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Age- and weight-dependent susceptibility of rainbow trout *Oncorhynchus mykiss* to isolates of infectious haematopoietic necrosis virus (IHNV) of varying virulence

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ABSTRACT: The virulence of 5 European and 1 North American isolate of infectious haematopoietic necrosis virus (IHNV) was compared by infecting female sibling rainbow trout ('Isle of Man' strain) of different weights and ages (2, 20 and 50 g). The fish were exposed to 10^4 $TCID_{50}$ IHNV per ml of water by immersion, and the mortality was recorded for 28 d. Two new IHNV isolates from Germany were included in the investigation. One was isolated from European eels kept at 23°C (±2°C) and the other was not detectable by immunofluorescence with commercially available monoclonal antibodies recognising the viral G protein. The results showed that IHNV isolates of high or low virulence persisted in rainbow trout of all ages/weights for 28 d, with the exception of fish over 15 g in the eel IHNV (DF [diagnostic fish] 13/98)-infected groups from which the virus could not be reisolated on Day 28. The smallest fish were most susceptible to an infection with any of the IHNV isolates. The lowest cumulative mortality (18%) was observed in fingerlings infected with the North American isolate HAG (obtained from Hagerman Valley), and the highest mortality (100%) in DF 04/99 infected fish. The DF 04/99 and Ö-13/95 viruses caused mortality in fish independent of their weight or age. The isolates FR-32/87 and I-4008 were virulent in fish up to a weight of 20 g and caused no mortality in larger fish. In the IHNV HAG- and DF 13/98 (eel)-infected rainbow trout, no signs of disease were observed in fish weighing between 15 and 50 g. An age/weight related susceptibility of rainbow trout was demonstrated under the defined conditions for all IHNV isolates tested.

KEY WORDS: Susceptibility · Infectious haematopoietic necrosis · IHN · Infectious haematopoietic necrosis virus · IHNV · Virulence differences · Rainbow trout

INTRODUCTION

Infectious haematopoietic necrosis (IHN) and viral haemorrhagic septicaemia (VHS) are rhabdoviral diseases of rainbow trout *Oncorhynchus mykiss* which are of high economic importance. In Europe IHN was first detected in France (Baudin Laurencin 1987) and Italy (Bovo 1987) in 1987, and in Germany in 1992 (Enzmann et al. 1992). IHN virus (IHNV) often was isolated from farms where the fish were chronically infected but did not show abnormal mortality or clinical signs of the disease. Some salmonids probably are genetically more resistant to IHNV or VHS virus (VHSV) than others (LaPatra et al. 1994, Trobridge et al. 2000, Quillet et al. 2001). Furthermore, the virulence of IHNV isolates varies in different species of fish (LaPatra et al. 1990a,b, 1993, Basurco et al. 1993).

The manifestation of IHN disease also depends on the size/age of the fish, water temperature, season and environmental conditions — factors which can all

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heavily affect the immune response in fish (LaPatra 1998). In this study, rainbow trout resistant to a waterborne VHSV infection were kept under defined conditions and were infected with European and North American isolates of IHNV. The aims of this study were to determine the virulence of the different isolates and to compare the virulence of individual isolates in fish of different sizes and ages.

MATERIALS AND METHODS

Cell culture and viruses. *Epithelioma papulosum cyprini* (EPC) cells (Fijan et al. 1983) were grown in 75 cm^2 flasks (Sarstedt USA) at 26°C for 1 d and were then transferred to 15°C. Five IHNV isolates (Table 1) at low cell culture passages (9 to 11) were propagated in EPC cell monolayers at 15°C and 1 isolate (obtained from European eel *Anguilla anguilla*) was propagated at 20°C. Each isolate was harvested after 4 to 6 d post infection (p.i.), frozen once at –20°C, and titrated 3 times in EPC cells. All European virus isolates descended from outbreaks with symptoms and heavy losses (DF 13/98, Ö-13/95, FR-32/87), some of up to 100% (DF 04/99, I-4008). The American IHNV isolate HAG (LaPatra et al. 1990a,b) was found to be virulent in steelhead trout *Oncorhynchus mykiss*, kokanee *O. nerka* and chinook *O. tshawatscha* of different ages and weights (LaPatra et al. 1990a,b, 1993).

Fish and schedules of the infection trials. Female rainbow trout *Oncorhynchus mykiss* of the Isle of Man strain were obtained from a commercial hatchery free from infectious pancreatic necrosis virus, VHSV, IHNV and *Aeromonas salmonicida salmonicida*, and were kept in 400 l tanks with a circulating freshwater system (1.8 m^3 h^{-1}) and 40 l water exchange per day at 9 ± 1°C. Commercially available rainbow trout food suitable for fish of each size and age was used. Small fish up to a weight of 10 g were fed 4 times daily. Feeding of larger fish was reduced to twice daily. The fish were raised from 2 g to a weight of 50 g under these defined conditions. After a 2 wk adaptation period, duplicate groups of fish weighing 2.5 to 3 g (n = 40) were infected by immersion in 10^4 $TCID_{50}$ ml^{-1} of each IHNV isolate for 1 h. Thereafter, the fish were held at a water temperature of 9°C (±1°C) for 28 d. All infected survivors were anaesthetised with 2% Benzocaine in water and killed by decapitation. Virus reisolation from organ pools (spleen, heart, kidney) of individual dead fish was carried out in EPC cells and virus was identified by immunofluorescence according to Commission Decision 96/240/EC (Anonymous 1992, Fichtner et al. 2000). Two months later, duplicate groups of fish grown up to 15–20 g (n = 15) were infected by immersion with the same IHNV isolates under identical conditions as described above. Siblings (n = 20) from the same stock were also infected by immersion when they had reached a weight of 40 to 50 g. For each experiment, negative control fish were kept separately from the infection trials, but under the same conditions.

Virus identification. Prior to the infection trials, all IHNV isolates were identified according to Commission Decision 96/240/EC (Anonymous 1992) by immunofluorescence with monoclonal antibodies recognising the viral G (Fichtner et al. 2000) or N protein (Bergmann et al. 2002), especially for the German isolate DF 04/99. Virus was reisolated in EPC cells from pooled organs of fish that had died during the experiments and from survivors on Day 28 p.i., and subsequently were identified by immunofluorescence or the virus neutralisation test using neutralising rabbit antisera from the CRL (Community Reference Laboratory for Fish Diseases, Århus, Denmark).

Statistical analysis. The χ^2 test ($p < 0.05$) was used to show significant differences in cumulative mortality induced by the virulence of each IHNV isolate. In the groups of fish with 15 to 20 and 40 to 50 g body weight, the Yates correction was used for determining the significance of χ^2 for data with small values (n ″ 60) (Yates 1984).

Table 1. Isolates of infectious haematopoietic necrosis virus (IHNV) used to compare virulence by infecting female sibling rainbow trout *Oncorhynchus mykiss.* Stock virus titration according to Spearman & Kaerber (Kaerber 1931). EPC: *Epithelioma papulosum cyprini*

Isolate	Country of origin	Year of isolation	Host species	Total passages in EPC cell	Stock virus titre	Source
HAG	USA	1983	Rainbow trout	11	$10^{7.75}$ $TCID_{50}$ ml^{-1}	LaPatra et al. (1990b)
FR-32/87	France	1987	Rainbow trout	11	$10^{7.0}$ $TCID_{50}$ ml^{-1}	Baudin Laurencin (1987)
I-4008	Italy	1987	Rainbow trout	10	$10^{7.25}$ $TCID_{50}$ ml^{-1}	Bovo et al. (1987)
DF 04/99	Germany	1999	Rainbow trout	10	$10^{7.0}$ $TCID_{50}$ ml^{-1}	Fichtner et al. (2000)
DF 13/98	Germany	1998	European eel	9	$10^{7.0}$ $TCID_{50}$ ml^{-1}	Bergmann et al. (2002)
Ö-13/95	Austria	1995	Rainbow trout	8	$10^{7.5}$ $TCID_{50}$ ml^{-1}	Bergmann et al. (2002)

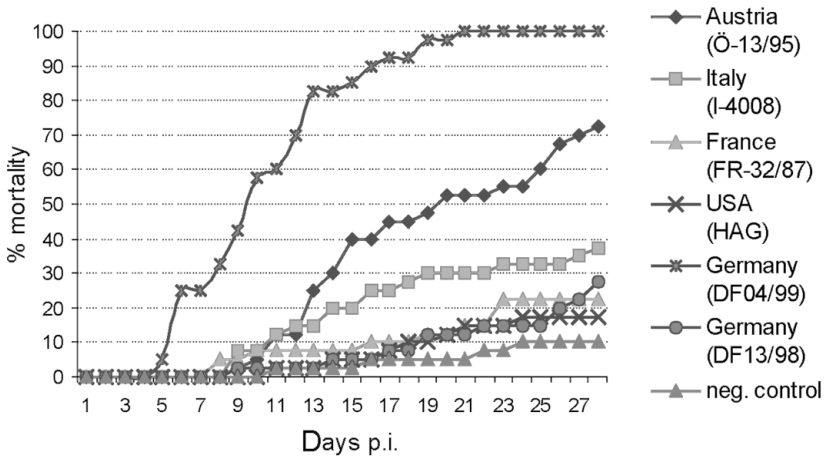

Fig. 1. *Oncorhynchus mykiss.* Cumulative mortality of rainbow trout fingerlings (2.5 to 3.0 g) infected with different infectious haematopoietic necrosis virus (IHNV) isolates. See Table 1 for details of isolates

RESULTS

Rainbow trout weighing 2.5 to 3 g

Mortality started on Day 4 p.i. in fish infected with the German IHNV isolate DF 04/99. The DF 04/99-infected rainbow trout showed typical clinical signs, e.g. apathy without food intake, dark colouration of the skin, ascites and exophthalmus. In the tanks where rainbow trout infected with the other 5 IHNV isolates were kept, the first dead fish were observed between Days 6 and 10 p.i. Clinical signs of IHN were observed in fish infected with the Austrian IHNV isolate Ö-13/95, the French isolate FR-32/87 and the Italian isolate I-4008. Additionally, isolate FR-32/87 was the only one that induced petechial haemorrhages in the skin. However, no clinical signs were observed following infection with isolate DF 13/98 from European eel and with the USA isolate HAG. The highest mortality was found in the groups of fish infected with the German isolate DF 04/99, of which 100% died within 18 d (Fig. 1). The Austrian isolate Ö-13/95 had induced a mortality of 73% by Day 28 p.i. A considerably lower mortality was caused by the IHNV isolates obtained from Italy (38%), France (23%) and the USA (18%). The German eel isolate DF 13/98 killed 28% of the rainbow trout fingerlings. At the end of the experiment (Day 28) all IHNV isolates were detected and identified from organ pools of the survivors, except from pools of trout infected with DF 13/98 virus. A significant difference in mortality compared to the control groups occurred in fish infected with isolates DF 04/99, I-4008, Ö-13/95 and DF 13/98.

Rainbow trout weighing 15 to 20 g

Six days after infection by immersion, mortality was observed in the groups of fish infected with the German IHNV isolate DF 04/99. Mortality occurred on Days 10 and 16 p.i. in fish infected with the Austrian isolate Ö-13/95 and with the French isolate FR-32/87, respectively. By Day 28 p.i., the German isolate DF 04/99 had caused 53% mortality; the Austrian isolate Ö-13/95, 23%; and the French isolate FR-32/87, 8% (Fig. 2). None of the other isolates induced symptoms or mortality. Severe clinical signs of IHN only were observed in fish infected with isolate DF 04/99.

The viruses were reisolated from fish infected with all of the IHNV isolates except for fish infected with the German eel isolate DF 13/98 sampled on Day 28 p.i.

A significant difference in mortality compared to the control groups was only observed in fish infected with isolate DF 04/99.

Rainbow trout weighing 40 to 50 g

Again, the German IHNV isolate DF 04/99 induced severe clinical signs of IHN and the highest cumulative mortality (20%), after 28 d. The Austrian isolate Ö-13/95

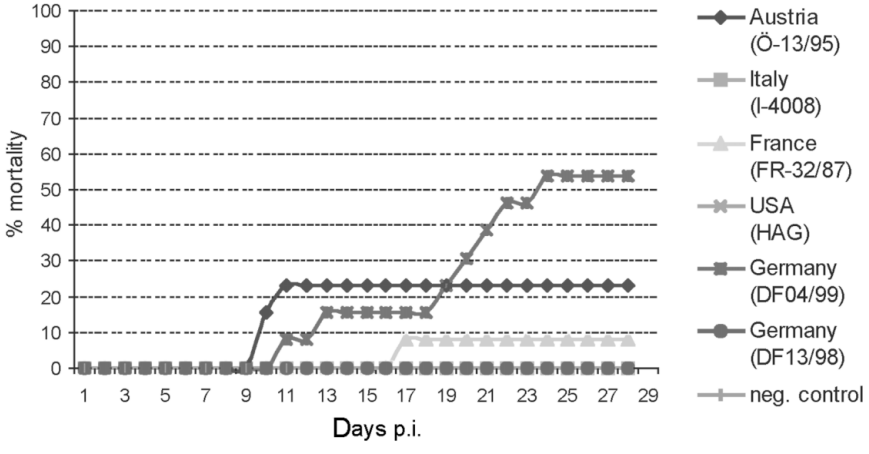

Fig. 2. *Oncorhynchus mykiss.* Cumulative mortality of rainbow trout (15 to 20 g) infected with different infectious haematopoietic necrosis virus (IHNV) isolates. See Table 1 for details of isolates

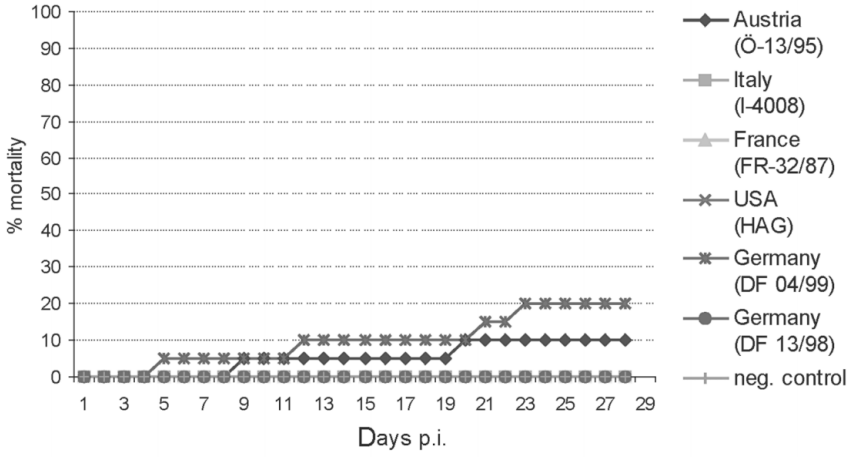

Fig. 3. *Oncorhynchus mykiss.* Cumulative mortality of rainbow trout (40 to 50 g) infected with different infectious haematopoietic necrosis virus (IHNV) isolates. See Table 1 for details of isolates

caused a mortality of 10% (Fig. 3). In each of the groups infected with the Italian isolate I-4008, the German eel isolate DF 13/98, and the French isolate FR-32/87, 1 fish died before Day 3, without any clinical sign of disease. In contrast to all other dead fish, neither virus nor pathogenic bacteria and general parasites were detectable. Therefore, those fish were not included in the calculation of the cumulative mortality. Neither IHNV-induced mortality nor clinical signs of IHN were observed in fish infected with HAG (USA), FR-32/87 (France), I-4008 (Italy) or DF 13/98 (Germany).

At the end of the experiment (Day 28), IHNV could not be detected in fish infected with the German eel isolate DF 13/98. However, IHNV was identified by immunofluorescence in the organ pools of all other survivors.

A significant difference in mortality compared to the control group of the same weight and age was only observed in fish infected with isolate DF 04/99.

DISCUSSION

Antigenic relationships, differences in virulence and electropherotyping of the N proteins among North American IHNV isolates have been reported (Rucker et al. 1953, McCain et al. 1971, LaPatra et al. 1993, 1994). However, no information is available on differences in virulence between European IHNV isolates in fish of different ages and sizes. For these experiments the 'Isle of Man' rainbow trout strain was selected because of its low susceptibility to a waterborne VHSV infection (10^4 $TCID_{50}$ ml^{-1}) (data not shown). The results of these experiments confirmed and expanded the results of LaPatra et al. (1990a,b, 1991, 1993, 1994). In fish of all ages and weights, larger differences in virus virulence were observed after waterborne infection with the selected IHNV isolates (5 European and 1 North American), although the cumulative mortality decreased with increasing age/weight of the fish. In all 3 groups the highest mortality was induced by the German isolate DF 04/99 (Fig. 4). The virus had killed 100% of the 2.5 to 3 g fish, 53% of the 20 g fish and 20% of the 50 g fish by Day 28 p.i. This virus isolate was also the only one that was not detectable by immunofluorescence with commercially available MAbs directed against the G protein (Fichtner et al. 2000). It is presumably a new variant which contains changes in the amino acid sequence of the G protein. In contrast, all viruses were identified by immunofluorescence using MAbs against the N protein (Bergmann et al. 2002).

Fish infected with the Austrian isolate Ö-13/95 succumbed to the virus in the same way as they did to isolate DF 04/99, but the former isolate induced a relatively lower cumulative mortality. In fingerlings, a cumulative mortality of 72% occurred. When the fish had grown to larger sizes, the cumulative mortality decreased to 23% in fish of 20 g and to 10% in fish of 50 g.

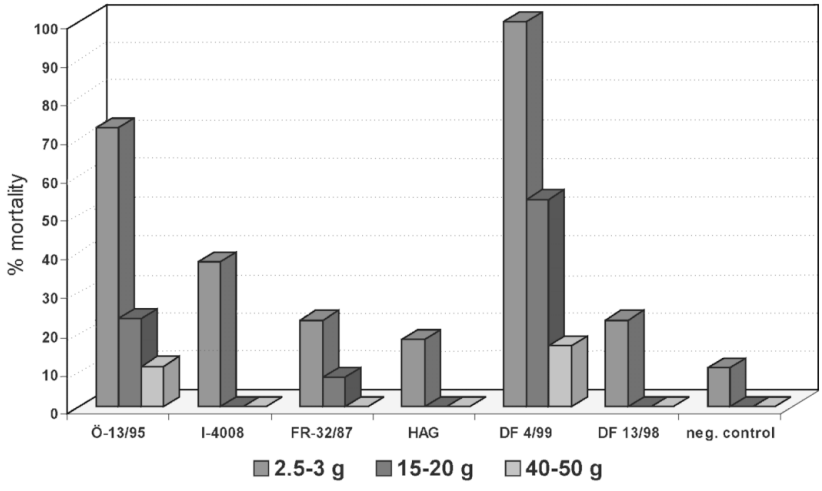

Fig. 4 *Oncorhynchus mykiss.* Comparison of cumulative mortality (%) of fish infected with 5 European infectious haematopoietic necrosis virus (IHNV) isolates and 1 North American IHNV isolate at 28 d post infection

This pattern of mortality, decreasing with increasing size or age, also occurred in experiments with the other isolates, although the observed mortality rates were lower. No mortality occurred in fish of 50 g infected with the Italian and the French IHNV isolates.

In all groups, the lowest mortality occurred when the USA isolate HAG and the German isolate DF 13/98 were used for infection of fish. In fingerlings, a very low mortality was caused by these 2 isolates, and no mortality occurred in larger fish. This contradicts the findings of LaPatra et al. (1990a,b), where a higher HAG-induced mortality in smaller and larger rainbow trout was observed. This can be explained by the serial passage of the HAG virus (11 passages) in a non-salmonid cell line (EPC) and the obviously natural resistance of the Isle of Man rainbow trout strain to IHNV infection. The same considerations also might apply to the French (FR-32/87) and Italian (I-4008) isolates.

With the exception of fish infected with German eel isolate DF 13/98, all viruses could be reisolated in EPC cells from fish that had died by, or were sampled on, Days 7 and 28. It was not possible to reisolate virus from fish infected with the German eel isolate DF 13/98 on Day 28 p.i., but on Day 7 p.i. the virus was isolated in EPC cells at a low titre (10^1 $TCID_{50}$ ml^{-1}). These differences between the isolates were reflected *in vitro* since the DF 13/98 replicated poorly at 15°C, but very well at 20°C in EPC cells, and did not replicate in cells of rainbow trout origin such as the rainbow trout gonad (RTG-2) cell line (data not shown). The replication rate of this isolate in fish kept at 10°C and in cells incubated at 15°C obviously was very low. Thus, DF 13/98 seems to be a representative of a new type of IHNV found in Europe.

The cumulative mortality was significantly different from the control groups ($p < 0.05$) in all groups (2.5 to 50 g) infected with DF 04/99 and in the fingerling groups (2.5 to 3 g) infected with Ö-13/95, I-4008 and DF 13/98.

Further studies should focus on antigenic and genetic differences of the virus G protein, which contains the neutralising epitopes and which, to all appearances, is responsible for the virulence of the virus (Engelking & Leong 1989). The highly virulent isolate DF 04/99 or the 'exotic' DF 13/98 eel isolate should be given priority. For diagnostic purposes, the N gene and the viral N protein have to be included in the investigation with the aim of identifying newly emerging viruses. This information will be important for the understanding of viral pathogenesis and immunogenesis in fish, for the development of effective vaccines and for the reproducibility of IHNV challenge models or investigations on virus virulence.

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