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Evaluation of BIONOR Mono-kits for rapid detection of bacterial fish pathogens

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ABSTRACT: The accuracy of the latex agglutination-based BIONOR Mono-kits for the rapid detection of different bacterial fish pathogens was evaluated. In addition, the usefulness of these commercial kits was compared with that of conventional slide agglutination techniques using specific monovalent antisera. The Mono-Yr (for detection of *Yersinia ruckeri*) and Mono-Va (for *Vibrio anguillarum*) kits specifically detected the isolates belonging to the serotypes included in the kit's composition (serotypes O1, O2, and O3 of each species). Moreover, the Mono-Va kit was also able to detect some strains of the environmental serotypes of *V. anguillarum* (O4, O5, and O7). The Mono-Pp kit (against *Pasteurella piscicida*) reacted with all the *P. piscicida* strains included in this study, with no cross-reactions with other bacterial groups. However, non-specific agglutinations were observed in both, test and negative control reagents, when isolates of *V. splendidus* or motile *Aeromonas* were analyzed with the Mono-Va and between some clinical members of the *Pasteurellaceae* family and the Mono-Pp. The kit for detection of *Renibacterium salmoninarum* (Mono-Rs) recognized all the strains possessing the predominant p57 antigen, but failed to agglutinate some isolates lacking this surface protein in their cell envelopes. These findings indicate that, although the BIONOR Mono-kits can be easily improved (i.e. including more antigens in heterogeneous groups, changing the inert protein in some of the kits, etc), they are applicable for a rapid screening of the fish pathogens studied. In addition, the kits present an adequate cost-effectiveness ratio, with a simple test performance and interpretation, which make easy their use by aquaculture facilities and laboratories.

KEY WORDS: Latex agglutination kits · *Yersinia ruckeri* *Vibrio anguillarum* · *Pasteurella piscicida* · *Renibacterium salmoninarum*

INTRODUCTION

Serological procedures, in particular agglutination tests, have been developed in recent years in response to the need for rapid tools for disease diagnosis in aquaculture. With these techniques, not only has the time required for recognition of many of the major infectious diseases been reduced from days to hours, but also the accuracy of diagnosis has been increased.

Since the introduction of a simple slide agglutination test for the identification of *Aeromonas salmonicida* by Rabb et al. (1964), this procedure has been applied to a great number of bacterial fish pathogens with few problems of cross-reactivity (Eurell et al. 1979, Toranzo et al. 1987a). However, the appearance of autoagglutinating strains has reduced the use of the slide agglutination tests in some bacterial species. The use of latex agglutination procedures (McCarthy 1975) has solved this problem, enabling the identification of smooth and autoagglutinating strains.

Recently, a Norwegian diagnostic company (BIONOR AS) developed latex agglutination kits (Mono-kits) for the rapid detection of several fish pathogens with the aim of avoiding the fairly laborious and time-consuming classical methods for diagnosis of fish diseases and to facilitate the identification of pathogens in the field.

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The purpose of this study was to evaluate the specificity and sensitivity of these Mono-kits in the detection of some important pathogens causing disease problems in continental and marine aquaculture over the world, namely *Yersinia ruckeri*, *Vibrio anguillarum*, *Pasteurella piscicida* and *Renibacterium salmoninarum*. In addition, the usefulness of these commercial kits was compared with that of conventional slide agglutination techniques using specific antisera produced in our laboratory.

MATERIALS AND METHODS

Bacterial strains. To evaluate the accuracy of the 4 BIONOR Mono-kits, a collection of 85 strains from 4 bacterial species pathogenic for fish, with different geographical origin and host species, and representative of the different serotypes within each species, were employed. Accordingly, this study included 25 *Yersinia ruckeri* isolates, 26 *Vibrio anguillarum* isolates, 18 *Pasteurella piscicida* strains, and 16 *Renibacterium salmoninarum* isolates. The identity of each isolate was confirmed with standard biochemical procedures (Toranzo et al. 1987b, Fouz et al. 1990, Romalde et al. 1990, Romalde & Toranzo 1991, Bandín et al. 1992, Magariños et al. 1992). In addition, in order to assess the specificity of the detection kits, 52 isolates belonging to species taxonomically related to those cited above were also utilized: *Yersinia intermedia*, *Y. kristensenii*, *Y. enterocolitica*, *Vibrio ordalii*, *V. tubiashii*, *V. damsela*, *V. splendidus*, *V. pelagius*, *Aeromonas hydrophila*, *A. sobria*, *A. caviae*, *A. salmonicida* subsp. *salmonicida*, atypical *A. salmonicida*, *Pasteurella multocida*, *P. haemolytica*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Enterococcus* sp., *E. seriolicida*, *E. fecalis*, *Lactococcus garvieae*, *Carnobacterium piscicola* and *Corynebacterium aquaticum* (Tables 1 to 4).

Representatives of *Yersinia* and *Aeromonas* genera were routinely grown in tryptic soy agar (TSA; Difco Lab., Detroit, MI, USA). For the culture of the halophilic *Vibrio* species and *Pasteurella piscicida* strains this medium was supplemented with 1% (wt/vol) NaCl. With the exception of *Renibacterium salmoninarum*, the remaining strains were cultured in Blood Agar (bioMerieux, Madrid, Spain). The incubation was conducted at 22 or 37°C (depending on the strain) over 24 to 48 h. In the case of the fastidious *R. salmoninarum* strains, Mueller-Hinton agar (Difco) supplemented with 1% (wt/vol) cysteine (MH-C) was employed, being incubated at 15°C for at least 15 d. Stock cultures of the strains were maintained at -70°C in appropriate media with 15% (vol/vol) glycerol.

Detection systems. ***BIONOR Mono-kits:*** These kits utilize latex particles coated with specific polyvalent sheep antisera against various bacterial pathogens such as *Yersinia ruckeri* (Mono-Ys), *Vibrio anguillarum* (Mono-Va), *Pasteurella piscicida* (Mono-Pp), and *Renibacterium salmoninarum* (Mono-Rs). Mono-Yr and Mono-Va include antisera against 3 strains from the main pathogenic serotypes (O1, O2 and O3) of each species. In the kits Mono-Pp and Mono-Rs, the latex particles are coated with the serum raised against a single strain of the respective species. When incubated with microorganisms obtained from a pure culture, the specific antiserum bind to the organism, resulting in an agglutination of the latex particles and thus providing the identity of the bacterial culture. The kits also contain a negative control reagent consisting of monodispersed latex particles coated with non-specific protein.

Kits were used following the manufacturer's instructions. Positive results were recorded only if clear agglutination was seen within 30 s after mixing the test reagent with the bacteria and if no reaction was observed with the negative control reagent. To estimate the sensitivity of these commercial kits, 10-fold serial dilutions of the antigen preparations were made and the detection limit was expressed as number of bacteria ml^{-1} needed to obtain agglutination.

Conventional slide agglutination tests: Selected strains of *Yersinia ruckeri*, *Vibrio anguillarum*, *Pasteurella piscicida* and *Renibacterium salmoninarum* were employed as antigens for immunization. The isolates chosen on the basis of the serological heterogeneity of the bacterial species were: *Y. ruckeri* 11.4 (serotype O1), 11.29 (serotype O2) and 1533/87 (serotype O3); *V. anguillarum* R 82 (serotype O1), RV 22 (serotype O2) and ATCC 43307 (serotype O3); *P. piscicida* DI 21; *R. salmoninarum* ATCC 33209 (possessing the p57 antigen) and R 1 (lacking the p57 surface protein) (Tables 1 to 4).

Immunization was performed following basically the procedures of Toranzo et al. (1987b). Briefly, suspensions of formalin-killed bacteria (approximately 10^9 cells ml^{-1}) were injected intravenously in New Zealand rabbits in consecutive daily doses of 0.25, 0.5, 1.0, and 2 ml followed by a single 1.5 ml injection 2 wk later. One week after the last injection, the rabbits were bled by cardiac puncture. Blood was allowed to clot and the separated sera were stored at -30°C until used.

Agglutination assays were performed by mixing a drop of bacterial suspension of about 10^9 cells ml^{-1} (antigen preparations) with a drop of antiserum. A distinct agglutination in 30 s. was registered as positive, and a weak agglutination after 5 min was considered as a negative test. Controls were made with saline and serum from non immunized rabbits. Sensitivity was determined as above.

Somatic 'O' antigens were prepared as previously described (Toranzo et al. 1987b) by heating the bacterial suspensions at 100°C for 1 h and the agglutination results obtained compared with those of whole cell preparations.

RESULTS

The results obtained in the comparative study of accuracy between the Mono-kits and the conventional slide agglutination test using specific antisera produced in our laboratory are compiled in Tables 1 to 4. The effectiveness achieved by each kit in the detection of the different pathogens, as well as their sensitivities are described below.

Yersinia ruckeri

The Mono-Yr kit (containing particles coated with antisera against the classical serotypes O1, O2 and O3) detected all the strains belonging to serotypes O1 and O3 using 24 h cultures (Table 1). With the exception of

Table 1. Results obtained in the comparative analysis between BIONOR Mono-Yr and slide agglutination test using specific monovalent antisera. In parenthesis: detection limits of the Mono-kit and the specific monovalent antisera as no. bacteria ml^{-1}

Strain	Serotype	Origin	Mono-Yr	Antisera against *Yersinia ruckeri* strain		
				11.4 (serotype O1)	11.29 (serotype O2)	1533/87 (serotype O3)
Yersinia ruckeri						
NCIMB 1316	O1	Rainbow trout, UK	+ (10^8)	+ (10^7)	–	+ (10^7)
FP-13	O1	Rainbow trout, Spain	+ (10^8)	+ (10^7)	–	+ (10^8)
PP-31	O1	Rainbow trout, Spain	+ (10^8)	+ (10^7)	–	+ (10^7)
AG-71	O1	Rainbow trout, Spain	+ (10^9)	+ (10^7)	–	+ (10^7)
AG-143	O1	Rainbow trout, Spain	+ (10^9)	+ (10^7)	–	+ (10^7)
YF-1	O1	Rainbow trout, Spain	+ (10^8)	+ (10^7)	–	+ (10^7)
15R	O1	Rainbow trout, Spain	+ (10^8)	+ (10^7)	–	+ (10^7)
AF-1	O1	Water, Spain	+ (10^9)	+ (10^7)	(+)[a]	+ (10^8)
11.4	O1	Rainbow trout, USA	+ (10^9)	+ (10^7)	(+)[a]	+ (10^8)
11.40	O1	Rainbow trout, USA	+ (10^8)	+ (10^7)	(+)[a]	+ (10^7)
RS2	O2	Rainbow trout, Canada	–	–	+ (10^9)	–
RS6	O2	Brook trout, Canada	+ (10^{10})	–	+ (10^9)	–
11.29	O2	Chinook salmon, USA	+ (10^8)	(+)[a]	+ (10^7)	–
11.31	O2	Rainbow trout, USA	+ (10^9)	–	+ (10^7)	–
1622/87	O2	Atlantic salmon, Norway	+ (10^8)	–	+ (10^8)	–
1638/87	O2	Atlantic salmon, Norway	+ (10^9)	–	+ (10^7)	–
87/09/700	O2	Rainbow trout, UK	+ (10^9)	–	+ (10^7)	–
1533/87	O3	Atlantic salmon, Norway	+ (10^8)	+ (10^8)	–	+ (10^7)
RS54	O3	Brook trout, Canada	+ (10^8)	+ (10^7)	–	+ (10^7)
11.45	O3	Rainbow trout, Australia	+ (10^9)	+ (10^7)	–	+ (10^7)
11.47	O5	Rainbow trout, USA	–	–	–	–
RS24	O5	Rainbow trout, Canada	–	–	–	–
RS25	O5	Rainbow trout, Canada	–	–	–	–
11.73	O6	Rainbow trout, USA	+ (10^8)	–	–	–
RS80	O6	Rainbow trout, Canada	–	–	–	–
Yersinia intermedia						
IP-16835		Water, Italy	–	–	–	–
Yersinia kristensenii						
IP-16832		Water, Italy	–	–	–	–
Yersinia enterocolitica						
Ye 8001		Human, USA	–	–	–	–
Vibrio anguillarum						
R-82	O1	Turbot, Spain	–	–	–	–
RV-22	O2	Turbot, Spain	–	–	–	–
Aeromonas hydrophila						
B-32	O3	Rainbow trout, Spain	–	–	–	–
80-A1	O24	Rainbow trout, Spain	–	–	–	–

[a]Cross-reaction between serotypes O1 and O2 of *Y. ruckeri* when whole cell antigens were employed. These cross-reactions were eliminated using the somatic 'O' antigens

the strain RS2, the kit also showed positive results with the serotype O2 strains, but to obtain a clear reaction it was necessary to subculture the colonies or to perform the tests with 48 h cultures (data not shown). As expected, on the basis of its composition, the kit was unable to detect *Yersinia ruckeri* strains of serotypes O5 and O6. On the other hand, sensitivity assays showed that a minimum concentration of 10^8 bacteria ml^{-1} is needed in the reaction to get a positive agglutination.

Regarding the conventional slide agglutination test using specific antisera, both anti-serotype O1 and anti-serotype O3 reacted with all the strains belonging to these 2 serotypes (Table 1). The anti-serotype O2 serum detected only the isolates of this serotype. In addition, some cross-reactions were observed among serotypes O1 and O2 when whole cell preparations were used as antigen, but not with the somatic 'O' antigens (data not shown). The detection limit of the rabbit sera anti-*Yersinia ruckeri* was 10^7 cells ml^{-1} (Table 1).

No cross-reactivity with other environmental and clinical *Yersinia* species or with other fish pathogens such as *Vibrio anguillarum* and motile aeromonads was detected by any of the procedures utilized, BIONOR Mono-kits or slide agglutination test.

Vibrio anguillarum

As in the case of *Yersinia ruckeri*, this Mono-Va kit contains latex particles coated with antisera against serotypes O1, O2 and O3 of *Vibrio anguillarum*. Therefore, the kit was highly effective for the detection of strains of these 3 serotypes. Moreover, it was able to react with some strains of *V. anguillarum* belonging to the environmental serotypes O4, O5 and O7 (Table 2). Non-specific agglutinations occurred in both test and control reagents when *Vibrio splendidus* and motile *Aeromonas* strains were tested, but not with other *Vibrio* species like *V. damsela* or *V. pelagius*. However, no cross-reactions were observed with other related fish pathogens such as *Pasteurella piscicida* or *Aeromonas salmonicida*. No differences were observed in the results obtained after 24 or 48 h of incubation.

The 3 monovalent antisera (against serotypes O1, O2 and O3) employed in the slide agglutination tests, detected specifically the isolates belonging to the homologous serotype. No cross-reactivity was observed with other serotypes of *Vibrio anguillarum* or with different species of the genera *Vibrio* and *Aeromonas* (Table 2).

The sensitivity assays showed, as in the case of *Yersinia ruckeri*, a difference of about 1 to 2 log units among the titers obtained with the Mono-Va kit and those yielded by our specific antisera. Thus, a bacterial concentration of 10^8 cells ml^{-1} was needed to obtain a positive reaction with the latex particles while 10^7 bacteria ml^{-1} was enough to obtain agglutination with the rabbit antisera (Table 2).

Pasteurella piscicida

The Mono-Pp kit was able to identify all the *Pasteurella piscicida* strains included in this study (Table 3). Cross-reactions with representative isolates of related species such as *Vibrio anguillarum* or *Aeromonas salmonicida* were not detected. Interestingly, although other members of the family Pasteurellaceae did not react with the test reagent, some non-specific agglutinations were elicited for *Pasteurella multocida*, *Haemophilus parasuis* and *Actinobacillus pleuropnemoniae* strains when the nega-

Table 2. Specificity and sensitivity of the BIONOR Mono-Va kit for the detection of *Vibrio anguillarum*. In parenthesis: detection limits of the Mono-kit and the specific antisera as no. bacteria ml^{-1}

Strain	Serotype	Origin	Mono-Va	Antisera against *Vibrio anguillarum* strain		
				ATCC 43305 (serotype O1)	ATCC 43306 (serotype O2)	ATCC 43307 (serotype O3)
Vibrio anguillarum						
ATCC 43305	O1	Rainbow trout, Denmark	+ (10^8)	+ (10^7)	–	–
R-82	O1	Turbot, Spain	+ (10^8)	+ (10^7)	–	–
96-F	O1	Striped bass, USA	+ (10^8)	+ (10^7)	–	–
775	O1	Pacific salmon, USA	+ (10^9)	+ (10^7)	–	–
SO-86.3	O1	Pacific salmon, Spain	+ (10^9)	+ (10^7)	–	–
TM-14	O1	Rainbow trout, Spain	+ (10^8)	+ (10^7)	–	–
RG-111	O2α	Turbot, Spain	+ (10^9)	–	+ (10^7)	–
ATCC 14181	O2α	Brown trout, UK	+ (10^9)	–	+ (10^7)	–
ATCC 19264	O2α	Cod, Denmark	+ (10^9)	–	+ (10^7)	
ATCC 43306	O2α	Cod, Denmark	+ (10^9)	–	+ (10^7)	–

Table 2 (continued)

Strain	Serotype	Origin	Mono-Va	Antisera against *Vibrio anguillarum* strain ATCC 43305 (serotype O1)	ATCC 43306 (serotype O2)	ATCC 43307 (serotype O3)
Vibrio anguillarum (continued)						
43-F	O2β	Striped bass, USA	+ (10^8)	–	+ (10^7)	–
RV-22	O2β	Turbot, Spain	+ (10^8)	–	+ (10^7)	–
6828C	O2β	Turbot, Denmark	+ (10^8)	–	+ (10^7)	–
ATCC 43307	O3	Rainbow trout, Denmark	+ (10^9)	–	–	+ (10^7)
13A5	O3	Sea water, Spain	+ (10^9)	–	–	+ (10^7)
PT-493	O3	Ayu, Japan	+ (10^8)	–	–	+ (10^7)
RPM-5701	O3	Turbot, Spain	+ (10^8)	–	–	+ (10^7)
11008	O3	Seabass, France	+ (10^9)	–	–	+ (10^7)
ET-208	O3	Japanese eel, Japan	+ (10^8)	–	–	+ (10^7)
ATCC 43308	O4	Cod, Denmark	+ (10^9)	–	–	–
ATCC 43309	O5	Cod, Denmark	+ (10^{10})	–	–	–
ATCC 43310	O6	Cod, Denmark	–	–	–	–
ATCC 43311	O7	European eel, Denmark	+ (10^9)	–	–	–
ATCC 43312	O8	Cod, Denmark	–	–	–	–
ATCC 43313	O9	Cod, Denmark	–	–	–	–
ATCC 43314	O10	Cod, Denmark	–	–	–	–
Vibrio ordalii						
NCIMB 2167		Pacific salmon, USA	–	–	–	–
Vibrio tubiashii						
ATCC 19109		Clam, USA	–	–	–	–
EX1		Oyster, Spain	–	–	–	–
Vibrio damsela						
ATCC 33539		Angel fish, USA	–	–	–	–
RG-91		Turbot, Spain	–	–	–	–
RM-71		Turbot, Spain	–	–	–	–
Vibrio splendidus						
ATCC 25914		Sea water, USA	–[a]	–	–	–
RG-52		Turbot, Spain	–[a]	–	–	–
RM-51		Turbot, Spain	–[a]	–	–	–
RI-42		Turbot, Spain	–[a]	–	–	–
TR-4		Rotipher, Spain	–[a]	–	–	–
Vibrio pelagius						
ATCC 25916		Sea water, USA	–	–	–	–
RG-165		Turbot, Spain	–	–	–	–
RA-21		Turbot, Spain	–	–	–	–
ST-11		Atlantic salmon, Spain	–	–	–	–
NF-182		Rainbow trout, Spain	–	–	–	–
Aeromonas hydrophila						
B-32	O3	Rainbow trout, Spain	–[a]	–	–	–
B-35	O3	Rainbow trout, Spain	–[a]	–	–	–
80-A1	O24	Rainbow trout, Spain	–[a]	–	–	–
Aeromonas. sobria						
P-33	O3	Rainbow trout, Spain	–[a]	–	–	–
Aeromonas caviae						
1.25	O3	Human, USA	–	–	–	–
Aeromonas salmonicida subsp. *salmonicida*						
ATCC 33658		Atlantic salmon, USA	–	–	–	–
MT-004		Atlantic salmon, Scotland	–	–	–	–
SEG-10.1		Atlantic salmon, Spain	–	–	–	–
Pasteurella piscicida						
ATCC 17911		White perch, USA	–	–	–	–
DI-21		Seabream, Spain	–	–	–	–
10831		Seabass, France	–	–	–	–
MP-7801		Yellowtail, Japan	–	–	–	–

[a]Positive reaction with both test and control reagents

tive control reagent was utilized and for *Pasteurella haemolytica* with both test and control reagents. On the other hand, our rabbit antiserum raised against *P. piscicida* DI 21 strain, specifically recognized this bacterial group with no cross-reactions with other species.

The detection limit of the Mono-Pp kit was 10^{10} bacteria ml^{-1}. This limit decreased to 10^7 cells

Table 3. Effectiveness of the BIONOR Mono-Pp kit in the detection of *Pasteurella piscicida*. In parenthesis: detection limits of the Mono-kit and the specific antisera as no. bacteria ml^{-1}

Strain	Origin	Mono-Pp	Antiserum against *Pasteurella piscicida* DI-21
Pasterurella piscicida			
DI-21	Seabream, Spain	+ (10^{10})	+ (10^7)
DI-71	Seabream, Spain	+ (10^{10})	+ (10^7)
DI-91	Seabream, Spain	+ (10^{10})	+ (10^7)
B-32	Seabream, Spain	+ (10^{10})	+ (10^7)
B-51	Seabass, Spain	+ (10^{10})	+ (10^7)
B-52	Seabass, Spain	+ (10^{10})	+ (10^7)
10831	Seabass, France	+ (10^{10})	+ (10^7)
IT-1	Seabream, Italy	+ (10^{10})	+ (10^7)
IT-2	Seabream, Italy	+ (10^{10})	+ (10^7)
MP-7801	Yellowtail, Japan	+ (10^{10})	+ (10^7)
KP 9006	Yellowtail, Japan	+ (10^{10})	+ (10^7)
EPOY-8803-II	Red grouper, Japan	+ (10^{10})	+ (10^7)
P-3333	Amberjack, Japan	+ (10^{10})	+ (10^7)
P-3334	Yellowtail, Japan	+ (10^{10})	+ (10^7)
AT LIT 2	Striped bass, Israel	+ (10^{10})	+ (10^7)
AT LIT 18	Seabream, Israel	+ (10^{10})	+ (10^7)
ATCC 17911	White perch, USA	+ (10^{10})	+ (10^7)
ATCC 29690	Yellowtail, Japan	+ (10^{10})	+ (10^7)
Pasteurella multocida			
CECT 902	Bovine, Spain	–[a]	–
04041	Porcine, The Netherlands	–[a]	–
Pasteurella haemolytica			
CECT 924	Ovine, Spain	+[b]	–
Haemophilus parasuis			
94388	Unknown, The Netherlands	–[a]	–
Actinobacillus pleuropneumoniae			
WST 25	Porcine, The Netherlands	–[a]	–
Vibrio anguillarum			
R-82	Turbot, Spain	–	–
775	Pacific salmon, USA	–	–
96-F	Striped bass, USA	–	–
SO-86.3	Pacific salmon, Spain	–	–
ATCC 19264	Cod, Denmark	–	–
RV-22	Turbot, Spain	–	–
RG-111	Turbot, Spain	–	–
Aeromonas salmonicida subsp. *salmonicida*			
SR-19.1	Rainbow trout, Spain	–	–
SEG-10.1	Atlantic salmon, Spain	–	–
E-8101	Pacific salmon, Japan	–	–
H-22/77	Brown trout, UK	–	–
MT-004	Atlantic salmon, UK	–	–
3.101	Brook trout, USA	–	–
ATCC 33658	Atlantic salmon, USA	–	–
Atypical *Aeromonas salmonicida*			
2F9-81/A	Flounder, Finland	–	Autoagglutination
26F23-21/1	Flounder, Finland	–	Autoagglutination

[a] Positive reaction with the negative control reagent
[b] Positive reaction with both test and control reagents

ml^{-1} when the rabbit antiserum was employed (Table 3).

Renibacterium salmoninarum

A similar pattern was observed using the Mono-kit and the conventional slide agglutination test with anti-*Renibacterium salmoninarum* ATCC 33209 (typical strain possessing intact p57 antigen), detecting the majority of *R. salmoninarum* isolates. However, both tests failed to agglutinate 3 strains of this bacterial species that lacked the p57 surface protein, namely R1, K57 and K76 (Table 4). Interestingly, our antiserum raised against R1 strain only detected these 3 isolates. On the other hand, independently of the procedure performed, no cross-reactions were seen with other Gram-positive pathogens belonging to *Corynebacterium* species and the group of lactic acid bacteria.

As for the other fish pathogens included in this work, differences in sensitivity of about 1 log unit were observed between the Mono-kit and the specific rabbit antisera (Table 4).

DISCUSSION

The development of serological procedures in the last decades has been associated with major advances in practically all facets of fish health (Schill et al. 1989).

Table 4. Comparative analysis of the BIONOR Mono-Rs kit and conventional slide agglutination test for detection of *Renibacterium salmoninarum*. In parenthesis: detection limits of the Mono-kit and the specific antisera as no. bacteria ml^{-1}

Strain	Origin	Mono-Rs	Monovalent sera anti-*R. salmoninarum* ATCC 33209	R-1
Renibacterium salmoninarum				
ATCC 33209	Pacific salmon, USA	+ (10^8)	+ (10^7)	–
ATCC 33739	Brook trout, USA	+ (10^8)	+ (10^7)	–
NCIMB 1111	Salmonid fish, UK	+ (10^8)	+ (10^7)	–
NCIMB 1113	Brook trout, USA	+ (10^8)	+ (10^7)	–
MT-239	Atlantic salmon, UK	+ (10^8)	+ (10^7)	–
MT-251	Atlantic salmon, UK	+ (10^8)	+ (10^7)	–
MT-417	Atlantic salmon, UK	+ (10^8)	+ (10^7)	–
MT-421	Atlantic salmon, UK	+ (10^8)	+ (10^7)	–
MT-422	Atlantic salmon, UK	+ (10^8)	+ (10^7)	–
MT-444	Atlantic salmon, UK	+ (10^8)	+ (10^7)	–
MOC	Brook trout, Canada	+ (10^8)	+ (10^7)	–
466	Pacific salmon, Canada	+ (10^8)	+ (10^7)	–
RS-92	Pacific salmon, Spain	+ (10^8)	+ (10^7)	–
R-1	Salmonid fish, Canada	–	–	+ (10^7)
K-57	Atlantic salmon, UK	–	–	+ (10^7)
K-76	Brook trout, Canada	–	–	+ (10^7)
Enterococcus sp.				
RA-99.1	Turbot, Spain	–	–	–
RM-207.1	Turbot, Spain	–	–	–
AZ-12.1	Turbot, Spain	–	–	–
AZ-64.1	Turbot, Spain	–	–	–
TW-1	Rainbow trout, Spain	–	–	–
Enterococcus seriolicida				
YT-3	Yellowtail, Japan	–	–	–
SS-91014	Yellowtail, Japan	–	–	–
Enterococcus faecalis				
ATCC 19433	Human, France	–	–	–
Lactococcus garvieae				
NCDO 2155	Bovine, UK	–	–	–
Carnobacterium piscicola				
ATCC 35586	Cutthroat trout, USA	–	–	–
HB-425	Striped bass, USA	–	–	–
PT-3.1	Rainbow trout, Spain	–	–	–
Corynebacterium aquaticum				
ATCC 14665	Distilled water, USA	–	–	–
RB-968 BA	Striped bass, USA	–	–	–

In particular, those regarding accuracy and time required for diagnosis have been significantly improved. Moreover, the possibility of application of these methods under field conditions is of great importance since few fish farms possess their own diagnostic laboratories on site. However, the non-availability of standardized antigens and antisera of known quality can constitute a limitation since they are important requirements for serological procedures to be of value and results of tests reliable. Commercial reagents are now becoming available for use in monitoring fish health. Among these products, rapid latex test kits for the detection of the most important fish pathogens have been developed in the late 1980s. Since then, few studies on their effectiveness, sensitivity and specificity have been performed (Kusser et al. 1988).

Yersinia ruckeri, the causal agent of Enteric Redmouth (ERM) disease, was initially considered an homogeneous bacterial group (Ewing et al. 1978, O'Leary et al. 1979). Soon, serological variations among strains were demonstrated and 5 serotypes were established (O'Leary et al. 1982, Stevenson & Airdrie 1984, DeGrandis et al. 1988, Davies & Frerichs 1989). The Mono-Yr kit contains latex particles coated with antisera against the classical serotypes O1, O2 and O3 which have been mainly associated with the disease outbreaks. Further studies (Romalde et al. 1993) made possible updates in the serological classification of *Y ruckeri*. Thus, the former serotypes O1 and O3 were joined into a new serotype O1, and the former serotype O2 was divided into 3 subgroups (2a, 2b, and 2c). The results obtained here with our specific monovalent antisera raised against representatives of the old serotypes O1, O2 and O3 supported the new scheme previously reported by the authors (Romalde et al. 1993).

The Mono-Yr kit, as expected, detected all the isolates of the former serotypes O1 and O3. However, and due to the serological heterogeneity demonstrated within the former serotype O2, some of the representatives of this serotype, particularly those belonging to the subgroup O2a (Romalde et al. 1993), did not react with the latex particles. This fact does not constitute major problems, since isolates of this serological group are very scarce and their relation to fish mortalities and/or disease outbreaks is unclear

For *Vibrio anguillarum*, 10 serotypes (O1 to O10) are defined in the European serotyping scheme (Sørensen & Larsen 1986), but only serotypes O1, O2 and O3 have been associated with fish mortalities (Toranzo & Barja 1990, 1993). Members of the remaining serotypes have been considered environmental strains without pathogenic importance. These environmental serotypes are closely related to other *Vibrio* species such as *V. splendidus* and *V. pelagius* (Pazos et al. 1993). The results obtained with the Mono-Va kit revealed a good specificity for the pathogenic serotypes O1, O2 and O3. In addition, the detection of some of the environmental serotypes (O4, O5 and O7) can be explained by the presence of common minor antigens among these serogroups (Bolinches et al. 1990). On the other hand, some reactions were observed when members of *V. splendidus* and motile *Aeromonas* were assessed with both test and negative control reagents, even after being subcultured and re-tested 2 d later following the kit instructions. The appearance of these agglutinations, however, fits better (according to the manufacturer's description) with a positive agglutination than with spontaneous agglutination. Therefore, they can be due to non-specific agglutination to the inert protein and/or latex particles. The option of cross-reactivity between related groups with common antigens, although possible in this case, should be ruled out since agglutination also occurred with the negative control reagent.

In contrast, using the conventional slide agglutination test and our monovalent *Vibrio anguillarum* antisera, no cross-reactions were observed either with environmental serotypes of this species (from O1 to O10) or with other *Vibrio* and *Aeromonas* species.

In contrast to the 2 pathogens already mentioned (*Yersinia ruckeri* and *Vibrio anguillarum*), *Pasteurella piscicida* is a highly homogeneous group, without different serogroups (Magariños et al. 1992). This homogeneity also contrasts with the serological heterogeneity described for other members of the family Pasteurellaceae such as *P. multocida* and *P. haemolytica* (Biberstein 1978, Hofacre & Glisson 1986). Consequently, the Mono-Pp kit exhibited a great specificity for the detection of *P. piscicida* strains with no cross-reactions with other bacterial groups. However, agglutinations were displayed only by the negative control reagent when other members of the family such as *P. multocida*, *Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* were tested. This fact could be explained by certain reactivity among the inert control protein and these bacterial species. On the other hand, the rabbit serum against the Spanish isolate DI-21 specifically detected all the *P. piscicida* strains without problems of cross-reactions with the other species studied.

The major *Renibacterium salmoninarum* antigen detected in fish sera and tissues in monitoring programs is a protein of 57 kd (Griffiths et al. 1991). This protein can be found as the predominant antigen in both, cell surface and extracellular products (Wiens & Kaattari 1989). However, instability of this protein has been recently described due to a possible autolytic nature (Griffiths & Lynch 1991) and the existence of a serin protease activity associated with the cell surface

(Rockey et al. 1991) The degradation of the major antigen and/or the existence of some strains that never exhibit an intact 57 kd protein could indicate antigenic heterogeneity within *R. salmoninarum* (Bandín et al. 1992). The results obtained in this work with the Mono-Rs latex particles support this hypothesis, since some strains, possessing a degraded p57 protein, were not detected with this kit. The same results were observed when our specific antisera against *R. salmoninarum* were used. The Mono-Rs kit could, therefore, be improved if bacterial antigens of this second group of strains (without 57 kd protein) were also included in the kit. In addition, although the presence of the 57 kd antigen in strains belonging to *Corynebacterium aquaticum* and *Carnobacterium piscicola* has been demonstrated by western-blot experiments (Bandín et al. 1993, Toranzo et al. 1993), no cross-reactions were achieved with these 2 species or other members of the lactic acid bacteria by using the Mono-Rs kit or the conventional slide agglutination test with our specific antisera.

Regarding the sensitivity of the commercial Mono-kits, these always showed higher detection limits than our specific rabbit antisera by at least 1 log unit. Thus, while in the majority of the cases the specific antisera detected concentrations of 10^7 bacteria ml^{-1}, the Mono-kits recognized 10^8 to 10^9 cells ml^{-1} This difference was significantly higher in *Pasteurella piscicida* strains for which the kit only detected values of 10^{10} bacteria ml^{-1}. These differences in sensitivity can be due to the different host species in which the antisera were raised (sheep in the kits and rabbit in the monovalent antisera) and/or variations in the immunization procedure. Although the Mono-kits showed a great accuracy when applied to bacterial colonies grown in agar plates (for which they were devised), their sensitivity limits hinder the possibility of detection of the pathogens directly in fish fluids or tissues which would represent important time and equipment saving.

In summary, from a practical point of view, we consider that, although the BIONOR Mono-kits can be improved (i.e. by including more antigens in heterogeneous groups, changing the negative control inert protein in some kits, etc), they are applicable for a rapid preliminary screening of the pathogens and disease diagnosis. Moreover, since this test is relatively simple and possesses an adequate cost-effectiveness, it can be used by most aquaculture facilities and laboratories. However, in order to determine the predominant serotype(s) in a given geographical area, and their possible changes in time, specific antisera against the different serotypes within each pathogen must be utilized. This fact is of special importance when performing epidemiological studies and to design effective vaccines.

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