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Indoor Study on the Immunization of Red Tilapia: *Oreochromis niloticus* x *O. mossambicus* Against Aeromonad and Pseudomonad Septicemias

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Abstract: Monovalent, killed and live attenuated vaccines of *Aeromonas hydrophila* and *Pseudomonas putida* were used in the immunization of red tilapia against Motile Aeromonad and Pseudomonad septicemias. There were 4 treatments and a 5th control group with 3 replicates per each. A 4th replicate was kept for replacement of natural mortality among the experimented fish. The 4 treatments included, Heat-killed vaccine of *A. hydrophila*, Live-attenuated vaccine of *A. hydrophila* (using herbs), Heat-killed vaccine of *P. putida* and Live-attenuated vaccine of *P. putida*. A total of 160 brood stocks of *O. niloticus* with 250 g average body weight were used for all treatments (8 fish per each glass aquarium). Vaccination was conducted via the Intra Peritoneal route (I/P) as an initial dose followed by 2 booster doses every 2 weeks. The last dose was applied via the immersion route. The evaluation of vaccination was carried out through periodical antibody titration of the serum of the examined fish (every 2 weeks) using direct agglutination method as well as by the experimental challenge 3 months after the initial immunization. Results revealed that there were a significant difference between the vaccinated and non vaccinated fish of the control group regarding antibody titers and Relative Percent Survival (RPS) of the challenge test. Differences in immunity levels within the vaccinated groups themselves were demonstrated.

Key words: Tilapia, *Aeromonas hydrophila*, *Pseudomonas putida*, vaccination, immunization

INTRODUCTION

Fish diseases caused by *Aeromonads* and *Pseudomonads* considered to be the major bacterial problems facing the aquaculture development causing mass mortalities, reduced production and low quality of aquatic organisms (Ghittino, 1976; Abdel-Hadi, 2004; Laila *et al*., 2004).

Both *Aeromonas* sp. (*A. hydrophila*, *A. sobria* and *A. caviae*) and *Pseudomonas* sp. (*P. fluorescens*, *P. putida* and *P. aeruginosa*) were incorporated in severe outbreaks among *O. niloticus* in fish hatcheries (Ahmed and Shoreit, 2001) in intensive culture farms (Eisa *et al*., 1993) in earthen ponds and in floating cages (Gamal *et al*., 2002; Abu El-Attah, 2003; Abdel-Hadi, 2004).

Motile Aeromonas Septicemia (MAS) is one of the most economic diseases affecting fish farms in Egypt (Atallah *et al*., 1999).

Aeromonas hydrophila is considered among the most pathogenic organisms to both homothermic and poikilothermic hosts including tilapia species (Amin *et al*., 1985; Zaki, 1991). It affects not only *O. niloticus* but also, causes severe outbreaks in *Cyprinus carpio* and *Mugil cephalus* (Marzouk and Nawal, 1991; Eisa *et al*., 1993).

Pseudomonas fluorescens, *P. putida*, *P. aeruginosa*, *P. chlorophis* and *P. anguilliseptica* were recognized as the causative agents of bacterial hemorrhagic septicemia in different species of fish (Robert, 1989; Schaperclaus, 1992; Abu El-Attah, 2003; Abdel-Hadi, 2004).

Resistance of *Aeromonas* and *Pseudomonas* sp. against the most commonly used antimicrobials in aquaculture has developed greatly in the recent years (Inglis *et al*., 1997; Kampfer *et al*., 1999; Ahmed and Shoreit, 2001; Taylor, 2003; Abdel-Hadi, 2004). Those antimicrobials have been so abused by fish farmers that they have been accumulated in the edible muscles and different internal organs of the treated fish (Somsiri *et al*., 1997). So, other environmentally safe alternatives including vaccination are recommended (Abdel-Hadi, 2004).

Indeed, the concept of vaccinating fish has been the subject of considerable research efforts world-wide (Wiegertjes, 2001). Vaccination is an important disease management strategy used to maintain human and animal

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health. Vaccines developed for aquaculture have reduced antibiotic use in fish production. Work in the 1990s showed the use of various strategies to develop modified live vaccines for use in fish. Modified live vaccines are advantageous in that they can be easily delivered (i.e., by immersion to young fish) and stimulate both humoral and cellular immunity of long duration. Disadvantages include issues with modified live vaccine safety to the host and environment (Craig *et al*., 2009).

Thus, this study was conducted to tackle the following objectives:

- Developing suitable and effective vaccines against Aeromonad and Pseudomonad septicemias using monovalent killed and live attenuated vaccines of *A. hydrophila* and *P. putida*, respectively
- Comparing the efficacy of both killed and live attenuated vaccines on the specific immune responses and antibody titers of the examined red tilapia
- Evaluating the immunity levels obtained via the I/P injection route of administration succeeded by the direct immersion route
- The ultimate goal is to reach a feasible and an environmentally safe system for tilapia vaccination to be applied in fish hatcheries

MATERIALS AND METHODS

Fish: A total of 160 red tilapia fish with average body weight of 250 g were used in this study. The fish were divided into 5 groups (groups A-D and control). Each group had 3 aquaria representing 3 replicates where 8 of the examined fish were kept in each aquarium (150×45×70 cc). One group was kept as control. The fish of the 4th replicate were kept for the replacement of natural mortality among the experimented fish. Water temperature was maintained to 27°C, dissolved Oxygen to 5.5 mg L^{-1} and pH to 7.2.

Preparation of vaccines: Heat-killed vaccines were prepared according to Chandran *et al*. (2002).

Live attenuated vaccine: Using the crude extract the natural herb of *Oreganum vulgare* via using bacterial colonies attenuated by the Minimal Inhibitory Concentration (MIC) of the herb.

Sterility and safety: Sterility and safety of the prepared vaccines were tested according to Ward (1982).

Treatments

I/P immunization of tilapia with heat-killed and live-attenuated vaccines: The examined fish were first anaesthetized using MS222 in the rate of 100 mg L^{-1}. The fish in group A and B were injected Intraperitoneally (I/P) with 0.1 mL containing 9×10^9 cfu mL^{-1} (Zaki, 1998) of heat-killed and live attenuated *A. hydrophila* vaccines respectively (Azad *et al*., 1997). Similarly, fish in groups C and D were injected I/P with 0.1 mL containing 9×10^9 cfu mL^{-1} of heat-killed and live attenuated *P. putida* vaccines. Two booster doses were given, 4 and 6 weeks, after the initial dose. Fish of the control group were I/P injected with 0.1 mL of Phosphate Buffer Saline (PBS).

Direct immersion vaccination: This dose was applied 8 weeks after the initial I/P immunization (and 2 weeks after the 2nd I/P booster dose) The examined fish were anaesthetized using MS222, immersed for 2 min in an ice box containing a hypertonic solution (1.5% sodium chloride) and directly immersed in 4 ice boxes containing dechlorintaed tap water with a final concentration of 1.5×10^9 cell mL^{-1} of killed, live attenuated *A. hydrophila*, killed and live attenuated *P. putida* vaccines, respectively for 2 min (Ahmed *et al*., 1995).

The fish of the control group were immersed in an ice box containing dechlorintaed water plus (PBS) only. The residual water-containing vaccines for both *A. hydrophila* and *P. putida* were disinfected by chlorine as a bactericidal agent to avoid any environmental pollution.

Periodical antibody titration in the sera of the examined fish: Six samples were taken in a regular basis; every 2 weeks. Blood samples were taken from the caudal vein of 3 fish per each treatment and serum samples were separated by centrifugation (Chandran *et al*., 2002). Antibody titers for both *A. hydrophila* and *P. putida* were detected in the sera using the direct agglutination test (10 Double-fold dilutions from 1:1 until 1:512) according to Ayub *et al*. (1997). Ab titers were also detected in 12 pre-immunized tilapia (6 fish for *A. hydrophila* and 6 for *P. putida*).

Activation of the bacterial isolates for the challenge test: The *A. hydrophila* and *Pseudomonas putida* strains, which were used for the challenge test were activated (Azad *et al*., 1997) 10 days before the challenge by serial I/P passage through live juvenile red tilapia fish for 3 times. Thus, they restored their virulence. Eighteen fish were used for the activation (3 fish were used per each passage per isolate).

Experimental challenge: Hot strains of *A. hydrophila* for the fish immunized by *A. hydrophila* vaccines and *P. putida* for the fish immunized by *P. putida* vaccines were injected I/P into the examined fish. Ten fish from each of the 4 treatments were taken and kept in 4 aquaria.

Twenty fish were taken from the control group and were kept in 2 aquaria with 10 fish per each (1 aquarium for *A. hydrophila* and the other for *P. putida*), where they were used as controls for the challenge test. The used challenging dose was 0.1 mL containing 7×10^7 cfu mL^{-1} (Zaki, 1998). The fish were noticed for 10 days (Azad *et al*., 1997) and the daily mortalities were recorded.

Statistical analysis: Was carried out using SPSS Repeated Measures ANOVA.

RESULTS AND DISCUSSION

The results revealed that antibody titers of the direct agglutination method were higher in fish vaccinated with live attenuated *A. hydrophila* vaccine than those obtained in fish vaccinated with heat killed *A. hydrophila* vaccine but with no significant difference. This is logic and agreed with the same fact in other farm animals and human (Craig *et al*., 2009).

However, Ab titers in the vaccinated fish with both types of vaccines were significantly, higher than those of the non vaccinated or control group (Table 1 and Fig. 1).

On the contrary, antibody titers were higher in fish vaccinated with heat killed *P. putida* vaccine than in fish vaccinated with live attenuated *P. putida* vaccine but also with no significant difference and both of them were significantly, higher than those of the non vaccinated tilapia (Table 2 and Fig. 2).

The I/P injection route induced higher Ab titers than obtained by immersion route, where Ab titers decreased dramatically after application of the immersion vaccination in both *A. hydrophila* and *P. putida* vaccines (Fig 1 and 2). However, these Ab titers

Table 1: Ab titers estimated by direct agglutination in sera of red tilapia vaccinated against *A. hydrophila*

Sample No.	Treatments	Time of blood sample	Antibody titers (Ab)	Mean Ab±SE
1	Heat-killed *A. hydrophila* vaccine	2 weeks after the initial	8	
2		immunization	8	10.6667±2.6667
3			16	
4	Live-attenuated *A. hydrophila* vaccine		8	8.0000±0.0000
5			8	
6			8	
7	Control		4	1.3333±1.3333
8			0	
9			0	
10	Heat-killed *A. hydrophila* vaccine	4 weeks after the initial	16	12.0000±4.0000
11		immunization	4	
12		(time of the 1st I/P	16	
13	Live-attenuated *A. hydrophila* vaccine	booster dose)	8	8.0000±0.00000
14			8	
15			8	
16	Control		0	5.3333±5.3333
17			16	
18			0	
19	Heat-killed *A. hydrophila* vaccine	6 weeks after the initial	32	32.0000±0.00000
20		immunization	32	
21		(time of the 2nd I/P	32	
22	Live-attenuated *A. hydrophila* vaccine	booster dose)	64	53.3333±10.6667
23			64	
24			32	
25	Control		0	5.3333±5.3333
26			16	
27			0	
28	Heat-killed *A. hydrophila* vaccine	8 weeks after the initial	32	32.0000±0.00000
29		immunization	32	
30		(time of the	32	
31	Live-attenuated *A. hydrophila* vaccine	immersion dose)	64	53.3333±10.6667
32			64	
33			32	
34	Control		0	2.6667±2.6667
35			0	
36			8	
37	Heat-killed *A. hydrophila* vaccine	10 weeks after the	16	10.6667±2.6667
38		initial I/P immunization	8	
39		And 2 weeks after	8	
40	Live-attenuated *A. hydrophila* vaccine	vaccination by	16	10.6667±2.6667
41		immersion	8	
42			8	
43, 44 and 45	Control		4, 8 and 0	4.0000±1.6924

Table 2: Ab titers estimated by direct agglutination in the sera of red tilapia vaccinated against *P. putida*

Sample No.	Treatments	Time of blood sample	Antibody titers (Ab)	Mean Ab±SE
1	Heat-killed *P. putida* vaccine	2 weeks after the initial	4	2.6667±1.3333
2		immunization	4	
3			0	
4	Live-attenuated *P. putida* vaccine		4	3.3333±0.6667
5			4	
6			2	
7	Control		0	0.0000±0.00000
8			0	
9			0	
10	Heat-killed *P. putida* vaccine	4 weeks after the initial	0	3.3333±2.4037
11		immunization	2	
12		(time of the 1st I/P	8	
13	Live-attenuated *P. putida* vaccine	booster dose)	32	24.0000±8.0000
14			32	
15			8	
16	Control		0	0.0000±0.00000
17			0	
18			0	
19	Heat-killed *P. putida* vaccine	6 weeks after the initial	64	37.3333±14.1107
20		immunization	32	
21		(time of the 2nd I/P	16	
22	Live-attenuated *P. putida* vaccine	booster dose)	32	32.0000±0.00000
23			32	
24			32	
25	Control		0	16.0000±9.2376
26			32	
27			16	
28	Heat-killed *P. putida* vaccine	8 weeks after the initial	64	42.6667±10.6667
29		immunization	32	
30		(time of the	32	
31	Live-attenuated *P. putida* vaccine	immersion dose)	32	32.0000±0.00000
32			32	
33			32	
34	Control		0	0.0000±0.00000
35			0	
36			0	
37	Heat-killed *P. putida* vaccine	10 weeks after the	4	5.3333±1.3333
38		initial I/P immunization	8	
39		And 2 weeks after	4	
40	Live-attenuated *P. putida* vaccine	vaccination by	8	10.6667±2.6667
41		immersion	16	
42			8	
43, 44 and 45	Control		0, 16 and 8	8.0000±4.6188

Table 3: Mortality and RPS of the examined fish challenged with *A. hydrophila* and *P. putida*

No. of days after challenge	Killed *A. hydrophila* vaccine	Live *A. hydrophila* vaccine	Control for *A. hydrophila*	Killed *P. putida* vaccine	Live *P. putida* vaccine	Control for *P. putida*
Daily mortalities						
1	-	-	-	-	-	-
2	-	-	-	-	-	1
3	-	-	2	-	-	-
4	-	1	5	2	-	3
5	-	2	1	1	-	2
6	-		2	1	-	1
7	-	1	-	1	1	1
8	1	-	-	-	1	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-
Total	1	4	10	5	2	8
RPS	90%	60%	0%	50%	80%	20%

RPS = Relative Percent Survival

induced by the immersion route were still significantly higher than non vaccinated fish of the control group. Similar results were recorded by Badran (1987), Ayub *et al*. (1997) and Rongxing *et al*. (2008).

On the other hand, all examined 12 pre-immunized tilapia had no Ab titers (0) against both *A. hydrophila* and *P. putida* except 1 fish with +ve Ab titer (1:4) against *A. hydrophila* and 1 fish with +ve Ab titer (1:1) against

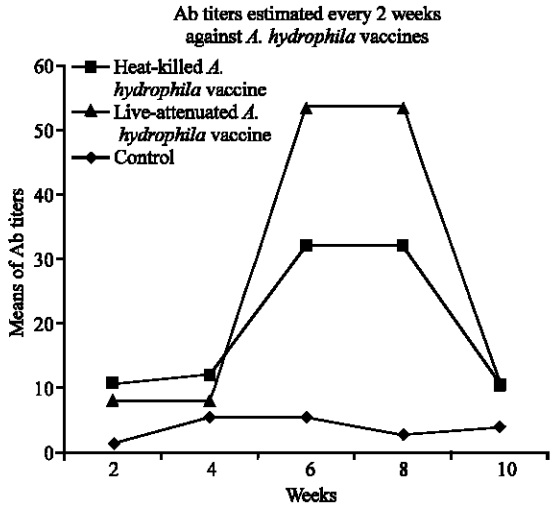

Fig. 1: Ab titers of *A. hydrophila* vaccines compared with the control group

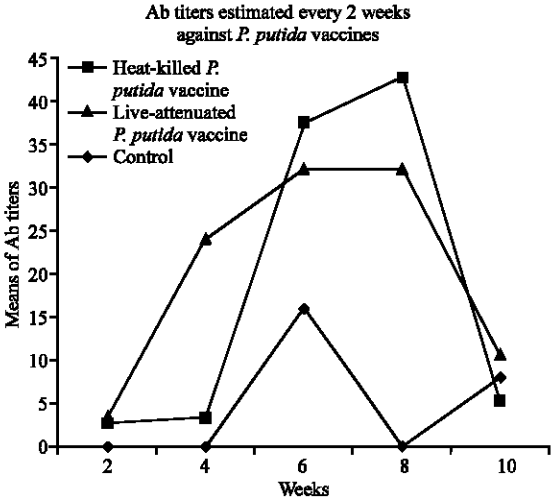

Fig. 2: Ab titers of *P. putida* vaccines compared with the control group

P. putida. This may be attributed to the fact that *A. hydrophila* is one of the natural aquatic flora, where it lives in water and gets its name referring to water. On the other hand, 1 of the pre-immunized fish had the antibodies of *P. putida*. This may be due to a sporadic infection.

Regarding the challenge test, results showed that heat killed *A. hydrophila* vaccine gave a higher level of Relative Percent Survival (RPS) (90%) for the vaccinated fish than the live attenuated vaccine (60% of RPS) and both had higher protection than fish of the control group (0% RPS). On the contrary, live attenuated *P. putida* vaccine produced a higher RPS (80%) for the vaccinated fish than heat killed vaccine (50%) and both had higher

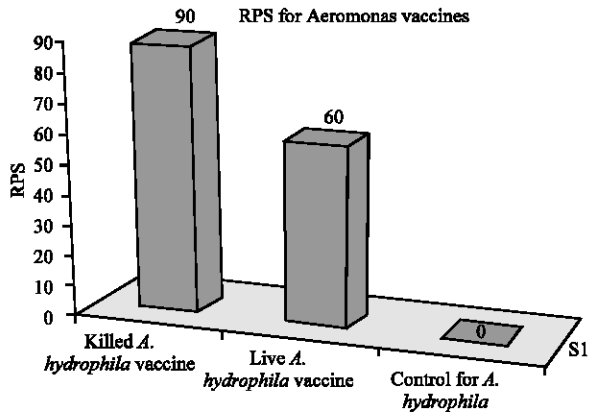

Fig. 3: RPS of *A. hydrophila* vaccines compared with the control group

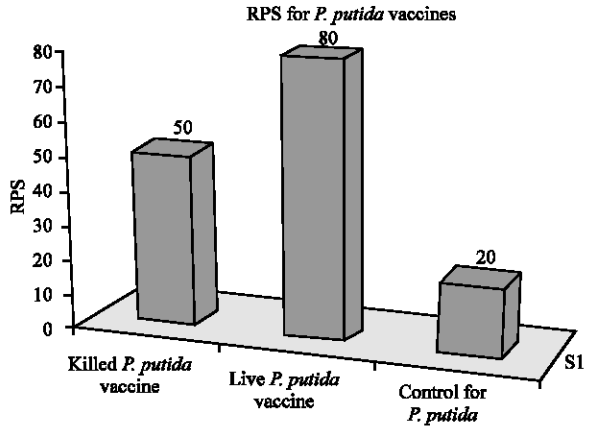

Fig. 4: RPS of *P. putida* vaccines compared with the control group

RPS than fish of the control group where RPS was 20% (Table 3, Fig. 3 and 4). This indicates the effective immunization induced by the experimented vaccines.

CONCLUSION

Heat-killed *A. hydrophila* and *P. putida* vaccines are recommended for the immunization of red tilapia against Aeromonad and Pseudomonad septicemias respectively. They are safer for the aquatic ecosystem than live-attenuated vaccines. Besides, no significant difference was found in this study between the heat-killed and live-attenuated vaccines for both bacterial species, regarding the antibody titers in the vaccinated fish.

The I/P injection is suitable for vaccination of the brood stock in fish hatcheries. However, an insulin syringe with thin needle should be strictly used to avoid excessive traumatic effects of the larger or thicker needles.

Direct immersion could be used for vaccinating frys and fingerlings in fish hatcheries especially, prior to release to the rearing ponds.

Further study on the development and evaluation of recombinant vaccines against *A. hydrophila* and *P. putida* is recommended.

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