Detection of koi herpesvirus using real-time PCR targeting the polymerase gene

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Central Veterinary Institute
Central Veterinary Institute (CVI)

- National reference institute for animal diseases in the Netherlands
- Diagnostic testing, advice and support for government, vets, farmers and zoos.
- Fish and shellfish disease unit, 5 people
Diagnosis of KHV in the Netherlands

- Positives thus far restricted to ornamental koi
- No outbreaks in ‘wild’ carp
- Relative large percentage positives (~50%)
- Challenge for diagnostics!

<table>
<thead>
<tr>
<th>Year</th>
<th>PCR positive / total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>2 / 5</td>
</tr>
<tr>
<td>2002</td>
<td>3 / 9</td>
</tr>
<tr>
<td>2003</td>
<td>29 / 69</td>
</tr>
<tr>
<td>2004</td>
<td>39 / 83</td>
</tr>
<tr>
<td>2005</td>
<td>27 / 68</td>
</tr>
<tr>
<td>2006</td>
<td>70 / 140</td>
</tr>
<tr>
<td>2007</td>
<td>61 / 132</td>
</tr>
</tbody>
</table>

*excluding import/export controls
PCR methods used for KHV diagnosis

- Gilad et al. 2002 (KHV9/5)
  - 2002 - 2004
- Bercovier et al. 2005 (TK)
  - 2005 - 2006
- Gilad et al. 2004 (86f/163r real time)
  - 2005 tested for potential use in diagnostics
  - However, …
  - Overlap target KHV9/5 and real time
- In house real time
  - 2006 - now
Advantages of real-time PCR system
- Short run time
- Elimination of toxic ethidium bromide
- Does not require post-PCR analysis
- Limiting carry-over contamination:
  - Reaction and measurement takes place in closed tube
  - dUTP – UDG system
Development of in-house real-time PCR for clinical and sub-clinical detection of KHV

Target: KHV polymerase gene
- Conserved gene, identical between KHV-I, KHV-U and KHV-J
- Primer-probe set developed with Primer Express software
- Two target sites within polymerase gene:
  1) Taqman chemistry  KHV.F07/R07/p07
  2) SYBR Green chemistry  KHV.F08/R08
Results of KHV polymerase gene real-time

- Amplification and dissociation plots
  - KHV.F07/R07/p07
  - KHV.F08/R08

![Amplification and dissociation plots](image)
Specificity

- **In silico**
  - At least two mismatches with CyHV-1 and CyHV-2 in each primer / probe.

- **In vitro**
  - No amplification of CyHV-2 DNA (provided by A. Goodwin)
  - No amplification of CyHV-1 suspected sample (needs verification)
Comparative sensitivity

Comparative sensitivity of the real-time assay vs. TK for detection of KHV

<table>
<thead>
<tr>
<th>Dilution</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt;/ml</th>
<th>Average cycle threshold Ct (Std Dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KHV.F07/R07/p07</td>
</tr>
<tr>
<td>10-1</td>
<td>2.09 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>15.52 (0.54)</td>
</tr>
<tr>
<td>10-2</td>
<td>2.09 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>18.34 (0.42)</td>
</tr>
<tr>
<td>10-3</td>
<td>2.09 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>21.88 (0.59)</td>
</tr>
<tr>
<td>10-4</td>
<td>2.09 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>24.92 (0.72)</td>
</tr>
<tr>
<td>10-5</td>
<td>2.09 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>28.05 (0.60)</td>
</tr>
<tr>
<td>10-6</td>
<td>2.09 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>31.10 (0.80)</td>
</tr>
<tr>
<td>10-7</td>
<td>2.09 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>35.91 (0.12)</td>
</tr>
<tr>
<td>10-8</td>
<td>2.09 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>36.23</td>
</tr>
<tr>
<td>water</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Linearity and efficiency of amplification

- 10 fold serial diluted KHV DNA from cultured virus
Linearity and efficiency of amplification

- 10 fold serial diluted KHV DNA
- Cultured virus vs. spiked negative carp tissue

![Graph showing linearity and efficiency of amplification](image)

- SYBR Green spiked
  \[ R^2 = 0.998 \]
- SYBR Green
  \[ R^2 = 0.9977 \]
Preliminary inter-laboratory comparison

- 6 positive field samples compared between two laboratories in the Taqman assay

### Extracted DNA

<table>
<thead>
<tr>
<th>Sample nr</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVA Ct</td>
<td>24</td>
<td>16</td>
<td>22</td>
<td>24</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>CVI Ct</td>
<td>26</td>
<td>18</td>
<td>25</td>
<td>26</td>
<td>33</td>
<td>30</td>
</tr>
</tbody>
</table>

### Tissue-samples:

<table>
<thead>
<tr>
<th>Sample nr</th>
<th>1 1:1000</th>
<th>2 1:1000</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVA Ct</td>
<td>32</td>
<td>29</td>
<td>22</td>
<td>33</td>
<td>28</td>
<td>35</td>
</tr>
<tr>
<td>CVI Ct</td>
<td>35</td>
<td>27</td>
<td>25</td>
<td>26</td>
<td>33</td>
<td>30</td>
</tr>
</tbody>
</table>

Åsa Hagström is acknowledged for the data from the National Veterinary Institute in Sweden
Evaluation of Taqman with conventional PCR

- Evaluation of the Taqman and conventional TK primers for detection of KHV DNA in diagnostic samples (2005/2006)
- Kappa value of 0.77 indicates substantial agreement between both tests
- * 25 / 28 TK neg, Taqman pos have Ct > 35

<table>
<thead>
<tr>
<th></th>
<th>TK positive</th>
<th>TK negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>KHV.F07/R07/p07</td>
<td>91</td>
<td>28*</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>158</td>
<td>161</td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>186</td>
<td>280</td>
</tr>
</tbody>
</table>
Results of real-time with the CEFAS ring-trial

- Ring trial

2006

2007

KHV07

TK
Current use of real-time PCR in diagnostics

- Taqman based assay used as standard in our lab for KHV diagnostics
  - First round 1:1000 dilution of the extracted DNA
  - Negative samples and Ct ≥ 35 re-tested undiluted
  - Gilad et al. 2004 primers for carp glucokinase are used as control of the DNA extraction

- SYBR Green based assay used for confirmation
  - Undiluted samples with Ct ≥ 35
Summary and discussion

- A real time PCR was developed to detect clinical and sub-clinical infections of KHV in carp tissue
  - Specific for CyHV-3
  - Similar to higher sensitivity compared with Bercovier TK primers
  - Further validation is ongoing
  - Results show potential for diagnostic use