World Journal of Fish and Marine Sciences 3 (6): 564-569, 2011
ISSN 2078-4589

Efficacy of *Pseudomonas fluorescens* as Biological Control Agent against *Aeromonas hydrophila* Infection in *Oreochromis niloticus*

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Abstract: The objective of this study was to evaluate previously isolated non pathogenic *Pseudomonas fluorescens* biovars inhibitory effect against pathogenic *A. hydrophila in vitro* and *in vivo*. The results showed that *P. fluorescens* biovars have *in vitro* antibacterial inhibitory effect against *A. hydrophila*. Also, feeding *O. niloticus* diet containing *P. fluorescens* biovars showed resistance against *A. hydrophila* infection. The hematological parameters, total protein and globulin were significantly increased in groups which were fed on diet containing *P. fluorescens* strains. It can be concluded that *P. fluorescens* biovar I, II and III are beneficial for *O. niloticus* when administered as a feed additive through enhancing fish resistance against *A. hydrophila* infection.

Key words: *Pseudomonas fluorescens* % Antibacterial % *Aeromonas hydrophila* % *Oreochromis niloticus* % Physiological Parameters

INTRODUCTION

Aquaculture is considered an important source of high nutritive value and cheap animal proteins and, it becomes an important economic activity in many countries [1]. Bacterial fish disease is a major problem facing fish farming industry, which is currently fast growing with annual increase approximately 12% [2]. *A. hydrophila* is a pathogen that can infect human, animal, bird and fish. As a fish pathogen, *A. hydrophila* can infect a wide variety of freshwater and marine fish, causing hemorrhagic septicemia [3, 4]. In Egypt, Tilapia has attained great economic importance [5]. Nile tilapia, *Oreochromis niloticus* is an important species for freshwater aquaculture and disease-resistance is a major problem facing the improving of its culture and fish culturists [6].

In aquaculture practices bacterial infectious diseases prevention by antimicrobial compounds are used intensively. In the process of antimicrobial treatment, drug resistance patterns can develop within the pathogenic microbial community. Therefore, alternatives to the use of antimicrobials are gaining importance in many countries [7]. So the use of microbial communities in aquaculture for controlling pathogenic bacteria shows promises. Several non-pathogenic bacterial strains are capable of inhibiting fish pathogenic bacteria and or fungi *in vitro* assay [8]. Fluorescent pseudomonads have been evaluated as biological control agents in aquatic animals, its have inhibitory effect against *Aeromonas salmonicida* [9], *Staphylococcus aureus* and *Aeromonas sobria* [10], Saprolegnia and other fungi [11, 12], *Vibrio* [13-16] and *A. hydrophila* [8, 17]. Since, the use of beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment.

Therefore, it was of great importance to direct the aim of this study to evaluate the antibacterial efficiency of non pathogenic *Pseudomonas* strains against *A. hydrophila* infection in Nile tilapia *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial Strains: Previously isolated and identified non pathogenic *Pseudomonas* strains *P. fluorescens* biovar I, *P. fluorescens* biovar II and *P. fluorescens* biovar III were subjected for this study [18].

Determination of Antibacterial Activity: The pure *P. fluorescens* biovars were examined for its inhibitory effects against the pathogenic *A. hydrophila*, which was obtained from Aquatic Diseases Lab., National Institute of Oceanography and Fisheries (NIOF), Egypt. The *in vitro* antibacterial activity was assessed according to the

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method of Irianto and Austin [19]. Briefly, the strains were cultured on TSB for 24 hr at 25°C, then *P. fluorescens* biovar I, II and III were streaked followed by cross re-streaking of pathogenic *A. hydrophila* on TSA plates (each three replicate) and incubated for 24 hr at 25°C; then inhibitory effect of *P. fluorescens* biovars against pathogenic strains was demonstrated either by over growing or interrupted growth.

Safety of *P. fluorescens* Strains*:* The safety of isolates, that showed antibacterial activity against the pathogenic *A. hydrophila in vitro*, was evaluated by using 120 healthy Nile tilapia (70 ± 5 g/fish) obtained from Fish Farm Research Station, NIOF, Egypt. The fish were acclimatized for two weeks in aquaria. The fish were divided into 4 equal groups with three replicates per each. Groups (1, 2 and 3) were intra-peritoneally (IP) injected by 0.1 ml of saline containing 3 x 10^7 cells /ml of 24 hr grown *Pseudomonas* sp., respectively. The fourth group was IP injected by 0.1 ml of sterile saline (0.85% NaCl) as control [19]. All groups were kept under observation for 14 days and the mortality and morbidity rates were recorded. The fish were subjected to laboratory examination and bacterial re-isolation.

Fish Feeding Experiment

Feed Ingredients: A commercial diet (Zoocontrol®, Egypt) contained 30% crude protein, 3.7 Kcal/g of metabolizable energy, 3.4% fiber and 7.03% fat as well as vitamins and minerals in the form of dry pellets. Bacterial colonies (*P. fluorescens* biovars I, II, III) were grown on TSB, harvested by centrifugation at 1000 RPM for 10 min, washed by saline and resuspended in saline to 10^{10} cells mL^{-1}. Then, volumes were mixed thoroughly in 100 g of the commercial dry feed to achieve a dose equivalent to 10^8 bacterial cells g^{-1} of feed [19].

Experiment: A total of 75 healthy Nile tilapia (70 ± 5 g/fish) was obtained from Fish Farm Research Station, NIOF, Egypt. The fish were acclimatized in indoor glass aquaria for two weeks and then randomly stocked at a rate of 15 fish / aquarium. Fish received incorporated feed for 7 days then injected with the pathogenic strain. All experimental groups were kept under daily observation for 7 days post inoculation. Signs, lesions and mortality rate were recorded. Recently Dead and morbid fish were examined for the presence of bacterial pathogens. Groups, dose, route of inoculation were represented and tabulated (Table 1).

Physiological Analysis: At the end of the experiment, 5 fish of each aquarium were anaesthetized and blood samples were collected from the caudal vein by sterile syringe using EDTA-disodium as an anticoagulant. The blood samples were used for determination of RBCs, Hb, PCV, WBCs and differanital leukocytic count according to Schalm [20]. Serum samples were obtained by blood centrifugation at 3000 R.P.M for 15 minutes for estimation of the total protein content colorimetrically according to method described by Henry [21] and albumin content by a colorimetric method at wave length 550 nm according to Dumas and Biggs [22].

Statistical Analysis: The mean values and standard errors were calculated. Analysis of variance (ANOVA) was applied to make comparison between the mean values of groups to check the significance which was set at $P > 0.05$ and $P > 0.01$ according to Feldman *et al.* [23].

RESULTS

***In vitro* Antibacterial Activity:** *P. fluorescens* biovars II and III resulted in larger inhibition zones than *P. fluorescens* biovars I against *A. hydrophila.*

Safety of *P. fluorescens* Strains: The I/P challenge of fish with the three bacterial isolates didn't induce any signs of morbidity or mortalities and *Pseudomonas* strains were safe to fish in comparison with the control which have 20 % mortality rate.

Table 1: Feeding experiment design to evaluate antibacterial efficiency of isolated non pathogenic *Pseudomonas* strains

Groups		Inoculating organisms			Inoculating bacteria (pathogenic)		
Group No.	No. of Fish	Species of bacteria	Dose	Route	Species of bacteria	Dose	Route
1st group	15	*P. fluorescens* biovar I	10^8 cells /g	Feeding	*A. hydrophila*	0.1ml (3 x 10^7 cells)	I/P
2nd group	15	*P. fluorescens* biovar II	10^8 cells /g	Feeding	*A. hydrophila*	0.1ml (3 x 10^7 cells)	I/P
3rd group	15	*P. fluorescens* biovar III	10^8 cells /g	Feeding	*A. hydrophila*	0.1ml (3 x 10^7 cells)	I/P
4th group	15	Control infected	Normal diet	Feeding	*A. hydrophila*	0.1ml (3 x 10^7 cells)	I/P
5th group	15	Control non infected	Normal diet	Feeding	Saline 0.9%	0.1 ml	I/P

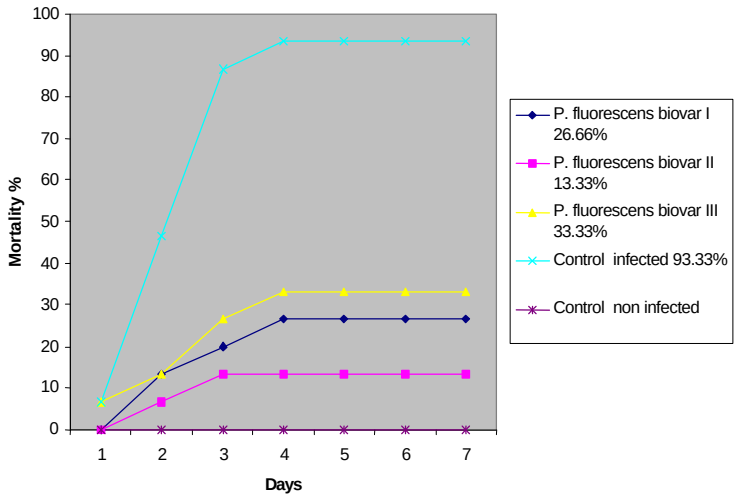

Fig. 1: Cumulative Mortality rate of *O. niloticus* fed with diet containing 10^8 cells /g P. *fluorescens biovar I, II and III* for 7 successive days and then challenged intraperitoneally with 0.1 ml (3 x 10^7 cells) of *A. hydrophila*

Table 2: Hematological parameters of *O. niloticus* at the end of experiment

parameters

Studied groups	Hb g/100ml	PCV %	RBCs10^6/mm^3	WBCs10^3/mm^3	Lymphocytes 10^3/mm^3	Monocytes 10^3/mm^3
Control	4.1± 0.077^a	14.95 ± 0.11^a	1.14 ± 0.015^a	56.32 ± 0.96^a	46.24 ± 0.58^a	4.71 ± 0.039^a
Pseudomonas fluorescens biovar I	5.58 ± 0.19^b	22.28 ± 0.4^b	1.57 ± 0.038^b	70.82 ± 0.60^b	54.98 ± 0.6^b	11.02 ± 0.31^b
Pseudomonas fluorescens biovar II	5.38 ±0.19^b	23.22 ± 0.31^c	1.44 ± 0.12^b	71.2 ± 0.92^b	55.52 ± 0.74^b	11.18 ± 0.25^b
Pseudomonas fluorescens biovar III	5.32 ±0.12^b	23.22 ± 0.32^c	1.50 ± 0.15^b	72.88 ± 0.81^c	55.96 ± 0.59^b	11.3 ± 0.41^b

C The mean difference is significant at the 0.01 level.

C Means with the same letter at the same column are not significantly different.

Table 3: Total protein, albumin and globulin of *O. niloticus* at the end of experimental period

parameters

Studied groups	Protein g/100ml	Albumin g/100ml	Globulin g/100ml
Control	2.82 ± 0.124^a	0.73 ± 0.02^a	2.09 ± 0.10^a
P. fluorescens biovar I	3.25 ± 0.073^b	0.64 ± 0.019^b	2.61 ± 0.80^b
P. fluorescens biovar II	3.77 ± 0.23^c	0.53 ± 0.03^c	3.24 ± 0.032^c
P. fluorescens biovar III	3.63 ± 0.035^d	0.53 ± 0.028^c	3.09 ± 0.049^d

C The mean difference is significant at the 0.05 level.

C Means with the same letter at the same column are not significantly different.

Efficacy of the non Pathogenic *Pseudomonas* Strains Fed with Diet to *O. niloticus* in Controlling *A. hydrophila*: Fish fed incorporated diet with *P. fluorescens* biovars *I, II and III* resisted *A. hydrophila* infection and showed mortality rate of 26.66, 13.33 and 33.33% respectively. While the control group didn't resist *A. hydrophila* and showed 93.33% mortality rate. The fish mortality increased by time until the 4th day then stopped as shown in figure (1).

Physiological Parameters: The hematological parameters, total proteins and globulin were significantly high in the groups which were fed *P. fluorescens* biovars I, II and III incorporated diet in comparison to the control non infected group (Tables 2 and 3).

DISCUSSION

This study evaluated the efficacy of isolated non pathogenic *P. fluorescens* biovars isolated and identified

by Eissa *et al.* [18] as biological control agent against *A. hydrophila*. It has been found that several species of *Pseudomonas* are pathogenic to the fish and others are non pathogenic and can be used for control of some fish bacterial pathogens [24].

The present work revealed that *P. fluorescens* biovars I, II and III have an inhibitory effect *in vitro* against pathogenic *A. hydrophila.* These results agreed with those of Abd El-Rhman *et al.* [17] who reported that *Pseudomonas* species showed inhibitory effects *in vitro* against *A. hydrophila*. Gram *et al.* [25] who proved that *in vitro* antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* doesn't confer protection of salmon against furunculosis. Also, the author reported that strain AH2 inhibits the growth of *A. salmonicida* in defined glucose-casamino acid media in both agar-well-diffusion assay and in broth cultures. The authors also explained that inhibition is significantly enhanced by iron limited conditions as compared to iron surplus conditions. It has been found that, the inhibitory action among *Pseudomonas strains* is attributed to production of siderophores and the presence of iron eliminates the antibacterial effect of the inhibitory strains. Siderophore mediated competition for iron may explain the inhibitory activity of these strains [10]. Also, the antimicrobial activity of *Pseudomonas* species has been also attributed to a several antibiotic-like substances which have been identified, including bacteriocins (notably pyocin from *Pseudomonas aeruginosa*), a phenazine antibiotic [26] and non-nitrogen-containing compounds such as 2,4-diacetylphloroglucinol [27]. Of further interest, the isolated *Pseudomonas fluroscens* biovars I, II and III in this study were non pathogenic and safe to *O. niloticus*, this result was supported by Abd El-Rhman *et al.* [17].

The fish feeding experiment revealed that *O. niloticus* fed on incorporated diet with *P. fluorescens biovar I, II and III* resulted in resistance against *A. hydrophila* infection showing mortality rate of 26.66, 13.33 and 33.33% respectively in comparison control infected group didn't resist *A. hydrophila* and showed 93.33% mortality rate. Similarly, Smith and Davey [9] found reduction in diseases caused by *A. salmonicida* in Atlantic salmon after challenged with *P. fluorescens* and Wanga *et al.* [28] detected strong protection against *A. hydrophila* infections in Japanese flounder injected with *P. fluorescens.* Meanwhile, these results disagreed with those of Abd El-Rhman *et al.* [17] who observed high mortality rate in Nile tilapia feed on diets supplemented with *Pseudomonas* species and challenged with *A. hydrophila.*

Concerning, the effect of non pathogenic *Pseudomonas* strains on the health status of Tilapia, our results indicated a positive effect represented by significant increase in RBCs count, PCV%, Hb concentration, WBCs, Lymphocytes and monocytes in all treated groups in comparison to control group. These could be attributed to the fact that, probiotics increased blood parameter values as a result of hemopiotic stimulation [29, 30] and supported by Nayak, [31] who reviewed the immunomodulatory activity of probiotics and evaluated the factors that regulate for the optimum induction of immune responses in fish. Our results are in complete agreement with the finding of Irianto and Austin [19] who found an increase in the RBCs count in fish fed on supplemented diet with probiotic bacteria than the control group. Moreover, the hematocrite values were significantly higher in the group fed on diet supplemented with probiotic as compared with the control group [32]. The various hematological and biochemical parameters decreased or increased in fish fed on diets supplemented with *Pseudomonas* species, this mean, that *Pseudomonas* species might be changed to pathogenic-version resulting from the three month feeding on diet supplemented with *Pseudomonas* species [17, 33, 34].

A significant increase in total protein and globulin with decreased albumin in all groups treated with *Pseudomonas fluorescens* in comparison to the control group, this is attributed to the modulatory effect of *Pseudomonas fluorescens* as probiotic on liver cells which activate the anabolic capacity of hepatocytes to produce blood proteins especially globulin [17, 31]. Total plasma-protein was decreased, in rainbow trout, when injected intramuscularly with *Vibrio anguillarum* extra-cellular products. The fluctuation in the biochemical activities could be due to the severe damage of some organs as liver, spleen, muscle and kidney [35].

It can be concluded that *P. fluorescens* biovars I, II and III are beneficial for Nile tilapia when administered as a feed-additive through enhancing fish resistance against *A. hydrophila* infection.

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