Viral diseases in Indian freshwater and marine water pisciculture

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Intensification of aquaculture allows emergent and re-surgent viral pathogens to cause large die-offs in the wild and farms. The inherent capability of viruses to exist in multiple forms outside the hosts gives them an edge for easy transmission and translocation increasing the chances of infection. More efforts are needed for an in-depth understanding of viral epidemiology. Quantification of factors determining the virulence mechanisms and variability in disease expression is necessary to strengthen the basic knowledge on virology. The article is an update on the current understanding on viral diseases in fish causing loss to Indian aquaculture systems.

Keywords: Aquaculture, fish, freshwater, marine water, viral diseases.

THE aquaculture sector has exhibited an upsurge during the past years. The prime contributor in aquaculture production is China followed by other developing countries within the Asia-Pacific region; accounting for 89% by volume of global production and 77% by value[1]. In order to meet the rising demand, farmed fish production has increased tremendously qualitatively and quantitatively in both freshwater and marine water regimes. Indian pisciculture encompasses a diverse range of fishes, including carps (Indian major carps, minor carps, catfishes and barbs), tilapia, climbing perch and murrels. Moreover, aquariculture (culturing of aquarium fishes) has also gained importance worldwide because of its aesthetic value as well as economic profit. In India, ornamental fish farming is mainly practiced in West Bengal, Tamil Nadu, Kerala, Karnataka and states of the North East[2], and the country possesses a great potential in contributing to the global ornamental sectors. Concurrently, the increase in routine aquaculture operations has provided a thruway for pathogens to infiltrate into the fish populations by either being a carrier or through transboundary movements. These aquatic pathogens, especially viruses, are associated with episodic mortalities and morbidities in their natural hosts. With the ability to persist and adjust to the changing environmental conditions, viruses display their ubiquitous nature and have been successful to evade non-natural hosts as well on numerous occasions. This property of prevalence and subsequent emergence of viruses has been a major bottleneck in sustainable aquaculture production[3,4]. The presence of viruses in the latent stage and limited knowledge about viral etiology hinder their detection.

This article gives an extensive insight into some of the significant viral pathogens known to affect freshwater fishes in India, the existing diagnostics as well as procedures for disease prevention, and the way forward to avert prospective diseases in wild and farmed fish.

Ranavirus

Frog virus-3 (FV-3), the first ranavirus belonging to the family Iridoviridae, was a serendipitous discovery that paved the way for more information on the virus family[5]. At the outset, infected cases due to FV-3 and other iridoviruses were observed less in number and this prevented the researchers to view ranaviruses as a potential threat until more mortalities caused by them came to light[6]. Among the five genera of the family Iridoviridae, *Ranavirus* (in addition to *Megalocytivirus* and *Lymphocystivirus*) affects only poikilothermic vertebrates, viz. fish, amphibians and reptiles. They are emerging pathogens with a diverse host range possessing a large double-stranded DNA[7]. Ranaviruses have been reported from both freshwater and marine fish. Three species of *Ranavirus* infecting finfishes as mentioned by the International Committee on the Taxonomy of Viruses (ICTV) are EHNV (epizootic haematopoietic necrosis virus), LMBV (Largemouth Bass Virus) and ECV (European catfish virus)[8].

Aetiological agent of the disease

Ranaviruses approximately range from 150 to 200 nm in diameter in an enveloped form with icosahedral symmetry[9] (Figure 1) and have a genome size of 105–140 kb with 92–139 putative open reading frames (ORFs)[10]. The genome of EHNV, another species of *Ranavirus*, is 127 kb with 55% guanine–cytosine (GC) content, which is larger than the amphibian Ranavirus genomes that are typically 105 kb (ref. 9).

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Clinical signs

Infected fish are often seen with loss of buoyancy, erratic swimming, anorexia and red swollen gills. Haemorrhages in internal organs like fat bodies[11,12] and swim bladder may occur along with inflation of swim bladder[13].

Histopathology

Histopathological examinations comprise multifocal necrosis of renal haematopoietic tissue, spleen and gastrointestinal epithelium. In addition, acute focal hepatocellular necrosis and basophilic inclusions in spleen, kidney and hepatocytes are observed[14].

Host range

Ranaviruses are described as one of the emerging pathogens due to their ability to infect a large array of species at a given time[8]. The host range of *Ranavirus* is not restricted to a single taxonomic class and may infect other poikilothermic vertebrates[15]. Although the disease has not been reported in the wild, EHNV challenge studies have shown a minimum of 14 fishes to be susceptible, excluding the two natural hosts. The major bottleneck in EHNV detection has been the non-availability of infected living samples with proper reporting[16,17].

Geographical distribution

The members of *Ranavirus* have been detected from Australia (EHNV), Europe (ECV), North America and Asia (Santee Cooper Ranavirus)[16].

Outbreaks in India

The first case of ranavirus from India was reported in 2015 in koi (*Cyprinus carpio* L.) farms of South India with severe mortality. The isolated virus resembled the Santee Cooper Ranavirus[10]. Furthermore, a second detection of Ranavirus in the country in carps (*Puntius sarana* and *Osteobrama belangeri*) was from North East India[18]. In contrast to the above reports from freshwater, Ranavirus from marine ornamental fish *Pomacentrus similis* was isolated and characterized from ornamental farms in South India[19]. From the above reports, it seems that the virus is widespread and endemic to India.

Epidemiology

Epidemiology of ranaviruses has not been established and understood properly due to lack of detection methods in isolated places and deep waters. Little information on this aspect is available from studies conducted on EHNV in Australia and LMBV in Southeast Asia[14]. Despite being from the same genus, the patterns of mortality event caused by LMBV vary slightly from EHNV. LMBV affects adults and older largemouth bass (*Micropterus salmoides*) as noticed in 1990, but the occurrence of diseased or moribund fish is a rare event[13]. Thus, the common feature in comprehending the epidemiology of both the above-mentioned ranaviruses is their pattern of outbursts at a particular life-cycle stage at high temperatures (EHNV in juveniles and LMBV in adults). Ranaviruses transmission is contact-dependent. The affected fish may transfer the disease through water or fomites as well as by eating diseased fish[15].

Pathogenesis

Iridovirus replication has been best studied using the FV-3 model, a type species of the genus *Ranavirus*. The nucleic acid synthesis occurs in both nucleus and cytoplasm. The virus entry of both enveloped and non-enveloped virions occurs through a cellular unknown receptor. Between both virion types, the non-enveloped ones first interact with the plasma membrane and then core DNA is released into the cytoplasm as a result of uncoating, whereas the enveloped virions make their way into the cells through receptor-mediated endocytosis and are then released into the cytoplasm[20]. Consequently, virions are translocated into the nucleus and hijack the macromolecular machinery of the host followed by synthesis of viral polymerases to synthesize viral copies of DNA and transcript. Replication is controlled by viral gene expression, particularly
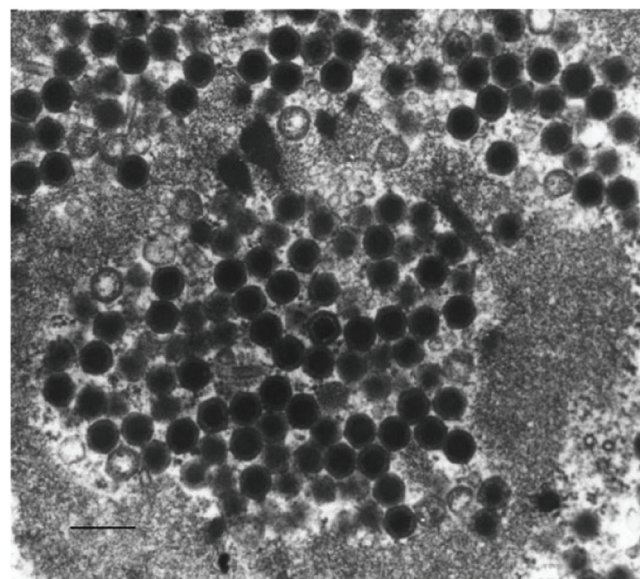
Figure 1. Transmission microscopy of KIRV (Koi ranavirus) propagated in SNKD2a cells showing icosahedral particles of 100–120 nm (bar = 200 nm). (From George *et al.*[10], reproduced with permission from John Wiley and Sons, License No. 4684781350278.)

in three distinct phases, viz. immediate–early, early and late early[21]. DNA copies formed are transported to the cytoplasm with concurrent DNA methylation and second stage of viral DNA synthesis[21]. Virus assembly of DNA and capsids occurs in a distinct location of the cytoplasm known as viral assembly sites. If the virions exit via budding, they form an envelope, or they are released without an envelope in case the cell is lysed[21].

Diagnosis

The diagnosis of Ranavirus should be done based on OIE protocols involving PCR, virus isolation, electron microscopy, immunohistochemistry, *in situ* hybridization, q-PCR and antigen-capture enzyme-linked immunosorbent assay (Ag-capture ELISA). Conventional PCR generally targets the *MCP* (major capsid protein) gene which is highly conserved and preferred for ranavirus detection, but phylogenetic differences among *Ranavirus* species cannot be achieved using this gene[20]. Other genes for PCR include neurofilament triplet H1 protein, DNA polymerase[22] and an intergenic variable region[23]. In recent times, q-PCR has provided more sensitivity compared to conventional PCR in terms of viral loads.

Prevention and control

Prior knowledge about viral disease distribution in various regions and disease surveillance are prerequisites when considering a regional control. Disease control requires efficient diagnostic tests, destocking to eliminate the occurrence in required areas, modification of guidelines to more stringent rules for facilitating trade, and mandatory health certification for imported and exported samples[16]. The disease can be managed by monitoring the stocking rates and water quality. Employment of specific pathogen-free (SPF) stocks as a biosecurity measure should be done to avoid transmission of infections among the farms.

Future research needs

Although the virus seems to be endemic to India, more emphasis on its surveillance needs to be given to identify carriers and susceptible species. Future research should focus on probable mechanisms favouring Ranavirus persistence in the environment outside the host, identification of host reservoirs and latent existence within the hosts using sensitive tools.

Megalocytivirus

Megalocytivirus (MCV) belongs to the family Iridoviridae along with genera *Iridovirus*, *Chloriridovirus*, *Rana-*

virus and *Lymphocystivirus*. Megalocytivirus is the newest addition to the family and infects cold-blooded vertebrates[24], viz. amphibians, fishes[25] and reptiles[26]. First evidence of megalocytivirus-induced mortality was seen in Japan in 1990 and was referred to as red sea bream iridovirus disease (RSIVD)[27]. Megalocytivirus has received much attention in the past few years due to its wide host range and mortality events[28].

Aetiological agent of the disease

The genome of megalocytivirus is a linear, double-stranded DNA molecule and belongs to the family Iridoviridae. The virus is enveloped with icosahedral symmetry. The average diameter is in the range 140–200 nm (Figure 2). Despite having a number of members in the genus, infectious spleen and kidney necrosis virus (ISKNV) and red sea bream iridovirus (RSIV) are the two most important representatives of Megalocytivirus[29]. Phylogenetic analysis of various megalocytivirus isolates, using MCP and ATPase genes, has revealed three clusters within it, namely RSIV, ISKNV and TRBIV (Turbot reddish body iridovirus)[24,30]. The genome size of RSIV is 112.4 kb with an approximate G + C content of 53%, and has similarity to Ranaviruses[31].

ISKNV, the other member of the genus, has a genome size of 111.4 kb with a G + C content of 54.78% (ref. 32). TRBIV consists of 110 kb genome with 55% G + C content. In total, 115 ORFs were detected coding 40–11468 amino acids[33].

Clinical signs and histopathology

Clinical signs and histopathological observations of all the three clusters (RSIV, ISKNV and TRBIV) are almost
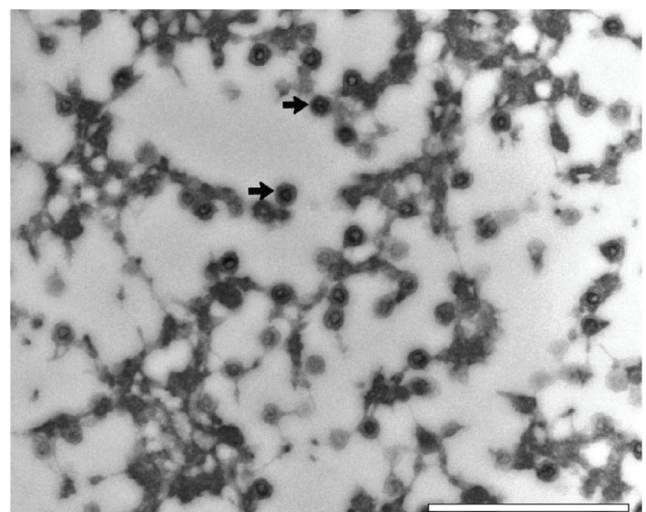
Figure 2. Transmission electron photomicrograph of intestinal submucosal cells of Nile tilapia showing intracytoplasmic polygonal-shaped virions (scale bar = 1 μm). (Reprinted from Subramaniam *et al.*[101], © Inter-Research 2016.)

the same. The diseased fish are lethargic, swim helplessly and exhibit severe anaemia, petechiae of the gills and enlargement of the spleen with 20–60% mortality (Figure 3). Histopathological examinations of RSIV include eosinophilic degenerated cells and basophilic enlarged cells in the spleen, heart, kidney, liver and gills. The pathognomonic sign includes appearance of inclusion body-bearing cells[34,35].

Histopathological studies of ISKNV were characterized by cell hypertrophy in the spleen, kidney, cranial connective tissue and endocardium[36].

Histopathological changes of TRBIV consist of enlarged basophilic cells in the splenic pulps and sheathed tissue along with such observation in the kidney, gills, intestine, stomach and heart[37].

Host range and geographical distribution

Megalocytiviruses are known to infect marine as well as freshwater fishes such as brown spotted groupers, Malabar groupers, orange chromide cichlid, red sea bream, angelfish, sea bass and African lampeye[38]. RSIV showed mortalities in red sea bream whereas ISKNV was highly pathogenic to mandarian fish (*Siniperca chuatsi*) and largemouth bass (*M. salmoides*). Moreover, TRBIV caused severe infections and mass mortality in turbot when compared to rock bream (*Oplegnathus fasciatus*) and Japanese flounder (*Paralichthys olivaceus*)[28].

RSIV seems to be present in tropical countries like Indonesia, Thailand, Singapore and Taiwan as well as temperate areas, namely Japan and Korea[39]. ISKNV-like viruses have been reported from 13 cultured fish species and 39 wild fish species including both freshwater and marine fish. Though RSIV, ISKNV and TRBIV belong to the *Megalocytivirus* as different members of the genus, there is need to resolve the ambiguity in their taxonomic classification based on multiple parameters apart from host species or geographical prevalence[28].
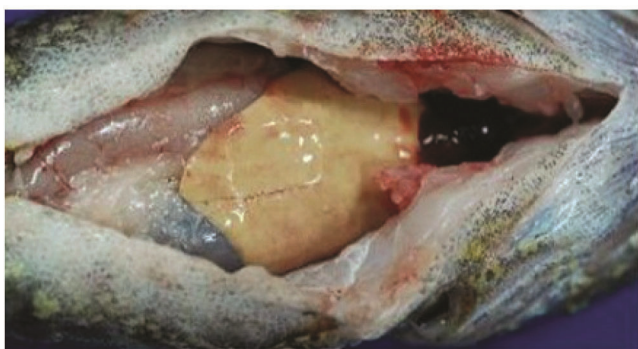
Figure 3. Image showing fatty liver of marble goby infected with red sea bream iridovirus. (Reprinted from Chen *et al.*[102], Copyright 2013.)

Outbreak in India

While the disease has been reported from Asian countries, there had been no report of MCV with a first preliminary report of 71% incidence in India from molly and angelfish samples obtained from ornamental fish market of Kurla East, Mumbai[30,40].

Pathogenesis

Being from family Iridovirus, *Megalocytivirus* viral replication follows the same replication mechanism as of FV-3 (ref. 41). At the onset of viral infection, the envelope protein is involved in virus attachment to the host receptors followed by fusion with host cell membranes. Previous studies indicate the entry of MCV via receptor-mediated endocytosis until a recent study in mandarian fish fry provided evidence of caveola-dependent endocytosis[28]. Virion DNA is transported to the nucleus where signal transduction of immediate early and delayed early genes is initiated. Progeny DNA is again transported back to the cytoplasm as rough granules and few more rounds of replication result in large concatemeric structures. The DNA undergoes methylation as is suggestive of the fact that methylation protects degradation of viral genome and prevents host innate immune response from getting stimulated. Late genes are activated once replication has started encoding structural proteins of virions for virus assembly[41].

Transmission

Global trade of ornamental fishes is the most common portal of viral entry in other regions of the world. ISKNV-infected mandarin fish transmit the disease in the natural hosts through contaminated water or by tissue ingestion from an asymptomatic carrier[42]. Moreover, in a cohabitation study on TRBIV, no signs were observed when Japanese flounder and rock bream were injected with TRBIV-infected homogenate from turbot but were detected PCR-positive, indicating that these species can serve as carriers[28].

Diagnosis

Histopathology and electron microscopy do not provide sufficient and precise information for identification of different Megalocytivirus species. The recommended method by Office International des Epizooties (OIE) is the use of molecular diagnostics (PCR) to differentiate RSIV and ISKNV employing two primer sets as a confirmation assay. In addition, an indirect fluorescent antibody test (IFAT) has also been used and recommended to identify RSIV and ISKNV in suspected fish[43]. For propagation

and isolation of RSIV, ISKNV and TRBIV specific cell lines have been used, namely CRF-1, MFF-1 (mandarin fish fry) and FEC (flounder embryonic cell line) respectively[43,44].

Recently, LAMP has been used for RSIV detection employing primers directed towards *Pst*-I restriction fragment of RSIV along with DNA microarray for characterization of the viral gene expression profiles in red sea bream infected with RSIV homogenate[28].

Prevention and control

Megalocytivirus has a wide host range with mostly similar or no clinical signs. The steps towards disease prevention include achieving alkaline pH around 11 by disinfecting the reservoirs such as ponds and tanks with sodium hypochlorite or potassium permanganate at 100–200 mg/l or higher. Further, for achieving sterile conditions, small tanks may be irradiated with UV radiation to reduce drastically the pathogenic flora[28].

Future research needs

The collection of adequate data about all known genotypes of megalocytiviruses and practising advanced techniques for obtaining optimized results should be implemented in concerned research laboratories. Active targeted surveillance would be able to point towards incidence and any loss to this virus.

Herpesviral hematopoietic necrosis

The causative agent of herpesviral hematopoietic necrosis (HVHN) is cyprinid herpesvirus-2 (CyHV-2), which belongs to the family Alloherpesviridae, besides other two members of the same family infecting fish, i.e. carp pox (CyHV-1) and koi herpes virus (CyHV-3). It is intriguing to note that CyHV-2 which primarily affects goldfish (*Carassius auratus*), does not infect koi (*C. carpio* L.) and common carp though both are hosts for CyHV-1 and CyHV-3 (ref. 45). CyHV-2 is a linear, double-stranded DNA virus of 290 kb genome (approx.) with 51.7% GC content that multiplies in the nucleus, and consists of 154 predicted ORFs and terminal direct repeat of 15,353 bp (ref. 46).

Viral morphology

Ultrathin sections of spleen and kidney from virus-infected goldfish revealed round, enveloped virions measuring 170–220 nm, in addition to hexagonal nucleocapsids of diameter 115–117 nm (ref. 47). In another study, similar hexagonal viral particles with nucleocapsid of 100–110 nm in diameter were observed[48]. Studies conducted on virus-infected Prussian carp, *Carassius gibelio* showed many hexagonal, enveloped virions of slightly larger average diameter of 170–220 nm (ref. 49).

### Clinical signs and histopathology

The diseased fish exhibit apathy, anorexia, pale gills and pale skin patches[50,51]. Internal signs include enlarged kidney and distended spleen with white focal nodules[52] (Figure 4). Histopathological studies have demonstrated that the infected fish exhibit necrosis of hematopoietic tissues of spleen and kidney.

Species susceptibility and geographical range

CyHV-2 causes large-scale mortality in its host, goldfish (*C. auratus*). CyHV-2 has also been associated with mortalities of Prussian carp (*C. gibelio*)[53] and crucian carp (*Carassius carassius*)[51]. Recent co-infection studies have shown that the disease can be transmitted to bighead carp (*Aristichthys nobilis*), culter alburnus (*Erythroculter ilishaeformis*), silver carp (*Hypophthalmichthys molitrix*) and black carp (*Mylopharyngodon piceus*)[54]. The disease was first reported in Japan[55]. It appears to be widespread and has been detected in a number of countries across the globe, including USA, UK, Australia, Switzerland, Taiwan, China, Italy, Czech Republic and France[4].

Outbreaks in India

Till now, there have not been catastrophic losses due to viral diseases in India, until recently an outbreak of CyHV-2 associated with multi-drug resistant *Aeromonas hydrophila* caused mortality in goldfishes which were cultured in commercial Indian farms from Hooghly district, West Bengal[4]. The infection has now been established in India in many states since then. A recent survey from goldfish samples obtained for viral screening in our laboratory recorded an incidence of 35.71% of CyHV-2 infections in Odisha and West Bengal (P. K. Sahoo, pers. commun.). An experimental study revealed that the Indian major carps and koi carp are not susceptible to this virus infection[4].
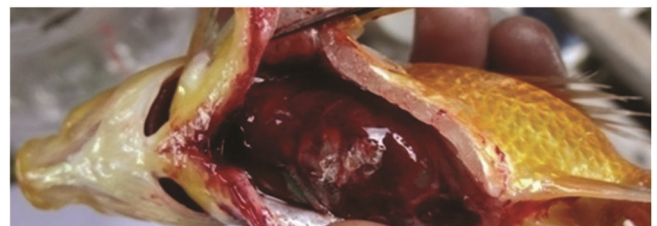
Figure 4. Internal examination of goldfish showing enlarged spleen with white focal nodules.

Diagnosis

Quantitative-PCR have been found to be a more appropriate diagnostic than conventional PCR, as it distinguishes asymptomatic virus from replicating virus which eventually leads to mortalities on exposure to any environmental stressor. Additionally, PCR may produce faulty results giving cross-amplification with that of the CyHV-1 size amplicon[45,56]. Isolation of the virus in susceptible cell lines, viz. KF-1 or any other cell lines of goldfish remains to be the gold standard along with histopathology.

Prevention and control

Most of the herpes-viral infections are temperature-specific, and can only be prevented by managing stress and temperature. In CyHV-2, diseases can be prevented if the temperature is raised to 27°C or above, as this helps the fish immune system to overcome the disease. General prevention practices include quarantine, hygienic maintenance of hatcheries and tanks, antibiotic treatments against secondary pathogens and regular observation for clinical signs, besides biosafety measures.

Future research needs

As the virus is endemic to the country, it is essential to prevent this infection in goldfish farms in terms of developing suitable vaccines and adoption of biosecurity measures at the farm level. As this goldfish species is traded in both ways (import and export), it is essential to monitor quarantine facilities to prevent the entry of new strains. In this regard, development of rapid field-level diagnostics is required, which can be used at the farm site. Further extending research scopes, scientists may initiate with the production of resistant hybrid species.

Carp Edema Virus

A historical view

Carp edema virus was first reported in Japan in the 1970s (ref. 57). The two most characteristic features of the disease include lethargy and gill necrosis, accompanied by a high mortality rate. Viral edema of carp (VEC) and koi sleepy disease (KSD) caused by carp edema virus are different despite having mostly similar pathological signs, which are governed by age and size of the fish[58]. In KSD, the fish generally lie at the bottom, show less edema, congested gills, alterations in skin near mouth and fin base, and infection of respiratory epithelium of gill lamellae[59,60]. VEC is characterized by severe oedema and swollen trunk in small fries. Adult fish exhibit enophthalmia and swollen gills followed by death in a day or two. Juveniles in VEC often congregate beneath the water surface in contrast to KSD[61].

Aetiological agent of the disease

Carp edema virus belongs to family of Poxviridae and subfamily Chordopoxvirinae[62]. It is a mulberry-shaped, enveloped, double-stranded DNA virus of about 250–280 nm diameter[63]. Transmission electron microscopic (TEM) studies revealed virions in enlarged epithelial cells of gill lamellae[60]. In contrast, ovoid virions with comparatively larger diameter of 360 nm in the cells of the gill epithelium were observed[64]. Phylogenetic analysis resulted in two genogroups–I and II sharing a 93–96% identity. A third genogroup (IIb) was assigned to the left sequences having 95.4–96.5% and 97.5–98.6% identify to genogroup I and IIa respectively[59].

Clinical signs and histopathology

Fish infected with the disease show skin edema of underneath tissue. Other signs include excess of mucus in gills, sunken eyes, anorexia, ulcerations near mouth and fin base[60,65]. Histological studies with respect to carp edema exhibit mucus cell disappearance, edema and atrophy of the epidermal cells, whereas in KSD, erosion and ulceration of epidermal cells occur[58].

Environmental factors favouring disease occurrence

Water temperature is one of the principal factors associated with occurrence of the disease in koi and common carp. The virus can get detached or sloughed-off from epithelial cells of the gill filament[60]. The disease is typically observed at water temperatures between 15°C and 25°C (59–77°F) in koi, and at 6°C and 10°C (43°–50°F) in common carp, and can kill up to 75–100% of juvenile koi during an outbreak[64,66]. In contrast, water temperature of 6–9°C was seen to trigger KSD in England, UK[59].

Species susceptibility and resistance

The natural hosts for CEV infection are common carp and koi. Jung-Schroers *et al.*[67] observed no signs of disease in goldfish and sturgeon reared along with infected koi in the same farm in Germany. In another experiment, co-cultured fishes (resident koi carp, goldfish and Indian major carps) along with infected koi showed no signs of disease[65].

Geographical distribution

Carp edema virus was initially characterized from koi carp in Japan in 1974. The first report of CEV in England

was from imported koi in 2009, followed by more KSD cases in common carp and koi. Outbreaks of the disease were reported from other European countries and India[66].

Outbreaks in India

Through previous years, koi carp culture has rapidly progressed majorly in Kerala, Tamil Nadu, West Bengal and Maharashtra in India. The first outbreak of carp edema virus was reported in 2016 in Ernakulam district, Kerala, causing 100% mortality[60,65]. Another study reported CEV infections from koi ornamental farms in Odisha with 100% mortality[68]. Widespread occurrences of CEV have been detected in European countries over the past years and similar reports might come up in future in India in the states involved in koi and common carp trading.

Pathogenesis

Poxviruses replicate in the host cytoplasm unlike other large DNA viruses without utilization of the host machinery for gene expression[69]. They have rapid adaptability towards variable hosts attributed to rearrangements of the terminal sequences which provides evolution of variable functions[70]. The probable reasons pertaining to infection in specific organs at the initiation of pathogenesis and subsequent spread to the entire body is quite unique due to the versatility of poxvirus multiplication strategies.

Diagnosis

Since the first outbreak until the recent reported cases, researchers have used conventional PCR for the detection of CEV for partial 4a gene and 5′-UTR fragment gene. Partial 4a gene is vital in virus detection because it is necessary for assembly of nucleoprotein complex over the host cell[71]. However, real-time PCR and end-point PCR detection prove to be more specific and sensitive. According to the recent advancements, PCR has been revolutionized by modified versions such as RPA (Recombinase Polymerase Amplification) possessing high specificity which can readily differentiate CEV from KHV DNA in the tissue sample[59,71].

Prevention and control

Sudden fluctuations with respect to seasonal changes or transportation conditions may cause a state of stress, making them prone to virus invasion. Therefore, it is advisable to maintain clean environments for keeping fish away from viruses by regular cleaning through disinfectants and drying out the tanks before stocking. In case of CEV symptoms, 0.3–0.5% of salt treatment is found to suppress the infection to certain extent as this removes secondary pathogens, including bacteria and parasites from the host body, inducing antiviral immunity against the virus[72].

Future research needs

Of all the existing known viruses of fish, accurate detection and prevention should be the matter of concern for researchers. Challenges in vaccine development can be overcome by getting a clear picture of the infection mechanism of the virus through various experimental studies. Identifying a susceptible cell line is a big challenge and this needs to be addressed.

Tilapia Lake Virus

Tilapines are one of the most widely farmed fish species. Viruses were not considered as a major threat to tilapia aquaculture until the 2009 outbreaks in Ecuador and Israel due to Tilapia Lake Virus (TiLV). Both countries experienced massive mortalities in farmed tilapia. TiLV is the infectious agent that affects both wild and farmed tilapines[73].

Aetiological agent of the disease

TiLV is an enveloped virus with an average diameter range between 55 and 100 nm (ref. 74) (Figure 5). It has a genome size of 10.323 kb and consists of 10 genome segments, each of which possesses an ORF. The ORF of segment 1 shows weak sequence homology with the PB1 subunit of influenza C virus. The remaining nine segments possess no homology to other viruses. However, the
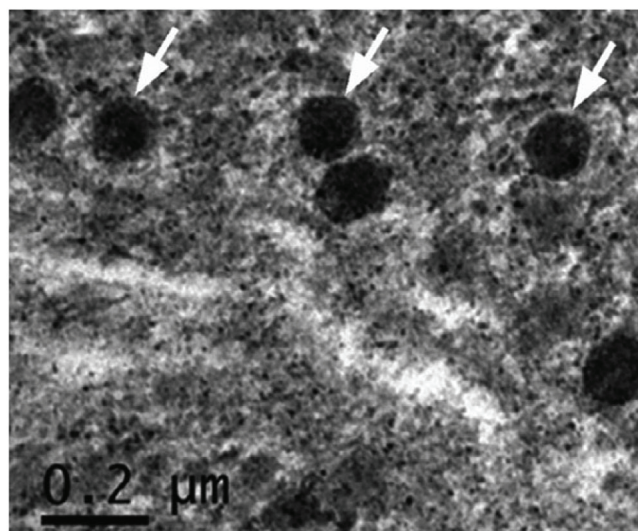
Figure 5. Transmission electron photomicrograph of TiLV-infected fish liver showing cytoplasmic viral particles. (Reprinted from Jansen et al.[76], Copyright 2018.)

complementary sequences at the 5′ and 3′ termini were conserved and consistent with the genome organization found in orthomyxoviruses. Due to this homology with orthomyxo-like viruses, TiLV was classified under family Orthomyxoviridae[73,74]. Recently, a taxonomic proposal stating the reclassification of TiLV has been submitted to ICTV for a new unassigned genus *Tilapinevirus*, where TiLV is a representative isolate[75].

Clinical signs

The fishes infected with the disease exhibit lethargy, ocular alterations, abdominal distension, scale protrusion, gill necrosis, exophthalmia, abdominal swelling, skin erosions and discoloration[76].

Histopathology

Histopathological examinations of brain displayed edema, focal haemorrhages in meninges, white and grey matter congestion, foci of gliosis and encephalitis[74,77]. Liver of infected fish showed edema of hepatocytes, syncytial giant cells, multifocal areas of necrosis and the spleen displayed eosinophilic intracytoplasmic inclusion bodies along with increased melano-macrophage centres[78].

Host range

Susceptible wild fish species detected to be positive for TiLV include *Sarotherodon galilaeus*, redbelly tilapia (*Tilapia zilli*), blue tilapia (*Oreochromis aureus*) and wild tilapia (*Tristamellasimonis intermedia*), wild black (*Oreochromis placidus*) and Nile tilapia (*Oreochromis niloticus*). The virus also causes large die-offs in farmed fishes like hybrid tilapia (*Oreochromis niloticus* × *O. aureus* hybrid), Nile tilapia and red tilapia[76]. There are no reports of TiLV infecting co-cultured fishes with tilapines, as was evident from mass mortality in Israel causing no disease in grey mullet (*Mugil cephalos*) and carp (*C. carpio*)[74]. It affects all developmental stages of tilapia; however, fingerlings and juveniles were more susceptible to TiLV[79]. Various cohabitation experiments show that TiLV is transmitted horizontally between infected and unaffected fish. Vertical transmission is also potential evidence of transmission of TiLV from broodstock to progeny[80].

Geographic distribution

The disease was first reported from freshwater in Israel in 2014 (ref. 74). Subsequently, the virus was also reported in freshwater and brackish water of Ecuador, Colombia, Egypt, Thailand, Malaysia and India[60,77].

Outbreaks in India

The first outbreak of TiLV was 2016 from West Bengal, followed by more breakouts in two geographically isolated states of India, namely West Bengal (South Paraganas) and Kerala (Ernakulam) in July 2017 (ref. 77). TiLV caused 85% mortality.

Replication

In situ hybridization (ISH) studies have revealed liver and central nervous system to be the sites of replication and transcription in infected tilapia. In the brain, hybridization affinity of segment 1 of genomic RNA and mRNA was restricted to the leptomeninges, mostly adjacent to the blood vessels. Hybridization signal for segment-3 mRNA was detected in hepatocytes along with numerous clustering of nuclei. TiLV mRNA was detected in both the nucleus and cytoplasm of multiple cells[73].

Diagnosis

Initially, RT-PCR was used for TiLV detection with TiLV-specific primers[74]. The nested RT-PCR assay was found to detect TiLV in both fresh and preserved diseased fish samples from Israel, Ecuador and Colombia. Real-time PCR for quantifying and detecting TiLV was found to be most reliable and sensitive to detect asymptomatic carriers in wild and available broodstock, field samples and experimentally challenged fish[81]. Furthermore, a rapid, one-step reverse transcription, loop-mediated isothermal amplification (RT-LAMP) method was developed having high specificity and sensitivity than RT-PCR[82].

Prevention and control

There is no treatment for TiLV. It has to be managed through farm-level management interventions, stricter biosecurity controls, proper quarantine and restricted/regulated movement of animals from the affected farms[78]. A risk analysis should be conducted for any live tilapia entering the country as seed or brood fish for aquaculture purposes. Moreover, an emergency preparedness should be planned to deal with any covert outbreak.

Future research needs

Various cohabitation experiments must be performed to get an insight into the current knowledge on real geographical distribution of TiLV. More studies regarding the epidemiological aspects of TiLV could help in preventing future mortality events. There is an urgent need for analyzing genetically improved strains of tilapia to overcome the problem of its susceptibility to viral pathogens along with development of effective vaccines.

Viral Nervous Necrosis Virus

Viral Nervous Necrosis, also known as viral encephalopathy and retinopathy is caused by an RNA virus which belongs to family Nodaviridae, genus *Betanodavirus*. It affects a broad host range due to its high pathogenicity and is often associated with mass mortality in fish juveniles[83]. The first nodavirus infection associated with brain lesions in barramundi (*Lates calcarifer*) was detected in 1987 (ref. 84).

Aetiological agent of the disease

Viral Nervous Necrosis Virus is a non-enveloped virus with icosahedral symmetry, and the diameter of the capsid falls in the range 25–34 nm with bisegmented RNA genome (Figure 6). The genome is 4.5 kb in size, made up of two single-stranded positive-sense RNA segments, RNA1 (3.1 kb with 49.6% G + C content) and RNA2 (1.4 kb with 53.24% G + C content), which encode two viral replicase proteins and a capsid protein respectively[85]. In addition, during virus replication which occurs in the cytoplasm, a subgenomic transcript known as RNA3 originating from the 3′-terminus of RNA1 helps in viral RNA accumulation into the host cells[86].

Phylogenetic studies on the T4 variable region within the RNA2 segment have classified betanodavirus into four genotypes, namely red-spotted grouper nervous necrosis virus (RGNNV), barfin flounder nervous necrosis virus (BFNNV), striped jack nervous necrosis virus (SJNNV) and tiger puffer nervous necrosis virus (TPNNV). Among these, the most commonly occurring and widespread is RGNNV[85,86]. Moreover, two additional genotypes have been proposed based on the characterization of partial RNA2 sequences, viz. the Atlantic cod nervous necrosis virus (ACNNV) and the turbot nodavirus (TNV)[86].
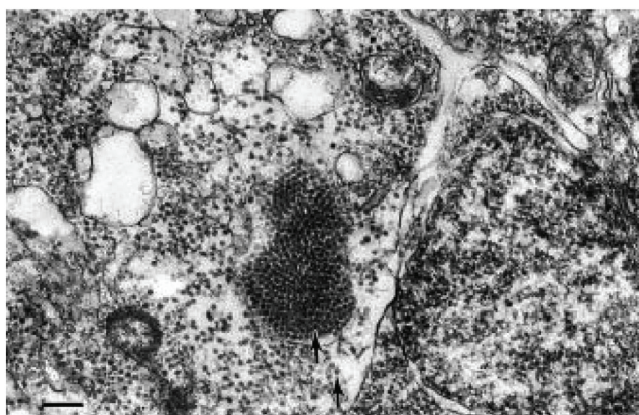
Figure 6. Transmission electron photomicrograph of betanodavirus particles in the brain of a barramundi larva (bar = 250 nm). (From Munday *et al.*[83]; reproduced with permission from John Wiley and Sons, License No. 4686450741036.)

Clinical signs

Fish infected with betanodavirus show symptoms like loss of appetite, darkened body, abnormal swimming pattern, swim bladder inflation and neurological dysfunction[85].

Histopathology

Histopathological examinations include retinal lesions, degeneration, vacuolation and gliosis throughout the central nervous system of the fish. Degree of vacuolation is high in the grey matter of the optic cerebellum[83].

Host range

Betanodavirus has a broad host range and has been reported from both the wild and cultured farm (freshwater as well as cold water marine fish) fishes[86]. The virus affects more than 40 fish species which include Japanese parrotfish, red spotted grouper, striped jack, barramundi, turbot, tilapia and European seabass. Fish in larval or juvenile stage are more susceptible to the disease[83]. Due to diverse host range, various betanodavirus from freshwater fishes have been detected in Chinese catfish (*P. asotus*); Australia catfish (*Tandanus tandanus*), barramundi (*L. calcarifer*), medaka (*Oryzias latipes*), guppy (*Poicelia reticulata*) and zebrafish (*Danio rerio*)[85].

Geographical distribution

The disease has been reported in Japan, Korea, Taiwan, China, the Philippines, Thailand, Vietnam, Malaysia, Singapore, Indonesia, Brunei, India, Australia, Israel, Croatia, Greece, Italy, France, Spain, Portugal, Norway, USA and Canada[83,86].

All the four genotypes display specificity towards different optimal growth temperatures when cultured *in vitro*: 25–30°C for RGNNV, 20–25°C for SJNNV, 20°C for TPNNV and 15–20°C for BFNNV[86]. It has been reported that temperature differences influence the host specificity, occurrence and distribution of betanodaviruses and its variants[87]. Also, there have been few evidences of co-occurrence of SJNNV and RGNNV genotypes in a different variant having RNA1 of SJNNV genotype and RNA2 molecule from RGNNV-type[88].

Outbreaks in India

The first report of piscine nodavirus in seabass (*Lates calcarifer*) larvae was from Chennai[89]. Consequently, isolation and characterization of VNNV were performed from Asian seabass (*L. calcarifer*) collected from hatcheries located in Tamil Nadu, India, and its larvae experienced mass mortality[90]. Another nodavirus infection was reported from freshwater aquarium fishes, namely gold fish

(*Carassius auratus auratus*) and rainbow shark (*Epalzeorhynchos frenatum*) near Chennai, India[91]. A similar detection of VNNV was reported from hatchery-reared larvae of clownfish, *Amphiprion sebae* Bleeker[92], and experimental lot of goldfish and zebrafish in India[93]. Recently, Banerjee *et al.*[94] reported nodavirus-associated mortalities in Asian seabass from Kasargode district, Kerala, collected from four different cage cultures. Furthermore, suspected seabass samples collected from Kakdwip, West Bengal, accounted for 19.51% incidence of betanodavirus infection, according to screening results from our laboratory (P. K. Sahoo, pers. commun.). The wild seeds of seabass collected from both the east and west coast of India were also found to be positive for RGNNV in a recent study, thus proving the latency or persistence of virus in healthy seeds for further increasing the chance of infection in the culture system[95].

Transmission

The route of transmission of betanodavirus can either be horizontal or vertical. Horizontal transfer occurs if there is direct contact with the infected fish, infected water or any equipment which is contaminated[85]. Horizontal spread is also affected by factors like temperature, virulence of the viral strain and stocking density[83]. Vertical transmission has been described from the brooder to the eggs in striped jack (*Pseudocaranx dentex*), sea bass (*Dicentrarchus labrax*) and barfin flounder (*Verasper moseri*)[96].

Diagnosis

Various techniques have been employed for detection of betanodaviruses, like conventional PCR, nested PCR, electron microscopy, serological assays and cell culture-based isolation[83]. However, real-time PCR is the most rapid and sensitive detection tool for viral detection, and has been used to study the transmission routes and developmental methods of betanodavirus infections in fish juveniles[97]. ELISA is also a popular method when testing for unknown fish sera and a large number of samples can be screened quickly. The level of sensitivity of the technique may vary and therefore it is helpful when applied to epidemiological studies and zonal outbreaks[83]. Another method of betanodavirus confirmation is the use of immunofluorescent antibody testing with polyclonal and monoclonal antibodies[85]. Recently, efforts have been made to develop a sensitive automated microfluidic chip and a lateral flow paper biosensor for detecting nodavirus in groupers and European seabass respectively[98].

Prevention and control

To devise a definite control strategy against VNN infections, knowledge about the transmission mechanism is a prerequisite. Asymptomatic broodstock or larvae could serve as reservoirs for horizontal transfer. In this case, infected fish and suspected carriers should be kept in confinement to prevent any introduction or episodes of mass mortality in the hatchery or farm. Some studies have revealed virus inactivation and disease prevention (horizontal transfer) on application of disinfectants. Vertical transfer can be avoided either by eliminating the use of infected spawners in production or using ozone-disinfected fertilized eggs[99]. Moreover, a wide range of vaccines have been used against betanodavirus, such as inactivated whole viruses, recombinant capsid protein, viral like particles and recombinant DNA[100]. In agreement with the above reports, the best way to prevent betanodavirus introduction or spread is to abide by strict biosecurity measures, in addition to periodic disinfection.

Future research needs

All the reported cases of VNNV from wild and hatchery-reared fish indicate an alarming concern regarding the expanding host range of betanodavirus in India and other countries. Despite the availability of current and efficient diagnostic methods, undiagnosed carriers typically pose a major problem in virus spread, and require attention. Furthermore, additional experimental studies need to be performed on fish which are not natural hosts for the disease, as this may help in the identification and critical assessment of risks associated with disease translocation in aquaculture production systems. The underlying mechanism of salinity-tolerant strains of marine origin causing infection in freshwater milieu needs to be unravelled. Thus, understanding epidemiology, pathogenesis and transmission patterns in detail can provide a helping hand in controlling the disease.

Conclusion

Transboundary infections are transmitted easily through extensive trade networking. The current scenario depicts wide distribution of multiple viral pathogens in freshwater aquaculture systems. Apart from all the reported cases of outbreak in India, there still exist chances of persistent carriers in the wild as well as in farms. In recent years, few virus occurrences have come to light in India, where no reports of mass moralities due to viruses were accounted before. Using the information and data compiled till now of all fish viruses, this article concludes that viruses as pathogens have similar mechanisms of transmission and infection within variable hosts. On this ground, it is essential for researchers to concentrate on resolving the role of viral genes involved in replication and immune evasion strategies, and conducting knockout experiments to decipher the host spectrum of specific fish viruses. There must be more focus on vaccine development for
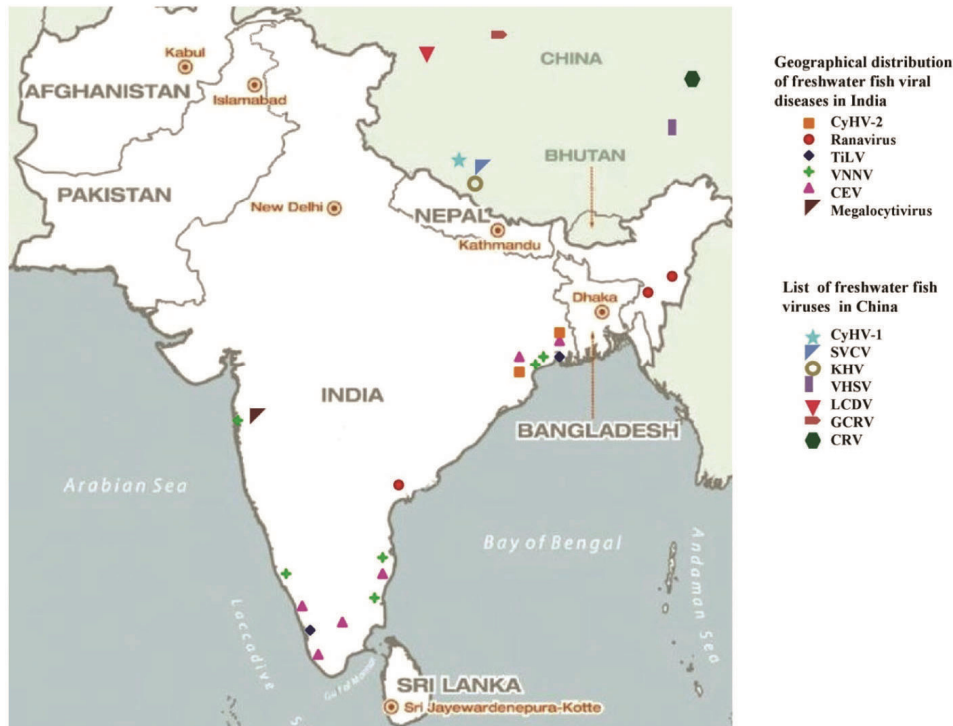
Figure 7. Map showing regions of India positive for freshwater fish viral pathogens and a list of detected viruses in China.

commercially and economically important fishes. This increases the necessity of enhancing and strengthening the surveillance studies and quarantine in the freshwater culture system. Further, the porous border or illegal trade with the neighbouring countries harbouring an array of reported viruses has put a big question mark on the country's effective surveillance system. It needs to be strengthened to identify any other persisting or latent infections in healthy fish. Considering the above scenario, there is a constant threat of transboundary migrations of viruses from neighbouring countries like China (Figure 7).

Conflict of interest: The authors declare no conflict of interest.

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