

Further observations on the epidemiology and spread of epizootic haematopoietic necrosis virus (EHNV) in farmed rainbow trout *Oncorhynchus mykiss* in southeastern Australia and a recommended sampling strategy for surveillance

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ABSTRACT: Epizootic haematopoietic necrosis virus (EHNV) is an iridovirus confined to Australia and is known only from rainbow trout *Oncorhynchus mykiss* and redfin perch *Perca fluviatilis*. Outbreaks of disease caused by EHNV in trout populations have invariably been of low severity, affecting only 0+ post-hatchery phase fingerlings <125 mm in length. To date the virus has been demonstrated in very few live in-contact fish, and anti-EHNV antibodies have not been found in survivors of outbreaks, suggesting low infectivity but high case fatality rates in trout. During an on-going study on an endemically infected farm (Farm A) in the Murrumbidgee River catchment of southeastern New South Wales, EHNV infection was demonstrated in 4 to 6 wk old trout fingerlings in the hatchery as well as in 1+ to 2+ grower fish. During a separate investigation of mortalities in 1+ to 2+ trout on Farm B in the Shoalhaven River catchment in southeastern New South Wales, EHNV infection was demonstrated in both fingerlings and adult fish in association with nocardiosis. A 0.7% prevalence of antibodies against EHNV was detected by ELISA in the serum of grower fish at this time, providing the first evidence that EHNV might not kill all infected trout. EHNV infection on Farm B occurred after transfer of fingerlings from Farm C in the Murrumbidgee river catchment. When investigated, there were no obvious signs of diseases on Farm C. 'Routine' mortalities were collected over 10 d on Farm C and EHNV was detected in 2.1% of 190 fish. Tracing investigations of sources of supply of fingerlings to Farm B also led to investigation of Farm D in Victoria, where the prevalence of anti-EHNV antibodies in 3+ to 4+ fish was 1.3%. The results of this study indicate that EHNV may be found in trout in all age classes, need not be associated with clinically detectable disease in the population, can be transferred with shipments of live fish, can be detected in a small proportion of 'routine' mortalities and may be associated with specific antibodies in a small proportion of older fish. Sampling to detect EHNV for certification purposes should be based on examination of 'routine' mortalities rather than random samples of live fish. Antigen-capture ELISA can be used as a cost effective screening test to detect EHNV on a farm provided that sampling rates conform with statistical principles.

KEY WORDS: Epizootic haematopoietic necrosis virus · Iridovirus · Certification · Rainbow trout · *Oncorhynchus mykiss* · ELISA

INTRODUCTION

Epizootic haematopoietic necrosis virus (EHNV) is an iridovirus and 1 of only 5 fish disease agents notifi-

able to the Office International des Epizooties (OIE). The significance of EHNV lies in its high virulence in certain hosts, lack of host specificity and apparent geographic restriction to the Australian mainland (Langdon et al. 1986, Langdon 1989, Whittington et al. 1996, Whittington & Hyatt 1998). Iridoviruses closely related

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to EHNV are emerging as an important group of pathogens for cultured and wild finfish in many countries (Ahne et al. 1997), as are iridoviruses of less certain taxonomic status (Fraser et al. 1993, Chua et al. 1994, LaPatra et al. 1994, Plumb et al. 1996, Jung et al. 1997).

In contrast to the epizootics that occur in wild redfin perch *Perca fluviatilis*, EHNV causes a disease of low severity in populations of farmed rainbow trout *Oncorhynchus mykiss*, with daily mortality of less than 0.2% and total mortality up to only 4% (Whittington et al. 1994). While EHNV can be found in most clinically affected fish, only a small proportion (4%) of in-contact, clinically normal fish were found to be infected during an outbreak, and the virus could not be found at all in surviving cohorts after an outbreak (Whittington et al. 1994). It appeared that most of the infected fish had died. This was supported by serological data: anti-EHNV antibodies could not be found in surviving cohorts even though the virus was shown to be immunogenic (Whittington et al. 1994). The poor infectivity of EHNV in trout has been confirmed experimentally (Whittington & Reddacliff 1995). The low prevalence of infection, high case fatality rate and apparent absence of carriers in rainbow trout make it difficult to prescribe sampling protocols for disease certification purposes.

In this report we present the results of further investigations of EHNV infection in rainbow trout. These findings indicate that infection of rainbow trout with EHNV is not always fatal, that fish of any age may be affected, that there may be serological evidence of infection in older fish on an infected farm and that detection of the virus in the absence of obvious clinical signs of the disease in a population is possible by examination of 'routine' mortalities. Recommendations for certification testing protocols can therefore now be made.

MATERIALS AND METHODS

Collection of specimens. Moribund and dead fry and fingerlings were collected individually or in lots of 20 to 50, placed in plastic bags and immediately frozen at -20°C , or held overnight at 4°C before freezing, pending examination. Live healthy fry and fingerlings were treated similarly after killing by immersion in benzocaine (200 mg l^{-1}). Grower fish were dissected on the farm and organs were placed either in 10% buffered neutral formalin or in sterile jars which were placed at 4°C overnight then at -20°C pending examination. Blood was collected from the caudal vein of grower fish that were anaesthetised in benzocaine (40 mg l^{-1}). After standing for 5 to 18 h at 4°C or at room temperature and centrifugation at $1400 \times g$ for 20 min,

serum was harvested, diluted 1:10 in 25 mM Tris-HCl pH 7.4, 0.15 M NaCl, 50% glycerol and stored at -20°C pending examination.

Pathology. Formalin-fixed tissues were embedded in paraffin, sectioned at $5\text{ }\mu\text{m}$ and stained with haematoxylin and eosin. One or more of the following lesions were considered to be consistent with EHNV infection: acute focal or diffuse renal haematopoietic necrosis, acute focal hepatocellular necrosis, and acute focal or diffuse splenic necrosis, with or without intracytoplasmic inclusions (Reddacliff & Whittington 1996).

Bacteriology. Samples were collected aseptically from parenchymal organs and cultured on 5% sheep blood agar at 22 and 37°C . Bacterial colonies were subcultured and identified using phenotypic characteristics (Barrow & Feltham 1993).

Preparation of fish tissues, antigen capture ELISA and cell culture. Whole fry, small fingerlings with head and tail removed, or pooled liver, kidney and spleen from larger fish were homogenised in disposable 1.5 ml tubes with fitted pestles. Clarified homogenates were prepared from individual fish as described previously (Whittington & Steiner 1993) and stored at -80°C . EHNV infection was detected by examining individual or pooled ($n = 5$) homogenates by culture in BF-2 cells (Farm A) as well as by antigen-capture ELISA on individual homogenates (Farm B), or by screening individual homogenates by antigen-capture ELISA and undertaking cell culture only on ELISA positive homogenates (Farm C). Cell culture and ELISA were undertaken as described by Whittington & Steiner (1993). Briefly, for ELISA, polystyrene microtitre plates were coated with affinity purified rabbit-anti-EHNV antibodies. Clarified homogenate was added and, after washing, the plates were reacted with sheep-anti-EHNV antibodies, rabbit-anti-sheep immunoglobulin antibodies conjugated to horseradish peroxidase and the chromogen ABTS. Those homogenates considered to be ELISA positive by developing a signal greater than that of an appropriate control were then cultured in BF-2 cells at 22°C to obtain an isolate of EHNV (Whittington & Steiner 1993). The cause of the characteristic lytic EHNV cytopathic effect was confirmed by antigen-capture ELISA on the culture supernatant.

Detection of anti-EHNV antibodies in trout serum. The ELISA for detection of antibodies against EHNV in serum was as described by Whittington et al. (1994) except that antigen was stored at -80°C immediately after preparation. Briefly, polystyrene microtitre plates were coated with affinity purified rabbit-anti-EHNV antibodies then undiluted EHNV antigen. After incubation and washing, fish serum diluted 1:100 was added. After further incubation and washing, the plates were reacted with mouse monoclonal anti-trout

IgM, rabbit-anti-mouse immunoglobulin antibodies conjugated to horseradish peroxidase and the chromogen ABTS. Each serum was tested in duplicate in wells with and without EHNv antigen to ensure that binding activity was genuine.

Definition of 'background' or 'routine' mortality. The poorly defined concept of 'background' or 'routine' mortality refers to the mortality rate considered to be acceptable by farm managers and below which a disease investigation would not be initiated. 'Routine' mortality rates have not generally been measured and probably vary considerably from farm to farm.

RESULTS

Farm A

An outbreak of low grade mortality commenced in September 1993 in the outdoor circular ponds on a farm, described in detail previously (Whittington et al. 1994), in the Murrumbidgee River catchment in south-eastern New South Wales. Histopathological examination of 3 fish confirmed the cause of death as being EHNv infection. On a farm visit in October 1993 there were 30 000 individuals, 60–67 mm FL (fork length) (range), 3.54 g (mean), 20–22 wk old, per 5400–8100 l, representing a biomass of 13–20 kg m⁻³, with a water exchange of 1.3–3 times h⁻¹. Mortality was noted also in 4–10 wk old, 35–50 mm FL, 0.44–1.42 g (ranges) fingerlings in races in the hatchery. These were stocked at a rate of 15 000 individuals per 180 l, biomass 40–120 kg m⁻³, with a water exchange of 4.3 times h⁻¹. We measured mortality rates of 0.02 and 0.06 % d⁻¹ in circular ponds and hatchery races, respectively.

EHNv was isolated from 60 % of 60 individual dead fingerlings from the circular ponds and from 78 % of 65 individual dead fingerlings collected from the hatchery races. EHNv was not isolated from any of 143 live, clinically normal, in-contact fingerlings from the circular ponds nor from any of 150 live, clinically normal, in-contact fingerlings from the hatchery races, all tested as pools of 5.

Four dead 1+ to 2+ grower fish were collected from concrete flumes; these were thought to represent 'routine' mortalities. EHNv was isolated from a pool of liver, kidney and spleen from 1 of these fish.

These results represented the first isolation of EHNv from the hatchery races on this farm and confirmed that disease due to EHNv may develop in very young fingerlings. Furthermore, the detection of EHNv in 1+ to 2+ growers was the first observation of EHNv infection in fish other than 0+ fingerlings <125 mm FL. Thus EHNv has now been confirmed from all locations/age classes other than broodstock on Farm A.

Farm B

This farm is located in the catchment of the Upper Shoalhaven River in southeastern New South Wales. Redfin perch do not occur in this catchment (Harris & Gehrke 1997, Marsden et al. 1997). The farm consists of outdoor concrete raceways in series and parallel, earthen ponds in series and a small indoor hatchery with circular tanks and races, all supplied from a reservoir deriving water from a stream. Partial water recirculation from the farm outlet to the reservoir was practised. During February 1996 mortality rates exceeded 'routine' rates in 1+, 200–280 mm FL (range) grower rainbow trout. This change was associated temporally with an increase in water temperature from 16 to 20°C and a sudden reduction in flow rates due to unscheduled maintenance work on the water recirculation system. Water quality data collected on a farm visit after restoration of flows but during the disease outbreak were: water temperature 20°C, total ammonia and nitrite low to not detectable, pH 6.5 and dissolved oxygen 4.2–7 mg l⁻¹ in ponds compared to 8.8 mg l⁻¹ in the main water inlet. Stocking densities in the grow-out facility averaged 3000 individuals per 10 000 l but biomass ranged from 30 to 180 kg m⁻³ in different sections.

In a sample of 9 fish from the affected cohort that was submitted to the laboratory for examination at the start of the outbreak, EHNv infection was confirmed histologically and by cell culture and antigen-capture ELISA on pools of liver, kidney and spleen in each of 3 fish, while granulomatous lesions due to *Nocardia asteroides* were found in the abdominal organs of another fish. Although the owner originally had reported losses of up to 1 % d⁻¹, we measured a daily mortality rate of about 0.1 % in 1+ to 2+ fish on a farm visit several weeks later. In addition, there was a high prevalence of acute and chronic fin and tail erosions in most groups in all age classes of fish on the farm. Seven moribund, 210–280 mm FL fish from an affected unit were necropsied. Nocardiosis was confirmed histologically in 5 fish. EHNv infection was confirmed by virus isolation and ELISA in 5 fish, 1 of which had granulomas throughout its kidney and liver due to nocardiosis but no obvious necrotic lesions consistent with EHNv. One 150 mm fingerling from the hatchery area had histological evidence of EHNv and the virus was found in pooled liver, kidney and spleen by ELISA and cell culture. One female broodstock that had died with chronic peritonitis did not have lesions consistent with EHNv and was negative by ELISA and cell culture. Overall, 4 of the 9 fish subjected to detailed examination had concurrent EHNv infection and nocardiosis.

Sera from 149 fish selected randomly from the affected 1+ to 2+ age class were tested for antibodies against EHNv. One serum had an optical density (OD)

of 0.90 when tested with antigen and 0.19 without antigen and was classified as positive. Equivocal results were obtained with sera from another 2 fish (OD 0.21 and 0.22 with antigen, both 0.10 without antigen).

The source of fish on Farm B was ascertained. In addition to home bred fingerlings, consignments of fingerlings from Farms C and D had been received 6 mo earlier; neither of these farms was known to be infected with EHNV.

Farm C

This farm is located in the Murrumbidgee River catchment close to Farm A, but uses water from a different river. Upon questioning, the owner could recall no unusual mortality events. In view of the lack of evidence of a disease outbreak, 'routine' mortalities from all age classes of trout were collected over a period of 10 d in April 1996 under the supervision of a government Fisheries Officer; these were stored at -20°C pending submission to the laboratory. Of 190 individual 60–250 mm FL, 1.9–177 g (ranges) trout, 7 were strongly positive for EHNV antigen when pooled liver, kidney and spleen was tested by ELISA, and 4 of these subsequently yielded EHNV in cell culture. The observed prevalence of live EHNV in routine mortalities was thus 2.1% (95% confidence limits: 0.6 to 5.3%), although the true prevalence may have been higher as ELISA was used as a screening test and is less sensitive than cell culture.

Farm D

This farm is located on an isolated stream in north-east Victoria, with no fish farms upstream in the catchment. The farm consists of concrete raceways and earthen ponds and has had no history of significant disease events. Blood samples were collected in July 1996 from 3+ to 4+ rainbow trout weighing 2 to 4 kg. Two of 159 sera contained antibodies against EHNV. OD for the 2 sera were 0.70 and 0.47 with antigen and 0.12 without antigen. Samples for virological examination were not submitted.

DISCUSSION

Prior to this study EHNV infection was known from only 2 trout farms in New South Wales and on the better-studied farm (Farm A) was thought most likely to be associated either with annual reinfection from a reservoir in wild redfin perch or persistence of the virus in sediments on the farm (Whittington et al.

1994). The present study has confirmed that EHNV infection in trout need not be derived from infection in wild redfin perch and can be spread from farm to farm with movements of fingerlings, a factor suspected by Langdon et al. (1988) in the original occurrence of the disease in trout.

Disease in rainbow trout caused by EHNV infection has previously been detected only in 0+ fingerlings <125 mm FL (Whittington et al. 1994) and has only been recorded in fingerlings stocked into ponds after the hatchery phase. However it is now clear that disease due to EHNV can occur in young fingerlings prior to stocking out for growth and can also occur in large grower fish several years of age. It is reasonable therefore to assume that EHNV infection and mortalities due to the virus may occur in trout in any age class.

The findings of this study support the earlier suggestion (Whittington et al. 1994) that disease in trout due to EHNV is associated with poor water quality and intercurrent diseases, including external parasites and systemic bacterial infection.

Significantly, it has now been shown conclusively that EHNV may persist in trout populations and not be suspected by farm managers because the mortality rate may not exceed the 'routine' mortality rate that managers have come to accept as being a normal part of rearing fish. This is likely to be a significant factor leading to underreporting of EHNV infection in trout.

It is still uncertain whether a true EHNV carrier state exists in trout. Where histopathology was undertaken in grower fish in this study, lesions consistent with EHNV were found, suggesting that there was active infection and disease rather than a carrier state. It must be acknowledged that, in the presence of intercurrent infections such as nocardiosis which produce substantial lesions, subtle lesions associated with early EHNV infection may be difficult to detect grossly and histologically; this was the case in 1 fish.

In this study antibodies were detected for the first time in a trout population known to be infected with EHNV. In contrast to an earlier study where anti-EHNV antibodies were not found in 0+ fingerlings after an outbreak of EHNV infection (Whittington et al. 1994), in this study blood samples were collected from older fish where perhaps there may have been more time available for immune responses to develop after exposure to the virus. The prevalence of antibody positive fish was low (0.7%, 95% confidence limits: 0.02 to 3.7%) on Farm B, consistent with low infectivity and a high case fatality rate (Whittington et al. 1994), but the finding supports the view that carriers of EHNV might exist in trout populations. To prove this, further longitudinal studies in which virus isolation, serology and histopathology are undertaken on large numbers of individual fish will be required. There are currently

insufficient data from field collections to comment on the length of time required for seroconversion to occur in survivors of EHNV infection, and similarly too few fish have been examined under experimental conditions (Whittington & Reddacliff 1995).

It was shown previously that the chances of detecting EHNV infection in apparently healthy fish is extremely low, even where disease is active in the same population, because there is a high case fatality rate (Whittington et al. 1994). This was confirmed again on Farm A in the present study. For practical purposes EHNV can only be detected in fish that are clinically affected or that have died with the infection. Thus routine certification practices based on the examination of large random samples of the entire population are not appropriate for EHNV. From a random sample of live fish it would be possible to misclassify a farm as being free of EHNV even during an outbreak of the disease because the prevalence of infection is generally very low. However, there are 2 possible methods for detecting trout populations infected with EHNV: virological examination of 'routine' mortalities and serology.

During an outbreak of disease, the prevalence of EHNV among mortalities may be 60 to 80% compared to only 2% when the disease is not apparent. However, we have shown in this study that the contribution of EHNV to 'routine' mortality on an endemically infected farm is high enough to enable detection of the virus in the absence of overt disease in the population. For EHNV detection and certification purposes the population of real interest is 'the population of mortalities' and sampling rates can be selected to detect at least 1 EHNV-infected individual at a given level of confidence given a certain prevalence of infection and test sensitivity (Cannon & Roe 1982, Simon & Schill 1984).

For EHNV detection and certification in New South Wales we assume a prevalence of 2%, based on the data from Farm C which suggested that at least 2% of 'routine' mortalities were associated with EHNV infection. The size of the population of 'routine' mortalities is difficult to measure as it increases daily, but if it is assumed to be very large a conservative outcome with respect to confidence will be obtained. The antigen-capture ELISA used to screen tissue homogenates for EHNV has a sensitivity of at least 60% compared to cell culture (Whittington & Steiner 1993, Whittington & Hyatt unpubl.). The sample size required from a very large population of 'routine' mortalities to provide 95% confidence in detecting at least 1 infected individual using a test of 60% sensitivity is approximately 250 (Cannon & Roe 1982, Simon & Schill 1984). In practice we specify that 'routine' mortalities be collected daily and stored in plastic bags at -20°C until a sufficient sample has been gathered. Where possible we target

young age classes to simplify dissections and tissue processing. Individual clarified homogenates that are positive in antigen-capture ELISA are then subjected to cell culture to obtain an isolate of EHNV. This is an economical approach as it greatly reduces the number of cell cultures required.

It appears that serology might also play a useful role in surveys to identify infected trout populations. On Farm D the prevalence of fish with anti-EHNV antibodies was 1.3% (95% confidence limits: 0.15 to 1.3%), a value comparable to that from Farm B, and it is unfortunate that samples from this farm were not available for virological examination. Assuming that the prevalence of seropositive grower fish is 1% on an endemically infected farm, a sample of 300 fish would be required to be 95% certain of detecting at least 1 infected individual (Cannon & Roe 1982). Further research is required to confirm the validity of this approach.

The distribution of EHNV in southeastern Australia is incompletely understood. As the virus is believed to be pathogenic for a wide range of fish species, including several that are rare or endangered (Langdon 1989), further spread of EHNV through movements of farmed salmonids is undesirable. For this reason, farms in the known-infected catchments are subject to restrictions that preclude movement of live fish to putatively uninfected catchments (Whittington & Hyatt 1996). Field surveys are planned to accurately map the distribution of EHNV in catchments in New South Wales.

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