A COMPARATIVE STUDY OF THE IMMUNOLOGICAL PROPERTIES OF

EXTRACELLULAR PRODUCTS BETWEEN VIRULENT AND

LESS VIRULENT *EDWARDSIELLA TARDA*

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A COMPARATIVE STUDY OF THE IMMUNOLOGICAL PROPERTIES OF

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VITA

Alyssa Ann Wiedenmayer, daughter of George and Patricia (Meek) Wiedenmayer, was born August 1, 1977, in Chestertown, Maryland. She graduated from Kent County High School as Salutatorian in 1995. She entered Washington College in Chestertown, Maryland in August 1995, and graduated *magna cum laude* with a Bachelor of Science degree in Biology in May, 1999. Upon graduation, she worked as a Graduate Assistant Rowing Coach and attended classes in the graduate program in Psychology at Washington College. She received a Masters of Arts degree in May, 2002. After working as a Laboratory Technician at the USDA-ARS Aquatic Animal Health Research Laboratory in Chestertown, Maryland until December, 2002, she transferred to the USDA-ARS Aquatic Animal Health Research Laboratory in Auburn, Alabama where she entered the graduate program in the Department of Fisheries and Allied Aquacultures in January, 2003.

DISSERTATION ABSTRACT

A COMPARATIVE STUDY OF THE IMMUNOLOGICAL PROPERTIES OF EXTRACELLULAR PRODUCTS BETWEEN VIRULENT AND LESS VIRULENT *EDWARDSIELLA TARDA*

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The immunological properties of the extracellular products (ECP) of the *Edwardsiella tarda* parent FL6-60 and mutant RET-04 isolates were investigated in Nile tilapia *Oreochromis niloticus*. Non-purified ECP derived from the FL6-60 parent and RET-04 mutant stimulated *in vitro* migration of macrophages and the movement was demonstrated to be predominantly chemokinetic. Semi-purification of the ECP by high pressure liquid chromatography (HPLC) identified components with molecular weights of 31.62, 3.19, and 0.60 kDa for the FL6-60 parent, and 5.65, 0.55, and 0.10 kDa for the RET-04 mutant ECP. Analysis of chemoattractant activity for both the semi-purified FL6-60 parent and RET-04 mutant ECP revealed primarily chemotactic migration and macrophage chemotaxis was increased over the non-purified fractions. In assays of

both non-purified and semi-purified ECP fractions, differences between macrophage movement induced by the FL6-60 parent and RET-04 mutant ECP were minimal. Additionally, protective immunity conferred by passive immunization was investigated. Hyperimmune serum was obtained from fish actively exposed to and challenged with *E. tarda* FL6-60, while nonimmune serum was acquired from fish IP administered tryptic soy broth (TSB). Fish were passively immunized by IP injection with pooled hyperimmune or nonimmune serum, or phosphate buffered saline (PBS), as a control, and sera were collected at 72 h after immunization. Antibody titer was significantly ($P < 0.0001$) higher in fish obtained from the hyperimmune group (1:25585) than in the nonimmune (1:606) or PBS (1:399) groups. Mean cumulative percent mortality was significantly ($P < 0.0001$) lower in the hyperimmune group (6.7%) than in the nonimmune (26.7%) or PBS (17.9%) groups. Immunization by intraperitoneal (IP) injection of FL6-60 parent and RET-04 mutant ECP was also studied. Following challenge, differences in cumulative percent mortality of the FL6-60 parent (64.6%), and RET-04 mutant (63.8%) ECP immunized groups, and TSB control group (79.5%) were not significant ($P < 0.3541$). Characterization of the FL6-60 parent and RET-04 mutant ECP by electrophoretic analysis revealed differences in banding profiles between the FL6-60 parent and RET-04 mutant ECP, most noticeably with a band of approximately 175 kDa resolved in the RET-04 mutant ECP absent in the FL6-60 parent ECP.

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I. INTRODUCTION AND LITERATURE REVIEW

The aquatic environment contains approximately 60 to 70 bacterial species capable of causing disease in fish populations (Plumb 1999). *Edwardsiella tarda*, the causative agent of *Edwardsiella* septicemia, is a bacterium found in marine and freshwater environments in both bottom substrate and water. This pathogen is responsible for major economic losses worldwide in many species of cultured fishes such as Japanese eel *Anguilla japonica*, with 7 million dollars loss reported for the year 1984 (Salati 1988), red sea bream *Pagrus major*, Japanese flounder *Paralichthys olivaceus*, channel catfish *Ictalurus punctatus*, and Nile tilapia *Oreochromis niloticus* (Hoshina 1962; Meyer and Bullock 1973; Austin and Austin 1987; Plumb 1999).

Taxonomy

Edwardsiella tarda is a member of the family Enterobacteriaceae, commonly known as the enteric or intestinal bacteria. The genus *Edwardsiella*, named in honor of microbiologist P.R. Edwards of the United States Centers for Disease Control and Prevention (CDC), presently contains three distinct species, *E. hoshinae*, *E. ictaluri*, and *E. tarda*. The genus was established based on the simultaneous discovery, in 1959, of related isolates obtained from human feces in the United States (Ewing et al. 1965) and snakes in Japan (Sakazaki 1967). *Edwardsiella hoshinae*, isolated from birds, reptiles, and water, was first described in 1980 (Grimont et. al 1980). This species was distinguished from *E. tarda* by its utilization of malonate as a carbon source and

production of acid from D-mannitol, as well as through DNA hybridization studies (Grimont et al. 1980). *Edwardsiella ictaluri*, originally isolated by Hawke in 1976**,** is the pathogen responsible for enteric septicemia of catfish (ESC) (Hawke 1979; Hawke et al. 1981)**.** *Edwardsiella ictaluri* was differentiated from *E. tarda* based upon negative reactions in several biochemical analyses, including production of indole from tryptophan and hydrogen sulfide (H_2S) from glucose on triple sugar iron agar, and a lack of significant DNA homology (Hawke et al. 1981). Recent research has also recognized interspecies differences between *E. ictaluri* and *E. tarda*. Random fragment length polymorphism (RFLP) comparison of the 16S-23S rRNA intergenic spacer regions substantiated the close genetic relationship of *E. ictaluri* and *E. tarda*, but also identified specific restriction sites enabling differentiation between species (Panangala et al. 2005). Enteric septicemia of catfish is recognized as the most economically devastating bacterial disease threat to the cultured catfish industry in the United States, responsible for losses in 52.9% of all fry/fingerling operations totaling millions of dollars annually (Hawke et al. 1998; Veterinary Services Centers for Epidemiology and Animal Health 2003).

The first *Edwardsiella* species to be described, *E. tarda*, has been identified as the etiological agent responsible for chronic to acute *Edwardsiella* septicemia in a variety of fish species globally. *Edwardsiella* septicemia was probably initially observed, in fish, in cultured eel in Japan, with the pathogen responsible reported as a new bacterium named *Paracolobactrum anguillimortiferum* (Hoshina 1962). Subsequent controversy arose with the taxonomic classification of this isolate as it appeared biochemically and biophysically identical to groups of strains, previously known as bacterium "1483-59" (Ewing et al. 1965), the "Asakusa group" (Sakazaki and Murata 1962), and the

"Bartholomew group" (King and Adler 1964), combined and designated *E. tarda* by Ewing et al. (1965). As a *P. anguillimortiferum* type strain was never registered and the original culture lost, a change from the specific epithet of *tarda* to *anguillimortiferum* was proposed for the organism commonly known as *E. tarda* since *anguillimortiferum* had been published first (Sakazaki and Tamura 1975). Although this dispute has never been formally resolved, *E. tarda* remains the accepted name and type species for this bacterium due to its more common usage (Sakazaki and Tamura 1975; Farmer et al. 1976; Abbott and Janda 2001).

Organism characteristics

Edwardsiella tarda is a gram-negative, rod shaped bacterium typically 2-3 μm in length. The bacterium is nonencapsulated and usually motile by uniformly distributed flagella (Farmer and McWhorter 1984). It is facultatively anaerobic, catalase positive, cytochrome oxidase negative, and capable of reducing nitrate to nitrite. Several distinctive characteristics of *E. tarda*, including indole production, fermentation of glucose, and production of H_2S gas, are also useful in presumptive identification of this bacterium and differentiation from other members of the genus (Plumb 1999). In an investigation of 116 isolates collected from the United States and Taiwan, Waltman et al. (1986) determined the species was essentially homogeneous in its biochemical and enzymatic characteristics, although Farmer and McWhorter (1984) described both a wild type and an additional biogroup 1. Biogroup 1 strains of *E. tarda* ferment sucrose, mannitol, and arabinose as well as glucose, but do not produce H_2S (Farmer and McWhorter 1984; Abbott and Janda 2001). A recent investigation examining intraspecific characteristics of nine fish pathogenic *E. tarda* isolates found high similarity

(95%) in comparison of fatty acid methyl ester (FAME) profiles, but reported much lower similarity (30%) through protein analysis of the isolates (Panangala et al. 2006). These results corroborated previous findings of phenotypic diversity within the species (Nucci et al. 2002; Tan et al. 2002). Also, Park et al. (1983) described four serotypes, identified as A, B, C, and D, recognized in isolates obtained from eel, water, and sediment samples. They observed serotype A to be the most virulent in fish infected experimentally. These findings were supported by a study examining *E. tarda* isolated from diseased Japanese flounder where 28 isolates associated with *Edwardsiella* septicemia were categorized as serotype A (Rashid et al. 1994a). Currently, the internationally accepted system of serotyping *E. tarda*, based on unique somatic (O) and flagellar (H) antigens, has recognized 61 O groups and 45 H antigens within the species (Tamura et al. 1988).

Epidemiology

Edwardsiella tarda is considered a ubiquitous organism and has been identified in animal hosts and environmental samples of water and sediments worldwide. Though the geographic range of this bacterium is extensive, countries where isolation has been significant include Israel, Japan, Taiwan, Thailand, the United States, and many areas deemed "developing" (Plumb and Evans 2006). The natural reservoir of *E. tarda* is thought to be the intestinal tract of animals from which deposition of feces allows the dissemination of the organism into the ecosystem and infection most likely occurs through injured epithelium and the intestine (Farmer and McWhorter 1984; Plumb and Evans 2006). White et al. (1973) reported detection of *E. tarda* from freshwater lakes and streams. Wyatt et al. (1979) found 75% of 86 water and 64% of 86 sediment samples

obtained from catfish ponds positive for *E. tarda*. In an investigation of Japanese flounder farms, *E. tarda* was isolated from 86% and 22% of water and 44% and 0% of sediment samples in two ponds, respectively, however; no clinical disease was observed at either location (Rashid et al. 1994b). Ishihara and Kusuda (1982) assessed the ability of *E. tarda* to survive in environmental water from multiple sources possessing varying pH levels and NaCl concentrations at different temperatures. It was determined that *E. tarda* remains viable at pH of 4.0-10.0, NaCl content of 0-4.0%, and temperatures from 15-45° C (Ishihara and Kusuda 1982). While the versatile nature of *E. tarda* is apparent, studies have suggested variations in environmental conditions (e.g. salinity and temperature) impact the virulence of the bacterium (Darwish et al. 2001; Zheng et al. 2004). Yasnobu et al. (2006) have suggested hemagglutinating activity and the expression of a 19.3 kDa fimbrial protein are increased at higher NaCl concentrations and virulence of *E. tarda* enhanced.

Edwardsiella tarda is widely considered an opportunistic pathogen, and although not necessarily critical to the onset of *Edwardsiella* septicemia, the addition of environmental stressors such as temperature fluctuations, degraded water quality, and crowding can increase the incidence and severity of the disease in fish species (Meyer and Bullock 1973; Plumb 1999). Temperature appears to influence the development of *Edwardsiella* septicemia, with an optimal range of approximately 20-30° C for transmission in most fish species (Plumb and Evans 2006). In cultured channel catfish, epizootics occur most often at temperatures exceeding 30° C (Meyer and Bullock 1973; Plumb 1999). Egusa (1976) found increased levels of infection in Japanese eels were observed during the summer months when temperatures were highest. *Edwardsiella*

septicemia also developed in cultured brook trout during periods of warmer temperatures (Uhland et al. 2000). In several reports, seasonal temperature fluctuations were implicated as a potential contributor to disease outbreaks in wild fish populations (Amandi et al. 1982; Francis-Floyd et al. 1993; Baya et al. 1997). Water quality has also been identified as a factor in the development of *Edwardsiella* septicemia. Walters and Plumb (1980) reported the development of *E. tarda* infections in 43% of experimentally environmentally stressed juvenile channel catfish. Japanese eels displayed less resistance to *E. tarda* infection when concentrations of copper in experimental water ranged from 100-250 µg/L. The concentrations were sublethal, but yielded an increase in susceptibility to *E. tarda* comparable to that observed with administration of corticosteroids, hormones related to the stress response (Mushiake et al. 1984). Uhland et al. (2000) suggested high organic loads in culture ponds contributed to the occurrence of disease in brook trout, while Wyatt et al. (1979) observed a noticeable increase in the presence of *E. tarda* in catfish ponds when organic content was higher. Mortalities from *E. tarda* infection have also been attributed to the exposure of fish to crowded conditions. Meyer and Bullock (1973) reported a less than 5% occurrence of *E. tarda* in cultured channel catfish ponds, but confinement in holding tanks following harvest resulted in rapid disease onset with nearly 50% loss.

Hosts

Edwardsiella septicemia is most often seen in warmwater environments, though mortalities in coldwater species such as wild chinook salmon *Oncorhynchus tshawytscha* in the northwestern United States have been documented (Amandi et al. 1982). In addition, *E. tarda* has been implicated in disease outbreaks of farmed rainbow trout

Oncorhynchus mykiss in Australia (Reddacliff et al. 1996) and broodstock brook trout *Salvelinus fontinalis* in Canada (Uhland et al. 2000) occurring during periods of increased temperature and suboptimal environmental conditions. Natural infections in wild largemouth bass *Micropterus salmoides* (Francis-Floyd et al. 1993) and striped bass *Morone saxatilis* (Baya et al. 1997) have also been reported. Warmwater cultured fish are often susceptible to infections, especially channel catfish, Japanese eel, and Nile tilapia (Plumb 1999). *Edwardsiella* septicemia was first observed in channel catfish on farms located in the southeastern United States (Meyer and Bullock 1973). In Japan, *Edwardsiella* septicemia has been responsible for considerable losses in several species including eels, Japanese flounder, and red sea bream (Kusuda and Salati 1993; Igarashi and Iida 2002). *Edwardsiella* spp. infection has been found in Nile tilapia reared through integrated systems utilizing pig manure as fertilizer (Muratori et al. 2000) or exposed to environmental stressors (Benli and Yildiz 2004). Fifty of 200 randomly selected Nile tilapia sampled from a rearing facility in Egypt demonstrated clinical signs of *Edwardsiella* septicemia (Saleh 2005). *Edwardsiella tarda* was also identified in kidneys of outwardly healthy tilapia tetrahybrids *Oreochromis mossambicus* x *O. niloticus* x *O. urolepis hornorum* x *O. aureus* farm-reared in Venezuela (Clavijo et al. 2002). Additionally, *E. tarda* isolation from ornamental aquarium fish, including freshwater angelfish *Pterophyllum scalare* and blue gourami *Trichogaster trichopterus*, has been described (Vandepitte et al. 1983; Dixon and Contreras 1992).

Edwardsiella tarda is not restricted to fish and is often considered normal commensal flora of numerous aquatic organisms (Wyatt et al. 1979; Vandepitte et al. 1983). Reptiles and amphibians have been identified as important reservoirs of the

bacterium. *Edwardsiella tarda* has been isolated from snakes, lizards, alligators, and turtles, as well as frogs and toads (Sakazaki 1967; Wallace et al. 1966; White et al. 1969; White et al. 1973; Kourany et al. 1977; Nagel et al. 1982). Birds are also recognized as potential carriers of the bacterium and may be an integral component in dissemination. White et al. (1973) reported the presence of *E. tarda* in several aquatic bird species such as the brown pelican. In another study, gull feces was implicated as a possible source of contamination at seafood processing facilities (Berg and Anderson 1972). *Edwardsiella tarda* is also capable of infecting a variety of mammalian species. The presence of this organism has been reported in monkeys (Kourany et al. 1977), cattle (Ewing et al. 1965), swine (Owens et al. 1974), and marine mammals including sea lion and porpoise (Wallace et al. 1966; Coles et al. 1978).

Edwardsiella tarda infection in humans, while relatively infrequent, yields several different pathological conditions. The continuum of disease caused by *E. tarda*, comparable to that of *Salmonella* spp., includes an asymptomatic intestinal carrier state, gastroenteritis of varying severity, typhoid-like enteric fever, and extra-intestinal infections (Jordan and Hadley 1969; Vandepitte et al. 1983). In greater than 80% of cases, the presence of the bacterium is correlated with gastrointestinal illness, however, reports of extra-intestinal infections have become more common (Slaven et al. 2001). It has been suggested that underlying immunosuppressive conditions, such as hematologic or hepatic disease, may increase risk for *E. tarda* infection though disease may appear in healthy individuals (Sonnenwirth and Kallus 1968; Slaven et al. 2001). Bockemühl et al. (1971) observed a more severe disease course in both infants and adult patients over 50 years of age. A relationship between the incidence of *E. tarda* septicemia and preexisting

conditions associated with iron overload including red cell sickling, neonatality, leukemia, and cirrhosis has been reported (Janda and Abbott 1993). One of the earliest publications describing *E. tarda* detailed the characteristics of a previously unnamed bacterium obtained from a patient displaying enteric fever and gastroenteritis (King and Adler 1964). *Edwardsiella tarda* associated gastrointestinal disease has been observed globally occurring most often in tropical and subtropical regions (Kourany et al. 1977; Vandepitte et al. 1983; Janda and Abbott 1993). Often, exposure to the aquatic environment and its inhabitants appears to be a precursor to *E. tarda* related disease and the zoonotic impact of this bacterium is important to consider. Potentially serious soft tissue infections, resulting from injuries occurring in water or related to aquatic animals, have been documented. Typically, these wounds involve the formation of abscesses or penetrating injuries of epithelial surfaces (Janda and Abbott 1993). Clinical signs range from mild cellulitis to severe necrosis (Jordan and Hadley 1969; Clarridge et al. 1980; Hargreaves and Lucey 1990; Janda and Abbott 1993; Slaven et al. 2001). *Edwardsiella tarda* has also been isolated from rectal, gynecologic, and hepatic abscesses (Clarridge et al. 1980; Slaven et al. 2001). In rare cases, *E. tarda* infection has been implicated in meningitis, sepsis, and even death (Sonnenwirth and Kallus 1968; Clarridge et al. 1980; Slaven et al. 2001). Due to its common presence in the environment, *E. tarda* is recognized as a potential food borne pathogen. Consumption of raw or improperly handled fish or seafood products processed in contaminated facilities may lead to illness (Berg and Anderson 1972; Meyer and Bullock 1973; Wyatt et al. 1979; Van Damme and Vandepitte 1980; Slaven et al. 2001).

Edwardsiella septicemia in eel

The clinical signs and severity of *Edwardsiella* septicemia varies between fish species. The infection is also known as hepatonephritis in eel and emphysematous putrefactive disease, or fish gangrene in channel catfish (Plumb 1999). In eel, affected individuals display lethargic behavior and may float near the water surface. External signs include hyperemia of the fins, petechia on the body surface, especially the belly, and a hyperemic and swollen anal region. Infected eels may also develop gas filled pockets between the dermis and underlying muscle. Internal signs typically include whitish coloration of and abscess development and swelling in the liver, kidneys, and spleen (Egusa 1976; Miyazaki and Kaige 1985; Plumb 1999; Plumb and Evans 2006). Study of *Edwardsiella* septicemia in Japanese eel has indicated the condition exists in both a renal and hepatic form. The renal form is identified grossly by an enlarged trunk kidney and presents histopathologically as suppurative interstitial nephritis. Abscesses of the trunk kidney initially form in the sinusoids of the hematopoietic tissue and increase in size, subsequently involving the adjacent hematopoietic tissue and nephrons. Enlarged abscesses are surrounded by fibrin and filled with neutrophils containing phagocytized bacteria. Other abscesses are liquefied and likely aid the invasion of *E. tarda* into nearby tissues and formation of emboli in blood vessels. Additional abscesses and necrosis are observed in the spleen, and liver, and occasionally in the heart and submucosal layer of the digestive tract, as well as lateral musculature adjoining the kidney (Miyazaki and Egusa 1976a; Miyazaki and Kaige 1985; Plumb 1999). The hepatic form, characterized macroscopically by an enlarged liver and the presence of leaked pus in the body cavity, presents histopathologically as suppurative hepatitis. Abscesses of the liver, similar to

kidney lesions observed in the nephric form, affect both the hepatocytes and blood vessels resulting in the development of emboli and pyemia with pronounced bacterial multiplication (Miyazaki and Egusa 1976b; Miyazaki and Kaige 1985). Hepatocytes uninfected with *E. tarda* display atrophy and fatty degeneration. Some abscesses appear to liquefy, leading to the release of exudates onto the digestive tracts, serosa, and peritoneum. Organs covered by exudates exhibit extensive bacterial invasion and subsequent necrosis. In the hepatic form of *Edwardsiella* septicemia, spleens with minimal bacterial infiltration display the proliferation of splenocytes and neutrophils, while spleens with increased bacterial infiltration demonstrate severe necrosis and hemorrhage. Kidneys also show the presence of neutrophils and bacterial dissemination in the sinusoids of hematopoietic tissue, while necrosis may be observed in the gill lamellae (Miyazaki and Kaige 1985).

Edwardsiella septicemia in channel catfish

Channel catfish infected with *E. tarda* initially develop scattered petechiae and cutaneous lesions, characteristically located dorsolaterally, approximately 1-5 mm in diameter (Meyer and Bullock 1973; Darwish et al. 2000; Plumb and Evans 2006). These small lesions may progress into larger abscesses in the musculature of the caudal portion of the body. These abscesses produce swollen areas of depigmentation and include copious amounts of necrotic tissue that release putrid gas when incised. In advanced infections, fish lose mobility of the caudal region. Internally, infected channel catfish exhibit hyperemia and occasionally, accumulation of bloody ascitic fluid, while the kidney is enlarged, the liver mottled, and both organs soft (Miyazaki and Kaige 1985; Plumb 1999; Plumb and Evans 2006). In both the head and trunk kidneys, necrosis and

hemorrhage of the hematopoietic tissue occurs and macrophages accumulate. The liver displays hepatocellular necrosis and extensive infiltration of bacteria laden macrophages. Multifocal necrosis is often visible in the white and red pulp of the spleen (Darwish et al. 2000).

Edwardsiella septicemia in Nile tilapia

In Nile tilapia, diseased fish show whitish discoloration of the body with a swollen abdomen. Exophthalmia, opacity, or hemorrhage of the eyes may also be present (Miyazaki and Kaige 1985; Plumb 1999). Clinical signs observed by Saleh (2005) include scale loss, excessive production of mucous, petechial hemorrhage, and large areas of necrosis concentrated in the caudal region of the body. Internal signs include the presence of yellowish ascitic fluid and nodular lesions visible on the liver, spleen, kidney, and possibly the intestine (Miyazki and Kaige 1985). Hemorrhagic and gas filled cavities may be present in the muscle (Plumb 1999; Saleh 2005). Additionally, blood parameters, such as hematocrit, erythrocyte, leukocyte, and thrombocyte counts, protein and hemoglobin concentrations, and ion content, were evaluated in Nile tilapia spontaneously infected by *E. tarda* and found similar to values obtained for other bacterial infections in various fish species (Benli and Yildiz 2004). Histopathological features of *Edwardsiella* septicemia in Nile tilapia are concentrated in the kidneys, liver, spleen, and intestine. The infection is characterized by liquefactive necrosis, extensive bacterial dissemination, and the presence of macrophages. Bacteria engorged macrophages associated with *E. tarda* lesions show bacterial multiplication within the cells and nuclear degeneration. As the disease progresses, granulomas form in the kidneys, liver, and spleen. The granulomas enclose coagulated and caseous debris and decrease bacterial accumulation in the

surrounding tissue. Additionally, in the gills, lesions resulting in necrosis, the infiltration of macrophages, and epithelial hyperplasia may occur (Miyazaki and Kaige 1985).

Edwardsiella septicemia in other fish

Disease signs of *Edwardsiella* septicemia in other fish species include loss of pigmentation, exophthalmia, opaqueness of the eye, necrosis of the skin and muscle, rectal protrusion, and abnormal swimming and behavioral patterns (Meyer and Bullock 1973; Plumb 1999). Herman and Bullock (1986) described *E. tarda* infection observed in hatchery reared striped bass. Lesions tend to be focused in the lateral line and head kidney. The lateral line canals exhibit epithelial hyperplasia, necrosis, inflammation, and accumulation of gram-negative bacteria. Histopathologic lesions of the head kidney are characterized by multiple areas of necrosis and the presence of gram-negative bacteria. In severe lesions, the necrotic foci are caseous (Herman and Bullock 1986). Padrós et al. (2006) detailed pathological changes seen in cultured turbot *Scophthalmus maximus* (L.). Lesions are observed in the head and trunk kidneys, and spleen. The kidneys and spleen display numerous abscesses and extensive purulent inflammation. Affected areas are also characterized by large numbers of macrophage aggregates. The liver is impacted to a lesser extent with a few bacteria filled macrophages present (Padrós et al. 2006). Histopathological evaluation of *E. tarda* afflicted Japanese flounder found suppurative inflammation and the presence of abscesses in the liver and kidneys (Miyazaki and Kaige 1985). In red sea bream, lesions are initially characterized by necrosis and the aggregation of bacteria laden macrophages. The development of granulomas is regularly seen in the liver, kidneys, spleen, and lateral musculature of fish with advanced *E. tarda* infections (Miyazaki and Kaige 1985).

Virulence factors

Numerous features and strategies of bacteria determine virulence within a host. A critical aspect in the success of a bacterial pathogen is the ability to attach to and penetrate host surfaces. Bacteria have evolved several mechanisms to aid in the crucial initial step of adherence. Proteinaceous, rod-like structures such as pili and fimbriae are produced by bacteria, especially gram-negative species, to maintain contact with host cell surfaces (Lyczak and Pier 2000; Salyers and Whitt 2002; Pizarro-Cerdá and Cossart 2006). Other surface proteins, known as afimbrial adhesins, may not arrange structurally in the manner of pili or fimbriae, but still function in the adherence of the bacterium to a host cell (Salyers and Whitt 2002). Outer surface polysaccharide molecules forming the glycocalyx also contribute to adherence of bacterial cells to the host and other bacteria (Madigan et al. 1997). Once a bacterial pathogen has adhered to the host, it must acquire access to the tissues. Often penetration is achieved through breaches in epithelial surfaces (Madigan et al. 1997; Salyers and Whitt 2002). Some bacterial pathogens are able to invade the host cells through the activity of cytotoxins and surface proteins like invasins that allow the manipulation of host cell membranes (Ellis 1999; Salyers and Whitt 2002). Invasins can induce the uptake of bacteria by typically non-phagocytic host cells. Specifically, enteric bacteria, including *Salmonella* spp., *Shigella* spp., and possibly *E. tarda*, may derive much of their virulence from the ability to invade epithelial cells (Janda et al. 1991a; Salyers and Whitt 2002). In addition, some bacterial pathogens have developed mechanisms to allow survival and multiplication intracellularly within professional phagocytic cells through the prevention of the phagosome-lysosome complex, escape from the phagosome by disruption of the membrane, or direct resistance

to killing (Russell 2000; Salyers and Whitt 2002). Typically, a bacterial pathogen must proliferate within the host to cause illness, as the initial inoculating dose is seldom substantial enough to result in disease. The process of bacterial colonization requires environmental and nutritional conditions conducive to growth and the ability to avoid or surmount immune responses of the host. While environmental parameters such as temperature, pH, and salinity can substantially affect bacterial growth, the availability of certain nutrients is often the limiting factor in bacterial establishment within a host. In particular, the importance of iron for growth of most pathogenic bacteria has been well established (Neilands 1995; Madigan et al. 1997; Salyers and Whitt 2002). As levels of accessible iron in the environment are commonly low, high affinity iron chelators, known as siderophores, are produced by many pathogenic bacteria to enhance survival and possibly increase virulence (Neilands 1995; Salyers and Whitt 2002). A potential host possesses a multifaceted system of innate and acquired immune defenses the potential bacterial pathogen must overcome in order to cause disease. Numerous bacterial pathogens may rely on the production of extracellular proteins, especially enzymes, to evade immune responses and inflict damage to host cells. Several enzymes implicated in the virulence of bacterial pathogens include catalase, hemolysins, and hydrolytic enzymes (Madigan et al. 1997; Ellis 1999; Salyers and Whitt 2002). Others secrete specific exotoxins that act to modify function of or injure host cells and result in pathologic changes characteristic of disease (Madigan et al. 1997; Barbieri and Pederson 2000; Salyers and Whitt 2002). Finally, in the consideration of gram-negative bacteria, the cell wall may function in virulence. Gram-negative bacteria have a complex cell wall comprised of the cytoplasmic membrane, peptidoglycan layer, and an outer membrane.

The outer membrane, composed of lipopolysaccharides (LPS), phospholipids, and proteins, may act as an endotoxin within the host and play a role in the regulation of other known virulence factors including hemolysins (Hirono et al. 1997, 1998; Madigan et al. 1997; Brogden 2000; Darwish et al. 2001).

The virulence factors of *E. tarda* have yet to be fully elucidated. Numerous mechanisms have been proposed and investigated in describing the pathogenicity of *E. tarda*. Some researchers suggest motility, invasion tactics, and adherence contribute significantly to the pathogenesis of *E. tarda* (Wong et al. 1989; Janda et al. 1991a, 1991b; Ling et al. 2000; Sakai et al. 2003; Matsuyama et al. 2005; Yasunobu et al. 2006). *Edwardsiella tarda* has been identified as being chemotactically motile (Janda et al. 1991a, 1991b). Matsuyama et al. (2005) explored differences in pathogenicity between motile and non-motile *E. tarda* strains in several marine fish species. Through intraperitoneal (IP) injection and immersion challenge, both motile and non-motile strains were lethal to yellowtail *Seriola quinqueradiata* and Japanese flounder, while only non-motile strains were lethal to red sea bream. Janda et al. (1991a) illustrated the ability of *E. tarda* to penetrate and replicate within monolayers of the HEp-2 line of epithelial cells. The invasion was reported to be microfilament dependent, as exposure to inhibitors of microfilament development, including cytochalasins B and D, resulted in a significant reduction in the invasion capability of *E. tarda* (Janda et al. 1991a). Green fluorescent protein was employed by Ling et al. (2000) in assessing the ability of *E. tarda* to adhere to and invade epithelioma papillosum of carp (EPC) monolayers. Successful adherence and invasion of EPC cells for several *E. tarda* isolates of varying virulence was observed. Wong et al. (1989) identified two major classes of adhesins produced by *Edwardsiella*

spp., mannose-sensitive hemagglutinin inhibited by D-mannose, and mannose-resistant hemagglutinin, typically associated only with *E. tarda*, not inhibited by D-mannose. Sakai et al. (2003) further investigated the hemagglutinating ability of *E. tarda* and cloned a gene responsible for a 19.3 kDa protein implicated in hemagglutinating behavior. Recently, Yasunobu et al. (2006) identified an association between hemagglutinin production and expression of the 19.3 kDa protein and reported both were enhanced by increased NaCl concentrations in the culture medium.

Other reports propose that avoidance of and resistance to host immune responses play a major role in the virulence of this pathogen (Ainsworth and Chen 1990; Janda et al. 1991b; Iida and Wakabayashi 1993; Mathew et al. 2001; Srinivasa Rao et al. 2001). Ainsworth and Chen (1990) found that channel catfish neutrophils demonstrated minimal bactericidal effects on *E. tarda*. Janda et al. (1991b) reported *E. tarda* isolates are resistant to killing by human serum, while the research of Mathew et al. (2001) explored potential loss in virulence when genes related to serum resistance were attenuated. In addition, Iida and Wakabayashi (1993) suggested *E. tarda* resists opsonization and phagocytosis as a means of manifesting disease, citing the success of this species in avoiding the bactericidal activity of eel complement. Srinivasa Rao et al. (2001) described the ability of virulent *E. tarda* isolates to not only adhere to blue gourami phagocytes, but also survive and replicate within the phagocytes. The virulent isolates investigated also failed to elicit levels of phagocyte produced reactive oxygen intermediates comparable to levels stimulated by non-virulent isolates, thus implying virulence of *E. tarda* could be related to the ability to resist killing by this process (Srinivasa Rao et al. 2001).

In further analyses of potential virulence factors, secreted products such as enzymes and siderophores have been implicated in the disturbance of host cell function (Ullah and Arai 1983a; Kokubo et al. 1990; Chen et al. 1996; Hirono et al. 1997; Mathew et al. 2001; Srinivasa Rao et al. 2003; Verjan et al. 2005). Recently, through the use of comparative proteomics and Tn*pho*A mutagenesis, gene clusters have been identified encoding both a type III secretion system (TTSS) and putative secretion system in *E. tarda* (Tan et al. 2002; Srinivasa Rao et al. 2003; Srinivasa Rao et al. 2004; Zheng et al. 2005). The TTSS, a multicomponent secretory pathway highly conserved among gram-negative bacteria, enables the transportation of effector molecules from the bacterial cytoplasm to the cell surface. Secretion is dependent on close proximity of the pathogen to host cells and release of the effector molecules function to facilitate survival and proliferation of the bacteria (Mecsas and Strauss 1996). The genes implicated in the TTSS exhibit homology to the recognized TTSS of other pathogenic bacteria including *Escherichia coli*, *Salmonella enterica* serovar typhimurium, and *Yersinia enterocolitica*. The putative secretion system, also linked to the delivery of *E. tarda* virulence proteins, shares homology with secretory genes of other plant and animal pathogens and symbionts of the genera *Escherichia, Salmonella*, *Vibrio*, *Yersinia*, *Rhizobium*, and *Agrobacterium* (Zheng et al. 2005). Verjan et al. (2005) identified the genetic loci of seven antigenic proteins of *E. tarda* including several secreted and exported proteins involved in transport of metabolites, stress response, and motility. Ullah and Arai (1983a) described the detection of hemolysins through the inoculation of horse red blood cell containing media with *E. tarda*. More recently, Hirono et al. (1997) reported the successful cloning and sequencing of the specific gene locus responsible for the secretion of hemolysin.

The amino acid sequence generated by the identified gene locus showed homology to the previously described hemolysin and activation/secretion proteins of *Heamophilus ducreyi*, *Proteus mirabilis*, and *Serratia marcescens* (Hirono et al. 1997). Chen et al. (1996) also detailed the cloning of an *E. tarda* β-hemolysin gene, into a plasmid expressed by *Escherichia coli*. It was noted in this investigation that the hemolysin producing sequence of *E. tarda* appears similar to sequences described for other bacterial species, but the mechanism of transportation across the cell envelope could be different (Chen et al. 1996). Catalase is another enzyme thought to contribute to the virulence of *E. tarda*. *Edwardsiella tarda* strains possessing mutations at the *katB* gene, involved in production of catalase, were observed to have higher median lethal dose (LD_{50}) values than wild-type isolates in blue gourami (Srinivasa Rao et al. 2003). In a study of attenuated *E. tarda*, mutated by insertion of Tn*phoA* transposons, Mathew et al. (2001) reported decreased virulence in strains deficient in the production of catalase. Kokubo et al. (1990) investigated the possibility of siderophore production as a virulence factor of *E. tarda*. These products have been implicated in the pathogenicity of other microbes, namely strains of *E. coli* and *Y. enterocolitica* (Neilands 1995).

In addition, a few specific toxins potentially involved in the virulence of *E. tarda* have been described (Ullah and Arai 1983a, 1983b; Suprapto et al. 1995; Suprapto et al. 1996; Tan et al. 2002). Nineteen isolates investigated by Ullah and Arai (1983a, 1983b) were capable of producing dermatonecrotic exotoxins as assessed using rabbits. Another toxin, estimated to have a molecular weight of 37 kDa, lethal to Japanese eel and Japanese flounder has been identified and purified from the extracellular products (ECP) and intracellular components (ICC) of *E. tarda* (Suprapto et al. 1995; Suprapto et al.

1996). Tan et al. (2002) employed a comparative proteomic approach to identify two proteins potentially involved in virulence from the ECP of virulent and non-virulent *E. tarda* isolates.

The properties of LPS and outer membrane proteins (OMP) contributing to the virulence of *E. tarda* have been investigated (Salati et al. 1983, Salati et al. 1984; Salati and Kusuda 1986; Salati et al. 1987; Kawahara et al. 1990; Hirono et al. 1997; Hirono et al. 1998; Darwish et al. 2001). The antigenic nature of LPS and its components have been investigated in several species including Japanese eel and red sea bream. The results of these studies suggested LPS is an important immunogen of *E. tarda* and could play a role in inducing immune protection (Salati et al. 1983, Salati et al. 1984; Salati and Kusuda 1986; Salati et al. 1987). Kawahara et al. (1990) examined the accumulation of injected LPS in Japanese eel and detected rapid deposition of antigens occurring primarily in the liver and kidney. Darwish et al. (2001) suggested the success of this bacterium in numerous hosts and environmental substrates may be attributed to certain proteins located within the organism's outer membrane. This theory may also be supported by evidence of protection elicited from exposure to purified OMP, of 37 kDa, in Japanese eels challenged by IP injection of live *E. tarda* (Tu and Kawai 1999). Also, certain OMP have been implicated in the activation and function of hemolysin (Hirono et al. 1997; Hirono et al. 1998).

Treatment and prevention

As the complete elimination of pathogens from the environment is not possible, efforts have been made to develop methods of treatment and prevention of *E. tarda* infection in cultured fish. The use of immunostimulants, including β-glucans, vitamins E

and C, and tuftsin, has been explored for protection against *Edwardsiella* septicemia with some promising results (Park and Jeong 1996; Sahoo and Mukherjee 2002; Misra et al. 2006a, 2006b). The oral administration of lysozyme-galactomannan and lysozyme-palmitic acid conjugates have also exhibited potential as therapeutic agents in studies conducted with common carp *Cyprinus carpio* L. (Nakamura et al. 1996). Currently, antibiotic treatment of *E. tarda* infection in fish farm reared in the United States occurs through oral application of oxytetracyline and a potentiated sulphonamide in the feed. While both agents are approved by the United States Food and Drug Administration (FDA) for the management of other bacterial fish diseases, neither drug is specifically designated for therapy of *Edwardsiella* septicemia (Center for Veterinary Medicine 2003; Plumb and Evans 2006). Additionally**,** antibiotic resistance has been reported in *E. tarda*, especially in isolates of Asian origin (Aoki et al. 1977; Waltman and Shotts 1986; Aoki and Takahashi 1987; Thune et al. 1993). With the ever increasing intensity of fish culture, immunoprophylaxsis, as a method of stimulating non-specific and specific immunity, has become the most important method of bacterial disease prevention (Gudding 1999; Håstein et al. 2005). Vaccines can be administered to fish by injection, typically intraperitoneally, by immersion in a vaccine solution, or orally, with the selection of immunization route based on factors such as feasibility, level of protection conferred, potential side effects, and cost (Gudding et al. 1999). Critical to the design of an efficacious vaccine is the initiation and optimization of host immune responses and recognition of possible virulence factors of the target pathogen. Vaccine formulations consisting of inactivated cell bacterins, live, attenuated cells, and DNA recombinant products have been developed for use in many fish species (Gudding et al. 1999).

At the present time, licensed vaccines against approximately 15 bacterial pathogens are employed in aquaculture worldwide, while numerous others are currently being researched (Håstein et al. 2005).

Although many potential vaccines have been evaluated, one effective in the prevention of *Edwardsiella* septicemia has yet to be developed. Early attempts were made by Song and Kou (1981) to immunize eels by immersion in a killed cell solution with lower mortality observed in immunized fish than in control fish. Salati et al. (1983) immunized eels by intramuscular (IM) injection with LPS, culture filtrates, and formalin killed whole cells (FKC) and found LPS immunogen yielded the greatest survival in challenged fish. In another experiment, IM injection of LPS and FKC, in eels challenged orally with virulent *E. tarda*, did elicit limited protection that may possibly be enhanced by macrophage phagocytosis of bacteria (Gutierrez and Miyazaki 1994). Also, Swain et al. (2002) have described significant protection against challenge achieved by bath immunization of young rohu *Labeo rohita* and catla *Catla catla* with an FKC suspension. As ECP and ICC have been implicated in the pathogenicity of *E. tarda* (Ullah and Arai 1983a, 1983b; Suprapto et al. 1995, Suprapto et al. 1996; Tan et al. 2002), some work has focused on the potential of extracellular and intracellular components in achieving protection through immunization. Salati et al. (1983) established that cell culture filtrates had minimal impact on the development of a protective immune response in Japanese eel. Mekuchi et al. (1995) investigated vaccination by FKC, intracellular components, and ECP in the Japanese flounder, and found increased antibody titers in fish exposed to FKC orally and by IM injection, while the diluted intracellular components and ECP proved lethal. This study did not find any treatment resulted in effective protection.

In trials using red sea bream, Salati et al. (1987) determined IM injected LPS and FKC were able to induce limited immunological responses and that macrophage activity could be an important factor in defense against *E. tarda* infection. Other research has focused on the potential protective capabilities of a conserved 37 kDa OMP of *E. tarda*. Kawai et al. (2004) observed protection in Japanese flounder IM injected with the OMP and challenged with several strains of *E. tarda* of varying serotypes. Similarly, studies conducted by Liu et al. (2005) found immunization of Japanese flounder with recombinant glyceraldehyde-3-phosphate dehydrogenase, a molecule showing high homology to the 37 kDa OMP of *E. tarda* and conserved in many gram-negative pathogens, provided effective protection against IP challenge. Evaluation of vaccination by a live attenuated strain of *E. tarda* was performed by Igarashi and Iida (2002). In this study, a mutant was developed from a virulent wild-type isolate by decreasing siderophore production. It was reported that IP injection of the mutant strain provided better protection than IP injection of FKC, although both treatments elicited antibody production. As a result, the authors concluded immunological protection against *E. tarda* may be a function of cell mediated mechanisms rather than humoral immunity (Igarashi and Iida 2002). Gutierrez et al. (1993) researched the protective capabilities of passive immunization. In this study, eels were orally administered immunoglobulin (Ig) Y obtained from hens vaccinated by injection with FKC and lower mortality was observed in challenged fish. Kwon et al. (2006) explored immunization of Mozambique tilapia *Oreochromis mossambicus* using both FKC and *E. tarda* cells inactivated by the expression of the cloned bacteriophage PhiX174 lysis gene *E* to produce a ghost vaccine and reported the ghost vaccine provided greater protection than an FKC preparation.

Most recently, Evans et al. (2006) have developed a rifampicin-resistant attenuated isolate of *E. tarda* for use as a live vaccine administered by injection or bath immersion. Currently, a number of antigens, including LPS, ECP, and OMP, are thought to be involved in inducing protection against *E. tarda* in fish, however; an effective, commercial vaccine is not presently available.

Edwardsiella septicemia in fish typically involves inflammation of several internal organs, including the liver, kidneys, and spleen, with the presence of numerous macrophages (Miyazaki and Kaige 1985; Darwish et al. 2000). Inflammation occurs as a response by the immune system to microbial invasion and tissue injury (Obenauf and Smith 1985; Nash et al. 1986; Weeks et al. 1988; Weeks-Perkins and Ellis 1995). The early process of inflammation is characterized by the migration of leukocytes from blood and other tissues to areas affected by microbial invasion or other injury. This directional movement, known as chemotaxis, is due to the activity of chemical attractants produced by the microbe or the host. Nondirectional movement of a more random nature, or chemokinesis, has also been described in the migration of fish leukocytes (Weeks-Perkins and Ellis 1995; Klesius and Sealey 1996). Previously, Klesius and Sealey (1996) reported the ability of ECP generated by *E. ictaluri* to attract macrophages in channel catfish through chemotactic and chemokinetic activity. While ECP have been implicated in the pathogenicity of *E. tarda* (Ullah and Arai 1983a, 1983b; Suprapto et al. 1995; Suprapto et al. 1996; Tan et al. 2002), the potential stimulatory effect of ECP on fish macrophage migration has yet to be fully investigated. In addition, Weeks-Perkins and Ellis (1995) noted differences between the behavior of Atlantic salmon *Salmo salar* macrophages in the presence of live and formalin killed virulent *Aeromonas salmonicida*

and non-virulent *A. salmonicida.* It is unclear whether virulence impacts the ability of *E. tarda* or its secreted products to attract macrophages, although Srinivasa Rao et al. (2001) did report a difference in phagocyte killing between virulent and non-virulent strains. It is conceivable that the migration of macrophages to the site of *E. tarda* infection may play an important role in the defense against *Edwardsiella* septicemia (Salati et al. 1987; Gutierrez and Miyazaki 1994). As it has also been suggested that *E. tarda* acts intracellularly and invasion could possibly be enhanced by macrophage activity (Srinivasa Rao et al. 2001), a better comprehension of the interactions between the secreted products of *E. tarda* and the fish immune system may aid in understanding protective immunity against this pathogen.

The objectives of the present study were to 1) evaluate the chemotactic response of macrophages obtained from Nile tilapia to extracellular products from a virulent parent and less virulent mutant strain of *E. tarda*, 2) determine if passive immunization with immune antibodies elicits protection from challenge in Nile tilapia, and 3) assess protection elicited by immunization with and describe the immunological properties of *E. tarda* extracellular products in Nile tilapia.

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II. THE MACROPHAGE CHEMOTACTIC ACTIVITY OF *EDWARDSIELLA TARDA* EXTRACELLULAR PRODUCTS (ECP)

Abstract

An *in vitro* study was conducted to evaluate the chemoattractant capabilities of extracellular products (ECP) derived from a virulent parent and less virulent mutant isolate of *Edwardsiella tarda*. The virulent isolate, FL6-60, was obtained from a morbid striped bass *Morone saxatilis*, while the RET-04 isolate is a less virulent rifampicin-resistant mutant of FL6-60. Macrophage chemotaxis and chemokinesis were assayed using blind well chemotaxis chambers with predominately macrophage populations elicited from the peritoneal cavity of Nile tilapia *Oreochromis niloticus* 5 d following injection with squalene. Non-purified ECP derived from both FL6-60 parent and RET-04 mutant *E. tarda* stimulated migration of macrophages and the movement was demonstrated to be predominantly chemokinetic. Only slight differences between the ability of the two ECP to elicit migration were observed. Additionally, ECP from both isolates were semi-purified by high pressure liquid chromatography and differences between the ECP were illustrated. Components with molecular weights of 31.62, 3.19, and 0.60 kDa, and 5.65, 0.55, and 0.10 kDa were identified for the FL6-60 and RET-04 fractions, respectively. Chemotactic and chemokinetic capabilities were analyzed using the semi-purified ECP and both fractions exhibited chemotactic and chemokinetic activity. Chemotactic activity for both the FL6-60 parent and RET-04 mutant ECP was increased over the non-purified ECP and migration was primarily chemotactic.

Differences between migration induced by the semi-purified FL6-60 parent and RET-04 mutant ECP were minimal. Exposure to ECP derived from a virulent and less virulent isolate promoted chemokinetic movement of macrophages that may be involved in inflammatory responses of Nile tilapia to *E. tarda* infection.

Introduction

Edwardsiella septicemia occurs in numerous freshwater and marine fish species including Japanese eel *Anguilla japonica*, red sea bream *Pagrus major*, Japanese flounder *Paralichthys olivaceus*, channel catfish *Ictalurus punctatus*, and Nile tilapia *Oreochromis niloticus* (Hoshina 1962; Meyer and Bullock 1973; Austin and Austin 1987; Plumb 1999). The causative agent, *Edwardsiella tarda*, is recognized as a common inhabitant of aquatic ecosystems and the bacterium has been isolated globally from animal hosts and environmental samples of water and sediments (Plumb and Evans 2006). *Edwardsiella* septicemia may vary in severity and clinical manifestations in different species, but this bacterium has been implicated in extensive losses of aquacultured fish throughout the world (Thune et al. 1993; Plumb and Evans 2006). Despite the potentially serious threat posed by *E. tarda* to fish, the nature of the host-pathogen interaction has yet to be fully elucidated.

In order to develop effective prevention and treatment strategies, an understanding of the factors contributing to the virulence capabilities of a pathogen and the immune responses leading to protection is crucial. Factors such as production of extracellular proteins, cell associated toxins, and avoidance of host cell immune mechanisms have been associated with the pathogenesis of *E. tarda* (Thune et al. 1993; Plumb and Evans 2006). Fish possess numerous humoral and cell mediated innate and acquired mechanisms to defend against bacterial infection (Ellis 1999; Magnadóttir 2006).

Edwardsiella septicemia in fish is characterized by inflammation of several organs including the head kidney, liver, and spleen (Miyazaki and Egusa 1976a, 1976b;

Miyazaki and Kaige 1985). Inflammation is a series of complex and interrelated events produced by the immune system of an organism in response to microbial invasion or other injuries. Macrophages are crucial to inflammatory reactions and other immune responses through their ability to function in effector, helper, and suppressor capacities (Nash et al. 1986; Zelikoff et al. 1991). The migration of macrophages from the blood and adjacent tissues to the site of microbial infiltration or injury is a critical initial step in the inflammatory process (Griffin 1984; Weeks et al. 1988; Weeks-Perkins and Ellis 1995; Klesius and Sealey 1996; Ellis 2001). The movement of macrophages and other leukocytes may involve chemotactic and chemokinetic mechanisms. Chemotaxis describes the directed migration of cells induced by chemoattractant molecules of either microbial or host origin, while chemokinesis refers to an increase in nondirectional, random movement of cells (Obenauf and Smith 1985; Nash et al. 1986; Weeks et al. 1988; Weeks-Perkins and Ellis 1995; Mañes et al. 2005).

Numerous studies detailing the *in vitro* chemotactic response of fish leukocytes to microbially derived chemoattractant substances have been reported (Griffin 1984; Obenauf and Smith 1985; Sharp et al. 1991; Zelikoff et al. 1991). Klesius and Sealey (1996) investigated the potential chemotactic and chemokinetic activity of channel catfish macrophages exposed to extracellular products (ECP) from *Edwardsiella ictaluri*. Assays conducted both *in vivo* and *in vitro* determined the ECP are capable of attracting channel catfish macrophages and these interactions could enhance the understanding of *E. ictaluri* pathogenesis (Klesius and Sealey 1996). Recently, Klesius et al. (2006) described the chemoattractant properties of ECP obtained from the fish pathogens *Streptococcus agalactiae* and *S. iniae* on peritoneal macrophages of Nile tilapia.

This research found *S. agalactiae* and *S. iniae* ECP preparations displayed chemotactic and chemokinetic activity, though *S. agalactiae* showed significantly greater ability to induce both chemotaxis and chemokinesis in macrophages (Klesius et al. 2006). Although production and excretion of ECP have been implicated in the virulence of *E. tarda* (Ullah and Arai 1983a, 1983b; Suprapto et al. 1995; Suprapto et al. 1996; Tan et al. 2002), information regarding the interactions between these ECP and fish macrophages is lacking. The aim of the present study was to investigate the possible ability of non-purified and semi-purified ECP prepared from a virulent parent and less virulent mutant of *E. tarda* to influence chemotactic and chemokinetic migration of Nile tilapia macrophages *in vitro*.

Materials and Methods

Fish

Nile tilapia *Oreochromis niloticus* (60±5 g, mean weight±standard deviation) were produced and maintained at the USDA-ARS Aquatic Animal Health Research Laboratory (AAHRL), Auburn, Alabama. The fish were fed a commercial diet (Aquamax Grower 400, Brentwood, Missouri) daily to satiation. Two weeks prior to the experiments, the fish were acclimated in 57 L glass aquaria. The aquaria were provided with flow-through dechlorinated tap water at 26±1° C at a rate of 0.5 L/min and aeration with air stones. For the duration of the study, the dissolved oxygen, temperature, pH, salinity, hardness, ammonia, and nitrite levels were monitored and maintained within acceptable ranges. During the experiment, the mean±standard deviation of dissolved oxygen was 5.8±0.5 mg/L, temperature was 26.2±0.8° C, pH was 7.1±0.3, salinity was 0.1±0.0%, and hardness was 110±10mg/L ($CaCO_3$). Ammonia and nitrite concentrations

were consistently below the detection limit (0 mg/mL) during the experiment. The fish were exposed to light and dark intervals of 12:12 h. Feeding was discontinued 1 d preceding collection of squalene-induced peritoneal macrophages.

Preparation of *E. tarda* non-purified extracellular products

The preparation of *E. tarda* extracellular products (ECP) was guided by the procedures presented by Klesius et al. (1999) describing the formulation of a killed *Streptococcus iniae* vaccine, and the resulting products used to characterize the biological properties of ECP from a virulent parent and less virulent mutant isolate of *E. tarda*. Specifically, the FL6-60 parent and RET-04 mutant isolates of *E. tarda* were utilized. The virulent FL6-60 parent isolate was obtained from a morbid striped bass *Morone saxatilis* (Baya et al. 1997). This isolate was received courtesy of Dr. A.M. Baya (Animal Health Diagnostic Laboratory, Maryland Department of Agriculture, College Park, Maryland). RET-04 is a mutant of FL6-60 developed through a rifampicin-resistance strategy as described by Schurig et al. (1991), Klesius and Shoemaker (1999), and Evans et al. (2006). The FL6-60 parent isolate was determined to be more virulent than the RET-04 mutant isolate through previous studies (unpublished; Wiedenmayer et al. 2006). Briefly, 1 mL aliquots of the FL6-60 parent and RET-04 mutant were inoculated, separately, into 5 L of sterile tryptic soy broth (TSB, Difco Laboratories, Sparks, Maryland) and incubated at 27° C for 72 h with shaking. The plate counts of the FL6-60 and RET-04 preparations after 72 h incubation were 3.02×10^9 and 1.68×10^9 colony forming units per mL (CFU/mL), respectively. The cultures were treated with 10% neutral buffered formalin (NBF) to yield a final concentration of 3% and allowed to stand at 27° C for 24 h. After 24 h, the culture was streaked onto sheep blood agar (SBA,

Remel, Inc.) and then incubated at 27° C for 72 h to ensure the preparation had been killed. The NBF treated cultures were centrifuged at 7000 x g for 30 min and the cell pellet and culture fluid separated. The culture fluid, containing ECP, was concentrated to 1 L on a 3 kDa Amicon column (S3Y3) using a Millipore Proflux M12 (Millipore, Billerica, Massachusetts) and filter sterilized with a 0.22 μm 1 L microbiological filter (Corning, Corning, New York). The protein concentration of the non-purified ECP was determined by the bicinchoninic acid (BCA) method and estimated to be approximately 7.74±1.30 and 6.64±1.66 mg/mL for the FL6-60 and RET-04 preparations, respectively (BCA Protein Assay Kit, Pierce, Rockford, Illinois). The whole cell fraction and concentrated culture fluid were stored separately at -80° C until needed in the study.

Semi-purification of extracellular products

For additional characterization, non-purified ECP were semi-purified via a high-pressure chromatography system (Waters, Milford, Massachusetts) outfitted with a Shodex® Protein KW-804 column (Showa Denko K.K., Tokyo, Japan). The total volume of the column (Vt) was 2400 mL while the void volume (Vo) was determined to be 4.5 mL. Tryptic soy broth was analyzed as a control. Elution was performed in phosphate buffered saline (PBS) at a flow rate of 0.5 mL/min. The elution profile was detected at a wavelength of 280 nm (Waters 996 Photodiode Array Detection, Milford, Massachusetts). Following the proper elution period, the semi-purified ECP were collected and pooled by isolate. The molecular weights of the semi-purified ECP were estimated by comparison to molecular weight standards ranging from 1.35 to 670 kDa (Bio-Rad 151-1091, Hercules, California). The determination of protein concentrations of the semi-purified ECP was attempted by the BCA method; however, the protein

amounts fell below the range of detection for the assay. The semi-purified ECP were stored at -70° C until use.

Macrophage collection

Macrophage enriched exudate was collected from Nile tilapia through the method of Klesius and Sealey (1996). Five Nile tilapia (60±5 g, mean weight±standard deviation) were intraperitoneally (IP) injected with 250 µL of squalene (Sigma Chemical Co., St. Louis, Missouri). The fish were maintained at the previously described conditions for 5 d, with feeding discontinued 1 d prior to the harvest of the peritoneal exudate. After 5 d, the fish were euthanized by immersion in a 300 mg/mL buffered tricaine methanesulfonate (MS-222, Finquel, Argent Chemical Laboratories, Redmond, California) solution. The peritoneal cavity of each fish was washed three times with 15 mL of cold sterile phosphate buffered saline (PBS) using a 20 gauge needle connected to a 3-way valve. The collected peritoneal exudates were pooled and centrifuged at 300 x g for 10 min at 4° C. The supernatant was removed and discarded and the reserved cells suspended in calcium and magnesium free Hanks' balanced salt solution without phenol red (HBSS, Gibco, Grand Island, New York). The cells were washed once in HBSS as described above and the resulting cell pellet resuspended in 2 mL HBSS. The cell solution was diluted with 9 mL of sterile deionized water to lyse the red blood cells for 20 s, after which 1 mL of 10 X HBSS was added. The cells were washed twice in the previously described HBSS at 300 x g for 10 min at 4° C. The collected cells were enumerated and assessed for viability using a hemacytometer and the trypan blue exclusion assay.

Chemotaxis and chemokinesis assays of non-purified extracellular products

Chemotaxis assays were performed using the lower surface method detailed by Boyden (1962) as adapted by Klesius and Sealey (1996) for their evaluation of channel catfish macrophage response to *E. ictaluri* derived exoantigen. Nile tilapia macrophage migration in the presence of *E. tarda* ECP was assayed in duplicate with blind well chemotactic chambers (Corning CoStar, Cambridge, Massachusetts) and 8 μm-pore-diameter polycarbonate membrane filters (Nucleopore, Pleasanton, California). The filters were treated prior to macrophage analysis with RPMI-1640 (Gibco BRL, Grand Island, New York) containing 1% horse serum. To evaluate directional movement of macrophages, the lower chamber compartment was filled with 200 μL of various concentrations of sterile ECP or RPMI-1640 with 1% horse serum as a control. ECP concentrations contained in the lower compartment were 0, 10, 30, 50, or 70%. The upper chamber compartment was filled with 200 μL of exudate cells with a concentration of approximately 1.0×10^5 cells/mL. In addition, the checkerboard assay of Zigmond and Hirsch (1973), also employed by Klesius and Sealey (1996), was used to determine chemokinetic and chemotactic activity of Nile tilapia macrophages in the presence of *E. tarda* ECP. For this analysis, ECP were placed in the upper chamber compartment with exudate cells, as well as various concentrations of ECP in the upper, lower, or both compartments for a volume of 200 μL in each compartment. The ECP concentrations placed in the upper, lower, or both chamber compartments were 0, 10, 30, 50, or 70%. For both the lower surface and checkerboard procedures, the chambers were incubated at 25° C on a horizontal platform shaker at 100 revolutions/min. After 90 min incubation, the filters were removed from the chamber, inverted, and placed on a precleaned slide.

The filter was attached to the slide using clear fingernail polish and the slide stained with Protocol Hema 3 stain (Fisher Scientific Co., LLC, Kalamazoo, Michigan), in a manner similar to Weeks et al. (1988) and Klesius and Sealey (1996)**.** Enumeration of migrating macrophages was achieved by counting five fields of view on the bottom surface of the filters with a light microscope at 400 X magnification. The mean number of macrophages per field of view and standard error were calculated.

Chemotaxis and chemokinesis assays of semi-purified extracellular products

Chemotaxis and chemokinesis assays were performed utilizing semi-purified ECP obtained from both the FL6-60 parent and RET-04 mutant *E. tarda*. The procedures detailing the lower surface and checkerboard assays were executed as described previously for analysis utilizing non-purified ECP, with the exception of the ECP concentrations. For this study, the ECP concentrations placed in the upper, lower, or both chamber compartments were 0, 50, or 70%.

Statistical analysis

Chemotactic and chemokinetic data were analyzed using SAS software (SAS Institute, Cary, North Carolina) by one-way analysis of variance (ANOVA) with Duncan's multiple range test for significance and by *t*-test. Significant differences were determined at $P < 0.05$.

Results

Determination of chemotactic activity for FL6-60 and RET-04 non-purified extracellular products

The FL6-60 parent ECP induced greater migration of macrophages as the concentration of ECP in the lower chamber was increased. ECP concentrations of 30, 50,

and 70% in the lower chamber produced mean macrophage counts differing significantly ($P < 0.0001$) from those obtained from ECP concentrations of 0 and 10% in the lower chamber (Table 1). The RET-04 mutant ECP also induced greater migration of macrophages as the concentration of ECP in the lower chamber was increased. ECP concentrations of 10, 30, 50, and 70% produced mean macrophage counts differing significantly ($P < 0.0001$) from those obtained from an ECP concentration of 0% in the lower chamber (Table 2). No significant differences were observed for mean macrophage counts between the FL6-60 parent and RET-04 mutant ECP in the lower surface assay (Tables 1 and 2, Figure 1).

Determination of chemokinetic activity for FL6-60 and RET-04 non-purified extracellular products

The checkerboard assay was employed to distinguish chemokinetic from chemotactic activities of the non-purified ECP preparations of the FL6-60 parent and RET-04 mutant. With analysis of both ECP, macrophage migration seemed to be primarily chemokinetic in nature. Increase of ECP concentration in the lower chamber did appear to stimulate movement of macrophages across the membrane, but movement of macrophages was also greater when ECP concentrations were higher in the upper chamber (Tables 1 and 2). While concentrations of 10, 30, and 50% of both ECP and 70% of the RET-04 mutant ECP in both the lower and upper chambers did not elicit significantly different mean macrophage movements, these means were significantly ($P < 0.0001$) different than those of the 0% ECP concentrations of the control suggesting a lack of neutralization (Tables 1 and 2). Additionally, mean migration of macrophages observed where the FL6-60 parent ECP concentration was 70% in both the lower and

upper chambers was similar to movement seen in chambers where ECP were only contained in the lower chamber (Table 1). Significant differences were observed for mean macrophage counts between the FL6-60 and RET-04 treatments with 30 ($P < 0.0168$) and 50% ($P < 0.0062$) ECP in the upper chamber with 0% ECP in the lower chamber, and with 70% ECP in the upper chamber with 10 ($P < 0.0029$) and 70% ($P < 0.0017$) ECP in the lower chamber (Tables 1 and 2).

Comparison of semi-purified *E. tarda* extracellular product molecular weight

The molecular weights of the semi-purified ECP of the FL6-60 parent and RET-04 mutant preparations were determined based on the elution times of five molecular weight standards. The elution times were recorded as 18.629, 23.367, 25.466, 27.199, and 30.241 min for the 670, 158, 44, 17, and 1.35 kDa standards, respectively. The elution profile of the FL6-60 parent ECP preparation revealed one major peak at 26.278 min and two minor peaks at 31.952 and 36.099 min (Figure 2). The molecular weights were estimated to be approximately 31.62 kDa for the major peak and 3.19 and 0.60 kDa for the minor peaks, respectively, though the third peak was outside the range of the standards. The elution profile of the RET-04 mutant preparation revealed one major peak at 30.537 min and two minor peaks at 36.278 and 40.56 min (Figure 3). The molecular weights were estimated to be approximately 5.65 kDa for the major peak and 0.55 and 0.10 kDa for the minor peaks, respectively, although the second and third peaks were outside the range of the standards. For both ECP preparations, all peaks were collected at the appropriate elution periods and pooled by isolate for the subsequent migration assays. As a control, TSB was also analyzed by HPLC. The elution profile showed two major peaks at 26.517 and 31.055 min and one minor peak at 35.366 min (Figure 4).

The molecular weights were estimated to be approximately 28.70, 4.58, and 0.80 kDa, respectively, again the third peak was outside the range of the standards.

Determination of chemotactic activity for FL6-60 and RET-04 semi-purified extracellular products

The lower surface assay was utilized to assess the chemoattractant capabilities of semi-purified ECP of the FL6-60 parent and RET-04 mutant. The semi-purified products were prepared, as previously described, by HPLC and pooled by isolate. The FL6-60 parent ECP elicited significantly ($P < 0.0001$) greater migration of macrophages as the concentration of ECP in the lower chamber was increased from the 0% control to 50 and 70%. Additionally, 70% ECP in the lower chamber produced significantly ($P < 0.0001$) more macrophage movement than did 50% ECP (Table 3). Similarly, the RET-04 mutant ECP also elicited significantly ($P < 0.0001$) greater migration of macrophages as the concentration of ECP in the lower chamber was increased from the 0% control to 50 and 70%. As with the FL6-60 parent ECP, 70% ECP in the lower chamber produced significantly ($P < 0.0001$) more macrophage movement than did 50% ECP (Table 4). No significant differences were observed for mean macrophage counts between the FL6-60 parent and RET-04 mutant for the lower surface assay (Tables 3 and 4, Figure 5).

Determination of chemokinetic activity for FL6-60 and RET-04 semi-purified extracellular products

The semi-purified FL6-60 parent and RET-04 mutant ECP were incorporated in a checkerboard assay to distinguish chemokinetic from chemotactic activities. Both the FL6-60 parent and RET-04 mutant ECP appeared to exhibit chemotactic and chemokinetic activities. In the checkerboard analysis, both ECP preparations showed

neutralization of macrophage migration when the ECP concentrations were equal in the lower and upper chambers and mean macrophage counts were not significantly different to those of the 0% ECP concentration control (Tables 3 and 4). Additionally, increased macrophage migration was observed for both ECP when concentrations were 50 or 70% in the upper chamber and 0% ECP in the lower chamber (Tables 3 and 4). Significant differences did not occur in the comparison of both ECP in replicates with 50 or 70% concentrations in either the upper or lower chambers to the control, with the exception of significantly ($P < 0.0001$) greater macrophage migration observed with 70% RET-04 mutant ECP in the upper and 50% in the lower chamber over the control (Tables 3 and 4). Significant differences were seen between the FL6-60 and RET-04 treatments with 50 ($P < 0.0029$) or 70% ($P < 0.0083$) ECP concentrations in the upper chamber and 0% ECP concentration in the lower chamber, although the migration behavior observed for both the FL6-60 parent and RET-04 mutant appeared consistent with chemokinesis for these replicates (Tables 3 and 4).

Discussion

This study evaluated the ability of ECP derived from a virulent parent and less virulent mutant isolate of *E. tarda* to induce chemotactic and chemokinetic movement of Nile tilapia macrophages. Both non-purified and semi-purified ECP fractions were analyzed by varying concentrations of the ECP in blind well chemotactic chambers and assessing macrophage migration. In the lower surface assay of non-purified ECP, results indicated both the FL6-60 parent and RET-04 mutant ECP enhanced movement of macrophages toward increased concentrations of ECP (Tables 1 and 2). Weeks et al. (1988) reported a potential correlation between chemotactic activity and virulence in

studies of *Legionella pneumophila,* suggesting lowered chemoattractant capability of a pathogen was related to increased virulence. In the present study, both the FL6-60 parent and RET-04 mutant ECP did appear capable of stimulating chemotaxis, however; no significant differences in level of migration were observed between the virulent and less virulent fractions (Figure 1). To determine possible chemokinesis, non-purified FL6-60 parent and RET-04 mutant ECP were applied to the chambers in a checkerboard pattern of varying concentrations in both the upper and lower wells. For both ECP, migration appeared to be largely chemokinetic. As the quantity of ECP in the upper compartment was increased, macrophage migration was enhanced, and when ECP concentrations were equal in the upper and lower chambers movement of macrophages was not neutralized. Additionally, as observed in the lower surface assay of the non-purified ECP, few differences between the chemokinetic activity of the virulent FL6-60 parent and less virulent RET-04 mutant ECP were revealed (Tables 1 and 2).

Differences existed between the elution profiles of the FL6-60 parent and RET-04 mutant ECP as generated by HPLC analysis. Three peaks were eluted from the non-purified FL6-60 parent ECP with molecular weights estimated to be 31.62, 3.19, and 0.60 kDa, while the three peaks eluted from the non-purified RET-04 mutant ECP were considerably smaller at estimated molecular weights of 5.65, 0.55, and 0.10 kDa (Figures 2 and 3). Because of the small size of most of the respective ECP components, it is possible the molecular weights estimated for these components may not be accurate as the elution times and estimated molecular weights fell outside the range established by the standards. Additionally, the estimated molecular weights of the 0.60, 0.55, and 0.10 kDa components are smaller than the molecular weight cutoff of the column used to

concentrate the ECP. The 31.62 kDa component detected in the elution profile of the FL6-60 parent ECP was not observed for the RET-04 mutant ECP (Figures 2 and 3), but was comparable in elution time and molecular weight to the first peak identified for the TSB control (Figure 4). This fraction probably did not contribute considerably to the chemoattractant capabilities of the semi-purified FL6-60 parent ECP given the generally similar macrophage migration (Tables 3 and 4) elicited by both ECP despite the lack of this component in the RET-04 mutant ECP profile. These results suggest the chemoattractant properties of the FL6-60 parent and RET-04 mutant ECP may be attributed to the small molecular weight components revealed in the semi-purification of both ECP preparations. Notwithstanding the apparent distinction in molecular weight of the semi-purified ECP, few differences between the semi-purified FL6-60 parent and RET-04 mutant ECP were exhibited in the stimulation of macrophage migration as evaluated by the lower surface and checkerboard assays. Similar chemoattractant activity was observed for both semi-purified ECP for all treatments, with the exception of the 50 and 70% ECP concentration in the upper chamber over 0% ECP concentration in the lower chamber replicates where significantly higher migration was seen with the FL6-60 semi-purified ECP over the RET-04 semi-purified ECP at the same concentrations (Tables 3 and 4). While similar quantities of protein were detected in the non-purified FL6-60 parent and RET-04 mutant ECP, BCA analysis of the semi-purified ECP revealed no protein content. Additional analysis is necessary to determine the chemical nature of the chemoattractant factors contained in the semi-purified ECP. As such, differences in chemotactic and chemokinetic activity between the semi-purified FL6-60 parent and RET-04 mutant may be due to varying amounts of chemoattractant constituents contained

in the semi-purified fractions. Further chemotactic blind well chamber comparisons of the ECP, standardized by weight, should be conducted to ensure analogous quantities of chemoattractant components are present in both semi-purified ECP. Also, Weeks-Perkins and Ellis (1995) suggested a major chemotactic component of *A. salmonicida* was the 50 kDa cell associated proteins comprising the A-layer, and differences were noted in levels of macrophage migration induced by an A-layer positive virulent isolate and an A-layer negative attenuated isolate. In a study conducted by Arias et al. (2003), compositional differences in LPS expressed by the virulent EILO parent and attenuated RE-33 rifampicin-mutant *E. ictaluri* isolates were analyzed. It was reported the RE-33 mutant was deficient in the production of high molecular weight LPS exhibited by the EILO parent (Arias et al. 2003). It is possible cell associated factors, such as LPS, could be involved in the chemoattractant activity of the FL6-60 parent and RET-04 mutant and potential differences between the isolates could be illuminated through further investigation and characterization of these components.

Overall, both semi-purified ECP induced primarily chemotactic migration as illustrated by the neutralization of migration seen in replicates with equal ECP quantities in both the upper and lower chambers (Tables 3 and 4). In the lower surface assay, higher mean macrophage migration was observed for both semi-purified ECP in comparison with their non-purified counterparts at 50 and 70% ECP concentrations in the lower chamber. Additionally, for both ECP fractions the activity of the semi-purified preparations appeared more chemotactic in the checkerboard assay than did the non-purified fractions, especially considering the neutralization of migration displayed in this trial. However, direct comparisons of macrophage behavior elicited by the non-purified

and semi-purified fractions may need further consideration given the greater amount of macrophage movement observed for the control replicates in the semi-purified ECP study (Tables 3 and 4). Additional study of the differences in chemoattractant properties between the non-purified and semi-purified ECP is warranted.

Early stages of the inflammatory response are characterized by the migration of leukocytes from the blood and surrounding tissues to the site of injury. The initial activity of phagocytes often determines the outcome of a microbial infection (Griffin 1984; Weeks et al. 1988; Weeks-Perkins and Ellis 1995; Klesius and Sealey 1996). The defense against *Edwardsiella* septicemia in fish is significantly impacted by the activity of macrophages (Miyazaki and Egusa 1976a, 1976b; Miyazaki and Kaige 1985; Padrós et al. 2006). Mutoloki et al. (2006) reported *A. salmonicida* derived ECP induced inflammatory reactions and may have enhanced the production of chemotactic signals in Atlantic salmon *Salmo salar* L. Similarly, the excretion of ECP by *E. tarda* could contribute to the localized effects of this bacterium on specific organs through chemoattractant abilities and proinflammatory action. The results of the present study found *E. tarda* ECP exhibited some chemotactic impact on Nile tilapia macrophages, though most activity appeared to be chemokinetic. Stimulation by *E. tarda* ECP could, to some extent, directly lead to the migration of macrophages toward the site of bacterial invasion, but more importantly could aid in initiating the production and release of host derived chemoattractant substances, such as components of the complement system, involved in the mediation of the inflammatory response (Nash et al. 1986; Klesius and Sealey 1996; Ellis 2001).

The pathogenesis of *E. tarda* in fish has yet to be fully elucidated. Numerous virulence factors, including extracellular toxin production (Ullah and Arai 1983a, 1983b; Suprapto et al. 1995; Suprapto et al. 1996; Tan et al. 2002), have been implicated in *Edwardsiella* septicemia. As previously mentioned, the stimulation of phagocyte migration by the excreted products of *E. tarda* to the source of microbial invasion is potentially an important defense mechanism of the host against this disease. Nevertheless, the interaction of this bacterium with macrophages could actually play a role in the manifestation of disease. *Edwardsiella tarda* has been reported to successfully invade host phagocytic cells and avoid killing by phagocyte mediated processes (Ainsworth and Chen 1990; Srinivasa Rao et al. 2001; Srinivasa Rao et al. 2003). Potentially, attraction of macrophages by *E. tarda* and its secreted products could function to advance the dissemination of the pathogen and contribute to the development of septicemia (Klesius and Sealey 1996; Srinivasa Rao et al. 2001; Padrós et al. 2006). While this investigation indicates ECP of FL6-60 parent and RET-04 mutant *E. tarda* are capable of stimulating Nile tilapia macrophage migration, further study is necessary to determine the specific nature of these host-pathogen interactions and the potential contributions to immune defense and virulence.

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Table 1. Effect of varying the concentration of non-purified extracellular products (ECP) obtained from the FL6-60 parent isolate of *Edwardsiella tarda* in the upper and lower assay chambers on the number of Nile tilapia macrophages migrating to the lower chamber.

Percent concentration of ECP in the lower chamber	**Percent concentration of ECP in the upper chamber**				
	0	**10**	**30**	**50**	**70**
0	6.9±0.6^{i}	15.6±2.1^{f,g,h}	**9.3±1.5^{h,i}**	**23.2±2.9^{b,c,d,e,f}**	22.4±2.2^{b,c,d,e,f}
10	8.8±2.2^{h,i}	17.8±1.2^{f,g}	22.5±2.6^{b,c,d,e,f}	18.6±1.6^{f,g}	**29.4±0.8^{a,b,c}**
30	23.0±1.5^{b,c,d,e,f}	23.2±2.1^{b,c,d,e,f}	16.4±1.2^{f,g}	26.8±2.4^{a,b,c,d,e}	30.0±1.9^{a,b}
50	29.8±3.5^{a,b}	22.0±3.3^{c,d,e,f}	13.4±0.7^{g,h,i}	19.2±1.0^{e,f,g}	23.3±2.9^{b,c,d,e,f}
70	33.4±2.3^{a}	27.6±3.3^{a,b,c,d}	22.4±3.0^{b,c,d,e,f}	20.6±4.4^{d,e,f,g}	**30.0±2.8^{a,b}**

Mean±standard error of duplicate filters, each read in five fields of view, with a light microscope at 400X. Macrophages added to the upper chamber and migration to ECP in lower chamber was measured. Macrophage values without a letter in common are significantly different ($P < 0.05$).

Macrophage values in bold are significantly different ($P < 0.05$) from values observed for RET-04 mutant ECP at the same concentrations.

Table 2. Effect of varying the concentration of non-purified extracellular products (ECP) obtained from the RET-04 mutant isolate of *Edwardsiella tarda* in the upper and lower assay chambers on the on the number of Nile tilapia macrophages migrating to the lower chamber.

Percent concentration of ECP in the lower chamber	**Percent concentration of ECP in the upper chamber**				
	0	**10**	**30**	**50**	**70**
0	6.9±0.6^{h}	11.2±2.4^{g,h}	**18.0±2.1^{e,f,g}**	**7.3±2.9^{h}**	20.4±2.7^{c,d,e,f}
10	15.3±2.2^{f,g}	15.0±1.9^{f,g}	17.4±1.6^{e,f,g}	19.0±1.7^{d,e,f,g}	**16.0±3.1^{e,f,g}**
30	21.0±3.5^{c,d,e,f}	17.8±3.2^{e,f,g}	15.0±1.7^{f,g}	30.2±1.0^{a,b}	27.0±1.8^{b,c}
50	25.6±1.2^{b,c,d}	27.0±2.9^{b,c}	17.5±4.5^{e,f,g}	20.2±4.0^{c,d,e,f}	23.6±1.8^{b,c,d,e}
70	36.2±1.4^{a}	29.8±3.3^{a,b}	17.8±2.0^{e,f,g}	22.8±0.5^{b,c,d,e,f}	**16.2±0.1^{e,f,g}**

Mean±standard error of duplicate filters, each read in five fields of view, with a light microscope at 400X. Macrophages added to the upper chamber and migration to ECP in lower chamber was measured. Macrophage values without a letter in common are significantly different ($P < 0.05$).

Macrophage values in bold are significantly different ($P < 0.05$) from values observed for FL6-60 parent ECP at the same concentrations.

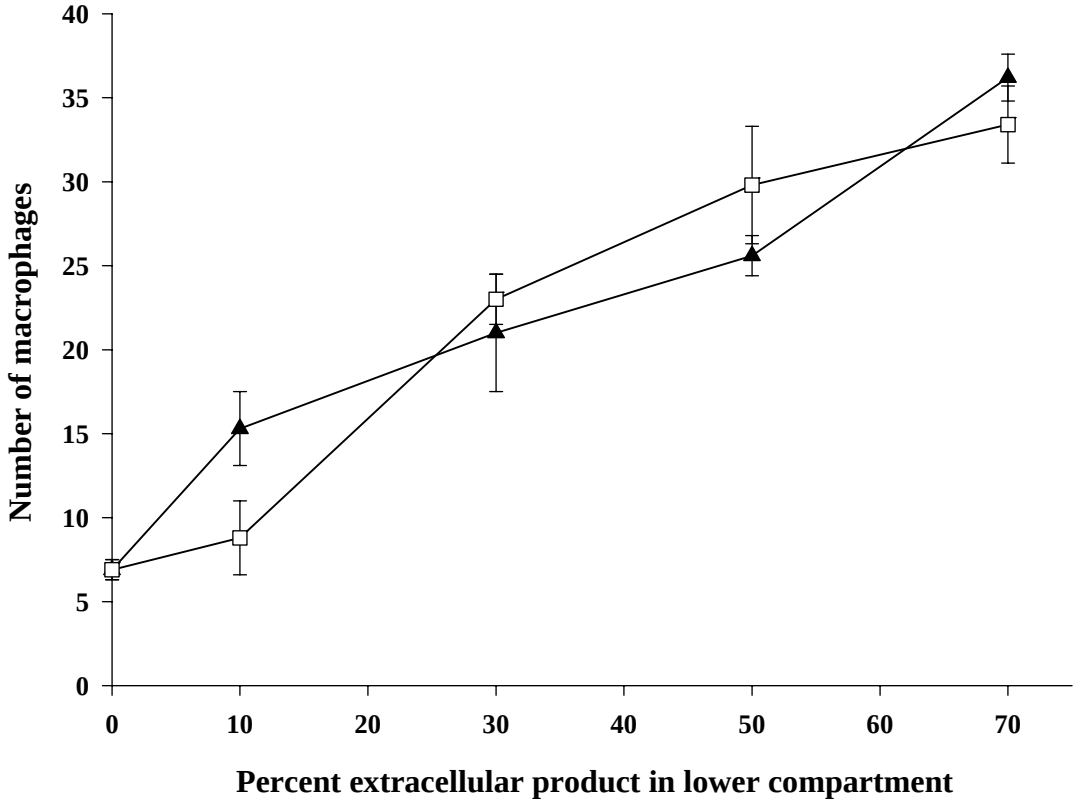

Figure 1. Nile tilapia macrophage chemotaxis (mean migration±standard error) in response to non-purified *Edwardsiella tarda* FL6-60 parent (□) and RET-04 mutant (▲) extracellular products (ECP).

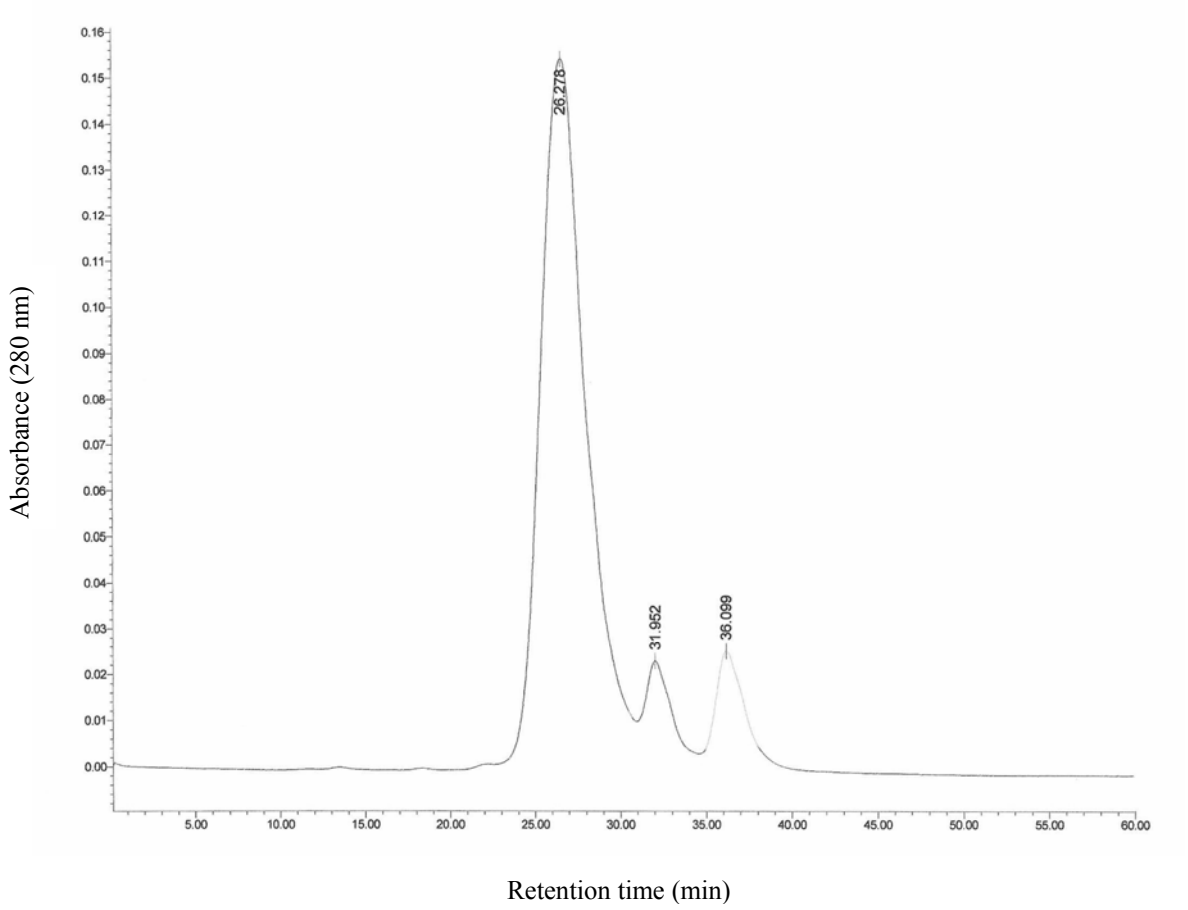

Figure 2. Retention profile (detection at 280 nm) of *Edwardsiella tarda* FL6-60 parent extracellular products (ECP) from high pressure liquid chromatography (HPLC) in 0.1 M phosphate buffered saline (PBS).

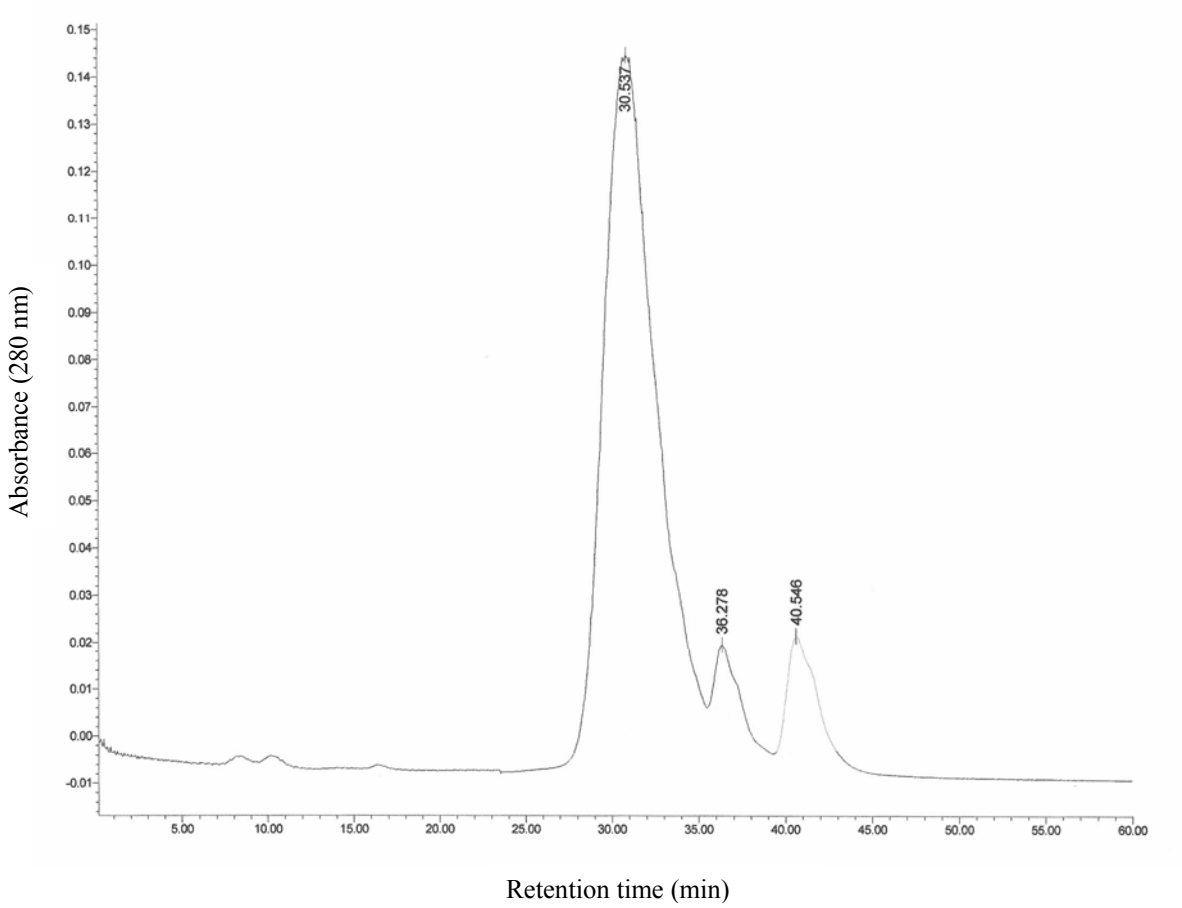

Figure 3. Retention profile (detection at 280 nm) of *Edwardsiella tarda* RET-04 mutant extracellular products (ECP) from high pressure liquid chromatography (HPLC) in 0.1 M phosphate buffered saline (PBS).

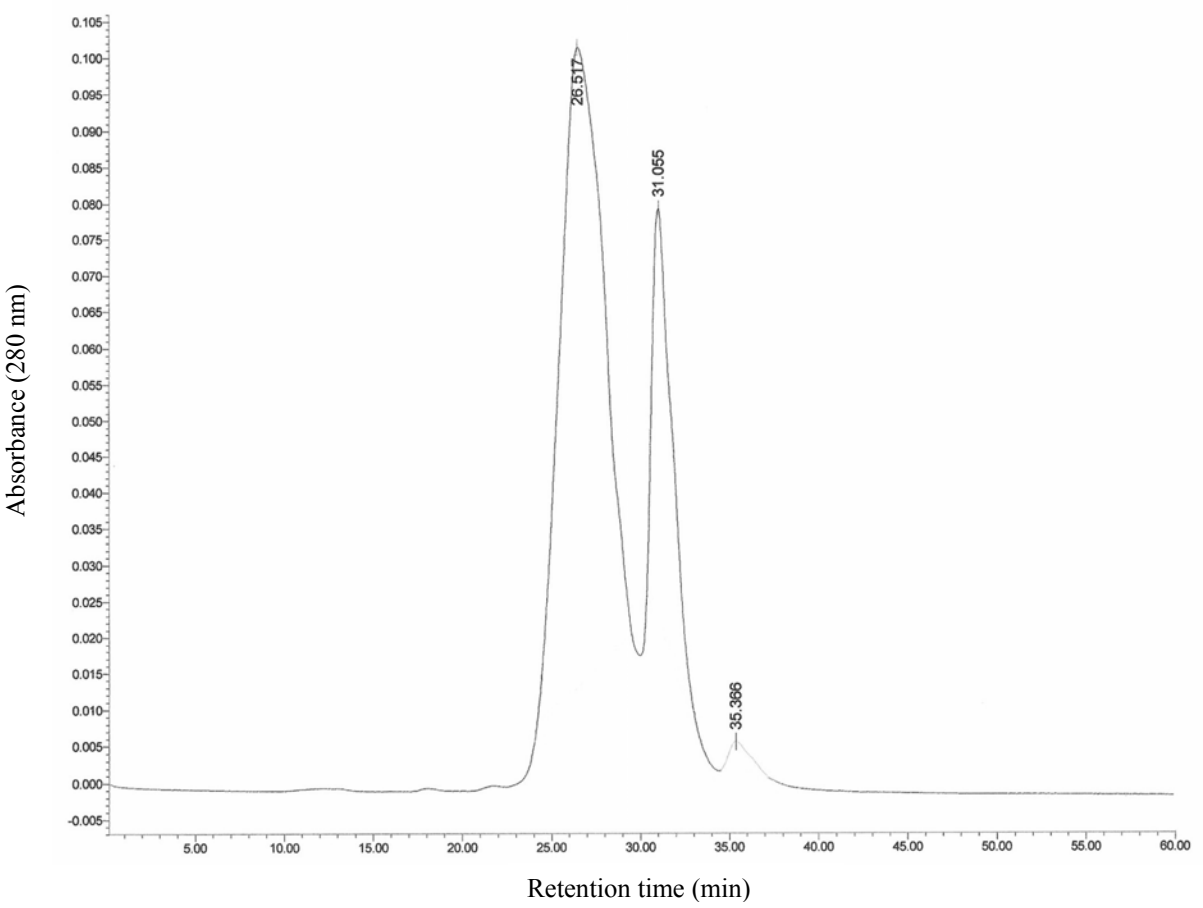

Figure 4. Retention profile (detection at 280 nm) of tryptic soy broth (TSB) from high pressure liquid chromatography (HPLC) in 0.1 M phosphate buffered saline (PBS).

Table 3. Effect of varying the concentration of semi-purified extracellular products (ECP) obtained from the FL6-60 parent isolate of *Edwardsiella tarda* in the upper and lower assay chambers on the number of Nile tilapia macrophages migrating to the lower chamber.

Percent concentration of ECP in the lower chamber	**Percent concentration of ECP in the upper chamber**		
	0	**50**	**70**
0	20.6±1.6[c]	**38.9±2.6[b]**	**50.7±1.5[a]**
50	37.8±2.2[b]	22.2±2.6[c]	26.3±3.0[c]
70	47.4±2.3[a]	26.4±3.0[c]	20.5±1.6[c]

Mean±standard error of duplicate filters, each read in five fields of view, with a light microscope at 400X. Macrophages added to the upper chamber and migration to ECP in lower chamber was measured. Macrophage values without a letter in common are significantly different ($P < 0.05$).

Macrophage values in bold are significantly different ($P < 0.05$) from values observed for RET-04 mutant ECP at the same concentrations.

Table 4. Effect of varying the concentration of semi-purified extracellular products (ECP) obtained from the RET-04 mutant isolate of *Edwardsiella tarda* in the upper and lower assay chambers on the number of Nile tilapia macrophages migrating to the lower chamber.

Percent concentration of ECP in the lower chamber	**Percent concentration of ECP in the upper chamber**		
	0	**50**	**70**
0	20.6±1.6[e]	**25.4±2.7[d,e]**	**30.7±4.2[c,d]**
50	37.4±1.6[b]	19.8±1.2[e]	32.3±1.6[b,c]
70	43.8±2.7[a]	22.4±4.5[e]	23.4±1.0[e]

Mean±standard error of duplicate filters, each read in five fields of view, with a light microscope at 400X. Macrophages added to the upper chamber and migration to ECP in lower chamber was measured. Macrophage values without a letter in common are significantly different ($P < 0.05$).

Macrophage values in bold are significantly different ($P < 0.05$) from values observed for FL6-60 parent ECP at the same concentrations.

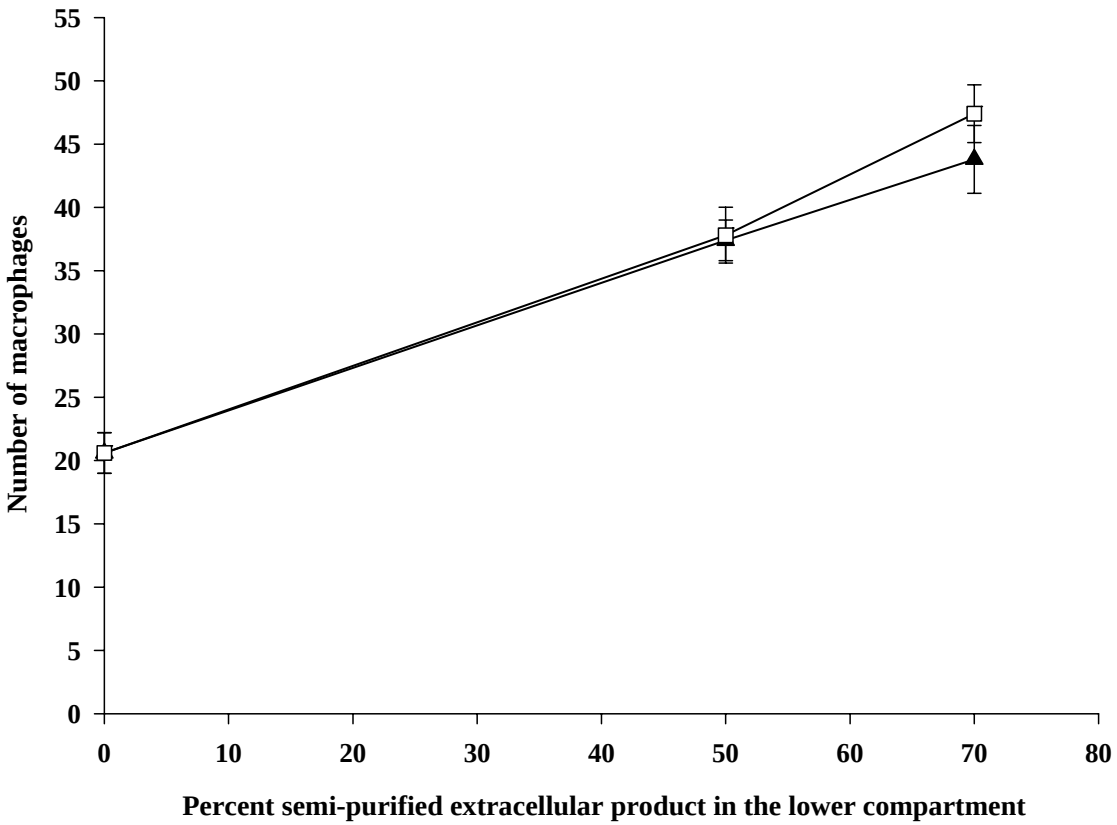

Figure 5. Nile tilapia macrophage chemotaxis (mean migration±standard error) in response to semi-purified *Edwardsiella tarda* FL6-60 parent (□) and RET-04 mutant (▲) extracellular products (ECP).

III. PASSIVE IMMUNIZATION OF NILE TILAPIA *OREOCHROMIS NILOTICUS* AGAINST *EDWARDSIELLA TARDA*

Abstract

A study was conducted to determine if passive immunization of Nile tilapia *Oreochromis niloticus* with *Edwardsiella tarda* immune serum could elicit protection against experimental infection. The *E. tarda* immune serum was obtained from fish intraperitoneally (IP) injected with 1.5 x 10^8 CFU/mL *E. tarda* FL6-60 or tryptic soy broth (TSB), as a control. Serum was obtained at 14 d after injection (immune and nonimmune) and at 14 d following challenge with 3.0 x 10^8 CFU/mL *E. tarda* FL6-60 (hyperimmune). An antibody response was identified in the immunized fish by enzyme-linked immunosorbent assay (ELISA) and immunity confirmed by 100% survival of actively infected and challenged fish. Fish were passively immunized by IP injection with pooled nonimmune or hyperimmune serum, or phosphate buffered saline (PBS), as a control, and challenged with 7.0 x 10^7 CFU/mL *E. tarda* FL6-60 72 h after immunization. Serum obtained at 72 h after immunization from fish passively immunized with hyperimmune serum illustrated a significantly higher antibody titer than observed for fish immunized with nonimmune serum or PBS. Cumulative percent mortality was 6.7, 26.7, and 17.9% for the hyperimmune, nonimmune, and PBS treatments, respectively. Differences in cumulative percent mortality were significant ($P < 0.0001$). The results of this investigation suggest specific antibody titers against *E. tarda* following passive immunization may play a role in protection of Nile tilapia against experimental infection.

Introduction

Edwardsiella tarda, the pathogenic bacterium responsible for *Edwardsiella* septicemia in fish, has been implicated in significant losses of commercially cultured freshwater and marine species throughout the world (Thune et al. 1993; Plumb 1999; Plumb and Evans 2006). Despite the potentially serious threat posed by *E. tarda* to farmed fish populations, few effective methods of treatment or prevention are presently available. Chemotherapy is often ineffective against *Edwardsiella* septicemia due to the limited selection of drugs available for use in aquaculture (Thune et al. 1993; Center for Veterinary Medicine 2003) and the reported occurrence of antibiotic resistance in *E. tarda* isolates (Aoki et al. 1977; Waltman and Shotts 1986). Use of immunostimulants such as β-glucans in the prevention of *Edwardsiella* septicemia has been applied with some success (Park and Jeong 1996; Sahoo and Mukherjee 2002; Misra et al. 2006a, 2006b). In recent years, vaccination has become a preferred method for the prevention of infectious disease in fish production systems (Gudding et al. 1999). Although numerous vaccine approaches have been investigated for the prevention of *Edwardsiella* septicemia in a variety of fish species (Salati 1988; Plumb 1999; Plumb and Evans 2006), a commercial vaccine for the prevention of *E. tarda* has yet to be developed.

Immunoprophylaxis is characterized by stimulation of the innate and acquired immune mechanisms of the fish (Gudding et al. 1999). The production of specific antibodies and the activation of the humoral immune system are often crucial in eliciting protection against bacterial pathogens (Ellis 1988; Ellis 1999). Passive immunization has been investigated as a possible method of disease prevention and used to determine the role of specific antibodies in eliciting immunity against fish pathogens such as

Aeromonas salmonicida (Spence et al. 1965; Marquis and Lallier 1989), *Flavobacterium psychrophilum* (LaFrentz et al. 2003), *Streptococcus* spp. (Akhlaghi et al. 1996; Shelby et al. 2002; Pasnik et al. 2006), *Vibrio anguillarum* (Harrell et al. 1975; Akhlaghi 1999), and *Yersinia ruckeri* (Olesen 1991). Varying levels of protection and reduction of mortality were observed in these evaluations of passive immunization. Additionally, Klesius and Sealey (1995) evaluated the ability of antibodies obtained from *E. ictaluri* immune channel catfish to passively immunize naïve channel catfish. While the administration of serum antibodies failed to reduce mortality in the experimentally challenged fish, *E. ictaluri* circulating antibody levels were observed to be significantly increased prior to challenge (Klesius and Sealey 1995). Gutierrez et al. (1993) demonstrated reduced mortality of Japanese eel through oral administration of anti-*E. tarda* chicken egg yolk immunoglobulin (Ig) Y. This study concluded oral immunization with *E. tarda* specific antibodies could be effective in the prevention of disease outbreaks, but not beneficial to already diseased fish (Gutierrez et al. 1993). The present study was conducted to evaluate the role of *E. tarda* immune antibodies, administered by passive immunization, in protection of Nile tilapia against experimental *E. tarda* infection.

Materials and Methods

Bacteria

Edwardsiella tarda isolate FL6-60 was grown in tryptic soy broth (TSB, Difco Laboratories, Sparks, Maryland) for 24 h at 28° C. The virulent FL6-60 isolate was obtained from a morbid striped bass *Morone saxatilis* (Baya et al. 1997). This isolate was received courtesy of Dr. A.M. Baya (Animal Health Diagnostic Laboratory,

Maryland Department of Agriculture, College Park, Maryland). Virulence of the FL6-60 isolate was determined through previous studies (unpublished; Wiedenmayer et al. 2006). The cultures were adjusted to approximately 1.5×10^8, 3.0×10^8, and 7.0×10^7 colony forming units (CFU)/mL for the active immunization, active immunization challenge, and passive immunization challenge inocula, respectively. The fish were immunized or challenged by intraperitoneal (IP) injection of 0.1 mL of a particular inoculum.

Fish

Nile tilapia *Oreochromis niloticus* were produced and maintained at the USDA-ARS Aquatic Animal Health Research Laboratory (AAHRL), Auburn, Alabama. Prior to experiments, brain, liver, intestine, and head kidney samples were taken aseptically from 20 fish, and shown to be culture negative for *E. tarda* on *Salmonella Shigella* agar (SSA, Remel, Inc., Lenexa, Kansas) incubated for 24 h at 28° C in an atmosphere of air. Two weeks before the beginning of the study, the fish were acclimated in 57 L glass aquaria. The fish were fed a commercial diet (Aquamax Grower 400, Brentwood, Missouri) daily to satiation. The aquaria were provided with flow-through dechlorinated tap water at 26±1° C at a rate of 0.5 L/min and aeration with air stones. For the duration of the study, the dissolved oxygen, temperature, pH, salinity, hardness, ammonia, and nitrite levels were monitored and maintained within acceptable ranges. During the experiment, the mean±standard deviation of dissolved oxygen was 5.8±0.5 mg/L, temperature was 26.2±0.8° C, pH was 7.1±0.3, salinity was 0.1±0.0%, and hardness was 110±10mg/L ($CaCO_3$). Ammonia and nitrite concentrations were consistently below the detection limit (0 mg/mL) during the experiment. The fish were exposed to light and dark intervals of 12:12 h. Feeding was suspended the day of immunization or challenge.

Production of *E. tarda* hyperimmune and nonimmune sera

Two aquaria, of 35 and 20 Nile tilapia (38±7 g, mean weight±standard deviation), were used to generate sera for the passive immunization trials. To produce active immunization by infection, the group of 35 fish was IP injected with 0.1 mL of 1.5 x 10^8 CFU/mL inoculum, while the group of 20 fish was IP injected with 0.1 mL of sterile TSB to yield nonimmune serum. For 13 d following immunization, the fish remained in separate aquaria and were maintained as previously described. They were monitored daily for clinical signs of disease and mortality. At 13 d after immunization, 20 immune and 20 nonimmune fish were bled from the caudal vein and the samples retained for enzyme-linked immunosorbent assay (ELISA) analysis and passive immunization trials. Blood samples were allowed to clot for 1 h at 25° C. The serum was separated by 5 min centrifugation at 300 x g and stored at -80° C until use.

On d 14 after immunization, the surviving fish were challenged by IP injection with 0.1 mL of 3.0 x 10^8 CFU/mL inoculum and monitored as described for an additional 14 d. During this period, approximately 20% of moribund and dead fish were sampled aseptically to confirm the presence of *E. tarda* from the brain, head kidney, liver, and intestine. Samples were cultured on SSA and incubated at the aforementioned conditions. Mortality was recorded. At 14 d after challenge, 20 hyperimmune (actively infected and challenged) fish were bled as described and the serum retained for ELISA and passive immunization trials.

Passive immunization and *E. tarda* challenge infection

For the passive immunization study, three tanks of 40 fish (6±1 g, mean weight±standard deviation) were used. Fish were passively immunized by IP injection

with 50 µL hyperimmune serum, nonimmune serum, or sterile PBS as a control. At 72 h after immunization, 10 fish from each treatment were bled from the caudal vein and serum collected to determine antibody titers against *E. tarda* by ELISA. The remaining 30 fish in each treatment were challenged with 0.1 mL of 7.0 x 10^7 CFU/mL inoculum by IP injection. Fish were monitored and sampled as previously described for an additional 14 d.

Enzyme-linked immunosorbent assay (ELISA)

Acquired Nile tilapia sera were tested to determine antibody titers against an *E. tarda* fraction by an indirect ELISA procedure, based on the methodology of Shelby et al. (2002). Blood samples obtained at 72 h after immunization were prepared as previously described. The ELISA coating antigen was prepared from whole cell FL6-60 *E. tarda*. The cells were harvested from a 24 h culture through centrifugation at 3000 x g. The cells were sonicated for 2 min and the resulting sonicate centrifuged at 4000 x g for 20 min. Following centrifugation, the supernatant was separated and removed from the cellular debris. The total protein content of the sonicate fraction was determined by the bicinchoninic acid (BCA) method (BCA Protein Assay Kit, Pierce, Rockford, Illinois) and adjusted to 500 µg/mL. A 1:100 dilution of the fraction was made in 0.5 M sodium carbonate buffer and 100 µL of the dilution added to each well of a 96-well microtiter plate (Maxisorp, Nunc, Roskilde, Denmark). The plates were incubated overnight at 25° C, and then washed five times with phosphate-buffered saline plus 0.05% tween-20 (PBS-T). The wells were blocked with 100 µL SuperBlock® blocking buffer (Pierce) for 1 h at 25° C and washed as previously described. Serum samples were initially diluted 1:100 in phosphate buffered saline (PBS) and 100 µL of each preparation added to the

wells and diluted serially 1:2 on the vertical axis of the microtiter plate to provide a range of 1:100 to 1:102400 dilutions. The plate was incubated for 30 min at 25° C and washed as previously described. Mouse anti-tilapia IgM heavy chain specific monoclonal antibody 1H1 (Shelby et al. 2002) was diluted 1:5000 in PBS and 100 µL added to each well. The plate was incubated for 30 min at 25° C and washed as previously described. Peroxidase-conjugated rabbit anti-mouse IgG (Pierce) was diluted 1:5000 in PBS and 100 µL added to the wells. Following 30 min incubation at 25° C and washing five times in PBS, 50 µL of One Step Ultra TMB-ELISA® was added to the wells. The ELISA reaction was stopped at 5 min by adding 50 µL 3 M H_2SO_4 to each well. The optical density (OD) of the ELISA reactions was read spectrophotometrically (Model 680 microplate reader, Bio-Rad**,** Hercules, California) at 450 nm. Antibody titer for the samples was reported as the logarithmic (log_{10}) transformation of the reciprocal of the highest serum dilution yielding an OD of 0.100 at 450 nm. Negative controls were included on each plate and consisted of wells coated with antigen and no serum sample.

Statistical analysis

Mortality data and ELISA results were analyzed using SAS software (SAS Institute, Cary, North Carolina) by one-way analysis of variance (ANOVA) with Duncan's multiple range test for significance. Significant differences were determined at $P < 0.05$.

Results

Production of *E. tarda* hyperimmune and nonimmune sera

Fish were immunized by IP injection with 1.5 x 10^8 CFU/mL *E. tarda* FL6-60 inoculum or TSB as a control and subsequently bled after 13 d to obtain serum for use in

the passive immunization study. Following immunization, 5.7 and 0% cumulative percent mortality was observed in the *E. tarda* and TSB treated tanks, respectively. An additional 17.1 and 10.0% mortality occurred after blood collection from the *E. tarda* and TSB treated tanks, respectively (Figure 1). At 14 d after immunization, both groups of fish were challenged by IP injection with 3.0 x 10^8 CFU/mL *E. tarda* FL6-60 inoculum. After this second injection, the fish were monitored for an additional 14 d. In fish initially injected with *E. tarda*, clinical disease signs were absent and no further mortality was observed after the second injection of *E. tarda*. Signs of *Edwardsiella* septicemia, including lethargy and refusal of food, were displayed by the TSB treated fish. Mortality in the TSB treated fish commenced within 48 h of IP challenge and totaled 30.0% (Figure 1). Samples were obtained for microbiological analysis after challenge and cultured on SSA for 24 h at 28° C. Cultures considered positive displayed small (0.5-1.0 mm) diameter colonies that were white to translucent in color with black centers indicative of H_2S production. The primary isolates were also cytochrome oxidase negative (Farmer and McWhorter 1984). All dead fish were culture positive for *E. tarda* from the brain, intestine, liver, and head kidney. Blood was collected from the *E. tarda* actively infected-challenged fish at 14 d after challenge to serve as hyperimmune serum in the passive immunization study.

Sera acquired from the hyperimmune, immune, and nonimmune fish from the active immunization study was pooled and five replicate samples analyzed by ELISA. The antibody titer was determined to be 5.010±0.000 (1:102329) for the pooled hyperimmune serum, 3.927±0.165 (1:8452) for the pooled immune serum, and 2.843±0.252 (1:696) for the pooled nonimmune serum. Of the serum utilized in the

passive immunization study, the antibody titer of the pooled hyperimmune serum (1:102329) was observed to be significantly ($P < 0.0001$) higher than that of the pooled nonimmune serum (1: 696).

Passive immunization and *E. tarda* challenge infection

In the passive immunization study, three groups of 40 fish were administered hyperimmune serum, nonimmune serum, or sterile PBS as a control. At 72 h after immunization, 10 fish were selected from each group for blood collection, while the remaining 30 fish in each treatment were challenged by IP injection with 7.0×10^7 CFU/mL *E. tarda* FL6-60 inoculum. At 24 h after challenge, fish in each group were lethargic and refused food. Mortality also commenced at 24 h after challenge, although most occurred between 48 to 96 h following challenge (Figure 2). Cumulative percent mortality was 6.7% for the hyperimmune group, 26.7% for the nonimmune group, and 17.9% for the PBS group (Table 1). Significant ($P < 0.0001$) differences between cumulative percent mortality were observed between all treatments. All dead fish were culture positive for *E. tarda* from the brain, intestine, liver, and head kidney.

Sera acquired from experimental fish 72 h following passive immunization were analyzed by ELISA. The mean antibody titer was determined to be 4.408±0.246 (1:25585) for the hyperimmune group, 2.783±0.638 (1:606) for the nonimmune group, and 2.602 ±0.394 (1:399) for the PBS group. Antibody titer was significantly ($P < 0.0001$) higher in fish obtained from the hyperimmune group (1:25585) than in either the nonimmune (1:606) or PBS (1:399) groups (Table 1). No significant ($P < 0.4946$) difference in antibody titer was observed in comparison of the nonimmune and PBS groups (Table 1).

Discussion

In the present study, Nile tilapia were actively immunized and subsequently challenged with the FL6-60 isolate of *E. tarda* to produce hyperimmune serum. The results illustrated an antibody response following initial infection and a secondary antibody response after challenge infection. This observation was supported by mean antibody titers of pooled hyperimmune and immune serum higher than that of nonimmune serum as obtained by ELISA analysis. The pooled hyperimmune and nonimmune sera were selected to immunize fish against *E. tarda* for the passive immunization study.

For the passive immunization experiment, passively immunized fish were challenged 72 h after immunization with 0.1 mL of *E. tarda* FL6-60 7.0 x 10^7 CFU/mL inoculum by IP injection. Mortality was initially seen at 24 h after challenge in the PBS treated group, although most deaths in all treatments were recorded at 48-96 h after challenge (Figure 2). The lowest cumulative percent mortality, 6.7%, was observed in fish passively immunized with hyperimmune serum, followed by the PBS treated fish with 17.9% mortality (Table 1). The nonimmune serum immunized group exhibited 26.7% cumulative mortality, and differences in cumulative percent mortality between all treatments were significant (Table 1). Small (6±1 g) sized fish were selected for this trial, based on the observations of greater effectiveness of passive immunization against *Lactococcus garviae* and *S. iniae* due to higher relative amounts of challenge inoculum contributing to increased susceptibility to disease (Muzquiz et al. 1999; Shelby et al. 2002). It is possible the small size of the experimental fish contributed to the significant differences in cumulative percent mortality observed between treatment groups.

As the data acquired from the passive immunization study revealed a 20% increase in survival in the hyperimmune serum treated fish over the nonimmune serum treated fish, measurements of *E. tarda* specific antibody titer of sera collected 72 h after immunization also reflected differences. The mean antibody titers of 10 fish sampled from the PBS and nonimmune groups were 2.602±0.394 (1:399) and 2.783±0.638 (1:606), respectively, and were not significantly different (Table 1). Conversely, the mean antibody titer of 10 fish obtained from the hyperimmune group was significantly higher than both the PBS and nonimmune treatments at 4.408±0.246 (1:25585) (Table 1). These findings are in accordance with those of Pasnik et al. (2006) in their observation of significantly increased levels of specific *S. agalactiae* antibodies 72 h following passive immunization of Nile tilapia with anti-*S. agalactiae* serum. Further studies would need to be conducted to determine the duration of these elevated antibody levels as decreases in levels of protection, attributed to decline in antibody titer, conferred by passive immunization over time have been reported (Akhlaghi et al. 1996).

Passive immunization has been investigated as a method of prophylaxis or therapy against a number of fish pathogens. Immune protection of limited duration has been observed with passive immunization of salmonids against *A. salmonicida* (Spence et al. 1965; Marquis and Lallier 1989). Effective protection against *Streptococcus* spp. has been reported for rainbow trout *Oncorhynchus mykiss* and Nile tilapia (Akhlaghi et al. 1996; Shelby et al. 2002; Pasnik et al. 2006). Harrell et al. (1975) and Akhlaghi (1999) found passive immunization successful in the prevention of vibriosis in rainbow trout. Olesen (1991) also indicated humoral immunity is an important component in defense against enteric redmouth disease resulting from *Y. ruckeri* infection. Other studies have

found passive transfer of immune serum yields marginal or no protection. In immunization trials against *F. psychrophilum* in rainbow trout, LaFrentz et al. (2003) observed minimal immunity conferred by passive immunization and theorized that significant protection required exposure to high titer serum (≥51,200). Klesius and Sealey (1995) reported antibodies to *E. ictaluri* were not capable of eliciting protection against ESC mortality in channel catfish *Ictalurus punctatus*. Presently, few analyses have evaluated the potential of passive immunization in protection of fish against *Edwardsiella* septicemia. Gutierrez et al. (1993) reported oral administration of anti-*E. tarda* chicken egg yolk IgY in Japanese eel prevented systemic infection via the intestine. It was suggested that passive oral immunization with anti-*E. tarda* IgY could be an advanced method of *Edwardsiella* septicemia prophylaxis, especially useful when disease episodes are anticipated and time is insufficient for the development of active immunity in the endangered population (Gutierrez et al. 1993; Gutierrez and Miyazaki 1994; Akhlaghi et al. 1996). The results of the present study also appear to support the potential value of passive immunization to prevent *Edwardsiella* septicemia outbreaks in vulnerable fish.

The importance of humoral factors in immune protection of fish against *E. tarda* is uncertain. In the present study, levels of specific *E. tarda* antibody were observed to be elevated in Nile tilapia passively immunized with hyperimmune serum over those administered PBS or nonimmune serum, and a significant decrease in mortality was observed. However, reports detailing trials in Japanese eel, Japanese flounder, and Nile tilapia reported increases in the production of antibodies specific for *E. tarda* induced by various vaccine formulations, though the presence of antibodies did not necessarily elicit

complete protection against challenge (Salati et al. 1983; Mekuchi et al. 1995; Igarashi and Iida 2002). Klesius and Sealey (1995) indicated the passive transfer of immune serum did not prevent *E. ictaluri* related mortality in channel catfish and that specific antibodies do not function unassisted in protection against enteric septicemia of catfish (ESC). The researchers suggested the intracellular nature of *E. ictaluri* limited the contribution of antibodies to defense against this pathogen (Klesius and Sealey 1995). As *E. tarda* is also believed to function intracellularly (Miyazaki and Kaige 1985; Gutierrez and Miyazaki 1994; Rashid et al. 1997; Padrós et al. 2006), similar conclusions may be drawn in the interpretation of the present study. While immune protection was observed in the passive immunization trial, it is likely cell mediated immune responses are also required to induce protection against *E. tarda*. It is possible the reduction in mortality witnessed here could be related to increased macrophage uptake and killing due to bacterial opsonization by *E. tarda* specific antibodies. Shoemaker et al. (1997) reported an increase in the ability of macrophages elicited from immune channel catfish to kill opsonized *E. ictaluri* in *in vitro* assays. Additionally, stimulation of nonspecific innate immune factors could be important in defense against *E. tarda* as reported for other intracellular pathogens (Portnoy 1992); although the increased mortality observed in this study following immunization with nonimmune serum suggests non-specific mechanisms do not contribute greatly to immunity against *E. tarda*. Similar results reported by Shelby et al. (2002) and Pasnik et al. (2006), in the analysis of passive immunization against *Streptococcus* spp., were attributed to a lack of complement or cytokine function in developing immunity to streptococcosis.

In conclusion, the present trials show that active infection of Nile tilapia with *E. tarda* does induce the production of specific antibodies against this pathogen, however; these antibodies alone may not confer complete protection in naïve fish by passive immunization. It is probable that humoral factors play a role in immunity against *E. tarda*, but cell mediated and possibly nonspecific responses are required for significant protection to develop (Miyazaki and Kaige 1985; Portnoy 1992; Gutierrez and Miyazaki 1994; Marsden et al. 1996; Rashid et al. 1997; Daly et al. 2001; Padrós et al. 2006).

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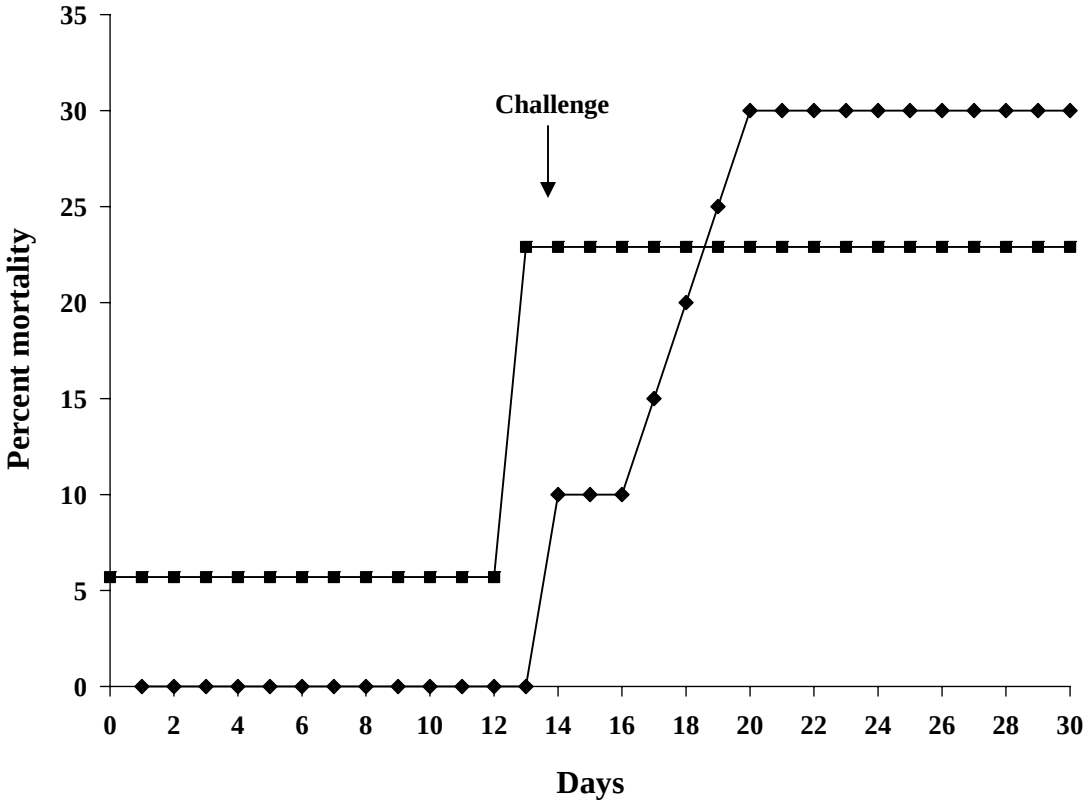

Figure 1. Cumulative percent mortality of Nile tilapia actively immunized with 1.5 x 10^8 CFU/mL *Edwardsiella tarda* FL6-60 (■) or administered tryptic soy broth (TSB) as a control by intraperitoneal (IP) injection (♦) and challenged at 14 d after immunization with 3.0 x 10^8 CFU/mL FL6-60 inoculum by IP injection. On d 13 after immunization, 20 fish from each treatment were bled for serum collection and returned to their respective tanks.

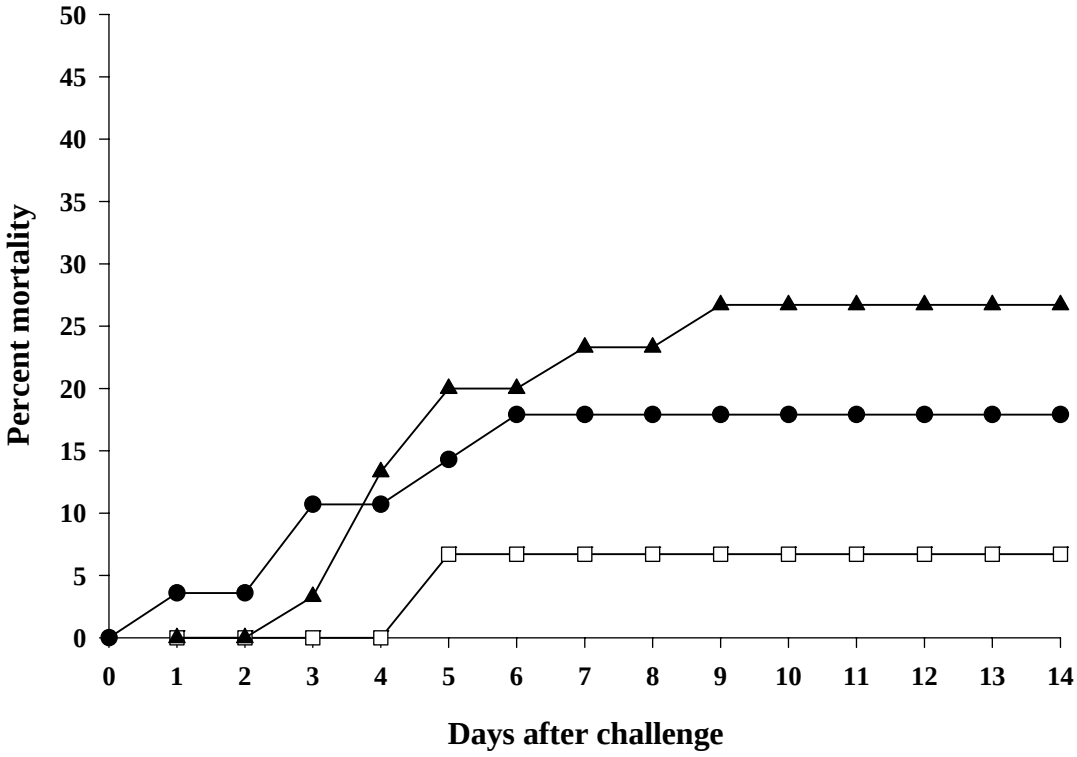

Figure 2. Cumulative percent mortality of Nile tilapia passively immunized with hyperimmune (□) or nonimmune (▲) serum or administered phosphate buffered saline (PBS) as a control (●) by intraperitoneal (IP) injection and challenged at 72 h after immunization with 7.0 x 10^7 CFU/mL *Edwardsiella tarda* FL6-60 by IP injection.

Table 1. *Edwardsiella tarda* isolate FL6-60 antibody titer and cumulative percent mortality of Nile tilapia passively immunized with *E. tarda* hyperimmune or nonimmune serum or administered phosphate buffered saline (PBS) as a control.

Serum administered	*E. tarda* antibody titer (Logarithmic transformation of mean reciprocal±SD)[a]	Cumulative percent mortality[b]
Hyperimmune	4.408±0.246[y]	6.7[x]
Nonimmune	2.783±0.638[z]	26.7[y]
None (PBS)	2.602±0.394[z]	17.9[z]

a Logarithmic (log_{10}) transformation of mean specific antibody titer detected in the sera of 10 passively immunized fish at 72 h after immunization. Mean values with different letters indicate significant difference ($P < 0.05$).

b Cumulative percent survival observed in *E. tarda* challenged fish monitored for 14 d after exposure. Mean values with different letters indicate significant difference ($P < 0.05$).

IV. EFFICACY OF *EDWARDSIELLA TARDA* EXTRACELLULAR PRODUCTS (ECP) IMMUNIZATION OF NILE TILAPIA *OREOCHROMIS NILOTICUS* AND CHARACTERIZATION OF ECP

Abstract

A study was conducted to evaluate protection from experimental challenge elicited in Nile tilapia *Oreochromis niloticus* by intraperitoneal (IP) immunization with extracellular products (ECP) derived from a virulent parent and less virulent mutant isolate of *Edwardsiella tarda*. The virulent isolate, FL6-60, was obtained from a morbid striped bass *Morone saxatilis*, while the RET-04 isolate is a less virulent rifampicin-resistant mutant of FL6-60. Nile tilapia were administered 0.1 mL FL6-60 parent ECP, RET-04 mutant ECP, or tryptic soy broth (TSB), as a control, and were challenged 30 d after immunization with 4.4 x 10^8 CFU/mL FL6-60 parent by IP injection. Cumulative mortality was 64.6%, 63.8%, and 79.5% for the FL6-60 parent ECP immunized, RET-04 mutant ECP immunized, and the control groups, respectively, following challenge. The mortality patterns did not display any significant differences among treatment groups. The relative percent survival (RPS) was 18.7 and 19.7 for the FL6-60 parent and RET-04 mutant ECP immunized groups, respectively. Enzyme-linked immunosorbent assay (ELISA) analyses, incorporating both the FL6-60 parent and RET-04 mutant ECP as the target antigen, were conducted to assess specific antibody titer of sera obtained on d 30 after immunization prior to challenge. Results of the first ELISA analysis, using the FL6-60 parent ECP as the coating antigen, revealed that titers were not significantly different.

The second ELISA analysis, with the RET-04 mutant ECP as the coating antigen, indicated numerically higher antibody titers in serum obtained from both the FL6-60 parent (2.662±0.579) and RET-04 mutant (3.224±0.847) ECP immunized groups over the TSB (2.151±0.301) treated group. Serum acquired from the RET-04 mutant ECP exhibited a significantly ($P < 0.0427$) higher titer as compared to the TSB control. Results of both ELISA analyses suggest a lack of immunodominant antigens present in the FL6-60 parent and RET-04 mutant ECP. Electrophoretic analysis revealed differences in banding profiles between the FL6-60 parent and RET-04 mutant ECP, with a major difference identified as an approximately 175 kDa band resolved in the RET-04 mutant ECP absent in the FL6-60 parent ECP. Despite some apparent dissimilarity in the protein content of the FL6-60 parent and RET-04 mutant ECP, the results of this study found neither ECP fraction capable of eliciting significant protection against virulent *E. tarda* challenge.

Introduction

Edwardsiella tarda, the agent responsible for *Edwardsiella* septicemia of fish, is a gram-negative, rod shaped bacterium commonly found in marine and freshwater environments in both bottom substrate and water. The occurrence of *Edwardsiella* septicemia in cultured fish and economic losses resulting from *E. tarda* infection has motivated researchers to actively pursue methods of preventing this potentially serious disease. Numerous attempts have been made to develop vaccine preparations effective in immune stimulation of fish against *E. tarda* (Salati 1988; Plumb 1999; Plumb and Evans 2006), although a commercial vaccine is presently not available.

Secreted extracellular products (ECP) have been identified as potential sources of virulence for numerous bacterial pathogens of fish including *E. tarda* (Ullah and Arai 1983a, 1983b; Suprapto et al. 1995; Suprapto et al. 1996; Tan et al. 2002; Pasnik et al. 2005). Ullah and Arai (1983a, 1983b) identified 19 isolates capable of producing dermatonecrotic toxins. A 37 kDa protein lethal to Japanese eel *Anguilla japonica* and Japanese flounder *Paralichthys olivaceus* has been purified from the ECP and intracellular components (ICC) of *E. tarda* (Suprapto et al. 1995; Suprapto et al. 1996). Tan et al. (2002) used a comparative proteomic approach to identify two proteins possibly related to pathogenicity obtained from the ECP of virulent and avirulent *E. tarda* isolates.

Vaccine formulations incorporating antigenic ECP have been reported for several bacteria pathogenic to fish. Chandran et al. (2002) observed increased survival of Indian major carps *Catla catla, Labeo rohita,* and *Cirrhinus mrigala* challenged with virulent *Aeromonas hydrophila* following immunization with *A. hydrophila* ECP preparations.

In sole *Solea senegalensis*, effective immune protection has been elicited against *Photobacterium damselae* subsp. *piscicida* and *Vibrio harveyi* through the application of ECP supplemented vaccine formulations (Zorrilla et al. 2003; Arijo et al. 2005). Additionally, whole cell bacterin vaccine preparations enhanced with concentrated ECP have been shown effective in the prevention of streptococcosis caused by *S. agalactiae* and *S. iniae* infection (Klesius et al. 1999; Klesius et al. 2000; Evans et al. 2004).

Several antigens, including LPS (Salati et al. 1983, 1984; Salati and Kusuda 1986; Salati et al. 1987; Gutierrez and Miyazaki 1994), outer membrane proteins (OMP) (Tu and Kawai 1999; Kawai et al. 2004; Liu et al. 2005), and secreted ECP (Salati et al. 1983; Mekuchi et al. 1995) have been implicated in developing immune protection against *E. tarda* in fish. The purpose of the present study was to characterize the immunogenic properties of ECP derived from a virulent parent and less virulent mutant *E. tarda* isolate. Specifically, the ability of the *E. tarda* ECP to induce protection from challenge in Nile tilapia was assessed and the protein profile of the ECP was compared by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Materials and Methods

Fish

Nile tilapia *Oreochromis niloticus* were produced and maintained at the USDA-ARS Aquatic Animal Health Research Laboratory (AAHRL), Auburn, Alabama. Prior to experiments, brain, liver, intestine, and head kidney samples were taken aseptically from 20 fish, and shown to be culture negative for *E. tarda* on *Salmonella Shigella* agar (SSA, Remel, Inc., Lenexa, Kansas) incubated for 24 h at 28° C in an atmosphere of air.

Two weeks before the beginning of the study, the fish were acclimated in 57 L glass aquaria. The fish were fed a commercial diet (Aquamax Grower 400, Brentwood, Missouri) daily to satiation. The aquaria were provided with flow-through dechlorinated tap water at 26±1° C at a rate of 0.5 L/min and aeration with air stones. For the duration of the study, the dissolved oxygen, temperature, pH, salinity, hardness, ammonia, and nitrite levels were monitored and maintained within acceptable ranges. During the experiment, the mean±standard deviation of dissolved oxygen was 5.8±0.5 mg/L, temperature was 26.2±0.8° C, pH was 7.1±0.3, salinity was 0.1±0.0%, and hardness was 110±10mg/L ($CaCO_3$). Ammonia and nitrite concentrations were consistently below the detection limit (0 mg/mL) during the experiment. The fish were exposed to light and dark intervals of 12:12 h. Feeding was suspended the day of immunization or challenge.

Preparation of *E. tarda* extracellular products

The preparation of *E. tarda* extracellular products (ECP) was guided by the procedures presented by Klesius et al. (1999) describing the formulation of a killed *Streptococcus iniae* vaccine, and the resulting products used to characterize the biological properties of ECP from a virulent parent and less virulent mutant isolate of *E. tarda*. Specifically, the FL6-60 parent and RET-04 mutant isolates of *E. tarda* were utilized. The virulent FL6-60 isolate was obtained from a morbid striped bass *Morone saxatilis* (Baya et al. 1997). This isolate was received courtesy of Dr. A. M. Baya (Animal Health Diagnostic Laboratory, Maryland Department of Agriculture, College Park, Maryland). RET-04 is a mutant of FL6-60 developed through a rifampicin-resistance strategy as described by Schurig et al. (1991), Klesius and Shoemaker (1999), and Evans et al. (2006a). The FL6-60 parent isolate was determined to be more virulent than the RET-04

mutant isolate through previous studies (unpublished; Wiedenmayer et al. 2006). Briefly, 1 mL aliquots of the FL6-60 parent and RET-04 mutant were inoculated, separately, into 5 L of sterile tryptic soy broth (TSB, Difco Laboratories, Sparks, Maryland) and incubated at 27° C for 72 h with shaking. Following incubation, the optical density was obtained at 540 nm and colony forming units per mL (CFU/mL) was determined by plate count. The concentrations of the FL6-60 and RET-04 preparations after 72 h incubation were 3.02×10^9 and 1.68×10^9 CFU/mL, respectively. The cultures were treated with 10% neutral buffered formalin (NBF) to yield a final concentration of 3% and allowed to stand at 27° C for 24 h. After 24 h, the culture was streaked onto sheep blood agar (SBA, Remel, Inc.) and then incubated at 27° C for 72 h to ensure the preparation had been killed. The NBF treated cultures were centrifuged at 7000 x g for 30 min and the cell pellet and culture fluid separated. The culture fluid, containing ECP, was concentrated to 1 L on a 3 kDa Amicon column (S3Y3) using a Millipore Proflux M12 (Millipore, Billerica, Massachusetts) and filter sterilized with a 0.22 µm 1 L microbiological filter (Corning, Corning, New York). The protein concentration of the ECP was determined by the bicinchoninic acid method and estimated to be 7.74±1.30 and 6.64±1.66 mg/mL for the FL6-60 and RET-04 preparations, respectively (BCA Protein Assay Kit, Pierce, Rockford, Illinois). The whole cell fraction and concentrated culture fluid were stored separately at -80° C until use.

Extracellular product immunization

For the ECP immunization study, nine tanks of 15 fish (mean weight 18 ± 4 g) were used. Three replicate groups of 15 fish were immunized with 0.1 mL of ECP

preparations of *E. tarda* FL6-60 parent and RET-04 mutant by intraperitoneal (IP) injection. Three replicate groups of 15 fish were injected with 0.1 mL of sterile TSB to serve as a control. For 30 d following immunization, the fish remained in separate aquaria and were maintained as previously described. On d 30 after immunization, five fish per replicate group were bled from the caudal vein and the samples retained for ELISA analysis. Blood samples were allowed to clot for 1 h at 25° C. The serum was separated by 5 min centrifugation at 300 x g and stored at -80° C until use.

E. tarda experimental challenge infection

On d 30 after immunization, the fish were challenged by IP injection with 0.1 mL of the *E. tarda* FL6-60. Briefly, the *E. tarda* FL6-60 parent isolate was grown in TSB for 24 h at 28° C. The culture was adjusted to approximately 4.4 x 10^8 CFU/mL for the challenge inoculum. Following challenge, the fish were monitored daily for the presence of clinical signs of disease and mortality for 14 d. During this period, approximately 20% of moribund and dead fish were sampled aseptically to confirm the presence of *E. tarda* from the brain, head kidney, liver, and intestine. Samples were cultured on SSA and incubated at the aforementioned conditions. Cumulative mortality was determined for the 14 d observation period. Relative percent survival (RPS) was calculated according to the methods of Amend (1981):

$$RPS = 1 - \frac{\%\ \text{vaccinate mortality}}{\%\ \text{control mortality}} \times 100$$

Enzyme-linked immunosorbent assay (ELISA)

Nile tilapia sera were tested to determine antibody titers against the *E. tarda* FL6-60 parent and RET-04 mutant ECP by an indirect ELISA procedure, based on the

methodology of Shelby et al. (2002). Blood samples obtained on d 30 after immunization were prepared as previously described. The ELISA coating antigens were prepared from the FL6-60 parent and RET-04 mutant ECP. The total protein content of the concentrated ECP preparations was determined by the bicinchoninic acid (BCA) method (BCA Protein Assay Kit, Pierce, Rockford, Illinois) and adjusted to 500 µg/mL with 0.5 M sodium carbonate buffer and 100 µL of the dilution added to each well of a 96-well microtiter plate (Maxisorp, Nunc, Roskilde, Denmark). The plates were incubated overnight at 25° C, and then washed five times with phosphate-buffered saline plus 0.05% tween-20 (PBS-T). The wells were blocked with 100 µL SuperBlock® blocking buffer (Pierce) for 1 h at 25° C and washed as previously described. Serum samples were initially diluted 1:100 in phosphate buffered saline (PBS) and 100 µL of each preparation added to the wells and diluted serially 1:2 on the vertical axis of the microtiter plate to provide a range of 1:100 to 1:102400 dilutions. The plate was incubated for 30 min at 25° C and washed as previously described. Mouse anti-tilapia IgM heavy chain specific monoclonal antibody 1H1 (Shelby et al. 2002) was diluted 1:5000 in PBS and 100 µL added to each well. The plate was incubated for 30 min at 25° C and washed as previously described. Peroxidase-conjugated rabbit anti-mouse IgG (Pierce) was diluted 1:5000 in PBS and 100 µL added to the wells. Following 30 min incubation at 25° C and washing five times in PBS, 50 µL of One Step Ultra TMB-ELISA® (Pierce) was added to the wells. The ELISA reaction was stopped at 5 min by adding 50 µL 3 M H_2SO_4 to each well. The optical density (OD) of the ELISA reactions was read spectrophotometrically (Model 680 microplate reader, Bio-Rad, Hercules, California) at 450 nm. Antibody titer for the samples was reported as the logarithmic (log_{10})

transformation of the reciprocal of the highest serum dilution yielding an OD of 0.100 at 450 nm. Negative controls were included on each plate and consisted of wells coated with antigen and no serum sample.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The potential antigens of the *E. tarda* FL6-60 parent and RET-04 mutant ECP were analyzed using sodium SDS-PAGE. The SDS-PAGE assay was performed in a manner similar to that of Pasnik et al. (2005) in their study of the antigenicity of *S. agalactiae* ECP. Prior to SDS-PAGE analysis, the ECP were dialyzed, in dialysis tubing with a membrane molecular weight cutoff of 6-8K, for 72 h against 1X Tris glycine buffer and distilled water to remove small contaminants. The SDS-PAGE procedure was executed based on the technique of Laemmli (1970) using Criterion 4-15% precast slab gels (Bio-Rad). The FL6-60 parent and RET-04 mutant ECP were combined 1:2 with SDS-PAGE sample buffer (Bio-Rad) without 2-β-mercaptoethanol and heated in a 95° C water bath for 5 min. Three replicate 30 µL samples of each ECP solution were loaded into the gel and separated at 175 V for approximately 1 h in a Criterion Midi electrophoresis cell (Bio-Rad). After the resolving the ECP, the gel was stained with silver nitrate (GelCode-SilverSNAP staining kit, Pierce).

Statistical analysis

Mortality data and ELISA results were analyzed using SAS software (SAS Institute, Cary, North Carolina) by one-way analysis of variance (ANOVA) with Duncan's multiple range test for significance. Significant differences were determined at $P < 0.05$.

Results

Extracellular product immunization and experimental challenge

The fish were challenged with *E. tarda* FL6-60 parent on d 30 after immunization and subsequently monitored for clinical signs of *Edwardsiella* septicemia and mortality. At approximately 24 h after challenge, fish in all treatments began to exhibit disease signs including lethargy and anorexia. Mortality was initially observed 48 h after challenge. Most deaths in all treatments occurred within 72 h after challenge. During the 14 d period following challenge, cumulative mortality was 64.6% for the FL6-60 parent ECP immunized group, 63.8% for the RET-04 mutant ECP immunized group, and 79.5% for the control group (Figure 1). The mortality patterns did not display significant ($P < 0.3541$) differences between treatment groups. The RPS was calculated to be 18.7 for the FL6-60 parent ECP immunized group and 19.7 for the RET-04 mutant ECP immunized group at 14 d after challenge.

Samples were obtained from nine fish per treatment group for microbiological analysis after challenge and cultured on SSA for 24 h at 28° C. Cultures considered positive displayed small (0.5-1.0 mm) diameter colonies that were white to translucent in color with black centers indicative of H_2S production. The primary isolates were also cytochrome oxidase negative (Farmer and McWhorter 1984). All fish from each treatment were culture positive for *E. tarda* in samples acquired from intestine, liver, and head kidney. One fish from the RET-04 mutant treatment was culture negative from a sample acquired from the brain, although all other fish tested yielded brain samples positive for the presence of *E. tarda*.

ELISA

Serum samples obtained on d 30 after ECP immunization were analyzed by ELISA on substrates with the FL6-60 parent and RET-04 mutant ECP fractions as the target antigen. In the first ELISA analysis, utilizing plates coated with FL6-60 parent ECP, mean antibody titers were 3.312±1.202 (1:2051), 3.104±0.695 (1:1270), and 2.731±0.670 (1:538) for FL6-60 parent ECP treatment, RET-04 mutant ECP treatment, and the control treatment, respectively. Serum obtained from the FL6-60 parent ECP treated fish yielded the highest mean titer, however; no significant ($P < 0.4908$) differences were observed between treatments (Table 1). In the second ELISA analysis, utilizing plates coated with RET-04 mutant ECP, mean antibody titers were 2.662±0.579 (1:459), 3.224±0.847 (1:1674), and 2.151±0.301 (1:141) for FL6-60 parent ECP treatment, RET-04 mutant ECP treatment, and the control, respectively. Both the FL6-60 parent and RET-04 mutant ECP treatments produced higher mean titers than the control, but only the RET-04 mutant ECP treatment serum was significantly ($P < 0.0427$) greater (Table 1).

SDS-PAGE

ECP fractions prepared from the FL6-60 parent and RET-04 mutant were resolved by SDS-PAGE and silver stained for comparison. Electrophoresis elucidated differences in banding patterns between the FL6-60 parent and RET-04 mutant samples (Figure 2). The FL6-60 parent ECP exhibited bands at approximately 66, 47, 44, and 31 kDa. The RET-04 mutant ECP exhibited a predominant band at approximately 175 kDa that was not present in the FL6-60 parent ECP. The RET-04 mutant ECP fraction

displayed additional bands at approximately 61, 42, 35, 32, and 28 kDa. Banding patterns at molecular weights below 25 kDa appeared generally similar for both ECP.

Discussion

In the first portion of the investigation, the effectiveness of intraperitioneally immunizing Nile tilapia with ECP preparations obtained from virulent and less virulent isolates of *E. tarda* was evaluated, while the second portion of the investigation was focused on identifying the potential immunogenic components of the ECP. The results of the immunization trials suggested the concentrated ECP of both the FL6-60 parent and RET-04 mutant were ineffective in eliciting significant protection against challenge. Cumulative mortality was observed to be 64.6%, 63.8%, and 79.5% for the FL6-60 parent ECP exposed group, RET-04 mutant ECP exposed group, and the control group, respectively, and no significant differences were detected between treatments in mortality (Figure 1). Additionally, RPS values calculated for administration of the FL6-60 parent and RET-04 mutant ECP did not indicate significant protection was conferred by either ECP preparation. Analysis of sera collected 30 d after immunization was conducted to determine antibody response of Nile tilapia to IP injection with the FL6-60 parent and RET-04 mutant ECP using both ECP as target antigens. Although the results of the ELISA investigation appear to indicate antibody production elicited by injection of the *E. tarda* ECP, the response does not seem to be particularly strong. This could suggest a lack of immunodominant antigens contained in the ECP fractions. It is also possible the inactivation of the ECP, by treatment with formalin, may have altered or destroyed any immunogenic components present in the fractions (Norqvist et al. 1994; Hernanz Moral et al. 1998; Evans et al. 2006a). This could explain the lack of immune protection

conferred by the ECP immunization, although a direct correlation between antibody response and increased protection from disease has not been identified for *E. tarda* in fish (Salati et al. 1983; Salati 1988; Mekuchi et al. 1995; Igarashi and Iida 2002). Additionally, cross reactivity between the ECP treatments occurred when the serum acquired from the FL6-60 parent ECP immunized fish was analyzed against the RET-04 mutant ECP by ELISA and vice versa, possibly illustrating the presence of similar antigenic components in the ECP fractions.

The ECP of the FL6-60 parent and RET-04 mutant were compared through resolution by SDS-PAGE and subsequent silver staining. Analysis of banding patterns reflected dissimilarities between the fractions. Most striking was the presence of a predominant band at approximately 175 kDa in the RET-04 mutant ECP that was not visible in the FL6-60 parent ECP (Figure 2). Furthermore, while both fractions displayed similar banding in the 60 kDa range, differences appeared to occur at lower molecular weights. The FL6-60 parent ECP showed two bands at approximately 47 and 44 kDa, while the RET-04 ECP displayed only one band at 42 kDa. The FL6-60 parent ECP exhibited one light band at approximately 31 kDa, while the banding profile exhibited by the RET-04 mutant ECP showed multiple, darker bands within this same range at approximately 35, 32, and 28 kDa. In one dimensional PAGE analysis of ECP fractions acquired from five virulent and seven avirulent *E. tarda* strains, Tan et al. (2002) reported two major bands at 55 and 21 kDa unique to three fish derived virulent isolates, although few differences were observed in background banding patterns between the virulent and avirulent isolates. Additionally, considerable heterogeneity has been observed in one dimensional PAGE comparison of whole-cell protein extracts obtained from nine fish

pathogenic *E. tarda* isolates (Panangala et al. 2006). Further comparison of the FL6-60 parent and RET-04 mutant ECP with ECP obtained from other virulent and avirulent *E. tarda* isolates could provide more information regarding the possible significance of observed banding profile differences. As the RET-04 mutant was derived from the FL6-60 parent through a rifampicin-resistance strategy, the presence of the 175 kDa band could be due to the attenuation process and account for the lessening of virulence displayed by the RET-04 isolate. However, the loss of virulence caused by rifamipicin-resistance mutation reported in an attenuated isolate of *E. ictaluri* is attributed to changes in the LPS (Klesius and Shoemaker 1999; Arias et al. 2003). In order to more completely characterize differences between the FL6-60 parent and RET-04 mutant isolates of *E. tarda* and their antigenic properties, analysis of LPS is necessary.

Extracellular products are understood to be important virulence factors for a number of bacterial pathogens of fish and have been shown to contribute to development of immune protection in several vaccine formulations (Klesius et al. 1999; Klesius et al. 2000; Chandran et al. 2002; Zorrilla et al. 2003; Evans et al. 2004; Arijo et al. 2005). In some instances, administration of ECP alone was sufficient to reduce mortality in challenged populations (Chandran et al. 2002; Zorrilla et al. 2003), while others selected and concentrated ECP antigens to augment the performance of bacterin vaccine preparations (Klesius et al. 1999; Klesius et al. 2000; Evans et al. 2004; Arijo et al. 2005). Although whole cell bacterin vaccines have shown limited success, singularly, in protection against *Edwardsiella* septicemia (Salati and Kusuda 1986; Salati et al. 1987; Gutierrez and Miyazaki 1994; Mekuchi et al. 1995; Swain et al. 2002), the inclusion of the whole cell would allow exposure to additional potential antigens such as LPS and

OMP (Salati et al. 1983; Salati et al. 1984; Salati et al. 1986; Salati et al. 1987; Gutierrez and Miyazaki 1994; Tu and Kawai 1999; Chandran et al. 2002; Kawai et al. 2004; Liu et al. 2005). It is possible the ECP fractions investigated in this study could function to enhance the immune response afforded by a whole cell preparation and could contribute to the development of an efficacious ECP enriched bacterin vaccine formulation.

Route of administration has been identified as a key factor in the ability of vaccine formulations to elicit immune protection. Vaccines can be presented to fish by IP or IM injection, by immersion in a vaccine solution, or orally, with the selection of immunization route based on considerations including feasibility, level of protection conferred, potential side effects, and cost (Gudding et al. 1999; Klesius et al. 2006). Klesius et al. (2006) suggested IP injection to be a generally more efficacious means of exposure than IM injection of ECP modified bacterin vaccines against streptoccocosis. Conversely, Evans et al. (2006b) reported increased survival of subclinically infected hybrid striped bass *Morone chrysops* x *M. saxatilis* IM injected over IP injected with a *S. iniae* ECP preparation. The researchers proposed longer retention of ECP antigens in the musculature versus the peritoneal cavity may account for the greater survival observed (Evans et al. 2006b). It is conceivable the FL6-60 parent and RET-04 mutant ECP preparations evaluated in the present study may have yielded improved immunostimulation by an alternate route of administration.

Several recent investigations have reported some success in eliciting immune protection against fish pathogens through the application of attenuated, live vaccine formulations (Vaughan et al. 1993; Norqvist et al. 1994; Marsden et al. 1996; Hernanz Moral et al. 1998; Klesius and Shoemaker 1999; Daly et al. 2001). Crucial antigenic

factors are often lost during the formulation of conventional bacterin preparations due to the process of inactivation (Norqvist et al. 1994; Hernanz Moral et al. 1998; Evans et al. 2006a). Additionally, as observed with intracellular pathogens such as *E. ictaluri*, cell mediated immune responses are required to elicit immune protection and these cellular mechanisms can be stimulated through the application of attenuated live vaccines (Klesius and Shoemaker 1999; Shoemaker et al. 1999). A live *E. tarda* isolate, mutated to lower siderophore production, was reported capable of reducing mortality in vaccination trials in Nile tilapia, although attenuation did not completely eliminate virulence of the mutant isolate (Igarashi and Iida 2002). Recently, the RET-04 mutant isolate has been recognized as a live vaccine isolate effective in the prevention of *Edwardsiella* septicemia when administered by injection or bath immersion and further study could contribute to a more thorough understanding of the mechanisms of immunity against *E. tarda* (Evans et al. 2006a).

Overall, this study determined ECP preparations derived from the FL6-60 parent and RET-04 mutant did not induce immune protection in Nile tilapia through IP injection, despite slight increases in specific antibody titers. Characterization of the fractions by SDS-PAGE revealed unique banding profiles, however; the mortality data did not display a significant difference in protection conferred between immunization with the FL6-60 parent or the RET-04 mutant ECP. Although neither the FL6-60 parent nor RET-04 mutant ECP appeared capable of eliciting immune protection individually, the potential role of these ECP in defense against *Edwardsiella* septicemia warrants further investigation.

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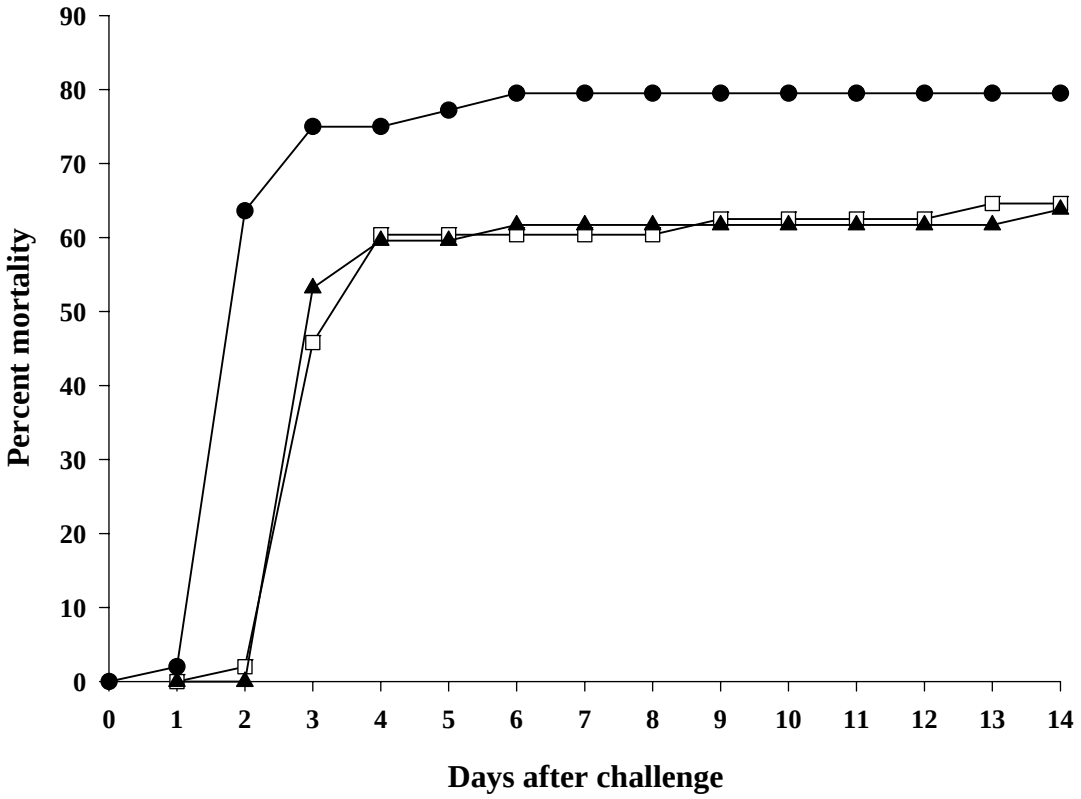

Figure 1. Mean percent cumulative mortality of Nile tilapia administered *Edwardsiella tarda* FL6-60 parent (□) or RET-04 mutant (▲) extracellular products (ECP) or tryptic soy broth (TSB) as a control (●) by intraperitoneal (IP) injection and challenged at 30 d after immunization with 4.4 x 10^8 CFU/mL *E. tarda* FL6-60 parent by IP injection.

Table 1. Mean specific antibody titer of Nile tilapia immunized with *Edwardsiella tarda* FL6-60 parent or RET-04 mutant extracellular products (ECP) or administered tryptic soy broth (TSB) as a control.

Treatment	Antibody titer against FL6-60 ECP (Logarithmic transformation of mean reciprocal±SD)[a]	Antibody titer against RET-04 ECP (Logarithmic transformation of mean reciprocal±SD)
FL6-60 ECP	3.312±1.202[y]	2.662±0.579[y,z]
RET-04 ECP	3.104±0.695[y]	3.224±0.847[z]
TSB	2.731±0.670[y]	2.151±0.301[y]

a Logarithmic (log_{10}) transformation of mean specific antibody titer detected in the sera of 15 immunized fish at 30 d after immunization. Mean values with different letters were significantly different ($P < 0.05$).

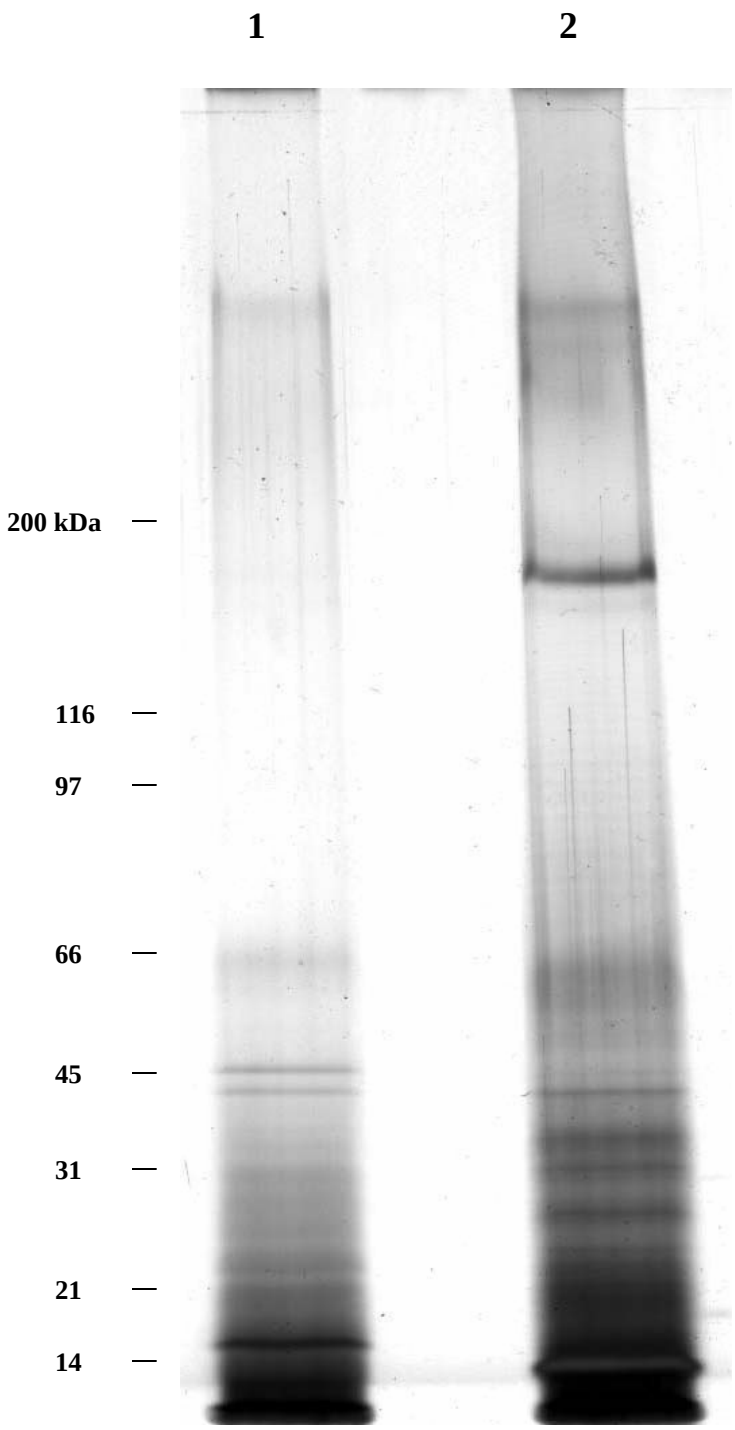

Figure 2. Silver stained SDS-PAGE profiles of *Edwardsiella tarda* extracellular product (ECP) fractions used in the study. Lane 1 FL6-60 parent; lane 2 RET-04 mutant.

V. SUMMARY

The purpose of this study was to 1) evaluate the chemotactic response of macrophages obtained from Nile tilapia to extracellular products (ECP) from a virulent parent and less virulent mutant isolate of *Edwardsiella tarda*, 2) determine if passive immunization with immune antibodies may elicit protection from challenge in Nile tilapia, and 3) assess protection conferred by immunization with ECP and describe their immunological properties. The experiments were conducted using two isolates of *E. tarda*, the virulent FL6-60 parent, isolated from a morbid striped bass *Morone saxatilis*, and the less virulent RET-04 mutant, attenuated from the FL6-60 parent through a rifampicin-resistance strategy. Analysis of the chemoattractant ability of non-purified ECP derived from both the FL6-60 parent and RET-04 mutant indicated a predominantly chemokinetic effect on Nile tilapia intraperitoneal macrophages by *in vitro* assays. Marginal differences were noted in chemokinesis induced by the FL6-60 parent and RET-04 mutant ECP. Semi-purification of the ECP by high pressure liquid chromatography revealed differences between the constituent components of the FL6-60 parent and RET-04 mutant ECP, however; as observed in migration assays utilizing non-purified ECP, minimal differences were illustrated in the relative abilities of the FL6-60 parent and RET-04 mutant to elicit movement of macrophages. The semi-purified ECP fractions were found to induce primarily chemotactic activity and chemotactic migration was increased for both ECP over the non-purified fractions. The results suggest ECP, as

derived from both the virulent FL6-60 parent and less virulent RET-04 mutant, presumably influence the inflammatory reactions displayed by Nile tilapia infected with *E. tarda*. Two vaccination trials were also performed to assess the protective capabilities of the FL6-60 parent and RET-04 mutant and their ECP. In the passive immunization experiment, an antibody response was observed and immunity confirmed in Nile tilapia actively infected and challenged with live *E. tarda* FL6-60 parent. Subsequent passive immunization with hyperimmune or nonimmune serum, or phosphate buffered saline (PBS), as a control, indicated significantly higher antibody titers at 72 h after immunization in the hyperimmune serum treated group over the nonimmune serum or PBS treated groups. Mortality data reflected a significant increase in survival following challenge infection in the hyperimmune serum treated group. The results of this trial imply specific antibody titers against *E. tarda* following passive immunization may play a role in protection of Nile tilapia against experimental infection. In the ECP immunization experiment, fish immunized with either FL6-60 parent or RET-04 mutant ECP exhibited an increase in specific antibody titers to *E. tarda* ECP fractions, although the titers were generally not significantly different from those of the tryptic soy broth (TSB) treated controls. The mortality data did not reflect a significant increase in survival of either FL6-60 parent or RET-04 mutant ECP immunized fish over the TSB treated group. Despite the lack of ability by either ECP fraction to elicit protection from challenge, characterization of the ECP by electrophoretic analysis indicated differences in the banding profiles of the FL6-60 parent and RET-04 mutant ECP. The results of this experiment demonstrated that immunization with the FL6-60 parent and RET-04 mutant

ECP led to an increase in specific antibody production, but a significant decrease in mortality was not observed.

The research conducted suggests *E. tarda* infection stimulates the humoral immune system of fish, although the production of specific antibodies does not appear to yield complete immunity against *E. tarda*. The results of the passive immunization study indicated some protection against experimental challenge was conferred by the administration of hyperimmune serum; however, immunization with the FL6-60 parent and RET-04 mutant ECP also resulted in an increase in *E. tarda* specific antibodies, but significant protection from experimental challenge was not observed. The chemoattractant activity displayed by Nile tilapia macrophages exposed to the FL6-60 parent and RET-04 mutant ECP suggests a probable role of secreted ECP in the pathogenesis of *E. tarda*. Additionally, the interaction of the macrophages and ECP witnessed here may support the necessity of cellular immune mechanisms in the development of complete protection against *Edwardsiella* septicemia.

The broad host range and pervasive presence of *E. tarda* in the aquatic environment and fish production systems results in a continual threat of disease from this pathogen. Losses of economically important cultured species, such as Nile tilapia, attributed to *E. tarda* infection have highlighted the need for effective prevention strategies. While passive immunization with hyperimmune serum and immunization with FL6-60 parent and RET-04 mutant ECP may not have provided total immune protection against experimental challenge from *E. tarda*, the results of the current experiments yielded information regarding the mechanisms of immunity against *Edwardsiella* septicemia in Nile tilapia. Furthermore, analysis of the virulent FL6-60 parent and less

virulent RET-04 mutant ECP and their chemoattractant properties illustrated few differences between isolates. Despite the established difference in virulence between the FL6-60 parent and RET-04 mutant isolates, the results of this study may indicate comparable ability to induce macrophage migration that could contribute to the immune response against *E. tarda* infection. As previous work with other piscine pathogens has proven, the potential for successful disease prevention through the application of bacterial ECP and exposure to live, attenuated bacterial isolates exists. It is hoped the information gained by this investigation can contribute to the elucidation of mechanisms of immunity against *Edwardsiella* septicemia in fish and the development of efficacious control strategies.

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