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NOTE

Isolation of *Aeromonas salmonicida* from salmon lice *Lepeophtheirus salmonis* and marine plankton

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ABSTRACT: *Aeromonas salmonicida* was isolated from salmon lice *Lepeophtheirus salmonis* and zooplankton in samples taken at a fish farm with an outbreak of furunculosis. Approximately 10^4 cells of *A. salmonicida* per salmon louse and 600 cells of *A. salmonicida* per ml of homogenized plankton suspension were detected. By use of immunomagnetic beads coated with monoclonal antibodies against the lipopolysaccharides of *A. salmonicida*, the bacteria were retrieved in almost pure culture from certain of the heavily 'contaminated' environmental samples.

Aeromonas salmonicida, the etiological agent of furunculosis, causes severe losses in cultured fish in many parts of the world (Austin & Austin 1987). Since 1985, furunculosis has spread epizootically in Norway. Furunculosis has now been recorded in most fish farms on the western coast of Norway, where outbreaks result in the highest mortalities experienced on the farms.

The survival potential of *Aeromonas salmonicida* in water has been thoroughly investigated (McCarthy 1977, Sakai 1986, Rose et al. 1990). The bacterium has never been isolated from any environmental sources but it has been detected in high numbers by application of immunofluorescence in samples from sediments, seawater, and surface films from fish farms stocked with salmon suffering from furunculosis (Enger & Thorsen 1992).

Although fish have been regarded as the main vector in the transmission of furunculosis, ectoparasites may also serve as vectors because they have been shown to be likely vectors in the transfer of viral diseases (Ahne 1985, Mulcahy et al. 1990). Salmon lice may therefore be important in introducing pathogens such as *Aeromonas salmonicida* into fish farms (Cusack & Cone 1986, Nylund et al. 1991).

In this study we report immunofluorescence detection of *Aeromonas salmonicida* in high numbers in sediments, seawater, and in surface films. In addition, we have been able to isolate the bacterium from salmon lice and from marine plankton. We also discuss the benefits that immunomagnetic beads provide in the isolation of *A. salmonicida* from environmental samples.

Materials and methods. ***Sampling:*** Atlantic salmon *Salmo salar* suffering from furunculosis were killed by a blow to the head. Salmon lice were picked from the fish with sterile tweezers, care being taken not to collect lice from bloody areas on the fish. Plankton samples were collected by dragging a plankton trawl (mesh size 40 µm) through the upper 50 cm in a net-cage. The samples were kept in the dark and on ice until they were processed in the laboratory on the same day. Water and sediments were sampled and processed as described by Enger & Thorsen (1992).

Antibodies: Two monoclonal antibodies, AL2E6 and AL1B2, directed towards the lipopolysaccharide (LPS) and the outer protein layer (A-layer), respectively, of *Aeromonas salmonicida* were kindly donated by R. Nilsen, Apothekerenes Laboratorium, Tromsø, Norway.

Double staining of samples: The abundance of *Aeromonas salmonicida* and a total count in the samples were obtained by a combination of a fluorescent antibody technique (FA) and staining of bacterial DNA by 4',6-diamidino-2-phenylindole (DAPI) (Enger et al.

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1989). This method allows simultaneous enumeration of total number of bacteria and number of fluorescent antibody-stained cells in the same sample.

Fifteen ml of surface sample, 20 ml of water samples, 1 ml of plankton sample or 1 ml of a 1:1000 dilution of sediment were filtered through Nuclepore filters with a pore size of 0.2 µm. The monoclonal antibody directed against the LPS of *Aeromonas salmonicida* (AL2E6) was used in the immunofluorescence assay.

Immunomagnetic beads: Monodispersed latex beads, 2.8 µm in diameter, with covalently linked sheep anti-mouse IgG antibodies (Dynabeads, M-280 sheep anti-mouse IgG, Dynal A/S Oslo, Norway) were used in the experiment by following the manufacturer's instructions.

To coat the beads with mouse antibodies specific to *Aeromonas salmonicida*, about 10^9 beads were transferred to a tube containing 12 ml of monoclonal antibody preparation [anti-LPS diluted 1:500 in phosphate-buffered saline (PBS), pH 7.5, with 0.5 % (v/w) bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA)] (PBS-BSA). The beads were incubated with the monoclonal antibodies overnight at 5 to 7 °C on an end-over-end rotator (4 rpm) to avoid settling. After the immunomagnetic beads had been drawn towards the inner wall of the tube by a magnet, the residual liquid was aspirated and the immunomagnetic beads were suspended in 5 ml of sterile PBS-BSA.

Sample preparation: Salmon lice were counted and homogenized (Thommas Tissue Grinders, 3431-E04, Swedesboro, NJ, USA) in sterile physiological saline (0.9 % NaCl). One ml of the suspension was incubated with immunomagnetic beads (2×10^8 beads sample^{-1}) for 1 h. To remove unbound bacteria, the immunomagnetic beads were washed 5 times (1 ml of physiological saline containing 0.1 % Tween 20). Three replicate samples of 0.1 ml of the bead suspension were transferred to agar plates [Brain Heart Infusion Broth (Oxoid) + 1.5 % agar (Difco) with 0.01 mg ml^{-1} Coomassie Brilliant Blue (Serva) and 0.015 mg Oleandomycin (Oxoid) ml^{-1} (BHIA-CBB Ol 15)]. Colonies were counted after 48 h of incubation at 21 °C.

Surface sterilization of the salmon lice was carried out by dipping them twice in 70 % ethanol. Between the dips the lice were washed in sterile physiological saline.

Plankton samples of 3 ml were centrifuged ($90 \times g$ for 5 min) and the supernatants were removed. The centrifuged plankton samples (ca 1 ml) were homogenized in 1 ml of physiological saline and then incubated with immunomagnetic beads for 1 h. The samples were then washed and inoculated on agar plates as described earlier.

Immuno-colony-blot: *Aeromonas salmonicida* was identified by the technique described by Rodrigue et al. (1989). Nitrocellulose membranes (BioRad, Richmond, CA, USA) were deposited on BHIA-CBB plates containing possible *A. salmonicida* colonies. The membranes were removed and treated with an antibody solution (AL1B2, diluted 1:4000) followed by an incubation with peroxidase-conjugated goat anti-mouse antibodies (BioRad, diluted 1:2000). After incubation with peroxidase substrate (0.06 % 4-chloro-naphthanol (BioRad) with 20 % ethanol and 0.015 % H_2O_2 in tris-buffered saline (20 mM Tris, 0.5 M NaCl, pH = 7.5) colonies of *A. salmonicida* was observed as purple spots on the membranes.

Results and discussion. Fluorescent antibody detection showed that *Aeromonas salmonicida* comprised approximately 0.1 and 0.02 % of the total bacterial numbers in the water samples and in the sediment, respectively (Table 1). The high number of *A. salmonicida* found in the sediment (Table 1) suggests that

Table 1. *Aeromonas salmonicida*. Number of bacteria detected using different detection methods. The samples were collected in a fish farm during an outbreak of furunculosis. Total: total bacterial counts after staining with DAPI. FA: counts with the fluorescent antibody technique. IMB: immunomagnetic beads. ND: not determined

Samples	Microscope counts (cells ml^{-1})		Plate count before IMB (CFU ml^{-1})		Plate count after IMB (CFU ml^{-1})		Colony blot (CFU ml^{-1})
	Total	FA	Other bact.	*A. salmonicida*	Other bact.	*A. salmonicida*	*A. salmonicida*
Surface	5.3×10^6	6.3×10^3	5.6×10^2	–	20	–	ND
Water at 10 cm	3.3×10^5	3.0×10^2	33	–	–	–	ND
Water at 3 m	3.0×10^5	3.9×10^2	66	–	13	–	ND
Sediment[a]	1.3×10^9	2.9×10^5	4.5×10^4	–	20	–	ND
Plankton	9.0×10^5	1.1×10^4	ND	–	ND	ca 300	620
Lice[b]	ND	ND	4.4×10^3	6.8×10^3	93	1.1×10^4	1.3×10^5
Surface-sterilized lice[b]	ND	ND	4.8×10^2	4.0×10^4	3	1.6×10^3	1.7×10^3

–: No colonies
[a] Results given as cells per gram
[b] Results given as cells per louse

the pathogen had been accumulating there for many weeks. In the plankton samples, *A. salmonicida* comprised 1.2 % of the total bacterial counts, which is one order of magnitude higher than the relative abundance observed in the samples from the water column. Despite the fact that *A. salmonicida* was detected by FA in water and sediment samples in numbers well within the detection limits of the immunomagnetic beads method, attempts to isolate the bacterium from the water and sediment samples were unsuccessful. This is in agreement with previous reports, indicating that *A. salmonicida* does not survive indefinitely when it occurs outside of its host (McCarthy 1977, Rose et al. 1990).

Aeromonas salmonicida was isolated in high numbers from marine plankton and salmon lice (Table 1). In direct plate counts from homogenized salmon lice, *A. salmonicida* comprised 60 % of the colony forming units (cfu). Using immunomagnetic beads, however, *A. salmonicida* accounted for 98 to 99 % of the cfu. *A. salmonicida* has not previously been isolated from animals other than fish (Austin & Austin 1987). Because the louse can swim between different hosts (Bruno & Stone 1990), the association of *A. salmonicida* with salmon lice suggests that the lice may be important vectors in the spread of furunculosis.

Disinfection of the salmon lice in 70 % ethanol reduced the number of bacteria isolated. However, the successful isolation of *Aeromonas salmonicida* from lice that had been surface-disinfected suggests that a significant proportion of the *A. salmonicida* cells must have been residing inside the lice, probably in the gastrointestinal tract. Detection of live cells of *A. salmonicida* in the plankton samples also indicates that marine plankton should be considered as a vector of potential importance in the spread of furunculosis.

The isolates were identified as *Aeromonas salmonicida* subsp. *salmonicida* based on the API 20 E (Analytical Profile Index Systems S.A., La Balme les Grottes, France) assay.

It is important to underscore the qualitative differences between the 2 detection methods applied in this study. While the immunofluorescence technique provided a useful tool for microscopic enumeration of *Aeromonas salmonicida* in preserved samples, the isolation techniques based on the use of immunomagnetic beads made it possible to grow the detected bacterium on non-selective media. The ability to isolate and grow specific bacteria from environmental samples heavily 'contaminated' with other bacteria is an essential prerequisite for further work with the specific bacteria. Our results show that the often disturbing influence of the background bacterial flora can be reduced substantially by use of this method.

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