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REVIEW ARTICLE

Viruses Associated with Epizootic Ulcerative Syndrome: An Update

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Abstract Epizootic ulcerative syndrome is one of the most serious infections noticed in the finfish of Asia-Pacific during the last three decades. Different viral agents and a consistent fungus were isolated from the EUS infected fishes from various countries. Rhabdoviruses are by far the most isolated group of viruses followed by birnaviruses. One reovirus and a ranavirus have also been isolated from ulcerated fishes of which the ranavirus was capable of reproducing the clinical signs of the disease. Albeit heterogenic, due to frequent isolation, range of areas from which isolates have been obtained and ability to induce dermal lesions in experimental infectivity studies make rhabdoviruses one of the likely primary aetiological agents that could be triggering the initiation to EUS. However, further investigations may be required to fully establish the role of these viral agents in the induction of EUS. Viruses observed as persistent infections in fishes evidenced by their presence in cell cultures also require further investigation for their likely role in predisposing the fishes to EUS.

Keywords Epizootic ulcerative syndrome · Birnavirus · Rhabdovirus · Reovirus · Ranavirus · *Aphanomyces invadans*

Introduction

Epizootic ulcerative syndrome (EUS) was a term coined to describe a severe cutaneous ulcerative epizootic condition affecting many species of wild and farmed freshwater and estuarine finfish [64] which has spread across Southeast Asia, India and later to Pakistan and Africa. Due to the consistent presence of the fungus in the infected fishes, EUS was later defined to include the presence of invasive *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response [63]. Now it is suggested to rename EUS as epizootic granulomatous aphanomycosis [6]. EUS is currently recognised as similar to Red Spot Disease (RSD) in Australia and ulcerative mycosis in Western Atlantic estuaries and mycotic granulomatosis (MG) of cultured Ayu (*Plecoglossus altivelis*) in Japan. EUS infected fish is characterised by the presence of single or multiple lesions of varying shapes with acute dermatitis, hyperaemia and oedema leading to the development of typically large shallow or deep ulcers with a haemorrhagic necrotic base anywhere in the body with muscular inflammation and multiple granuloma [43] (Figs. 1, 2). EUS is one of the listed diseases of fishes as per OIE, 2012. The disease has been found to infect more than 100 species of fishes and cause huge economic losses. It causes an annual loss of 0.7 million USD in Australia, while in Thailand, EUS caused a loss of 100 million USD during the 10 year period from 1983 to 1993. Bangladesh suffered a revenue loss of 4.8 million USD during the 2-year period of 1988–89 due to EUS. The disease also caused a loss of 0.3 million USD loss in Pakistan in 1996, while in Indonesia the loss was to the tune of 0.235 million USD during 1980–87. Several pathogens were isolated from EUS infected fishes including viruses [See 19 for a review]. In this review we attempt to cover the viral agents thenceforth identified in association with EUS and the studies involving viral agents isolated from EUS.

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Fig. 1 Snakehead fish infected with EUS showing ulceration in the head region and onset of the lesion on the side

Fig. 2 Typically EUS infected snakehead fishes with deep ulcers and heavy loss of muscle tissue (Courtesy S.D. Millar)

Geographical Spread of EUS

After the eruption of MG in farmed freshwater ayu (*P. altivelis*) in Japan in 1971 [18] and RSD in estuarine fish, grey mullet (*Mugil cephalus*) in eastern Australia in 1972 [53], a progressive spread of a syndrome associated with dermal ulceration and involving large scale mortalities in a number of freshwater and estuarine fish species have been observed westwards across Asia. An ulcerative disease outbreak of the nature of EUS occurred in the rivers of southern Papua New Guinea during 1975–76 [25]. The first confirmed report of the characteristic manifestation of severe ulcerative disease was in peninsular Malaysia in 1979–80 [69]. High mortalities have been observed in gudgeon (*Ophieleotris aporos* and *Oxyeleotris heterodon*) from inland areas and mullet from estuaries in northern Papua New Guinea region during 1982–83 [12]. EUS further spread through southeast Asian countries to south Asia since seventies through Singapore in 1977 [64], Thailand in 1981 [72], Myanmar, the Lao PDR and Cambodia in 1984 [46], The Philippines in 1985 [54], Srilanka in 1987 [13], Bangladesh in 1988 [7], Nepal [70], Bhutan [62] and north India in 1989 [16], Indonesia in 1990 [65] reaching peninsular India in 1991 [15, 56, 67]. Later it has been spread into Pakistan in 1996 [11, 35] in USA in 1997 [8] and in 2007 in Botswana, Namibia and Zambia in southern Africa [4] (Fig. 3). EUS has now been reported from 24 countries in four continents: North America, southern Africa, Asia and Australia in which finfishes in wild and aquaculture farms are prone to the attack of EUS [57].

Species Affected

A wide range of freshwater and brackishwater wild and cultured fish involving over 100 species have been affected by EUS [46]. Snakeheads were by far the most seriously affected species, even though species like *Puntius* spp., catfishes (*Heteropneustes fossilis*, *Clarias batrachus*), Indian major carps (*Catla catla, Cirrhinus mrigala, Labeo rohita*), climbing perch (*Anabas testudineus*), mullet (*Mugil cephalus*), gobies (*Glossogobius giurus*, *Oxyeleotris marmoratus*), spiny eel (*Mastacembalus armatus*), swamp eel (*Fluta alba*), gouramis (*Trichogaster pectoralis*) were also among those seriously affected. Among snakeheads, *Channa striatus* was the most severely affected while *C. marulius* and *C. punctata* were affected to a slightly lesser extent [21]. A few commercially important freshwater and brackish water species including milkfish (*Chanos chanos*), tilapias, Chinese carps were found consistently resistant to EUS [2, 63].

Aetiology

Although the role of the pathogenic fungi, *Aphanomyces invadans* has been proved in the spread of EUS [1, 45, 68, 73], the agent by itself has not been hither to shown to invade and induce the disease. Hence the role of different agents including infectious and non infectious has been implicated in the onset of the disease. Bacterial pathogens have been consistently found associated with EUS infected fishes [9, 10, 30, 39, 52, 58, 60]. Experimental infections using *Aeromonas hydrophila* isolated from EUS infected fishes were found to induce EUS –like lesions in fish [5, 40, 47, 51]. A recent report suggested possible entry of the zoospores of *A. invadans* through intact skin of Atlantic menhaden (*Brevoortia tyrannus*) [42]. However, the study indicated that the entry could have been through the epidermal cells which have lost its integrity. Role of

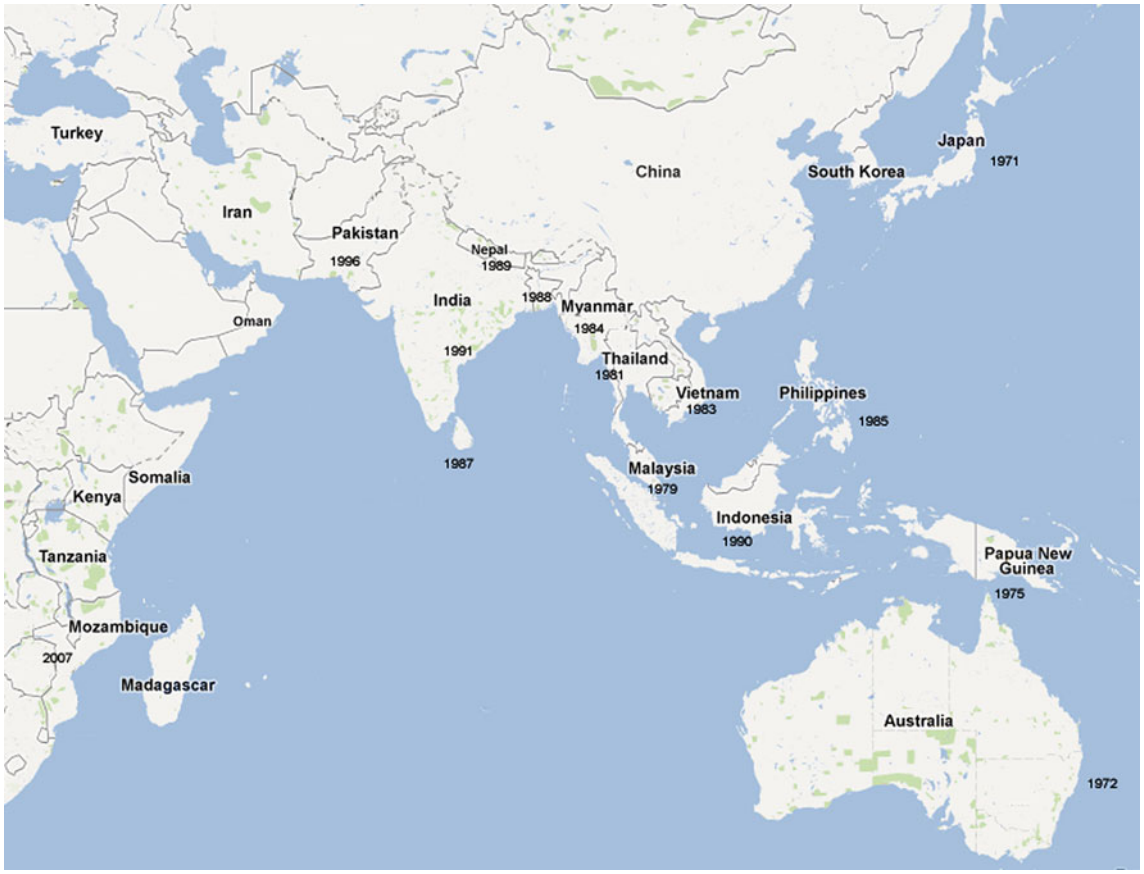

Fig. 3 Map showing the geographical spread of EUS in the last three decades

environmental factors like high rainfall, poor water quality, low pH in the induction of EUS has been documented [14, 59]. Low salinity and sudden rainfall were implicated in EUS induction in mullets in estuaries [55]. They noticed that an increase in salinity increases healing of ulcers.

Viral Association in EUS Infection of Finfishes

Since 1983, several types of viruses have been observed and isolated from fishes infected with EUS [19]. These viral isolates were principally rhabdoviruses and birnaviruses. A single isolation of a reovirus-like agent was also made during the 1992 Thailand epizootic [63]. A ranavirus isolation is also reported recently from an ulcerated fish from China [17].

Viruses Observed in Electron Microscopy

Presence of virus particles in the tissues of EUS infected fishes was first demonstrated by electron microscopy. The initial observations of viral association with EUS were noted by transmission electron microscopic studies of tissues of diseased striped snakehead fish and walking catfish specimens obtained from the epizootic in Thailand during 1983 and 84. Presence of virus-like particles was reported in hepatocytes, spleen, kidney and blood cells, capillary endothelium and muscle myofibrils taken from the site of ulceration in diseased specimens [74–76, 78]. These particles observed in the cytoplasm were of approximately 70 nm diameter but could not be precisely identified as they were pleomorphic with shapes varying from round, oval, elongate to kidney shaped. Particles of rhabdovirus-like morphology were noticed in skin lesions of infected barramundi (*Lates calcarifer*) [61]. Presence of rhabdovirus and infectious pancreatic necrosis virus like particles in cell cultures inoculated with tissue extracts from EUS infected snakeheads was noted by Wattanavijarn et al. [77]. Rhabdoviruses were also observed in cell cultures inoculated with tissue extracts from a variety of infected fishes including that of a barramundi, which showed extensive cytopathic effect [29].

Viruses Isolated in Cell Culture

Birnaviruses

Birnaviruses belonging to the coldwater *Infectious pancreatic necrosis virus* (IPNV) serotypes and different from

the existing reference strains (Sp, Ab, VR299 and TV-1) were isolated from EUS infected fishes. The first isolation of a virus associated with EUS was from an ulcerated sand goby (*Oxyeleotris marmoratus*) in Thailand [27]. The agent named sand goby virus (SGV) was found to belong to the family *Birnaviridae* although the biological, serological and biochemical characteristics of the virus differed from the existing reference strains of IPNV VR299, Sp and Ab. Although SGV was found to replicate well at temperatures ranging from 5 to 30 °C, its role in inducing EUS was not determined. SGV was later reported to be a mixture of two strains Sp and Ab of IPNV based on a comprehensive reciprocal cross- neutralisation study [28]. Second isolation of another birnavirus was from EUS infected snakehead fish (*Channa striata*) in Thailand in 1986 [66]. The snakehead fish virus was found similar to IPNV on the basis of morphometric characteristics and biophysical properties such as resistance to chloroform and heat treatment, but was not serologically compared with the existing strains of IPNV. Third birnavirus isolation was made from pooled organ extracts of EUS infected snakehead fishes from Myanmar and Thailand and eye spot barb (*Hampala dispar*) from Lao PDR in southeast Asia in 1988 [79]. The isolate was serologically compared and identified as Sp serotype of IPNV. A fourth isolation of an aquatic birnavirus was reported in 1993 from the spleen of an ulcerated giant snakehead (*C. micropeltes*) in Singapore [71]. The isolate differed from the existing strains of IPNV in the relative mobility patterns of nucleic acid and structural polypeptides. Further comparative studies of the isolate, however, were not reported. None of the above viruses have been reported to have any causal role in the pathogenesis of EUS.

Rhabdoviruses

Since its first isolation from striped snakeheads and a freshwater eel (*Fluta alba*) in 1986 in Northern Thailand and Myanmar [23], rhabdoviruses were further isolated from infected snakehead fish. The five ulcerative rhabdovirus isolates (UDRV) obtained grew in AS, BF-2, snakehead (*O. striatus* and *O. micropeltis*) and climbing perch (*Anabas testudineus*) cell lines [20]. CHSE-214, RTG-2, EPC, nile tilapia (*Oreochromis niloticus*) and grass carp (*Ctenopharyngodon idella*) cell lines were all refractory to infection. Characterisation of these isolates by cross-neutralisation showed that they were serologically distinct from the common pathogenic fish rhabdoviruses VHSV, IHNV, EVA, EVX, SVCV, PFRV and perch Rhabdovirus. Rhabdovirus isolates were subsequently isolated from infected snakehead fish (SHRV) in central Thailand in 1986 [77]. UDRV and SHRV were distinguishable by serology and the relative mobility pattern of their structural polypeptides [3]. Both viruses were serologically unrelated to the other known fish rhabdoviruses [41] and phylogenetic analysis of the SHRV G protein indicated that the SHRV belonged to the genus *Novirhabdovirus* [34]. The rhabdovirus isolates obtained from EUS infected fishes have been found to grow well in the cell lines established from the susceptible fishes [38]. The UDRV failed to induce any clinical lesions in snakeheads subjected to intraperitoneal inoculation or bath exposure [22]. EUS lesions were however induced in snakeheads (*C. striata*) held at 20 °C injected intramuscularly with a rhabdovirus (strain T9412) followed by bath challenge with *A. invadans* spores [37]. Rhabdovirus injection alone induced only small haemorrhagic lesions at the injection site most of which had healed by the end of the experiment. They did not get any EUS induction in a similar experiment conducted at 29 °C indicating the combinatorial conditions that lead to the development of EUS lesions. Cell culture grown EUS-associated rhabdovirus was experimentally shown to induce slight to moderate skin lesions in all tested naive snakeheads at rearing water temperatures of 20–22.5 °C, but not at 28–32 °C [48]. LioPo et al. [49] transmitted the rhabdovirus isolated from the Philippines horizontally from virus infected fish to naive snakehead (*C. striata*) by cohabitation and lesions appeared in 6–14 days on the naive fish. Naive fish exposed to lake water only developed lesions in 16–21 days. Rhabdovirus was isolated from fish in both treatments suggesting transmission via water from infected fish. Fish in aquifer water only without contact with EUS-infected fish did not develop the disease.

Further isolation of rhabdoviruses was reported during the later incidences of EUS in Thailand, Sri Lanka and the Philippines [21, 36, 38, 44, 50] making this group of viruses the most isolated from EUS infected fishes. Though the rhabdoviruses isolated were heterogenic, their frequent isolation, range of areas from which isolates have been obtained and ability to induce dermal lesions in experimental infectivity studies make rhabdoviruses one of the likely potential primary aetiological agents that could be triggering the onset of EUS paving way for the invasive fungus *A. invadans* to develop characteristic dermal lesions.

Reoviruses

A reovirus has been isolated from EUS infected snakehead fish in 1992 following a cohabitation experiment [63]. EUS was induced in six healthy snakehead fish by cohabitation with six characteristically EUS infected snakehead fish. All six of the EUS infected fish died within 5 days of cohabitation trial. Tail ulcerations developed in the healthy snakehead fish 12 days post co-habitation were sampled for virological examination by inoculating the tissue homogenate onto SSN-1 and BF-2 cells. The cytopathic effect

(CPE) was developed in SSN-1 cells incubated at 25 °C after 8 days. Virus was further passaged onto fresh SSN-1 cells and CPE observed in 2 days. This isolate (*Snakehead reovirus*, SKRV) was characterised in comparison with other European and American aquareoviruses and observed to be different due to a 10-segmented dsRNA genome, lack of syncytium formation and inability to multiply in the coldwater fish cell line CHSE-214 [32]. The snakehead reovirus (SKRV) was found to belong to the *Orthoreovirus* genus and is the first orthoreovirus to be isolated from a fish host. SKRV was found to grow in SSN1 and SSN3 cell lines at 25-30 °C. Serologically the virus was distinctly different from GSV (golden shiner virus), CRV (catfish reovirus), TNRV (tench reovirus) and CHRV (chub reovirus). Pathogenicity studies indicated that the virus was not capable of producing EUS associated lesions in snakehead juveniles [31].

Ranaviruses

A virus belonging to *Ranavirus* of *Iridoviridae* family was isolated from cultured largemouth bass (*Micropterus salmoides*) experiencing extensive mortality in the Guangdong Province, China in 2008 [17]. Though not categorised into EUS, affected fish had ulcerations on the skin and muscle. The virus grew well in EPC cell cultures and the transmission electron microscopy of the ulcerative muscle tissue and the infected EPC cells revealed cytoplasmic, icosahedral virions of 145 nm in diameter. The virus resembled *Doctor fish virus* and closely related to *Largemouth bass virus* up on sequence analysis of viral major capsid protein and DNA methyltransferase. Intramuscular injection of the virus resulted in clinical signs of the disease and caused 100 % mortality of healthy largemouth bass.

Cell Culture Associated Viruses

Some of the cell cultures developed from tropical warmwater fishes were found to harbour persistent retrovirus [24] and birnavirus-like infections [31]. Spontaneous production of C-type retrovirus particles was noticed in cell lines developed from healthy striped snakehead, snakeskin gourami (*T. pectoralis*) and climbing perch (*A. testudineus*), which are known to be susceptible to EUS. Similarly a cell line developed from blotched snakehead (*Channa lucius*) was found infected with a birnavirus, which was found to be a new serotype (Serotype C) of the IPNV group [33]. Pathogenicity studies indicated that both the snakehead retrovirus and blotched snakehead virus were unable to induce the disease condition in snakeheads [22, 31]. Role of these persistent viral infections in acting as potential reservoirs of viruses and their effect on the immune system of the carrier fish has not been fully investigated.

Concluding Remarks

Investigations so far conducted on EUS infected fishes have shown that the spread of the disease is due to *A. invadans,* a fungus, which is capable of producing a proteolytic enzyme that helps it to penetrate the fish tissue to cause the shallow to deep ulcers. However, the fungus itself was not able to initiate the infection unless the integrity of the epidermis is compromised due to either biological or non biological agents. The report of a pathogenicity study conducted on Atlantic menhaden [42] indicated that the fungus could cause the infection in experimental fish not subjected to any stress conditions. However, the electron microscopic observations revealed that the germinating spores could enter the epidermal layer of the fish, which has lost its integrity.

Cohabitation studies involving the striped snakehead fish (*C. striata)* was found to transmit the EUS to healthy snakehead fish in two separate investigations [49, 63], where virus isolates were recovered in both the cases. The ranavirus isolated from ulcerated largemouth bass also produced skin and muscle ulcerations in a pathogenicity study conducted in the same fish without the presence of an invading fungus [17]. Continued investigations may lead to recovery of more viral agents from EUS infected fishes and throw more light on the involvement of viruses in the initiation of the disease either by lowering the immune status of fish and consequent compromising of the integument integrity or inducing dermal ulcerations independently. Presence of persistent viral infections in the cell lines developed from some of the EUS susceptible tropical fishes also point to the possible role of viruses in the induction of EUS. Although the retrovirus isolated from the SSN1 cells differed from human and other vertebrate retroviruses [26], what role such viral persistence would play in the development of EUS is not clear. Incidences of EUS have been on a decline in the last few years and role of viruses was not heavily pursued. Further investigations are therefore necessary with a panel of additional susceptible fish cell lines for isolating viruses, if any, from the infected fishes to delineate the role of viruses in EUS.

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