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Original

Replication and survival traits of spring viremia of carp virus (SVCV) isolated in Mexico

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ABSTRACT

Objective. To perform the isolation of spring viremia of carp virus (SVCV) in common carp (*Cyprinus carpio*) and evaluate its growth in different cell types and viral survival at different temperatures. **Materials and methods.** Ten carps of between 400-500 grams of a lagoon in central Mexico were processed for diagnosis of SVCV by isolation in cell culture and by RT-PCR. The virus obtained was inoculated into EPC, BF-2, CHSE-214 and RTG-2 cells to determine differences in virus growth; the survival of virus stored at room temperature (TA 20-25°C), refrigeration (REF 4°C) and freezing (CONG -80°C) up to eleven months was also evaluated. Internal organ samples were processed for histological analysis. **Results.** The fish analyzed did not show external signs suggestive of disease but internally and histopathologically lesions suggestive of systemic infection were observed. SVCV was isolated in EPC and BF-2 cells and confirmed by semi-nested RT-PCR. SVCV only induced CPE in EPC and BF-2 cells and was negative in RTG-2 and CHSE-214. The virus conserved at TA lost viability after four months post-infection (mpi), being total at six mpi; while REF and CONG were stable during the eleven months. **Conclusions.** Subclinical SVCV infection was confirmed in carp that presented histological lesions associated with this infection; SVCV only caused CPE in EPC and BF-2 cells; and the virus kept in refrigeration and at -80°C retained its viability up to eleven months; while TA was lost in six months.

Keywords: *Cyprinus carpio*; fish; disease; RT-PCR; infection; cells (*Source: AGROVOC*).

RESUMEN

Objetivo. Realizar el aislamiento del virus de la viremia primaveral de la carpa (SVCV) en ejemplares de carpa común (*Cyprinus carpio*), evaluar su crecimiento en diferentes tipos de células, así como la supervivencia viral a diferentes temperaturas. **Materiales y métodos**. Diez carpas de entre 400-500 gramos de una laguna del centro de México fueron procesadas para el diagnóstico de SVCV mediante aislamiento en cultivo de células y RT-PCR semianidado. El virus obtenido se inoculó en células EPC, BF-2, CHSE-214 y RTG-2 para determinar diferencias de crecimiento de SVCV. Además, se evaluó la supervivencia del virus conservado a temperatura ambiente (TA 20-25°C), refrigeración

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(REF 4°C) y congelación (CONG -80°C) hasta once meses. Los órganos internos se procesaron para análisis histológico. **Resultados.** Los peces analizados no presentaron signos externos sugestivos de enfermedad, pero interna e histopatológicamente se observaron lesiones sugestivas de infección sistémica. SVCV fue aislado en células EPC y BF-2 y confirmado por RT-PCR semianidado. SVCV únicamente indujo CPE en células EPC y BF-2 y fue negativo en RTG-2 y CHSE-214. El virus conservado a TA perdió viabilidad después de cuatro meses post infección (mpi), siendo total a seis mpi; mientras REF y CONG fueron estables durante los once meses de estudio. **Conclusiones.** La infección subclínica por SVCV fue confirmada en carpas que presentaron lesiones histológicas asociadas a esta infección. SVCV únicamente causó CPE en células EPC y BF-2 y el virus conservó su viabilidad a 4ºC y -80°C hasta once meses; mientras que a TA se perdió en seis meses.

Palabras clave: *Cyprinus carpio*; peces; enfermedad; RT-PCR; infección; células (*Fuente: AGROVOC*).

INTRODUCTION

Spring viremia of carp (SVC) is a systemic infectious disease that primarily affects cyprinids and is listed as a required notifiable disease by the Office International des Epizooties (OIE) of the World Organization for Animal Health. This disease is considered one of the primary economic and health risks for carp (*Cyprinus* spp.) maintained under intensive farming conditions. In wild fish, SVC generally presents sub-clinically, meaning that infection can go unnoticed (1,2).

SVC principally occurs in young fish (i.e. <1 year) and can reach mortality rates of 90%. Mortality and clinical signs can be influenced by a number of factors, notable among which are the age of the fish, the presence of secondary infections, and the water temperature (2). The most common clinical signs occur at temperatures between 10 and 17°C. Accumulated mortality can likewise occur in this temperature range, with the fastest mortalities occurring at 17°C (1,2).

Septicemic infection in fish evidences nonspecific clinical symptoms, such as pale gills; exophthalmia; hemorrhaging and petechiae of the skin, gills, and eyes; and abdominal distention. Necropsy can further reveal serous fluid or fluid mixed with blood or necrotic material; focal hemorrhages or petechiae in the muscle and fat tissue, swim bladder, and other abdominal organs; and a usually swollen, thickly textured spleen (1). Chronic cases of infection present pleural adherences between internal organs. Infected animals that do not die or those that are infected without clinical disease act as carriers (2,3).

SVC was first reported in Yugoslavia in 1970 (1) and has since been reported in various countries across Europe (1,2), the Middle East (4), and China (2). In America, SVC has been reported in the USA (2,3), as well as Canada and Brazil (2). This disease has not previously been officially recognized in Mexico (5,6).

SVC virus (SVCV) has been replicated in primary cultures of the gonads and swim bladder of carp, in various fish cell lines, and, even, in cell lines of other vertebrates (1,2). However, epithelioma papulosum cyprini (EPC) and fathead minnow (FHM) cells are the primary candidates (1,2). Furthermore, SVCV presents differences in growth and survival under different temperature conditions. According to the OIE Manual of Diagnostic Tests for Aquatic Animals (2), SVC should be diagnosed through more than one technique. In clinically affected fish, these techniques include viral isolation or direct detection in tissues by the indirect fluorescent antibody technique (IFAT), the enzyme-linked immunosorbent assay, or polymerase chain reaction (PCR). Furthermore, direct identification should be confirmed through isolation plus neutralization or through RT-PCR together with sequencing of the obtained product.

Regarding fishing output in Mexico, carps (*Cyprinus* spp.) rank 8th in terms of volume (i.e. 53,421 tons) and 14th in terms of economic value (7). Since being introduced to Mexico, carps have been distributed in bodies of water across nearly the entire country, with the primary purpose being extensive production (7,8,9). Regarding sanitary concerns, reports of clinical disease are rare, and no studies exist determining the health status in Mexican carps based on a comprehensive health diagnosis (5).

While specimens did not present signs of disease, SVCV was initially reported by our group in common carp (*Cyprinus carpio carpio*) from a

lagoon in central Mexico at the end of 2015 (5). Despite the results of isolation (5), the respective health authorities ruled out the presence of a virus through molecular analysis based only on RT-PCR. As such, SVCV is still considered exotic for Mexico (6). For the present study, new carp specimens were collected, and cell culturing and semi-nested PCR were used to detect the presence of SVCV in Mexican carp. Additionally, described are the growth traits of the virus in different cell lines and viral survival at different temperatures.

MATERIALS AND METHODS

Sample collection. In October 2015, the aquatic animal health authorities used molecular diagnosis to rule out the presence of SVCV in the Tecocomulco lagoon where the virus has been previously identified (5). Ninety days later, an additional 10 common carp (400-500 g) were captured by local fishermen from the Tecocomulco Lagoon. The fish were originally captured for consumption purposes for visitors to the lagoon. Ten specimens were taken for a comprehensive health diagnosis.

Cell-culture isolation. Kidney samples (≈0.5 cm^3) were collected from each fish. Pooled samples from two specimens were submerged in a Falcon tube (15 mL) containing the Leibovitz's L-15 Medium (Cat. No. 11415-064, Gibco-BRL) supplemented with fetal bovine serum and stored at 4°C. Within 24 h, the contents of the tube were macerated, centrifuged, and filtered with membranes (0.22 µm). Monolayer EPC cells at ≥90% confluence were inoculated with a 1:10 or 1:100 dilution of the filtered contents. As a positive control, two wells were inoculated with 100 µL of SVCV. The negative control consisted of two wells inoculated with 100 µL of the L-15 medium supplemented with 2% fetal bovine serum. The plates were incubated at 20 or 25°C for up to seven days. Manifestation of the cytopathic effect (CPE) was recorded microscopically daily.

Replication of SVCV in different cell lines. The supernatant of the primary isolation was collected and diluted to 1:10 and 1:100. From these dilutions, 100 µL was inoculated in monolayers of the following cell lines: EPC; Bluegill fry (*Lepomis macrochirus*) (BF-2); Chinook salmon (*Oncorhynchus tshawytscha*) embryo (CHSE-214); and rainbow trout (*Oncorhynchus mykiss*) gonad (RTG-2). The samples were incubated at 20°C for 7 days to determine which cells evidenced viral multiplication.

Survival of SVCV under maintenance at three temperatures. The supernatant from primary isolate cultures was divided into three aliquots. One was kept at room temperature (i.e. 20-25°C), another under refrigeration (i.e. 4°C), and another under freezing conditions (i.e. -80°C). Survival of the conserved virus over an 11-month period (i.e. March 206 to January 2017) at different temperatures was assessed by determining the maximum dilution at which the virus was able to induce the CPE. The viral titer was calculated using serial dilutions (10^{-1} to 10^{-8}) in EPC cells at 90% confluence on a 96-well plate. The plate was sealed and incubated at 25°C, and the presence or absence of the CPE was recorded up to 7 days post-inoculation to determine viral titer.

Histopathology. Samples of the spleen, liver, kidney, and intestine were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin blocks using standard procedures. Each tissue was sectioned to 5 µm and dyed with Hematoxylin-Eosin to describe any histopathological changes observed under different magnifications with a light microscope (Olympus BH-2).

RNA extraction and RT-PCR to confirm SVCV. The presence of SVCV in samples evidencing the CPE was confirmed through RT-PCR for four of the five supernatants. RNA was extracted using 200 µL of supernatant from cultures evidencing the CPE, as per instructions for the FavorPrep™ Viral Nucleic Acid Extraction Kit (Taiwan). The cDNA was synthesized through reverse transcription using the PrimeScript™ RT Reagent Kit (Perfect Real Time, Takara, Japan), adding 1 ng of total RNA for each reaction in a total volume of 20 µL.

A semi-nested PCR analysis was conducted (2). The first step used the oligos VVPC F1 5'-TCTTGGAGCCAAATAGCTCARRTC-3' and VVPC R2 5'-AGATGGTATGGACCCCAATACATHACNCAY-3', which amplified 714 base pairs (bp) in a 25 µL reaction volume containing the GoTaq® Green Master Mix (Promega, USA) and 200 ng of cDNA. The obtained PCR product was used as a template for a second reaction, which amplified a 606 bp product using the oligos VVPC F1 5'-TCTTGGAGCCAAATAGCTCARRTC-3' and SVCR4: 5'-CTGGGGTTTCCNCCTCAAAGYTGY-3' in a 25 µL reaction volume with concentrations similar to the first PCR. Amplifications were

revealed through electrophoresis on 1.5% agarose gel.

To detect if carp were carriers for the infectious pancreatic necrosis virus (IPNV), cells evidencing the CPE were subjected to IFAT and RT-PCR according to OIE Manual protocols (2).

RESULTS

Clinical analysis and macroscopic examination. The reviewed specimens did not present signs of external injuries associated with any disease (Figure 1A). Furthermore, the local fishermen did not report any instances of sickness or mortalities among the fish in the lagoon. During necropsy, however, moderate congestion and adhesions between internal organs and with the abdominal wall were observed (Figure 1B).

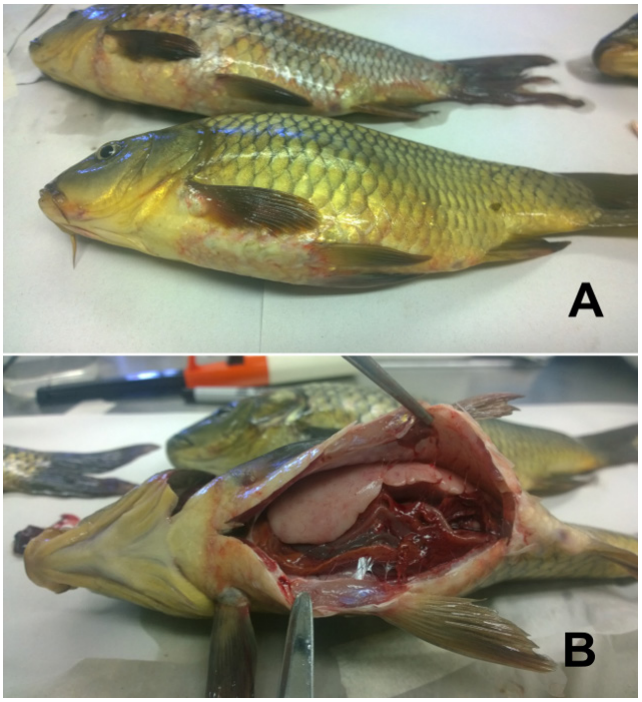

Figure 1. Common carp naturally infected with SVCV. (1A) External body surface without apparent injuries. (1B) Necropsy revealed adherences between internal organs and between organs and the abdominal wall.

Isolation of SVCV. Four of the five inoculations in EPC cells evidenced the CPE, at dilutions of 1:10 and 1:100. In samples incubated at 25°C, the CPE was evident at both dilutions from 48 h post-inoculation (hpi) (Figure 2B). Samples incubated at 20°C presented the CPE at 72 hpi (Figure 2C). In both cases, the CPE was characterized by the formation of areas with the rounding and detachment of cells, rupturing of the monolayer, and cellular lysis (Figure 2D).

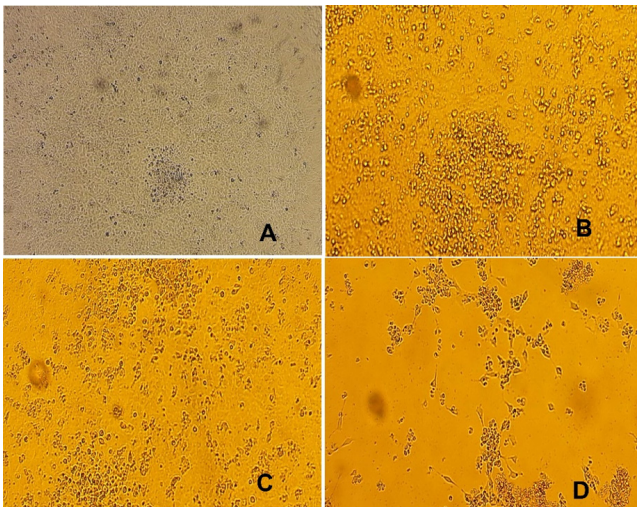

Figure 2. EPC cells infected with SVCV in a 1:10 dilution and incubated at 25°C. (A) Cells without infection. (B) Cells incubated at 25°C showing the CPE at 48 hpi. (C) Cells incubated at 20°C showing the CPE at 72 hpi. (D) Final destruction of the cell monolayer.

Multiplication of SVCV in EPC, RTG-2, BF-2, and CHSE-214 cells. Following the infection of different cell lines, SVCV only induced the CPE in EPC and BF-2 cells. The CPE occurred 48 hpi in EPC cells, independent of dilution. By contrast, the CPE manifested at 72 hpi in BF-2 cells only when using a 1:10 concentration. The CPE was not recorded in the RTG-2 or CHSE-214 lines (Figure 3).

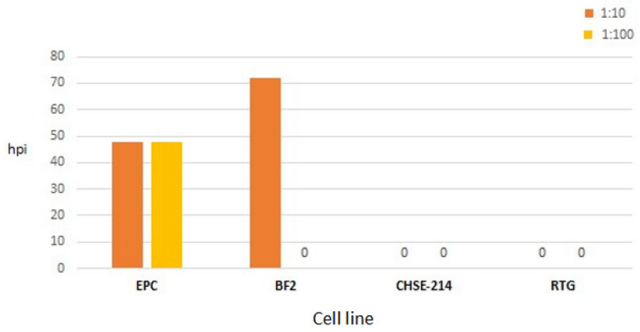

Figure 3. Manifestation of the CPE in EPC, BF-2, CHSE-214, and RT-2 cells infected with SVCV at 1:10 and 1:100 dilutions and incubated at 20°C.

Survival of SVCV under maintenance at three temperatures. Figure 4 evidences the survival of SVCV when maintained at room (20-25°C), refrigeration (4°C), and freezing (-80°C) temperatures. At room temperature, virus survival was generally lower. The CPE was observed during March and April up to a dilution of -6. For May and early June, up to a -7 dilution resulted in the CPE, but for late June and early July, this decreased to -1. No infection by SVCV at room temperature was recorded from late July. In turn, refrigerated SVCV demonstrated the CPE with a -7 dilution in March and April, but from the third reading, this increased to a -8 dilution until the end of the study. Finally, the frozen aliquot was the most stable. From the first until the last inoculation point (i.e. 12 total), SVCV maintained at -80°C caused the CPE up to a -8 dilution.

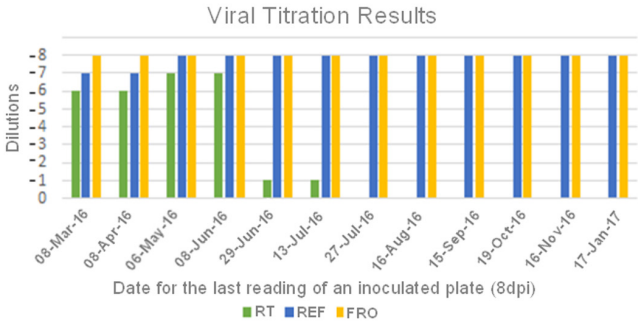

Figure 4. Viral titers obtained from three SVCV aliquots conserved for 11 months at room temperature (RT), refrigeration (REF), or frozen (FRO). Analyses were conducted using EPC cells for inoculation and using microtiter plates (11).

Histopathology. The principal histological injuries were detected in the kidney, which presented sinusoidal congestion and interstitial hemorrhages; the proliferation of macrophages; tubular degeneration and necrosis with vacuolization; peritoneal hemorrhaging; and steatites (Figure 5A). The liver presented degeneration of the vascular walls and multifocal hepatic necrosis (Figure 5B); moderate perivascular hemorrhages; pancreatitis and peri-pancreatic infiltrations (Figure 5C); and multifocal mononuclear hepatitis (Figure 5D). The intestines showed granulomatous ulcerative enteritis (Figure 5E), whereas the spleen evidenced splenic degeneration; congestion; hemorrhaging and splenic hemosiderosis; and lymphoid depletion (Figure 5F).

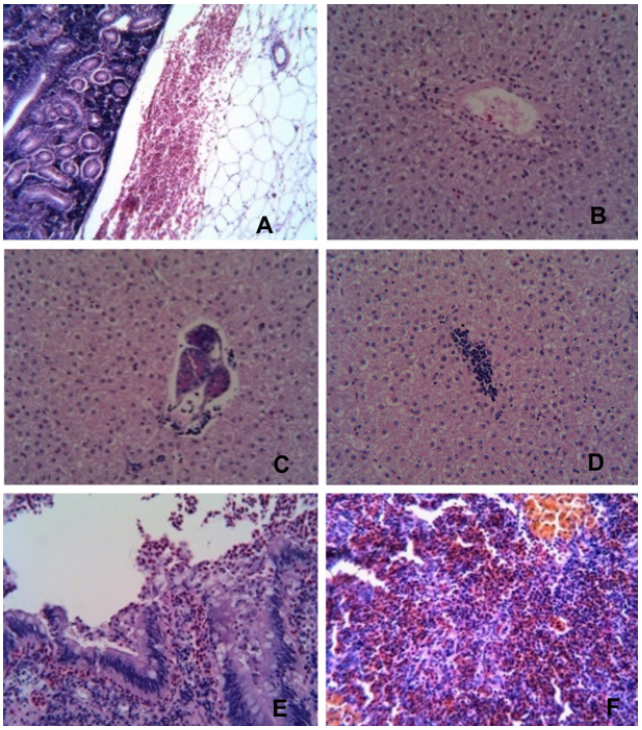

Figure 5. Histological injuries in common carp infected by SVCV. Kidney: (A) interstitial infiltration; peritoneal hemorrhaging and steatites; and melanomacrophage proliferation, 100x. Liver: (B) degeneration of vascular walls, multifocal degeneration and necrosis, 250x; (C) mononuclear peri-pancreatitis and necrotic pancreatitis; and (D) multifocal lymphocytic infiltration. Intestine: (E) necrotic cryptitis, ulcerative hemorrhagic enteritis, 250x. Spleen: (F) multifocal congestion and hemosiderosis, 250x.

Detection of SVCV through RT–PCR. Analysis through RT-PCR confirmed the presence of SVCV in the four analyzed samples. As expected, the first reaction resulted in a 716 bp product, whereas the second reaction amplified a specific product of 606 bp (Figure 6).

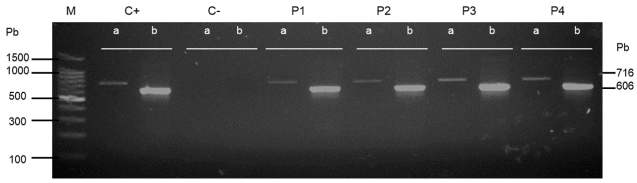

Figure 6. Detection of SVCV through a semi-nested RT-PCR using the OIE protocol. M, indicator of molecular weight; C+, positive control; C-, negative control; P1-P4, pooled samples obtained from analyzed fish.

Detection of the IPNV through IFAT and RT-PCR analyses provided negative results (data not shown).

DISCUSSION

Early SVC diagnosis is one of the main measures for detecting and removing infected fish to prevent further viral exposure (1,2). In line with this, the general recommendation for the diagnosis of infectious diseases is to obtain samples from clinically symptomatic or moribund individuals. This is due to the difficulty of isolation from sub-clinical individuals, even from animals that had survived previous outbreaks (1,3).

The ten fish assessed in the present study did not show signs of disease, despite presenting internal injuries suggestive of systemic infection. While the observed histopathological injuries were non-specific, they were consistent with lesions described in cases of SVC. Such injuries include, among others, vascular damage in internal organs that translates into hemorrhages and the formation of adherences, as described herein (2,3,10). These observations are reinforced by the lack of another known viral disease affecting common carp in Mexico (5,6,10). Other rhabdovirus, such as hemorrhagic septicemia and infectious hematopoietic necrosis, cause similar histological injuries, but these are exotic to Mexico and use the rainbow trout as a host (1,2,11).

Despite not showing outward signs of disease, this did not exclude the fish from the lagoon possibly being carriers for SVCV. To increase the chances of successful isolation, the collected samples were inoculated in four cell lines. The CPE was observed in EPC and BF-2 cells, which are permissive not only to SVCV, but also to IPNV, which is endemic to Mexico (6,11). The possibility of the CPE being due to fish acting as carries for IPNV was ruled out following negative RT-PCR and IFAT tests. On the other hand, the semi-nested RT-PCR for SVCV provided positive results.

SVCV can replicate in the cells of fish and other animals (2,12,13), but the reasons for this permissiveness and the mechanisms of virus-cell interaction are unknown (12,14). One proposal for birnavirus is that the virus uses cell receptors common to distinct species or multiple receptors that adhere to cells, but this does not guarantee penetration into non-permissive cells since a co-receptor that probably does not present would be required (16). In grass carp (*Ctenopharyngodon idella*) ovary cells, SVCV enters by clathrin-mediated endocytosis and macropinocytosis requiring the participation of dynamin II, actin microfilaments, microtubules, and endosomal acidification (13). However, the cellular elements that favor cell permissiveness towards endocytosis and replication remain to be determined (12,13,15,16).

The clinical manifestation of SVC is associated with various factors, important among which is temperature (1,2). In the assay to evaluate viability at different temperatures, maintenance at room temperature resulted in a drastic loss of viability over the course of three months and the disappearance of the virus by six months. In a prior study, SVCV remained viable for up to five weeks in river water at 10°C and for more than six weeks in pond scum at 4°C (2). This evidences that when the virus is in contact with organic material, it inactivates quicker. This might explain why the virus has not been detected sometime after being originally informed. The longer survival time at room temperature reported herein could be due to a probable protector effect of the fetal bovine serum incorporated into the culture medium (2).

Further related to temperature, when SVCV was kept refrigerated or at -80°C, the viral titer was practically sustained over the 11 months. Literature on this point describes storage of the virus by freezing at -30 or 74°C for more than six months without titer loss if the medium contains 2-5% fetal bovine serum (1,2). However, the present study did not evaluate the possible effect of fetal bovine serum.

Isolation can be difficult when the temperature is different to that associated with the clinical manifestation of disease (2). Ahne (17) reported 90% mortality and high viral titers in fish kept in water at 10-12 °C, whereas between 20-22°C, no mortalities were recorded, and repeated isolation of the virus was not possible. The loss of viral titer due to temperature increases could explain why health authorities did not detect the virus in samples collected during March, when the average temperature of the lagoon is 16.6°C. By contrast, the samples assessed in the present study were collected in October, when the average temperature of the lagoon is 13.5°C (18).

The type of sample and the diagnostic techniques used for the detection of infectious agents are determinants for obtaining reliable results (1,2,16,19). SVCV can be isolated with some ease from clinically affected fish, but the direct detection of antigens or nucleic acids decreases if using tissues from fish that are sub-clinical

carriers. Nevertheless, not finding the viral agent in such samples does not guarantee the absence of the virus in either the analyzed animals or in the population (1,2). In the present study, cell cultures permitted the isolation of SVCV and subsequent confirmation via RT-PCR, the diagnosis of which aligned with OIE recommendations. A positive PCR result requires isolation to be considered definitive, but a positive result does indicate the presence of SVCV in cell cultures (2).

In conclusion, this study determined that, among four cell lines, SVCV causes the CPE in EPC and BF-2 cells, with the effect presenting quicker in EPC. Likewise, the virus was found to conserve its viral titer up to 11 months if maintained at -80°C. While SVCV was clearly identified per the diagnostic techniques recommended by international health authorities (2), as well as through molecular characterization (5), the molecular or cellular mechanisms that influenced the results presented herein require further study.

Conflict of interest

The authors of this study declare no conflict of interest in relation to the publication of this manuscript.

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