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Molecular epidemiology of *Epizootic haematopoietic necrosis virus* (EHNV)

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ABSTRACT

Low genetic diversity of *Epizootic haematopoietic necrosis virus* (EHNV) was determined for the complete genome of 16 isolates spanning the natural range of hosts, geography and time since the first outbreaks of disease. Genomes ranged from 125,591–127,487 nucleotides with 97.47% pairwise identity and 106–109 genes. All isolates shared 101 core genes with 121 potential genes predicted within the pan-genome of this collection. There was high conservation within 90,181 nucleotides of the core genes with isolates separated by average genetic distance of 3.43×10^{-4} substitutions per site. Evolutionary analysis of the core genome strongly supported historical epidemiological evidence of iatrogenic spread of EHNV to naïve hosts and establishment of endemic status in discrete ecological niches. There was no evidence of structural genome reorganization, however, the complement of non-core genes and variation in repeat elements enabled fine scale molecular epidemiological investigation of this unpredictable pathogen of fish.

1. Introduction

Epizootic haematopoietic necrosis virus (EHNV) was first isolated during an investigation of mass mortality affecting juvenile redfin perch (*Perca fluviatilis*) in freshwater impoundments in Victoria, Australia, in summer 1984 (Langdon et al., 1986). This pathogen has continued to cause fish kills affecting wild redfin perch, but the occurrence is sporadic and the disease is restricted to south-eastern mainland Australia (Whittington et al., 2010). Additionally, EHNV has caused less severe disease characterized by low grade mortality in farmed rainbow trout (*Oncorhynchus mykiss*) in the same regions. Epizootic haematopoietic necrosis (EHN) was one of the first diseases of finfish that was attributed to an iridovirus, a virus family including many emerging pathogens that are now a frequent cause of disease in fish (Hick et al., 2016). It is predicted that EHNV could severely impact recreational fisheries, aquaculture and ecosystems of conservation value in locations beyond the geographic range of the virus where there are populations of susceptible hosts in environments conducive to disease (Peeler et al., 2009). For this reason The World Organization for Animal Health (OIE) lists infection with EHNV to facilitate biosecurity measures (OIE, 2015). Present disease control measures are based on tests for freedom from infection prior to translocation of fish and general biosecurity using effective disinfection protocols (Hick et al., 2017). Upon first isolation of EHNV, Langdon et al. (1986) remarked at the apparent inexplicability of the origin and emergence of the virus. The same question is pertinent 25 years later, there having been no reports of spread of EHNV beyond the endemic catchments since 1996 and only sporadic recurrence of disease events within these regions (Whittington et al., 2010). The lack of broader impact of EHNV on aquatic animal health could be considered surprising given the wide range of potential translocation scenarios and potentially susceptible host species (Becker et al., 2013; Langdon, 1989). Thus, greater understanding of the biology and evolution of this pathogen will benefit effective disease management in a changing environment.

The disease EHN is observed as mass mortality in wild redfin perch. Experimental infections indicate an incubation period of 10–28 days with a brief period of anorexia, darkened coloration and abnormal swimming preceding death (Langdon, 1989; Whittington and Reddacliff, 1995). Affected fish have hemorrhage at the base of the fins, peritoneal effusion, enlargement of the spleen and kidney with pale foci on the liver (Langdon and Humphrey, 1987; Reddacliff and Whittington, 1996). This reflects systemic necrosis consistent with the tropism of EHNV for haematopoietic and vascular endothelial cells (Whittington et al., 2010). Basophilic intra-cytoplasmic inclusion bodies are most evident in

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hepatocytes adjacent to necrotic foci (Becker et al., 2013; Reddacliff and Whittington, 1996). Evidence for subclinical infection of redfin perch was evident by isolation of EHNV from some individuals that survived experimental challenge and from apparently healthy wild fish after a disease outbreak, although the duration of persistent infection is not known (Becker et al., 2013; Langdon and Humphrey, 1987). By contrast, natural cases of EHN in rainbow trout result in low mortality (< 0.05% per day) with variation from 100% morbidity evident as reduced feeding and abnormal swimming to inapparent disease (Langdon et al., 1988; Whittington et al., 1999). Recent experimental challenges of redfin perch have resulted in lower mortality, and higher infective doses were required compared to earlier studies in Australia (Becker et al., 2013; Langdon, 1989; Whittington and Reddacliff, 1995). Similarly, it was noted recently that European stocks of redfin perch were relatively refractory to experimental challenge with moderate mortality (Ariel and Jensen, 2009; Borzym and Maj-Paluch, 2015). It is not known if this reflects differences between EHNV in the various inoculums, or increased resistance of the fish due to age, selection pressure, genotype or their husbandry.

The known geographical range of EHNV is restricted to south-east mainland Australia and the occurrence of EHN has been limited to constructed freshwater impoundments or aquaculture facilities (Whittington et al., 2010). The only two naturally affected hosts were introduced to Australia from Europe in 1861 (redfin perch, Cadwallader & Backhouse 1983) and 1894 (rainbow trout, Roughly 1966). The potential host range of EHNV is greater than that which has been has been observed in natural disease outbreaks. Several species of fish present in EHNV endemic regions including introduced (*Gambusia affinus holbrooki*) and native Australian fish of conservation value (e.g. *Macquaria australasica*) were susceptible to infection with EHNV in experimental settings (Becker et al., 2013; Langdon, 1989). Some European fishes, *Ameiurus melas*, *Esox lucius* and *Sander lucioperca* were also susceptible to EHNV experimentally (Jensen et al., 2009, 2011). The occurrence of EHN has been discontinuous over time and space with discrete disease events occurring within the endemic regions without a long-term pattern of recurrence. Disease is limited to summer months, consistent with the observations that transmission of infection and disease are limited to water temperatures > 12 °C in experimental settings and increased water temperature is conducive to pathogenic disease (Ariel and Jensen, 2009; Whittington and Reddacliff, 1995). Seasonal outbreaks in summer months are coincident with the recruitment of large populations of susceptible young fish which may lack an adaptive immune response (Whittington et al., 1994). Upstream spread in the Murrumbidgee and Murray River systems increased the initial geographic range of EHNV (Whittington et al., 1996), however, the virus has remained restricted to south-eastern mainland Australia for > 30 years (Whittington et al., 2010). Subclincial infection of wild fish is a feature of the disease that could contribute to this pattern of irregular recurrence and spread. Experimental infection by cohabitation and immersion indicate the potential for indirect horizontal transmission in wild fish (Becker et al., 2013; Langdon, 1989). Translocation of fingerling stock with clinically inapparent infection has spread EHNV between rainbow trout farms (Whittington et al., 1994, 1999). Although there are precedents for transmission of ranaviruses between amphibian and piscine hosts (Brenes et al., 2014; Mao et al., 1999), there is presently no evidence of a potential amphibian reservoir host for EHNV. Additionally, the resistance of EHNV is conducive to iatrogenic spread via fomites and persistence in the environment between outbreaks without a host; EHNV remained infectious for periods of approximately 100 days at 15 °C in water or a dried form (Langdon, 1989).

Epizootic haematopoietic necrosis virus (EHNV) is a species within the genus *Ranavirus* (Jancovich et al., 2012). This is a diverse genus that includes pathogens with reptile, amphibian and fish hosts, including viruses that are capable of infection across these classes (Brenes et al., 2014). Molecular characterization of the complete genomes of ranaviruses has rapidly advanced and includes several from fish hosts: sheatfish (*Silurus glanis*), Germany 1989 (Mavian et al., 2012); Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*), Denmark 1979 and 1989, respectively (Ariel et al., 2016); short-finned eel (*Anguilla australis*) imported to Italy from New Zealand in 1999 (Subramaniam et al., 2016); pike-perch (*Stizostedion lucioperca*) in Finland 1995 (Holopainen et al., 2016). Diversity within this genus is evident through more detailed phylogenetic characterizations (Jancovich et al., 2015a) with four unique genome organizations (Jancovich et al., 2015b). A number of complete genomes have been determined for ranaviruses from fish which are represented in the three linages of ranavirus identified by Jancovich et al. (2015a) as well as *Santee Cooper ranavirus* and *Singapore grouper ranavirus*. The complete genome of an EHNV isolate that was putatively isolated from redfin perch during the initial disease investigation was significantly larger than the type species *Frog virus 3*, at 127 kb in length but contained a similar number of predicted genes with 100 potential open reading frames (Jancovich et al., 2010). EHNV is most closely related to *European catfish virus* (ECV) which has 88% complete genome sequence identity and similar organization (Mavian et al., 2012). ECV causes a disease similar to EHN that is restricted to select freshwater fish species in continental Europe (Hick et al., 2016). Beyond taxonomic classification, these detailed genetic characterizations can infer evolutionary information, for example, an ancestral ranavirus of fish was proposed to have undergone recent host shifts to generate virus species with amphibian hosts (Jancovich et al., 2010). Molecular epidemiology enables inferences about these diseases when large numbers of isolates from a virus species are characterized (Epstein and Storfer, 2015). At present, the ecological aspects of EHN are insufficiently understood to make inferences about the evolutionary pressure on EHNV.

The genomes of 16 isolates of EHNV were characterized using full genome sequencing. These were selected to include isolates from both known natural host species and to span the 25 year history of occurrence within New South Wales and watersheds shared with the Australian Capital Territory and with reference to a preceding outbreak in Victoria. The objectives of this study were: to document the diversity within the virus species; evaluate differences in the pathogen that might be associated with host specificity, severity of disease; to make inferences about patterns of spread and possible environmental reservoirs for the virus; and identify evidence of response of EHNV to evolutionary pressures in changing ecosystems.

2. Materials and methods

2.1. Origin of virus isolates

The isolates used in this study were obtained opportunistically during disease investigations and were cryopreserved at low passage level in a collection curated by the OIE reference laboratory for EHNV at the University of Sydney (Table 1). Information about the location and disease scenarios were obtained from the original laboratory records (Fig. 1).

2.1.1. Cluster 1, reference genomes, Victoria

Isolate 1 was obtained from redfin perch during a disease outbreak in Glastonbury Dam, Victoria in 1985 (Table 1) (Langdon, Humphrey, 1987). This sequence was determined to complement information about the virus close to the time of the first detection and description from December 1984 in the nearby Lake Nillahcootie, north eastern Victoria (Langdon et al., 1986), along with the reference EHNV genome (Genbank NC028461). Isolate 2 was obtained from rainbow trout at a farm in Dandenong, Victoria in 1988. This represents an early detection of EHNV in rainbow trout in Victoria, 2 years after the first detection in this host (Langdon et al., 1988) and after 4 years of annual recurrence of the disease in redfin perch in the region.