

CpG motif in synthetic ODN primes respiratory burst of olive flounder *Paralichthys olivaceus* phagocytes and enhances protection against *Edwardsiella tarda*

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ABSTRACT: Effects of synthetic cytidine-phosphate-guanosine (CpG) oligodeoxynucleotide (ODN) on respiratory burst activity of olive flounder (*Paralichthys olivaceus*) head-kidney phagocytes and on protection against lethal infection with *Edwardsiella tarda* were investigated. Phagocytes pre-cultured with a CpG ODN showed significantly higher chemiluminescence (CL) responses than phagocytes pre-cultured with guanosine-phosphate-cytidine (GpC) ODN or culture medium alone (control) at all concentrations. Supernatants produced from leucocytes, which were pulsed with CpG ODN, induced significantly higher respiratory burst activity than supernatants produced by GpC ODN or culture medium alone. In an *in vivo* experiment, respiratory burst activities of the head kidney phagocytes in the groups injected either 0.25 or 0.5 µg fish⁻¹ of CpG ODN were significantly higher than those in the groups injected with GpC ODN or HBSS (control) at 3, 5 and 7 d after injection. The groups of fish injected with 0.25 or 0.5 µg of CpG ODN showed higher survival rates (83.3%) than groups treated with GpC ODN (33.3%) and a control group (8.3%) after challenge with *E. tarda*. The present *in vitro* and *in vivo* experiments have demonstrated the ability of synthetic CpG ODN to increase phagocyte respiratory burst activity and disease resistance in olive flounder.

KEY WORDS: Synthetic CpG ODN · Olive flounder · Respiratory burst · Phagocytes · Disease resistance · *Edwardsiella tarda*

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INTRODUCTION

The innate immune system of vertebrates recognizes unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides flanked by specific bases in bacterial DNA as a danger signal (Krieg et al. 1995, 2000). Such sequences are approximately 20 times more common in microbial than in mammalian DNA due to differences in the utilization frequency and methylation pattern of CpG dinucleotides in prokaryotes versus eukaryotes (Bird 1980, 1987). Synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG ODN) mimic the activity of bacterial DNA (Yamamoto et al. 1992, Krieg et al. 1995, Ballas et al. 1996, Klinman et al.

1996). A possible molecular mechanism whereby bacterial DNA activates immune cells is revealed with the discovery of Toll-like receptor 9 (TLR9) in mice, a transmembrane receptor capable of recognizing unmethylated CpG oligonucleotides in bacterial DNA. The motifs are internalized into acidified endosomal vesicles (Hemmi et al. 2000), and released to trigger immune stimulation through a signaling cascade that involves the up-regulation of NF-κB (Krieg et al. 2000). Antigen presenting cells (APCs) triggered by CpG-ODN up-regulate their expression of co-receptor molecules and secrete a variety of cytokines, including IL-12, IL-6, IL-1 and TNF-α (Stacey et al. 1996, Chase et al. 1997, Lipford et al. 1997).

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To date, there is limited information concerning the biological effects of CpG ODN on fish. Kanellos et al. (1999) reported that plasmids co-injected with a recombinant protein potentiated antibody responses to the protein in goldfish *Carassius auratus*. Recently, Jørgensen et al. (2001a,b) demonstrated that plasmid DNA and synthetic CpG ODN induced production of IFN-like cytokine and IL-1b in Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* leucocytes. Oumouna et al. (2002) demonstrated activation of nonspecific cytotoxic cells of catfish *Ictalurus punctatus* with synthetic ODN and bacterial genomic DNA. Tassakka & Sakai (2002) reported that intraperitoneal injection of CpG ODN in carp *Cyprinus carpio* enhanced the nonspecific immune responses, including phagocytic and nitroblue tetrazolium (NBT) activity in kidney phagocytes and serum lysozyme activity. Meng et al. (2003) observed an increase of respiratory burst activity, acid phosphatase and bactericidal activity of grass carp (*Ctenopharyngodon idellus*) head-kidney macrophages by *in vitro* incubation with CpG ODN. However, the effect of CpG ODN on disease resistance in relation to enhanced non-specific immune responses has not been elucidated so far.

In the present study, we investigated *in vitro* and *in vivo* effects of synthetic CpG ODN on the respiratory burst activity of olive flounder (*Paralichthys olivaceus*) head-kidney phagocytes. In addition, the effect of intraperitoneal injection of CpG ODN on the disease resistance was examined by challenge with *Edwardsiella tarda*.

MATERIALS AND METHODS

Fish. In *in vitro* experiments, a total of 30 olive flounder *Paralichthys olivaceus* weighing 350 to 400 g, obtained from a local fish farm, were kept in 500 l fiberglass tanks containing filtered, aerated sea water at a temperature of $20 \pm 2^\circ\text{C}$. Fish were acclimated to these conditions for at least 2 wk before use, and fed commercial flounder pellets. In *in vivo* experiments, juvenile olive flounder, weighing 35 to 40 g, were obtained from a local fish farm. Fish were stocked into 10×50 l aquaria at a density of 12 fish per aquarium, and were acclimated 2 wk prior to initiating the experiment.

ODNs. Nuclease-resistant phosphoro-thioate ODNs were purchased from Bioneer. The sequence of CpG ODN 1668 was: 5'-TCCATGACGTTCCTGATGCT-3' (CpG motif is underlined). GpC ODN 1720 was synthesized by replacing a CpG dinucleotide in ODN 1668 with a GpC dinucleotide.

Isolation of head-kidney phagocytes. Fish were anaesthetized with tricaine methanesulfonate (MS222;

Sigma). The head kidney was extracted by ventral incision and transferred to L-15 medium (Sigma) supplemented with 2% foetal calf serum (FCS; Sigma), heparin (10 U ml^{-1} , Sigma), penicillin ($100 \mu\text{g ml}^{-1}$, Sigma) and streptomycin (100 U ml^{-1} , Sigma). To get leucocytes, the cell suspensions obtained by forcing the organ through a nylon mesh were layered over a 51% Percoll (Sigma). After centrifugation at $400 \times g$ for 30 min at 4°C , the leucocytes fraction was removed from the Percoll-medium interface, washed 3 times, counted and adjusted to 5×10^6 cells ml^{-1} . To get phagocytes, head-kidney cell suspensions, obtained as described above, were layered over a 34/51% Percoll density. After centrifugation at $400 \times g$ for 30 min at 4°C , the phagocyte-enriched interphase was collected and washed 3 times. Then, the cells were resuspended in culture medium, and dispensed into flat-bottomed 96-well plates. After 2 h at 20°C , wells were washed with culture medium to remove non-adherent cells. The remaining phagocytes were detached from the plates by incubating for 1 h at 4°C . Cell viability was examined with trypan blue exclusion and evaluated to be greater than 95%. The number of phagocytes were adjusted to 1×10^6 cells ml^{-1} .

Chemiluminescence (CL) assay. The reactive oxygen species (ROS) produced by stimulated phagocytes was quantified using an automatic photoluminometer (Bio-Orbit 1251). Each test cuvette contained 0.7 ml luminol (Sigma) made according to the method of Scott & Klesius (1981), 0.4 ml cell suspension, and 0.3 ml zymosan (Sigma), which was added just prior to measurement. The measurements were made for 1 h and the assay was carried out in triplicate.

Direct priming effects of CpG ODN on respiratory burst activity of phagocytes. Head kidney phagocytes (1×10^6 cells ml^{-1}), obtained as described above, were precultured for 6, 12 and 24 h with 0 (control), 1.25, 2.5, 5 and $10 \mu\text{g ml}^{-1}$ of CpG or GpC ODN at 20°C . Cells were then washed twice with Hanks' balanced salt solution (HBSS, Sigma) to remove any residual ODN and analyzed for ROS production by CL as described above.

Priming effects of supernatants produced from leucocytes pulsed with CpG ODN on respiratory burst activity of phagocytes. Five ml of the leucocytes suspension (5×10^6 cells ml^{-1}) were seeded into tissue culture flasks (25 cm^2), and were pulsed for 3 h at 20°C with 1.25, 2.5, 5.0 and $10.0 \mu\text{g ml}^{-1}$ of CpG or GpC ODN. After that, the cells were gently washed 3 times with HBSS to remove any residual ODN, and cultured in L-15 medium for 48 h. The supernatants were then harvested, centrifuged and stored at -70°C until use. A supernatant collected from leucocytes that had not been pulsed with ODN was used as a control. The supernatants were diluted to a 1:8 or

1:16 ratio with culture medium, and were added to the phagocytes monolayer in culture plates for 4, 8, 12 and 24 h at 20°C. At each time point, the cells were washed twice with HBSS and analyzed for ROS production by CL.

In vivo stimulating effects of CpG ODN on respiratory burst of phagocytes. Acclimated juvenile olive flounder were divided into 5 groups, each containing 12 fish. CpG and GpC ODN were diluted with HBSS and injected intraperitoneally (i.p.) at a dose of 0.25 or 0.5 $\mu\text{g fish}^{-1}$. Control fish received HBSS alone. Three fish of each group were sampled at 1, 3, 5 and 7 d after injection. Head kidney phagocytes were isolated as described above and analyzed for ROS production by CL.

Effects of CpG ODN on disease resistance. Acclimated juvenile olive flounder were divided into 5 groups, each containing 12 fish, and were treated i.p. with CpG or GpC ODN at a dose of 0.25 or 0.5 $\mu\text{g fish}^{-1}$. Control fish received HBSS alone. *Edwardsiella tarda* FSW 910410, isolated from an episode of edwardsiellosis of olive flounder on a local farm in Korea, was obtained from the Laboratory of Fish Diseases Prevention, Pukyong National University. To enhance the virulence, the bacteria had been passaged in 5 naive olive flounders by i.p. injection. The bacterium was reisolated from the kidney of moribund fish 3 d later and cultured on trypticase soy agar (TSA; Sigma) plates supplemented with 1.5% NaCl for 24 h at 27°C. The bacterial cell suspension was adjusted to 1.35 $\times 10^6$ colony forming units (cfu) l^{-1} . Fish were challenged by immersion into 50 l of the bacterial suspension for 30 min at 2 d post injection of either ODN or HBSS injection. Deaths were recorded over 15 d. Dead fish were collected daily and necropsied. Kidney samples were streaked on TSA to confirm the presence of *E. tarda*.

Statistical analysis. Statistical significance was evaluated using Student's *t*-test, and $p < 0.05$ was considered statistically significant.

RESULTS

Direct priming effects of CpG ODN on respiratory burst activity of phagocytes

Head-kidney phagocytes precultured with CpG ODN for 6, 12 and 24 h showed significantly higher CL responses than phagocytes precultured with GpC ODN or culture medium alone (control) at all concentrations (Fig. 1). Phagocytes primed with 10 $\mu\text{g ml}^{-1}$ of CpG ODN for 24 h showed significantly lower CL responses than those primed with lower concentrations of CpG ODN. Although phagocytes precultured

with GpC ODN for 6 h at all concentrations and for 12 or 24 h at 1.25 $\mu\text{g ml}^{-1}$ showed significantly higher CL responses than controls, the extent of CL enhancement was extremely low compared to CpG ODN.

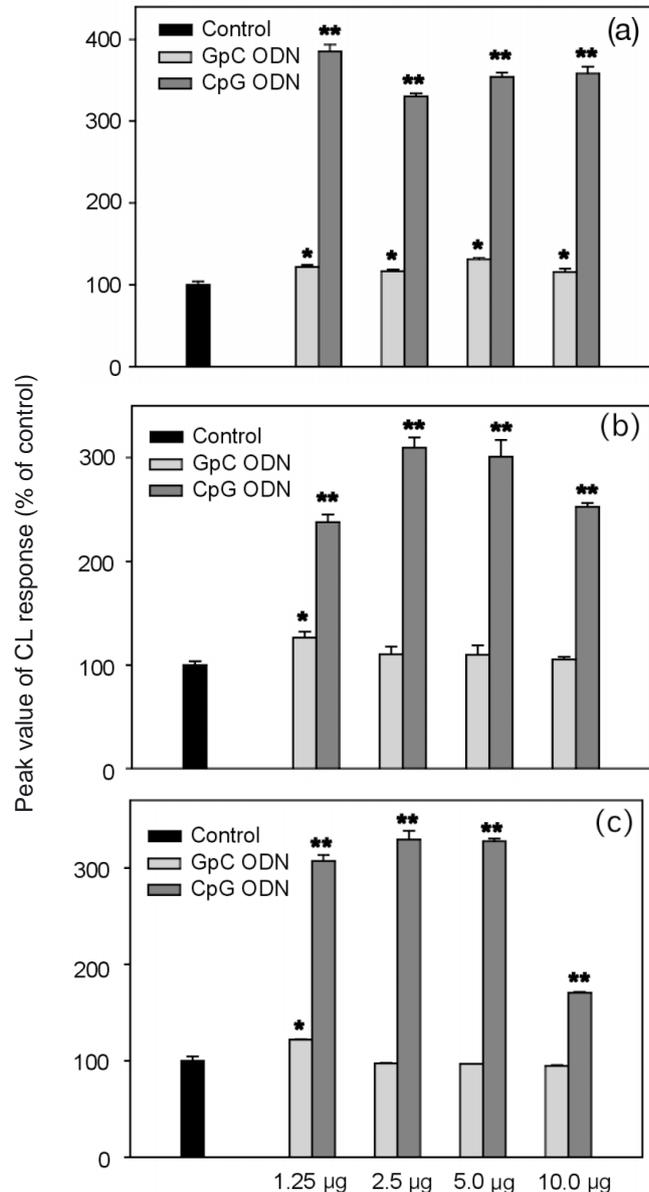


Fig. 1. *Paralichthys olivaceus*. Effects of synthetic oligodeoxynucleotides (ODN) on direct priming of respiratory burst activity of olive flounder head-kidney phagocytes. Phagocytes were precultured with various concentrations (1.25, 2.5, 5.0 and 10.0 $\mu\text{g ml}^{-1}$) of each ODN or medium alone (control) for (a) 6, (b) 12 and (c) 24 h, then analyzed for reactive oxygen species (ROS)-production by chemiluminescence (CL) assay. Results are means of triplicate samples \pm SD. *: Significantly different from control; **: significantly different from control and GpC ODN; $p < 0.05$. CpG: cytidine-phosphate-guanosine; GpC: guanosine-phosphate-cytidine

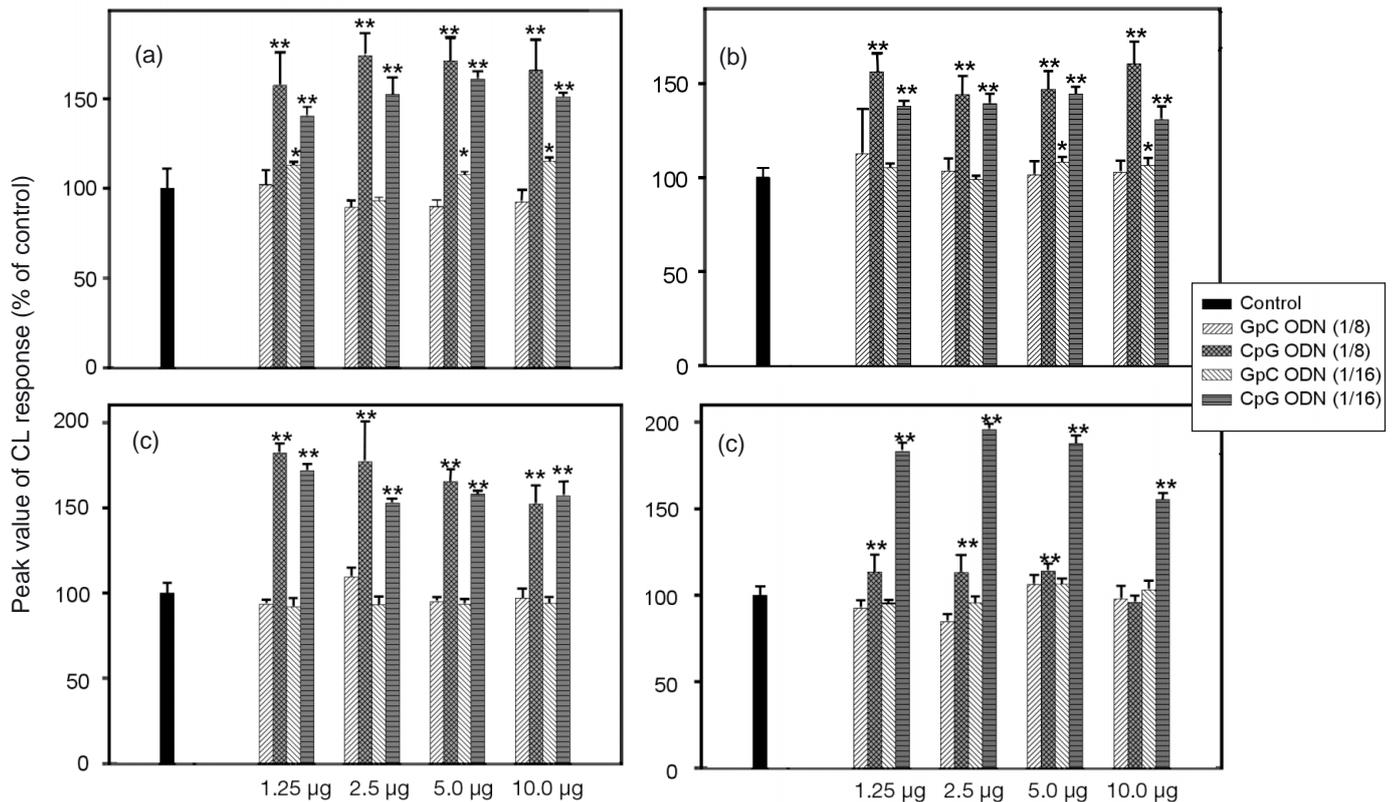


Fig. 2. *Paralichthys olivaceus*. Effects of supernatants (1:8 or 1:16 dilution) produced from leucocytes pre-primed with synthetic oligodeoxynucleotides (ODN) on the respiratory burst activity of olive flounder head-kidney phagocytes. Leucocytes were pulsed with various concentrations (1.25, 2.5, 5.0 and 10.0 $\mu\text{g ml}^{-1}$) of each ODN or medium alone (control) for 3 h, and then washed gently. Supernatants were harvested after incubation of the washed leucocytes for 48 h at 20°C. Target phagocytes were incubated with each diluted supernatant for (a) 4, (b) 8, (c) 12 and (d) 24 h, and then analyzed for reactive oxygen species (ROS)-production by chemiluminescence (CL) assay. Results are mean of triplicate samples +SD. *: Significantly different from control; **: significantly different from control and GpC ODN; $p < 0.05$

Priming effects of supernatants produced from leucocytes pulsed with CpG ODN on respiratory burst activity of phagocytes

Supernatants produced from leucocytes pulsed with various concentrations of CpG ODN increased CL responses of head-kidney phagocytes significantly when compared to control, irrespective of dilution ratio, except for phagocytes primed by a supernatant produced by 1:8 dilution of 10 $\mu\text{g ml}^{-1}$ for 24 h (Fig. 2). In contrast, supernatants produced by GpC ODN did not enhance significantly, or slightly, CL responses of phagocytes.

In vivo stimulating effects of CpG ODN on respiratory burst of phagocytes

Respiratory burst activities of head kidney phagocytes in the groups injected with either 0.25 or 0.5 μg of

CpG ODN were significantly higher than those in the groups injected with GpC ODN or HBSS (control) at 3, 5 and 7 d after injection (Fig. 3). The group of fish injected with GpC ODN 1720 showed no significant differences with control in CL responses.

Effects of CpG ODN on disease resistance

The groups of fish injected with 0.25 or 0.5 μg of CpG ODN showed higher survival rates (83.3%) than groups treated with GpC ODN (33.3%) and the control group (8.3%) after challenge with *Edwardsiella tarda* (Fig. 4). All dead fish were positive for *E. tarda*.

DISCUSSION

In this study, olive flounder head-kidney phagocytes precultured with various concentrations of CpG ODN

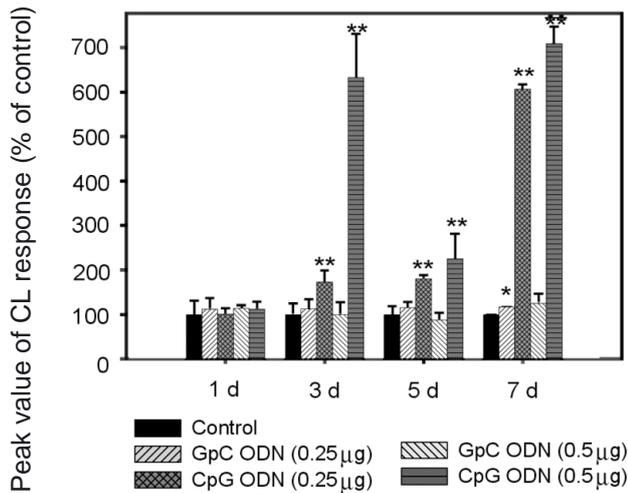


Fig. 3. *Paralichthys olivaceus*. Respiratory burst activity of head-kidney phagocytes of olive flounder injected intraperitoneally with 0.25 or 0.5 ml of synthetic oligodeoxynucleotides (ODN) or HBSS alone (control). The respiratory burst activity was measured by chemiluminescent response (CL) and analyzed at 1, 3, 5 and 7 d after injection. Values are mean of triplicate samples + SD. *: Significantly different from control; **: significantly different from control and GpC ODN; $p < 0.05$

for certain times (6, 12 and 24 h) showed significantly higher CL responses than phagocytes precultured with GpC ODN or with medium alone. This result suggests that synthetic ODN containing the CpG motif can enhance respiratory burst activity of fish phagocytes, and this priming effect is not by nonspecific unmethylated DNA but by ODN containing the CpG motif. As the phagocytes were isolated by 2-step manipulation including Percoll gradient centrifugation and plate adherence, phagocytes of fish are considered as effective target cells to respond to CpG ODN. In mammals, macrophages are involved in the production of Th1 cytokines as well as antigen processing/presentation, and thus play an important role in the response elicited by CpG DNA (Stacey et al. 1996, Sparwasser et al. 1997a,b). In fish, Jørgensen et al. (2001a,b) demonstrated that the adherent cell population (macrophages were the dominant cell type) was the main source for CpG ODN-mediated expression of IFN-like cytokines and IL-1b. The CpG ODN 1668 used in this study has been shown to stimulate immune responses not only in mammals (Krieg et al. 1995, Lipford et al. 1997, Sparwasser et al. 1998, Yi & Krieg 1998) but also in fish (Jørgensen et al. 2001a,b). Therefore, it can be considered that CpG ODN 1668 has an immunostimulating effect over a wide variety of vertebrates.

The present results demonstrate that olive flounder head-kidney leucocytes release a soluble phagocyte-

activating factor(s) able to enhance the respiratory burst activity of target phagocytes. Supernatants produced from leucocytes, which were pulsed with CpG ODN for 3 h and subsequently cultured for 48 h with culture medium alone, induced significantly higher head-kidney phagocyte respiratory-burst activity than supernatants produced by GpC ODN or culture medium alone (control). Although there are several features that distinguish the CpG motifs' activation of immune cells from mitogen activation, the stimulating effect of CpG ODN in this study was similar to that of macrophage activating factor (MAF), which is produced by various mitogens, in that it primes the respiratory burst response of phagocytes after stimulation (Nagakawara et al. 1982, Graham & Secombes 1988, 1990a,b).

In the present *in vitro* experiments, the highest concentration ($10 \mu\text{g ml}^{-1}$) of CpG ODN showed relatively lower phagocyte-activating activities compared to lower concentrations. Klinman et al. (1996) reported that the production of cytokines, including IL-12 and IFN-g from murine leucocytes, induced by CpG ODNs was higher at a concentration 0.10 to $0.33 \mu\text{g ml}^{-1}$ than that at $3.0 \mu\text{g ml}^{-1}$. Additionally, it has been demonstrated that MAF supernatants often show inhibitory effects on macrophage respiratory burst activity, possibly due to the presence of suppressive factors (Graham & Secombes 1990a,b, Mulero & Meseguer 1998).

In this study, groups of fish injected with a CpG ODN showed significantly higher respiratory burst activity of phagocytes. Similarly Tassakka & Sakai (2002) reported that carp *Cyprinus carpio* injected intraperitoneally with CpG ODN showed significantly higher nitroblue tetrazolium (NBT) activity in kidney phagocytes.

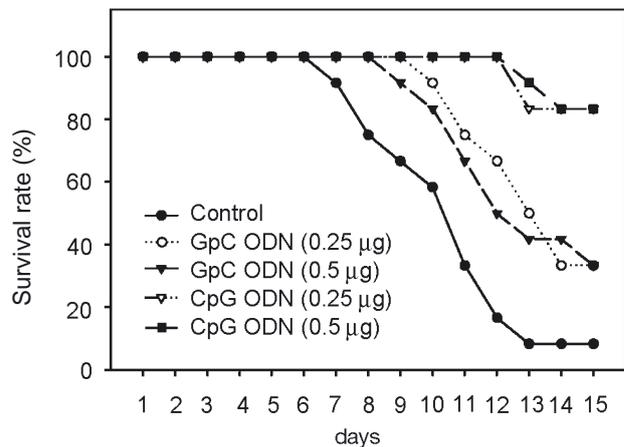


Fig. 4. *Paralichthys olivaceus*. Survival rate of olive flounder injected intraperitoneally with 0.25 or 0.5 ml of synthetic oligodeoxynucleotides (ODN) or HBSS alone (control) after challenge with *Edwardsiella tarda*

The rapid induction of an innate immune response is critical in controlling the early spread of intracellular pathogens. Although previous studies showed that CpG ODNs have significant *in vitro* biological activity, the *in vivo* significance of these effects has been unclear. The present results demonstrated that treatment of fish with DNA containing the CpG motif confers protection against lethal bacterial infection. Fish treated with a CpG ODN showed considerably higher survival rates than fish in control and GpC ODN-injected groups against *Edwardsiella tarda* challenge. Thus, the stimulation of innate protective immunity by host recognition of a synthetic ODN-containing CpG motif would contribute to the higher survival rate.

In conclusion, the present *in vitro* and *in vivo* experiments have demonstrated the ability of synthetic CpG ODN to increase phagocytes' respiratory burst activity and disease resistance in olive flounder *Paralichthys olivaceus*.

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