

Responses of monosex Nile tilapia (*Oreochromis niloticus*) to intraperitoneal challenge by *Streptococcus iniae* after vaccination with ghosts of the bacterium

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Abstract

The vaccine potential of *Streptococcus iniae* ghosts produced by gene E mediated lysis was investigated using tilapia (*Oreochromis niloticus*). Tilapia immunized with *S. iniae* ghosts (SIG) and formalin killed *S. iniae* (FKC) vaccines showed significantly higher serum agglutination titres than control fish. Fish immunized with SIG showed no significant differences with fish immunized with FKC in serum agglutination titres, but showed significantly higher bactericidal activity than fish immunized with FKC. Furthermore, fish immunized with SIG showed higher protection than fish immunized with FKC. As this promising type of a non-living whole cell envelope preparation seems to be favorable over conventional vaccines, we suggest *S. iniae* ghosts as a new vaccine candidate. (*Bangl. vet.* 2012. Vol. 29, No. 1, 31 - 37)

Introduction

S. iniae is a Gram-positive, oxidase-negative, catalase-negative, sphere-shaped bacterium. It is the main etiological agent of streptococcosis also caused by *Streptococcus* and *Lactococcus* spp., in a variety of fresh and saltwater fish species in worldwide and results in estimated losses \$150 million in annually (Shoemaker and Klesius, 1997). Because, antibiotic resistance of *S. iniae* has been reported widely in the world (Park *et al.*, 2009), there is urgent need to find other approaches for the treatment and prevention of this infection. Over the last decade vaccination has become important for the prevention of infectious diseases in farmed fish (Gudding *et al.*, 1999). Although, several attempts have been made to induce protection against *S. iniae* (Eldar *et al.*, 1997; Kvitt and Colorni, 2004) and the protection efficiency was variable among the studies. Recently, the outer membrane proteins of pathogenic bacteria have been studied in relation to inducing protective humoral and cell-mediated immunity (Heckels *et al.*, 1989; Kawai *et al.*, 2004). However, traditional inactivation of bacteria by heat or formalin may influence the physico-chemical

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characteristics of surface antigens, and immune responses against the modified antigens may not be protective against live bacteria. The genetic inactivation of pathogenic Gram-positive bacteria by the controlled expression of cloned bacteriophage Phi X 174 lysis gene E offers a promising new approach in non-living vaccine technology (Eko *et al.*, 1996; Szostak *et al.*, 1996). Expression of plasmid-encoded gene E leads to the formation of a transmembrane tunnel structure through the cell envelope of Gram-positive bacteria, which consequently leads to the loss of cytoplasmic contents. The resultant ghosts have been known to retain the functional and antigenic determinants of the envelope with their living counterparts and thus represent ideal vaccine candidates (Witte *et al.*, 1992). In the present study, we investigated the vaccine potential of *S. iniae* ghosts in monosex Nile tilapia.

Materials and Methods

Fish

Juvenile tilapia, weighing 95 ± 20 g was obtained from the fish farm in South-West Area Integrated Water Resources Planning and Management Project (SAIWRPMP) funded by Asian Development Bank (ADB), Ministry of Water Resource and Bangladesh Water Development Board (BWDB), Bangladesh. For the immunization experiments, fish were stocked into either three 150 litre aquaria at a density of 30 fish per aquarium, or a single 100 litre aquarium at a density of 100 fish. Fish were acclimated for two weeks prior to initiating the experiments.

Bacterial strain

Streptococcus iniae SI01 provided by SAIWRPMP, was grown in Brain heart infusion (BHI) medium (broth and agar) at 27°C. Transformed *S. iniae* was grown in BHI containing 50 mg/mL ampicillin (Sigma Chemical Co., St Louis, MO, USA). Incubation temperatures for repression and expression of lysis gene E in transformants were 27°C and 42°C, respectively. Growth and lysis of bacterial cultures were monitored by measuring the optical density at 600 nm (OD₆₀₀).

Production of S. iniae ghosts (SIG)

Lyophilized *S. iniae* ghosts were produced as described previously (Kwon *et al.*, 2005). Briefly, *S. iniae* harboring the lysis plasmid pλPR-cl-Elysis was induced for lysis after growth under culture conditions by elevation of temperature from 27°C and 42°C. At the end of lysis, ghosts were harvested, washed and resuspended in phosphate buffered saline (PBS) and then lyophilized. The efficiency of E-mediated killing of *S. iniae* was estimated by plating samples of appropriate dilutions of lyophilized SIG on BHI agar containing 50 mg/mL ampicillin and results were compared with those from samples obtained prior to onset of lysis. Results indicated a 100% killing efficiency as no colony-forming units were found on plates with lyophilized SIG preparations at any dilution.

Production of formalin-killed S. iniae (FKC)

S. iniae was grown for 24h at 27°C in tryptic soy broth (TSB, Sigma, USA) containing 1.5% NaCl. For FKC preparation, formalin was added to a 24h culture of the bacterium to make the final concentration 0.5%. After 24h incubation, cells were washed three times with PBS (pH 7.2) and resuspended in 10 mL PBS. The suspensions were streaked on tryptic soy agar containing 1.5% NaCl for checking sterility and stored at 4°C until use.

Immunization and collection of blood samples

SIG and FKC preparations were reconstituted with PBS. In vaccine experiment I, groups of fish (25 fish/group) stocked into three aquaria were immunized twice intraperitoneally (ip), two weeks apart, with 1.4×10^6 cells of either SIG or FKC in 50 mL of PBS. A group that received PBS (50 mL) ip represented the control. Two weeks after the last vaccine dose (day 28), 10 fish in each group were randomly sampled and blood specimens were collected from the caudal vein for agglutination and bactericidal tests. In vaccine experiment II, fish stocked into a single aquarium were randomly divided into three groups (25 fish/group) by pectoral fin clipping and were cohabitated in a single aquarium to eliminate tank effects. The immunization regime of experiment II was the same as that of experiment I.

Agglutination activity of serum

The agglutination test was conducted in 'U'-shaped microtitre plates. The anti-sera raised in fish and test sera were serially diluted two-fold, to which a constant amount of FKC (approximately 4 mg/mL) were added and kept overnight at room temperature. The agglutination activity was determined as the first serum dilution where no agglutination occurred, and expressed as the reciprocal of that dilution.

Bactericidal activity of serum

The serum bactericidal activity was determined according to Yin *et al.* (1996) with some modifications. The bacterial cultures were pelleted (3000g, 10 min) and washed three times with sterile PBS. The bacterial suspension was adjusted to 4×10^9 cells/mL. A volume of 25 μ L bacterial suspension and 25 μ L serum of fish in each group were mixed in sterile Eppendorf tubes. They were incubated at room temperature for 1h, and subsequently, were used to determine colony forming units (CFU)/mL by plating the mixtures on TSA containing 1.5% NaCl.

Challenge test

To enhance the virulence, *S. iniae* had been passaged in naive tilapia by intraperitoneal injection. The bacterium was reisolated from the kidney of moribund fish three day later and cultured on TSA plates supplemented with 1.5% NaCl for 24h at 27°C. The bacterial cell suspension was adjusted to 5×10^7 cells/mL. Fish (20 fish/group in experiment I and 25 fish/group in experiment II) were challenged by 100 mL (5×10^6 cells/mL cells/fish) of the bacterial suspension ip two weeks after

the booster dose. Deaths were recorded over 25 days. Dead fish were collected daily and necropsied and kidney samples were streaked on Salmonella Shigella agar (SS agar, Difco) to confirm the presence of *S. iniae*.

Statistical analysis

Serum agglutination and bactericidal data were analyzed by the Student's t-test, and challenge test data were analyzed by the Chi-square test. Significant differences were determined at $P < 0.05$.

Results and Discussion

Agglutination and bactericidal activities of serum

Fish immunized with SIG or FKC showed significantly higher agglutination titre than control fish (Fig. 1). Although, fish immunized with SIG showed higher agglutination titre than fish immunized with FKC, there were no significant differences between these two groups of fish. In serum bactericidal activity, fish immunized with SIG showed significantly higher activity than fish immunized with FKC (Fig. 2). The fish in the control group showed the lowest serum bactericidal activity.

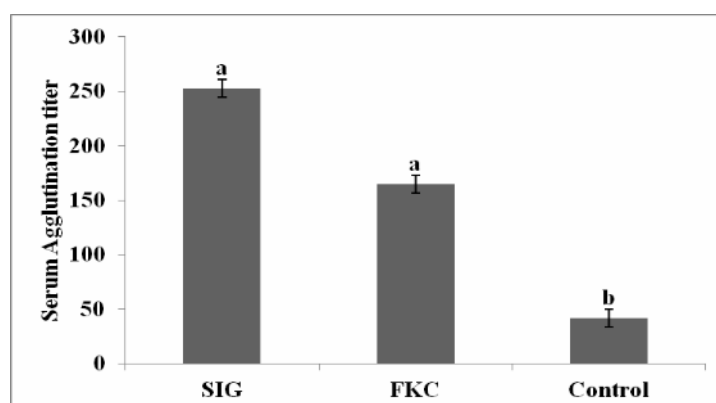


Fig. 1. Serum agglutination titre of tilapia immunized intraperitoneally with *Streptococcus iniae* ghosts (SIG), and formalin-killed *S. iniae* (FKC) or PBS alone (Control) after two weeks of the boost immunization. Values are mean \pm standard error. Different letters on the bar indicate statistically significant differences at $P < 0.05$

Protective efficacy of SIG vaccine

In vaccine experiment I, groups of fish immunized with SIG and FKC showed significantly higher survival rate than control fish (Fig. 3). Furthermore, fish vaccinated with SIG showed significantly higher survival rate than fish vaccinated with FKC. In vaccine experiment II, the cumulative mortalities of control, FKC and SIG immunized groups were 20%, 8% and 0%, respectively. All dead fish were positive for *S. iniae*.

Our new approach to produce a non-living *Streptococcus iniae* ghosts vaccine is based on the gene E-mediated lysis of cells, which has been extensively investigated in *E. coli* (Witte and Lubitz, 1989; Witte *et al.*, 1992; Schön *et al.*, 1995; Witte *et al.*, 1997). Lysis of bacteria by controlled expression of gene E leading to empty cell envelopes is suggested as an alternative method for inactivation of bacteria without chemical or physical stress, which can reduce antigenicity. The potential usefulness of this technology has recently been reported in mammalian pathogenic Gram-negative and positive bacteria (Eko *et al.*, 1994; Eldar *et al.*, 1997; Katinger *et al.*, 1999; Panthel *et al.*, 2002; Marchart *et al.*, 2003; Kvitt and Colorni, 2004). In the present study, we have tested the degree of protection and immune response induced after immunization of tilapia with *S. iniae* ghosts vaccine.

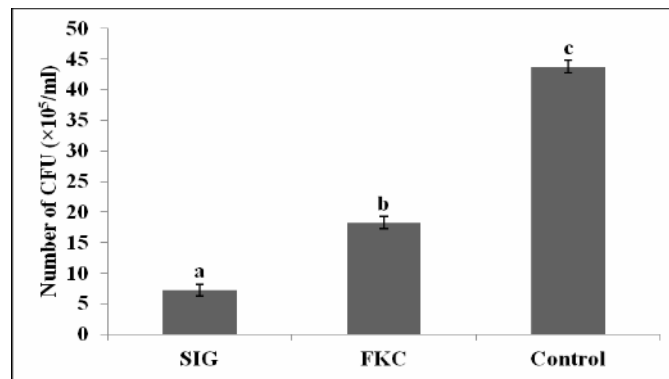


Fig. 2. Serum bactericidal activity of tilapia immunized intraperitoneally with *Streptococcus iniae* ghosts (SIG), and formalin-killed *S. iniae* (FKC) or PBS alone (Control) after two weeks of the boost immunization. The bactericidal activity of the serum was expressed as the number of colony forming unit (CFU)/mL of *S. iniae* after 1 h incubation with each serum. Values are mean \pm standard error. Different letters on the bar indicate statistically significant differences at $P < 0.05$

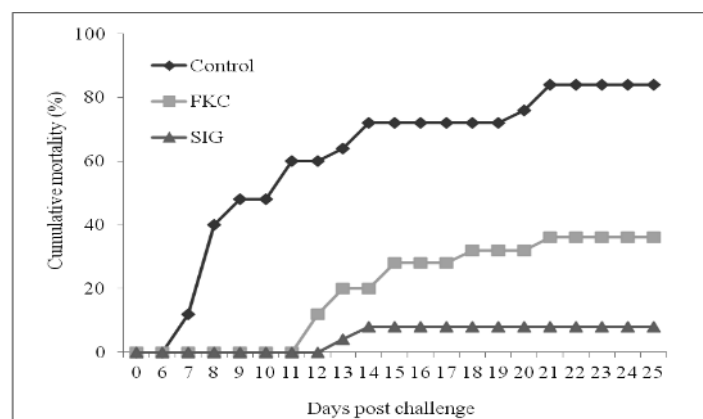


Fig. 3. Cumulative mortality of tilapia immunized intraperitoneally with *Streptococcus iniae* ghosts (SIG), and formalin-killed *S. iniae* (FKC) or PBS alone (Control) after challenge with *Streptococcus iniae*

In the present study, significantly higher serum agglutination titres in SIG or FKC immunized fish than control fish indicate, that both SIG and FKC induced specific humoral immunity in tilapia. Fish immunized with SIG showed significantly higher serum bactericidal activity and higher survival rates than FKC immunized fish. These results suggest that SIG has higher potential to induce protective antibodies than FKC. Compared to heat or formalin killed bacterial vaccines, the adjuvant effect of ghost bacterial vaccines has been reported in mammalian pathogenic bacteria (Jalava *et al.*, 2002). 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 *S. iniae* ghost's vaccine (Eldar *et al.*, 1997; Haslberger *et al.*, 1997; Haslberger *et al.*, 2000).

As this promising type of a non-living whole cell envelope preparation seems to be favorable over conventional vaccines, we suggest *S. iniae* ghosts as a new vaccine candidate.

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References

- Eko FO, Szostak MP, Wanner G, Lubitz W 1994: Production of *Vibrio cholerae*-ghosts (VCG) by expression of a cloned phage lysis gene: potential for vaccine development. *Vaccine* **12** 1231-1237.
- Eko FO, Witte A, Huter V, Kuen B, Fu¨rst-Ladani S 1996: New strategies for combination vaccines based on the extended recombinant bacterial ghost system. *Vaccine* **17** 1643-1649.
- Gudding R, Lillehaug A, Evensen O 1999: Recent developments in fish vaccinology. *Veterinary Immunology and Immunopathology* **72** 203-212.
- Haslberger A, Mader HJ, Schmalnauer M, Kohl G, Messner P, Sleytr UB 1997: Bacterial cell envelopes (ghosts) and LPS but not bacterial S-layers induce synthesis of immune-mediators in mouse macrophages involving CD14. *Journal of Endotoxin Research* **4** 431-441.
- Haslberger AG, Kohl G, Felnerova D, Mayr UB, Fu¨rst-Ladani S, Lubitz W 2000: Activation stimulation and uptake of bacterial ghosts in antigen presenting cells. *Journal of Biotechnology* **83** 57-66.
- Heckels JE, Fletcher JN, Virji M 1989: The potential protective effect of immunization with outer membrane protein I from *Neisseria gonorrhoeae*. *Journal of General Microbiology* **135** 2269-2276.

- Jalava K, Hensel A, Szostak M, Resch S, Lubitz W 2002: Bacterial ghosts as vaccine candidates for veterinary applications. *Journal of Control Release* **85** 17-25.
- Katinger A, Szostak MP, Stadler M, Klein R, Huter V, Lubitz W 1999: Pigs aerogenously immunized with genetically inactivated (ghosts) of irradiated *Actinobacillus pleuropneumoniae* are protected against a homologous aerosol challenge despite differing in pulmonary cellular and antibody responses. *Journal of Biotechnology* **72** 247-256.
- Kawai K, Liu Y, Ohnishi K, Oshima S 2004: A conserved 37 kDa outer membrane protein of *Edwardsiella tarda* is an effective vaccine candidate. *Vaccine* **22** 3411-3418.
- Kvitt K, Colorni A 2004: Strain variation and geographical endemism in *Streptococcus iniae*. *Disease of Aquatic Organism* **61** 67-73.
- Kwon SR, Nam YK, Kim SK, Kim DS, Kim KH 2005: Generation of *Edwardsiella tarda* ghosts by bacteriophage PhiX174 lysis gene E. *Aquaculture* **250** 16-21.
- Marchart J, Dropmann G, Lechleitner S, Schlapp T, Wanner G, Szostak MP 2003: *Pasteurella multocida*- and *Pasteurella haemolyticaghosts*: New vaccine candidates. *Vaccine* **21** 3988-3997.
- Pantel K, Jechlinger W, Matis A, Rohde M, Szostak M, Lubitz W 2002: Generation of *Helicobacter pylori* ghosts by PhiX protein E-mediated inactivation and their evaluation as vaccine candidates. *Infection Immunology* **71** 109-116.
- Park YK, Nho SW, Shin GW, Park SB, Jang HB, Cha IS, Ha MA, Kim YR, Dalvi RS, Kang BJ, Jung TS 2009: Antibiotic susceptibility and resistance of *Streptococcus iniae* and *Streptococcus parauberis* isolated from olive flounder (*Paralichthys olivaceus*). *Veterinary Microbiology* **136** 76-81.
- Schoen P, Schrot G, Wanner G, Lubitz W, Witte A 1995: Two-stage model for integration of lysis protein E of bacteriophage PhiX174 into the cell envelope of *Escherichia coli*. *FEMS Microbiology Review* **17** 207-212.
- Shoemaker C, Klesius P 1997: *Streptococcal disease problems and control: a review*. In: Fitzsimmons K (Edn), *Tilapia Aquaculture* vol. 2 Northeast Regional Agricultural Engineering Service-106, Ithaca, NY, USA, pp. 671-680.
- Szostak MP, Hensel A, Eko FO, Klein T, Mader H, Haselberger A 1996: Bacterial ghosts: non-living candidate vaccines. *Journal of Biotechnology* **4** 161-70.
- Witte A, Lubitz W 1989: Biochemical characterization of ϕ X174-protein-E-mediated lysis of *Escherichia coli*. *European Journal of Biochemistry* **180** 393-398.
- Witte A, Schrot G, Schoen P, Lubitz W 1997: Proline 21, a residue within the α -helical domain of ϕ X174 lysis protein E, is required for its function in *Escherichia coli*. *Molecular Microbiology* **26** 337-346.
- Witte A, Wanner G, Lubitz W 1992: Dynamics of PhiX174 protein E-mediated lysis of *Escherichia coli*. *Archive Microbiology* **157** 381-388.
- Yin Z, Lam TJ, Sin YM 1996: The role of specific antiserum of catfish, *Clarias gariepinus*, as a defence against *Aeromonas hydrophila*. *Fish and Shellfish Immunology* **6** 57-69.