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Serological Characterization of Atypical Strains of *Edwardsiella tarda* Isolated from Sea Breams

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The serological relationships between a non-motile variant of *Edwardsiella tarda* isolated from sea breams, *Pagrus major* and *Evynnis japonica*, and typical strains of *E. tarda* isolated from Japanese eel, *Anguilla japonica* and Japanese flounder, *Paralichthys olivaceus*, were studied. Cross-absorption test revealed that all strains shared a similar O-antigen. Agglutination tests showed the presence of heat-labile antigens on the cell surface of the non-motile strains from sea breams. Immunoelectrophoretic analysis demonstrated the relatedness of cell surface antigens among the strains from various fish species. The possibility of usage of a common vaccine for edwardsiellosis among sea breams and other fish species is suggested.

Key words: *Edwardsiella tarda*, edwardsiellosis, sea bream, O-antigen, cellular antigen, lipopolysaccharide

Edwardsiella tarda was firstly described by Ewing *et al.* (1965), but it was first isolated in Japan in 1954 and previously described by Hoshina (1962) as a fish pathogen, *Paracolobactrum anguillimortiferum*. It has been isolated from Japanese eel, *Anguilla japonica* (Wakabayashi and Egusa, 1973; Kanai *et al.*, 1977), channel catfish, *Ictalurus punctatus* (Meyer and Bullock, 1973), tilapia, *Oreochromis nilotica*[*5], yellowtail, *Seriola quinqueradiata* (Yasunaga *et al.*, 1982), Japanese (olive) flounder, *Paralichthys olivaceus* (Nakatsugawa, 1983), etc., and a large variety of animals, including reptiles and human (Ullah and Arai, 1983).

In recent years, a group of non-motile *E. tarda* has been isolated from red sea bream, *Pagrus major* and crimson sea bream, *Evynnis japonica* (Kusuda *et al.*, 1977; Yasunaga *et al.*, 1982; Baxa *et al.*, 1985), and was classified as a variant of *E. tarda* (Kusuda *et al.*, 1977). Epizootic occur in August to November among 2–3 year-old cultured sea breams (Hatai, 1989). The external signs of diseased fish were ulcerative lesions on the body surface, and the histopathological changes of infected lesions were initially characterized by necrosis and accumulation of macrophages with bacterial invasion (Miyazaki and Kaige, 1985).

Until now serological studies have been carried out on *E. tarda* from eel (Minagawa *et al.*, 1983; Park *et al.*, 1983) and flounder (Mamnur Rashid *et al.*, 1994), but not yet on isolates from sea breams. Serological characterization of a pathogen is useful not only in diagnosis and epidemiological studies, but also for development of vaccines. Moreover, for planning an effective program of vaccination, it is important to know what kinds of antigens the pathogen possesses.

The present study was undertaken to determine the serological relationship between atypical strains of *E. tarda* from sea breams and typical strains from other fish species, and also to investigate the nature of the cell surface antigens.

Materials and Methods

Organisms

Eight strains of atypical *E. tarda* isolated from red sea bream and crimson sea bream were studied. Other strains were from Japanese eel, tilapia, Japanese flounder, and greenling, *Hexagrammos otakii* (Table 1). All strains were preserved in liquid nitrogen. Working cultures were maintained on yeast extract (YE) agar, consisting of 1% polypepton, 0.5% Bacto-yeast extract,

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Table 1. Strains used in this study

Strain		Used for		Source
		AGGL	IE	
Atypical *Edwardsiella tarda*	NUF 9[a]			red sea bream, 1983, Nagasaki, Japan
Atypical *E. tarda*	NUF 10	*	*	red sea bream, 1983, Nagasaki, Japan
Atypical *E. tarda*	NUF 500	*	*	red sea bream, 1991, Nagasaki, Japan
Atypical *E. tarda*	NUF 512	*	*	red sea bream, 1991, Nagasaki, Japan
Atypical *E. tarda*	NUF 523	*	*	red sea bream, 1991, Kagoshima, Japan
Atypical *E. tarda*	NUF 623	*		red sea bream, 1992, Nagasaki, Japan
Atypical *E. tarda*	NUF 624	*		red sea bream, 1992, Nagasaki, Japan
Atypical *E. tarda*	NUF 483	*		crimson sea bream, 1990, Nagasaki, Japan
Atypical *E. tarda*	NUF 696	*	*	crimson sea bream, 1993, Nagasaki, Japan
E. tarda	MZ 8202		*	Japanese eel, 1982, Miyazaki, Japan
E. tarda	NUF 49	*	*	Japanese eel, 1984, Nagasaki, Japan
E. tarda	NUF 69		*	Japanese eel, 1984, Nagasaki, Japan
E. tarda	KG 8401		*	Japanese eel, 1984, Kagoshima, Japan
E. tarda	NUF 116		*	Japanese eel, 1985, Nagasaki, Japan
E. tarda	KGE 8201	*	*	tilapia, 1982, Kagoshima, Japan
E. tarda	NUF 251	*	*	Japanese flounder, 1986, Nagasaki, Japan
E. tarda	NUF 511		*	Japanese flounder, 1991, Nagasaki, Japan
E. tarda	NUF 702		*	Japanese flounder, 1993, Oita, Japan
E. tarda	NUF 804		*	Japanese flounder, 1997, Kagoshima, Japan
E. tarda	NUF 805		*	Japanese flounder, 1997, Kumamoto, Japan
E. tarda	NUF 705	*	*	greenling, 1993, Oita, Japan

[a] NUF (Nagasaki University, Fisheries) 9 was used only for antiserum preparation; AGGL = Agglutination; IE = Immunoelectrophoresis.

0.5% NaCl and 1.5% agar at pH 7.2.

Preparation of antigens

Bacteria were grown in YE broth at 28°C for 24 h. A portion of the cells harvested was killed by heating at 121°C for 90 min, and another part was killed by adding formalin to a final concentration of 0.5% and kept at room temperature for 2 nights. Both heat- and formalin-killed bacteria were washed five times with phosphate buffered saline (PBS), pH 7.2, suspended in PBS at 100 mg/m*l* and stored in a refrigerator.

Preparation of antisera

Antisera were prepared by immunizing rabbits with formalin-killed cells (f) of *E. tarda* strains NUF10, NUF49 and NUF251, and with heat-killed cells (h) of NUF9 and NUF251. The antigen was diluted 1:10 with PBS, mixed with Freund's complete adjuvant at a ratio of 1:1, and 2 m*l* of the mixture (5 mg antigen/m*l*) were injected subcutaneously twice, with an interval of two weeks, into the back of a rabbit. In the fourth week 1.0 m*l* of the antigen (10 mg/m*l*) was injected into the rabbit ear vein, (NUF9(h), NUF10(f), NUF49(f) and NUF251(h)) or intraperitoneally (NUF251(f)), and after one week the agglutinin titer of the serum was tested. The blood was collected 6 weeks after the first injection from the carotid artery.

Agglutination test

The microtiter technique (Toranzo *et al.*, 1987; Roberson, 1990) was used to examine the serological relationship between the strains from sea breams and the representative strains from other fish (Table 1). Concentration of each antigen used for agglutination was standardized to approximately 10^9 cells/m*l* (Roberson, 1990). Titration of the antisera in PBS started from 2-fold dilution. Antigen and antiserum were mixed in a 96-well microtiter plate, incubated at 37°C for 2 h and placed in a refrigerator overnight.

Absorption test

To determine the antigenic structure of the strains

NUF10, NUF49 and NUF251, an antibody absorption test was conducted. About 100 mg of packed bacterial cells of each strain was added to 1.5 m*l* of antiserum (NUF251) or antiserum diluted 1 : 10 with PBS (NUF10 and NUF49). The cells and antiserum were mixed, incubated at 37°C for 2 h and placed in a refrigerator overnight, and the cells were removed by centrifugation (10,000 × *g*/10 min). Each antiserum was absorbed twice and tested for agglutinin titers against absorbing strains. If agglutinins remained, the antiserum was absorbed again.

Extraction of cellular antigens

Extraction of cellular antigens was carried out by the method used for the extraction of cell surface antigens of *Escherichia coli* (Ørskov and Ørskov, 1970) with some modifications. Bacteria were grown on YE agar at 28°C for 48 h. The cells were washed off the plates with PBS, collected by centrifugation (10,000 × *g*/10 min) and suspended at 0.3 g/m*l* in PBS. The extraction of all isolates were performed by heating the bacterial suspensions at 60 or 100°C for 30 min. The supernatants of the heated samples were designated 60°C extracts and 100°C extracts, respectively. To test the heat stability of the antigens, a part of the 60°C extracts was also heated to 100°C for 30 min.

Proteinase digestion

One hundred microliter of the 60°C extracts from NUF10, NUF49 and NUF251 were digested with 4 μ*l* of proteinase K (10 mg/m*l*) at 50°C for 1 h.

Preparation of lipopolysaccharide (LPS)

The LPS of NUF10 was extracted by Westphal's phenol-water method (Westphal and Jann, 1965). The water layer was dialyzed to distilled water and the LPS was purified by repeated ultracentrifugation (150,000 × g/6 ~ 8 h), and the precipitated LPS was lyophilized and kept at 4°C until used. The supernatant of the first ultracentrifugation was also kept for immunoelectrophoretic analysis.

Immunoelectrophoresis (IE)

The electrophoresis of the cell extracts or LPS was carried out in 1% agarose, at 10 mA per slide (5 cm × 7.5 cm), for 90 min, with barbiturate buffer (pH 8.6). Purified LPS was dissolved in PBS at 5 mg/m*l* and heated at 60°C for 1 min. After electrophoresis the troughs were filled with antisera, and the slides were incubated in humid atmosphere at room temperature. The precipitation arcs could be observed after 12 ~ 20 h, and the results were recorded after 24 h.

Results

The agglutinin titers of antisera for formalin-killed and heat-killed cells are shown in Tables 2 and 3. When formalin-killed cells of the strains were used, they were divided into two groups by the titers of anti-NUF10(f) and anti-NUF49(f) sera; high titer group, all

Table 2. Agglutinin titers of rabbit anti-*E. tarda* sera for formalin-killed cells

Antigen	Antiserum				
	NUF9(h)	NUF10(f)	NUF49(f)	NUF251(f)	NUF251(h)
NUF 10(f)	8	16384	131072	256	16
NUF 500(f)	8	32768	262144	256	64
NUF 512(f)	8	32768	131072	128	64
NUF 523(f)	16	131072	131072	128	64
NUF 623(f)	8	131072	131072	128	64
NUF 624(f)	16	32768	65536	512	64
NUF 483(f)	8	65536	131072	128	16
NUF 696(f)	8	32768	32768	128	32
NUF 49(f)	64	512	16384	512	256
NUF 251(f)	32	512	4096	512	64
NUF 705(f)	32	1024	4096	512	128
KGE 8201(f)	16	2048	16384	128	128

h = Heat-killed cells; f = Formalin-killed cells.

Table 3. Agglutinin titers of rabbit anti-*E. tarda* sera for heat-killed cells

Antigen	Antiserum				
	NUF9(h)	NUF10(f)	NUF49(f)	NUF251(f)	NUF251(h)
NUF 10(h)	128	512	2048	256	512
NUF 500(h)	256	1024	2048	512	256
NUF 512(h)	128	1024	2048	256	256
NUF 523(h)	512	1024	2048	512	512
NUF 623(h)	256	1024	1024	256	512
NUF 624(h)	128	512	4096	256	512
NUF 483(h)	512	1024	2048	512	512
NUF 696(h)	256	256	2048	256	512
NUF 49(h)	256	512	2048	512	512
NUF 251(h)	256	1024	4096	256	512
NUF 705(h)	256	256	2048	512	512
KGE 8201(h)	512	512	2048	512	512

h = Heat-killed cells; f = Formalin-killed cells.

Table 4. Agglutinin titers of antisera absorbed with heat-killed cells

Antiserum	Absorbed with	Agglutinin titer for		
		NUF10 (h)	NUF49 (h)	NUF251 (h)
NUF10(f)[a]	NUF 49(h)	< 4	< 4	< 4
	NUF 251(h)	< 4	< 4	< 4
	None	64	64	64
NUF49(f)[a]	NUF 10(h)	< 4	< 4	< 4
	NUF 251(h)	< 4	< 4	< 4
	None	256	256	256
NUF251(f)[b]	NUF 10(h)	< 4	< 4	< 4
	NUF 49(h)	< 4	< 4	< 4
	None	256	512	512

[a] 1/10 dilution; [b] 1/1 dilution; h = Heat-killed cells; f = Formalin-killed cells.

strains isolated from sea breams, and low titer group, strains isolated from other fish species. These groups were not clearly distinguished by the other antisera (Table 2). When heat-killed cells were used, five antisera respectively showed similar titers for all strains, and so they belonged to one group (Table 3). Agglutinin titers of antisera raised against heat-killed cells, anti-NUF9(h) and anti-NUF251(h), were lower for formalin-killed cells than for heat-killed cells.

The results of cross-absorption tests with heat-killed cells indicated that NUF10, NUF49 and NUF251, the representative strains of sea breams, eel and flounder, respectively, belonged to the same O-serotype (Table 4). Anti-NUF10(f) and anti-NUF49(f) sera absorbed with antigens other than NUF10(f) presented high agglutinin titers for formalin-killed cells of NUF10. Anti-NUF251(f) serum after absorbtion with NUF251(f) still agglutinated heat-killed cells of NUF251, and after absorbtion with NUF251(h) still agglutinated formalin-killed cells of NUF10, NUF49 and NUF251. Anti-NUF10(f) serum after absorbtion with NUF10(f) agglutinated heat-killed cells of NUF10, too (Table 5).

Generally similar immunoelectrophoresis (IE) patterns were observed among NUF10, NUF49, and NUF251. When 60°C extracts were applied, distinct precipitation arcs appeared toward the anode at the position close to and far from the application well. On the other hand, when 100°C extracts were applied, the

Table 5. Agglutinin titers of antisera absorbed with formalin- and heat-killed cells

Antiserum	Absorbed with	Agglutinin titer for					
		NUF10(f)	NUF10(h)	NUF49(f)	NUF49(h)	NUF251(f)	NUF251(h)
NUF10(f)[a]	NUF10(f)	< 4	16	NT	NT	NT	NT
	NUF10(h)	2048	< 4	4	NT	4	NT
	NUF49(f)	2048	NT	< 4	NT	4	NT
	NUF251(f)	2048	NT	< 4	NT	< 4	NT
	None	2048	64	64	64	64	64
NUF49(f)[a]	NUF10(f)	< 4	NT	< 4	NT	< 4	NT
	NUF49(f)	2048	NT	< 4	< 4	NT	NT
	NUF49(h)	4096	NT	4	< 4	4	NT
	NUF251(f)	2048	NT	< 4	NT	< 4	NT
	None	2048	256	256	256	512	256
NUF251(f)[b]	NUF10(f)	< 4	NT	8	NT	8	NT
	NUF49(f)	8	NT	< 4	NT	< 4	NT
	NUF251(f)	NT	NT	NT	NT	< 4	128
	NUF251(h)	32	< 4	32	< 4	32	< 4
	None	256	256	512	512	512	256

[a] 1/10 dilution; [b] 1/1 dilution; h = Heat-killed cells; f = Formalin-killed cells; NT = not tested.

arc far from the well was observed faintly and an arc which migrated to neither direction from the well became obvious in NUF10 and NUF251 against anti-NUF251(f) serum. Besides these arcs several faint arcs also appeared around and distant from the well (Fig. 1). When 60°C extracts were heated to 100°C for 30 min, the distinct arc far from the application well disappeared, except for NUF49 in which the arc was only faintly formed with anti-NUF10(f) serum (Fig. 2).

Protein digestion of 60°C extracts with proteinase K resulted in the disappearance of the arcs, but the distinct arcs close to and far from the application well were still observed (Fig. 3). There were no changes in the IE patterns upon treating 60°C extracts with DNase or RNase (Data not shown).

Purified LPS of NUF10 formed a precipitation arc close to the application well toward the anode against anti-NUF10(f) serum. The supernatant after ultracentrifugation of the water layer of the LPS purification process also formed an arc near the well (Fig. 4). Purified LPS and the supernatant also formed the arcs against anti-NUF251(h) serum (Data not shown).

IE patterns of 60°C extracts from the representative strains against various kinds of anti-NUF251 sera are shown in Fig. 5. Although many arcs were formed against anti-NUF251(f) serum, only one arc which located close to the application well toward the anode was observed against anti-NUF251(h) serum. When anti-NUF251(f) serum absorbed with heat-killed cells was used, only this arc disappeared. When anti-NUF251(f) serum absorbed with formalin-killed cells was used, no arcs were seen in the extracts from NUF10, but some of the arcs could still be observed in the extracts from NUF49 and NUF251.

IE patterns of 60°C extracts of *E. tarda* isolated from various fish species are shown in Fig. 6. Regardless of source all the isolates formed two distinct arcs which located close to and far from the application well.

Discussion

Minagawa *et al.* (1983) reported that virulent strains of *E. tarda* found in eel ponds shared common O-antigens and were divided into 2 serotypes based on antibody absorption tests. Park *et al.* (1983) tested 445 isolates of *E. tarda* collected in eel culture ponds, and 270 strains were classified, according to O-agglutination test, into 4 serotypes, with predominance of serotype A. Mamnur Rashid *et al.* (1994) tested 28 strains of *E. tarda* isolated from diseased flounder using anti-NUF251

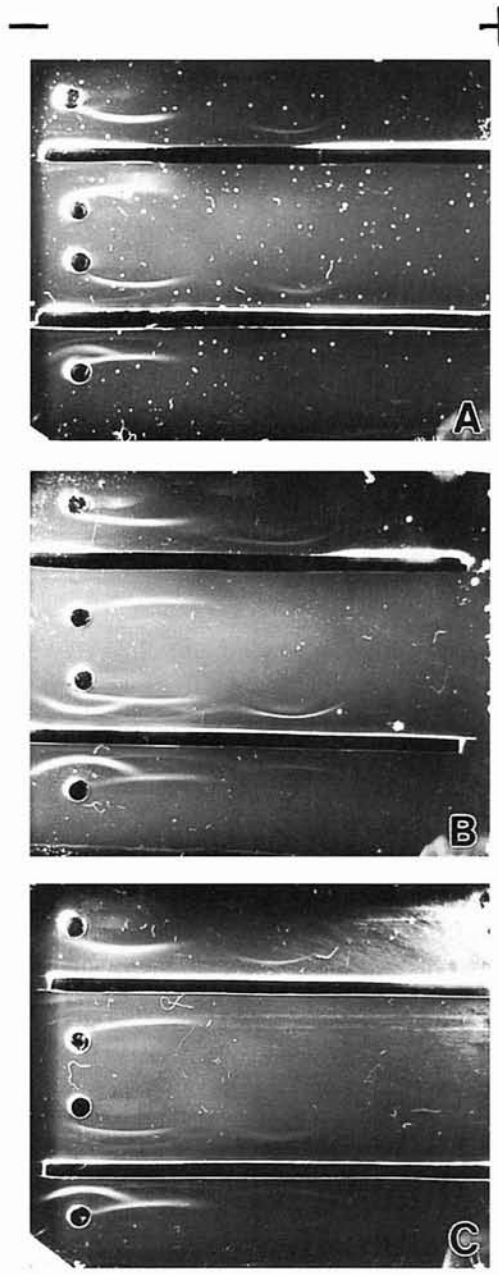

Fig. 1. Immunoelectrophoretic patterns of 60°C (in the well above trough) and 100°C extracts (in the well below trough) of cells of *E. tarda* NUF 10(A), NUF 49(B), and NUF 251(C). In the upper trough anti-NUF 10(f) serum and in the lower trough anti-NUF 251(f) serum.

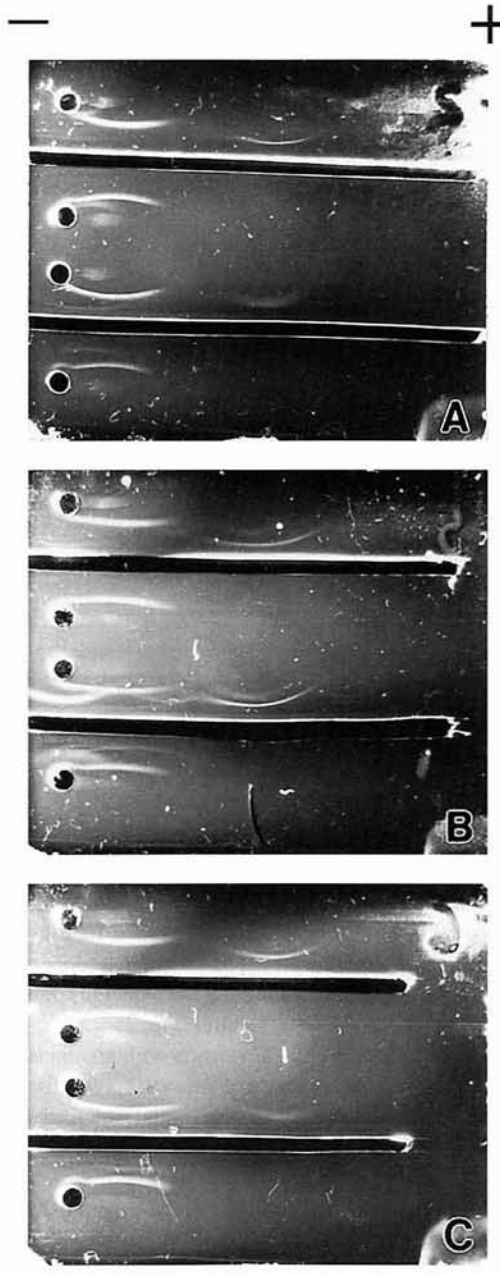

Fig. 2. Immunoelectrophoretic patterns of 60°C extracts of cells of *E. tarda* NUF 10(A), NUF 49 (B), and NUF 251 (C) before (in the well above trough) and after heated at 100°C for 30 min (in the well below trough). In the upper trough anti-NUF 10(f) serum and in the lower trough anti-NUF 251(f) serum.

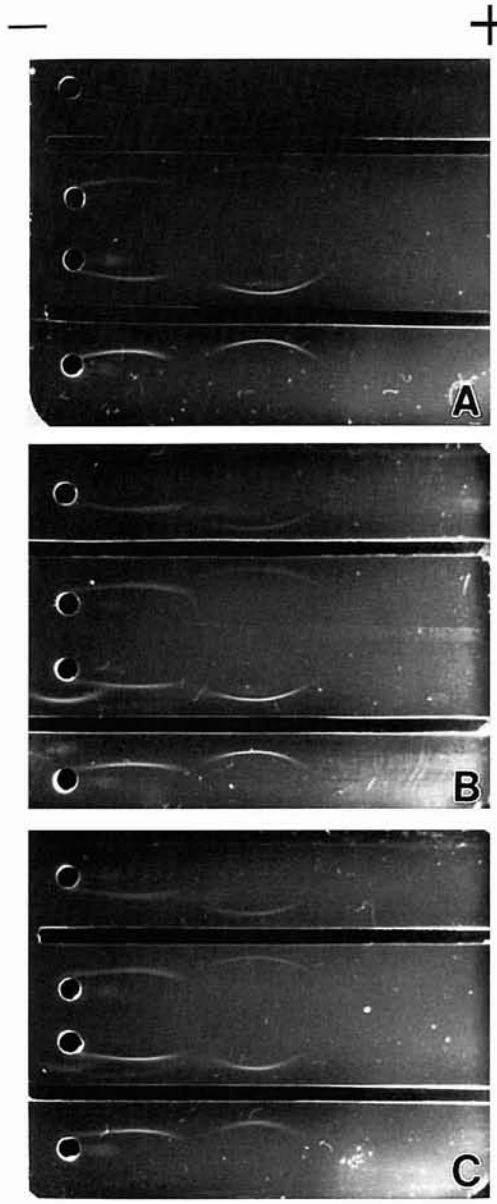

Fig. 3. Immunoelectrophoretic patterns of 60°C extracts of cells of *E. tarda* NUF 10(A), NUF 49(B), and NUF 251(C) before (in the well above trough) and after protein digestion (in the well below trough). In the upper trough anti-NUF10(f) serum and in the lower trough anti-NUF 251(f) serum.

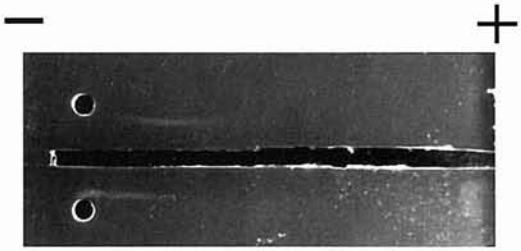

Fig. 4. Immunoelectrophoretic patterns of LPS of NUF 10. In the well above trough LPS in the supernatant after ultracentrifugation of the water layer (see Text), in the well below trough purified LPS. In the trough anti-NUF10(f) serum.

serum and concluded that all the strains were serologically homogeneous and belonged to one O-serotype, which was serologically identical to type A of eel strains. In the present study, according to agglutination and antibody absorption tests, all the strains from sea breams belonged to the same O-serotype as NUF49 and NUF251, representative strains isolated from eel and flounder, respectively (Tables 3 and 4).

Agglutinin titers of anti-NUF10(f) serum for formalin-killed cells of the strains from sea breams were higher than the titers for the heat-killed cells (Table 2), and anti-NUF10(f) serum absorbed with heat-killed cells of NUF10 still presented high titer for the formalin-killed cells (Table 5). These results indicate the existence of heat-labile antigens on the cell surface of the strains of sea breams. Since atypical strains from sea breams are non-motile, it is not likely that these heat-labile antigens include H-antigens (flagellar antigens). And some of these antigens may inhibit O-agglutination of the strains, because agglutinin titers of anti-NUF9(h) and anti-NUF251(h) sera were lower for formalin-killed cells than for heat-killed cells (Tables 2 and 3). The same phenomena were also seen in the strains from other fish species. It is interesting that the titers of anti-NUF49(f) serum for formalin-killed cells of the strains from sea breams were higher than the titer for NUF49. Low agglutinin titers of anti-NUF251(f) serum are probably due to poor production of heat-labile surface antigens by the immunized strain and/or poor response of the rabbit to the antigens.

Immunoelectrophoretic analyses demonstrated the relatedness of cell surface antigens among the strains from various fish species. As shown in Fig. 6 all strains studied commonly presented two distinct arcs against anti-NUF10(f) and anti-NUF251(f) sera, one appeared

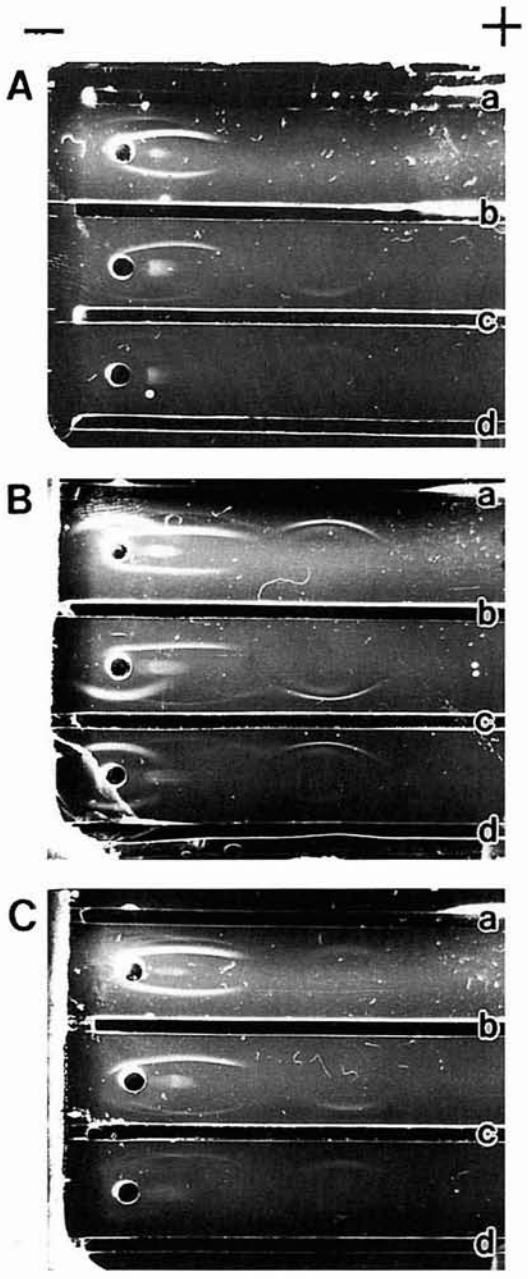

Fig. 5. Immunoelectrophoretic patterns of 60°C extracts of cells of *E. tarda* NUF 10(A), NUF 49(B), and NUF 251(C) against; a, anti-NUF 251(f)serum; b, anti- NUF 251(h) serum; c, anti-NUF 251(f) serum absorbed with NUF 251 heat- killed cells; and d, anti-NUF 251(f) serum absorbed with NUF 251 formalin- killed cells.

toward the anode at the position close to the application well and the other appeared far from the well. The former arc was probably due to O-antigen of the strains, because it appeared against anti-NUF251(h) serum and did not appear against anti-NUF251(f) serum absorbed with heat-killed cells (Fig. 5), and because purified LPS formed precipitation arc at the same position (Fig. 4). The latter arc was due to the antigen which was anionic, inactivated by heating at 100°C (Fig. 2) and resistant to proteinase K digestion (Fig. 3) as well as RNase and DNase. Further study is needed to clarify whether this antigen is a polysaccharide K-antigen or not.

The strains from eel formed more arcs than the strains from other fish species (Fig. 6). This was probably not for qualitative difference of antigens between the strains from eel and other fish species, but for quantitative difference in antigen productions, because the strains from eel formed the arcs against antisera raised against *E. tarda* isolated from red sea bream and flounder, which also possessed the same antigens as the strains from eel.

From the present study it was revealed that non-motile variant of *E. tarda* isolated from sea breams belonged to the same O-serotype and possessed similar surface antigens as *E. tarda* isolated from eel and flounder. This suggests the possibility of usage of a common vaccine for edwardsiellosis among sea breams and other fish species. Further study is needed to clarify which antigens are involved in the pathogenicity and which are the protective antigens.

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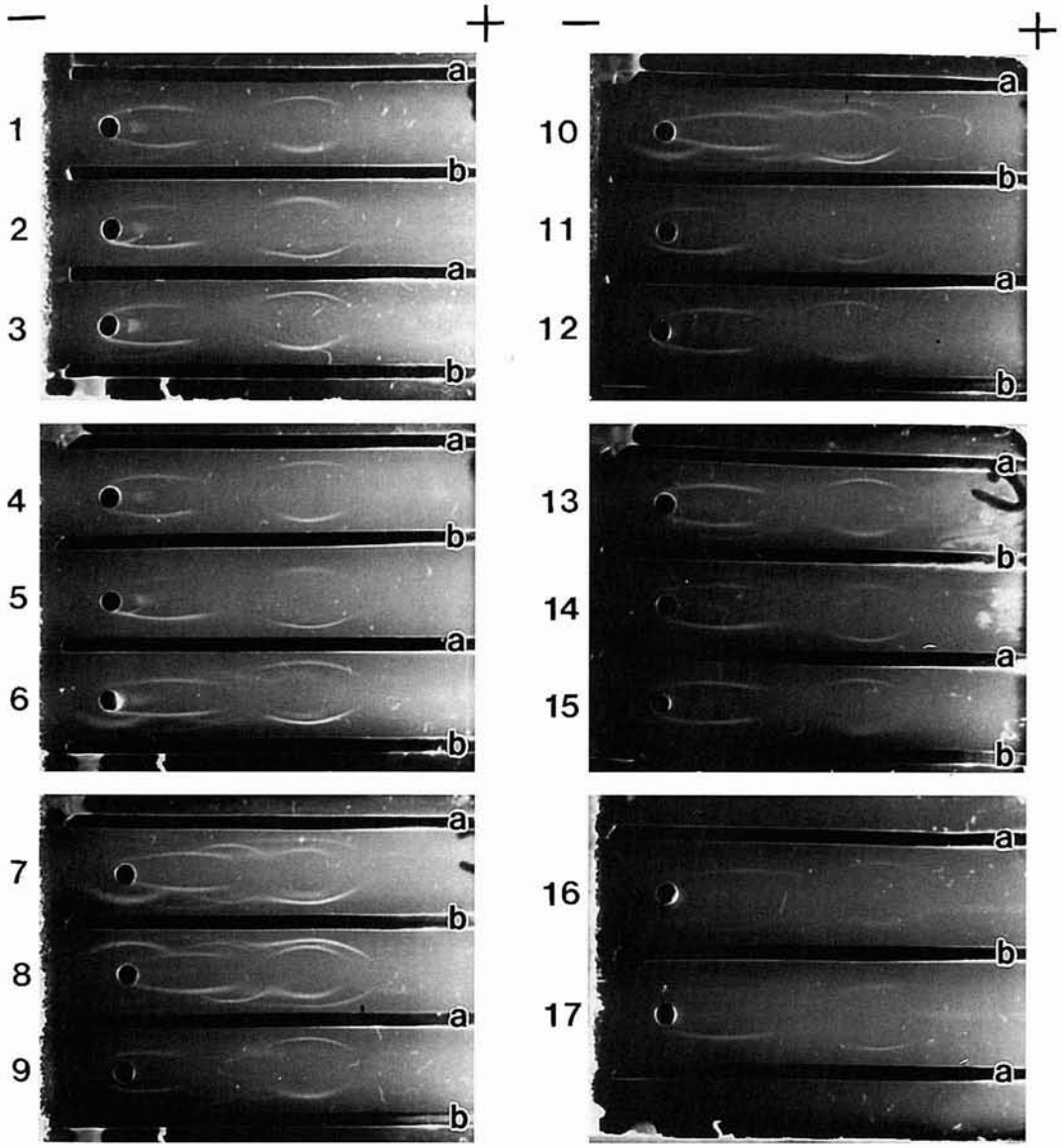

Fig. 6. Immunoelectrophoretic patterns of 60°C extracts of *E. tarda* isolated from red sea bream (1, NUF 10; 2, NUF 500; 3, NUF 512; 4, NUF 523), crimson sea bream (5, NUF 696), eel (6, NUF 49; 7, NUF 69; 8, NUF 116; 9, KG8401; 10, MZ 8202), tilapia (11, KGE 8201), flounder (12, NUF 251; 13, NUF 511; 14, NUF 702; 15, NUF 804; 16, NUF 805), and greenling (17, NUF 705). a, anti-NUF 10(f) serum; b, anti-NUF 251(f) serum.

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