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Eighteen years of vaccination against viral haemorrhagic septicaemia in France

P de Kinkelin 1*, M Béarzotti 1, J Castric 2, P Nougayrède 3, F Lecocq-Xhonneux 4, M Thiry 4

1 INRA, laboratoire de virologie et immunologie moléculaires, 78352 Jouy-en-Josas;  
2 CNEVA, BP 70, 29280 Plouzané;  
3 Laboratoire départemental, 40000 Mont-de-Marsan, France;  
4 Pharos, 4102 Seraing, Belgium

Summary — Viral haemorrhagic septicaemia (VHS) has been considered for many years to be a major cause of loss in the French trout industry. The high prevalence of VHS in certain geographic areas made a control strategy based on control policy unfeasible. This provided the impetus for immunoprophylaxis development that resulted in 3 successive types of vaccines: inactivated, live attenuated and recombinant vaccines. When delivered by intraperitoneal injection, the 2 propiolactone-inactivated VHS virus was immunogenic and/or protective for trout all of sizes, but it was not suitable for the practical immunization of alevin, the trout life stage that is the most sensitive to VHS. A carp cell-passed, attenuated variant of the VHS virus was effective after both immersion or injection delivery and met the practical requirements of juvenile vaccination. However, this vaccine was discarded because it retained some virulence that discouraged the launching of its commercialization. Then came the era of genetically engineered vaccines. The recombinant glycoprotein of VHSV produced in Escherichia coli or in Saccharomyces cerevisiae failed to protect fish whatever the route of delivery. A recombinant baculovirus vaccine was found to be immunogenic and protective against VHS, but only when delivered by injection. Due to its cost and route of delivery, the latter vaccine was not licensed. Simultaneously, the sudden occurrence of another rhabdovirosis, infectious haematopoietic necrosis (IHN), in France, rendered vaccination against VHS questionable. Indeed, no cross-protection between these 2 rhabdoviruses exists. If vaccination is still believed to be an effective control method for VHS, it should be based in the future upon an autoreplicative vaccine. Protection against IHN will also have to be taken into consideration. It is also possible that certain technical devices will, some day, render the injection of inactivated IHN and VHS viruses acceptable for the trout farming industry.

salmonid / vaccination / viral haemorrhagic septicaemia / France

Résumen — Dix-huit années de vaccination contre la septicémie hémorragique virale (SHV) en France. La présence endémique de la septicémie hémorragique virale (SHV) dans certaines zones géographiques rendait irréaliste son éradication par la seule prophylaxie sanitaire à laquelle par ailleurs les pisciculteurs étaient hostiles. La vaccination a donc suscité des travaux soutenus pour trouver un produit adapté à l'administration de masse mais répondant également, pour un prix bas, aux critères généraux des vaccins des mammifères. Cette quête d'un idéal vaccinatoire a conduit présentement à l'absence de vaccin commercialisé. Les vaccins inactivés étaient apathogènes, protecteurs, immu-

* Correspondence and reprints
nogènes, d'un prix de production acceptable mais devaient être injectés et ne convenaient donc pas à l'administration aux jeunes poissons, cibles privilégiées des virus. Les vaccins vivants (virus atténués) étaient efficaces administrés par bainéation mais avaient une virulence variable et des bases d'atténuation inconnues. Les protéines virales recombinantes furent des échecs et un seul baculovirus recombinant fut protecteur et immunogène mais seulement par injection et d'un prix bien supérieur à celui des vaccins inactivés. Seul un produit autorépliquant, appliqué par voie orale ou balnéatoire, peut apporter une solution vaccinale à la SHV et il faudra sûrement y associer une méthode de prévention de la nécrose hématoïétique infectieuse, autre rhabdovirose nouvellement implantée en Europe.

salmonidés / vaccination / septicémie hémorragique virale / France

INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a cold water rhabdovirus infection of rainbow trout (Oncorhynchus mykiss) and several other fish species. VHS is a matter of concern chiefly because of its economic consequences to rainbow trout farming. VHS results in a strong protective immunity in the survivors, inducing the synthesis of circulating antibodies as well as an asymptomatic virus carrier-state, which can be the source of further contamination for naive trout.

When the vaccination venture began, the only control method proposed for VHS was control policy. Because of its drastic constraints for the live fish trade, French trout farmers were reluctant to employ it. They thus combatted the VHS problem by growing more fish than they lost and the prices of trout still support this defensive strategy.

Nevertheless, many trout farmers also considered vaccination to be a desirable control method for VHS, based on its effectiveness against vibrioses and yersiniosis, 2 bacterioses of salmonid fish (Fryer et al, 1976). Insofar as the juveniles are the most sensitive fish to VHS infection, there was an impetus for a mass-delivered vaccine. The contamination usually occurred once the fish were transferred from the indoor hatcheries to the outdoor ponds, which are supplied with river water, ie around the age of 1 500 degrees x d. After this time, overt VHS was mainly recorded in fingerlings.

Although exhibiting wide variations according to individual fish, the virulence of virus and sometimes the environment, the usual rates of losses due to VHS ranged from 50 to 80% of the infected fish group. For these reasons, the trout farmers, understandably, believed that it would be a significant economic improvement if the mortality rates could be decreased to the range 20–50% (or lower) following vaccination by an easy-to-use procedure.

Preliminary, encouraging (but non-reproducible) results reported that infectious haematopoietic necrosis (IHN), another rhabdovirosis inducing clinical signs similar to those of VHS, was prevented using a water-borne delivery for an inactivated virus vaccine preparation (Amend, 1976). At the same time, it was demonstrated that a water-borne delivery of an attenuated VHS virus generated fair protection to experimental challenge with the wild virus (Vestergaard-Jorgensen, 1976).

We thus undertook investigations aiming at achieving a vaccine to VHS that would result in an economically significant reduction in loss to a level of 30% for trout farmers. Three types of vaccines were successively developed and used: inactivated (killed), live attenuated and recombinant vaccines.

We present here a certain number of examples that demonstrate how, taking into account the requirements of potency, safety, ease of delivery, cost and attenuation mechanisms, our vaccines always
induced significant levels of protection in fish but never met all of the above requirements.

MATERIALS AND METHODS

**Inactivated vaccines**

The inactivated vaccine preparations were derived from wild VHSV isolates propagated in the epithelioma papulosum cyprini (EPC) cell line (Fijan et al., 1983). The virus isolate 07.71 from rainbow trout belonged to serotype 1 (Vetergaard-Jorgensen, 1972) and isolate 23.75 from brown trout (Salmo trutta) (de Kinkelin and Le Berre, 1977), belonged to serotype 3 (Le Berre et al., 1977). Clarified virus-infected cell supernatants were treated with 2-propiolactone 1:5 000 for 24 h at 15°C and formalin was later added to a final dilution of 1:2 000. The vaccine dose for the intraperitoneal (ip) injection was 2 × 10⁶ inactivated (i) pfu/g of fish delivered as dual (1 × 10⁶ ipfu/g) or single (full dose) injection. The vaccine concentration of the immersion delivery was 5 × 10⁴-10⁵ ipfu/ml water for 3 h at 9-12°C. The vaccine preparations were usually stored at -20°C but they also retained their efficacy for at least 1 year at 4°C. Control fish were injected ip with a placebo made of uninfected disrupted cell culture supernatant treated with 2 propiolactone and formalin as previously described.

**Attenuated vaccines**

The live, attenuated vaccines to VHS were obtained through successive passages of VHSV in either trout or carp cells (EPC), part of these subcultures being achieved at progressively increasing temperatures (up to 25°C). After a first vaccine (de Kinkelin and Béarzotti, 1981) was thus derived from the Danish VHSV isolate F1 (Jensen, 1965), a certain number of other attenuated virus variant preparations were derived from the French VHSV isolate 07.71 (Bernard et al., 1985) and provided the results reported here. The attenuated variants were delivered by the immersion of trout, around 1 500 degrees x d old, for 15 min at 10°C in an aqueous vaccine suspension titering 5 × 10⁵ pfu/ml. The vaccine stocks were stored frozen at -70°C and retained their protective properties for more than 2 years.

**Recombinant vaccines**

A copy of the RNA encoding for G protein of VHSV was primarily cloned and sequenced (Thiry et al., 1991). The VHSV G protein expressed in *Escherichia coli* as a fusion protein with the bacterial enzyme Trp E, however, failed to protect the trout, whatever the route of delivery (unpublished results). A recombinant glycoprotein was thus prepared in the eukaryotic cells SF9 (*Spodoptera frugiperda*), infected with recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV). This virus was obtained after the co-infection of SF9 cells with 1-type AcNPV and a recombinant transfer vector, p BacSHVG, which contained the VHSV G protein gene under the control of the AcNPV polyhedrin promoter (Lecocq-Xhonneux et al., 1994). The fish were immunized with 1 × 10⁶ recombinant baculovirus infected IP mixed with an adjuvant (Suvaxyn, Solvay Duphar Animal Heath, Weesp, The Netherlands) or by immersion in an aqueous suspension containing 2 × 10⁵ disrupted SF9 cells. The mock immunized group was made of fish injected with SF9 cells or immersed in an aqueous suspension containing 2 × 10⁵ disrupted cells/ml.

**Fish, experimental groups and statistics**

Almost all the vaccination trials reported here were performed with duplicates encompassing a minimum number of 30 x 2 fish per group (a and b). The target fish species was mainly rainbow trout although brown trout was used once. The work was conducted both under laboratory and trout farming conditions (see below). All the laboratory challenges were performed using wild VHS virus type 1.

The statistics were computed according to the chi square table with the Yates correction. The relative percentage survival (RPS) (Amend, 1981) was established from the following formula:

\[
\text{RPS} = \frac{1 - \% \text{ loss of immunized fish}}{\% \text{ loss of mock-immunized controls}} \times 100
\]
RESULTS

Inactivated virus (killed vaccines)

The dual IP injection (d0 and 23) of inactivated vaccines to groups of rainbow trout alevins (2 x 100 fish/group, groups a and b) resulted in a strong protection against a water-borne challenge performed at d 63 (fig 1). In this early work, the survival in the group of fish vaccinated by immersion also differed significantly ($\chi^2 = 12.46; \alpha < 0.001$) from that of the mock-immunized fish. Nevertheless, this decrease in mortality failed to appear in many further assays and was too low to constitute any economic advantage.

For these reasons, the immunization assays with inactivated vaccines were restricted to certain situations such as further transfer to sea water of high value fish or protection of the large trout that are currently produced in many French trout farms. Some examples of the above vaccination trials are given in table I. They all provided evidence for the potency of the inactivated vaccines that resulted in the decreased mortality ≥ 30%, ie economically significant.

Attenuated vaccines

The results reported in figure 2 offer a typical example of a vaccination trial using attenuated virus variant vaccines conducted on 8 duplicate groups of juvenile rainbow trout (60 x 2 fish/group).

The vaccine preparations differed in their number of passes to last cloning they had undergone. These ranged from 2 (vaccines No 4, 5) to > 15 (vaccines 3, 6, 7), their overall number of passes in cell culture (94 for No 5, 7; 100 for No 6; 111 for No 3, 4) and also their incubation temperature (14°C for No 4, 7; 25 for No 3). The group 1 fish, mock-immunized uninfected controls, underwent a background mortality of 5%. The group 8 fish, mock-immunized and challenged on day 45, only presented a survival rate of 15%. In contrast, the fish from immunized groups 3–7 presented overall survival rates ranging from 60 to 42.5 (mean 52; $\alpha < 0.0001$). The survival rate of the group 3 fish differed significantly ($\alpha < 0.001$) from those of the other 4 groups. The group 2 fish that survived at 10% were infected with wild VHSV on d0 and d45 permitting the evaluation of the safety of live vaccine preparation and mim-

Fig 1. Vaccination of rainbow trout against VHS using an inactivated vaccine. Survival of immunized (1 and 2) and mock-immunized (3) alevins to water-borne challenge with wild VHSV virus type 1. Age of fish at vaccination: 1 000 d x degrees (900 mg each); No of fish: 100 x 2 (a,b)/group. Delivery: 1 and 3 intraperitoneal injection; 2, immersion. Immunizations were performed on day 0 and 24 and challenge on day 63.
The course of vaccination trial had 2 stages: immunization from d0 to d45 and challenge from d45 to d85. During the first step, vaccine preparations 4 and 5 were safe, whereas vaccines 6 and 7 killed 53% of the fish. Between these 2 extremes, the attenuated vaccine virulence appeared to be 4.5 to 6.5 times lower than that of wild VHSV.

Table 1. Vaccination of trout against VHS using inactivated vaccines: some examples of fish responses recorded.

<table>
<thead>
<tr>
<th>Fish</th>
<th>Inactivated virus</th>
<th>Challenge</th>
<th>Survival (%)</th>
<th>( \chi^2 ) test</th>
<th>RPS</th>
<th>Neutralizing antibody responding fish (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 g rainbow trout</td>
<td>0.71</td>
<td>d50, IP</td>
<td>86/50</td>
<td>(a &lt; 0.0001)</td>
<td>78</td>
<td>nd</td>
</tr>
<tr>
<td>(40 x 2 fish/group)</td>
<td></td>
<td>2 x 10^8 pfu/fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5 g brown trout</td>
<td>23.75</td>
<td>d21, water-borne</td>
<td>80/31.7</td>
<td>(a &lt; 0.0001)</td>
<td>60</td>
<td>nd</td>
</tr>
<tr>
<td>(30 x 2 fish/group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>900 g rainbow trout</td>
<td>0.71</td>
<td>d112, IM</td>
<td>100/60b</td>
<td>nd</td>
<td>nd</td>
<td>d0: 0</td>
</tr>
<tr>
<td>(500 fish)</td>
<td></td>
<td>6 x 10^6 pfu/fish</td>
<td></td>
<td></td>
<td></td>
<td>d83: 55 ± 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>d112: 80 ± 35c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>d140: 62.5 ± 17</td>
</tr>
</tbody>
</table>

RPS: relative percentage survival; IP: intraperitoneal; IM: intramuscular; nd: not done; a: after transfer to seawater; b: as estimated from infection trials performed on 5 and 10 fish; c: as estimated from 36 immunized fish, the non-immunized fish did not respond.

The course of vaccination trial had 2 stages: immunization from d0 to d45 and challenge from d45 to d85. During the first step, vaccine preparations 4 and 5 were safe, whereas vaccines 6 and 7 killed 53% of the fish. Between these 2 extremes, the attenuated vaccine virulence appeared to be 4.5 to 6.5 times lower than that of wild VHSV.

Although vaccine 3 had retained some degree of virulence, its use resulted in obvious potency. Due to the great need for a vaccine, it was decided to check its safety under trout farming conditions, in a farm

**Fig 2.** Live attenuated virus vaccines against VHS: survival of the water-route immunized (day 0) rainbow trout to a water-borne challenge, given as mean value in each duplicate. Day 0: vaccination. Age of the fish: 1 400 degrees x d; No of fish 60 x 2/group; 1: mock-immunized, non-challenged fish; 2: wild VHSV infected (day 0) fish; 3–7: attenuated VHSV variant immunized fish; 8: mock-immunized fish. Day 45: challenge (arrow) of groups 2–8 with wild VHSV type 1.
with an established history of VHS (table II). Surprisingly, throughout 4 successive trials, the safety was fair. A 5th trial, encompassing mock-immunized fish was then conducted, again revealing an acceptable vaccine safety (2.8% losses). Unfortunately, after the trout were transferred to the outdoor ponds, they became infected with IHNV and the vaccine potency could no longer be assessed.

Recombinant baculovirus vaccine

The vaccination trials conducted at the laboratory on duplicated groups of 1 400 degrees x d old rainbow trout (50–55 x 2 fish/group), clearly demonstrated the potency and immunogenicity of the recombinant baculovirus vaccine in comparison with those of the inactivated and attenuated vaccines. The percentage survival for the recombinant, inactivated and attenuated vaccine groups and their controls were 68 vs 52.8, 70 vs 31.1 and 72.5 vs 35.2, respectively. All these differences were significant after $\chi^2$ analysis ($\alpha < 0.05$) but an obvious non-specific protection was generated by the injection of Sf9 cells, resulting in a survival rate of 52.8 vs 31.1 in the group of trout previously infected with cell culture medium.

A second series of trials provided similar results except that the overall survival rates were higher 95.2 vs 75.8 in the controls. Again, the survival in the groups of fish that did not receive the Sf9 cells was lower (60%). No protection could be induced following the immersion and oral routes of vaccine delivery.

DISCUSSION

All 3 groups of anti-VHS vaccines proved their potency and immunogenicity. In terms of production costs, 2 vaccines were satisfactory, the inactivated vaccines and attenuated vaccines. However, the inactivated vaccines were not suitable for mass delivery and were discarded. In contrast, the attenuated vaccines were well suited for practical delivery, but their residual virulence under experimental conditions discouraged a vac-
cine manufacturer, who had formerly been interested, from launching any commercial production, as the attenuation mechanisms of VHSV are unknown. Moreover, the French trout farmers who had proclaimed the need for a vaccine against VHS so often in the past, then failed to maintain their interest in it. For these reasons, we attempted to demonstrate the practicability of using an attenuated variant vaccine under trout farming conditions. Unfortunately, when it became clear that variant No 3 was safe for fish, an IHN infection suddenly occurred in the trout population and doomed the anti-VHS vaccination to failure insofar as there is no cross-protection between the 2 rhabdoviruses (Hattenberger-Baudouy et al, 1989). The occurrence of intercurrent infections (LaPatra et al, 1995) remains a major limitation to overcome in the assessment of the vaccine potency in fish (de Kinkelin, 1988). Indeed, such infections can hinder both the immunization of fish and further challenges.

Nevertheless, during the periods when IHN was detected in France, fish disease control methods were entering the era of genetic engineering and biotechnology. It was thus reported that vaccination to IHN could be achieved experimentally after water-borne delivery, using a recombinant virus protein expressed in E coli (Gilmore et al, 1988). A similar construction made with the VHSV G protein was uneffective and did not provide protection (unpublished) and we continued our quest for the ideal vaccine to VHS. Our efforts resulted in a recombinant baculovirus vaccine that was only potent after delivery by immersion and thus led us back, at a much higher cost, to the inactivated vaccines 17 years earlier! The objective of recombinant baculovirus vaccine to VHS was abandoned.

Investigations on vaccines to VHS that were conducted in Denmark resulted in both a live-attenuated vaccine and a recombinant protein one. The former (Vestergaard-Jorgensen, 1982) provided protection, but was not used in practice for several possible reasons relevant to fish health legislation, environment conservation and lack of interest by the vaccine industry. The recombinant protein vaccine (Lorenzen et al, 1993), which is made of an almost entire VHSV G protein, elicited the synthesis of neutralizing antibodies in trout after IP injection. This recombinant protein had to be renatured prior to its injection and, in contrast with our findings, its generated neutralizing activity was heat-labile. Despite the amount of work undertaken to investigate vaccination to VHS, there is no vaccine currently available in practice. There are several reasons to account for this. Developing a vaccine can be a complicated problem, resulting in a long delay between the original demand by fish farmers for a vaccine and the final availability of the product. It is obvious that if an attenuated virus variant, which may remain slightly virulent, but which is able to lower the mortality rate of fish by 30%, had existed during the early seventies, it would have been in systematic use before the limiting regulations were enforced. Since this was not the case, the achievement of a recombinant subunit vaccine, the fruit of genetic engineering techniques, raised hopes that a VHS vaccine had been found that combined potency, safety and cheapness. This approach just overlooked the fact that many recombinant subunit vaccines to mammal viroses are less potent than their conventional counterparts even after repeated administrations and, more precisely, that the mode of vaccine delivery is critical for fish vaccination.

Besides the problem of concurrent infections, the design of a vaccine to VHS was faced with several factors affecting its fate. To name a few, these included host species and strain, individual and age (expressed as degrees x d), pathophysiological state, water temperature, oxygen concentration, photoperiod season, diet, virulence of the
challenging virus and non-viral components of the vaccine preparation. The overall effect of these factors often results in a decrease in fish sensitivity induced by non-specific responses, as those observed with the inactivated vaccine delivered by immersion (fig 1) and injected Sf9 cells (Lecocq-Xhonneux et al, 1994). Such responses may give an incorrect notion of the potency of certain vaccines, as was the case for both delivered, inactivated vaccines to IHN (Amend, 1976). All the mechanisms leading to low rates of losses in the mock-immunized fish group may also render the vaccine effectiveness unacceptable when assessed according to the dogmatic criteria (Amend, 1981) of international legislation governing the marketing of fish vaccines.

Another reason that slowed down the trend towards making a commercial vaccine, came from the existing strategy of animal disease control. According to this strategy, vaccination is only used to diminish disease losses until a reasonably low number of farms are infected. Then, eradication of the disease and control policy measures are implemented. Vaccination thus appears as a provisional control method resulting, for vaccine manufacturers, in vaccines with commercial lives of less than 10 years. Considering the small size of the VHS vaccine market, it appeared that the break-even point of industrializing such a vaccine manufacture, would never be reached. This discouraged anyone from launching the venture. Moreover, there is no commercially available viral vaccine for aquaculture to date (Leong and Fryer, 1993).

The fate of vaccination for VHS in France generates some feelings of frustration because of the effort that was made and that the knowledge gathered now appears somewhat useless. Indeed, due to the recent availability of an automatic injection device for fish, the immunization to VHS by injection is now believed to be achievable insofar as the fish could be stored under a virus-free condition until they weighed around 15 g each. The immunizing preparation would come out in the guise of an autovaccine made with inactivated VHSV that would thus bypass the difficulties and cost of licensing procedures. However, the existence of IHN in France, as in many European countries, demands that such a vaccine be multivalent.

Nevertheless, if fish vaccination to viroses is to become a practical reality someday, future vaccines to salmonid fish rhabdoviroses and, even more so, to fish viroses, it will have to be based upon the use of autoreplicative agents inducing protection early in the fish life. There may be viruses which are deleted for virulence (Béarzotti et al, 1995) or which are better gene carrier viruses, but they remain to be constructed. On the other hand, practical antiviral vaccination of fish appears more frequently as a control method, which is based on the use of safe, inexpensive and readily accessible products and that should be available soon after the discovery of any viral disease. Such a venture appears increasingly to be a mere challenge to fish pathologists and scientists.

Moreover, once a vaccine becomes available, its acceptance for use by professionals still depends on the state of their minds and on the fish culture management methods existing at the time. In both humans and fish, there is a place and time for anything.

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