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EPIZONE Work package 6.1
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Marc Engelsma[1], Olga Haenen[1], Søren Kahns[2], Helle Frank Skall[2], Sven M. Bergmann[3], Laurent Bigarré[4], Åsa Hagström[5], Guiseppe Bovo[6], Erika Rampazo[6], Ewa Borzym[7], Keith Way[8]

1. CVI: Central Veterinary Institute of Wageningen UR, Lelystad, the Netherlands
2. DTU vet: National Veterinary Institute, Technical University of Denmark, Hangøvej 2, DK-8200 Århus N, Denmark
3. FLI: Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute for Infectiology, Suedufer 10, 17493 Greifeswald - Insel Riems, Germany
4. AFSSA: (Agence Française de Sécurité Sanitaire des Aliments), Laboratoire d'Etudes et de Recherches en Pathologie des Poissons, BP 70 -Technopôle Brest – Iroise, F-29280 Plouzané, France
5. SVA: National Veterinary Institute, Ulls väg 2B, SVA, SE-751 89 Uppsala, Sweden
6. IZS-Ve: Istituto Zooprofilattico Sperimentale delle Venezie Centro di Referenza Nazionale delle Malattie dei Pesci, Molluschi e Crostacei. Viale dell'Università, 10 35020 Legnaro- PADOVA, Italy
7. NVRI: Zaklad Chorób Ryb, Pan'stwowy Instytut Weterynaryjny, Pan'stwowy Instytut Badawczy, al. Partyzantów 57, 24-100 Pulawy, Poland
8. CEFAS: Aquatic Health and Hygiene Division, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, England

Introduction

In medical and veterinary diagnostics there have been rapid developments in the use of molecular based methods for detection of disease agents. There are a number of advantages in the use of PCR based methods to detect DNA or RNA from pathogenic bacteria, viruses, and parasites. In general these methods are highly specific and sensitive. Furthermore, results can be obtained in a matter of hours and the tests are relatively labour extensive. In contrast, the methodology allows detection of the agent but not detection of disease and gives no information on the viability of the agent. The specificity of the assay is dependent on the choice of the primer set and stringency of the annealing temperature. A clear advantage of the PCR assays is their high sensitivity. But inevitably a higher sensitivity evokes a higher risk of cross-contamination.
For diagnosis of viral diseases in fish, isolation of the virus by cell culture is often regarded as the gold standard. Unfortunately in the case of koi herpesvirus (KHV) the available cell lines lack the required sensitivity to be used for diagnostic purposes. Current diagnostics of KHV are primarily based on a number of PCR assays. The methods which have been published are often scarcely supported by validation data. Furthermore, the reliability of the diagnosis is not only dependent on the test itself, as it is also dependent on the processing and handling of the samples and prevention of cross-contamination between samples. This was demonstrated especially in the latest ring trial organised by CEFAS in 2008. The participating laboratories were challenged to detect KHV in five ampoules by PCR. The results showed a considerable number of labs having problems of false positive samples. This report establishes a general protocol for handling diagnostic specimens prior to PCR analyses in order to limit the risk of cross-contamination. Secondly, the report gives an overview and remarks on the currently used PCR assays for detection of KHV, as discussed and concluded at the EPIZONE workshop, November 12-13 2009, at CVI, Lelystad.

Precautions in handling of diagnostic specimens for PCR based methods

In the general section of the OIE diagnostic manual a general procedure for validation of diagnostic assays and precautions is described (OIE, 2009). However, specific precautions and pitfalls in using PCR assays are not described. In the past a number of papers have described these issues in more detail and these can be used as guidelines (Dieffenbach and Dveksler, 1993; Dragon, 1993; Hartley and Rashtchian, 1993; Kwok and Higuchi, 1989).
In order to prevent cross-contaminations control of the routing and knowledge on handling of diagnostic samples for PCR in the laboratory is essential. A key component in this is the creation of separate working spaces for the different steps involved in the PCR assays. The work areas can consist, for example, of different rooms or separate laminar flow hoods. Four different steps can be distinguished in the process as shown in figure 1: 1) Sampling, 2) preparation of reagent mix (PCR I), 3) DNA extraction and preparation of reaction tubes or plate (PCR II) and 4) PCR run and agarose gel electrophoresis (PCR III). The working direction is from PCR I -> PCR II -> PCR III. Working in the reverse direction should be strictly avoided. Below are some considerations for each of the different steps.

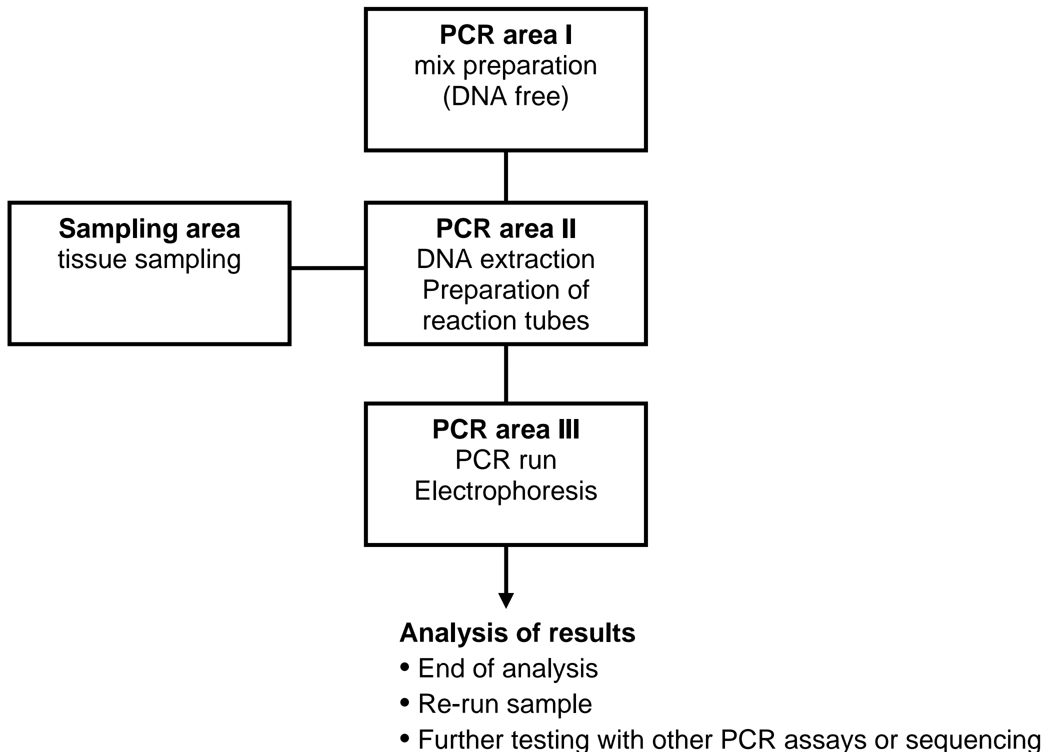

Figure 1. Schematic presentation of a general workflow for PCR diagnostics.

General considerations working with molecular methods:

- Use laboratory coat and nitril of latex gloves.
- Use tips with filters for pipettes.
- Keep separate sets of pipettes for each area.
- Change laboratory coats and gloves between areas.

Sampling area:

- Use a clean set of dissection tools per fish/sample.

- Use new gloves after handling each sample.
- Clean table thoroughly with detergents & hot water, and expose overnight to UV light.
- Non-disposable dissection material: clean mechanically and disinfect with one or a combination of methods (e.g. chloramin-T, detergents, autoclave, UV treatment).

PCR area I (preparation of reagent mix):
- Limit entrance of DNA, keep only reagents and primers.
- By using commercial master mixes including for example Mg and dNTP's pipetting mistakes can be limited.

PCR area II (DNA extraction and plate preparation):
- Replace gloves after handling of each individual sample or batch.
- Decontaminate surface afterwards by mechanical cleaning and use of a disinfectant (for example with LTK-008, Biodelta GmbH, Germany). A flow cabinet equipped with UV can be considered as working station.
- Mechanically clean the used dissection tools and treat with one or a combination of DNA / RNA destroying methods (e.g. detergents, autoclave, UV treatment).
- Inclusion of proper controls in each assay is essential:
  A positive control to verify the capability of the used reagents to amplify the target. A (diluted) DNA extract from cultured virus or a confirmed positive sample can be used as template for this reaction. Alternatively, to minimise the risk of a false positive result, because of the positive control, an artificial construct can be used of target DNA having the same primer-binding sequences but generating an amplicon of different size.
  A mix control to verify the purity of the used reagents. Consists of the reagents mix and PCR grade water as template.
  Negative controls to be able to verify potential cross-contaminations between samples from different owners, systems or batches during the DNA extraction and/or the PCR assay. Consists of water or non-infected tissue processed along with the samples in the DNA extraction and PCR reaction.
- Integrity of the sample and DNA extraction can be controlled by a separate PCR assay targeting DNA of the host or an artificially produced internal control (as for example described in (Hoffmann et al., 2006)). For real-time PCR assays the described assay targeting carp glucokinase gene can be used (Gilad et al. 2004). For conventional PCR assays, an artificial construct can be used as internal control consisting of target DNA having the same primer-binding sequences but generating an amplicon of different size. The internal control can be added to the sample taking into account the conditions where co-amplification of template and internal control occur in the same tube under minimal competition.

PCR area III (PCR run and agarose gel electrophoresis):
- Opening tubes is major source of contamination.
- DNA is persistent: amplicon can accumulate over time.

Sample collection and DNA extraction

Results obtained with the KHV PCR assays will be dependent on the circumstances under which the samples were taken. Most reliable results will be obtained from fish kept for prolonged time at permissive temperatures for the virus (for 2-3 weeks at 20°C to 26°C). There are indications that stress (e.g. transport of the fish) can evoke the virus in fish with a carrier status, thus increasing the chance of detection. It is not recommended to implement stress as a standard as it is negatively affecting the welfare of the fish. However, if possible samples should be collected after an

unavoidable stress condition such as transport or netting and transfer to a new system to enhance the chance of KHV detection.

In general during clinical infections a high viral load can be observed in most of the organs, in particular in gill and kidney (Gilad et al., 2004). Most often gill, kidney and spleen are used as source for KHV detection. This is also recommended in the OIE diagnostic manual (OIE, 2009). Until now only a few studies have carried out a comparison between different tissues. In an experimental KHV bath infection study, Gilad et al. (2004) reported that in addition to gill and kidney also brain of surviving fish was still positive for viral DNA by PCR at 62 days post-infection. Uchii et al. (2009) used a PCR that amplified part of the major capsid protein, developed by Dishon et al. (2007), and specific KHV amplicons were detected in brain (16 carp) and gill tissue (6 carp) from 76 collected carp, two years after the first occurrence of KHV disease in Lake Biwa. Furthermore, there are indications that other organs (encephalon, intestine) and separated peripheral blood leucocytes may contain the highest concentration of viral particles at sub-clinical or "latent / persistent" stages of the infection (unpublished results). Based on this, other tissues may need to be considered in the future but this will need further validation. The use of gill swabs or separated peripheral blood leucocytes could be an alternative non-lethal source for detection however, their sensitivity compared to (lethal) use of kidney and gill tissue needs to be further validated.

Pooling of tissue samples

Pooling samples can reduce the work considerably but it should be taken into consideration that If the KHV levels are close to detection level of the test pooling might dilute the positive KHV signal to a level below the limit of detection. In a recent investigation by Bergmann et al. (2010), carp from a KHV exposed population were sampled. Six of 10 carp were positive for KHV when tested individually by Gilad Taqman real-time PCR. When tested as 2 pools of 5 carp both pools tested negative (were below limit of detection). This suggests a reduced sensitivity of the method by pooling of the samples. No details of the Ct values from the positive signals were provided. We recommend that in acute cases up to 5 fish can be pooled and in case of surveillance no pooling is recommended. If pooling is necessary, the number of fish in the pool should be as little as possible.

DNA extraction

From the KHV PCR ring trial results, it can be concluded that in general the most clean and correct results were obtained with the silica matrix based methods compared to salt based extraction methods. The drawback of most of these column based methods is that only a very limited amount of tissue is used as the source material for the DNA extraction, biasing the diagnostic sensitivity of the assays. A possible suggested solution to this is to take one gram of tissue, homogenize it and use this as source for the extraction. An alternative option used by SVA is to cut the organ with a scalpel and swab the organ with a cotton swab and use the swab as source material.

Currently available PCR methods for KHV detection

There is a range of PCR methods published for the detection of KHV (see Table 1). Four different methodologies can be distinguished: conventional PCR, (semi-)nested PCR, real-time PCR and isothermal PCR. In a recent paper the isothermal PCR shows a high sensitivity and the advances are promising (Soliman and El-Matbouli,

2009). However, to our knowledge the isothermal PCR technique seems to be limitedly used in diagnostic laboratories and is not further considered in this overview. The methodologies differ in their suitability for use as a diagnostic method (clinical cases) and a surveillance method (for disease freedom). The specificity of the assay is most important for diagnostic use while the sensitivity is most important for surveillance.

Conventional PCR

Conventional PCR assays are widely used for detection of KHV. Most frequently used assays are the method described by Gray et al. (2002) with the modifications of Yuasa et al. (2005) and the assay targeting the thymidine kinase (Tk) gene (Bercovier et al., 2005). Both methods are recommended methods for the OIE (2009). The conventional PCR assay targeting the thymidine kinase (Tk) gene shows a very high sensitivity. However, a possible limitation of the Bercovier Tk assay is addressed in a recent publication of Bergmann et al. (2010) where KHV variants seem to be detected by several KHV PCR methods with the exception of the Tk assay. The possible genetic variation in the KHV Tk gene needs to be further investigated.
General primers for detection of a wide range of cyprinid herpesviruses are of practical use in confirmation of the identity. The method has not been published yet but was supplied by CEFAS to the labs participating in the KHV PCR ring trial of 2008.

Nested PCR

With nested PCR the sensitivity of the PCR assay can be increased up to a detection limit of 1-5 copy numbers. This is potentially very useful for surveillance purposes. However, a potential risk in the use of nested or semi-nested PCR assays is the necessity to open the tubes between and after the reactions and the high copy numbers generated in the assays, considerably increasing the risk of cross-contamination.

Bergmann et al. (2010) used a newly developed one-tube semi-nested PCR (sn PCR) and reported the detection level to be equal to real-time PCR while the risk of contamination was significantly reduced compared to (semi-)nested PCR. However, the sn PCR is a recently published method that has not been used by other laboratories.

Real-time PCR

For diagnostic use real-time PCR has considerable advantages. It has a high sensitivity with a detection limit of 1-5 copy numbers. There is no need to open the reaction tube after the PCR run which eliminates a major source of cross contamination. In addition, carryover contamination is further controlled by the inclusion of a dUTP - uracil DNA glycosylase (UDG) system in most real-time PCR settings. A drawback of the method is the high cost of the PCR machine, reagents and probes used.
Thus far only one real-time assay has been published (Gilad et al., 2004) for the detection of KHV. The results of the KHV PCR ring trial of CEFAS suggest that the real-time assays are less prone to contamination. A concern of this particular assay is that the target region is within the target region of one of the published conventional PCR assays (Gilad et al., 2002). This could lead to contamination when this method is used in laboratories that have previously used the conventional PCR assay of Gilad et al. (2002).

In conclusion

In case of surveillance: As KHV can be present in very low copy numbers, the detection of KHV can be challenging. Sampling strategies (site selection, number, time, test) are of prime importance for the results obtained. Of the used PCR methodologies, the real-time PCR is the assay that shows the most optimal combination between the highest sensitivity and restriction of carryover contamination. It is therefore recommended to use for detection of KHV for surveillance purposes. Presently only one real-time PCR targeting KHV has been published (Gilad et al., 2004). The proficiency test provided by the OIE reference laboratory for KHV disease confirms that of the assays listed in this report, the real-time PCR assay gives the highest sensitivity and specificity of the methods used by the participating laboratories. Not all laboratories are, however, in a situation where real-time PCR is available. In this case conventional PCR may be applied. The conventional PCR assays described in table 1 are methods that might be used although they are less sensitive compared to the real-time PCR. Adjusting sampling strategies (e.g. number of animals sampled) can be considered to increase the overall sensitivity. The PCR assays described by Bercovier et al. (2005) and by Yuasa et al. (2005) have both been tested in proficiency tests. The newly published semi-nested one tube PCR assay (Bergmann et al., 2010) has a very high sensitivity and also restricts the problem of carryover contamination. If the sensitivity and specificity of this method can be reproduced in more laboratories in future proficiency tests is would be an alternative for the use of the real-time PCR.

In acute cases: Although varying in sensitivity and specificity the PCR assays listed in table 1 are suitable for diagnostic detection of KHV during outbreaks. We recommend using the most sensitive assay possible (see above: In case of surveillance).

In order to be able to verify the results obtained with a routine assay a testing laboratory should, in addition to the use of appropriate positive and negative controls, consider using a second assay with similar sensitivity but targeting a different position on the KHV genome.

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Table 1. Overview described KHV PCR detection assays*.

	Reference	Primer name	Validation (from paper)		Comments
Conventional PCR	**(Gilad et al., 2002)**	KHV9/5F – KHV9/5R	*Specificity*: *Sensitivity*:	no amplification of CHV, CCV up to 1 pg of KHV DNA	
	(Gray et al., 2002) Modified by **(Yuasa et al., 2005)**	*Sph*I-5	*Specificity*: *Sensitivity*:	no amplification of CHV, CCV, HVS, WSV, SNSV (Gray et al) up to 100 fg (600 DNA copies; Gray et al)	OIE (Yuasa modification)
	(Bercovier et al., 2005)	KHV-TKf – KHV-TKr	*Specificity*: *Sensitivity*:	no amplification of CHV, CCV up to 10 fg (30 virons)	OIE
	(Hutoran et al., 2005)	AP1-AP2, AP1-AP3 or NH1-NH2	*Specificity*: *Sensitivity*:	not indicated not indicated	
	(Bigarre et al., 2009)	oPVP53, oPVP54, oPVP55, oPVP56	*Specificity*: *Sensitivity*:	no amplification of CyHV1, CyHV2 As sensitive as Bercovier et al	
(Semi-)nested PCR	**(Bergmann et al., 2006)**	KHV-1Fn – KHV-1Rn nested on Gilad et al 2002	*Specificity*: *Sensitivity*:	no amplification of CCV, CyHV-1, CyHV-2, HVA** 10 – 100 copies**	
	(El-Matbouli et al., 2007)	based on MCP	*Specificity*: *Sensitivity*:	No amplification of CyHV1, CyHV2 10 times more sensitive than Gilad et al 2002	
	(Bergmann et al., 2010)	Sn1F – Sn1R Sn1F – Sn2R / Sn3R	*Specificity*: *Sensitivity*:	CyHV-1, GHV, CCV, HVA tested. Positive signal at a level between 1-5 genomic KHV copy numbers	based on ORF 56, gp gene
Real-time PCR	**(Gilad et al., 2004)**		*Specificity*: *Sensitivity*:	No amplification of CyHV1, CyHV2, IcHV1 Down to 10 KHV plasmid copies	

* Isothermal PCRs were not included. Their use in diagnostic laboratories seems to be limited.

** Personal communication S. M. Bergmann

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