

Emergence of carp edema virus in cultured ornamental koi carp, *Cyprinus carpio koi*, in India

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A disease outbreak was reported in adult koi, *Cyprinus carpio koi*, from a fish farm in Kerala, India, during June 2015. The clinical signs were observed only in recently introduced adult koi, and an existing population of fish did not show any clinical signs or mortality. Microscopic examination of wet mounts from the gills of affected koi revealed minor infestation of *Dactylogyrus* sp. in a few koi. In bacteriological studies, only opportunistic bacteria were isolated from the gills of affected fish. The histopathological examination of the affected fish revealed necrotic changes in gills and, importantly, virus particles were demonstrated in cytoplasm of gill epithelial cells in transmission electron microscopy. The tissue samples from affected koi were negative for common viruses reported from koi viz. cyprinid herpesvirus 3, spring viraemia of carp virus, koi ranavirus and red sea bream iridovirus in PCR screening. However, gill tissue from affected koi carp was positive for carp edema virus (CEV) in the first step of nested PCR, and sequencing of PCR amplicons confirmed infection with CEV. No cytopathic effect was observed in six fish cell lines following inoculation of filtered tissue homogenate prepared from gills of affected fish. In bioassay, the symptoms could be reproduced by inoculation of naive koi with filtrate from gill tissue homogenate of CEV-positive fish. Subsequently, screening of koi showing clinical signs similar to koi sleepy disease from different locations revealed that CEV infection was widespread. To our knowledge, this is the first report of infection with CEV in koi from India.

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INTRODUCTION

In the international ornamental fish trade, more than 90% of the freshwater fishes (by volume) are farm bred, and about 4000 species of freshwater ornamental fishes are traded every year (Whittington & Chong, 2007). Goldfish (*Carassius auratus*) and koi carp (*Cyprinus carpio koi*) are the most extensively traded fishes species (Ariel, 2005). Importantly, the global ornamental fish trade relocates large quantities of live fish species between countries, and

therefore can be a potential source for spread of exotic pathogens, particularly viral pathogens, which have been associated with high morbidity and mortality in fishes, in the importing countries (Oyamatsu *et al.*, 1997a; Amita *et al.*, 2002).

Some of the important viral diseases affecting koi carp, *C. carpio koi*, are caused by infection with cyprinid herpesvirus 3 (CyHV-3, also known as koi herpesvirus), spring viraemia of carp virus (SVCV), ranavirus (George *et al.*, 2015) and carp edema virus (CEV) (Oyamatsu *et al.*, 1997a). CEV is an unclassified virus belonging to the family *Poxviridae* and has two main lineages: lineage 1 (CEV) from koi carp which is more similar to original Japanese CEV and lineage 2 (CEV-like) from common carp (Way & Stone, 2013).

The GenBank/EMBL/DDBJ accession numbers for the new sequences reported in this paper are KX503806 and KX503807 (will be released to public database after July 2017).

Infection with CEV, also known as koi sleepy disease (KSD), was originally described from koi carp in Japan in 1974 (Murakami *et al.*, 1976) and has recently been reported from many European countries including UK, France (Way & Stone, 2013), The Netherlands (Haenen *et al.*, 2013), Germany (Jung-Schroers *et al.*, 2015), Italy (Pretto *et al.*, 2015), Czech Republic (Vesely *et al.*, 2015) and Austria (Lewisch *et al.*, 2015). Recently, a highly specific and sensitive quantitative PCR protocol was developed to improve CEV detection in koi by Adamek *et al.* (2016). Importantly, there is no report of KSD from India, till date.

The disease is characterized by typical sleepy behaviour, enophthalmia, generalized oedematous condition and gill necrosis (Lewisch *et al.*, 2015). The affected juvenile carp usually congregate near the surface of pond or water inlet, whereas the older fish tend to lie on the bottom of the pond and eventually die of anoxia (Miyazaki *et al.*, 2005) and the mortality may reach 80–100%. The koi carp culture in India is growing vigorously and it is localized in selected states viz. Kerala, Tamil Nadu, West Bengal and Maharashtra. In the present study, we report an outbreak in koi carp in Ernakulam District, Kerala, India, resulting in 100% mortality and, to our knowledge, this is the first report of infection with CEV from India. In subsequent studies, the infection appears to be quite widespread in koi in the country.

RESULTS AND DISCUSSION

The disease outbreak was reported in an ornamental fish farm in the month of June 2015 which is known to be a rainy season in Kerala, India. The affected fish exhibited lethargic behaviour and were seen lying at the bottom of the pond. The other clinical signs included haemorrhages on the surface, sunken eyes and swelling of body and gills



Fig. 1. CEV-affected fish with swollen body and gills from koi carp showing pale appearance, swelling and abundant mucus.

(Fig. 1). Importantly, these clinical signs were observed only in introduced adult koi, 3 days after stocking, and the number of fish displaying these signs increased daily. The mortality started on the fifth day after stocking and 100% mortality (a total of 800 fish) was reported within 8 days of stocking. The water temperature of the affected pond was 18–20 °C.

The clinical signs viz. anorexia, lethargy, skin erosions, enophthalmos and gill necrosis, as observed in the present outbreak, have been reported earlier as well (Miyazaki *et al.*, 2005; Haenen *et al.*, 2013; Way & Stone, 2013; Lewisch *et al.*, 2015). Interestingly, the disease was not observed in existing population of koi (250 fish), goldfish (300 fish) and Indian major carps (IMCs; *Labeo rohita* and *Catla catla*) (500 fish) reared in the same pond. Previously, Jung-Schroers *et al.* (2015) observed that an existing population of koi in a German farm was affected by the disease, but the disease was not observed in goldfish and sturgeon kept in the same farm. In conformity with the findings of the present study, very high mortality ranging from 80 to 100% has also been reported previously in affected koi (Oyamatsu *et al.*, 1997a; Miyazaki *et al.*, 2005). Similar to the observation of the present study, CEV outbreak in koi ponds has been reported at 15–25 °C (Oyamatsu *et al.*, 1997a; Amita *et al.*, 2002). It has been reported that water temperature and acute stress conditions (restocking, transport, etc.) play a crucial role in the onset of disease outbreaks in fish populations (Miyazaki *et al.*, 2005; Magnadottir, 2010). Therefore, it can be assumed that stress of transportation could have precipitated KSD outbreak in adult koi in the present outbreak. In accordance with our findings, Lewisch *et al.* (2015) reported that restocking is an important predisposing factor responsible for KSD outbreaks (Lewisch *et al.*, 2015). Similarly, Jung-Schroers *et al.* (2015) also reported that KSD outbreaks are generally observed when koi are moved from earthen ponds to cemented tanks. In the outbreak reported here, the lack of transmission of disease to the previously resident fish might be due to lack of handling stress in those fish or, possibly, immunity due to previous sublethal exposure to the virus.

Microscopic examination of wet mounts from the gills revealed minor infestation of *Dactylogyrus* sp. in a few koi. Furthermore, bacteria viz. *Aeromonas sobria*, *Klebsiella pneumoniae*, *Proteus penneri*, *Shewanella decolorationis* and *Escherichia coli* were isolated from the gills of affected fish. However, bacteria could not be isolated from internal organs viz. spleen and kidney of affected fish. Since the parasites and bacteria were not observed consistently in the diseased koi, therefore, it was presumed that the aetiological agent of disease might be other than parasites and bacteria.

The histopathological examination of the affected fish revealed severe necrosis of gills, proliferation of the epithelial cells and adhesion of gill lamellae. In addition, infiltration of eosinophilic granular cells was observed between the secondary gill lamellae along with large number of mononuclear cells and a few erythrocytes (Fig. 2a). In the kidney,

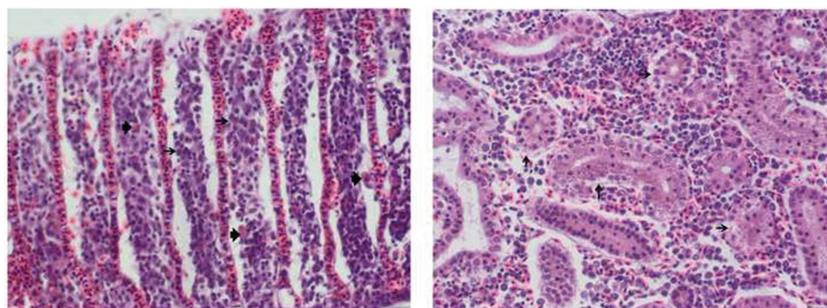


Fig. 2. Histopathological studies of gills from CEV-affected koi carp. (a) Extensive infiltration of mononuclear cells (arrows) with a few eosinophilic granular cells (arrowheads) and erythrocytes in the interlamellar region of affected gill tissue. Bar, 20 μ m. (b) Section of kidney showing degenerative changes (arrows) in tubular epithelial cells. Bar, 20 μ m.

degenerative changes were observed in tubular epithelial cells (Fig. 2b). The histopathological alterations, as observed in the present case, are consistent with previous reports of KSD outbreaks (Ono *et al.*, 1986; Miyazaki *et al.*, 2005; Jung-Schroers *et al.*, 2015; Lewisch *et al.*, 2015). As the virus is reported to replicate in gill epithelial cells (Ono *et al.*, 1986; Miyazaki *et al.*, 2005), this would have resulted in severe histopathological alterations in the gills. However, atrophy of splenic pulp as reported by Miyazaki *et al.* (2005) was not observed in affected koi. In a previous study, multifocal necrotic areas in hepatopancreas and myocarditis due to infection with CEV have been reported (Lewisch *et al.*, 2015). However, in the present study, these tissues were not examined for histopathological alterations. It has been suggested previously that cellular changes in organs other than gills are due to effect of hypoxia caused by severe damage to gills (Lewisch *et al.*, 2015).

Importantly, virus particles were demonstrated in cytoplasm of gill epithelial cells of affected koi carp and these

were spherical and electron dense in transmission electron microscopy (TEM) analysis (Fig. 3). However, no virus particles were observed in spleen and kidney tissue sections of the infected koi carp. These findings are consistent with previous reports (Ono *et al.*, 1986; Oyamatsu *et al.*, 1997b; Miyazaki *et al.*, 2005; Jung-Schroers *et al.*, 2015). In contrast, Way & Stone (2013) failed to demonstrate virus particles in gill tissue from diseased carp. In accordance with our results, the virus particles have not been demonstrated in tissues other than gills (Oyamatsu *et al.*, 1997a; Miyazaki *et al.*, 2005).

PCR screening of the tissue samples (gills, spleen and kidney) from affected koi revealed that these were negative for CyHV-3, SVCV, koi ranavirus and red sea bream iridovirus. However, gill tissue (37 samples) from affected koi carp was positive for CEV in nested PCR developed by Oyamatsu *et al.* (1997b). Interestingly, other co-habiting fishes viz. resident koi carp, goldfish and IMCs collected from the affected farm were not positive even in the second-step PCR (five fish tested for each species). The sequence analysis of 548 bp fragment of randomly selected 10 PCR amplicons from CEV-positive fish revealed greater than 96 and 98 % sequence identity with the original sequences submitted by Oyamatsu *et al.* (1997b) and Jung-Schroers *et al.* (2015) (accession number KM283182), respectively. This confirmed the identity of CEV as the probable cause of the mortality in affected koi. The sequences have been submitted to NCBI GenBank (accession numbers KX503806 and KX503807). Way & Stone (2013) also reported that amplification products from the majority of CEV-positive cases of koi in UK share 97.5–99.5 % nucleotide sequence identity with the original Japanese CEV sequence. Further, all 37 fish samples were re-tested with a second nested PCR assay (recent assay better at detecting CEV in carp) targeting 478 bp partial 4a gene sequence from CEV. The sequence analysis of 10 partial 4a gene sequences (analysed in the previous PCR) revealed 95 and 99 % identity with sequence of CEV isolated from common carp (KX254010, KX254011, KX254019 and KX254020) and koi carp (KX254000, KX254001, KX254021, KX254022 and KX254024),

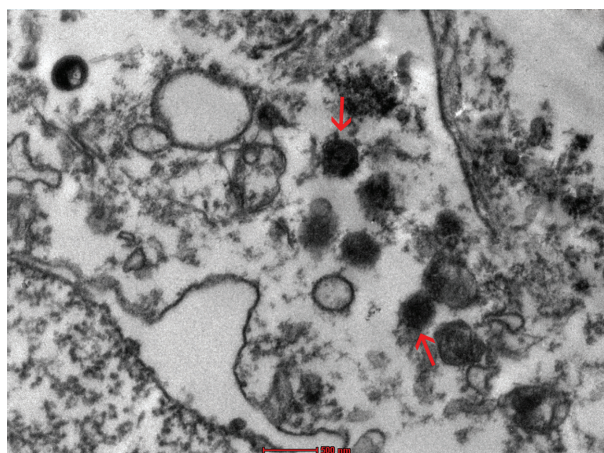


Fig. 3. Transmission electron photomicrograph of a koi gill epithelial cell containing large intracytoplasmic spheroid CEV virions (arrows). Bar, 500 nm.

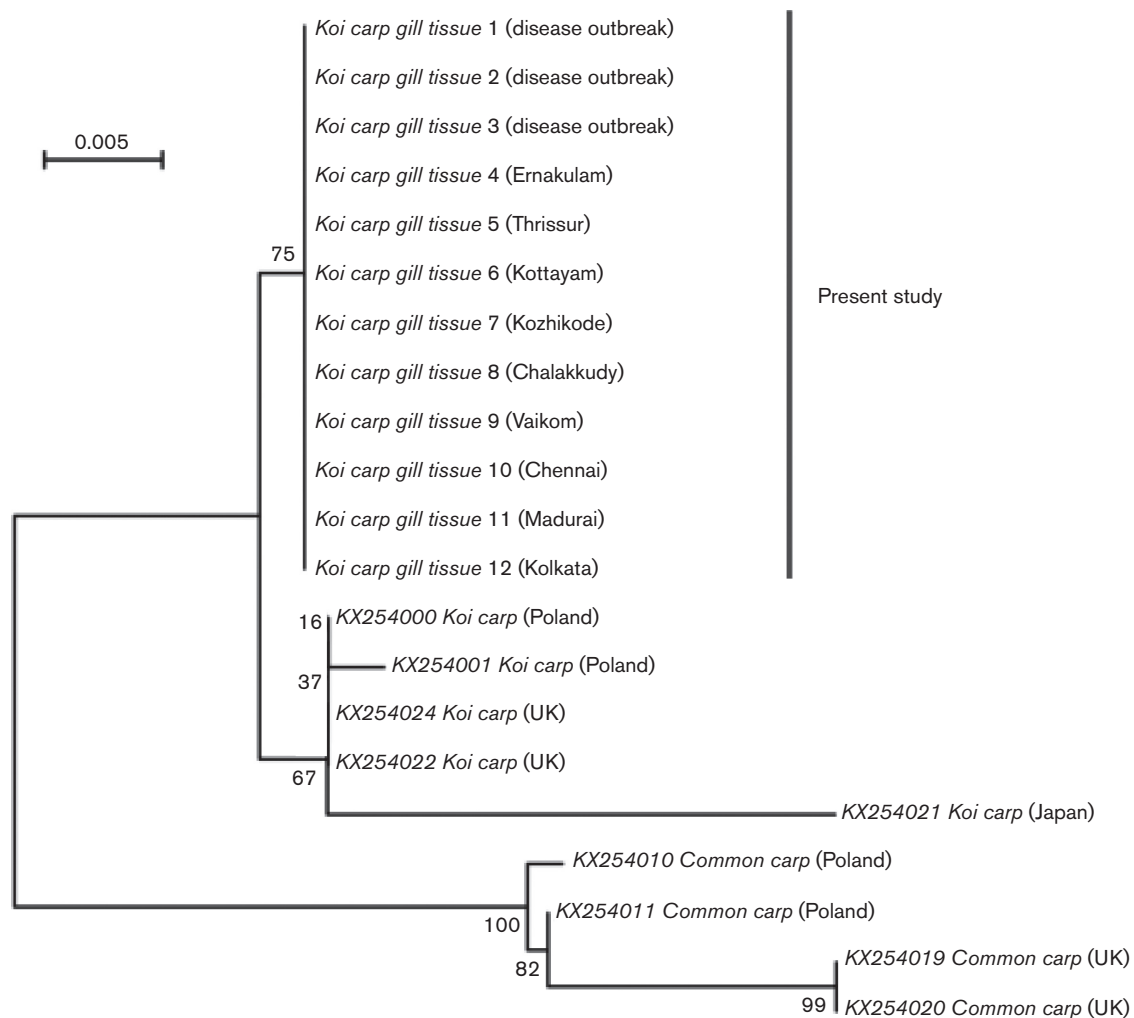


Fig. 4. Unrooted neighbour-joining tree based on 478 bp partial 4a gene sequence of CEV. Except the Indian CEV sequences, all other CEV sequences of common carp and koi carp were obtained from NCBI. The scale bar represents substitutions per nucleotide site.

respectively. No substitutions of the nucleotide were found among all 12 Indian CEV sequences and showed 100 % sequence identity. The sequences have been submitted to NCBI GenBank. Phylogenetic analysis of 478 bp fragment of 4a gene revealed two distinct clusters viz. CEV sequences from koi carp and common carp and suggested that there was a high relatedness between the Indian koi carp CEV sequence and koi carp CEV sequences reported in UK, Poland and Japan (Fig. 4). Thus, the koi carp CEV identified in India had highest identity with koi carp CEV detected in UK, Poland and Japan. The positive results in the first-step PCR indicate high virus loads in gills of adult koi undergoing mortality patterns and corroborate severe damage observed in gill tissue sections. Earlier, Lewisch *et al.* (2015) could detect high virus load in pooled tissues (gills, brain, head kidney and spleen) in a few cases with terminal stages of the disease and even in blood of koi not

exhibiting any clinical signs of disease except for lethargy. Similar to our results, Jung-Schroers *et al.* (2015) detected CEV in a large number of tested samples, and Miyazaki *et al.* (2005) observed CEV-specific amplicon from gills and erosive skin of moribund koi while no amplification was derived from liver and kidney.

No cytopathic effect was observed in six cell lines following inoculation of filtrate from gill tissue homogenate of CEV-positive koi, after 15 days of inoculation and even after five blind passages. The cell lines tested were derived from koi, goldfish and four other ornamental fish species cultured in India (Swaminathan *et al.*, 2010, 2013, 2015, 2016a, b). The results are in accordance with earlier report of Oyamatsu *et al.* (1997a) who were not able to isolate virus using 31 cell lines including 9 cyprinid, 8 salmonid, 4 anguillid and 10 other teleostean cell lines inoculated with filtered



Fig. 5. Collection sites of koi carp (*C. carpio koi*) in the study for the survey of CEV.

homogenate of gills. Similarly, Jung-Schroers *et al.* (2015) did not observe any cytopathic effect following inoculation of filtrate from pooled tissues (gills, kidney, gut and brain) in fathead minnow and common carp brain cell lines. Therefore, it is imperative to develop *in vitro* cultivation methods to better understand host–pathogen interaction.

In bioassay, koi inoculated with filtrate from gill tissue homogenate of CEV-positive fish showed clinical signs 4 days post-inoculation (dpi) onwards and infected fish began to die from 6 dpi and during experimental period of 10 dpi; 5 out of 15 inoculated fish died. PCR of gill tissue of dead fish was positive for CEV in the first step of nested PCR. However, in the control group, there was neither morbidity nor mortality and all the tested fish were negative in PCR.

Survey was carried out to detect presence of CEV in koi exhibiting clinical signs similar to sleepy disease from other locations in India (Fig. 5). The samples were screened for the presence of CEV as per Matras *et al.* (2016). A total of 53 out of 320 koi carps (16.56 %) were positive for CEV in the first step, whereas additional 9 samples (2.8 %) were positive in the second-step PCR (Table 1). CEV was detected at nine locations during active surveillance of KSD/CEV. All the nine sequences generated from the survey of CEV in India were identical with each other and also with the disease outbreak sequences. Three sampling locations viz. Chennai, Madurai and Kolkata (hubs of ornamental fish farming in India) had higher prevalence levels of 31.58, 27.78 and 28.57 %, respectively. Shifting of the koi

fingerlings from the cemented tanks to the earthen ponds for growout is a common practice followed in these three locations. In other sampling locations, KSD/CEV was observed mainly when the koi carps were under stress due to transport and restocking in a different farming system, i.e. cemented tanks to earthen ponds and vice versa.

In the present study, screening of koi showing symptoms similar to sleepy disease from different locations revealed that CEV prevalence was widespread and the virus was detected in all the 10 locations sampled. Interestingly, all the partial 4a gene sequences generated from India were identical, suggesting high genetic similarity among Indian CEV. Previously, several cases of mortality in koi exhibiting gill necrosis were negative for CyHV-3 in PCR. In the context of present findings, it may be assumed that some of these cases could have been due to infection with CEV and would require testing to confirm in future studies. Importantly, CEV-like virus has been detected in clinically healthy koi carps imported from Israel and Japan to the UK (Way & Stone, 2013). So, trading apparently healthy koi may pose a threat of spreading CEV to unaffected regions, which emphasizes the need of precautionary approach to prevent its spread.

METHODS

Fish samples. During May 2015, a disease outbreak was reported from an ornamental fish farm in Ernakulam District, Kerala, India, with a cumulative mortality up to 100 % in adult koi carp ($n=800$; 40–42 cm

Table 1. Detection of CEV in koi collected from different locations and exhibiting clinical signs similar to sleepy disease

Sample no.	Sampling location	No. of samples tested	No. of samples positive in first-step PCR	Additional number of samples positive in second-step PCR	Remarks
1	Thrissur	30	2	0	Samples were collected from a farm
2	Ernakulam	33	3	0	Samples were collected from a retail shop
3	Kottayam	28	2	0	Samples were collected from a farm
4	Kozhikode	24	4	1	Adult koi with symptoms were purchased and transported recently from Madurai, Tamil Nadu
5	Paravoor	26	0	0	Samples were collected from a farm
6	Chalakkudy	34	7	2	Adult koi with symptoms were purchased and transported recently from Chennai
7	Vaikom	36	3	0	Positive samples were collected from a retail shop
8	Chennai	38	12	2	Samples were collected when the adult koi were shifted from cemented tank to earthen ponds
9	Madurai	36	10	2	Samples were collected when the adult koi were shifted from cemented tank to earthen ponds
10	Kolkata	35	10	2	Samples were collected when the adult koi were shifted from cemented tank to earthen ponds
Total		320	53	9	

total length with 0.85–1.12 kg body weight). The adult koi had been purchased from an other farm located about 200 km away and stocked in an earthen pond, with existing population of koi carp ($n \approx 250$; 0.3–0.35 kg body weight), goldfish ($n \approx 300$) and IMCs ($n \approx 500$), 3 days prior to disease outbreak. The fishes ($n = 37$ affected koi; $n = 5$ resident koi; $n = 5$ goldfish and $n = 5$ IMCs) were transported to the laboratory for diagnosis on the second day of onset of mortality.

On arrival, the affected fish were observed for clinical signs and gross lesions. Fresh smears of gills and skin were examined under a microscope for ectoparasites. Thereafter, the fish were euthanized with MS222 (Sigma-Aldrich) and gill, spleen and kidney tissues were collected aseptically for bacterial and virus isolation. The tissues were also fixed in 10 % neutral buffered formalin and in 2.5 % glutaraldehyde (Sigma-Aldrich) in phosphate buffer for histopathology and TEM, respectively. Gill, spleen and kidney tissue samples were also preserved separately in 99 % ethanol for molecular diagnosis.

Bacteriological investigation. Bacterial isolation was attempted from gills and kidney of the affected fish, using standard methods. Briefly, swabs from the gill and kidney of affected koi carps were plated onto brain–heart infusion agar (HiMedia) and the plates were incubated at 28 °C for 48 h. Pure bacterial colonies were biochemically characterized as per *Bergey's Manual of Determinative Bacteriology* and also by 16S rRNA gene sequencing.

PCR and sequence analysis. Extraction of nucleic acid (RNA and DNA) was done by GeneJET RNA purification kit (Thermo Scientific) and DNAeasy Blood and Tissue kit (Qiagen), respectively, following the manufacturer's instructions. The tissue samples viz. gills, spleen and kidney were pooled from individual fish for the isolation of nucleic acids. The cDNA synthesis was done using Verso cDNA kit (Thermo Scientific). Samples were analysed for the presence of nucleic acid sequences specific for SVCV (Stone *et al.*, 2003), CyHV-3 (Bercovier *et al.*, 2005), koi ranavirus (George *et al.*, 2015) and red sea bream iridovirus (Kurita *et al.*, 1998). Further, DNA samples were examined for the presence of CEV-specific DNA sequences (an undefined segment of the CEV

genome) by PCR (548 bp in the first step and 181 bp in the second step) as described by Oyamatsu *et al.* (1997b) and another PCR protocol recently developed based on the partial 4a gene of CEV (528 bp in the first step and 478 bp in the second step) in the Centre for Environment, Fisheries and Aquaculture Science, Weymouth laboratory, UK (Matras *et al.*, 2016), for further confirmation.

Isolation of CEV on different fish cell lines. The tissue homogenate for infecting the cell culture monolayer was prepared from pooled gill, spleen and kidney of CEV-positive koi carps, using the standard protocol. Briefly, pooled tissues were homogenized using sterile Leibovitz's L-15 medium supplemented with 1000 IU ml⁻¹ penicillin, 1000 µg ml⁻¹ streptomycin and 2.5 µg ml⁻¹ amphotericin B (Life Technologies) without FBS. In order to release the virus particles from the infected cells, the homogenized samples were frozen and thawed alternately, thrice. The homogenate was centrifuged at 300 g for 30 min at 4 °C and the supernatant was filtered through a 0.22 µm filter (Millipore). The filtrate (500 µl) was inoculated onto a confluent monolayer of the *C. carpio* koi fin cell line (Swaminathan *et al.*, 2015) in a 25 cm² flask (Nunc) which was incubated at 28 °C. After adsorption for 1 h at room temperature, 5 ml of a maintenance medium (L-15 medium with 2 % FBS) was added. In control flasks, 500 µl of maintenance medium was used. Cells were monitored for any cytopathic effect daily under an inverted microscope (Nikon) for 15 dpi. Similarly, the filtrate was inoculated in fish cell lines that have been developed in our laboratory viz. pearl spot fin (Swaminathan *et al.*, 2010), catopra fish fin (Swaminathan *et al.*, 2013), *Horabagrus brachysoma* fin (Swaminathan *et al.*, 2016a), *C. carpio* koi fin (Swaminathan *et al.*, 2015), angelfish fin (Swaminathan *et al.*, 2016b) and goldfish fin (unpublished data) for viral isolation. Blind passage of inoculated cell lines was carried out five times on all cell lines at an interval of 2 weeks. In addition, DNA was isolated from supernatant as well as cells of the inoculated cell lines and checked for CEV-specific sequences using PCR.

Histopathology. For histology, gill, spleen and kidney tissues fixed in 10 % neutral buffered formalin were dehydrated in ascending grades of ethanol, cleared in chloroform and embedded in paraffin wax. Tissue

sections of 5 µm thickness were cut using a rotary microtome, stained with haematoxylin and eosin and examined under the light microscope.

Electron microscopy. The fixed tissues were processed as per the standard methods for ultra-thin sections for TEM analysis with modifications. Briefly, the infected gill, spleen and kidney tissues fixed with 2.5% glutaraldehyde were post-fixed in 2.0% osmium tetroxide prepared in 0.1 M phosphate buffer for 1 h at 4 °C. The tissues were dehydrated in ascending grades of ethanol and were embedded in Araldite CY212. Ultra-thin sections (60–70 nm) of tissues were cut using a microtome (Leica Ultracut UCT), mounted onto copper grids and stained with uranyl acetate and alkaline lead citrate. Sections were examined and documented under a Tecnai T12 Spirit transmission electron microscope at 60 kV.

Experimental infection. Apparently healthy koi ($n=30$, 20–26 g) were procured from local ornamental fish shops and acclimatized in glass aquaria for 10 days, and subsequently, 15 koi were injected intra-peritoneally with 0.5 ml filtrate of pooled gill homogenate from CEV-positive koi and kept in three different aquaria with five fish each as the infected group. Another group of 15 carp was similarly injected with 0.5 ml of L-15 medium and constituted the control group. The fish were observed daily for development of clinical signs as well as mortality for a period of 10 days. During the experiment, temperature, dissolved oxygen, pH, nitrite and ammonia were 19.0 ± 1.4 °C, 6.23 ± 0.81 mg l⁻¹, 7.9 ± 0.56 , 0.012 ± 0.009 mg l⁻¹ and 0.11 ± 0.027 mg l⁻¹, respectively. Moribund or dead fish in the infected group and healthy fish in the control group were randomly collected for the PCR in order to detect CEV.

Survey of CEV. A total of 320 koi carps (12–15 cm total length with 25–30 g total body weight) were collected from 10 different locations, from either farms or retail shops in India during the last 1 year. The samples of gills from these fish were screened for CEV using second PCR assay described by Matras *et al.* (2016).

Phylogenetic analysis of the Indian CEV sequences. The PCR products amplified from gill samples collected from the disease outbreak cases as well as from different locations during survey were cleaned and sequenced in Applied Biosystems 3730 XL capillary sequencer at a private sequencing facility in Kochi, Kerala. The raw DNA sequences were aligned and edited using BioEdit sequence alignment editor version 7.0.5.2. A neighbour-joining tree was also constructed using the partial 4a gene of Indian CEV sequences generated from the disease outbreak (three sequences) and from survey (nine sequences) together with corresponding sequences of CEV sequences obtained from both koi and common carp from the UK, Poland and Japan by the Kimura two-parameter method at 1000 bootstrap replications using MEGA 4.0 (Tamura *et al.*, 2007).

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