

Phenotypic, Serological and Genetic Characterization of *Pseudomonas anguilliseptica* Strains isolated from cod (*Gadus morhua*) in North Europe.

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Abstract

In the present work, the biochemical, serological and genetical characteristics of six strains of *Pseudomonas anguilliseptica* isolated from cod (*Gadus morhua*) in Scotland were analyzed in comparison with previous well characterized isolates with different origin of this bacterial species. Biochemical and physiological analyses showed that this group of isolates is highly homogeneous, their characteristics matching previous descriptions of the pathogen. Similar results were obtained for the six cod isolates in the serological assays, all of them belonging to the serotype O1. A high homogeneity was observed also in the genetical study, analyzed by means of RAPD, ERIC-PCR and REP-PCR procedures, showing that they are similar to isolates from gilthead seabream (*Sparus aurata*), black spot seabream (*Pagellus bogaraveo*) and turbot (*Scophthalmus maximus*). Virulence assays demonstrated that cod isolates are highly virulent for turbot and sole with LD₅₀ between 7.6×10^4 and 5×10^7 bacterial cells/fish.

Introduction

Pseudomonas anguilliseptica is an opportunistic pathogen for a variety of fish species cultured in marine and brackish waters worldwide (Daly 1999). This microorganism was originally described as the causal agent of red spot disease of Japanese eel (*Anguilla japonica*) cultured in Japan (Wakabayashi & Egusa 1972). Since then, the pathogen has been isolated in different countries from a variety of cultured and wild fish species such as European eel (*Anguilla anguilla*), black seabream (*Acanthopagrus schlegeli*), ayu (*Plecoglossus altivelis*), Atlantic salmon (*Salmo salar*), sea trout (*S. trutta*), rainbow trout (*Onchorhynchus mykiss*), whitefish (*Coregonus* sp.), Baltic herring (*Clupea harengus membras*), Striped jack (*Pseudocaranx dentex*), orange-spotted grouper (*Epinephelus coioides*), gilthead seabream (*Sparus aurata*), turbot (*Scophthalmus maximus*) and black spot seabream (*Pagellus bogaraveo*) (Austin & Austin 1999; Daly 1999; Al-Marzouk 1999; López-Romalde, Núñez, Toranzo, & Romalde 2003c). More recently, *P. anguilliseptica* has been isolated in farmed cod (*Gadus morhua*) in UK and Canada (Ferguson Collins, Moore, Coles, & MacPhee 2004).

Pseudomonas anguilliseptica is a microorganism with high phenotypic homogeneity but serological and genetical analysis have demonstrated the existence of intraspecific variability within the species (López-Romalde, Magariños, Ravelo, Toranzo, & Romalde 2003b; López-Romalde 2005; Blanco, Gibello, Vela, Moreno, Domínguez, & Fernández-Garayzábal 2002), with epidemiological significance.

Serologically two major groups have been established on the basis of their O- antigen, the serotype O2 includes the majority of eel isolates, whereas serotype O1 includes the isolates of all other species (López-Romalde et al. 2003b). Genetical analysis by RAPD also demonstrated the existence of these two groups. Results recently obtained using ERIC-PCR and REP-PCR techniques supported the existence of these clonal lineages, although these PCR-based procedures showed higher discriminatory power than RAPD, allowing a further discrimination among the isolates from seabream and turbot (López-Romalde 2005).

The genetic heterogeneity of this pathogen was also demonstrated in a study with seabream isolates of different origin using PFGE (Pulse Field Gel Electrophoresis) (Blanco et al. 2002). This procedure allowed to distinguish four different profiles within

these seabream isolates, which authors claim to help in distinguishing high virulent strains, although no pathogenicity assays were included in their study.

In the present work, we describe the biochemical, serological and genetical characteristics of six isolates of *P. anguilliseptica* from cod, as well as their virulent capacity for other important cultured marine fishes.

Methods

Bacterial strains

A total of 6 Scottish strains of *P. anguilliseptica* were studied, all of them isolated from cod. In addition, representative isolates, previously well characterized (López-Romalde, Magariños, Núñez, Toranzo, & Romalde 2003a; López-Romalde 2003c) of the different clonal lineages and serotypes previously described within *P. anguilliseptica* (López-Romalde et al. 2003b) were included for comparison. The *P. anguilliseptica* strains used, as well as their host and geographic origins, are listed in Table 1.

The bacteria were routinely aerobically grown on Columbia Sheep Blood agar (CBA; Oxoid, Ltd., Madrid) and on Tryptone Soy agar supplemented with 1% NaCl (TSA-1; Difco Laboratories, Detroit, Michigan) at 22-25°C for 48 h. Stock cultures were maintained frozen at -70°C in Tryptone Soya broth with 15% glycerol.

Phenotypic test

All the strains were subjected to taxonomical analysis by standard morphological, physiological and biochemical plate and tube test (MacFaddin 1983, Wicklund & Bylund 1990; Thoesen 1994; Berthe, Michel, & Bernadet 1995). Arginine dihydrolase (ADH) activity was evaluated under anaerobic conditions using Thornley's semisolid medium and Moller's broth base supplemented with 1% L-arginine monohydrochloride (Sigma Chemical Co., St. Louis, Missouri).

The commercial API20NE and APIZYM systems (Biomerieux, Madrid) were used following the recommendations of López-Romalde et al. (2003a) using standardized inocula from CBA plates adjusted spectrophotometrically to A=1.0 (A₅₈₀). In all cases the temperature of incubation was set at 22°C.

The drug sensitivities of the strains were determined by the disk diffusion method on Muller-Hinton agar (Oxoid) with 1% NaCl. The following chemotherapeutic agents (micrograms per disk) were used: Oxitetracycline (30), oxolinic acid (10),

Trimethoprim-sulfamethoxazole (25), Enrofloxacin (5), Florphenicol and Amoxiciline (25).

Serological analysis

For the serological characterization, slide agglutination test were conducted as described by Toranzo, Baya, Roberson, Barja, Grimes & Hetrick (1987) using antisera against representative strains of both serotypes within *P. anguilliseptica*, the Spanish isolate TW-P1 (serotype O1) and the reference strain CECT 899^T (serotype O2) (Romalde et al. 2003b). Whole cells preparations as well as heat stable O antigens of the *P. anguilliseptica* isolates were employed in the serological assays performed.

Genetical characterization

DNA isolation was performed using the Insta-Gene Matrix (Bio-Rad) following the manufacturer's instructions. Identificative PCR analysis was performed using Ready-To-Go PCR Beads (Pharmacia Biotech, Barcelona, Spain) as previously described (Romalde, López- Romalde, Ravelo, Magariños & Toranzo 2004). RAPD typing analysis was performed using Ready-To-Go RAPD (Pharmacia Biotech, Barcelona, Spain) following the protocol described by López-Romalde et al. (2003a). ERIC-PCR and REP-PCR analysis were performed as previously described (Rodríguez, López-Romalde, Beaz, Alonso, Castro, & Romalde 2006), using Ready-To-Go PCR Beads (Pharmacia Biotech, Barcelona, Spain)

All the amplifications were carried out in a T Gradient thermocycler (Biometra), and the PCR products were electrophoresed in a 1,5% agarosa gel and stained with ethidium bromide (Bio-Rad). A 50-2000 basepair (bp) ladder (Sigma) was used as a molecular mass marker. Gels were scanned and the images captured by a Gel-Doc-2000 Gel Documentantion System (Bio-Rad 1998). Data analyses were performed using the Diversity Database software.

Virulence assays

Virulence assay was performed in turbot, a fish species in which *P. anguilliseptica* was previously isolated and in sole to determine the pathogenic potencial of the pathogen for this commercially imported cultured fish.

In both cases, the virulence was tested “*in vivo*” using fry fishes (4 g) that were held in tanks supplied by a filtered, flow-through water supply and maintained at 14°C, following the methodology previously described by Toranzo, Barja, Colwell, Hetrick, & Crosa (1983).

Two representative cod isolates, TW 150/04 and TW 153/04, were chosen for the virulence assay. In addition, strain TW 444, isolated from gilthead seabream, was used as positive control whereas sterile PBS buffer was used as negative control. In addition another two strains were used for the challenge on sole: the type strain CECT 899^T isolated from Japanese eel (serotype O2) and RQ 174.1, isolated from turbot (serotype O1).

The inocula were prepared by suspending cells in tubes containing sterile PBS (saline phosphate buffer) and adjusting them to a concentration of 10^9 cells/ml using McFarland scale to compare the opacity of suspension. Three doses were inoculated, 10^3 , 10^6 , 10^8 cells/ml, of each strain, using 5 fishes per dose. The inoculation was done via intraperitoneal, using 0,1 ml of each bacterial suspension. Virulence degree was expressed as lethal dose 50% (LD50), and was calculated by means of Reed & Muench method (1983). The strains were distributed in three different categories according to their virulence degree (Santos, Lallier, Bandín, Lamas & Toranzo 1991).

Results

The biochemical characterization of the bacterial isolates obtained in pure culture from infected fishes showed that they were Gram-negative, motile rods and oxidase, catalase and citrate positives. In addition, the isolates were positive for arginine dihydrolase in Thornley's semisolid medium and were non-haemolytic on CBA plates. These characteristics allowed their presumptive identification as *Pseudomonas anguilliseptica* (Table 2).

The utilization of the miniaturized systems API 20NE and API ZYM in all cases the result was the same. In API 20NE all the cod isolates exhibited the same profile 010054. In the API ZYM system, complete homogeneity was observed among the isolates, being the only difference the intensity of the colours generated because of the enzyme activity. Activity was detected for esterase, esterase lipase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and acid fosfatase. All the cod isolates were sensitive against all the chemotherapeutic agents used.

In the specific PCR protocol, the six cod isolates rendered the characteristic amplification band of 418 pb by PCR, confirming their identification as *P. anguilliseptica* (Fig 1).

Regarding serological analysis, both whole cells and O antigens from all the strains isolated from cod agglutinated with the antiserum made against the Spanish strain TW-P1, but not with the antiserum raised against the reference strain CECT 899^T which confirmed their inclusion in the serotype O1.

RAPD analysis showed that this new isolates from cod rendered the same profile which is similar to the profile produced by Spanish and French gilthead seabream, black spot seabream and turbot isolates (Fig. 2a).

ERIC-PCR and REP-PCR results were similar. Using REP-PCR, cod isolates rendered a pattern formed by 13 bands, identical to the turbot and black spot seabream isolates profile (Fig. 2b). Profiles obtained using ERIC-PCR showed a profile formed by 10 bands, very similar to the profile obtained for turbot and black spot seabream isolates, but some minimal differences in the lowest molecular weight bands could be observed (Fig. 2c).

For turbot the Letal Doses were comprised between 5×10^7 and $6,3 \times 10^5$ bacterial cells/fish, being considered moderately and highly virulent respectively. For sole the results were comprised between 7.6×10^4 and 3.8×10^7 bacterial cells/fish (Table 3).

In all cases the reisolation of the pathogen in pure culture from kidney and brain was possible. In some fishes, some external symptoms typical in pseudomonadiosis were observed (Fig 3).

Discussion

Intensive culture of cod is in its beginning, so the knowledge of potential pathogens for this fish specie is very limited. In 2004 the first pseudomonadiosis outbreaks in cod from UK and Canada were reported (Ferguson et al. 2004). The causative agent of this disease, *P. anguilliseptica*, is considered a limiting factor for the culture of a number of fish species with great economic importance (Barnabe 1990; Domenech, Fernández-Garayzábal, Lawson, García, Cutili, Blanco, Gibello, Moreno, Collins, & Domínguez 1997; Romalde, López-Romalde, Ravelo, Magariños & Toranzo 2003; López-Romalde 2003c).

In this work, we characterize a representative group of six Scottish isolates of cod, that were presumptively identified as *P. anguilliseptica*.

The results obtained by biochemical techniques used both plate and tube test as well as miniaturized API systems, showed that they were highly homogenic (Wiklund & Bylund 1990; Michel Bernadet. & Dinand 1992; Lönnström, Wiklund & Bylund 1994; Berthe et al. 1995; Doménech et al. 1997; López-Romalde et al. 2003a).

As with many other fish pathogens, *P. anguilliseptica* is not included in the API data base. However, these miniaturized systems are commonly used in fish pathology laboratories (Romalde & Toranzo 1991; Furones, Rodgers & Munn 1993; Austin & Austin 1999; Ravelo, Magariños, Toranzo & Romalde 2001). Particularly, the utility of the miniaturized systems API 20NE and API ZYM, when employed under optimized conditions has been previously described for the identification of *P. anguilliseptica* (López-Romalde et al. 2003a).

The results obtained in this work using API systems were completely homogeneous, being the only difference the intensity in the colour intensity for enzyme activities in the API ZYM. According to the API database, the *P. anguilliseptica* isolates could be misidentified as *Comamonas testosteroni* or *P. pseudoalcaligenes*. While *P. pseudoalcaligenes* has been described as fish pathogen and, therefore, its isolation from a fish is possible, it can be easily differentiated from *P. anguilliseptica* based on their capacity to grow at 41°C, since *P. pseudoalcaligenes* is able to grow at this temperature whereas *P. anguilliseptica* is not (Austin & Austin 1999). *C. testosteroni* has never been isolated from fish and can be easily differentiated by its capacity to hydrolyze Tween 80.

On the other hand, false-negatives results were detected in the API 20NE for the utilization of citrate, in comparison with the results obtained using Simmon's citrate test, as well as for the arginine dihydrolase. The existence of false-positive and/or false negative tests on API 20NE were previously described for fish isolated bacterias (Kent 1982; Grisez, Ceuster & Ollivier 1991; Santos, Romalde, Bandín, Magariños, Núñez, Barja, & Toranzo 1993) and specifically for *P. anguilliseptica* a false-negative result in the arginine dihydrolase test were detected in comparison with the result obtained when used Thornleys semisolid medium (López-Romalde 2005). These facts can explain the of citrate and ADH

All the strains were sensitive to all the chemotherapeutics agents used, even to the trimethoprin-sulfomethoxazol, which some of the strains of *P. anguilliseptica* showed to be resistant in other works (López-Romalde 2005). This fact can be

explained on the basis of the little development of the culture of cod and therefore for the lack of use of chemotherapeutics agents used for the control of diseases.

Regarding to the serological analysis, all the cod isolates belong to serotype O1. These result is in agreement with previous studies that propose that serotypes of *P. anguilliseptica* depends on their their host origin and not on geographical origin (López-Romalde 2005; Nakai, Muroga & Wakabayashi 1981; Nakai, Hanada & Muroga 1985a, Nakai, Kanemori, Nakajima & Muroga 1985b).

Despite the high phenotypic homogeneity of new cod isolates of *P. anguilliseptica* respect of the rest of isolates of this species, genetical analysis using typing methods as RAPD, ERIC-PCR and REP-PCR showed some minor differences between cod isolates and the other isolates of this pathogen.

They belong, therefore, to RAPD type one of genetical group A defined previously for *P. anguilliseptica*.

Previous studies showed the epidemiological utility of this test to differentiate the *P. anguilliseptica* isolates by this host origin (López-Romalde et al. 2003a; López-Romalde 2005). Our results by RAPD are in concordance with these previous studies, belonging to the RAPD type I of genetical group A, compound by all the isolates of *P. anguilliseptica* except these isolates from eel.

On the other hand, ERIC-PCR and REP-PCR techniques allow more precise identification, permitting differentiation between turbot, gilthead seabream and black spot seabream (López-Romalde 2005). Profiles obtained using REP-PCR is identical to the turbot profile, while these obtained by ERIC-PCR showed some little differences in the lowest molecular weight bands. Thus, cod isoates belong to profile II of *P. anguilliseptica*.

For the turbot virulence assays, only two of the cod isolates were used due to the biochemical, serological and genetical similarity of the six strains. The strains TW 150/04, showed to be moderately virulent, while strain TW 153/04 result to be highly virulent with a $LD_{50} = 16,3 \times 10^5$ bacterial cells/fish. It's necessary more studies to clarify the virulence degree of these isolates.

In the sole virulence assay, the strain TW 153/04 results to be highly virulent, as well as in turbot assay. Similar results were obtained with the turbot isolate RQ 174.1 and with gilthead seabream isolate TW 444. These strains were classified as highly virulent in previous studies performed in turbot (López-Romalde 2005). In the same way, results obtained with the type strain CECT 899^T are agree with results obtained in turbot. These

results supports the hypothesis that the specie *P. anguilliseptica* doesn't show host specificity.

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Table 1: Strains used in the study.

Strain	Host	Geografical origin	Isolation date
CECT 899 ^T	<i>Anguilla japonica</i>	Japan	1971
TW-P1	<i>Sparus aurata</i>	Spain	2000
TW 444	<i>Sparus aurata</i>	Spain	2001
AZ 208.2	<i>Scophthalmus maximus</i>	Spain	2002
RQ 174.1	<i>Scophthalmus maximus</i>	Spain	2001
TW 150/04	<i>Gadus morhua</i>	Scotland	2004
TW 151/04	<i>Gadus morhua</i>	Scotland	2004
TW 152/04	<i>Gadus morhua</i>	Scotland	2004
TW 153/04	<i>Gadus morhua</i>	Scotland	2004
TW 154/04	<i>Gadus morhua</i>	Scotland	2004
TW 155/04	<i>Gadus morhua</i>	Scotland	2004

Table 2: Results of biochemical characterization of cod isolates in comparison with previous descriptions of *P. anguilliseptica*.

PRUEBA	López-Romalde (2005)	TW 150/04	TW 151/04	TW 152/04	TW 153/04	TW 154/04	TW 155/04
Gram	-	-	-	-	-	-	-
Morfología ^a	B	B	B	B	B	B	B
Movilidad	+	+	+	+	+	+	+
Oxidasa	+	+	+	+	+	+	+
Catalasa	+	+	+	+	+	+	+
O/F	O	O	O	O	O	O	O

ADH	NC	NC	NC	NC	NC	NC	NC
LDH	NC	NC	NC	NC	NC	NC	NC
ODC	NC	NC	NC	NC	NC	NC	NC
Indol	-	-	-	-	-	-	-
V.P	-	-	-	-	-	-	-
Nitrato	-	-	-	-	-	-	-
Citrato	V	+	+	+	+	+	+

Table 3: Results of virulence of *P. anguilliseptica* in turbot and sole.

Strain	DL ₅₀	
	Turbot	Sole
899	ND	3.8 x 10 ⁷
RQ 174.1	ND	<3.2 x 10 ⁵
TW 444	ND	<7.6 x 10 ⁴
TW 150/04	5 x 10 ⁷	ND
TW 153/04	6,3 x 10 ⁵	3.02 x 10 ⁵

Figure legends

Fig. 1: PCR results.

Fig. 2: Genetical characterization of cod isolates of *P. anguilliseptica*. 2a: RAPD profile. 2b: REP-PCR profile. 2c: ERIC-PCR profile. Lanes: MW, Molecular Weight; 1, *P. anguilliseptica* CECT 899^T; 2, *P. anguilliseptica* TW-P1; 3, *P. anguilliseptica* AZ-208.2; 4-9, Scottish isolates TW 150/04, TW 151/04, TW 152/04, TW 153/04, TW 154/04, TW 155/04; 10, negative control (no DNA); Numbers at left show sizes in base pair of molecular weight.

Fig. 3: Moribund fishes showing typical symptoms (arrows) of pseudomonadiosis after inoculation of *P. anguilliseptica*: swollen abdomen in soles (3a) and ventral heamorrhages in turbot (3b).







