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Original Article

Pathogenicity and molecular characterization of cyprinid herpesvirus-2 (CyHV-2) infecting *Hypophthalmichthys molitrix* in India

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Abstract
Cyprinid herpesvirus-2 (CyHV-2), a linear double-stranded DNA virus of the genus *Cyprinivirus* (family Alloherpesviridae), is a significant pathogenic agent responsible for herpesviral hematopoietic necrosis disease (HVHND), causing severe mortality in cyprinid fishes. While CyHV-2 is well documented in goldfish and crucian carp, information on its pathogenicity in Chinese carps remains limited. The present study investigated a severe disease outbreak in carp polyculture systems, with a focus on *Hypophthalmichthys molitrix*, integrating epidemiological observations, pathology, molecular characterization, and experimental infection trials. Affected fish exhibited clinical signs, including lethargy, hemorrhages, and respiratory distress, accompanied by extensive gross and histopathological lesions involving the gills, kidney, spleen, and musculature. CyHV-2 was detected by PCR in both diseased and apparently healthy fish, suggesting subclinical infection or transient viral presence. Sequence analysis of the partial DNA polymerase gene showed 94.95% identity with the virulent Asian strain CNDF-TB2015 and clustered within the CyHV-2 clade, confirming the virus identity. Experimental infection using tissue-derived inocula showed differential pathogenicity, with kidney (upto 80%) and gill-derived inocula (70%) inducing significantly higher mortality (log-rank test, $p < 0.005$) than liver-derived inocula (10%). Bacteriological analysis revealed concomitant *Aeromonas* infection, suggesting viral-bacterial synergism during disease outbreaks. Collectively, the findings implicate CyHV-2 as a primary viral agent involved in the outbreak and highlight kidney and gill tissues as critical targets for pathogenesis. This study provides the first integrated pathological, molecular, and experimental evidence of CyHV-2 pathogenicity in carp polyculture systems and underscores the need for routine surveillance to mitigate emerging viral threats in aquaculture.

Keywords: *Aeromonas*; cyprinid herpesvirus-2; CyHV-2; molecular characterization; pathogenicity; tissue tropism

1 | INTRODUCTION

Aquaculture is one of the fastest-growing food production sectors worldwide. Yet, its rapid intensification, characterized by high stocking densities and chronic husbandry stress, consistently promotes the emergence and spread of infectious diseases (FAO 2024). Recent estimates indicate that bacterial pathogens account for 54.9% of aquatic disease outbreaks, followed by viral (22.6%), parasitic

(19.4%), and fungal (3.1%) agents (Lakshmi *et al*. 2019; Elabd *et al*. 2024). Among these, viral diseases are particularly detrimental because of their high transmission rates, limited treatment options, and resulting economic losses (Thangaraj *et al*. 2021).

Cyprinid herpesvirus-2 (CyHV-2), a member of the genus *Cyprinivirus* within the family Alloherpesviridae, was first recognized following outbreaks of severe mortality among cultured goldfish (*Carassius auratus*) in Japan during the spring of 1992 and 1993 (Jung and Miyazaki 1995). Since then, CyHV-2 has been established as one of the most impactful viral pathogens affecting cyprinid aquaculture worldwide (Hedrick *et al*. 2006). The virus possesses an icosahedral nucleocapsid (110 – 120 nm) enveloped within a lipid membrane, yielding a total virion diameter of 170–200 nm (ICTV 2023). A notable challenge in CyHV-2 management is its capacity to establish long-term latency, enabling asymptomatic carriers to disseminate the virus across farms and regions (Padhiary *et al*. 2023; He 2025). The thymidine kinase (TK) gene has been implicated in virulence in several herpesviruses; however, its role in pathogenicity appears to be context-dependent and may vary among viral species and experimental systems. (Qian *et al*. 2023; Cui *et al*. 2025).

CyHV-2 is the etiological agent of herpesviral hematopoietic necrosis (HVHN), a severe, frequently fatal disease characterized by 50–100% mortality (Dharmaratnam *et al*. 2023). Infected fish typically show lethargy, anorexia, systemic and gill hemorrhages, and pronounced lesions on internal organs (Liang *et al*. 2015; Zhu *et al*. 2019; Jiang *et al*. 2020). Hemorrhages of the swim bladder and congested viscera are hallmarks of the disease, and distinct patterns of gill bleeding may be observed during stress events, although these signs vary among individuals (Liang *et al*. 2015). The severity of these pathological manifestations, coupled with the absence of vaccines or effective therapeutics, underscores the urgent need for rapid detection and robust surveillance strategies in aquaculture (Preena *et al*. 2022; Chen *et al*. 2023).

Environmental conditions play a crucial role in CyHV-2 epidemiology. Outbreaks in *Carassius gibelio* (gibel carp) were reported in 2018, typically occurring during spring and autumn at water temperatures of 20–25°C (Ouyang *et al*. 2020), though the virus can induce mortality across a broader thermal range from 15–32°C (Claudio *et al*. 2017) and has been associated with mortality at temperatures as low as 10–16°C (Goodwin *et al*. 2009). Stressors such as handling, overcrowding, and transportation further exacerbate infection risk (Chen *et al*. 2023). CyHV-2 has now been documented across multiple continents, including Europe, Asia, North America, and Oceania, largely driven by global trade of asymptomatic goldfish and the persistence of latent infections (Becker *et al*. 2014; Ito *et al*. 2017; Adamek *et al*. 2018; Panicz *et al*. 2019). In India, the virus was first identified in 2014 during a major outbreak in goldfish farms in West Bengal, causing severe economic losses (Abraham *et al*. 2020).

While CyHV-2 is well established in goldfish (Goodwin *et al*. 2009; Boitard *et al*. 2016; Jiang *et al*. 2020; Piewbang *et al*. 2024) and gibel carp (Xu *et al*. 2013; Ouyang *et al*. 2020), accumulating evidence indicates its capacity for cross-species transmission. A notable outbreak in Kunshan, Jiangsu Province, China, revealed CyHV-2-like symptoms in multiple cyprinid species, including silver carp, black carp, bighead carp, spotted barbel, and top mouth culter, highlighting the virus's broad host range and capacity for interspecific spread (Zhu *et al*. 2019).

Despite this growing global recognition, no confirmed reports exist on CyHV-2 infection in *H. molitrix* from India, a major carp species central to South Asian aquaculture. This gap is concerning, given the species economic significance and susceptibility of related cyprinids to CyHV-2. Therefore, the present study was undertaken to evaluate the pathogenicity and conduct molecular characterization of CyHV-2 isolated from *H. molitrix*. By providing the first evidence of CyHV-2 involvement in this species in India, this work aims to refine the current understanding of CyHV-2 host range, inform disease surveillance efforts, and support the development of improved management strategies in regional aquaculture.

2 | METHODOLOGY

2.1 Sampling

During the first week of July 2024, a severe disease outbreak with approximately 70% cumulative mortality (over a period of 3 days) was reported from a commercial fish farm in Dhaliarkandi, Kailashahar, North Tripura, India (24.359009°N, 92.007818°E). The outbreak affected adult populations of silver carp (*H. molitrix*), rohu (*Labeo rohita*), catla (*L. catla*), and mrigal (*Cirrhinus cirrhosus*) (Figure 1). A total of ten moribound specimens were collected for laboratory analyses, as recommended for diagnostic investigations during disease outbreaks and in accordance with OIE guidelines (OIE 2019a; OIE 2019b). Samples were transported in oxygenated polythene bags to the laboratory. Fish were euthanized using clove oil at 50 µL L^{-1} (Devi and Kamilya 2019), following the American Veterinary Medical Association (AVMA) guidelines, prior to packing or dissection (AVMA 2013). Whole moribund fish intended for further analyses were preserved at –20°C. Additionally, water quality parameters such as temperature, pH, dissolved oxygen (DO), alkalinity, hardness, and ammonia levels were measured following standard protocols (APHA 2005).

2.2 Clinical and pathological examination

The etiology of the disease outbreak was investigated using bacteriological, parasitological, and virological approaches. Clinically, affected fish exhibited excessive mu-

cus accumulation on the skin, pronounced cutaneous and gill hemorrhages (Figure 2a, 2b). Multiple hemorrhagic patches were visible along the body surface. Internal examination revealed severe lesions and extensive hemorrhaging in the liver, kidney, and intestine (Figure 3).

FIGURE 1 The sampling pond at Dhaliarkandi, Kailashahar, North Tripura, India (24.359009°N, 92.007818°E).

FIGURE 2 Gross clinical lesions in *Hypophthalmichthys molitrix* associated with CyHV-2 infection showing (a) extensive focal hemorrhages and scale degeneration and (b) severe gill hemorrhages accompanied by excessive mucus secretion and gill tissue degeneration.

A complete necropsy following the procedure described by Noga (2010). Initial examinations focused on external clinical signs and gross pathological changes, followed by the preparation of wet mounts from gill and skin tissues for parasitological assessment under microscopy.

Aseptic sampling of kidney, liver, gill, and spleen tissues was performed to facilitate bacterial and viral isolation as described by Soto *et al*. (2019). Portions of these tissues were fixed in 10% neutral buffered formalin and in 2.5% glutaraldehyde (Sigma-Aldrich) prepared in phosphate buffer for histopathological evaluation. For bacteriological isolation, swabs were streaked onto Rimler-Shotts agar, followed by Nutrient Agar plates (Himedia, India) and incubated at 30°C. Kidney, gill, and liver tissues intended for viral screening were preserved in DNA diluent (Molecular grade, Himedia, India) and stored at –20°C until further processing.

FIGURE 3 Gross pathological findings showing exudates and hemorrhages on intraperitoneal organs of *Hypophthalmichthys molitrix* infected with CyHV-2.

2.3 Histopathology

For histological examination, tissues from the gills, spleen,

and kidneys, initially fixed in 10% neutral buffered formalin, were processed following the method described by Bisai *et al.* (2025). They were dehydrated through ascending grades of ethanol, cleared with xylene, and subsequently embedded in paraffin wax. To identify histopathological changes, 5-µm-thick sections were cut using a rotary microtome, stained with hematoxylin and eosin, and observed under a light microscope.

2.4 DNA extraction, PCR assay and sequencing

2.4.1 Viral DNA extraction & PCR amplification

Total genomic DNA was extracted from kidney, gill, and liver tissues of moribund fish following the method described by Cano *et al*. (2007). Six independent biological samples (n = 6 fish) were analyzed for each tissue type. DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the extracted DNA were determined using UV–visible spectrophotometry. PCR amplification was carried out using the virus-specific primers listed in (Table 1).

TABLE 1 Oligonucleotide primers used for the amplification of virus and bacteria.

Primer	Sequence	Annealing temp (°C)
CyHV-2	**Forward:** CCCAGCAACATGTGCGAG **Reverse:** CCGTARTGAGAGTTGGCA	55
CyHV-3	**Forward:** GGGTTACCTGTACGAG **Reverse:** CACCCAGTAGATTATGC	52
Aerolysin	**Forward:** CTCAGTCCGTGCGAC-CGACT **Reverse:** GATCTCCAGCCTCAGGCCTT	60
A. hydrophila 16s	**Forward:** GAAAGGTTGATGCCTAA-TACGTA **Reverse:** CGTGCTGG-CAACAAAGGACAG	58
CEV	**Forward**: CTCTTCAC-TATTGTGACTTTG **Reverse:** ATGGAG-TATCCAAAGTACTTAG	49

2.4.2 PCR screening for virus

PCR screening for Cyprinid herpesvirus-2 (CyHV-2), Cyprinid herpesvirus-3 (CyHV-3), and carp edema virus (CEV) was performed using virus-specific primers. CyHV-2 detection was carried out using the primer pair CyHV-2 FP (5′-CCCAGCAACATGTGCGAG-3′) and CyHV-2 RP (5′-CCGTARTGAGAGTTGGCA-3′), while CyHV-3 was screened using thymidine kinase (TK) gene-specific primers TKf (5′-GGGTTACCTGTACGAG-3′) and TKr (5′-CACCCAGTAGATTATGC-3′) (Bercovier *et al*. 2005). CEV screening employed the primer pair CEV-F (5′-CTCTTCACTATTGTGACTTTG-3′) and CEV-R (5′-ATGGAGTATCCAAAGTACTTAG-3′). All PCR reactions were performed in a final volume of 25 µL containing 12.5 µL of 2× PCR Taq Mixture (HiMedia, India), 1.0 µL each of forward and reverse primers (10 pmol μL^{-1}), 1.0 µL of template DNA, and 9.5 µL of molecular-grade water, with components mixed by brief centrifugation. Thermal cycling conditions for CyHV-2 consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. For CyHV-3, amplification involved an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min. PCR amplification for CEV was performed using an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 49°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min.

2.4.3 Agarose gel electrophoresis

PCR products were analyzed via electrophoresis on a 1.5% agarose gel (HiMedia, India). The gel was prepared with 1X Tris-acetate-EDTA (TAE) buffer and stained with 0.5 µg mL^{-1} ethidium bromide (Himedia, India) to visualize the DNA fragments.

2.4.4 Sequencing and analysis

The confirmed CyHV-2 sequence was submitted to M/s. Bioserve Biotechnologies Pvt. Ltd., India, for sequencing, and subsequently validated using Chromas Lite software. Using a BLAST (basic local alignment search tool) 2.13.0+ program, the DNA sequence of the isolate was compared to those found in the GenBank NCBI (National Centre for Biotechnology Information) database to determine its identity (Zhang *et al*. 2000). ClustalW was used to accomplish pairwise alignment of the sequences and the MEGA 11 software (Tamura *et al*. 2021) was utilized to create a phylogenetic tree using the neighbour-joining technique (Saitou and Nei 1987). BLAST similarity metrics (E-value and percentage identity) and phylogenetic analysis were used to assess the sequence identity and phylogenetic placement of the isolate.

2.5 Bacteriology of diseased fish

Bacteria were isolated from gill and kidney swabs of diseased fish using standard microbiological procedures following the method described by Noga (2010). Samples were streaked onto Rimler-Shotts Agar (TSA; HiMedia, India) plates and incubated at 30°C for 24–30 h (Noga 2010). Predominant colonies were subcultured repeatedly on nutrient agar to obtain pure isolates. Preliminary phenotypic characterization was performed based on colony morphology, Gram staining, and a series of biochemical tests following standard protocols described in Bergey's Manual of Determinative Bacteriology.

For molecular identification, genomic DNA was extracted from representative isolates, and PCR amplification of the bacterial 16S rRNA gene was performed using *Aeromonas*-specific primers (annealing temperature: 58°C). In addition, the presence of the aerolysin-encoding gene (aerA) was screened using gene-specific primers (annealing temperature: 55°C) to assess the occurrence of a virulence-associated marker (Table 1). PCR products were visualized by agarose gel electrophoresis. The combined phenotypic and molecular data were used to support the identification of *Aeromonas* spp. associated with diseased fish.

2.6 Experimental infection with suspected virus to *H. molitrix*

Fingerlings of *H. molitrix* (average length: 19.20±0.57 cm; average weight: 30.50±1.30 g) were procured from a local fish farm and acclimatized in 500 L fiber-reinforced plastic (FRP) tanks with continuous aeration for 10 days. During acclimatization, fish were fed a commercial pelleted diet twice daily at 3% of body weight. Water quality parameters were maintained uniformly throughout the experiment.

To prepare the viral inoculum, gill, liver, and kidney tissues were collected separately from naturally infected *H. molitrix* confirmed positive for CyHV-2 by PCR. Each tissue was homogenized in sterile phosphate-buffered saline (PBS) to obtain a 10% (w/v) suspension, centrifuged at 5,000 rpm for 5 min at 4°C, and the supernatant was passed through a 0.22 µm membrane filter to remove cellular debris. The clarified homogenates were stored at –20°C until use.

For the challenge experiment, 120 healthy fingerlings were randomly divided into four experimental groups. Fish in the treatment groups were intraperitoneally injected with 0.1 mL of gill (T1), liver (T2), or kidney (T3) tissue homogenate, respectively, while the control group (C) received 0.1 mL of sterile PBS. Each group consisted of three replicate tanks containing ten fish per tank. Fish were monitored daily for 10 days for the development of clinical signs and mortality. Moribund and freshly dead fish from each group were collected and screened for CyHV-2 by PCR to confirm the presence of viral DNA and to assess the association between tissue-derived viral exposure and disease manifestation. However, conventional PCR alone cannot confirm active viral replication. Future investigations employing quantitative PCR, virus isolation, or localization approaches such as in situ hybridization (ISH) or immunohistochemistry (IHC) will be necessary to further elucidate viral replication dynamics and tissue tropism.

2.7 Data analysis

Survival data following CyHV-2 challenge were analyzed using Kaplan-Meier survival curves, and differences among groups were assessed using the log-rank (Mantel-Cox) test. Statistical significance was set at $p < 0.05$. All experiments were conducted in triplicate. Data analysis was performed using IBM SPSS Statistics version 27.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

3.1 Water quality parameetrs

The water quality parameters recorded at the affected fish farm during the sampling period are presented in Table 2. The measured values of pH, dissolved oxygen, alkalinity, hardness, nitrite, nitrate, ammonia, and temperature showed moderate variation among sampling points but remained within the ranges generally reported for carp aquaculture systems.

TABLE 2 Water quality parameters recorded at the affected fish farm during sampling. Values are expressed as mean ± SD (n = 3).

Parameter	Results
pH	6.70 ± 0.67
Dissolved Oxygen (mg L^{-1})	7.01 ± 2.22
Alkalinity (mg L^{-1} as $CaCO_3$)	58.73 ± 32.11
Hardness (mg L^{-1} as $CaCO_3$)	48.02 ± 30.46
Nitrite (NO_2^- mg L^{-1})	0.195 ± 0.055
Nitrate (NO_3^- mg L^{-1})	0.102 ± 0.045
Ammonia (NH_3 mg L^{-1})	0.086 ± 0.017
Temperature (°C)	26.04 ± 2.63

3.2 Epidemiology and gross pathological signs

A disease outbreak was observed in multiple freshwater ponds, with silver carp (*H. molitrix*) exhibiting the highest mortality, averaging approximately 15–20% per day during the acute phase of the outbreak. Farmers also reported concurrent mortalities among *L. rohita*, *C. catla*, and *C. cirrhosus* reared in the same ponds. Affected fish displayed lethargy, erratic swimming behavior, and frequent surface gasping prior to death, with individuals across different size classes being affected.

External examination revealed pale gills, sunken eyes, swollen vent, and multiple hemorrhagic patches distributed over the body surface, particularly around the gills, caudal peduncle, and fin bases (Figure 2a, 2b). Protrusion of scales was frequently noted. In *H. molitrix*, a rapid loss of pigmentation resulted in a distinct whitish discoloration of the body.

Necropsy of moribund fish revealed prominent internal lesions. Gills appeared pale with areas of necrosis, while the kidney and spleen were enlarged and congested. The spleen frequently showed whitish necrotic foci, and hemorrhagic fluid was observed within the abdominal cavity (Figure 3), indicating extensive systemic tissue involvement.

3.3 Histopathology

Histopathological examination of tissues from *H. molitrix* exhibiting clinical disease revealed pronounced pathological alterations in multiple organs (Figure 4a–4c). Kidney tissue (Figure 4a) showed severe degeneration of renal tubules, tubular nephritis, and extensive necrosis. Tubular epithelial cells exhibited fusion, pyknosis, karyomegaly, and the presence of structures consistent with intranuclear inclusion bodies. Marked congestion, hemorrhage, and inflammatory cell infiltration were observed, along with degeneration of hematopoietic tissue and disruption of normal renal architecture.

Gill sections (Figure 4b) exhibited extensive epithelial hyperplasia, necrosis, and inflammatory cell infiltration. Lamellar fusion and accumulation of cellular debris within interlamellar spaces were frequently observed, indicating substantial structural damage to respiratory tissues.

Liver sections (Figure 4c) demonstrated multifocal hepatocellular degeneration and necrosis, accompanied by sinusoidal congestion and hemorrhage. Aggregates of inflammatory cells were distributed throughout the hepatic parenchyma. Hepatocytes exhibited karyomegaly and intranuclear inclusion bodies, consistent with severe cellular stress and degeneration.

3.4 Molecular characterization and prevalence of CyHV-2 in affected carp populations

CyHV-2 DNA was detected by PCR in both clinically diseased and apparently healthy fish collected during the outbreak from a carp polyculture farm in Kailashahar, North Tripura, during the first week of July 2024. The proportion of PCR-positive samples varied among examined fish, with detection rates ranging from 30% to 100% depending on tissue type and sampling group.

PCR amplification using CyHV-2–specific primers yielded the expected ~296 bp product from multiple tissue samples of *H. molitrix* (Figure 5). The purified amplicon was subjected to Sanger sequencing, and the resulting partial DNA polymerase gene sequence was analyzed using the NCBI BLAST tool. The sequence shared 94.95% nucleotide identity with the Cyprinid herpesvirus 2 strain CNDF-TB2015 (GenBank accession no. MN201961.1), indicating close genetic similarity to previously reported Asian CyHV-2 isolates.

Multiple sequence alignment performed using Clustal Omega revealed several conserved regions within the analyzed gene fragment when compared with representative CyHV-2 sequences retrieved from GenBank. Minor nucleotide substitutions were observed relative to reference strains, reflecting sequence variability within the CyHV-2 group. Phylogenetic analysis based on the partial DNA polymerase gene sequence was conducted using the neighbor-joining method with the Kimura 2-parameter model and 1,000 bootstrap replicates (Figure 6).

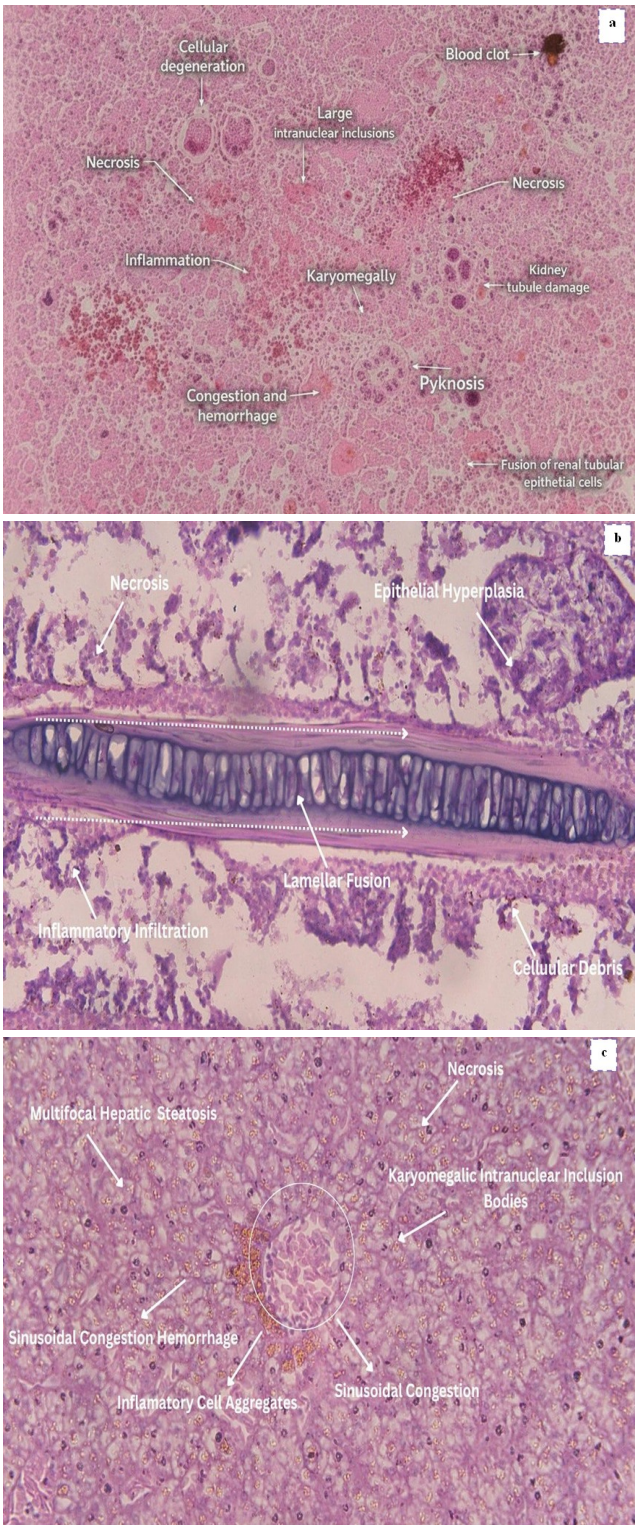

FIGURE 4 Histopathological lesions in CyHV-2–infected *H. molitrix*: (a) kidney showing tubular degeneration, necrosis, karyomegaly, intranuclear inclusion bodies, inflammation, congestion, and hemorrhages; (b) gill exhibiting epithelial necrosis, hyperplasia, inflammatory infiltration, lamellar fusion, and cellular debris; (c) liver showing multifocal steatosis, necrosis, inflammatory cell aggregates, sinusoidal congestion, hemorrhages, and intranuclear inclusion bodies.

The Tripura isolate clustered within the CyHV-2 clade alongside previously reported Asian isolates, including strains CNDF-TB2015 (MN201961.1) and ST-J1 (NC019495.1). Bootstrap support values for internal nodes ranged from 21% to 53%, indicating moderate phylogenetic resolution within the analyzed sequence fragment. The Indian isolate showed close clustering with a previously reported CyHV-2 isolate from West Bengal (KT900136.1), suggesting genetic relatedness among CyHV-2 sequences detected in eastern India.

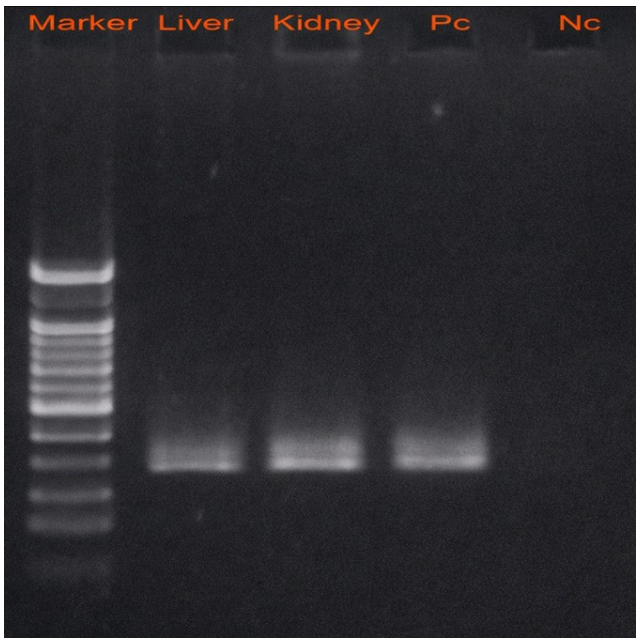

FIGURE 5 PCR amplification of Cyprinid herpesvirus-2 (CyHV-2) visualized by agarose gel electrophoresis. Lane 1: DNA marker; lanes 2 and 3: liver and kidney samples from diseased *Hypophthalmichthys molitrix* showing CyHV-2–specific amplicons; lane PC: positive control; lane NC: negative control.

3.5 Experimental infection with suspected virus to *H. molitrix*

Distinct differences in cumulative mortality were observed among *H. molitrix* fingerlings following intraperitoneal injection with tissue homogenates prepared from CyHV-2 positive fish (Figure 7). Fish receiving kidney-derived homogenates (T3) exhibited the highest cumulative mortality, reaching approximately 80% by 8 days post-injection (dpi). The group injected with gill-derived homogenates (T1) showed comparatively lower mortality, with cumulative mortality reaching 70% over the 10-day observation period. In contrast, fish challenged with liver-derived homogenates (T2) displayed limited mortality, with cumulative mortality not exceeding 10% by the end of the experiment. No mortality or abnormal clinical signs were observed in the control group injected with sterile phosphate-buffered saline.

Mortality in the T1 and T3 groups was observed during the early post-injection period and progressed throughout the trial, whereas mortality in the T2 group was sporadic and minimal. Moribund fish collected from all treatment groups tested positive for CyHV-2 by PCR, whereas control fish remained PCR-negative.

3.6 Bacteriology and parasitology

Parasitological examination of gill and skin wet mounts from diseased fish did not reveal the presence of ectoparasitic or endoparasitic organisms. Microscopic screening across multiple visual fields consistently failed to detect parasitic stages in any of the examined samples, suggesting that parasitic infection was unlikely to be a contributing factor in the observed disease outbreak.

In contrast, bacteriological analysis revealed the frequent isolation of *Aeromonas* spp. from internal organs of diseased fish. Pure bacterial colonies exhibiting morphological characteristics consistent with *Aeromonas* were recovered from kidney, and gill tissues. Biochemical profiling of the isolates yielded uniform phenotypic characteristics consistent with *Aeromonas* spp. (Table 3). Molecular identification by PCR further confirmed the isolates as *Aeromonas* spp. through amplification of a genus-specific 16S rRNA gene fragment. In addition, PCR amplification of the aerolysin (aerA) gene indicated the presence of a virulence-associated genetic marker in all isolates (Figure 8).

TABLE 3 Biochemical characteristics of the suspected *Aeromonas* isolates.

Biochemical test	*Aeromonas* spp. (*n* = 5)
Motility	+
Gram staining	–
Indole production	+
Urea hydrolysis	–
Esculin hydrolysis	+
Gelatin hydrolysis	+
p-Nitrophenyl-β-D-galactopyranoside (ONPG)	+
Glucose assimilation	+
Arabinose utilization	–
Mannose utilization	+
Mannitol utilization	+
Maltose utilization	+
Nitrate reduction	+
Oxidase	+
Catalase	+
Voges–Proskauer (VP)	+
Glucose gas production	+
Sucrose	+

4 | DISCUSSION

Cyprinid herpesvirus-2 (CyHV-2) is a well-recognized viral pathogen responsible for significant economic losses in cyprinid aquaculture, particularly in farmed *Carassius*

species, where herpesviral hematopoietic necrosis disease (HVHND) is associated with severe and often unpredictable mortality (Hedrick *et al*. 2006; Ouyang *et al*. 2020). Despite its established importance, information on the occurrence, molecular characteristics, and pathogenicity of CyHV-2 in *H. molitrix* and other carp species cultured in India has remained limited (Thangaraj *et al*. 2021). In the present study, we provide pathological, molecular, and experimental evidence supporting the involvement of CyHV-2 in a severe disease outbreak affecting *H. molitrix* within a polyculture system in northeastern India. These findings contribute new regional data on CyHV-2 circulation and highlight its potential relevance to Indian carp aquaculture. However, whole-genome sequencing would be required for robust phylogeographic and evolutionary inference.

4.1 Clinical manifestations and tissue pathology associated with CyHV-2 infection

Field observations and experimental infection trials demonstrated that affected *H. molitrix* exhibited lethargy, hemorrhages, depigmentation, erratic swimming, respiratory distress, and elevated mortality, which are broadly consistent with clinical manifestations previously described for HVHND in goldfish and gibel carp (Goodwin *et al*. 2009; Jiang *et al*. 2020). Gross pathological findings, including severe gill necrosis, splenomegaly with whitish necrotic foci, renal congestion, and hemorrhagic ascites, indicate extensive systemic involvement and are comparable to lesions reported in CyHV-2–infected cyprinids elsewhere (Wu *et al*. 2013; Jiang *et al*. 2015; Gao *et al*. 2023).

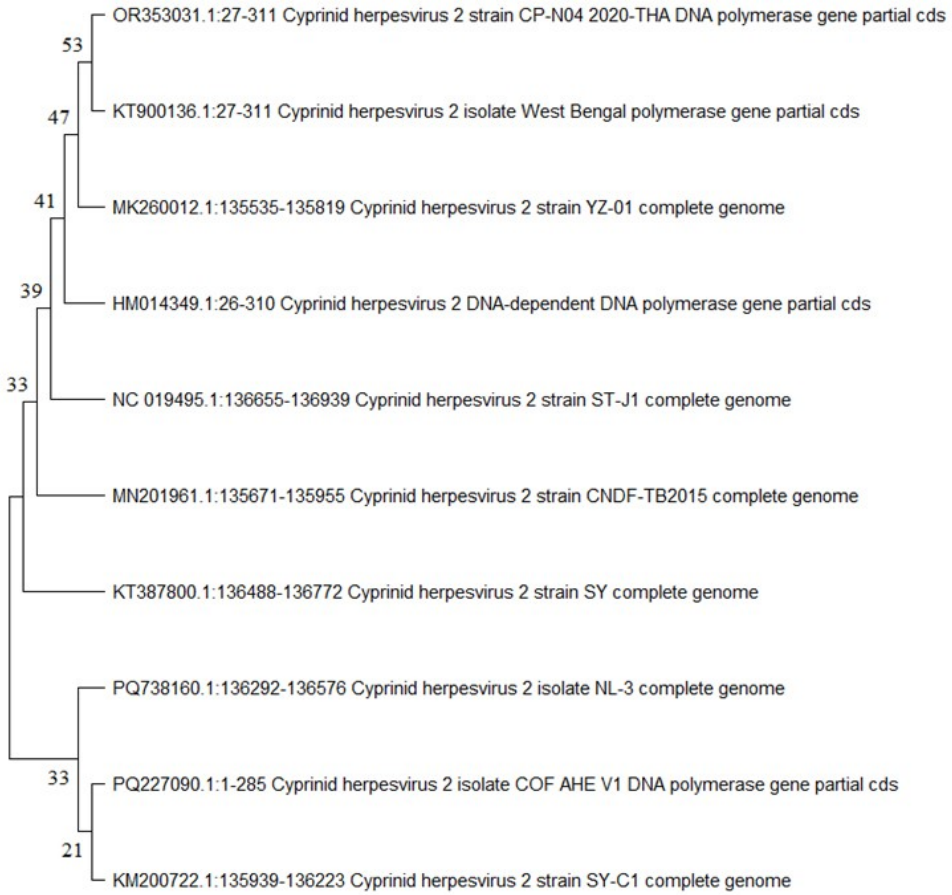

FIGURE 6 Phylogenetic analysis of Cyprinid herpesvirus-2 (CyHV-2) based on partial DNA polymerase gene sequences. The tree was constructed using the neighbor-joining method, including representative CyHV-2 strains retrieved from GenBank. Bootstrap values (1,000 replicates) are shown at branch nodes. The CyHV-2 isolate obtained in the present study clustered within the CyHV-2 clade and showed close genetic relatedness to Asian reference strains.

Histopathological examination further revealed pronounced tissue damage, particularly in the kidney and gills, characterized by tubular nephritis, hematopoietic tissue degeneration, necrosis, inflammatory cell infiltration, epithelial hyperplasia, and lamellar fusion. Intranuclear inclusion bodies observed in renal and hepatic tissues are consistent with active herpesviral replication and have been widely documented in CyHV-2 infections (Zhu *et al*. 2019; Ouyang *et al*. 2020). Collectively, these findings support the capacity of CyHV-2 to induce severe multi-organ pathology in *H. molitrix* under natural and experimental conditions.

4.2 Phylogenetic relationships, host range expansion, and molecular characterization of Cyprinid herpesvirus 2 (CyHV-2)

PCR-based screening detected CyHV-2 DNA in both clinically diseased and apparently healthy fish, suggesting the presence of subclinical or latent infections within the affected population. Latency and asymptomatic carriage

are well-recognized features of cyprinid herpesviruses and contribute to silent viral dissemination within aquaculture systems (Ito *et al*. 2017; Adamek *et al*. 2018; Yang *et al*. 2024). Sequence analysis of a partial DNA polymerase gene fragment revealed high nucleotide similarity (94.95%) to previously reported CyHV-2 strains from Asia, confirming the molecular identity of the virus detected in this study.

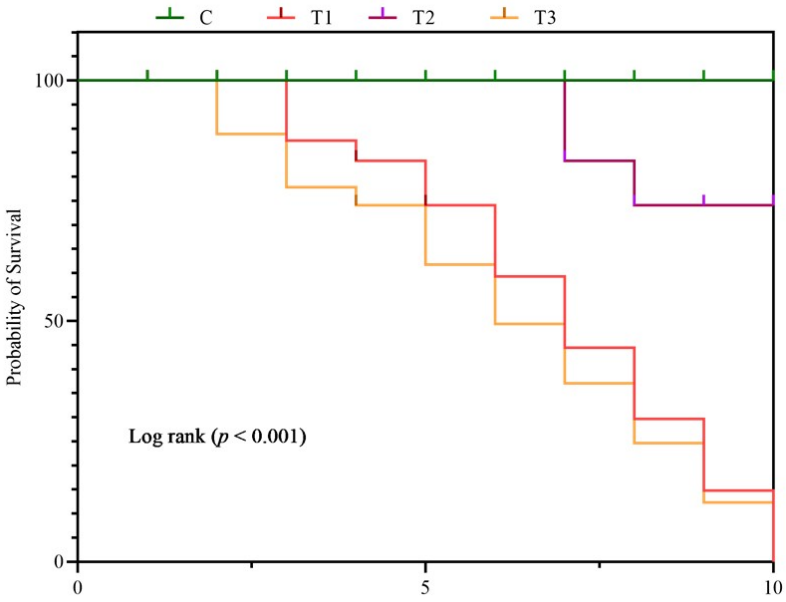

FIGURE 7 Cumulative mortality of *Hypophthalmichthys molitrix* fingerlings following intraperitoneal challenge with Cyprinid herpesvirus-2 (CyHV-2) tissue homogenates. Fish were injected with gill (T1), liver (T2), or kidney (T3) homogenates and monitored for 10 days post-infection. A control group received sterile phosphate-buffered saline. Mortality was recorded daily till 10 days.

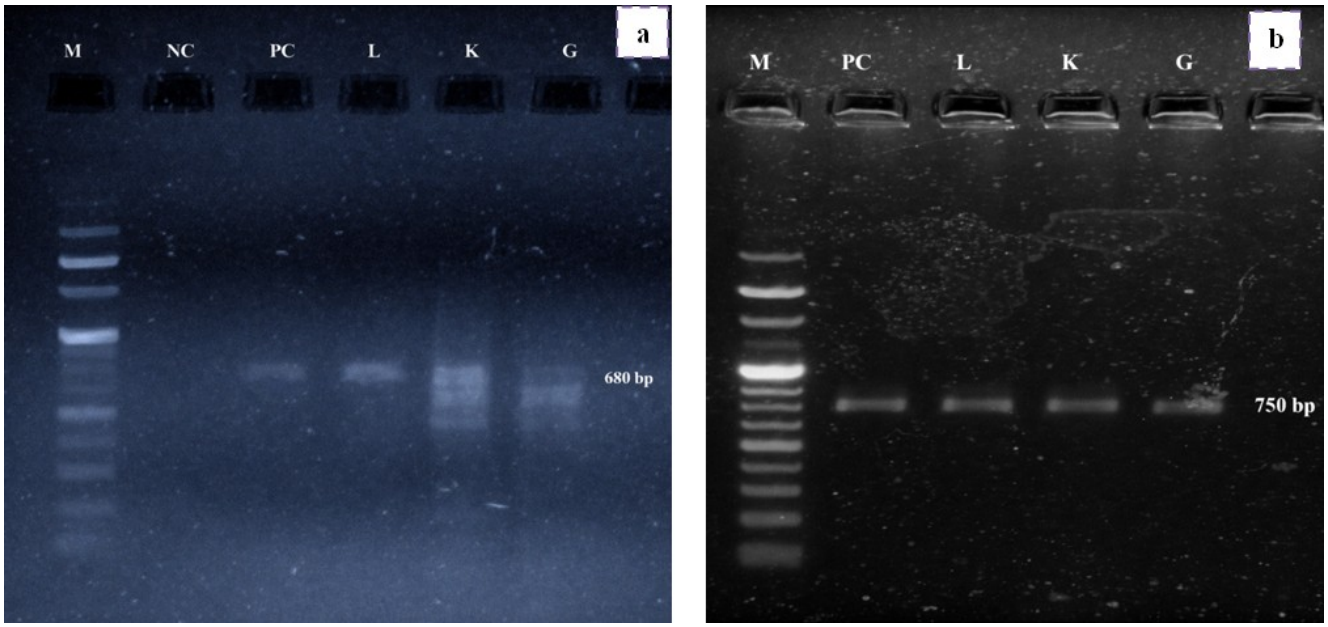

FIGURE 8 PCR amplification of (a) Aerolysin and (b) A. hydrophila 16s gene, visualized by agarose gel electrophoresis. Lane M: 100 bp DNA marker; lane PC: positive control; lane NC: negative control, lane L: liver, lane K: Kidney and lane G: Gill, samples from diseased *Hypophthalmichthys molitrix* showing CyHV-2–specific amplicons; lane PC: positive control; lane NC: negative control.

Phylogenetic analysis placed the Indian CyHV-2 sequence within the CyHV-2 clade, clustering near Asian reference strains, including CNDF-TB2015 and ST-J1. However, bootstrap support values were moderate to low, reflecting the limited resolution of partial gene sequences for fine-scale evolutionary inference. Accordingly, while the phylogenetic data confirm clade-level placement and close genetic relatedness, they should not be interpreted as evidence of strong evolutionary affinity or strain-level assignment. Whole-genome sequencing would be required to more accurately resolve phylogeographic relationships and assess viral evolution within

Indian aquaculture systems.

Detection of CyHV-2 in *H. molitrix* supports the notion that this species is susceptible to infection under field conditions. However, PCR-based detection alone does not establish host range expansion in a strict virological sense. Further studies incorporating viral isolation, transmission experiments, and quantitative replication analyses are necessary to definitively determine the epidemiological significance of *H. molitrix* as a host for CyHV-2.

4.3 Concomitant *Aeromonas hydrophila* infection during CyHV-2 outbreaks

Bacteriological analyses consistently identified *Aeromonas* spp. in all diseased fish examined, while parasitological screening yielded negative results. Molecular screening targeting the 16S rRNA gene together with the aerolysin (aerA) virulence gene indicated the presence of potentially virulent *Aeromonas* strains. However, because 16S rRNA-based identification alone does not provide sufficient resolution for definitive species-level discrimination within the genus *Aeromonas*, the isolates are conservatively referred to here as *Aeromonas* spp. Given the absence of parasitic agents and the opportunistic nature of *Aeromonas* infections in stressed fish, their involvement in the outbreak is most plausibly secondary to the primary viral infection.

Cyprinid herpesviruses are known to induce immunosuppression through lymphoid depletion and hematopoietic dysfunction, thereby increasing host susceptibility to secondary bacterial infections (Adamek *et al*. 2018; Yang *et al*. 2024). The consistent recovery of *Aeromonas* isolates from diseased fish in this study supports a scenario in which CyHV-2 infection predisposed fish to opportunistic bacterial septicemia, exacerbating disease severity and mortality. Similar viral-bacterial coinfections have been widely reported during cyprinid herpesvirus outbreaks and are recognized as important contributors to complex disease outcomes in aquaculture (Ren *et al*. 2021; Shen *et al*. 2022; Vega-Heredia *et al*. 2024).

4.4 Experimental infection with suspected virus to *H. molitrix*

Experimental infection trials demonstrated marked tissue-dependent differences in pathogenicity, with kidney-derived homogenates inducing the highest cumulative mortality, followed by gill-derived homogenates, while liver-derived homogenates caused minimal mortality. These findings are consistent with previous reports identifying the kidney as a principal replication site for CyHV-2, given its central role in teleost hematopoiesis and immune function (Hedrick *et al*. 2006; Jiang *et al*. 2020). Elevated viral loads in renal tissue are likely to facilitate systemic dissemination and immunosuppression, thereby contributing to high mortality.

Similarly, the gills serve as a major portal of viral entry and replication, and CyHV-2–associated gill necrosis has been linked to respiratory dysfunction and mortality (Wang *et al*. 2012; Ito *et al*. 2017; Ouyang *et al*. 2020). The comparatively low mortality observed following liver-derived inoculation suggests that the liver is not a primary site of CyHV-2 replication but is affected secondarily during systemic infection, as reported in other cyprinid hosts (Wei *et al*. 2020; Piewbang *et al*. 2024).

PCR confirmation of CyHV-2 in moribund fish from experimentally challenged groups, together with the absence of mortality in control fish, provides strong experimental support for CyHV-2 as the etiological agent associated with the observed disease. In the present study, the viral inoculum was prepared from tissue homogenates derived from infected fish without prior viral titration or genome copy quantification. Although this approach is commonly employed in preliminary pathogenicity and transmission studies when purified viral stocks are unavailable, the absence of standardized viral dose measurements represents a limitation of the experimental design. Furthermore, the intraperitoneal challenge model used in this study does not fully replicate natural transmission routes and therefore reflects relative tissue-associated pathogenicity rather than natural infection dynamics.

Future studies employing immersion-based challenge models, quantitative viral load assessments, and comprehensive genomic analyses will be essential to more accurately characterize transmission dynamics, tissue tropism, and virulence determinants of CyHV-2 in carp aquaculture.

5 | CONCLUSIONS

This study provides pathological, molecular, and experimental evidence supporting the involvement of Cyprinid herpesvirus 2 (CyHV-2) in a severe disease outbreak affecting cyprinid fishes, including *Hypophthalmichthys molitrix*, under both field and experimental conditions. Integration of epidemiological observations, characteristic gross and histopathological lesions, PCR detection, sequence analysis, and challenge experiments support the association of CyHV-2 with the observed clinical signs and mortality. Pronounced lesions and tissue-dependent mortality further indicate that kidney and gill tissues play key roles in disease progression.

Detection of CyHV-2 in polyculture systems suggests the potential for viral persistence and circulation among cohabiting carp species, where environmental stress and interspecies contact may facilitate transmission. Frequent co-isolation of *Aeromonas* spp. indicates that secondary bacterial infections may exacerbate disease severity during CyHV-2 outbreaks, highlighting the need for integrated health management. Prioritizing kidney and gill tissues for routine molecular surveillance may improve early detection and outbreak response.

Although partial gene sequencing and intraperitoneal challenge models limit inference of natural transmission dynamics, this study establishes a foundation for understanding CyHV-2–associated pathology in Indian carp aquaculture. Future studies incorporating whole-genome sequencing, quantitative viral analyses, and natural exposure models are warranted.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHORS' CONTRIBUTION

SM: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. HS: Writing – review & editing, Validation, Supervision, Funding acquisition. LG: Investigation, Formal analysis, Methodology, Investigation, Validation.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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