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A review of current understanding on carp edema virus (CEV): A threatful entity in disguise

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Abstract
Aquaculture has been an integral part of food consumption and aesthetics since advent of agricultural industry with increasing demand to meet the needs of expanding population. Fish trading among the nations have widened the pathogenic window towards their susceptible hosts. Immediate attention is required with respect to budding and persisting pathogens in waters across the world. Extensive and efficient surveillance can help in preventing loss of fish in huge quantity. One of the emerging infectious viral pathogens that have caused a huge loss to aquaculture particularly to carp industry is carp edema virus belonging to Poxviridae family. It is known to affect only freshwater koi and common carp with symptoms resembling those of other viruses. Experiments and observations drawn have made a positive approach towards understanding the infection mechanism of the pathogen, host range, clinical signs, preventions and its treatment.

Keywords: Carp edema virus, diagnosis, distribution, immunity, pathology, review

1. Introduction
Since ages, pathogens particularly viruses have been in association with the vertebrate and invertebrate hosts raising their own survival efficiency. To achieve such mechanisms these have undergone transformations and transitions with changing environment as well as modifications in host immune functions. Some of the viruses are easily detected while others remain buried within the possible host species for years in latency. Many viruses infecting carps and ornamental species have afflicted tremendous loss to the aquaculture industry and thus to livelihood of the people involved. For tackling such loss, vaccine development along with suitable methods of detection have been the demanding tools to check cases of the mass mortality prevalent with viral diseases.
About 4000 freshwater ornamental species are known to be traded every year as per the international ornamental trade status excluding high quantity (by volume) bred in farms [1]. Amongst those, goldfish and koi carp are commonly traded ornamental species [2]. Random relocations of huge quantities of fishes between countries can be potential source of pathogenic transmission; particularly viruses responsible for sudden outbreaks [3, 4]. Of many known viruses, the poxvirus is one of the emerging virus groups through years in aquaculture. Carp edema virus disease (CEVD) or koi sleepy disease (KSD) are caused by carp edema virus belonging to poxvirus group, having koi carp and common carp as the two most suitable hosts for multiplication. These are known to affect the gills and skin causing hyperplasia of gills, anoxia, ulcerations around mouth and fin bases, and also inflammation of anal opening [5].

2. Global distribution
Carp edema virus (CEV) was the first poxvirus known to infect finfishes, specifically to the ornamental varieties of the common carp (*Cyprinus carpio*) as reported for the first time in Japan in 1974 [6, 7]. The first ever outbreak in European nations was reported in England followed by France and Netherlands [8]. In subsequent years, frequent outbreaks have been reported in Italy [9], Czech Republic [10], Germany and Austria [11], and in southern hemisphere [12]. With the expansion of area of CEV invasion, new countries have been added to the list (Table 1). Reports from India confirms CEVD outbreak in south eastern states. According to the first report of CEV outbreak in India [13], out of the positive nine places, Chennai, Madurai

and Kolkata showed the highest prevalence of the virus infection. Further, CEV was being reported from ornamental farms of Odisha in India [14]. A similar temperature range of 19-24 °C has been reported in the outbreaks of Japan and India [15]. A report from two north western districts of Serbia has been published having around 20% mortality in common carp at 9 to 15 °C and in Prussian carp without any external clinical signs with high mortality at 26 °C of temperature in 2017 [16].

Table 1: The distribution of CEV in various countries under different periods of report at different water temperatures.

Country	Water temperature	Persistence duration (years)	Reference
Japan	19- 24 ℃	1986	[17, 5]
North American countries	Low temperature	1996-2015 (in various states)	[18]
Czech Republic	13-15 ℃	2013-16	[10]
France	Outbreaks in winter and May or July	2013-15	[19]
Germany	17-22 ℃	Numerous linked (first case in koi in 2009) and unlinked cases (2014-2016)	[11, 20]
Italy	13-16 ℃ 2010 case; 6-7 ℃ and 23 ℃ also.	Several outbreaks in 2015 and 2016	[9]
Austria	7-15 ℃	2014	[21]
Netherlands	6-7℃ (2010 case); 20-23 ℃	2015/2016	[8, 22]
Poland	16-19 ℃	2015 and 2017	[23]
Switzerland	January with high mortality.	2016	[24]
UK	Greater than 16 ℃ in juveniles; 6-9 ℃ in adult carps.	2009-2016	[25, 26]
India	19-24 ℃	2015	[13]
China	24 ℃	2015 -2017	[5]
Hungary	Winter and early Spring	2016-2017	[27]
Serbia	9-15 ℃	2017	[16]

3. Virus structure and mechanism of replication

Poxviruses are A+T rich, double stranded DNA viruses which possess a large complex genome. Transmission electron microscopy studies reveal its oval or brick shape of around 200-400 nm in length. These have a variety of hosts. Virions are enveloped when present outside the host and have capsule or pill like flattened shape inside. Enveloped virions inside host cell are also infectious. Although this shape varies among different species, the basic oval structure remains similar. There are about 100 proteins encoded by the virions which are present in four structures such as core, membrane, lateral body and envelope. The virus has partial lipid bilayer with arbitrary distribution of surface tubule elements (STE) on the outer surface [28]. Isolation of fish poxviruses through cell culture have not been made possible so far due to poor understanding on the mechanisms of infection [29].

The peculiarity exhibited by viruses of this class is their ability to replicate in the cytoplasm of the host cell which deviates from usual replication mechanism of other double stranded DNA viruses. Fluorescence microscopy studies illustrate the detection of poxviruses roughly within 2 hours of virus entry into the host cell [30]. Glycosaminoglycans (GAGs) are the receptors present on the host cell required for attachment of the virions and forcible entry into the cell with subsequent removal of envelope. Replication continues inside the host cell cytoplasm by virus' own transcriptional machinery and RNA polymerase enzyme until the host dies [31]. Sequential events occurring in replication mechanisms of poxviruses seemingly comprise of virus entry, uncoating, early gene expression, DNA replication, late gene expression, virus assembly and virus diffusion with few deviations within species [28].

4. Host range

As per the reported data, CEV is known to reside in specific host namely, *C. carpio*, and ornamental koi and produces gill necrosis and skin lesions as significant symptoms. Few other DNA and RNA viruses are also known to affect numerous carp species, usually the ornamental koi carp and common carp (*C. carpio*) viz., CyHV-3 (Cyprinid herpesvirus 3), spring viraemia of carp virus (Rhabdovirus), CyHV-1 (Cyprinid herpesvirus 1), and common carp from paramyxovirus (CCPV) [32, 33, 34, 35] producing mortality and/or similar clinical signs, thus making the diagnosis bit confusing.

5. Phylogenetic status

CEV is a double stranded DNA virus belonging to the family Poxviridae with unclassified generic status. Complete CEV genome has been recovered de novo by NGS (next generation sequencing) technique. Phylogenomic analyses have confirmed that CEV and the salmon gill poxvirus (SGPV) are sister species, and together form the deepest branch of the Chordopoxvirinae within the family Poxviridae [26, 29]. Further on outbreaks reported in China, phylogenetic comparison indicated that CEV of Chinese strain has a strong genetic link with CEVs detected in Japan and the UK. Phylogenetic analysis of the partial core protein p4a has revealed existence of three genogroups I, IIa and IIb showing 6-10% genetic diversity [36]. CEV has two main lineages; lineage 1 from koi similar to that found in Japanese koi and lineage 2 belonging to common carp found in Poland. Further reports obtained from Polish outbreaks suggested the existence of the third lineage having similarity with both the earlier discovered lineages. Hungary has later reported presence of all the three strains with different outbreaks [23, 37, 38].

6. Gross pathology and clinical signs

CEV is known to exhibit two different pathological facets of the disease viz., Carp Edema (CE) and Koi Sleepy Disease (KSD). The symptoms of edematous body and hyperplasia of gill epithelial cells are characteristic to CE seen in koi carp (0.1- 1.2 g) during early summer. Affected fish moves close to the water surface or to the pond walls raising the mortality up to almost 80% within a few days [39, 40]. In case of KSD, yearlings weighing around 20 to 30 g are found to be affected after transfer from earthen ponds to cement-lined ponds in winter season. Lowering of temperature between 15 to 25 ºC in the spring and autumn leads to severe outbreaks bringing lethargy in greater numbers of carp making them lay at the pond bottom. If disturbed, the fish swims for a short while

before going back again to sleeping or inactive state at the bottom. Subsequent days of persisting infection results in death, and mass mortality occur within next two weeks [17, 29]. Definite pathognomonic signs exhibited by carp infected with CEV include lethargy, and other external signs such as swollen gills, enophthalmia, vasocongestion of the caudal fin, swelling and ulceration of anus [21, 22].

Fig 1a): Haemorrhagic body of CEV infected koi carp. b) CEV infected gills of koi showing swelling of primary lamellae (Black arrow) and necrotic tissue (White arrow). [Reproduced with permission from BioMed Central Ticket ID#93198 under CC-BY license [11].

7. Susceptible organs of infection

The most vulnerable organ of CEV infection is gill because it is an external organ primarily coming in contact with the pathogens providing a path to internal organs. In addition, skin, fin, spleen and kidney can also be probable infection holders responsible for invasion into the host immune system but with less viral load than gill making it the prime target organ that can be used for specific virus detection [24].

8. Transmission

From the previously gathered data, temperature is known to be one of the most sensitive factors in the absolute occurrence of this disease. With increase in the temperature up to 28 °C, a remarkable decrease in morbidity and mortality was observed. These data indicate that temperature acts as an important abiotic predisposing factor in the onset of disease outbreaks in fish populations by modulating the immune system [5, 41]. Fish, being a poikilothermic organism, is mostly influenced by change in environmental temperature. However, with fluctuations in temperature during transport gives an unwanted shock to the organism's physiological state delaying the acclimatization time in a new environment. Apart from temperature, unhygienic maintenance, overstocking and trading off across countries are probable reasons of severe mortality and morbidity. As per the reports of Germany which imported koi population, harboured high number of CEV specific DNA in gills but did not exhibit the clinical signs. This might be a stress-related reactivation of a persistent CEV infection, as also suspected in fowl pox infections [42], showing a potential dispersal route of CEV. Further, a link between Spring Carp Mortality Syndrome (SCMS) and KSD was suspected [25]. Nitrite concentration is also an important parameter found to cause mortality in infected fish. The carp displays severe methemoglobinemia having high nitrite content as compared to those treated with 0.5% salt water [43, 44]. This acted as a stress factor for the koi, enhancing KSVD propagation according to the studies. The disease also spreads directly during transfer through encounter of the infected carps in the same tank or natural pond being exposed to excess mucus and worn out particles from infected carp body. Additionally, vertical transmission cases have not been reported in the literature which seems nearly impossible as the juvenile carriers tend to develop the disease and die after sometime [45]. Recently, it has been reported in experimental transmission studies that co-habitational spread of CEV from infected common carp to bleak, crucian carp, European perch, Prussian carp, roach and tench within Cyprinids is possible [46].

9. Co-infection

On transfer of the newly purchased koi into the affected ponds already having koi kept for a couple of years, development of typical clinical signs was observed as per the experimental studies carried out [21]. The case from a German farm was found to differ when species, like goldfish, and sturgeons, kept into the same affected pond were unaffected; might be a cause of infection susceptibility towards a specific host [11]. Experimental studies of cohabitation with related species such as IMCs (Indian Major Carps) have clarified it not to be the probable host species of the virus [13]. However, it is essential to understand the carrier effects of the virus through experimental studies in co-cultured fish with susceptible host species.

10. Histological study

Multiple tissue sections have been examined to identify visible changes for more clarity of symptoms [5, 47]. Multifocal mononuclear cell infiltrates, hyperaemia, and necrotic changes in the gill sections are mostly observed those taken from infected carps. Hyperplasia of the gill lamellae specific to this poxvirus is observed as a unique clinical sign. Peculiar characteristic signs include clubbing of gill filaments occurring probably due to proliferation of epithelial cells in the filaments [17]. Several opportunistic bacterial species viz., *Aeromonas sobria, Klebsiella pneumoniae, Proteus penneri, Shewanella decolorationis* and *Escherichia coli* as secondary pathogens are found to be associated with CEV [13]. The bacterial infestation associated specifically with severe gill necrosis and occlusion of interlamellar spaces is *Flavobacterium branchiophilum* along with reported parasites like *Ichthyobodo necator* and *Argulus foliaceus* [15, 48]. Multiple parasites viz., *Gyrodactylus* sp., *Ichthyobodo necator*, *Dactylogyrus* sp., *Trichodina* sp., *Ichthyophthirius multifiliis*, *Bothriocephalus* sp., *Capillaria* sp. and *A. foliaceus* have been reported in CEVD cases in an Austrian survey [21, 26].

11. TEM examination

The electron microscopic studies demonstrate the measurements of immature virions which range up to 450 nm in diameter and mature ones are roughly oval of size 400 × 413 nm. The characteristic feature of the mature virions is the presence of surface globules (capsomeres) and a dense dumbbell shaped core enclosed by a prominent membrane. Electron microscopy demonstrates mulberry like enveloped herpes virus with mature nucleocapsids measuring up 117 nm and mature enveloped nucleocapsids up to 180 nm in the affected cells [24, 49].

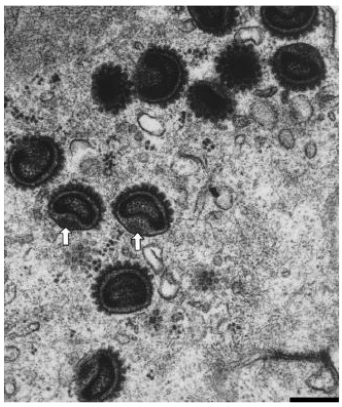

Fig 2: Transmission electron microscopy picture of CEV mature virions in gill epithelial cell: 333-400 x 400-413 size mature virions having surface globular units and thickened core membrane with electron dense core. White arrows indicate the lateral body with an unsharp outline beside the core. Scale bar = 350 nm. [Reproduced with permission from Original publisher "© Inter-Research 2005" [17].

12. Isolation using cell lines

Numerous cell lines have been attempted for CEV isolation including fathead minnow (FHM), koi fin (CCKF), pearl spot fin (PSF), catopra fish fin (CFF), common carp brain (CCB), *Horabagrus brachysoma* fin (HBF), goldfish fin (GFF), angelfish fin (AFF) and many other teleostean cell lines [11, 13]. However, cytopathic effect (CPE) could not be detected in any of the above cell lines nor isolation of the virus has been made possible. This makes virus isolation and purification in large numbers a difficult task further impeding other outputs of molecular and virological research.

13. Detection methods

As most of the natural cases are presented with concomitant secondary infections associated with bacteria and parasites, the detection method needs to very specific and sensitive. Confirmed identification of a specific host could be achieved by employing a variety of techniques conducting parasitological, bacteriological, histopathological and virological examinations. In case of CEV, skin and gill scrapings are usually collected from pectoral fin for parasitological detections. Bacterial examinations are performed with use of TSB (Tryptic Soy Broth) culture. Further, molecular screening was performed using host virus gene specific primers [47]. Viruses like CyHV-3 and SVCV have pathognomonic signs similar to CEV infected fishes. Therefore, specific and sensitive primers may be used for detection of CEV infection only. The polymerase chain reaction or nested PCR primers used with confirmed results in few studies are mentioned in the table below.

Table 2: Standardized nested PCR primer conditions for CEV detection for 5'- UTR region and partial 4a gene [3, 5, 23, 50].

Sl. No.	Fragment Name	Nested Primers	Primer sequences	PCR conditions	Expected band size
11.	5'-UTR	CEV F1	5'-GCTGTTGCAACCA-3'	Initial denaturation: 95 °C - 3min for 40 cycles. Denaturation: 95 °C – 30 s Annealing: 60 °C - 30 s, Extension: 72 °C - 30 s followed by a final extension step at 72 °C for 10 min.	548 bp
		CEV R1 1st step PCR	5'-TGCAGGTTGCTCCTAATCCT-3'		
		CEV F2	5'-GCTGCTGCACTTTTAGGAGG-3'	Initial denaturation: 95 °C - 3min for 40 cycles. Denaturation: 95 °C – 30 s Annealing: 48 °C – 30 s Extension: 72 °C - 30s followed by a final extension step at 72 °C for 10 min.	181 bp
		CEV R2 2nd step PCR	5'-TGCAAGTTATTTCGATGCCA-3'		
2.	Partial 4a gene	CEV For B	5'-ATGGAGTATCCAAAGTACTTAG-3'	Initial denaturation: 95 °C - 3 min for 40 cycles. Denaturation: 95 °C – 30 s Annealing: 55 °C - 30 s Extension: 72°C- 30s, followed by a final extension step at 72 °C for 10 min	528 bp
		CEV rev J 1ststep PCR	5'-CTCTTCACTATTGTGACTTTG-3'		
		CEV ForB-int	5'-GTTATCAATGAAATTTGTGTATTG-3'	Initial denaturation: 95 °C - 3 min for 40 cycles. Denaturation: 95 °C – 30 s Annealing: 55 °C - 30 s Extension: 72 °C- 30s, followed by a final extension step at 72 °C for 10 min	478 bp
		CEV RevJ-int 2nd step PCR	5'-TAGCAAAGTACTACCTCATCC-3'		

A probe based real time PCR (qPCR) is used for quantitative estimation of the core protein p4a DNA in the susceptible target organs developed by CEFAS in Wemouth, UK. The primers used were CEFAS qF: AGTTTTGTAKATTGTAGCATTTCC, CEFAS_qR: GATTCCTCAAGGAGTTDCAGTAAA and the double-labelled probe was ATGGAGTATCCAAAGTACTTAG [FAM]-AGAGTTTGTTTCTTGCCATACAAACT-[BHQ1]. The amplification program includes initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s. Further for sequencing purposes, a larger fragment of p4a gene amplification was performed by using end point PCR primers, CEFASF: ATGGAGTATCCAAAGTACTTAG; and CEFASR: CTCTTCACTATTGTGACTTTG and the KAPA2G Robust Hot Start PCR kit (Peqlab, Germany). PCR amplification includes an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. Additionally, a final elongation step for 7 min at 72 °C was performed at the end of each run [23, 27]. For faster screening of the virus a new approach of detection using nested PCR primers has been introduced which is known to be Recombinase Polymerase Amplification assay (RPA). This assay is advantageous to molecular diagnostics by the fact that it requires low concentration of nucleic acid consuming less time with high sensitivity. Single plex and multiplex RPA for detection of CEV and CyHV-3 have been standardized [51].

14. Insight into Poxvirus

Various diagnostic techniques have facilitated our understanding of the viral morphogenesis. Along with structure, several gene expression studies have revealed the likely mechanism of poxvirus invasion into host cell. A peculiarity in clinical signs includes increased mucin in CEV infected carps providing a platform for rise of secondary infections. This shows that persisting virus decreases mucus production over skin which upregulates host's immune system leading to increased expression of mucus for avoiding further infections [51]. Poxviruses have a complicated mechanism of infecting its host. The mechanisms vary among different groups which might be acquired from different hosts. Poxvirus deviates from other DNA viruses by exhibiting replication in the cell cytoplasm. Mostly, poxviruses cause necrosis and few apoptotic cases are found because of their ability to inhibit apoptotic factors [52]. The gill damage induced by poxvirus leads to secondary infections mostly by making the host immunocompromised.

15. Antiviral immunity

The antiviral immunity is established in the host on virus replication. Replication occurs in the cytoplasm of host cells causing a permissive infection, altering the host's antiviral immune system, specifically the innate immune response [53]. Antiviral immunity may be present within the organism prior to viral infection known to be induced by certain molecules such as interferons released from immune cells in the body leading to viral restriction or preventing further invasion into host cells. Temperature is an important factor that affects viral replication in the ectothermic vertebrates [54]. As per the method practiced by some koi producers, artificially elevated water temperatures could limit KHV infections by inducing antiiral immunity [55]. This influences the onset and severity of fish virus infections directly by altering virus replication and indirectly by augmenting the efficacy of the host immune response [56, 57]. However, a lot need to be explored that leads to virus latency in carp for prolong period.

16. Prevention and treatment of CEV

For careful maintenance and prevention of viral infection in fish, certain factors may be taken into consideration. Raised water temperature is to be maintained to protect the fish against cold stress and kept indoors to provide favourable ambience. Suspected fishes may be kept after quarantine in separate stocking tanks to avoid spread [45, 58]. In case of mild infections, 0.3-0.5% with maximum limit of 3% (in severe infections) of salt bath may be given to the stocks followed by regular monitoring as inferred from the Japan case [24, 40]. Although salt treatment reduces osmotic stress slowing down the virus spread, it does not assure of the cure as there has been insufficient information regarding the effectiveness of salt treatment on killing the virus [59]. Freezing, drying out and disinfection of the pond bottom with lime are still very important prophylactic measures [60]. Some of the farmers avoid harvesting of carps during temperature conditions favourable for infection. Fishes must be checked every day for secondary infection or common parasites such as bacteria or ectoparasites (protozoans and fluke infections) and fungal species. The water quality is a vital parameter to be checked as a preventive measure including pH, dissolved oxygen, as well as nitrates, nitrites and ammonia [61]. Hygienic maintenance of tanks, aerators and its filters are also necessary which might be house to common pathogens. Overfeeding should be avoided to prevent aggregation of secondary infectious agents from the leftover food exposed to the tank or pond bottoms, also affecting the water quality. Along with hygiene, nutrition should also be administered for capacitating the fishes with strengthened immunity. Avoidance in overstocking is another potential strategy towards prevention of viral infection. Biosecurity principles and disease surveillance can be applied to CEVD, when the diagnostic tests are easily available. For successful treatment of the disease, one must understand the pathogen well in aspects including its mechanism of infection, phylogenetic status, sensitivity towards antibiotics, antimicrobial peptides and naturally found molecules.

17. Conclusion

CEV has been in reports through years of surveillance covering almost all the European countries and still is on the rage of rapid spread. Isolated environmental conditions or quarantine may bring changes in response towards infection with monitoring under specific temperatures. Salt treatment also minimizes the infection during transport within countries. Surveillance in countries existing as part of aquaculture industry could be involved in strict sampling and monitoring of the available cases in order to make susceptible nations virus free. Final conclusions can be drawn from the above collected data that salinity affects virus replication which might be a possible cause of marine fishes not carrying this particular virus keeping in contradiction to the fact that other fish viruses belonging to Poxviridae can affect marine fishes. This might be a probable effect of changing strains in the virus. Continuous review of literature and experimentation can help in predicting probable aspects of variable viral infections. Further speculations for finding out possible random mutations occurring in viruses through aquatic sources might be placed in context of vaccine development.

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