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Ribotyping and plasmid profiling of *Yersinia ruckeri*

J.A. Garcia[1,2], L. Dominguez[2], J. L. Larsen[1] and K. Pedersen[1]

[1]*Laboratory of Fish Diseases, Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, Frederiksberg, Denmark, and* [2]*Departamento de Patología Animal I (Sanidad Animal), Facultad de Veterinaria, Universidad Complutense, Madrid, Spain*

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J.A. GARCIA, L. DOMINGUEZ, J.L. LARSEN AND K. PEDERSEN. 1998. A total of 183 strains of *Yersinia ruckeri*, isolated from a wide variety of sources, were studied with respect to their plasmid profile and ribotype patterns. Eight plasmid profiles and 11 ribotypes were demonstrated, with one profile being predominant by both typing methods. The results suggest a clonal structure for this species, with a predominant clone being responsible for most of the outbreaks worldwide. The results of a long-time survey in several fish farms in Spain and Denmark seem to support this idea.

INTRODUCTION

Yersinia ruckeri is the causative agent of enteric redmouth (ERM) disease, an acute to chronic stress-related disease of salmonids (Busch 1983; Austin and Austin 1993; Stevenson *et al.* 1993). It was first isolated in the early 1950s in the Hagerman Valley of Idaho (USA) from rainbow trout by Ross and co-workers and has now spread throughout the world (Busch 1983; Austin and Austin 1993; Stevenson *et al.* 1993). This spread has been explained by the import of infected fish (Busch 1983; McArdle and Dooley-Martin 1985; Michel *et al.* 1986) but this explanation appears to be insufficient as ERM has been diagnosed in places with no known history of recent introductions of fish (Bullock *et al.* 1978). The isolation of *Y. ruckeri* not only from salmonids but also from feral and ornamental fish (McArdle and Dooley-Martin 1985; Michel *et al.* 1986; Willumsen 1989), birds (Willumsen 1989), muskrat (Stevenson and Daly 1982) and even humans (Farmer *et al.* 1985), suggests the existence of different sources in nature (Willumsen 1989) that could play an important role in the epidemiology of this disease.

Different techniques have been used to characterize strains of *Y. ruckeri*. Serotyping has been the most widely used and several serogroups have been described (Stevenson and Airdrie 1984; Daly *et al.* 1986; Davies 1990; Romalde *et al.* 1993). However, the actual situation is confusing as the different systems do not correlate well (Austin and Austin 1993; Furones *et al.* 1993; Romalde *et al.* 1993). There appears to be more agreement among different authors about the existence of plasmids (De Grandis and Stevenson 1982; Toranzo *et al.* 1983; Stave *et al.* 1987; Romalde *et al.* 1993). All these studies have concluded that most of the strains in serovar O1, the most pathogenic, carry a large plasmid of 40–70 MDa, and some have also found a small plasmid of 20–30 MDa (De Grandis and Stevenson 1982) or 12–15 MDa (Stave *et al.* 1987; Romalde *et al.* 1993). In contrast, strains from the other serotypes carried no, or very small plasmids. The similarity in weight of the large plasmid and the virulence plasmid of other *Yersinia* species led to the suggestion that these plasmids might be related, but no calcium requirement has been observed in *Y. ruckeri* (De Grandis and Stevenson 1982), and later work has shown different restriction patterns between the plasmid of *Y. ruckeri* and that in the other *Yersinia* species (Guilvout *et al.* 1988). However, some role for this plasmid in the virulence of *Y. ruckeri* seems likely, but so far has not been investigated (De Grandis and Stevenson 1982; Toranzo *et al.* 1983; Stave *et al.* 1987; Furones *et al.* 1993).

Other techniques used have been outer membrane protein (OMP) profiles and genetic methods. Davies (1991a) described five different OMP types but these have not been found by later authors (Romalde *et al.* 1993). Finally, although few attempts have been made to characterize this bacterium genetically (Schill *et al.* 1984; De Grandis *et al.* 1988; Romalde *et al.* 1993), all these authors have concluded that *Y. ruckeri* has a very low genetic intraspecies diversity, forming a genetically homogeneous group.

Ribotyping has proved to be a very helpful tool for both taxonomy and epidemiology (Bingen *et al.* 1994), and it has been applied in a number of different studies (Grimont and Grimont 1986; Saunders *et al.* 1988; Andersen and Saunders 1990; Moyer *et al.* 1992; Bingen *et al.* 1994; Mendoza *et al.*

Correspondence to: Jose A. Garcia, Departamento de Patología Animal I (Sanidad Animal), Facultad de Veterinaria-UCM, Avda Puerta de Hierro s/n, 28040-Madrid, Spain (e-mail: g.cabrera@eucmax.sim.ucm.es).

1996). The aim of this investigation was to explore the use of ribotyping as a typing method that could differentiate *Y. ruckeri* into groups, and compare and evaluate the use of plasmid profiling and ribotyping to relate different strains of this bacterium from several origins.

MATERIALS AND METHODS

Bacterial strains

A total of 183 strains from nine different European countries, the USA and Chile, and reference strains, were used in this investigation (Table 1). These strains were stored as Luria-Bertani (LB, Gibco) broth cultures with 15% (v/v) sterile glycerol at −80 °C.

Plasmid profiling

Overnight cultures in Marine broth (MB; Difco) were used for the determination of plasmid profiles. The plasmids were extracted by the method of Kado and Liu (1981) and separated in 0·8% agarose gels (Seakem GTG, FMC Bioproducts) by gel electrophoresis in TAE buffer, at pH 8·0. After electrophoresis, the gels were stained with ethidium bromide, 2 $\mu g\,ml^{-1}$ (Fluka), and photographed in u.v. light. The size of the plasmids was determined from the length of their migration compared with plasmids from *Escherichia coli* V517 (Macrina *et al.* 1978) and 39R861 (Threlfall *et al.* 1986).

Table 1 Number and origin of the strains used in this study

Country	Number of strains
Type strains (Ty)	5
Denmark (DK)	101
Spain (SP)	38
Italy (I)	9
Norway (N)	9
UK	5
Germany (D)	4
USA	4
France (F)	3
Portugal (PL)	2
Sweden (S)	2
Chile (CH)	1
Total	183

Type strains: NCTC (National Collection of Type Cultures)10476, NCTC 10478, CECT (Coleccion Española de Cultivos Tipo) 955, CECT 956 and NCMB (National Collection of Marine Bacteria) 1316.

Nicked forms were confirmed by the method of Hintermann *et al.* (1981).

DNA extraction, digestion and ribotyping

Chromosomal DNA was isolated essentially as previously described by Pedersen and Larsen (1993). Briefly, 1·5 ml of an overnight broth culture grown in LB was harvested by centrifugation in a microfuge tube, resuspended in 500 μl Tris:ethylenediaminetetraacetate (EDTA) 50:50 $mmol\,l^{-1}$, pH 8·0, and lysed by the addition of 25 μl lysozyme (20 $mg\,ml^{-1}$), 40 μl of 10% SDS and 5 μl proteinase K (20 $mg\,ml^{-1}$). The lysates were extracted with phenol:chloroform:isoamylalcohol, 25:24:1, and chloroform:isoamylalcohol, 24:1, precipitated with isopropanol, washed twice with 70% ethanol, and resuspended in 40 μl Tris:EDTA 10:1 $mmol\,l^{-1}$, pH 7·6.

The extracted DNA solution (5 μl) was digested with the restriction enzyme according to the manufacturer's instructions. Digested DNA was precipitated with 22 μl 7·5 $mol\,l^{-1}$ ammonium acetate and 130 μl 96% ethanol, centrifuged, and resuspended in 20 μl distilled water. Then, 10 μl of the loading buffer (EDTA 2 $mmol\,l^{-1}$, pH 8·0, bromophenol blue 0·1%, glycerol 30%) were added to the resuspended material and the mixture was electrophoresed in 0·8% agarose gels in TAE buffer, pH 7·8. Subsequently, gels were stained and photographed as described above.

After separation by electrophoresis, DNA fragments were transferred onto a nylon hybridization membrane (Micron Separation Inc.) and fixed to the membrane by incubation at 80 °C for 1 h. Southern blots were then hybridized overnight at 56 °C using a digoxigenin-labelled probe made from 16S and 23S rRNAs of *E. coli* (Boehringer). Labelled fragments were visualized by incubation of the membrane with alkaline phosphatase-labelled antidigoxigenin immunoglobulin (Boehringer), followed by the addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer).

RESULTS

Plasmid profiles

Eight plasmid profiles were detected (Fig. 1) and given arbitrary letters from A to H. Most of the strains (162, 88·5%) carried a large plasmid of approximately 75 MDa. In addition to this large plasmid, 155 of the strains also carried a smaller plasmid of 12·7 MDa, while five of the other eight strains carried a plasmid of 15·5 Mda. Nineteen strains had no plasmids, two carried a small plasmid of 3·1 MDa, one had a 35 MDa plasmid and two presented a plasmid of 64 MDa.

The largest group was group A (84·15% of all strains studied); this group presented two plasmids (75 and 12·7

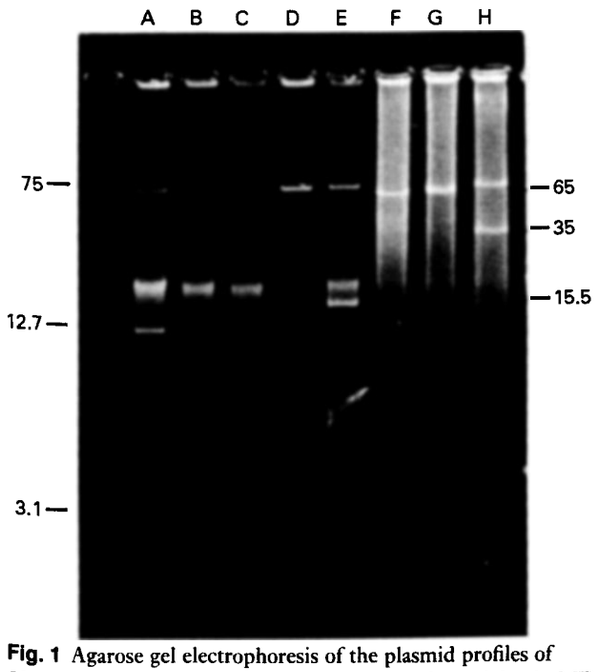

Fig. 1 Agarose gel electrophoresis of the plasmid profiles of *Yersinia ruckeri*. Numbers indicate the molecular weights in MDa

MDa). This profile included most of the O1 reference strains used (Table 2) The last O1 reference strain (CECT 955) was included in group E, also carrying two plasmids but a 15·5 plasmid instead of the 12·7 Mda plasmid of group A. Group A included most of the strains from Denmark (97%), Spain (100%), Italy (100%), France (66·7%), Portugal (100%) and the USA (50%) (Table 2). Group E included another four strains from the UK (80%) (Table 2).

The second largest group was group B (10·4%). This was shown to have no plasmids; it included some strains from Denmark (2%), UK (20%) and France (33·3%), and most isolates from Norway (66·7%), Germany (75%), Sweden (100%), the USA (50%) and Chile (100%). The O2 type strain (CECT 956) was also included in this profile (Table 2).

Profile F included three strains (1·64%), two from the UK (40%) and one from Norway (11·1%). Profiles C and D had very low frequencies (0·55% each) as they included only one strain. These strains presented one single plasmid, but while the plasmid in group C was very small (3·1 MDa), the plasmid in group D was of the same molecular range as the large plasmid in groups A and E (75 MDa); the plasmid in group F was slightly smaller than this (64 MDa). The strain in group C had been isolated in Germany (25% of the strains from this country), whereas the strain in group D had been isolated in Denmark (1·0%) and the strain in group F, in Norway (11·1%) (Table 2).

The two remaining profiles, G and H, presented two plasmids and included one strain (0·55%) each. Profile G harboured a large plasmid of 64 MDa together with a small one of 3·1 MDa, and the strain was isolated from Norway (11·1%). The strain from profile H, also isolated from Norway (11·1%), carried a large plasmid of 75 MDa and a second of 35 MDa.

Ribotyping

Ten strains were randomly chosen for initial tests with an array of 11 restriction enzymes (*Eco*RI, *Bam*HI, *Hin*dIII, *Xba*I, *Mlu*I, *Bgl*I, *Kpn*I, *Sma*I, *Pst*I, *Ava*I and *Hae*III) prior to choosing the definitive enzyme. The best results were achieved with *Eco*RI and *Mlu*I (data not shown), so *Eco*RI was chosen to perform the ribotyping.

When all 183 isolates were examined, 11 different ribotypes were detected, and given an arbitrary number from 1 to 11 (Fig. 2). The different ribotype patterns showed a varying number of bands, but three bands were common to all patterns (Fig. 2). Pattern 1 was the most frequent (84·2%), with 154 strains, and included most of the strains from the same countries as plasmid profile A, i.e. Denmark (95·1%), Spain (100%), Italy (100%), France (66·7%), Portugal (100%) and the USA (50%), as well as one strain from the UK (20%) and all the O1 reference strains. The rest of the ribotypes were each demonstrated in one or two countries, except for type 2 that was present in four countries and 5 in three (Table 2); most of them included only one to four strains except, again, group 2 (Table 2).

The second most abundant pattern was group 2 with 10 strains (5·46%), two from Norway (22·2%), two from Germany (50%), three from the UK (60%), two from the USA (50%) and one reference strain. Group 5 included five strains (2·73%), three from Norway (33·3%), one from Denmark (0·99%) and one from France (33·3%). Ribotypes 6 and 7 both included three strains (1·64%). Pattern 6 included two strains from Denmark (1·98%) and one from Norway (11·1%), while ribotype 7 included two from Sweden (100%) and one from Norway (11·1%). Groups 3 and 8 included two strains each (1·09%). Group 3 included one from Denmark (0·99%) and one from Germany (25%), and type 8, one from Germany (25%) and one from Chile (100%).

Finally, groups 4, 9, 10 and 11 were each represented by only one strain (0·6%). In the case of ribotype 4, the strain was Danish (1·0%), type 9 British (20%), and groups 10 and 11 Norwegian (11·1% each).

DISCUSSION

The observation of a large plasmid in most of the strains (89·13%) was not surprising as several authors (De Grandis and Stevenson 1982; Toranzo *et al.* 1983; Stave *et al.* 1987;

Table 2 Number and origin of strains in each of the groups described

		Country											
Rb	Pp	DK	SP	I	N	UK	D	USA	F	PL	S	CH	Ty
1	A	96	38	9				2	2	2			3
	E					1							1
2	B				2		2	2					1
	F					2							
	I					1							
3	B	1					1						
4	A	1											
5	A	1											
6	B				3				1				
	B	1											
	D	1											
	H				1								
7	B				1						2		
8	B											1	
	C						1						
9	B					1							
10	F				1								
11	G				1								

Rb = ribotype; Pp = plasmid profile.

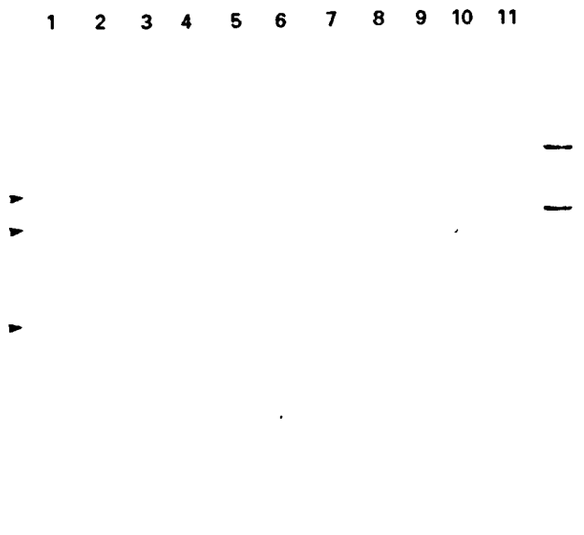

Fig. 2 Ribotypes of *Yersinia ruckeri* isolates with *Eco*RI as restriction enzyme. Last column on the right is a DNA molecular weight marker (Boehringer Mannheim)

Guilvout *et al.* 1988; Romalde *et al.* 1993) have described the existence of a large plasmid in the majority of the strains studied, independently of their geographical origin. According to these authors, this plasmid is common to all clinical strains. This agrees with the fact that most of the strains in the present study had been isolated from clinical outbreaks. The existence of a smaller plasmid of about 12·7 MDa, which was present in most of the strains with the 75 MDa plasmid, also agrees with reports from other authors (De Grandis and Stevenson 1982; Stave *et al.* 1987; Romalde *et al.* 1993). In this respect, different authors have observed a small plasmid in strains of serovar O1. However, this small plasmid has received much less attention than the large one, probably because of the attempts to correlate the latter with the virulence plasmid of other *Yersinia* species (De Grandis and Stevenson 1982; Stave *et al.* 1987; Guilvout *et al.* 1988). The observation of strains with no plasmids, or plasmids of very low molecular weight, also agrees with observations by the same authors (De Grandis and Stevenson 1982; Stave *et al.* 1987; Romalde *et al.* 1993). No descriptions of large plasmids in serogroups other than O1 have been found in the literature. The only references are to the presence of small plasmids if there are any in these serotypes. However, De Grandis and Stevenson (1982), studying 12 serotype O1 and five serotype O2 strains, found that two serogroup O1 strains had no plasmid, so strains without plasmids cannot be excluded from this serotype.

From these results, it is obvious that plasmids, although of some help in typing *Y. ruckeri* strains, cannot be used to characterize this pathogen clearly because they are not present in all the strains and most strains seem to carry the same plasmids.

Using the ribotyping method it was possible to distinguish 11 different groups of *Y. ruckeri*. Pattern 1 was the most frequent type (84·2%). It was also the most widely distributed ribotype, appearing both in Europe and in the USA, and included all serotype O1 reference strains. This ribotype 1 included none of the strains from Norway, Sweden or Germany and only one from the UK. Although these results agree with the observations by Davies and Frerichs (1989) of a clear-cut geographical distribution of strains from the UK, Norway and the rest of Europe using morphological and biochemical criteria, we, like these authors, also think that the strains have been selected by the contributors and are not necessarily representative samples of the strains of *Y. ruckeri* isolated in these countries. Another fact that needs to be kept in mind when analysing results from these strains is the low number of strains from the UK, Sweden and Germany. More strains should be studied from all these countries, including Norway, before concluding that ribotype 1 is lacking. The rest of the ribotypes described in the present work were limited to more restricted areas in Europe. An exception to this was ribotype 5 which was isolated from countries in Northern Europe (Denmark and Norway) and from France.

The ribotyping results were confirmed when both ribotyping and plasmid profiles were taken into account, although in this case a slightly higher number of groups (Hintermann *et al.* 1981) was found. There was a predominant group which included almost all the strains in ribotype 1 and most of the strains with the plasmid profile A. However, differences were seen in some groups. Strain CECT 955 differed from the rest of the ribotype 1 strains by carrying a larger small plasmid (15·5 MDa instead of 12·7 MDa) and one strain carried a second large plasmid of about 35 MDa together with the 'typical' large plasmid (75 MDa). Whether the 15·5 MDa plasmid is merely a derivative of the 12·7 MDa plasmid is at present unknown but thought to be very likely.

Ribotypes 2, 5, 6 and 8 were the only ribotypes to include strains with and without plasmids. Ribotype 2 included 10 strains, seven of which were profile B (no plasmids) and three of which were profile E (75 and 15·5 MDa plasmids). These three strains were all isolated from the UK while the other seven had been isolated from Norway, Germany and the USA, and the last one was an O2 reference strain (CECT 956). Ribotype 5 included five strains, four of which carried no plasmids (profile B) while the fifth presented the 75 and 12·7 MDa plasmids (profile A). Ribotype 6 included three strains, each one with a different profile; one was profile B, a second was profile D (75 MDa plasmid only) and the last harboured the 75 and a 35 MDa plasmids (profile H). Finally, ribotype 8 included one strain from profile B and a second with a very low (3·1 MDa) plasmid (profile C). However, it is not known whether the strains without plasmids had carried plasmids when they were isolated but had lost them later by laboratory handling. At present, there is no information on the stability of plasmids in *Y. ruckeri*. The impact of the plasmids on virulence should be investigated using strains with and without plasmids, but with identical ribotypes, or strains cured of the plasmids.

The existence of different subtypes has also been observed by other authors. Davies and Frerichs (1989) found that European strains of *Y. ruckeri* could be divided into two groups according to their biochemical characters. One of these groups was predominant in the UK and Norway (non-motile and Tween-80 negative) and the second, in the rest of Europe (motile and Tween-80 positive). However, these authors established the need for further studies concerning this clear-cut geographical distribution. A possible relation between motility and virulence is at present unknown and needs further investigation. Davies (1991b) also found different clonal groups within serotype O1 according to biotypes, serotypes and OMP profiles. This author reported that two of these clonal groups were predominant, one in the UK and the other in the rest of Europe. Romalde *et al.* (1993) also observed great serological homogeneity among the isolates in Europe, with the exception of some isolates from the UK and the Baltic countries. The same authors also found a high degree of homogeneity among the Spanish strains used in their study, regardless of the year of isolation. This homogeneity was based on serology, LPS, OMP, total DNA fingerprints and plasmid profiles. The same results were achieved by ribotyping and plasmid profile of the Spanish strains in the present study. All of them showed the same pattern (1 A), which confirms the high degree of homology among these strains, and the existence of one group responsible for most of the outbreaks (Schill *et al.* 1984).

Compared with the results of Romalde *et al.* (1993), our groups 1 A, 1E, 4 A and 5 A would represent strains from their serogroup O1a, while group 6D would represent strains from their serotype O1b. The group 6B should then, according to Romalde *et al.* (1993), be included in one of the other serotypes. In certain other bacteria, e.g. *Vibrio anguillarum* (Pedersen *et al.* 1996) and *Vibrio vulnificus* (Biosca *et al.* 1997), plasmids belonging to the same homology group seem to be restricted to the same O-serogroup. The relationship between serotype and plasmid content of *Y. ruckeri* needs further clarification, but from the present results it seems that the 75 MDa and 12·7 (or 15·5) MDa plasmids are only present in serotype O1 strains. Whether strains without plasmids also belong to this serotype is at present unknown.

The fact that almost 85% of the strains studied have shown the same pattern suggests the existence of a predominant group and a high degree of homogeneity within the species. This is in accordance with the results of Schill *et al.* (1984) using multilocus isoenzyme electrophoresis. According to these authors, the genetic structure of *Y. ruckeri* is clonal, with one predominant clonal group. The predominant group in the investigation of Schill *et al.* (1984) was widespread, as

was our group 1 A. In addition, the finding that all the isolates from different rainbow trout farms controlled for at least 3 years in Spain, and 1 in Denmark, showed a high level homogeneity, belonging always to the same ribotype 1 and the same plasmid profile A in both countries, suggests that only one group is responsible for most of the outbreaks worldwide. It is not known whether *Y. ruckeri* maintains the same homogeneity in the environment as it has been found among the clinical isolates, and although several selective media have been described (Waltman and Shotts 1984; Rodgers 1992), no references have been found in the literature about the isolation of the different serogroups from the environment.

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