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Evidence for the Presence of a K Antigen in Strains of *Vibrio anguillarum*

Henrik Berg Rasmussen

Laboratory of Aquatic Pathobiology, Royal Veterinary and Agricultural University, Frederiksberg, Denmark

Abstract. Eleven *Vibrio anguillarum* O group 1 strains, isolated from different species of diseased fish, were selected for an immunoelectrophoretic study. Antigenic preparations for immunoelectrophoresis were simple water extracts boiled for 1 h. Using O and OK antisera, immunoelectrophoretic patterns suggested the presence of a K antigen; there is evidence that examined strains possess a common K antigen.

Vibrio anguillarum, causing hemorrhagic septicemia in feral and cultured fish, is considered the most important fish pathogenic bacterium in the marine environment [1]. Several O groups of *V. anguillarum* have been reported [6, 7]. Recently, existing O grouping schemes were coordinated [13]. Capsule polysaccharides, K antigens, are considered important virulence factors, providing bacteria with antiphagocytic and anticomplementary activities [3, 4]. A K antigen determination is included in the serological characterization of several bacterial species [9]. However, the eventual presence of K antigens in *V. anguillarum* has, apparently, not been examined. The present investigation suggests the existence of a K antigen in O group 1 strains of *V. anguillarum*, isolated from diseased fish in different countries.

Materials and Methods

Strains. Examined strains are listed in Table 1. All strains were serotyped O1 according to the serogrouping scheme reported recently [13].

Production of rabbit antisera. OK antiserum was produced against strain 6018/1 and 820901-2/8. Formalinized bacteria were injected intravenously. Injections were given three times weekly for 4 consecutive weeks, in doses ranging from 10^7 to 10^{10} bacterial cells. The rabbits were bled 1 week following the last injection.

O antiserum was produced against strain 6018/1. Bacterial cells were autoclaved (120°C, 2 h), then washed in PBS (pH 7.2), and finally resuspended in PBS to an OD value of 1.0 (590 nm). Two intravenous injections of these suspensions were given weekly, starting with 0.1 ml. Gradually the dose was increased, ending with 2.0 ml after 7 weeks of immunization. The rabbits were bled 1 week after the last injection.

Purification of antisera. A partial purification was carried out by mixing equal volumes of antiserum and a solution of saturated $(NH_4)_2SO_4$. The pH was adjusted to 8.0, and after stirring for 1 h, the mixture was centrifuged (1500 *g* for 15 min). The supernatant was discarded; the precipitate was redissolved to initial volume of antiserum, with PBS (pH 7.2). Finally, dialysis against PBS was carried out overnight.

Preparation of antigenic extracts for immunoelectrophoretic procedures. Strains were grown on Marine agar (Difco) containing 5% citrated calf blood. After incubation at 20°C for 48 h, plates were harvested and antigens extracted. Upon centrifugation, antigenic extracts were boiled for 1 h [9, 10].

Immunoelectrophoretic techniques. Gels, cast in a thickness of 1.5 mm, were prepared from 1% agarose HSA (Litex, Copenhagen). Gel Bond plastic film support was used (FMC, Maine, USA). The electrophoresis buffer was Tris/barbital buffer pH 8.6, as described by Svendsen et al. [14]. Addition of calcium lactate was, however, omitted. The ionic strength was 0.05 for the micromethod of immunoelectrophoresis, whereas an ionic strength of 0.02 was used for other immunoelectrophoretic techniques. Gels were stained in a 0.5% wt/vol solution of Coomassie brilliant blue R 250 (Hopkin and Williams, England).

Micromethod of immunoelectrophoresis. Principles outlined by Ørskov et al. [10] and Scheidegger [12] were employed. After electrophoretic separation of antigens, the longitudinal basin was filled with partially purified antiserum. Gels were placed in a humid chamber at 20°C for 36 h. After washing and staining, results were recorded by drawing.

Crossed immunoelectrophoresis of protease-treated antigenic extract. A 200-μl extract from strain 820901-2/8 was added to 100 μl of a 2 mg/ml protease solution (Proteinase K from *Tritirachium album*, Sigma, St. Louis) or 100 μl distilled water (control). The mixtures were incubated at 37°C for 24 h and then boiled for 5 min; 2 μl of these mixtures were applied for

Address reprint requests to: Dr. Henrik Berg Rasmussen, Laboratory of Aquatic Pathobiology, The Royal Veterinary and Agricultural University, 13 Bülowsvej, DK-1870 Frederiksberg C, Denmark

Table 1. *Vibrio anguillarum* O group 1 strains

Strain	Origin	Source
820901-2/8	*Salmo gairdneri*, Sweden	I. Dalsgaard
820617-1/8	*Salmo gairdneri*, Denmark	I. Dalsgaard
6018/1	*Salmo gairdneri*, Denmark	J. L. Larsen
408 F	*Salmo gairdneri*, France	F. B. Laurencin
PT 213	*Plecoglossus altivelis*, Japan	T. Aoki and K. Muroga
FT 1642	*Salmo salar*, Norway	T. Håstein
1359 A	*Salmo gairdneri*, Italy	G. Giorgetti
1359 B	*Salmo gairdneri*, Italy	G. Giorgetti
T 265	*Salmo salar*, England	B. Austin
53-507	*Oncorhynchus kisutch*, Canada	K. A. Johnson
B 2381/80	*Salmo gairdneri*, Sweden	O. Ljungberg

a crossed immunoelectrophoretic analysis. The 1st-dimensional electrophoresis was performed at 4 V/cm for 75 min. Antibody-containing gels were prepared (100 μl homologous OK antiserum/ml agarose); 2nd-dimensional electrophoresis was performed at 2.5 V/cm for 20 h.

Crossed line immunoelectrophoresis. Four μl of antigenic extract from strain 820901-2/8 were applied and 1st-dimensional electrophoresis was carried out (4 V/cm for 75 min). An antigen-containing gel with 40 μl extract from strain 820901-2/8, 6018/1, or 1359 A/ml agarose was situated on the anodic side of the application well. Antibody-containing gel was prepared with 100 μl OK antiserum against 820901-2/8/ml agarose; the 2nd-dimensional electrophoresis was performed at 2.5 V/cm for 20 h.

Rocket line immunoelectrophoresis. An antigen-containing gel (40 μl antigenic extract from strain 1359 A/ml agarose) was situated on the anodic side of the application wells. Antibody-containing gel was prepared with 100 μl OK antiserum against strain 820901-2/8/ml agarose. Antigenic extracts (1.2 μl dil. 1 : 2) were placed in wells. Electrophoresis was carried out at 2.5 V/cm for 20 h.

Results

Immunoelectrophoresis of extracts from strain 6018/1 and 820901-2/8 with OK antiserum against 6018/1 demonstrated the presence of an electrophoretic, almost nonmigrating antigen and an anodic migrating antigen (Fig. 1). A similar result was recorded with respect to these strains when OK antiserum against 820901-2/8 was applied. The O anti-

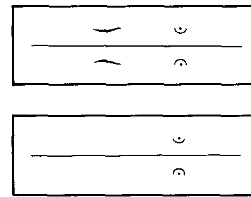

Fig. 1. Immunoelectrophoresis (micromethod). Amounts of 1.5 μl antigenic extracts from strain 820901-2/8 and 6018/1 were applied (dots illustrate application wells). After electrophoretic separation, the longitudinal basin was filled with OK antiserum against 6018/1 (drawing at the top) or with O antiserum against the same strain (drawing at the bottom). Anode at the left.

serum against 6018/1 precipitated only the antigen, which exhibited the low electrophoretic migration (Fig. 1).

Incubation with protease did not affect the precipitation patterns of the antigens under analysis (Fig. 2).

Crossed line immunoelectrophoretic patterns with extract from homologous strain (Fig. 3) or from strain 6018/1 (not shown) for preparation of the antigen-containing gel demonstrated the presence of a distinct line, elevated by an anodic peak. An indistinct broad line close to the application wells was observed. Crossed line immunoelectrophoresis with extract from strain 1359 A for preparation of the antigen-containing gel exhibited a similar pattern. However, an additional anodic line (arrow), not elevated by the peak, was noted (Fig. 4).

The rocket line immunoelectrophoretic pattern demonstrated that all examined extracts displaced the faster anodic electrophoretic migrating line. Elevations below peaks of the faster anodic line or "spurs" below this line were not apparent. Elevations of a slow migrating line (arrow) could be distinguished (Fig. 5).

Discussion

Antigenic extracts prepared by extraction of bacterial suspensions in saline followed by boiling of the extracts have proven valuable for immunoelectrophoretic investigations of O and K antigens. With these antigenic extracts, evidence for the eventual presence of K antigens can be provided when immunoelectrophoretic patterns, produced by O and OK antiserum, are compared [9–11]. By

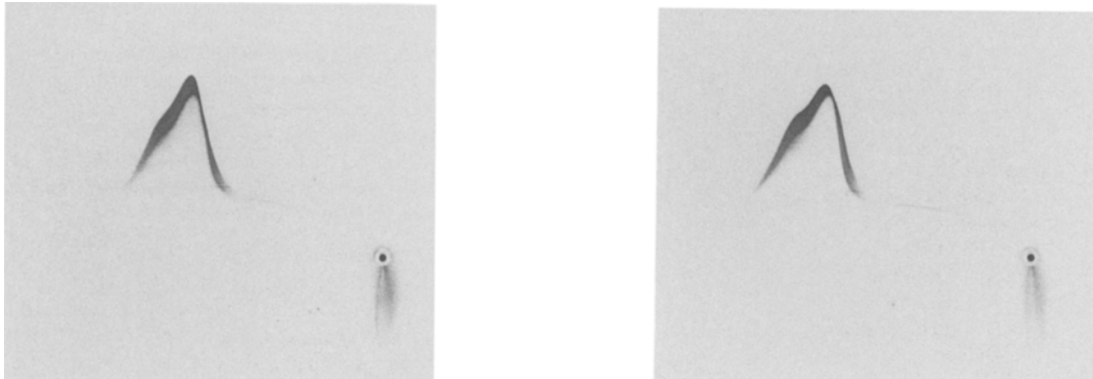

Fig. 2. Crossed immunoelectrophoresis of protease-treated antigenic extract. Antigenic extract from strain 820901-2/8, incubated with water for control (left) or protease (right), was applied. Antibody-containing gel was prepared with homologous OK antiserum. The 1st-dimensional electrophoresis, anode at the left; and the 2nd-dimensional electrophoresis, anode at the top. Application wells are marked with dots.

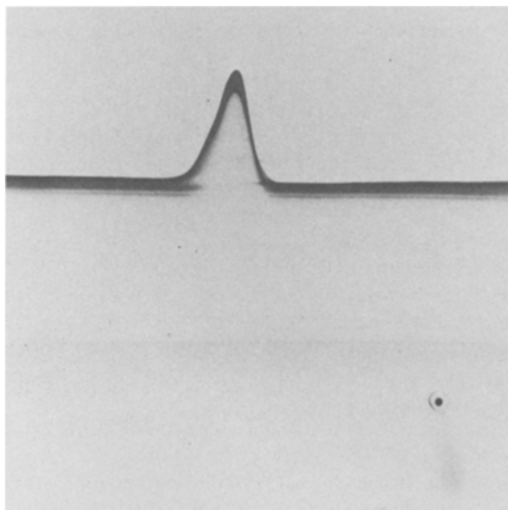

Fig. 3. Crossed line immunoelectrophoresis. Extract from strain 820901-2/8 was placed in the well and submitted for electrophoresis. Antigen-containing gel was formed with antigenic extract from same strain. Antibody-containing gel was prepared with homologous OK antiserum. The 1st-dimensional electrophoresis, anode at the left; and the 2nd-dimensional electrophoresis, anode at the top. Application well is marked with a dot.

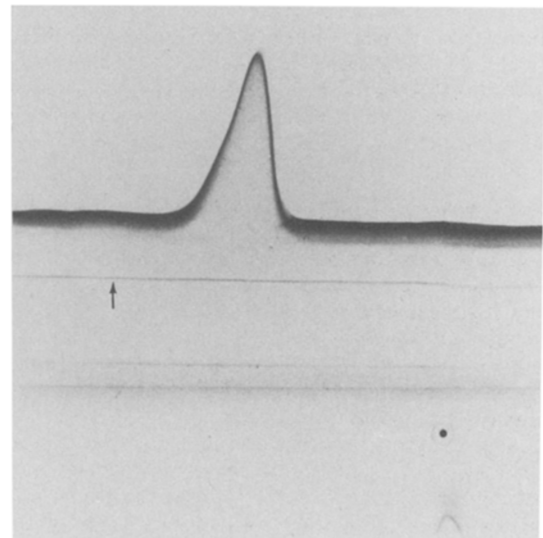

Fig. 4. Crossed line immunoelectrophoresis. Extract from strain 820901-2/8 was placed in the well and submitted for electrophoresis. Antigen-containing gel was formed with extract from strain 1359 A. Antibody-containing gel was prepared with OK antiserum against 820901-2/8. The 1st-dimensional electrophoresis, anode at the left; and 2nd-dimensional electrophoresis, anode at the top. Application well is marked with a dot.

employing similar principles, the present study suggests that examined *V. anguillarum* strains exhibit a fast anodic migrating K antigen and a mainly nonmigrating O antigen. The identification of these antigens as polysaccharides is supported by the demonstration of their resistance to treatment with proteinase K.

The crossed line and rocket line immunoelectrophoretic analyses demonstrated reactions of serological identity, suggesting that examined strains, although derived from different sources, possess a common K antigen. Studies in progress show the existence of several distinct *V. anguillarum* K antigens (H.B. Rasmussen, unpublished results). It is

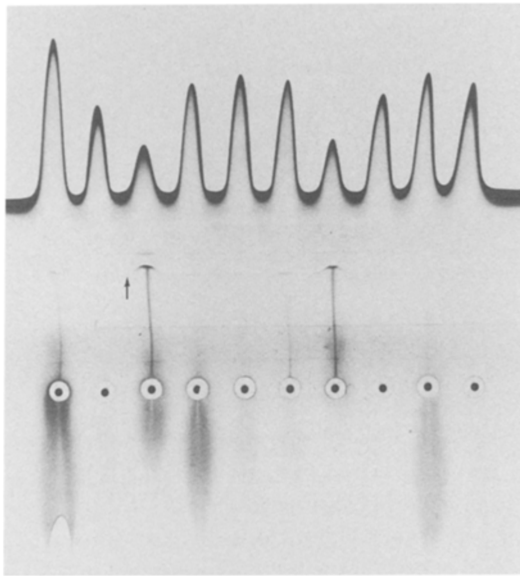

Fig. 5. Rocket line immunoelectrophoresis. In wells (from left to right) were placed antigenic extract from strain 820617-1/8, 408 F, PT 213, FT 1642, 1359 B, 1359 A, T 265, 53-507, B 2381/80, and 820901-2/8. Antigen-containing gel was formed with extract from strain 1359 A. Antibody-containing gel was prepared with OK antiserum against 820901-2/8. Anode at the top. Application wells are marked with dots.

suggested that the K antigen, reported in the present paper, will be named K1.

Iron-sequestering ability [2], in addition to hemolysin and other extracellular products [5, 8], probably contributes to the pathogenicity and virulence of *V. anguillarum*. Further investigations on K antigens can prove valuable in the selection of strains for vaccine purposes and provide detailed information on the pathophysiological processes underlying infections with *V. anguillarum*.

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