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METHOD

Propagation of koi herpesvirus using embryonated chicken eggs: a potential substitute method for fish vaccine production?

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Koi herpesvirus disease (KHVD) is caused by a large DNA virus that commonly infects carp, *Cyprinus carpio* (koi and common carp). KHVD has spread rapidly across the globe and caused high mortality in all ages of common carp and koi. Until now, no effective treatment has been applied to prevent KHV infection impacting the mass production of koi and common carp . An environmentally friendly alternative strategy for controlling fish disease is vaccination. One of the challenges facing conventional viral vaccine production is the requirement for koi or common carp cell cultures, which must be frequently maintained with expensive materials required for virus propagation. This study aims to obtain KHV that has been propagated in embryonated chicken eggs (ECE) to formulate an affordable KHV vaccine for the aquaculture industry. This research consisted of three stages; the first stage was virus inoculation into various parts of eggs (allantoic fluid, chorioallantoic membrane, amniotic fluid, and egg yolk). The second stage was observing viral growth and collecting ECE fluid and membranes. The third stage involved quantitatively determining the viral genomic copy numbers using the quantitative Polymerase Chain Reaction (qPCR) assay. KHV propagation in various parts of ECE resulted in varying viral genomic copy numbers with a high DNA copy number reported in allantoic fluid on the third day after inoculation. Further work is required to monitor virus titer in later passages and optimize methodology for using ECE as the potential alternative to cultured cells as the medium for virus propagation. In the future the system could be developed to produce promising vaccine candidates with more affordable vaccine prices for the Indonesian fish farming industry.

Introduction

Koi Herpesvirus (KHV) infection in koi and common carp caused a loss of 14.200 Euro in the first two years of the outbreak (2002-2004) (Sunarto and Cameron 2005). In Indonesia, the spread of this disease has crossed almost all carp production areas. Indonesia routinely encounters outbreaks of KHV in common carp (*Cyprinus carpio*) every year. The symptoms of KHV as a primary infection are aggravated by secondary infections, for

example with *Aeromonas hydrophila*. For example: The phenomenon of mass deaths ofup to around 150 tons of common carp encountered in the Koto Panjang Hydropower Reservoir was confirmed to be due to KHV and *A. hydrophila* on February 8, 2023, as reported by the Kampar Fisheries Service (Indriani and Putri 2023). The available KHV vaccine is an imported vaccine which is very expensive for common carp fish farmers. Besides the application of the vaccination is difficult because the fish must be soaked in water containing ice cubes, and the seed virus strain from the imported KHV vaccine is not necessarily protective against the type of KHV virus strains in Indonesia. In addition to this, the imported vaccine only contains KHV, not *A. hydrophila* which always appears as a secondary infection. In some countries, according to Bergmann et al. (2020), autogenous inactivated vaccines are produced and applied to protect common carp against KHV. However, due to the constraints in Indonesia, the production of KHV vaccines are less economical, because the ingredients within the formulation are very expensive so the price of the vaccine cannot be afforded by the carp fish farmers. So the idea arose to grow the local KHV virus strains from fish in embryonated chicken eggs (ECE) so that it could be mass-produced making the price of the vaccine more affordable.

Intensive fish farming, koi fish exhibitions, and trading both domestically and internationally, without strict regulations and restrictions in examining and implementing quarantine programs, are the causes of the rapid spread of this disease globally (Shahin et al. 2020). Cases of this disease in carp and koi cause high mortality of up to 90-100%, and until now, it has not been controlled effectively, either through prevention or treatment. Various efforts to control fish diseases have long been carried out, such as administering medications, antibiotics, and the application of antiviral medications. However, using drugs for medications and antibiotics to treat fish and shrimp diseases raises the problem of bacterial resistance and antibiotic residues, which has led to the rejection of many fishery products from Indonesia by importing countries due to the presence of antibiotic residues.

Vaccination is one way to lessen the need for antibiotics (Bergmann et al. 2010; Boutier et al. 2015). The potency and efficacy of the inactivated vaccine against KHV combined with an adjuvant had a positive prospect for carp and koi (Solichah et al., 2016). Several studies are increasingly facilitating the rational design of attenuated vaccines against CyHV-3 by targeting various open reading frames (ORFs) through a cell culture approach (Embregts et al. 2019; Boutier et al. 2017). Since it is costly to generate a viral vaccine, applying vaccinations to koi and common carp would not be economically viable. A common problem with mass production of KHV using whole virus vaccines is the low titer of virus obtained from fish cell cultures which makes conventional inactivated vaccines expensive. Embryonated chicken eggs (ECE) have the option for vaccine materials in large quantities at a significantly lower cost. However, the question arises can fish viruses be

propagated in embryonated chicken eggs? ECE costs less than cell culture (Blyden and Watler 2010; Lin et al. 2015). The other reason is the submission of IPR with US Number 4783411A, which claims that the Influenza-A vaccine shows that the Influenza virus can be propagated in fish cell cultures. This raises hope for this study's observation that the KHV virus can propagate in embryonated chicken eggs. This study aims to obtain KHV propagation in ECE as an alternative to KHV vaccine preparations for preventing potential diseases in koi and common carp. This research aims to understand the proliferation of KHV and test the membrane and liquid of ECE that would have to be produced as a source of antigen with high titers for the KHV-*Aeromonas hydrophila* (KHV-Aero) vaccine candidate. It is hoped that the results of this study can be packaged into an effective and affordable vaccine formulation that can prevent potential KHV and Motile Aeromonas Septicemia (MAS) diseases in koi and common carp as an effort to develop better strategies to succeed in disease control programs in freshwater aquaculture.

Materials and Methods

Virus stock for inoculation

The virus had previously been propagated in a fish cell line, KF-1 cells (Lusiastuti et al. 2021). Leibovitz's medium (L-15), and 2% fetal bovine serum were used to culture the virus on KF-1 cells. The appearance of vacuoles in the cytoplasm of cultured cells indicated virus growth by developing a cytopathic effect (CPE), and virus harvesting was carried out on the fifth day post-inoculation of the virus. The collected infected cell culture supernatant was stored in a deep freezer at -80 °C until used in the study.

Virus Inoculation in Embryo Chicken Eggs (ECE)

Virus inoculation was carried out on ECE by injection through the allantoic fluid (AF), the chorioallantoic membrane (CAM), the amniotic cavity (AMN), and the yolk sac (Y). ECE was examined by candling, and egg shells were disinfected with 70% alcohol before being injected in a volume of 0.2 mL of inoculum/egg. Injection holes were sealed, and eggs were labelled (isolates, dates, and treatment methods) before being incubated at two incubation temperatures (30 and 37 º C), which contained five eggs for each group and observed daily for five days by candling. Egg mortality in less than 24 h was disregarded and considered as contaminated eggs. AF was collected on the first hour and the first, third, and fifth-day post-inoculation, then stored at -86 º C. On the fifth day, CAM, AMN, and Y from eggs were harvested and stored at -86 º C.

KHV detection on ECE by Conventional PCR and quantitative PCR (qPCR) assay

DNAzol reagents were used for extraction of DNA using the manufacturer's instructions. Samples (250μL) were mixed with 500μL of DNAzol into a microtube and centrifuged at 12,000 rpm for 10 min at room temperature. 400μL of supernatant was transferred onto a new tube and then filled with 500μL of 100% alcohol. The microtube was inverted 3-6 times and then incubated for 1-3 min at room temperature. Centrifugation was carried out at 8,000 rpm for 3 min at room temperature. The DNA pellet obtained was washed with 500μL of 95% alcohol, and then the microtube was inverted 3-6 times before being centrifuged at 8,000 rpm for 5 min at room temperature. This washing process was carried out twice. The DNA pellet was eluted with100-200μL ddH_2O. The amplification process was carried out using the primer Sph I-5 (Yuasa et al. 2005) to detect KHV with the following nucleotide sequences: Sph I-5 F: 5'-GAC ACC ACA TCT GCA AGG AG-3' and R: 5 '-GAC ACA TGT TAC AAT GGT CGC-3'. Amplification was carried out by the following reaction conditions: pre-denaturation at 94 º C for 30 sec, 40 cycles of denaturation at 94 º C for 30 sec, annealing at 63 º C for 30 sec, and extension at 72 º C for 30 sec. Final elongation was carried out at 72 º C for seven min. Reagent preparation and composition for each sample were carried out in a total reaction volume of 25 μL as follows: 12.5 μL of master mix (MyTaq HS Red Mix 2x, Bioline); 2 μL of each primer reverse & forward (20 pMol), 8.5 μL of RNAse/DNAse free water and 2 μL of template DNA (100 – 200 ng μL^{-1}). Optimization of the temperature setting and duration of DNA amplification includes pre-denaturation temperature, then DNA amplification was carried out in 40 cycles consisting of denaturation, annealing, elongation, and final elongation, and the samples were subjected to agarose gel electrophoresis. qPCR amplification was carried out using extracted DNA samples. The total qPCR reaction of 25μL consisted of the SensiFAST SYBR® Hi-ROX Kit (Bioline) master mix. Sample amplification was performed by including three to five serial dilutions of standard gblock (Genetica Science) and No Template Control (NTC) as negative control. The total reaction was 10μL consisting of 5 μL master mix, 0.4μL each primer (10 pmol), 2.2μL nuclease-free water and 2μL template DNA. The reaction cycles used were as follows: pre-incubation cycle at 95 º C for 2 min, 40 cycles (denaturation and annealing) at 95 º C for 10 sec, 60 º C for 10 sec, 72 º C for 20 sec, and finally, the melting curve stage at 95 º C for 10 sec, 60 ºC for 1 min and 95 º C for 15 sec.

Table 1. The presence of KHV in ECE after virus inoculation

Detection Methods	Incubation Temperature									
	30 °C					37 °C				
	AF	CAM	AMN	Y	C	AF	CAM	AMN	Y	C
PCR	+	+	+	+	-	+	+	+	+	-
qPCR	+ (day 1-5)	+	+	+	-	+ (day 1-5)	+	+	+	-

Data analysis

The level of viral DNA from ECE in AF, CAM, AMN and yolk sac was calculated using software on the Bio-Rad qPCR machine CFX96 Touch Real-Time PCR Detection System, following the manufacturer's instructions, and compared to the standard copies of DNA used. PCR and real-time PCR data were analyzed descriptively.

Result and Discussion

Presence of KHV on ECE

Cell cultures have some drawbacks, such as requiring extra handling and expensive equipment and materials, so ECE provides an alternative to obtain viruses to use for vaccine production. According to Benzarti et al. (2020), ECE is low-cost, easy to manipulate system, and an ethically accepted model for many areas of research, vaccine development, and production. The efficacy of a culture-based influenza vaccine compared to embryonated eggs-based vaccines showed there were no significant differences between effectiveness of using both vaccines. Both methods have their advantages and disadvantages in terms of production capacity, production facilities, and the possibility of the emergence of allergic reactions in vaccinates and mutations (Álvarez Aldeán et al. 2022). KHV was propagated in cell lines with a Ct value of 17 or 1.4 x 10^9 copies of virus, and were used as the antigen source. The virus titer was $10^{3.7}$ $TCID_{50}/mL^{-1}$ (data not shown). The products of the KHV qPCR assays were positive at a size of 290 bp – the expected size of the target band (Table 1). Table 1 presents the results of the presence of KHV following a 0.2 mL virus inoculation on ECE.

Our data showed that KHV proliferates in ECE through AF, CAM, AMN, and Y inoculation by forming a plaque on CAM based on PCR confirmation. Certain viruses, such as the Usutu virus (USUV), can replicate highly in allantoic fluid and embryonic tissue, resulting in chicken embryo mortality (Benzarti et al. 2020). Benzarti et al. (2020) also showed that CAM is the preferred location for viral propagation. Nagai et al. (1979), using a pathogenic and non-pathogenic strain of the Newcastle Disease Virus to infect chicken and chick embryos, showed that CAM is an entry point for the virus into the embryo. Inoculation of a pathogenic strain at the endodermal site of CAM led virus spreading into all embryonic tissues; inoculation of a

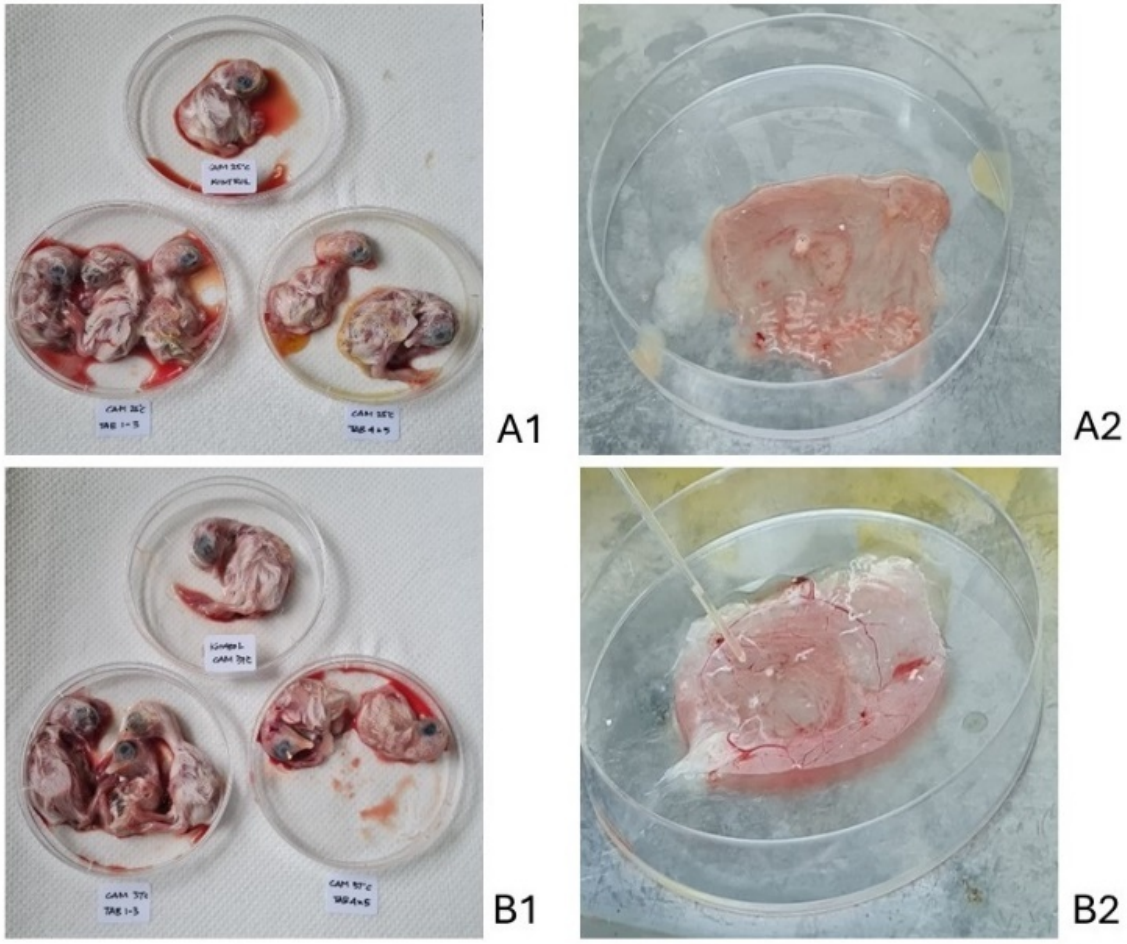

Figure 1. Embryonated Chicken Eggs (ECE) condition after being inoculated with Koi herpesvirus (KHV). Haemorrhage, dwarfism and lesions or plaques in KHV-inoculated ECE. A1 and A2: Group of embryos and lesions/plaques from 30°C incubation; B1 and B2: Group of embryos and lesions/plaques from 37°C incubation.

pathogenic strain through the endodermal site has generated a high virus titre in the allantoic membrane; ectodermal inoculation with pathogenic strains resulted in replication in the CAM mesodermal and ectodermal layers; and ectodermal infection with a pathogenic strain resulted in an infection in a small area of ectoderm and was not lethal to the embryo. KHV appears pathogenic to ECE and non-lethal, resulting in moderately high virus titres in allantoic fluid, a small number of plaques on CAM and no embryo mortality in this research. However, as shown in Figure 1 and Figure 2, some KHV-inoculated embryos appeared to suffer bleeding (haemorrhages) and dwarfism compared to control embryos.

Koi herpesvirus in ECE detection by conventional PCR (PCR) assay

The KHV test on ECE yielded positive results for all materials tested, including AF, CAM, AMN, and Y (Table 2).

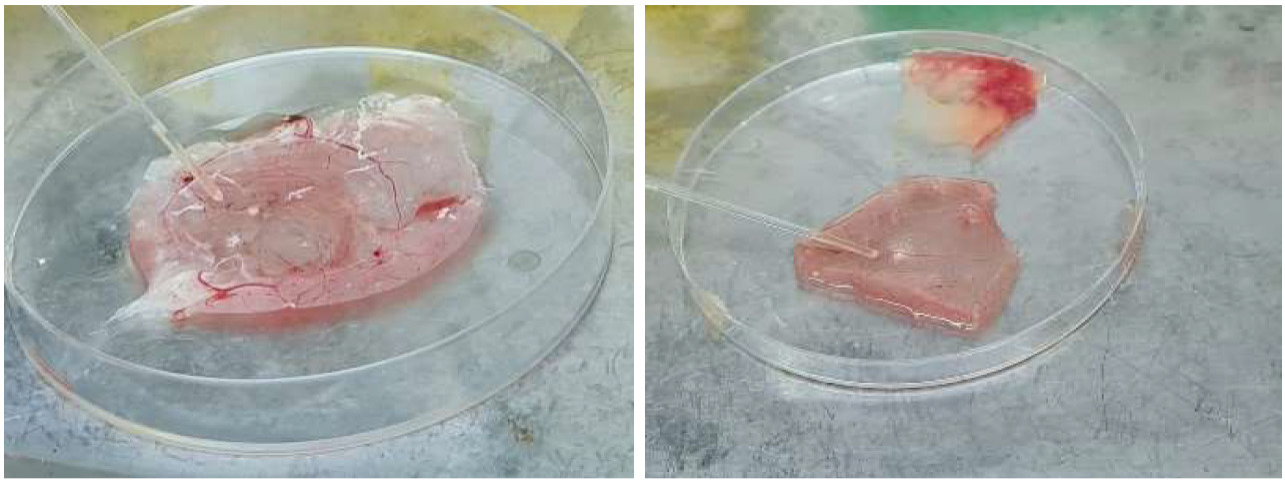

Figure 2. Chorioallantoic membrane showing 1-2 mm pocks at third passage level.

Table 2. PCR results of KHV propagation in ECE

No.	Sample	PCR* Result
1	Control AF	-
2	AF Hour-1 at 30 ℃	+
3	AF Hour-1 at 30 ℃	+
4	AF Day-1 at 30 ℃	+
5	AF Day-1 at 30 ℃	+
6	AF Day-3 at 30 ℃	+
7	AF Day-3 at 30 ℃	+
8	AF Day-5 at 30 ℃	+
9	AF Day-5 at 30 ℃	+
10	AF Hour-1 at 37 ℃	+
11	AF Hour-1 at 37 ℃	+
12	AF Day-1 at 37 ℃	+
13	AF Day-1 at 37 ℃	+
14	AF Day-3 at 37 ℃	+
15	AF Day-3 at 37 ℃	+
16	AF Day-5 at 37 ℃	+
17	AF Day-5 at 37 ℃	+
18	CAM Day-5 at 30 ℃	+
19	CAM Day-5 at 30 ℃	+
20	CAM Day-5 at 37 ℃	+
21	Control CAM	-
22	CAM Day-5 at 37 ℃	+
23	AMN Day-5 at 37 ℃	+
24	Control AMN	-
25	AMN Day-5 at 37 ℃	+
26	AMN Day-5 at 30 ℃	+
27	AMN Day-5 at 30 ℃	+
28	Yolk Day-5 at 30 ℃	+
29	Yolk Day-5 at 37 ℃	+

*PCR primers according to Yuasa et al. (2005)

Table 3. Results of quantification of KHV in ECE samples using the qPCR assay

No.	Treatment	Ct	Average of a copy of DNA (KHV load) from three replicated	Results
1	KHV from cell culture	17	$1.43x10^9$	+
2	Control (one hour post-injection)	37	$2.1x10^2$	-
3	AF Hour-1 at 30 ℃	27	8.4×10^5	+
4	AF Day-1 at 30 ℃	26	1.6×10^6	+
5	AF Day-3 at 30 ℃	27	5.3×10^6	+
6	AF Day-5 30 ℃	31	1.7×10^5	+
7	AF Hour-1 at 37 ℃	33	3.2×10^3	+
8	AF Day-1 at 37 ℃	33	5.2×10^3	+
9	AF Day-3 at 37 ℃	30	8.6×10^5	+
10	AF Day-5 at 37 ℃	28	1.6×10^5	+
11	CAM Day-5 at 30 ℃	28	2.6×10^5	+
12	CAM Day-5 at 37 ℃	25	3.4×10^6	+
13	AMN Day-5 at 30 ℃	29	7.8×10^5	+
14	AMN Day-5 at 37 ℃	29	2.3×10^5	+
15	Yolk Day-5 at 30 ℃	32	6.5×10^3	+
16	Yolk Day-5 at 37 ℃	31	1.8×10^4	+

We extended the detection of KHV on ECE using qPCR to obtain the number of viral loads at each injection site. The standard curve was generated from 10-fold serial dilutions of the synthetic nucleotide (gBlock) consisting of DNA sequences of KHV, ranging from 7.5 x 10^2 to 7.5 x 10^8 DNA copies. The level of viral DNA from ECE in AF, CAM, AMN and yolk sac were calculated using software on the Bio-Rad qPCR machine and compared to the standard copies of DNA used. Data from Bio-Rad qPCR yielded a graph in the form of an amplification curve; the standard curve (Figure 3) with a value of R2 = 0.924 at a slope value (Y = -2.807) and an amplification efficiency (E) of 127.1%. Table 3 shows that KHV multiplication on ECE depends on the Ct value and the amount of DNA copies or viral copies 50 ng μL^{-1} in each viral DNA template. The highest Ct value of AF was identified at 30°C on day 3, while the Ct value of the CAM was detected at 25 in the sample at 37°C. The Ct value of AMN, 30°C, is 29, and the Ct value of yolk is 32 in the yolk sample (37°C). The KHV isolate derived from cell culture medium that was inoculated into ECE, has a Ct value of 17 (1.43 x 10^9 copies) and the ECE treatment at one hour post-injection (control) has an average Ct value of 37 (2.14 x 10^2 copies) (Figure 3).

KHV was detected in AF, CAM, AMN, and yolk (Y). KHV was most frequently found in allantoic fluid with an incubation period of three days. The number of KHV DNA obtained by virus propagation in ECE is much smaller than of viral DNA copies produced in cultured cells, which can reach 14.3 x 10^9 (data not shown). The viral supernatant from allantoic fluid was inoculated into CCB cell culture, but CPE did not form for up to 2 weeks. The maximum result obtained in virus propagation on embryonated chicken eggs was only 5.3 x 10^6 DNA copies of KHV. The growth of the virus in

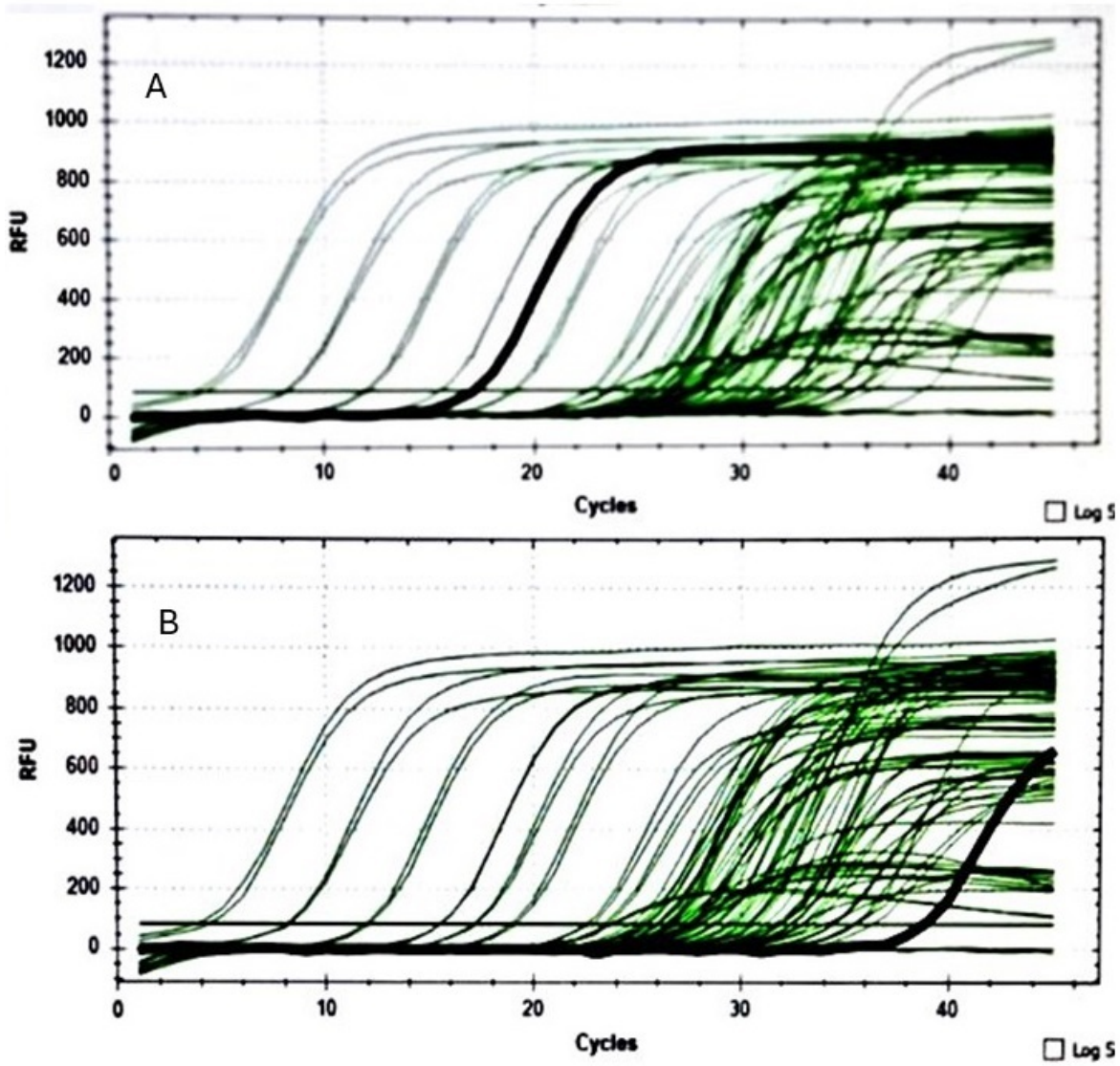

Figure 3. (A) The amplification of qPCR KHV from cell culture. The bold line refers to the amplification curve of KHV from cell culture with an average Ct value of 17 (1.43 x 10^9 copies). (B). The amplification of qPCR from ECE. The bold line refers to the amplification curve of KHV from ECE treatment at one hour post-injection with an average Ct value of 37 (2.14 x 10^2 copies)

ECE may lose its virulence so it does not cause CPE effects on cell culture. Further research is needed, from the ECE supernatant, carried out by Koch's postulates in fish before being inoculated into cell culture to increase its virulence. The results were obtained because the virus was still in the first passage in the chicken eggs; therefore, further study is required to carry out the next passage so that KHV can adapt effectively. KHV is not a zoonotic disease. As described in early publications (Haenen et al. 2004; Pikarsky et al. 2004), the main infection route for *C. carpio* is horizontal, from fish to fish including waterborne infections. According to Bergmann et al. (2016) in the aquatic environment, there is no species specificity for herpesviruses but for the occurrence of the disease only in common carp and koi. To our

knowledge, there is no report on the mutation of KHV from non-zoonotic to zoonotic virus. Further study is required to determine the potential zoonotic mutation.

ECE is a complex structure consisting of the cells of an embryo and its supporting membranes that provides a variety of cell types required for the successful replication of a wide range of microbial pathogens (Anjum et al. 2010; Clavijo et al. 2000; Crespo et al. 2009; Guy 2008; Jacobsen et al. 2010). Embryonic Chicken Eggs have long been used as a host or system for virus isolation and propagation (Crespo et al. 2009; Guy 2008), but there is little information on whether this system can be used as a host for KHV - a fish virus. The Herpes simplex virus has been tested on ECE, where it adapted well to ECE. According to Richman and Murphy (1979), viruses that replicate well at low temperatures (permissive) and poorly replicate at higher temperatures (restrictive) are defined as temperature-sensitive. This means that a higher temperature for the virus is the normal temperature of the host and the virus can replicate but not as well as at the permissive temperature, therefore replicating poorly and relatively restrictive, but nonetheless they can grow. To replace the culture cell approaches that are commonly used, KHV was tested to propagate ECE.

Microorganisms can adapt beyond conditions comfortable for their survival, although they tend to replicate poorly and restrictively. According to Bergmann et al. (2020), KHV exhibits an unexpected ability to quickly adapt to different conditions, such as different temperatures, as well as grow in different cell cultures and hosts. This is evidenced by the research results of Klafack et al. (2017) and Dong et al. (2011) that DNA viruses often experience genomic changes in the form of deleted, inserted or incomplete replication or substitution to adapt to different conditions. This supports the possibility fos KHV to replicate in ECE in this study, that the KHV can grow outside its comfort zone temperature of >30°C, namely at >37°C within ECE. Moreover, the other possibility is that ECE, especially CAM tissue, has a receptor-binding site which is compatible with the KHV. Hardy et al. (1995) said CAM tissue has sialyloligosaccharides cells which contain Sialic Acid (α 2-3) Galactose Linkages [(SA (α 2-3) Gal] in its cell surface. However, further research is required to ensure the capacity of the virus to be replicated at high temperatures and its correlation with the virus virulence. This will likely require more serial passages for better adaptation of the virus to show a CPE. Homogenate pock lesions on CAM should be continued to investigate whether the virus is present.

Predicting the possibility of KHV growing in ECE, and whether it could potentially become a zoonosis, there are two viral traits termed zoonotic potential (the ability of an animal virus to infect a human host) and epidemic potential (the ability of a zoonotic infection to cause disease and transmit onward in human populations) (Carlson et al. 2021). Even if KHV could

replicate at 37°C, the reason why KHV is not zoonotic, is that to date there have been no reported incidents of transmission of KHV from fish to humans. Viruses need to adapt to their new host by forming specific proteins such as lock and key interaction systems to replicate through genetic mutations. The virus should live fully in the body of the new host. However, the KHV is a DNA virus, making it more difficult to mutate.

Conclusions

KHV can propagate ECE through the allantoic fluid and yield the highest titer on the third day post-infection at 30°C. Further research with several combination treatments is required to determine the potential of embryonated chicken eggs as an alternative for maintaining KHV propagation with high viral genomic copy numbers similar to KHV titers grown in cell culture.

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REFERENCES

Álvarez Aldeán, J., I. Salamanca, D. Ocaña, J. L. Barranco, and S. Walter. 2022. "Effectiveness of Cell Culture-Based Influenza Vaccines Compared with Egg-Based Vaccines: What Does the Literature Say?" *Revista Espanola de Quimioterapia : Publicacion Oficial de La Sociedad Espanola de Quimioterapia* 35 (3): 241–48. https://doi.org/10.37201/req/117.2021.

Anjum, A. A., I. Hussain, S. M. Mahmood, and M. I. Anwar. 2010. "Adaptation of Infectious Bursal Disease Virus by Cultivation in Embryonated Chicken Eggs and Evaluation as a Potential Candidate for Local Live Attenuated Vaccine." *Pakistan Journal of Life and Social Sciences* 8 (1): 30–34.

Benzarti, Emna, José Rivas, Michaël Sarlet, Mathieu Franssen, Nassim Moula, Giovanni Savini, et al. 2020. "Usutu Virus Infection of Embryonated Chicken Eggs and a Chicken Embryo-Derived Primary Cell Line." *Viruses* 12 (5). https://doi.org/10.3390/v12050531.

Bergmann, S. M., M. Cieslak, D. Fichtner, J. Dabels, S. J. Monaghan, Q. Wang, W. Zeng, and J. Kempter. 2016. "Is There Any Species Specificity in Infections with Aquatic Animal Herpesviruses? The Koi Herpesvirus (KHV) – an Alloherpesvirus Model." *Fish and Aquacult J* 7:2–6. https://doi.org/10.4172/2150-3508.1000169.

Bergmann, S. M., Y. Jin, K. Franzke, B. Grunow, Q. Wang, and S. Klafack. 2020. "Koi Herpesvirus (KHV) and KHV Disease (KHVD) – a Recently Updated Overview." *Journal of Applied Microbiology* 129:98–103. https://doi.org/10.1111/jam.14616.

Bergmann, S. M., J. Sadowski, M. Kielpinski, M. Bartlomiejczyk, D. Fichtner, R. Riebe, M. Lenk, and J. Kempter. 2010. "Susceptibility of Koi · Crucian Carp and Koi · Goldfish Hybrids to Koi Herpesvirus (KHV) and the Development of KHV Disease (KHVD)." *Journal of Fish Diseases* 33:267–72. https://doi.org/10.1111/j.1365-2761.2009.01127.x.

Blyden, B. E. R., and P. K. Watler. 2010. "New Approaches to Improved Vaccine Manufacturing in Embryonated Eggs." *BioPharm Int. Suppl.*, 4–9.

Boutier, M., Y. Gao, Catherine Vancsok, Nicolás M. Suárez, Andrew J. Davison, and Alain Vanderplasschen. 2017. "Identification of an Essential Virulence Gene of Cyprinid Herpesvirus 3." *Antiviral Research* 145:60–69. https://doi.org/10.1016/j.antiviral.2017.07.002.

Boutier, M., M. Ronsmans, P. Ouyang, G. Fournier, Anca Reschner, Krzysztof Rakus, et al. 2015. "Rational Development of an Attenuated Recombinant Cyprinid Herpesvirus 3 Vaccine Using Prokaryotic Mutagenesis and in Vivo Bioluminescent Imaging." *PLoS Pathogens* 11 (2): e1004690. https://doi.org/10.1371/journal.ppat.1004690.

Carlson, C. J., M. J. Farrell, Z. Grange, B. A. Han, N. Mollentze, A. L. Phelan, A. L. Rasmussen, et al. 2021. "The Future of Zoonotic Risk Prediction." *Phil. Trans. R. Soc. B* 376:20200358. https://doi.org/10.1098/rstb.2020.0358.

Clavijo, A., R. A. Heckert, G. C. Dulac, and A. Afshar. 2000. "Isolation and Identification of Bluetongue Virus." *Journal of Virological Methods* 87:13–23.

Crespo, R., H. L. Shivaprasad, M. Franca, and P. R. Woolcock. 2009. "Isolation and Distribution of West Nile Virus in Embryonated Chicken Eggs." *Avian Diseases* 53 (4): 608–12. https://doi.org/10.1637/8899-040209-ResNote.1.

Dong, C., S. Weng, W. Li, X. Li, Y. Yi, Q. Liang, and J. He. 2011. "Characterization of a New Cell Line from Caudal Fin of Koi, *Cyprinus carpio Koi*, and First Isolation of Cyprinid Herpesvirus 3 in China." *Virus Res* 161:140–49. https://doi.org/10.1016/j.virusres.2011.07.016.

Embregts, Carmen W. E., Roni Tadmor-Levi, Tomáš Veselý, Dagmar Pokorová, David Lior Wiegertjes, and Geert F. Forlenza. 2019. "Intra-Muscular and Oral Vaccination Using a Koi Herpesvirus ORF25 DNA Vaccine Does Not Confer Protection in Common Carp (Cyprinus carpio L.)." *Fish & Shellfish Immunology* 85:90–98. https://doi.org/10.1016/j.fsi.2018.03.037.

Guy, J. S. 2008. "Isolation and Propagation of Coronaviruses in Embryonated Eggs." In *SARS and Other Coronaviruses: Laboratory Protocols*, edited by D. Cavanagh, 109–17. New York: Human Press.

Haenen, O. L. M., K. Way, S. M. Bergmann, and E. Ariel. 2004. "The Emergence of Koi Herpesvirus and Its Significance to European Aquaculture." *Bull Eur Assoc Fish Pathol* 24:293–307. https://doi.org/10.1006/viro.1995.1405.

Hardy, T. C., S. A. Young, R. G. Webster, C. W. Naeve, and R. J. Owens. 1995. "Egg Fluids and Cells of the Chorioallantoic Membrane of Embryonated Chicken Eggs Can Select Different Variants of Influenza A (H3N2) Viruses." *Virology* 211:302–6. https://doi.org/10.1006/viro.1995.1405.

Indriani, C., and G. S. Putri. 2023. "Suspected of Being Infected with the KHV Virus, 15 Tons of Carp Fish in the Koto Panjang Hydroelectric Power Reservoir, Riau, Die Every Day." 2023. https://regional.kompas.com/read/2023/02/01/220417378/diduga-terinfeksi-virus-khv-15-ton-ikan-mas-di-waduk-plta-koto-panjang-riau.

Jacobsen, Ilse D., K. Grosse, S. Slesiona, B. Hube, A. Berndt, and M. Brock. 2010. "Embryonated Eggs as an Alternative Infection Model to Investigate Aspergillus Fumigatus Virulence." *Infection and Immunity* 78 (7): 2995–3006. https://doi.org/10.1128/IAI.00268-10.

Klafack, S., Q. Wang, W. Zeng, Y. Wang, Y. Li, S. Zheng, J. Kempter, P. Y. Lee, et al. 2017. "Genetic Variability of Koi Herpesvirus in Vitro—a Natural Event?" *Front Microbiol* 8:982. https://doi.org/10.3389/fmicb.2017.00982.

Lin, J., Y. Liu, X. Wang, B. Yang, P. He, Z. Yang, J. Xie, L. Zou, J. Zhao, J. Pan, et al. 2015. "Efficacy Evaluation of an Inactivated Duck Tembusu Virus Vaccine." *Avian Dis* 59:244–48. https://doi.org/10.1637/10960-101514-Reg.

Lusiastuti, A. M., H. Novita, L. Gardenia, T. Taukhid, and S. M. Bergmann. 2021. "Combination Vaccines against Koiherpes Virus and *Aeromonas Hydrophila* Co-Infection in Koi and Common Carp." *Indonesian Aquaculture Journal* 15 (2). https://doi.org/10.15578/iaj.15.2.2020.93-102.

Nagai, Y., K. Shimokata, T. Yoshida, M. Hamaguchi, M. Ilnuma, K. Maeno, T. Matsumoto, H.D. Klenk, and R. Rott. 1979. "The Spread of a Pathogenic and Apathogenic Strain of Newcastle Disease Virus in the Cick Embryo as Depending on the Protease Activity of the Virus Glycoproteins." *J.Gen.Virol* 45:263–72. https://doi.org/10.1099/0022-1317-45-2-263.

Pikarsky, E., A. Ronen, J. Abramowitz, B. Levavi-Sivan, M. Hutoran, Y. Shapira, M. Steinitz, A. Perelberg, et al. 2004. "Pathogenesis of Acute Viral Disease Induced in Fish by Carp Interstitial Nephritis and Gill Necrosis Virus." *J Virol* 78:9544–51. https://doi.org/10.1128/JVI.78.17.9544-9551.2004.

Richman, D.D., and B.R. Murphy. 1979. "The Association of the Temperature-Sensitive Phenotype with Viral Attenuation in Animals and Humans: Implications for the Development and Use of Live Virus Vaccines." *Rev Infect Dis* 1 (3): 413–33. https://doi.org/10.1093/clinids/1.3.413.

Shahin, K., E. Soto, B. Martínez-López, and S. Barnum. 2020. "Genetic Diversity of Cyprinid Herpesvirus 3 from Different Geographical Locations during 1999-2019 in the United States of America." *Journal of Aquatic Animal Health*, 50–56. https://doi.org/10.1002/aah.10098.

Sunarto, A., and A. Cameron. 2005. "Response to Mass Mortality of Carp: An Indonesian Experiences." In *Regional Workshop on Preparedness and Response to Aquatic Animal Health Emergencies in Asia*, edited by R. P. Subasinghe and J. R. Arthur, 87–106. FAO Fisheries Proceeding 4.

Yuasa, K., M. Sano, J. Kurita, and T. Lida. 2005. "Improvement of a PCR Method with the Sph 1-5 Primer Set for the Detection of Koi Herpesvirus (KHV)." *Fish Pathology* 40:37–39. https://doi.org/10.3147/jsfp.40.37.