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Original Research Article

Experimental Infection with Koi Ranavirus (KIRV) of Common Carp (*Cyprinus carpio* L.) and Rohu (*Labeo rohita* H.)

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The aim of this study was to determine the pathogenicity of koi ranavirus (KIRV) in common carp (*Cyprinus carpio* L) and rohu (*Labeo rohita* H). In the present study, common carp and rohu fingerlings were experimentally infected with KIRV by intraperitoneal injection with 50 µl of the virus preparation having an infectious dose of $10^{6.9}$ TCID50/ml. After injection, tissues such as spleen and kidney of both challenged fishes with the virus were collected at 3, 7, 14, 21 and 28 dpi for investigation by polymerase chain reaction (PCR), cell culture assay and histopathological technique. KIRV was detected in pooled samples of kidney and spleen collected from both challenged fishes after 3 dpi by PCR, while no positive results were obtained from all other time points. In cell culture assay no cytopathic effect was observed in the EPC (*Epithelioma papulosum cyprini*) cell line at all time points of both fish species for virus reisolation. The results of the histopathological analysis showed necrosis and hypertrophy in kidney tissue of common carp and necrosis and detachment of glomeruli from the epithelium of tubules in kidney tissue of rohu at 3 and 7 dpi, while no typical pathological changes were observed in spleen tissue of common carp at all time points and melanomacrophage cells were observed in spleen tissue of rohu at 3 dpi. On the whole, results reveal that there is no clear clinical pathology observed in common carp and rohu fingerlings following experimental infection with KIRV.

Introduction

Aquaculture production has increased

prominently in South East Asian countries during the last decade. Viral diseases cause serious problems in finfish aquaculture and

are one of the most critical factors in the fish farming sector. Viruses in the family *Iridoviridae* have emerged over the last two decades to become important pathogens in the intensively raised finfish aquaculture sector. Ranaviruses belonging to the family *Iridoviridae* causes significant morbidity and mortality among the ectothermic vertebrates like amphibians, fish and reptiles (Chinchar *et al.,* 2011). Ranavirus is now recognized as major emerging threat to economically and ecologically important cold-blooded vertebrates (Williams *et al.,* 2005) infecting at least 14 families with about 70 amphibian species, more than 100 fish species and dozens of reptiles (Chinchar, 2002; Robert *et al.,* 2011). Ranavirus infection cause significant morbidity and mortality in ectothermic vertebrates like fish, amphibians, and reptiles for the period of past 30 years (Chinchar *et al.,* 2009, 2011). Ranavirus are large icosahedral, double-stranded DNA (dsDNA) viruses, 120-200 nm in diameter in size and with a genome size of 105 and 140 kbp that can infect a broad range of lower vertebrates (Holopainen, 2012).

The genus *Ranavirus* consists of six officially identified species such as *Ambystoma tigrinum* virus (ATV), Bohle iridovirus (BIV), Epizootic haematopoietic necrosis virus (EHNV), European catfish virus (ECV), Frog virus 3 (FV3) and Santee-Cooper ranavirus (SCRV) with FV3 being recognised as the type species of the genus (Jancovich *et al.,* 2012). EHNV was the first ranavirus isolated from fish during a disease outbreak of redfin perch (*Perca fluviatilis*) and rainbow trout (*Salmo gairdneri*) in Australia (Langdon, 1989; Langdon *et al.,* 1988). Recently, a marine ranavirus (similar damselfish virus, SRDV) has been reported to be causing high mortalities in seabass (*Lates calcarifer*) and similar damselfish (*P. similis*) fingerlings (Sivasankar *et al.,* 2017). The first documentation to the isolation of virus resembling Santee-Cooper ranavirus infection

in koi was reported from India (George *et al.,* 2015), where the corresponding viral agent was detected and successfully isolated and named as koi ranavirus (KIRV). The aim of the study was to determine the susceptibility of common carp and rohu to KIRV. The study involved experimental infection with clinical pathology, confirmation of the results by PCR, reisolation of the virus in cell culture and histopathology.

Materials and Methods

Virus strain and propagation

Koi ranavirus (KIRV) isolated from koi (*Cyprinus carpio* L) was used for (George *et al.,* 2015) experimental study. *Epithelioma papillosum cypini* (EPC) cell line was used for isolation, propagation and infectivity assay of the virus. The cell line was maintained in Leibovitz-15 (L-15) medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1x antibiotic-antimycotic solutions (Gibco, USA) in 25 cm^2 cell culture flasks (Thermo, Korea) at 27° C. On completion of CPE, the infected culture supernatant was harvested and clarified by centrifugation at 1500 g for 15 min (Fig. 1). Clarified supernatant held at 4°C was titrated in EPC cells.

Experimental fish

Common carp (*Cyprinus carpio* L) fingerlings (mean length of 7.59 cm and mean weight 6.54 g) and rohu (*Labeo rohita*) fingerlings (mean length of 7.14 cm and mean weight 6.32 g) were obtained from procured from national fish seed farm at Manimuthar, Tamil Nadu and maintained in the laboratory until non-specific mortalities had stopped. Fish were acclimatized to the glass tank of 100L capacity maintained with 50 L freshwater at a temperature of 29 $\pm$ 1°C and fed with commercial pelleted feed twice daily. Tanks were provided with continuous aeration and

cleaned daily by siphoning out waste material along with partial water exchange (10–20%). The fish was tested for the presence of koi ranavirus by PCR and was conformed negative before the experimental infection started.

Experimental challenge

Two groups consisting of 30 fish each in duplicate were used for experimental infection (Fig. 2). Prior to challenge, the fish was sedated using benzocaine at a concentration of 40 mg/l. Experimental fishes were administered 50 µl of cell culture grown KIRV stock ($10^{6.9}$ TCID$_{50}$/volume inoculated) by intraperitoneal (i.p) injection while control group received same amount of L-15 medium from the supernatant of an uninfected cell culture medium was injected. Tissues samples such as kidney and spleen were taken from three fish in each treatment at 3, 7, 14, 21 and 28 days after injection and samples were analysed by molecular method, cell culture assay and histopathological technique.

Virus reisolation

Samples of kidney and spleen tissue from common carp and rohu were aseptically removed, pooled and examined for after experimental infection. Pooled tissue extracts were inoculated into the EPC cells following standard virus isolation procedures (Amos, 1985).

Homogenised tissue samples were diluted to 1/10 (w/v) in L-15 medium containing 10 % FBS and 1x antibiotic-antimycotic mix. Homogenised tissue extract was clarified by centrifugation at 3000 g for 15 min and the supernatant passed through a 0.22 µm sterile disposable filter. The pellet with little amount of supernatant was subjected to PCR analysis with diagnostic primer developed from KIRV-MCP to assay the presence and the

absence of the virus in both group of experimental fish sample.

Detection of viral DNA from experimental fish by PCR

DNA from KIRV injected fish tissues from the experimental challenge study was extracted using 1ml of DNA extraction solution (Himedia) as per the manufacturer's instructions. The extracted DNA was dissolved in 100 µl of sterilized deionised water (Thermo) and stored at 4° C before use. The DNA extracted from KIRV injected and control fish were used for PCR in 25 µl reaction mixture with Smart Prime Mix (Ampliqon, Denmark). Specific primers and cycling conditions to KIRV; MCPFORW and MCPREV set (George *et al.,* 2015) were used for PCR amplification. The PCR amplified products along with molecular markers stained by ethidium bromide were visualized in 1.5% agarose gels by a gel documentation system (Biorad, U.S.A).

Histopathology

Tissues of gill, kidney and spleen from experimental fish without clinical signs and from control fish were excised and fixed in 10% neutral buffered formalin for at least 24 h or preserved until process of histology. The tissues were processed routinely, embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin (HE) and and examined by bright-field microscopy.

Results and Discussion

Ranaviruses are recognized as important and emerging multi-host pathogens with very broad host ranges (Gray *et al.,* 2009). The movement of animals out from original geographical region brings together with new host species and potential pathogens, facilitates host-switching and continues the

cycle for disease emergence (Peeler *et al.,* 2011). Being a virus of recent origin in a commercially important fish in India, there was urgent need to find the susceptible hosts among the cultured species in India. In this study therefore we determined the experimental transmission and pathogenicity of koi ranavirus (KIRV) in common carp and rohu widely cultivated carp species in India.

Clinical pathology

Host susceptibility to viral infection is not a specific trait for a susceptible animal because susceptibility to disease is influenced by a complex interaction of host, pathogen and environmental factors (Tweedell and Granoff, 1968). Infection by ranavirus results in mortality and morbidity different from species to species. Some of the experimental challenge study and field data show that mortality can range from low (e.g. 0%) to 100% of infected animals of an experimental test group depending on species, virus and age and health condition of the host following short infection times (Hyatt *et al.,* 1998; Pearman *et al.,* 2004; Harp and Petranka, 2006). In the present study, we observed no specific external clinical signs and mortality during the experimental period. The target viral DNA was detected from the tissues of both experimental fish species after virus injected fish sampled at 3 dpi by PCR. However, we could not detect specific DNA of KIRV from tissues of both experimental fish species sampled at later periods. Experimental infections and clinical signs may vary according to various factors. In general, differences on the time of transmission, age of fish and route of infection are the important factors for pathogenicity of virus (Aranguren *et al.,* 2002).

Ranaviruses infection differs due to the varied dynamics among the host species and viral isolate and the detailed molecular mechanisms by which ranaviruses are able to cause disease and circumvent the host immune response are not completely understood (Allen *et al.,* 2017). Not all diseases caused by ranaviruses always result in mortality (Miller *et al.,* 2009). However, some field studies confirmed that individuals can be sublethally infected with ranaviruses (Brunner *et al.,* 2004, Pearman *et al.,* 2004, Greer *et al.,* 2009). While the relationship between viral load and ranaviral disease is currently not well understood (Gray *et al.,* 2009), there is less evidence that they are correlated (Hoverman *et al.,* 2010). High variation in susceptibility of amphibians, fish, and chelonians to ranaviruses has been reported by (Hoverman *et al.,* 2011). Variations in host susceptibility to different ranavirus pathogens create an ideal scenario for the pathogen to move between hosts, utilizing highly susceptible species and also low susceptible hosts for amplification and for persistence respectively (Gray *et al.,* 2009).

Cell culture assay

Ranavirus isolated from marine damselfish was found to be growing well in marine and freshwater fish cell lines derived from seabass and snakehead (Sivasankar *et al.,* 2017). They have also re-isolated ranavirus using cell culture from the pooled homogenates of the spleen and kidney of each of the dead and moribund seabass fingerlings which had experimentally infected. In contrast, in our study, the virus could not be reisolated from the spleen and kidney of the both experimental virus infected fish species indicating that the virus could have been either eliminated by the fish or reduced the load that evaded detection using cell cultures.

PCR detection

Tissues such as spleen and kidney of common carp and rohu challenged with KIRV were

collected at 3, 7, 14, 21 and 28 dpi and diagnosed for the presence of KIRV by PCR. PCR detected the target MCP amplicon indicating the presence of KIRV in pooled samples of kidney and spleen from experimental group fishes of common carp and rohu after 3 dpi. However, no positive results were obtained from other sampling time points of 7, 14, 21 and 28 dpi (Fig. 3). PCR detection of the infected fish tissues showed that the viral DNA is present in the infected tissues such as spleen and kidney of the koi (George *et al.,* 2015).

Fig.1 Cytopathic effect induced by KIRV in EPC cell lines (A) EPC Control (x 200) (B) KIRV infected EPC cell line (x 200)
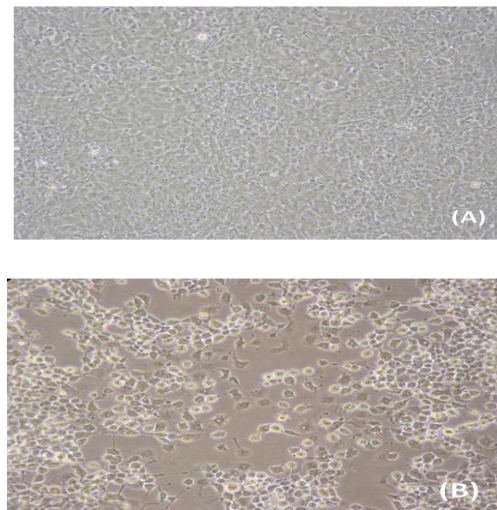
Fig.2 Experimental setup for koi ranavirus (KIRV) experimental infection to common carp and rohu
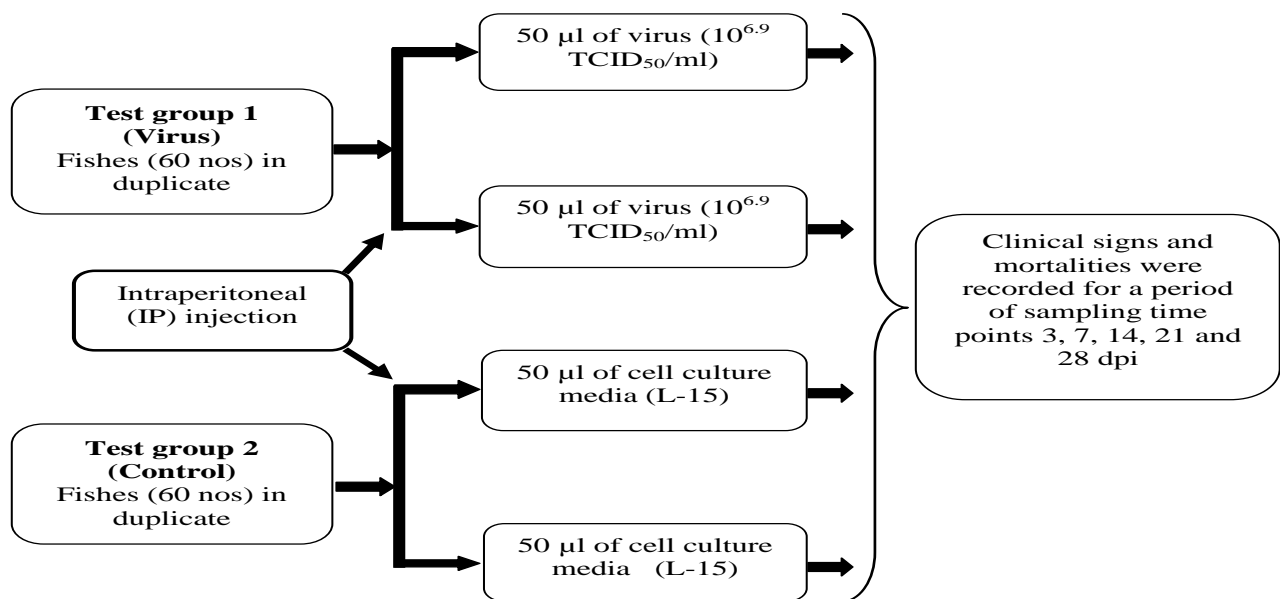
Fig.3 Agarose gel electrophoretic analysis for detection of KIRV in tissues samples collected from control and virus groups at 3, 7, 14, 21 and 28 dpi by PCR. (A) Common carp experiment - Lane 3&4 positive sample (virus group in duplicate sample), Lane 13&24- Positive control, Lane 14&25- Negative 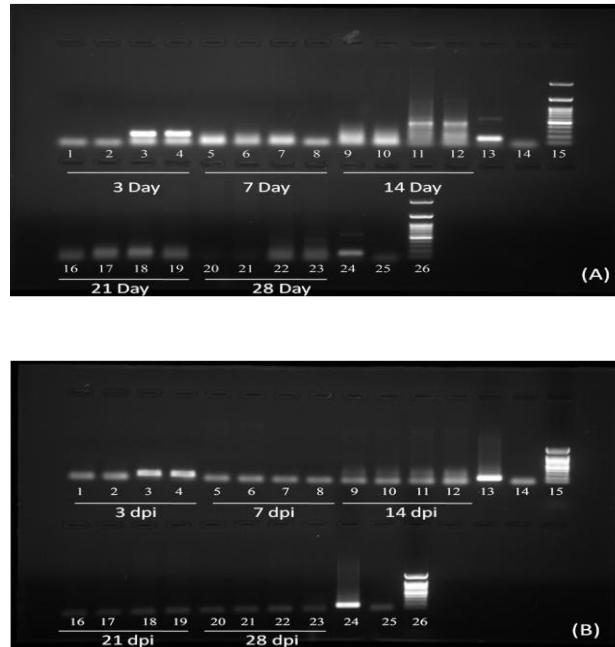control, Lane 15&26- 100bp marker and (B) Rohu experiment - Lane 3&4 positive sample (virus group in duplicate sample), Lane 13&24- Positive control, Lane 14&25- Negative control, Lane 15&26- 100bp marker

Fig.4 Common carp kidney: (A) necrosis and hypertrophy in tubules at 3 dpi and (B) necrosis and hypertrophy in kidney tubules at 7 dpi HE (100x)
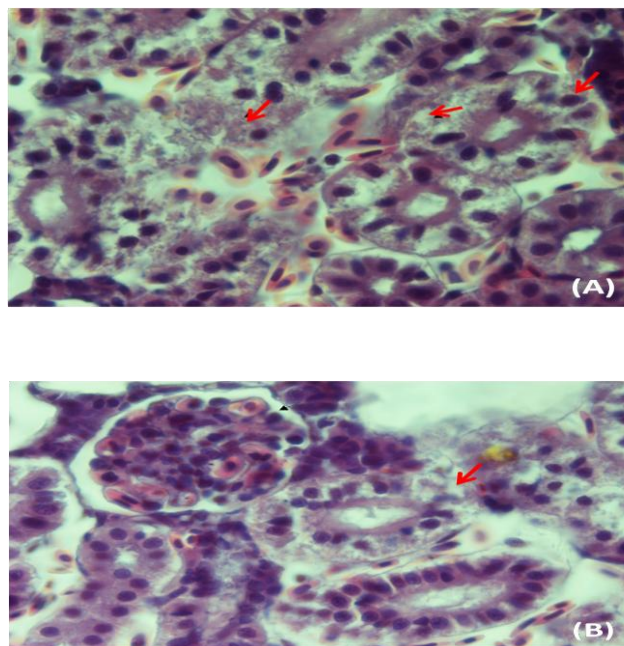
Fig.5 Rohu kidney: (A) Necrosis at 3 dpi and (B) detachment of glomeruli from epithelium of tubules at 7 dpi HE (60x)
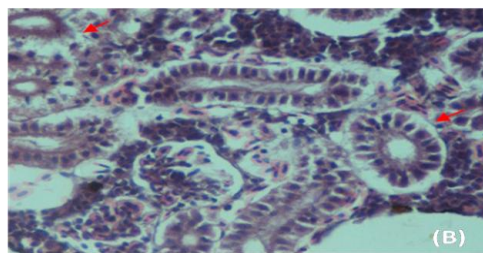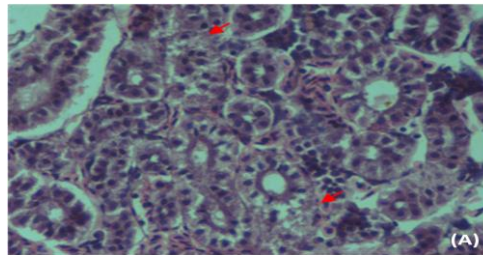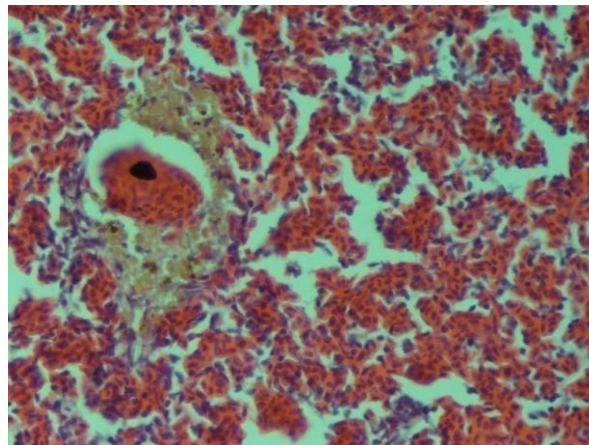

Fig.6 Rohu spleen: Melanomacrophage center observed in spleen at 3 dpi HE (60x)

Histopathology analysis

Gobbo *et al.,* (2010) have reported that histopathology was not always performed during disease outbreaks or in experimental trials with ranavirus. There are 10 different families represented among the 14 fish host species potentially susceptible to EHNV infection but the susceptibility does not appear to correlate with taxonomic relationships (Jensen *et al.,* 2011; Gobbo *et al.,* 2010). Zilberg *et al.,* (2000) reported necrosis of the gastrointestinal (GI) mucosal epithelium, gills, and heart in largemouth bass (*Micropterus salmoides*) experimentally infected with *Santee - Cooper ranavirus*. Our results of the histopathological study revealed the necrosis and hypertrophy in kidney tubules of common carp with infected KIRV (Fig. 4) and also necrosis and detachment of

glomeruli from the epithelium of tubules in kidney tissue of rohu infected with KIRV infected at 3 and 7 dpi (Fig. 5). Unfortunately, there was no any pathological change observed in kidney collected from both common carp and rohu at 14, 21 and 28 dpi. In spleen tissue also not showed typical histopathological changes at all the time points in common carp. While in rohu experiment, melanomacrophage centers were observed in the spleen of experimentally infected fish at 3 dpi (Fig. 6) and no typical histopathological changes observed in spleen at other time points. Reports indicate that there are 10 different families represented among the 14 fish host species potentially susceptible to EHNV infection but the susceptibility does not appear to correlate with taxonomic relationships (Gobbo *et al.,* 2010; Jensen *et al.,* 2011).

In conclusion the several diseases induced by members of the genera *Ranavirus* have been responsible for mortality in captive and wild amphibian, reptile and fish populations around the globe over the past two decades. Ranaviruses are now recognized as emerging pathogens causing acute and systemic diseases in various host animals like fishes, amphibians and reptiles. The present study was to provide evidence to determine the experimental transmission and pathogenicity of koi ranavirus (KIRV) to common carp, *Cyprinus carpio* L and rohu, *Labeo rohita*. This investigation indicated that there is no gross mortality or any specific external pathological changes observed in common carp and rohu fingerlings following experimental infection with KIRV.

Acknowledgments

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