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# Carp edema virus in ornamental fish farming in India: A potential threat to koi carps but not to co-cultured Indian major carp or goldfish

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Koi sleepy disease caused by carp edema virus (CEV) of pox virus is a potential carp killer and poses a constant threat to co-cultured fish. Only limited studies are available on its occurrence from the eastern part of India. Here, we report a large-scale mortality of koi carps (*Cyprinus carpio koi*) in ornamental fish farm of Choudwar, Cuttack, Odisha. The fish showed clinical signs of ulcers on body, patchy haemorrhages, massive necrosis of gills, and observed sleeping at the bottom of tanks before death. However, the goldfish maintained in the same tanks did not show any clinical signs or mortality. We noticed *Trichodina* infestation in the gills of affected animals and secondary bacterium *Aeromonas hydrophila* in kidney tissue. The gills, liver and kidneys of koi carp were found positive for carp edema virus (CEV) (Poxviridae) in PCR followed by sequencing and blast search, and phylogenetic analysis. The damages to the gills and kidney along with the presence of intracytoplasmic eosinophilic inclusions in gill epithelial cells upon histopathology suggested viral association. Experimental challenge with CEV failed to reproduce the disease in Indian major carp and goldfish, the commonly co-cultured fish species with koi carp, though, the disease was reproduced in koi carp. To our best knowledge, it is the first report of CEV infection from the farm of the eastern part of the country. Another mortality incidence in ornamental shop retailer in the same locality also revealed the presence of CEV in koi carp and pointed possibly towards its wide spread. Observations from this study suggest the need for CEV disease surveillance in the country.

**Keywords:** Aquarium, *Carassius auratus*, CEV, *Cyprinus carpio koi*, Experimental transmission, Goldfish, Koi sleepy disease, *Labeo rohita*, Rohu

Viral diseases are major challenges in fish farming and cause severe morbidity and mortality throughout the world[1]. The ornamental fish trade is one of the major causes for transboundary spread of aquatic animal diseases in different parts of the globe. Out of above 2500 species of ornamental fish traded every year[2], goldfish (*Carassius auratus*) and koi carp (*Cyprinus carpio koi*) are the most commonly traded ornamental fish species[2,3]. In koi carp, *Cyprinid herpes virus 3* (CyHV-3) has been the major viral disease of concern[4-6] causing koi herpes virus (KHV) disease, although other viral diseases *viz*., spring viraemia of carp[7], carp edema virus (CEV)[8] and ranavirus[9] are not uncommon. KHV has seriously affected koi trade worldwide after its appearance in 1990s, although it is not reported from India. Infection with CEV, known as koi sleepy disease (KSD), a pox virus, is known in koi and common carp with restricted geographic spread from at least three continents Asia, North America and Europe, particularly from Germany[10,11], India[12], China[13], Korea[14] and Iraq[15] from common carp and koi carp disease outbreaks. CEV was also reported as an emerging disease in central eastern Europe[16]. The infected fish show typical signs *viz*., enophthalmia, congested and edematous gills with hyperplasia, necrosis and clubbing of lamellae causing hypoxic conditions[8,17,18] and skin lesions around base of the mouth and base of the fins[19]. Affected fishes exhibit lethargic behaviour and lye at the bottom of tanks or ponds. The stressed fish when transferred from earthen ponds to concrete tanks show mortality of 80-100% with a prevalence of 87.5% between water temperature 15 and 25°C[18]. Although koi carp were more susceptible at 24°C causing 100% mortality within 10 days of experimental challenge, it also caused infection and late mortality of 26.67% within 14 days even at 28°C[13].

The electron microscopic study and partial core protein P4a (in the absence of whole genome information) along with phylogenomic analysis

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revealed CEV belonging to *Chordopoxvirinae* subfamily of *Poxviridae*[11,19]. There exists two or three genogroups or lineages of the virus based on the 6% divergence in the partial P4a nucleotide sequence information[20,21]. The genogroup I was mostly found in farmed common carp, genogroup IIa was predominantly reported from koi while genogroup IIb was recently described from infected common carps of Poland[20,21]. Recently, two more new genogroups (IIIa and IIIb) were described to classify Austrian isolates[22]. The possible worldwide trade with improper quarantine might be one of the factors for spread of genogroup IIa in koi carps. In the absence of cell line (where virus could be replicated *in vitro*)[11,12], PCR remains to be gold standard for detection of this virus.

CEV has been reported to be a major concern to ornamental koi breeders as well as common carp farms in India[12]. Here we report a large scale mortality of koi carps in the farms of Choudwar, Cuttack, Odisha, investigated for CEV infection, and also the carrier status of this virus in other co-cultured food fish species, if any.

## Material and Methods

### Background

A large-scale mortality of koi carps [*Cyprinus carpio* var. *koi* (Linnaeus, 1758)] in ornamental fish farms of Choudwar, Cuttack district, Odisha, India, Latitude- 20º 5391' 679'' N, Longitude- 85º 9151' 2109'' E was reported during February, 2017. The cemented tanks were mostly stocked with koi carp and goldfish. An onset of disease appeared when new stocks from Kolkata, West Bengal market were brought and added to the existing tanks within 8-15 days. Juvenile and adult koi carps (30-200 g) displayed clinical signs of ulcers on body, patchy haemorrhages, depigmentation and massive necrosis of gills (Fig. 1), lethargy, and found lying at the bottom of tanks before death. The cumulative mortalities of koi carps in the affected tanks were in the range of 90-100%. The goldfish maintained in the same farm and tanks did not reveal any clinical signs and mortality. The source of water was from bore-well and fish were being fed with commercial pellet feed. The water temperature during the period was ~28ºC. Further during June 2017, 100% koi carp mortality was observed in aquaria of a major retailer shop at Bhubaneswar, Khurda, Odisha, India (Latitude 20º 33'6481" N, Longitude 85º 81'0134"E) with similar clinical signs. Water temperature recorded in tanks during mortality ranged from 32-34ºC.

### Collection of infected samples

The moribund koi samples from both cases were bought to the laboratory, euthanized with MS222 (Sigma-Aldrich), and tissue samples (skin, gills, kidney tissues for microscopic smears/squash preparations for parasitology; gills, liver, kidney, brain and spleen tissues in 100% ethanol (EMSURE, Merck) for PCR screening of viruses; gill, liver and kidney tissues in 10% neutral buffered formalin for histopathology, and kidney tissues for bacteriology) were collected for further investigations. The tissue samples collected in 10% neutral buffered formalin were processed further for histology following routine protocol with haematoxylin and eosin sections.

### Molecular identification of virus

Different tissues (liver, gills, kidney, spleen and brain) of infected koi carp from the field samples were processed for DNA isolation. Tissue samples (approximately 100 mg) were treated with proteinase K in lysis buffer (50 mM Tris/HCl, 100 mM NaCl, 100 mM EDTA, 1% [w/v] SDS, pH 8.0) and subjected to extraction with phenol/chloroform/isoamyl alcohol, followed by ethanol precipitation. The DNA was diluted in TE (50 mM Tris/HCl, 1.0 mM EDTA, pH 7.5) buffer. Concentration and purity of the extracted DNA were determined by measuring OD at 260 and 280 nm using a NanoDrop ND1000 spectro-photometer (NanoDrop Technologies Inc., USA). The samples were stored at −20ºC for further analysis. PCR was performed using five sets of published oligonucleotide primers for confirmation of CEV. Infected tissue samples were also screened for the presence of koi herpes virus (KHV) and CyHV2 (Cyprinid herpes virus 2) by PCR using published primers and PCR conditions[23].

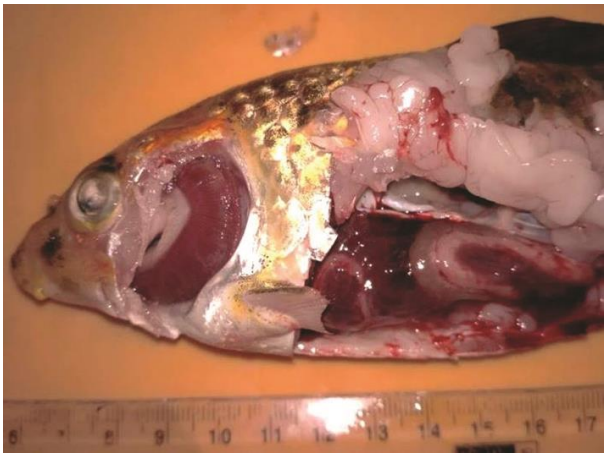

Fig. 1 — Koi infected with carp edema virus showing damage to the gills

For CEV, PCR was performed using five sets of published oligonucleotide primers, as detailed in Table 1. In the first set, CEV For B and CEV Rev J[21] primers were used in a final volume of 25 µL containing 1.0 µL of total DNA, 1.5 µL (10 pmol) of each primer, 0.25 µL of *Taq* DNA polymerase (5 U/µL), 2.5 µL of 10X *Taq* buffer A, 0.5 µL of dNTPs (2 mM) and $ddH_2O$ to make final volume to 25 µL. The reaction mix was subjected to 35 temperature cycles (1 min at 95°C, 1 min at 55°C and 1.0 min at 72°C) after an initial denaturing step (5 min at 95°C) followed by a final extension step of 10 min at 72°C in a Veriti thermal cycler (Applied Biosystem). A nested PCR was performed using second set primers CEV For B-int and CEV Rev J-int using similar conditions[21]. In the third set, F1-R1 primers[8] were used in a final volume of 25 µL containing 1.0 µL of total DNA, 1.5 µL (10 pmol) of each primer, 0.25 µL of *Taq* DNA polymerase (5 U/µL), 2.5 µL of 10X *Taq* buffer A, 0.5 µL of dNTPs (2 mM) and $ddH_2O$ to make final volume to 25 µL. The reaction mix was subjected to 35 temperature cycles (30 s at 94°C, 60 s at 60°C and 60 s at 72°C) after an initial denaturing step (120 s at 95°C) followed by a final extension step of 300 s at 72°C in a Veriti thermal cycler. Semi-nested PCRs were performed using fourth and fifth set primers F1-R2 and F2-R1, respectively using above PCR conditions[8].

Table 1 — Details of the primers used in this study with expected size of amplicons (bp)

| Patho-gen | Primer | Nucleotide base sequence (5′–3′) | Ampl. size (bp) |
|---|---|---|---|
| CEV | CEV-F B | ATGGAGTATCCAAAGTACTTAG | 528 |
| | CEV-R J | CTCTTCACTATTGTGACTTTG | |
| | CEV-F B-int | GTTATCAATGAAATTTGTGTATTG | 478 |
| | CEV-R J-int | TAGCAAAGTACTACCTCATCC | |
| | F1 | GCTGTTGCAACCATTTGAGA | 548 |
| | R1 | TGCAGGTTGCTCCTAATCCT | |
| | F1 | GCTGTTGCAACCATTTGAGA | 481 |
| | R2 | TGCAAGTTATTTCGATGCCA | |
| | F2 | GCTGCTGCACTTTTAGGAGG | 248 |
| | R1 | TGCAGGTTGCTCCTAATCCT | |
| KHV | CyHV-3Gray Sph-F | GACACCACATCTGCAAGGAG | 292 |
| | CyHV-3Gray Sph-R | GACACATGTTACAATGGTCGC | |
| CyHV -2 | CyHVpol-F | CCCAGCAACATGTGCGACGG | 362 |
| | CyHVpol-R | CCGTARTGAGAGTTGGCGCA | |
| For bactm. | 16S-F | AGAGTTTGATCATGGCTCAG | 1500 |
| | 16s-R | GGTTACCTTGTTACGACTT | |
| Aero-lysin | AHAero-F | CAAGAACAAGTTCAAGTGGCC | 309 |
| | AHAero-R | ACGAAGGTGTGGTTCCAGT | |
| Hemo-lysin | AHH/F | GCCGAGCGCCCAGAAGGTGAGTT | 130 |
| | AHH/R | GAGCGGCTGGATGCGGTTGT | |
| Elastase | AHEL/F | ACACGGTCAAGGAGATCAAC | 513 |
| | AHEL/R | CGCTGGTGTTGGCCAGCAGG | |
| Lipase | AHLI/F | ATCTTCTCCGACTGGTTCGG | 382 |
| | AHLI/R | CCGTGCCAGGACTGGGTCTT | |
| Flagellin | AHFLA/F | TCCAACCGTYTGACCTC | 608 |
| | AHFLA/R | GMYTGGTTGCGRATGGT | |
| Cytoen | AHCYTO/F | GAGAAGGTGACCACCAAGAACAA | 232 |
| | AHCYTO/R | AACTGACATCGGCCTTGAACTC | |
| Omp TS | AHOmp/F | GCAGTGGTATATGACAAGGAC | 1008 |
| | AHOmp/R | TTAGAAGTTGTATTGCAGGGC | |
| β hemo-lysin | AHBH/F | GCTATGAAAAAACTAAAATAACTG | 1600 |
| | AHBF/R | CAGTATAAGTGGGGAAATGGAAAG | |
| T3SS | AHT3/F | ATGGACGGCGCCATGAAGTT | 710 |
| | AHT3/R | TATTCGCCTTCACCCATCCC | |

**Sequencing and phylogenetic study**

The DNA amplicons (using primers CEV For B and CEV Rev J of CEV4a protein gene) from three positive samples of each location were purified using Bangalore GeNei gel purification kit and PCR purified products were commercially sequenced (Agri Genome Labs Pvt Ltd, Kochi, India). The nucleotide sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) of NCBI (http://www.ncbi.nlm.nih.gov /blast) to find out the homology. The amino acid sequences for both location samples were derived. Available amino acid sequences of CEV 4a protein of different isolates were retrieved from the NCBI database and aligned with the amino acid sequences of Odisha samples. Multiple alignment was performed with MEGA 6 using ClustalW algorithm[24]. Phylogenetic analysis of CEV4a protein sequences were performed through Maximum Likelihood method available in MEGA 6 and the phylogenetic tree was constructed using Maximum Likelihood method.

**Experimental transmission study with CEV infected tissue homogenate**

Gill and liver tissues obtained from diseased koi carp (confirmed as CEV positive in first step PCR) were pooled and homogenized in phosphate buffer saline (PBS) to make a 10% (w/v) suspension. The homogenate was centrifuged at 5000 rpm for 5 min at 4ºC. The supernatant was filtered (0.22 µm filter) and the suspension (inoculum) stored in a sterile tube at −20ºC until required. Apparently, healthy rohu, koi carp and goldfish juveniles were obtained from nearby farm without any previous history of infection; and koi carp representative samples from the lot were screened for CEV and found to be negative in nested PCR as described earlier. The fish were divided into five groups (Table 2) and kept in fibre-reinforced plastic tanks in a wet laboratory in semi-controlled

Table 2 — Experimental transmission study using different fish species and different methods

| Group | Viral inoculum (CEV infected tissue homogenate) | Fish species (numbers/average size in g) | Intraperitoneal (i.p.)/immersion route/co-habitation |
|---|---|---|---|
| I | 100 µL | Goldfish (5/10) | i.p. |
| II | 200 µL | Rohu (5/25) | i.p. |
| III | 100 µL/l L of water | Rohu (5/25) and koi carp (3/30) | immersion |
| IV | 100 µL | Rohu (5/25) and koi carp (3/30) along with challenged goldfish (3/10) | Co-habitation with i.p. injected goldfish (3 nos.) |
| V | 100 µL PBS | 5 fish from each of the three species | i.p |

condition under continuous aeration. The water temperature during the experiment was 32ºC and care was taken to maintain normal physicochemical parameters of water in the tank. The fish were fed with commercial pellet fed twice daily. The fish were challenged by different routes of exposure with viral inoculum (Table 2). The fish were observed for any mortality for 10 days and then live/moribund fish were euthanized, and gill and liver tissues were collected. The tissues were processed for nested PCR analysis (using primers CEV For B, CEV Rev J, CEV For B-int and CEV Rev J-int[22] F1- R1, F1-R2 and F2-R1[8]) to detect the presence of virus as described earlier. MS222 (Sigma, USA) was usedduring injection and euthanization.

#### Bacteriological investigation

Inoculum from the kidney tissues of naturally infected koi was streaked onto tryptone soy agar (HiMedia, India) and pure colonies obtained were processed for DNA isolation following phenol-chloroform extraction method of Sambrook & Russell[25]. The DNA was subjected to 16S rDNA PCR followed by sequencing to identify the individual isolates[23]. The consensus 16S rRNA gene sequences obtained from the sequencing were analyzed with the other *Aeromonas* strains 16S rRNA gene sequences available in GenBank using Basic Local Alignment Search Tool (BLAST) algorithm provided by National Centre for Biotechnology Information (NCBI) for bacterial identification.

Based on the results, further PCR for aerolysin and beta-haemolysin genes specific to *Aeromonas hydrophila* were carried out for reconfirmation[26] (Table 1). OmpTs and AHCYTOEN gene-based primers were also used to determine whether the strain involved was pathogenic or not[27] (Table 1). Further, elastase, lipase, and flagellin, T3SS and haemolysin gene based primers were used to study the degree of infection and pathogenicity of the bacterium in koi carp[28,29] (Table 1).

#### Bacterial challenge study

Healthy koi carp, *Cyprinus carpio* showing no signs of disease were taken for the experiment. The fish weighing approximately 20 g were stocked in four tanks (five fish in each) in a wet laboratory under continuous aeration and commercial feed was provided. They were acclimatized for a week in the tank before conducting the experiment. Two tanks fish were challenged with isolated *A. hydrophila* strain at $10^7$ CFU/20 g fish intraperitoneally to check its virulence. The fish of other two tanks served as control and received only PBS injection. The fish were observed for any mortality up to day 10 post-challenge.

## Results

#### Microscopy

The gill tissues collected from the farm koi carp samples revealed infection with parasite *Trichodina*. Other tissue samples were found to be parasite-free.

#### Histopathology

Upon histology of gills, massive necrosis, clubbing and fusion of secondary gill lamellae due to proliferation and hyperplasia of epithelial cells (Fig. 2A) were evident along with infiltration of few eosinophilic granular cells and accumulation of cellular debris. There was evidence of intracytoplasmic eosinophilic viral inclusions in gill epithelial cells (Fig. 2B). The posterior kidney revealed degeneration and necrosis of tubular epithelia along with massive congestion of blood vessels (Fig. 2C).

#### Identification of virus in the samples

Using primers CEV F B/R J and F B int/R J int of CEV4a gene, amplicons of 528 and 478 bp were

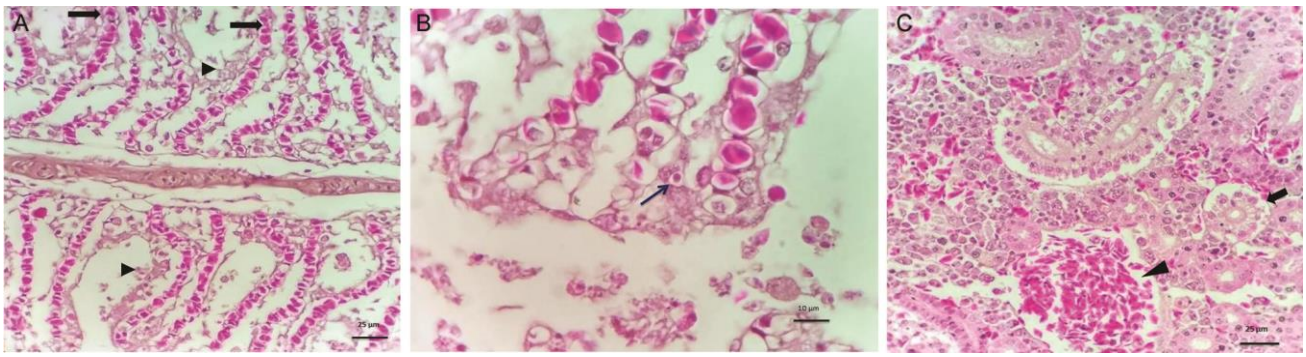


Fig. 2 — (A) Gill lamellae showing massive necrosis (arrow head), clubbing and fusion (arrow) of secondary lamellae (H & E; Bar = 25 µM); (B) Intracytoplasmic eosinophilic viral inclusion (arrow) in gill epithelial cell (H & E; Bar = 10 µM); and(C)- Posterior kidney revealed degeneration and necrosis (arrow) of tubular epithelia along with massive congestion (arrow head) of blood vessels (H & E; Bar = 25 µM)

obtained in first and second step PCRs, respectively (Fig. 3A). For further confirmation of CEV, the gill and liver tissues were amplified with F1 and F2 primers. The amplified products of 548 bp (in the 1st round) and 478, 481 and 248 bp in the 2nd round (with primers F1-R1, F1-R2 and F2-R1, respectively) (Fig. 3B) were obtained for CEV.

All the samples collected from natural infected cases were found to be negative for KHV and goldfish haematopoietic necrosis virus (CyHV-2) using respective primer sets.

**Sequence analysis and phylogeny**

The CEV 4a gene amplicons obtained were sequenced and a BLAST search of the sequence revealed 100% identity with known previously published 4a genes of CEV. The 4a protein gene sequence amplicon of 477 bp encoding 159 amino acids has been submitted to GenBank (GenBank accession number: MF326541). The multiple alignment of twelve isolates of CEV disclosed that our two sequences obtained in this study from two different locations of the Odisha are 100% similar or homologous with each other. The sequence similarity between different isolates is shown in Fig. 5A. There was a marked variation at position corresponding to amino acids $12^{(Tyr- His)}$, $30^{(Ala- Val)}$, $45^{(Ser-Asn)}$, $62^{(Tyr-Cys)}$ and $91^{(Thr-Ala)}$ between Odisha and UK (KX254027 and KX254021) isolates. Apart from these changes at position $65^{(Thr-Val)}$, $79^{(Phe-Tyr)}$, $91^{(Ser-Thr)}$ amino acids were replaced between Odisha isolates with UK (KX254020) and Poland (KX254012). Both the isolates of Odisha also showed 100% sequence similarity with UK (KX254022) and Poland (KX254003) isolates. The present CEV isolate was more closely linked to the branch of CEVs detected

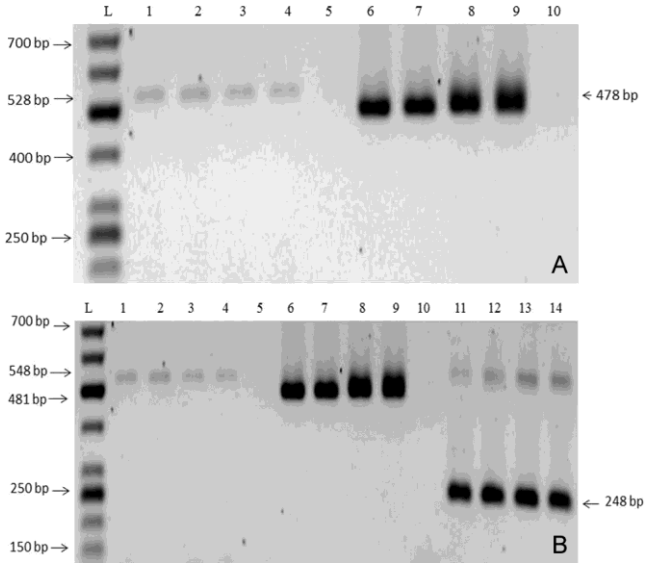


Fig. 3 — Samples amplified with (A) CEV F B/R J and CEV F B-int/R J-int primers, with expected product sizes of 528 and 478 bp, respectively. [Lanes 1-4 represent infected samples from Choudwar, Odisha, amplified with CEV F B/R J primer; Lanes 6-9 represent 2nd step PCR product, amplified with CEV F B-int /R J-int primer; and Lanes 5 and 10 represent negative controls; 50 bp Ladder (Thermo Scientific)]; and (B) CEV F1/R1, CEV F1/R2 and CEV F2/R1 primers with expected product sizes of 548, 481 and 248 bp, respectively. [Lanes 1-4, represent infected samples from Choudwar, Odisha, amplified with CEV F1/R1; Lanes 6-9 and 11-14 represent 2nd step PCR product, amplified with CEV F1/R2 and CEV F2/R1 primer, respectively; and Lanes 5, 10 represent negative controls; 50 bp Ladder (Thermo Scientific)]

in the UK (KX254022) and Poland (KX254003) (Fig. 4 A & B).

A phylogeny tree based on CEV 4a gene amino acid sequence was constructed (Fig. 5B) and the present isolate clustered within all reported CEV isolates. It also revealed significant separation between CEV 4a

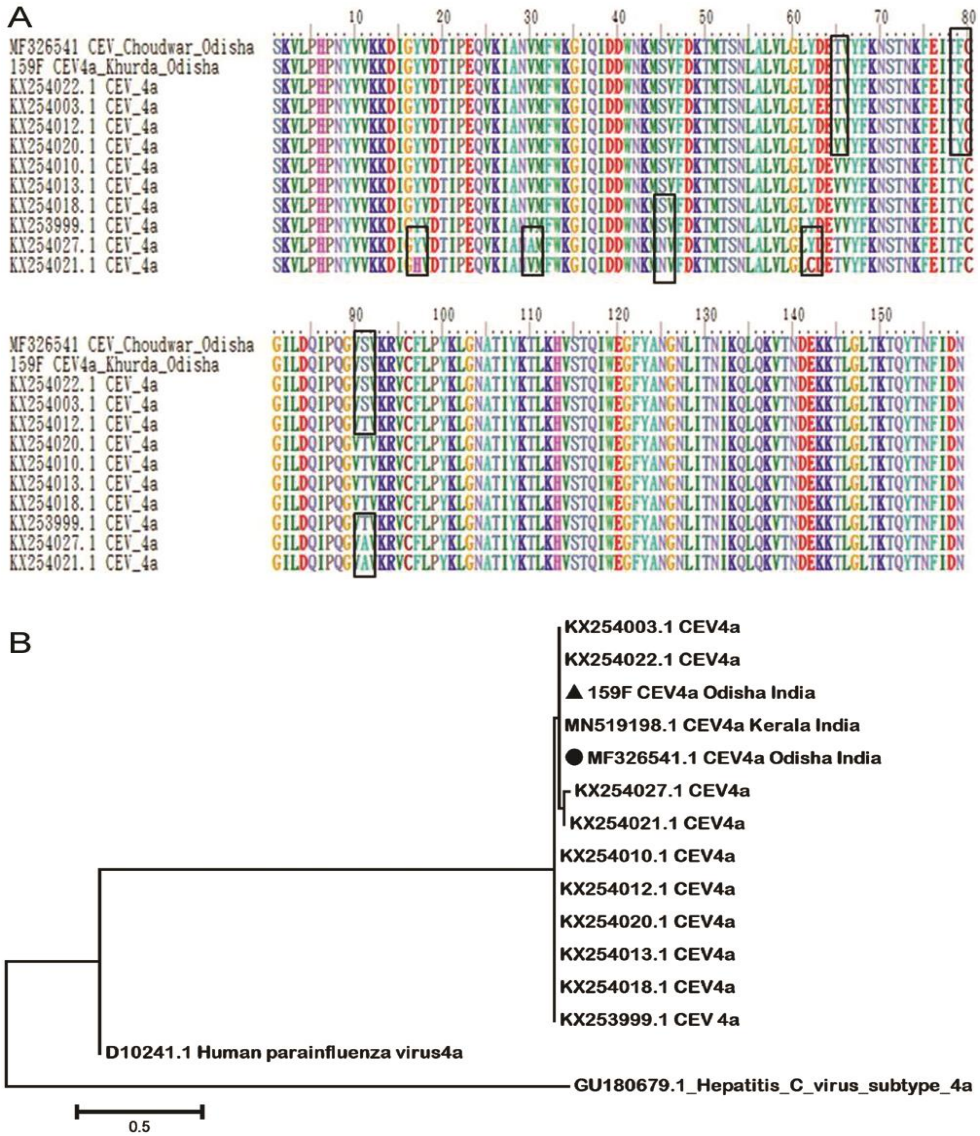


Fig. 4 — (A) Amino acid alignment of CEV 4a sequences (1-159) of Odisha and other available isolate retrieve from NCBI. [The aa variations are outlined. The alignment result is obtained by graphic view of Bio Edit Sequence Alignment Editor]; and (B) Phylogenetic tree based on the amino acid sequence of Carp edema virus homologues. [The tree was constructed using Maximum likelihood method of Clustal W. MF326541: Carp edema virus, Choudwar, Odisha, India isolate 4a protein gene, partial cds, GU180679.1: Hepatitis C virus 4a protein gene, D10241.1: Human parainfluenza virus 4a gene]

gene of Odisha isolate with human parainfluenza virus 4a gene and hepatitis C virus subtype 4a gene, as indicated by their positions in different clusters. Phylogenetic comparison of the partial 4a gene fragments of this virus with various other representative CEVs indicated that the present isolate was most closely related to CEV detected in koi in Poland and the UK.

**Experimental transmission study with CEV infected tissue homogenate**

Rohu and goldfish challenged either by intra-peritoneal or immersion or co-habitational methods were found to be negative for CEV using first step and nested PCR of gill and liver tissues. Koi carps challenged by co-habitational route with intraperitoneally injected goldfish were found negative for CEV in their tissues, whereas koi carps challenged by CEV infected tissue homogenate by immersion method were found to be positive. On the other hand, rohu challenged by only immersion method, and also maintained with koi carp challenged by immersion in the same tank (co-habitation) were found to be negative in nested PCR for carrying the virus. There

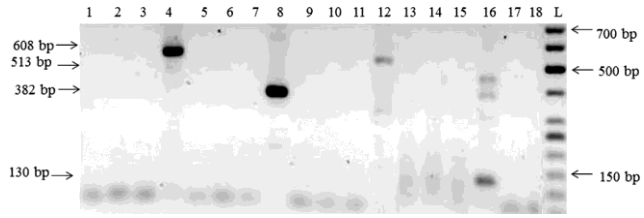


Fig. 5 — Gel showing PCR amplification of *Aeromonas hydrophila* different gene. [Lanes 2,4,6,8,10,12,14,16,18 represent amplified product of primer AHAEROF/R, AHFLF/R, AHBHF/R, AHLIF/R, AHT3F/R, AHELF/R, AHOmpF/R, AHHF/R and AHCYTOF/R, respectively; Lanes 1,3,5,7,9,11,13,15,17 represent the negative control of respective primers. Bands obtained for flagelin, lipase, elastase and hemolysin genes represents lane 4,8,12 and 16, respectively. L, 50 bp ladder (Thermo Scientific)]

was no mortality in any of the fish species during the experiment.

**Bacteriology**

A gram negative bacterium was isolated from the kidney samples of infected fish of the affected farm and was confirmed to be *A. hydrophila* in 16s rDNA PCR followed by sequencing and blast. The bacterium was further characterized for its virulence associated genes and found to be positive for carrying hemolysin, elastase, lipase, and flagellin genes, and but negative for aerolysin, T3 secretion system, OmpTS, β hemolysin and cytoen genes (Fig. 5). The koi carps experimentally challenged with *A. hydrophila* revealed signs of septicaemia with haemorrhages on ventral parts of body whereas there was no mortality observed in the challenged fish. The affected fish recovered from the infection after 10 day post-challenge as noticed from absence of gross clinical signs.

## Discussion

The results of our study confirmed the presence of CEV infection in koi carps cultured or traded in eastern part of India, Odisha state. There is lack of knowledge on the pathogenesis of this virus, and the role of other co-cultured or traded fish being acting as vectors or carriers of infection is poorly understood. In the present study, an attempt was made to infect the Indian major carp, rohu, and goldfish (fish being co-cultured or traded with koi) by various methods of exposure (intraperitoneal, immersion and co-habitational routes) and it was noticed that neither of the routes of exposure could able to establish the infection in both rohu and goldfish. Earlier report also mentioned about similar findings in natural infection case with koi tanks that were infected with CEV without infecting goldfish or rohu[12]. Thus, it is confirmed that there exists no or limited threat to co-cultured goldfish or Indian major carps to CEV infection. However, further detailed study is needed to say whether any other ornamental fish are susceptible to this infection or not. Earlier studies have noticed infection of koi with CEV at a temperature below 25°C causing 100% mortality, and 26.67% mortality at 28°C[13,18]. However, in the present investigation mortality in koi carps were recorded at temperature as high as 32-34°C. However, it needs further investigation to find out suitable temperature for this virus replication in the absence of any susceptible cell lines for this virus.

Although water temperature was not found to be a major stress factor as noticed in these two cases as contrary to previous reports[8,13], transportation stress and/or restocking in tanks played crucial role in disease outbreak in these two cases. The koi carps revealed clinical signs of ulcers on body, patchy haemorrhages, depigmentation and massive necrosis of gills, lethargy, and sleeping at the bottom of tanks before death as noticed earlier reported outbreaks[12,13,,1618]. However, there was no evidence of sticky mucus deposits on skin as described in carps infected with CEV previously[16]. The gills showed massive clubbing and fusion of secondary gill lamellae due to proliferation and hyperplasia of epithelial cells along with infiltration of few eosinophilic granular cells and accumulation cellular debris. The posterior kidney revealed degeneration and necrosis of tubular epithelia. Similarly, massive necrosis and hyperplastic changes in gill tissues with infiltration of eosinophilic granular cells besides mononuclear cellular infiltration, hyperemia and necrosis in posterior kidney tissues were noticed in CEV cases in koi carps[13,16]. The burden of concomitant infections, at least with ectoparasite and possibly even with bacteria, might suggest a strong immunesuppressive action played by CEV[16]. This finding could explain the chronic character of this disease during natural occurring outbreaks, despite the previously suggested acute form of outbreaks[8,18]. The degree of stress might be main governing factor for influencing the nature of disease.

PCR is a reliable molecular detection method for detection of CEV[11]. As CEV is new to India, five sets of primers were used to confirm the case followed by sequence analysis and challenge study. All the five sets of primers could successfully amplified the desired amplicons in two cases of CEV recorded here. A strong amplification product indicated high virus

load in infected samples. Phylogenetic analysis revealed that our sequence having 100% similarity with other reported Indian sequence and these isolates are more closely related to CEV isolates from Poland, Japan and UK forming a single cluster. The minute variations at amino acid levels in 4a gene sequence among the isolates needs further investigations for their probable roles in viral pathogenicity, particularly causing high mortality at water temperature as high as 32-34°C.

The wet mount microscopic examination of gill tissues revealed presence of *Trichodina* parasite infection in few of koi carps. However, there was absence of any parasite in aquarium koi carp samples. Further, the farmed koi tissue revealed the presence of *A. hydrophila* in kidney tissue. On the other hand, the fish received from aquaria were free from bacteria in their kidney tissues. The bacterium was further characterized for its virulence associated genes and found to be positive for carrying hemolysin, elastase, lipase, and flagellin genes, but negative for aerolysin, T3 secretion system, OmpTS, β hemolysin, cytoen toxin genes. An experimental challenge with this bacterium also failed to reproduce the disease in infected koi although the koi revealed signs of septicaemia with haemorrhages on ventral parts of body without causing any mortality. Hence, it was concluded that the CEV as the primary etiological agent to cause koi sleepy disease and bacterium being secondary to aggravate the condition. Similarly, earlier researchers have also noticed association of bacteria *viz*., *Aeromonas sobria*, *Klebsiella pneumonia*, *Proteus penneri*, *Escherichia coli* and *Shewanella decolorationis* in gills of naturally infected koi carps with CEV infection[12] and *Aeromonas hydrophila*, *A. sobria*, an array of ectoparasites, and spring viraemia of carp virus and koi herpes virus in infected common carp[14-16]. It is also common to find bacterial infections being superimposed with primary viral etiological agents in farmed ornamental fish as noticed in our earlier study in goldfish infected with CyHV-2 infection[23]. Further, the presence of few toxin genes of *A. hydrophila* might not be sufficient for causing any mortality until unless fish are stressed to certain level, thus further confirming the bacterium to be an opportunistic pathogen in koi carps. Thus, intensive investigation in each case is needed to ascertain or delineate concomitant opportunistic parasitic or bacterial infections that hide viral infections.

## Conclusion

Observations of present investigation on large scale mortality of koi carps from selected farms of Choudwar, Cuttack, and also retail market at Bhubaneshwar, Odisha, suggest edema virus (CEV) affecting both farmed and trade koi from the eastern part of India. Infected koi carps did not pose any major threat to either goldfish or food fish like Indian major carps that are co-cultured or traded with koi carps. Further, the results support earlier findings on immunosuppression by CEV infection which leads to infestation by ectoparasites and other bacteria. High mortality at 32-34°C has not been reported earlier for CEV. We recommend strong surveillance and a pan-India study to understand the persistence of virus to prevent further mortality of this important ornamental species and also economic loss in ornamental fishery.

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## Conflict of interest

None to declare.

## Ethical approval

The work has been carried out following approval of Institute Ethics Committee

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