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Original article

Effect of dietary supplementation with *Echinacea purpurea* on vaccine efficacy against infection with *Flavobacterium columnare* in zebrafish (*Danio rerio*)

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Abstract

The effect of dietary *Echinacea purpurea* (EP) on the response of zebrafish (*Danio rerio*) to a *Flavobacterium columnare* vaccine was investigated. Two hundred *D. rerio* with an average weight of 290 ± 40 g were selected and fed different levels of *E. purpurea* (5 g kg^{-1} diet – group 1, 10 g kg^{-1} diet – group 2, 20 g kg^{-1} diet – group 3, 30 g kg^{-1} diet – group 4, and 0 g kg^{-1} diet – group 5). Experimental feeding was begun 3 weeks prior to bath immunization and continued until the end of the experiment. Twenty-eight days after immunization the fish were challenged by bath immersion with *F. columnare* at a concentration of 1x106 CFU/ml. The relative percent survival of the experimental groups (1, 2, 3, 4 and 5) was 5.0, 6.0, 30.0, 36.0 and 5.0, respectively. In conclusion, diet supplementation with *E. purpurea* may effectively enhance the response of zebrafish to a *F. columnare* vaccine.

Key words: *Flavobacterium*, *Echinacea*, fish, zebrafish

Introduction

Echinacea purpurea (EP) has been shown to have non-specific immunostimulatory properties, including increased phagocytosis, cytokine production (TNFh, IL-1, IL-6 and IL-10), natural killer cell activity, chemotaxis and oxidative burst of either neutrophils or macrophages (Bauer 2002, Stevenson et al. 2005, Oskoii et al. 2012). The glycoproteins, polysaccharides, caffeic acid derivatives, isobutylamides and alkylamides in EP have been shown to have immunostimulatory activity (Burger et al. 1997). In fish, EP has been reported to increase specific growth rate, survival rate, resistance to challenge infection, total and differential white blood cell counts, lysozyme activity, and hematocrit and nitroblue tetrazolium values (Aly and Mohamed 2010, Kasiri et al. 2011, Oskoii et al. 2012). The effects of *Echinacea* supplementation on disease resistance have been studied in Nile tilapia (*Oreochromis niloticus*) (Aly and Mohamed 2010) and in the ornamental fish *Poecilia reticulata* (Guz et al. 2011).

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Flavobacterium columnare is an important pathogen of wild, farmed and ornamental freshwater fish. Prevention of columnaris disease by vaccination is an important goal of fish producers worldwide. Columnaris disease is mainly controlled by antibiotics in fish farming, but their continuous use often leads to the development of drug resistance. Vaccine development for columnaris disease has been in progress for a number of years (Shoemaker et al. 2007, 2009, Bebak et al. 2009). However, no study has been reported in the literature on the vaccination effect of dietary EP in fish.

Danio rerio have been suggested as an immunological model system and as a system for bacterial pathogenesis (Neely et al. 2002, Yoder et al. 2002, Moyer and Hunnicutt 2007). Recent reports of *F. columnare* infection of fish raise the possibility that infection of zebra fish might occur (Moyer and Hunnicutt 2007).

The main objective of the study was to examine the effect of EP in the diet of *D. rerio* on the effectiveness of vaccination against columnaris disease.

Materials and Methods

The study was approved by the Local Ethical Committee in Lublin (permit No. 56/2013).

A total of 200 adult zebrafish (*Danio rerio*) with an average weight of 290 ± 40 mg were obtained from a private farm in Lublin, Poland, and acclimatized for 7 days. The fish were kept in 20 L aquariums at 24 ± 1°C in filtered water. Water pH and dissolved oxygen were constant (7.3 – 7.6 and 80 – 90% saturation). The fish were divided into ten duplicate groups (20 fish/group) and fed according to five regimes.

Echinacea purpurea plants were grown at a local plant garden in the Lublin region in Poland. The aerial parts of the plants were collected, washed with tap water, air-dried and ground. An artificial diet was prepared using wheat flour (85, 81, 71, 61 and 91 g kg^{-1} diet in groups 1, 2, 3, 4 and 5, respectively), yellow lupine (80 g kg^{-1} diet), soybean meal (80 g kg^{-1} diet), corn meal (80 g kg^{-1} diet), soybean oil (20 g kg^{-1} diet), fish oil (30 g kg^{-1} diet), fishmeal (610 g kg^{-1} diet), vitamin C (1 g kg^{-1} diet), II-calcium phosphate (8 g kg^{-1} diet) and vitamin/mineral mix (IU or mg kg^{-1} diet): vitamin A, 4 400.00 IU; vitamin D3, 680.00 IU; vitamin E, 6.00 mg; thiamin, 0.60 mg; riboflavin, 1.20 mg; pyridoxine, 0.80 mg; vitamin B12, 6.00 mg; folic acid, 0.16 mg; biotin, 0.04 mg; niacin, 20.00 mg; Ca, 4.00 mg; Mn, 16.00 mg; Zn, 20.00 mg. Five diets were used, each differing in EP content (5 g kg^{-1} diet – group 1, 10 g kg^{-1} diet – group 2, 20 g kg^{-1} diet – group 3, 30 g kg^{-1} diet – group 4, and 0 g kg^{-1} diet – control group 5). The control diet was prepared using the same composition of ingredients, but without the plant EP. The ingredients were ground, milled, mixed and pelleted. The feeds were air dried and all diets were stored at -20°C until use. The fish were fed three times a day, at a rate of 2% of their biomass daily. During the acclimatization the fish were fed with the control diet. Experimental feeding was begun 21 days prior to the immunization and continued until the end of the experiment.

A virulent strain of *F. columnare* DR1 was isolated in our lab from the ascitic fluid of diseased zebrafish. After biochemical confirmation and virulence tests, the bacteria were maintained at -80°C as glycerol stock for further use. The *F. columnare* DR1 strain was grown in tryptic soy broth (Sigma) at 28°C for 24 h and then inactivated with 0.3% formalin and incubated for 14 h at 28°C. The strain was then centrifuged at 1,000 g for 30 min at 4°C. The supernatant was discarded and the bacterial pellet was suspended in PBS to obtain a concentration of 1 x 10^7 CFU/ml. After 21 days of feeding, both the vaccine groups (control fish and those fed experimental feed) were immunized by bath immersion for 10 minutes in a solution containing formalin-inactivated whole cells of *F. columnare* at a concentration of 1x10^7 CFU/ml. The unvaccinated groups were sham-bath vaccinated in pure water. For evaluation of efficacy, 28 days after immunisation the vaccinated and unvaccinated fish were challenged by bath immersion with a freshly grown culture of *F. columnare* suspended in PBS at a concentration of 1x10^6 CFU/ml. Mortalities were observed for 10 days to record mortality. Kidney samples were taken aseptically from dead and moribund fish, plated on tryptic soy agar (Sigma) and *Aeromonas* medium base (Rayan) to confirm the specific cause of death and morbidity. The potency of the vaccine was calculated as the relative percent survival (RPS) using the formula RPS = [l-(% mortality in vaccinated fish/% mortality in control fish)] x 100. Kaplan-Maier curves and a log-rank test were carried out using Statistica software for survival analysis of all challenge trials.

Results

Relative percent survival (RPS) of the experimental groups (1, 2, 3, 4 and 5) challenged with *F. columnare* (1x10^6 CFU/ml) were determined to be 5.0, 6.0, 30.0, 36.0 and 5.0, respectively (Table 1). The protection level was found to be dependent on the dose of EP in the diet. The log-rank test showed a significant difference in probability of survival between the vaccinated groups 3 and 4, and unvaccinated groups

Table 1. Protection of *Danio rerio* from *Flavobacterium columnare* challenge in laboratory aquarium trial

Protection rate (dead fish/total fish)	Groups				
	1	2	3	4	5
Vaccinated:					
Challenge 1	9/10	8/10	6/10	5/10	9/10
Challenge 2	9/10	9/10	6/10	4/10	10/10
Cumulative mortality (%)	90	85	60	45	95
Unvaccinated:					
Challenge 1	10/10	9/10	9/10	7/10	10/10
Challenge 2	9/10	9/10	8/10	7/10	10/10
Cumulative mortality (%)	95	90	85	70	100
RPS (%)	5	6	30	36	5
P-value	0.488	0.625	0.025	0.039	0.208

3 and 4, with P-values of 0.025 and 0.039, respectively (Table 1).

The log-rank test showed a significant difference in probability of survival between unvaccinated control group 5 and unvaccinated group 4, with P-values of 0.025. There was no significant difference between the unvaccinated group 5 and groups 1 (P=0.947), 2 (P=0.674), or 3 (P=0.723) (data not shown).

The log-rank test showed a significant difference in probability of survival between vaccinated control group 5 and vaccinated groups 3 and 4, with P-values of <0.0001 and <0.0001, respectively. There was no significant difference between group 5 and groups 1 (P=0.990) or 2 (P=0.912) (data not shown).

Discussion

F. columnare is the causative agent of columnaris disease, a common bacterial disease affecting the skin and gills of freshwater fish which may cause significant mortalities. Preventive methods against columnaris disease are scarce. Several immunomodulators have been used to prevent losses in fish farms (Sakai 1999). In previous research, dietary supplementation with EP has been found to enhance disease resistance against *A. hydrophila* in Nile tilapia (*O. niloticus*) (Aly and Mohamed 2010), and against *A. bestiarum* in guppy (*P. reticulata*) (Guz et al. 2011). Stevenson et al. (2005) demonstrated that *Echinacea* is an effective modulator of the macrophage immune response. Moreover, EP is used as a helper substance (adjuvant) in veterinary rabies vaccines to activate antigen presenting cells and to stimulate these cells to produce more cytokines which activate lymphocytes producing specific antibodies (Liu et al. 2012). Previous studies have evaluated the efficiency of different vaccine preparations against *F. columnare* infection and the humoral immune response induced by them (Grabowski et al. 2004, Shoemaker et al. 2007, 2009, Leal et al. 2010). Grabowski et al. (2004) reported that formalin-killed *F. columnare* administered intraperitoneally and by immersion could not stimulate a specific immune response in Nile tilapia without Freund;s complete adjuvant. At present, a modified live vaccine, AQUAVAC-COL™ (Merk & Co., Inc.), is commercially available to protect farm raised catfish against columnaris disease in the US. According to Kirkland (2010) no significant differences were found in survival rates of pond-raised catfish between those vaccinated with AquaVac-COL™ and unvaccinated fish. Recently, Mohammed et al. (2013) reported the efficacy of a new modified live genomovar II vaccine in channel catfish, zebrafish and Nile tilapia. Also in our study the administration of a vaccine by itself did not efficiently reduce the mortality of fish. The present study is the first to show that EP administered *per os* together with a vaccine against columnaris disease exhibits adjuvant activity by reducing mortalities of fish in a challenge test with *F. columnare*.

In conclusion, supplementation of feed with 20 and 30 g EP kg^{-1} diet enhanced the strength of vaccination against columnaris disease in the fish. The results of this experiment suggest that EP has potential for use as an adjuvant for vaccines when provided as a dietary supplementation.

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