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THE USE OF THYME; *Thymus vulgaris* EXTRACT TO DEVELOP INACTIVATED VACCINES AGAINST PATHOGENIC *Pseudomonas Putida* OF CULTURED RED TILAPIA; (*Oreochromis niloticus x O. mossambicus*)

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Abstract

This study aimed to investigate antibody titers and relative percent survival (RPS) of red tilapia vaccinated with Biofilm-coated vaccine (BFV) of *P. putida* with and without thyme extract. For this experiment, 375 red tilapia (25 fish/tank) have been used in 5 treatments including BFV of *P. putida* with 40 and 100 mg per ml thyme extract, BFV of *P. Putida* alone, thyme extract alone and control. The fish were kept in glass tanks for 12 weeks. Two booster doses of vaccines and other treatments were given at weeks 4 and 8 respectively after the initial dose. Blood samples were taken at zero day to get the pre-immune serum and every two weeks from the initial dose for antibody titration in the fish sera, using ELISA. Experimental challenge with virulent strains of *Pseudomonas putida, P. fluorescens* and *P. aeruginosa* has been done to test the vaccines efficacy and level of protection. Daily mortality was recorded for 14 days to estimate the cumulative mortality and relative percent survival (RPS) of the fish in different treatments. Results revealed that antibody titers and RPS in BFV of *P. putida* vaccine alone and BFV with 100 mg^{-ml} gave the highest IgM titers and RPS.

Keywords: *Pseudomonas putida*, Biofilm-coated vaccine, *Thymus vulgaris*, thyme extract

INTRODUCTION

Pseudomonas is a Gram negative bacteria that belongs to family Pseudomonadaceae. Although it is regularly reported to affect human and plant in many cases, it is also among the major causative agents of bacterial fish diseases. Fish under stress or being attacked by other pathogen may be infected with Pseudomonas as a secondary invader. Fish is associated with Pseudomonas species frequently (Cahill, 1990) and had been isolated from three different parts; gill, intestine and also skin (Colwell, 1962; Horsley, 1973; Trust and Sparrow, 1974 and Tripathy *et al*., 2006).

Major targets in the aquaculture industry are to maintain fish health as well as to improve fish performance. The use of plant extracts in practical diets for fish is a very topical concept in aquaculture. The main constituents of thyme (*Thymus vulgaris* Linnaeus) belonging to the Lamiaceae family. It is an aromatic plant with a wide distribution throughout the Mediterranean area and Asia (Vokou *et al*., 1993). *Thymus* species are considered as medicinal plants due to their pharmacological and biological properties. In native medicine, flowering parts and leaves of *Thymus* species have been extensively used as herbal tea, tonic, carminative, antitussive, and antiseptic as well as for treating colds (Rota *et al*., 2008).

Vaccination is among the best solutions to prevent fish diseases in aquaculture. There are different types of vaccination available and each of them has its own advantage and disadvantage. Adjuvant may be used in elicitation of immune responses which will give better protection against certain pathogens and may also cause toxicity. Scientists nowadays defined adjuvant as group of structurally heterogeneous compounds that are able to modulate immunogenicity intrinsically of an antigen (Guy, 2007; Tafalla *et al.*, 2013). This study was done to compare biofilm-coated *P. Putida* vaccine with and without thyme extract as an adjuvant to inactivate bacteria.

MATERIALS AND METHODS

Fish:

For this experiment, 375 red tilapia (hybrid of *Oreochromis niloticus x O.mossambicus*) with mean weight 7.22±0.25 g and mean length 3.56 ± 0.28 cm have been used in 5 treatments including 3 replicates per each treatment. Fish were kept in 120 liters glass tanks for 12 weeks.

Herbal extract:

Thymus vulgaris methanollic extract (MeOH) was prepared according to Şahin *et al*., 2004 with slight modification. Dried leaves were crushed into small particles and 500g were weighed before being extracted with 1 litre of methanol using Soxhlet extractor at 80 ºC for 72 h. Whatman filter paper (No 1) was then used to filtrate the extract before being concentrated using a rotary evaporator at 40 ºC.

Preparation of Biofilm-coated vaccines:

BF vaccines of *Pseudomonas putida* were prepared according to Azad *et al.,* 1997. *P. putida* isolate was grown on chitin flakes for 6 h a day for total of 4 days. Enumeration was done to confirm the desired concentration. BF-coated vaccines were heated for 90 ºC for 40 min to inactivate and confirm the sterility of the vaccines using Tryptic soy broth by zero growth on plate tested. For BF-coated vaccines of *P. putida* with 100 mg^{-ml} and 40 mg^{-ml} thyme extract, the bacteria were killed using the aforementioned concentrations of thyme extract. Control feed without any addition of vaccines was also prepared. Vaccines were then incorporated in feed to 10^{10}CFU $g^{-1}$by mixing with feed powder using homogenizer and loaded into the pelleting machine to obtain suitable pellet size before drying up in the oven at 65 ºC.

Oral vaccination:

375 tilapia (25 fish/tank) have been used in 5 treatments including BF of *P. putida* with 40 and 100 mg^{-ml} thyme extract, BFV of *P. Putida* alone, thyme extract alone and control (without addition of any herbs or vaccines to the

feed).The fish were fed with BFV incorporated diet at 10^{10}CFU g^{-1}concentration. Two booster doses were given at weeks 4 and 8 post immunization respectively. Blood samples were taken every two weeks starting from the initial dose for antibody titration using ELISA.

Enzyme-linked immunosorbent assay (ELISA):

Pre and post-vaccination sera were collected for ELISA in order to determine the level of antibody titers. ELISA was done (Kits and manual of Aquatic Diagnostics, Stirling University, Scotland) according to Ma *et al.*, 2006.

Challenge test:

Experimental challenge with hot strains of the *P. putida, P. aeruginosa and P. fluorescens* strains by I/P. injection of 0.1.ml/fish of $7x10^7$ of 24 hour broth culture (Abdelhadi *et al.,* 2009) of three strains has been done to test the vaccines efficacy and level of protection. Daily mortality was recorded for 14 days to estimate the cumulative mortality and relative percent survival (RPS) of the fish in different treatments.

RESULTS AND DISCUSSION

Pseudomonas sp. is commonly reported to affect human worldwide compared to fish. *P. aeruginosa* leading the Pseudomonas species that was reported causing in several cases pneumonia that may cause life-threatening. *P. putida* had also reported as a responsible human pathogen bacteria that may cause bacteraemia and sepsis in cancer patients, neonatal and neutropenia besides urinary tract infections (Martino *et al.,* 1996; Ladhani and Bhutta, 1998; Lombardi *et al.,* 2002; Perz *et al.,* 2005 and Altinok *et al.,* 2006). However, these bacteria are not primary causative agents of diseases and often play a role as secondary agents. Use of adjuvant is one of the ways to improve vaccines although its efficacy is still being doubt.

Immunization by BF vaccines of *Pseudomonas putida* without thyme extract gave higher antibody level of IgM compared to BF vaccines of *P. putida* with 100 mg and 40 mg^{-ml} thyme extract, thyme extract only and control group.

However, the additions of higher concentrations of thyme extract or oil in future work may be promising as it will protect tilapia from Pseudomonas septicemia and at the same time, the herbal extract will address and stimulate the non-specific immune response and general health status of fish.

Results indicated that there was no significant difference in using thyme extract as an adjuvant compared to BF vaccines without any addition of the herbal extract. However, the concentration (40 and 100 mg^{-ml}) used in the preparation of vaccines does give slightly different result of the two types of vaccination groups. This indicated that higher concentration might be needed in order for the herbs to be effective for the vaccination. Serum antibody produced in Biofilm of *P. putida* with 100 mg^{-ml}thyme extract is higher compared to 40 mg^{-ml} thyme extract vaccinated group (Figure 1) which resulted in lowest protection during challenge test (Figure 2). The Relative Percent Survival (RPS) in the BF vaccines of *P. putida* alone was greater compared to other vaccinated group in 14 days of challenge test. Immunized fish in BF-coated *P. putida* and BF-coated *P.putida* with 100 $mg^{-m\ l}$ thyme extract concentration showed higher RPS than non-immunized group. In this study, small size of fish had been used and this also confirmed that oral vaccination method is an efficient method of administration.

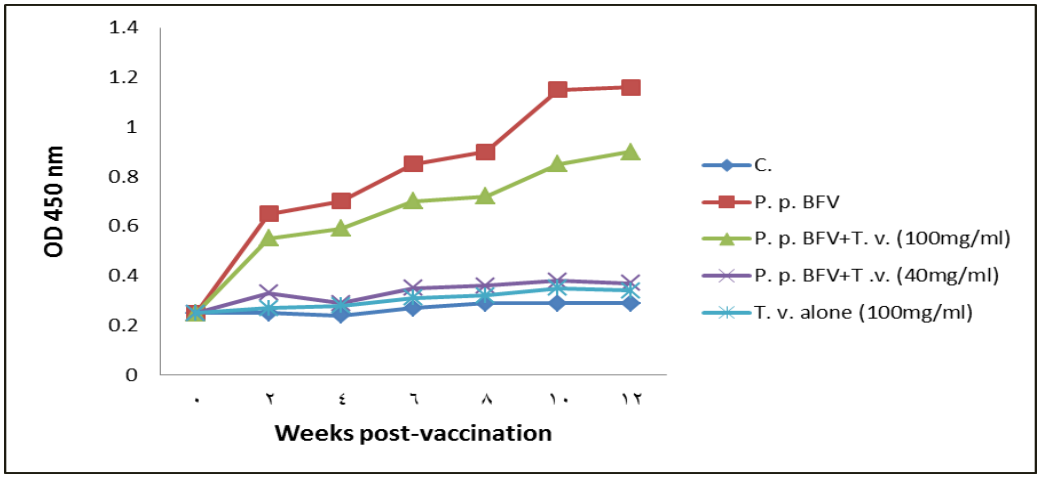

Figure 1. Antibody titers (IgM) of red tilapia vaccinated with *P. putida* BFV, *P. putida*BFV+T.v. (100mg/ml and 40 mg/ml) compared to control and T.v. alone (T.V.=*Thymus vulgaris*, P.p.= *Pseudomonas putida*).

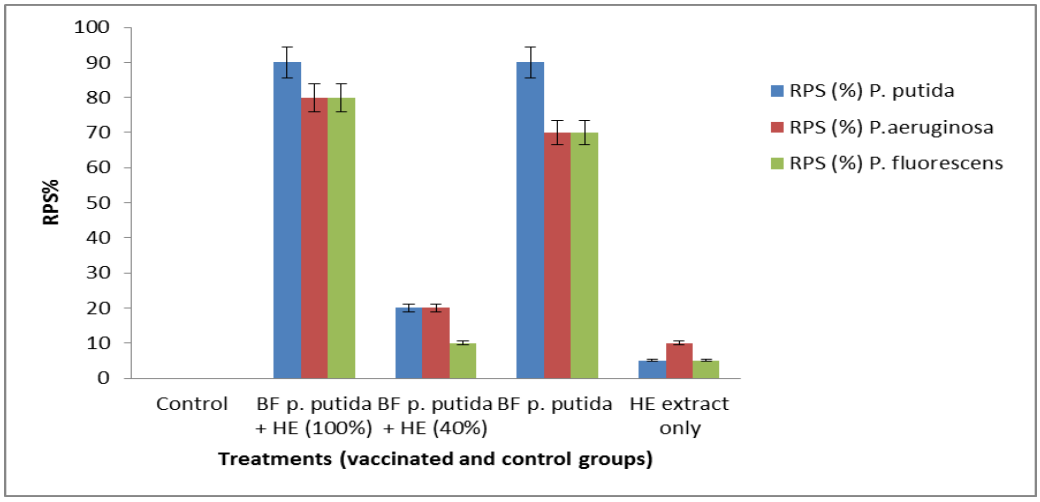

Figure 2. Relative percent survival (RPS) of vaccinated red tilapia post-challenge with *P. putida, P. aeruginosa and P. fluorescens*.

BFV of *P. putida* alone and addition of 100 mg^{-ml} herbs provided an excellent protection of red tilapia following oral vaccination. Hamdan et al., 1991 and Nour and Abou El-Ghiet, 2011 reported that antimicrobial activity of Pseudomonas species had contributed to several identified antibiotic-like substances including bacteriocins (*Pseudomonas aeruginosa* notably pyocin), a Phenazine antibiotic.

CONCLUSION AND FUTURE WORK

In conclusion, the biofilm (BF) vaccines alone without addition of any *Thymus vulgaris* adjuvant gave an excellent protection against *P. putida*, *P. fluorescens* and *P. aeruginosa*.

Biofilm-coated *Pseudomonas putida* with 100 mg^{-ml} thyme extract is promising as a novel vaccine with herbal adjuvant to cultured tilapia.

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استخدام خلاصة الزعتر (ثايمس فولجاريس) فى تطوير لقاحات ميتة لبكتيريا السيدوموناس الممرضة لأسماك البلطى الأحمر المستزرعة (هجين البلطى النيلى مع البلطى الموزمبيقى)

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الملخص العربى

أجريت هذه الدراسة لتحديد مستوى الأجسام المضادة ونسبة الإعاشة للبلطى الأحمر المطعم بلقاحات مغلفة وممزوجة بخلاصة الزعتر أو بدونه. وتمت هذه التجربة على عدد ٣٧٥ سمكة (٢٥ سمكة لكل حوض) فى ٥ معاملات أو مجموعات هى لقاح السيدوموناس بيوتيدا المغلف مضاف إليه خلاصة الزعتر بمعدل ٤٠ ، ١٠٠ ميللى جم على التوالى ثم مجموعة لقاحالسيدوموناس بيوتيدا المغلف بدون خلاصة الزعتر ومجموعة الأسماك التى غذيت على عليقة بها خلاصة الزعتر فقط بالإضافة إلى أسماك المجموعة الضابطة. وضعت الأسماك فى أحواض زجاجية لمدة ١٢ أسبوع. تم حقن الأسماك باللقاحات والمعاملات المذكورة بجرعتين مقويتين فى الأسبوع الرابع والثامن على التوالى بعد الجرعة الإبتدائية. أخذت عينات الدم من عدد ٦ سمكات قبل بداية التجربة ثم كل أسبوعين من الجرعة الإبتدائية لقياس مستوى الأجسام المضادة فى السيرم باستخدام الإليزا. وتم عمل اختبار التحدى التجريبى ليختبر مدى كفاءة اللقاحات ومستوى حماية الأسماك.وكان يتم تسجيل عدد الأسماك النافقة يوميا لمدة ١٤ يوم لتقدير النافق التراكمى والإعاشة النسبية للأسماك فى المعاملات المختلفة. وقد أظهرت النتائج أن مستوى الأجسام المضادة والإعاشة النسبية للأسماك المطعمة باللقاح المغلف للسيدوموناس بيوتيدا (بدون خلاصة الزعتر) وتلك الممزوجة بخلاصة الزعتر بتركيز ١٠٠ ملللى جم لكل مللى لتر هى التى أعطت أعلى قيم للأجسام المضادة والإعاشة النسبية.