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NOTE

Ribotyping of *Aeromonas salmonicida* subsp. *salmonicida*

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ABSTRACT: A collection of 124 strains of the bacterial fish pathogen *Aeromonas salmonicida* subsp. *salmonicida* isolated from diseased salmonids in Denmark (n = 57), Norway (n = 28), Scotland (n = 20), and North America (n = 19) was characterized by ribotyping. For reference, the type strain NCMB 1102 was likewise examined. Digestion of total DNA with the enzymes *Eco*R1, *Hin*dIII and *Sma*1 resulted in 2, 1 and 5 ribotypes, respectively, with a total of 6 ribotypes in combination. The type strain NCMB 1102 belonged to the most common of the ribotypes, irrespective of the enzyme used. Although Danish and North American strains were genetically very homogeneous, Norwegian and Scottish isolates showed some genetic diversity. In general, ribotyping was not found to be applicable as an epizootiological typing system for *A. salmonicida* subsp. *salmonicida* due to low discriminatory power. Norwegian and Scottish strains contained a common *Sma*1 ribotype suggesting a connection between isolates from these 2 countries.

KEY WORDS: *Aeromonas salmonicida* · Ribotyping · Genotyping

Furunculosis, caused by the fish pathogenic bacterium *Aeromonas salmonicida*, is one of the major diseases among farmed salmonids in both freshwater and seawater aquaculture. First isolated almost 100 yr ago by Emmerich & Weibel (1894), *A. salmonicida* is today found worldwide (Austin & Austin 1987).

Phenotypic systems for subspecies characterization such as biotyping, serotyping, and resistotyping have been used within *Aeromonas salmonicida* without success because of the very homogeneous nature of the species (Austin & Austin 1987). Phage typing has proved to be useful (Popoff 1971a, b, Rogers et al. 1981) but this system has not yet been applied to larger investigations on the epizootiology of furunculosis.

Genotypic methods such as plasmid profiling, restriction endonuclease analysis (RE), and ribotyping have proved to be effective tools for subspecies characterization of many pathogenic bacteria (Farrar 1983, Saunders 1991). In this report, results from ribotyping of strains of *Aeromonas salmonicida* subsp. *salmonicida* from Denmark, Norway, Scotland, and North America are presented with the aim of evaluating the possible use of this method as a tool for typing of *A. salmonicida*, and to elucidate the genetic variation within this bacterial subspecies.

Materials and methods. ***Bacterial strains and growth conditions:*** A collection of 124 strains of *Aeromonas salmonicida* subsp. *salmonicida* from Denmark, Norway, Scotland, and North America together with a reference strain, *A. salmonicida* subsp. *salmonicida* NCMB 1102, were investigated. The strains are listed in Table 1. All strains were isolated from diseased salmonids.

The bacteria were grown on brain heart infusion (BHI) agar (Difco, Detroit, MI, USA). After 48 h of incubation at 20 °C, 5 to 8 colonies were harvested, mixed in 1 Eppendorf tube, and used for DNA isolation.

Isolation of total DNA and ribotyping: Isolation of total DNA, quantification of DNA, and ribotyping were performed as previously described by Olsen et al. (1992). Briefly, 1 µg of purified DNA was digested with one of the enzymes *Bam*H1, *Cla*1, *Eco*R1, *Hin*dIII, *Pst*1, *Sac*1 or *Sma*1 (Boehringer, Mannheim), separated in 0.8% agarose gels (Litex, LSL), stained with 2 mg l^{-1} ethidium bromide (Sigma), and photographed under ultraviolet light. The DNA was transferred to nylon-hybridization membranes (Hybond-N, Amersham) by vacuum blotting and hybridized with a digoxigenin (Boehringer, Mannheim) labelled DNA probe complementary to 16S and 23S rRNA of *Escherichia coli* (Sigma).

Results. ***Choice of restriction enzymes for characterization:*** Total DNA of the reference strain NCMB 1102 was separately digested with the enzymes *Bam*H1, *Cla*1, *Eco*R1, *Hin*dIII, *Pst*1, *Sac*1, and *Sma*1. When ribotyping was performed, the selected enzymes gave patterns with 7 to 9 clear bands and 2 to 5 weak

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Table 1. Source and year of isolation of the examined strains of *Aeromonas salmonicida* subsp. *salmonicida* from Denmark, North America, Norway, and Scotland. Numbers refer to number of strains isolated

Host	Year of isolation										
	1970	80	82	83	84	85	86	87	88	89	90
Denmark[a]											
Oncorhynchus mykiss			8	7	8	8	4	4	2		
Salmo trutta				1	2	1	2	1	3		
Salvelinus fontinalis				2	1			1			
Salmo salar				1		1					
North America[b]											
Salmo salar		1			1		1			1	6
Salmo trutta			1	1			1				
Salvelinus fontinalis						1				1	
Oncorhynchus mykiss						1				1	
Oncorhynchus tshawytscha	1						1				
Norway[c]											
Salmo salar										24	
Oncorhynchus mykiss										4	
Scotland[d]											
Salmo salar										20	

Strains provided by: [a]Dr I. Dalsgaard, Danish Institute for Fisheries and Marine Research, Fish Disease Laboratory, Royal Veterinary and Agricultural University, Copenhagen, Denmark; [b]Dr G. Olivier, Dept of Fisheries & Oceans, Halifax, Canada; [c]Dr E. Myhr, National Veterinary Institute, Oslo, Norway; [d]Dr T. Hastings, The Scottish Office Agriculture and Fisheries Dept, Marine Laboratory, Aberdeen, Scotland

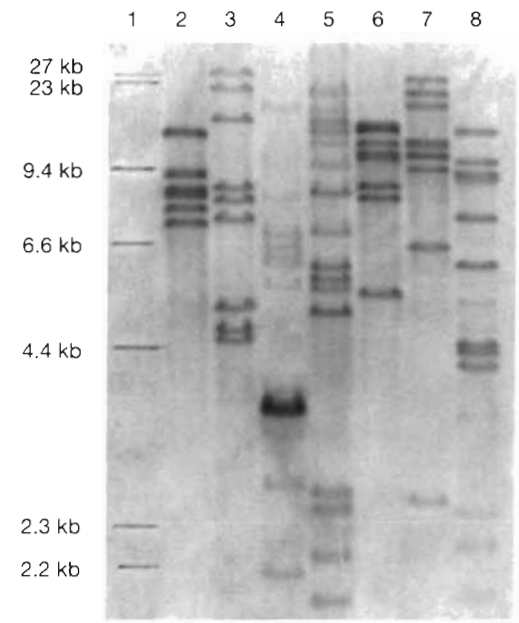

Fig. 1 Ribotypes of *Aeromonas salmonicida* subsp. *salmonicida* NCMB 1102 obtained after digestion with the enzymes: (2) *Bam*H1, (3) *Cla*1, (4) *Eco*R1, (5) *Hin*dIII, (6) *Pst*1, (7) *Sac*1 and (8) *Sma*1. Lane 1 represents the molecular weight marker with bands at 27, 23, 9.4, 6.6, 4.4, 2.3 and 2.2 kb

bands (Fig. 1). With some of the enzymes (*Bam*H1 and *Pst*1), the bands were very close to each other, while the bands were well separated with other enzymes (*Cla*1, *Eco*R1, *Hin*dIII, *Sac*1 and *Sma*1). Based on these results, the relatively inexpensive enzymes *Eco*R1, *Hin*dIII, and *Sma*1 were chosen for ribotyping of the full strain collection.

Ribotyping: Table 2 summarizes the results of the ribotyping. The enzyme *Hin*dIII gave 1 ribotype among all 124 strains examined, while *Eco*R1 resulted in 2 different ribotypes (Fig. 2).

All strains from Denmark and Scotland, 23 strains from Norway (82%), and 18 North American strains (95%) belonged to *Eco*R1 ribotype no. 1, while 5 Norwegian strains (18%) and 1 North American strain (5%) belonged to *Eco*R1 ribotype no. 2.

As seen from Fig. 3, 5 different ribotypes were observed with the enzyme *Sma*1. All strains from Denmark and North America belonged to *Sma*1 ribotype No. 1. Norwegian strains could be divided into 2 *Sma*1 ribotypes, with 24 strains (86%) in *Sma*1 ribotype no. 1 and 4 strains (14%) in *Sma*1 ribotype no. 2. The 5 Norwegian strains of the *Eco*R1 ribotype no. 2 all belonged to *Sma*1 ribotype no. 1. The 20 Scottish strains could be divided into 5 *Sma*1 ribotypes. Six strains (30%) belonged to *Sma*1 ribotype no. 1, 7 strains (35%) to *Sma*1 ribotype no. 2, 1 strain (5%) to *Sma*1 ribotype no. 3, 2 strains (10%) to *Sma*1 ribotype no. 4 and 4 strains (20%) to *Sma*1

Table 2. Ribotypes of 124 strains of *Aeromonas salmonicida* subsp. *salmonicida*. All strains had an identical *Hin*dIII pattern. The table shows the *Sma*1 distribution according to the observed *Eco*R1 type of the strains. The total number of different types was 6

Enzyme:	*Eco*R1	*Sma*1				
	No. of strains with type	No. of strains with type				
		1	2	3	4	5
Denmark	1: 57	57	0	0	0	0
	2: 0	0	0	0	0	0
Norway	1: 23	19	4	0	0	0
	2: 5	5	0	0	0	0
North America	1. 18	18	0	0	0	0
	2: 1	1	0	0	0	0
Scotland	1: 20	6	7	1	2	4
	2: 0	0	0	0	0	0

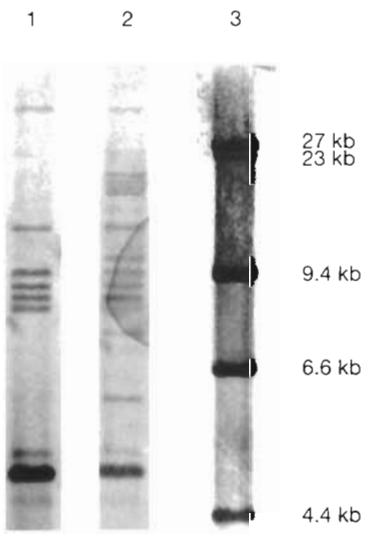

Fig. 2. *Eco*R1 ribotypes demonstrated among the 124 examined isolates of *Aeromonas salmonicida* subsp. *salmonicida*. Lane 1 demonstrates the common *Eco*R1 ribotype; Lane 2 demonstrates the rare *Eco*R1 ribotype. Molecular weight marker with bands at 27, 23, 9.4, 6.6, and 4.4 kb is shown in Lane 3

ribotype no. 5. Ribotypes were found to be reproducible on up to 8 repeating typings. No correlation with host species was demonstrated. The reference strain NCMB 1102 belonged to *Hin*dIII, *Eco*R1, and *Sma*1 ribotypes no. 1.

Discussion. Ribotyping of the reference strain NCMB 1102 by use of 7 different enzymes showed that some enzymes were more suitable than others. In the ribotypes produced by *Cla*1, *Eco*R1, *Hin*dIII, *Sac*1, and *Sma*1 the bands were well separated and the ribotype was easily read. Based on this knowledge and the price of the enzymes, *Eco*R1 and *Hin*dIII were chosen for ribotyping of the strain collection. The enzyme *Sma*1 was also chosen, as this enzyme has proved useful for ribotyping of other Gram-negative bacteria (Olsen et al. 1992).

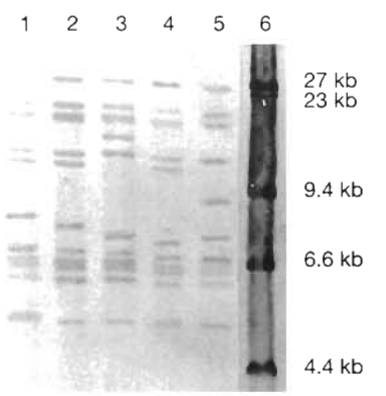

Fig. 3. *Sma*1 ribotypes demonstrated among the 125 examined strains of *Aeromonas salmonicida* subsp. *salmonicida*. Lanes 1 to 5 show the respective 5 *Sma*1 ribotypes; Lane 6 shows the molecular weight marker with bands at 27, 23, 9.4, 6.6 and 4.4 kb

A total of 6 ribotypes were detected in the present study. Two ribotypes were produced with *Eco*R1, but only 5% of the strains belonged to *Eco*R1 ribotype no. 2. In line with results published for ribotyping of *Salmonella enteriditis* (Martinetti & Altwegg 1990) and *S. berta* (Olsen et al. 1992) ribotyping by use of *Sma*1 resulted in the highest degree of polymorphism. Danish and North American strains showed only 1 ribotype, and in general, Danish and North American strains of *Aeromonas salmonicida* subsp. *salmonicida* appeared to be very homogeneous as they, with the exception of 1 North American strain, produced only 1 ribotype after digestion with all 3 enzymes.

A higher degree of ribotype diversity was observed among strains originating from Norway and Scotland. The 20 Scottish strains, all isolated in 1990, resulted in 5 *Sma*1 ribotypes.

The plasmid content has previously been reported (Nielsen et al. 1993) and using plasmid profiling as a typing system, the Scottish strains showed a higher degree of variation than the Danish and North American ones.

The appearance of *Sma*1 ribotype no. 2 among both Scottish and Norwegian strains may indicate a link between these 2 countries. A similar indication has been noted by us using plasmid profiles as an epizootiological marker, e.g. a plasmid of 8.5 kb was detected only among Scottish and Norwegian strains (Nielsen et al. 1993). Other indications of links between geographically separated populations are indicated from the results. The appearance of *Eco*R1 ribotype no. 2 in 5 strains from Norway and 1 strain from North America may indicate a connection, either directly or through a common third source. This relationship is possible because a large number of rainbow trout have been imported from North America to Europe (Austin & Austin 1987).

In view of the observed distribution of ribotypes (Table 2), ribotyping, in general, must be considered of limited value and less useful than phage typing and plasmid profiling for epizootiological investigations of furunculosis outbreaks. However, the demonstration of 5 *Sma*1 ribotypes among 20 Scottish isolates shows that ribotyping in certain geographical areas may be useful. The demonstration of 5 *Sma*1 ribotypes among the 20 strains examined makes it seem likely that additional *Sma*1 ribotypes among Scottish *Aeromonas salmonicida* subsp. *salmonicida* strains will be demonstrated if a larger strain collection is examined.

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