Notes

Comparative isolation of infectious salmon anaemia virus (ISAV) from Scotland on TO, SHK-1 and CHSE-214 cells.

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Abstract

The TO cell line was tested for the isolation of infectious salmon anaemia virus (ISAV) from Atlantic salmon organs. Isolations were made from stored liver and mixed organ samples from a confirmed case of ISA in Scotland. TO, SHK-1 and CHSE-214 cells were used. The TO cell line was faster to show a cytopathic effect than SHK-1 or CHSE-214 cells and also isolated virus from more samples than the other cell lines. These results indicate that TO cells can be used to improve the sensitivity of virus isolation tests for Scottish isolates of ISAV.

A new cell line (TO) has been developed that is susceptible to ISAV (Wergeland & Jakobsen, 2001). The isolation of ISAV from fish organs was first demonstrated with the use of the SHK-1 cell line (Dannevig et al., 1995) and is currently in use for virus isolation in Scotland and Norway. By contrast, the CHSE-214 cell line is used by some laboratories in Canada for the isolation of ISAV (Bouchard et al., 1999, Kibenge et al., 2000). The ASK cell line (Devold et al. 2000) has also been shown to replicate ISAV and show cytopathic effect (CPE). Another cell line that has been shown to grow ISAV is the AS line (Sommer & Mennen, 1997) which was reported as non-cytopathic. ISA virus replication without CPE can be detected by haemadsorption (Sommer & Mennen, 1997, Smail et al. 2000) which improves the detection in samples with no CPE. There is also some evidence that cell culture adaptation can be enhanced by the addition of trypsin (Kibenge et al. 2001). The aim of this study was to enhance our isolation rate of ISAV by cell culture, improve the sensitivity and shorten the time scale of our diagnostic test. The first aim was to compare 4 cell lines for speed of CPE on passage of the Scottish reference isolate 390/98. A second aim was to compare SHK-1, TO and CHSE-214 cells for isolation rate, using frozen tissue material from a case previously confirmed positive by isolation and indirect fluorescent antibody technique (IFAT) on SHK-1 cells.

Stock CHSE-214 cells (provided by Dr F Geoghegan, Fisheries Research Centre, Department of Marine, Dublin, Ireland) were grown in E-MEM medium supplemented with 10% FCS and 1% L-Glutamine and for the tests were maintained in the same medium.
with 5% FCS. For inoculation the pass number was in the range 262-471.

Stock SHK-1 cells (provided by Dr B. Dannevig, National Veterinary Institute, Oslo, Norway) were grown and inoculated in L-15 medium supplemented with 5% FCS, 2% L-glutamine and 0.08% 2-mercaptoethanol. For inoculation the pass number was in the range 57-69.

Stock TO cells (provided by Dr H. Wergeland, Department of Fish. and Marine Biology, University of Bergen, Norway, in agreement with Alpharma A/S) were grown and inoculated with Earles MEM with Hanks BSS (H-MEM) medium supplemented with 5% FCS, 1% L-Glutamine and 1% non-essential amino acids (NEAA). In open well plates 1M HEPES buffer was added to the medium to a final concentration of 1.4% v/v. For inoculation the pass number was in the range 57-69.

Stock ASK-1 cells (provided by Dr B. Krossøy, Department of Fish. and Marine Biology, University of Bergen, Norway) were grown and inoculated in L-15 medium supplemented with 5% FCS and 1% L-Glutamine. For inoculation the pass number was 30.

**Development of CPE in 4 cell lines.**

The Scottish reference isolate 390/98, grown in SHK-1 cells, was passed in 4 cell lines at a range of dilutions to assess the development of CPE. Virus was inoculated into 25cm² flasks of TO, SHK-1, ASK and CHSE-214 cells at dilutions from $10^{-3}$ to $10^{-5}$ and incubated at 15°C. On days 1-13 the extent of cell lifting and necrosis was estimated for cultures at the $10^{-2}$ to $10^{-5}$ dilutions as a percentage of the whole cell monolayer. The estimate was made by a single analyst over the whole period. CPE was read after 17 days for ASK and TO cells and 21 days for SHK-1 cells when no further CPE was observed at the endpoint.

**Isolation comparison, Liver tissue**

10 liver homogenates from individual fish stored at −80°C, from case 388/98 previously found positive for ISAV on SHK-1 cells, were used to evaluate ISAV replication in TO cells versus CHSE-214 and SHK-1 cells. Samples were preincubated with IPNV-Sp antiserum diluted 1:50, then inoculated directly into 24-well plates to a final dilution of 200x w/v and serially diluted (10x) through 12 wells for each cell line. TO & SHK-1 cultures were incubated at 14°C and CHSE-214 at 15°C for 14 days then screened by haemadsorption (Smail et al., 2000) using rabbit red blood cells. The supernatants were passed onto fresh cells at 1:10 dilution, incubated for a further 2 weeks and read by haemadsorption. Positive samples were confirmed by IFAT using the Mab 3H6F8 (Falk et al., 1998) and goat anti mouse IgG FITC conjugate (Sigma).

**Isolation comparison, from mixed organs**

10 pooled organ samples (heart, liver, kidney and spleen) from individual fish stored at −80°C, from case 388/98 used in the isolation from liver, were processed to simulate a diagnostic ISAV sample. Samples were preincubated with IPNV-Sp antiserum diluted

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of pools +ve</th>
<th>IFAT id.</th>
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<tbody>
<tr>
<td>CHSE-214</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td>SHK-1</td>
<td>7/10</td>
<td>all</td>
</tr>
<tr>
<td>TO</td>
<td>10/10</td>
<td>all</td>
</tr>
</tbody>
</table>

Table 1. Liver isolation: nos. of pools positive for CPE and virus identification by IFAT.
1:50 and inoculated in duplicate to give a tissue dilution of 1000x w/v in the first well then serially diluted (1:10) across 8 wells containing either TO, SHK-1 or CHSE-214 cells in 12 well plates. In a parallel test each pooled organ sample was titrated by end point dilution on 96-well plates starting with a dilution of 200x w/v. TCID\textsubscript{50} titres were calculated by the method of Reed & Muench (1938). The cultures were incubated and tested as for the liver isolation.

Growth of supernatant virus
Fig 1. shows the development of CPE in the different cell lines. At the low dilution of virus (10\textsuperscript{-2}) there was no difference between the ASK and the TO cell lines which both performed slightly better than the SHK-1 cell line. As the dilutions increased up to 10\textsuperscript{-5} the TO cell line showed a markedly earlier CPE compared to the other cell lines. Throughout the test the CHSE-214 cells did not show any CPE.

Liver isolation
The CHSE-214 cell line did not show any CPE and therefore did not detect any virus from the homogenates. The SHK-1 cell line showed CPE in 7 pools, which were all confirmed as ISAV by IFAT. The TO cell line showed CPE in all 10 pools, which were all confirmed as ISAV by IFAT (Table 1). The SHK-1 cell line showed the earliest CPE after 10 days in the

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Fig 1. The development of CPE in 4 cell lines after inoculation of ISAV at 4 dilutions, A) 10\textsuperscript{-2}, B) 10\textsuperscript{-3}, C) 10\textsuperscript{-4}, D) 10\textsuperscript{-5}. 

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passage culture compared to the TO cell line which by contrast showed CPE after 12 days in the primary culture.

**Mixed organ isolation**

The CHSE-214 and the SHK-1 cell lines did not show any CPE and no virus was detected from the homogenates. The TO cell line showed CPE in 4/10 samples with the standard isolation method. The parallel titration of the organ homogenates on TO cells detected 5/10 samples positive all with low titres (Table 2.)

The CHSE-214 cell line did not replicate ISAV from tissue material used in this study showing no CPE for 2 cases where isolation was successful on other cell lines. Haemadsorption was used in addition as a check on intra-cellular replication without CPE. Kibenge *et.al.* (2000) also reported that the reference isolate 390/98 caused no CPE on CHSE-214 cells, a property also of some Canadian isolates, showing phenotypic divergence of viral host range in culture. The majority of Canadian ISAV isolates show CPE in CHSE–214 cells, reflecting the genetic dissimilarity of the Canadian isolates from the Norwegian and Scottish isolates analysed (Cunningham & Snow, 2000). However we have found that the Scottish reference isolate 390/98 will grow on CHSE-214 cells with limited CPE 14 days post inoculation after repeated passage following primary isolation on SHK-1 cells.

Testing of the organ samples was intended to show how sensitive TO cells were to fish organ samples. Using the liver samples, the TO cell line showed a greater sensitivity to virus, detecting 3 more positives than the SHK-1 cell line. The time to detection is also very important for our diagnostic tests. In this study, the TO cells detected virus by CPE 12 days before the SHK-1 cells. The mixed organ samples were used to imitate a normal diagnostic sample. In this case, only the TO cells detected any virus. One more pool was detected by titration, which may be due to a lower starting dilution of 200x or the greater number of replications used for this method in contrast to the 1000x starting dilution used for the isolations.

It was surprising that the mixed organ samples were negative on SHK-1 cells since most of the individually tested organ homogenates were positive on the initial diagnosis (e.g. 9/10 heart, 2/10 kidney, 7/10 liver & 7/10 spleen). The sensitivity of the test was 500 infectious units per g pooled tissue for the isolation. Perhaps there was significant loss of titre due to pooling of tissues and also due to freeze/thawing of the homogenates after storage for 22 months at –80°C. However, since the homogenate dilution used was the same

<table>
<thead>
<tr>
<th>Fish No.</th>
<th>CPE (TO Cells)</th>
<th>Virus titre (TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</th>
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<tr>
<td>1</td>
<td>-</td>
<td>&lt;10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>&lt;10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>&lt;10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>4.2 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>1.57 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>2.04 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>&lt;10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>&lt;10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>1.43 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>1.0 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
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Table 2. Isolation of ISAV from mixed organ samples by detection of CPE in TO cells and corresponding virus titres.
for all the cell lines tested the comparison for CPE and haemadsorption was real and showed greater sensitivity of the TO cell line at the end point tested.

Comparing the results from the three experiments there is an increase in the sensitivity of detection when using TO compared with SHK-1 cells. In addition there is a shorter time to CPE using TO cells, in agreement with the results of Wergeland & Jackobsen (2001). Furthermore, TO cells replicate ISAV to a higher titre than SHK-1 cells (Wergeland & Jakobsen 2001).

Several reports now show that the TO cell line is sensitive to ISAV isolates e.g. for Chile (Kibenge et. al. 2001), Norway (Wergeland & Jakobsen, 2001) and this study using the Scottish isolate 388/98.

The TO cell line appears to be stable and does not alter in sensitivity to ISAV after up to 120 passages. This is in contrast to the SHK-1 cell line, which loses its ISAV susceptibility assessed by CPE at around pass 80 (unpublished results & B.Dannevig, pers.comm.). A cell line that is pass number stable has an advantage for both experimental and diagnostic work, making it easier to compare results and maintain the sensitivity of testing. The TO cell line would appear to be a useful cell line for the isolation of ISAV in cases of clinical disease and in support of clinical inspection and surveillance programmes. For virus diagnostics, application for use of the TO cell line should be made to Alpharma A/S; consideration will be given on a case by case basis.

Acknowledgements
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References


Reed L J, Muench H (1938) A simple method of estimating 50% end points. Amer. J. Hyg. 27: 493-497

