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In vitro studies and in vivo immunisation with the first viral haemorrhagic septicaemia viruses isolated in Spain compared to international reference serotypes

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The first five viral haemorrhagic septicaemia virus (VHSV) isolates found in Spain were examined for in vitro growth characteristics, neutralisation by trout antiserum and immunisation challenge of trout fingerlings by water immersion. The viruses had come from different host species in various geographical locations in different years. Three reference VHSV serotypes (F_1, F_2 and 23·75) were also included in the study. There appeared to be little relationship between the in vivo delay of mortality or the protection of the immunised trout after challenge and the in vitro characteristics studied. In contrast to the in vitro results, the in vivo delay of mortality suggested a closer relationship of the Spanish VHSV isolates to the F_2 serotype than to the F_1 or the 23·75 serotypes. If the final protection figures are analysed, however, there could be three or four groups of viruses.

VIRAL haemorrhagic septicaemia (VHS) is a disease of some cold water salmonids in northern Europe (De Kinkelin 1972). The mortalities in trout farms in Spain during 1984 to 1986 (Jimenez et al 1988) made it possible to recover and isolate five VHS viruses (VHSV) from different geographical locations, at various dates and from different host species (Basurco and Coll 1989a,b).

Even though mammalian antiserum against VHSV serotype F_1 reacted against all VHSV isolates in an immunoblot assay, the antiserum neutralised efficiently only the homologous serotype (McAllister and Owens 1987). Three serological types of VHSV (F_1, F_2 and 23·75) have been described to date by serum neutralisation (Jensen 1963, Vestergaard-Jørgensen 1972, Le Berre et al 1977, De Kinkelin and Le Berre 1977b). However, the ability of VHSV serotypes to induce an in vivo cross-protective immunity to other VHSV has not yet been sufficiently studied (Gerard 1977, De Kinkelin and Bearzotti 1981).

In this work, the five Spanish isolates and the three serotypes of VHSV were tested in vivo to determine their capacity to induce protective immunity against one isolate of VHSV. It was also of interest to determine whether or not the VHSV isolates from Spain fell into different patterns that would correlate with differences in in vitro virulence, host or geographical distribution as has been shown for another fish rhabdovirus, the infectious haematopoietic necrosis virus (Mulcahy et al 1984).

Materials and methods

Viruses

The strains of virus used were VHSV F_1, and VHSV F_2, provided by Dr P. E. Vestergaard-Jørgensen, and VHSV 23·75 and VHSV 07·71 provided by Dr P. De Kinkelin. Five VHSV isolates (Jimenez et al 1988) had been obtained from fish tissue samples in Spain and isolated and studied (Basurco and Coll 1989 a,b). The viruses isolated are described in Table 1.

Cells, media and virus titration

Epithelioma papillosum cyprini (EPC) cells were used throughout the experiments. Cell culture technique (EMEM medium, Flow) was essentially as reported by De Kinkelin (1972) and modified by Basurco and Coll (1989 a,b). Virus was titrated by the TCID50 method in EPC monolayers in 96-well plates (200 µl per well) at fivefold serial dilutions in quadruplicate (Reed and Muench 1983, Basurco 1990).

TABLE 1: Spanish isolates and international reference serotypes of VHSV

Virus	Location	Year	Host species	Reference
689	Galicia	1984	*Oncorhynchus mykiss*, W	Basurco and Coll 1989b
471	Navarra	1986	*Oncorhynchus mykiss*, W	Basurco and Coll 1989b
144	Salamanca	1984	*Oncorhynchus mykiss*, W	Basurco and Coll 1989b
472	Cantabria	1986	*Salmo salar*, L	Basurco and Coll 1989b
798	Aragón	1986	*Barbus graellsi*, S	Basurco and Coll 1989b
F_1*	Denmark	1963	*Oncorhynchus mykiss*, W	Jensen 1963
F_2*	Norway	1972	*Oncorhynchus mykiss*, W	Vestergaard-Jørgensen 1972
23·75†	France	1977	*Salmo trutta*, L	De Kinkelin and Le Berre 1977b, De Kinkelin 1977

Spanish VHSV isolates were made in the EPC cell line as described
*Provided by Dr P. E. Vestergaard-Jørgensen, National Veterinary Laboratory, Hangovej, Aarhus, Denmark
†Provided by Dr P. De Kinkelin, Centre de Recherches Jouy-en-Josas, INRA, Domaine de Vilvert, France

To obtain the in vitro growth rates, the viruses were inoculated in EPC cells at 0·1 multiplicity of infection and the cultures maintained at 14°C. Every eight to 16 hours, 40 µl of the supernatant were harvested and kept at –70°C. After 100 hours, all the samples were simultaneously titrated by TCID50. The results were expressed as the minimal time needed to get 10^7 TCID50 ml^{-1}.

Neutralisation

Trout anti-serum against VHSV F_1 07·71 was a gift of Dr P. De Kinkelin in 1989. Rabbit antiserum against VHSV F_1 was a gift of Dr Vestergaard-Jørgensen in 1987.

Each virus (100 µl with 2000 PFU ml^{-1}) was mixed with 50 µl of fourfold serial dilutions of trout antiserum and incubated for one hour at 14°C. Then 50 µl of 10-fold diluted fresh trout serum was added as a source of complement and incubated an additional 30 minutes. Each mixture was added (25 µl) to EPC cell cultures in 96-well plates and adsorbed for one hour at 14°C. Afterwards, 100 µl of EMEM, 0·5 per cent methylcellulose (Sigma) were added and left at 14°C for five days. To count plaques, the cultures were fixed with 10 per cent formaldehyde and stained with 0·5 per cent toluidine blue. Neutralisation titre was defined as the reciprocal of trout anti-serum dilution needed to reduce to half the initial virus titre.

Rabbit anti-serum (50 per cent neutralisation titre of 1/1600) was 50-fold diluted (100 µl) with 10^{-3} virus and tested (100 µl) by a similar procedure as described above.

Trout immunisation and challenge

Trout (*Oncorhynchus mykiss*, Walbaum) were purchased from commercial farms (0·2 to 1 g bodyweight) after several annual tests indicated they were free of infectious pancreatic necrosis (Jimenez et al 1988). Thirty-six trout were held in 30 litre aquaria with dechlorinated free-flowing water at 10 to 14°C.

To immunise trout by infection with the VHSV isolates and reference strains or to challenge the surviving trout with VHSV-144, the water level of the aquaria was decreased to 1 to 2 litres, cooled to 8 to 10°C, viruses were added and the trout held for two hours with strong aeration (De Kinkelin and Bearzotti 1981). Then the flow was restored to 1 litre min^{-1} and mortality was recorded daily. Dead fish were removed from each tank, weighed and deaths recorded daily. Dead fish were frozen at –40°C and processed for virus isolation.

Pools of three fish were homogenised in 2 ml of cell culture medium and tested for the presence of virus in EPC monolayers in 96-well plates by adding 50 µl of homogenates to 200 µl of cell culture. After seven days of incubation at 14°C, the plates were frozen and thawed several times and inoculated into fresh EPC monolayers. After an additional seven days incubation, cultures were screened for cytopathic effect.

Trout were immunised and challenged as above and as described (De Kinkelin and Bearzotti 1981, Neukirch 1984, 1986, Neukirch and Glass 1984). Briefly, trout were first immunised by infecting them with 10^5 TCID50 ml^{-1} of each VHSV, attenuated by at least 10 passes in EPC, and held at 12 to 14°C. All the surviving trout from each virus infection were given a second exposure or challenge to 10^6 TCID50 ml^{-1} of VHSV recovered from a dead trout with only two passes in culture and held at 10 to 11°C. The time of half maximum mortality of each isolate was defined by dividing the time to reach the maximum mortality by two.

The delay of mortality relative to control during challenge was obtained by taking away the time of half maximum mortality in control trout from the time of half maximum mortality in immunised trout. Protection was defined by the formula, number of fish surviving challenge/initial number of fish after immunisation but before challenge multiplied by 100.

Results

In vitro characterisation of the VHSV isolates

Table 2 shows that the time in EPC culture needed to obtain a virus titre of 10^7 TCID50 was between 42 and 48 hours for all the viruses used except for the F_2 which was 65 hours. All five Spanish VHSV isolates were completely neutralised by anti-VHSV F_1 rabbit antiserum. The neutralisation by anti-VHSV 07·71 (F_1) trout antiserum showed significantly different neutralisation titres for the 471 and the 472 isolates.

Immunisation by infection with different VHSV

To study the possibility of immunising trout with the VHSV isolates, preliminary experiments were conducted to adjust dosage, time of exposure and temperatures of infection and maintenance to obtain a mild infection causing low mortality. Trout (36 per aquarium) were immunised by infection with viruses attenuated by 10 passes on EPC, relatively low dosages (10^5 TCID50 ml^{-1}) and moderately high temperature (12 to 14°C). No signs of VHS could be observed during the first four days, but after seven to eight days the trout immunised with VHSV developed typical symptoms of the disease (mainly darkening of the skin, exophthalmic eyes and haemorrhagic fins) and began to die. After 23 days, mortality varied between 10 and 50 per cent, depending on the virus (Fig 1). No deaths were observed in control unimmunised trout during the same period. Virus could be isolated from pools of three dead trout obtained from each of the viruses with a titre of 10^6 to 10^7 TCID50 g^{-1} of trout. The isolation of infectious virus from the dead trout sometimes failed probably because of VHSV inactivation during storage although more or less marked specific signs of VHSV disease had been observed in these trout.

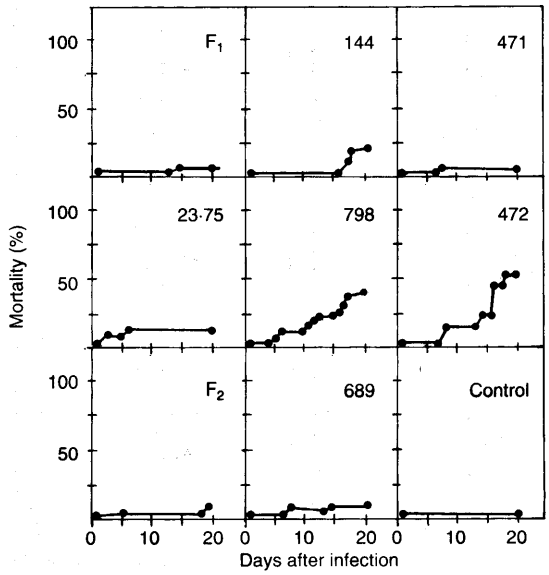

FIG 1: Cumulative mortality of trout immunised by infection with Spanish isolates and reference strains of VHSV. VHSV were attenuated by 10 serial passages in the EPC cell line. Healthy trout (36 per VHSV) were exposed to 10^5 TCID50 of each VHSV ml^{-1}. Control trout received EPC supernatant. Water temperature was kept at 12 to 14°C

TABLE 2: Comparison of VHSV isolates by in vitro growth rate, trout serum neutralisation titre and in vivo delay of mortality after challenge

Virus	Growth rate (h)	Neutralisation titre $\times 10^{-3}$	Delay of mortality (days)
F_1	43	1	0·0
23·75	42	1	0·0
F_2	65	1	5·0
144	48	2	5·5
798	43	2	5·0
689	42	1	4·5
471	48	10	5·0
472	45	4	5·5
Control	–	–	0·0

Challenge of survivors with virulent VHSV-144

Twenty-three days after the immunisation by infection, all the surviving trout were challenged with the heterologous or homologous virus VHSV 144 with a 10-fold higher virus concentration, lower temperature (10 to 11°C) and virulent virus freshly isolated from infected trout. Twenty days later, mortality was higher than during the first infection but a significant level of protection was afforded by immunising with the 144, 472, F_2, 798 and 689 VHSV isolates ranging from 25 to 60 per cent depending on the virus (Fig 2).

A comparison of the time required to produce half of the maximum deaths in the challenged

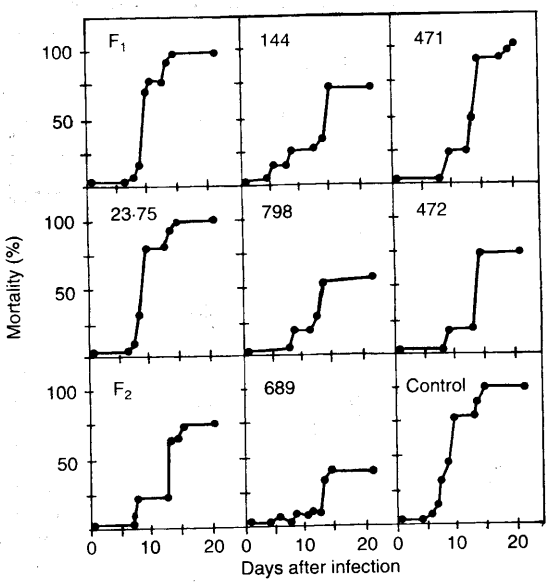

FIG 2: Cumulative mortality of surviving trout to challenge with virulent VHSV-144. VHSV-144 recovered from infected trout were passed two times in the EPC cell line. Each of the surviving trout from the immunised group (Fig 1) were exposed to 10^6 TCID50 VHSV 144 virus ml^{-1}. Water temperature was kept at 10 to 11°C

fish was made (Table 2). Half of the control fish (unimmunised but challenged) that died, did so after 8·5 days together with the fish immunised with the VHSV F_1 and 23·75. The rest of the fish (immunised either with the F_2 serotype or with each of the Spanish isolates) died with half of their maximal deaths occurring between 12·5 and 13·5 days (delay of four to five days relative to control). Trout which survived the challenge showed no signs of VHS and have been maintained for 12 months showing normal growth rates.

Discussion

Only serotype F_2 showed a significantly different in vitro growth rate (Mulcahy et al 1984), slower than all the other viruses (Table 2). Even though rabbit antiserum against F_1 neutralised all Spanish isolates, trout antiserum against 07·71 (F_1) neutralised isolates 471 and 472 much more effectively and was unable to distinguish between the three serotypes. Isolates 471 and 472 came into the laboratory the same week in 1986 although from two different Spanish locations and two different host species. The present authors were recently able to distinguish isolates 471 and 472 from all the rest by their lack of reactivity with monoclonal antibodies (F. Sanz, and J. M. Coll, personal communication). According to the rate of mortality during the challenge experiments, the viruses studied could be divided into at least two groups, one of them with all the Spanish isolates and F_2 and the other with F_1 and 23·75. There appeared to be little relationship between cross-immunisation of trout and any of the in vitro characteristics of the virus isolates studied here.

A critical question in the development of any viral vaccine is whether cross-immunity will arise from vaccination with a vaccine derived from a single virus serotype (Gerard 1977, De Kinkelin and Le Berre 1977a). To study this question, trout were immunised by natural infection with attenuated VHSV isolates and serotypes under condition of 40 to 60 per cent of survival (De Kinkelin and Bearzotti 1981, Vestergaard-Jørgensen 1982) and then the survivors were challenged with a virulent virus isolate under conditions of no survival in unvaccinated trout. Protection against the challenge was demonstrated by a two to three-fold reduction in mortality of some of the immunised trout relative to unvaccinated trout. Similar partial protection has been reported earlier for VHSV challenged trout (Vestergaard-Jørgensen 1976, De Kinkelin and Bearzotti 1981). Immunisation by infection with cell cultured VHSV isolates did not give complete protection, probably because the young trout used, known to be more susceptible to VHSV, were not fully competent immunologically (Johnson et al 1982). During challenge, the virus dose has to be sufficient to produce some mortality but not so great as to overwhelm the immune system. Difficulties of this kind have been reported with the infectious haematopoietic necrosis virus during vaccination assays (Engelking and Leong 1989).

The difference of death rates (delay of mortality) and total deaths (protection) between the immunised (Fig 1) and challenged groups (Fig 2) can be explained by the 10-fold increase in virus dosage, the lower number of passages in cell culture of the virus and the lower temperature, all used during the challenge experiment (Hetrick et al 1979, De Kinkelin and Bearzotti 1981, Neukirch 1986). The results showed that the death rates caused by the Spanish isolates and the F_2 were delayed four to five days with respect to control fish and the other two serotypes. These results suggest that the immunological system of

the trout sees the VHSV isolates as more like the F_2 virus than the F_1 or 23·75 virus. These results might have implications for vaccine development because they show that cross-immunisation, determining the extent to which protection of trout occurs, is more dependent on the trout serotypes than on the rabbit serotypes. However, since trout serum neutralised isolates 471 and 472 more effectively (Table 2) and no protection was obtained by immunising with F_1, 23·75 and 471, about 25 per cent protection was obtained by immunising with 144, 472 and F_2 and 40 to 60 per cent protection was obtained by immunising with 798 and 689 (Fig 2), there could be three to four or more groups of VHSV for trout. More work is needed to establish trout serological virus relationships for the possible development of vaccines.

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