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An ELISA technique for rapid diagnosis of vibriosis in sea bass *Dicentrarchus labrax*

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ABSTRACT: Three batches of rabbits were immunized against 2 strains of *Vibrio anguillarum* (Va V 62 and Va V 408), which cause vibriosis in sea bass *Dicentrarchus labrax* (L. 1758) Serranidae. Homologous and heterologous reactions were obtained against these *Vibrio* strains and other bacteria using an improved indirect ELISA technique. Antibody and antigen titrations revealed high immunogenecity (high antibody titers for the antisera) of the 2 *Vibrio* strains. They correspond to serotype I and show close affinity. The specificity and sensitivity of 1 selected antiserum were tested on brain extracts from healthy sea bass and those infected artificially with *V. anguillarum* serotypes I and III. Preliminary results confirm the reliability of the technique and warrant its use for improving disease diagnosis.

INTRODUCTION

Vibriosis is one of the main causes of high mortality in farmed populations of sea bass *Dicentrarchus labrax* (L. 1758). In acute cases, the fish die of hemorrhagic septicemia and the mortality rate often exceeds 50 % in farmed populations, particularly juvenile fish (Roberts 1975, Breuil & Haffner 1989). In chronic cases, integument lesions of variable intensity on the body surface are accompanied by haemorrhaging in the abdominal cavity and by hemolytic anemia. Early diagnosis of the disease would ensure rapid treatment or elimination of infected individuals to prevent its spread.

At present, the only diagnostic method, apart from histopathological examination, is the identification of bacterial cells collected from integument fragments. The cells are seeded in an identification gallery and subjected to an agglutination test (Skøge Jonsen 1977, Simonson & Siebeling 1988). However the first phase of this technique is too long and the test is not adequately sensitive to provide conclusive results.

Meanwhile, certain indirect ELISA techniques have produced satisfactory results for detecting *Vibrio parahaemolyticus* (Honda et al. 1985, 1989) and *V. salmonicida* (Espelid et al. 1988).

The objective of this study was to characterize the rabbit antisera produced for the ELISA tests according to their titer and specificity, and to assess the reliability of the indirect ELISA technique for detecting local *Vibrio* strains in farmed fish.

MATERIALS AND METHODS

Bacterial strains. Two *Vibrio anguillarum* strains corresponding to serotype I were used to obtain rabbit antisera. Strain V 62 (Va V 62), API E code 3247524 (Breuil & Haffner 1989), was isolated from Mediterranean sea bass and strain V 408 (Va V 408), API 20E code 3247527 (Baudin-Laurencin 1981), from trout *Salmo gairdneri* Richardson. Both strains are highly pathogenic and cause septicemia in their respective hosts.

The specificity of the antisera produced against these 2 strains was studied by reacting them with other bacterial strains: *Vibrio anguillarum*, API 20E code 3247527, serotype III (Va V III); *V. harveyi*, V 605, API 20E code 4144527 (Vh V 605); *V. parahaemolyticus*, API 20E code 4146125 (Vp); and *Pasteurella pisciscida*, API 20E code 2004004 (Pp). These strains were supplied by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), France.

All the bacterial cells used for immunization and corresponding ELISA tests were isolated from infected fish. They were cultured on marine agar (Difco) and incubated at 25 °C for 24 h. Their biochemical properties were identified using API 20E galleries. Cells were harvested after centrifugation, suspended in physiological saline at a concentration of 10^9 cells ml^{-1}, and heat-inactivated (2 h at 18 °C). Reserve antigen suspensions were maintained in buffers at 4 °C. Concentration rates of the bacterial suspensions were determined by colony forming unit (CFU) counts on bacteria cultured for 24 h on agarose.

Preparation of antisera. New Zealand female rabbits were inoculated with the 2 inactivated bacterial extracts (Va V 62 and Va V 408) according to the following procedure:

- 1 intradermal injection containing 10^9 cells ml^{-1} in Freund's incomplete adjuvant at 2 ml per rabbit;
- 30 d later, a subcutaneous booster containing 2×10^9 cells ml^{-1} in Freund's incomplete adjuvant at 2 ml per rabbit;
- 15 d later, a final booster identical to the preceding one.

The 5 rabbits used for immunization were divided into 3 batches: Batch 1: A, 1 rabbit immunized against Va V 62; Batch 2: B, B', 2 rabbits immunized against Va V 408, Batch 3: C, C', 2 rabbits immunized against a mix of Va V 62 and Va V 408. 1 non-immunized rabbit, R_0S, served as the control.

The rabbits were bled 15 d after the final booster. Serum was separated by centrifugation after coagulation. It was then aliquoted and stocked at −80 °C.

Artificial infection, challenges. The sea bass (30 g) were reared at IFREMER in 500 l tanks in which the water was maintained at 20 °C and 35 ‰ salinity and renewed at the rate of 500 l h^{-1}.

Two strains belonging to different serotypes were used for inoculation: Va V 62 from serotype I and Va V III from serotype III. For each strain, 36 fish were artificially infected by intraperitoneal injection of 0.1 ml of bacterial suspension (1×10^7 cells ml^{-1} for Va V 62 and 2×10^7 cells ml^{-1} for Va V III). The controls were injected with 0.1 ml of physiological saline.

After 10 d, samples of brain and kidney tissue from dead fish were checked for the presence of *Vibrio* spp., using standard techniques. Agglutination tests were carried out on samples cultured on marine agar (Difco) and seeded on API 20E identification galleries.

Healthy and infected brain extracts were used for assessing the indirect ELISA technique. In all cases healthy fish were used as controls.

Indirect ELISA technique. Certain modifications were made to the ELISA technique to obtain reliable results. These included fixing agents (different concentrations of poly L-lysine), microtitration plates (Greiner, Nunc, with normal or hyperabsorption), and saturation buffers (BSA, Régilait, casein, etc.).

Titration of antisera. The procedure used for antiserum titration was as follows:

(1) Wells of a flat-bottomed Nunc-ELISA plate were coated with 50 µl of a bacterial suspension at the rate of 10^7 cells ml^{-1} in phosphate-buffered saline (PBS), pH 7.2. The plate was then incubated at 60 °C for 3 h (patented modification, Guillet et al. 1983).

(2) The plate was washed 3 times by adding 200 µl of PBS to each well. The wells were emptied by suction, using an automatic plate washer (Nunc). Any excess solution after the last wash was removed by quickly overturning the plate on blotting paper.

(3) Saturation was obtained by adding 300 µl of 0.3 % BSA/PBS (w/v) in each well. The plate was covered and incubated at 37 °C for 1 h. During incubation, dilutions of each antiserum sample were prepared in 0.2 % BSA/PBS (w/v). The dilutions generally ranged between $1:10^3$ and $1:10^6$ (v/v). The nonspecific solution (control) was prepared using 0.2 % BSA/PBS (w/v).

(4) The saturation solution was removed by suction and the plate was dried.

(5) Each well was washed 3 times with PBS (300 µl the first time and 200 µl the next 2 times). The plate was then dried.

(6) Fifty µl of one of the following were deposited in each well:

(a) antiserum from immunized rabbits at different dilutions in 0.2 % BSA/PBS (w/v) ranging between $1:10^3$ and $1:10^6$;

(b) serum from the nonimmunized rabbit at the same dilutions as in (a);

(c) buffer solution of nonspecific 0.2 % BSA/PBS (w/v).

The plate was incubated for 2 h at room temperature (20 ± 2 °C).

(7) The solution was removed, each well was washed 3 times with 200 µl of PBS, and the plate was dried.

(8) Fifty µl of conjugate made up of anti-rabbit antibody diluted to 1:4000 (v/v) in 0.2 % BSA/PBS (w/v) was added to each well. The plate was covered and incubated at 37 °C for 1 h.

(9) The solution was removed by suction. The plate was washed 3 times with 200 µl of PBS and dried.

(10) Fifty µl of chromogenic substrate (orthophenylenediamine: R8 Rapid Elavia reagent and R9 Monolisa AgHBS, Eria Diagnostic Pasteur), prepared just before use, was added to each well. The plate was incubated for 10 min in the dark and at room temperature.

(11) The reaction was stopped, without washing, by adding 25 µl of H_2SO_4 4N to the solution.

(12) Optical density was measured immediately at 492 nm by means of a plate reader (Titertek-Multiskan).

Titration of antigens. The same procedure was used for antigen titration except for Steps 1 and 6; they were replaced by:

(1') Wells of a flat-bottomed Nunc ELISA plate were coated with 50 µl of bacterial suspensions *at different concentrations* (10^9 to 10^3 cells ml^{-1} in PBS); they were incubated and dried at 60 °C for 3 h.

(6') Fifty µl of one of the following were deposited in each well:

(a) antiserum from immunized rabbits, at *constant dilution* (1:2 × 10^4, v/v) in 0.2 % BSA/PBS (w/v);

(b) serum from the nonimmunized rabbit at the same dilution as in (a);

(c) buffer solution of nonspecific 0.2 % BSA/PBS (w/v).

The plate was incubated for 2 h at room temperature (20 ± 2 °C).

Assessment of the ELISA technique. The ELISA technique was tested by checking its ability to detect *Vibrio anguillarum* antigens – serotypes I (Va V 62) and III (Va V III) – in brain extracts of infected fish. The indirect ELISA technique used for this purpose was similar to that for antiserum titration except for Steps 1 and 6, which were replaced by:

(1") Wells of a flat-bottomed Nunc ELISA plate were coated with 50 µl of infected (or healthy) brain extracts. These were previously homogenized (Dounce) for 2 min in 600 µl of sterile PBS and then diluted at *different concentrations* (1:10, 1:30, 1:150, 1:1500, 1:15 000) in sterile PBS. The plate was then incubated at 60 °C for 3 h.

(6") Fifty µl of one of the following were deposited in each well:

(a) antiserum from rabbits immunized against Va V 62, at *constant dilution* (1:10^4, v/v) in 0.2 % BSA/PBS (w/v);

(b) serum from the nonimmunized rabbit at the same dilution as in (a);

(c) buffer solution of nonspecific 0.2 % BSA/PBS (w/v).

The plate was incubated for 2 h at room temperature (20 ± 2 °C).

RESULTS

Antiserum titration: homologous and heterologous reactions (Table 1, Fig. 1)

In this test, the antibody concentration varied, whereas that of the antigen was constant (1 × 10^7 cells ml^{-1} or 2 × 10^6 cells per well). Results showed that:

- Optic density (OD) was low (0.15) owing to background interference (nonspecific buffer).
- Regardless of the antigen, serum from the nonimmunized rabbit (R_0S) at both dilutions (1:10^3 and 1:10^4, v/v) gave very low OD: <0.3 at 1:10^3 and close to 0 at 1:10^4.
- The antibody titer* of antisera from immunized rabbits was higher for homologous reactions than for heterologous reactions. For example, the antiserum produced by rabbit A against Va V 62 gave a high titer (2 × 10^5 and 1.6 × 10^5 respectively) when reacted with strains Va V 62 and Va V 408, which belong to the same serotype. In comparison, antibody titers obtained in the presence of other antigens were: 7 × 10^4 with Vh V 605, 2.3 × 10^4 with Va V III and 1.2 × 10^3 with Pp. The same phenomenon was observed for homologous and heterologous reactions of antisera from B, B', C, and C' rabbits.
- As expected, the Va V 62 and Va V 408 strains, which belong to the same serotype, showed greater affinity between themselves than with Va V III or Vh V 605.
- In one case, antisera from rabbits C and C' immunized against both Va V 62 and Va V 408 appeared to produce a slightly stronger reaction to the 2 antigens presented separately. The antiserum C titer (3 × 10^5) is higher than that of antisera A, B, and B'

* The antibody titer of an antiserum was defined as being equal to the dilution at which its OD was 3 times the background interference (Mourton 1991)

Table 1. Antibody titers (inverse value of dilution) of antisera from rabbits immunized against 2 *Vibrio anguillarum* strains, as determined by indirect ELISA. ND: not determined. Results of homologous reactions are in bold

Antigen	Antiserum A	Antiserum B	Antiserum B'	Antiserum C	Antiserum C'	Serum R_0S (control)
Va V 62	**2 × 10^5**	2 × 10^5	8 × 10^4	**3 × 10^5**	**1.5 × 10^5**	0
Va V 408	1.6 × 10^5	**1.7 × 10^5**	**8 × 10^4**	ND	**8 × 10^4**	0
Vh V 605	7 × 10^4	1.6 × 10^4	1.6 × 10^4	2.8 × 10^4	2.4 × 10^4	0
Va V III	2.3 × 10^4	9.5 × 10^3	ND	ND	ND	0
Vp	2.8 × 10^3	1.4 × 10^3	ND	ND	3.5 × 10^3	0
Pp	1.2 × 10^3	<1 × 10^3	ND	ND	2.2 × 10^3	0

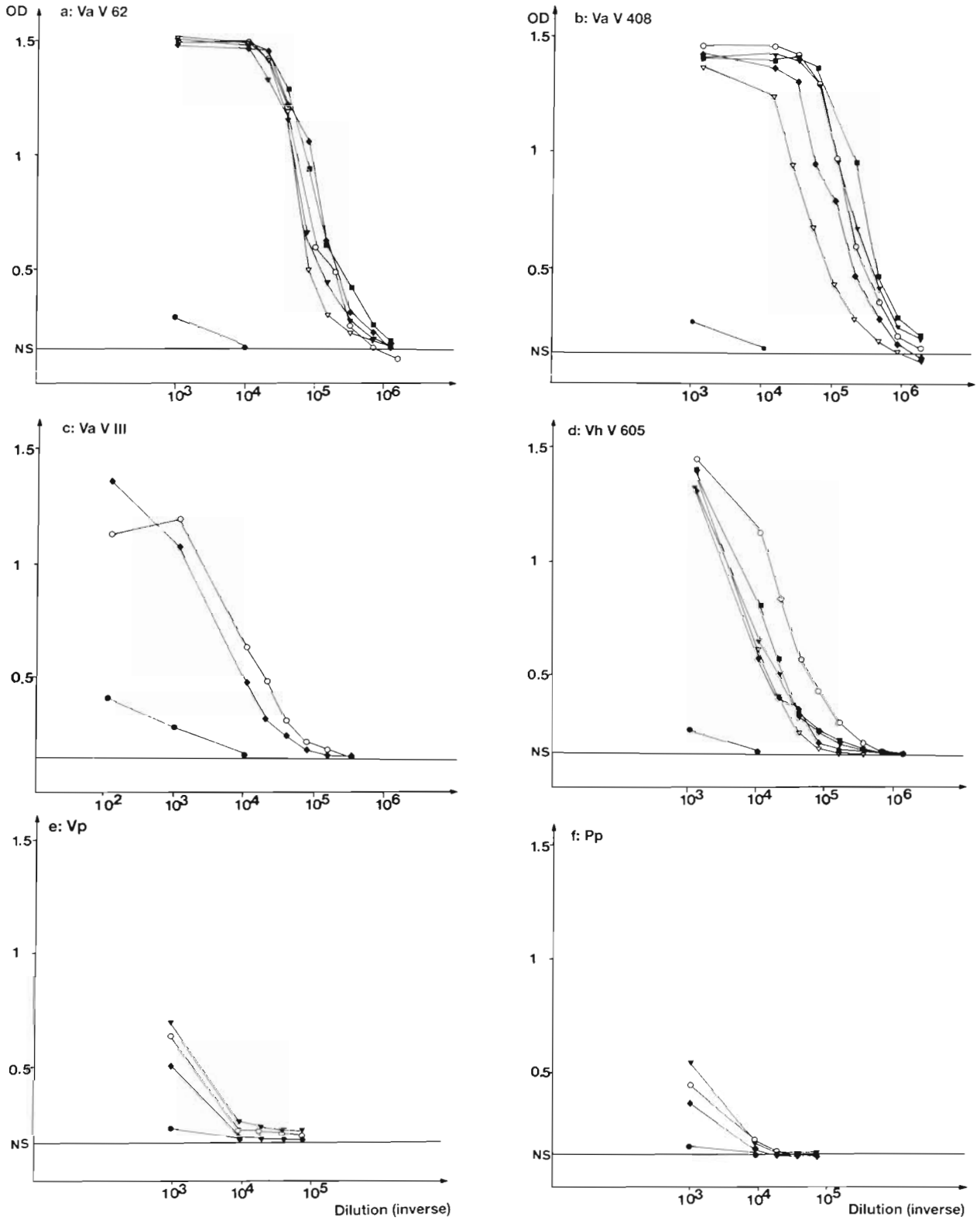

Fig. 1. Antiserum titers as determined by indirect ELISA. Antigens (10^7 cells ml^{-1}): (a) Va V 62 and (b) Va V 408: *Vibrio anguillarum* serotype I; (c) Va V III: *V. anguillarum* serotype III; (d) Vh V 605: *V. harveyi*; (e) Vp: *V. parahaemolyticus*; (f) Pp: *Pasteurella pisciscida*. Antisera. (○) A: rabbit immunized against Va V 62; (◆) B, (▽) B': rabbits immunized against Va V 408; (■) C, (▼) C': rabbits immunized against Va V 62 and Va V 408; (●) R_0S: serum of nonimmunized rabbit (control) • OD: optical density; NS: nonspecific

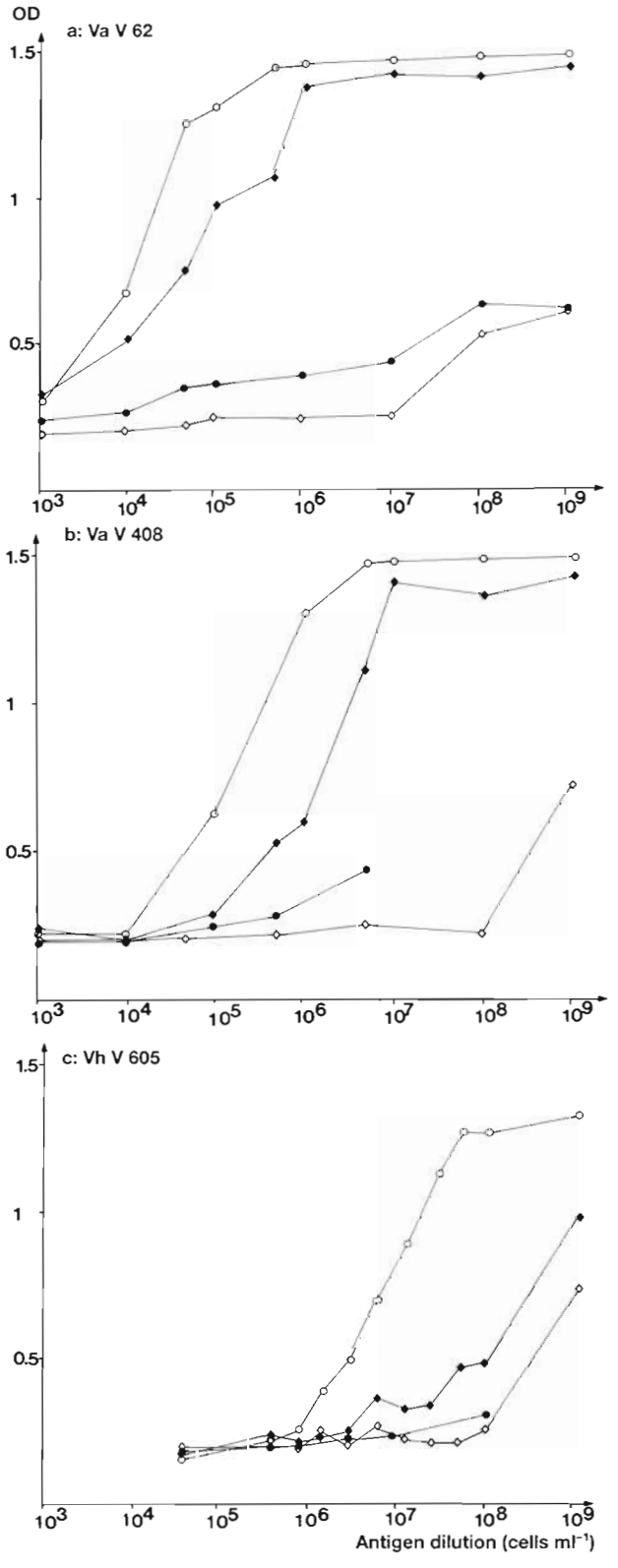

Fig. 2. Bacterial antigen titers as determined by indirect ELISA. Antigens: (a) Va V 62: *Vibrio anguillarum* serotype I; (b) Va V 408: *V. anguillarum* serotype I; (c) Vh V 605: *V. harveyi*. Antisera (constant dilution 1:2 × 10^4, v/v): (○) A: rabbit immunized against Va V 62; (●) B: rabbit immunized against Va V 408; (◇) R_0S: serum of non-immunized rabbit (control) (◇) non-specific; OD: optical density

Table 2. Antigen titration tests. No. of bacterial cells per ml required to obtain optical density 3 times the background interference, using different antisera (constant dilution, 1:2 × 10^4). Results of homologous reactions are in bold

Rabbit antisera	Va V 62	Va V 408	Vh V 605
	(bacterial cells ml^{-1})		
A	**3 × 10^4**	7 × 10^5	0.15 × 10^7
B	1 × 10^5	**3.5 × 10^6**	1 × 10^9

(2×10^5, 2×10^5, 8×10^4 respectively) when reacted with Va V 62.

- The antisera were absorbed by corresponding heterologous bacterial strains to increase antiserum specificity, but this also reduced their sensitivity.

Antigen titration (Table 2, Fig. 2)

In this indirect ELISA test antisera from A and B rabbits were used at a given constant dilution (generally 1:2 × 10^4, corresponding to an OD of 1) on plates coated with variable concentrations of Va V 62, Va V 408, and Vh V 605 (dilutions from 10^9 to 10^3 cells ml^{-1}).

The results obtained with the 3 strains and 2 antisera confirmed earlier results:

- The number of bacterial cells per ml required to obtain the predetermined OD level (3 times the background interference) was lower for homologous reactions compared with heterologous reactions. Antiserum A was generally more sensitive than antiserum B.
- Strains Va V 62 and Va V 408, which belong to the same serotype, appeared to have a larger number of common antigen determinants between themselves than with Vh V 605.

Assessment of the indirect ELISA technique using brain extracts of artificially infected fish (Fig. 3)

The final stage was devoted to an assessment of the indirect ELISA technique. The objective was to determine whether the technique developed by us could be used to detect pathogenic *Vibrio anguillarum* in artificially infected sea bass.

The mortality rate 10 d after artificial infection was 70 % for fish infected with Va V 62 and 74 % for fish infected with Va V III. It was 0 % in healthy controls.

All the dead fish carried bacterial cells; these were isolated from brain and kidney tissue and identified

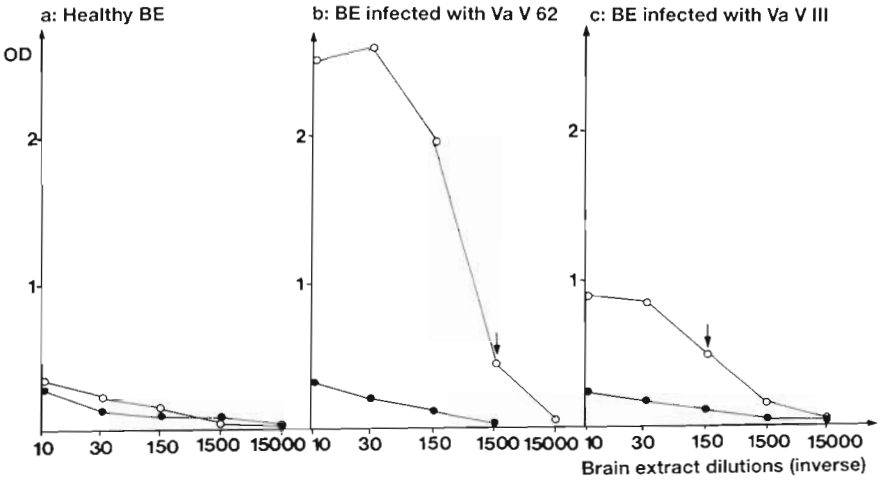

Fig. 3. Reaction of (a) healthy brain extracts (BE), and those infected with (b) Va V 62 and (c) Va V III to (○) antiserum of rabbit A immunized against Va V 62 (dilution $1:10^4$, v/v) and (●) control serum (dilution $1:10^4$). OD: optical density. For each antigen extract, background interference was subtracted from the OD value of each antiserum. See text for explanation of arrows

using standard techniques. No bacterial cells were found in brain and kidney tissue of the healthy controls.

Using the improved indirect ELISA technique, we studied the reactions of antiserum A (antibodies against Va V 62) and serum from the nonimmunized rabbit (R_0S) to healthy brain extracts and those from fish infected with Va V 62 and Va V III. Antiserum A and serum R_0S were used at a constant dilution of $1:10^4$, whereas the brain extracts were deposited at different dilutions in microtitration plate wells. The results showed that:

- Regardless of the type of brain extract (healthy, infected by Va V 62 or Va V III) and its dilution, R_0S serum gave no significant OD.
- Antiserum A containing antibodies against Va V 62 did not detect any challenge in healthy brain extracts (Fig. 3a) but gave a significant OD in reaction to Va V 62-infected brain extracts for dilutions equal to or less than 1:1500 (Fig. 3b, arrow). Antiserum A weakly recognized Va V III in brain extracts infected with the corresponding antigen, but only at dilutions equal to or less than 1:150 (Fig. 3c, arrow).

The results confirmed the high sensitivity of the antiserum to Va V 62 both *in vitro* (buffered antigen suspension) and *in vivo* (infected brain extract). The reactions are specific to the bacterium because OD was 0 with healthy brain extract. A slight crossed reaction of the antiserum with Va V III suggests that the strain presents certain antigenic affinities with Va V 62.

These preliminary results confirm the validity of the indirect ELISA technique for detecting *Vibrio anguillarum* in infected fish brain extracts.

DISCUSSION

This study complements research already accomplished on various fish vibrioses. Earlier work on cold water vibriosis (Hitra disease) caused by *Vibrio salmonicida*, which was based on the use of polyclonal (Holm & Jørgensen 1987) and monoclonal (Espelid et al. 1988) antibodies, led to the development of taxonomic classification and diagnoses of the initial stages of the disease.

In the present study on vibriosis in farmed sea bass, high titers were obtained for the different rabbit antisera: between 8×10^4 and 3×10^5 in homologous reactions for an antigen concentration of 10^7 cells ml^{-1}. This clearly demonstrates the high immunogenicity of the 2 inactivated *Vibrio anguillarum* strains. It also confirms results obtained by other authors for these strains using agglutination tests (Baudin-Laurencin 1981, Sørensen & Larsen 1986, Breuil & Haffner 1989).

There is a definite antigenic affinity between the selected strains Va V 62 and Va V 408, which belong to serotype I. They differ from certain other *Vibrio* strains and other genera of bacteria with which they were compared in this study. The extreme sensitivity of the indirect ELISA technique enabled us to identify cross reactions between the different antisera and certain other *Vibrio* strains, particularly Va V III which is also pathogenic in farmed sea bass. These cross reactions are usually not clearly perceptible in agglutination tests. We concluded that the strains selected from different serotypes may present certain antigenic affinities.

To increase the specificity of the test antisera we undertook absorption reactions of each antiserum by corresponding heterologous bacterial strains. Although their specificity increased, their sensitivity was reduced.

Agreement between the antiserum and antigen titrations validates the use of Va V 62 antisera for detecting *Vibrio* spp. in infected sea bass tissue using a selected polyclonal rabbit antiserum.

Artificial infection of fish with other bacterial strains (*Vibrio ordali, Pasteurella pisciscida*) using different techniques are planned. They will be useful for developing field-usable diagnosis kits to detect various pathogens of sea bass.

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