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Fish rhabdoviruses

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ABSTRACT

Fish viruses belonging to many different genuses have been isolated during the last decades. Among those, rhabdoviruses are of the most abundant and the most virulent. Fish rhabdoviruses infect not only species actually farmed (trout, salmon) but also species with increasing farming capacities (sea bass, turbot, eel) as well as wild species found in fresh (carp, perch) or in salt (cod) water including some crustacean (shrimp). This work reviews the actual state of fish rhabdovirus research. It focus on designing new methods for detection of asymptomatic rhabdovirus fish carriers, sequencing of the fish rhabdovirus genomes for phylogenetic studies, making infectious cDNA copies of its RNA genomes for reverse genetics, investigating the early steps of infection, studying of the fish immunological responses and designing of recombinant and/or DNA vaccines.

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Introduction to fish viruses. The studies about fish viruses began about 40 years ago with the first isolation of a birnavirus causing infectious pancreatic necrosis (IPN) and with the development of the first fish cell line [1]. From there, the number of identified fish viruses have been growing continuously and now there is an impressive number of RNA or DNA viruses, with or without envelope. The majority of the isolated fish viruses belong to the families rhabdoviridae, birnaviridae, aquareoviridae, retroviridae, herpesviridae and iridoviridae [2]. Some of these viruses are only found in fish, like the iridoviridae linfocistis [3] or the aquareoviridae, now being used to induce unspecific responses against rhabdoviruses [4, 5]. Some low pathogenic fish viruses have been studied to try to develop possible vectors such as the catfish [6] or the salmon (project FAIR CT95-0850) herpesviruses.

Many other potential viruses remain unclassified or are suspected to be associated to clinical fish diseases [2, 7, 8]. Furthermore, in marine sea water samples, many other viruses whose aquatic host are unknown can be easily isolated by filtration [9].

Among all the fish viruses actually known, the rhabdoviruses [10] together with the birnaviruses [11] are the most numerous. The rhabdoviruses are the most virulent and therefore cause the highest economic impact. It is not surprising that their presence follows the development of intensive aquaculture during the last decades throughout the world (Fig 1). About

Figure 1. Geographic distribution of the most important fish rhabdoviruses. IHNV [■] and VHSV [●]. About 30 years ago, the IHNV was first detected in USA and Japan and the VHSV in Europe. More recently both viruses are being spread to either Europe or the USA, respectively [136]. The relative sizes of the symbols express the differences in prevalence.

20-40% of the annual production of salmonids is being lost by rhabdovirosis world wide (Europe, USA, Japan) (Fig 2). No other pathology either infectious or not, is causing such losses in Aquaculture. There are no commercial vaccines [10, 12], nor therapeutic methods and it is very difficult to detect asymptomatic carrier fish [10, 13, 14]. The European Union began and continues an eradication program developed a few years ago (Doc. 90/495/EF) [15].

Even though more than 20 different types of fish rhabdoviruses have been described the majority have not been further studied [16]. Among the most studied are the viral haemorrhagic septicaemia virus (VHSV) of Europe and the infectious haematopoietic necrosis virus (IHNV) of USA and Japan of cold water fish followed by the carp viral spring viremia (SCV) of warm water fish.

The VHSV also known as the Egtved

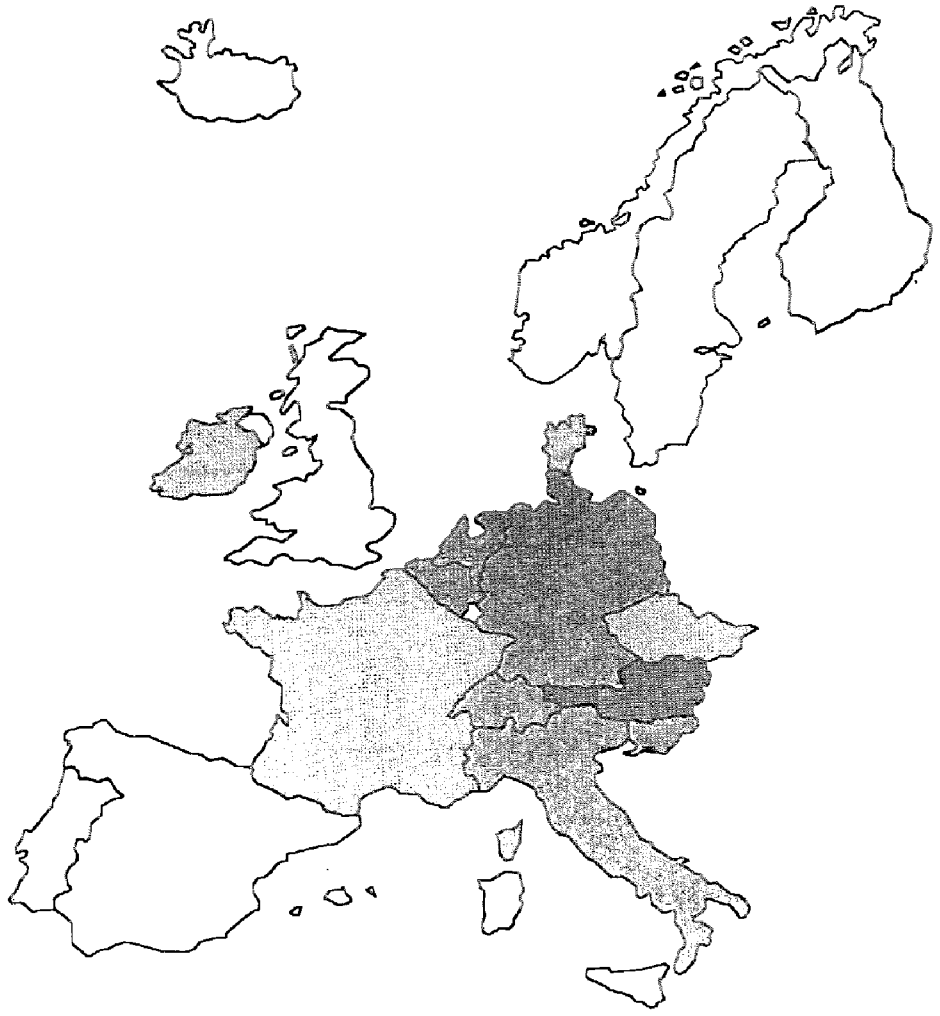

Figure 2. Prevalence of rhabdovirus in Europe. Results from the European Union (EU) survey of 1998 [137, 138]. □ 0% of farms with rhabdoviruses. ▒, < 5% of farms with rhabdoviruses. ▒, 15-25% of farms with rhabdoviruses. ▒, ≃ 50% of farms with rhabdoviruses.

virus, was first isolated in Europe in cold water salmonids. There have been experimental infections reported in sea bass and turbot, as well as isolation in cultured species like turbot [17, 18], eels and shrimps [19]. More recently it has been isolated in North America from salmon and cod [20-22]. The VHSV, is similar to the IHNV [23] from North America and/or Japan, however they have no crossneutralization and very low sequence homology. The IHNV was first detected in France [24] and Italy [25] in Europe and has been spread since then. There were a few cases of VHSV reported in Spain in 1985 [26] and in 1993 there was a single case of coinfection with IPNV reported in Spain [27-29], but there were no other cases described since then.

In the group of SCV there are isolates from carp and other fresh water fish from Europe. Some crossreactivity has been reported between SCV and pike fry rhabdovirus (PFR), although their sequences are not homologous [30]. The irame rhabdovirus isolated in Japan is different from IHNV and shows some crossneutralization with VHSV. The glycoprotein G of the snakehead rhabdovirus has been characterized [31]. In the group of eel rhabdovirus there are many viruses isolated from eels. The first one isolated was the eel virus american (EVA). In this group, there are vesiculovirus-like (EVA, EuropX, C30, B44, D13) and lyssavirus-like (L59, B12, C26) [32] eel rhabdoviruses. There have been also rhabdoviruses isolated from shrimp [33, 34].

Isolation and detection of fish rhabdoviruses. Because most fish rhabdoviruses can be cultured in many different fish cell lines, they are relatively easy to detect. For instance, VHSV can multiply in mammalian (BHK-21), reptile (GL-1 y TH-1) or insect (H5) cell lines but always at 14 °C [16, 35]. Different fish rhabdoviruses replicate at different temperatures (10-30 °C), each one having its own optimal temperature (Table 1), according to its host fish specie. The cytopathic effect consists in lysis of the cells producing visible plaques 5 days after in vitro infection. During infection, infective virions and many incomplete defective particles are produced. Defective particles are not infective but interfere with replication of infective virions [16].

To detect fish rhabdoviruses, cell culture techniques to amplify the virus and identification techniques such as neutralisation [36] or immunofluorescence, are still being used. Those techniques are being substituted by other new methodologies. Due to the development of monoclonal antibodies (Mab) and of DNA amplification (PCR) techniques, an increase in specificity and sensibility has been made possible. The use of capture of rhabdovirus with solid-phase anti-rhabdovirus MAbs followed by PCR in the same well could contribute to the automation of these new assays so they could be applied to rutinary diagnostics [37]. Other methods such as the estimation of anti-viral antibodies by ELISA to detect rhabdoviral fish carriers (see later) [38, 39] or the estimation of fish cellular memory responses are not yet developed in the laboratory to the point of possible use in routine diagnosis [40]. The use of recombinant highly antigenic fragments of the glycoprotein G of VHSV to increase sensitivity of the detection of trout anti-VHSV antibodies, could be one solution to the problem of detecting fish anti-rhabdoviral antibodies [41-43] (see immunological responses, later).

Molecular structure of fish rhabdoviruses. Like any mammalian rhabdovirus (rabies or VSV), fish rhabdoviruses have a bullet morphological appearance. The fish rhabdoviruses contain one interior nucleocapsid and an exterior envelope made of a lipidic membrane in which the glycoprotein G trimers are embedded. The proteins which form the virus are: L, G, N, M1 (ó P) and M2 (ó M). The protein L (190 KDa) is associated to the

Table I
Fish rhabdoviruses

Virus	fish specie	Temp °C	Cells
VHSV Viral haemorrhagic septicaemia	trout *Oncorhychus mykiss*	6-18	R,F,E,C
IHNV Infectious haematopoietic necrosis	salmon *Oncorhynchus nerka*	13-18	R,F,E,C
SVC Spring viraemia carp	Carp *Cyprinus carpio*	4-32	R,F,E,C
PFRV Pike Fry rhabdo	Tench *Exos lucius*	21-28	R,F,E
EVA Eel Virus A	Eel *Anguilla rostrata*	10-29	R,F,E,B
EVEX Eel Virus Europe X	Eel *Anguilla anguilla*	10-29	R,F,E,B
CUSR Cod ulcus-syndrome rhabdo	Haddock *Gadus morhua*	14-16	PS (R,F,B)
RGPR Rio Grande Perca	Perch *Cichlasoma cyanogutatum*	23-33	R,F,B
EVB Eel Virus B_{12}	Eel *Anguilla anguilla*	10-20	E (R)
RS Rhabdovirus salmonis	trout *Oncorhynchus mykiss*	18-20	R,F,E
PR Perch rhabdovirus	Perch *Perca fluviatilis*	13-15	R (E)
HRV Hirame rhabdovirus	Hirame *Paralichthys olivaceus*	5-20	R,F,E,B (C)
UDRV Ulcerative disease rhabdo	Striped snakehad *Ophicephalus striatus*	25-30	B (C,E)

Susceptible cell lines: R = RTG2. E = EPC. F = FHM. C = CHSE. B = BF2. Resistant cell lines in parenthesis [16].

viral RNA and it has transcriptase and replicase activity. The glycoprotein G (65 KDa) constitutes the trimeric protruding spicula which are responsible for the induction of neutralizing antibodies (Abs). The phosphoprotein N (40 KDa) of the nucleocapsid is the most abundant protein. In VHSV, a Nx antigenically related to the N has been also described with an unknown function [44]. The protein M1 or P (19 KDa) is found associated to the polymerase L. All the proteins N, M1 and L are required for transcription and replication (the nucleocapsid complex). The protein M2 or M can be located either around the lipid membrane or in the interior of the nucleocapsid [45]. In VHSV and IHNV an additional non structural protein of

about 12 KDa has been described, the Nv [46-49]. The Nv has not been found in any mammalian or plant rhabdoviruses.

Molecular biology of fish rhabdoviruses. The molecular biology of fish rhabdoviruses is not as widely known as that of the mammalian rhabdoviruses. However in the last few years, the complete RNA genome (~12 Kb) of IHNV [50-54], important parts of the genomes of VPC or HRV [30] and the complete genome of VHSV [55] have been elucidated. Several incipient phylogenetic studies have been already made comparing partial sequences of a few isolates [47, 50, 51, 53, 56, 57].

The first steps towards the development of infectious cDNA clones of IHNV and VHSV are been taken as it was first reported for rabies and VSV a few years ago [58-60]. Rescue with infectious IHNV of synthetic salmonid rhabdovirus DNA minigenomes [61] have been accomplished and the gen Nv has been knockout from the cDNA of IHNV [62]. The presence of Nv increases the replication of IHNV from its cDNA. Furthermore the recovery of infectious Nv knockout IHNV expressing foreign genes has been accomplished (project EU FAIR CT984398). This work opens a wide avenue for many kinds of manipulation of the fish rhabdoviruses [63]. For instance, multiple point mutant viruses with low probabilities of reversion can now be made to be used as vaccines. Also the phospholipid binding domain of VHSV [64] is now being mutated to produce low virulence strains of VHSV (work in progress). Other possibility is to design rhabdovirus carrying in their genome other G proteins from other rhabdoviruses or viruses or other proteins from pathogens other than viruses. It will now be possible to develop viral vectors which could transcribe but not replicate to increase security. Alternatively viral vectors with new restriction sites to differentiate vaccinated animals from infected animals could be designed.

Early steps of rhabdoviral infections. These studies are needed if we want to know the mechanisms of viral entry and the possibilities of interference with the first steps of the infection to take an advantage of the accessibility of possible drugs through the water (reviewed by Coll) [65]. At the level of the fish organism, little is known about the rhabdovirus entrance tissue. By immunohistochemistry, a few experiments suggest the oesophagus tissue as the main site by which the fish rhabdoviruses go into the fish [66], but these studies are still preliminary and they have to be confirmed.

Very few studies have been made to define the cellular targets of IHNV or VHSV, however it is known that both of these viruses replicate in leukocytes [66-68].

At least one receptor of VHSV in salmonid cells has been identified as species-specific fibronectin by using anti-trout cell Mabs that will inhibit the binding of VHSV to the cells [69].

Respect to the rhabdovirus, it is not yet known what are the regions that interact with the receptor. However, neutralisation resistant Mab mutants have been described for IHNV [70] and for VHSV [50, 71]. In both cases most of the Mab resistant mutants (MAR) showed at least 2 different mutations located far apart in the G sequence (aa 140 and 433 for VHSV), confirming the conformational nature of the binding site which most probably is the site of the G protein that would interact with the receptor. Other MAR mutants map outside this site.

After binding to its receptor, fish rhabdoviruses are internalised and fused with the cellular membranes when the pH is lowered. Fusion defective mutants in VHSV have predicted the position of the fusion domain in two distant segments aa 110-118 and 144-154 maintained together by a disulphide bridge between cysteins 110 and 152 [72]. These results partially confirm the domain predicted by

alignment of many rhabdoviral sequences (aa 141-160) [73].

On the other hand, at least two regions containing hydrophobic heptad repeats were located in the glycoprotein G of rhabdoviruses including fish rhabdoviruses [74]. Interactions of these heptad repeats with phospholipid liposomes have been demonstrated not only in VHSV [75, 76] but also in VSV and rabies [77]. There is increasing evidence that phospholipid-binding regions in the glycoprotein G of the VHSV might be related to fusion after the specific binding of VHSV to fibronectin [69] reviewed by Coll [65]. Thus, the major phospholipid-binding peptide p2 (aa 82-107) contained some hydrophobic heptad repeats [76, 77] and anti-p2 antibodies recognized VHSV only at the pH of fusion [76], inhibited phospholipid-binding to VHSV [75] and inhibited VHSV-induced cell to cell fusion [78]. In addition, the p2 peptide mediates low pH dependent phospholipid aggregation, lipid mixing and leakage of liposome contents [79]. Furthermore, phospholipid-binding to VHSV [75] and fusion induced in glycoprotein G transfected cells [80] showed similar pH dependence profiles. Some mutants affecting the pH treshold for fusion of VHSV mapped adjacent to p2 [72], some fusion defective mutants of vesicular stomatitis virus (VSV) mapped adjacent to its p2-like regions [77, 81, 83] and hydrophobic photolabeling labeled both adjacent and p2-like regions in VSV and rabies [84].

Synthetic peptides corresponding to hydrophobic heptad repeats in other enveloped viruses are inhibitory [85] and it is possible that something similar could occur in fish rhabdoviruses [65, 86]. However, in vitro enhancement of infectivity at low pH was shown by frg#11 (aa 56-113) from VHSV (frg#11 contains the p2 peptide). Frg#11 mediated low pH dependent phospholipid vesicles aggregation with a 5-fold higher specific activity than its p2 peptide component, induced spreading and fusion in uninfected cells and it showed pH dependent β conformational changes. Although it is not yet clear what these results mean in terms of understanding the viral infection early steps, the functional and structural data mentioned above suggests that a β-sheet conformation of frg#11 interacts with phospholipid membranes (viral and/or cellular) to enhance VHSV infectivity [64].

If frg#11 were part of the fusion peptide of VHSV, an all β-conformation could show an U structure with an estimated length of 41-46 Å according to computer modelling [86]. The bending of the all β-conformation would thus explain how frg#11 could penetrate a membrane phospholipid bilayer without any cleavage of the glycoprotein sequence. Frg#11 would insert into the membrane phospholipids by the p2 sequence [79], leaving the rest of the molecule in the outside. Further work by using mutations of the G incorporated into whole virus (by using reverse genetics) should clarify if the mechanisms implicated in the frg#11 in vitro enhancement effect are also relevant during the VHSV early steps of the infection cycle.

Immunological responses to the rhabdovirus infections. Fish have IgM but not IgG, have a hystocompatibility system not yet well characterized and have B and T lymphocytes. However, the actual state of knowledge of fish immunology is still in its beginnings. Both antibody and cellular responses have been studied to help improvement of both viral detection and vaccination methods.

The difficulty to detect anti-rhabdoviral Abs in fish is due to the low affinity of these Abs because of its IgM nature [87]. The traditional assays based on in vitro virus neutralisation only detect neutralizing antibodies. However, only 50% of VHSV survivor trout will have detectable titres of neutralizing Abs by using these methods [36, 42, 88, 89]. Because fish rhabdoviral neutralizing Abs only recognise conformational epitopes, it is possible that the

rest of the fish Ab response to lineal epitopes would remain undetectable by the neutralization assays [38]. Thus, capture ELISA for anti-VHSV [89, 90], for anti-SCV [91], for anti-IHNV [92] and even for birnavirus IPNV [93] have been described. However they all have low sensibility. Present efforts are based upon the use of recombinant small fragments of the G to increase the epitopic density on the solid-phase (work on progress).

Other efforts have been concentrated in mapping the antigenicity of different rhabdoviral proteins, specially of the glycoprotein G. Mapping of the G of IHNV antigenicity by using recombinant fragments [10, 94-96] and of the G of VHSV antigenicity by using pepscan peptides [39] have been reported.

The in vitro immunoproliferative responses of lymphocytes from trout survivors of VHSV to inactivated rhabdovirus [97], to purified rhabdoviral proteins [98-100], to recombinant fragments of the G of VHSV expressed in E.coli, Y.ruckeri (a pathogen bacteria to trout) and S.cerevisiae [101] and to a pepscan of 50 peptides from the G of VHSV [102, 103] have been reported. These studies have found that each individual trout recognises a different domain of the G. Result that could be expected because it was obtained from a heterogeneous fish population. Confirming the immunological nature of these responses, it has been possible to isolate the first antigen dependent cells from any fish in trout. Using a recombinant fragment of the G of VHSV as antigen and homologous trout adherant cells

Prevention methods to rhabdovirosis which are being developed. Due to the lack of vaccines [10, 107], the fight against fish rhabdoviruses is actually reduced to periodic controls and desinfection of the rhabdovirus-positive farms [12].

During the last 20 years, several European and american laboratories have studied the possibility of fish immunisation to VHSV and IHNV. Attenuated rhabdoviral vaccines protected but could not be used in the field because of possible reversion. Whereas, inactivated vaccines only by injection would confer some protection. On the other hand, attempts to develop trout strains resistant to VHSV had a limited success [108]. There have also been some reports about the viability of passive immunisation using trout antibodies [109] or DNA injection of neutralizing mouse antibody genes [110] or even describing immunity transfer to IHNV from mother to sons through the eggs [111 1205]. However all these methods and their results should be further studied and/or confirmed. That is why the actual efforts are centred in recombinant subunit vaccines, DNA-vaccines or new viral vectors.

Infection of purified G proteins from IHNV or VHSV [112] will confer some degree of protection against lethal dosages of the viruses [23]. Furthermore neutralizing Mabs against IHNV [113] and against VHSV [40, 114] recognized epitopes localised in the G [41, 115]. Most probably due to the conformational nature of the epitopes as demonstrated with the mapping of MAR mutants [70, 71] there have been only 3 neutralising Mabs anti VHSV described in Denmark [41, 88], France [116] and Spain [40, 114]. Similarly

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