International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2025; SP-9(2): 435-443
www.biochemjournal.com
Received: 11-12-2024
Accepted: 14-01-2025

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Determining lethal dose (LD_{50}) and pathological effects of ISKNV in monosex gift tilapia (*Oreochromis niloticus*)

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DOI: https://doi.org/10.33545/26174693.2025.v9.i2Sf.3791

Abstract
This study investigates the lethal dose (LD_{50}) and pathological changes associated with Infectious Spleen and Kidney Necrosis Virus (ISKNV) infection in monosex genetically improved and farmed tilapia (GIFT) (*Oreochromis niloticus*). The virus was propagated in the Sahul Indian Seabass Spleen (SISS) cell line, displaying notable cytopathic effects. The LD_{50} assay determined the lethal dose of ISKNV for GIFT tilapia to be 1.7×10^5 copies/µl with mortality rates ranging from 15% to 85% across different viral dilutions. Infected fish exhibited clinical signs such as splenomegaly, significant haemorrhaging, and behavioural changes like erratic swimming and anorexia. The infection was confirmed by Polymerase chain reaction (PCR) assays using major capsid protein (MCP) gene The successful experimental infection of ISKNV in GIFT tilapia, demonstrates high mortality rates and significant pathological changes. Histopathological analysis further substantiates these findings by revealing characteristic features of ISKNV infection, such as degenerated epithelium, melano macrophage centres (MMC), increased interstitial spaces, and ruptured glomeruli and renal tubules. This study provides valuable insights into the severity and progression of ISKNV infection in GIFT tilapia, offering a foundation for developing effective disease control strategies in aquaculture.

Keywords: ISKNV, lethal dose 50, GIFT tilapia, pathology

Introduction
Due to its adaptability, rapid growth, and high reproductive rate, tilapia is a popular species in global aquaculture. With a production of around 5.3 Million Metric Ton (MMT), Nile tilapia (*Oreochromis niloticus*) is the fourth highest produced species in the world [1]. Average annual fish production growth rate in India is 7.98%, inland production (13.11 MMT) being the major contributor to the total fish production. The Food and Agriculture Organization (FAO) forecasts a 26% increase in India's fish production from 2018 to 2030. This growth rate is 6.8% faster than the projected average for Asia and 11.5% higher than the global average [1]. The demand for the freshwater and estuarine fishes have been increasing since 1960s, which resulted in the increased share of tilapia like fishes to the total per capita consumption of fish [2]. Due to its hardy nature, tilapia is one of the important freshwater fish species in India. Introduction of tilapia has given a significant boost to the aquaculture sector through the implementation of intensive culture systems like biofloc and cage culture. Although the current production of tilapia is minimal compared to the total fish production, there is enormous potential for growth in the coming years [3]. Intensifying culture systems can indeed make fish more susceptible to various diseases, both infectious and non-infectious. Viral diseases are particularly problematic, often leading to significant losses for farmers [4].

One of the major viral diseases affecting fish, leading to significant economic losses, is caused by the *Iridoviridae* family of viruses. These viruses also impact amphibian populations. As an emerging, re-emerging, and transboundary pathogen, the *Iridoviridae* family poses a global threat, and was recently identified in India as Red Seabream Iridovirus (RSIV) infection in Asian seabass (*Lates calcarifer*) [5] and ISKNV infection in various ornamental fishes [6]. The Iridovirus family, characterized by double-stranded DNA with an icosahedral structure having a diameter ranging in-between 120-200 nm.

The family *Iridoviridae* consists of 5 genera: *Chloriridovirus*, *Iridovirus*, *Lymphocystivirus*, *Ranavirus* and, last but not least, *Megalocytivirus*. Within the *Megalocytivirus* genus, the viruses can be classified into Red Seabream Iridovirus (RSIV), Infectious spleen and kidney necrosis virus (ISKNV), and Turbot Reddish Body Iridovirus (TRBIV), the Scale drop disease virus (SDDV) being a newly identified member. *Megalocytivirus* infect a variety of freshwater and tropical marine fish, such as groupers, seabass, gourami, cichlids, red seabream, angel fish, and lamp eyes. Such infections in aquaculture can result in diseases with high mortality rates, causing substantial economic losses for the industry [7].

ISKNV is a double stranded DNA, non-enveloped virus having a genome length of approximately 110 kb. It was first reported in Mandarin fish (*Siniperca chuatsi*) with high economic losses, infected fish showed clinical signs like anorexia, petechial haemorrhages, abnormal swimming, pale coloration and mortality ranging from 50-90% [8, 9]. ISKNV has been associated with systemic disease and high mortality in many freshwater and marine ornamental and cultured food fishes [8, 10]. The virus has a vast host range and can infect nearly 50 different freshwater, brackish water, and marine species including both food fish and ornamental fish [11]. The internal organs showed liver haemorrhage, splenomegaly and kidney enlargement (nephromegaly) in ISKNV infected fish. In ornamental fishes, ISKNV can sometimes present with or without symptoms (asymptomatic) [6]. Histopathology of ISKNV infection is characterized by cell hypertrophy with melano-macrophage centres (MMCs) in the spleen and kidney tissues. Transmission electron microscopy analysis of the infected cells revealed presence of large numbers of icosahedral viral particles in the cytoplasm [12]. Recently, there have been several reports of infectious spleen and kidney necrosis virus (ISKNV) infections in ornamental fish from Karnataka [6, 13] and Kerala [14, 15]. Whole genome sequencing of the virus has also been conducted to understand its pathogenicity and infective characteristics [16]. The potentially high mortality rates observed in some economically important fish species highlight the need for a better understanding of the pathogenesis of ISKNV infections in order to develop effective new preventive measures.

Materials and Methods

Experimental fish

In this experiment, monosex (all male) genetically improved farmed tilapia (GIFT) (*Oreochromis niloticus*) were utilized. GIFT fry were sourced from the Zonal Agricultural & Horticultural Research Station in Mudigere, Karnataka and reared in cement tanks at the Instructional Fish Farm, College of Fisheries, Mangaluru, until they reached the fingerling stage. The ISKNV negative fish were then acclimatized in 50L rectangular glass tanks in the wet lab facility of the Department of Aquatic Animal Health Management (AAHM), College of Fisheries, Mangaluru, for one week prior to the experiment. Each tank was aerated, and one-third of the water was replaced every two days. During the acclimatization period, the fish were fed with commercial pellets. Dead fish were promptly removed, and debris was siphoned from the aquarium bottom.

Virus and growth media

For determining the LD_{50} of the ISKNV virus, a previously isolated strain from angel fish was utilized which was stored in the Department of Animal Health Management (AAHM), College of Fisheries, Mangaluru. Sahul Indian Sea bass Spleen (SISS) fish cell line cultured in Leibovitz's L-15 medium (Himedia, India) with 10% fetal bovine serum (FBS) (Himedia, India) media was used to propagate the ISKNV [16, 17]. ISKNV infection study was performed with 2% FBS L-15 media and incubated at 25 °C till 80% of the cells were infected.

Virus propagation and confirmation

The virus was taken from the frozen storage, thawed and passed through 0.45µ syringe filter. The filtered virus was propagated in SISS cell line. The infection was monitored daily by observing the cytopathic effect (CPE) on the SISS cells. When CPE was observed in 80% of the cells, the virus-infected cells were scraped and reinfected to confirm the true CPE caused by the ISKNV virus. After third passage, the virus was subjected to alternative freezing (at-80 °C) and thawing (at 30 °C). The fish cell line supernatant was centrifuged at 6000 g for 10 min and the supernatant was filtered using a 0.45 µm filter. One millilitre of the filtered virus was used to extract the viral DNA by following standard protocol [18], and later on subjected to PCR amplification by targeting the ISKNV MCP gene (F: 5'CGTGAGACCGTGCGTAGT3'; R: 5'AGGGTGACGGTCGATATG3') (563 bp) to confirm the presence of ISKNV infection [13].

Confirmation of ISKNV

The DNA from the experimentally infected fish was isolated using the standard protocol [18] with minor modifications. Initially the tissue sample were macerated with digestion buffer (10 mM Tris-HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% Triton-X100; 6 M Guanidine hydrochloride; 0.1 M Sodium acetate) and the supernatant was mixed with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The aqueous phase containing DNA was precipitated using 2-3 volumes of 100% ethanol, and washed with 70% of ethanol. Finally, DNA pellet was dissolved in 100 µl of 1X TE buffer pH 8.0 and was stored at-20 °C for later use, or used directly for PCR analysis. PCR was carried out for the extracted DNA in 30 µl reaction mixture containing 2.0 µl template DNA, 1X assay buffer (10 mM Tris –HCl, pH 9.0; 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Gelatin), 10 µM of each four deoxyribonucleotide triphosphates (dATP, dCTP, dTTP, dGTP), 10 pM of each primer (MCP gene – 563 bp) and 0.6 U of Taq DNA polymerase (Genei, Bangalore).

Quantification of the virus

Plasmid containing major capsid protein (MCP) gene (1362 bp) was used to plot the standard curve. The concentration of the plasmid was measured in Nano drop 1000 TM spectrophotometer (Thermo fisher scientific, USA) at 260 nm. The copy numbers were calculated using the following equation [19].

$$\text{No. of copies/µl} = \frac{6.02 \times 10^{23}(\text{copies}) \times \text{plasmid concentration (g/µl)}}{\text{number of bases (bp)} \times 660(\text{daltons/base pair})}$$

After the calculations of the copy number, serial dilutions of the plasmids were prepared until the copy number reaches single digit. Then the real time PCR assay was carried out using SYBR Green PCR Master Mix (Takara). For each real time PCR reaction 10 µl SYBR Green PCR Master Mix (with 40 µl of ROX/ml of SYBR Green), 1 µl of each ISKNV MCP rt forward (5'ATCCCCTCCATCACATCCAGCAAG3') and reverse primers (5'CATGCAGGCGTTCCAGAAGTCAAG3') (2.5 pM), 7 µl of sterile water and 1 µl of plasmid dilutions. Serial dilutions of the ISKNV DNA to be tested were also prepared and used as templates. All the reactions were performed in triplicates with non-template control (NTC) as negative control. The cycling conditions used for the PCR are as follows, 10 minutes at 95 °C, followed by 40 cycles at 90 °C for 15 seconds, 60 °C for 30 seconds, followed by melt curve [20]. At the end of the real time PCR assay, a standard curve was generated and the quantification of the unknown viral DNA sample was done by measuring the Ct value and interpolating in the standard curve. To avoid the nonspecific amplification and primer-dimer formation during the qPCR assay, the melt curve was analysed.

Lethal dose 50 (LD_{50}) of ISKNV in GIFT tilapia

Ten healthy GIFT tilapia fish, each averaging 14.54 ± 1.64g in weight, were randomly allocated into six distinct groups, five groups with ISKNV at varying dilutions, while one group served as the control. The experiment was conducted in duplicate. Five serial dilutions of ISKNV were prepared using L-15 medium, and the fish were intraperitoneally (i.p.) injected with 0.1ml of the viral suspension using a 1ml syringe. The control group was injected with 0.1ml of sterile L-15 medium. Infection signs were monitored by observing clinical symptoms and, mortality rates were recorded twice daily. Moribund fish were closely observed for clinical signs, and dead fish were promptly removed from the aquaria and used for histopathological studies. After 10 days, the percentage cumulative mortality and the lethal dose 50 (LD_{50}) for the population were determined using the Reed–Muench method. Proportionate distance and LD_{50} was calculated using the below mentioned formulas [21].

$$\text{Proportionate distance (PD)} = \frac{\%\ \text{positive above 50\%} - 50\%}{\%\ \text{positive above 50\%} - \text{positive below 50\%}}$$

$$\text{Log LD}_{50} = \text{Log (dilution with} > 50\%\ \text{positive)} + \text{PD} \times [-\log(\text{dilution factor})]$$

The morality graph was plotted using GraphPad Prism ver.8.0, where the percentage mortality of the fish in different groups was plotted against time interval of the experiment.

Histopathology

For histopathological examination, tissue samples from spleen and kidney were fixed in 10% neutral buffered formalin (pH 6.8) for 24-72 hours. Following fixation, the tissues were dehydrated through a series of ascending alcohol concentrations (70%, 80%, 90% and 100%), embedded in paraffin, and sectioned to a thickness of 5 µm using a microtome. The tissue sections were then stained with haematoxylin and eosin (H&E) and examined under a light microscope using 40X magnification objective. All histological procedures were conducted according to the methods outlined by [22, 23].

Results

Propagation of ISKNV in fish cell line

The ISKNV-infected SISS cell line exhibited notable cytopathic effects, such as rounded and elongated bulged cells, which became prominently visible 3-4 days post-infection. By the eighth day of post-infection, the SISS cells were scraped and transferred to another flask containing a monolayer of SISS cells. The second passage demonstrated significant cytopathic effects, with initial cell rounding occurring on the fourth day post-infection. During the third passage, cytopathic effects were observed as early as the second day post-infection. Control SISS fish cell lines showed no cytopathic effects even after 10 days post-infection (Fig. 1).

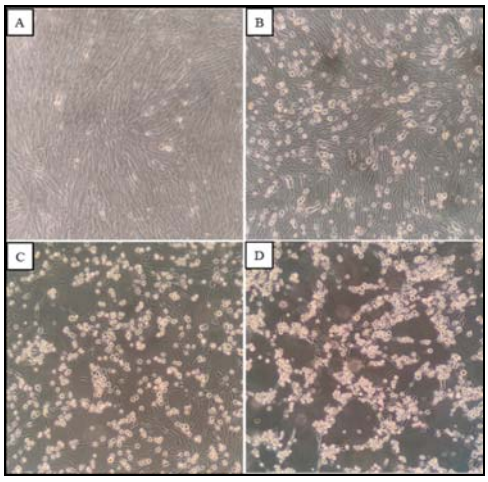

Fig 1: Propagation of ISKNV in SISS cell lines. A) Control SISS Cell Line: Demonstrating excellent confluency without the presence of any cytopathic effects (CPE). B) ISKNV Infected Cell Lines at 3 dpi: Exhibiting rounding CPE, indicative of the early stages of viral infection. C) Severely Infected SISS Cell lines with 80% infected cells at 6 dpi. D) ISKNV infected SISS cell lines at 10 dpi: Characterized by extensive CPE, resulting in the detachment of cells from the culture flask.

Quantification of ISKNV

The amplification plot was generated by mapping fluorescence intensity against the cycle number and the Ct value was obtained for each dilution. The melt curve was derived by plotting the derivative of fluorescence against dissociation temperature. The single peak observed in the melt curve confirms the presence of a single amplicon. The standard curve was obtained by plotting the mean Ct value against plasmid copies per µl with the slope of-3.212, Y-intercept 38.469, R^2 value of 0.994 and efficiency percentage of 104.778% (Fig. 2). ISKNV DNA dilutions showed amplification similar to that of standard curve and the Ct was interpolated in the standard curve (Fig. 3). The copy number of the ISKNV was calculated to be 5.4 x 10^7 viral copies per microliter. All the figures mentioned in this section was generated by using StepOnePlus real-time PCR System (Applied Biosystems, USA).

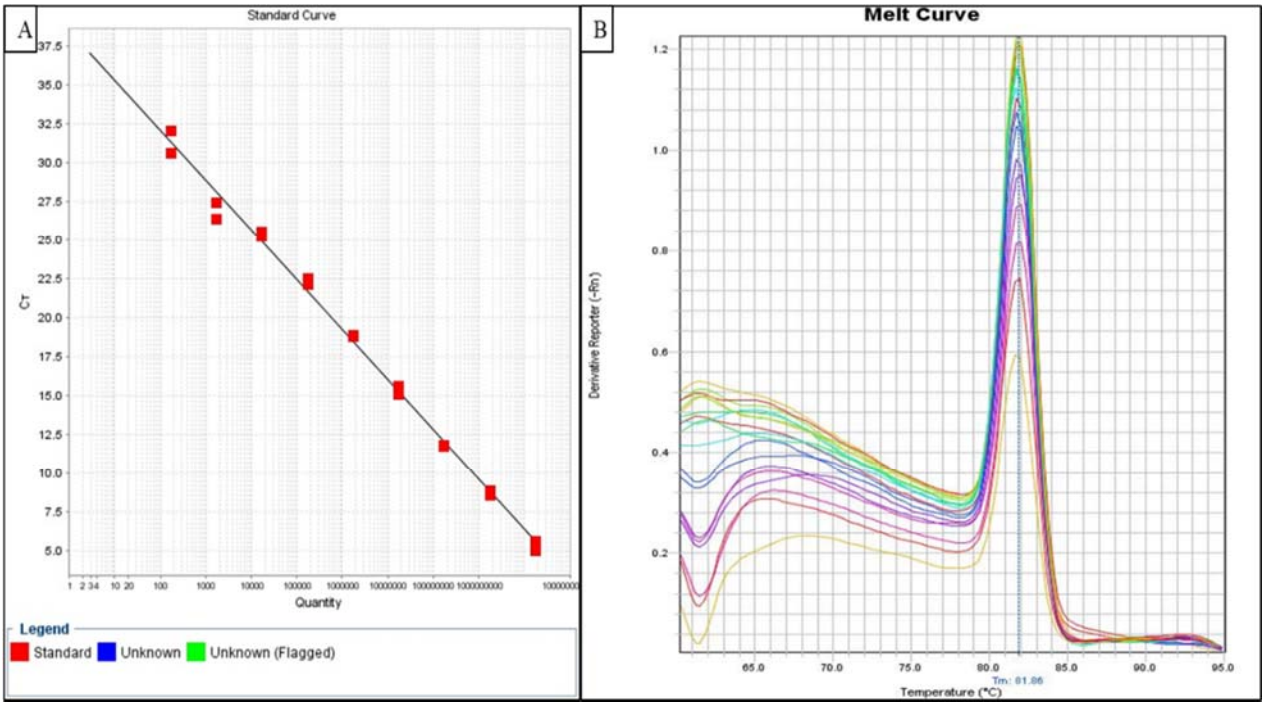

Fig 2: Standard curve plot using ISKNV MCP gene plasmids. A) Standard curve of ISKNV MCP plasmid through real-time PCR assay. B) Melt curve showing single sharp peak of the plasmid dilutions which are used to plot standard curve.

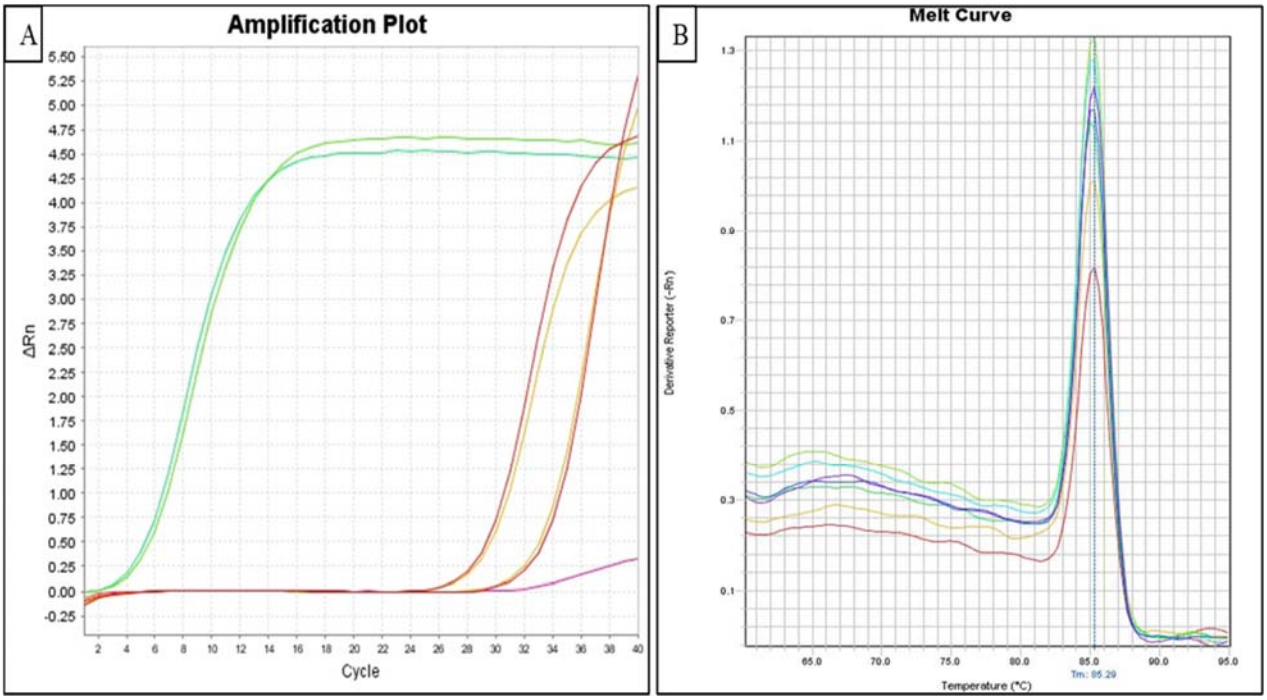

Fig 3: Quantification of ISKNV using the StepOnePlus real-time PCR system. A) Amplification plot of ISKNV with positive control (PC) and negative control (NC), B) Melt curve showing a single sharp peak for MCP gene.

LD_{50} of ISKNV in GIFT tilapia

Mortality rates were recorded daily across all study groups. The mortality rate ranged from 85% in the lowest dilution (5.4 x 10^6 copies/µl) to 15% in the highest dilution (5.4 x 10^2 copies/µl). Mortality began within 24 hours post-infection in the three groups with higher dilutions (5.4 x 10^6, 5.4 x 10^5, and 5.4 x 10^4 copies/µl). In the 5.4 x 10^3 and 5.4 x 10^2 groups, mortality was observed on the second and third days, respectively. The control group experienced no mortality throughout the experiment. By the end of the fifth day, cumulative mortalities across the groups were as follows: 60%, 35%, 25%, 10%, and 10% mortality were observed in 5.4 x 10^6, 5.4 x 10^5, 5.4 x 10^4, 5.4 x 10^3 and 5.4 x 10^2 copies/µl respectively. Detailed mortality data can be found in Table 1. The cumulative percentage mortality, plotted against the time interval, highlighted mortality trends among different groups. Using the Reed-Muench formula, the LD_{50} was calculated to be 1.7 x 10^5 copies/µl, with a proportionate distance (PD) of 0.5 (Fig. 4).

Table 1: Concentration of ISKNV and number of mortalities observed in during the experiment in different groups.

SL. No.	Viral concentrations (copies/µl)	Initial No. of fish	Days										Total Mortality	Cumulative Percentage Mortality (%)
			1	2	3	4	5	6	7	8	9	10		
1.	5.4 X 10^6	20	4	3	1	3	1	2	1	2	0	0	17	85
2.	5.4 X 10^5		2	1	0	2	2	1	0	3	0	1	12	60
3.	5.4 X 10^4		1	1	2	0	1	1	0	1	1	0	8	40
4.	5.4 X 10^3		0	1	0	1	0	0	2	1	0	0	5	25
5.	5.4 X 10^2		0	0	1	0	1	1	0	0	0	0	3	15
6.	Control		0	0	0	0	0	0	0	0	0	0	0	0

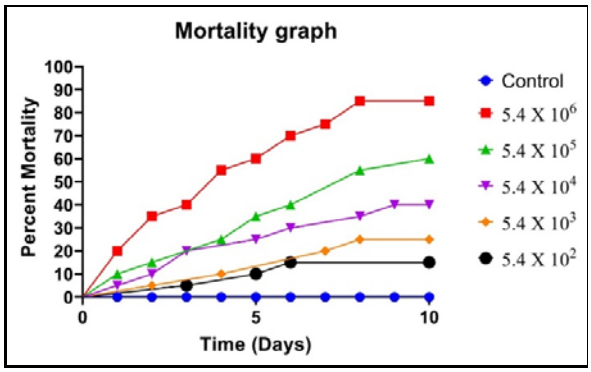

Fig 4: Mortality graph of the ISKNV in GIFT tilapia was observed over a 10-day period. The cumulative percentage mortality across different groups is plotted against the time interval in days

Clinical signs, gross pathology

Fish infected with ISKNV exhibit reddening and discoloration on their body surface, particularly in the abdominal region. Internally, they show typical clinical signs such as an enlarged spleen and kidney (Fig. 5). The development of clinical signs in the spleen and kidney occurred earlier than the onset of morphological and behavioural changes in ISKNV-infected fish. The infected fish showed behavioural symptoms like erratic swimming and anorexia when compared with the control fish.

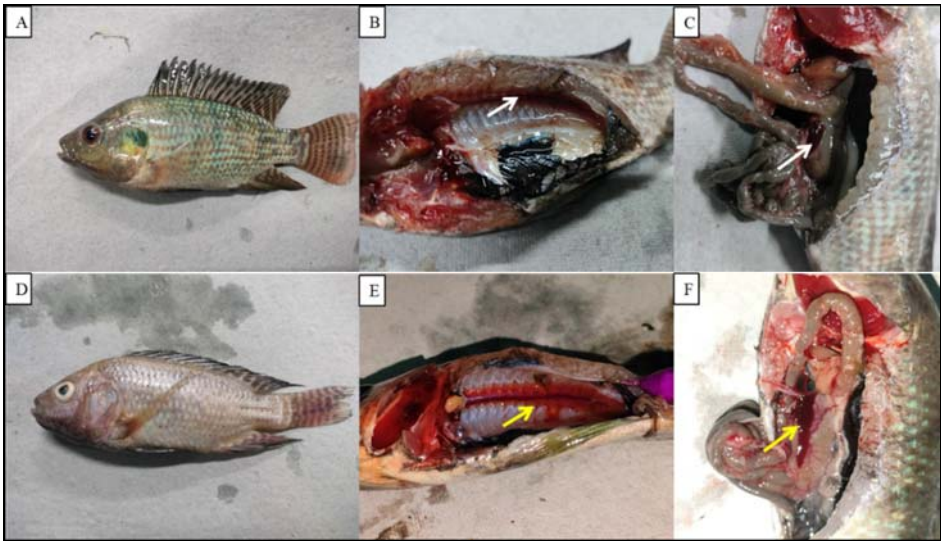

Fig 5: Comparison of the pathological changes in experimental fish. Control fish showing normal coloration and body morphology (A), kidney (white arrow) (B) and spleen (white arrow) (C). ISKNV infected fishes showing clinical signs like-discoloration and reddening on body surface (D), nephromegaly (yellow arrow) (E) and splenomegaly (yellow arrow) (F).

Confirmation of the ISKNV infection

The PCR assay performed for the DNA extracted from the ISKNV infected fish showed a positive result. The PCR product of both neat and diluted DNA under UV illuminator showed an amplicon size of 563 bp which is similar to that of the positive control (Fig. 6).

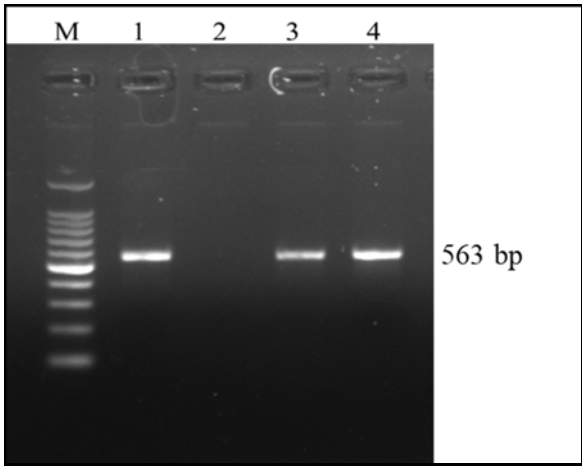

Fig 6: Confirmation of ISKNV in the extracted virus using ISKNV MCP primers (Lane M: 100bp DNA ladder; Lane 1: Positive control; Lane 2: Negative control; Lane 3: DNA Neat; Lane 4: DNA dilution)

Histopathology

The haematoxylin-eosin staining of the histological sections allowed us to identify the histopathological changes in spleen and kidney of ISKNV infected GIFT tilapia. ISKNV infected fish tissues displayed numerous basophilic hypertrophied cells. There were several necrotic areas with reduced red blood cells (RBCs) in the ISKNV-infected group when compared to the control splenic tissue. The epithelial cells of kidney found to be degenerated and lost its normal shape. The interstitial space in the glomeruli and renal tubules was enlarged with few or no red blood cells observed (Fig. 7).

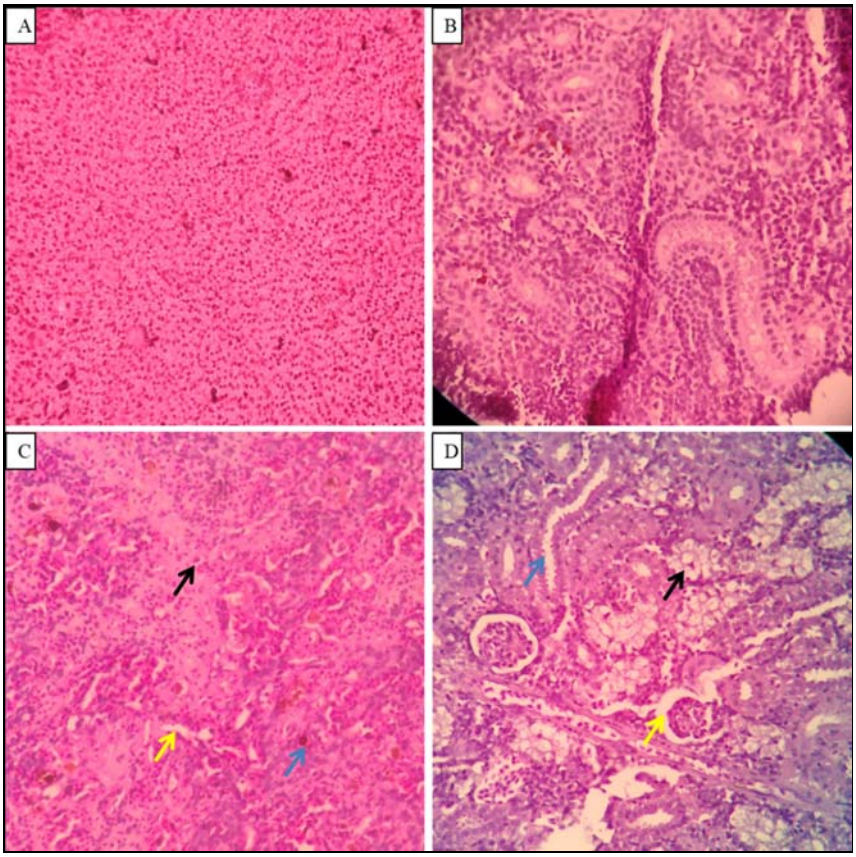

Fig. 7: Histopathological changes observed in spleen and kidney tissues of ISKNV infected in comparison with control. A) Control spleen B) Control Kidney C) ISKNV infected spleen: displayed numerous basophilic hypertrophied cells (black arrow), several necrotic areas (yellow arrow) and reduced red blood cells (RBCs) (blue arrow) in the ISKNV-infected group when compared to control. D) ISKNV infected kidney: epithelial cells of kidney found to be degenerated (blue arrow), interstitial space in the renal tubules and glomeruli got enlarged (yellow arrows) and glomerular atrophy (black arrow).

Discussion

Disease is a significant challenge for the cultivation of aquatic species, hindering economic growth and social progress in both developed and developing nations. This study aims to evaluate the severity of infection by identifying the lethal dose of the Infectious Spleen and Kidney Necrosis Virus (ISKNV) in mono sex GIFT tilapia. The pathological changes observed in the ISKNV infected tilapia during the study was examined and the findings were compared with the control group. The *in-vitro* propagation of the ISKNV in fish cell lines will help in understanding the cellular pathogenic mechanism [24]. In the present study, the SISS cell line was used to propagate the virus. Cytopathic effects like rounding, elongation and detachment of infected cells were observed during the infection. Common CPEs found in ISKNV infection are rounding of cells and surface detachment [6, 26], which suggests that the CPE found in the present study infers the ISKNV infection. In an *In-vitro* study conducted in MFF-1 cells, the ISKNV infected cells showed shrinkage and rounding at 2 days post infection (dpi) and extensive rounding at 3 dpi. The CPE was varied during initial three passages and stabilized in later passages [26]. Similarly, in the present study, the CPE was observed at 3 dpi in the first passage, at 4 dpi in second passage and 2 dpi in the 3rd passage. After 10 dpi, the SISS propagated ISKNV was used to extract the genomic DNA using the standard protocol [18]. The dilutions of the genomic DNA were subjected to real time PCR assay for the quantification of the viral copy number. The Ct value obtained after the quantification was interpolated with standard curve and the virus titre was calculated. The obtained titre value was as high as 5.4 x 10^7 copies/µl. Similarly, when ISKNV DNA was extracted using two different methods, the viral titer was measured, with magnetic bead extraction, the viral titer was found to be 5.85×10^7 and while with spin column extraction, it was 2.26×10^7 [20].

The LD_{50} assay assesses a fish species' tolerance to pathogenic viral suspensions at a defined concentration. It determines the virus's pathogenicity by causing 50% mortality in the fish population over a specified period. To enhance the virulence of the virus, the ISKNV cell line extract from cell culture was used for challenge studies in the present study. Lethal dose 50 of the ISKNV against GIFT tilapia in the present study was determined as 1.7 x 10^5 copies/µl. During the 10 days duration of the experiment, mortality rates of all the six groups (five different dilutions from 10^6 to 10^2 and one PBS control) were noted down. A mortality graph was produced using cumulative percentage mortality of the tilapia for all the groups. The cumulative percentage mortality reached its peak (85%) at the lowest dilution of ISKNV (5.4×10^6 copies/µl). In contrast, the lowest cumulative percentage mortality (15%) was observed at the highest dilution, (5.4×10^2 copies/µl). Similar results were observed in an LD_{50} experiment conducted over 14 days with Nile tilapia, in which the fish were injected with different dilutions of ISKNV. The percentage survival rate in the ISKNV-infected group was 20% (indicating an 80% mortality rate), compared to the control group with 90% survival [27]. The clinical manifestations like discoloration & reddening on body surface, splenomegaly and nephromegaly were observed in this study after the ISKNV infection, which was similar to the typical clinical signs observed during *Megalocytivirus* (ISKNV) infections [6, 23 28]. The presence of these clinical signs is a hallmark of ISKNV infection and provides strong evidence supporting our findings.

Histopathology plays a vital role in diagnosing viral infections in fish by examining thin, stained tissue sections under a microscope. This process helps identify pathogen-induced changes in the tissues. In the present study, haematoxylin and eosin (H&E) method was used to look into the pathological changes occurred in spleen and kidney of ISKNV infected with respect to control. ISKNV infected fish tissues displayed numerous basophilic hypertrophied cells. There were several necrotic epithelial areas with reduced red blood cells (RBCs) in the spleen tissue of ISKNV-infected group when compared to the control. Degenerative and necrotic cells were found in ISKNV infected kidney tissue which had lost its normal shape. The interstitial space in the glomeruli and renal tubules was enlarged with reduced or no red blood cells. A study based on histopathological examinations of tropical food and ornamental fishes were conducted. All the fish species consistently revealed pale to intensely basophilic hypertrophied virus-infected cells in spleen, kidney, liver, brain and intestine [29]. Several ISKNV infected fishes like pearlspot [15], Nile tilapia [30], mandarin fish [9] and zebrafish [31] exhibited similar histological changes in both liver and spleen tissues. In the liver, the hepatic parenchyma displayed enlarged basophilic cells, while the spleen showed an increase in red pulp with enlarged basophilic cells. Inclusion bodies in spleen and kidney can be also found during the megalocytiviral infections [32 The reason for the hypertrophy of endocardium and vascular endothelium in organs such as the spleen and kidney, possibly by the reaction of antigen-sensitive cells to an intense viraemia or as a result of toxaemia [29]. The evidences suggest that the infection, along with the observed clinical signs and pathological changes, was due to ISKNV infection.

Conclusion

In conclusion, this study highlights the profound impact of ISKNV infection on mono-sex GIFT tilapia, resulting in severe pathological changes and significantly elevated mortality rates. The determination of the lethal dose (LD_{50}) and the identification of cytopathic effects provide valuable insights into the virus's pathogenicity. The findings emphasize the importance of histopathological examinations in diagnosing viral infections in fish and understanding the cellular mechanisms involved. The study also highlights the need for effective disease management strategies to mitigate the economic and social challenges posed by viral infections in aquaculture, both in developed and developing nations. These results contribute to the growing body of knowledge on ISKNV and its effects on aquatic species, paving the way for future research and potential interventions to control and prevent the spread of this virus.

Acknowledgment

This work was carried out with funding support from the National Surveillance Programme for Aquatic Animal Diseases under Pradhan Mantri Matsya Sampada Yojana (No. 35028/05/2021-Fy (Trade) (E-3603) dated 7th June 2022) from the Department of Fisheries, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India is thankfully acknowledged.

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