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Phaya, Xolani; Zhou, Jing-Xiang; Wang, Zhuo Yu; Wang, Hao; and Liang, Jie (2021) "Carp edema virus a rising threat to global carp population," The Thai Journal of Veterinary Medicine: Vol. 51: Iss. 3, Article 1.
DOI: https://doi.org/10.56808/2985-1130.3135
Available at: https://digital.car.chula.ac.th/tjvm/vol51/iss3/1

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Carp edema virus a rising threat to global carp population

Xolani Phaya¹* Jing-Xiang Zhou¹ Zhuo Yu Wang¹ Hao Wang¹ Jie Liang¹

Abstract

Carp edema virus (CEV), a poxvirus from the family Poxviridae, was first detected in 1974 in Japan and has since spread to most parts of the world. CEV is the etiological agent of koi sleepy disease (KSD), which has adverse health effects on both koi and common carp fish species. Equally, KSD leads to detrimental financial distress to carp farmers and further threatens both food security and ecology. The onset and severity of CEV/KSD is influenced by water temperature and handling stress. The prominent clinical sign of KSD in both koi and common carp is lethargy, which is manifested when the water temperature is in the range of 15 – 25 °C and 6 – 9 °C, respectively. Much advancement is needed to curb the spread of CEV. Since the early years of CEV, the immersion of diseased fish into a 0.5% salt-water solution has been shown to be efficacious in treating the clinical signs but ineffective in eradicating the virus in infected fish. Therefore, infected asymptomatic fish become CEV carriers. This is further exacerbated by the limited knowledge of the transmission pathways of CEV. This paper aims to collate the current knowledge on CEV/KSD, to give an insight into the nature and characteristics of CEV.

Keywords: HACCP, Koi sleep disease, Polymerase Chain Reaction, Recombinase Polymerase Amplification

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Received: September 23, 2020.
Accepted: April 21, 2021.
doi: https://doi.org/10.14456/tjvm.2021.51

Introduction

Carp edema virus (CEV) is a double-stranded DNA poxvirus that infects both the common and koi carp. CEV is an etiological agent to a clinical disease called koi sleepy disease (KSD) when conditions are conducive to an outbreak (Way and Stone, 2013; Way et al., 2017; Adamek et al., 2018a). All age groups of common and koi carp species are susceptible to CEV/KSD infection. CEV is assumed to be a member of the family Poxviridae (Oyamatsu et al., 1997a; Miyazaki et al., 2005; Adamek et al., 2018a). Infected fish develop lethargic behaviour, displaying sleepiness and sluggishness, hence the clinical disease was named “koi sleepy disease”. Initially, fish infected with CEV, suffering from KSD, were observed in Japan in 1974 (Ono et al., 1986; Oyamatsu et al., 1997a). Outbreaks occurred mainly when fish were moved from earth ponds to cement-lined ponds in the spring and autumn seasons (Jung-Schoeners et al., 2015; Lewisch et al., 2015). CEV establishes a persistent infection and later, when the water temperature changes due to the change in season, this leads to activation of the persistent CEV, which manifests itself as KSD in the infected fish host both in farmed fish and/or wild populations. Therefore, with CEV infection handling stress and temperature are crucial factors in activating persistent CEV infection, therefore significantly influencing the morbidity and mortality of the infected carp (Miyazaki et al., 2005; Lewisch et al., 2015; Zhang et al., 2017).

Studying CEV is not a straightforward task as it comes with some challenges, one of which is that efforts to culture CEV in the laboratory using current cell culture methods such as in vitro cultivation have been unsuccessful (Jung-Schoeners et al., 2015; Lewisch et al., 2015; Adamek et al., 2017a) and this hinders progress in the determination of the CEV virus titer, accurate virulence research and molecular virological research (Jung-Schoeners et al., 2015; Lewisch et al., 2015). Currently, researchers such as Oyamatsu et al., (1997) and others have been successful in visualizing poxvirus-like particles in the gill epithelial cells of CEV infected fish using transmission electron microscopy (ETM) (Ono et al., 1986; Hedrick et al., 1997; Oyamatsu et al., 1997a; Miyazaki et al., 2005; Zhang et al., 2017). Therefore, the detection of CEV infection in carp predominantly relies on the testing of gill tissue for the presence of CEV-specific DNA sequences using PCR (Matras et al., 2017; Adamek et al., 2017a), mainly due to the above-mentioned challenge with studying and understanding CEV.

The sequencing of the known DNA fragment encoding p4A the core protein from CEV infected fish from different locations in Europe and Asia discovered a genetic diversity of 6-10% (Matras et al., 2017; Adamek et al., 2017b). The observed diversity allowed for the identification of up to three different genetic lineages (genogroup I; Ila & IIb) (Matras et al., 2017; Adamek et al., 2017a). CEV viruses from the genogroup I infect farmed common carp, while CEV viruses from the genogroup IIa infect koi carp (Matras et al., 2017; Adamek et al., 2017a). CEV viruses from genotype IIb can infect both koi and common carp (Adamek et al., 2017a).

CEV infection has a high mortality rate of up to 80-100% in juvenile koi fish stock (Ono et al., 1986; Oyamatsu et al., 1997a; Miyazaki et al., 2005). This has a detrimental financial effect on koi and common carp farmers as they lose both their stock and profits. Further, the mortality rate of CEV poses a risk to both local and global food security as common carp is mainly cultured for food purposes. The damage and loss caused by CEV infection are further exacerbated by the scarcity of knowledge on CEV (Adamek et al., 2017a), which would contribute greatly towards the mitigation and/or elimination of further spread and the scourge of CEV/KSD. Hence it is imperative to scrutinize and fully comprehend the characteristics of CEV. This paper aims to collate the current knowledge on CEV/KSD, thereby giving an insight into the nature and characteristics of CEV and further outlining challenges with studying the CEV virus.

Signs of CEV

Clinical signs: The most prominent external clinical sign of active CEV infection (KSD) in diseased fish is the display of lethargic behaviour, which is inactivity and sluggishness, hence the name “koi sleepy disease”. Other commonly observed external clinical signs of KSD include pale oedematous gills due to epithelial hyperplasia of the gill filaments (Miyazaki et al., 2005); encephalomalacia (Haenen et al., 2014; Lewisch et al., 2015); haemorrhages, often around the mouth and at the base of the fins; edema of the anal vent and loss of appetite (Oyamatsu et al., 1997a; Miyazaki et al., 2005; Way and Stone, 2013; Haenen et al., 2014; Jung-Schoeners et al., 2015; Swaminathan et al., 2016; Zhang et al., 2017); Overproduction of mucus on the skin and gills is often observed (Lewisch et al., 2015; Pretto et al., 2015; Zhang et al., 2017).

Most KSD diseased fish show enlargement of some of the internal organs, which include the spleen; kidneys and heart. The heart also displays some noticeable colour changes, as it becomes bright brown coloured. The gastrointestinal tract becomes oedematous with no presence of intestinal contents (Lewisch et al., 2015).

Extensive erosions and haemorrhages of the skin with edema of the underlying tissue are often observed particularly in diseased juvenile koi (Miyazaki et al., 2005). Juvenile carp suffering from KSD usually gather together near the surface of a pond or water inlet, whereas older fish tend to lie on the bottom of the pond and eventually die of anoxia, as they cannot take up oxygen due to gill necrosis (Miyazaki et al., 2005; Lewisch et al., 2015). Mortality may reach 80-100% (Ono et al., 1986; Oyamatsu et al., 1997a; Miyazaki et al., 2005).

The clinical signs of CEV and Cyprinid herpesvirus 3 (CyHV-3) are so similar that even a trained eye cannot distinguish between the two diseases (Jung-Schoeners et al., 2015; Ouyang et al., 2018). Therefore, one needs to be very cautious about making diagnoses based on clinical signs displayed by the diseased fish.

Histopathological signs: The target organs of CEV infection are the gills of the infected, therefore the gills will have a higher CEV viral load per DNA unit than
in any other organ of the infected fish (Miyazaki et al., 2005; Swaminathan et al., 2016; Zhang et al., 2017; Adamek et al., 2017a). Therefore, KSD moribund fish exhibit the most severe microscopic damage in the gill tissue. The moribund fish exhibit hypertrophy and severe hyperplasia of branchial epithelial cells resulting in the fusion of adjacent secondary lamellae and the clubbing of the gill filaments (Ono et al., 1986; Miyazaki et al., 2005). The diseased fish become less active and show signs of lethargy (Miyazaki et al., 2005; Lewisch et al., 2015). Subsequently, the fish die due to oxygen deficiency, as the damaged gills retard the intake of oxygen. A concentration of eosinophilic cells is often observed in gill lamellae (Adamek et al., 2018a).

The other histopathological signs include the muscle cells in the lateral musculature of moribund fish becoming cloudy with milky-white colouration. Moribund fish in the 6-7 days post-infection, produce blood with a slight brown colouration signifying that the blood has started producing an abnormal amount of methemoglobin causing methemoglobinemia (Miyazaki et al., 2005).

Changes occur in intra-cytoplasmic organelles in the gills of fish infected with CEV. The cytoplasm of infected fish are enlarged and also contain many glycogen particles, ribosomes, mitochondria, reticula and Golgi apparatuses releasing many small vesicles and inclusions compared to the cells of fish free of CEV. These changes in the cytoplasmic organelles highlight that cells of fish infected with CEV are hypertrophied. The other observation is that infected cells have fine desmosomes, preventing separation from neighbouring cells (Miyazaki et al., 2005).

It is noteworthy to mention that the findings of an epidemiological study carried out in China contradict the observation that gills of CEV/KSD infected fish have a higher CEV viral load per DNA unit than in any other organ of the infected fish, as has been recorded by other researchers such as Ono et al., 1986; Oyamatsu et al., 1997a; Miyazaki et al., 2005; Lewisch et al., 2015; Adamek et al., 2018a. The results from the analysis of samples from an outbreak of carp disease that occurred in a koi pond in the southwest of China showed that the kidneys (8.46x10^6 copies per microgram of total DNA) had a high average viral load when compared with the gills (4.52x10^6 copies per microgram of total DNA), during acute phases of infection in koi fish (Ouyang et al., 2018).

### Epidemiology of CEV infection

#### CEV global distribution and its genetic lineages:
CEV was first discovered in Japan in 1974 from the koi carp (Ono et al., 1986; Oyamatsu et al., 1997a). The virus has since spread throughout the globe and has been recorded in several continents such as Asia, America and Europe. In Asia, where the virus was first discovered, it has been recorded in several countries, namely, Japan as early as the 1970s (Oyamatsu et al., 1997a; Miyazaki et al., 2005); India (Swaminathan et al., 2016); the Republic of Korea (Kim et al., 2017); China (Zhang et al., 2017; Ouyang et al., 2018); Thailand (Pikulkaew et al., 2009; Pikulkaew et al., 2020) and Iraq (Tofsan et al., 2020). In the United States of America CEV has been reported (Hedrick et al., 1997; Habibi et al., 2015; Viadanna et al., 2015; Lovy et al., 2018; Stevens et al., 2018). In Europe the virus has been recorded in several countries such as the United Kingdom (Way and Stone, 2013); the Netherlands (Haenen et al., 2014); Austria (Lewisch et al., 2015); Italy (Pretto et al., 2015); Germany (Jung-Schroers et al., 2015); France (Bigarré et al., 2016); Poland (Matras et al., 2017); Hungary (Adamek et al., 2018a); Ireland (Braithwaite, 2018); the Czech and Slovak Republics (Matějíčková et al., 2020); and Croatia (Zrnčić et al., 2020). Each one of the reported CEV/KSD outbreaks varies in magnitude but they all cause huge monetary and stock losses to farmers. The geographical distribution of CEV infection may widen as climate change is occurring at an alarming rate (Pankhurst and Munday, 2011) as changing climates come with the great migration of aquatic animal species and their pathogens.

Owing to the high diversity of CEV-specific DNA fragment encoding, the core protein p40, three genotypes I, IIa & IIb have been recognized. CEV viruses from genogroup I infect farmed common carp, while CEV viruses from genogroup IIa mainly infect koi (Matras et al., 2017; Adamek et al., 2017a; Su and Su, 2018). The results of an epidemiological study have revealed that both koi and common carp are susceptible to CEV viruses from genogroup IIb (Adamek et al., 2017a). Recently, two new additional CEV genotypes, namely IIa and IIIb, have been reported. The discovery was made in a study that sought to understand the phylogenetic relationship between the three already known CEV genotypes (I; IIa & IIb) and the Austrian isolates (Soliman et al., 2019). However, a proposal for the addition of the two new Austrian CEV genotypes was disputed, as the two additional genotypes were due to an analytical error. The sequences belonging to the proposed new Austrian CEV genotypes, which were submitted to the GenBank, showed the sequence of nucleotides in a reverse order and, after reversing the order, these sequences belonged to the already established and existing genotypes I and II (Zrnčić et al., 2020).

#### Composition and structure of CEV:
CEV has two stages, immature virions and mature virions found in the cytoplasm of the gill epithelial cells (Miyazaki et al., 2005). Mature virions are oval-shaped with a length of 288–337 nm (mean; 313 ± 16 nm) and width of 238–300 nm (mean; 273 ± 19 nm) as recently reported by Lovy et al. (2018), while Miyazaki et al. (2005) reported a length of 400-413 nm and breadth of 333-400 nm. Mature virions are pleomorphic in shape (Miyazaki et al., 2005). The surface membrane of the mature virions is covered with 10 to 13 cylindrical projections on one side (Lovy et al., 2018), that are about 50 nm in diameter (Miyazaki et al., 2005) and about 31–56 nm (mean; 41 ± 8.4 nm) in length (Lovy et al., 2018). Mature virions enclose a unilateral inward-curved core (dumbbell formed) that is most electron-dense at the boundary and a single lateral body with an unclear outline at the invaginated point on the side opposite to the cylindrical projections (Miyazaki et al., 2005; Lovy et al., 2018).

#### Activators of the persistent CEV:
Many researchers have reported that water temperature and handling
stress (restocking; transporting & transferring) are crucial factors that lead to the activation of persistent CEV to outbreaks of KSD in both wild and cultured common carp and koi fish populations (Miyazaki et al., 2005; Lewisch et al., 2015; Zhang et al., 2017). Water temperature is known to influence the onset and severity of viral infection by altering virus replication and indirectly by augmenting the efficacy of the host immune response (Alcorn et al., 2002). Further, temperature is a key influence in controlling the immune-compentence of fish (Köllner and Kotterba, 2002; Bowden, 2008; Gorgoglione et al., 2013). The functions of both cellular and humoral immunity in fish is greatly affected by water temperature (Pokorova et al., 2005). Therefore, water temperature affects fish (both common and koi carp) morbidity and mortality in the case of CEV infection (Miyazaki et al., 2005; Magnadottir, 2010; Lewisch et al., 2015; Zhang et al., 2017). Temperature may also directly influence the degree of parasite proliferation and development (Noe and Dickerson, 1995; Karvonen et al., 2010). Most recorded cases of KSD outbreaks in koi carp populations occurred in the Spring and Autumn seasons, when the water temperature was within the range of 15 to 25 °C (Miyazaki et al., 2005; Jung-Schroers et al., 2015; Matras et al., 2017; Way et al., 2017). In common carp populations, KSD outbreaks occur mostly at low water temperatures when the water temperature falls to the range of 6 to 9 °C (Miyazaki et al., 2005; Matras et al., 2017) and the disease has a lower mortality rate but with a longer course than that in the koi population (Miyazaki et al., 2005). This is because temperature significantly controls the course of the disease in poikilothermic vertebrates (Ahne et al., 2002).

It has been reported that handling stress, resulting from restocking, transferring and/or transportation is also an important influencing factor responsible for CEV/KSD outbreaks (Lewisch et al., 2015). This is evident as KSD outbreaks have generally been observed when cultured fish are moved from earthen ponds to cemented tanks (Jung-Schroers et al., 2015). The transfer of fish stresses the animals. Subsequently, the high level of stress in fish triggered the activation of persistent CEV leading to the outbreaks that were observed.

**CEV and Flavobacteria co-infection:** A recent study by Adamek et al. (2018b), sought to understand the relationship between CEV and other co-pathogens such as parasites and bacteria species present on gills, the skin or internal organs in the development of KSD. In the study, the researchers examined selected field samples from Germany and Hungary and confirmed the presence of CEV and flavobacteria co-infections in the subset of samples. The study concluded that *Flavobacterium branchiophilum* is a possible co-pathogenic infection to CEV but CEV is the etiological agent of KSD as *Flavobacterium branchiophilum* is not required for the development of clinical KSD (Adamek et al., 2018b). The results of a recent study found *Flavobacterium succinicans* and *Flavobacterium sasangense* as predominant bacterial co-pathogens in fish infected with CEV (Adamek et al., 2019). The results of the study suggest that copies of flavobacteria DNA were most abundant in the gills followed by the fins and kidneys. In Korea, *Flavobacterium granuli* has long been identified as the main colony in KSD moribund fish (Aslam et al., 2005) but no clinical incident of infection by these microorganisms has been reported (Kim et al., 2017).

The down regulation of mucin mRNA expression in the gills and gut of pathogenic virus-infected common carp, such as CEV infection, causes severe distress to the mucosal tissue in infected carp (Adamek et al., 2017c). This helps to increase the susceptibility of virus-infected carp to secondary bacterial infection, as the expression of mucin is reduced (Adamek et al., 2017c; Adamek et al., 2018b). In the case of CEV infected fish, Flavobacteria takes advantage of the weakened defence abilities of the fish and uses the gills as an entry point.

**Transmission pathways:** CEV is transmitted through cohabitation via water media in the wild or in tanks. The virus is shed from the gills of the infected fish and contaminates the water in which both the infected and naïve fish cohabits (Oyamatsu et al., 1997a; Adamek et al., 2017a). Subsequently, the virus gets deposited onto the gills of naïve fish, as the gills act as an entry point, therefore infecting the fish with the CEV virus. It is speculated that there may be other biotic carriers of CEV, such as other fish species (Adamek et al., 2017a; Matras et al., 2019); fish lice or fish-eating birds that wade from one stretch of water to another in search of food but there is no evidence of this at present. In laboratory experiments, CEV virus is transmitted by the cohabitation of both infected and naïve fish in the same water or by inoculating naïve fish with CEV positive gill tissue homogenate filtrate or by introducing CEV positive gill tissue homogenate filtrate into the water with naïve fish (Oyamatsu et al., 1997a; Zhang et al., 2017; Adamek et al., 2017a; Matras et al., 2019).

There are still huge knowledge gaps concerning the transmission pathways of CEV (Matras et al., 2019). Way et al. (2017), suggests that addressing the following knowledge gaps amongst others would shed some light on the transmission pathways of CEV. He states that some of the gaps in knowledge include (i) understanding the survival mechanism of the CEV virus outside of the host; (ii) discovery of other susceptible aquatic species (carriers) other than carp; (iii) does the virus exist as a low-level persistent infection and are there aquatic vectors?; (iv) what is the prevalence in carp populations?; (v) and discovering other important environmental factors responsible for triggering CEV/KSD outbreaks (Way et al., 2017). The other tricky question is whether the CEV virus can be vertically transmitted from an infected parent fish to off-springs through eggs or/and sperm?

Recently, it has been reported that other fish species are a potential vector of the CEV virus when they cohabit with CEV-infected koi and/or common carp (Adamek et al., 2017a; Matras et al., 2019). The DNA of the CEV virus has only been detected on the gills and skin of the vector fish species, with no apparent signs and mortality of vector species from CEV infection reported. The optimal cohabitation period is 12 hours.
for the transmission of the CEV virus, from the infected koi and common carp fish to vector fish species.

**Prevention and Treatment of CEV/KSD:** The application of prophylactic salts in water is the most used prevention method, with 0.5 % (5 g/L) salt immersion in the fish pond/tank water, which researchers have recommended as an effective method to prevent outbreaks of KSD (Oyamatsu et al., 1997a; Seno et al., 2003; Miyazaki et al., 2005). The other encouraging prevention method is the adjustment of water temperatures to temperatures that are non-permissive for the activation of CEV since KSD is temperature-dependent. This method inactivates the virus but does not eradicate the virus in fish infected with CEV. The temperature adjustment should be species-dependent as CEV permissive temperature ranges are different for koi carp to the common carp, 15–25 °C and 6–9 °C, respectively. Currently, CEV infection/KSD does not have a cure and the application of prophylactic salts in pond or tank water is used to treat the clinical signs of KSD but does not cure the disease (Stevens et al., 2018). This method has been used since the early cases of KSD were recorded in Japan. Currently, it is common practice to treat receiving ponds/tanks with salt and allow them to dry before transferring fish (Seno et al., 2003; Hesami et al., 2015; Jung-Schroers et al., 2015; Stevens et al., 2018). Salt has a prophylactic effect, therefore, salt treatment is a supportive treatment rather than a curative treatment (Seno et al., 2003; Stevens et al., 2018). The main benefit of prophylactic salt treatment is that it alleviates the osmotic stress caused by gill and skin lesions but does not eradicate and/or inactivate the CEV virus in infected fish (Seno et al., 2003; Stevens et al., 2018). When an infected fish is in an advanced diseased state, salt treatment may be ineffective (Stevens et al., 2018). In surviving fish, CEV persists in the population for some time even after the clinical signs have subsided. A study done by Lovy et al. (2018), demonstrated that after 5 months CEV became negative as a realtime qPCR was unable to detect CEV in the DNA extracted either directly from gill tissue or from gill tissue processed to purify/concentrate CEV particles of surviving carp after CEV fish mortality. Further, the results of Adamek et al. (2017a), demonstrated the presence of the virus could not be confirmed in survivors of clinical KSD by qPCR one month after the last clinical signs had been recorded. Therefore, KSD survivors do shed off the virus and do not develop a persistent sub-clinical infection (Adamek et al., 2017a). Also, Stevens et al. (2018), reported that gill samples collected 11 months post-KSD diagnosis and the initiation of 0.3-0.5% salt treatment showed fish that had previously been CEV-infected were clear of virus, as all the samples tested negative for CEV by quantitative PCR. More research is needed to confirm the exact length of CEV persistence before the virus is cleared in surviving fish after a mortality event.

Good hygiene practices (farm biosecurity) are generally important to prevent the spread of disease in aquatic farms regardless of their size (Subasinghe et al., 2001; Peeler and Taylor, 2011). Effective farm biosecurity means that good hygiene practices are in place on the entire farm especially in the aquatic animal holdings. Generally, biosecurity programs advise that farmers should buy stock (eggs, larva and broodstock) from registered suppliers, which have biosecurity measures in place for disease-free stock. A farmer must ensure that there is no mixing of animals from different supplier farms on delivery vehicles. Once the stock has arrived at the farm, it has to be quarantined and monitored for a certain period to ensure that it is disease-free and to prevent the introduction and spreading of infectious diseases throughout the farm (Lee and O’Bryen, 2003). That is also the case with CEV infected fish stocks, even after a successful salt treatment, since the CEV virus is not eradicated and can be still passed to naïve fish.

Application of HACCP (Hazard Analysis and Critical Control Point) strategy at the grow-out areas and ponds can greatly help in the prevention of CEV spreading from infected fish to naïve fish or through fomites. The application of HACCP in this instance requires identifying and controlling the hazard (CEV) by eliminating cross-contamination. This is achieved through proper cleaning and disinfecting of fish habitats; personnel hands and protective clothing; handling equipment and gear; checking water quality in the holding tanks and/or ponds; separating and quarantining newly purchased stock and purchasing stock from certified sellers; fencing and netting outside ponds to eliminate contact with foreign organisms such as birds which might introduce CEV by wading from one pond to another.

**Detection methods of CEV:** Currently, it is not possible to detect CEV infection by re-isolation in cells, as in vitro cultivation of CEV is not feasible with currently available fish cell lines (Jung-Schroers et al., 2015; Lewisch et al., 2015; Adamek et al., 2017a; Adamek et al., 2018a). Therefore, detection of CEV is possible by these types of methods: (i) observation of CEV clinical signs; (ii) PCR-based detection methods; and (iii) Recombinase Polymerase Amplification (RPA) based method.

**Laboratory-based methods:** At the moment, there are five different most used PCR-based detection methods (assays) that have been developed by various researchers for the detection of CEV-specific DNA. All five of these different PCR-based detection methods were designed using sequences of CEV DNA fragments coding protein pka (Adamek et al., 2017a) in the development of these detection assays some were based on virus-specific sequences obtained from CEV infected koi or common carp species respectively, while some used sequences obtained from both CEV infected koi and common carp species. Each of the five different PCR-based detection methods have their advantages and/or disadvantages when it comes to the efficacy of detection, specificity and quantification of CEV-specific DNA viral load in the infected fish. The high genetic variability of the CEV pka gene used in these PCR-based detection methods could be a serious obstacle to successful and reliable detection of virus infection in field samples when an assay employing a double-label probe is used (Adamek et al., 2017b). This is due to some of the PCR-based detection methods having low specificity and sensitivity and therefore
only being able to detect one specific genotype of CEV-specific DNA in diseased fish.

The five commonly used PCR-based detection methods for the detection of CEV-specific DNA are:

(i) the end-point PCR designed by Oyamatsu et al. (1997b);
(ii) the CEFAS end-point PCR assay developed by CEFAS and published by Matras et al. (2017);
(iii) the CEFAS quantitative (probe) PCR assay published by Matras et al. (2017);
(iv) the TiHo quantitative (probe) PCR assay published by Adamek et al. (2016);
(v) the TiHo SYBRGreen quantitative PCR assay designed by Adamek et al. (2017a).

Each of these PCR-based detection methods is discussed in depth below.

<table>
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<th>Table 1</th>
<th>Sequences of primers and probes used in the PCR methods employed in the detection of CEV.</th>
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<tr>
<td><strong>PCR assay</strong></td>
<td><strong>Primer name</strong></td>
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<tr>
<td>Oyamatsu end-point</td>
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<td>Oyamatsu nested end-point</td>
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<td>CEFAS nested end-point</td>
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<td>CEFAS quantitive probe qPCR</td>
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<td>CEFAS qR</td>
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<td>TiHo probe qPCR</td>
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<td>TiHo_qR</td>
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<td>TiHo_q_Probe</td>
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<td>TiHo SYBRGreen qPCR</td>
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<td>TiHo_SYbbr_qR</td>
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**The CEFAS end-point PCR assay developed by CEFAS and published by Matras et al. (2017):** The CEFAS end-point PCR assay was developed by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in the United Kingdom, this assay was developed using CEV-specific DNA extracted infected koi fish and common carp samples from United Kingdom (Matras et al., 2017; Adamek et al., 2017a). The advantage of CEFAS quantitative PCR assay is that it has a high specificity; sensitivity and quantitative nature as it can detect CEV-specific DNA in a sample regardless of fish species (koi and common carp) and CEV genogroup, but it is a non-quantitative essay (Adamek et al., 2017a).

In Table 1, below in the Table section are the sequences of the primers and probes used in the CEFAS end-point PCR assay mentioned above. This assay was also developed using CEV-specific DNA extracted infected koi and common carp samples from the United Kingdom (Matras et al., 2017; Adamek et al., 2017a). The advantage of CEFAS quantitative PCR assay is that it has a high specificity; sensitivity and quantitative nature as it can detect CEV-specific DNA in a sample regardless of fish species and CEV genogroup (Adamek et al., 2017a).

In Table 1, below in the Table section are the sequences of the primers and probes used in the CEFAS quantitative (probe) PCR assay developed by CEFAS and published by Matras et al. (2017).

**The TiHo quantitative (probe) PCR assay published by Adamek et al. (2016):** The TiHo quantitative (probe) PCR assay was developed at the University of Veterinary Medicine in Hannover (TiHo) based on CEV sequences from koi samples in Germany (Adamek et al. 2016). According to the results of Adamek et al. (2017b), this assay is somewhat effective for the detection of CEV virus from genogroup IIa and genogroup IIb (Adamek et al., 2017a), which are predominately found in koi species.
In Table 1, below in the Table section are the sequences of the primers and probes used in the TiHo quantitative (probe) PCR assay published by Adamek et al. (2016).

The TiHo SYBRGreen quantitative PCR assay designed by Adamek et al. (2017a): The TiHo SYBRGreen quantitative PCR assay was designed by Adamek et al. (2017a), based on CEV sequences from koi samples in Germany (Adamek et al., 2017a). The TiHo SYBRGreen quantitative PCR assay was designed to improve on the shortcomings of the TiHo quantitative (probe) PCR assay, by introducing the use of an intercalating dye – SYBRGreen. This PCR assay was developed at the University of Veterinary Medicine in Hannover (TiHo).

The results of a study done by Adamek et al. (2017b) showed that the TiHo SYBRGreen qPCR assay had a significantly higher analytical inclusivity and diagnostic sensitivity in samples infected with CEV from genogroup I but also genogroups Ila and IIb (Adamek et al., 2017b).

In Table 1, below in the Table section are the sequences of the primers and probes used in the TiHo SYBRGreen quantitative PCR assay designed by Adamek et al. (2017a).

Conclusion on laboratory-based CEV detection methods: Amongst these PCR-based CEV detection methods discussed above, the CEFAS quantitative (probe) PCR assay is the most effective (Adamek et al., 2017a). The CEFAS quantitative (probe) PCR assay has a high specificity; sensitivity and quantitative nature as it can detect CEV-specific DNA in a sample regardless of carp fish species and CEV genogroup.

Field detection methods: Currently, there are not many field methods available for the accurate and precise detection of CEV infection in fish populations. The most used method in the field is the observation of clinical signs displayed by the diseased fish in an event of an outbreak. The problem with using clinical signs to identify CEV infection is that fish suffering from CEV infection display similar clinical signs to those of fish suffering from KHV (CyHV-3) (Miyazaki et al., 2005; Jung-Schroers et al., 2015; Soliman and El-Matbouli, 2018). Therefore, it is very easy to make an incorrect diagnosis of the cause of sickness in the diseased fish population, even for a trained eye.

Soliman and El-Matbouli (2018) developed and optimized rapid and accurate single and multiplex isothermal diagnostic tools, based on Recombinase Polymerase Amplification (RPA), for the detection and differentiation of CEV (Soliman and El-Matbouli, 2018). Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification technique, which relies on a combination of recombinase, single-strand binding protein and strand-displacing DNA polymerase for DNA amplification at a constant temperature between 37 and 42°C (Piepenburg et al., 2006). Due to RPA running at low temperatures and not following Watson–Crick DNA base-pairing rules, it has minimal undesired amplification products that could otherwise impede amplification of the target (Sharma et al., 2014). CEV-RPA assays are specific to the target virus and have lower detection limits similar to those of PCR-based detection methods for CEV.

CEV-RPA assay was developed by Soliman and El-Matbouli (2018) to be used along with a lateral flow dipstick to allow for visual detection of the amplification products and also to allow a post-amplification analysis of the products. The use of a lateral flow dipstick gives the CEV-RPA detection method an advantage to be used in the field for CEV screening of fish and acquiring diagnostic results in a short time (Soliman and El-Matbouli, 2018), in approximately 50 minutes, without the need to send samples to the laboratory, which consumes time and money. Due to the recency of its development, the CEV-RPA detection method is currently not approved by the FDA (Lobato and O’Sullivan, 2018). Error! Reference source not found. below, in the Table section, shows the summarized knowledge on the recombinase polymerase amplification (RPA) technique.

Table 2 Summary of recombinase polymerase amplification (RPA) technique.

<table>
<thead>
<tr>
<th>Isothermal technique</th>
<th>Target</th>
<th>Primers needed</th>
<th>Initial heating</th>
<th>Incubation temperature (°C)</th>
<th>Amplification time (min)</th>
<th>Detection Limit (copies)</th>
<th>Multiplexing</th>
<th>Lyophilised reagents</th>
<th>FDA approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPA</td>
<td>DNA</td>
<td>Yes</td>
<td>No</td>
<td>37-42</td>
<td>20-40</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Table adapted from (Lobato and O’Sullivan, 2018)

Conclusion and future directions: In conclusion, CEV is a highly detrimental infection, which leads to huge monetary loss and further threatens both food supply and ecology. Therefore, it is imperative to find alternative methods that not only prevent the spread of CEV but also totally eradicate the CEV virus in infected fish. More research is needed to fully comprehend the genome of CEV, which will allow for advancement in coming up with effective and efficient field methods for the detection, specificity and quantification of CEV. Subsequently, this will help with the quick detection and prevention of CEV in the wild koi and common carp fish populations. To achieve the above, firstly, the transmission pathways, including carriers, of CEV need to be fully understood to be able to curb the CEV spread along with the disaster that comes with the infection and disease. Therefore, this knowledge will go a long way into facilitating innovation of coming up with various alternative treatment methods.

CEV/KSD should be listed in the World Organization for Animal Health (OIE) to create global awareness given the destructive nature of the disease and the financial risk it is to the farmers. Farmers along with a team of multi-disciplinary specialists need to come up with effective biosecurity programs to alleviate the scourge of CEV/KSD. Such a biosecurity program may also include or follow HACCP guidelines to eliminate cross-contamination between
fish stocks and gear used in the farming activities. Biosecurity programs will protect the farmers against the introduction of the infection and disease and this will promote optimum environments and conditions for healthy fish and protects the financial investments of farmers. Human beings are spared from CEV infection as fish diseases do not infect humans, thus CEV is not considered harmful to humans (Hesami et al., 2015).

The use of RPA with dipsticks, as developed by Soliman and El-Matbouli (2018), is a promising method for the detection of all three CEV genotypes, the method has a high sensitivity and specificity degree that is on par if not slightly higher to those of PCR-based methods. The quick turnaround time to acquire results and field usability makes the RPA method ideal when making a diagnosis in the field in the urgent case of an outbreak.

References


