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Short communication

Laboratory validation of a lateral flow device for the detection of CyHV-3 antigens in gill swabs

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A B S T R A C T

Cyprinid herpesvirus-3 (CyHV-3) induces the highly contagious koi herpesvirus disease (KHVD) and may result in significant economic losses to the ornamental and food-producing carp industry. Suspicion of KHVD is triggered by clinical signs and confirmed using laboratory techniques. The latter are labour- and time-consuming, require specialised equipment and trained personnel. For rapid, on-site detection of CyHV-3, a lateral flow device (LFD) was developed using two monoclonal antibodies directed towards the viral glycoprotein ORF65. The LFD was highly specific with analytical and diagnostic specificities of 100%. Analytical sensitivity ranged between 1.25×10^2 and 2.40×10^4 plaque forming units per ml for isolates originating from geographically distinct regions. In experimentally infected carp, CyHV-3 was detected as early as 4–5 days post infection. Diagnostic sensitivities of 52.6% and 72.2% relative to PCR were recorded, depending on the viral isolate used. When onset of mortality was taken as reference, diagnostic sensitivities increased to 67.0% and 93.3%. The diagnostic sensitivity for freshly found-dead animals was 100%, irrespective of the virus isolate used. Given the high specificity and ease-of-use for on-site detection of CyHV-3, the LFD was regarded fit for purpose as a first-line diagnostic tool for the identification of acute CyHV-3 infections in KHVD affected (koi) carp.

Cyprinid herpesvirus-3 (CyHV-3, also known as koi herpesvirus or KHV) is the causative agent of koi herpesvirus disease (KHVD), a highly contagious disease of common carp (*Cyprinus carpio*) and its colourful ornamental varieties (koi) (Waltzek et al., 2005). KHVD may result in mortality rates as high as 70–100%. The disease was first reported in Israel in the late 1990s; then rapidly spread throughout the world (Michel et al., 2010a). This rapid spreading was attributed to international fish trade and to koi shows organised around the world. Outbreaks of KHVD are often associated with considerable economic damage to both the food-producing and the ornamental carp industry (Haenen et al., 2004). In order to adequately control KHVD outbreaks, rapid diagnosis is crucial. In general, suspicion of CyHV-3 infection in naive carp populations is triggered by the observation of clinical signs of disease. Even though KHVD does not induce pathognomonic signs, lethargy, overproduction of mucus, discolouration of the gills, and herpetic lesions of the skin have often been associated with CyHV-3 infection. Neurological signs such as disorientation and loss of equilibrium may be observed at a later stage (OIE, 2012).

Diagnosis of CyHV-3 mainly relies on laboratory techniques, whereby the virus is detected either directly (*e.g.* CyHV-3 isolation on susceptible cell cultures or PCR-based techniques) or indirectly (detection of virus-induced antibodies by ELISA or neutralisation assays). Particularly, highly sensitive PCR-based techniques have been suggested as the gold standard for CyHV-3 diagnosis (Bergmann et al., 2010), although the World Organisation for Animal Health (OIE) recommends a combination of detection methods (OIE, 2012). A common drawback of all laboratory techniques is that they are labour- and time-consuming, require specialised equipment and trained personnel. A rapid assay, suitable for use under field conditions, could thus be a valuable tool for carp and koi breeders as well as veterinarians in diagnosing CyHV-3 "on-site". The present study aimed at developing and validating an immunochromatographic test for the specific detection of CyHV-3 antigen using a non-invasive approach.

A characterised panel of monoclonal antibodies (mAbs), originating from mice immunised with CyHV-3, were screened by ELISA for reactivity against CyHV-3. Two highly reactive mAbs, hereafter

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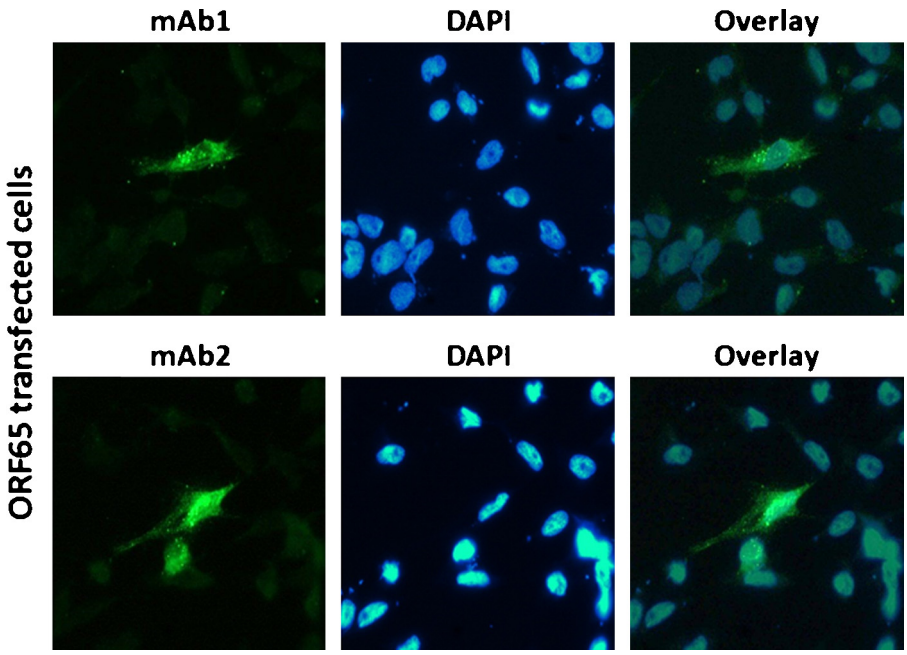

Fig. 1. Indirect immunofluorescence staining of CCB cells transfected with a pcDNA3 derived vector expressing ORF65. Left panels show immunoreactivity of mAb1 and mAb2 with ORF65 as revealed by Alexa fluor probe 488 (green signal). The blue fluorescence represents the DAPI nuclear counterstain (middle panels). An overlay of both fluorescent signals is depicted in the right panels.

called mAb1 and mAb2, both specific for the structural ORF65 protein (Michel et al., 2010b) were selected for incorporation into a lateral flow device (LFD). The molecular target of these mAbs was verified by indirect immunofluorescence staining of common carp brain cells (CCB) transfected with pcDNA3 derived vector expressing CyHV-3 ORF65 (Invitrogen). Cells were transfected using the Lipofectamine Plus reagent (Invitrogen). Transfected cells were stained using either mAb1 or 2 as primary antibody and an Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G as the second conjugate as published earlier (Costes et al., 2008). In parallel, a nuclear counterstain (DAPI) was performed according to the manufacturer's protocol (Invitrogen). Microscopic analysis, carried out as described earlier (Costes et al., 2008), revealed a positive immunoreactivity of mAb1 and mAb2 with ORF65 (Fig. 1).

Using a proprietary method (Coris BioConcept SPRL), mAb1 was coupled to colloidal gold particles, adsorbed on a conjugate pad and applied to the base of a plastic-backed nitrocellulose membrane. The mAb2 was immobilised on the nitrocellulose membrane at the test line (T) and a control antibody (*in casu* a goat anti-mouse antibody) was immobilised at the control line (C). The LFD was assembled by embedding the test strip in a plastic cassette (see Fig. 2A).

To determine the LFD's analytical sensitivity, two-fold serial dilution series of *in vitro* cultured isolates of CyHV-3 were eluted in 250 μl of a proprietary elution buffer (Coris BioConcept SPRL). The test was carried out by transferring 100 μl of eluent to the sample pad of the LFD. Once applied, the gold-conjugated mAb1 is reconstituted in the conjugate pad. When CyHV-3 antigen is present in the eluent, an antigen/mAb1 complex is formed. This complex migrates through the nitrocellulose membrane by capillary force and encounters the immobilised mAb2 at *T*. Consequently, the antigen/mAb1 complex is captured at *T* and a reddish-brown colour appears (Fig. 2B). Remnants of the conjugated complex migrate further and are captured by the goat anti-mouse control antibody resulting in a similar signal at *C*, substantiating the test result (Fig. 2B). Results of the test are read 15 min following application of the eluent to the sample pad. The analytical sensitivity was evaluated with four CyHV-3 strains originating from three countries. The FL (Costes et al., 2008) and the M3 strains were isolated from the kidney of carp that died of KHVD in two distinct geographical regions of Belgium. A German isolate (G) (kindly provided by Dr. Fuchs, FLI, Germany) and an Israeli isolate (I) (kindly provided by Dr. Kotler, The Hebrew University Hadassah Medical School, Israel) were also used. All CyHV-3 strains were produced on CCB cells. Clarified cell supernatants were stored at −80 °C and titrated before use. The LFD was able to detect all tested isolates with an analytical sensitivity ranging from 1.25×10^2 to 2.40×10^4 plaque forming units per ml (pfu/ml) (Table 1).

The analytical specificity of the LFD was evaluated using other known viral pathogens of carp, namely *Spring viraemia of carp virus* (SVCV; kindly provided by Dr. Bremont, INRA, France) and *Cyprinid herpesvirus-1* (CyHV-1; UKG364 P2 isolate kindly provided by Dr. Keith Way, CEFAS, UK). SVCV and CyHV-1 were produced on Epithelioma Papulosum Cyprini (EPC) cells and KF-1 (koi fin) cells, respectively. Clarified cell supernatants were stored at −80 °C and titrated before use. SVCV was chosen because this carp pathogen, like CyHV-3, can induce mortality without associated pathognomonic clinical signs; whereas CyHV-1 is genetically the most closely-related aquatic pathogen to CyHV-3 able to infect common carp. No cross-reactivity or false positive results were observed on the LFD when the analyses where performed on a twofold dilution series in elution buffer of the virus stock of SVCV (3.5×10^8 pfu/ml) and CyHV-1 (2.4×10^6 pfu/ml); indicating an analytical specificity of 100% for the LFD and ensuring the correct diagnosis of KHVD in case of a positive LFD result.

To define the diagnostic sensitivity of the LFD, two identical experimental infection studies in carp were carried out using

Table 1
Analytical sensitivity of the LFD on CyHV-3 isolates from different geographical regions.

CyHV-3 isolate	Origin	Detection limit (pfu/ml)
I	Israel	1.5×10^3
FL	Belgium	2.4×10^4
M3	Belgium	3.1×10^3
G	Germany	1.25×10^2