An Outline of Testing Procedures Used for the Detection of Porcine Circoviruses in Cell Substrates and Starting Materials

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Introduction
The purpose of this document is to indicate how BioReliance currently tests cell substrates and raw materials for freedom from contamination by porcine circovirus 1 & 2 (PCV1 & 2), including additional custom approaches that may be of value in specific circumstances.

Porcine Circovirus (PCV) is a member of the Circoviridae, a family of small unenveloped, closed circular ssDNA, viruses. PCV-1 is ubiquitous in pig populations worldwide and appears to be non-pathogenic. In contrast, pathogenic forms of PCV-2 have emerged over the last 2 decades and are believed to be a major causal agent of the economically important, post weaning multisystemic wasting syndrome. While PCV 1 is regarded as non pathogenic in pigs, there are grounds for caution in assuming that it will always be non-pathogenic in other species. There are many examples of viruses that are non pathogenic in their hosts but which cause serious diseases in heterologous hosts, for instance the non-pathogenic ovine herpesvirus OHV-2 causes fatal, malignant catarrhal fever in cattle. Secondly, single stranded DNA viruses have shown a propensity to change hosts and pathogenicity following relatively few mutations, as evidenced by the evolution of the canine parvovirus, the Kresse strain of porcine parvovirus and the emergence of pathogenic PCV-2 strains.

PCV has been reported to infect Vero and HeLa cells as well as porcine, bovine and ovine cells (Allan et al 1994, Hattermann et al 2004, Kiupel et al 2005). The ability of PCV to infect pluripotent cells should be considered when evaluating the safety of products derived from stem cells (Steiner et al 2008). PCV-1 often establishes persistent, non-cytopathic infections and most banks of the widely used PK-15 cells are infected. In contrast, PCV-2 can be cytopathic, for instance in HeLa cells.

It should be noted that a bovine circovirus has been described that has high nucleotide similarity to PCV-2 strains. It is not known if this virus is truly endemic in cattle populations or results from occasional cross species transmission. The serological data are conflicting but, as described below, we have occasionally detected circovirus sequences in pooled bovine serum. Very recently a fatal haemorrhagic diathesis in calves has been described which may be aetiologically associated with BCV infection (Kappe et al 2010). Tischer (1995) reported the presence of antibody cross reactive with PCV in sera of humans, cattle and mice but, in a more recent survey, Ellis et al (2000) did not detect antibody to PCV-2 in veterinarians and others exposed to infected pigs. Porcine circovirus sequences have been detected in porcine pepsin intended for human use (Fenaux et al 2004) but the significance of this observation has not been established.

BioReliance recommends testing cells for a range of porcine viruses if they have been exposed to porcine trypsin at any point in their history; these
viruses are listed in the document *Porcine Viruses of Concern in Biotechnology*. Trypsin is widely used in tissue culture but is also employed to activate certain viruses and, in the case of rotaviruses, infectivity is probably dependent on trypsin treatment of the virus. Proteolytic activation of the haemagglutinin (HA) protein is indispensable for influenza virus infectivity and trypsin has been used to activate influenza virus.

Where it is known that irradiation has been used, it may not be necessary to test for all of the viruses listed in *Porcine Viruses of Concern in Biotechnology* but a particular feature of porcine circoviruses is their high radioresistance, as shown in Figure 1, based on a study sponsored by Life Technologies, BioReliance’s former parent company (Plavsic & Bolin 2001). Consequently irradiated trypsin may still be a PCV hazard.

Circoviruses, like parvoviruses also display high resistance to other physicochemical treatments and are not inactivated by treatment at pH 3.0.

Recently, a new genus of closed circular ssDNA viruses has been described, the anelloviruses. These include TTV virus of humans and the porcine torque teno viruses. Until recently little attention has been paid to these viruses but tests for these viruses are now requested by European authorities, when evaluating the safety of cell substrates used for veterinary vaccines. Recently, porcine torque teno viruses have been detected in a human drug product as well as in veterinary vaccines (Kekarainen et al 2009). In 2010 Li et al. described a new putative genus the Cyclovirus genus with multiple species widely distributed in animals and humans.

The role of these viruses in disease remains to be determined.

**Testing Regimes**

The testing regime for cells is essentially straightforward. BioReliance utilises a validated, real time, QPCR which detects PCV 1 & 2 and BCV. Independent confirmation is provided by immunofluorescence assays for viral capsid antigens. Where necessary, further evidence of replication can be obtained by electron microscopy, although this is not a sensitive method for persistent infections, and by analysis of spliced transcripts of the replicase gene.

**PCR Detection**

BioReliance currently employs a validated QPCR assay (protocol 107031GMP.BUK) for the detection of porcine circovirus 1 & 2 and bovine circovirus. The assay is validated to detect 100 genome copies per reaction but trend analysis indicates it will regularly detect 10 genome copies.

Key points to be kept in mind when using PCR to detect PCV in cell substrates are:

- If possible the cells should be at the end of log phase of growth when harvesting for PCR analysis.
- Porcine trypsin should not be used in the culture period as residual PCV sequences from the high level present in porcine trypsin can give false positives. There is also a theoretical possibility of detection of
residual BCV sequences although, in our experience the levels of this virus are low or absent in most bovine sera. The cells should be washed several times in non-serum medium and removed by scraping or with a recombinant trypsin like Life Technologies’ Tryple™.

While there has been a recent example of a PCV positive cell substrate used in a veterinary vaccine, we have not detected a positive Vero cell bank. We are currently tracing through our data but of the four most recent commercial Vero cell banks none were positive. One of the banks was identified as CCL81 but the origin of the other 3 was not specified by the client. During the validation of our assay we included CCL81 cells and they were negative on that occasion. While sequences in porcine trypsin are abundant, the relatively infrequent infection of non-porcine cell lines is likely attributable to the reduced, albeit not complete, inactivation by irradiation and the observation that high virion numbers are required to infect cells (Tischer et al 1987). This is supported by findings that in vivo infection of gnotobiotic pigs can be orders of magnitude more sensitive than in vitro assays (Allan, Personal Communication).

**Immunofluorescent detection**

Immunofluorescence for capsid antigens provides the simplest method to confirm infection of cell substrates. We utilise both monoclonal antibodies to PCV-2 and polyclonal antibodies reactive with PCV-1. This assay was regularly employed in the past but was discontinued based on commercial demand. However, it is currently being re-established. Infected PK-15 cells are used as a positive control with appropriate negative cells. Key factors in using IF confirmation are:

- The cells must be mitotically active.
- Porcine trypsin should not be used in preparing the cells and irradiated bovine serum or serum free medium should be used during pre-test culture.
- Treatment with 300 mM glucosamine for 1 hour, 3 to 6 hours after replating the cells (or after infecting the cells) can enhance antigen expression by up to 7 fold ~20 hours later (Tischer et al 1987).
- Intense nuclear staining is observed in positive cultures.

**Alternative assay to detect replicating PCV**

When low level PCV PCR signals are obtained it is possible to investigate whether active replication is occurring through detection of splice variants of the rep gene (Bratanich et al 2002). Replicase gene products of 1kb, 600bp and 250bp, resulting from alternative spliced transcripts are observed in cells with actively replicating virus.

**Analysis of raw materials for PCV & BCV and infectivity assays**

In our experience PCV sequences are present at a high level in most batches of porcine trypsin. Illustrative examples are shown in Table 1, p. 4. The positive bovine products giving signals below the limit of quantification, were confirmed as positive on re-amplification but note the much lower level of sequences compared to porcine trypsin. It is unlikely the origin of the positive bovine serum is of relevance.
A standard PCR positive assay does not indicate whether or not the genomes are encapsidated or present within infectious particles. It is possible to evaluate:

- If the genomes are protected within capsids by centrifugation through gradients, followed by nuclease treatment to destroy free nucleic acid. The capsids are then disrupted and the genomes detected by PCR.
- Using rolling circle amplification of the genomes it is possible to determine if the genomes are full length or have been fragmented by irradiation.

Neither of these procedures are routine and they are only used in investigations. An infectivity assay provides a method of directly detecting infectious virus but there are several factors to be considered:

- As indicated above *in vitro* infectivity assays are relatively insensitive.
- Permissive cells like PCV negative clones of PK-15 cells should be used.
- Mitotically active cells are required and if an IF endpoint is employed, glucosamine treatment should be considered.
- PCR endpoints are sensitive alternatives but great care is required to ensure that residual input virus is not being detected. An early post infection sample should be taken and an increase in genome copy number demonstrated during the culture period.

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**Table 1. QPCR detection of PCV and BCV in Materials**

<table>
<thead>
<tr>
<th>Material</th>
<th>Quantity of Starting Material In Reaction</th>
<th>Result</th>
<th>Genome Equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine trypsin</td>
<td>3.2 ml</td>
<td>Positive</td>
<td>4,673 ± 2,098</td>
</tr>
<tr>
<td>Bovine trypsin</td>
<td>5.31 mg</td>
<td>Positive</td>
<td>9.62 ± 0*</td>
</tr>
<tr>
<td>Bovine insulin</td>
<td>3.2 mg</td>
<td>Positive</td>
<td>2.71 ± 0*</td>
</tr>
<tr>
<td>BSA</td>
<td>1.4 mg</td>
<td>Negative</td>
<td>0.00</td>
</tr>
<tr>
<td>FBS 1 (US)</td>
<td>3.2 ml</td>
<td>Negative</td>
<td>0.00</td>
</tr>
<tr>
<td>FBS 2 (US)</td>
<td>3.2 ml</td>
<td>Negative</td>
<td>0.00</td>
</tr>
<tr>
<td>FBS (French)</td>
<td>3.2 ml</td>
<td>Negative</td>
<td>0.00</td>
</tr>
<tr>
<td>FBS 4 (South American)</td>
<td>3.2 ml</td>
<td>Positive</td>
<td>5.74 ± 0*</td>
</tr>
<tr>
<td>FBS 5 (New Zealand)</td>
<td>3.2 ml</td>
<td>Negative</td>
<td>0.00</td>
</tr>
<tr>
<td>FBS 6 (Australian)</td>
<td>3.2 ml</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Sentinel extraction control</td>
<td></td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>PCR negative control</td>
<td></td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Positive standard 10² target copies</td>
<td></td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Positive standard 10¹ target copies</td>
<td></td>
<td>Positive</td>
<td></td>
</tr>
</tbody>
</table>

*Below level of quantification. Samples confirmed positive on re-amplification*
References.


FNV2#FN2 Journal of Virology, February 2010, p. 1674-1682, Vol. 84, No. 4


