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Identification of atypical *Aeromonas salmonicida*: inter-laboratory evaluation and harmonization of methods

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I. DALSGAARD, B.K. GUDMUNDSDÓTTIR, S. HELGASON, S. HØIE, O.F. THORESEN, U.-P. WICHARDT AND T. WIKLUND. 1998. The atypical isolates of *Aeromonas salmonicida* are becoming increasingly important as the frequency of isolation of bacteria belonging to this group continues to rise. The primary object of this study was to compare and evaluate the results obtained in various laboratories concerning the biochemical identification of atypical *Aer. salmonicida* before and after standardization of media and methods. Five laboratories examined 25 isolates of *Aer. salmonicida* from diverse fish species and geographical locations including the reference strains of *Aer. salmonicida* subsp. *salmonicida* (NCMB 1102) and *Aer. salmonicida* subsp. *achromogenes* (NCMB 1110). Without standardization of the methods, 100% agreement was obtained only for two tests: motility and ornithine decarboxylase. The main reason for the discrepancies found was the variation of the incubation time prior to reading the biochemical reactions. After standardization, improvement was obtained with the identification; however, disagreement was still observed between the different laboratories. These findings demonstrate the difficulties involved in a proper identification of atypical *Aer. salmonicida* and also that data presented in the literature on various strains of *Aer. salmonicida* are not readily comparable. This paper seems to be the first on standardization of microbiological tests for identification of fish pathogens and the results obtained show the need for standardization of methods both within and between laboratories.

INTRODUCTION

An increasing number of atypical strains of *Aeromonas salmonicida* have been implicated as the cause of serious disease problems in both wild and cultivated fish in the Nordic countries. In Iceland serious outbreaks of the disease have been recorded in cultivated Atlantic salmon (*Salmo salar*), Arctic char (*Salvelinus alpinus*) and Atlantic cod (*Gadus morhua*). Furthermore, the organism is frequently isolated from wild salmonids (Gudmundsdóttir *et al.* 1996). The species mainly affected in Sweden are brown trout (*Salmo trutta* f. *fario*) and Arctic char (Wichardt *et al.* 1989). In Finland atypical strains have been isolated from ulcerated wild pike (*Esox lucius*), flounder (*Platichthys flesus*) (Wiklund 1990; Wiklund *et al.* 1994) and farmed salmonids (Rintamäki and Valtonen 1991). In Norway atypical *Aer. salmonicida* have been associated with disease in farmed Atlantic salmon and farmed turbot (*Scophthalmus maximus*). Atypical strains have also been isolated from various wild fish, comprising sea- and freshwater species (Lunder and Håstein 1990). In Denmark the disease has so far caused minor problems in fish farms (Pedersen *et al.* 1994). However, atypical *Aer. salmonicida* strains have been isolated from ulcerated wild fish from Danish coastal waters (Dalsgaard and Paulsen 1986; Wiklund and Dalsgaard 1995).

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Two workshops on atypical *Aer. salmonicida* were held in 1992 and 1993, sponsored for the most part by the Nordic Academy for Advanced Study (NorFA). The intention of the workshops was to review knowledge about infections caused by atypical *Aer. salmonicida* in farmed and wild fish in the Nordic countries. One of the conclusions drawn from the first workshop was that a proper identification of strains belonging to the 'atypical group' of *Aer. salmonicida* is becoming increasingly important as the frequency of isolation of bacteria belonging to this group continues to rise. Consequently, it was decided that a collection of strains of atypical *Aer. salmonicida* should be characterized biochemically by different laboratories in the Nordic countries.

The primary objective of this study was to compare and evaluate the results obtained in the various laboratories concerning the identification of atypical *Aer. salmonicida* using biochemical tests before and after standardization of the methods.

MATERIALS AND METHODS

Participants

Five laboratories, viz. the Fish Disease Laboratory, Denmark, the Institute of Parasitology, Finland, the Fish Disease Laboratory, Iceland, the Central Veterinary Laboratory, Norway and the National Veterinary Institute commissioned by the Fish Health Control Program, Sweden, participated in two trials, which were performed as blind tests.

Bacterial strains

Isolates of atypical *Aer. salmonicida* were collected from the participating countries by the co-ordinating Danish laboratory. Each laboratory examined 23 strains isolated from diverse fish species and geographical locations (Table 1). Reference strains of *Aer. salmonicida* subsp. *salmonicida* NCMB 1102 and *Aer. salmonicida* subsp. *achromogenes* NCMB 1110 were included.

Taxonomic criteria

The criteria essential for the identification of the strains were agreed by the participants. Each strain was examined for 19 characters (Tables 2 and 3). Additionally, the serological examination of the bacteria and the susceptibility to novobiocin (5 μg) and vibriostatic agent O/129 (10 and 150 μg) (Neo-Sensitabs®, Rosco Diagnostica, Taastrup, Denmark) were tested by the co-ordinating laboratory (Dalsgaard *et al.* 1994). Cultivation of atypical *Aer. salmonicida* was achieved on media enriched with blood (Ishiguro *et al.* 1986): tryptic soy agar (TSA), brain heart infusion agar (BHIA) or blood agar base supplemented with 5% horse or calf blood. All experiments were performed at 20 °C. If no growth was obtained in the medium used for the biochemical identification, the test was repeated with the same medium supplemented with serum (1% to broth and 5% to agar).

First trial

Each strain was given a code number (1–25) and distributed in Stock culture agar (Difco, Detroit, MI, USA) to all participating laboratories. In the first trial all laboratories examined the isolates, using their routine methodology, with either conventional biochemical test media or commercial kits (API 20E; bioMérieux, Marcy l'Etoile, France).

Second trial

For the second trial the strains were given a new number and redistributed to the participants. The same biochemical tests as in the first trial were performed. However, the methods and media were standardized after agreement by the participants. Unless indicated otherwise, the methods and media were taken from *Cowan and Steel's Manual for the Identification of Medical Bacteria* (Barrow and Feltham 1993). A brief description of the tests follows.

Fundamental reactions. Haemolysin production was tested on BHIA supplemented with 5% horse blood. The plates were observed for 7 d. The presence of pigment production was tested on BHIA as well as Columbia agar (Oxoid, Basingstoke, UK) (Altmann *et al.* 1992). The plates were incubated for 7 d.

Motility was examined by the hanging drop technique or other wet preparation with a young broth culture (48 h). A negative reaction could be confirmed by observing the growth in semi-solid agar (3 g agar l^{-1}; BHI). Catalase production was tested with the slide method with a bacterial culture (age 48 h or as young as possible) grown on agar medium without blood and 3% H_2O_2 (microscopical confirmation of the reaction, if necessary). The presence of cytochrome oxidase was determined by Kovács' method using bacteria from an agar plate incubated for 48 h (or as young as possible) and filter paper soaked with 1% *N,N,N,N*-tetramethyl-*p*-phenylenediamine dihydrochloride + 0·1% ascorbic acid. The reaction was read within 30 s.

The fermentative metabolism of glucose was determined in Hugh and Leifson's medium (OF basal medium). The inoculated tubes were observed for 14 d. If no growth was obtained, the test was repeated in Hugh and Leifson's medium supplemented with serum or in Modified Leifson's medium (MOF) (Leifson 1963).

Secondary reactions. Arginine dihydrolase, lysine and orni-

Table 1 Bacterial cultures included in the two trials

Strain	Original code	Host	Location
1	909/81	Atlantic salmon (*Salmo salar*)	Norway
2	2013/81	Atlantic salmon (*Salmo salar*)	Norway
3	1977/88	Atlantic salmon (*Salmo salar*)	Norway
4	1777/92	Wolf fish (*Anarhichas lupus lupus*)	Norway
5	2656/92	Halibut (*Hippoglossus hippoglossus*)	Norway
6	M45/89	Arctic char (*Salvelinus alpinus*)	Iceland
7	S226/90	Brown trout (*Salmo trutta* f. *fario*)	Iceland
8	M283/89	Atlantic salmon (*Salmo salar*)	Iceland
9	T233/91	Atlantic cod (*Gadus morhua*)	Iceland
10	T3-A1	Haddock (*Melanogrammus aeglefinus*)	Iceland
11	No. 1	Sea trout (*Salmo trutta* f. *trutta*)	Finland
12	No. 2	Brown trout (*Salmo trutta* f. *lacustris*)	Finland
13	No. 3	Grayling (*Thymallus thymallus*)	Finland
14	3-15	Pike (*Esox lucius*)	Finland
15	921203-2/3	Flounder (*Platichthys flesus*)	Denmark
16	850319-1/4	Sand-eel (*Ammodytes lancea* and *Hyperoplus lanceolatus*)	Denmark
17	920225-1/2	Eel (*Anguilla anguilla*)	Denmark
18	860613-1/1	Atlantic salmon (*Salmo salar*)	Faroe Islands
19	920720-2/5	Turbot (*Scophthalmus maximus*)	Denmark
20	329/89	Atlantic salmon (*Salmo salar*)	Sweden
21	298/89	Arctic char (*Salvelinus alpinus*)	Sweden
22	420/88	Brown trout (*Salmo trutta* f. *fario*)	Sweden
23	261/89	Brown trout (*Salmo trutta* f. *fario*)	Sweden
24	NCMB 1102	Atlantic salmon (*Salmo salar*)	Scotland
25	NCMB 1110	Sea trout (*Salmo trutta* f. *trutta*)	Scotland

Strain 10 isolated by Eva Benediktsdottir, University of Iceland, Reykjavik, Iceland; strains 11, 12 and 13 provided by Päivi Rintamäki, University of Oulu, Oulu, Finland. NCMB, National Collection of Marine Bacteria; NCMB 1102, *Aeromonas salmonicida* subsp. *salmonicida*; NCMB 1110, *Aeromonas salmonicida* subsp. *achromogenes*.

thine decarboxylase production were performed using Moeller's decarboxylase base with 1% of the amino acids (L-forms). The inoculated tubes were observed for 14 d before being discarded as negative. Indole production was assayed after 7 d growth in 1% tryptone + 0·5% NaCl with Kovács' indole reagent. The culture was divided into two halves before adding reagent to one of them. If the test was negative after 7 d, the remaining half of the broth culture was reincubated for an additional week. Before the test is performed it is important to observe growth of the bacteria in the medium.

The Voges–Proskauer test was performed on 7 d cultures grown in MR-VP broth by adding 0·6 ml 5% α-naphthol and 0·2 ml 40% KOH to half of the broth. The reaction was repeated after incubation of the other half of the broth for an additional week if negative. The ability to hydrolyse aesculin was shown by a blackening following growth in 1% peptone broth containing 0·5% NaCl, 0·1% aesculin and 0·05% ferric citrate. The tubes were kept for 14 d before being discarded as negative.

Acid production from sucrose, mannitol and salicin was determined in Bacto phenol red broth base (PRB) with filter-sterilized sugar added to a final concentration of 1%. Gas production was detected in PRB + 1% glucose with an inverted Durham tube. The tubes were observed for 14 d.

Gelatin liquefaction was tested on BHIA with 1% gelatin. The culture was streak inoculated with a heavy inoculum. Following incubation for 14 d 20% sulphosalicylacid (article no. 691; Merck, Darmstadt, Germany) was added to the plate (clear zone indicates a positive reaction). An alternative method without adding reagent was also tested by the co-ordinating laboratory: nutrient agar + 1% gelatin (clear zone indicates a positive reaction).

The susceptibility to ampicillin (33 μg) and cephalothin (66 μg) was determined on Mueller–Hinton agar using the agar diffusion method with commercial discs (Neo-Sensitabs®; Rosco Diagnostica, Taastrup, Denmark). The appropriate inoculum should yield a dense but not completely confluent growth. Strains with inhibition zones equal to or exceeding 20 mm were scored as sensitive.

Table 2 Biochemical fundamental reactions in the first and second trial of 23 atypical and two reference strains of *Aeromonas salmonicida* performed by five laboratories

Strain	Haemolysin ($1^{st}/2^{nd}$ trial)	Brown pigment ($1^{st}/2^{nd}$ trial)	Motility ($1^{st}/2^{nd}$ trial)	Catalase ($1^{st}/2^{nd}$ trial)	Cytochrome oxidase ($1^{st}/2^{nd}$ trial)	Glucose fermentation ($1^{st}/2^{nd}$ trial)
1	−*/−	v/−*	−/−	+/+	+/+	+/+
2	−*/−	+/+	−/−	+/+	+/+	+/+
3	−/−	−/−	−/−	v/+*	+/+	+/+
4	−*/−	+/+	−/−	+/+	+/+	+/+
5	−/−	+/+	−/−	+/+	+/+	+/+
6	−/−	+/+	−/−	+/+	+/+	+/+
7	−/−	+/+	−/−	+/+	+/+	+/+
8	−/−	+/+	−/−	+*/+	+/+	+/+
9	−/−	+/+	−/−	+/+	+/+	+/+
10	−/−	+/+	−/−	+*/+	+/+	+/+
11	−*/−	+/+	−/−	+*/+	+/+	+/+*
12	−/−	−/−	−/−	+*/+	+/+	+/+
13	−/−	−/−	−/−	+*/+	+/+	+/+
14	−*/−	+/+	−/−	+*/+	+/+	+/+
15	+*/+	−/−	−/−	+*/+	v/−	+/+
16	−/−	+/+	−/−	+/+	+/+	+/+
17	−/−*	−/−	−/−	+*/+	+/+	v/v
18	−/−	+/+	−/−	+/+	+/+	+/+
19	v/v	−/−	−/−	v/+	−*/−	v/v
20	−/−	−/−	−/−	−*/+*	+/+	+/+*
21	−*/−	−/−	−/−	+/+	+/+	+/+
22	−*/−	+/+	−/−	+/+	+/+	+/+
23	−*/−	+/+	−/−	+/+	+/+	+/+
24	+/+	+/+	−/−	v/+	+/+	+/+
25	−*/−	−/−	−/−	+/+	+/+	+/+

+ or −, All five laboratories obtained the same reaction; +* or −*, four laboratories obtained the same reaction; v, two or three laboratories obtained the same reaction.

RESULTS

The taxonomic analysis of the atypical *Aer. salmonicida* strains revealed that all were Gram-negative rods. They showed a positive reaction in the slide agglutination test with rabbit antiserum raised against a Danish strain of *Aer. salmonicida* subsp. *salmonicida* (830809–3/5) and all were resistent to novobiocin and the vibriostatic agent O/129. The results from the two trials are shown in Tables 2 and 3.

First trial — fundamental reactions

In the first trial (Table 2) full agreement with six strains (5, 6, 7, 9, 16 and 18) in all six reactions was obtained by all laboratories. Most strains showed discrepancies in one or two tests except for strains 15 (from flounder) and 19 (from turbot) which deviated in three and four tests, respectively. The discrepancies were observed mainly in haemolysin production and catalase production. The haemolysin production was read after 2 d by all laboratories. Three laboratories used horse blood and two used cow and calf blood, respectively. The variability was observed both with the use of cow and horse blood. Full agreement was observed in one test, the motility test.

Second trial — fundamental reactions

The identification made after standardization (second trial) showed full agreement with 19 strains, including the two reference strains (Table 2). Improved agreement was obtained with the flounder (15) and turbot (19) strains. The results from one of the laboratories deviated from the others in catalase and pigment production and glucose fermentation. Full agreement for all the strains was obtained in two tests, motility and cytochrome oxidase. One test, glucose fermen-

Table 3 Biochemical secondary reactions in the first and second trial of 23 atypical and two reference strains of *Aeromonas salmonicida* performed by five laboratories

Strain	Arginine dihydrolase ($1^{st}/2^{nd}$ trial)	Lysine decarboxylase ($1^{st}/2^{nd}$ trial)	Ornithine decarboxylase ($1^{st}/2^{nd}$ trial)	Indole production ($1^{st}/2^{nd}$ trial)	Voges–Proskauer ($1^{st}/2^{nd}$ trial)	Aesculin hydrolysis ($1^{st}/2^{nd}$ trial)	Sucrose acid ($1^{st}/2^{nd}$ trial)	Mannitol acid ($1^{st}/2^{nd}$ trial)	Salicin acid ($1^{st}/2^{nd}$ trial)	Glucose gas ($1^{st}/2^{nd}$ trial)	Gelatin liquefaction ($1^{st}/2^{nd}$ trial)†	Ampicillin 33 μg ($1^{st}/2^{nd}$ trial)	Cephalothin 66 μg ($1^{st}/2^{nd}$ trial)
1	−*/+	−*/−	−/−	+*/+	−*/+*	−/−	+/+	v/+	−/−	−/−	v/+*	R/R	R/R
2	−/−*	−/−	−/−	+/+	+*/+	+*/+	+*/+	+*/+	−*/v	v/−	+*/+	R/R	R/R
3	−*/v	+*/+	−/−	+*/+*	−/−	−/−*	+*/+	v/+	−/−	−/−	−*/+	R/R	R/R
4	−/−*	−/−	−/−	+/+	v/+	+/+	+/+	+*/+	+*/+	−/−	+/+	R/R	R/R
5	+*/+	−*/−	−/−	v/+	v/+*	−/−	+/+	+*/+	−/−	−/−	v/+*	R/R	R/R
6	+*/+*	−/−	−/−	v/+	v/v	−/−	+/+	+*/+	−/−	−/−	+*/+	R/R	R/R
7	+*/+	−*/−	−/−	v/+	v/v	−/−	+/+	+*/+	−/−	−/−	+*/+	R/R	R/R
8	v/+*	−*/−	−/−	v/+	v/v	−/−	+/+	+*/+	−/−	−/−	+*/+	R/R	R/R
9	+*/+	−*/−	−/−	+*/+	v/v	−/−	+/+	+*/+	−/−	−/−	v/+*	R/R	R/R
10	+/+*	−*/−	−/−	v/+	v/v	−/−	+/+	+*/+	−/−*	−/−*	v/+*	R/R	R/R
11	+*/+*	−/−	−/−	−/−	−*/+	−/−	+/+	+/+	−/−	−/−	+*/+	R/R	R/R
12	−*/v	−/−	−/−	−/−	−/−*	−/−*	+/+	+*/+	−/−	−/−	−*/+	S/S	S*/S
13	−/−	−/−	−/−	−/−	−/−	−/v	+/+	+*/+	−/−	−/−	−*/+	S/S	S*/S
14	v/v	−/−	−/−	−/−	v/v	−/−	+/+	+/+	−/−	−/−	+*/+	S/S	S/S
15	v/v	−/−	−/−	−*/−	+*/+*	+*/+	−*/+*	−*/+*	−/+	−/−	v/+	S*/S*	S*/S*
16	−/−*	−/−	−/−	+/+	+*/+	−/−	+/+	+*/+	−/−	−/−	−*/+*	R/R	R/R
17	−/−	−/−*	−/−	+/+	−*/−	−/−	v/v	−/−*	−/−*	−/−	−*/+*	S*/v	S*/S*
18	+*/+	−/−	−/−	+*/+	v/v	−/−	+/+	+*/+*	−/−	−/−	−*/−*	R/R	R/R
19	+*/v	−/−	−/−	−/−	−*/v	v/v	−/−*	−/−*	−/v	−/−	v/+	S/S*	R/R*
20	v/v	v/+	−/−	v/v	−*/v	−/−	+*/+	v/+*	−/−	−/−	−*/+	R/R	R/R
21	v/v	+*/+	−/−	+/+	v/+	−/−*	+/+	+*/+	−/−	−/−*	+/+	R/R	R/R
22	v/+*	−/−	−/−	−/−	v/+*	−/−	+*/+	+/+	−/−	−/−	+*/+	S*/S	S*/S
23	+/v	−/−	−/−	−/−	−*/v	−/−	+*/+	+/+	−/−	−/−	+*/+	S/S	S/S
24	+/+	−*/v	−/−	−/−	−/−	+*/+	−*/−	+/+	−*/v	v/+*	v/+	S/S	S/S
25	v/+*	−/−*	−/−	+/+	v/+*	−/−	+/+	+/+*	−/−	−/−	v/+	R/R	R/R

+ or −, All five laboratories obtained the same reaction; +* or −*, four laboratories obtained the same reaction; v, two or three laboratories obtained the same reaction.
† 2^{nd} trial results only obtained from four laboratories.
R, Resistant; S, Sensitive.

tation, gave variable results between the laboratories for four strains. Two reactions, glucose fermentation and haemolysin production, which gave full agreement in the first trial, gave discrepancies in the second trial with three strains. Agreement was obtained with pigment production tested both on BHIA and Columbia agar. Some of the strains produced pigment earlier and more strongly on Columbia agar than on BHIA.

First trial — secondary reactions

Table 3 shows the results of the secondary reactions from the first trial. The five laboratories were not able to obtain full agreement with any strain tested. The strain isolated from flounder showed the highest discrepancy in the different tests (nine of 13 tests) and 10 other strains showed discrepancies in six or seven tests out of 13. The incubation time before reading the results of the different tests varied between 2 and 14 d for the five laboratories. A great variability was observed in the individual tests. Full agreement was only obtained for one reaction, ornithine decarboxylase. Gelatin liquefaction and Voges–Proskauer reaction showed the most variability between the different laboratories with discrepancies in more than 20 strains. Production of arginine dihydrolase, lysine decarboxylase, indole and acid from mannitol showed discrepancies for 10 or more strains. The laboratory which read the reactions after 2 d incubation obtained a large number of discrepancies while other discrepancies were due to the use of API 20E by another laboratory.

Second trial — secondary reactions

The secondary reactions from the second trial are listed in Table 3. Despite the standardization of methods, the five laboratories were not able to obtain full agreement with any of the strains tested. The highest discrepancies (five to eight tests) were obtained with four strains (10, 15, 17 and 19), including the two cytochrome oxidase-negative strains isolated from flounder (15) and turbot (19). Two of these strains (17 and 19) gave higher disagreement in the second trial compared with the first one. Large disagreements still occurred in arginine dihydrolase and Voges–Proskauer reactions. Improved agreement was obtained with the following reactions: lysine decarboxylase, indole production, acid from mannitol and sucrose and gelatin liquefaction. The remaining reactions showed the same discrepancies as in the first trial with variations with two to five strains. Improvement was obtained with the characterization of the reference strain *Aer. salmonicida* subsp. *salmonicida* (24) and the reference strain *Aer. salmonicida* subsp. *achromogenes* (25).

DISCUSSION

Before the trials were initiated the identity of the included strains was confirmed by showing agglutination to anti-*Aer. salmonicida* serum (Dalsgaard *et al.* 1994) and resistance to vibriostatic agent O/129 and novobiocin (Magariños *et al.* 1992; Dalsgaard *et al.* 1994). The cultivation of these organisms was successfully made on media enriched with blood. The major part of the collection could have been grown on media without blood, but two of the strains, the isolates from flounder (Wiklund and Dalsgaard 1995) and turbot (Pedersen *et al.* 1994), were fastidious and slow-growing, and it was necessary to enhance the growth with media enriched with blood (Ishiguro *et al.* 1986).

After the results of the first trial were compiled, and it was demonstrated that there were large discrepancies in the data generated by different laboratories, the background information about the methodology used was collected. The principal cause of the variability in the results appeared to be the duration of the incubation time before the reactions were read, confirming the work by Sneath and Johnson (1972). The most homogeneous results were obtained after incubation of the test media for 7 d. Large discrepancies were seen when the results were recorded after only 2 d of incubation. Major disagreement was also revealed in the results from the laboratory using API 20E. Only the laboratory using API 20E had difficulties in evaluating the aesculin hydrolysis and acid production from carbohydrates of the strains examined. These results indicate that API 20E was not a suitable system for the identification and characterization of atypical *Aer. salmonicida* strains. From the results of the first trial it was concluded that standardization of methods was essential in order to reduce discordance between the laboratories.

The fundamental reactions, such as motility, catalase and oxidase production and the method of carbohydrate breakdown, are very important. Most bacteria can be identified to genus level by using the results of the limited number of important selected fundamental reactions (Barrow and Feltham 1993). After the second trial improvement was obtained with the most important tests used in the early stages for identification of bacteria. Until recently the positive reaction of the cytochrome oxidase test has been one of the most consistently used in the identification of *Aer. salmonicida* isolates. However, reports on the isolation of cytochrome oxidase-negative *Aer. salmonicida* strains from herring (*Clupea harengus pallasi*) (Traxler and Bell 1988), farmed coho salmon (*Oncorhynchus kisutch*) (Chapman *et al.* 1991), turbot (Pedersen *et al.* 1994) and flounder (Wiklund *et al.* 1994) show that the oxidase reaction is not 100% reliable for identification. The variability of the fermentation reaction of glucose was probably due to the differences in inoculation technique. The inocula were not specially standardized in the two trials. However, the experience from the first trial showed that a heavy inoculum is needed, especially for the slow-growing fastidious bacteria.

Production of brown pigment is a property used in the classification of *Aer. salmonicida* subspecies, and it was found

that the pigment production could be determined on several media with good results, but the use of Columbia agar (Altmann *et al.* 1992) made the reading easier. The pigment production of the type strain of *Aer. salmonicida* subsp. *achromogenes*, which was observed in both trials by all laboratories, is in conflict with the classification of this strain. However, a weak pigment production by the same strain was also observed by McCarthy and Roberts (1980) and Paterson *et al.* (1980). According to Altmann *et al.* (1992) pigment production depends on the composition of the media.

The secondary biochemical reactions have been chosen primarily from Popoff (1984) and the resistance or sensitivity to ampicillin and cephalothin was included due to the results of Dalsgaard *et al.* (1994) which showed that the reference strain *Aer. salmonicida* subsp. *salmonicida* was sensitive to both antibiotics and the reference strain *Aer. salmonicida* subsp. *achromogenes* was resistant to both antibiotics. Most of the strains (15 of 23) in this study were resistant to both antibiotics. Only one strain (isolated from turbot) was found to be sensitive to ampicillin and resistant to cephalothin.

Despite the standardization of media and methods disagreement was still obtained in the second trial between the participating laboratories. The decarboxylase patterns and the Voges–Proskauer reaction of the different subspecies could be taxonomically useful. However, the results in the present study revealed that these tests need further standardization. The media used for these tests were without NaCl and a way to overcome the problems with these tests might be to add NaCl to the media. This suggestion is based on the optimum growth of the strain from flounder, which occurred in 0·5–2·5% NaCl (Wiklund *et al.* 1994), and the fact that the majority of the strains studied were isolated from marine fish species. The errors found indicate that 'false-negative' reactions were far more likely to occur. The 'false-negative' reactions were observed with the gelatin reaction where all strains were expected to be positive, as reported by Gudmundsdóttir (1996). Gelatin liquefaction was shown to be difficult to read because the 'clear zone' was hidden by pigment production. However, changing the agar from BHIA to nutrient agar showed that all the strains were proteolytic.

In reading the tests it was necessary to ascertain that there was good growth in the media. If not, the test had to be repeated. The general principle was that a medium had to be heavily inoculated in order to obtain good growth. The cultures were examined for growth daily for up to 7 d but, because the strain collection also included slow-growing fastidious bacteria, the examination was done both after 7 and 14 d.

The most extensive discordance between the laboratories was revealed for strains 15, 17 and 19. The discrepancy in the identification of the strains isolated from turbot and flounder was expected because these strains belong to the slow-growing fastidious forms of *Aer. salmonicida* (Pedersen *et al.* 1994; Wiklund *et al.* 1994). The discrepancies in the results with the reference cultures are problematic. These strains are needed in any study to make sure that the methods are performed properly. To obtain appropriate identification procedures it is necessary to emphasize the importance of including reference cultures with well-known characters as controls for the characterization tests used.

The large disagreement demonstrated in the trials may partly be attributed to the fact that the experience in characterization of the atypical strains of *Aer. salmonicida* varied between the participating laboratories. If uniform identification of fish pathogens is to be achieved in the future, it is recommended that the methodology (e.g. media, reagents and incubation time) should be standardized. Despite standardization, the data presented in this work indicate that discrepancies can always be expected when two or more laboratories examine identical bacterial strains. These observations are in agreement with the findings of Sneath and Johnson (1972), who illustrated that reproducibility between laboratories is usually a good deal worse than between replicates within laboratories. Proficiency testing, in which samples are distributed by mail, has for many years served as a basis for evaluating the performance of clinical laboratories (Peterz 1992). Laboratories that have been participating in proficiency testing for a long time produced, on average, fewer false and outlying results, and obtained more accurate and precise results than those that had recently joined the testing (Peterz 1992). According to these observations by Peterz (1992) and the experience from the present trials the laboratories which participated in the identification of the fish pathogens improved in performance during the time period studied. This paper seems to be the first on standardization of microbiological tests for identification of fish pathogens, and the results obtained show the need for standardization of test methods both within and between laboratories.

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