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Short Communication

Dot-Enzyme-Linked Immunosorbent Assay (Dot-ELISA) for the Diagnosis of *Edwardsiella tarda* Infection in Fish

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Abstract

A dot-Enzyme-Linked Immunosorbent Assay (ELISA) for rapid and confirmatory identification of *Edwardsiella tarda* in infected and dead fish through detection of its antigen in the tissues like liver, spleen and kidney was developed. The hyperimmune sera (HIS) raised in rabbit against *E. tarda* was adsorbed with other related fish bacteria to eliminate possible cross reactions in the test. The test significantly and specifically detected the *E. tarda* antigen in decayed tissues within 48 to 72 h upon death. It is suggested that this test be used for the detection and diagnosis of *E. tarda* infection in fish.

Introduction

Edwardsiella tarda is a common aquatic bacterium responsible for mass mortality in many fish species in Asia especially in Japan, in India and in the USA (Herman and Bullock 1986; Sahoo and Sahoo 1997). In India, the disease occurs frequently in adult as well as in fry and fingerling and the current trends of diagnosis are based on clinical signs, gross and histopathological lesions and elaborate procedures of bacterial isolation and

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characterization, that are not only time consuming but also expensive and require elaborate laboratory facilities. These tests moreover, cannot be applied to diagnose the disease in dead and putrefied fish received in the laboratory from far away places of outbreaks. The ELISA is widely used in human and veterinary medicine and has also been used in fish disease diagnosis (Klesius and Johnson 1991; Lewis 1986; Rodak et al. 1995). Since reports in detecting the bacterial antigen in tissues of infected fish are scanty, the present study was therefore attempted to develop and standardize an ELISA technique to detect *E. tarda* specific antigen in tissues of dead/moribund fishes since a diagnostic tool has been made.

Materials and Methods

A virulent strain of *E. tarda* maintained at the Bacteriology Laboratory of the Aquatic Animal Health Division of the Central Institute of Freshwater Aquaculture, Bhubaneswar, India was grown in brain heart infusion (BHI) broth (Hi Media; Mumbai) at 30°C for 24 h and washed in phosphate buffer saline (PBS, pH 7.2). The number of bacterial cells in PBS were adjusted to 10^9 cells·ml using Mcfarland's standard and were further checked through colony count in nutrient agar plates. The 24 h old culture of virulent *Aeromonas hydrophila* and *Pseudomonas fluorescens* were obtained from the same division for their use in adsorption test. These two organisms were selected for absorption with anti *E. tarda* serum because of their co-existence and cross reactivity with *E. tarda*.

The hyperimmune sera (HIS) against *E. tarda* were raised in 6 mo old rabbits that were injected intramuscularly with 10^9 cells·ml with equal volume of Freund's complete adjuvant (FCA) followed by a booster with Freund's incomplete adjuvant (FIA) at every alternate week. The animals were bled for serum through the ear vein, seven days after the 2nd booster and serum samples were inactivated at 56°C for 30 min. The HIS was then adsorbed with *A. hydrophila* and *P. fluorescens* cells to avoid possible cross reaction. For this purpose 1 ml of serum was added to twice the volume of washed and packed *A. hydrophila* and *P. fluorescens* cells. The mixtures were incubated for 1 to 2 h with continuous mixing and the bacteria was removed by centrifugation. The antibody titre in adsorbed HIS and the presence of further antibodies to *A. hydrophila* and *P. fluorescens* were checked using the agglutination test (Klesius and Johnson 1991).

Fingerlings of Rohu (*Labeo rohita*) with an average weight of 30 ± 5 g) and *Anabas testudineus* (ave. wt. 17.5 ± 2.5 g) were reared separately in 300 l tanks with controlled feeding and water was changed regularly. The water temperature and oxygen content were monitored regularly. Ten fish of each species were injected intraperitoneally with 0.1 ml of suspension containing 10^8 cells·ml. Five fish of each species were reared separately as control and were injected intraperitoneally with 0.1 ml of PBS. These fishes were kept under constant observation for a period of seven days for any clinical signs or gross lesions. From the dead fishes, tissues were collected immediately after death

and at 24, 48 and 72 hours upon death, were minced with equal volume of PBS and stored at -20°C till further use. Likewise, the tissues collected from live healthy fishes (control) were prepared in a similar manner and used as the negative control.

Nitrocellulose paper (NCP) strips of 5 x 5 mm² were coated separately with 2 to 3 µl of tissue preparations for the test and *E. tarda* bacterial suspension as positive control. The negative controls included the NCP coated with preparation from healthy tissue, *A. hydrophila* and *P. fluorescens*, respectively. The coated NCP strips were dried at 65°C for 2 h in an incubator and then blocked in PBS containing 0.05% Tween – 20 (PBS-T) and incubated with 1:100 dilution of HIS, washed in PBS-T and incubated further with anti rabbit horse radish peroxidase conjugate (Genei, Bangalore, India) at 1:2000 dilution for 1 h at 37°C. The strips were washed several times before putting them on the substrate solution consisting of 5 mg of 3,3-diaminobenzidine tetrahydrochloride (DAB), 10 µl of 38% hydrogen peroxide and 5 ml of 50 mM Tris buffer (pH.7.6) till development of brown coloration. These were then washed in running tap water and dried at room temperature before evaluation.

All the fish experimentally infected with *E. tarda* died within 24 to 48h and characteristic septicaemic cutaneous lesions in rohu were observed whereas in *Anabas* sp no marked gross lesion was noticed but *E. tarda* could be reisolated from the organs of the dead fish to confirm death due to *edwardsiellosis*. The adsorbed HIS used in this study had an agglutination titre of 1:256 and did not cross react with *A. hydrophila* and *P. fluorescens* either in the agglutination test or ELISA.

In ELISA, brown coloration was developed on the NCP coated with infected tissues such as liver, spleen and kidney (Fig. 1). No color development was seen in the negative samples; suggesting that the test is very specific.

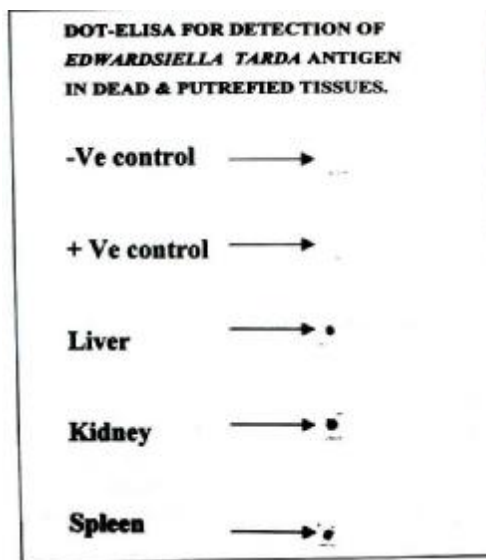


Fig. 1. Detection of *E. tarda* specific antigen in tissues of infected fish 48 hours after death.

Drying of the samples at 65°C for 2 h eliminated the possibility of false color development due to destruction of endogenous peroxidase activity as suggested by Ahmad et al (1992). The test could detect *E. tarda* specific antigen in these organs of all experimentally infected dead fish but only in seven out of eight suspected cases of *edwardsiellosis* received from field outbreaks. The result also coincided with the isolation of bacteria from the tissues. It was possible to detect the *E. tarda* antigen in the putrefied organs up to 72 h upon death. This is unsuitable for bacteriological tests thus justifying the suitability of the test over other conventional tests in detecting the *E. tarda* infection in fish.

The color matching of the NCP coated with positive antigen (10⁹ cells/ml) with those coated with test materials made on the basis of visual observation revealed differences in color intensity with different tissues which directly depended on the antigenic concentration. Antigen concentration was higher in the kidney tissue followed by the spleen and liver, hence kidney may be the tissue of choice for diagnostic purposes. This is the first attempt to detect *E. tarda* antigen in tissues, although ELISA has been used to detect the *E. ictaluri* antibodies in channel catfish by several workers (Klesius and Johnson 1991; Waterstrat et al. 1989).

Conclusion

Since the diagnosis of a particular infection based on the detection of its causative agent always proves to be the best method, this test therefore, can be very well utilized for the diagnosis of *E. tarda* infection in clinical cases and in dead fish received at the laboratory from far away places. Tissue samples preserved on ice will be sufficient for this purpose. Similar tests for other economically important bacterial infection of freshwater fish are in the process of development.

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