after 16-18 h incubation and proceeded with RNA extraction using Epicentre RNA Purification Kit (All Eights, Malaysia). The successful RNA were then reverse transcribed using ProtoScript II RT-PCR Kit (New England Biolabs Incorporation) to obtain the cDNA and were then used for Reverse Transcriptase-PCR (RT-PCR). Products of treated and untreated bacteria from RT-PCR were then sent for commercial sequencing. The results were then analyzed using BLAST analysis and compared to GenBank and Biology Workbench.

Preparation of fish pellet incorporated with attenuated and non-attenuated cells of a *V. alginolyticus*: Preparation of attenuated *V. alginolyticus* cells was done according to the Minimal Inhibitory Concentration (MIC) method. In brief, treated and non-atenuated *V. alginolyticus* was promoted to grow about 16-18 h provided with Luria Bertanii broth supplemented with 2% sodium chloride. The cells were then centrifuged briefly at 3500 rpm for 10 min, washed twice with sterile phosphate buffered saline (PBS 0.1 M, pH 7.2) and then resuspended in PBS to the desired concentration of 108 cfu mL^{-1}. Under sterile conditions, the bacteria were manually incorporated into commercial dry pellets at rates of 108-1010 cfu g^{-1}, respectively. The attenuated bacteria were injected into fish to validate the attenuation process compared to non-attenuated bacteria. Fish fed only commercial dry pellets served as a control. For control, fish pellet were incorporated with sterile Phosphate Buffered Saline (PBS). Prepared fish pellet were kept in 4°C until further use.

Fish stock maintenance: Healthy seabass (*Lates calcarifer*) with an average weight of 29 g (±0.32) were purchased from a hatchery in Johor and reared in several aquaria using artificial seawater at a final salinity in the range of 16-18 ppt. Fish were acclimatized in the aquarium condition for three weeks before carrying out experiments and were maintained at 25-28°C. Fish were fed commercial dry pellet before treatment.

Oral immunization of fish: After 3 weeks of acclimatization, fish were fed twice a day with fish pellet incorporated with attenuated and non-attenuated bacterial fish pellet for a period of 30 days.

Attenuated feed performance: Feed incorporated with attenuated whole cells was assessed for fish health improvement efficacy, involving body weight and immune response. Fish were bled weekly at the same time to obtain serum to perform agglutination titer method to see the antibody response. Fish were anaesthetised and bled from their caudal veins with non-heparanised disposable syringes. Blood was allowed to clot at 4°C overnight. The clot free serum was s eparated following centrifugation at 3500 rpm for 10 min and stored at -80°C until further use. Fish weights were measured weekly to observe fish growth. Fish were challenged by injecting 0.6 OD (1×10^7 cfu mL^{-1}) of pathogenic *V. alginolyticus* intraperitoneally after 4 weeks treatment to see the protective efficacies against disease vibriosis.

RESULTS AND DISCUSSION

Lipopolysaccharide (LPS) is one of the major components of the bacterial outer membrane (Nikaido and Vaara, 1985). It is composed of lipid A and a core oligosaccharide and in many species of bacteria also, has a polysaccharide chain termed the O-specific antigen (Whitfield and Valvano, 1993; Werner *et al*., 1997). The O-antigen is a major contributor to the antigenic variability of the bacterial cell surface. The O-antigen promoted the activation of complement and formation of the complement membrane attack complex away from its site of insertion in the outer membrane (Joiner, 1988). The early research has been focused on the role of LPS as a virulence determinant and on its use as a vaccine candidate. Since that time, studies have expanded to include analysis of the chemistry and biosynthesis of the O-antigenic region due to its immunogenicity, serotype specificity and serum resistance properties. *In vitro* attenuation efficacy plate assay showed that several

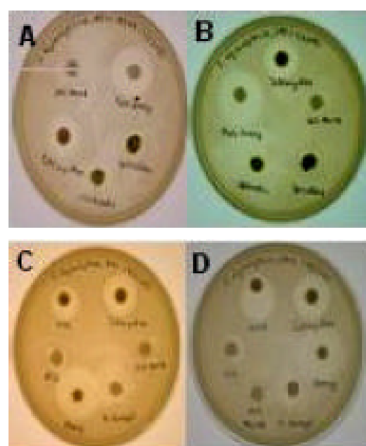

Fig. 1: Pictures A, B, C and D show growth inhibition zone of *V. alginolyticus* around disc impregnated with various natural product

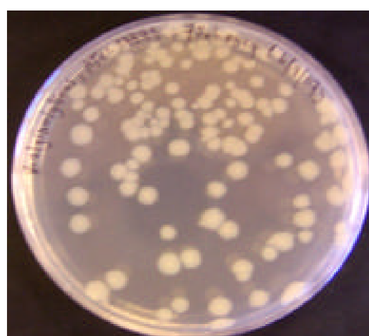

Fig. 2: Bacterial growth for MIC determination

naturally derived products gave clear growth inhibition zones when tested on *V. alginolyticus*. The product that showed the biggest inhibition zone (Fig. 1) was the naturally derived acidified fructose. The product at calculated MIC value of 7% (v v^{-1}) inhibited growth of ATCC 17749 (Fig. 2). Attenuation of *Vibrio alginolyticus* isolates was hypothesized to be achieved through treatment with a growth inhibitory agent at a dosage below the MIC value.

RNA was successfully extracted upon treatment of *V. alginolyticus* with 7% agent (v v^{-1}). RT-PCR amplification of a selected infective gene at 1056 bp was successfully amplified from treated and untreated ATCC17749. The bands upon purification and commercial sequencing showed changes in the nucleotide sequences of treated bacteria and no significant changes were seen in bacteria exposed to agent giving smaller zone of inhibition and also in strain treated with the agent at a concentration lower than the MIC value (Table 1 and Fig. 3). The molecular assay of a selected surface structure gene of treated strain also indicated alteration in the partially expressed gene upon RT-PCR analysis.

Table 1: Percentage of homology on untreated and treated *V.alginolyticus* (ATCC 17749)

Isolates	Homology (%)
ATCC DNA	96
ATCC untreated	95
ATCC marine extract treatment	96
ATCC spirullina treatment	96
ATCC marine invertebrate extract treatment	95
ATCC naturally derived acidified fructose	88

The bacteria was confirmed attenuated after injecting the bacterium into fish where, the survival rate was 100% compared to fish injected with non-attenuated bacteria giving a 50% survival. The present finding confirmed attenuation of a virulent *V. alginolyticus* isolate when fish injected with attenuated strain showed high survival rate in comparison to those injected with untreated strain and the control, with 100% mortality. Fish growth measured by fish weight showed fish fed with attenuated bacteria gained weight up to 38 g compared with other treatments (Fig. 4). Fish fed with attenuated bacteria gave a 90% survival compared to non-attenuated at 30% survival and control at 0% survival after challenge with *V. alginolyticus* (Fig. 5). As for the antibody response, fish fed with attenuated bacteria showed high antibody titer at 128 (2.1 mean antibody titer (log)) compared to non-attenuated at 16 (1.2 mean antibody titer (log)) and control fish at 2 (0.3 mean antibody titer (log)) (Fig. 6).

The efficacy of feed incorporated with attenuated strain also showed favorable effect based on the significant weight gain and agglutination antibody titer. The groups of fish fed with feed incorporated with attenuated strain were highly protected when challenged with virulent strain since high survival rate were observed. Overall, orally vaccinated fish with attenuated bacterium showed higher antibody titers compared to unvaccinated controls. Based on fish weight gained, antibody titer and survival rate, fish fed with attenuated bacteria showed comparable difference from the other treatments. The present findings imply that feed incorporated with attenuated bacteria potential to improve fish health. These findings thus are in accordance to the hypothesis that attenuated bacteria are good immuno stimulant and are beneficial to the aquaculture industry. Evaluation of immune status after oral vaccination may not be complete by restricted the measurement to serum parameters. The assessment need to be carried out at the

```
ATCC_treated   ACTTGATGGCTACAAATCACTT------CAGCTGAAAGG-----AGTTTG
Non-treated    ACTTGATGGCTACAAATCACTTTGGCGACAGCTGAAAGGCAGAGAGTTTG
               **********************      ***********     ******

ATCC_treated   ATGCGCTATTGCATATGCAG---GCGATTCGAG--AGTGTCGC---GTTG
Non-treated    ATGCGCTATTGCATATGCAGTACGCGATTCGAGCGAGTGTCGCAACGTTG
               ********************   **********  ********   ****

ATCC_treated   GGCGTTGAA-----ATACAAACTTGGTT---CTTCTGATAGAAGCCAGGA
Non-treated    GGCATTAAAGCAAAATACAAACTTGGTTTCTCTTCTGATAGAAGCCAGGA
               *** ** **     **************   *******************
```

Fig. 3: Alteration in sequences of surface structure gene of ATCC 17749 upon treatment with naturally derived acidified fructose

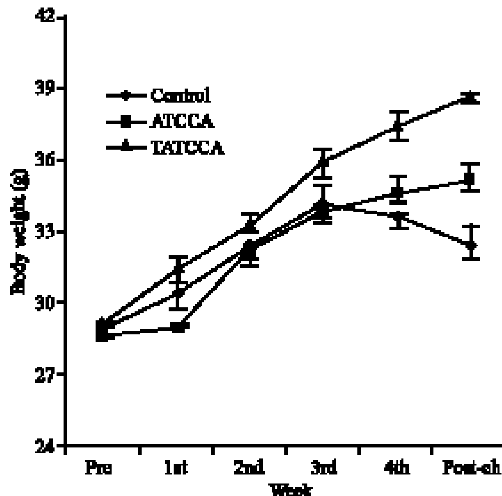

Fig. 4: Body weight of fish fed with attenuated bacteria (TATCCA), non-attenuated (ATCCA) and PBS (control)

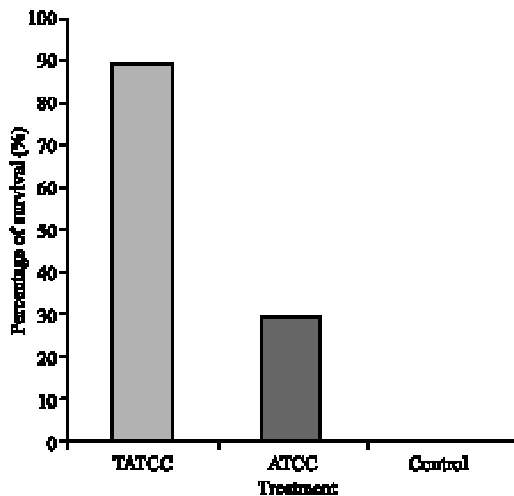

Fig. 5: Percentage of survival after challenge with pathogenic *V. alginolyticus*

integumental/entero-mucosal surfaces considered as the primary sites of pathogen attack and entry. The

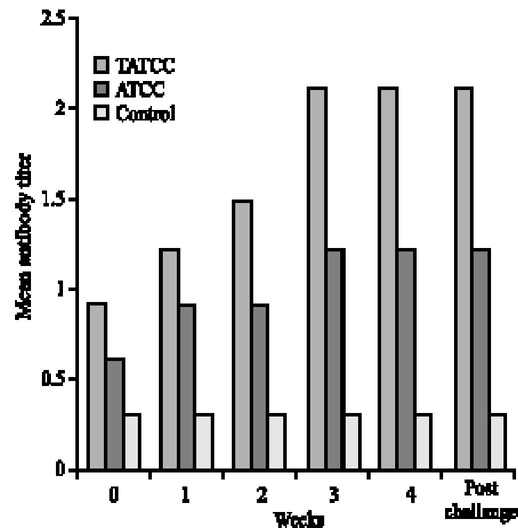

Fig. 6: Mean antibody titer (log) of siakap fed with safe attenuated bacteria (TATCC), non-attenuated bacteria (ATCC) and PBS (control)

interactions of fish Gut Associated Lymphoid Tissue (GALT) and oral vaccines needs to be evaluated. The concept of safe attenuated bacterium for oral vaccination, besides having application in aquaculture, may have potential for oral prophylaxis for enteric diseases in veterinary and human medicine.

ACKNOWLEDGEMENT

Authors acknowledge the funding support from IRPA grant number 5484100, Government of Malaysia.

REFERENCES

Gladys, P., H. Nadine, P. Kalinka, A. Miguel and D. Gerard, 2006. An *in vitro* screen of bacterial lipopolysaccharide biosynthesis enzymes identifies an inhibitor of ADP-Heptose biosynthesis. Chem. Biol., 13: 437-441. PMID: 16632256. DOI: 10.1016/j.chembiol.2006.02.010.

Joiner, K.A., 1988. Complement evasion by bacteria and parasites. Ann. Rev. Microbiol., 42: 201-230. PMID: 3059994.

Kawakami, H., N. Shinohara, Y. Fukuda, H. Yamashita, H. Kihara and M. Sakai, 1997. The efficacy of LPS mixed chloroform-killed cells (LPS-CKC) bacterin of *Pasteurella piscida* on Yellowtail, *Seriola quinqueradiata*. Aquaculture, 154: 95-105.

Miranda, C.D. and R. Zemelman, 2002. Bacterial resistance to oxytetracycline in Chilean Salmon farming. Aquaculture, 212: 31-47. DOI: 10.1016/S0044-8486(02) 00124-2.

Nikaido, H. and M. Vaara, 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev., 49: 1-32. PMID: 2580220.

Raetz *et al*., 1998. Enzymes of lipid A biosynthesis targets for the design of new antibiotic. Prog. Clin. Biol. Res., 397: 1-14. PMID: 9575543.

Sabine, G., B. Werner and B. Helmut, 2000. Comparative functional characterization *in vitro* of heptosyltransferase I (WaaC) and II (WaaF) from *Escherichia coli*. Eur. J. Biochem., 267: 6602-6611. PMID: 11054112.

Vine, N.G., W.D. Leukes, H. Kaiser, S. Daya, J. Baxter and T. Hecht, 2004. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria of fish intestinal mucus. J. Fish Dis., 27: 31-47. PMID: 15189372.

Werner, B., M.L. Sven, H. Otto and B. Helmut, 1997. Deletion of the heptosyltransferase genes rfaC and rfaF in *E. coli* K-12 results in a re-type lipopolysaccharide with a high degree of 2-aminoethanol phosphate substitution. Eur. J. Biochem., 247: 416-724. PMID: 9266718.

Whitfield, C. and M.A. Valvano, 1993. Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. Adv. Microb. Physiol., 35: 135-246. PMID: 8310880.