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Susceptibility of juvenile sole *Solea senegalensis* to marine isolates of viral haemorrhagic septicaemia virus from wild and farmed fish

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ABSTRACT: The susceptibility of sole *Solea senegalensis* to infection with 3 viral haemorrhagic septicaemia virus (VHSV) strains obtained from wild Greenland halibut *Reinhardtius hippoglossoides* and farmed turbot *Psetta maxima* was demonstrated. Fish were infected by an intraperitoneal (i.p.), immersion or cohabitational route, and maintained at 16°C. Infection trials showed that VHSV isolates were pathogenic for sole fingerlings by i.p. injection and waterborne exposure causing moderate levels of mortality (10 to 55%). In addition, the mortality observed in fish cohabitating with i.p.-infected sole confirms horizontal transmission of the virus. However, the low rates of mortality registered in this challenge suggest that there is a low dissemination of virus by the i.p.-infected sole, which results in lower secondary challenge of the cohabitating fish. External signs of disease included haemorrhaging of the ventral area and ascitic fluid in the body cavity. Dead fish were tested for VHSV by both cell culture and RT-PCR assay, using pools of kidney and spleen from 10 individuals. Virus was recovered from most of the pools composed of dead fish. The results obtained in this study not only demonstrate the susceptibility of sole to the VHSV strains employed but also indicate that wild VHSV marine isolates represent a potential risk for sole aquaculture.

KEY WORDS: Viral haemorrhagic septicaemia virus · VHSV · Susceptibility · Sole · Greenland halibut · Turbot

INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a well-known disease of farmed rainbow trout *Oncorhynchus mykiss*, caused by a member of the family *Rhabdoviridae,* genus *Novirhabdovirus*. However, in recent years, the occurrence of VHS virus (VHSV) in wild marine fish has become increasingly evident and has led to the conclusion that VHSV is enzootic in the marine environment (Skall et al. 2005). In addition, disease outbreaks in cultured turbot have been reported (Ross et al. 1994), and a genetic link has been established between the isolates responsible for those epizootics and VHSV isolates recovered from wild fish caught in the North Sea (Stone et al. 1997, Snow et al. 1999, 2004). Therefore, the existence of marine reservoirs of VHSV represents a potential risk for the marine fish farming industry in Europe and highlights the importance of establishing the susceptibility to marine VHSV strains of any candidate fish species for aquaculture.

Since the 1990s, there has been an increasing commercial interest focused on sole (both Senegalese sole *Solea senegalensis* and common sole *S. solea*) production due to its high market potential. Most of the technical problems regarding sole rearing are being solved, but disease problems continue to threaten the commercialisation of these species. Although viral diseases have not yet achieved the prominence of bacterial diseases, some viral agents such as aquabirnaviruses and betanodaviruses have been detected or associated with disease outbreaks in both species (Hill 1982, Rodriguez et al. 1997, Starkey et al. 2001, Thiéry et al. 2004, Cutrín et al. 2007, Olveira et al. 2009). In

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addition, lymphocystis virus (Alonso et al. 2005) has been isolated from cultured Senegalese sole. All of these isolations and/or detections point out the need to investigate the susceptibility of this fish species to other viral agents, especially if the potential pathogens are enzootic in European waters, as is VHSV (Skall et al. 2005).

We have previously reported high levels of mortality in experimentally infected turbot using 2 VHSV strains obtained from wild Greenland halibut *Reinhardtius hippoglossoides* and farmed turbot *Psetta maxima*, both grouped in Genotype III (López-Vázquez et al. 2007). The aim of the present study was to investigate the susceptibility of Senegalese sole to those 2 strains together with another strain also obtained from wild Greenland halibut using intraperitoneal (i.p.), immersion and cohabitational routes.

MATERIALS AND METHODS

Fish. Senegalese sole (average weight 20 g) were obtained from the Instituto Español de Oceanografía (IEO) in Vigo, Spain. Fish were acclimatised for 15 d after arrival at the aquarium of the Universidad de Santiago de Compostela and maintained at 16°C. Prior to challenge, 20 fish were examined for the presence of infectious pancreatic necrosis virus (IPNV), betanodaviruses and VHSV by inoculation in cell culture and RT-PCR.

Cell culture and virus. The epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983) was used to propagate the virus. The cell line was grown at 25°C in minimum essential medium (MEM) plus 10% foetal bovine serum (EMEM-10), 100 IU ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. For virus production, the serum content of the medium was reduced to 2% and the temperature to 15°C. The 3 strains used in the present study belonged to Genotype III (López-Vázquez et al. 2006a); 2 of them (GH40 and GH44) were obtained from apparently healthy Greenland halibut caught in the Flemish Cap (Newfoundland) (Dopazo et al. 2002), and the other strain (SM2897) was isolated from farmed turbot showing ulcerative lesions in the skin (López-Vázquez et al. 2007) The 3 viral strains were thawed before the challenge and were not subjected to more than 2 to 3 passages in cell culture.

Experimental infection. ***Intra-peritoneal and cohabitation infection:*** Sole were held in 125 l aquaria and stocked at a density of 40 fish per tank in each of 6 tanks (A1, A2, B1, B2, C1 and C2). Fish from Tanks A1 and A2 were inoculated with strain GH40, those from Tanks B1 and B2 with strain GH44, and those from Tanks C1 and C2 with strain SM2897, in order to obtain 2 replicates of each experimental inoculation. From each tank, 20 fish were removed and injected i.p. with an inoculum volume of 100 µl representing a dose of 10^5 $TCID_{50}$ per fish. These injected fish were marked by clipping the caudal fin, and returned to their original tank to cohabit with the remaining 20 fish. A control group of 10 fish was injected with 100 µl of MEM with no virus.

Infection by immersion: Sole were distributed in 6 tanks (20 fish each, 2 tanks per strain, Tanks D1, D2, E1, E2, F1 and F2), as indicated above, and exposed to the 3 different viruses at a concentration of 10^5 $TCID_{50}$ ml^{-1} in a total volume of 5 l for 3 h. As negative controls, 1 tank containing 10 fish was also set up using MEM containing no virus.

Both experiments were terminated after 60 d. Mortality was recorded, and tissue samples (spleen and kidney) from both dead fish and those surviving at the end of the challenge were examined for the presence of virus.

Virological examination. ***Virus isolation:*** Tissue samples (spleen and kidney) were processed in pools of 10 fish. Samples corresponding to each dead fish were included in the pool following the sequence of deaths. When the number of dead fish was insufficient, the pool was completed up to 10 with tissue samples from fish sacrificed at the end of the experiment. Samples were homogenised as previously described (López-Vázquez et al. 2007) and inoculated onto a monolayer of EPC cells (100 µl per well) cultured in 24-well plates. The plates were incubated at 15°C and monitored for cytopathic effect (CPE). After 7 d, positive and negative monolayers (cultures showing CPE or no CPE, respectively) were subjected to a second or blind passage by inoculating 0.1 ml of the scraped cell suspensions onto new cultures and monitored for CPE for a further 7 d. Identity of viral isolates was confirmed by RT-PCR.

RT-PCR detection: Total RNA was extracted using Trizol® LS Reagent (Life Technologies), according to the manufacturer's instructions. The primer pair used for RT-PCR was *cm3* (López-Vázquez et al. 2006b), developed to amplify a 358 nt sequence partially corresponding to the initial sequences of the nucleoprotein (N) coding region of VHSV. Complementary DNA (cDNA) synthesis and amplification rounds were carried out using SuperScript™ III Platinum® One-Step Quantitative RT-PCR System Kit / SuperScript™ One-Step RT-PCR (Life Technologies) as previously described (López-Vázquez et al. 2007). PCR products were analysed by electrophoresis on a 2% agarose gel.

Statistical analysis: Chi-squared tests of independence implemented in Statgraphics® Centurion were performed in order to test for significant differences between routes of infection and replicates within challenges.

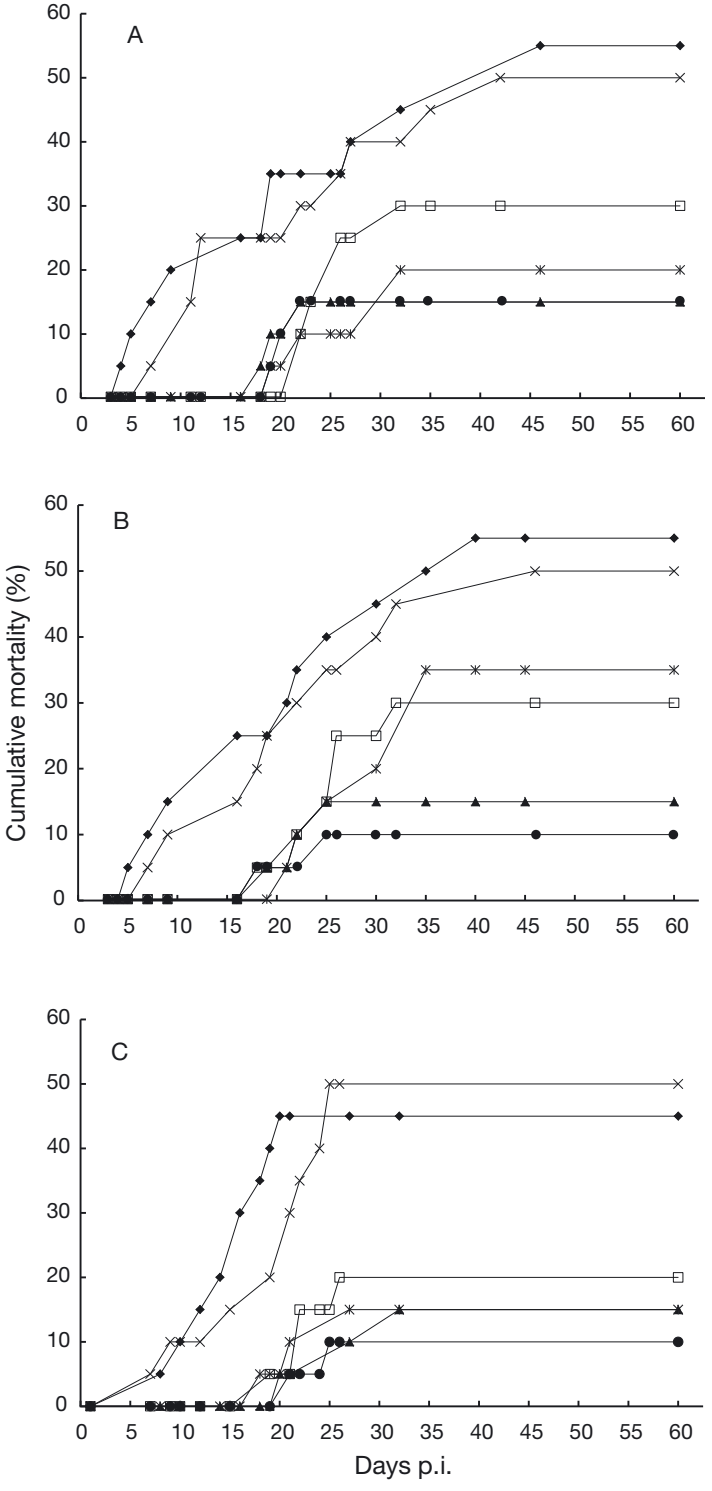

Fig. 1. *Solea senegalensis.* Mortality curves for Senegalese sole experimentally infected with viral haemorrhagic septicaemia virus marine isolates by intra-peritoneal (i.p.) injection (Tank 1, –◆–; Tank 2, –×–), cohabitation (Tank 1, –▲–; Tank 2, –●–) and immersion (Tank 1, –✳–; Tank 2, –□–) using an inoculum dose of 10^5 $TCID_{50}$ $fish^{-1}$ (i.p.) or 10^5 $TCID_{50}$ ml^{-1} (immersion) at 15°C. (A) GH40 (B) GH44 (C) SM2897

RESULTS

Sole were confirmed to be free from VHSV, IPNV and nodaviruses prior to infection. No mortalities were observed in the non-challenged fish, and no virus was detected in these control fish at the end of the trials.

Cumulative mortalities and mortality kinetics of i.p., cohabitation and immersion-challenged sole are shown in Fig. 1 and Table 1.

Table 1. *Solea senegalensis.* Summary of results obtained from intra-peritoneal (i.p.), cohabitational (Coh) and immersion (Imm) challenges of sole with 3 isolates (GH40, GH44, SM2897) of viral haemorrhagic septicaemia virus obtained from farmed and wild marine fish. Route: route of infection; dpi: days post-infection; %: percentage cumulative mortality; no. dead fish: number of dead fish in each pool of 10 fish; RT-PCR: detection by RT-PCR and visualisation of amplification products under UV light; CC: development of cytopathic effect in epithelioma papulosum cyprini (EPC) cells

Route	Tank	Dpi	%	Pool no.	No. dead fish	RT-PCR	CC
GH40							
i.p.	A1	4–46	55	1	10	+	+
				2	1	+	–
	A2	7–42	50	1	10	+	+
				2	0	+	–
Coh	A1	18–22	15	1	3	+	+
				2	0	–	–
	A2	19–22	15	1	3	+	+
				2	0	–	–
Imm	D1	19–32	20	1	4	+	+
				2	0	–	–
	D2	22–32	30	1	6	+	+
				2	0	–	–
GH44							
i.p.	B1	5–40	55	1	10	+	+
				2	1	+	–
	B2	7–46	50	1	10	+	+
				2	0	+	–
Coh	B1	19–25	15	1	3	+	+
				2	0	–	–
	B2	18–25	10	1	2	+	–
				2	0	–	–
						–	–
Imm	E1	21–35	35	1	7	+	+
				2	0	+	–
	E2	18–32	30	1	6	+	+
				2	0	–	–
SM2897							
i.p.	C1	8–20	45	1	9	+	+
				2	0	+	–
	C2	7–25	50	1	10	+	+
				2	0	+	–
Coh	C1	20–32	15	1	3	+	+
				2	0	–	–
	C2	21–25	10	1	2	+	–
				2	0	–	–
Imm	F1	18–27	15	1	3	+	+
				2	0	–	–
	F2	19–26	20	1	4	+	+
				2	0	–	–

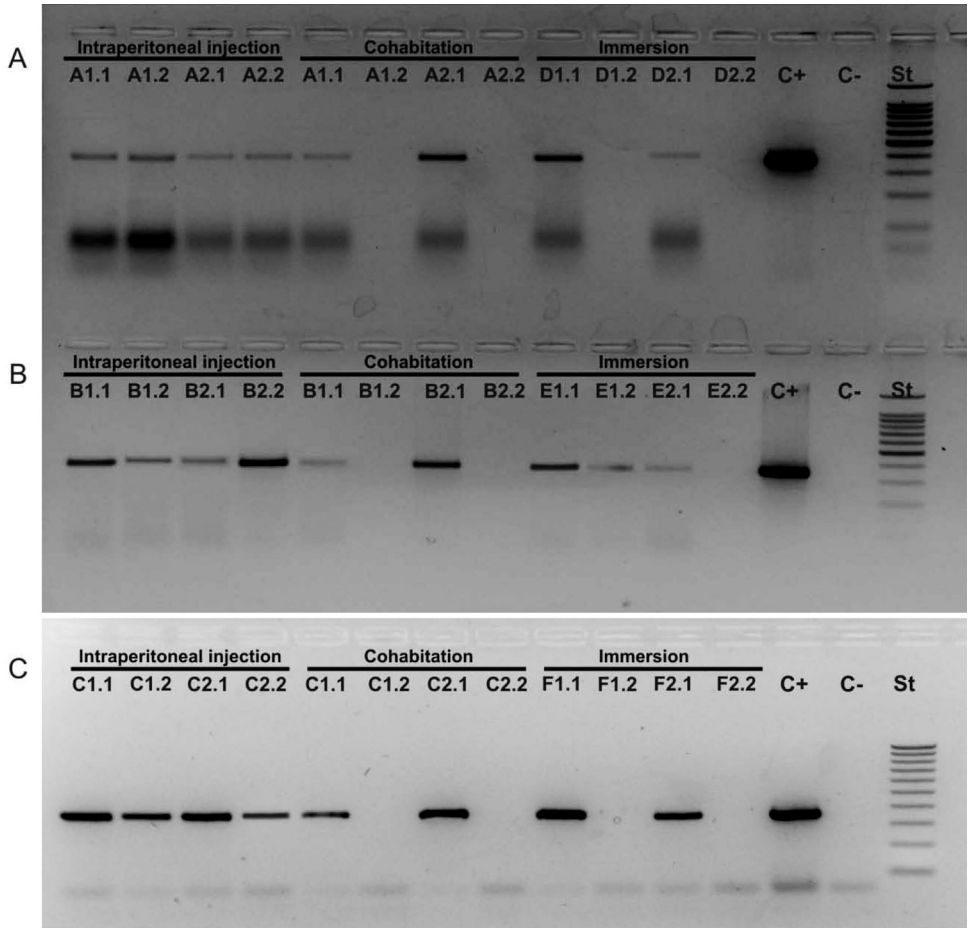

Fig. 2. *Solea senegalensis.* RT-PCR detection of viral haemorrhagic septicaemia virus (VHSV) in experimentally infected Senegalese sole. Lane names correspond to tank and pool number as indicated in Table 1. RT-PCR amplification products (10 µl) were analysed on an agarose gel stained with ethidium bromide. (A) GH 40, (B) GH 44, (C) SM2897. C+: RT-PCR applied to RNA from VHSV strain UK860/94; C–: RNase-free water. In (A) and (B), St: bench-top 100 bp DNA ladder (Promega) with 11 fragments ranging from 100 to 1000 bp in 100 bp increments, with an additional band of 1500 bp. In (C), St: Simply Load® 100 bp DNA ladder (Lonza) with 10 fragments ranging from 100 to 1000 bp in 100 bp increments

Intra-peritoneal and cohabitation infection

Mortalities were first recorded 4 to 5 d post infection (dpi) in the fish injected with both Greenland halibut strains and were registered up to 42 to 46 dpi. However, in the fish injected with the turbot strain, mortalities were slightly delayed (earliest death at 8 dpi) and concentrated in a shortened period of time (up to 25 dpi). Cumulative mortalities were about 50% in all experiments (2 replicates per strain). No significant differences were observed between replicates ($p > 0.05$).

In the cohabitation experiment, mortalities (10 to 15%) were much lower than in the i.p.-infected fish and were recorded between Days 18 and 25 of cohabitation with the i.p.-infected fish in the case of the fish infected with the Greenland halibut strains and from Days 20 to 32 in fish infected with the turbot strain.

The lesions exhibited by dead fish collected in the i.p.-infected groups coincided with the reported pathology for VHS and included reddened foci (0.5 mm in diameter) in the ventral skin and/or accumulation of ascitic fluid in the body cavity. These lesions were more frequent in the fish infected with Greenland halibut strains, especially GH40 (60% of the dead fish with lesions, results not shown). The lowest percentage was observed in the fish inoculated with strain SM2897 (10% of the fish with lesions).

Most of the dead fish cohabitating with sole infected with both Greenland halibut strains showed the typical signs of VHS. However, in the case of the fish cohabitating with sole challenged with the turbot strain, 5 dead fish were collected, and although VHSV was recovered in cell culture, none of the fish showed macroscopic lesions.

Results from PCR detection and cell culture isolation are detailed in Table 1. In the i.p.-infected groups, corresponding to the highest percentages of mortalities, re-isolation of the virus was effective from pools composed mainly of dead fish. Nevertheless, the virus was detected by RT-PCR in all pools, even in those exclusively composed of fish sacrificed at the end of the experiment. In the cohabitating sole, PCR detection was positive in all pools containing dead fish, and re-isolation of the virus was attained only from those pools containing at least 3 dead fish (Fig. 2). All pools were tested for the presence of IPNV and nodaviruses by PCR and were negative (results not shown).

Infection by immersion

Mortalities were detected between 18 and 35 dpi, reaching 20 to 35% in the fish challenged with halibut strains GH44 and GH40 and 15 to 20% in the fish challenged with the turbot strain SM2897 (Fig. 1). This difference was statistically significant ($p = 0.001$). Most of the dead fish showed external and internal haemorrhages. Both RT-PCR detection and viral isolation were achieved only from pools containing dead fish, with the exception of 1 pool containing 10 fish that had survived challenge with strain GH44 and were positive by RT-PCR (Table 1, Fig. 2). As above, all pools were tested by PCR and considered negative for the presence of IPNV and nodaviruses (results not shown).

DISCUSSION

Infection trials showed that the 3 marine VHSV isolates tested were pathogenic for Senegalese sole fingerlings by i.p. injection with cumulative mortalities of 50%. Sole were also challenged via cohabitation and immersion routes that better reflect natural VHSV infection, which is thought to occur by the horizontal transmission of waterborne virus (Wolf 1988). The mortalities observed using these latter infection methods confirm that these VHSV strains are able to infect sole via waterborne exposure as we had previously reported in experimental infections performed with turbot (López-Vázquez et al. 2007). However, the mortalities decreased considerably, suggesting that non-specific immune mechanisms at epidermal and epithelial surfaces of the fish may be involved in the protection of the sole against VHSV. In fact, epidermal tissues have been reported to exert an important role in facilitating or denying the entry of virus in susceptible or resistant fish, respectively (Yamamoto et al. 1990, Dorson & Torhy 1993). Another explanation could be the low level of virus in the water used for infection/challenge. However, the dose we used is high enough and has commonly been used in VHSV challenges (King et al. 2001, Brudeseth et al. 2002, Snow et al. 2005). It could be also argued that the level of virus in the water could have dropped drastically during the 3 h of the challenge, although Kocan et al. (2001) reported that only 50% of inactivation of VHSV was reached in sea water after 10 h at 15°C.

Although the 3 isolates tested resulted in low to moderate mortalities in sole in the immersion challenge, there was a significant difference between the virulence of the Greenland halibut strains, especially GH44 (32.5%) and the turbot strain SM2897 (17.5%). It has been reported that the virulence of VHSV for turbot is genotype specific (Snow et al. 2005), but all VHSV isolates used in this study belonged to Genotype III, which is one of the most virulent genotypes for turbot. The virulence for turbot has also been related to the geographic distribution of the isolates rather than the host species from which they were recovered (King et al. 2001, Snow et al. 2005). This could also be true for Senegalese sole. The 2 GH isolates which were obtained from fish caught in the western Atlantic were more virulent for sole, which is distributed along the eastern Atlantic and in a small area of the Mediterranean, than the SM2897 strain, which was isolated from a turbot in Spain. However, a higher number of isolates will be needed to determine a possible relationship between geographic origin of the VHSV isolates and virulence in sole.

On the other hand, there was also a difference in the mortality between cohabitation and immersion challenges, suggesting that additional factors such as the level of viral shedding by i.p.-infected sole may result in a lower water concentration of virus compared with immersion experiments.

The passage of the VHSV strains through i.p.-injected sole did not appear to increase the virulence for this species of any of the VHSV strains assayed. This finding is in contrast to our previous observations, which indicated an increase in the virulence of one of the VHSV strains used in the present study (GH40) after i.p. injection in turbot (López-Vázquez et al. 2007).

VHSV was detected by RT-PCR from pools containing dead fish and even from all of the pools exclusively composed of surviving sole infected by i.p. and one pool infected by immersion. This may indicate that in these surviving fish a persistent infection has been established, and if so a carrier state may represent a risk of infection to other fish in the same population. Alternatively, the PCR may have simply detected residual inocula in the i.p.-infected fish.

Viral recovery in cell culture was attained only from pools containing at least 3 dead fish. These results con-

firm the lower sensitivity of the viral isolation in cell culture when compared with RT-PCR, as previously reported for this and other viruses (Devold et al. 2000, López-Vázquez et al. 2007)

The present study has demonstrated the susceptibility of sole fingerlings to infection with marine VHSV strains. Although moderate to low mortalities were obtained by immersion or cohabitation challenges, the selection pressures associated with intensive aquaculture together with the adaptability of RNA viruses highlight that VHSV marine isolates can become a real threat for sole farming.

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