Immunohistochemical identification of *Vibrio salmonicida* in stored tissues of Atlantic salmon *Salmo salar* from the first known outbreak of cold-water vibriosis ('Hitra disease')

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ABSTRACT: Immunohistochemistry based on the avidin-biotin complex method was employed to identify *Vibrio salmonicida* or its products, in formalin-fixed, paraffin-embedded tissue specimens from Atlantic salmon *Salmo salar* L. suffering from experimentally induced cold-water vibriosis. The technique was also used to establish the presence of the bacterium in stored tissue specimens collected in 1977 during the first known outbreak of the disease in Norway. Antigens of *V. salmonicida* were specifically identified in tissue specimens from heart, liver, spleen, kidney, and gut by incubation with monoclonal antibodies specific for the bacterium. These findings offer strong evidence for *V. salmonicida* as the causative agent in the first outbreak of the disease, and are consistent with other reports identifying it as the aetiologic agent of cold-water vibriosis.

INTRODUCTION

'Hitra disease', or cold-water vibriosis (CV) was first reported in 1977 from the northern part of Norway (Poppe et al. 1985). No causative agent was identified at that time, and a multifactorial aetiology was suggested, the disease being referred to as a 'haemorrhagic syndrome', and later as 'Hitra disease' (Poppe et al. 1985). In subsequent discussions on the aetiology of the disease, a nutritional deficiency was suggested, especially a lack of dietary vitamin E and selenium (Fjølstad & Heyeraas 1985, Poppe et al. 1985, 1986). Other workers have postulated that the condition is infectious, basing their views on the isolation of *Vibrio salmonicida* from diseased fish (Egidius et al. 1981, Egidius et al. 1984, Holm et al. 1985), and on infection studies in which the disease has been successfully reproduced using the bacterium (Bruno et al. 1985, Holm 1985). Recently, it has been demonstrated that vaccination against *V. salmonicida* gives very good protection against the disease (Holm & Jørgensen 1987, Lillehaug 1990). At present the term cold-water vibriosis is generally accepted as the name for the condition.

Vibrio salmonicida exhibits a species-specific antigen called VS-P1, and 2 monoclonal antibodies (MAbs) raised against the bacterium (derived from clones 2B5 and 7F3) have been reported to identify an epitope on VS-P1 unique for *V. salmonicida* (Espelid et al. 1988, Bøgwald et al. 1990).

The objectives of the present study were (1) to develop an immunohistochemical technique to identify *Vibrio salmonicida* or its products in formalin-fixed and paraffin-embedded tissue by the application of monoclonal antibodies raised against the VS-P1 antigen, and (2) to establish the presence of the same bacterium in stored tissue specimens collected during the first known outbreak of the disease.

MATERIALS AND METHODS

Controls. Organ samples from positive control fish, Atlantic salmon *Salmo salar*, were collected during an infection experiment with *Vibrio salmonicida* carried out in 1989. Atlantic salmon with an average weight of 55 to 60 g were used in the experiment. The fish were held in 1 m^3 tanks with a water temperature of 7 to
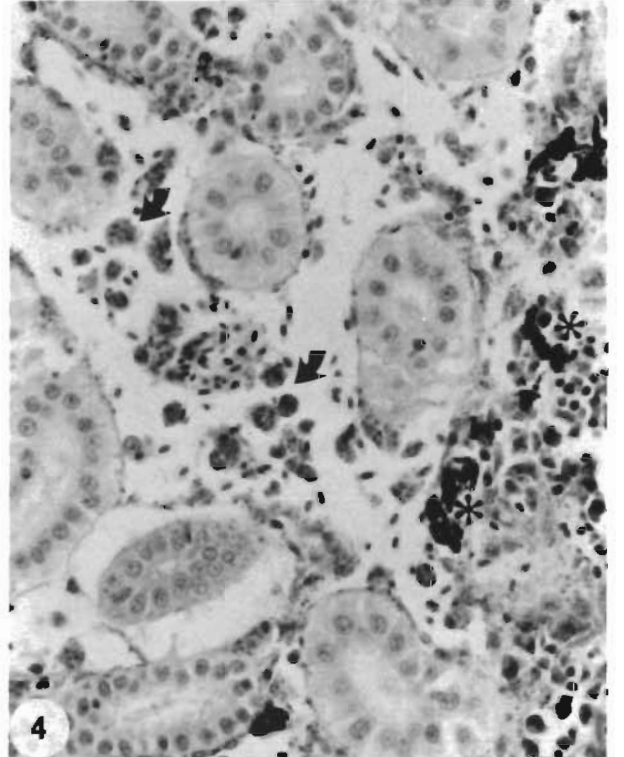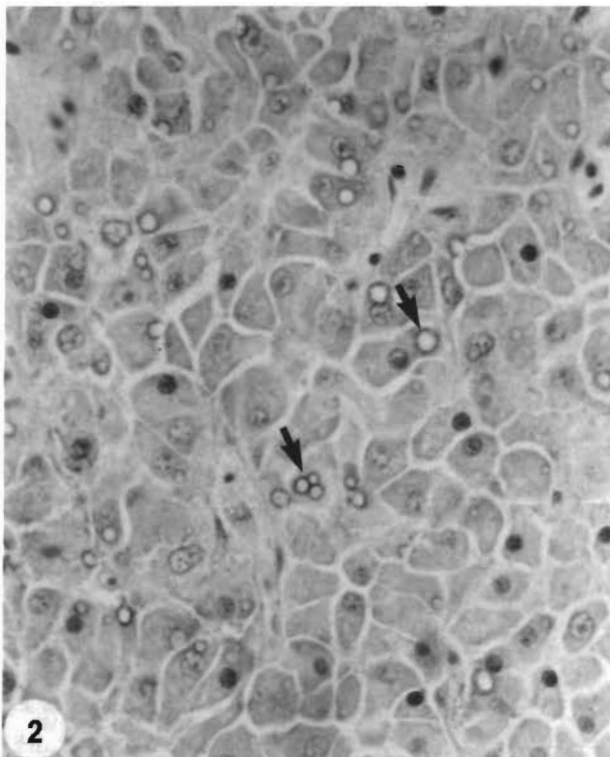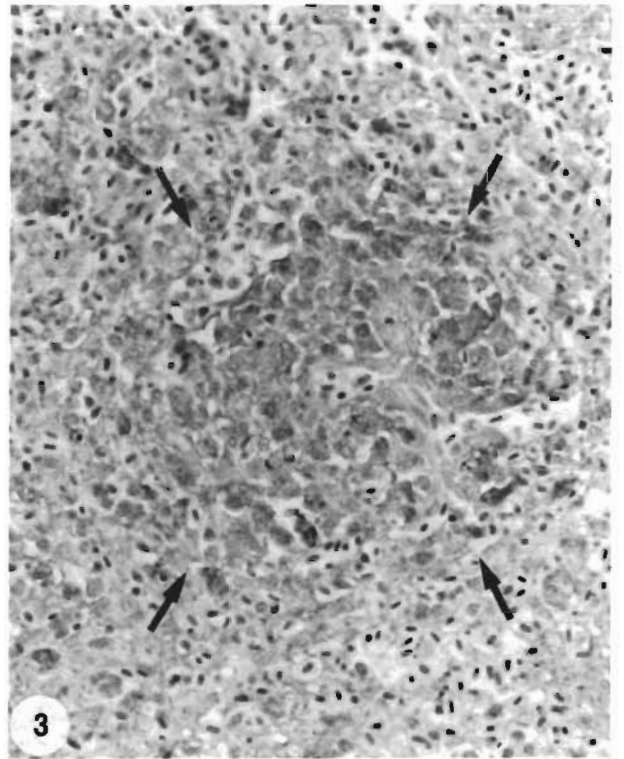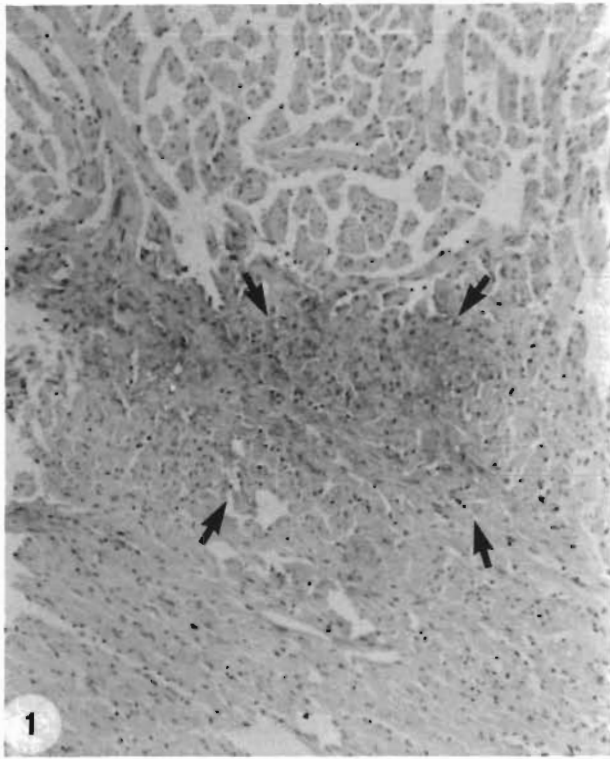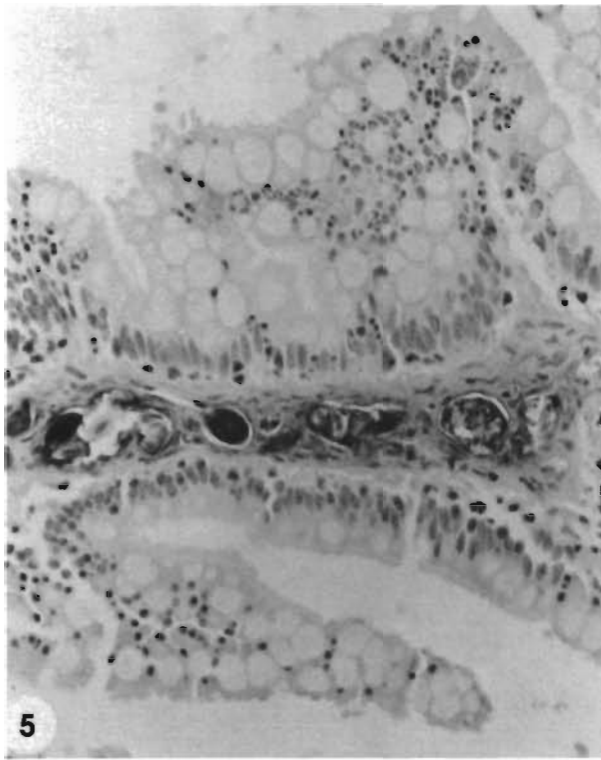
Figs. 1 to 5. *Salmo salar.* Stored tissue specimens collected during the first known outbreak of CV in 1977. All specimens counterstained with haematoxylin. Fig. 1. Heart. Positive focal reaction extracellularly between the compact and the spongeous part of the myocardium (arrows). Single bacteria could not be identified. × 100. Fig. 2. Liver. Positive reaction of hepatocyte vesicular membranes (arrows). The vesicles are probably fat vacuoles. × 400. Fig. 3. Spleen. Positive reaction intra- and extracellularly in the white pulp (arrows). × 250. Fig. 4. Kidney. Intracellular positive reaction of intertubular cells (possibly macrophages) (arrows). Melanomacrophages are also evident (*) but no reaction was observed in these cells. There is marked intercellular edema. × 400. Fig. 5. Fore-gut. An intense reaction is present intra- and perivascularly in fore-gut lamina propria. No reaction is observed in the epithelium. × 250

8 °C. Salinity was 29 to 30 ‰. *V. salmonicida* (88/09/173 of National Veterinary Institute Culture Collection) was grown on blood-agar plates (containing 2 % NaCl) for 4 d at 15 °C, harvested, and suspended in sterile 0.1 M phosphate-buffered saline (PBS). Each fish was injected intraperitoneally with 10^6 bacteria (0.1 ml PBS suspension per fish). Fish were killed on Days 5, 4, 7, and 11 after injection. Organ samples were collected from heart, liver, spleen, kidney, fore-gut, and musculature. The samples were fixed immediately in 10 % phosphate-buffered formalin, and processed routinely. Negative controls (from seawater) were collected from the same lot of fish as that used in the experiment, but prior to the experiment. Negative controls were also collected from parr (ca 30 g) held in fresh water.

Stored tissue. Tissue samples were collected from heart, liver, spleen, kidney, fore-gut, and musculature. Samples from moribund and dead fish involved in the first outbreak of the disease were fixed in 10 % phosphate-buffered formalin and stored in paraffin. For immunohistochemical studies, samples were cut at 5 to 6 μm, and placed on gelatin-coated slides.

Monoclonal antibodies. MAbs, 2B5 and 7F3, produced as previously described (Espelid et al. 1988), were used for immunohistochemistry. Both MAbs were of the IgGl, kappa isotype. Hybridoma ascites was produced by injecting 10^5 to 10^6 *Vibrio salmonicida* cells intraperitoneally into mice. Ascitic fluid was collected 7 to 21 d later (Espelid et al. 1988). The MAbs used in this study showed a specificity for VS-P1, or more precisely, the lipopolysaccharide (LPS) moiety of the VS-P1 complex (Bøgwald et al. 1990).

Heterologous MAbs of the same isotype were used as a negative control.

Bacteriological examination. Bacteriological examination was carried out on kidney samples from all of the control fish included in the experiment. Cultivation was performed on blood-agar plates with 2 % NaCl at 15 °C for at least 6 d. *Vibrio salmonicida* was identified by an ELISA technique performed as previously described (Espelid et al. 1988).

Immunohistochemistry. The immunohistochemical procedure was performed according to the avidin-biotin complex (ABC) method (Hsu et al. 1981). The sections were deparaffinized at 58 to 59 °C for 30 min, washed in 2 xylene baths, and rehydrated through a series of ethanols (100, 96, and 70 %). Endogenous peroxidase activity was blocked by incubating for 20 min with methanol (100 %) containing 3 % H_2O_2. Sections were then washed in PBS for 10 min. Following this, the sections were incubated with 25 % bovine serum albumin (BSA) in PBS for 20 min. They were then blotted dry and reacted with primary antibody (2B5 and 7F3 diluted 1/5000) in PBS containing 12.5 % BSA as diluent. After 10 min washing in PBS, the secondary antibody [antimouse immunoglobulins (Dako) conjugated to biotin] was applied to the specimens at a dilution of 1/300, and incubated for 30 min. After 10 min washing in PBS, the avidin-biotin-peroxidase complex (diluted 1/300) was applied and incubated for ca 30 to 60 min. The sections were then washed in PBS for 10 min, and incubated for 10 min with 0.03 % H_2O_2 and 0.06 % 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M imidazole buffer, pH = 7.4. After ca 1 min washing in tap water, the section were counterstained with Harris haematoxylin (H) for 2 min, washed in tap water, and dehydrated through an ethanol series (70, 96, and 100 %), and 2 xylene baths. Coverslips were mounted with Eukitt.

RESULTS

Vibrio salmonicida in the positive controls was iden-
tified by bacteriological examination and an ELISA-
technique. It was also identified in situ in tissue sam-
ples collected from the infection experiment in 1989,
using clone 2B5 and 7F3. *V. salmonicida*, or its pro-
ducts, were also identified in stored paraffin-embedded
tissue specimens from 1977. The results obtained for
tissues from the 2 different sources were comparable.

In heart tissue a positive reaction was visualized as a
brown colour, mostly extracellularly, between the com-
pact and the spongeous part of the myocardium
(Fig. 1). The reaction occurred in both a diffuse and a
focal form. It was not possible to identify single bac-
teria. In the liver, distinct coloration of the hepatocyte
intracellular vesicle membranes was observed (Fig. 2).
A positive reaction was present within phagocytic cells
of the splenic white pulp and the intertubular cells of
the kidney. In addition, positive extracellular reactions
were present in both of these organs (Figs. 3 & 4). The
positively coloured cells in both organs, were judged to
be phagocytic cells (macrophages).

A focal to diffuse positive reaction was present within
and around vessels of the foregut lamina propria, as
well as in the lamina propria per se. (Fig. 5).

No reaction was observed in tissue specimens from
negative control fish held in sea or fresh water. A
heterologous MAb of the same isotype was also applied
on positive and negative control tissues and on stored
tissue specimens from the 1977 outbreak. All gave
negative results.

DISCUSSION

This study showed that *Vibrio salmonicida*, or its
products, could be identified by immunohistochemistry
in formalin-fixed, paraffin-embedded tissue specimens
from Atlantic salmon suffering from experimentally
induced cold-water vibriosis, and also in stored tissue
specimens collected during the first outbreak of the
disease in Norway in 1977. These findings are consist-
ent with other reports concerning the aetiology of the
1977 outbreak of the disease.

The nutritional aspects of 'Hitra disease' or CV have
been thoroughly discussed by various authors (Fjølstad
& Heyeraas 1985, Poppe et al. 1986), deficiencies in
dietary vitamin E and selenium being hypothesized as
the cause of the disease. This possibility had been
suggested because of the similarity between the lesions
seen in mammals with such deficiencies (Hulland 1985)
and those appearing in Atlantic salmon with 'Hitra
disease' (Poppe et al. 1986). *Vibrio salmonicida*
recovered from diseased fish was considered as a sec-

ondary invader (Poppe et al. 1986). However, a study of
plasmid profiles of *Vibrio*-like isolates from Atlantic
salmon suffering from 'Hitra disease' has shown that
these bacteria exhibit an extensive homology in their
content of cryptic plasmids (Sørum et al. 1988). These
findings do not support the hypothesis that *V. sal-
monicida* is an occasional secondary invader. Further-
more, the disease can be reproduced under experimen-
tal conditions (Bruno et al. 1985), and vaccination
against *V salmonicida* provides a very high level of
protection against the disease (Holm et al. 1987, Lil-
lehaug 1989). There thus seems to be good evidence
that *V. salmonicida* is the causative agent of cold-water
vibriosis.

Over the last 2 decades, immunohistochemistry
utilizing enzymes to produce coloured reactants has
supplied important investigative and diagnostic his-
tological tools (Rickert & Maliniak 1989). The tools
permit the in situ identification of tissue constituents by
means of antigen-antibody reactions, and there seems
to be no limit to the range of substance that may be
detected in this way (Noorden 1986). In this study, it
was not possible to identify single bacteria in the tissue
specimens, and the specific coloration in heart speci-
mens was focal or diffuse, and was located extracellu-
larly between the compact and the spongeous tissues.
Similar diffuse extracellular staining was seen in organ
specimens from the spleen and kidney. *Vibrio sal-
monicida* produces an LPS-containing surface layer
complex, VS-P1 (Bøgwald et al. 1990), and VS-P1 is
released into the surroundings during growth in the
host (Espelid et al. 1988). Furthermore, VS-P1 can be
detected in high concentrations in sera from Atlantic
salmon suffering from cold-water vibriosis (Hjelmeland
et al. 1988). The diffuse staining pattern demonstrated
in specimens from the heart, spleen, and kidney seems
to reflect the above mentioned observations.

Negative controls including fish held in either sea
water or in fresh water gave no reactions. Similar
negative reactions were obtained when a heterologous
MAb was applied. The tissue reactions were easy to
read and no interpretative difficulties were experi-
enced. The specific identification of the VS-P1 antigen
in the tissues by clone 2B5 and 7F3 provides strong
additional evidence that *Vibrio salmonicida* was the
causative agent of the first outbreak of the disease.
Furthermore, based on this investigation one might
state that immunohistochemical techniques can be
used by anyone on stored, fixed material who wants to
study the history of a pathogen in a particular location.
The only requirement is that specific polyclonal or
monoclonal antibodies are available.

In conclusion, the present study showed that anti-
gens specific for *Vibrio salmonicida* could be identified
in formalin-fixed, paraffin-embedded tissue specimens

from Atlantic salmon suffering from cold-water vibriosis, and also in tissue specimens collected during the first outbreak of the disease in 1977. The findings offer additional evidence for the bacterium as the causative agent of 'Hitra disease'.

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