Girijakumari Nisha, Rajagopalan; Rajathi, Vellaikannu; Manikandan, Ramar; Marimuthu Prabhu, Narayanan
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Acta Scientiae Veterinariae, vol. 42, núm. 1, enero, 2014, pp. 1-7
Universidade Federal do Rio Grande do Sul
Porto Alegre, Brasil

Acta Scientiae Veterinariae,
ISSN (Printed Version): 1678-0345
ActaSciVet@ufrgs.br
Universidade Federal do Rio Grande do Sul
Brasil

Acta Scientiae Veterinariae, 2014. 42: 1195.

RESEARCH ARTICLE
Pub. 1195

ISSN 1679-9216

Isolation of *Plesiomonas shigelloides* from Infected Cichlid Fishes using 16S rRNA Characterization and its Control with Probiotic *Pseudomonas* sp.

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ABSTRACT

***Background*:** Bacterial diseases are reported to cause heavy mortalities in both cultured and wild fishes throughout the world. In any field, diagnosis of disease plays a main role in curing the disease. In aquaculture, antibiotics are used to control the infectious diseases caused by bacteria. The use of antibiotics has been questioned as it experiences problems of bacterial resistance to antimicrobials and unacceptable residues in aquaculture products and environment. Recently, many studied have been performed in the application of probiotic as a control measure of infectious diseases. Therefore, this study has designed for the isolation of possible probiotic bacteria which inhibit the growth of specific pathogenic bacteria isolated from a fresh water ornamental fish.

***Materials, Methods & Results*:** Diseased cichlid fishes were brought to the laboratory for the isolation of causative bacteria. A total of three strains were isolated (APG01, APG02 and FA3) from the serially diluted samples of gut, gill and spleen. The isolated pathogenic bacteria were injected at the concentration of 10^5 cfu/mL to healthy cichlid fishes to find out its virulency *in vivo* at varying time duration (12, 24, 36 and 48 h) of exposure for 15 days. Among the three isolates, only APG01 caused 100% mortality of cichlid fishes at 48 h of exposure. APG01 was subjected to 16S rRNA characterization and confirmed as *Plesiomonas shigelloides*. The nucleotide sequence was submitted to NCBI Banklt and provided with the accession number. To study the *in vitro* inhibition of *Plesiomonas shigelloides*, a possible probiotic bacteria (*Pseudomonas* strain BPH2) was isolated from fermented rice. *In vitro* antibacterial activity was studied by agar well diffusion assay with 10, 50 and 100 µL of probiotic strain at the concentration 10^7 cfu/mL. Maximum zone of inhibition of 23 ± 0.78 mm was produced by 50 µL and 100 µL *Pseudomonas* strain BPH2. Also, the antibacterial effect of cell free supernatant was studied and observed the zone of inhibition of 21 ± 1.00 mm against *P. shigelliodes*. The susceptibility and reproducibility of probiotic bacteria at varying pH was tested by observing the growth in the media with the pH ranging from 5 to 9. The growth was measured by absorbency at 600 nm for every 8 h. *Pseudomonas* strain BPH2 was found susceptible at the pH ranging from 6 to 9.

***Discussion*:** In agar well diffusion assay, *Pseudomonas* strain BPH2 isolated from fermented rice showed maximum inhibitory activity against *P. shigelloides* APG01. In the report of *Lactococcus lactis* isolated from fresh milk showed a maximum inhibitory effect of 14.77 ± 1.17 mm against the pathogenic strain *Aeromonas hydrophila* in the agar well diffusion assay. While comparing this result, the inhibitory activity of *Pseudomonas* strain BPH2 is maximum. The pH tolerance study revealed that pH could significantly influence the viability and growth of probiotic *Pseudomonas* strain BPH2. *Lactobacillus mesenteroides* showed survival and growth at the pH 3 - 7 with highest viability and growth rate at neutral conditions. The probiotic strain used in this study showed viability between the pH 6 to 8. Hence *Pseudomonas* strain BPH2 failed to show susceptibility at the acidic pH, it might be questionable to use as a gut associated probiotic. Other than that this strain can survive in fresh aquatic environment and this strain might be effectively used in the aquaculture system like water and soil to control the bacterial disease.

Keywords: fish pathogen, pH tolerance, *Plesiomonas shigelloides*, Probiotics, *Pseudotropheus socolofi*, well diffusion.

Received: 20 January 2014 Accepted: 12 June 2014 Published: 23 June 2014

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INTRODUCTION

In large scale ornamental fish production facilities, high stocking density, excess feed may cause diverse ecological impacts in culture which lead to diseases, resulting in serious economic losses [8]. Next to the viral, bacterial diseases are reported to cause heavy mortalities in both cultured and wild fishes throughout the world. Major disease causing bacteria are gram-negative *Aeromonas hydrophila*, *Aeromonas salmonicida, Flavobacterium columnare*, *Vibrio*, *Pseudomonas* species and gram-positive *Streptococcus*.

When the disease outbreak encounter, the most common response moves towards the application of antimicrobial drugs. As the excessive usage of antibiotics and chemotherapeutic substances will cause damage to the environment and also a microbial imbalance in host gut [12,20], the uses of beneficial bacteria to manage the pathogenic organism through a variety of mechanisms are increasingly viewed as an alternative method. Use of probiotics in aquaculture is increasing [9] with the demand for environment-friendly as it enhance the resistance of the host animal against pathogens and prevent subsequent disease outbreak.

In this present study, the bacteria were isolated from disease outbreak cichlid ornamental fishes (fresh water) to find out the causative bacteria by 16S rRNA sequencing. To confirm the pathogenesis, virulence of the isolated strain was studied *in vivo* with cichlid fishes. Furthermore, to ascertain the possible control measures, probiotic strains were isolated from the food materials and its efficacy was studied against the isolated pathogenic strain for the benefit of ornamental fish aquarium holders and farmers.

MATERIALS AND METHODS

Isolation and identification of pathogenic bacteria

Diseased fishes were collected from an aquarium and brought to the laboratory. Internal organs (gut, gill and spleen) were dissected, serially diluted upto 10^{-9} and cultured in nutrient agar[1] and incubated at 37°C for 24 to 48 h. The isolated bacteria were purified by subculture on nutrient agar and identified based on its morphological feature followed by biochemical analysis [5].

Virulence study

Healthy cichlid fishes (*Pseudotropheus socolofi* albino snow white) with an average body weight of 610 ± 3.21 mg were purchased from the ornamental fish farm, Tamilnadu, India. These fishes were acclimatized for 30 days in laboratory condition in 90 L rectangular plastic tubs (64×45×32 cm) at 31 ± 1°C. Fishes were fed with commercial (Crude protein - 35%, Crude fat - 3 %, Crude fiber - 5% and Moisture - 10 %) diet of 3% body weight twice a day. For the virulence study, these healthy fishes were evenly (30 nos each) distributed into four distinct groups for each bacterial strain (APG01, APG02 and FA3) with two replicates and one control was maintained. Cichlid fishes were exposed to all the three isolated bacteria individually with the cell density of 10^5 cfu/mL for different time duration (12, 24, 36 and 48 h). The relative percentage of survival (%) was recorded over 15 days of post infection.

16S rRNA sequencing

The virulent strain was selected for 16S rRNA sequencing to identify the bacteria. DNA of bacterial strain was extracted by alkaline lysis method[2] [3]. A complete 16S rRNA was amplified by using universal primers[3], namely forward primer 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer - 5' TACGGCTACCTTG TTACGACTT 3' [2]. PCR reaction[4] was performed in Eppendorf Master Cycler with the initial denaturation for 5 min at 94ºC, there were 40 cycles consisting of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 2 min and final extension of 10 min at 72ºC [17]. The amplification of 16S rRNA of the isolate was confirmed by running the amplification product in 1% agarose gel electrophoresis in 1X TAE buffer. The PCR product of 16S rRNA of the isolates was sequenced with primer set 518F-5´ CCAGCAGCCGCGGTAATACG 3´ and 800R-5´ TACCAGGGTATCTAATCC 3´. Further comparison was made with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Blast Local Alignment Search Tool).

Isolation and primary screening of probiotic strains

Serially diluted samples of healthy fish gut and fermented rice were platted on Bacillus medium[5] and King's medium A base[6] (for isolation of *Bacillus* spp. and *Pseudomonas* spp. respectively). For the confirmation, pure isolates were subjected to biochemical analysis and screened for its antagonistic activity against the pathogen which was isolated from diseased ornamental fishes. The bacterial isolates showing higher zone of inhibition were selected and maintained separately for

further studies [15]. Additionally, the selected probiotic strains were inoculated at different concentration (10^3, 10^4, 10^5, 10^6 and 10^7 cfu/mL) in cichlid fish (10 fish each concentration) tank and observed for 15 days to confirm the probiotic efficacy by observing the behaviour and mortality of fish.

Preparation of cell free supernatant

Overnight culture of isolated probiotic bacteria was grown in the Luria bertani[7] (LB) broth (LB). The culture in the broth was centrifuged at 800X *g* for 10 min. Cell free extract was filtered using a syringe with 0.2 µm acetate cellulose filter [16]. The cell free extract was stored for further study.

Agar well diffusion assay

A pre-screened bacterial strain isolated from fermented rice was further tested for antagonistic activity in agar well diffusion method. The pathogenic bacterium was grown overnight at 37°C in LB broth and seed on LB agar plates (10, 50 and 100 µL) at the cell density of 10^5 cfu/mL. In this medium, 6 mm wells were made and filled with 50 µL of probiotic culture (BPH2) with the cell density of 10^7 cfu/mL and cell free supernatant of probiotic strain. The petriplates were allowed to incubate at 37°C ± 1 for 24 h to observe the zone of inhibition.

Determination of pH tolerance of probiotic strains

Acid and alkaline tolerance test was investigated for this probiotic strain BPH2 to find out the efficacy of field application by determining the bacterial growth at different pH of media. LB broth with different pH including 5, 6, 7, 8 and 9 were prepared using 1% HCl and 1 N NaOH and divided in flasks [19]. Overnight culture of probiotic strain was inoculated in the prepared LB medium and incubated at 30°C. The growth rate of bacteria was measured by UV-vis spectrophotometer (Shimadzu UV-1800, Japan) at 600 nm for every eight hours and recorded up to 36 h [4,6,14].

Statistical analysis

Data are expressed as mean ± SD. The significant difference between the varying concentrations of *P. shigelloides* in the well diffusion assay and varying time duration of pathogenic exposure in virulence study were done by one-way ANOVA. The significant difference between probiotic and cell free extract of probiotic in the well diffusion assay was done by two-way ANOVA.

RESULTS

Isolation and identification of pathogenic bacteria

A total of three bacterial strains (APG01, APG02 and FA3) were isolated from infected fish which showed abnormal behaviour like circling and 70% of cumulative mortality within one week. All the three strains were able to grow at 37°C in nutrient agar with the morphology of round, milky white, elevated and mucoid colonies at the pH 8.0 ± 0.2. Isolated strains (APG01, APG02 and FA3) were motile, gram negative and showed positive results for oxidase, and catalase and glucose fermentation test (Data not shown).

Virulence study

In vivo mortality of cichlid fishes challenged with APG01, APG02 and FA3 were observed for 30 days. Among the three, only the strain, APG01 showed behavioural changes and mortality in cichlid fishes and no mortality was recorded in APG02 and FA3 bacterial strains. APG01 (10^5 cfu/mL) strain caused 100% mortality of fish at 48 h of exposure. A 71.7 and 70% mortalities rates were observed at 36 and 24 h respectively and only 31.7% fatality rate was recorded at 12 h exposure. Significant difference ($P < 0.05$) found in the mortality rate of varying durations of pathogenic exposure (Figure 1). Before 24 h of the fatality, behavioural changes of the infected chichilid fishes were less feed consumtion, spiral and circular movements.

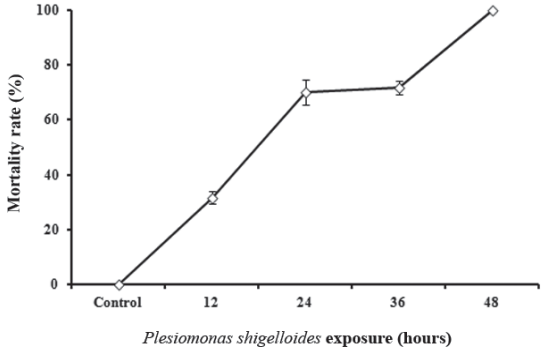

Figure 1. Percentage of cichlid fishes' mortality at different time of exposure to *P. shigelloides* APG01 (10^5 cfu/mL). Values are expressed as mean ± SE. A significant difference ($P < 0.05$) was found between the mortality rate and different exposure time of pathogen.

16S rRNA characterization

Based on the virulent study result, APG01 strain was selected for the 16S rRNA sequence analysis and identified as *Plesiomonas shigelloides*, showing similarity of 1479/1484 base pair (99%) with the sequence NR_044827.1 (*Plesiomonas shigelloides* NCIMB9242) by BLAST analysis in the NCBI database. The nucleotide sequence of *Plesiomonas shigelloides* was submitted in the NCBI BankIt (Acc. No: KF769536).

Screening for probiotic strains

The primary screening of 56 bacterial strains, isolated from the healthy fish gut and fermented rice were selected for antagonistic activity against pathogenic strain. Only one bacterium isolated from fermented rice (BPH2) grown in King's medium A base exhibited antibacterial activity against *Plesiomonas shigelloides* APG01. The result of biochemical analysis of *Pseudomonas* strain (BPH2) showed that they were gram negative, motile, oxidase positive, catalase positive and glucose fermentation negative (Data not shown). The *in vivo* probiotic efficacy experiment, no mortality was observed even after 15 days at the *Pseudomonas* cell density of 10^7 cfu/mL inoculated in cichlid fish culture tank. Consequently, this *Pseudomonas* strain was selected for well diffusion assay to confirm the antagonistic potential.

Agar well diffusion assay

The zone of inhibition was significantly increased when the concentration of *Plesiomonas shigelloides* APG01 decreases. *Pseudomonas* strain BPH2 (10^7 cfu/mL) exhibited the maximum zone of inhibition of 23 ± 0.78 mm for 50 µL and 100 µL of *Plesiomonas shigelloides* APG01 (10^5 cfu/mL) strain. No significant difference ($P > 0.05$) was found among the varying concentrations of *P. shigelloides* (Figure 2). The cell free supernatant (CFS) of *Pseudomonas* strain BPH2 (10^7 cfu/mL) also exhibited zone of inhibition of 21 ± 1.00 mm against this pathogen. There was a significant difference ($P < 0.05$) found between the *Pseudomonas* strain BPH2 and its CFS (BPH2-CFS) [Figure 2].

pH tolerance

Probiotic strain *Pseudomonas* strain BPH2 showed potential viability at the pH 6, 7, 8 and 9 (Figure 3). A complete disability was observed at the acidic pH (pH 5).The narrow growth rate was observed for the pH 7 throughout 36 h of incubation period. BPH2 showed a higher growth rate even after 30th h at the pH 6, 7 and 8 respectively. Whereas, at pH 9 the lesser growth rate was observed when comparing to 6, 7 and 8.

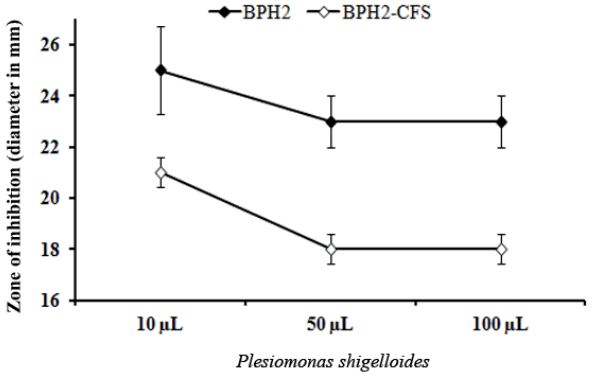

Figure 2. Antagonistic activity of *Pseudomonas* strain BPH2 as zone of clearance in mm against different concentrations (µL) of *P. shigelloides* (10^5 cfu/mL) in well diffusion assay. Values are expressed as mean ± SE. No significant difference ($P > 0.05$) in the zone of inhibition was found between the different concentrations of *P. shigelloides*. A significant difference ($P < 0.05$) was found between the *Pseudomonas* strain BPH2 and its cell free supernatant (BPH2-CFS).

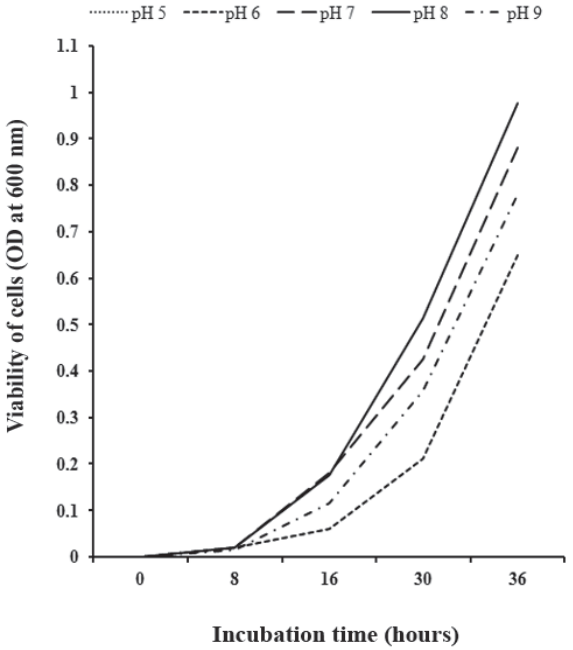

Figure 3. Viability of *Pseudomonas* strain BPH2 at different pH levels. The graph showed the viability of cells at different hours of incubation time.

DISCUSSION

Microorganisms are naturally occurring opportunistic pathogens which invade into the fish tissue and cause disease [18]. Bacterial infections are fairly common in freshwater aquarium fish, especially where the water quality is low this may due to overfeeding, inadequate filtration or a number of other environmental factors. When the water quality deteriorates, fish become stressed and increases their susceptibility to disease. Isolation and identification of bacterial communities and pathogenic taxa associated with the aquatic animals would broadly benefit to the aquaculture industry including ornamental fish culture.

16S rRNA gene sequencing is one of the most attractive methods for identification of bacterial isolates, which provide genus and species confirmation that do not corresponding to any recognized biochemical profiles. In this study 16S rRNA characterization was performed to identify the pathogenic bacteria, isolated from diseased cichlid fish and revealed that the strain APG01 was *Plesiomonas*. This bacterium is gram-negative, motile, non spore forming bacilli, which are facultative anaerobes and oxidase positive [11]. Fresh water habitats are the primary reservoirs of *P. shigelloides* [22] and this microorganism have been recognized as potential fish pathogens [10] and also caused mortality in Asian Arowana, recorded in an aquarium, Seoul Korea [13]. Cruz *et al*., reported that *P. shigelloides* caused 40% mortality in 1-2 year old rainbow trout in Portugal [7]. In the present study, isolated *P. shigelloides* APG01 exhibited virulence on cichlid fishes at 10^5 cfu/mL. A 100% rate of mortality of cichlid fishes was found in 48 h exposure of *P. shigelloides* APG01 (10^5 cfu/mL). A 71.7% and 70% rates of mortalities at 36 and 24 h, 31.7% fatality rate at 12 h were recorded respectively. This shows that, when the duration of exposure increases percentage of mortality was also increased significantly ($P < 0.05$).

Further, this pathogenic *P. shigelloides* APG01 bacteria was inhibited with *Pseudomonas* strain BPH2 isolated from fermented rice. Probiotic competes with pathogenic bacteria through the production of inhibitory compounds, improvement of water quality, enhancement of immune response and nutrition of host species through the production of supplemental digestive enzymes [21,23]. In the present study, out of 56 bacterial isolates, one strain BPH2 isolated from fermented rice was found antagonistic activity against this pathogenic bacterium.

In agar well diffusion assay, *Pseudomonas* strain BPH2 isolated from fermented rice showed maximum inhibitory activity against *Plesiomonas shigelloides* APG01 (100 µL) with the cell density 10^5 cfu/mL and the zone of inhibition was 23 ± 0.78 mm. In earlier reports, *Lactococcus lactis* isolated from fresh milk showed a maximum inhibitory effect of 14.77 ± 1.17 mm against the pathogenic strain *Aeromonas hydrophila* in the agar well diffusion assay [24]. Probiotic bacteria isolated from marine sediments exhibited maximum antibacterial activity of 15-34 mm zone against 100 µL of pathogenic *Vibrio* species and there is a significant difference in the zone of inhibition when the concentration of pathogen increases [17]. In the present study, no significant difference ($P > 0.05$) was found among the varying concentrations (10 µL, 50 µL and 100 µL) of *P. shigelloides* APG01. Hence, there is significant difference ($P < 0.05$) found between the probiotic strain (BPH2) and CFS (BPH2-CFS). However, there were no previous studies to compare the antibacterial activity of probiotic against *Plesiomonas shigelloides*.

Additionally, to find out the viability of isolated probiotic strain, pH tolerance study was conducted and the highest survival and growth of *Pseudomonas* strain BPH2 (the probiotic strain isolated from fermented rice) was observed at 6 to 9 pH. The maximum growth rate was observed when the time of incubation increased. The pH tolerance study revealed that pH could significantly influence the viability and growth of probiotic strain BPH2. *Lactobacillus mesenteroides* showed survival and growth at the pH 3 - 7 with highest viability and growth rate at neutral conditions [1]. According to the earlier reports, one of the most important criteria for the selection of probiotic species is excellent viability at low pH [14]. Although, *Pseudomonas* strain BPH2 showed disability in growth at the pH 5, maximum growth was obtained at the pH 6 - 9. In general, the freshwater ornamental fish pond water pH varied from 6.5 to 9 and soil 6 to 8. Hence, the present result confirms that the strain can survive in fresh aquatic environment and this strain can be effectively used in the aquaculture system like water and soil to control the bacterial disease.

The result revealed that, bacteria isolated from diseased cichlid fish was *Plesiomonas shigelloides* and its virulence was confirmed *in vivo*. This pathogenic strain was effectively inhibited by *Pseudomonas* spe-

cies isolated from fermented rice. Furthermore, a pH tolerance study also confirmed that the isolated probiotic strains from fermented rice showed maximum viability at pH 6 to 9. Therefore, this probiotic bacterium can be used effectively in aquarium tank water for controlling the ornamental fish pathogens.

SOURCES AND MANUFACTURERS

[1]Nutrient agar - HIMEDIA, Mumbai, India.

[2]Chemicals for DNA isolation - Sisco Research Laboratories Pvt. LTD, Mumbai, India.

[3]16S rRNA primers - Acme Progen Biotech (India) Pvt. Ltd. Salem, TN, India.

[4]Taq DNA Polymerase - Synergy Scientific Services, Chennai, TN, India.

[5]Bacillus medium - HIMEDIA, Mumbai, India.

[6]Kings medium - HIMEDIA, Mumbai, India.

[7]Luria bertani Broth - HIMEDIA, Mumbai, India.

***Funding*.** The authors gratefully thank University Grant Commission (UGC), India for providing fund to pursue this work.

***Declaration of interest*.** Authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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