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SEROLOGICAL INVESTIGATION OF THE FISH PATHOGEN *EDWARDSIELLA ICTALURI*, CAUSE OF ENTERIC SEPTICEMIA OF CATFISH

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ABSTRACT: The serological relationships among 32 isolates of *Edwardsiella ictaluri* obtained from fish were studied. The strains were extremely homogeneous in protein and lipopolysaccharide preparations as observed by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis. Only minor variations were observed in the structural O-side chain subunits in three isolates; however, such variation did not preclude antigenic recognition by two *E. ictaluri* antisera in either microagglutination or Western blot immunoassays. The antigenic homogeneity of *E. ictaluri* was further demonstrated by microagglutination assays with both formalin-killed and heat inactivated cellular antigens. The minimal degree of antigenic variability observed suggested that most isolates of *E. ictaluri* compose a single antigenic serotype.

Key words: *Edwardsiella ictaluri*, serology, agglutination, protein, lipopolysaccharide, antigens, enteric septicemia of catfish.

INTRODUCTION

Enteric septicemia of catfish (ESC), caused by the bacterium *Edwardsiella ictaluri* (Hawke et al., 1981), is a significant problem associated with the culture of ictalurid fishes (Hawke, 1979). In the United States, ESC is almost exclusively a disease of channel catfish (*Ictalurus punctatus*) although infections have been reported also in the brown bullhead (*I. nebulosus*) and white catfish (*I. catus*) (Plumb and Schwedler, 1982). In fact, Plumb and Sanchez (1983) indicated that a number of other warmwater species, including tilapia (*Sarotherodon aureus*), largemouth bass (*Micropterus salmoides*), golden shiner (*Notemigonus crysoleucas*) and bighead carp (*Aristichthys nobilis*), were highly resistant to experimental infections. Despite its general prevalence for ictalurids, there have been limited reports on the isolation of *E. ictaluri* from certain ornamental fishes, including the green knife fish (*Eigenmannia virescens*) (Kent and Lyons, 1982); the danio (*Danio devario*) (Waltman et al., 1985); the walking catfish, *Clarius batrachus* (Kasornchandra et al., 1987); and the rosy barb, *Puntius conchonius* (Humphrey et al., 1986).

Isolates of *E. ictaluri* exhibit homogeneous biochemical (Waltman et al., 1986), plasmid (Lobb and Rhoades, 1987) and antimicrobial sensitivity (Waltman and Shotts, 1986) patterns. Unlike *E. tarda*, a closely related bacterium, *E. ictaluri* is considered to be more homogeneous serologically, and such speculation has led to the adaptation of enzyme immunoassays and fluorescent antibody techniques for the serodiagnostic detection of this pathogen (Rogers, 1981; Ainsworth et al., 1986). Despite the development of such assays, little documented information exists on the serological relatedness among strains of *E. ictaluri*. Such documentation was the major intent of this study.

MATERIALS AND METHODS

Bacterial isolates

Bacteria used in this study were maintained in the culture collection at the National Fish Health Research Laboratory (Box 700, Kearneysville, West Virginia 25430, USA). The history and source of isolation for each of the 32 isolates of *E. ictaluri* were described elsewhere (Bertolini, 1986). Briefly, all but two of the iso-

lates were obtained from ictalurid hosts. Isolates 10.15 and 10.16 were obtained from the danio and green knife fish, respectively. Isolate 10.12, from an ictalurid host, was selected as a reference strain because its biochemical characteristics were typical for the species (Waltman et al., 1986).

Serological analysis

Both formalin-killed and heat-stable somatic antigens from each isolate were used in serological investigations. Bacteria were incubated for 24 hr at 30 C on tryptic soy agar (Difco, Detroit, Michigan 48201, USA) and harvested by centrifugation at 12,000 × *g* for 5 min in sterile phosphate buffered saline (PBS; pH 7.2). Bacteria were washed three times in sterile PBS and adjusted in PBS to an optical density of 0.52 at 525 nm. Standardized bacterial suspensions served as stock solutions for the preparation of formalin-killed (FK) and heat-killed (HK) antigens. The FK antigens were prepared by adding formalin to a final concentration of 0.5% in a specific volume of the standardized bacteria, and the HK antigens by autoclaving a specific volume of the standardized bacteria at 121 C and 15 psi, for 30 min. Bacteria were pelleted by centrifugation and resuspended in sterile PBS to an optical density of 0.52 at 525 nm.

Antisera were produced in New Zealand white rabbits (Hazelton Laboratories Corporation, Vienna, Virginia 22180, USA), against whole cell antigens of two bacterial strains, isolate 10.12 from an ictalurid host and 10.16 from a green knife fish. The FK antigens from each isolate were emulsified in an equal volume of Freund's incomplete adjuvant for primary and booster injections. Rabbits were immunized with multiple subcutaneous and intradermal injections (0.1 to 0.2 ml) delivered across several sites on their backs and necks. After primary immunization, rabbits were rested for 28 days and then given two booster injections at 2-wk intervals. Rabbits were exsanguinated 14 days after the second booster injection, sera were collected, passed through a 0.45 μm filter (Gelman Sciences, Ann Arbor, Michigan 48201, USA), and lyophilized. Prior to use in serological test, 1 ml aliquots of antisera were reconstituted with sterile distilled water.

In initial experiments, antisera were reacted in standard microagglutination experiments (Maisse and Dorson, 1976) with both the homologous and heterologous antigens from each of the two reference strains, 10.12 and 10.16. Cross adsorption experiments were then performed to further assess the relatedness of the two antisera. The FK antigens used for adsorption studies were prepared from stock solutions of reference cells that, when diluted 1:10 (v/v) in PBS, were equivalent to an optical density of 1.00 at 525 nm. The cell pellet derived from 1 ml of concentrated adsorbing antigen was resuspended in 0.2 ml of antiserum. Antisera were adsorbed twice at 25 C for 4 hr and then again at 4 C for 16 hr. After the final adsorption, antisera were reacted in microagglutination assays with FK antigen of both reference isolates.

Further agglutination studies were conducted by reacting both antisera with the FK and HK antigens derived from each of the 32 isolates of *E. ictaluri*. Antibody titers were expressed as the reciprocal of the last $\log_{(2)}$ dilution yielding positive agglutination. Mean serum antibody titers were calculated for the reaction of each antiserum with the collective groups of FK and HK antigens. Statistical analysis was performed on a Hewlett-Packard 9845B computer (Hewlett-Packard, San Diego, California 92127, USA) that was programmed to validate statistical differences ($P < 0.05$) between groups by the least significant difference of the means test (Hicks, 1973).

Electrophoretic analysis

Each isolate was standardized in sterile PBS to an optical density of 0.52 at 525 nm for comparison of protein and lipopolysaccharide (LPS) characteristics by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples for protein analysis (1.5 ml) were centrifuged at 12,000 × *g* for 5 min and the cell pellet was resuspended in 100 μl of sterile deionized water. The suspension was frozen at −70 C for 24 hr and thawed by sonification until the suspension was fully melted.

Samples were again centrifuged and the supernatant was resuspended in 100 μl of sample buffer (Laemmli, 1970) and boiled for 5 min. Samples treated with proteinase K were prepared according to the procedure of Hitchcock and Brown (1983). Briefly, 1.5 ml of the standardized bacterial suspensions were centrifuged at 12,000 × *g* for 5 min. The cell pellet was dissolved in 60 μl of sample buffer and boiled for 10 min. Boiled cells were treated with 25 μg of proteinase K (Sigma Chemical Co., St. Louis, Missouri 63178, USA) and dissolved in 10 μl of sample buffer; the resulting mixture was incubated at 60 C for 60 min.

SDS-PAGE was conducted as described by Laemmli (1970), using a 12% acrylamide resolving gel and a 4% acrylamide stacking gel. Bacterial samples, reference molecular weight markers (Sigma Chemical Co.) or reference LPS samples from *Salmonella typhimurium* (Sigma Chemical Co.) were loaded (20 μl) into gel slots and electrophoresis was conducted as described by Cipriano and Pyle (1985). After electrophoresis, gels were stained either for protein in 0.01%

TABLE 1. Microagglutination of *E. ictaluri* reference antisera reacted and cross-adsorbed with formalin-killed antigens derived from a channel catfish (10.12) and a non-ictalurid host (10.16).

Antiserum	Adsorbing antigen	Antigen 10.12	Antigen 10.16
10.12	—	9.0 ± 0.0*	5.5 ± 0.7
	10.16	8.0 ± 0.0	0.0
	10.12	0.0	0.0
10.16	—	7.0 ± 0.0	9.0 ± 0.0
	10.12	0.0	8.5 ± 0.7
	10.16	0.0	0.0

* Mean $\log_{(2)}$ geometric antibody titer ± SE derived from duplicate assays.

Coomassie Brilliant Blue (Bio Rad, Richmond, California 98404, USA), or for LPS according to the modified silver-stain procedure of Tsai and Frasch (1982).

The antigenic specificity of antisera reactions was further characterized by Western Blot immunoassays. In these assays, whole cell lysates (WC; representing the total antigenic composition of the bacteria) and proteinase K digests (representing the LPS constituents of the bacteria) were analyzed by SDS-PAGE and electrotransfer onto nitrocellulose. Antigens transferred to nitrocellulose were visualized with enzyme-labeled antisera as described by Towbin et al. (1979) in which a 1:100 dilution of a respective *E. ictaluri* antiserum was used as the primary antibody. In addition to reactions with both *E. ictaluri* antisera, WC and PK samples were also reacted with normal rabbit serum that was obtained from the Biologics Section at the National Fish Health Research Laboratory.

RESULTS

Antisera to *E. ictaluri* isolate 10.12 from the channel catfish, and isolate 10.16 from the green knife showed strong agglutination patterns with both the homologous and heterologous reference antigens. However, titers against the heterologous antigens were lower than those reported in the homologous systems. For example, the mean geometric antibody titer was 9.0 ± 0.0 (standard deviation) for duplicate assays in which antiserum to isolate 10.12 was used against its homologous 10.12 antigen (Table 1). By comparison, this same antiserum produced a mean titer of 5.5 ± 0.7 against the heterologous antigen 10.16. Conversely, the mean geometric titer of antiserum to isolate 10.16 was 9.0 ± 0.0 against homologous antigen and 7.0 ± 0.0 against the heterologous 10.12 antigen. Overall patterns observed in cross adsorption studies indicated that both antisera reacted with antigens common to the reference isolates. Each antiserum also had additional antibody activity that was expressed exclusively against the homologous antigen from which it was derived (Table 1).

The two *E. ictaluri* antisera were further tested against the FK and HK antigens derived from each of the 32 isolates used in this study. In these tests, the mean (±standard error) $\log_{(2)}$ antibody dilution titer to the collective group of FK antigens was 9.3 ± 0.2 for antiserum against isolate 10.12 and 7.0 ± 0.1 for antiserum against 10.16. When the HK antigens were assayed, results were similar. The mean agglutinin response to HK antigens was 9.3 ± 0.1 for antiserum prepared against isolate 10.12 and 6.5 ± 0.2 for the antiserum to 10.16. A comparison of these means (Fig. 1) indicated (1) there was no significant difference between the reactivity of a specific antiserum with either the FK or HK antigens, and (2) the overall reactivity of the two antisera differed significantly (ANOVA; P ($F > 78.1$) = 0.000). Alternatively stated, the antisera responded similarly to FK and HK inactivated antigens, but not with similar intensity.

SDS-PAGE electrophoresis indicated that there was much structural homogeneity in both the protein and LPS composition among the 32 isolates examined. No structural protein differences were visually observed among any of the isolates, including the two isolates (10.15 and 10.16) derived from non-ictalurid hosts (Fig. 2). The LPS electrophoregrams of the 32 *E. ictaluri* isolates were also homogeneous, although several minor variations were noted (Fig. 3). These variations appeared as a slightly out-of-phase difference in the O-antigen side chain migration of isolates

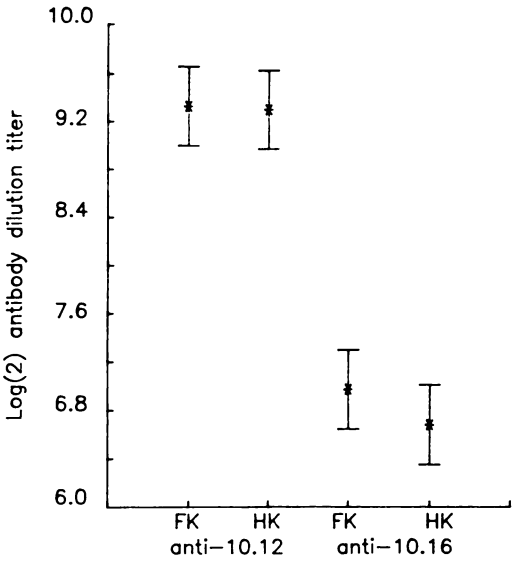


FIGURE 1. Mean geometric $\log_{(2)}$ antibody dilution titers of antisera to formalin-killed cells of *E. ictaluri* isolates 10.12 and 10.16 reacted in microagglutination assays with each of the 32 formalin-killed (FK) and heat-killed (HK) antigens used in this study. Least significant difference of the means test indicated that there was no significant difference ($P > 0.05$) in the reactivity of a specific antiserum with either the FK or HK antigens. However, the antisera themselves differed significantly. Vertical bars at each data point show the standard error of the mean.

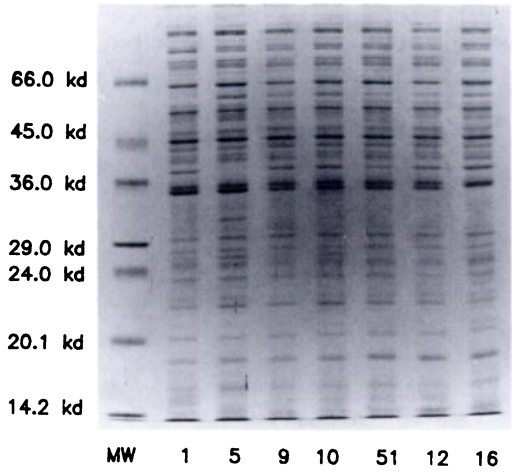


FIGURE 2. SDS-PAGE of soluble whole cell protein sonicates from each of seven isolates of *E. ictaluri*—isolates 1, 5, 9, 10, 51, and 12 from ictalurid hosts and isolate 16 from a green knife fish. The gel demonstrates considerable homogeneity among the structural proteins of this organism. Protein samples are compared to reference molecular weight standards (MW; Sigma Chemical Co., St. Louis, Missouri 63178, USA).

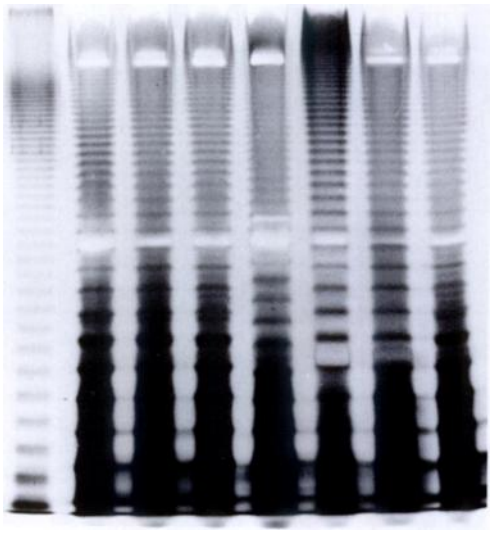
10.16 and 10.42 (not shown), and a more prominent O-side chain region in isolate 10.05. A single isolate, 10.81, lacked the O-side chain completely and was considered a rough mutant.

Protein, LPS, and antigenic analysis of WC and PK preparations from *E. ictaluri* 10.12 and 10.16 (Fig. 4) were performed to evaluate the antigenic specificity for each antiserum. These experiments showed that antisera 10.12 and 10.16 both reacted strongly with the WC and PK extracts from the homologous and heterologous antigens. Although slight variations appeared in the migration distance of the bands representing the O-side chain regions of the LPS, as evidenced in the PK treatments of isolates 10.12 and 10.16, these differences did not preclude antigenic recognition by either antiserum (Fig. 4).

Some antibody or non-specific binding was evident when WC preparations reacted with normal rabbit serum; however, antiserum reactions were more intense and reacted with a greater number of protein bands. Normal rabbit serum did not react with the PK preparations. The reaction of normal rabbit serum with protein constituents of both isolates 10.12 and 10.16 could not be definitively explained but were attributed to either non-specific binding or to previous exposure of the rabbit used in immunization experiments to bacteria that share an antigen common to *E. ictaluri*. Despite such reactivity, it is important to note that the lack or reactivity of the normal rabbit serum with PK treatments of both isolates 10.12 and 10.16 emphasizes the specificity of antigen/antibody interaction for the LPS of *E. ictaluri*.

DISCUSSION

Isolates of *E. ictaluri* obtained from ictalurid hosts composed a very tight and homogeneous serological group, despite some minor differences in the structural



lipopolysaccharide antigens among isolates. When Kent and Lyons (1982) originally isolated *E. ictaluri* from the green knife fish, they suspected that the new isolate might be a second serotype. We ob-

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FIGURE 3. SDS-PAGE of proteinase K treated bacterial sonicates stained to visualize structural lipopolysaccharide characteristics as discussed by Hitchcock and Brown (1983). The *E. ictaluri* isolates 3, 9, 12, 51, and 77 were derived from ictalurid hosts and represent the typical profile observed for most of the 32 isolates examined. Isolate 5 from a catfish and isolate 16 from green knife fish show some of the minor structural variations observed in the electrophoretic migration of the repeating subunits of the O-antigen side chain. Reference LPS from *Salmonella typhimurium* (S; Sigma Chemical Co., St. Louis, Missouri 63178, USA) was run as a standard control.

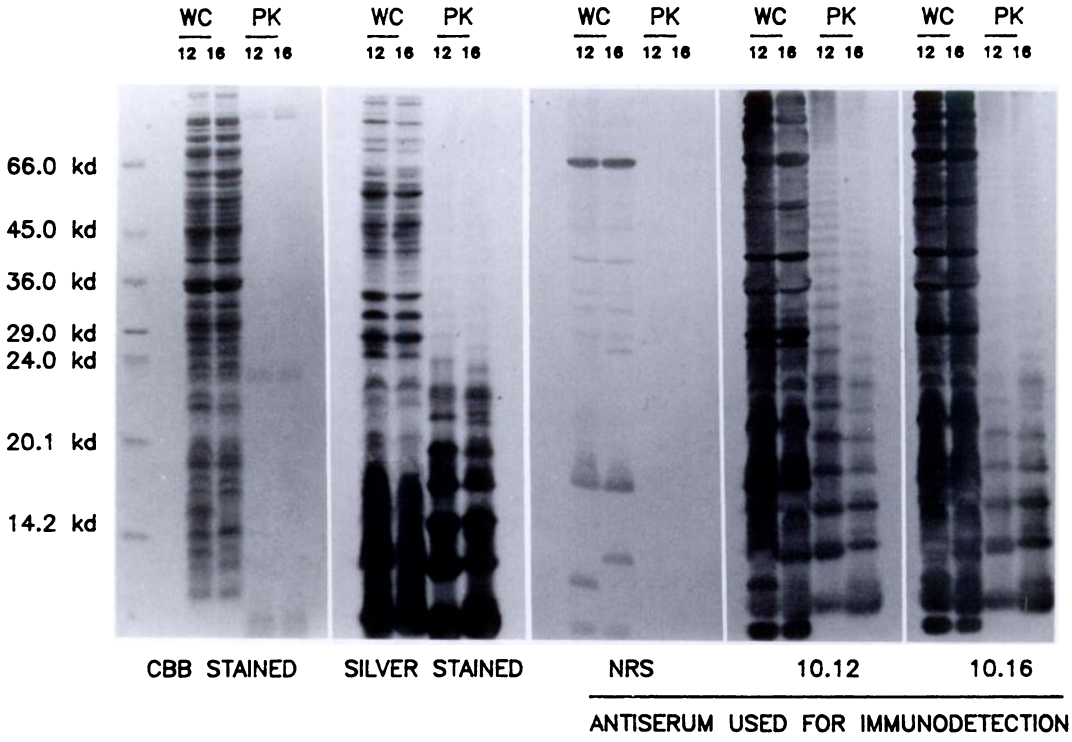

FIGURE 4. Western blot immunoassays of whole cell (WC) protein lysates and proteinase K (PK) digests of *E. ictaluri* isolates 12 and 16, stained for protein with Coomassie Brilliant Blue (CBB), silver-stained for lipopolysaccharide analysis, and probed with normal rabbit serum (NRS), and antisera against *E. ictaluri* isolates 10.12 and 10.16 (see text for details).

served certain minor variations in the structural composition of the LPS in this isolate, but these differences were no greater than other minor LPS variations observed in isolates from ictalurid hosts. For example, similar variations were observed in the lipopolysaccharide compositions of isolates 10.05 and 10.42; but these variations did not preclude immunodetection by the 10.12 and 10.16 antisera in either microagglutination or immunoblotting analyses.

Despite an overall difference between antiserum prepared against the catfish isolate (10.12) and the green knife fish isolate (10.16) in the intensity of reaction with the total collection of HK and FK antigens, both antisera reacted strongly with each isolate used in this study. Because no statistical differences were noted in the mean reactions of a specific antiserum with either formalin-killed or heat stable somatic antigens, we suspect that the antigenic homogeneity detected was based on the somatic O-antigen.

This study, therefore, established a strong basis to support the claim of Rogers (1981) that *E. ictaluri* is a serologically homogeneous organism. It also indicates that, as suggested by Plumb (1984), *E. ictaluri* is indeed an excellent candidate for vaccine development. Our data appears to indicate that a vaccine such as the experimental vaccine developed by Saeed (1983), which was based on the LPS of a single *E. ictaluri* isolate, might afford immunity to a broad spectrum of *E. ictaluri* isolates inherent in ictalurids. Similarly, serodiagnostics that have been developed to detect *E. ictaluri* may be effective with most isolates. Because of the strong cross reactivity between the non-ictalurid isolate 10.16 with both homologous and heterologous antisera, we find no conclusive evidence that establishes this isolate as a second serotype.

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