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Experimental infection models and susceptibility of Atlantic salmon *Salmo salar* to a Scottish isolate of infectious salmon anaemia virus

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ABSTRACT: Infection models for both fresh and seawater salmon were established using a Scottish isolate of infectious salmon anaemia virus (ISAV). Modes of infection were intra-peritoneal injection, cohabitation and immersion exposure, and a range of doses was tested. Development of these models using a Scottish isolate of ISAV provided an approximation of the minimum infective dose leading to mortality under different infection regimens. The models also allow prediction of the time to first mortality and an estimation of expected total mortality following the various routes of infection. Such knowledge is important to the development of anti-ISAV vaccines and to future studies aimed at understanding the biology of ISAV in general.

KEY WORDS: Infectious salmon anaemia virus · Atlantic salmon · *Salmo salar* · Experimental infection

INTRODUCTION

Infectious salmon anaemia (ISA) is a serious disease of Atlantic salmon that is responsible for significant losses to the Atlantic salmon farming industry. Although epizootics have occurred in Norway since 1984, the disease has only recently been diagnosed in Canada (Mullins et al. 1998, Bouchard et al. 1999, Lovely et al. 1999), Scotland (Rodger et al. 1998, Stagg et al. 1999, Turnbull 1999), Chile (Kibenge et al. 2001), Faroes (Anonymous 2000) and the USA (Bouchard et al. 2001). Typical pathological changes are severe anaemia, leucopenia, ascites and haemorrhagic liver necrosis (Thorud & Djupvik 1988, Evensen et al. 1991). The aetiological agent has been identified as an orthomyxovirus-like enveloped virus with an RNA genome comprising 8 RNA segments (Mjaaland et al. 1997).

The development of standardised infection models is a fundamental requirement for research using experimental transmission of important fish pathogens such as ISA virus (ISAV). Of particular importance is the establishment of models that mimic natural routes of experimental infection; natural infection with ISAV is thought to occur largely by passive waterborne transmission of the virus in seawater (Vågsholm et al. 1993). The development of such infection models for ISAV is important to a number of areas of research including determination of the efficacy of experimental vaccines, identification of species susceptible to ISAV and identification of potential ISAV carrier fish species. Such models may also facilitate investigation into the progression of pathology following exposure to natural levels of ISAV and identification of organs important for virus replication and persistence. These findings might also contribute to the development of improved diagnostic methods to assist in surveillance programmes.

The aim of the current study was to develop comprehensive infection models to form the basis for future research into the Scottish isolate of ISAV. Infection studies were conducted using immersion infection, intra-peritoneal infection (i.p.) and cohabitation infection routes in both salmon parr in freshwater and in salmon post-smolts held in seawater.

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MATERIALS AND METHODS

Cell culture and virus propagation. The isolate of ISAV used in this study was 0390/98, which originated from a clinical outbreak of ISAV on a salmon farm in west Scotland. The salmon head kidney-1 (SHK-1) cell line was used to propagate virus for transmission experiments according to previously described methods (Dannevig et al. 1995). Harvested virus was stored at –80°C following 2 passages in SHK-1 cells. A single aliquot of virus was titrated using the tissue culture infectious dose method ($TCID_{50}$) (Reed & Muench 1938, Burleson et al. 1992) following a single freeze-thaw cycle.

Pathogen-free fish. Atlantic salmon parr (mean weight 27.5 g) and Atlantic salmon post-smolts (mean weight 273 g) were reared at the FRS Marine Research Unit, Aultbea, Ross-shire, Scotland. Before the infection experiments fish were screened for the presence of ISAV, viral haemorrhagic septicaemia virus, infectious haematopoietic necrosis virus and infectious pancreatic necrosis virus by standard virology tissue culture methods as previously described (Dannevig et al. 1995, Snow & Smail 1999) and, for ISAV, by reverse transcription polymerase chain reaction (RT-PCR) (Mjaaland et al. 1997).

Experimental infection with ISAV. Fish were allowed to acclimate for 7 d and starved for 24 h before all experimental infections. Water temperatures were maintained at 10°C for the duration of each experimental infection. Freshwater was supplied from Aberdeen city drinking water, which originates from the River Dee and passes through an activated carbon filter and UV treatment before entering fish tanks. Seawater was extracted from the North Sea at a salinity of 32‰ and passed through a sand filter and UV disinfection treatment before entering fish tanks.

Experimental infection of freshwater Atlantic salmon parr with ISAV. Atlantic salmon parr were stocked in 30 l aquaria supplied with flow rates of 40 l h^{-1} freshwater, at a density of 15 fish per tank. Experimental infections were obtained via immersion, i.p. and cohabitation routes. Fish were anaethetised before i.p. infection by immersion in methane tricaine sulphonate (3-aminobenzoic acid ethyl ester of MS-222; Sigma) at a concentration of 100 mg l^{-1}.

Immersion infection. Groups of 15 Atlantic salmon parr held in duplicate tanks were exposed to virus at concentrations of 2.5×10^5, 2.5×10^4, 2.5×10^3, 2.5×10^2 and 2.5×10^1 $TCID_{50}$ ml^{-1} in a total volume of 10 l for 4 h. An equal volume inoculum of Leibovitz (L-15) medium containing no virus was added to 2 further tanks of 15 fish, which served as control groups. After 4 h, flow was restored to all tanks at 40 l h^{-1}, and the volume of water in the tanks was allowed to return to normal (30 l).

Intra-peritoneal and cohabitation infection. Groups of 15 Atlantic salmon parr held in duplicate tanks were anaesthetised and injected i.p. with virus in an inoculum volume of 100 µl. Fish in replicate tanks were infected with doses of 2.5×10^7, 2.5×10^6, 2.5×10^5, 2.5×10^4, 2.5×10^3 and 2.5×10^2 $TCID_{50}$ $fish^{-1}$. A control group, set up in duplicate, consisted of groups of 15 fish injected with 100 µl of L-15 medium containing no virus. Before being returned to their respective tanks, all i.p infected fish were marked for future identification by clipping of the adipose fin. After 48 h, a further 15 salmon parr were added to each tank containing i.p infected and control fish to serve as cohabitees.

Fish infected via all 3 routes were observed, and dead fish were removed and sampled for the duration of the experimental infection period (93 d).

Experimental infection of seawater Atlantic salmon with ISAV. Atlantic salmon post-smolts were stocked in 360 l aquaria supplied with flow rates of 150 l h^{-1} seawater, at a density of 11 fish per tank. Experimental infections were obtained via i.p. and cohabitation routes.

Intra-peritoneal and cohabitation challenge. Groups of 11 Atlantic salmon held in replicate tanks were anaesthetised and injected i.p. with an inoculum volume of 200 µl. Fish in replicate tanks were infected with doses of 5×10^7, 5×10^5, 5×10^4, 5×10^3, 5×10^2 and 5×10^1 $TCID_{50}$ $fish^{-1}$. Duplicate control groups consisting of groups of 11 fish were injected with 200 µl of L-15 medium containing no virus. Before being returned to their respective tanks, all i.p. infected fish were marked ventrally near the pelvic fin for future identification using a panjet. After 48 h, a further 11 salmon were added to each control tank and each tank containing i.p. infected fish to serve as cohabitees. Fish were monitored and dead fish were removed and examined daily for the duration of the experimental infection period (96 d).

Virus identification in mortalities. The minimum sample size from the salmon parr mortalities required to be tested from each tank to determine, with 95% confidence, the true proportion of deaths due to ISAV, with a maximum error of ±0.1, was calculated (Thompson 1992). All fish within the sample were tested for ISAV by RT-PCR (Mjaaland et al. 1997). All seawater salmon mortalities, being fewer than for salmon parr, were tested for the presence of ISAV by RT-PCR.

Statistical analysis. Generalised linear models were fitted to the mortality data, assuming a binomial distribution, to test for significant differences between treatment doses. Significant differences are described in the text together with probability values.

RESULTS

Virus identification in mortalities

Control fish

Thirteen control salmon parr died during the study at Days 60 and 90, all of which were cohabiting fish from a single tank. Ten seawater fish also died in the control groups. No ISAV was detected by RT-PCR from any of these control fish. A single death in the seawater fish was attributable to procedural stress 3 d after injection, with a further 7 attributable to an interruption of the water supply to a single tank on one day.

Infected fish

Three hundred and forty-four parr died in the infected groups with only 2 out of 310 (number required for 95% confidence) being negative when tested for ISAV by RT-PCR. The PCR method is a more sensitive method than tissue culture for diagnosing ISAV (Devold et al. 2000, Mikalsen et al. 2001, this issue) and, therefore, was chosen for confirmation of whether mortality could be attributed to ISA. The deaths of the 2 fish that tested negative by RT-PCR could not be attributed to ISAV and were not included in further analysis.

Two hundred and nine seawater salmon mortalities were tested by RT-PCR with 43 testing negative. These negative fish were also not included in subsequent analyses.

Experimental infection of freshwater Atlantic salmon parr with ISAV

Immersion infection

Average cumulative percentage mortalities of 80% were recorded for viral doses ranging from 2.5×10^3 to 2.5×10^5 $TCID_{50}$ ml^{-1} (Table 1). No ISAV-specific mortality occurred with the lowest infection dose (2.5×10^1 $TCID_{50}$ ml^{-1}) or in either of the control tanks included in this study (data not shown). Following exposure to a dose of 2.5×10^2 $TCID_{50}$ ml^{-1}, mortalities recorded in individual tank replicates were 0 and 80% respectively. The pattern of mortality in the latter tank replicate was consistent with the establishment of a secondary infection resulting from a single death caused by the initial viral exposure (data presented in Fig. 1).

Table 1. Time to first mortality and final cumulative mortality in salmon parr infected with ISAV by the immersion route. No ISAV-specific mortality was recorded in control tanks. Estimated 70% lethal dose (LD_{70}) is $>2.5 \times 10^2 < 2.5 \times 10^3$ $TCID_{50}$ ml^{-1}. Numbers of fish shown in brackets

Dose ($TCID_{50}$ ml^{-1})	Time to first mortality (d)			Final cumulative % mortality		
	Replicate 1	Replicate 2	Mean	Replicate 1	Replicate 2	Mean
2.5×10^1				0	0	0
2.5×10^2		26	26[a]	0	80 (12)	80[a]
2.5×10^3	25	20	22.5	60 (9)	87 (13)	73.5
2.5×10^4	15	18	16.5	80 (12)	73 (11)	77
2.5×10^5	18	15	16.5	100 (15)	80 (12)	90

[a]Using replicate with mortalities

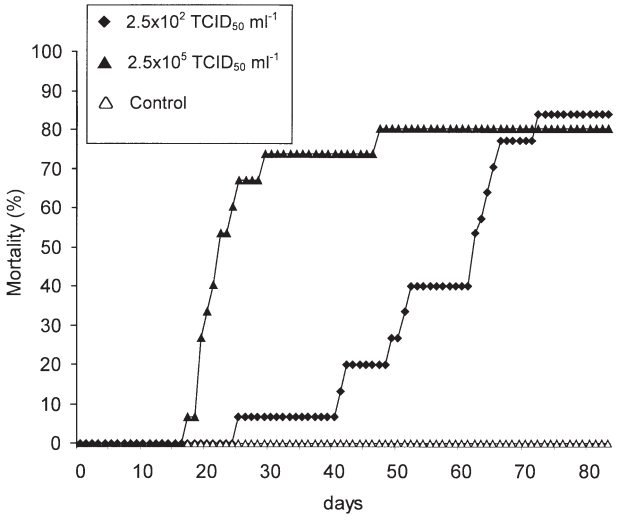

Fig. 1. Cumulative mortality for salmon parr infected with infectious salmon anaemia virus (ISAV) by the immersion route at doses of 2.5×10^2 $TCID_{50}$ ml^{-1} and 2.5×10^5 $TCID_{50}$ ml^{-1}. The pattern of mortality due to the lower dose is consistent with secondary cohabitation effects compared with the higher rate of mortality due to the higher dose

Average time to first mortality ranged from 26 d with the lowest dose causing mortality (2.5×10^2 $TCID_{50}$ ml^{-1}) to 16.5 d with the highest doses (2.5×10^4 and 2.5×10^5 $TCID_{50}$ ml^{-1}) (Table 1).

Intra-peritoneal and cohabitation infection

Average cumulative percentage mortalities of over 85% were recorded for all viral doses administered by i.p. injection (Table 2). The maximum level of mortality achieved in individual tank replicates was 100% (doses 2.5×10^5 and 2.5×10^7 $TCID_{50}$ $fish^{-1}$).

Final mean cumulative percentage mortalities in cohabiting fish were lower than those recorded in i.p. infected fish in all cases, and ranged from 46.7 to 80%.

Mean time to first mortality in i.p. infected fish ranged from 13 d at the highest dose (2.5×10^7 $TCID_{50}$ $fish^{-1}$) to 18.5 d at the lowest dose (2.5×10^2 $TCID_{50}$ $fish^{-1}$) (Table 2), with these 2 mean times being significantly different ($p < 0.05$). In cohabiting fish, the first mortalities occurred 14 to 23 d following the first mortality in i.p. infected fish with the mean time of first mortality of the i.p. inoculated fish ranging between 30 and 37 d post-infection (Table 2).

Experimental infection of seawater Atlantic salmon with ISAV

Intra-peritoneal and cohabitation infection

Following i.p. infection, mean percentage cumulative mortality reached 86.4% with doses of 5×10^5 and 5×10^7 $TCID_{50}$ $fish^{-1}$ (Table 3). Mean cumulative percentage mortality increased (not significant, $p > 0.05$) between the lowest dose of 5×10^1 $TCID_{50}$ $fish^{-1}$ and 5×10^4 $TCID_{50}$ $fish^{-1}$ from 54.5 to 81.8%, respectively.

Similarly, mortality in cohabiting fish increased with the dose administered to the i.p. fish within the dose range 5×10^1 $TCID_{50}$ $fish^{-1}$ to 5×10^4 $TCID_{50}$ $fish^{-1}$ (Table 3). Above these doses cumulative percentage mortalities in cohabiting fish decreased (63.6% at 5×10^5 $TCID_{50}$ $fish^{-1}$ and 40.9% at 5×10^7 $TCID_{50}$ $fish^{-1}$).

Time to first mortality in i.p. infected fish ranged from a mean of 17 d at the highest dose (5×10^7 $TCID_{50}$ $fish^{-1}$) to 28 d in the tank with mortalities at the lowest dose (5×10^1 $TCID_{50}$ $fish^{-1}$) (Table 3). In cohabiting fish, the first mortalities occurred at an average of 21.5 d following the first mortality in fish i.p. infected with the highest dose of 5×10^7 $TCID_{50}$ $fish^{-1}$. In the fish cohabiting with the i.p. fish that received the lowest dose (5×10^1 $TCID_{50}$ $fish^{-1}$) time of first mortality was 53 d in the tank with mortalities (Table 3).

Estimation of 70% lethal dose (LD_{70})

Estimating LD_{70} is required for establishing challenges to determine vaccine efficacy. To assist in future trials, the doses above which 70% mortality occurs (as estimated from the mortality data in Tables 1 to 3) are shown in the legends of Tables 1 to 3.

Table 2. Time to first mortality and final cumulative mortality in salmon parr infected with ISAV intra-peritoneally and by cohabitation. No ISAV-specific mortality was recorded in control tanks. Estimated LD_{70} is $< 2.5 \times 10^2$ $TCID_{50}$ injected i.p. $fish^{-1}$. Numbers of fish shown in brackets

Dose ($TCID_{50}$ $fish^{-1}$)	Time to first mortality (d)			Final cumulative mortality (%)			Time to first mortality (d)			Final cumulative mortality (%)		
	Replicate 1	Replicate 2	Mean	Replicate 1	Replicate 2	Mean	Replicate 1	Replicate 2	Mean	Replicate 1	Replicate 2	Mean
2.5×10^2	19	18	18.5	93.3 (14)	53.3 (8)	73.3	39	35	37	40 (6)	53.3 (8)	46.7
2.5×10^3	17	17	17	93.3 (14)	93.3 (14)	93.3	36	36	36	60 (9)	40 (6)	50
2.5×10^4	16	18	17	86.7 (13)	86.7 (13)	86.7	34	36	35	53.3 (8)	53.3 (8)	53.3
2.5×10^5	18	16	17	66.7 (10)	100 (15)	83.3	33	30	31.5	86.7 (13)	73.3 (11)	80
2.5×10^6	16	13	14.5	93.3 (14)	66.7 (10)	80	32	28	30	60 (9)	73.3 (11)	66.7
2.5×10^7	13	13	13	100 (15)	86.7 (13)	93.3	36	30	33	53.3 (8)	53.3 (8)	53.3

Table 3. Time to first mortality and final cumulative mortality in seawater salmon infected with ISAV intra-peritoneally and by cohabitation. No ISAV-specific mortality was recorded in control tanks. Estimated LD_{70} is $< 2.5 \times 10^2$ $TCID_{50}$ injected i.p. $fish^{-1}$. Numbers of fish shown in brackets

Dose ($TCID_{50}$ $fish^{-1}$)	Time to first mortality (d)			Final cumulative mortality (%)			Time to first mortality (d)			Final cumulative mortality (%)		
	Replicate 1	Replicate 2	Mean	Replicate 1	Replicate 2	Mean	Replicate 1	Replicate 2	Mean	Replicate 1	Replicate 2	Mean
2.5×10^1		28	28[a]	0	54.5 (6)	54.4[a]		53	53	0	45.5 (5)	45.5[a]
2.5×10^2	23	24	23.5	81.8 (9)	54.5 (6)	68.2	57	48	52.5	54.5 (6)	54.5 (6)	54.5
2.5×10^3	20	24	22	54.5 (6)	81.8 (9)	68.2	35	54	44.5	90.9 (10)	55.5 (5)	68.2
2.5×10^4	22	22	22	81.8 (9)	81.8 (9)	81.8	39	40	39.5	100 (11)	72.7 (8)	86.4
2.5×10^5	18	22	20	100 (11)	72.7 (8)	86.4	37	41	39	54.5 (6)	72.7 (8)	63.6
2.5×10^7	16	18	17	90.9 (10)	81.8 (9)	86.4	38	39	38.5	72.7 (8)	9.1 (1)	40.9

[a]Using replicate with mortalities

DISCUSSION

Transmission of ISAV to both freshwater and seawater salmon was shown to be highly efficient following i.p. infection with isolate 0390/98, in support of previous studies using both Norwegian (Dannevig et al. 1993, Totland et al. 1996) and Canadian isolates (Jones et al. 1999, Lovely et al. 1999). Thus, the absence of disease in freshwater salmon farms is likely to be due to insufficient infectious pressure at these farms. This emphasises the importance of addressing the risk factors for ISA, which were identified by Jarp & Karlsen (1997).

It was not possible to establish the minimum infective dose for i.p. infection due to the severity of challenge, in either fresh or seawater salmon, with the range of doses used in this study. However, the variable mortality of between 0 and 54.5% recorded for replicates of the lowest i.p. dose used in seawater salmon (5×10^1 $TCID_{50}$ $fish^{-1}$) indicates that this dose is approaching the minimum required for infection by the i.p. route. Such lower infectious doses were not used in freshwater salmon due to limitations in achieving the accurate delivery of low virus doses in these smaller fish. The injection of a dose of 5 $TCID_{50}$ in a volume of 0.1 ml is prone to big variation as 1 $TCID_{50}$ represents 20% of the sample and even distribution of virus at these concentrations is not certain. Larger numbers of fish would need to be injected to overcome the inherent variation of a low dose and this was not possible during this study.

ISA disease developed in fish cohabiting with salmon i.p. infected with all of the doses used in this study, in both fresh and seawater, with the exception of one tank at the lowest dose where no deaths occurred in i.p. infected post-smolts due to ISAV. Mortalities obtained in cohabiting fish were generally more variable than those obtained in corresponding i.p. injected groups. Infection by cohabitation is by its very nature more variable than injection challenge, which is generally regarded as a highly reproducible and reliable method (Nordmo 1997). Although every effort was made to standardise flow rates and the ratio of infected to cohabiting fish, it is not possible to quantify the dose or duration of challenge received by individual cohabiting fish. This dose may vary according to the level and duration of viral shedding by i.p. infected fish. Furthermore, secondary cohabitation effects following the infection of cohabiting fish cannot be determined. Secondary cohabitation effects probably skewed the data recorded for the bathing challenge at the lowest dose (2.5×10^2 $TCID_{50}$ ml^{-1}) that resulted in mortality. Secondary cohabitation effects are inherent in studies of infection.

Although time to first mortality decreased between the lowest dose tested and the highest dose tested for all 3 infection routes, only in i.p. infected salmon parr was the difference statistically significant ($p < 0.05$). In the case of immersion infection at the 2 highest doses, the time of first mortality was comparable with that obtained following i.p. infection. Onset of mortality in cohabiting salmon post-smolts occurred, on average, 22 d following the first mortality in i.p. injected fish, supporting previous studies (Totland et al. 1996), while in parr in freshwater the corresponding time was, on average, 18 d. With the lowest i.p. injected dose in seawater fish (5×10^1 $TCID_{50}$ $fish^{-1}$), replicate variability was greatest. This indicates proximity to the minimum infective dose required to induce death.

The strengths and weaknesses of different challenge methods have been reviewed by Nordmo (1997). Immersion and cohabitation more closely mimic natural routes of infection, which, for ISA, is considered to be principally waterborne transmission (Vågsholm et al. 1993). Where transmission occurs passively through the water column, individual mortalities cannot be treated as independent events since the death of one fish may influence the survival of others. This effect will be more pronounced where viral doses are close to the threshold required to induce mortality. This explains the greater level of variability observed with the lower doses in the immersion and seawater i.p. and cohabitation infections apparent in this study. The estimation of LD_{70}s will, under the conditions used in this study, enable standardised challenge models that fulfil criteria set by Amend (1981), including control mortality in excess of 60%.

This work has established infection models for ISAV, including standardisation of an immersion infection model, for the first time. The development of reproducible models based on different modes of infection in seawater and freshwater salmon has allowed estimation of the minimum infective doses resulting in mortality. These models are essential in refining future experimental studies aimed at understanding the biology of ISAV, including testing the efficacy of anti-ISAV vaccines.

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