Monitoring of viral pathogens in the main Goldfish (*Carassius auratus*) farms using Immunochemical and Molecular Biology techniques

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Abstract

Diseases are among the most critical threats to the aquaculture industry so that their prevalence leads to major economic losses. Monitoring for viral pathogens in aquaculture has high importance due to their incurability, speed of transmission, the severity of pathogenesis, and difficulties of their diagnosis. According to reports of goldfish (*Carassius auratus*) losses with suspected viral etiology in farms of Gilan province this study designed to inquiring about the existence of selected viral agents. Thus, the spring viremia of carp (SVC) and Koi herpesvirus (KHV) which are among the notifiable infectious diseases (OIE, 2019) were investigated by cell culture, PCR, RT-PCR, and immunofluorescent antibody test (IFAT). The results showed the absence of the RNA of *Rhabdovirus carpio* and the DNA of Koi herpesvirus in tested samples. Accordingly, 470 bp band related to SVCV and 292 bp band related to KHV were not confirmed in any of the samples in PCR test as well as neither Cytopathic effect in cell culture nor positive reaction in IFAT were observed. Hence, it seems that non-viral agents are the main causes of periodic population loss of goldfish in the surveyed farms and can be controlled and prevented by the health management procedures.

Keywords: Goldfish (*Carassius auratus*), fish viruses, Koi herpesvirus (KHV), spring viremia of carp (SVC).

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Introduction

Ornamental fish propagation and trade is a thriving global industry in a way that about 1.5 billion fish of nearly five thousand different species are traded around the world. However, goldfish (*Carassius auratus*), as a member of the *Cyprinidae* family, is the most common ornamental fish cultured in Iran, which has a global distribution. According to the available data, Gilan province has taken the first place in the production of goldfish and the second place in the propagation of ornamental fish in the country. Although ornamental fish trade is developing as a global business, the risk of various diseases outbreak among provinces and even countries always threat. Many pathogens are imported into provinces and countries which may ultimately have a destructive effect on endemic aquatic animals of that region (Jung-Schroers et al. 2016; Mohr et al. 2015). Therefore, early detection of diseases in them through active surveillance, is the key to prevention and control of such diseases. Among the infectious agents, bacterial and viral pathogens are the most prevalent ones which the role of viral agents is much more significant due to their incurability, transmissibility, sever pathogenicity, difficult diagnosis and also their prevalence leads to major economic losses. According to the instructions of the World Organization for Animal Health (Office International des Epizooties), 9 notifiable infectious diseases have been determined which Spring Viremia of Carp (SVC) and Koi Herpes Virus disease (KHV) are among the viral diseases of warm-water fish species (Góchez et al. 2019).

Spring Viremia of Carp Virus (SVCV) is the cause of a fatal disease called spring viremia of carp, belongs to the genus *Vesiculovirus* of the *Rhabdoviridae* family; all members of the *Cyprinidae* family have been identified as susceptible to this epidemic agent (Ahne et al. 2002). The significant prevalence of SVC in Iran, Europe, United States and several Asian countries have been reported with obvious symptoms such as exophthalmia, in the form of petechiae on the skin, gills and bleeding in eyes and internal organs, abdominal distension, swollen spleen, degeneration of the gill lamellae, pericarditis, Enteritis and liver necrosis (Ashraf et al. 2016).

Another viral pathogen that causes pathogenicity in cyprinid fish is Koi Herpes Virus (KHV), which is a member of the *Alloherpesviridae* family (Haramoto et al. 2007) even though the partial sequencing analysis of its genome has shown that KHV is closely related to CyHV-1 and CyHV-2 (Waltzek et al. 2005). lethargy and food refusal three to nine days post infection (dpi), followed by an enormous increase in mucus production on skin and gill tissues, frequently enophthalmos later on focal and expanded necrosis of the gill tissues round to massively expanded skin necrosis, petechial to expanded bleeding of the skin and fins up to sandpaper skin due to the release of mucus has been reported as the most usual signs of this disease. the clinical symptoms can occur altogether or

individually, but can also be absent in the case of a preacute disease event (Bergmann et al. 2020).

Although the propagation and rearing of warm-water fish in Iran has outspreaded rapidly in recent years, the bio-security infrastructures and disease control have not been developed simultaneously. Many warm-water fish farmers reared goldfish in earthen ponds along with four species of Chinese carp. There have been several reports of mortalities in biggest goldfish rearing farms of Gilan Province; therefore, In this study, possible viral diseases were investigated, using the methods proposed by the World Organization of Animal Health (OIE 2017) like Cell culture, IFAT (Immunofluorescent Antibody Test),RT-PCR (Reverse Transcription Polymerase Chain Reaction) and PCR .

Materials and methods

Sampling and clinical diagnostics

Sampling was done during one year and each season separately from four main Goldfish rearing farms in Gilan province (Table 1), with an extensive loss in previous years. Fish with clinical symptoms such as lethargy, imbalance, abdominal distension, gill necrosis and bleeding, darkening of color and skin wounds were caught with fishing nets and transferred alive to Inland Waters Aquaculture Research Centre, Iranian Fisheries Science Research Institute (IFSRI), Bandar-e Anzali, Iran. After observation of the clinical signs, biometry was performed. Then Sampling of target organs including kidney, spleen and gills for virological examinations in a sterile condition was done. Tissue samples were kept in a -80°C freezer due to performing the cell culture and PCR.

Table 1: number of examined samples from each rearing farm.

Rearing farm	Number of examined samples			
	Spring	summer	Autumn	Winter
A	50	30	30	30
B	60	30	30	30
C	60	30	30	30
D	50	30	30	30

Cell culture

Preparation of EMEM and production of cell monolayer

In order to prepare one liter of culture medium according to the manufacturer's instructions, 9.6 grams of MEM powder, 1.2 grams of bicarbonate, 10 ml of penicillin-streptomycin and 100 ml of Fetal Bovine Serum were dissolved in 875 ml of deionized water and after complete dissolution, sterilized using a 25 mm diameter sterile syringe filter with a 0.22 µm pore size mixed Cellulose Esters membrane under a laminal flow hood.

The $25cm^2$ flasks were used to prepare fresh monolayer cell cultures. After trypsinization, cell monolayers were transferred from old flasks to new flasks along with 15-20 mL of EMEM medium. Then the flasks incubated at 28°C for 24 hours.

In vitro propagation of SVCV as positive control

In this study, the standard strain of spring viremia of carp virus, was used as the positive control of the experiments

with strain number 56/70 and gene registration number z37505/1(S30) (Stone et al. 2003), from the European Union Reference Laboratory for fish and crustaceans, Denmark.

In order to virus propagation, after thawing the lyophilized vial of SVCV sample using PBS, the prepared viral solution was filtered using 0.45 µm pore size syringe filter and then 1.5-2 ml of viral solution added to EPC containing flasks. All flasks were incubated at 15°C and examined during seven days to daily monitoring of cytopathic effect (CPE) of cells.

Virus Isolation

Sample inoculation on EPC and BF2 cell lines

The target tissues including kidney, spleen and gill were completely homogenized in EMEM medium containing 100 mg/mL streptomycin and 100 IU/mL penicillin using a sterile porcelain mortar.

The homogenized samples were transferred to conical tubes and centrifuged for 10 minutes at 4°C, 2000×g. The supernatant filtered using a 0.45 µm pore size syringe filter (CHMLab).

150 µl of filtered supernatant were inoculated into 24 well plates containing EPC and/or BF2 cells using a disposable filter pipette in three various dilutions triplicate and then incubated at a 15ºC. The inoculated cells were monitored for 7 days using an inverted microscope (Nikon-TS100) to observe the cytopathic effect (CPE). According to the OIE instructions, in case of no cell damage, secondary culture or blind passage was performed twice.

Median Tissue Culture Infectious Dose ($TCID_{50}$) assay

The virus stock was titrated in 96-well plates containing EMEM medium to verify the viral titer of tested viruses. 8 various dilutions of 10^0 to 10^{-7}, of the testing viral fluid were added to the wells based on Reed and Müench (Reed and Muench 1938) recommended method. After 7 days of incubation, virus titration calculated as $TCID_{50}$ /mL.

Indirect immunofluorescent antibody test (IFAT)

To confirm the viral particles existence, indirect immunofluorescent antibody test was applied, according to the procedure described by Qin et al. (2006). The KHV monoclonal antibody (Aquatic Diagnostics, United Kingdom) or SVC polyclonal antibody were used as the first layer antibody which test by a second layer anti mouse or anti rabbit antibody conjugated to fluorescein isothiocyanate and then, samples were observed under a fluorescence Inverted microscope (Nikon-TS100).

Polymerase Chain Reaction (PCR)

This experiment was performed in two methods; PCR and RT-PCR, for the amplification of the DNA of KHV and RNA of *Rhabdovirus carpio* using specific primer pairs (Table 2) according to the instruction of Qiagen'sOnestep RT-PCR kit and Recommended methods (Koutna *et al*., 2003; OIE, 2019).

Table 2: the sequence of primers used in this study.

Primer	Sequence	Reference
SVC $_{Forward}$	GCCTAAATGTGTTGATGGAACG	(Koutna et al. 2003)
SVC $_{Reverse}$	GGATAATATCGGCTTGGAAAGC	
KHV $_{Forward}$	GACACCACATCTGCAAGG	(OIE 2019)
KHV $_{Reverse}$	GACACATGTTACAATGGTCGC	

RNA extraction

For RNA extraction from liver, kidney, spleen, gill and intestine tissues, Qiagen's RNeasy kit was used according to the manufacturer's instructions.

DNA extraction

The required DNA was extracted using Qiagen commercial kit according to the manufacturer's instructions (DNeasy Blood & Tissue Kit (50), Qiagen).

Agarose gel electrophoresis from PCR product

The electrophoresis steps were performed in SCIE-PLAS model 13-HV. The final PCR product was electrophoresed on a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide for 60 minutes under a voltage of 120 V. TAE running buffer was added to the agarose-containing flask. Then 5 µL of RT-PCR product was mixed with 3 µL of loading buffer then was loaded into the gel. Considering 100 pairs markers (manufactured by Vivantis) were poured into the first well without adding a loading buffer. After Replacing the lid to the gel box, 120 voltage was applied to the gel running for 60 minutes. After the end of the electrophoresis, the gel was transferred to the UVIpro Gold gel detector and the formation of Expected bands at the 470 bp for SVCV and 292 bp for KHV was investigated by UV radiation in comparison with the positive and negative controls.

Results

Clinical observations

Sampling of 490 goldfish, preferably with clinical symptoms in a weight range from 2 to 8 g, was done. All farms were using splash for aeration. Clinical symptoms were varied from dark color, hyperemia of the gills, corrosion and necrosis of the gills and corrosion of the caudal fin to excessive emaciation. In the farms A, B and D, which had a long history of breeding goldfish, many deposits on the bottom of the pools and black sludge were clearly observable and the turbidity of the water was highly visible. Extreme foaminess on the water surface and gas leakage was observed in most pools of farm A. The growth of plants on the edge of the ponds in farm D and algal blooms were visible in all 4 farms in hot seasons. The presence of populations of fish-eating birds was significant, especially in farm A, which had several hectares of ponds and there was a history of night casualties in all farms (Fig. 1).

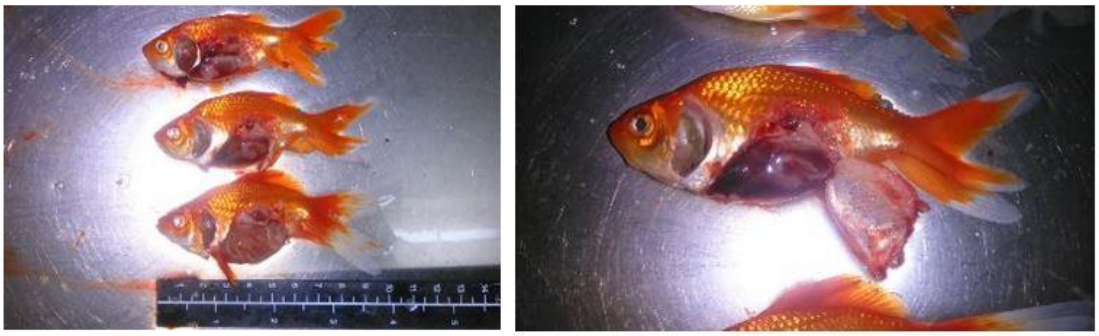
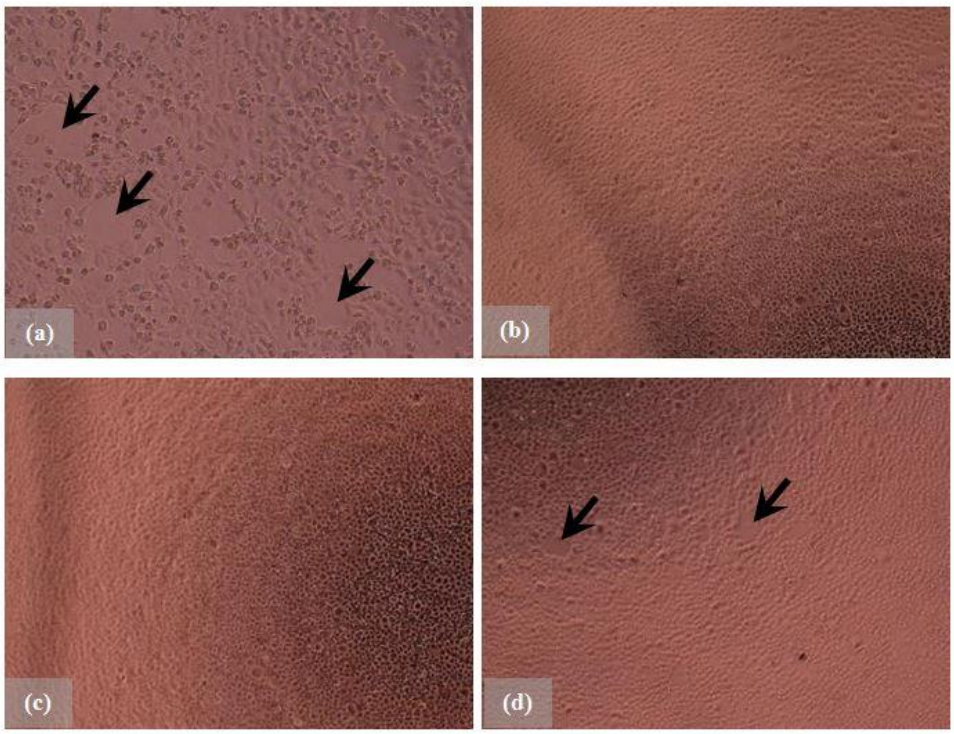


Figure 1: Goldfish (*Carassius auratus*), intestinal bleeding and gill corrosion.

Cell culture

In cell culture, slight cytopathic effects (CPE) like changes were observed in samples of farm A; however, no signs of cell damage were observed from samples of other farms following the inoculation of fish tissue homogenates on the EPC cell monolayer (Fig. 2).



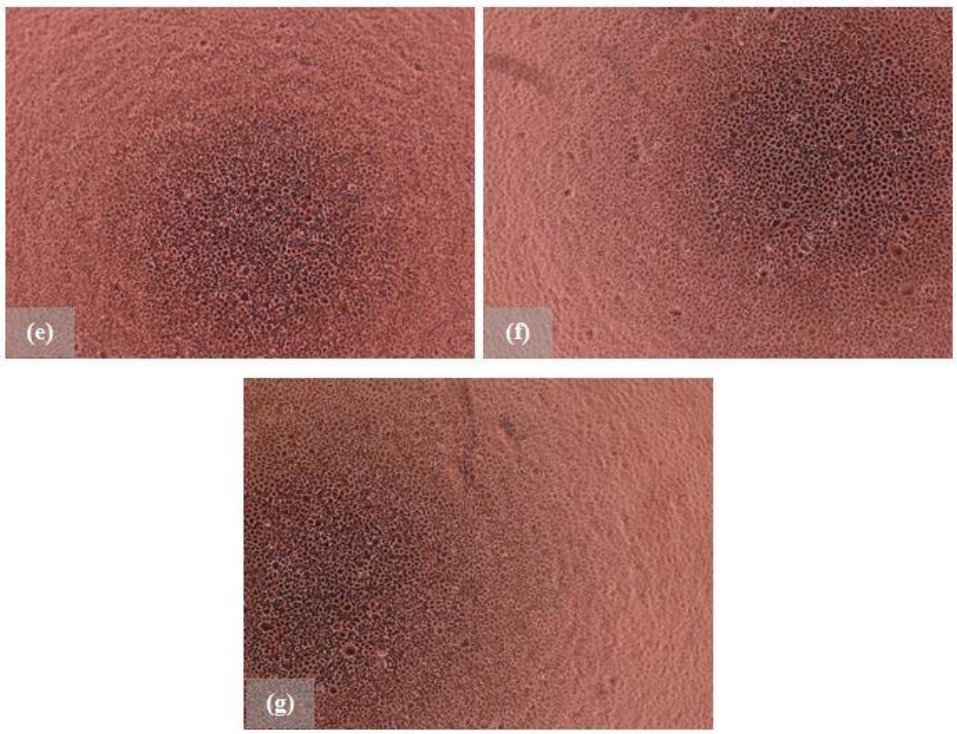


Figure 2: Positive control of SVCV on EPC monolayer, the signs of CPE showed with arrows (a) , 40X; The EPC cell line monolayer, negative control, 40X (b);The EPC monolayer inoculated with tissue homogenates from samples of farm A, no signs of CPE (c) , 40X; suspected signs of CPE from a sample of farm A (d) , 40X; The EPC monolayer inoculated with tissue homogenates from samples of farm B, no signs of CPE (e) , 40X; The EPC monolayer inoculated with tissue homogenates from samples of farm C, no signs of CPE (f) , 40X; The EPC monolayer inoculated with tissue homogenates from samples of farm D, no signs of CPE (g) , 40X.

Indirect Immunofluorescent Antibody Test (IFAT)

In the indirect immunofluorescent antibody test using a *Rhabdovirus carpio* polyclonal antibody, which was performed 72 hours following inoculation of tissue homogenates of goldfish samples on the EPC cell monolayer, no reaction was observed. While in the SVC positive control, traces of the antibody reaction with antigen were observed as bright phosphorus spots (Fig. 3). In order to perform IFAT for KHV disease, because the lack of a suitable cell line for this disease, the imprinting method of the target tissues and anti-KHV monoclonal antibody were used on a glass slide. The antibody conjugated with fluorescein isothiocyanate was used to identification that no traces of antibody reactions with antigen were observed in the form of bright phosphorus spots in the samples of 4 target farms (Fig. 4).

RT-PCR and PCR

In the PCR test using SVCV specific primer pairs that amplifies the 470 bp PCRproduct, except the positive control, no bands of the mentioned size were observed in any samples on the agarose gel. Also, by using the KHV specific primer to amplify a product with the size

of 292 bp, all tested samples were found negative (Figs. 5 and 6).

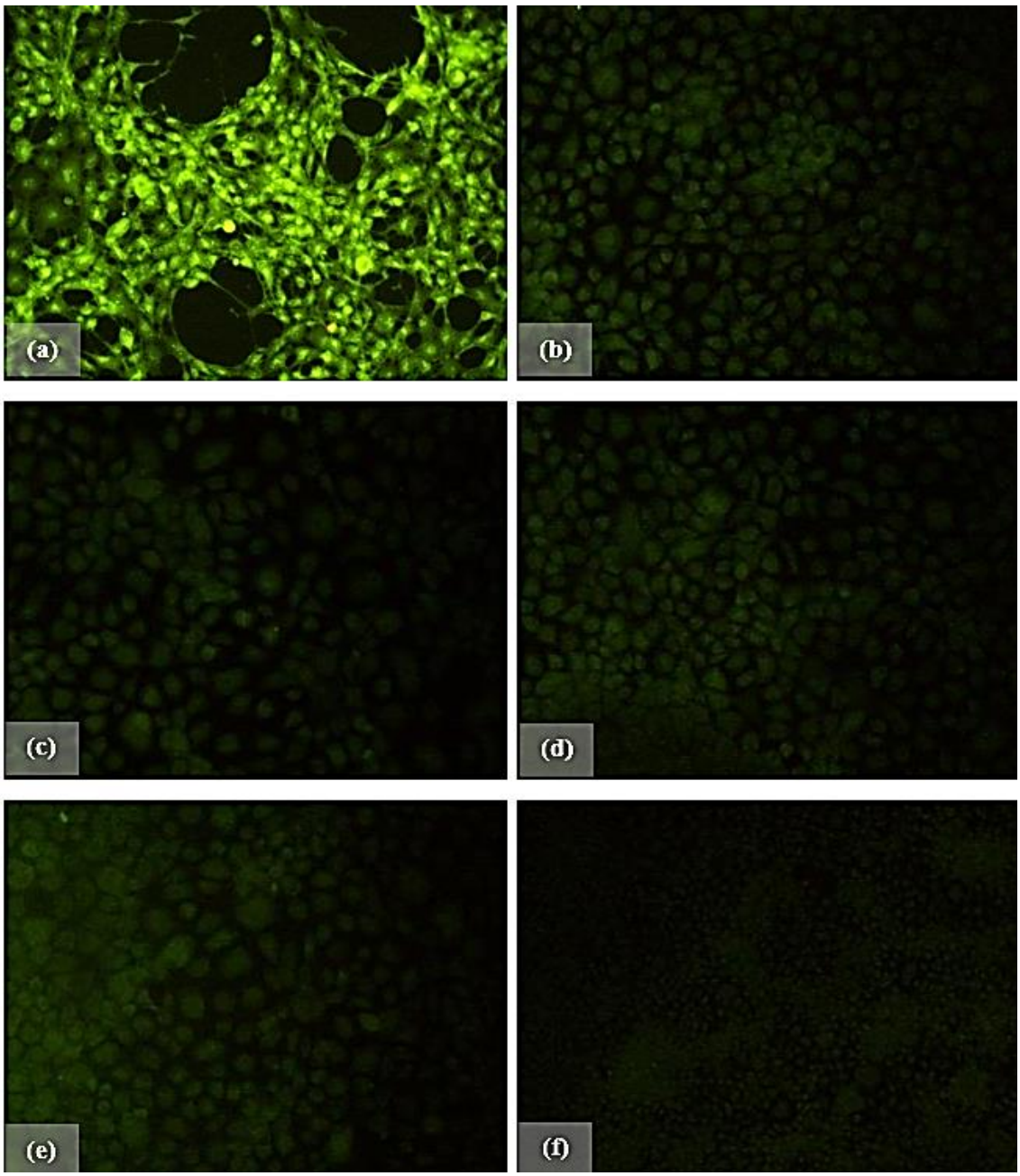


Figure 3: Results of IFAT: SVC positive control on EPC cell line monolayer, 200X (a); SVC negative control using SVC polyclonal antibody 200X (b); Results of IFAT from Inoculated tissue homogenates of samples of farm A on EPC monolayer using anti-SVC, 72hrs post inoculation, no signs of phosphorus spots 200X (c); Results of IFAT from Inoculated tissue homogenates of samples of farm B on EPC monolayer using anti-SVC, 72hrs post inoculation, no signs of phosphorus spots 200X (d); Results of IFAT from Inoculated tissue homogenates of samples of farm C on EPC monolayer using anti-SVC, 72hrs post inoculation, no signs of phosphorus spots 200X (e); Results of IFAT from Inoculated tissue homogenates of samples of farm D on EPC monolayer using anti-SVC, 72hrs post inoculation, no signs of phosphorus spots 200X (F).

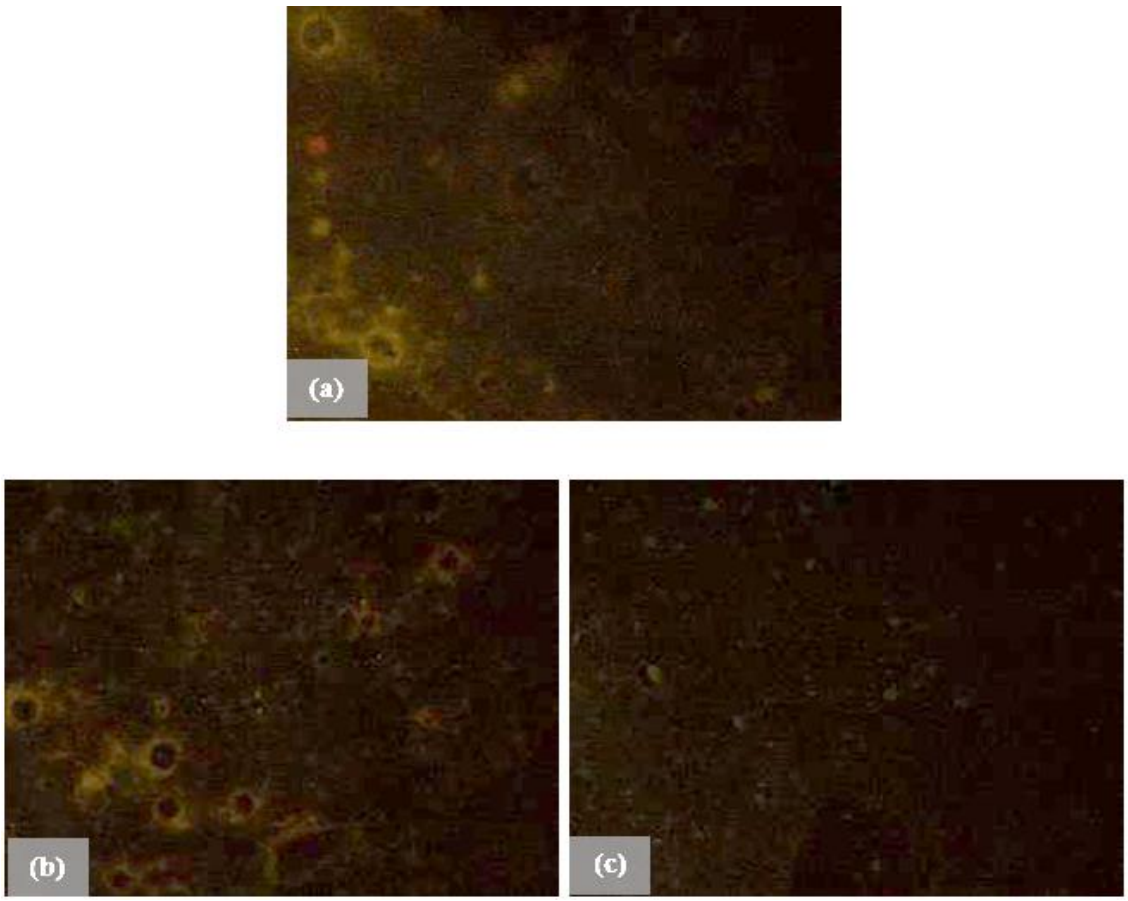


Figure 4: IFAT results: Negative control of KHVD through imprinting method using anti-KHV, no signs of phosphorus spots 200X (a); IFAT of KHVD through imprinting method of two samples using anti-KHV, no signs of phosphorus spots 200X (b, c).

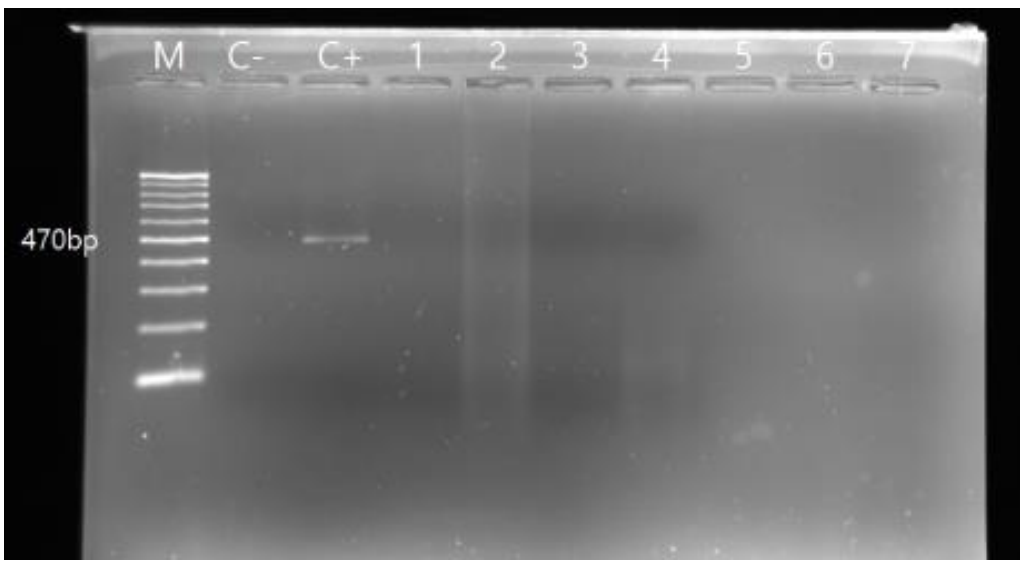


Figure 5: 1.5% agarose gel electrophoresis related to RT-PCR product using SVC specific primer; M=marker (100 bp by Vivantis); C- =negative control; C+ =positive control; Columns 1 to 7=goldfish samples.

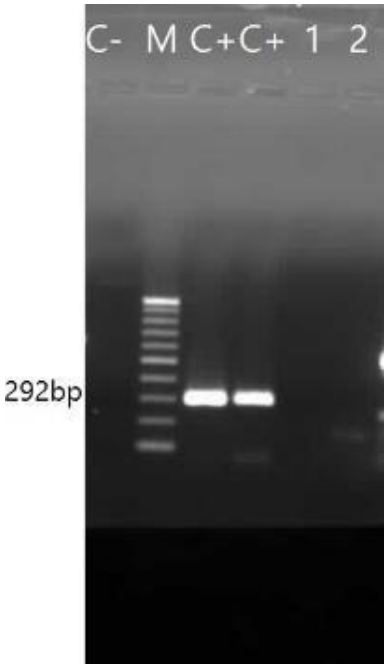


Figure 6: 1.5% agarose gel electrophoresis of the PCR product using KHV specific primer; C- =negative control; C+ =KHV positive control (292 bp), M=marker (100 bp by Vivantis); Columns 1 and 2=goldfish samples.

Discussion

The present study was carried out following the occurrence of major mortalities in goldfish earthen rearing ponds in Gilan province. Spring viremia of carp (SVC) and koi herpes virus (KHV) are contagious notifiable diseases (OIE, 2019) which cause pathogenicity in *Cyprinidae* family. Therefore, these two diseases were investigated using cell culture, PCR, RT-PCR and IFAT methods.

Since Gilan province is pioneer in the production of goldfish in the country (Iranian.Fisheries.Organization 2017) and some large scal farms are engaged in propagation and rearing of goldfish exclusive scale, even a small percentage of losses suspected to viral diseases can be worrying because if viral infections exist, quickly spread can occur which further lead to huge losses. On the other hand, since the SVC virus horizontally transfer to gills, simultaneous rearing along with Chinese carp in many farms of the province, provides the possibility of transmission of viral agent among species (Ellis 1988). Major losses of goldfish were reported at the end of the summer season and during the fall season. Occasional losses were also occurred when the weather got warm in the late spring and during the summer.

According to the recent studies, the *Carassius auratus* known as a susceptible host to KHV but not for the disease resulted from this virus. It means that, considering the ability of virus to propagate in goldfish tissues and detectability by IFAT or in-situ hybridization (ISH), the species showed resistance against mortality, even though the species preserve the virus transmissibility (Bergmann et al. 2010). However, it is clearly proven that *Carassius auratus* is a susceptible host to SVC (Dixon and Stone 2017).

In this pathogen-monitoring, clinical symptoms including corrosion of fins and gills, hyperemia of fins and gills, mild exophthalmia, lethargy, rarely subcutaneous bleeding and accumulation on the surface of the water and around the aerator were seen which they could observe in two mentioned viral diseases as well as improper farm water management conditions and some bacterial diseases.

Based on the instructions and recommendations of the OIE, various methods could be used for identifying

viruses including cell culture, RT-PCR, IFAT, NT and ELISA. In order to isolate SVCV, several cell lines have been proposed, but EPC and FHM cell lines have been suggested as the most appropriate and the most effective cell lines for SVCV isolation by the World Organization for Animal Health (OIE, 2019). The efficiency of these cell lines in propagation and isolation of SVCV has been previously reported (Björklund, Emmenegger, and Kurath 1995). Therefore, in this research, after the inoculation of the tissue homogenate on the EPC cell line, the results were also confirmed by RT-PCR and IFAT methods. To reach this, 4 days of post inoculation, these cells were examined using IFAT. This method is based on the use of specific antibodies against an antigen or several similar antigens. This method also previously hired by an antibody against SVCV glycoprotein (G protein) (Way 1991; Rodak et al. 1993). In addition cases of cross-reactivity between anti-SVCV with other rhabdoviruses including PFRV have been reported (Ahne *et al*., 2002).

On the other hand, environmental stress factors such as high temperature of water, low oxygen and parasitic pollution can cause the spread of bacterial diseases such as hemorrhagic septicemia caused by *Aeromonas* spp. and followed mortalities which reported as common disease in Chinese Carp rearing farms (Sun et al. 2016; Nielsen et al. 2001).

Algal blooms are another factor of the water quality reduction. It is also a critical parameter for weakness of the immune system which could resulted for the invasion of opportunistic agents such bacteria into the internal organs of fishes alike *Aeromonas hydrophila*, a pathogenic agent which more distributed in waters with high organic matter load (Elgendy et al. 2017; Oliveira et al. 2011). In septicemias caused by both motile and non-motile *Aeromonas* spp., similar clinical symptoms occur, which are similar to other bacterial and viral infections (Newaj-Fyzul et al. 2007). Another suspected pathogenic cause could be from *Pseudomonadaceae* family that widely distributed in soil and water environments and may be opportunistic pathogens for humans, animals and plants (Sujatha et al. 2011).

Based on the consequences of present study it seems that non-viral factors are the primary suspect of periodical and nocturnal losses of goldfish in target farms that the desired viral factors were not confirmed in any of the examined specimens.

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