

Infection and pathogenicity of *Myroides odoratimimus* (NIOCR-12) isolated from the gut of grey mullet (*Mugil cephalus* (Linnaeus, 1758))

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Abstract

Myroides sp. are gram negative aerobes and known for its opportunistic pathogenicity in humans. In the present study, *Myroides odoratimimus* isolated from the gut of *Mugil cephalus* showed potential infectivity to the experimental grey mullet and acted as an ultimate pathogen with significant symptoms. Furthermore, the inoculum isolated from the infected fishes were cultured and the selected colonies were reisolated and reinjected into healthy juveniles of *Mugil cephalus*. Characterizations of the re-isolated bacteria were the same as those of the isolated *M. odoratimimus* from naturally infected mullet. The median lethal dose (LD₅₀) of the bacteria was 3×10^6 CFU fish⁻¹. In order to assay the accuracy of infection, the production of reactive oxygen species (ROS), the respiratory burst activity of blood leukocytes of mullet before and after challenge was measured, an indicator of the innate immune system. The mullet infection increased the respiratory burst activity and superoxide dismutase activity. In addition, the innate immune response of TLR 9 expression against *M. odoratimimus* infection and CpG ODN treatment in disease model, zebrafish confirms the *M. odoratimimus* infection and pathogenicity.

Key words: innate immunity, Infection, *Myroides*, *Mugil cephalus*, Pathogen, zebrafish

1. Introduction

The genus *Myroides*, belongs to the family flavobacteriaceae [1] and its species have been found in various terrestrial and aquatic environments, and also as causative agents in various infections. *Myroides* comprises of two medically relevant species *Myroides odoratus* and *Myroides odoratimimus* [1]. Most strains of *M. odoratimimus* have been isolated from clinical specimens [2]. It has been recognized as an important human pathogen because of its ability to develop resistance to antibiotics [3] and an opportunistic pathogen in humans [4]. Further, *Myroides* spp. has also been reported in wet environments [3], insect guts [5] and sea water [6, 7]. Previously, *M. odoratus* was isolated from tilapia fish [8]. However, there is not a single report of isolation as well as experimental studies on *M. odoratimimus* infection and pathogenicity from fish species.

Fish gastrointestinal tract constitutes one of the major infection routes like the skin and the gills and allows a favorable site for bacteria proliferation. Thus, during the course of grey mullet gut microbiota studies, we isolated a *Myroides* sp. collected from government fish farm, Old Goa, India and characterized by various genotypic and biochemical methods. We experimentally infected *Mugil cephalus* with the *M. odoratimimus* isolate and re-isolated *M. odoratimimus* from the liver, muscle and gut of moribund fish. The *M. odoratimimus* infection and pathogenicity was confirmed with that of the innate immunity indicator tests such as respiratory burst activity, Super oxide dismutase (EC1.11.1.6) activity and as well as the CpG ODNs effects with that of the challenged fish. In addition we also used the disease model, Zebrafish for the infection and immune responses. Thus, this study infers multi-resistant *M. odoratimimus* strains may cause diseases that leads to mortality in aquaculture fish species.

2. Materials and Methods

2.1 Isolation and identification of pathogenic bacteria using biochemical and molecular assay

Fish (*M. cephalus*) was anaesthetized using clove oil (Merck) and dissected using the dissection kit under sterile condition for the gut regions (Fig. 1A, B). In order to retrieve the bacteria associated with the inner walls of the intestine, 2 mL of sterilized 0.9 % saline was gently flushed through the intestine from the anterior to posterior end with a syringe. Eluted saline sample was directly plated on nutrient agar (NA), Zobell marine agar (ZMA) and tryptic soy agar (TSA) (HiMedia, Mumbai, India) and pure bacterial strains were isolated. Biochemical tests like gram staining, motility, catalase, oxidase, all have been carried out as per the standard microbiological protocol suggested by the supplier (HiMedia, Mumbai, India). Isolates were subjected to antibiotic sensitivity tests, using

the disc diffusion method. Antibiotic discs (HiMedia, Mumbai, India) were placed on the seeded surfaces of Mueller-Hinton agar (HiMedia, Mumbai, India) plates.

Genomic DNA was extracted from the gut isolated strains using DNeasy Blood and Tissue kit (QIAGEN) according to manufacturer's instructions. PCR amplification of 16S rRNA gene and sequencing was performed as described earlier [9] The gene sequence (819 nt) was subjected to BLASTn on the EzTaxon 2.1 server [10]. The sequence was aligned using Clustal W alignment program [11] and phylogenetic tree was established with neighbour-joining [12] algorithm using MEGA 5.0 software [13] with bootstrap analysis based on 1,000 replications [14]. The Gen Bank accession number was KJ789856.

2.2. Reisolation of bacteria and Lethal dose (LD₅₀) test

The bacterial suspension was adjusted to 1.3×10^7 cfu/ml by plate counting. The suspension was then diluted into 1.3×10^3 , 1.3×10^4 , 1.3×10^5 , 1.3×10^6 , 1.3×10^7 cfu/ml with normal saline, respectively. The challenge tests for healthy fish against pathogen were performed using 6 groups of fish. Each group consisted of 10 fish weighing 5.5-7 g reared in an aquarium with a water temperature of 28 °C. Experimental fish were injected intraperitoneally (i.p.) with 0.5 ml of a bacterial suspension at a density ranging from 1.34×10^3 to $1.3^4 \times 10^7$ cfu/ml, respectively. Fish of the control group were injected i.p. with 0.5 ml of filter-sterilized saline. Salinity and temperature of the water were maintained at 28 ppt and at 28°C, respectively, throughout the experimental period. The fish were observed for lethargy, lack of feeding and mortality for 30 days from the day of bacterial administration. Samples of the liver, muscle and gut region were analysed. The fish were considered infected if only *M. odoratimimus* was recovered from assayed fish. The recovery or reisolation of bacteria was performed with moribund and dead fish of each group. The predominant bacterial growth was identified in accordance with plate culture, PCR and also by morphological, physiological, and biochemical characteristics. The LC₅₀, was calculated by the method of Reed and Muench [15].

2.3. Leukocytes Respiratory burst activity test

Blood was collected from the seven day old challenged fish and about 100 µL of blood was added to 100 µL of 0.2 % nitroblue tetrazolium solution (NBT, Himedia, India) and the final solution was homogenised and incubated for 30 minutes at 25 °C. The NBT solution was prepared in phosphate buffered saline (PBS), pH 7.4. After incubation and homogenisation, 50 µL from the solution were added to 1 ml of N, N-dimethyl formamide (DMF, FINAR, India) in a glass tube. This new solution was homogenised and centrifuged at 3000 g for 5 minutes. The optical density (OD) of supernatant

was determined on spectrophotometer at 540 nm. The blank consisted of the same components and steps except blood that was exchanged with distilled water.

2.4. Super oxide dismutase (SOD) activity assay

Superoxide dismutase (SOD) (EC1.11.1.6) activity of SOD in the gels was visualized by the activity staining procedure as described earlier [16]. The cell grounded intracellular extracts subjected with control and challenged fish were resolved on 12 % and 10 % non-denaturing PAGE in Tris-glycine buffer. The resolved gel was incubated in staining buffer (50 mmol/L potassium phosphate buffer, pH 7.8, 0.1 mmol/L EDTA, 28 mmol/L TEMED, 0.003 mmol/L riboflavin and 0.25 mmol/L NBT) for 30 min in the dark at room temperature. Gels were then exposed under two fluorescent tubes until the white SOD activity bands became visible.

2.2. Effects of CpG ODN in the respiratory burst and SOD activity

Nuclease-resistant phosphoro-thioate ODNs were purchased from Sigma, India. The sequence of CpG ODN 1668 was: 5'-TCCATGACGTTCCTGATGCT-3' (CpG motif is underlined). GpC-ODN 1668 (5'-TCCATGAGCTTCCTGATGCT-3') by replacing a CpG dinucleotide in ODN 1668 with a GpC dinucleotide [17]. ODNs (3 μ M) were injected after seven days of infection of bacterial cells and tested for their effects after 24 hours in respiratory burst activity and as well as SOD activity.

2.3. *M. odoratimimus* infection, RNA isolation and quantitative RT-PCR analysis in zebrafish (*Danio rerio*).

Young adult male zebrafish was infected with *M. odoratimimus* as described above and after seven days of infection, the CpG ODN (3 μ M) was treated for 24 hours and sampled for RNA extraction. Total RNA was extracted from the healthy control fish, bacterial challenged fish, bacterial challenged fish and CpG ODN 1668 treated fish, and only GpC ODN 1668 treated zebrafish. Total RNA contents were extracted from zebrafish using Tri Reagent according to the manufacturer's protocols (Sigma-Aldrich, USA). The RNA preparations were treated with ribonuclease-free deoxyribonuclease (Dnase) I (Invitrogen) to remove residual genomic DNA. Total RNAs were then subjected to quantitative RT-PCR for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primer for 1 h at 50 °C. Thereafter, equal amounts of cDNA products were amplified using the primers, beta actin (forward, 5'- CTC TTC CAG CCT TCC TTC CT -3'; reverse, 5'- GGT TGG TTC GTT CGT TTG AAT-3') [18] ; zTLR 9 F 5'- GGG GAA GAT GGC GCT TTC AG-3'; zTLR9 R 5'-CCA GAA GAG CGG CTG CAC TC-3') [19] with the required cycle numbers at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The intensities of the relative bands were measured using Image J software.

3. Results and Discussion

3.1 Isolation and identification of *Myroides odoratimimus*

Bacterial colonies of identical morphotype were collected from the gut of the moribund fish (Fig. 1A, B). The isolates thus obtained grew on nutrient agar as typical flavobacteria, being aerobic and yellow pigmented with fruity odor. Colonies were observed in MacConkey agar and TSA plates and there was no growth on TCBS agar plates. The colonies were yellowish orange, circular and convex with entire margins on marine agar. The biochemical tests revealed that they were Gram-negative non motile rods, catalase and oxidase positive. They were negative for H₂S production. Amplified 16S rRNA gene from this isolate was sequenced and blast analyzed. The 16S rRNA gene sequence obtained from the strain NIOCR-12 was found to be closely related to *Myroides odoratimimus* with high sequence similarity. Neighbour-joining phylogenetic analysis also confirms that the strain NIOCR-12 formed a phylogenetic lineage with *M. odoratimimus* (Fig. 2).

Myroides sp. are well known for its broad range of antibiotic resistance [3] thus, we analysed antimicrobial susceptibility test for the strain NIOCR-12. *M. odoratimimus*, (NIOCR-12), in the present case, showed wide resistance to the antibiotics such as β -lactams (ampicillin, cefadroxil, cefoperazone, ceftazidime, ceftriaxone), aminoglycosides (streptomycin, amikacin, netilmicin, gentamicin), Tetracycline's (Tetracycline) and some quinolones (levofloxacin). Whereas, isolate was sensitive to antimicrobials like gatifloxacin, vancomycin, ofloxacin, sparfloxacin, azithromycin, chloramphenicol, nitrofurantoin, norfloxacin (Table 1). These antimicrobial susceptibility test results of strain NIOCR-12 agrees with that of the earlier reports of *M. odoratimimus* [20, 21, 22, 23] (Table 1). Furthermore, while comparing the above results with that of the earlier reports we found antibiotic, chloramphenicol was distinctively sensitive with that of *M. odoratimimus* strains but resistant with that of *M. odoratum* species [3] (Table 1). Thus, chloramphenicol may be used to distinguish the two strains of *M. odoratimimus* and *M. odoratum*. Resistance to the above mentioned antibiotics by the isolate may be due to the presence of some virulence genes or involvement of secretion machinery of multi drug efflux proteins [21]. It has also been shown that resistance to β -lactams antibiotics is due to the production of chromosome-encoded metallo- β -lactamases (TUS-1 and MUS-1) [24]. However, the absence of metallo- β -lactamases (TUS-1 and MUS-1) genes in *Myroides* spp. have also been reported for their antibiotic resistance because of their intrinsic resistance mechanisms [21].

3.2. *M. odoratimimus* infection, reisolation and lethal dose

Infectivity assays showed abnormalities in fish behavioral characteristics by 1-2 days post inoculation (dpi) with displayed anorexic and head up swimming with rapid breathing. During 3-5 dpi the moribund fish was observed for swimming close to the surface or stationary at bottom and lethargic activity. Skin hemorrhage and reddening were found at the abdomen, mouth and pectoral fin regions at the initial stages of infection. At the advance stage of infection, fishes were found with symptoms of external black colour patches on the skin and also with eye hemorrhages. *M. odoratimimus* was recovered from all target organs liver, muscle and gut of all diseased mullet performed, while, the bacteria was not isolated from any control fish. To confirm the reproducibility of the disease in host fish, a challenge test was performed, and a positive result was obtained in the experimentally inoculated healthy grey mullet. Results are listed in table 2. Observations of challenged fish were extended to 37 days. When mullet were experimentally injected with 1.3×10^7 cfu/ml of bacteria, a total of 7 fish died. In addition, about five and three fishes died in the groups 1.3×10^6 and 1.3×10^5 cfu/ml respectively. Of the remaining groups, fish survived when concentrations of 1.3×10^3 , 1.3×10^4 was administered into the fish. Thus, the reisolated *M. odoratimimus* established the bacteria as ultimate pathogenic for grey mullet. The LC_{50} of the isolated *M. odoratimimus* to experimental grey mullet was 1.3×10^6 CFU fish⁻¹ (Table 2), which indicates the virulence of bacteria. Characterizations of the re-isolated bacteria were the same as those of the isolated *M. odoratimimus* from naturally infected mullet. Clinical symptoms and histopathological changes of the experimental fish induced by bacterial infection were similar to those of naturally infected fish (data not shown).

3.3. Leucocytes respiratory burst test in mullet challenged with *M. odoratimimus*.

The respiratory burst activity of phagocytes has been used frequently as an indicator of nonspecific immunity in fish against the pathogenic infections. Superoxide anion radical (O_2^-), a highly toxic reactive oxygen species (ROSs) are released during the phagocytosis process and act as a potent antibacterial system was analyzed in this study. Thus, we used the NBT assay method were the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anions. Samples were analyzed with two groups of fishes, one with before challenge and the other with after challenge. The leukocyte respiratory burst activity increased after the pathogen injection (Fig. 3), indicating that phagocytes enhanced the production of ROSs by activating the NADPH oxidase enzyme that produces oxygen peroxide. Thus, mullet challenged by *M. odoratimimus* have shown improvement in ROSs production and increased protection against pathogen. This activation of immune mechanisms of an important function in preventing disease in fish have been evaluated in

several studies of fish responses after pathogen infection. Furthermore, the present results demonstrated that treatment of fish with DNA containing the CpG motif confers protection against lethal bacterial infection by increasing the respiratory burst activity. Thus, fish treated with a CpG ODN showed considerably higher survival rates than fish in control and GpC ODN injected groups against *M. odoratimimus* challenge (data not shown). The results of effects of CpG ODN 1668 in fish of the present study was similar with that of previous studies [25, 26]. Thus, the stimulation of innate protective immunity by host recognition of a synthetic ODN-containing CpG motif would contribute to the higher survival rate [26].

3.4. SOD activity test in mullet challenged with *M. odoratimimus*

The high levels of ROSs compounds are remarkably toxic to both microorganisms and host cells since its action is usually nonspecific. Therefore, detoxification of residual ROS is performed by the host using antioxidant enzymes to protect itself from cell damage. Superoxide dismutase (EC1.11.1.6) enzymes scavenge radicals by catalyzing the conversion of radicals into hydrogen peroxide and molecular oxygen, thus being involved in the cellular defense strategies against oxidative damage by ROS [16]. Thus, we performed the SOD activity using the activity staining assay in this study. It was observed that a significant induction of SOD activity occurred at 7 h in post challenge (Fig. 4) than the uninfected one. The SOD electrophoretic profile showed two bands of the isoforms of Cu/zn SOD and Mn-SOD (Fig.4). Our results exhibited the similar expression pattern with that of several studies on infected fish and in vertebrates. Moreover, the challenged fish treated with a CpG ODN showed increased level of SOD when compared with that of the negative control, GpC ODN and uninfected, positive control (Fig.4). Thus, the superoxide generation against the *M. odoratimimus* infection and the detoxification SOD expression in post challenge provides the strong evidence of the pathogen, *M. odoratimimus* infection in mullet.

3.5. *M. odoratimimus* infection and innate immune response in zebrafish

It is well known that CpG-ODNs is a ligand of *TLR9* and induces the *TLR9* signaling pathway in a variety of cells. To examine whether *M. odoratimimus* infection and CpG-ODNs induced expression of *zTLR9*, we treated zebrafish with injecting *M. odoratimimus* and CpG ODN's intraperitoneally. Our results showed that the fish Toll like receptor 9 (*TLR 9*), receptor of innate immune system was triggered by the bacterial infection and CpG ODN 1668 (Fig.5). To investigate if the expression of *TLR9* genes was responsive to microbial infection, we compared expression levels between fish infected with only *M. odoratimimus*, *M. odoratimimus* and CpG ODN, and only healthy control fish (Fig. 5). RT-PCR analysis demonstrated that the *TLR9* genes were expressed at higher levels in the infected fish and CpG ODN treatment fish when compared with that of the controls (Fig. 5). The

results was similar with that of the *zTLR9* expression against the *Mycobacterium marinum* infection in zebrafish [19]. Thus, *M. odoratimimus*, the opportunistic human pathogen was demonstrated for its infection in zebrafish, which has close homology with that of the mammalian systems.

3.4. Conclusion

M. cephalus is cosmopolitan in the coastal waters of most tropical and subtropical zones and they are also known for catadromous, that is, they frequently found coastally in estuaries and freshwater environments. Furthermore, mullet is a diurnal feeder, consuming mainly zooplankton, dead plant matter and detritus, elucidating that the feed habit of fish may be an important factor for the entry of the *M. odoratimimus*.

Further, from the present study, it is clear that the *M. odoratimimus* is a serious pathogen with significant symptoms for grey mullet. To our knowledge this is the first description of *M. odoratimimus* infecting fish, grey mullet and as well as zebrafish. Thus, human enteric bacteria are able to infect fish, and also it is possible that fish may act as an intermediate vector in transferring this pathogenic bacterium back to humans. We also propose that the antibiotics like gatifloxacin, vancomycin, ofloxacin, sparfloxacin, azithromycin, chloramphenicol, norfloxacin may be used for treating the *Myroides* infections. Moreover, the present *in vivo* experiments have demonstrated the ability of synthetic CpG ODN 1668 to increase phagocytes respiratory burst activity and disease resistance in mullet and zebrafish.

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References

- Vancanneyt M, Segers P, Torck U, Hoste B, Bernardet JF, Vandamme P, Kersters K. Reclassification of *Flavobacterium odoratum* (Stutzer 1929) strains to a new genus, *Myroides*, as *Myroides odoratus* comb. nov. and *Myroides odoratimimus* sp. nov. Int J Syst Bacteriol. 1996;46:926–932.
- Holmes B, Snell JJS, Lapage SP. Revised description, from clinical isolates, of *Flavobacterium odoratum* Stutzer and Kwaschnina 1929, and designation of the neotype strain. Int J Syst Bacteriol. 1977;27:330–336.
- Holmes B, Snell JJS, Lapage SP. *Flavobacterium odoratum*: a species resistant to a wide range of antimicrobial agents. J Clin Pathol. 1979;32:73-77.
- Benedetti P, Rassu M, Pavan G, Sefton A, Pellizzer G. Septic shock, pneumonia, and soft tissue infection due to *Myroides odoratimimus*: report of a case and review of *Myroides* infections. Infection 2011;39:161-165.

Spiteller D, Dettner K, Bolan W. Gut bacteria may be involved in interactions between plants, herbivores and their predators: microbial biosynthesis of N-acylglutamine surfactants as elicitors of plant volatiles. *Biol Chem*. 2000;381:755-762.

Maneerat S, Bamba T, Harada K, Kobayashi A, Yamada H, Kawai F. A novel crude oil emulsifier excreted in the culture supernatant of a marine bacterium, *Myroides* sp. strain SM1. *Appl Microbiol Biotechnol*. 2006;70:254-259.

Yoon J, Maneerat S, Kawai F, Yokota A. *Myroides pelagicus* sp. nov., isolated from seawater in Thailand. *Int J syst Evol Microbiol*. 2006;56:1917-1920.

Jacobs A, Chenia HY. Biofilm-forming capacity, surface hydrophobicity and aggregation characteristics of *Myroides odoratus* isolated from South African *Oreochromis mossambicus* fish. *Appl Microbiol*. 2009;107:1957-1966.

Mohandass C, Rajasabapathy R, Ravindran C, Colaco A, Santos RS, Meena R. Bacterial diversity and their adaptations in the shallow water hydrothermal vent at D. João de Castro Seamount (DJCS), Azores, Portugal. *Cah Biol Mar*. 2011;53:65-76.

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. *Int J syst Evol Microbiol*. 2012;62:716-721.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 1994;22:4673-4680.

Saitou N, Nei M. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987;4:406-425.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 2011;28:2731-2739.

Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783-789.

Reed LJ, Muench H. A simple method of estimating fifty percent end points. *American J Hyg*. 1938;27:493-497.

Ravindran C, Varatharajan GR, Rajasabapathy R, Vijayakanth S, HarishKumar A, Meena RM. A role for antioxidants in acclimation of marine derived pathogenic fungus (NIOCC 1) to salt stress. *Micro Pathogenesis* 2012;53:168-179.

Ravindran C, Cheng YC, Liang SM. CpG-ODNs induces up-regulated expression of chemokine CCL9 in mouse macrophages and microglia. *Cell Immunol* 2010;260:113-118.

Jault C, Pichon L, Chluba J. Toll-like receptor gene family and TIR-domain adaptors in *Danio rerio*. *Mol Immunol* 2003;40:759-771.

Meijer AH, Krens SFG, Rodrigues IAM, He S, Bitter W, Snaar-Jagalska BE, Spaank HP. Expression analysis of the toll-like receptor and TIR domain adaptor families of zebrafish. *Mol Immunol*. 2004;40:773-783.

Yagci A, Çerikçioglu N, Kaufmann ME, Malnick H, Söyletir G, Babacan F, Pitt T.L. Molecular typing of *Myroides odoratimimus* (*Flavobacterium odoratum*) urinary tract infections in a Turkish hospital. *Eur J Clin Microbiol Infect Dis*. 2000;19:731–732.

Dharme MS, Gupta AK, Rangrez AY, Ghate HV, Patole MS, Shouche YS. Antibacterial activities of multidrug resistant *Myroides odoratimimus* bacteria isolated from adult flesh flies (Diptera: Sarcophagidae) are independent of metallo beta-lactamase gene. *Braz J Microbiol*. 2008;39:397-404.

Marakia S, Sarchianaki E, Barbagadakis S. *Myroides odoratimimus* soft tissue infection in an immunocompetent child following a pig bite: case report and literature review. *Braz J infect dis*. 2012;16:390–392.

Suganthi R, Shanmugapriya T, Saranya A, Kaleeswaran T. Relationship between plasmid occurrence and antibiotic resistance in *Myroides odoratimimus* SKS05-GRD isolated from raw chicken meat. *W J Microbiol Biotechnol*. 2013;29:983–990.

Mammeri H, Bellais S, Nordmann P. Chromosome-encoded β -lactamases TUS-1 and MUS-1 from *Myroides odoratus* and *Myroides odoratimimus* (formerly *Flavobacterium odoratus*), new members of the lineage of molecular subclass B1 metalloenzymes. *Antimicrob Agents and Chemother*. 2002;46:3561–7.

Jørgensen JB, Johansen A, Stenersen B, Sommer AI. CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar* L.) leucocytes to produce supernatants with antiviral activity. *Develop Comp Immunol*. 2001;25:313–321

Lee CH, Jeong HD, Chung JK, Lee HH, Kim KH. CpG motif in synthetic ODN primes respiratory burst of olive flounder *Paralichthys olivaceus* phagocytes and enhances protection against *Edwardsiella tarda*. *Dis Aquat Organ*. 2003;56:43–48.

Figure legends

Fig.1. A. Fish (*Mugil cephalus*) was anaesthetized for dissecting the gut region. **B.** The fish was dissected using the dissection kit under sterile condition for the gut regions. The obtained gut region was kept under sterile condition for the isolation of gut microbiota, where *M. odartimimus* was isolated and re-isolated. **C.** Control fish without any infection. **D.** Infected fish, arrow indicates fish with symptoms of reddening and skin hemorrhage due to infection.

Fig.2. Neighbour-joining tree showing the phylogenetic relationship of strain NIOCR-12 and phylogenetically related species (retrieved from EzTaxon database) based on 16S rRNA gene sequences. Bootstrap percentages (1,000 replications) are shown at branching points and the values >50 % are shown. *Sphingobacterium spiritivorum* DSM 2582 (AJ459411) was used as an out group. Scale bar 0.02 substitutions per nucleotide position.

Fig. 3. Leukocytes respiratory burst activity of grey mullet (*Mughil cephalus*) before and after challenge of *Myroides odartimimus* with treatment of immunostimulant CpG ODN and negative control GpC ODN. Data represent mean \pm SEM (n = 3).

Fig. 4. a). Superoxide dismutase (SOD) expression in grey mullet (*Mughil cephalus*) before challenge (a) and after challenge (b), CpG ODN treatment with challenge and d) GpC ODN treatment with challenge. The upper band represents the Cu-Zn SOD and the lower band for Mn-Zn SOD. **B).** Cu-zn SOD protein expression levels were quantified using Image J software to estimate the relative band intensities. Data represent mean \pm SEM (n = 3).

Fig. 5. A). TLR 9 expression in zebrafish before challenge (a), after challenge (b), CpG ODN treatment with challenge and d) GpC ODN treatment with challenge. **B).** Expression levels were quantified using Image J software to estimate the relative band intensities. GpC-ODN was used as the negative control. β -actin was used as an internal control. Data represent mean \pm SEM (n = 3).

Table 1 –Comparison of *In vitro* susceptibility of the *Myroides odoratimimus* isolate.

Antibiotics	<i>M. odoratimimus</i>						<i>M. odoratus</i>	
	Yagici et al. 2000	Maraki et al. 2012	Mammeri et al. 2002	Suganthi et al. 2013	Dharne et al. 2008	Present study	Holmes et al. 1979	Motwani et al. 2004
Ticarcillin	-	R	-	-	-	-	-	-
Piperacillin	R	R	R	R	-	-	-	-
Imipenem	R	I	R	R	-	-	-	R
Amoxicillin/clavulanic acid	-	S	R	-	-	-	-	-
Piperacillin/tazobactam	-	R	S	-	-	-	-	R
Cefuroxime	-	R	R	-	-	-	-	-
Cefotaxime	-	R	-	R	-	-	-	R
Ceftriaxone	R	R	R	R	R	R	-	-
Ceftazidime	R	R	-	R	R	R	-	R
Cefoperazone	R	-	-	R	R	R	-	-
Ampicillin	-	-	-	R	R	R	R	R
Netillin	-	-	-	R	R	R	-	-
Cefazolin	-	-	-	-	-	-	-	R
Cefotetan	-	-	-	-	-	-	-	R
Cefadroxil	-	-	-	R	R	R	-	-
Cefepime	-	-	R	-	-	-	-	R
Aztreonam	R	-	R	-	-	-	-	-
Streptomycin	-	-	-	R	R	R	R	-
Kanamycin	-	-	-	R	R	-	-	-
Tobramycin	R	-	-	-	-	-	R	R
Netilmicin	-	R	R	-	-	-	-	-
Amikacin	R	R	-	R	R	R	R	R
Gentamicin	R	R	-	R	R	R	R	R
Trimethoprim/sulfamethoxazole / Cotrimoxazole	R	S	-	-	-	-	I	S
Doxycycline hydrochloride	-	S	-	-	S	R	-	-
Tetracycline	R	R	-	I	S	R	R	-
Azithromycin	-	S	-	-	S	-	-	-
Vancomycin	-	S	-	-	S	S	-	-
Nitrofurantoin	-	S	-	-	S	S	-	-
Norfloxacin	-	S	-	-	S	-	-	-
Sparfloxacin	-	S	-	R	S	-	-	-
Ciprofloxacin	R	S	-	R	S	S	-	S
Levofloxacin	-	S	-	-	-	-	-	S
Moxifloxacin	-	S	-	-	-	-	-	-
Ofloxacin	-	S	-	-	S	S	-	-
Gatifloxacin	-	S	-	-	S	-	-	-
Chloramphenicol	-	S	-	S	S	S	R	R

S, susceptible; I, intermediate; R, resistant

Table 2 –LD₅₀ test and mortality rate of experimentally infected fish with reisolated *M. odartimimus*

Group	Pathogen concentration per fish	Total number of tested fish	Dead fish after 7 dpi (days)										No. of dead fish	Mortality rate %
			1	2	3	4	5	10	15	20	25	30		
1	1.3×10^7 (0.5 ml)	10	0	0	0	0	1	1	2	1	2	0	7	70
2	1.3×10^6 (0.5 ml)	10	0	0	0	0	0	1	1	2	1	0	5	50
3	1.3×10^5 (0.5 ml)	10	0	0	0	0	0	0	0	1	1	1	3	30
4	1.3×10^4 (0.5 ml)	10	0	0	0	0	0	0	0	0	0	0	0	0
5	1.3×10^3 (0.5 ml)	10	0	0	0	0	0	0	0	0	0	0	0	0
6	Control (PBS) (0.5 ml)	10	0	0	0	0	0	0	0	0	0	0	0	0

Figure 1



Figure 2

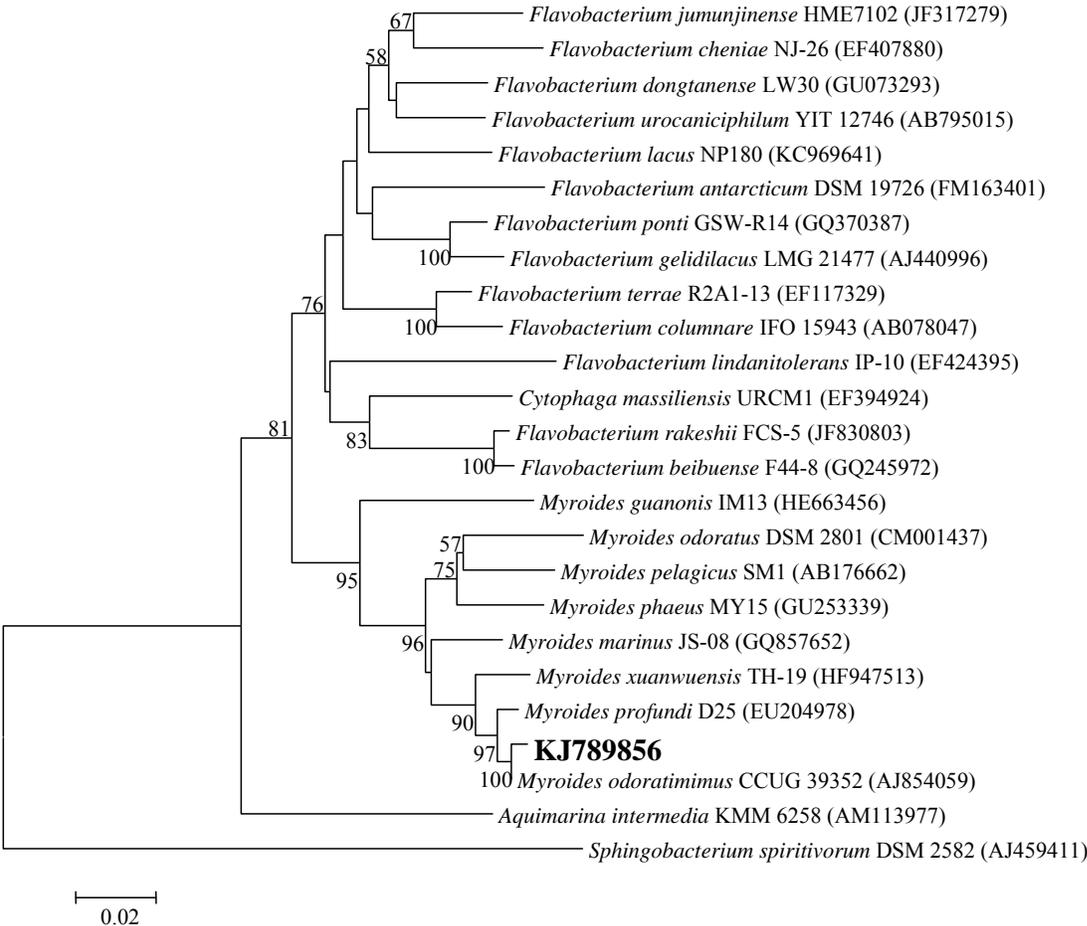


Figure 3

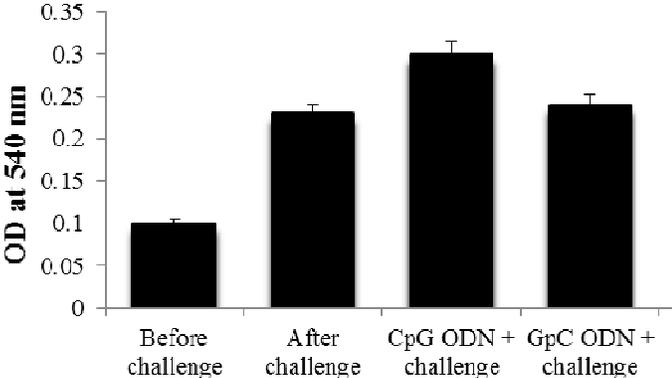


Figure 4

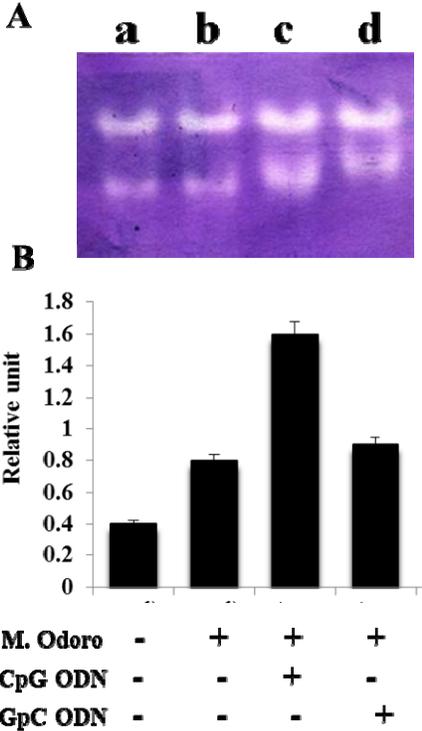


Figure 5

