

1 Short communication

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

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

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22

23 **Abstract**

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

38

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

40

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

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

## 53 1. Introduction

54 Koi herpesvirus (KHV), taxonomically classified as Cyprinid herpesvirus 3 (CyHV-  
55 3), is an *Alloherpesvirus* of the order *Herpesvirales* (Waltzek et al., 2005; Davison et al.,  
56 2009) and the causative agent of the highly infectious and lethal disease of Koi (*Cyprinus*  
57 *carpio* L.), koi herpesvirus disease (KHVD) (Hedrick et al., 2000). Koi herpes virus has  
58 affected not only farmed carp (Gotesman et al., 2013), but also fisheries and wild carp stocks  
59 (Uchii et al., 2009; Taylor et al., 2010).

60 A number of temperature manipulation studies have demonstrated latent- and / or  
61 persistent-like infection of KHV (Gilad et al., 2003, 2004; St-Hilaire et al., 2009; Eide et al.,  
62 2011; Reed et al., 2014), where low virus levels were detected without notable virus  
63 replication. This makes detection of KHV in apparently latently or persistently infected,  
64 clinically healthy fish, a challenge (Bergmann et al., 2010; Matras et al., 2012). The rapid  
65 spread of KHV worldwide may have been attributed to false negative results following  
66 screening of such fish by PCR, due to limitations in the sensitivity of molecular diagnostics  
67 for detecting KHV DNA (Bergmann et al., 2010; Matras et al., 2012; Monaghan et al., 2015).  
68 An alternative way to identify carrier fish may be through the detection of KHV-specific  
69 antibodies in the serum of fish by ELISA, indirectly indicating that the fish is or has been  
70 infected with the virus (Bergmann et al., 2017;; OIE, 2019; Soto et al., 2020).

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

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

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

87

## 88 **2. Materials and Methods**

### 89 **2.1 Control fish sera**

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

### 98 **2.2 Koi serum samples from Asian surveillance programme**

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

103 Ms. Yahui Wang (AVFA) and included sub-samples taken from a minimum of 30 non-  
104 selectively randomly collected fish from each import consignment. The source of country,  
105 clinical signs of koi and PCR results from all the cases were also recorded and provided to  
106 the University of Stirling prior to ELISA analysis (Table 1). Six organ pools (brain, gill,  
107 kidney, intestine, liver and spleen from 5 random non-selectively collected fish) for each  
108 submitted case (group of fish), were tested for KHV using a commercial PCR kit (KHV  
109 IQ2000 Test kit, GeneReach Biotechnology, Taiwan), the PCR described by Bercovier et al.  
110 (2005) and Yuasa et al. (2005) and / or real-time PCR developed by Gilad et al. (2004),  
111 providing 5 independent molecular testing outcomes. The analytical groups were divided into  
112 2 categorical groups depending on their KHV status. Group 1 consisted of samples taken  
113 from fish where KHV DNA had been detected in tissues by PCR as described or where  
114 sampled fish had been historically associated with KHV infected fish (PCR positive or  
115 clinically sick). Group 2 consisted of fish sampled from regions with no historic association  
116 with KHV and where no KHV DNA had been detected in tissues by PCR (Table 1).

### 117 **2.3 Virus antigen production**

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

### 124 **2.4 ELISA for detecting anti-KHV antibodies**

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

128 purified KHV or BSA (as a negative antigen control) at 0.3 µg per well in 0.05M carbonate-  
129 bicarbonate buffer, pH 8.6 (Sigma-Aldrich, St. Louis, MO), incubated overnight at 4°C.  
130 Wash buffers included low salt wash buffer (LSWB: 0.02 M Trisma base, 0.38 M NaCl,  
131 0.05 % Tween-20, pH 7.3) and high salt wash buffer (HSWB: 0.02 M Trisma base, 0.5 M  
132 NaCl, 0.1% Tween-20, pH 7.7). Control sera was included as described above.. All test sera  
133 were initially tested at 1/200 and 1/400 dilutions. Where remaining sera was available,  
134 positive serum samples were re-screened at 1/200, 1/400, 1/800, 1/1600 and 1/3200 dilutions.  
135 Fifty µL/well of mouse anti-carp IgM Mab (Aquatic Diagnostics Ltd., Stirling, UK), diluted  
136 1:73.3 in 0.1% BSA in PBS, was used as the secondary antibody. The plates were read at 450  
137 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, VT, USA).  
138 Fish were considered positive when the OD<sub>450nm</sub> was > 3 x greater than the mean background  
139 OD<sub>450nm</sub> (negative control wells) of that plate (sensitivity threshold/cut-off).

## 140 **2.6 Ethics statement**

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

## 144 **3. Results**

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

153 of koi from *Group 1* were seropositive (Figure 1B). In contrast, 52.9% of *Group 2* koi were  
154 seropositive, although these had previously been reported negative for the presence of KHV  
155 DNA by PCR (Figure 1C). Of the seropositive fish from *Group 1*, 12.1% were strong  
156 responders, 8.6% were moderate responders and 10.3% were weak responders (Figure 1B).  
157 The remaining fish were seronegative / non-responders. The majority of seropositive fish  
158 from *Group 2* were moderate (29.8%) or weak responders (20.1%) (Figure 1C), although a  
159 number of moderate responders from this group had much higher absorbance values  
160 ( $OD_{450nm}$ ) than the cut-off value when initially tested at a 1/400 dilution, and were later found  
161 to be strong seropositive responders when rescreened at a dilution of  $>1/1600$  ( $n = 9$ ).  
162 Furthermore, a number of fish had absorbance values greater than 2x the cut-off at a dilution  
163 of 1/400 ( $n= 6$ ), but were not titrated further due to lack of sera, thus were likely to have also  
164 been strong responders (Table 1). Taken together  $>34\%$  of *Group 2* fish (PCR-ve and no  
165 known association with KHV) were moderate/strong responders compared to  $<21\%$  of *Group*  
166 *1* fish (KHV PCR+ve and associated with infected farms/sites).

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

171 Most populations of fish suspected of KHV infection were PCR positive and  
172 seropositive (6/9), although there were two groups that were PCR positive but sero-negative  
173 (2/9), and one group that was PCR negative, but seropositive (1/9) (Table 3). This clinically  
174 healthy population belonged to group C3, a Malaysian farm previously associated with  
175 KHVD in previous years (C1 and C2, see Table 1).

176

#### 177 **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

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

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

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

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

238

## 239 **5. Conclusions**

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

245

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253

254 **Declaration of conflicts of interest**

255 All authors declare there are no potential conflicts of interest regarding the research,  
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344

345 28. **Figures**

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

348 (A) Cumulative antibody responses following screening by KHV antibody ELISA (n=162);

349 (B) Antibody responses of koi in **Group 1** (KHV +ve by PCR and associated with infected

350 farms/sites) (n=59); (C) Antibody responses of koi in **Group 2** (KHV –ve by PCR and no

351 association with infected farms/sites) (n=103). Bars represent different categories depending

352 on antibody titre of responders designated as: strong ( $\geq 1/800$ ), moderate (1/400), weak

353 (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 consignm ent 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
<b>C1</b> (12/08)	Clinically healthy koi.	Malaysia	Positive by IQ2000 test kit & Bercovier TK primers.	1-3 (n=3)	<b>2/3</b>	<b>1/800</b>  (*1/12800)	<b>1</b>
<b>C2</b> (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)	<b>1/4</b>	<b>1/200</b>	<b>1</b>
<b>C3</b> (03/09)	Clinically healthy koi associated with C1 and C2.	Malaysia	Negative by Bercovier TK primers.	8-15  (n=8)	<b>3/8</b>	<b>1/800</b>	<b>1</b>
<b>C4</b> (03/09)	Diseased. Koi were thin with reddened, dry skin, cloudy and sunken eye.	Malaysia	Positive by Bercovier TK primers.	16-27  (n=12)	<b>12/12</b>	<b>1/400</b>  (* high OD <sub>450</sub> )	<b>1</b>
<b>C5</b> (03/09)	Clinically healthy koi.	Malaysia	Positive by IQ2000 test kit and Yuasa-Grey Sph primers.	28-29  (n=2)	<b>0/2</b>	-	<b>1</b>

<b>C6</b> (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)	<b>0/2</b>	-	<b>1</b>
<b>C7</b> (03/09)	Diseased koi.	Unknown	Positive by Bercovier TK and Yuasa-Grey Sph primers.	32-36 (n=5)	<b>1/5</b>	<b>1/400</b>	<b>1</b>
<b>C8</b> (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)	<b>3/5</b>	<b>1/400</b>	<b>1</b>
<b>C9</b> (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)	<b>0/5</b>	-	<b>1</b>
<b>C10</b> (04/09) - <b>C11</b> (03/09)	Clinically healthy koi.	Malaysia	Positive by Bercovier TK primers.	47-53 (n=7)	<b>3/6; 1/1</b>	<b>1/400</b> (*1/1600)	<b>1</b>
<b>C12</b> (04/09)	Clinically healthy koi.	Malaysia	Negative by IQ2000 test kit and Yuasa-Grey Sph primers.	54-63 (n=10)	<b>6/10</b>	<b>1/400</b> (* high OD <sub>450</sub> )	<b>2</b>
<b>C13</b> (04/09)	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	64-65 (n=2)	<b>2/2</b>	<b>1/400</b>	<b>2</b>

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

<b>C27</b> (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)	<b>2/3</b>	<b>1/400</b>	<b>1</b>
<b>C28</b> (11/10)- <b>C30</b> (12/10)	Clinically healthy koi.	Singapore	Negative by Bercovier TK primers.	136-144 (n=9)	<b>2/3; 1/3; 0/3</b>	<b>1/400</b>	<b>2</b>
<b>C31</b> (12/10)	Diseased koi.	Singapore	Positive by real-time TaqMan PCR.	145-147 (n=3)	<b>0/3</b>	-	<b>1</b>
<b>C32</b> (12/10)	Clinically healthy koi.	Malaysia	Negative by real-time TaqMan PCR.	148-150 (n=3)	<b>1/3</b>	<b>1/400</b>	<b>2</b>
<b>C33</b> (12/10)	Clinically healthy koi.	Singapore	Negative by real-time TaqMan PCR.	151-152 (n=2)	<b>1/2</b>	<b>1/400</b>	<b>2</b>
<b>C34</b> (12/10)	Clinically healthy koi.	Malaysia	Negative by real-time TaqMan PCR.	153-154 (n=2)	<b>1/2</b>	<b>1/200</b>	<b>2</b>
<b>C35</b> (11/10)	Clinically healthy koi.	Japan	Negative by real-time TaqMan PCR.	155-162 (n=8)	<b>3/8</b>	<b>1/400</b>	<b>2</b>

(\* high OD<sub>450</sub>)

**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**

	<b>Clinically diseased fish group (8/35)</b>	<b>Clinically healthy fish group (27/35)</b>	<b>ELISA +ve</b>	<b>ELISA -ve</b>
<b>PCR positive group (n=6 organ pools from 5 fish per group; n=11/35 groups)</b>	8 (19/39 fish seropositive)	4 (6/12 fish seropositive)	8	3
<b>PCR negative group (n=6 organ pools from 5 fish per group; n=24/35 groups)</b>	0	23 (58/111 fish seropositive)	22	2
<b>ELISA +ve</b>	5	25		
<b>ELISA -ve</b>	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 <b>with</b> historic association with KHV (no. seropositive fish)		No. of groups from a farm/region <b>without</b> historic association with KHV (no. seropositive fish)		
	<b>PCR +VE</b>	<b>PCR-VE</b>	<b>PCR+VE</b>	<b>PCR-VE</b>	<b>TOTAL</b>
<b>ELISA +VE</b>	<b>6</b> (11/32)	<b>1</b> (3/8)	<b>2</b> (4/7)	<b>21</b> (55/100)	<b>30</b> (73/147)
<b>ELISA -VE</b>	<b>2</b> (0/5)	<b>0</b> (0)	<b>2</b> (0/7)	<b>1</b> (0/3)	<b>5</b> (0/15)
<b>TOTAL</b>	<b>8</b>	<b>1</b>	<b>4</b>	<b>22</b>	<b>35</b> (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.



