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Short communication

Serological analysis of historical field samples reveals major inconsistency between PCR and antibody ELISA for establishing KHV infection status of groups and individual koi

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Running head: Discrepancies between ELISA and PCR testing for KHV in the field

Abstract

Koi herpesvirus (KHV) is the causative agent of a highly infectious and notifiable disease of *Cyprinus carpio* L. Serology has the potential to identify koi or carp that have been previously exposed to KHV and which may be possible carriers of the virus. In the present study, sera (n=162) from groups of farmed koi carp, previously screened for KHV using a variety of molecular methods as part of a surveillance program in Asia from 2008-2010, were subsequently tested here individually by ELISA using plates coated with purified virus (American isolate KHV-I, H361). Only 31% of koi from PCR-positive KHV fish groups or populations associated with KHV disease (n=59/162) were seropositive when screened in the ELISA at a serum dilution of 1/200, in contrast to 52.9% of seropositive koi that were KHV-negative by PCR (n=103/162). Furthermore >34% of those seropositive/PCR negative fish had titres of >1/400 (moderate-strong responders). This field data highlights the concerns related to carp populations that have been screened for KHV using molecular methods alone and supports the need for serology to accompany molecular testing in carp for this notifiable virus.

Keywords: CyHV-3, serology, antibodies, ELISA, koi herpesvirus, field cases

Abbreviations: ARF, Aquatic Research facility; BCA, bicinchoninic acid assay; BSA, bovine serum albumin; CEFAS, Centre for Environment, Fisheries and Aquaculture Science; CyHV-3, CCB, common carp brain cells; CPE, cytopathic effect; Cyprinid herpesvirus 3; DNA, deoxyribonucleic acid; DPBS, Dulbecco's phosphate buffered saline; EMEM, Eagle's minimum essential medium; ELISA, enzyme-linked immunosorbant assay; FBS, foetal bovine serum; HRP, horse radish peroxidase; HSWB, high salt wash buffer; IoA, Institute of Aquaculture; KF-1, Koi fin cells; KHV, Koi herpesvirus; KHVD, Koi herpesvirus disease;

LSWB, low salt wash buffer; Mab, monoclonal antibody; NEAA, non-essential amino acids; OD, optical density; OIE, Office International des Epizooties; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SNT, serum neutralisation test; TCID, tissue culture infectious dose; TE buffer, Tris-sodium chloride buffer; TMB, 3,3',5,5'-Tetramethylbenzidine.

1. Introduction

Koi herpesvirus (KHV), taxonomically classified as Cyprinid herpesvirus 3 (CyHV-3), is an *Alloherpesvirus* of the order *Herpesvirales* (Waltzek et al., 2005; Davison et al., 2009) and the causative agent of the highly infectious and lethal disease of Koi (*Cyprinus carpio* L.), koi herpesvirus disease (KHVD) (Hedrick et al., 2000). Koi herpes virus has affected not only farmed carp (Gotesman et al., 2013), but also fisheries and wild carp stocks (Uchii et al., 2009; Taylor et al., 2010).

A number of temperature manipulation studies have demonstrated latent- and / or persistent-like infection of KHV (Gilad et al., 2003, 2004; St-Hilaire et al., 2009; Eide et al., 2011; Reed et al., 2014), where low virus levels were detected without notable virus replication. This makes detection of KHV in apparently latently or persistently infected, clinically healthy fish, a challenge (Bergmann et al., 2010; Matras et al., 2012). The rapid spread of KHV worldwide may have been attributed to false negative results following screening of such fish by PCR, due to limitations in the sensitivity of molecular diagnostics for detecting KHV DNA (Bergmann et al., 2010; Matras et al., 2012; Monaghan et al., 2015). An alternative way to identify carrier fish may be through the detection of KHV-specific antibodies in the serum of fish by ELISA, indirectly indicating that the fish is or has been infected with the virus (Bergmann et al., 2017;; OIE, 2019; Soto et al., 2020).

The use of ELISA for monitoring the infection status of aquatic animals is rare compared to terrestrial animals (La Patra, 1996; Denzin and Staak, 2000). Health certification based on OIE guidelines for KHVD (OIE, 2019) currently places more emphasis on the use of molecular methods due to difficulties in validating serological methods. Previous ELISAs have not been entirely specific for KHV, especially at serum dilutions of $\leq$1/400 (Adkison et al., 2005; St-Hilaire et al., 2009), limiting the conclusions that can be drawn from a positive outcome. Laboratory-based studies have highlighted the usefulness of serological diagnostics

where molecular methods may fail to detect subclinical infections (Matras et al., 2012; Soto et al., 2020). However, there is limited data published pertaining to comparison of molecular and serological KHV diagnosis discrepancies from field samples.

The aim of the present study was to determine the discrepancies between diagnostic testing using antibody serology from a 1/200 serum dilution, and molecular testing from samples of farmed fish groups previously screened by molecular methods. To achieve this, serum samples from a KHV surveillance program conducted in Asia between 2008 and 2010, including 35 independent groups from four different countries, were analysed using a whole virus ELISA.

2. Materials and Methods

2.1 Control fish sera

High titre anti-KHV sera (1/1600), pooled from experimentally infected koi (kindly provided by Dr. Keith Way, Centre for Environment, Fisheries and Aquaculture Science; CEFAS), were used as a positive control in the ELISA. This sera was previously used to develop and optimise an ELISA based on whole-purified KHV (St-Hilaire et al., 2009). Sera from mirror carp (30 – 40 g) obtained from a farm with no previous history of KHVD (Hampshire Carp Hatcheries, Hampshire, UK) were used as a negative control. These fish were negative when screened by ELISA (using protocol described below) and qPCR (Gilad et al., 2004) just prior to analyses (within 2 weeks).

2.2 Koi serum samples from Asian surveillance programme

Sera were collected from 162 individual koi imported from China, Japan, Malaysia and Singapore into Singapore by the Animal and Plant Health Laboratories, Agri-Food and Veterinary Authority of Singapore (AVFA Lorong Chencharu, Singapore), between December 2008 and December 2010. Sera used in the present study were kindly provided by

Ms. Yahui Wang (AVFA) and included sub-samples taken from a minimum of 30 non-selectively randomly collected fish from each import consignment. The source of country, clinical signs of koi and PCR results from all the cases were also recorded and provided to the University of Stirling prior to ELISA analysis (Table 1). Six organ pools (brain, gill, kidney, intestine, liver and spleen from 5 random non-selectively collected fish) for each submitted case (group of fish), were tested for KHV using a commercial PCR kit (KHV IQ2000 Test kit, GeneReach Biotechnology, Taiwan), the PCR described by Bercovier et al. (2005) and Yuasa et al. (2005) and / or real-time PCR developed by Gilad et al. (2004), providing 5 independent molecular testing outcomes. The analytical groups were divided into 2 categorical groups depending on their KHV status. Group 1 consisted of samples taken from fish where KHV DNA had been detected in tissues by PCR as described or where sampled fish had been historically associated with KHV infected fish (PCR positive or clinically sick). Group 2 consisted of fish sampled from regions with no historic association with KHV and where no KHV DNA had been detected in tissues by PCR (Table 1).

2.3 Virus antigen production

Common carp brain cells (CCB cells), derived from brain tissue of common carp, *C. carpio* (Neukirch et al., 1999) were kindly provided by the Friedrich-Loeffler-Institut, Greifswald, Germany. Virus culture and purification was undertaken as previously described (Monaghan et al., 2016) using an American KHV isolate (KHV-I, H361) (Hedrick et al., 2000) with a titre of $10^{4.4}$ $TCID_{50}$ mL^{-1}. The purified virus (0.9 - 1.4 mg mL^{-1} - quantified with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, USA) was stored at -70°C.

2.4 ELISA for detecting anti-KHV antibodies

The KHV ELISA protocol used was based on a previously published protocol (St-Hilaire et al., 2009) with modifications. Deviations to the published protocol included the use of Immulon-4 HBX 96-well microtitre plates (ThermoFisher Scientific) coated with 50 µL of

purified KHV or BSA (as a negative antigen control) at 0.3 µg per well in 0.05M carbonate-bicarbonate buffer, pH 8.6 (Sigma-Aldrich, St. Louis, MO), incubated overnight at 4^{o}C. Wash buffers included low salt wash buffer (LSWB: 0.02 M Trisma base, 0.38 M NaCl, 0.05 % Tween-20, pH 7.3) and high salt wash buffer (HSWB: 0.02 M Trisma base, 0.5 M NaCl, 0.1% Tween-20, pH 7.7). Control sera was included as described above.. All test sera were initially tested at 1/200 and 1/400 dilutions. Where remaining sera was available, positive serum samples were re-screened at 1/200, 1/400, 1/800, 1/1600 and 1/3200 dilutions. Fifty µL/well of mouse anti-carp IgM Mab (Aquatic Diagnostics Ltd., Stirling, UK), diluted 1:73.3 in 0.1% BSA in PBS, was used as the secondary antibody. The plates were read at 450 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, VT, USA). Fish were considered positive when the OD_{450nm} was > 3 x greater than the mean background OD_{450nm} (negative control wells) of that plate (sensitivity threshold/cut-off).

2.6 Ethics statement

All serological investigations, including anaesthesia and bleeding of fish, were performed with approval from the Animal Welfare and Ethics Review Body (AWERB) Committee of the University of Stirling, UK.

3. Results

When the sera from the groups of fish (defined field cases in Table 1) were screened by ELISA, 54.3% (88/162) were sero-negative, and 45.1% (74/162) were seropositive at a ≥1/200 dilution. Of the seropositive samples, 6.2% were categorised as strong (≥1/800) responders, 22.2% were moderate (1/400) responders and 16.7% were weak (1/200) responders (Figure 1). Fish were divided into groups based on the PCR results and their associations with KHV-positive sites, 36.4% (59/162) were allocated to ***Group 1*** (KHV +ve by PCR and associated with infected farms/sites), and 63.6% (103/162) were allocated to ***Group 2*** (KHV –ve by PCR and no association with infected farms/sites; Table 1). Only 31%

of koi from ***Group 1*** were seropositive (Figure 1B). In contrast, 52.9% of ***Group 2*** koi were seropositive, although these had previously been reported negative for the presence of KHV DNA by PCR (Figure 1C). Of the seropositive fish from ***Group 1***, 12.1% were strong responders, 8.6% were moderate responders and 10.3% were weak responders (Figure 1B). The remaining fish were seronegative / non-responders. The majority of seropositive fish from ***Group 2*** were moderate (29.8%) or weak responders (20.1%) (Figure 1C), although a number of moderate responders from this group had much higher absorbance values (OD_{450nm}) than the cut-off value when initially tested at a 1/400 dilution, and were later found to be strong seropositive responders when rescreened at a dilution of >1/1600 (n = 9). Furthermore, a number of fish had absorbance values greater than 2x the cut-off at a dilution of 1/400 (n= 6), but were not titrated further due to lack of sera, thus were likely to have also been strong responders (Table 1). Taken together >34% of ***Group 2*** fish (PCR-ve and no known association with KHV) were moderate/strong responders compared to <21% of ***Group 1*** fish (KHV PCR+ve and associated with infected farms/sites).

The presence of anti-KHV antibodies was found in a large proportion of the populations (69%) tested by ELISA, while testing negative by PCR (Table 2). When fish with no prior suspicion of KHV infection were screened, more groups of these fish were found to be seropositive (ELISA positive) (23/26) than were PCR positive (4/26) (Table 3).

Most populations of fish suspected of KHV infection were PCR positive and seropositive (6/9), although there were two groups that were PCR positive but sero-negative (2/9), and one group that was PCR negative, but seropositive (1/9) (Table 3). This clinically healthy population belonged to group C3, a Malaysian farm previously associated with KHVD in previous years (C1 and C2, see Table 1).

4. Discussion

178 Confirmation of KHV infection in the absence of clinical disease or mortality is still
179 only accepted based on virus detection using a combination of *in situ* hybridisation, PCR and
180 indirect fluorescent antibody test on carp tissues (OIE, 2019). However, 45% of all koi sera
181 screened from Asia against purified KHV by ELISA, in the present study, were seropositive
182 at a 1/200 dilution, despite a lack of clinical signs in the majority of fish (Figure 1A, Table 1,
183 2). This data supported the belief that KHV was present in apparently healthy koi populations
184 in Singapore and Malaysia in 2008, as previously reported for fish in Japan (Sano et al.,
185 2004). Nonetheless, many of the cases from the Asian surveillance program would otherwise
186 have been classified as sero-negative using published ELISA protocols at the time of
187 analysis. These protocols used high cut-off dilutions in order to prevent detection of cross-
188 reacting antibodies resulting from closely related aquatic herpesviruses such as CyHV-1 or
189 CyHV-2 and/or non-specific natural antibodies (Adkison et al., 2005; St-Hilaire et al., 2009;
190 Taylor et al., 2010). This represents a high level of risk of misclassifying latently infected fish
191 with low antibody titres.
192 The difference between KHV-specific antibody responses in fish from ***Groups 1*** and
193 ***2*** in the Asian surveillance program highlights the fact fish from the field are insufficiently
194 screened for KHV using molecular methods alone, which is commonly practiced.
195 Importantly, when ELISA testing was conducted on fish samples from farms with a previous
196 association with KHV, or suspected positive populations, those groups were subsequently
197 determined to be positive by either PCR, ELISA or both (Table 2). However, ELISA
198 screening of fish with no prior knowledge of their KHV-status, showed 23/26 groups to be
199 KHV-seropositive compared to only 4/26 by PCR (Table 3). Many of these seropositive fish
200 were moderate-strong (>1/400) responders, suggesting that these results were unlikely to be
201 due to false-positive reactions (Table 1, Figure 1C). Nonetheless, there were also populations
202 of fish testing positive by PCR that were seronegative, confirming that ELISA testing cannot

be substituted for molecular detection of virus DNA. Contrasting results between serological and molecular KHV testing have been reported previously from laboratory-based studies (Matras et al., 2012; Soto et al., 2020). Fish populations that were positive for anti-KHV antibodies by ELISA but negative for KHV DNA by various PCRs, may have resulted from low assay sensitivity (Bergmann et al., 2010; Monaghan et al., 2015). It should be noted that at the time of sampling in 2008, tissue pools were carried out for molecular detection, which is no longer advised (OIE, 2019). Dilution of viral DNA can occur by pooling tissue samples, as some tissues will contain lower concentrations of virus DNA, especially during potential latent or persistent infections (Gilad et al., 2004; Bergmann et al., 2010; Eide et al., 2011). Nonetheless, similar diagnostic discrepancies have been reported when using non-pooled gill and blood samples, and variable detection sensitivities of PCR may also be influenced by water temperatures during clinical KHVD (Matras et al., 2012; Soto et al., 2020).

KHV serology has proved useful for epidemiological screening of carp populations in fisheries and farms in the UK (Taylor et al., 2010). Previous studies of naturally exposed fish to KHV have also demonstrated greater numbers of KHV seropositive fish compared to PCR positive fish (Uchii et al., 2009), which indicates that persistent infections (with low viral loads) can be present that are undetected by PCR. Indirect detection of antibodies at a population level is effective, as antibody responses to KHV have been reported to be detectable for more than 1 year following transfer of infected fish to virus-free water (Ronen et al., 2003; Adkison et al., 2005). The high titre antibody responses of some healthy fish from ***Group 2*** could therefore be long-term responders that had recovered from KHVD and thus lacked detectable viral DNA by PCR.

Populations of fish from regions where KHVD outbreaks were present and/or fish were PCR positive, were often sero-negative. These fish may have been non-responders or experienced an acute KHV outbreak prior to sero-conversion, similar to findings from

experimental bath challenges (Matras et al., 2012). Nonetheless, at a population level, the use of ELISA appears to complement molecular testing as positive detection of only a single fish provided an indication of previous exposure of that farm to KHV where viral DNA was no longer present at sufficient levels for PCR detection. Therefore, control programs should implement antibody testing of small sub-samples of fish sera, avoiding pooling, in order to reliably screen for previous exposure of the population to KHV. More recently an ELISA based on fractionated KHV antigens (50% sucrose gradient fraction of purified virions) has avoided problems with antibody cross-reactivity with other aquatic herpesviruses (Bergmann et al., 2017), possibly due to removal of cross-reacting antigens, and this may facilitate more reliable and sensitive KHV sero-surveillance in order to improve KHVD control programs.

5. Conclusions

In conclusion, the results from the field samples analysed here, support previously reported laboratory-based results indicating antibody detection for KHV to be a valuable complementary tool for surveillance of KHV in conjunction with molecular methods, and should be applied routinely for control before transfer of fish to KHV negative sites and for management of KHVD in carp aquaculture.

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Declaration of conflicts of interest

All authors declare there are no potential conflicts of interest regarding the research, authorship or publication of this work.

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28. Figures

Figure 1. Antibody responses of koi carp to koi herpesvirus (KHV) in serum samples originating from a KHV surveillance programme in Asia (2008-2010)

(A) Cumulative antibody responses following screening by KHV antibody ELISA (n=162); (B) Antibody responses of koi in ***Group 1*** (KHV +ve by PCR and associated with infected farms/sites) (*n*=59); (C) Antibody responses of koi in ***Group 2*** (KHV –ve by PCR and no association with infected farms/sites) (*n*=103). Bars represent different categories depending on antibody titre of responders designated as: strong (≥1/800), moderate (1/400), weak (1/200), or negative at a 1/200 dilution. Sera screened against purified KHV virus by ELISA.

Table 1. Diagnostic grouping of fish extrapolated from Koi herpesvirus (KHV) surveillance program conducted by Agri-Food and Veterinary Authority of Singapore (AVFA), 2008 – 2010.

Fish group (Case; C) ID and consignment date	Clinical signs	Source Country	PCR Results and primers used on groups (n=5) of koi	Fish ID (no.)	ELISA positive fish at 1/200 from each consignment group	Highest measured antibody titre (*sera with higher titre when re-screened further or high ODs at 1/400)	KHV exposure group
C1 (12/08)	Clinically healthy koi.	Malaysia	Positive by IQ2000 test kit & Bercovier TK primers.	1-3 (n=3)	**2/3**	**1/800** (*1/12800)	**1**
C2 (12/08)	Diseased koi. Reddened skin and loose scales with ulcerated body, pale gills and cloudy eyes.	Malaysia	Positive by IQ2000 test kit	4-7 (n=4)	**1/4**	**1/200**	**1**
C3 (03/09)	Clinically healthy koi associated with C1 and C2.	Malaysia	Negative by Bercovier TK primers.	8-15 (n=8)	**3/8**	**1/800**	**1**
C4 (03/09)	Diseased. Koi were thin with reddened, dry skin, cloudy and sunken eye.	Malaysia	Positive by Bercovier TK primers.	16-27 (n=12)	**12/12**	**1/400** (* high OD_{450})	**1**
C5 (03/09)	Clinically healthy koi.	Malaysia	Positive by IQ2000 test kit and Yuasa-Grey Sph primers.	28-29 (n=2)	**0/2**	**-**	**1**

C6 (03/09)	Diseased koi with reddened skin, loose scales and pale gills.	Malaysia	Positive by IQ2000 test kit and Yuasa-Grey Sph primers.	30-31 (n=2)	**0/2**	**-**	**1**
C7 (03/09)	Diseased koi.	Unknown	Positive by Bercovier TK and Yuasa-Grey Sph primers.	32-36 (n=5)	**1/5**	**1/400**	**1**
C8 (04/09)	Clinically healthy when sampled but all 30 koi were dead on arrival. Pale gills and ascitic fluid in abdomen.	Malaysia	Positive by Bercovier TK and Yuasa-Grey Sph primers.	37-41 (n=5)	**3/5**	**1/400**	**1**
C9 (04/09)	Clinically healthy koi, 2 out of 30 koi dead on arrival.	Malaysia	Positive by Bercovier TK and Yuasa-Grey Sph primers.	42-46 (n=5)	**0/5**	**-**	**1**
C10 (04/09) - **C11** (03/09)	Clinically healthy koi.	Malaysia	Positive by Bercovier TK primers.	47-53 (n=7)	**3/6; 1/1**	**1/400** (*1/1600)	**1**
C12 (04/09)	Clinically healthy koi.	Malaysia	Negative by IQ2000 test kit and Yuasa-Grey Sph primers.	54-63 (n=10)	**6/10**	**1/400** (* high OD_{450})	**2**
C13 (04/09)	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	64-65 (n=2)	**2/2**	**1/400**	**2**

C14 (04/09) – **C15** (05/09)	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	66-79 (n=14)	**2/4; 3/10**	**1/400** (* high OD_{450})	**2**
C16 (01/10)	Clinically healthy koi.	China	Negative by Bercovier TK primers.	80 (n=1)	**1/1**	**1/400**	**2**
C17 (07/10)	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	81-86 (n=6)	**2/6**	**1/400**	**2**
C18 (05/09)	Clinically healthy koi with pale gills.	Malaysia	Negative by Bercovier TK primers.	87 (n=1)	**1/1**	**1/200**	**2**
C19 (07/10)	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	88-94 (n=7)	**7/7**	**1/400**	**2**
C20 (07/10)	Clinically healthy koi.	Singapore	Negative by Bercovier TK primers.	95-98 (n=4)	**4/4**	**1/400** (* high OD_{450})	**2**
C21-C23 (07/10)	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	99-118 (n=20)	**2/3; 7/8; 3/9**	**1/400**	**2**
C24 (07/10)- **C26** (11/10)	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	119-132 (n=14)	**1/9; 2/2; 3/3**	**1/400** (* high OD_{450})	**2**

C27 (11/10)	Diseased koi with abnormal swimming, showing respiratory distress and pale gills. Reddening of the body and mouth seen.	Singapore	Positive by real-time TaqMan PCR.	133-135 (n=3)	**2/3**	**1/400**	**1**
C28 (11/10)- **C30** (12/10)	Clinically healthy koi.	Singapore	Negative by Bercovier TK primers.	136-144 (n=9)	**2/3; 1/3; 0/3**	**1/400**	**2**
C31 (12/10)	Diseased koi.	Singapore	Positive by real-time TaqMan PCR.	145-147 (n=3)	**0/3**	**-**	**1**
C32 (12/10)	Clinically healthy koi.	Malaysia	Negative by real-time TaqMan PCR.	148-150 (n=3)	**1/3**	**1/400**	**2**
C33 (12/10)	Clinically healthy koi.	Singapore	Negative by real-time TaqMan PCR.	151-152 (n=2)	**1/2**	**1/400**	**2**
C34 (12/10)	Clinically healthy koi.	Malaysia	Negative by real-time TaqMan PCR.	153-154 (n=2)	**1/2**	**1/200**	**2**
C35 (11/10)	Clinically healthy koi.	Japan	Negative by real-time TaqMan PCR.	155-162 (n=8)	**3/8**	**1/400** (* high OD_{450})	**2**

Table 2. Results of PCR and ELISA screening of healthy or diseased koi from a koi herpesvirus surveillance programme in Asia (Agri-Food and Veterinary Authority of Singapore (AVFA), 2008 – 2010

	Clinically diseased fish group (8/35)	**Clinically healthy fish group (27/35)**	**ELISA +ve**	**ELISA -ve**
PCR positive group (n=6 organ pools from 5 fish per group; n=11/35 groups)	8 (19/39 fish seropositive)	4 (6/12 fish seropositive)	8	3
PCR negative group (n=6 organ pools from 5 fish per group; n=24/35 groups)	0	23 (58/111 fish seropositive)	22	2
ELISA +ve	5	25		
ELISA -ve	3	2		

PCR testing included protocols according to IQ2000 Test kit, Yuasa et al. (2005), Bercovier et al. (2005) and Gilad et al. (2004). The ELISA applied was a whole KHV antigen ELISA based on previously published protocols according to St-Hilaire et al. (2009) with modifications.

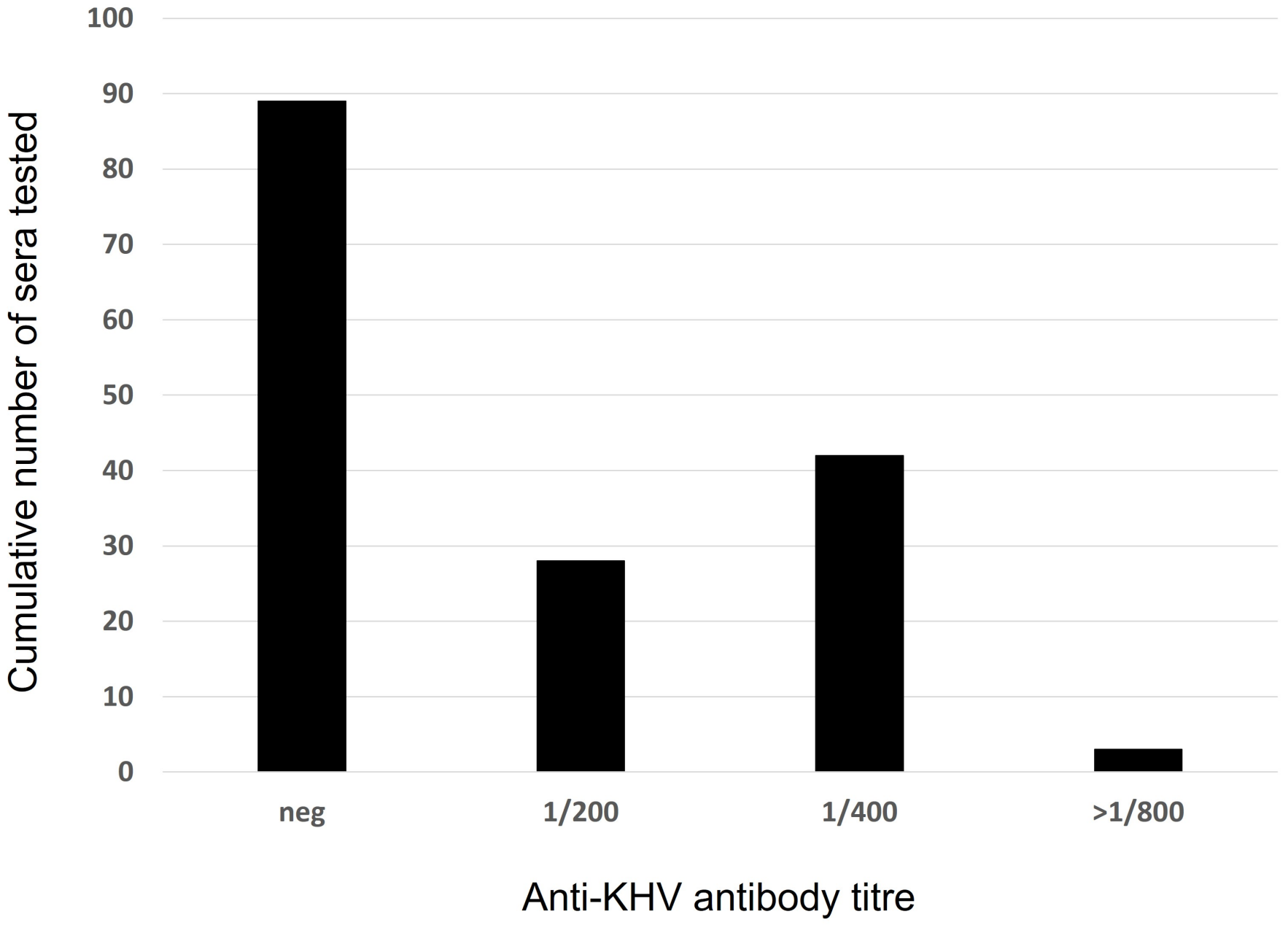
Table 3. Results for screening of 35 groups of koi carp for KHV genome in tissues (PCR) and anti-KHV antibodies in plasma (ELISA).

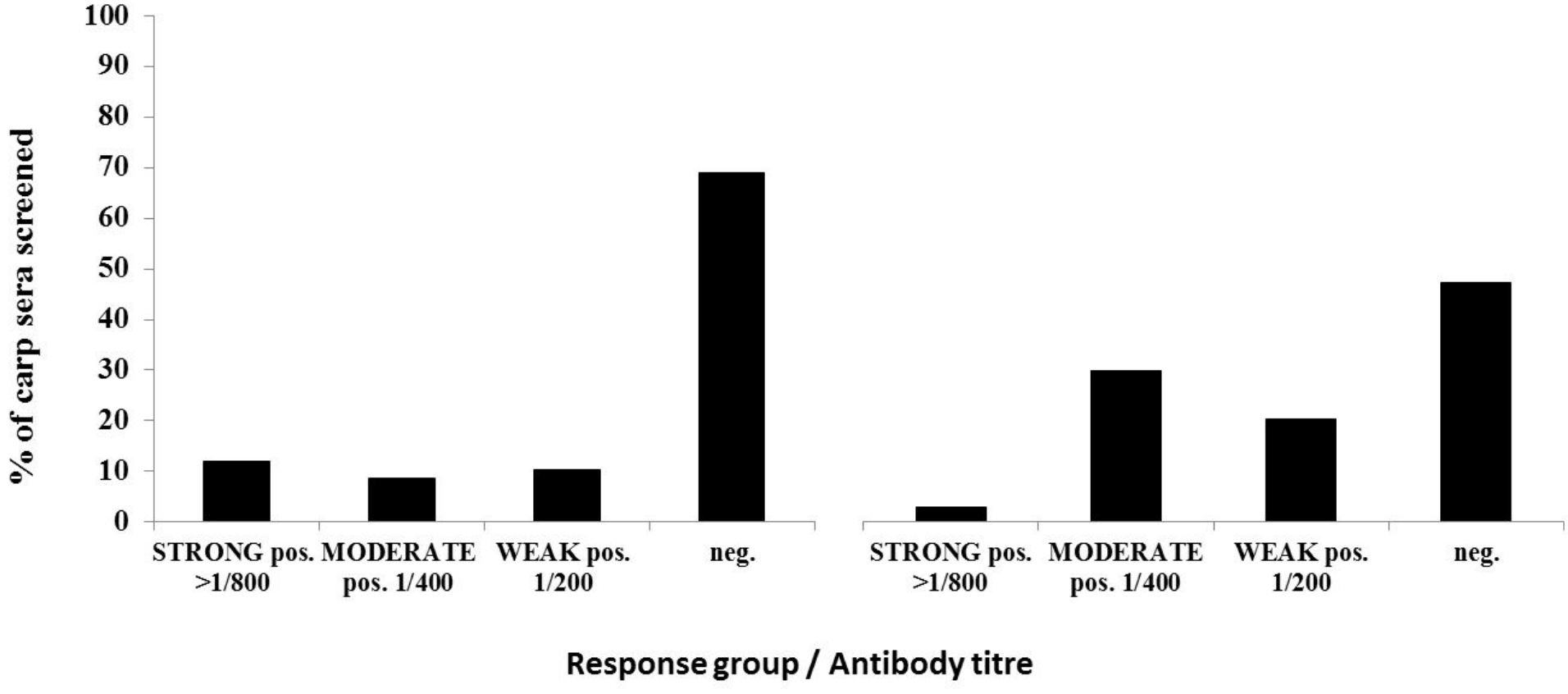
	No. of groups from a farm/region **with** historic association with KHV (no. seropositive fish)		No. of groups from a farm/region **without** historic association with KHV (no. seropositive fish)		
	PCR +VE	**PCR-VE**	**PCR+VE**	**PCR-VE**	**TOTAL**
ELISA +VE	**6** (11/32)	**1** (3/8)	**2** (4/7)	**21** (55/100)	**30** (73/147)
ELISA -VE	**2** (0/5)	**0** (0)	**2** (0/7)	**1** (0/3)	**5** (0/15)
TOTAL	**8**	**1**	**4**	**22**	**35** (162)

Note only ELISA data available for individual fish. PCR detection was based on tissue pools (n=6) of 5 random non-selectively collected fish from the respective group.

PCR testing included protocols according to IQ2000 Test kit, Yuasa et al. (2005), Bercovier et al. (2005) and Gilad et al. (2004).

The ELISA applied was a whole KHV antigen ELISA based on previously published protocols according to St-Hilaire et al. (2009) with modifications.


Cumulative number of sera tested
100
90
80
70
60
50
40
30
20
10
0
neg
1/200
1/400
>1/800
Anti-KHV antibody titre


100
90
80
70
60
50
40
30
20
10
0
% of carp sera screened
STRONG pos. >1/800
MODERATE pos. 1/400
WEAK pos. 1/200
neg.
STRONG pos. >1/800
MODERATE pos. 1/400
WEAK pos. 1/200
neg.
Response group / Antibody titre