VIBRIOSIS IN FISH:

a clinical, pathological and bacteriological study of the disease in Norwegian fishfarms

by

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ABSTRACT OF THESIS ON

VIBRIO ANGUILLARUM IN

NORWEGIAN FISH FARMS

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ABSTRACT

The work divides naturally into three sections, the first section dealing with the literature apposite to the study.

The literature review starts with the historical discovery of the disease, followed by description of morphology, metabolism, antibiotic sensitivity, serology and toxin production of Vibrio anguillarum. Literature on the normal and experimental pathogenesis of the disease is cited with descriptions of attempts to type Vibrio anguillarum into biotype groups. This section also includes a description of the host range and economic significance of the disease.

The second section of the study gives a short description of Norwegian fish farms in general, followed by a description of the methods of collection of pathological material from outbreaks of vibriosis in Norwegian fish farms.

This section also gives a description of the isolation procedures, bacteriological tests and histological techniques used in the study, followed by a description of

the pathology and treatment of the disease.

The fourth chapter of the second section gives the results of the bacteriological examinations carried out on 163 strains of Vibrio anguillarum obtained from outbreaks of vibriosis in Norway. This chapter includes a description of morphology, viability, antibiotic sensitivity, biochemical properties and production of specific proteinases identified by means of the caseinate precipitation inhibition test (CPI-test).

The third section of the work consists of a computer analysis study of the bacteriological data obtained in the second section of the work.

A review of the literature concerning numerical taxonomy is given, followed by a description of different methods for computation of the material. This includes a description of principal components analysis (PCA) and of numerical taxonomy by means of Single Link Listing (SLL).

Finally a description of the results obtained with these two methods is given. The PCA method gave two distinct groupings of the strains and allowed all strains to be included in the two groups. There seemed to be an even geographical distribution of both groups, and none of the groups could account for specific pathological findings. Two strains of Vibrio anguillarum obtained from the American Type Culture Collection fell into one group each and Vibrio metchnikovi (a human vibrion strain which

had been included in the study), was demarcated well outside group I. There seemed to be no specific group distribution of fish species, except in the case of isolates from saithe (Gadus virens) which almost all fell into one computer defined group.

Examination of the material by means of SLL gave five acceptable groups at 88.7%, leaving 23 strains ungrouped at this level.

Comparative study of the groups defined by PCA and by SLL showed that the SLL defined group I and II fitted into PCA group II, while SLL groups III, IV and V corresponded to PCA group I. It is therefore concluded that the PCA method provided the most suitable way to classify the material and that the taxonomic determinants allowing best definition of strains in the two PCA groups were citrate utilization together with arabinose, lactose, cellobiose and trehalose fermentation.

The two groups defined by the computer for Norwegian isolates of Vibrio anguillarum thus did not correspond to the groups proposed by Nybelin (1935) and Smith (1961) who based their grouping on indole production and mannitol and sucrose fermentation.

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VIBRIOSIS IN FISH, A CLINICAL, PATHOLOGICAL AND BACTERIOLOGICAL STUDY OF THE DISEASE IN NORWEGIAN FISH FARMS

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SECTION A

1. INTRODUCTION

Vibrio anguillarum is one of the most important pathogenic microorganisms to affect fish. It is the causative agent of vibrio diseases (Vibriosis).

It is found throughout the world and it is an important source of economic loss to both the marine fish farming industry and to the fishing industry.

The present study was instituted with five main objectives.

1.) To define the clinical and pathological features of vibrio disease in Norwegian fish farms.

2.) To study in detail strains of *Vibrio anguillarum* isolated from rainbow trout (*Salmo gairdneri*) and Atlantic salmon (*Salmo salar*) and to compare them with strains isolated from free living fish species.

3.) To utilize the information so obtained for estimation of the overall similarity of individual strains, and disposition of strains in a numerical classification according to their respective similarities.

4.) To see if any correlation obtained between such a classification and the species of host or the severity of the disease condition from which the strain originated.

5.) To assess the potential value of any of the procedures used with reference to rapid diagnosis of infections by *Vibrio anguillarum*.

The work may be conveniently divided into four sections, namely:

A) A review of the current literature on Vibrio disease in fish.

B) A broad study of Vibrio disease in Norwegian fish farms including a bacteriological survey of 163 strains of Vibrio anguillarum employing as many tests and measurements as was practically possible.

C) Codification of the data so obtained, for computer calculation using principal components analysis (PCA) and numerical taxonomy of the strains with reference to each other, and subsequent analysis of the information produced.

D) Conclusions.

2. REVIEW OF THE LITERATURE

2.1 Historical discovery

The microorganism Vibrio anguillarum and its association with disease of fishes have been recognized for nearly a hundred years.

In 1892-93 Canestrini isolated a Vibrio cholerae-like organism from eels (Anguilla anguilla) dying of an acute infection with a haemorrhagic syndrome along the Italian coast. The organism was pathogenic for fish and frogs, but not for homeothermic animals. However, it is possible that earlier records of disease in marine teleosts might have been due to vibrionic bacteria. Earlier records of such epizootics (Ozenam, 1823; Forel and Du Plessis, 1868) did not include full bacterial examinations so the cause could not be stated with certainty, but according to Hofer (1904) "red disease" of eels was described by Gian Franceses Bonaveri as early as 1718 from the Comachio lagoons of the East coast of Italy. Hofer also listed references to very similar disease in 1825, 1850, 1864, 1867, 1884, 1885 and 1889.

Canestrini named the bacteria associated with his disease Bacillus anguillarum, but his description was unfortunately so poor that it is impossible today to identify the bacteria with certainty. Therefore Bergman (1909), following his work in connection with an outbreak

of "red disease" in eels from the southern coast of Sweden, has been credited with the discovery and description of Vibrio anguillarum.

Evidence soon emerged that the bacterium could cause disease among species of fish other than eels, and in 1911 Bergman reported that similar bacteria caused a pathological condition in codlings (Gadus morhua) which he described as "keratomalaci". This disease condition started as a keratitis and progressed to total destruction of the eye tissues. Bergman also reported that the same bacterium caused an infection in the gingiva of the Northern pike (Esox lucius).

Aaser (1923) described a disease of Northern pike in Norway, from which he isolated a Vibrio and David (1927) recorded a disease of carp in which vibrio-like organisms were involved. However, these Vibrios appear from their description to differ from Vibrio anguillarum.

Infections due to Vibrio anguillarum have been described as the cause of disease in salmonids from many areas of the world.

In 1951 Earp described a Vibrio infection in salmon fingerlings, reared in saltwater. The disease was characterized by erythema of the fins and the sides of the fish, necrotic areas in the musculature, intestinal inflammation and a generalized septicaemia. Rucker and Ordal (1952) described a similar disease in rainbow trout.

Hoshina (1956) reported on an epidemic disease affecting rainbow trout in various districts in Japan. The investigations revealed a bacterium similar to Vibrio piscium as described by David (loc.cit.). The same author (1957) gave a more complete description of the epidemiology and the pathology of the disease and proposed that the bacteria should be named Vibrio piscium var. japonicus.

Although virtually all outbreaks of vibriosis have been recorded under marine conditions, Rucker et al. (1954) reported an incidence of vibriosis in a freshwater hatchery. This was the first time Vibrio anguillarum was described as causing disease among fish held in freshwater, but later Ross et al. (1968) also reported an outbreak of the disease in freshwater, this time from diseased juvenile rainbow trout reared at Willow Beach National Fish Hatchery, a freshwater hatchery in Arizona which uses drainage water from Colorado River.

Cisar and Fryer (1969) reported on Vibrio anguillarum infection in Pacific salmon (Oncorhynchus spp.), and Evelyn (1971) described the first record of the disease in cultured Pacific salmon from Canada.

In Europe several reports on the disease in salmonids have been reported (Holt 1970, Hastein and Holt 1972, McCarthy, 1974).

The first record of vibriosis in tropical freshwater

fish was reported by Hacking and Budd (1971). Vibrio anguillarum was identified as the causative agent and it was pathogenic for selected species of other freshwater fishes. The source of infection was not determined.

2.2 Morphology

The morphology of Vibrio anguillarum has been the subject of various studies by a number of workers. To the early investigators, the morphology was especially interesting because it was one of the few available taxonomic determinants and its characteristic nature was the main criterion for the eventual inclusion of the group into the Vibrio family.

Bergman (loc.cit.) was the first to suggest that the causative agent of "red pest" should be assigned to the vibrios and he named the bacteria Vibrio anguillarum because of its regular association with eels.

He described the bacterium as a short comma-shaped rod which was motile by means of a polar flagellum.

The length of the bacterium ranged from 1 - 3μ with an average of 1.5μ. Its width ranged from 0.1 - 0.5μ and was usually one quarter of the length.

He also described composite forms where two bacteria became attached, resulting in "S" or "3" forms, or less commonly, a strepto-vibrion of three or four bacteria linked together.

2.3 Metabolism

Determination of the biochemical characteristics of Vibrio anguillarum has engaged the attention of many workers. It is generally agreed that the organism grows easily in most bacterial substrates provided that they have a salt content exceeding 0.07%.

The possession of enzymes active against various carbohydrate substances is one of the classical criteria applicable to the differentiation of microorganisms.

Hendrie et al. (1971) found that the carbohydrate metabolism of strains of Vibrio anguillarum isolated from fish was fermentative and that acid but no gas was produced from glucose, fructose, mannose, sucrose, maltose, trehalose, mannitol, sorbitol, dextrin, glycogen and starch, while neither acid nor gas was produced from lactose, arabinose, xylose, ribose, sorbose, raffinose or dulcitol. The same authors found that acid may or may not be produced in cellobiose, rhamnose, glycerol, inositol and salicin.

The results of Holt's work (loc.cit.) are in full agreement with this, but the study by Håstein and Holt (loc.cit.) on twenty strains isolated mainly from salmonids showed minor differences. Biochemical investigations by Cisar and Fryer (loc.cit.), Evelyn (loc.cit.) and Hacking and Budd (loc.cit.) all yielded basically similar results although with some minor exceptions.

It is generally accepted that Vibrio anguillarum

causes liquefaction of gelatin and this was originally observed by Bergman (loc.cit.). Alteration of milk has been reported by several authors. Holt (loc.cit.) stresses that the organism produces a clot followed by proteolysis after forty-eight hours. Later work (Håstein, unpublished) showed that the coagulation was of enzymic nature due to proteinases.

Most of the workers cited also showed that Vibrio anguillarum is able to reduce nitrate to nitrite, but Holt (loc.cit.) remarked that the reaction was rather slow.

No authors have so far reported the production of hydrogen sulphide.

Håstein and Holt (loc.cit.) subjected twenty strains of Vibrio anguillarum to a number of biochemical tests which included citrate utilization as well as production of indole and acetyl-methyl-carbinol. All strains were shown to produce acetyl-methyl-carbinol while utilization of citrate and production of indole varied. This is largely in agreement with the report of Hendrie et al. (loc.cit.) although the latter workers supported the contention of Evelyn (loc.cit.) that citrate could not be used as the sole source of carbon. Hacking and Budd (loc.cit.) however were in agreement with Håstein and Holt (loc.cit.), that it was possible for the organism to use citrate as the sole carbon source.

2.4 Antibiotic sensitivity

Several authors have reported on antibiotic and chemo-therapeutic activity.

Hoshina and Chiba (1957) investigated the bacteriostatic activity of malachite green to vibrio disease, since this is a commonly available chemical on fish farms. They found that a concentration of 0.002 mg. malachite green in 10 ml. broth was completely ineffective in inhibiting growth of the organism but that 0.01 mg. prevented its replication.

Muroga and Egusa (1967) found, using sensitivity discs, that all their isolates of Vibrio anguillarum were highly sensitive to chloramphenicol, tetracycline, colistine and novobiocin, but were resistant to penicillin. These results were confirmed by Evelyn (loc.cit.), Holt (loc.cit.) and Håstein and Holt (loc.cit.).

Furanace (6 - hydroxymethyl - 2 [2 - (5 - nitro - 2 - furyl) vinyl] pyridine), a nitrofuran derivative*, has been reported to be most effective against vibriosis. Pearse et al. (1974) reported that seventeen strains of pathogenic vibrios isolated from marine fish and salmonids were highly sensitive to Furanace. Håstein (1974) described a few preliminary trials with Furanace against vibriosis in salmonids, but it was not found to be as effective as oxytetracycline.

Håstein (unpublished data) also found that Borgal,

* Dainippon Pharmaceutical Co.

a combination of trimethoprim and sulphadoxin was effective *in vitro* against the organism, and that Trafigal, a combination of trimethoprim and sulphadimethoxine was effective *in vivo*, and Withnell (Roberts, pers. comm., 1974) has found Tribrissen ** a combination of Trimethoprim and sulphadiazine also to be highly effective.

2.5 Serology

Serological examination has been carried out by several workers. Bergman (*loc.cit.*) reported on agglutination as a diagnostic method for *Vibrio anguillarum*. He prepared antisera in rabbits against two of his five isolates. From one of the antisera produced, only the homologous strain of the organism was agglutinated to high titre, but with the other antiserum both the homologous and one of the other strains were agglutinated. He also demonstrated agglutination with the latter serum with a strain isolated from a gingival lesion in a pike at dilutions of 1:10,000 and 1:8,000. He observed however that *Vibrio anguillarum* failed to agglutinate with a *Vibrio comma* antiserum from a rabbit (*Oryctolagus spp.*). Results obtained by Nybelin (1935) differed from those of Bergman in that there was greater variability in the degree of agglutination of his strains to antisera prepared from strains of *Vibrio anguillarum* isolated from diseased eels. Some of his strains showed agglutination to titre as high as 1:20,480, while others only agglutinated at a titre of 1:40.

** Burroughs Welcome Ltd.

Muroga and Egusa (1969) carried out vaccination experiments with antigens prepared from their Vibrio anguillarum strain PB-15 injected intramuscularly into eels (Anguilla japonica). The fish were starved throughout the course of the experiment which might be expected to affect the results. However, their work showed that there was no antibody production when the eels were held at water temperature of 11°C, but some antibody production at 15°C. This is in agreement with Nybelin's report (loc.cit.)., showing that European eels, which had been injected with heatkilled Vibrio anguillarum several times, did not produce agglutinating antibodies at 7 - 9.5°C, but did produce them at 16 - 19°C.

Hayashi et al. (1964) demonstrated natural immunity against vibriosis in sera of rainbow trout after a prevalence of the disease during the summer. The acquired immunity did not persist more than a short period. The same authors were also able to produce agglutinins after two weeks with daily administration of oral vaccines, but the highest titre obtained was 1:128 at the end of a four week period. With a non pathogenic Vibrio, they obtained a titre of 1:4096 by oral administration.

Cisar and Fryer (loc.cit.) reported that their three isolates LS-68 were agglutinated by antiserum which had been prepared against a Vibrio sp. obtained from a Pacific salmon. They did not observe any agglutination with anti-sera against A. salmonicida, A. punctata and an isolate of Vibrio spp. from Pacific Northwest herring (Clupea pallasi).

Hacking and Budd (loc.cit.) produced antiserum in rabbits with their three isolates of Vibrio anguillarum from tropical fish in a freshwater aquarium. Their strains did not agglutinate the ATCC 14181, Willow Beach, and NCMB 6 strains of Vibrio anguillarum, but cultures isolated from exposed and injected fish and from adult and young guinea pigs exposed to the organism were all agglutinated by this antiserum.

Kiehn and Pacha (1969) have suggested the existence of three serotypes of Vibrio anguillarum based on cultural and serological characters. Serotype 1 in their scheme comprised isolates from Pacific Northwest salmonids, serotype 2 European isolates, and serotype 3 isolates from Pacific Northwest herring.

The authors also indicated a pattern of relationships based on their investigations on deoxyribonucleic acid homology and base composition (Kiehn and Pacha, 1969).

Håstein (unpublished), using the casein precipitation test (CPT), showed a relationship between Vibrio anguillarum and Vibrio cholerae, while no relationship was shown to exist between Pseudomonas fluorescens or Aeromonas salmonicida.

In his study on the noncellular protective mechanism in rainbow trout, Harrell (1973) produced agglutinating antisera (titre 250,000) in steelhead trout by injection of heat killed Vibrio anguillarum in Freund's Complete Adjuvant and showed that specific antibody against Vibrio

could also be detected in fish body mucus. In the same study a specific serum antibody was demostrated which, together with a multifactorial protein substance, complement, appeared to play an important role in the protection against experimentally induced vibriosis in steelhead trout.

Conroy and Withnell (1974) investigated the use of a slide agglutination test in presumptive identification of strains of Vibrio anguillarum. They prepared monovalent and polyvalent antisera in sheep. The monovalent antisera were prepared by injection of oil emulsion vaccines prepared with sonication using an Arlacel and Tween 80 method at 60% sample inclusion rate. (Withnell, unpublished data). The initial viable cell count of the bacterial suspension ranged from 1 - 3 x 10^{10}/ml before sonication.

Ten ml. of each vaccine was injected by the intramuscular route (IM) three times at weekly intervals. Preparation of polyvalent serum for Vibrio anguillarum took place six weeks after treatment with vaccine by means of two intra-venous injections of a mixture of eleven Vibrio strains at an interval of seven days, each mixture containing 5 x 10^{9} viable cells/ml.

The slide and tube agglutination titres of the prepared antiserum ranged from 1:128 - 1:1024 and the positive reactions obtained included strains of European, Japanese and North American origin.

The authors concluded that the slide agglutination

technique using a polyvalent antiserum was a valuable tool for diagnosis of vibriosis in fish.

2.6 Toxin production

The haemolytic activity of Vibrio anguillarum has been studied in some detail by McArdle (1973). He found that a haemolysin was produced by the organism and attempted to purify it.

He tested:

1) Ether soluble extract
2) Crude NH_4SO_4 precipitate
3) Acetic acid supernatant
4) Acetic acid precipitate

and found that the only fractions showing significant activity were crude ammonium sulphate precipitate and the further purified acetic acid precipitate. On the basis of these investigations he suggested that the haemolysin was probably a protein. He was also able to show that there was a species difference in resistance to the haemolysin in that red blood cells of turbot (Rhombus maximus) were significantly more resistant than those of plaice (Pleuronectes platessa) to in vitro lysis. Experiments carried out to determine the heat stability of the toxin showed that activity was lost after exposure to 50 - 60°C for ten minutes. These findings are similar to those of Miwatana et al. (1972) who worked with haemolysin of Vibrio parahemolyticus, a closely related organism.

2.7 Normal disease pathology

The pathology of vibriosis has been described by several authors. Schäperclaus (1934) described the pathology of the disease in eels. In acute disease there was mortality without external pathological changes, although the eels were usually sluggish, showing spasmodic movements immediately pre mortem. In more chronically affected fish, localized red patches occurred on the lateral and ventral surfaces of the body, sometimes occurring as a generalized erythema of the fins or around the anus. Also characteristic red lesions in the musculature around the heart region were seen, especially in silver eels, i.e. eels which are adapted for the marine environment.

In more protracted cases, ulcers and swollen skin lesions were seen, often covered by a thick mucoid membrane. Internally the fish usually showed haemorrhages in the liver and acute inflammatory lesions in the gut. In the skin, haemorrhages were seen in the dermis. Microscopic sections showed that the main lesions were in the hypodermis where the vascular supply to the dermis was destroyed, with considerable haemorrhage especially around the scale beds.

Similar changes were also described by André *et al.* (1972), but in addition they described depletion and necrosis with destruction of the melanin macrophage tissue and considerable swelling and vacuolation of the

hepatic parenchymal cells. Small foci of Gram-negative bacteria were seen within the intestinal wall, but no evidence of a cellular response was found. They found however that the most characteristic lesions of the disease in eel were those involving the skin, which in early lesions showed intense oedema of the stratum compactum and stratum spongiosum. These layers were extremely congested but no haemorrhages or cellular response could be seen. In older lesions they found that the epidermis above the oedematous areas had sloughed to produce ulcers, and Gram negative bacteria and monocytes could be observed in the remaining areas of the spongiosum. The lesion showed a strong resemblance to the Erythema multiforme lesion as found in man.

Levin et al. (1972) reported on the pathology of Vibrio anguillarum in winter flounder (Pseudopleuronectes americanus). They found that the gross lesions of the disease were confined to the skin and musculature. Lesions in this fish species included petechia and ecchymoses in the acute phases, and ulceration in the more chronic stages of the disease. The ulcers often extended into the skeletal muscle, resulting in deep necrotic ulcers revealing the glistening white fibrous tissue between mytomes. When severe ulceration of the abdominal wall occurred, evisceration could take place while the fish was still alive. The dermal lesions were seen both on the pigmented and the unpigmented side of the fish. Necrosis was also observed in the fins, especially the anal and caudal fins. The histological description of

the pathological changes was similar to that described by André et al. (loc.cit.) in eels.

The gross pathology of the disease has been described in juvenile turbot and brill (Rhombus laevis) (Anderson and Conroy, 1970).

A very interesting and different set of pathological observations were obtained by Anderson, Hastein, Ferguson and Roberts (1974, unpublished) on acute lethal infections by Vibrio anguillarum in young wild (0^+) turbot subjected to transportation stress. These died very quickly within three or four hours of becoming depressed and dark coloured. As they died, they developed severe exophthalmos with retrobulbar oedema and abdominal swelling. At post mortem, Vibrio anguillarum was isolated in pure culture from blood, oedema fluid and most organs, but histologically the only site severely affected was the heart, where the atrial macrophages and cardiac muscle fibres were very necrotic. The putative pathogenesis was septicaemic infection with phagocytosis of bacteria by the heart which was then severely damaged by bacterial toxins.

In cod there seem to be two forms of Vibrio disease. One form that especially affects the eyes was described by Bergman (loc.cit.) as "Keratomalaci". In the early stages of this form, the cornea becomes opaque and gradually destroyed. Usually both eyes are affected. Inside the eye, the tissue appears to be greenish-red, marbled and more or less liquefied.

The ulcerative form of vibriosis in cod was described by Bagge and Bagge (1956) and their description of the pathological changes was very similar to those cited previously for eel and flounder.

Traxler and Li (1972) described an abscess associated with Vibrio anguillarum resulting in a purulent exudate in the nostrils of a cod. Histological examinations showed extensive destruction of the muscular and connective tissue and leucocytic infiltration.

The pathological findings produced by Vibrio anguillarum in marine salmonids bear some similarity to those of Aeromonas salmonicida, the causative agent of furunculosis in fresh water salmonids, and the disease has therefore often been referred to as "salt water furunculosis" (Rucker 1963).

The gross pathology of vibriosis in salmonids has been described by several authors [Hoshina 1956, 1957; Ross et al. 1968, Cisar and Fryer (loc. cit.), Fryer, Nelson and Garrison 1972, Evelyn (loc.cit.), Holt (loc. cit.)].

It is characterized by redness at the base of the fins, often associated with haemorrhages in the tissue between the finrays, ending up with destruction and necrosis of the fins.

External signs also include exophthalmos and abdominal distension [(Hoshina (loc.cit.), Ross et al.

(loc.cit.)].

Hoshina (loc.cit.), Ross et al. (loc.cit.) and Holt (loc.cit.) described ulcerative lesions and large unbroken vesicles in the musculature containing haemorrhagic necrotic material similar to that found in furunculosis. At autopsy, the liver, intestine and peritoneum were usually congested and the enlarged spleen was often in a liquefied state [Hoshina (loc.cit.)]. Ross et al. (loc.cit.) also reported that the kidney had a necrotic appearance. Ulcers in the skin and internal haemorrhages in the coelomic cavity and intestinal tract, together with a congested and friable spleen, were reported by McCarthy et al. (loc.cit.). They also found histopathological changes in rainbow trout which they described as characteristic for subacute vibriosis. Sections through the lesions described macroscopically, revealed marked muscle necrosis accompanied by inter-fibrillar haemorrhages and congestion of inter-fibrillar vessels; lack of leucocytic response was also noted and large numbers of bacteria could be seen.

2.8 Experimental pathogenicity

The pathogenicity of Vibrio anguillarum has been repeatedly demonstrated by various investigators.

Canestrini (loc.cit.) reported that his isolates were pathogenic to eels, sticklebacks (Gasterosteus aculeatus), carps (Carassius auratus), newts (Triturus sp.)

and frogs (Rana sp.), but not to warmblooded animals such as rabbits (Oryctolagus spp.), Guinea pigs (Cavia spp.), or mice (Mus musculus).

Muroga and Egusa (1969) infected ayu (Plecoglossus altivelis) and eels by intramuscular injection with the organism and showed that a dose of 1 mg. bacteria per 100 g. body weight of fish killed samples of ayu in thirty hours and eels within two days, when the temperature was 20-25°C.

Inoculated ayu showed haemorrhagic swelling at the site of injection which eventually became necrotic. Histological changes included alterations in the intestine, spleen and kidney.

In eels they found haemorrhagic lesions similar to those occurring in natural outbreaks of "red disease" but did not find definite evidence of internal pathological changes.

Evelyn (loc.cit.) reported that all fish experimentally infected died within 48 hours with symptoms typical of the natural disease except for a general absence of diffuse haemorrhages on the body surface.

Holt (loc.cit.) reported that experimental fish died within 18-24 hours when injected intramuscularly or intraperitoneally. The necropsies showed enlarged spleen, and haemorrhages in the dermis and myotomal muscle at the site of the injection.

Similar observations were also made by Håstein and Holt (loc.cit.), who also stated that when the organism was added to the water, the fish succumbed to the infection

within 10-27 days at a water temperature of 10°C.

Harell (loc.cit.) infected juvenile steelhead trout held at 17°C in a closed system with 0.1 ml. of a 1:1,000 solution of Vibrio anguillarum, containing approximately 2.5×10^5 bacteria. The fish died after forty eight hours, exhibiting haemorrhages both externally and internally. Mice, infected with the same culture of Vibrio anguillarum showed no pathological effects during two weeks of observation.

McArdle (loc.cit.) reported in his experimental study on vibriosis in turbot that the pathological changes were confined almost entirely to the haematopoietic tissue and circulating blood. These changes included generalized necrosis in the interstitial haematopoietic tissue and depletion of the haematopoietic tissue in the kidney with compensatory haematopoeisis in some areas. This was characterized by the presence of primitive stem cells and many mitotic figures.

In some places tubular destruction was observed. In the spleen there was marked necrosis and reduction of the white pulp and many of the blood vessels were lacking the collar of white pulp that normally surrounds them.

Externally he could observe no changes, but histological sections revealed loss of epithelium and large numbers of bacteria in the hypodermis and underlying muscle, the latter also showing haemorrhages, necrosis and cellular debris.

2.9 Classification

Vibrio anguillarum has never formally been recognized in any of the editions of Bergey's Manual of Determinative Bacteriology, the standard work on bacterial taxonomy, and uncertainty still exists as to the criteria defining the organism. Ross *et al*. (*loc.cit*.), however, recommended that *Vibrio anguillarum* should be included in the Eighth Edition of Bergey's Manual, which was also stated by Hendrie *et al*. (*loc.cit*.).

The generic name, *Vibrio*, is derived from the Latin word *vibrare* and the specific designation *anguillarum* is descended from the Latin name for eels, because the first isolation and description of the bacteria was made from eels (Bergman, *loc.cit*.).

When the first attempts at classification of *Vibrio anguillarum* were made, Nybelin (*loc.cit*.) based his classification on morphological and biochemical criteria. He divided *Vibrio anguillarum* into two biotypes which he called A and B. Type A produced acid without gas from saccharose and mannitol and was indole positive. Type B failed to produce acid or gas from saccharose and mannitol and was indole negative. In addition to these two biotypes mentioned by Nybelin, Smith (1961) proposed a third type, C, which produced acid, but not gas, from saccharose and mannitol and was indole negative.

A summary of the classification by Nybelin and Smith can be seen in Table 1.

Table 1

	A	B	C
Sucrose	+	-	+
Mannitol	+	-	+
Indole	+	-	-

Several authors placed strains of Vibrio anguillarum isolated from diseased fish within these three groups [Holt, (loc.cit.); Cisar and Fryer (loc.cit.); Muroga and Egusa (loc.cit)]. Håstein and Holt (loc.cit.) however, showed that their isolates required nine different biochemical groups, although characterization of the groups depended only on one or two minor criteria.

According to Conroy (cited by Collins, 1969) the material from Håstein and Holt (loc.cit.) could be categorized into four groups i.e. Vibrio anguillarum A, B, C and Vibrio ichthyodermis.

Hendrie et al. (loc.cit.) suggested that the genus Vibrio anguillarum should also include Vibrio piscium [David (loc.cit.)], Achromobacter ichthyodermis [(Wells and Zobell 1934)], Vibrio piscium var. japonicus [Hoshina (loc.cit.)] and Vibrio ichthyodermis (Shewan et al., 1960).

Based on serology and cultural characteristics, Kiehn and Pacha (1969) suggested the existence of three serotypes: serotype 1 comprised isolates from Pacific Northwest salmonids; serotype 2 comprised European isolates

and serotype 3 contained isolates from Pacific Northwest herring. DNA hybridization experiments carried out by the same workers confirmed and emphasized their contention that the marine vibrios could be divided into three groups.

Evelyn (loc.cit.) suggested an archetype for Vibrio anguillarum, but it remains to be seen whether the features he suggested for Vibrio anguillarum will prove stable and distinctive enough to prevent possible confusion with other marine vibrios.

2.10 Host range and economic significance of Vibrio anguillarum

During the years since it was first isolated, Vibrio anguillarum has been obtained from many morbid conditions of wild marine fish and reared fish throughout the world [e.g. (Hoshina (loc.cit.), Wolter 1960, Smith (loc.cit.), Lagard and Chakroun 1965, Ross et al. (loc. cit.), Cisar and Fryer (loc.cit.), Holt (loc.cit.), Evelyn (loc.cit.), Hastein and Holt (loc.cit.)], and the disease is an important source of economic loss in fish farms.

In this respect the situation seems very similar to that of furunculosis (McCraw, 1952) and vibriosis has been referred to as marine furunculosis (Rucker, (loc.cit.).

Arkwright (1912) described latent carriers as playing a major role in the epizootiology of furunculosis

and a similar way of maintaining the infectious agent of vibriosis in the marine environment is probable as the range of hosts in the disease is rather high. Although Kusuda (1966) described a condition in marine fishes which he diagnosed as ulcer disease, it is most probable that this was vibriosis as well. He described the disease in ayu, yellow tail (Seriola quinqueradiata), puffer (Fugu rubripes), "ishidai" (Oplegnathus fasciatus), wrasses (Pseudolabrus japonicus, Haliochoeres pocilopterus), "kisu" (Sillago sihama), horse mackerel (Trachurus japonicus) and file fish (Stephanolepsis cirrhifer).

Apart from the description of the disease among several marine tropical fish species, a case of vibriosis has also been reported in tropical freshwater fish (Hacking and Budd, loc.cit.). They isolated the pathogen from guppy (Lebistes reticulatus), tiger barb (Puntius spp.) and loach (Acanthrophthalmus spp.).

In Norwegian marine fish farms mortalities due to vibriosis range from 1% to 70% of the total number of fish on site with an average of approximately 13% (Håstein, unpublished data). If one considers the total production of rainbow trout and salmon for consumption to be approximately 1100 tons with an average price of £1 sterling per kg (1973), the losses amount to some £110,000 per year and are likely to increase as the industry expands and the value of the stock increases.

2.11 Differential diagnosis

Although some forms of *Vibrio* disease could be confused with furunculosis, when fish are raised in sea water, furunculosis can usually be eliminated from the differential diagnosis because as yet furunculosis has not been reported as a problem in sea water. In Norway there has been one outbreak of furunculosis of salmonids in sea water (Holt, unpublished) but as that occurred shortly after the fish had been stocked into sea water, the infection was probably already developing in the fish in freshwater prior to transfer.

Another differential diagnosis to be considered is Pasteurellosis (Håstein and Bullock, in press). This disease usually affects salmon raised in sea water or brackish water and then may show similar symptoms to those of vibriosis. Consequently, for certain diagnosis of *Vibrio* infections it is essential to isolate the aetiological agent in culture and verify its identity by bacteriological techniques.

2.12 Prophylaxis

To avoid vibriosis, in the long term, may be considered very difficult for the fish farmer, but use of a well balanced diet thus producing fish in good condition strengthens resistance of the fish to the disease. Reduction in the stocking density also reduces the infective rate of the disease because a heavy density of fish leads to stress phenomena, which increases the

susceptibility of the fish to the disease.

As well as other prophylactic measures, a successful vaccine would be of value. Promising reports on a useful form of oral vaccine, administered to the fish through their diet, have been published by Hayashi et al,(loc. cit.), Guymon 1972 and Fryer (personal communication) has also reported good results, but it seems probable that consistent results cannot be expected from this procedure under the very different circumstances of world mariculture.

Hayashi et al. (loc.cit.) found that agglutinins against virulent strains of Vibrio anguillarum were produced two weeks after administration of oral vaccines and they concluded that practical application of vaccines by injection or by administration of heavy doses in food was possible for prophylaxis and control of the disease.

Harrell (loc.cit.) suggested the possibility of treating vibriosis in salmonids with specific antisera as specific serum antibodies were shown to have an important protective role. Cellular protective mechanisms and the possibility of protective substances being transferred via eggs to susceptible offspring are important areas of research for future consideration.

Embody and Hayford (1925) and the Furunculosis Committee (1930) suggested that selective breeding of a resistant race to furunculosis might be feasible and a

similar selective breeding programme could be performed in the case of vibriosis. According to Gjedrem and Aulstad (1973) there exists a statistically significant difference between losses in different races of Atlantic salmon in a natural outbreak of the disease, but considerable further work is required on this aspect before it can be a usable means of controlling the disease.

SECTION B

VIBRIOSIS IN NORWEGIAN FISH FARMS

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   - 2.3 Isolation procedure
   - 2.4 Maintenance of the bacteria
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4.5 Proteinase production and serology

1. GENERAL DESCRIPTION OF NORWEGIAN FISH FARMS

Fish farming in Norway takes place in freshwater, brackish water, or in sea water.

The management in freshwater uses earthen ponds, concrete ponds, or troughs made partly of steel and partly of glass-fibre (figs. 1, 2, 3 and 4). The freshwater fish farms mainly produce fish for restocking of rivers and lakes, but also deliver smolts of Atlantic salmon or similar sized rainbow trout to marine fish farms, where rearing to edible sized fish (0.5 - 4 kgs.) takes place. At the moment only one freshwater fish farm produces fish in large scale for human consumption, but a few small ones produce semi-fermented trout (rakefisk) for sale. This is a Norwegian speciality which is produced by light curing of salmonids in vats under pressure to provide a high concentration of oxygen. The fish ferment by autolysis within two months at 5-10^{o}C.

The first attempts in Norway to rear fish in sea water were made in 1912 and were assisted by grants from Parliament. The attempts failed and it was not until the middle of the 1950's that rearing of fish under marine conditions was started again by, e.g., the Vik Brothers in Sykkylven.

According to Vik (1963) their experimental work was kept secret until 1960, but then the Norwegian Broadcasting Service (NRK) became aware of the results and there was

Fig. 1 Interior of a fish farm equipped with racks of glassfibre basins and automatic feeding units (Forsøksstasjon for fisk, Sunndalsøra).

Fig. 2 Interior of a fish farm with an alternative design to Fig. 1 (Settefiskanlegget, Hunderfossen).

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Fig. 3 Typical concrete basin (Nor Laks, Sykkylven).

Fig. 4 A large pond divided into raceways by means of wooden walls (Vestfold ørretfarm, Brunlanes).

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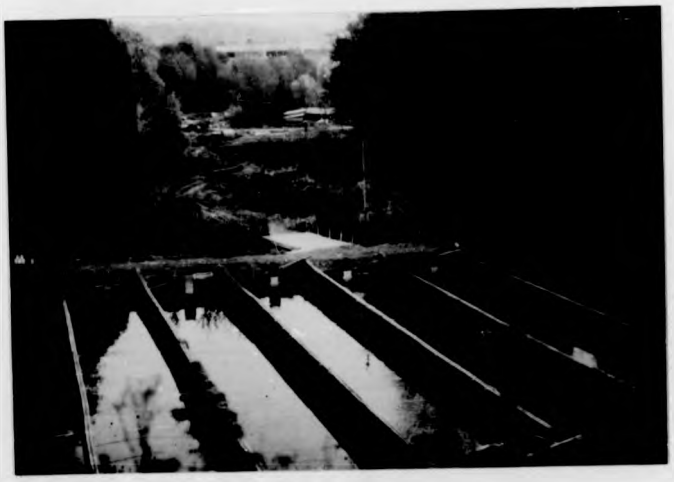

Fig. 5 The distribution of hatcheries and fish farms in Norway in 1973.


NORGE — NORWAY

then a sudden increase of interest in mariculture and today there is a large number of fishfarms along the Norwegian coastline up to Vesterålen. Fig. 5 shows the distribution of hatcheries and fishfarms in both freshwater and sea water (1973).

According to Sedgwick (1966), the reason for sea water utilization was the lack of sufficient freshwater supply and the low temperature of freshwater during the wintertime. Sedgwick (1970) also stated that a good reason for using sea water to rear rainbow trout was the stable water temperature allowing the fish to feed more or less the whole year round.

Today Norwegian rearing of edible salmonids (i.e. salmon and trout) for human consumption is a fast growing industry. The rationale for this is the fairly long coastal line with very many suitable fjords and a rather stable water temperature due to the Gulf Stream.

The design of the farming units in sea water varies to a great extent, but may be categorized as follows:

Floating Pens

The most common rearing units in marine fish farms in Norway at the present time comprise floating nets which may be linked together and as such may be adapted for small "hobby" farms or large industrial farms.

The size and shape of the nets varies; they can be round, rectangular, square or six-, eight- or ten-sided.

The depth of the nets varies from 2-8 m. and the surface area between 4-200 m^2. The average capacity of the nets is 198 m^3. (Anon, 1971; Braaten and Saethre, 1973).

Various types of flotation device are used, e.g. a floating collar of a material such as expanded polystyrene, empty barrels, prefabricated pontoons, or plastic tubes. From the floating collar the net hangs down into the water. The floating material is usually constructed to form a framework and a small gangway is erected on top. If wooden, the framework is usually deeply impregnated to prevent rotting.

From the corners of the floating unit, ropes for moving the nets either to land and/or to anchors are attached. A fence of fine meshed net is placed on top of the floating unit to cover the surface of the unit. This discourages predatory birds and prevents losses of fish due to their jumping out. The former is particularly important just after the fish have been put into the cages and are still small in size. Typical floating units are shown in figs. 6 and 7.

Fixed net enclosures

Another type of enclosure is one comprising a series of net walls secured to the sea bottom. Three sides of the unit consist of nylon netting, while the fourth side is the beach. A typical example of such a unit is shown in figs. 8 and 9.

Fig. 6 Typical floating pens (A/S Havlaks, Hitra).

Fig. 7 Close-up view of ten-sided floating pens (Frøya Edelfisk, Dyrvik).

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Fig. 8 Fish farm based on fixed nets (Eros Laks, Bjordal. Photo by courtesy of Dr. O. Ingebrigtsen).

Fig. 9 Fixed nets and an earthen pond on shore (Eros Laks, Bjordal. Photo by courtesy of Dr. O. Ingebrigtsen).

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One condition for use of this method is that the sea-bottom is evenly sloping to give a depth of about 10m. at a distance of 25m. from land.

The nets are mounted by means of wooden pillars which are placed at a distance of 3.5 - 5 m. from each other. Because of the tidal variation and spring flood, the pillars have to be sufficiently high to reach above the surface at the highest watermark.

Two sets of nets are fixed to the pillars. The inner net which encloses the fish, is surrounded by the outer net, which keeps wild fish at a distance from the farmed fish. The outer net also acts as a precautionary measure if the inner net bursts for some reason. The nets are loosely laid down on the sea-bottom and fixed there by means of a lead weighted hem at the base of the net. The nets are stretched fairly tightly between the pillars.

Coves, embayments etc.

A third alternative is to fence off small bays, sounds etc. This type of farming system is not common in Norway as yet.

The basic principle for this rearing method is to fence off the inlet of a creek, the inlet and outlet between small islands, rocks etc. The water exchange inside the enclosure is based on tidal exchange and the local currents.

The fencing is usually made of prestressed concrete bars or aluminium bars in a concrete frame. The size of

slits between the forms is approximately 15 mm.

The basic problem with this kind of farming system is that there is often insufficient replacement of the bottom water and the fish farmers have to install costly pumps either to pump freshly oxygenated water in, or suck out water and wastes from the bottom layers. A typical farm of this type is shown in figs. 10 and 11.

A few fish farmers have based their management on using both sea water and freshwater at different concentrations. Their ponds are usually of the Danish system and they pump sea water into the ponds and raceways (i.e. long narrow channels).

The main problem for these fish farmers is power failure to the pumps and they therefore have to install diesel generators as well as purchase extra insurance against such failures.

Fig. 10 Fencing off a small sound (A/S Mowi, Bergen. Photo by courtesy of Dr. Chr. Andersen).

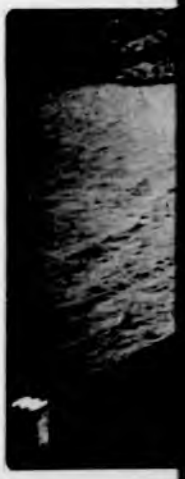

Fig. 11 Closer view of the fence seen from above (Photo by courtesy of Dr. Chr. Andersen).

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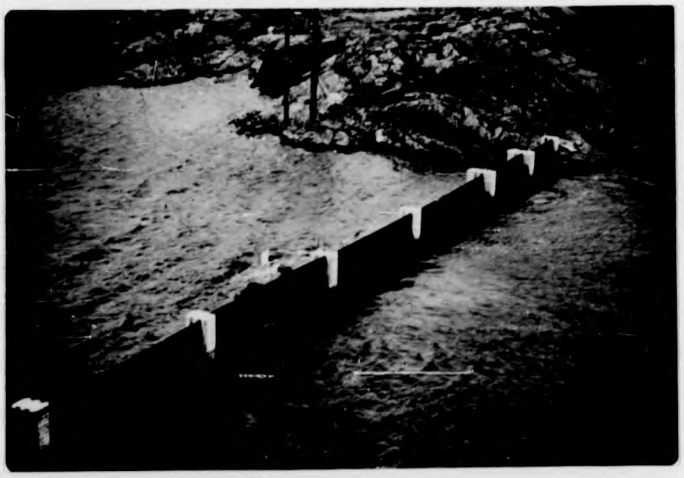


2. FIELD OBSERVATIONS AND COLLECTION OF PATHOLOGICAL MATERIAL AND STRAINS OF VIBRIO ANGUILLARUM

2.1 Collection of material

The outbreaks of vibriosis undertaken in this study have been investigated as part of the author's work as head of the fish disease research and diagnosis section at the National Veterinary Institute, Oslo. Clinical observations and material for pathological study were obtained on site when investigating outbreaks of disease in Norwegian fish farms during the period 1967-73. Bacterial isolates were made either on the farm or from material sent in from the farms to the National Veterinary Institute, in connection with losses on farms, in outbreaks of fatal disease in natural waters, or from fish showing lesions when caught. The information concerning the isolates is listed (see Table 2 in Appendix 1).

2.2 Specific diagnosis of the condition

All of the outbreaks documented in the clinical description, gross pathology and histopathology were cases in which Vibrio anguillarum was isolated, usually in pure culture and characterized as such by its morphology, colony morphology and reaction on biochemical tests.

2.3 Isolation procedure

The method of isolation of the bacteria from diseased fish was as follows:

For routine diagnostic purposes, material from organs showing pathological changes or from suspected organs of the fish was inoculated on blood agar plates containing 5% goat blood and 0.5% sodium chloride. The blood agar plates were then incubated for 48 hours at 22-24°C.

After 48 hours growth, the colonies of Vibrio anguillarum, if present, appeared greyish brown, semi-translucent, smooth, convex and with a haemolytic zone of β haemolytic character, usually in pure culture.

Thin smears from typical colonies were Gram stained and examined under an oil immersion objective. If the organisms appeared as Gram negative comma shaped rods, subculture from the selected colonies took place as mentioned above.

After subculture the organism was tested biochemically.

2.4 Maintenance of the bacteria

For stock cultures, each strain was grown as carpet growth on 5% goat blood agar plates, then the colonies were harvested and suspended in horse serumbroth and distributed into sterile freeze drying ampoules (10 mm. x 6 mm.). Each aliquot was processed and sealed off in vacuo in a Speedivac-gauge, Model B5 freeze drying machine (Edwards High Vacuum Ltd.). For immediate use, the cultures were held on 5% goat blood agar plates at 4°C

and subcultured when necessary, but before each sequential test new vials were opened, to prevent habituation or mutation of strains by artificial selection by media passage.

2.5 Bacteriological tests

The strains of Vibrio anguillarum were investigated with reference to:

2.5.1 Morphology

2.5.2 Biochemical characteristics

2.5.3 Antibiotic sensitivity

2.5.4 Proteinase activity

2.5.1 Morphology

a) Size

Gram-stained smears made from 48-hour cultures on goat-blood-agar plates incubated at 25°C were examined under an oil-immersion objective and 25 random samples of each strain were counted by means of the engraved scale of a stage micrometer graduated in units of 0.1 mm. The mean value of the 25 readings was calculated and multiplied by 0.082 (the magnification factor for the apparatus used) to give the size of the organism in microns.

b) Flagella

The method used for detection of flagella was the negative staining technique.

Growth from an 18-24 hours agar slope was harvested in 2.5% NaCl containing 5% neutral formalin. The fixed cells were then washed three times in 0.1 M ammonium acetate solution. To about 1.0 ml. of the washed cell suspension, two or three drops of 1% phosphotungstic acid (adjusted to pH 7 with KOH) were added. After standing for five minutes, one drop of the mixture was placed on a carbon-stabilized coated grid, dried and examined in a Siemens Elmiskop I A electron microscope.

2.5.2 Biochemical tests

a) Carbohydrate fermentation tests

The classical fermentation tests were performed in simple media containing 1% of the carbohydrate to be tested in peptone water, but in some pilot experiments Hugh and Leifson's medium was used as well. Bromthymol blue which is active over the pH range (6-7.6) was incorporated as an indicator to demonstrate the change of pH. The results of the fermentation tests were read after seven days. To obtain good growth on the sugars, one platinum loopful of 48 hours old bacteria grown on 5% goat blood-agar was suspended in approximately 3 ml. of sterile saline. The suspension was drawn into a sterile Pasteur pipette and two drops from the Pasteur pipette were put into each test tube.

Control inoculation of the suspension was carried out on 5% blood agar plates to verify pure culture of

the organism. If this test showed growth of a mixed bacterial culture, new tests were carried out with a fresh inoculum.

Table 3

Carbohydrates used in fermentation tests

Monosaccharides (pentoses):	arabinose, rhamnose, xylose
Monosaccharides (hexoses):	fructose, galactose, glucose, mannose
Disaccharides:	cellobiose, lactose, maltose, saccharose, trehalose
Trisaccharides:	raffinose
Polysaccharides:	dextrin, inulin
Sugar alcohols:	adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol
Glucosides:	aesculin, salicin

b) Utilization of citrate

Tubes containing crystalline tertiary citrate (Sandvik, 1972) were inoculated with one drop of the bacterial solution described previously under carbohydrate fermentation tests. A positive result was recorded when opacity of the medium occurred.

c) Digestion of gelatin

Gelatin is a semi-synthetic protein prepared from collagen. Gelatinolytic ability was determined in 5 ml. amounts of a medium containing nutrient gelatin fortified by serum and inoculated by deep stabbing with a straight platinum needle. Incubation was carried out for seven days at 24°C, whereupon the cultures were maintained at 4°C for one hour prior to reading the results. Gelatinolytic cultures produced liquefaction of the medium even at 4°C.

d) Production of hydrogen sulphide

Detection of hydrogen sulphide was carried out on triple sugar iron medium (Sandvik, loc.cit.). Incubation was performed at 24°C for seven days and production of hydrogen sulphide was detected, as blackening of the medium if the reaction was positive.

e) Production of indole

Cultures were made in tubes containing 5 ml. of casein peptone-phosphate-water and incubated for seven days at 24°C. After incubation, 0.5 ml Erlich's reagent was introduced, giving a red colour if the reaction was positive.

f) Production of methyl red

The methyl red test indicates the ability of a micro-organism to alter the reaction of a glucose peptone water solution medium from pH 7 to pH 4.4 - 4.7 or below. Each strain was cultivated in 5 ml. of Clark Lub's medium

(Sandvik loc.cit.). Incubation was carried out for seven days at 22-24°C and then one drop of methyl red was added. If a yellow colour occurred, the reaction was deemed negative.

g) Production of acetyl methyl carbinol. Voges-Proskauer reaction

Production of acetyl methyl carbinol was investigated according to the method of Kristiansen (pers. comm.). Thus strains were grown in glucose-phosphate peptone water containing 0, 0.9%, 1.5%, 2.5% sodium chloride. The Voges Proskauer (VP) reaction is based on the finding that certain bacteria produce acetyl methyl carbinol (CH_3 COHCO CH_3) which becomes oxidized in alkaline solution giving the diacetyl radical (CH_3 COCO CH_3) which reacts with the peptone in the medium, giving a red colour. The test was carried out by adding 1 ml. 40% sodium-hydroxide solution and some granules of creatinine. The reaction was read after 10 minutes.

h) Action on litmus milk

Ten ml. of litmus milk, prepared according to Sandvik (loc.cit.) were inoculated and incubated for seven days at 24°C and the reaction was observed.

i) Action on methylene blue milk

Ten ml. of methylene blue milk prepared according to Haugen (pers. comm.) were inoculated and incubated for

seven days at 24°C and the reaction was observed every day during the period.

j) Nitrate reduction

A peptone water solution containing nitrite free potassium-nitrate was inoculated and incubated at 24°C for seven days when Greiss-Ilsova reagent was added and the development of a red colour was taken to indicate a positive result.

k) Production of oxidase

Possession of oxidase was determined by the method of Kovacs (1956) in which one sheet of filter paper, impregnated with a few drops of a 1% solution of tetra-methyl-paraphenylene-diamine was smeared with a 48 hour culture of Vibrio anguillarum grown on blood agar.

A positive result was indicated by the appearance of a dark purple colour of the smear within thirty seconds. A culture of Escherichia coli was employed as a negative control.

l) Production of urease

Ability to decompose urea was determined after inoculation of strains into solid urea medium (Christensens medium).

The cultures were examined for the pink colouration indicating hydrolysis of urea after 48 hours and seven days.

m) Reaction on hippurate medium

Meat extract broth with 1% sodium hippurate was inoculated and incubated at 24°C for seven days. The reaction was deemed positive if crystals of benzoic acid were precipitated when 50% sulphuric acid was added.

2.5.3 Antibiotic sensitivity

The in vitro response of the strains to a variety of antibiotics was tested on the Mueller Hinton plates.

The organisms were cultured in serum broth for 48 hours and then poured into Mueller Hinton plate to get a uniform layer of bacteria on the surface of the plate. The excess of nutrient broth containing bacteria was discharged, leaving only a thin film of bacteria on the plate. The plates were allowed to stand at room temperature for two hours before the discs (impregnated with the antibiotic and bacteriostatic compounds being tested) were placed on the plates. The antibiotics used are listed in Table 4.

Borgal* sensitivity discs containing trimethoprim and sulphadoxin were used; the other sensitivity discs were "Sensitabs"** and Vibriostat 0/129.***

* A/S Norske Hoechst, Oslo, Norway.

** A/S Rosco Pharmaceutical Industries, Roskilde, Denmark.

*** Provided by Dr. R.J. Roberts, University of Stirling.

After incubation for 48 hours at 24°C, plates were examined under incident light and the width of any zone of inhibition measured from the edge of the sensitivity disc to where growth was seen.

Table 5 (Appendix 1) indicates the results of the antibiotic sensitivity tests. The symbols used are indicated as follows:

-	=	No inhibition
+	=	1.0 to 5 mm.
++	=	5.1 to 10 mm.
+++	=	10.1 to 15 mm.
++++	>	15 mm.

Table 4

Antibiotics contained in the sensitivity discs used in experiments on Antibiotic Sensitivity

Chloramphenicol	400 µg
Tetracycline	400 µg
Sulphadiazine	4 mg
Borgal (Trimethoprim)	1.25 µg
Borgal (Sulphadoxin)	62.5 µg
Vibriostat O/129	-
Penicillin low	10 I.U.
Penicillin high	350 I.U.

2.5.4 Proteinase activity

Extracellular proteinases are produced by numerous micro-organisms (Hagihara, 1960; Sandvik 1962, 1967) and the usual test for their presence is the ability to liquify gelatin. This test has been generally used in classification of bacteria (Breed et al., 1956). The gelatin liquifying enzymes have been shown to be identical with the so-called casein precipitating enzymes described by Sandvik (loc.cit.).

The proteinases are considered to be specific for the organism producing them and this is the basis for the serological enzyme classification introduced by Sandvik (loc.cit.) using the casein precipitating reaction (CP-test).

The principle for this test is that casein is incorporated in agar and the enzyme solutions to be tested are applied into wells in the agar. By this method the enzyme activity can thus be expressed as an increase in the opacity of the substrate in zones around the wells. This is considered to be due to a conversion of the κ casein by which the micelle stabilizing ability has been lost (Sandvik, loc.cit.).

Proteinase activity can also be demonstrated by applying filter paper moistened with the enzyme solution onto the agar surface.

A so-called electrophoretic precipitating inhibition test (CPI-test) which is also used for differentiation of

microbial proteinases was proposed by Fossum, (1971.) The main procedure of this method, which was also used in the present study is as follows:

Extracts of inhibitor containing materials such as rabbit antisera against Vibrio anguillarum (see Page 55) were subjected to paper electrophoresis [(Schleicher and Schüll filter paper No. 2043 bmgl 40 x 410 mm.)] in an LKB apparatus 3276 BN for 16 hours at 120 V, 4 m.A. using phosphate buffer 0.06 M. at pH 6.5 with merthiolate to a final concentration of 1: 10,000. The sera were applied in volumes of 8 - 12 µl.

The electrophoretic paper was then transferred to the surface of the casein containing agar and the extracts allowed to diffuse into the agar for approximately four hours. Paper strips 4 mm. broad were then moistened either with purified proteinases from Vibrio anguillarum or with five day old gelatin or litmus milk cultures of the same bacteria and placed onto the agar after removal of the electrophoretic papers. They were allowed to stand for 24 hours at 37°C before reading. Precipitating zones occurred along the enzyme-containing strips and specific inhibition was indicated by interruption of the white precipitation zone or by narrowing of the precipitation zone (Fig. 25,27).

The intention of this part of the work with the electrophoresis CPI-test was to see if there was any qualitative difference between the isolated Vibrio anguillarum species with regard to production of casein precipating enzymes. In order to achieve this ATCC strain

19264 of Vibrio anguillarum was chosen to act as the archetype.

This strain was cultured on semisolid skim milk agar in Roux bottles for five days. The milk agar was then frozen and thawed three times and then centrifuged at 2000 g. for 20 minutes. The precipitate was discarded and the supernatant filtered through a coarse millipore filter and then precipitated with ammonium sulphate to 80% saturation before centrifugation at 2000 g. for 20 minutes. This precipitate was dissolved in a small amount of distilled water and dialyzed against running tap water at 8 - 10°C for several hours. This rough enzyme concentrate was then used for antibody production in rabbits.

Rabbits were injected partly intradermally and partly subcutaneously with a mixture of equal parts of Freunds adjuvant (1.2 ml.) and the above mentioned preparation four times at weekly intervals. In the first injection, Freunds complete adjuvant was used, in the others, Freunds incomplete adjuvant, and the serum was collected and stored in small batches at -20°C until required for use.

The antiserum was then subjected to paper electrophoresis as described previously and the different strains of the isolated Vibrio anguillarum tested by means of the electrophoretic CPI-test.

2.6 Histological technique

Blocks (20 mm. x 3-4 mm.) were taken from skin lesions, muscular tissue and internal organs of moribund or newly dead specimens of salmon and rainbow trout from disease outbreaks. They were fixed in 10% formal-saline, processed and embedded in paraffin wax (Histowax) in embedding cassettes prior to sectioning at 6 µ.

The stains used were Haemalum Eosin, Giemsa and Humberstones-Gram method.

3. DESCRIPTION OF THE PATHOLOGY AND TREATMENT OF THE DISEASE

3.1 Epizootiology

As mentioned earlier, vibriosis normally occurs in sea water or brackish water (Håstein, loc.cit.). The infectious reservoir under natural conditions is thought to be infected marine fish, mainly small saithe (coalfish) (Gadus virens) which gather around the fish farms. The local fauna, including the tunicate (Ciona intestinalis) may harbour the bacteria. Infection may also arise when wet feed containing infected trash fish material is fed either because these trash species have been carriers of the organisms within their viscera or because they exhibit clinical disease.

When the disease is established, contact infection plays an important role in its spread within a holding facility.

As mentioned several factors influence the occurrence and development of an outbreak of vibriosis. Although occasional out-breaks of the disease have been recorded at any time of the year, most outbreaks occur in the summer and autumn when the water temperature exceeds 10°C.

Confirmed diagnoses of vibriosis at the National Veterinary Institute, Oslo in the period 1967 - 1973 are depicted in Fig. 12 in relation to the month of occurence and species involved.

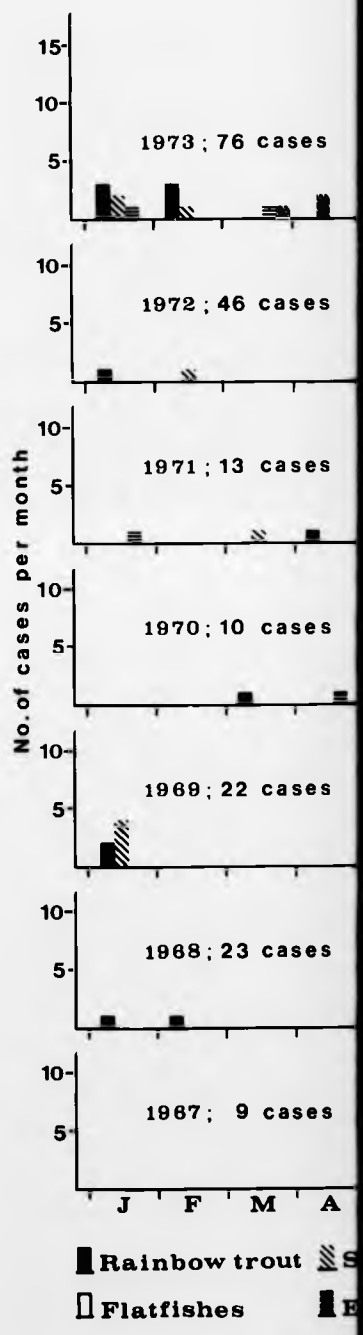
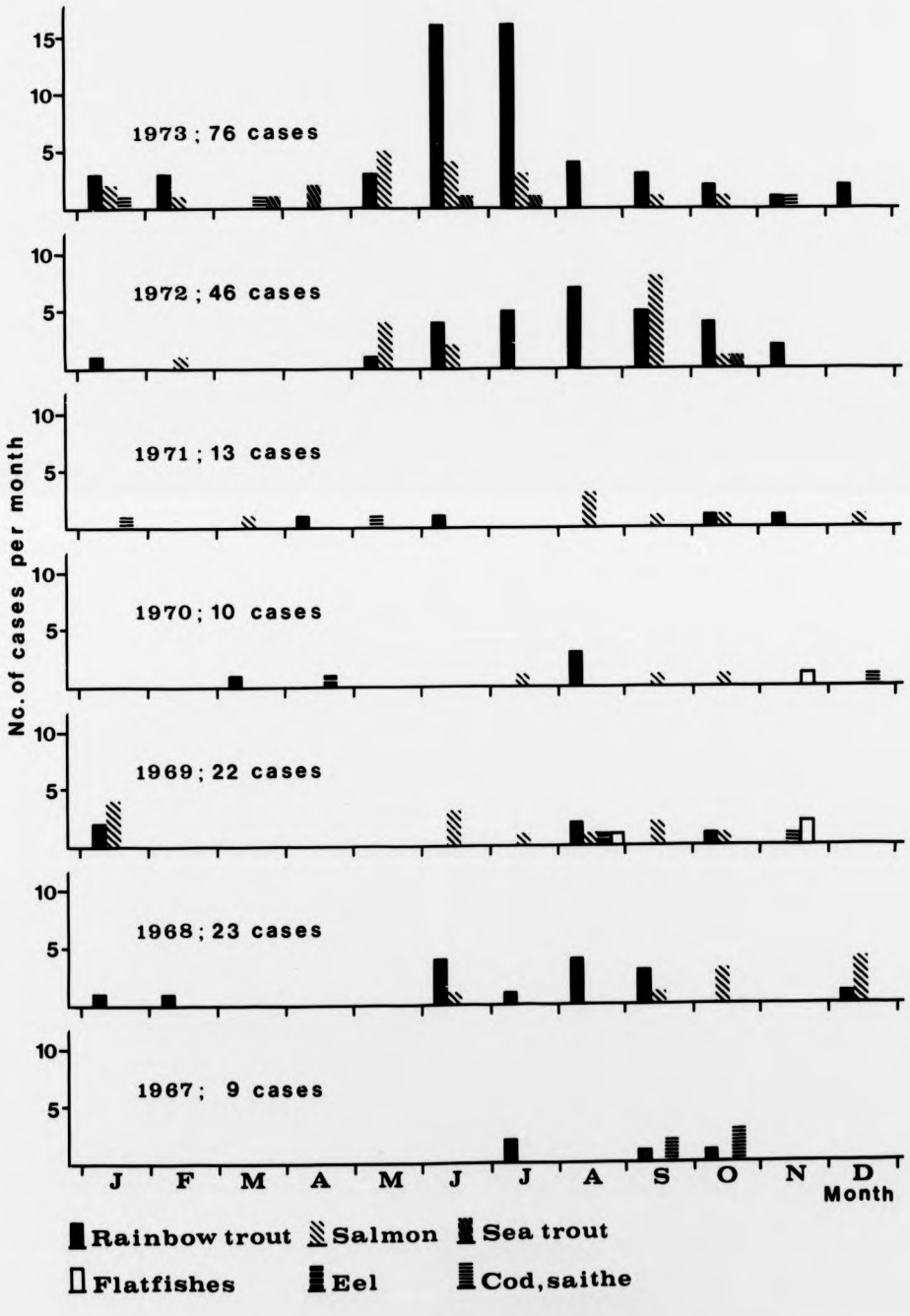


Fig. 12 The frequency of Vibriosis diagnosed at the National Veterinary Institute, Oslo, in the period 1967 - 1973


1973 ; 76 cases
1972 ; 46 cases
1971 ; 13 cases
1970 ; 10 cases
1969 ; 22 cases
1968 ; 23 cases
1967 ; 9 cases
No. of cases per month
J F M A M J J A S O N D
Month
Rainbow trout
Salmon
Sea trout
Flatfishes
Eel
Cod, saithe

Stress factors on the fish, such as heavy stocking rates, bad quality feed, transport stress, lack of oxygen or handling can all precipitate clinical outbreaks or increase losses.

3.2 Pathogenesis

The incubation period for the outbreaks of vibriosis studied, varied with temperature and other factors such as virulence of strain involved and handling stresses. It was usually between three to ten days. Experimental infections with virulent strains had incubation periods as short as 24 hours.

All weight classes of fish could be infected, but it seemed that rainbow trout in salt water were most susceptible at between 100 - 200 g. Atlantic salmon were most vulnerable at the parr-smolt stage, when they are first conditioned to salt water.

Mortality rate varied a great deal from 10% - 70% depending on the farm, the virulence of the organism and the stress factors. The average annual mortality due to vibriosis in Norwegian fish farms, based on interviews with the farmers, was approximately 13% (see Page 26).

The course of an outbreak usually followed a set pattern. First signs usually comprised a sudden and often dramatic increase in mortality of stock with no obvious external clinical features. In some cases anorexia, depression and reduced swimming activity were observed. After a few days of acute losses, or in more chronic outbreaks which developed slowly, skin lesions, taking the

form of raised swollen areas, usually occurred. These lesions were similar to those observed in furunculosis in salmonids in fresh water, although when incised appeared to be deeper. In more chronic stages or in fish surviving an outbreak, superficial lesions were seen on the sides of the body, on the operculum and the tip of the jaw (Figs. 13, 14, 15, 16, 17 and 18). The lesions were reddish in colour and surrounded by a small raised white halo at the junction with the normal skin.

The fins, especially the pectoral and pelvic fins, showed diffuse reddening with punctate haemorrhages at the base or between the fin rays.

3.3 Gross pathology

When the fish were opened up, the most common observation in those which had died suddenly was a slightly swollen spleen, the splenic tissues quite often being in a state of liquefaction, when no other pathological changes were apparent. In other cases, swollen liver and kidneys, congestion in the intestine and haemorrhages around the anus could be seen.

At this stage of the disease bacteriological examinations were essential for a correct diagnosis to be made.

In subacute stages extended petechiae could be seen in the liver, spleen and musculature, and kidney and spleen often exhibited a necrotic appearence.

Fig. 13 A typical superficial lesion on the body surface of a rainbow trout

Fig. 14 Petechiae in the liver of a rainbow trout suffering from vibriosis

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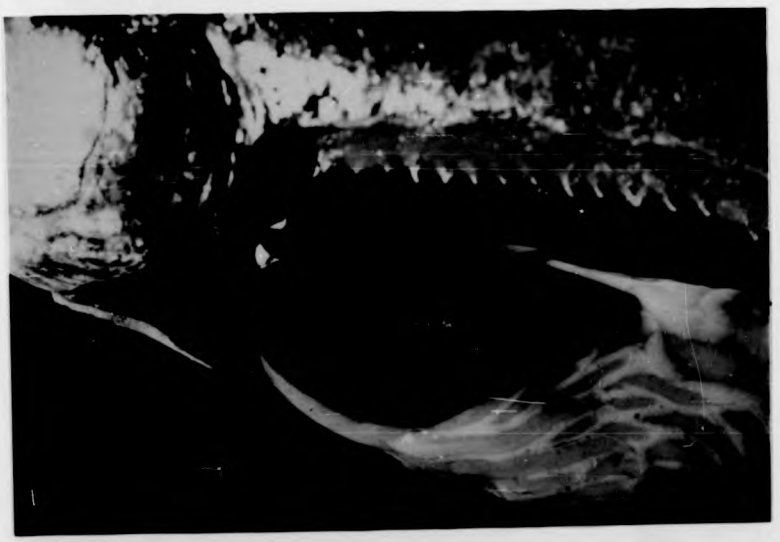

Fig. 15 Typical internal haemorrhagic changes of vibriosis in rainbow trout

Fig. 16 Close up of a deep muscle lesion

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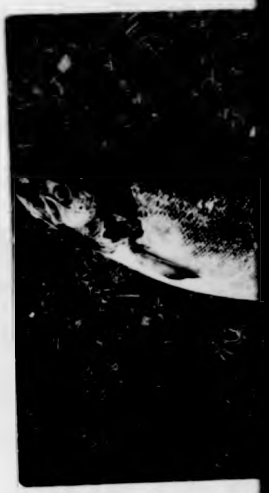
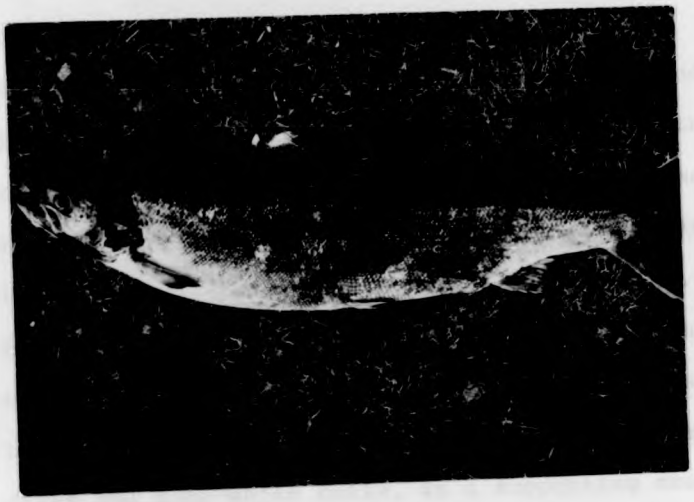


Fig. 17 Superficial ulcerations in the skin of Atlantic salmon suffering from vibriosis

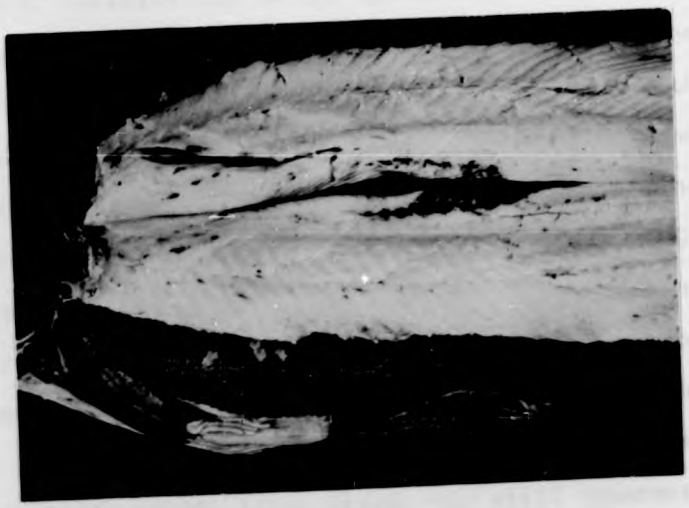
Fig. 18 A typical "boil" lesion in the musculature of an Atlantic salmon

skin of

vibriosis



musculature



In more chronic stages of the disease the syndrome was primarily oedematous with exophthalmos and abdominal swellings due to excessive intraperitoneal serous exudate, as well as the ulcers on the sides of the body and fine haemorrhages mentioned earlier. In such outbreaks many of the less severely affected fish would show changes similar to those described for subacute furunculosis (McCraw, loc. cit., Herman 1968). These comprised unbroken subcutaneous swellings, often relatively large, containing a sanguinous material comprised mainly of necrotic muscular tissue, red blood cells and some white cells, in a supporting mesh of inflammatory exudate and fibrin. Large numbers of bacteria were present in such lesions and rupture of these lesions released bacteria. This was considered by the author to be a major contribution to the spread of infection.

The 'boil" lesions occurred either just under the skin or in the deep layers of the muscular tissue. When they ruptured, the resultant ulcers had irregular edges and a necrotic appearance. Thus it was difficult to distinguish them grossly from furunculosis and pasteurellosis. Gram staining of the purulent content of the "boil" lesions usually permitted a rapid and accurate differentiation.

Internal changes in such cases included haemorrhages in the liver and intestine, and the swollen spleen, so characteristic of the acute stage, was still apparent. The posterior part of the intestine was usually filled with a yellowish mucoid material.

Where an epizootic had lasted for some time, with a reduction in losses and some clinical resolution, changes in the eyes began to be manifested. The first feature was an increasing opacity of the cornea, progressing to ulceration which extented over the whole surface of the eye, with subsequent coloboma and collapse of the entire orbit. Fish with eye lesions such as these died if they did not receive adequate treatment.

3.4 Histopathology

A number of sections from internal organs and skin lesions from fish at different stages of the disease were examined by light microscopy.

Integumental lesions

In acute cases where skin lesions were observed, histology showed that there had been complete destruction of the epidermis (Fig.19). The stratum spongiosum contained considerable numbers of bacteria which were scattered rather than in colonies. The stratum reticulare, a much more collagenous avascular tissue, was considerably less affected than the spongiosum. Melanophores of both spongiosum and hypodermis were relaxed or ruptured, with the result that mature melanosomes were scattered in the tissue. Often the surface of the ulcerated skin was invaded by Myxobacteria. The hypodermis was the most reactive tissue with considerable haemorrhage and necrosis of connective tissue.

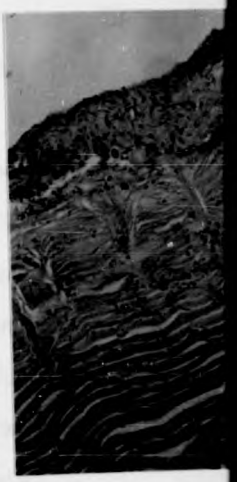


Fig. 19 Skin lesion with epithelium destroyed and underlying tissue invaded by Myxobacteria. Melanophores of stratum spongiosum and hypodermis relaxed (no reaction in stratum reticulare). (x 105) H.E.

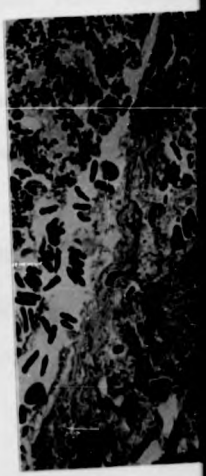


Fig. 20 Severe haemorrhages and destruction of the myofibrils. (x 105) H.E.

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From this focus the lesions extended down in association with the fascial planes of connective tissue into the myotomal muscles. In some cases these muscular lesions were very severe with complete destruction of myofibrils in the centre of the lesions resulting in an agglomerate of basophilic nuclear remnants, fibrin, sarcoplasmic debris and haemorrhages with bacteria on the edge. Myofibrils showed sarcoplasmic flocculation, nuclear karyorhexis and pyknosis and considerable hyperaemia.

Cellular infiltrates were not usually seen in such lesions, suggesting either aconsiderable suddenness in onset or some inhibiting factor precluding the expected cellular response although occasionally tissue histiocytic activity was observed.

In some less severe cases a cellular response could develop in the skin, the cell reaction usually comprising entirely monocytes of which a considerable number contained melanin and ceroid. In such cases there was also myophagia of muscle elements on the edge of the lesion.

In the centre all distinction between muscle or connective tissue was precluded by the degree of degeneration. A typical muscular lesion is shown in Fig. 20.

Liver

Focal necrosis of hepatic tissue was frequently seen and in all cases there was some evidence of toxic swelling

of the hepatocytes, sinusoidal congestion and occasionally destruction of the hepatic haematopoietic tissue around the portal triads. The small numbers of melanomacrophages in such areas were frequently ruptured (Fig. 21).

Spleen

The spleen was severely affected. In many cases it was unsuitable for examination as even in sacrificed moribund fish it was completely destroyed. There was considerable necrosis of the white pulp and melanomacrophage areas, with capsular oedema and, where they were apparent, destruction of the endothelial lining of the penicilliary arteries. Melanomacrophage centres were usually destroyed, but a feature was the considerable levels of ceroid in those cells remaining (Fig. 22).

Kidney

The kidneys showed a variable degree of nephrosis, with severe tubular necrosis evident in some cases but not in others. In all cases, however, severe destruction of haematopoietic elements with karyorhexis or pyknosis was observed. A feature of the large venous sinuses of the kidney was the much higher level of monocytes and melanin containing cells present that in normal kidney (Fig. 23).

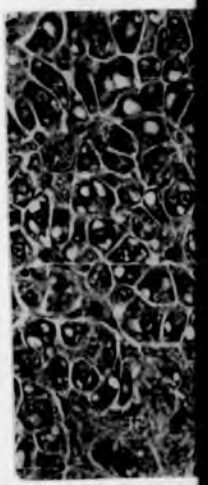


Fig. 21 Focal necrosis and swelling of hepatocytes in the liver of rainbow trout (x 105) H.E.

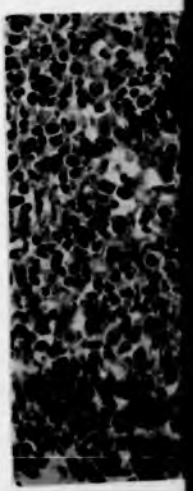
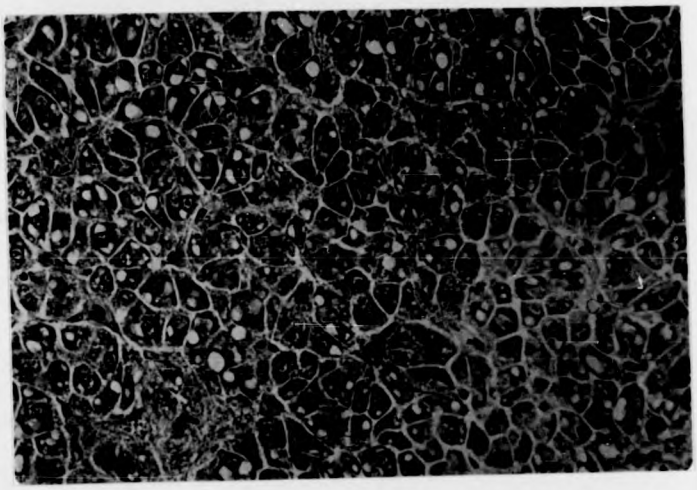


Fig. 22 Destruction of white pulp and melano-macrophage area in the spleen of rainbow trout (x 105) H.E.

Fig. 23 Destruction of haematopoietic tissue and incipient tubular necrosis, with unaffected glomeruli in the kidney of rainbow trout (x 105) H.E.

of hepatocytes

t (x 105) H.E.



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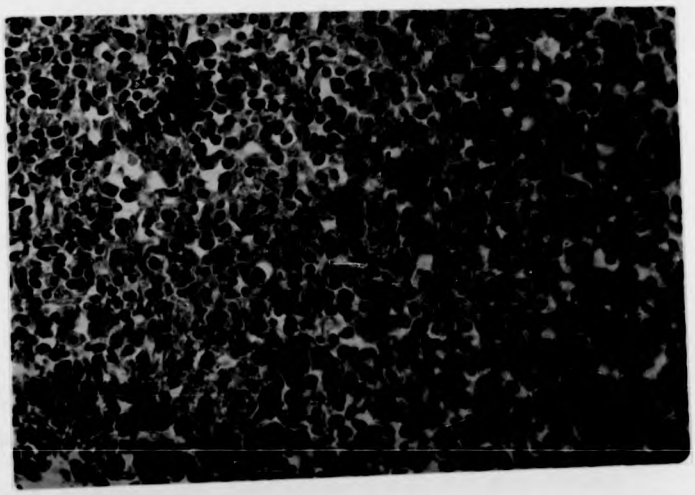

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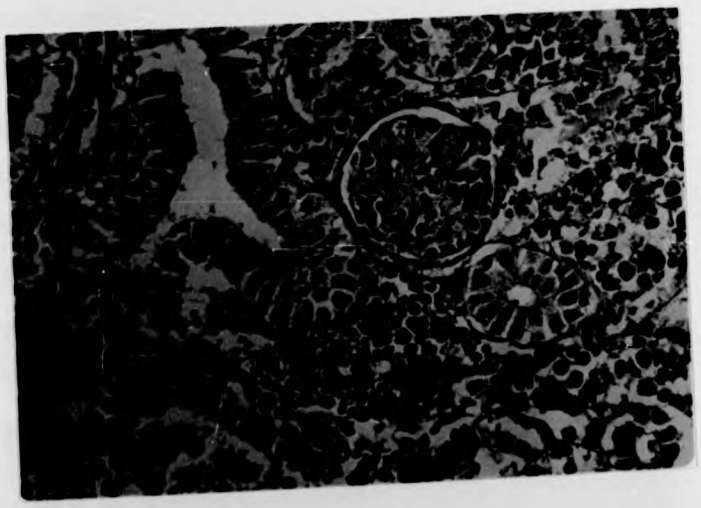


3.5 Prognosis

The prognosis was usually good if adequate treatment was given immediately after the first signs of disease had been observed.

Since treatment required mixing of the therapeutic agent in the food, the fish had to be still eating to have a chance of being cured and this clearly influenced the prognosis.

Despite application of adequate treatment methods, the disease could reappear several times in the same season. Questioning of the fish farmers indicated that recurrences often took place from two weeks to a month after the end of the previous attack, and some farmers reported up to five recurrences during the summer period.

3.6 Therapy

The treatment of vibriosis in Norwegian fish farms has comprised the use of chemotherapeutics and broad spectrum antibiotics.

Chemotherapeutics

The most common form of chemotherapy used, was a two part treatment with sulphamerazine in a dose of 0.2 g/kg fish/day. Treatment was given on three to four consecutive days, with suspension of treatment for one day, then treatment for another three days. This treatment was usually found successful in keeping the farm

free of the disease for a long period.

Small scale trials were carried out using two new chemotherapeutic agents - nifurpirinol, a nitrofuran derivative, and "Trafigal"*, a potentiated sulphonamide consisting of trimethoprim and sulphadimethoxine.

Nifurpirinol was given at a level of 4 mg/kg fish/day for five consecutive days, the highest level recommended by the manufacturer, but did not seem to give as good results as the drugs used at present.

"Trafigal" given in three field experiments at a dosage of 300 mg/kg fish/day for eight days had a good therapeutic effect compared with oxytetracycline.

Antibiotics

The most commonly used antibiotics were chloramphenicol and oxytetracycline. Both fed at a level of 50 mg/kg fish/day for five to six days, in serious cases up to ten days.

Chloramphenicol was the most effective drug, but because of its importance in human medicine, and the risk of infectious drug resistance, this antibiotic was replaced by oxytetracycline.

To avoid emergence of resistant strains of Vibrio anguillarum to different drugs, the current veterinary policy in Norway is to use a variety of drugs sequentially and thus far no sign of infectious drug resistance has been observed.

* A/S Norske Hoechst, Oslo, Norway

4. RESULTS OF BACTERIOLOGICAL EXAMINATIONS

4.1 Morphological examination

4.1.1 Dimension

The average length of Vibrio anguillarum grown on 5% goat blood agar plates at 24°C was found to be 1.41μ. The mean lengths of the individual strains from the different fish species are presented in Table 6.

Table 6

The mean length of isolates from the different sources

rainbow trout	1.29μ
Atlantic salmon	1.23μ
sea trout	1.43μ
saithe	1.25μ
cod	1.34μ
Ciona intestinalis	1.44μ
flatfishes	1.34μ
Vibrio ichthyodermis and ATCC strains of Vibrio anguillarum	1.99μ
Overall mean	1.41μ

When the Gram stained preparations of the bacteria were observed under the microscope at x 1000, the typical comma shaped structure of Vibrio bacteria was obvious.

4.1.2 Flagellation

All of the strains examined possessed a single polar flagellum which was twice as long as the bacterial cell (Fig. 24). In the electron microscope, cells with shorter rounder morphology, again equipped with one polar flagellum were also occasionally observed.

4.2 Viability of Vibrio anguillarum in different water qualities and at different temperatures

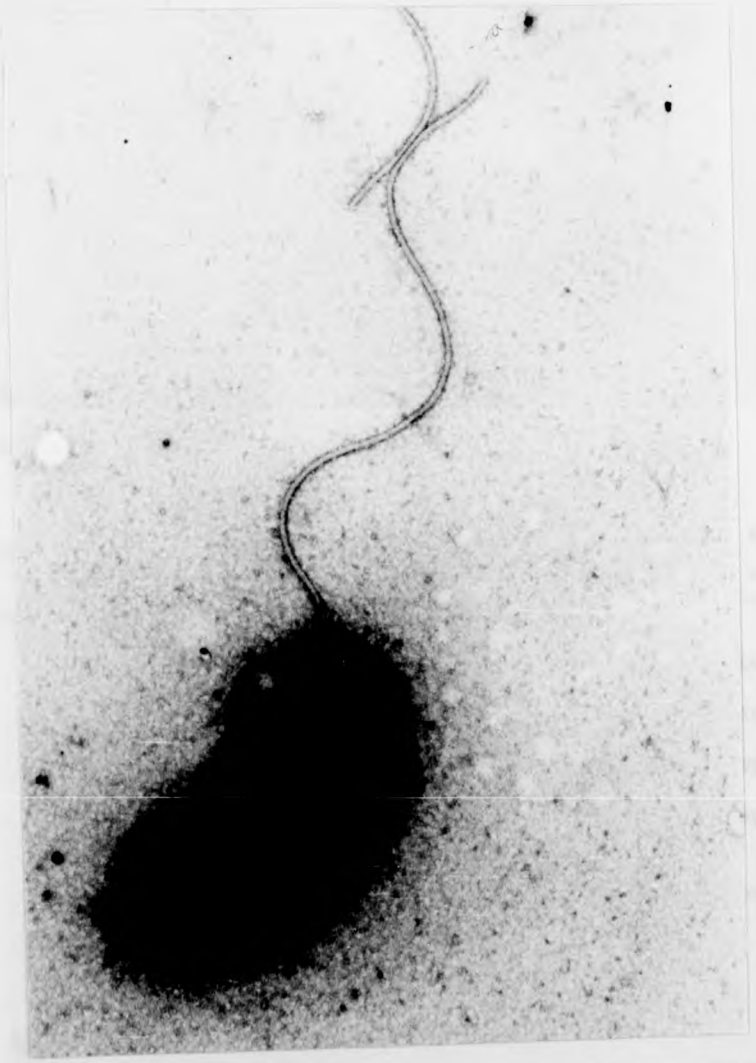
The viability of five strains of Vibrio anguillarum in distilled water, sterilized distilled water, sea water, sterilized sea water and sterile saline was tested at 4^{o}, 12^{o}, 22^{o}, 30^{o} and $37^{o}C$. The results are shown in Table 7.

48 hours colonies of the strains were inoculated onto 100 ml. flasks containing the media described above and during the first week after inoculation, daily samples from the bottles were streaked on blood agar plates, after thorough shaking to determine viability. The results of these viability studies showed conclusively that these strains of Vibrio anguillarum had no power of existing without some degree of salinity in the environment and were thus bound exclusively to sea water or brackish water.

Fig. 24 Electronmicrograph of Vibrio anguillarum

(x 30,000)

anguillarum



Although the organism was found to survive in distilled water up to seven days, its existence in fresh water was short thereby indicating that the disease can possibly be controlled by moving infected salmonids into fresh water to produce reduction in losses.

Table 7

Viability of Vibrio anguillarum in different media and temperatures

Temperature	Distilled water	Sterile distilled water	Sea water	Sterile sea water	Sterile saline
4°C	Up to 2d	1-7 days	1 month	2-6m	several m
12°C	approx 2d	approx 2d	approx 7d	3-4m	approx 2-4m
22°C	approx 2d	no growth	approx 7-8d	1-4m	approx 1-2m
30°C	approx 1-7d	approx 1d	approx 9-11d	13-22d	approx 10d
37°C	no growth	no growth	no growth	no growth	no growth

4.3 Antibiotic and chemotherapeutic sensitivity

The results of the antibiotic and chemotherapeutic sensitivity experiments of the isolated strains are listed in Table 8. For further details concerning the antibiotic sensitivity of the single isolates see Table 5 in Appendix 1.

All strains tested proved resistant to the effect of penicillin *in vitro*. Chloramphenicol was found to have a very potent inhibitory effect against all the isolated strains. Tetracyclines and sulphonamides were also found to exert a potent inhibitory effect although a few of the isolated strains exhibited no inhibition by sulphonamides. Borgal caused a slight to moderate inhibition of the majority of the strains (Fig. 25). All isolated strains except ten were inhibited by the specific Vibriostatic agent O/129.

Table 8

Graded Sensitivity *in vitro* of Different Antibiotics and Chemotherapeutics

Antibiotics	Sensitivity distribution of the strains					
	++++	+++	++	+	-	Total
Chloramphenicol	104	56	3	-	-	163
Tetracyclines	15	101	38	7	-	161
Sulphonamides	29	66	38	20	10	163
Borgal	-	30	108	17	3	158
Vibriostat O/129	1	6	79	67	10	163
Penicillin low/high	-	-	-	-	25	25

Fig. 25 *In vitro* sensitivity of *Vibrio anguillarum* to different antibiotics and chemotherapeutic agents

a) Chloramphenicol

b) Tetracycline

c) Sulphonamides

d) Borgal

e) Vibriostatic agent O/129

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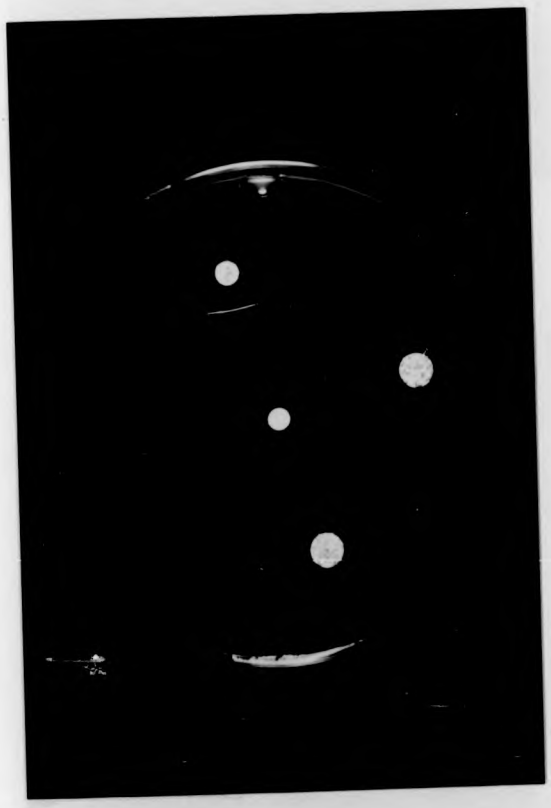


4.4 Motility and Biochemical reactions

4.4.1 Tests with constant results

The entire collection of Vibrio anguillarum strains was shown to be motile by means of a polar flagellum (Fig. 24). They also manifested their ability to produce gelatinase in serum gelatin, causing liquefaction of the medium.

All strains were shown to reduce nitrates to nitrites and produce oxidase.

All strains were shown to be negative in production of H_2S and splitting of hippurate.

4.4.2 Tests with varying results

The following tests gave variable results for the strains tested:

Carbohydrate fermentation

Litmus milk reaction

Methylene Blue milk reaction

Production of indole

Utilization of citrate

Production of urease

Methyl-red reaction

Voges-Proskauer reaction

The results for the individual strains are detailed in Table 9, Appendix 1.

4.4.3 Carbohydrate fermentation

A total of 22 substrates served to establish the fermentative reactions of the 163 isolated strains tested. The results are given in Table 9 and their relation to fish species is found in Table 2 (Appendix 1).

All strains produced acid in glucose, maltose, mannose amd dextrin, while all strains were negative with inulin, raffinose, rhamnose, adonitol, dulcitol and d-tartrate.

In the case of the other carbohydrates, the results varied to some extent and in some sugars only a slight acidification took place.

As pilot tests (20 strains) showed that gas production was insignificant, this test was abandoned for the rest of the strains.

4.4.4 Litmus milk reaction

The majority of the isolated strains (152) produced a reduction and peptonization of the medium within the seven day period, while one strain showed no reaction and the rest of the strains (10) showed only reduction.

4.4.5 Methylene Blue milk reaction

Of the 163 strains of Vibrio anguillarum, the majority (approximately 80%) showed no reaction on the medium after seven days.

Of a total of 38 positive strains, 33 caused complete reduction of the dye, while five strains showed both reduction and peptonization of the medium.

4.4.6 Production of indole

As shown in Table 10 a total of 43 strains did not produce indole, while the majority of the strains (118) were positive.

Table 10

Production of Indole

Species of origin	All negative	All positive		Variable reactions (negative without NaCl added, rest positive)	
		weak	strong	weak	strong
Rainbow trout	15	-	-	24	37
Atlantic salmon	13	1	4	15	23
Sea trout	3	-	-	1	1
Saithe	11	-	3	-	1
Cod	-	-	2	-	2
Flatfishes	1	-	-	1	1
Ciona intestinalis	-	-	-	-	2
Total	43	1	9	41	67

In addition to this, two strains of rainbow trout origin were positive with 0.9% NaCl, but negative with the others.

4.4.7 Utilization of citrate

35 strains utilized citrate as the sole carbon source, while the majority (128) were negative. Table 11 shows the distribution of the citrate positive strains.

Table 11

Distribution of citrate positive strains

Species	
Rainbow trout	18
Atlantic salmon	14
Sea trout	1
Saithe	2
Total	35

4.4.8 Urease production

Of the 163 strains tested 23 produced the enzyme urease. Table 12 shows the distribution of the positive strains.

Table 12

Distribution of urease positive strains

Species of origin	
Rainbow trout	5
Atlantic salmon	15
Sea trout	1
Cod	1
Ciona intestinalis	1
Total	23

4.4.9 Methyl red reaction

The vast majority of the isolated strains (154) were negative in the methyl red reactions.

The positive strains (10) included one strain from rainbow trout, one strain from Atlantic salmon, one strain from sea trout, three strains from saithe and two strains from flatfish. (Table 13).

Table 13

Methyl red reaction

Species of origin	All negative	All positive	Positive except without salt
Rainbow trout	72	1	-
Atlantic salmon	55	-	1
Sea trout	4	-	1
Saithe	11	1	3
Cod	4	-	-
Flat fishes	1	-	2
Ciona intestinalis	2	-	-
Total	154	2	7

4.4.10 Voges Proskauer reaction

The majority of the strains (136) were positive when NaCl was added to the medium. The distribution of positive and negative strains is shown in Table 14.

Table 14

Voges Proskauer Reaction

Species of origin	All negative	All positive	Positive except without salt
Rainbow trout	2	9	67
Atlantic salmon	1	8	47
Sea trout	-	1	4
Saithe	1	1	13
Cod	1	-	3
Flatfishes	1	2	1
Ciona intestinalis	-	-	2
Total	6	21	136

4.5 Proteinase production and serology

The results of these investigations revealed a close relationship between the suggested archetype for Vibrio anguillarum and the isolates from diseased fish from Norwegian fish farms, which all turned out to be enzymo-serologically closely related. The reactions obtained were that of complete inhibition or partial inhibition of the enzymes within the area of immunoglobulins (Figs. 26, 27). The specific proteinase antibodies are located in the area of the application line where the immunoglobulins are

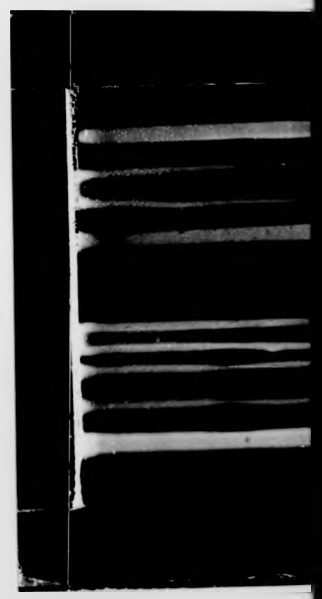
situated under the electrophoretic conditions used. The normally occurring proteinase inhibitors in the serum are seen on the anode side of the application line.

Similar tests with the same antiproteinase serum were carried out with other organisms like Aeromonas liquefaciens, Aeromonas salmonicida, Bacillus cereus, Pseudomonas fluorescens and Vibrio cholerae.

There was evidence of enzymoserological relationship as manifested by partial inhibition at the site of application between Vibrio anguillarum and Vibrio cholerae as shown in Fig. 26c, but there was no inhibition of the precipitates by any of the none vibrionic organisms tested. This has previously been mentioned by Håstein and Holt (loc.cit.). For the enzymoserological patterns see Figs. 26 and 27.

Fig. 26 Electrophoretic patterns for antiserum against the casein precipitating enzyme complex of Vibrio anguillarum (ATCC 19264) transferred to sodium caseinate agar. Developments were performed with different Vibrio anguillarum strains, Vibrio cholerae (ATCC 14734), Aeromonas liquefaciens, Pseudomonas fluorescens and Bacillus cereus. The enzymoserological reactions are, from the top:

a) Vibrio anguillarum, Collection No. 520, complete inhibition
b) Vibrio anguillarum, Collection No. 674, partial inhibition
c) Vibrio cholerae (ATCC 14734), partial inhibition
d) Aeromonas liquefaciens, no inhibition
e) Pseudomonas fluorescens, no inhibition
f) Bacillus cereus, no inhibition



A.P.: Line of application

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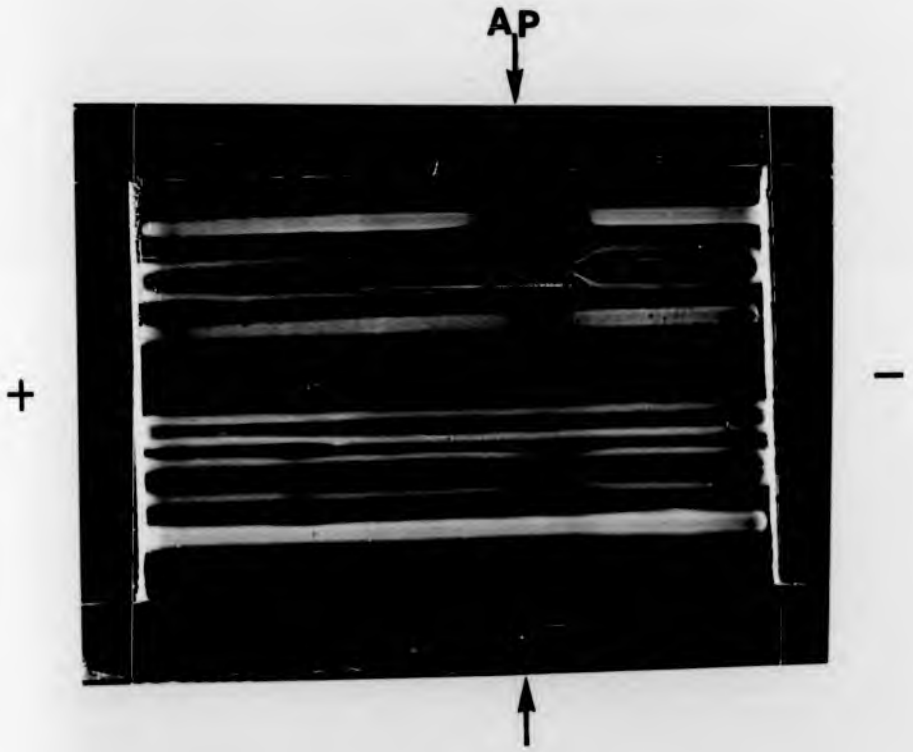

Fig. 27 Electrophoretic patterns for antiserum against the casein precipitating enzyme complex of Vibrio anguillarum (ATCC 19264) transferred to sodium caseinate agar. Developments were performed with different strains of Vibrio anguillarum isolated in connection with vibriosis in Norwegian fish farms and Bacillus cereus as a negative control. The enzymoserological reactions are from the top:

a) Partial inhibition indicating at least two enzymes
b) No inhibition (Bacillus cereus)
c) Complete inhibition
d) No inhibition (Bacillus cereus)
e) Complete inhibition
f) Complete inhibition

A.P.: Line of application

antiserum
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m (ATCC 19264)
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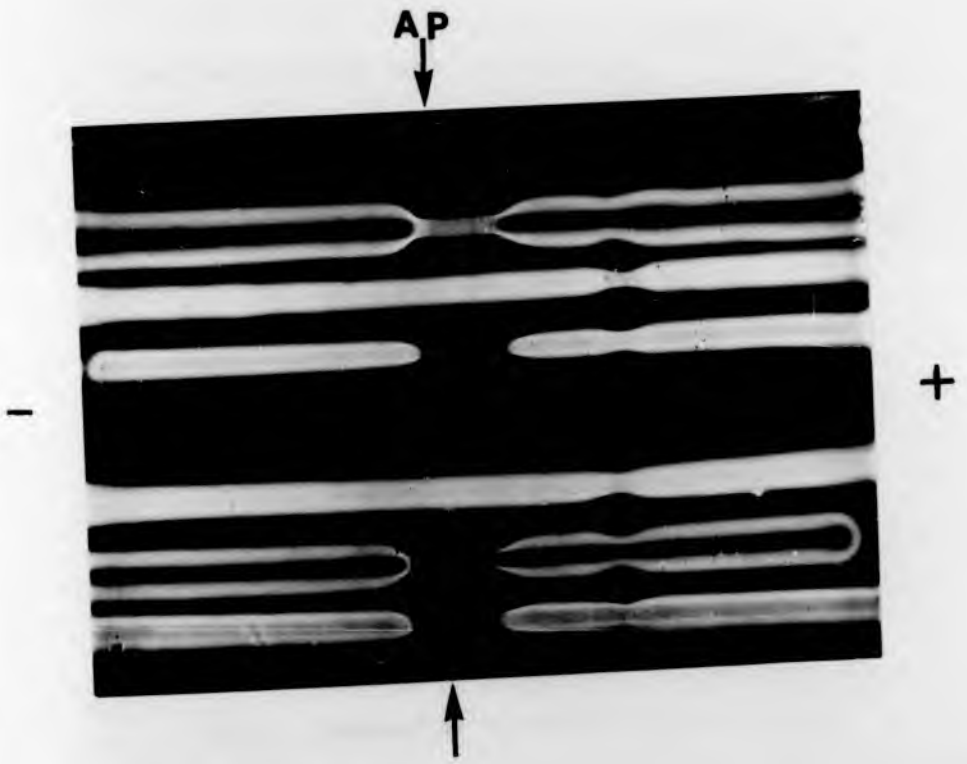

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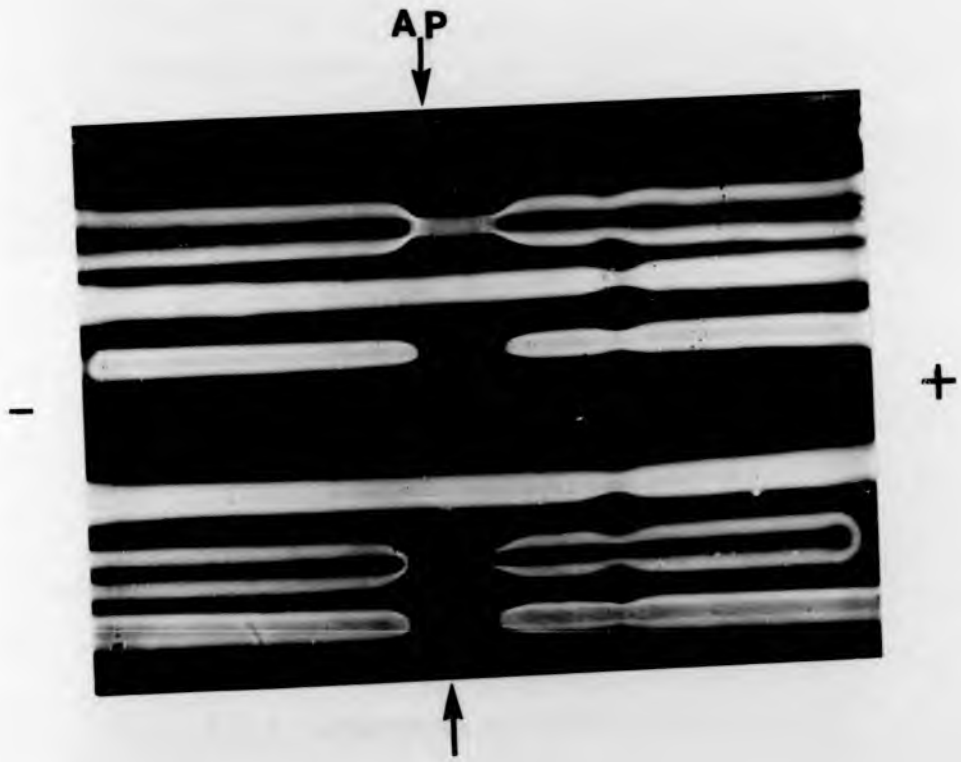

SECTION C

A COMPUTER ANALYSIS STUDY OF THE DATA OBTAINED IN SECTION B

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2. COMPUTER PROGRAMS USED IN THE STUDY
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1. REVIEW OF LITERATURE ON NUMERICAL TAXONOMY

Classification of bacteria is a difficult task because they have few obvious phenotypic characters. The first taxonomists working on bacteria, based their classification of micro-organisms on certain biochemical and morphological characteristics. However, such classifications may be somewhat arbitrary and easily break down because of the large number of exceptions to the proposed rules which may exist.

Thus Nybelin (loc.cit.) described two different varieties of Vibrio anguillarum, based on the organism's reaction in a few biochemical tests, and later Smith (1961) proposed a third variety of the same bacterium based on the same criteria (see Table 1, page 24).

Today classifications based on phenotypic features are no longer satisfactory for taxonomic purposes. Therefore microbiologists working with taxonomical problems have looked for alternative methods to improve the classification of bacteria.

An acceptable alternative to the conventional taxonomy was the development of so-called "Numerical Taxonomy" i.e. the use of mathematical methods to classify organisms on their overall similarity to one another. This method allows a more rational approach to the problem and is described in detail by Sokal and Sneath (1963).

The pioneer study on numerical taxonomy of micro-organisms was performed by Sneath (1957 a+b) and was

applied to the genus Chromobacterium.

Sneath was looking for a method of classifying this group and became aware of the work of Adanson (1763), a French botanist, (1726-1806) who had suggested that equal taxonomic weight should be given to all characteristics of an organism. Adanson put forward this suggestion because of difficulties in getting a satisfactory classification of the flora of Senegal, and therefore abandoned the current taxonomical system of his time, proposing instead what he called "la methode naturelle".

Sneath's acceptance of Adanson's earlier suggestions was not received with unanimous enthusiasm (Roberts 1968, Lockhardt and Liston 1970), and Leifson (1961) critically reviewed Sneath's and other workers' publications on numerical taxonomy.

The resulting classification system used by Sneath was designated "Adansonian" in recognition of its progenitor, but as Liston (1970) stresses, numerical taxonomy is not necessarily Adansonian.

According to Roberts (loc.cit.) this Adansonian classification system rests on five rules:

1) The taxonomy should be based on as many features as possible and provide greatest content of information.

2) In classification every feature is of equal importance.

3) Overall similarity depends on the proportion of total characteristics that are shared by the compared strains.

4) Distinct taxa are based on correlative features.

5) Affinity is to be considered independently of phylogenetic considerations.

Since Sneath's original work on Chromobacterium, many other workers have applied the method of numerical taxonomy for classification of bacteria and viruses.

Smith (1963) carried out a classification of Bacterium salmonicida on the basis of different features. Although several other authors, including Griffin et al. (1953), Eddy (1960, 1962), Ewing et al. (1961) and Schubert (1961) had classified the organism as belonging to the genus Aeromonas, she retained the name Bacterium salmonicida and compared 42 strains of this organism with 42 Aeromonas strains.

Evaluation of the tests showed that Bacterium salmonicida could be split up into two groups, i.e. pigmented and non-pigmented and that these groups, when compared with Aeromonas, failed to produce 2-3 butanediol and exhibited lack of vigorous gas production and motility.

She therefore suggested that a new genus should be formed in Pseudomonadaceae to accommodate Bacterium salmonicida and its nonpigmented variants, and she proposed the generic name Necromonas. However, that name has not yet gained acceptance among bacteriologists working with fish pathogens.

Notable among the later demonstrations of a successful application of numerical taxonomy is the work of Talbot and Sneath (1960), Smith and Thal (1965) on Pasteurella, Colman (1960) on Streptococci, and the clarification of the relationship between the strains of Influenza B virus obtained by Lee (1968).

Allen and Pelczar (1967) characterized a total of 169 cultures from white perch (Roccus americanus) by numerical taxonomy to determine phenotypic similarity. These cultures were sorted into seven major groups and were identified as belonging to Bacillus, Achromobacter, Pseudomonas, Aeromonas, Enterobacter and Micrococcus. A pasteurella-like bacterium implicated in a massive mortality among white perch was included in the analysis, but none of the above mentioned cultures gave an "S"-value greater than 67% when compared with the pasteurella-like strains, which had much higher mutual affinity levels.

A work of great importance to the current study is that of Simidu and Kaneko (1973) on the taxonomy of Vibrio and Aeromonas from normal and diseased fish.

They studied 114 strains of Vibrio and allied genera including 28 strains from diseased fish and shellfish. The results were determined as 190 two state features and pheno-typical similarity between the strains were calculated according to the matching coefficient of Sokal and Michener (loc.cit.). The mutual relationships among the strains were tested by two methods using hierarchial clusterings on the similarity values and examining the homogeneity by the method of Rogers and Tanimoto (loc.cit.). Both methods showed that the strains could be placed into two large distinct phenons of which both could be regarded as distinct species. Phenon 1 comprised mainly the isolates from diseased fish and shell-fish besides standard strains of Vibrio para-

hemolyticus and Vibrio alginolyticus. Phenon 2 was composed mostly of strains from intestine, gills and luminous organs of normal fish.

2. COMPUTER PROGRAMS USED IN THE STUDY

2.1 Methods

The analysis of the bacteriological data on the properties of the 169 strains tested in the course of the work detailed in Section B was carried out by means of Principal Component Analysis (PCA) as described by Whalstedt and Davis (1968) and by means of Numerical Taxonomy described by Deaville (1966).

Basically the intention was to classify the 163 strains of Vibrio anguillarum isolated from diseased fish from Norwegian fish farms into groups or clusters, and also to determine whether these strains showed any evidence of close relationship to the type cultured strains of Vibrio anguillarum (ATCC 19264 + 14181) or to Vibrio ichthyodermis to the cholera-like vibrion, Vibrio metchnikovi (ATCC 7708) and Cholera vibrion Vibrio cholerae (ATCC 14035), each comprising those strains which had the closest mutual similarity.

The analysis involved five separate processes.

a) Coding of the information acquired in Section B into a form acceptable to the computer programs employed.

b) Calculation of the correlation coefficients between the strains and calculation of the Euclidean distance coefficients between the strains from which the computer produced maps between the strains indicating the inter-relationships of the strains.

c) Calculation of "Coefficients of Similarity" for the strains and construction of a Similarity Matrix, again by use of electronic computation.

d) Grouping of the strains, on the basis of the results yielded by the computer, into clusters or groups of closely related strains.

e) Diagrammatic representation of the clusters in the form of a map of similarity or a dendrogram.

In the case of the PCA the serial numbers 1 - 169 in Table 2 listed in Appendix I were used to define the strains in the computer.

For the Numerical Taxonomy examination the collection of strains was reduced from 169 to 120, which was the maximum number of strains which could be accepted by the machine in the programme used. The elimination of strains for numerical taxonomy was carried out by choosing pairs which appeared close together on the PCA map and adding up the total numerical discrepancy in their coded properties. When a pair was found which had a low discrepancy, the strain having the lower reference was eliminated. Table 15 in Appendix 4 shows the eliminated and retained strains. After the reduction, the strains were renumbered from 1 - 120 and Table 16 in Appendix 4 shows the corresponding enumeration. The print-out in Fig.28 employs the computer enumeration, and in the dendrogram in Fig.32 this enumeration has again been used.

2.2 Coding of information

The computer accepted data in several forms.

a) Qualitative information, e.g. Voges-Proskauer reaction, an all or nothing type of reaction, scored by the computer as 1 or 0.

b) Quantitative information, e.g. measurement of cell length which was coded from 1 - 4 according to the description in Table 32.

c) Multistate characters, where rank is unimportant, e.g. the litmus milk reaction, where results are coded as 0, 1 or 2 (see Table 32).

Tests showing no variance, i.e. those tests which gave the same results for all of the strains, were eliminated. Twenty-eight tests remained and the complete list of tests used in the PCA and Numerical Taxonomy together with the form in which they were presented to the computer is listed in Table 9.

2.3 Computation by Principal Components Analysis (PCA)

a) Computation of the strains of Vibrio anguillarum surveyed by means of PCA was carried out by courtesy of Professor J.E. Smith, using an ICL 1905 F computer. The programme used was a Fortran IV programme for computation and display of principal components. (Wahlstedt and Davis, loc.cit.). A synopsis of this program appears in Appendix 2.

The principle of this method is that if several variables are measured on a set of samples, a linear trans-

formation of these variables can be obtained. This will result in new variables (eigenvectors) which are independent and account for, successively, as much of the total variation as possible. These new variables are called principal components and the process of computing the transformation is Principal Components Analysis (PCA).

By transforming new data into principal components, independence is achieved and the new transformed variates can be tested. Thus a few principal components may account for a large amount of the total variance in the data, and the nature of these important components can be deduced from an examination of the linear transformation, the so-called Euclidean distance.

2.4 Computation by numerical taxonomy

2.4.1. Similarity coefficients

This was also carried out by courtesy of Professor J.E. Smith, using the NUMTAX programme designated by Deaville (loc.cit.). A synopsis of the program appears in Appendix 3.

The NUMTAX programme causes the computer to calculate the percentage similarity coefficient between each strain and all of the others.

The first print out of results by the computer, was an unsorted "Similarity Matrix" correct to the first decimal place. Part of the "Similarity Matrix", ("S" values), for the present study is reproduced in Fig.28.

Fig.28 Example of a typical portion of the similarity matrix produced by the computer. Strains are listed both vertically and horizontally and "S" values for each pair of strains are thus found by cross reference.

NUMERICAL TAXONOMY

SIMILARITY MATRIX

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2.4.2 Cluster grouping

The grouping of the strains into sets based on their mutual similarity was carried out again by the NUMTAX computer programme, by the form of cluster analysis. In this process the strains are rearranged so that those having a high mutual similarity were grouped together.

Among methods commonly used for cluster analysis are Single Link Listing (S.L.L.) or Single Link Grouping (S.L.G.), both methods being built into the NUMTAX programme.

Although both methods were carried out on the material only the S.L.L. will be described in detail. However, the results gained in the S.L.G. will be dealt with in Chapter 3.6, in which the different methods are compared with each other.

The S.L.L. technique was devised by Smith (1964) and successfully used by Roberts (1968) in a study similar to the present one on Corynebacterium pyogenes.

The mechanics of Single Link Listing as described by Roberts (1968) were performed as follows: the unsorted similarity matrix stored in the computer was scanned horizontally and a list of all pairs of strains with the similarity values arranged from the highest mutual similarity value, was used to find nuclei for the delineation of clusters. At progressively falling similarity levels, those strains which had a similarity to the first or last strain in the cluster were added to the cluster, becoming themselves

"end lines". The procedure involves the simultaneous building up of several clusters from basic pairs, which are the pairs of strains showing the highest degree of mutual similarity.

When basic clusters have been established the remaining strains are added one by one to the cluster with whose end links they show the greatest similarity value.

Similarity with strains which are not end links, does not permit entry to that cluster. This is therefore the basic difference between S.L.L. and S.L.G., since the criterion for admission to a cluster by the latter method is similarity to any strain in the group, not only to the two terminal strains.

2.4.3 Diagrammatic presentation of cluster analysis

There are several ways of displaying the results of clustering e.g. bar diagram, shaded similarity matrix or dendrogram.

In the present study the "tree diagram" or dendrogram was used. Some programmes have a facility for plotting dendrograms, but the NUMTAX programme does not. The dendrograms were therefore drawn by hand. To construct a dendrogram the rearranged sequence of strains obtained by the cluster analysis was disposed along the abscissa.

A line was vertically drawn from each strain until it reaches the "S" level at which at which it joins another strain. A crossbar was drawn between these vertical

lines. When a pair or cluster was formed, a single line was dropped from it which in due course gave another fusion and another crossbar drawn. This procedure continued at appropriate falling "S" levels until finally all strains had been linked into one cluster and a single line drawn as desired.

The dendrogram was thus a taxonomic hierarchy and though having certain drawbacks, was a convenient summary such that the branches could be labelled with the strains in the hierarchy, and larger clusters could be recognised and designated as groups.

2.5 Calculation of group characteristics

This was done, again by computer; using a further programme, GROUP CHARACTERS (Harris, 1973).

The computer was used to characterize the PCA groups by showing, for each variable (test):

1) Frequency distribution of results
2) Mean value
3) Standard error
4) Standard deviation
5) Skewness
6) Kurtosis

The purpose of this was to provide information for comparing the groups and work out a summary of the major differences between the groups.

3. RESULTS OF COMPUTER ANALYSIS

3.1 Principal Components Analysis (PCA) maps

The principal component analysis was carried out by two different methods and the results so obtained were used to cause the computer to produce PCA maps.

The PCA maps produced, depicted the relationships of the tests and the position of the strains. The maps were written on the basis of eigenvectors. It seemed on inspection that in both correlation coefficient and Euclidean distance coefficient the best groupings of the strains were made by eigenvector 1 and 2.

The eigenvector 1 (horizontal) appeared to sort mainly on basis of chloramphenicol sensitivity and sensitivity to the vibriostatic agent O/129. This eigenvector also seemed to utilize the occasional positives to methyl red, urea, raffinose, dulcitol, xylose, salicin and inositol.

Eigenvector 2 (vertical) appeared to be a better taxonomic one. It used indole, arabinose, trehalose and cellobiose.

3.1.1 Definition of groups

The PCA chosen for subsequent work was the one using Euclidean distance coefficients: this method gave two groups compared with five groups yielded subsequently by numerical taxonomy and seemed better because it allowed inclusion of all the strains. Fig. 29 is a map showing distribution of strains between the two groups.

Fig. 29 PCA map of Vibrio anguillarum isolated from fish. Horizontal eigenvector = 1, vertical eigenvector = 2

rum isolated

envector = 1,

The groups were defined by inspection of the map and are thus semi-subjective.

As can be seen from Fig. 29, strain 155 and 158 could be recognized as intermediate strains.

The constituent strains of the two groups were as in Table 17.

Table 17

Group I

1	2	3	4	5	6	7	8	12	13	14	15	19
20	21	22	23	24	25	26	27	28	29	30	31	32
33	34	35	36	37	38	39	40	42	44	45	46	47
48	49	50	51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70	71	72	73
74	75	76	77	78	79	80	81	82	83	84	85	86
87	88	89	91	94	95	96	97	99	100	101	102	103
104	105	107	108	109	110	111	112	113	114	115	116	117
121	122	123	124	125	126	127	128	129	130	131	133	137
150	151	153	155	157	158	159	162	163	164	165	166	169

(130 strains)

Group II

9	10	11	16	17	18	41	43	90	92	93	98	106
118	119	120	132	134	135	136	138	139	140	141	142	143
144	145	146	147	148	149	152	154	156	160	161	167	168

(39 strains)

3.1.2 Information provided by the PCA map

a) Relationship between the computer defined groups and host species of origin

Table 18 indicates the distribution of the strains of Vibrio anguillarum from the different fish species among the PCA defined groups. The table also includes the two ATCC strains of Vibrio anguillarum, Vibrio ichthyodermis and the cholera vibrions in the groups (see Table 2).

The table shows that most of the strains from certain of the fish species fell in the same group, e.g. 90 per cent of strains from rainbow trout appeared in group I and 10 per cent in group II, while strains from saithe had the opposite distribution, 20% and 80%.

Such a distribution of the strains appears unlikely to have occurred by random distribution.

When, as shown in Table 19, the distribution of the strains from diseased wild fish is examined, it seems that there is a more even distribution of the strains, indicating that none of the groups should be recognized as specific for wild fish. The table also shows that there is an approximately equal distribution of the strains from the Atlantic salmon within the two groups.

Table 20 shows the distribution of strains from farmed fish amongst the PCA defined groups. The striking feature here is that approximately 90% of the strains from rainbow trout and Atlantic salmon are distributed in PCA

Table 18

Distribution of strains from different host species amongst the PCA defined groups

Host species	Total strains	PCA defined groups * I	II
Rainbow trout	78	70 (90)	8 (10)
Atlantic salmon	56	46 (82)	10 (18)
Sea trout	5	1 (20)	4 (80)
Saithe	15	3 (20)	12 (80)
Cod	4	3 (75)	1 (25)
Flatfishes	3	1 (33.3)	2 (66.7)
Ciona intestinalis	2	2 (100)	0 (-)
ATCC strains of *V. anguillarum*	2	1 (50)	1 (50)
Vibrio ichthyodermis	2	2 (100)	0 (-)
Vibrio cholerae spp.	2	1 (50)	1 (50)
	169	130	39

* The number of strains is given, followed in brackets by the percentage in relation to the total number of strains in the defined groups.

Table 19

Distribution of strains from different wild species of fish amongst the PCA defined groups

Fish species	Total strains	Groups I	Groups II
Atlantic salmon	11	6 (54.5%)	5 (45.5%)
Sea trout	1	1 (100%)	0 (-)
Saithe	15	3 (20%)	12 (80%)
Cod	4	3 (75%)	1 (25%)
Flatfishes	3	1 (33.3%)	2 (66.7%)
All species	34	14 (41.2%)	20 (58.8%)

Table 20

Distribution of strains from farmed fish amongst the PCA defined groups

Fish species	Total strains	Groups I	Groups II
Rainbow trout	78	70 (89.7%)	8 (10,3%)
Atlantic salmon	44	40 (90.9%)	4 (9.1%)
Sea trout	4	0 (0%)	4 (100%)
All species	126	110 (87.3%)	16 (12.7%)

group I, indicating that there may be a specific group of Vibrio anguillarum strains responsible for the disease in farmed fish.

In the 25 cases where several strains originated from the same farm site from different outbreaks of vibriosis, the PCA map in Fig. 29 showed that in almost all cases the strains belonged to the same group. Only in four cases was there a distribution of strains in both groups. In farm M strain 9 from a rainbow trout differed from the two strains from Ciona intestinalis (strain 162, 163) which had been taken from the nets enclosing the fish. In diseased wild Atlantic salmon from river G (strains no. 91, 92, 93, 97, 98) the distribution was two strains in group II and three strains in group I.

The PCA map also showed that the two ATCC strains were placed approximately in the middle of each group.

The ATCC strain 19264 of Vibrio anguillarum originating from Bagge and Bagge's isolates (loc.cit.) from ulcers in cod, fell into PCA group 1, the same group as that in which 75% of the isolates from cods in the present study were placed (Table 19). The ATCC strain 14181, originating from Isabel Smith's (loc.cit.) isolates from Atlantic salmon was placed in PCA group II.

The PCA also showed that Vibrio metchnikovi was far removed from either group, but that Vibrio cholerae was more closely connected to group I, although in the periphery of the group. Both strains of Vibrio ichthyodermis were placed in the outer region of the group I.

b) Geographical distribution of the strains according to PCA group.

As can be seen from Fig. 30, there is no specific geographical distribution of the strains in the different groups. Both groups are distributed along the coast from South to North and are also found close together in the same regions. However, Fig. 30 also shows that in the Frøya region where there is a heavy concentration of fish farms, group I is the only group found, suggesting that the infection may have spread from farm to farm.

c) Distribution of strains from different pathological conditions among the PCA defined groups

A further principal components analysis was carried out on individual Atlantic salmon using the pathological criteria listed in Table 21 in Appendix 4. The results of these tests are shown in Fig. 31. As can be seen, there is an even distribution of the two PCA groups which clearly indicates that there is no correlation between specific disease condition and any of the groups. Rationalisation of the print-out, however, indicates that vibriosis can be divided into four main clinico-pathological groups namely 1) no pathological changes, 2) boil lesions, 3) skin ulcers and 4) fin rot, but with many strains producing intermediate conditions which were not closely related to any of the major groups.

These four clinico-pathological criteria were used to scan the strains from rainbow trout in the PCA groups for correlation between pathological features and the groups.

Fig. 30 The map shows the distribution of the PCA defined groups I and II. Group I is marked red, group II white: in cases where the places of origin of the fish were not known, the strains from these fishes were not plotted on the map.
When several strains originated from the same fish farm, only one plot was made to indicate the position, thus accounting for the discrepancy between the number of isolated strains and the number of plots on the map.

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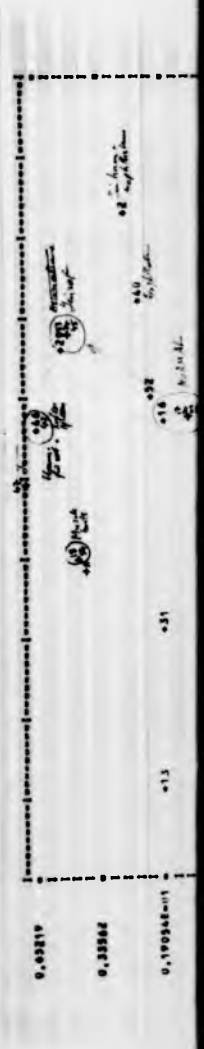


Fig. 31 Principal Components Analysis of disease symptoms from Atlantic salmon suffering from vibriosis

sis of disease

mon suffering

Although no satisfactory correlation was obtained, it was shown that PCA group II was considerably more common in fish showing no pathological changes, than in diseased fish with pathological changes i.e. 25 per cent against 2.7 per cent (Table 22).

Table 22

PCA group distribution of rainbow trout with and without pathological changes

Rainbow trout	Group I	Group II	Total
Diseased	36 (97.3%)	1 (2.7%)	37
No change	15 (75%)	5 (25%)	20

Diseased Atlantic salmon were most frequently disposed within the PCA group II. This was especially so with those Atlantic salmon showing boil lesions (Fig. 31).

Among saithe showing pathological changes, 30 per cent of the isolates fell into PCA group I and 70 per cent into PCA group II, while 100 per cent of those with no changes fell into PCA group II.

From the above, it must be concluded that none of the PCA defined groups allows simple correlation of group with specific clinico-pathological features among diseased fish.

3.2 Summary of major differences between the PCA defined groups

Table 23 shows the percentage distribution and the number of strains for the variables tested in the two PCA groups. As can be seen from the table, there is no difference between most of the variables tested, but a few tests showed considerable variance.

A more highly summarized differentiation between the groups is shown in Table 24.

Table 24

PCA groups: Summary of major differences

Variable	Group	
	I	II
Citrate	V	-
Arabinose	+	-
Lactose	V	-
Cellobiose	+	V
Trehalose	V	+

+ = 90% or more

- = 10% or less

V = between 10% and 90%

Table 23

Principal Components Analysis groups

Variable	I			II		
	No. +	% +	Mean	No. +	% +	Mean
IND O-2	116	89.2		9	23.1	
VP O-1	125	96.1		38	97.1	
MR O-1	4	3.1		8	20.5	
UREA O-1	20	15.4		2	5.1	
CITR O-1	35	26.9		0	0	
DULC O-2	1	0.77		0	0	
SORB O-2	126	96.9		35	89.7	
ARAB O-2	127	97.7		3	7.7	
XYLO O-2	3	2.3		1	2.6	
SALI O-2	1	0.77		0	0	
INOS O-2	1	0.77		1	2.6	
LACT O-2	45	34.6		1	2.6	
SUCR O-2	128	98.5		39	100	
MANN O-2	127	97.7		39	100	
RAFF O-2	0	0		1	2.6	
DEXT O-2	130	100		38	97.4	
GALA O-2	130	100		33	84.6	
CELB O-2	128	98.5		5	12.8	
TREH O-2	21	16.1		38	97.4	
GLYC O-2	129	99.2		37	94.9	
LM O-2	128	98.5		37	94.9	
MBM O-2	28	21.5		9	23.1	
VIBR O-4			1.47			1.69
CHLO O-4			3.58			3.67
TETR O-4			2.75			2.79
SULP O-4			2.44			2.72
BORG O-4			2.03			2.15
SIZE			1.85			1.90
No. in group	130			39		

Table 24 shows that the five variables of citrate, arabinose, lactose, cellobiose and trehalose should represent the major differences between the groups. Indole reaction might also however be included as a useful test.

3.3 Dendrogram

The data obtained in section B of this study, when processed by computer using the NUMTAX programme devised by Deaville, followed by manual cluster analysis by the Single Link Listing technique, provided information that could be used to construct the dendrogram shown in Fig. 32.

The computed similarity levels ranged from 70% to 100%.

These values are indicated in the dendrogram as horizontal lines at the correct level, linking the strain stems. Certain strains e.g. 63 and 12 were joined in the dendrogram at the 100% level indicating that they were identical.

3.3.1 Definition of groups

The 88.7 per cent level was selected as a convenient level for defining groups as at this level there are five "good" groups, ignoring three groups of 2 or 3 strains each and twelve ungrouped strains.

The constituents of the five groups were as in Table 25.

Table 25

Group I

16 17 98 118 120 136 143 167

Restored strains 41 92 93 119

(total 12 strains)

Group II

10 11 43 90 135 142 145 146 148 149 152 154 160

Restored strains 106 132 140 141 144 147

(total 19 strains)

Group III

8 23 32 35 37 39 40 45 58 60 62 71 73

82 89 95 99 105 113 129 130 150 151 153 155 157

158 159 163 166

Restored strains 13 21 29 31 51 56 57 72 78

94

(total 40 strains)

Group IV

25 36 42 67 127

Restored strains 1 24 52

(total 8 strains)

Group V

12 14 15 22 26 28 33 34 38 46 50 54 61

63 64 68 70 76 79 80 83 84 85 86 88 96

100 101 103 104 107 109 110 112 114 115 116 121 122

125 126 128 137 162

Restored strains 2 4 5 6 7 19 20 27 30

47 48 49 53 55 59 66 69 74 75 77 111 123

131

(total 67 strains)

Ungrouped at 88.7%

3 9 18 65 81 91 97 102 108 117 124 133 134

139 156 161 164 165 168 169

Restored 44 87 138

(total 23 strains)

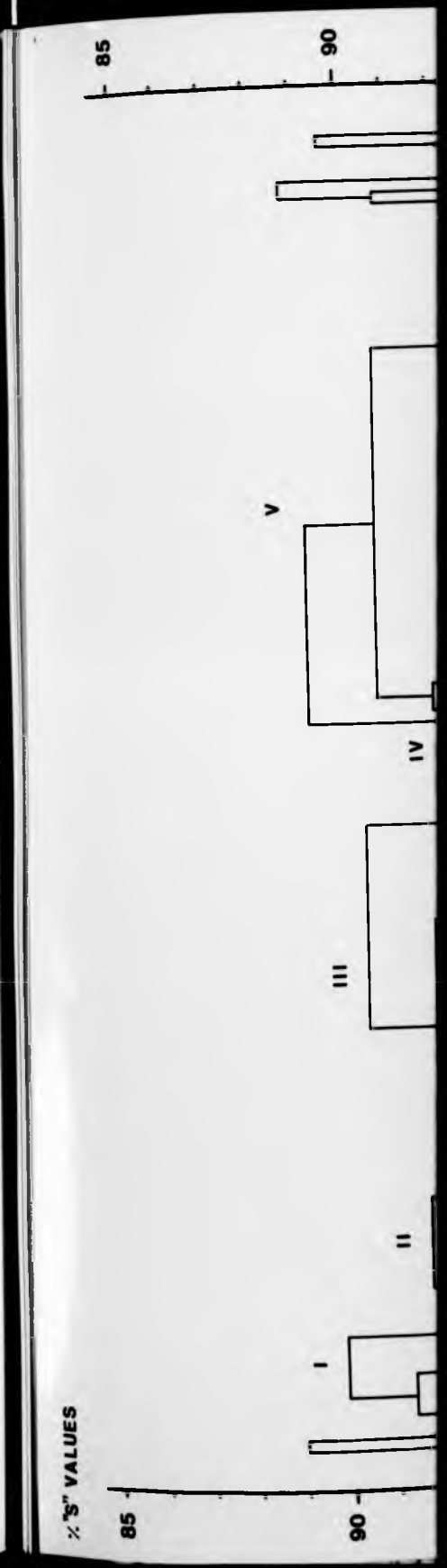
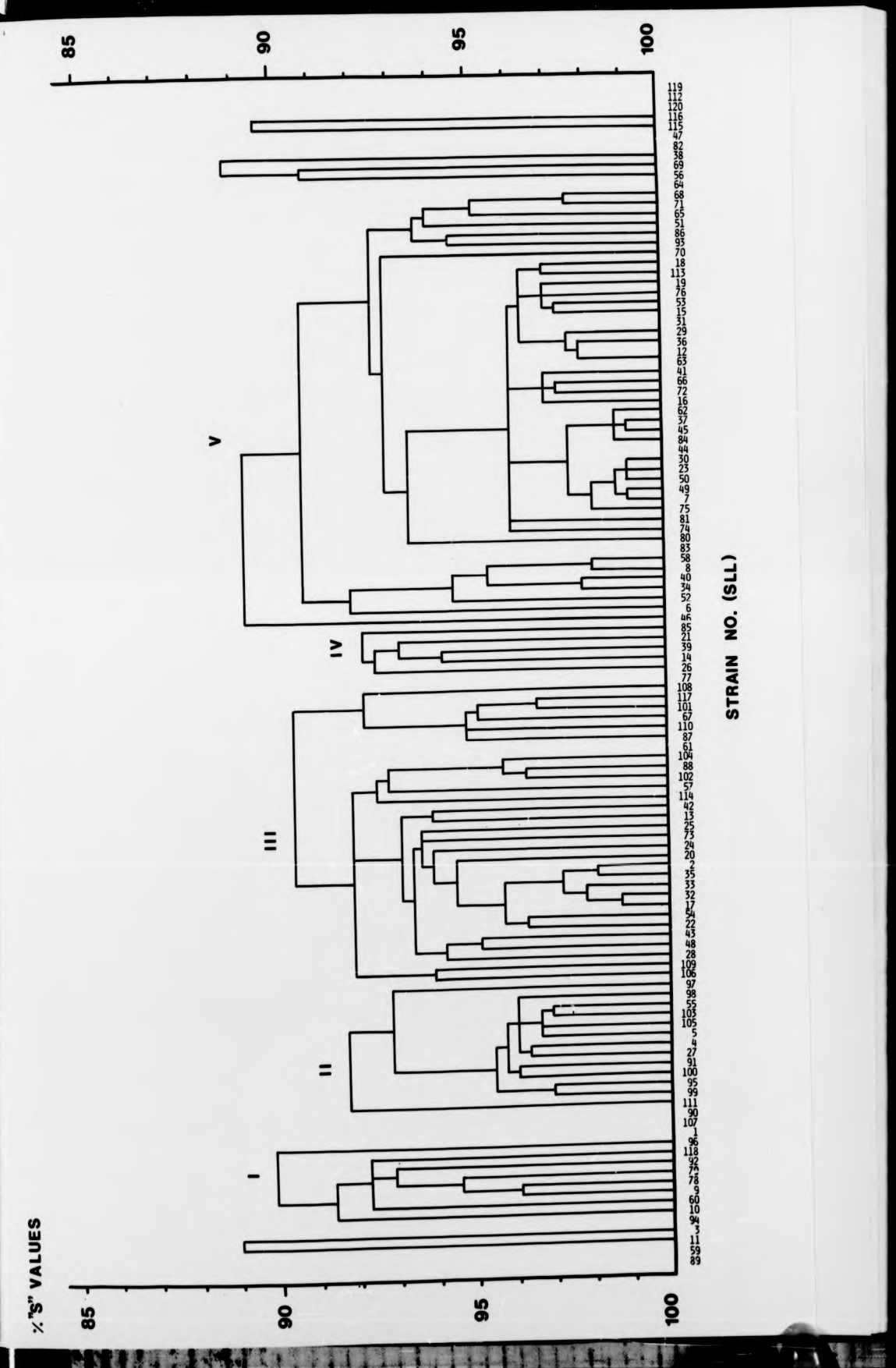


Fig. 32 The results of Single Link Listing of the computer defined "S" values for 163 strains of Vibrio anguillarum expressed in the form of a dendrogram

g of the

63 strains

n the form



3.3.2 Information provided by dendrogram

a) Relationship between computer defined groups and fish species of origin

Table 25 indicates the distribution of strains from the different fish species among the dendrogram defined groups.

One of the most striking features of that table was that almost all of the strains from saithe were placed in group II and accounted for more than fifty per cent of the total number of strains in that group.

The isolates from rainbow trout and Atlantic salmon were mainly distributed in groups III and V and in these groups the salmonids counted for approximately eighty and one hundred per cent respectively.

The few strains from flatfishes examined were evenly distributed, while most of the strains from cod (75%) were placed in group III.

Another striking feature was that both strains of Vibrio ichthyodermis fell into the ungrouped lot.

Both cholera vibrions also fell into the ungrouped lot; this would be expected since on taxonomic grounds its relationship to Vibrio anguillarum would be expected to be distant.

When examining the distribution of strains within the same farm, there does not appear to be the even distribution that was found in the PCA defined groups.

However, there was a systematic pattern in that strains of groups III + V, or IV + V were more frequently found together in any one site of origin. A list of the group distribution within farms is given in Table 27.

Table 26

Distribution of strains from different host species amongst the computer defined groups

Host species	Total species	Computer defined groups * I	II	III	IV	V	Ungrouped at 88.7% level
Rainbow trout	78	3(3.8)	3(3.8)	22(28.1)	7(9.-)	38(48.9)	5(6.4)
Atlantic salmon	56	6(10.7)	3(5.3)	9(16.-)	1(1.7)	27(48.5)	10(17.8)
Sea trout	5	1(20)	1(20)	0(-)	0(-)	1(20)	2(40)
Saithe	15	1(6.7)	11(73.3)	3(20)	0(-)	0(-)	0(-)
Cod	4	0(-)	0(-)	3(75.-)	0(-)	0(-)	1(25.-)
Flatfishes	3	0(-)	1(33.3)	1(33.3)	0(-)	0(-)	1(33.3)
Ciona intestinalis	2	0(-)	0(-)	1(50)	0(-)	1(50)	0(-)
ATCC type cultures of Vibrio anguillarum	2	1(50)	0(-)	1(50)	0(-)	0(-)	0(-)
Vibrio ichthyodermis	2	0(-)	0(-)	0(-)	0(-)	0(-)	2(100)
Cholera vibrions	2	0(-)	0(-)	0(-)	0(-)	0(-)	2(100)
All species	169	12	19	40	8	67	23

* The number of strains is given, followed in brackets by the percentage in relation to the total number of strains in the computer defined groups.

Table 27

Distribution of Numerical Taxonomy groups within farms

Fish farm	Total No. strains	Group distribution I	II	III	IV	V	Ungrouped
F.E.	4	-	-	2	-	2	-
M.E.	2	2	-	-	-	-	-
S.E.	2	-	1	-	-	-	1
A.F.	2	-	-	1	-	1	-
F.F.	5	-	-	1	-	3	1
F.S.F.	7	5	2	-	-	-	-
R.F.	4	-	-	-	2	2	-
S.F.	5	-	-	1	-	4	-
U.F.	2	-	-	-	1	1	-
River G.	5	3	-	-	-	-	2
E.H.	2	-	-	1	-	1	-
S.H.	2	-	-	2	-	-	-
W.H.	2	-	-	-	1	1	-
H.I.	2	-	1	1	-	-	-
R.F.I.	3	-	-	2	-	-	1
E.J.	3	-	-	-	-	2	1
N.L.H.	2	-	-	1	-	1	-
M.	3	-	-	1	-	1	1
T.M.	27	-	-	2	-	20	5
E.O.	4	-	1	1	-	2	-
E.P.	2	-	-	1	-	1	-
E.S.	3	-	-	1	-	1	1
H.S.	2	-	-	1	-	1	-
I.L.S.	4	-	-	2	-	2	-
R.S.	2	-	-	-	-	-	2

b) Geographical distribution of the defined groups

From Fig. 33 it can be seen that there is no definite geographical distribution of the strains although groups I and IV originated mainly from the Bergen area and northward. Groups II, III, V and the ungrouped strains originated from scattered loci widely distributed along the coastal line. The only three isolates from the northern region of Norway, however, all fell into group III, although it is not possible to draw conclusions from such a small sample.

c) Distribution of strains from different pathological conditions among the defined groups

No definite pathological changes could be related to the different groupings obtained by numerical taxonomy.

3.4 Summary of major differences between Single Link Listing (SLL) defined groups

The computer characterized the S.L.L. defined groups by showing for each variable (test):

1) Frequency distribution of result
2) Mean value
3) Standard error
4) Standard deviation
5) Skewness
6) Kurtosis

Table 28 shows the number of strains and distribution of positives, as a percentage, in each group.

Fig. 33 Geographical distribution of the SLL defined groups. (White: SLL group I, blue: SLL group II, lilac: SLL group III, green: SLL group IV, red: SLL group V and yellow: ungrouped strains.)

the SLL

group I,

L group III,

group V

.)

Table 28

Single Link Listing Groups

Variable	I			II			III			IV			V		
	No.+	% +	Mean	No.+	% +	Mean	No.+	% +	Mean	No.+	% +	Mean	No.+	% +	Mean
IND	4	50		2	15.4		30	100		3	60.0		40	90.9	
VP	7	87.5		13	100		29	96.7		5	100		43	97.7	
MR	1	12.5		3	23.1		0	0		0	0		0	0	
UREA	2	25.0		0	0		1	3.3		0	0		9	20.4	
CITR	0	0		0	0		12	40.0		0	0		10	22.7	
DULC	0	0		0	0		0	0		0	0		0	0	
SORB	8	100		13	100		30	100		5	100		44	100	
ARAB	0	0		0	0		27	90.0		5	100		44	100	
XYLO	0	0		0	0		0	0		0	0		0	0	
SALI	0	0		0	0		0	0		0	0		0	0	
INOS	0	0		0	0		1	3.3		0	0		0	0	
LACT	0	0		0	0		15	50.0		2	40.0		12	27.3	
SUCR	8	100		13	100		30	100		5	100		44	100	
MANN	8	100		13	100		30	100		3	60.0		44	100	
RAFF	0	0		0	0		0	0		0	0		0	0	
DEXT	8	100		13	100		30	100		5	100		44	100	
GALA	5	62.5		13	100		30	100		5	100		44	100	
CELB	0	0		1	7.7		30	100		5	100		44	100	
TREH	8	100		13	100		13	43.3		0	0		0	0	
GLYC	8	100		12	92.3		30	100		5	100		44	100	
LM	6	75.0		13	100		30	100		3	60.0		44	100	
MBM	0	0		3	23.1		9	30.0		0	0		7	15.9	
VIBR			1.00			2.15			1.47			1.20			1.32
CHLO			3.37			3.92			3.47			3.80			3.68
TETR			2.50			3.08			2.70			3.00			2.73
SULP			2.25			3.15			2.30			2.80			2.43
BORG			1.75			2.38			1.93			1.80			2.07
SIZE			2.12			1.85			1.93			2.00			1.86
No. in group	8			13			30			5			44		

Table 29 shows that the five parameters of citrate, arabinose, lactose, cellobiose and trehalose are the main criteria for differentiation.

Table 29

SLL groups: Summary of major differences

Variable	Group				
	I	II	III	IV	V
Citrate	-	-	V	-	V
Arabinose	-	-	+	+	+
Lactose	-	-	V	V	V
Cellobiose	-	-	+	+	+
Trehalose	+	+	V	-	-

+ = 90% or more +

- = 10% or less +

V = between 10% and 90% +

From the table one can see that there is no major difference between groups III, IV and V and that groups I and II are identical on these parameters.

Urea and galactose tests should also be included as a means of differentiation between groups I and II and similarly the urea and trehalose may also be included to give a better differentiation between groups III, IV and V.

3.5 Single Link Grouping

3.5.1 Definition of groups

The percentage similarity level ("S" level) of 92.9 per cent was chosen as probably the best level for examining major groups. This level gives two groups of unequal size plus a number of ungrouped strains.

Twenty eight strains were not grouped at this similarity level.

The constituent strains of the two main groups were as in Table 30.

Table 30

Group I

8	14	15	22	23	25	26	28	32	33	34	35	36
37	38	39	40	42	45	46	50	54	58	60	61	62
63	64	67	68	70	71	73	76	79	80	82	83	84
85	86	88	89	95	96	99	100	101	103	104	105	107
109	110	112	113	114	115	116	117	121	122	125	126	127
128	129	130	137	150	151	153	155	158	159	162	163	166

(78 strains)

Restored strains				1	2	4	5	6	7	13	19	20
21	24	27	29	30	31	44	47	48	49	51	52	53
55	56	57	59	66	69	72	74	75	77	78	94	111
123	131											

(37 strains)

Table 30 (contd.)

Group II

10 11 16 43 90 118 120 135 136 142 145
146 148 149 152 154 167

(17 strains)

Restored strains 41 92 106 119 132 140 141 144 147

(9 strains)

Ungrouped at 92.9%

3 9 12 17 18 65 81 91 97 98 102 108 124
133 134 139 143 156 157 160 161 164 165 168 169

(25 strains)

Restored strains 87 93 138

(3 strains)

3.6 Comparison of results obtained by principal components analysis (PCA) and numerical taxonomy by means of Single Link Listing (S.L.L.) and Single Link Grouping (S.L.G.)

A comparison between the groups within PCA, SLL and SLG was made to examine the concordance between these groupings.

A survey of this computation is shown in Table 31.

In the last part of the table, the equivalence of the groups is shown. As can be seen from this section, the PCA I group strains are equivalent to SLG I and SLL III + IV + V, while the PCA group II strains correspond to SLG II and SLL I + II.

Since the PCA grouping included all the strains, while SLG and SLL left some strains ungrouped, it might be

concluded that PCA is the best of the three methods when planning future work.

Table 31

Concordance between Principal Components Analysis groups and Numerical Taxonomy groups *

PCA group	SLG group *			
	I	II	Ungrouped	Total
I	115	0	15	130
II	0	26	13	39
Total	115	26	28	169

PCA group	SLL groups *						
	I	II	III	IV	V	Ungrouped	Total
I	0	0	40	8	67	15	130
II	12	19	0	0	0	8	39
Total	12	19	40	8	67	23	169

(entries in the concordance tables represent numbers of strains)

Equivalence of groups

PCA	SLG	SLL
I	I	III + IV + V
II	II	I + II

* Using the enlarged groups, with eliminated strains restored by adding them to the groups containing their pair.

CONCLUSIONS

The work described in the preceding pages was carried out to study in detail strains of Vibrio anguillarum isolated from diseased salmonids from Norwegian fish farms and from isolates from wild marine fishes obtained during the routine diagnostic work on fish diseases at the Norwegian National Veterinary Institute. An attempt was made to determine whether there were, within that species, any differences that could lead to a grouping of the strains and if such classification could be correlated to the species of the host fish or to production of particular morbid processes in the fish: also the relationship of Vibrio anguillarum to cholera vibrions was investigated.

As a result of the study, Vibrio anguillarum has been shown to be a closely knit group possessed of many features which are exhibited by all strains.

The results of the enzymoserological examinations showed that all strains contained common casein precipitating enzymes, and that by the casein precipitation inhibition test (CPI) the Vibrio group could rapidly be distinguished from other groups of bacteria such as Aeromonas spp. and Pseudomonas spp.

The CPI test also showed the existence of a relationship between Vibrio anguillarum and Vibrio cholerae.

Adaption of the CPI technique could therefore be a useful tool to facilitate immediate recognition of Vibrio spp. from diseased fish.

Future work should be carried out to elucidate the possibility of using material direct from diseased fish for this test which would be extremely useful from a diagnostic point of view.

Computer analysis of the results of the bacteriological examinations indicated that Vibrio anguillarum strains were linked together at high similarity values, giving good clusters at the 88.7% level.

According to Sneath as cited by Roberts (1968) the usual finding in this type of study is that strains of a single closely knit species are linked into coherent groups at "S" level of 75 - 80%.

The principal components analysis and numerical taxonomy by means of Single Link Listing did not show specific geographical distribution of the defined groups. It did not define any absolute relationship between the biotypes and the host of origin, although most of the salmonid isolates were placed in one PCA group while saithe isolates were mainly in the other PCA group. This does not exclude the possibility of a cross infection between saithe and salmonids taking place, but it does tend to reduce the validity of the fish farmers impression that saithe feeding around cages are the main vectors.

The pathological findings in diseased fish could not be correlated to any of the biotypes in PCA or SLL, but the computation did differentiate four main groups of the disease from a pathological point of view.

From these results a simplified model of the clinical features of the disease could be constructed which allows the syndrome to be delineated in four main forms 1) Fin lesions 2) Deep necrotic haemorrhage in muscle 3) Skin ulcers 4) No gross pathological changes.

In summary, the study has shown that the Vibrio anguillarum isolates from Norwegian fish farms were a close knit group which by means of principal components analysis (PCA) could be divided into two sub-groups, mainly differentiated by five parameters, clearly differing from the criteria put forward by Nybelin (loc.cit.) and Smith (loc.cit.) in their attempts to establish sub-groups within Vibrio anguillarum. (see Tables 1, 21 and 25).

The study indicates that Principal Components Analysis is the best taxonomical approach to such data as presented here since by this method it was possible to split the group into two sub-groups with the five variables citrate, arabinose, lactose, cellobiose and trehalose as key features which represented the major differences between those groups.

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APPENDICES

APPENDIX. TABULATED RESULTS OF SECTION B

Table 2

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
1	257	Rainbow trout	Not reported	W.H.	1964	Swollen kidneys, swollen spleen, skin ulcers
2	258	"	"	I.H.	1964	Swollen kidneys, greyish skin ulcers, swollen, liquified spleen
3	259	"	"	B.U.	1964	Haemorrhagic enteritis, haemorrhagic anus, swollen spleen, boil lesions in musculature
4	261	"	"	B.M.	1964	Swollen liquified spleen, haemorrhagic anus, boil lesions in musculature
5	262	"	"	E.R.	1964	Anemic, greyish skin ulcers, ascites, boil lesions in musculature
6	263	"	"	W.H.	1964	No changes, but cadaverous
7	313	"	"	N.L.H.	1966	Enteritis, haemorrhagic anus
8[x]	339	"	"	N.L.H.	1967	Swollen spleen, boil lesions in musculature, haemorrhagic skin ulcers
9	431	"	Kidney	M.	1968	No pathological changes, but cadaverous
10	433	"	"	L.Ø.	1968	Haemorrhagic anus
11[x]	435	"	"	M.B.	1968	No pathological changes, but cadaverous
12	488	"	Liver	I.L.S.	1968	Petecchiae in musculature, haemorrhagic skin ulcers

Table 2, cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
13	508	Rainbow trout	Not reported	I.L.S.	1969	Finrot with haemorrhage at the base, swollen, liquefied spleen, petecchiae in musculature
14	510	"	Kidney	I.L.S.	1969	Excoriation, but no other pathological changes
15	570	"	"	V.Ø.	1969	Haemorrhagic eye, swollen spleen, swollen liver with petecchiae
16[xx]	571	"	Spleen	M.E.	1969	Swollen spleen
17	575	"	Kidney	M.E.	1969	Swollen spleen
18	629	"	Musculature	E.S.	1970	Skin ulcers with central perforation to underlying tissue
19	630	"	Kidney	D.S.	1970	Boil lesions in musculature, exophtalmus
20	648	"	Liver	E.H.	1971	Liquefied spleen and kidneys
21	668	"	Kidney	N.L.	1971	Haemorrhagic anus, exophthalmos, swollen spleen
22	670	"	"	E.S.	1971	Liquefied spleen, Ichthyosporidium hoferi in heart and liver
23	672	"	"	E.S.	1971	Liquefied spleen, Ichtyosporidium hoferi in heart and liver
24	676	"	"	R.F.	1972	Haemorrhages between fin rays, liquefied spleen, boil lesion in musculature
25	677	"	"	R.F.	1972	Petecchiae in liver
26	694	"	"	R.F.	1972	Petecchiae in liver

Table 2, cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
27	698	Rainbow trout	Kidney	R.F.	1972	Greyish skin ulcers, swollen spleen
28	707	"	"	H.S.	1972	Swollen spleen, petecchiae in musculature
29	709	"	"	F.E.	1972	Swollen spleen
30	710	"	Not reported	U.F.	1972	Swollen spleen, haemorrhages in musculature
31	711	"	Kidney	D.F.	1972	Ichthyosporidium hoferi in heart and liver
32	714	"	Ulcer	H.S.	1972	Skin ulcers with perforations to underlying tissue
33	721	"	Kidney	A.E.	1972	Cadaverous, but no other pathological changes
34	723	"	Liver	F.F.	1972	Greyish skin ulcers
35	724	"	"	E.H.	1972	Haemorrhage in eye, greyish skin ulcers, enteritis, petecchiae in musculature
36	728	"	"	A.C.	1972	Greyish skin ulcers, liquefied spleen
37	731	"	Not reported	F.E.	1972	Skin ulcers with perforations to underlying tissue, liquefied spleen, petecchiae in liver and musculature
38	732	"	"	F.Y.	1972	Excoriation, but no other pathological changes
39	733	"	"	S.K.F.	1972	Ichthyosporidium hoferi in liver

Table 2, cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
40	734	Rainbow trout	Kidney	I.L.S.	1972	Boil lesions in musculature
41	737	"	Not reported	F.S.F.	1972	No pathological changes
42	740	"	"	U.F.	1972	Excoriation, skin ulcers with perfortions to underlying tissue, petecchiae in liver and musculature, liquefied kidneys
43	741	"	"	F.S.F.	1972	No pathological changes
44	743	"	Liver	F.F.	1972	No pathological changes
45	745	"	Kidney	S.F.	1972	Greyish skin ulcers, liquefied spleen and kidneys
46	746	"	"	S.F.	1972	Lipoid liver, liquefied kidneys
47	749	"	"	S.F.	1973	Excoriation, liquefied spleen, dorsal fin destroyed
48	751	"	Musculature	S.F.	1973	Excoriation, liquefied spleen, dorsal fin destroyed
49	754	"	Not reported	E.L.	1973	No pathological changes but cadaverous
50	757	"	Liver	S.F.	1973	Petecchiae in liver
51	794	"	Kidney	T.	1973	No pathological changes but cadaverous
52	796	"	Spleen	E.O.	1973	Haemorrhagic skin ulcers, haemorrhagic anus, liquefied spleen, petecchiae in liver, boil lesions in musculature

Table 2, cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
53	797	Rainbow trout	Kidney	E.P.	1973	Liquefied spleen, petecchiae in liver, boil lesions in musculature
54	801	"	Liver	E.O.	1973	Haemorrhagic skin ulcers, haemorrhagic fins, liquefied spleen
55	804	"	Kidney	T.J.	1973	Liquefied spleen, haemorrhages in musculature
56	806	"	"	N.S.	1973	Haemorrhagic anus, hyperaemia in oral cavity, liquefied spleen
57	807	"	Musculature	S.	1973	Swollen spleen, boil lesions in musculature
58	808	"	Kidney	V.A.	1973	Skin ulcers with perforations to underlying tissue, swollen, liquefied spleen
59	809	"	Musculature	K.E.	1973	Swollen, liquefied spleen, petecchiae in liver, boil lesions in musculature
60	810	"	Kidney	R.F.I.	1973	Swollen spleen, petecchiae in liver
61	811	"	Musculature	J.R.O.	1973	Boil lesions in musculature
62	812	"	Kidney	A.F.	1973	Boil lesions in musculature
63	816	"	Musculature	A.F.	1973	Swollen liquefied spleen
64	818	"	Kidney	H.+S.	1973	No pathological changes, but cadaverous

Table 2 cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
65	819	Rainbow trout	Kidney	S.F.O.	1973	Skin ulcers with perforations to underlying tissue, liquefied spleen, boil lesions in musculature
66	821	"	"	S.F.F.	1973	Excoriatio, caudal fin destroyed, skin ulcers with perforation to underlying tissue, liquefied spleen and kidney, boil lesion in musculature
67	822	"	"	H.+E.	1973	Excoriation
68	823	"	"	E.E.	1973	Excoriation, greyish skin ulcers
69	824	"	"	L.E.	1973	Excoriation
70	825	"	"	O.S.	1973	Excoriation, haemorrhagic eye, liquefied spleen, petecchiae in musculature
71	826	"	"	H.B.	1973	Salmon lice on skin, liquefied spleen, boil lesions in musculature
72	828	"	"	T.K.	1973	Ichthyosporidium hoferi in heart
73	831	"	Musculature	N.E.F.	1973	Excoriation, caudal fin destroyed, skin ulcers with perforation to underlying tissue, boil lesions in musculature
74	836	"	Kidney	G.E.	1973	Liquefied spleen and kidneys
75	839	"	"	A.R.	1973	Haemorrhagic anus, skin ulcers with perforations to underlying tissue, swollen liver, liquefied spleen, haemorrhages in musculature

Table 2 cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
76	840	Rainbow trout	Kidney	R.P.	1973	Skin ulcers with perforations to underlying tissue, swollen spleen
77	841	"	Musculature	L.H.	1973	Excoriation, haemorrhagic anus, haemorrhagic enteritis, liquefied spleen, boil lesions in musculature
78	842	"	Liver	R.F.I.	1973	Liquefied spleen and kidneys
79	285	Atlantic salmon	not reported		1965	Anemic, haemorrhagic skin ulcers, ascites
80	487	"	Kidney	T.M.	1968	Exophthalmos, haemorrhages at bases of fins
81	505	"	Ulcer	T.M.	1968	Fungused ulcers in skin, haemorrhages at bases of fins
82	511	"	Kidney	T.M.	1969	Haemorrhages between fin rays, petecchiae in liver, testis and musculature, liquefied spleen
83	513	"	"	T.M.	1969	Petecchiae in liver, boil lesions in musculature
84	516	"	Liver	T.M.	1969	Haemorrhagic ulcers in skin, degenerated liver, liquefied spleen
85	519	"	Kidney	T.M.	1969	Haemorrhagic skin ulcers, degenerated liver, liquefied spleen

Table 2 contd.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
86	521	Atlantic salmon	Liver	T.M.	1969	Haemorrhagic skin ulcers with perforation to underlying tissue
87	522	"	"	T.M.	1969	Haemorrhagic skin ulcers with perforation to underlying tissue
88	523	"	"	T.M.	1969	Haemorrhagic skin ulcers with perforation to underlying tissue
89	524	"	Ulcer	T.M.	1968	Haemorrhages at base of fins, greyish skin ulcers
90	537	"	Not reported	River S.E.	1969	Boil lesions in musculature
91	572	"	Spleen	River G.	1969	Haemorrhagic enteritis, swollen spleen, haemorrhages in musculature
92	576	"	Musculature	River G.	1969	Haemorrhagic enteritis swollen spleen, haemorrhages in musculature
93	577	"	Kidney	River G.	1969	Haemorrhagic enteritis swollen spleen, haemorrhages in musculature
94	578	"	Eye	Wild S.H.	1969	Keratomalacia
95	579	"	Musculature	Wild S.H.	1969	Keratomalacia
96	590[xx]	"	"	River E.	1969	Boil lesion in musculature
97	592	"	Kidney	River G.	1969	Haemorrhagic anus, liquefied spleen, boil lesions in musculature

Table 2 cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
98	593	Atlantic salmon	Spleen	River G.	1969	Haemorrhagic anus, liquefied spleen, boil lesions in musculature
99	631	"	Kidney	F.J.F.	1970	Karatomalacia intradermal haemorrhages
100	662	"	"	T.M.	1971	Greyish skin ulcers, liquefied spleen, haemorrhages in musculature
101	663	"	Ulcer	T.M.	1971	Greyish skin ulcers, liquefied spleen, haemorrhages in musculature
102	664	"	Spleen	E.J.	1971	Haemorrhagic skin ulcers, petecchiae in liver, liquefied spleen, boil lesions in musculature
103	666	"	Kidney	E.J.	1971	Skin ulcers
104	667	"	"	E.J.	1971	Skin ulcers
105	673	"	"	H.I.	1971	Exophthalmos, haemorrhages between finrays, Petecchiae in skin and liver
106	674	"	Ulcer	H.I.	1971	Exophthalmos, haemorrhages between finrays, petecchiae in skin and liver
107	685	"	Kidney	T.M.	1972	Finrot
108	688	"	"	T.M.	1973	No pathological changes
109	689	"	"	T.M.	1972	Haemorrhagic anus, swollen liver
110	690	"	"	T.M.	1972	Haemorrhagic skin ulcers, haemorrhages at base of fins

Table 2 cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
111	700	Atlantic salmon	Kidney	T.M.	1972	Finrot
112	701	"	"	T.M.	1972	Finrot
113	722	"	"	F.F.	1972	Greyish skin ulcers
114	725	"	Liver	T.M.	1972	Haemorrhagic skin ulcers, haemorrhagic fins, swollen liquefied spleen, boil lesions in musculature
115	726	"	Musculature	T.M.	1972	Haemorrhagic skin ulcers, petecchiae in liver, boil lesions in musculature
116	727	"	Kidney	T.M.	1972	Haemorrhagic skin ulcers, petecchiae in liver, boil lesions in musculature
117	729	"	Liver	T.M.	1972	Haemorrhagic skin ulcers, haemorrhagic fins, petecchiae in liver
118	738	"	Not reported	F.S.F.	1972	Exophthalmos, cachexia
119	753	"	Liver	F.S.F.	1972	Haemorrhagic skin ulcers
120	755	"	Kidney	F.S.F.	1973	Petecchiae in liver
121	762	"	Ulcer	F.F.	1973	Haemorrhagic skin ulcers, petecchiae in liver, swollen spleen, haemorrhages in musculature
122	763	"	Spleen	F.F.	1973	Haemorrhagic skin ulcers, petecchiae in liver, swollen spleen, haemorrhages in musculature
123	778	"	Kidney	T.M.	1973	Finrot

Table 2 cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
124	779	Atlantic salmon	Kidney	T.M.	1973	Finrot with haemorrhages at bases, swollen spleen
125	780	"	"	T.M.	1973	Finrot with haemorrhages at bases, swollen spleen
126	785	"	"	T.M.	1973	Punctate haemorrhages in musculature
127	791	"	Liver	F.E.	1973	No pathological changes
128	792	"	Kidney	E.O.	1973	Excoriation, greyish skin ulcers, swollen spleen
129	802	"	"	E.P.	1973	No pathological changes
130	815	"	Not reported	E.O.	1973	No pathological changes, but cadaverous
131	832	"	Kidney	T.M.	1973	No pathological changes, but *Trichodina* invasion
132	834	"	Musculature	River N.	1973	Boil lesions in musculature, *Aeromonas salmonicida* also isolated
133	843	"	Not reported	R.F.J.	1973	Liquefied spleen, petecchiae in liver
134	847	"	"	River S.E.	1973	Haemorrhagic skin ulcers, oedema in swimbladder, haemorrhages in musculature
135	739	Sea trout	Not reported	F.S.F.	1972	Exophthalmos
136	783	"	Kidney	F.S.F.	1973	Fungus$ed skin ulcers, swollen liver, liquefied spleen

Table 2 cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
137	793	Sea trout	Kidney	O.F.A.	1973	No pathological changes
138	827	"	Liver	R.S.	1973	No pathological changes
139	A626/73[xxx]	"	"	R.S.	1973	No pathological changes
140	281	Saithe	Intestine		1965	Not reported
141	282	"	Not reported		1965	Haemorrhagic skin ulcers
142	283	"	"		1965	Haemorrhagic skin ulcers
143	338	"	"		1967	Not reported
144	341	"	"	Kvalvig	1967	Haemorrhagic skin ulcers, haemorrhages in eyes
145	342	"	"	Kvalvig	1967	Haemorrhagic skin ulcers, haemorrhages in eyes
146	343[xx]	"	"		1967	No pathological changes
147	602[xx]	"	Kidney	Batalden	1969	Haemorrhagic skin ulcers, swollen spleen and kidneys
148	603	"	"	Batalden	1969	No pathological changes
149	604	"	"		1969	Haemorrhagic skin ulcers, swollen spleen and kidneys
150	606	"	"		1969	Haemorrhagic skin ulcers, swollen spleen and kidneys

Table 2 cont.

Scheme No.	My Collection No.	Host	Organ	Farm	Year	Pathological signs on fish
151	609	Saithe	Kidney		196[illegible]	Haemorrhagic skin ulcers, swollen spleen and kidneys
152	622	"	Not reported		1969	No pathological changes
153	634	"	"	Sogn	1970	Haemorrhagic skin ulcers, swollen spleen
154	636	"	Kidney	Kristvik	1970	Excoriation
155	646	Cod	Boil lesion	Larvik	1971	Boil lesions in pseudobranchs
156	761	"	"	Fredrikstad	1973	Boil lesions in pseudobranchs
157	781	"	"	Moss	1973	Cachectic, purulent, necrotic pus around eye
158	851	"	"	Oslofjord	1973	Boil lesions in pseudobranchs
159	573	Dab	Kidney	Oslofjord	1969	Swollen spleen
160	623	Flounder	"	Bergen	1969	Greyish skin ulcer, finrot, serous fluid in peritoneal cavity
161	635	Plaice	Not reported	Bergen	1970	Greyish skin ulcers, serohaemorrhagic fluid in peritoneal cavity, swollen spleen and kidneys, haemorrhages in musculature
162	481	Ciona intestinalis	Stomach	M.	1968	

Table 2 cont.

Scheme No.	My collection No.	Host	Organ	Farm	Year	Pathological signs on fish
163	483	Ciona intestinalis	Stomach	M.	1968	
164	759	Vibrio ichthyodermis			1973	From Shewan, Torry Research Station, Aberdeen, Scotland
165	760	"			1973	"
166	767	Vibrio anguillarum	ATCC 19264		1973	
167	768	"	ATCC 14181		1973	
168	805	Vibrio metchnikovi	ATCC 7708		1973	From Dr. Sandvik, Veterinary College, Oslo, Norway
169	835	Vibrio cholerae	ATCC 14035		1973	"

Strains marked ^{x}, ^{xx} and ^{xxx} died out before the testings were ended and therefore a few parameters for these strains are lacking.

Strains marked ^{x} lack size, flagellation and glycerol.

Strains marked ^{xx} lack size, flagellation, glycerol and Borgal.

Strains marked ^{xxx} lack size and flagellation.

Table 5

Antibiotic Sensitivity Results

Scheme No	Collection No	Chloramphenicol	Tetracyclines	Sulfonamides	Borgal	Vibriostat 0/129	Penicillin low/high
1	257	+ + + +	+ + + +	+ + +	+ +	+ +	
2	258	+ + + +	+ + + +	+ + +	+ + +	+ +	
3	259	+ + + +	+ + +	+ + +	+ +	+ +	
4	261	+ + +	+ + +	+ + +	+ +	+ +	
5	262	+ + + +	+ + +	+ + +	+ +	+	
6	263	+ + + +	+ + +	+ + +	+ +	+ +	
7	313	+ + + +	+ + + +	+ + +	+	+ +	
8	339	+ + + +	+ + +	+	+ +	+ +	-
9	431	+ + + +	+ + +	+ + +	+ +	+ + +	-
10	433	+ + + +	+ +	+ +	+ +	+ +	-
11	435	+ + + +	+ + +	+ + +	+ +	+ + + +	-
12	488	+ + + +		+ + +	+ +	+	-
13	508	+ + + +	+ + +	+ +	+ +	+ + +	-
14	510	+ + +	+ +	+	+ +	+	-
15	570	+ + + +	+ + +	+	+ +	-	-
16	571	+ + + +	+ + +	+ +		+	-

Table 5, cont.

17	575	+ + + +	+ + +	+ + +	+ +	-
18	629	+ + +	+ +	+ +	+	+
19	630	+ + +	+ + +	+ + +	+ + +	+ +
20	648	+ + + +	+ + +	+ + +	+ + +	+ +
21	668	+ + +	+ + +	+ + +	+ +	+ +
22	670	+ + + +	+ + + +	+ + +	+ + +	+ +
23	672	+ + + +	+ + +	+ + + +	+ + +	+ +
24	676	+ + + +	+ + +	+ + +	+ + +	+ +
25	677	+ + + +	+ + +	+ + + +	+ +	+ +
26	694	+ + +	+ +	+ + + +	+ +	+ +
27	698	+ + + +	+ + +	+ + +	+ + +	+ +
28	707	+ + + +	+ + +	+ + +	+ + +	+ +
29	709	+ + + +	+ + +	+ + +	+ +	+ +
30	710	+ + +	+ + +	+ +	+ +	+ +
31	711	+ + +	+ +	+ +	+ +	+ +
32	714	+ + +	+ +	+ +	+ +	+
33	721	+ + + +	+ +	+ +	+ +	+
34	723	+ + + +	+ + +	+	+ +	+
35	724	+ + + +	+	-	+	+
36	728	+ + +	+ + +	+	+ +	+

Table 5, cont.

37	731	+ + +	+ + +	+ +	+ +	+
38	732	+ + + +	+ +	+ +	+ +	+
39	733	+ + +	+ +	+ +	+	+
40	734	+ + + +	+ +	-	+ +	+
41	737	+ +	+	+	+	+
42	740	+ + + +	+ +	+ +	+ +	+
43	741	+ + + +	+ +	+	+ +	+
44	743	+ + +	+ +	+ +	+ +	+
45	745	+ + +	+ + +	+	+ +	+
46	746	+ + +	+ +	+ + +	+ + +	+
47	749	+ + + +	+ +	+ +	+ +	+ +
48	751	+ + +	+ +	+	+ +	+
49	754	+ + + +	+ +	+	+	+
50	757	+ + + +	+ +	+ + +	+ +	+
51	794	+ + + +	+ + +	+ + +	+ +	+ +
52	796	+ + + +	+ + +	+ + +	+ +	+ +
53	797	+ + + +	+ + +	+ + + +	+ +	+ +
54	801	+ + +	+ + +	+ + +	+ +	+
55	804	+ + + +	+ + +	+ + +	+ + +	+
56	806	+ + + +	+ + +	+ + +	+ +	+ +

Table 5, cont.

57	807	+ + + +	+ + +	+ +	+ +	+ +
58	808	+ + +	+ + +	+ +	+ +	+
59	809	+ + + +	+ + +	+ +	+ +	+
60	810	+ + + +	+ + +	+ + +	+ +	+
61	811	+ + + +	+ +	+ + +	+ +	+ +
62	812	+ + + +	+ + +	+ + +	+ +	+ +
63	816	+ + + +	+ + +	+ + +	+ + +	+
64	818	+ + + +	+ + +	+ + + +	+ + +	+ +
65	819	+ + + +	+ + + +	+ + + +	+ + +	+
66	821	+ + + +	+ + +	+ + +	+ +	+ +
67	822	+ + + +	+ + + +	+ + + +	+ +	+
68	823	+ + + +	+ + +	+ + + +	+ +	+ +
69	824	+ + + +	+ + +	+ + + +	+ +	+ +
70	825	+ + + +	+ + + +	+ + + +	+ +	+ +
71	826	+ + + +	+ + +	+ + + +	+ +	+ +
72	828	+ + + +	+ + +	+ + +	+ +	+
73	831	+ + +	+ + +	+ +	+ +	+
74	836	+ + +	+ + +	+ + +	+ +	+
75	839	+ + + +	+ + +	+ +	+ +	+ +
76	840	+ + +	+ + +	+ + +	+ +	+

Table 5, cont.

57	807	+ + + +	+ + +	+ +	+ +	+ +
58	808	+ + +	+ + +	+ +	+ +	+
59	809	+ + + +	+ + +	+ +	+ +	+
60	810	+ + + +	+ + +	+ + +	+ +	+
61	811	+ + + +	+ +	+ + +	+ +	+ +
62	812	+ + + +	+ + +	+ + +	+ +	+ +
63	816	+ + + +	+ + +	+ + +	+ + +	+
64	818	+ + + +	+ + +	+ + + +	+ + +	+ +
65	819	+ + + +	+ + + +	+ + + +	+ + +	+
66	821	+ + + +	+ + +	+ + +	+ +	+ +
67	822	+ + + +	+ + + +	+ + + +	+ +	+
68	823	+ + + +	+ + +	+ + + +	+ +	+ +
69	824	+ + + +	+ + +	+ + + +	+ +	+ +
70	825	+ + + +	+ + + +	+ + + +	+ +	+ +
71	826	+ + + +	+ + +	+ + + +	+ +	+ +
72	828	+ + + +	+ + +	+ + +	+ +	+
73	831	+ + +	+ + +	+ +	+ +	+
74	836	+ + +	+ + +	+ + +	+ +	+
75	839	+ + + +	+ + +	+ +	+ +	+ +
76	840	+ + +	+ + +	+ + +	+ +	+

Table 5, cont.

77	841	+ + +	+ + +	+ + +	+ + +	+ +	
78	842	+ + + +	+ + +	+ + +	+ +	+ +	
79	285	+ + + +	+ + +	+ + +	+ + +	+ +	
80	487	+ + + +	+ + +	+ +	+ +	+ +	-
81	505	+ + +	+	+ +	+ +	+ +	-
82	511	+ + + +	+ + +	-	+ +	+	
83	513	+ + +	+ +	+	+ +	+	
84	516	+ + + +	+ +	+	+ +	+	
85	519	+ + + +	+ + +	+ +	+ +	+	-
86	521	+ + + +	+ +	-	+ +	+	-
87	522	+ + +	+ +	+ +	+ +	+	
88	523	+ + +	+ + +	+ + +	+ +	+ +	
89	524	+ + +	+ + +	+ +	+ +	+	
90	537	+ + + +	+ + +	+ + + +	+ +	+ +	-
91	572	+ + + +	+ + +	+ + + +	+ +	+ +	-
92	576	+ + + +		+ +	+ +	-	-
93	577	+ + +	+ +	+ + +	+ +	+	
94	578	+ + +	+ + +	+ +	+ +	+ +	
95	579	+ + +	+ + +	+ +	+ +	+ +	

Table 5, cont.

96	590	+ + + +	+ + +	+		−	−
97	592	+ + + +	+ + +	−	+ +	−	−
98	593	+ + +	+ +	+ + +	+ +	+	
99	631	+ + +	+ + +	+ + + +	+ +	+ +	
100	662	+ + + +	+ + +	+ + + +	+ + +	+ +	
101	663	+ + + +	+ + + +	+ + + +	+ + +	+ +	
102	664	+ + + +	+ + +	+ + + +	+ +	+ +	
103	666	+ + + +	+ + + +	+ + +	+ +	+ +	
104	667	+ + + +	+ + +	+ + + +	+ +	+ +	
105	673	+ + + +	+ + +	+ + + +	+ + +	+ +	
106	674	+ + + +	+ + +	+ + +	+ + +	+ +	
107	685	+ + + +	+ + +	+ + +	+ + +	+ +	
108	688	+ + +	+ +	+ +	+ +	+	
109	689	+ + +	+ +	+ +	−	+	
110	690	+ + +	+ + +	+ + + +	+ +	+ +	
111	700	+ + +	+ + +	+ + +	+ +	+ +	
112	701	+ + + +	+ + +	+ + +	+ +	+ +	
113	722	+ + +	+ +	+	+	+	
114	725	+ + +	+ +	−	+	+	
115	726	+ + +	+ +	+ +	+ +	+	

Table 5, cont.

116	727	+ + + +	+ + +	+ +	+ +	+
117	729	+ + +	+ + +	+	+ +	+
118	738	+ + +	+ + +	+ +	+ +	+
119	753	+ + + +	+ +	+	+ +	+
120	755	+ + + +	+ +	+ +	+	+
121	762	+ + +	+ +	+	+	-
122	763	+ + +	+ +	+	+	+
123	778	+ + +	+	-	+	+
124	779	+ + +	+ +	-	+	-
125	780	+ +	+ +	-	+	-
126	785	+ + +	+	+	-	-
127	791	+ + + +	+ + +	+ + +	+	+
128	792	+ + + +	+ + +	+ + +	+ +	+
129	802	+ + +	+ + +	+ + +	+ +	+
130	815	+ + +	+ + +	+ + +	+ +	+
131	832	+ + + +	+ + +	+ + +	+ +	+ +
132	834	+ + + +	+ + +	+ + + +	+ +	+ +
133	843	+ + +	+ + +	+ + +	+ + +	+
134	847	+ + +	+ + +	+ + +	+ +	+ +

Table 5, cont.

135	739	+ + + +	+ + +	+ + +	+ + +	+	
136	783	+ +	+ +	-	+	+	
137	793	+ + + +	+ + +	+ + +	+ +	+	
138	827	+ + + +	+ + + +	+ + +	+ + +	+ +	
139	A 626/73	+ + + +	+ + + +	+ + +	+ + +	+ +	
140	281	+ + + +	+ + +	+ + + +	+ +	+ +	
141	282	+ + + +	+ + + +	+ + + +	+ + +	+ +	
142	283	+ + + +	+ + + +	+ + + +	+ + +	+ +	
143	338	+ + + +	+ + +	+ + +	+	+ +	-
144	341	+ + + +	+ + +	+ +	+ +	+ +	-
145	342	+ + + +	+ + +	+ + +	+ + +	+ +	-
146	343	+ + + +	+ + + +	+ + + +		+ + +	-
147	602	+ + + +	+ + + +	+ + +		+ + +	-
148	603	+ + + +	+ + +	+ + +	+ + +	+ + +	
149	604	+ + + +	+ + + +	+ + + +	+ + +	+	
150	606	+ + + +	+ + +	+ +	+ +	+ +	
151	611	+ + +	+ + +	+ +	+ +	+ +	
152	622	+ + + +	+ + +	+ + + +	+ +	+ +	
153	634	+ + + +	+ + +	+ + + +	+ +	+ +	

Table 5, cont.

154	636	+ + +	+ + +	+ + +	+ +	+ +
155	646	+ + + +	+ + +	+ + +	+ + +	+ +
156	761	+ + +	+	+ +	+ +	+
157	781	+ + +	+	+	−	−
158	851	+ + +	+ +	+ + +	+ +	+ +
159	573	+ + +	+ + +	+ + +	+ +	+ +
160	623	+ + + +	+ + +	+ + +	+ +	+ + +
161	635	+ + + +	+ + +	+ + +	+ + +	+ +
162	481	+ + + +	+ + +	+ +	+ +	+
163	483	+ + + +	+ + +	+ + + +		+ +
164	759	+ + +	+ +	+	+	+
165	760	+ + +	+ +	+ +	+ +	+
166	767	+ + + +	+ +	+ +	+ +	+
167	768	+ + +	+ +	+ +	+ +	+
168	805	+ + +	+ +	+ +	+ +	+
169	835	+ + + +	+ + + +	+ + + +	+ + +	+

Table 9

Biochemical and morphological properties of the isolates of Vibrio anguillarum as provided for the Computer

Table 9

Scheme No	Collection No	Indole	Indole + 0.9% NaCl	Indole + 1.5% NaCl	Indole + 2.5% NaCl	Voges Proskauer (V.P.)	VP + 0.9% NaCl	VP + 1.5% NaCl	VP + 2.5% NaCl	Methylred (M.R.)	MR + 0.9% NaCl	MR + 1.5 NaCl	MR + 2.5% NaCl	Nitrate	Gelatin	Urea	H_2S production	Citrate	Adonitol	Dulcitol	Sorbitol	Arabinose	Xylose	Rhamnose	Maltose	Salicin	Inositol	Lactose	Sucrose	Mannitol	Glucose	Raffinose	Dextrin	Inulin	Fructose	Mannose	Galactose	Cellobiose	Trehalose	d - tartrate	Glycerol	Litmus milk	Methyleneblue milk	Aesculin	Hippurate	Oxidase	Hemolysis	Size
1	257	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.62
2	258	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.43
3	259	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.54
4	261	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.39
5	262	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.52
6	263	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	0.99
7	313	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	2.37
8	339	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	-
9	431	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.31
10	433	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.78
11	435	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	-
12	488	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.72
13	508	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.43
14	510	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.35
15	570	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.12
16	571	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	-
17	575	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.17
18	629	0	0	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.03
19	630	0	0	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.16
20	648	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.19
21	668	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.39
22	670	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.38
23	672	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	0.90
24	676	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.31
25	677	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	2	0	0	2	0	0	2	2	2	2	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.16

Table 9, cont.

Scheme No	Collection No		Indole	Indole + 0.9% NaCl	Indole + 1.5% NaCl	Indole + 2.5% NaCl	Voges Proskauer (V.P.)	VP + 0.9% NaCl	VP + 1.5% NaCl	VP + 2.5% NaCl	Methylred (M.R.)	MR + 0.9% NaCl	MR + 1.5 NaCl	MR + 2.5% NaCl	Nitrate	Gelatin	Urea	H_2S production	Citrate	Adonitol	Dulcitol	Sorbitol	Arabinose	Xylose	Rhamnose	Maltose	Salicin	Inositol	Lactose	Sucrose	Mannitol	Glucose	Raffinose	Dextrin	Inulin	Fructose	Mannose	Galactose	Cellobiose	Trehalose	d - tartrate	Glycerol	Litmus milk	Methyleneblue milk	Aesculin	Hippurate	Oxidase	Hemolysis	Size
26	694		0	0	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.06
27	698		0	0	0	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.07
28	707		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.32
29	709		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	0.99
30	710		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.07
31	711		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.51
32	714		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.62
33	721		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.07
34	723		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.25
35	724		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.29
36	728		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.16
37	731		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.27
38	732		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.26
39	733		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.03
40	734		0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.14
41	737		0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.01
42	740		0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.36
43	741		0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	0.94
44	743		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.10
45	745		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.02
46	746		0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.05
47	749		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.02
48	751		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.09
49	754		0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.02
50	757		0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	0	0	2	2	2	2	2	0	2	2	2	2	0	2	2	0	0	0	1	1	1.07

Table 9, cont.

Scheme No	Collection No	Indole	Indole + 0.9% NaCl	Indole + 1.5% NaCl	Indole + 2.5% NaCl	Voges Proskauer (V.P.)	VP + 0.9% NaCl	VP + 1.5% NaCl	VP + 2.5% NaCl	Methylred (M.R.)	MR + 0.9% NaCl	MR + 1.5 NaCl	MR + 2.5% NaCl	Nitrate	Gelatin	Urea	H_2S production	Citrate	Adonitol	Dulcitol	Sorbitol	Arabinose	Xylose	Rhamnose	Maltose	Salicin	Inositol	Lactose	Sucrose	Mannitol	Glucose	Raffinose	Dextrin	Inulin	Fructose	Mannose	Galactose	Cellobiose	Trehalose	d - tartrate	Glycerol	Litmus milk	Methyleneblue milk	Aesculin	Hippurate	Oxidase	Hemolysis	Size
51	794	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.21
52	796	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.03
53	797	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.44
54	801	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.05
55	804	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.10
56	806	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.33
57	807	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.11
58	808	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.16
59	809	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.17
60	810	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.21
61	811	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.76
62	812	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.29
63	816	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.48
64	818	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.60
65	819	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.39
66	821	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.78
67	822	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.90
68	823	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.76
69	824	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.72
70	825	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.48
71	826	2	2	2	2	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.10
72	828	2	2	2	2	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.74
73	831	2	2	2	2	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.02
74	836	2	2	2	2	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	1.45
75	839	2	2	2	2	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	1	1	

Table 9, cont.

Scheme No	Collection No	Indole	Indole + 0.9% NaCl	Indole + 1.5% NaCl	Indole + 2.5% NaCl	Voges Proskauer (V.P.)	VP + 0.9% NaCl	VP + 1.5% NaCl	VP + 2.5% NaCl	Methylred (M.R.)	MR + 0.9% NaCl	MR + 1.5 NaCl	MR + 2.5% NaCl	Nitrate	Gelatin	Urea	H_2S production	Citrate	Adonitol	Dulcitol	Sorbitol	Arabinose	Xylose	Rhamnose	Maltose	Salicin	Inositol	Lactose	Sucrose	Mannitol	Glucose	Raffinose	Dextrin	Inulin	Fructose	Mannose	Galactose	Cellobiose	Trehalose	d - tartrate	Glycerol	Litmus milk	Methyleneblue milk	Aesculin	Hippurate	Oxidase	Hemolysis	Size
76	840	0	2	2	2	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.62
77	841	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.91
78	842	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.39
79	285	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.91
80	487	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.53
81	505	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.48
82	511	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.33
83	513	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.27
84	516	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.18
85	519	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.11
86	521	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.24
87	522	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	0.92
88	523	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.06
89	524	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.03
90	537	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.37
91	572	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.13
92	576	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.44
93	577	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.28
94	578	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.37
95	579	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.14
96	590	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	-
97	592	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.32
98	593	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.12
99	631	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.17
100	662	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0	2	0	0	2	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.03

Table 9, cont.

Scheme No	Collection No	Indole	Indole + 0.9% NaCl	Indole + 1.5% NaCl	Indole + 2.5% NaCl	Voges Proskauer (V.P.)	VP + 0.9% NaCl	VP + 1.5% NaCl	VP + 2.5% NaCl	Methylred (M.R.)	MR + 0.9% NaCl	MR + 1.5 NaCl	MR + 2.5% NaCl	Nitrate	Gelatin	Urea	H_2S production	Citrate	Adonitol	Dulcitol	Sorbitol	Arabinose	Xylose	Rhamnose	Maltose	Salicin	Inositol	Lactose	Sucrose	Mannitol	Glucose	Raffinose	Dextrin	Inulin	Fructose	Mannose	Galactose	Cellobiose	Trehalose	d - tartrate	Glycerol	Litmus milk	Methylenceblue milk	Aesculin	Hippurate	Oxidase	Hemolysis	Size
101	663	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.26
102	664	0	1	1	1	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	0.93
103	666	0	0	1	1	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.09
104	667	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.25
105	673	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.20
106	674	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.03
107	685	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.44
108	688	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.04
109	689	2	2	2	2	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.19
110	690	2	2	2	2	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.00
111	700	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.42
112	701	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.05
113	722	1	1	1	1	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.10
114	725	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	0.94
115	726	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.19
116	727	0	2	2	2	0	2	2	2	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.04
117	729	0	2	2	2	0	2	2	2	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.00
118	738	0	2	2	2	0	2	2	2	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.16
119	753	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.01
120	755	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.05
121	762	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.42
122	763	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.53
123	778	2	2	2	2	0	2	2	2	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.56
124	779	2	2	2	2	0	2	2	2	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.42
125	780	0	2	2	2	0	2	2	2	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0	2	0	0	0	2	2	2	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1.53
																																																1.56
																																																1.38

Table 9, cont.

Scheme No	Collection No	Indole	Indole + 0.9% NaCl	Indole + 1.5% NaCl	Indole + 2.5% NaCl	Voges Proskauer (V.P.)	VP + 0.9% NaCl	VP + 1.5% NaCl	VP + 2.5% NaCl	Methylred (M.R.)	MR + 0.9% NaCl	MR + 1.5 NaCl	MR + 2.5% NaCl	Nitrate	Gelatin	Urea	H_2S production	Citrate	Adonitol	Dulcitol	Sorbitol	Arabinose	Xylose	Rhamnose	Maltose	Salicin	Inositol	Lactose	Sucrose	Mannitol	Glucose	Raffinose	Dextrin	Inulin	Fructose	Mannose	Galactose	Cellobiose	Trehalose	d - tartrate	Glycerol	Litmus milk	Methyleneblue milk	Aesculin	Hippurate	Oxidase	Hemolysis	Size
126	785	0	2	2	2	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.10
127	791	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.34
128	792	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.34
129	802	0	2	2	2	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.30
130	815	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.37
131	832	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.12
132	834	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.10
133	843	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.30
134	847	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.31
135	739	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.64
136	783	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.44
137	793	0	2	2	2	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.16
138	827	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.49
139	A 626/73	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	-
140	281	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.34
141	282	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.71
142	283	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.33
143	338	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.14
144	341	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.17
145	342	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.04
146	343	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	-
147	602	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.19
148	603	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.27
149	604	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	1.36
150	606	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	2	2	0	0	0	2	0	0	2	2	2	0	2	2	2	2	2	2	2	2	2	0	0	1	1	-

Table 9, cont.

Scheme No	Collection No	Indole	Indole + 0.9% NaCl	Indole + 1.5% NaCl	Indole + 2.5% NaCl	Voges Proskauer (V.P.)	VP + 0.9% NaCl	VP + 1.5% NaCl	VP + 2.5% NaCl	Methylred (M.R.)	MR + 0.9% NaCl	MR + 1.5 NaCl	MR + 2.5% NaCl	Nitrate	Gelatin	Urea	H_2S production	Citrate	Adonitol	Dulcitol	Sorbitol	Arabinose	Xylose	Rhamnose
151	611	2	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	1	0	0	2	2	0	0
152	622	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	0	0	0
153	634	1	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	1	0	0	2	2	0	0
154	636	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	0	0	0
155	646	2	2	2	2	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	0	0	0
156	761	0	2	2	2	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2	0	0	0
157	781	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0
158	851	2	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	0	0	0
159	573	0	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0
160	623	0	0	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	2	0	0	0
161	635	0	2	2	2	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0
162	481	0	2	2	2	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	2	2	0	0
163	483	0	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0
164	759	0	2	2	2	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	2	0	0
165	760	0	2	2	2	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	2	0	0
166	767	1	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	2	0	0
167	768	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	2	0	0	0
168	805	2	2	2	2	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
169	835	0	2	2	2	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	2	0	0

Scheme No	Collection No	Maltose	Salicin	Inositol	Lactose	Sucrose	Mannitol	Glucose	Raffinose	Dextrin	Inulin	Fructose	Mannose	Galactose	Cellobiose	Trehalose	d - tartrate	Glycerol	Litmus milk	Methyleneblue milk	Aesculin	Hippurate	Oxidase	Hemolysis	Size
151	611	2	0	0	1	2	2	2	0	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1	1.29
152	622	2	0	0	0	2	2	2	0	2	0	2	2	2	0	2	0	2	2	0	0	0	1	1	1.04
153	634	2	0	0	0	2	2	2	0	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1	1.09
154	636	2	0	0	0	2	2	2	0	2	0	2	2	2	0	2	0	2	2	0	0	0	1	1	1.38
155	646	2	0	0	0	2	2	2	0	2	0	2	2	2	2	2	0	2	2	1	0	0	1	1	1.46
156	761	2	0	0	0	2	2	2	0	2	0	2	2	2	0	2	0	2	1	1	0	0	1	1	0.98
157	781	2	0	0	0	2	2	2	0	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1	1.23
158	851	2	0	1	1	2	2	2	0	2	1	2	2	2	2	2	0	2	2	1	0	0	1	1	1.78
159	573	2	0	0	0	2	2	2	0	2	0	2	2	2	2	2	0	2	2	1	0	0	1	1	1.22
160	623	2	0	0	0	2	2	2	0	2	0	2	2	2	2	2	0	0	2	1	0	0	1	1	1.34
161	635	2	0	0	0	2	2	2	0	2	0	2	2	2	1	1	0	0	1	0	0	0	1	1	1.58
162	481	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	2	2	0	0	0	1	1	1.44
163	483	2	0	0	2	2	2	2	0	2	0	2	2	2	2	0	0	-	2	1	0	0	1	1	-
164	759	2	0	0	0	2	2	2	0	2	0	2	2	2	2	2	0	2	2	2	0	0	1	1	2.02
165	760	2	0	0	0	2	2	2	0	2	0	2	2	2	2	2	0	2	0	0	0	0	1	1	1.33
166	767	2	0	0	0	2	2	2	0	2	0	2	2	2	2	2	0	2	2	0	0	0	1	1	2.32
167	768	2	0	0	0	2	2	2	0	2	0	2	2	2	0	2	0	2	0	0	0	0	1	1	2.18
168	805	2	0	2	2	2	2	2	1	0	2	2	2	2	0	2	0	2	1	0	0	0	1	1	1.30
169	835	2	0	0	0	0	2	2	0	2	0	2	2	2	2	2	0	2	1	0	0	0	1	1	1.46

APPENDIX 2. SYNOPSIS OF FORTRAN IV PROGRAMME FOR COMPUTATION AND DISPLAY OF PRINCIPAL COMPONENTS AS DEVISED BY DR. W.C. WAHLSTEDT AND DR. J.C. DAVIS

1. Function

The principal component analysis (PCA) consists of a series of operations on a covariance matrix which result in series of transformed variables each accounting successively for the most possible variance between the objects studied.

2. Restrictions

There were no restrictions on the program to number of objects or to the number of tests.

3. Mathematical development

Data are first read into the computer and converted into an m x m matrix of covariances or correlations, where m = number of variables. The covariance matrix is

$$A_{ij} = \left[a_{ij}\right] = \frac{n\sum_{i=1}^{m}\sum_{j=1}^{m} X_i X_j - \left(\sum_{i=1}^{m} X_i \sum_{j=1}^{m} X_j\right)}{j(n-1)}$$

The correlation matrix is formed by

$$A_{ij} = \left[r_{ij}\right] = \frac{n\sum_{i=1}^{m}\sum_{j=1}^{m} X_i X_j - \sum_{i=1}^{m} X_i \sum X}{\sqrt{\left(n\sum_{i=1}^{m} X_i^2 - \left(\sum_{i=1}^{m} X_i\right)^2\right)\left(n\sum_{j=1}^{m} X_j^2 - \left(\sum_{j=1}^{m} X_j\right)^2\right)}}$$

Associated with every square matrix [A] is a characteristic function

$$f(\lambda) = \left[A - \lambda \right] = \begin{bmatrix} a_{11}-\lambda & a_{12} & \cdots & a_{1m} \\ a_{21} & a_{22}-\lambda & \cdots & a_{2m} \\ --- & --- & --- & --- \\ a_{m1} & a_{m2} & \cdots & a_{mm}-\lambda \end{bmatrix}$$

which has the property $f(\lambda) = 0$. From this matrix equation, m roots called eigenvalues or latent roots, may be extracted. Associated with each eigenvalue is an eigenvector or latent vector which is a column vector $\left[X_i \right]$ having the property $\left[A-\lambda I\right] \cdot \left[X_i \right] = 0$. If the eigenvalues of a matrix are distinct (that is, not identical), the associated eigenvectors are independent. These vectors are the transformations desired in principal components analysis. In this program the eigenvalues and eigenvectors are found by a subroutine modified from one published by Cooley and Lohnes (1962, p. 187-189). Their procedure was based in turn on the Jacobi algorithm as developed by Greenstadt (1960, p. 84-91).

The trace (or sum of diagonal elements) of the matrix and the sum of the eigenvalues are identical and represent either the total variance or the total correlation in the matrix. Eigenvalues appear in the order of their magnitude, and the percentage of total variance or correlation accounted for each can be calculated by dividing the eigenvalues by the trace. In most instances, the first few eigenvalues will account for more variance or correlation than any of the original variables, and the last eigenvalues will account for less. If the first few account for an acceptable percent

of the total, the remaining eigenvalues may be discarded. The problem has then been reduced in dimensionality, from one of m variables to one of y m principal components.

The squared values of the terms of an eigenvector also may be summed, and the individual terms divided by the total. This yields the approximate percent contribution of the original variables to the principal component represented by that eigenvector. If only a few of the original variables account for most of a principal component, it may be interpreted by considering the nature of this combination, ignoring the contribution of other variables.

OPERATIONAL INSTRUCTIONS

This program is written in FORTRAN IV for the GE 625 Computer. Dimension statements are designed to allow up to 30 variables and 300 observations, although this may be altered by making simple adjustments within the program. The following control cards are necessary.

CONTROL CARD

Col.1 - 3 M, a right justified integer giving number of variables, up to 30.

Col.4 - 5 Blank .

Col.6 MANUAL, switch for co-variance correlation option.

1 = Use covariance matrix only.

2 = Use correlation matrix only.

3 = Use covariance and correlation matrices.

Col.7 OPDAT, switch for data listing option.

T = No list of input data.

Blank = List of input data.

VARIABLE NAMES CARD

This is a group of M cards containing the names of variables, listed one name to a card. A variable name may occupy columns 1 to 18. Names should appear in the same sequence as variables in the data set to avoid mis-labelling the output.

TITLE CARD

Col.1 - 72 Any alphanumeric information.

VARIABLE FORMAT CARD

Col.1 - 72 Format of data cards. The last field in the format should be L1, to read the last card signal.

DATA CARDS

Any number of data cards, up to 300, in the format specified above. The last data card must contain a "T" punched in the logic field defined in the variable format statement.

This completes one data set. If more that one set is to be processed, the sequence is repeated. A blank card should be placed after the final data set to provide normal termination of the program.

APPENDIX 3. SYNOPSIS OF THE NUMTAX PROGRAM, UNIVERSITY OF SURREY LIBRARY

1. Function of the programme

The programme accepts data in various forms regarding collections of objects. Percentage coefficients are calculated to express the similarity of each object with each other object, and may be printed in the form of a triangular matrix. The objects may then be sorted by stages into groups on the basis of their similarity coefficients using a method termed Single Link Grouping (SLG) or an alternative method termed Single Link Listing (SLL), or both, and the progress of sorting printed out at each stage until complete. The similarity matrix may then again be printed, this time with the objects in their rearranged order.

2. Limitations

There is no limitation to the number of tests, but the number of objects must not at present exceed 128.

3. Definitions

a) Objects are the items to be classified. They can also be called individuals units, OTU's and strains. They are numbered as shown in tables.

b) Tests are the properties of which classification is to be made. They may also be called characteristics or features.

c) The coefficient of similarity or similarity between objects is denoted S. The triangular matrix of off-diagonal elements is called the S-matrix.

d) Groups are exclusive sets of objects which may or may not cover the whole set of objects.

4. Calculation of Similarity Coefficient, S.

For any pair of objects:

$$S = \frac{t}{c}$$

Where t = total score and c = total count.

The total score is the sum of the individual scores awarded to each pair of test results; the total count is likewise the sum of the individual counts, every valid comparison being counted as 1. Individual scores and counts for each test are arrived at as follows:

a) Two-state qualitatives in the form 0,1

	Score	Count
Results for both objects = 0	1	1
Results for both objects = 1	1	1
Results for one object = 0, the other = 1	0	1
One or both results missing	0	0

b) Two-state qualitatives, in the form -,+

	Score	Count
Results for both objects = -	0	0
Results for both objects = +	1	1
Results for one object = -, the other = +	0	1
One or both results missing	0	0

d) Groups are exclusive sets of objects which may or may not cover the whole set of objects.

4. Calculation of Similarity Coefficient, S.

For any pair of objects:

$$S = \frac{t}{c}$$

Where t = total score and c = total count.

The total score is the sum of the individual scores awarded to each pair of test results; the total count is likewise the sum of the individual counts, every valid comparison being counted as 1. Individual scores and counts for each test are arrived at as follows:

a) Two-state qualitatives in the form 0,1

	Score	Count
Results for both objects = 0	1	1
Results for both objects = 1	1	1
Results for one object = 0, the other = 1	0	1
One or both results missing	0	0

b) Two-state qualitatives, in the form -,+

	Score	Count
Results for both objects = -	0	0
Results for both objects = +	1	1
Results for one object = -, the other = +	0	1
One or both results missing	0	0

c) Multistate qualitatives (form = A B C D ...)

	Score	Count
Result (= state) the same for both objects	1	1
Result (= state) different for the 2 objects	0	1
One or both results missing	0	0

d) Qualitatives (rescaled to the range 0 - 1)

	Score	Count
Result present for both objects	$1 - d^*$	1
One or both results missing	0	0

* Where d = the difference between the two values.

Example illustrating scoring and counting of 21 tests for a pair of objects, and calculation of S

Object No:	1	2	3	4*	5	6	7	8	9	10	11	12	13*	14	15	16	17	18	19	20	21
1	0	1	1	0	0	.6	F	C	E	+	-	+	-	1		0		23		+	
2	1	0	1	0	.8	1	B	D	E	-	+	+	-		1		0		F		
Score:	0	0	1	1	.2	.6	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
Count:	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0

$$S = \frac{\text{total score}}{\text{total count}} = \frac{4.8}{12} = .400$$

* compare: 0,1 and -,+ convention for two-state qualitatives and reaction tests.

5. There is no provision for differential weighting of tests, but individual tests may be given X2, X3, X4 weighting etc. by repeating the data for the test 2, 3, or 4 times.

6. SLG method of sorting

The S matrix is scanned for similarities at falling percentage levels, the user specifying the interval between these levels (e.g. 5 per cent). Objects which have mutual S values at a particular scanning level are paired or grouped together; objects are added to an existing group provided they are related, at current scanning level, to any object within the group, i.e. a grouping at 85 per cent S level of objects

1 - 2 - 32 - 21 - 25 -

would mean that object 1 had a similarity of at least 85 per cent to at least one of the other objects, 2, 32, 21 and 25.

7. SLL method of sorting

This is similar to the SLG method, except that objects are added to existing groups only if they are similar, at current scanning level, to one of the terminal members of the group, e.g. at 84.2 per cent similarity level, the addition of object 1 to an existing group, 2 - 32 - 21 - 25, giving a new group 1 - 2 - 32 - 21 - 25, implies that the S value of objects 1 and 2 is 84 per cent; object 1 would not be added to the group on the basis on an 84.2 per cent similarity with object 32 or 21.

The user does not specify a scanning interval for SLL; this is always 0.1 per cent.

When SLL is used, the sum of the percentage S values of every adjacent pair of objects in their final, re-arranged

order can be expected to be the highest (or nearly the highest) attainable by any arrangement of the objects.

Presentation of data

The first line of data should consist of four numbers:

1. The number of objects
2. The number of quantitative tests
3. The number of qualitative tests (two-state reaction or multistate)
4. The criterion for judging of these are sufficient comparisons to make an entry in the data matrix.

This should be followed by the results for the first quantitative test, in order of objects, and this followed by the results for the second quantitative test, in order of objects, and so on.

The results of the qualitative tests should now follow, reaction tests (-/+) first, then two-state (0/1), and finally multistate (RED, BLUE, GREY).

(For convenience in any later manipulations of the punched cards, begin a new line for each test or, if punching cards, begin a new card. Leave at least two spaces between items of numeric data).

The data should be followed on a new line by:

END ØF DATA

Then follow the processes required, with a new line for each.

ØMAT for printing of the similarity matrix, which can be done at any stage.

ØLG for the single link grouping process (and its printing). If this is specified, the scanning interval should be written after it, e.g. ØLG 5 (for 5 per cent scanning intervals). The scanning interval specified must not be less than 100 ÷ the total number of tests.

ØLL for the single link listing process (and its printing).

On a new line, conclude with

END ØF JØB

APPENDIX 4

CODING OF RESULTS FOR COMPUTATION

Table 15

Vibrios from fish. Elimination of strains for numerical taxonomy

Eliminated	Retained	Discrepancy
1	67	3
2	22	1
4	76	2
5	85	4
6	112	3
7	54,76	6
13	60	2
19	63	3
20	28	2
21	99	4
24	25	2
27	100	3
29	71	5
30	54	4
31	58	2
41	136	3
44	117	3
47	50	3
48	83	1
49	83	3
51	159	5
52	67	4
53	112	0

Table 15, contd.

Eliminated	Retained	Discrepancy
55	79	3
56	62	3
57	60	3
59	116	3
66	112	3
69	104	0
72	104	0
74	54,76	1
75	76	4
77	126	2
78	71	3
87	108	4
92	118	5
93	98	2
94	130	2
106	152	2
111	128	4
119	120	3
123	125	3
131	104	2
132	145	5
138	139	3
140	148	4
141	142	0
144	154	3
147	152	4

Table 16

Vibrios from fish: 120 strains selected for numerical taxonomy

Computer	Hästein	Computer	Hästein	Computer	Hästein
1	3	41	70	81	122
2	8	42	71	82	124
3	9	43	73	83	125
4	10	44	76	84	126
5	11	45	79	85	127
6	12	46	80	86	128
7	14	47	81	87	129
8	15	48	82	88	130
9	16	49	83	89	133
10	17	50	84	90	134
11	18	51	85	91	135
12	22	52	86	92	136
13	23	53	88	93	137
14	25	54	89	94	139
15	26	55	90	95	142
16	28	56	91	96	143
17	32	57	95	97	145
18	33	58	96	98	146
19	34	59	97	99	148
20	35	60	98	100	149
21	36	61	99	101	150
22	37	62	100	102	151
23	38	63	101	103	152
24	39	64	102	104	153
25	40	65	103	105	154
26	42	66	104	106	155
27	43	67	105	107	156
28	45	68	107	108	157
29	46	69	108	109	158
30	50	70	109	110	159
31	54	71	110	111	160
32	58	72	112	112	161
33	60	73	113	113	162
34	61	74	114	114	163
35	62	75	115	115	164
36	63	76	116	116	165
37	64	77	117	117	166
38	65	78	118	118	167
39	67	79	120	119	168
40	68	80	121	120	169

Table 21

Pathological criteria used for Principal Components Analysis in Atlantic Salmon

1. Skin lesion, absent/present, 0/1
2. Skin ulceration, 0/1
3. Skin haemorrhage, petecchiae, ulcer haemorrhage, 0/1
4. Skin excoriation, 0/1
5. Fin lesion, 0/1
6. Fin haemorrhage, 0/1
7. Fin rot, 0/1
8. Muscle lesions absent/present, 0/1
9. Muscle boil, 0/1
10. Muscle haemorrhage, 0/1
11. Eye changes, 0/1
12. Exophthalmos, 0/1
13. Changes in spleen, 0/1
14. Changes in liver, 0/1
15. Swellings of spleen, liver or kidneys, 0/1
16. Liquefaction of spleen, liver or kidneys, 0/1
17. Haemorrhage in spleen, liver or kidneys, 0/1
18. Intestinal changes including enteritis, haemorrhage, anal haemorrhage, 0/1
19. Anal haemorrhage, 0/1
20. Ascites or serohaemorrhagic fluid in peritoneum, 0/1
21. Loss of condition (e.g. "anemic", "cachectic", "cadaverous"), 0/1

Table 32

Coding of ranges of values, expressed in the Fortran IV and Numtax programmes used in the International Computer Limited 1905 F.

Variable	Reactions	Range Symbols
	-	0
Indole	Weak +	1
	Strong +	2
VP	-	0
	+	1
MR	-	0
	+	1
Urea	-	0
	+	1
Citrate	-	0
	+	1
	Negative	0
Litmus milk	Reduction	1
	Reduction + Peptonization	2
	Negative	0
Methyleneblue milk	Reduction	1
	Reduction + Peptonization	2
	-	0
Carbohydrates	Intermediate	1
	+	2
Antibiotic	0 mm	0
Sensitivity	1-5 mm	1
	5-10 mm	2
	11-15 mm	3
	16 mm	4

Table 32 contd.

Variable	Reactions	Range Symbols
Cell length	0 - 1μ	1
	1.1 - 2μ	2
	2.1 - 3μ	3
	3.1 - 4μ	4