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# Genetic Characterization of the Thymidine Kinase of Koi Herpesvirus 3 in Sulaymaniyah Province, Iraq

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**Abstract**

Koi herpesvirus 3 (KHV-3) is nominated as an emerging, highly devastating, and contagious viral disease with severe economic losses in the carp aquaculture industries worldwide. Carp rearing under intensive culture conditions is constantly vulnerable to infection by various pathogens in the water. Water temperature has a major influence on the onset and severity of the disease. Since common carp have gained importance in the Iraqi fish industry, the investigation and control of associated diseases, including KHV, are warranted. We confirmed the first detection of the KHV from the collected specimens from the suspected cages in the Sulaymaniyah region. Also, an analysis of the thymidine kinase (TK) gene sequence of the KHV showed that the virus strain had a point mutation (proline, P193) instead of threonine, T193. The isolated KHV strains were subdivided into three clades with three distinct genotypes: Asian (Iranian, Indonesian, Japanese, and Chinese), European (UK), and North American (USA and Mexico) genotypes. The virus sequences were more closely related to the Iranian and Indonesian genotypes. This report describes the first isolation and genetic characterization of koi herpesvirus (KHV) in the Sulaymaniyah province, which is important for efficacious management and vaccination programs in the future to control KHV infection in carp cages in Northern Iraq.

**Keywords:** Cyprinid herpesvirus 3, koi herpesvirus, koi carp, common carp, fish viral diseases

# التوصيف الوراثي لإنزيم الثيميدين كيناز لفيروس كوي الهربس 3 في محافظة السليمانية، العراق

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**الخلاصة**

تم ترشيح فيروس Koi herpesvirus 3 (KHV-3) باعتباره مرضًا فيروسيًا ناشئًا شديد التدمير ومعدٍ وله خسائر اقتصادية فادحة في صناعات تربية الاحياء المائية الكارب المائي في جميع أنحاء العالم. تربية كارب في ظروف الزراعة المكثف معرضة باستمرار للعدوى بمسببات الأمراض المختلفة في الماء. تؤثر درجة حرارة الماء بشكل كبير على ظهور المرض وشدته. بما أن الكارب الشائع يكتسب أهمية في صناعة الأسماك العراقية ، فإن البحث عن الأمراض المصاحبة بما في ذلك KHV ومكافحتها له ما يبرره. تأكدنا من الكشف الأول عن KHV من العينات التي تم جمعها من الأقفاص المشتبه بها في منطقة السليمانية. أيضا ، تحليل تسلسل الجين ثيميدين كيناز (TK) من KHV ميز سلالة الفيروس كذلك ، وصف تحليل تسلسل جين ثيميدين كيناز (TK) لـ KHV سلالة الفيروس بطفرة نقطية (Proline – P193) بدلاً من (Threonine – T193). تم تقسيم سلالات KHV المعزولة إلى 3 مجموعات مع ثلاثة أنماط وراثية متميزة: الأنماط الجينية الآسيوية (الإيرانية والإندونيسية واليابانية والصينية) والأوروبية (المملكة المتحدة) وأمريكا الشمالية (الولايات المتحدة والمكسيك). كانت تسلسل الفيروس أكثر ارتباطًا بالأنماط الجينية الإيرانية والإندونيسية. يصف هذا التقرير أول عملية عزل وتوصيف وراثي لفيروس الهربس koi (KHV) في محافظة السليمانية ، وهو أمر مهم للإدارة الفعالة وبرامج التطعيم في المستقبل للسيطرة على عدوى KHV في أقفاص المبروك في شمال العراق.

**الكلمات المفتاحية:** Cyprinid herpesvirus 3، koi herpesvirus، koi carp ، الكارب الشائع ، أمراض الأسماك الفيروسية.

## Introduction

In the last decades, Koi herpesvirus 3 (KHV 3) (recently known as Cyprinid herpesvirus 3) disease has become the most important virus disease in ornamental fish, food fish, and wild fish, with a very wide geographic distribution. As international fish trading increased, the common carp became among the most valuable aquaculture fish species worldwide, although their production is exposed to many infectious diseases [1]. Therefore, disease quarantine inspections of imported and exported fish need to be performed with greater precision. The quarantine system, which has been limited to assessing the Koi Herpes virus, should be expanded to include all carp diseases. Since it has a major economic impact, the rapid development of diagnostic methods and the production of vaccines have become necessary to control KHV infection [1]. A safe and effective vaccine is not yet commercially available, although live attenuated vaccines have been used to confer protection against the virus for more than 6 months [2].

The first reports of KHV infection were reported in Palestine, the UK, and Germany during the years 1996–1998, following the mass mortality of common and koi carp [3, 4]. Subsequently, KHV has been detected in many countries, and the global trading of live carp has possibly contributed to the rapid spread of the virus [5, 6].

The taxonomy of herpesviruses' order has recently been revised and split into three families of *Herpesviridae*: (1) *Herpesviridae* representing viruses of mammals, birds, and reptiles, which in turn are divided into subfamilies: *Alpha-*, *Beta-*, and *Gamm-herpesviral*; (2) a family of herpesviruses infecting fish and amphibians known as *Alloherpesviridae;* and (3) a family infecting oysters known as Ostreid herpesvirus 1 OsHV1 (Pacific oyster herpesvirus) [7].

The KHV has a double-stranded DNA genome that belongs to the family Alloherpesviridae. The virulence of the KHV is affected by the temperature of the water. The KHV infects fish of all ages at a water temperature between 16 and 25 °C, with a high

mortality rate of 80–100% [5]. KHV can survive in infected fish for a lifetime, i.e., fish that survive the KHV infection carry the virus and act as carriers [8, 9]. KHV is most prevalent in the gills, spleen, and kidneys of infected populations. The infection has rapid mortality, which starts within a few days of the onset of clinical signs [10].

Many viruses have been isolated from the carp family, but some of them have been shown to cause significant diseases, including KHV. At the moment, spring viremia of carp (SVC) and grass carp hemorrhagic disease (GCHD) are the only viral diseases of economic importance in cyprinid farming. Fish pox (or carp pox), which is common among fishery workers but less important, is another viral disease. In very recent reports, concern has been raised about diseases caused by herpes-like viruses and non-SVC rhabdoviruses [11].

All age groups of carp appear to be susceptible, but younger fish up to 1 year of age appear to be more susceptible to clinical KHV infection. Transmission of the virus occurs in the contaminated water with the feces, urine, sloughed gill cells, and skin mucus of the infected fish. Other fish species, invertebrates, piscivorous birds, and mammals may also contribute to the transmission of the virus. The virus remains active in the water for at least 4 hours at water temperatures of 23 °C to 25 °C. Currently, effective therapeutics and vaccines are not available. Therefore, preventing exposure to the virus, along with a good biosecurity and quarantine program, are still the best applicable methods for the prevention of KHV infection. Raising the water temperature above the submissive temperature has been shown to decrease mortalities, but survivors were reported as carriers of the virus. The depopulation of the remaining exposed individuals in an infected population needs to be considered. All systems and equipment exposed to the infection should be sanitized with an appropriate disinfectant [11–13].

As common carp become more important in the Iraqi fish industry, the identification and management of associated diseases have become a necessity, especially Koi herpesvirus infections, which have a high mortality rate in cages of low quality [14]. So, the goal of this study is to find out if the Koi herpes virus (KHV) is present in the carp cultures in the Sulaymaniyah province (Northern Iraq) by amplifying the virus's thymidine kinase (TK) gene using traditional polymerase chain reaction (PCR) and also to figure out the genetic relationship with the referenced TK gene sequence data in GenBank.

## Materials and Methods

The information on samples from different fish projects in Sulaymaniyah province is shown in **Table 1** below.

**Table 1:** Illustrates the data on fish samples collected from different projects

| Project type | No. of cage/ pond | Fish weight/gm | Mortality | Source of fingerlings |
|---|---|---|---|---|
| Cage | 24 | 800 – 1500 | 35000 | Babel |
| | 6 | 1500 | 10000 | Babel |
| | 8 | 800 – 1500 | 15000 | Babel |
| Earthen pond | 4 | 1800 - 2000 | 4000 | Babel |

For viral nucleic acid extraction and PCR detection, samples were taken from the skin and gills on the outside and from the spleen, intestine, liver, and kidneys on the inside. The pond water temperature was standard, between 20°C and 23.33°C (68 and 74 degrees Fahrenheit).

**DNA Extraction**

As described by the manufacturer, DNA was extracted from the specimens using a DNA extraction kit (Geneaid, Republic of Korea). The extracted DNA was stored at -20 ℃ for future applications.

**Primer Pairs**

Two sets of primer pairs were used to amplify different regions of the viral TK gene (409 bps and 1001 bps) (**Table 2**).

**Table 2:** Plasmid and primer pairs for quantitative PCR

| Plasmid Name | Primer Sequence (5'-3') | Reference |
|---|---|---|
| 1. Thymidine Kinase (TK) | F: GGGTTACCTGTACGAG<br>R: CACCCAGTAGATTATGC | [8] |
| 2. Thymidine Kinase (TK) | F: AACGCGGGCCAGCTGAACAT<br>R: TGTGTGTATCCCAATAAACG | [8] |
| F: forward; R: reverse | | |

**Polymerase Chain Reaction (PCR) and Sequencing**

The reactions were performed by using a PCR premix (GENET-BIO-Korea) of 20 µL total volume, including 10 µL supreme script PCR premix, 5 µL DNA, 1 µL forward (10 pmol), 1 µL reverse primers (10 pmol), and 3 µL free DNAse water to make up the final volume. The thermocycler temperature condition (Hercuvan, USA) was as follows: Initial denaturation at 95.0 ℃ for 5 minutes is followed by 40 cycles of 95 ℃ for 30 seconds, annealing at 60 ℃ for 35 seconds, extension at 72 ℃ for 30 seconds, and a final extension at 72 ℃ for 5 minutes. A similar condition was used for the complete CDS amplification process, except the extension step was up to 50 seconds.

Gel-electrophoresis was done using 1% agarose gel stained with a safe dye (Eurex-Poland) and 150 volts for 30 minutes. Ten microliters of each sample of amplified DNA were used. The amplified PCR products of the TK gene were sent for sequencing using the Sanger Sequencing Method (Macrogen Co., Rep. Korea). The BLAST and NCBI databases are used to describe the sequence homology.

**DNA Sequences Analysis**

The PCR data was aligned with the Koi herpes virus TK gene reference sequences in NCBI GenBank using the Clustal WI algorithm. Next, the neighbor-joining method and evolutionary analysis in MEGA X software were used to figure out the phylogenetic relationships using the Tarmura 3-parameter model with 1000 bootstrap replicates.

Multiple sequences of the partial TK gene of the virus were aligned with other reference strains' nucleotide residues using BioEdit software.

**Results and Discussion**

Based on previous studies, many biological, metabolic, or infectious diseases play a critical role in the morbidity and mortality of common carp aquaculture projects. For example, it is recommended that copper sulfate be applied at limited concentrations since high concentrations lead to gill tissue toxicity [15]. Also, bad management, such as poor nutrition, low oxygen application, and bad water quality, played a role in the outbreak of common carp aquaculture. Besides, other studies described the role of bacterial, fungal, and parasitic infections in common carp in different ratios compared with other countries and depending upon changes in climate and breeding habits. However, the gross and microscopic lesions were identical to those recorded worldwide [16].

Recently, viral infection has become a common cause of infection in carp farms in the surrounding countries and worldwide, especially with Koi herpesvirus (KHV) infection. A high mortality rate of KHV was commonly reported in cage projects that were not following the standard rules of the cage and fish density, reduced levels of water flow, irregular fish examination, and the throwing of infected dead fish directly into the river [14]. The incubation period of the virus is usually around 14 days following exposure to infected fish [17]. Although the appropriate temperature and the possibility of other factors may extend the incubation period to more than 14 days, the mortality rate of KHV infection typically occurs between 18 and 27 °C. In October 2018, 100% mortality was recorded in a dozen kilometers along the river course, with a loss of a thousand tons [14]. Clinical signs of severe gill pathology, such as patches of necrotic tissue, fins, and gill rot disease, were described in the cage projects of Northern Babylon down at Al-Musayab Electrical Station. The collected tissue samples of dead fish conveyed to international laboratories in Jordan, Italy, and the UK by the private sector and the FAO team indicated the presence of the Koi herpesvirus KHV infection in the suspected cages in the region [17].

The transportation of carp fish from the infected cages in the center or south of Iraq or even imported from neighboring countries to Sulaymaniyah province has raised the possibility of the spreading of the KHV-infected carp in the area. Therefore, it becomes necessary to confirm the presence and/or origin of the viral strain in the area as well as to produce or identify appropriate strain-specific viral vaccines available commercially. To find out, 100 samples were taken from different carp cages in the Sulaymaniyah area (see Table 1), and PCR amplification of the TK gene of the Koi herpesvirus genome was done using specific primer pairs that each target a different part of the TK gene of the virus genome (see Table 2). As shown in Figures 1 and 2, amplified bands of approximately 409 and 1001 bp lengths were yielded, respectively. After sequencing, the TK gene sequences were sent to GenBank, where they were given accession numbers (MW928743 and MW928744) and given the names Kurdistan/1/Koi-HV and Kurdistan/2/Koi-HV, respectively. In the end, the multiple sequence alignment tool and MEGA 11 software were used to look at the genetic relationship with other Cyprinid herpesvirus strains in the NCBI database and figure out how they were related.

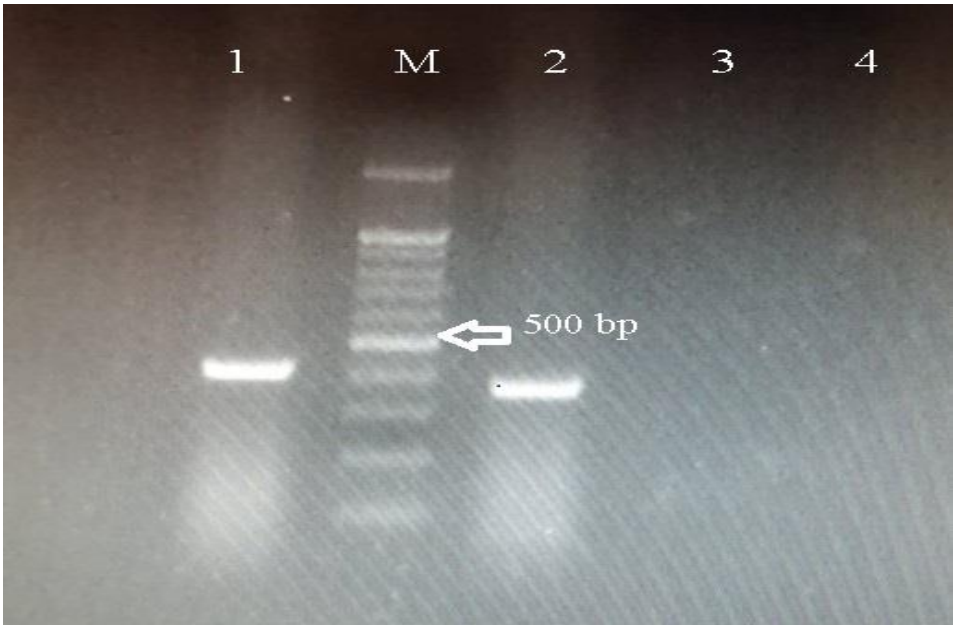


**Figure 1:** Agarose gel showing amplification of the partial thymidine kinase (TK) gene for KHV. (The M line is a DNA marker of 1000–100 bps; lanes 1 and 2 are positive samples for the partial CDS of TK genes = 409 bps; and lanes 3 and 4 are negative samples.)

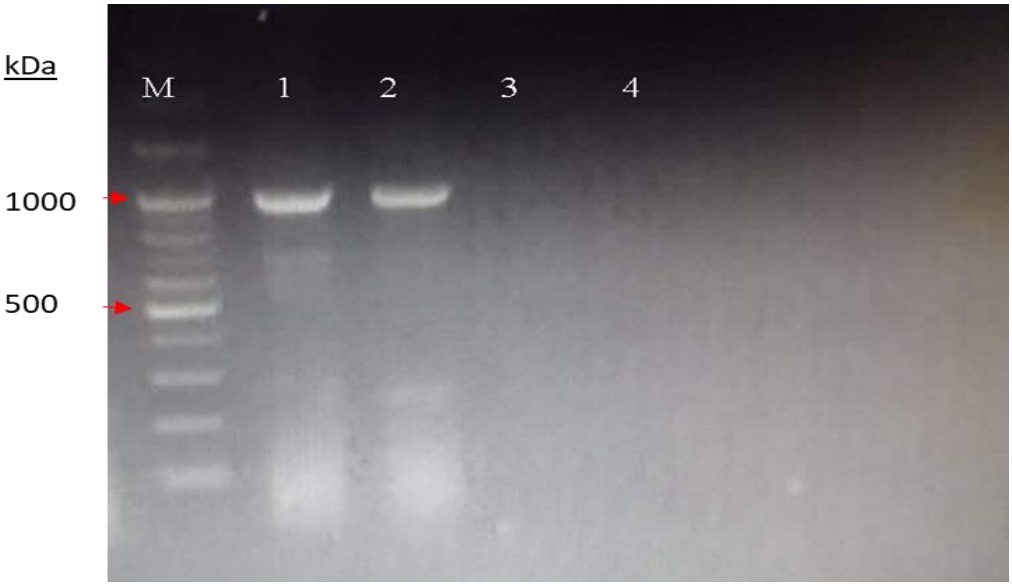


**Figure 2:** Agarose gel showing amplification of the complete thymidine kinase (TK) gene for KHV (The M line is a DNA marker of 1000–100 bp; lanes 1, 2, and 3 are positive samples for partial cds of the TK gene sequence = 1001 bps; and lanes 3 and 4 are negative samples.)
According to the BLAS sequencing, we demonstrate that the two field virus isolates (Kurdistan/1/Koi-HV and Kurdistan/2/Koi-HV) share 100% of their genetic structure but only 99.85% of their genetic makeup with the Asian genotypes of the Indonesian (AB375391) and Iranian (KX609547) strains.

Also, the neighbor-joining phylogenetic analysis of the TK nucleotide sequences separated the KHV strains into three different genotypes: Asian genotypes from Iran and Indonesia, European genotypes from the UK, and North American genotypes from the USA. As shown in Figure 3, the phylogenetic tree of the isolated virus sequences was more closely related to the Iranian and Indonesian isolates.

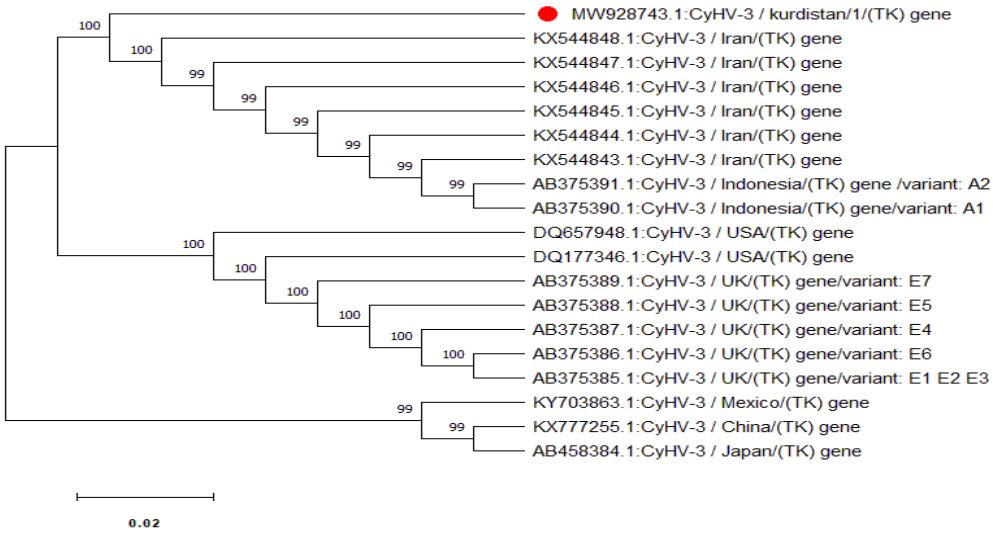


**Figure 3:** Cladogram showing the relationship among cyprinid herpesvirus-3 based on the conserved regions of the TK gene. Phylogenetic tree showing the evolution of common carp herpesviruses, depending on the TK gene sequences. Neighbor-joining method values are indicated above or below each node. The scale bar indicates branch lengths, which refer to the

number of errors or substitutions. The order sequence is in a red circle. Evolutionary analyses were conducted in MEGA X software.

**Protein Sequence Alignment**

Multiple sequences of the partial TK gene of the virus were aligned with other reference strains' amino acid residues, as shown in **Figure 4**. It describes that only one amino acid position was different from all references and was substituted by a new amino acid, such as the amino acid residue at position (proline, P193) instead of (threonine, T193). This substitution is possibly a point mutation in Sulaymaniyah's viral strain with other correlated reference strains.

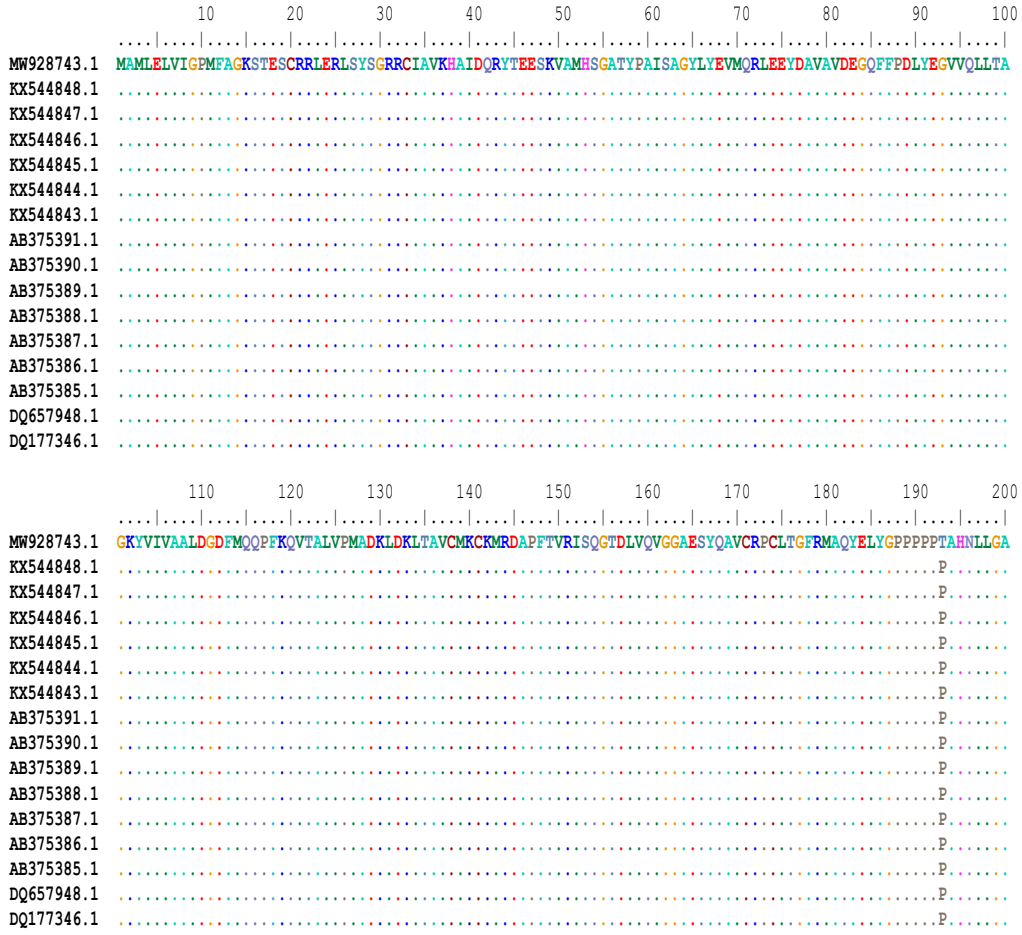


**Figure 4:** Multiple sequence alignment of the amino acid residue of the partial TK gene. The amino acid sequence alignment relationship of the isolate (Kurdistan/1/Koi-HV/2022) with other related reference strains

## Conclusion

In this report, KHV was found for the first time in the Sulaymaniyah province of northern Iraq. The genetic relationship with other references in GenBank was also described as being closely related to Iranian and Indonesian strains, with a possible single mutation in the TK gene protein sequence of the Sulaymaniyah KHV strain. This finding was important because it will help the carp farm in the area identify a specific control program and vaccines to confer protection against KHV infection in carp cages.

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**Declarations**

The authors declare no conflict of interest.

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