Evaluation of orf virus (ORFV) isolation in continuous lamb testis cells (OA3.Ts) and development of a co-culture method with infected cells to increase infectivity

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ABSTRACT
Continuous foetal lamb testis cells OA3.Ts was used to compare the isolation of a vaccine orf virus (ORFV) strain that had been adapted to primary lamb testes cells, with scab derived wild-type/field ORFV isolates. The wild type virus showed an accelerated and exaggerated cyto-pathic effect (CPE) than the vaccine virus as has been demonstrated by immunofluorescent detection of viral antigen using ORFV monoclonal antibodies. ORFV could be successfully isolated in OA3.Ts foetal lamb testes cells and can be used for direct isolation of the virus from clinical samples. Two different methods of cell culture infection were also compared during sub-culture, one using infected supernatant as the inoculum and the other using infected cells as a modified method for infecting healthy culture cells. The present study also indicates that a higher infection can probably be achieved with inoculating infected cells together with healthy ones as a co-culture method during propagation of ORFV. This has been revealed as visibly pronounced CPE and presence of larger and multiple aggregates of cytoplasmic inclusions than cells infected with infected supernatant alone.

Key words: Antigen, Cell culture, CPE, Immune-detection, Orf virus (ORFV).

INTRODUCTION
Orf is an exanthematous disease of sheep and goats, caused by orf virus (ORFV) of the family Poxviridae and it is the type species belonging to the genus Parapoxvirus (PPV). Virus isolation in cell culture is regarded as the ‘gold standard’ method for detection of poxviruses (Hautaniemi, 2012). Cultivation of Parapoxvirus in cell culture is sometimes regarded difficult because of many unsuccessful attempts to isolate the virus, but the reasons for this is unknown (Nagington, 1968; Suzuki et al., 1993). Plowright et al. (1959) initially used primary lamb testis and primary lamb kidney cells to isolate ORFV in tissue culture. Different primary cell cultures from ovine, bovine and caprine origin have been originally preferred, mainly because of their ability to support the replication of a variety of capripoxvirus isolates and the ability to obtain sufficient numbers of cells from the respective organs (Babiuk et al., 2007). These include ovine origin—primary lamb testis, lamb kidney, foetal lamb dermis cells, foetal lamb muscle cells (McInnes et al., 2001), ovine foetal turbinate cells (Delhon et al., 2004); bovine origin—foetal bovine lung cells (Inoshima et al., 2002), bovine foetal spleen (Hessami et al., 1979; Lard et al., 1991), bovine foetal muscle (Inoshima et al., 1999), primary bovine testis and lung (Rosenbusch and Reed, 1983; Suzuki et al., 1993; Mercer et al., 1994; Kuroda et al., 1999) etc. Chicken embryonated eggs have been reported to produce pathological changes in the form of odema, thickening, hemorrhages, small grayish white foci and pock lesions with clinically infected orf materials from sheep and goats (Ali et al., 2013).

Primary or secondary cell culture still have many of the detrimental, inherent characteristics exhibited by primary cultures, such as cell heterogeneity and the presence of potential contaminating elements such as endogenous viruses; besides, there is also variability in the ability to replicate virus at each passage level and virus susceptibility is still limited to within a few passages (Jassim and Keshavamurthy, 1981). This has prompted the alternate use of continuous cell lines for virus propagation (Babiuk et al., 2007). Established cell lines like Madin-darby bovine kidney (MDBK) (Klein and Tryland, 2005) and Madin-darby ovine kidney (MDOK) (Guo et al., 2003), Vero cells (Rohde et al., 2012) etc., have been used for ORFV isolation and propagation.

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American Type Culture Collection (ATCC) ovine foetal testis adherent cell line OA3.Ts cells with an epithelial morphology have been used to propagate both capripoxvirus and ORFV successfully (Babiuk et al., 2007). In the present study OA3.Ts was used to compare the isolation of a standard vaccine virus adapted to primary lamb testes cells with scab derived wild-type/field ORFV isolates.

After the initial blind passages, noticeable CPE in the continuous cells were very few. In a hypothetical attempt to increase the infectivity, two different methods of cell culture infection were also compared during sub-culture; one using infected supernatant as the inoculum and the other using infected cells as a modified method for infecting healthy culture cells.

MATERIALS AND METHODS

Viruses

Reference virus isolate: Live attenuated orf vaccine virus (Orf Muk 59/05, batch 06/09) isolated from goat and previously propagated in primary lamb testis cells (PLT) produced at Poxvirus Disease Laboratory, Division of Virology, Muktishwar was used as a reference ORFV strain.

Field/wild type virus isolates: Virus obtained from scabs of orf infected goats (BAG17) was isolated in cell culture system and included in the present study. Scab samples of affected goats were processed for virus isolation as described by Kottaridi et al. (2006) and Oem et al. (2013) with modification. Briefly, a 10-20% solution of the scab samples in PBS or HBSS was prepared by mechanical homogenization using a sterilized mortar pestle and freeze-thawed thrice at “70°C. To ensure release of intracellular viruses, the sample was pulse sonicated over three cycles of 10 seconds at 37% in an ultrasonic cell disrupter (SONICS VibraCell VCX750) modified from Klein (2004). Homogenized and sonicated solutions were centrifuged at 4°C at 3,000 x g for 15 min. The supernatant was then passed through a 0.45 im filter (Millipore) and incubated with 100X antibiotic/antimycotic solution (Sigma, #A5955) and supplemented with 10% foetal bovine serum and incubated at 37°C in 5% CO₂. The composition of the medium was approximately similar to the ATCC media (DMEM, ATCC 30-2002) recommended for the cell line. Two-four blind passages were performed until cytopathic effects (CPEs) were observed. A 2% serum concentration was used for maintenance of the cells. Similarly, processed clinical samples were subsequently used to inoculate the continuous ovine testis cells. About 1ml of processed antigen was used to infect a 75 cm² flask containing ~1.2 x 10^6 cells at 30-40% confluence (day 2). Changes were observed till 70-80% CPE for next subsequent passage.

Initial infection of culture cells: A 1 ml reconstituted suspension of the lyophilized, live attenuated vaccine virus (Orf Muk 59/05, batch 06/09) was propagated as a reference strain in ovine testis cells (OA3.Ts) at passage 8-11 (p8 to p11). The titre of standard reference vaccine virus was found to be 10^(5.75) TCID₅₀/ml (Bora, 2010). About 1 ml of reconstituted antigen was used to infect a 75 cm² flask containing ~1.2 x 10^6 cells at 30-40% confluency (day 2). Similarly, processed scab lysates derived clinical orf samples were also used as an inoculum to infect the culture cells at passage 8 (p8) for propagation of field/wild-type virus.

The adherent epithelial cells were propagated in Dulbecco’s modified Eagle’s medium containing high glucose (Gibco®, #12100-046) with added 1mM sodium pyruvate, 44.05 mM sodium bicarbonate, Antibiotic Antimycotic solution (100x) (Sigma, #A5955) and supplemented with 10% foetal bovine serum and incubated at 37°C in 5% CO₂. The composition of the medium was approximately similar to the ATCC media (DMEM, ATCC 30-2002) recommended for the cell line. Two-four blind passages were performed until cytopathic effects (CPEs) were observed. A 2% serum concentration was used for maintenance of the cells. Similarly, processed clinical samples were subsequently used to inoculate the continuous ovine testis cells. About 1ml of processed antigen was used to infect a 75 cm² flask containing ~1.2 x 10^6 cells at 30-40% confluency (day 2).

Subculture of infected cells: Subculture of healthy cells with infected culture supernatants: After the observation of 70-80% CPE, the infected culture cells were freeze-thawed thrice and the supernatant harvested. Approximately 1 ml of this culture supernatant after centrifugation at 3,000 x g for 5 min, was used to infect a fresh cell culture in a 75 cm² flask containing ~1.2 x 10^6 cells at 30-40% confluency (day 2). Changes were observed till 70-80% CPE for next subsequent passage.

Co-culture of healthy culture cells with infected cells: After the observation of 70-80% CPE, the infected culture cells were trypsinized and harvested. Healthy uninfected cells seeded ~1.2 x 10^6 cells and in approximately 30-40%
confluence were also harvested. Instead of infecting with the culture supernatant, equal volumes of infected culture was incubated along with the healthy cells in a 75 cm² flask as a co-culture method. Changes were observed till 70-80% CPE for next subsequent passage.

**Screening of infected culture cells:** The culture cells were regularly observed for characteristic cytopathic effects (CPE) caused by *Parapoxivirus*. Cells grown on chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System) were cytologically stained with Haematoxylin and Eosin stain or May-Grünwald Giemsa stain for cytomorphological changes and demonstration of inclusions.

**Indirect immunolabelling of ORFV in culture cells with FITC:** Fluorescent immunolabelling was used to demonstrate viral antigen in progressive infected cells using a mouse primary monoclonal antibody against orf virus (sc-101589, Santa Cruz Biotechnology Inc.) and goat anti-mouse antibody-FITC conjugate (sc-2010, Santa Cruz Biotechnology Inc.) to combine a maximum signal with a minimum background staining. The procedure described in EU diagnostic manual for CSF diagnosis (EU,2007) was followed with modifications. Briefly, cultures grown on chamber slides were rinsed once with washing buffer (0.01% PBS-Tween, pH 7.4) and incubated in blocking buffer (1% bovine serum albumin in PBS-Tween) for 1 hour in a humid chamber. The cells were overlaid with a 1:100 working dilution of orf virus mouse monoclonal antibody in blocking buffer and incubated overnight at 4°C in a moist chamber. The fixed cells were overlaid with a 1:30 working dilution FITC conjugate in blocking buffer. The conjugate was pre-filtered by 0.2μ pore syringe filter to remove FITC crystals. The cells were incubated for 60 min at 37ºC in a moist chamber. The cells were rinsed thrice for 5 min in washing buffer and once in distilled water. A drop of 20% glycerol in PBS was placed onto the cells and mounted for observation under a fluorescent microscope under UV illumination.

Culture cells at 12, 24, 48 and 96 hours post-infection (hpi) fixed and subsequently adsorbed with anti ORFV monoclonal antibody. PCR detection of *Parapoxivirus*: DNA isolated from both the reconstituted vaccine and from the clinical scab lysate was found to belong to *Parapoxivirus* by semi-nested PCR. The partial B2L gene which targets putative virion envelope was amplified by the primer set PPP1-PPP3 and PPP1-PPP4 (PPP1: GTG GTC CAC GAT GAG CAG CT; PPP3: GCG AGT CCG AGA AGA ATA CG; PPP4: TAC GTG GGA AGC GCC TCG CT) as described by Inoshima *et al.* (2000) for specific pan-parapoxvirus detection. For extraction of viral DNA, commercially available DNeasy Blood & Tissue Kit (Qiagen, #69504) was used according to manufacturer’s instructions. *Parapoxivirus* was also identified by electron microscopy and as ORFV species with immunoelectron microscopy using monoclonal antibody (data not shown).

**RESULTS AND DISCUSSION**

The qualitative and comparative assessment of vaccine strain and wild type orf virus (ORFV) infection in the continuous lamb testis (OA3.Ts) cells when sub-cultured with supernatant inoculum and co-culture with infected cell as inoculum is summarised in Table 1.

**OA3.Ts cells:** There was considerable heterogeneity in the cell morphology at different passage levels of the cells. Initially, when the cells were revived for the first time, they appeared short and plump. After third passage (p3) cells divided rapidly, achieving 80-90% confluency by 36 hours post seeding, and a complete monolayer was formed by 48 hours. After fifth passage, only 20% cells were short and plump, that further reduced to 10% by sixth passage. The plump cells were more difficult to detach and required longer incubation with trypsin. Subsequent passages presented cells with two distinct morphology: initial fibroblast-like elongate cells that tend to form whorls, and subsequently with more passages, cells had a characteristic less-elongated and epithelial-like morphology (Figure 1: A). Old cultures also tend to have increased cytoplasmic granularity. The cells were also very sensitive to pH changes in the media. Viability of the cells after cryopreservation was also found to be high.

Propagation of ORFV in OA3.Ts

**Table 1:** Qualitative assessment of continuous lamb testis (OA3.Ts) cells infected with vaccine strain and wild type Orf virus (ORFV) when sub-cultured with a modified co-culture method using infected cell as inoculum.

<table>
<thead>
<tr>
<th>Cyto-Pathic Effect (CPE)</th>
<th><strong>Vaccine virus</strong></th>
<th><strong>Wild type virus</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected inoculum</td>
<td>Infected inoculum</td>
<td>Co-culture with infected cells</td>
</tr>
<tr>
<td>Number of blind passages till visible CPE</td>
<td>3-4 days</td>
<td>2-3</td>
</tr>
<tr>
<td>First detection of CPE</td>
<td>2-3 days</td>
<td>2-3 days</td>
</tr>
<tr>
<td>Number of cells affected/ detached</td>
<td>Few; 30-40% by 6-7th day</td>
<td>More; 50-60% by 6-7th days</td>
</tr>
<tr>
<td>Intra-cytoplasmic inclusions</td>
<td>Small, often solitary</td>
<td>Large, often solitary</td>
</tr>
<tr>
<td>Detection of viral antigen</td>
<td>By 24 hpi; increased intensity by 48 hpi; vivid inclusions at 72 hpi</td>
<td>From 12 hpi; increased intensity by 24-48 hpi; vivid inclusions at 72 hpi</td>
</tr>
<tr>
<td>Immuno-fluorescence (hours post infection)</td>
<td></td>
<td>From 12 hpi; dense inclusion aggregates at 48-72 hpi</td>
</tr>
</tbody>
</table>
Propagation of vaccine virus: CPE with the live attenuated cell culture adapted vaccine virus were comparable to those in primary lamb testis cells. In the continuous cells (OA3.Ts), CPE was observed by 2-3 day post-inoculation after 3-4 blind passages as increased cytoplasmic granularity, cell rounding and detachment. Refractile inclusions were also sometimes observed with cells losing their normal epithelial morphology to become swollen and irregularly rounded. Retraction of their cytoplasm made the cells to attain bizarre shapes and gradually lose their cell-to-cell attachments, and finally detach from the surface (Figure 1: B). There was no cytolysis observed, and cells were affected in a diffuse manner without formation of distinct plaques. Cytological stained infected cells showed small eosinophilic intracytoplasmic inclusion (Figure 1: C) similar to those in the primary lamb testis culture cells (Figure 1: D). No CPE was observed when the vaccine was filtered through a 200 nm pore size syringe filter.

Propagation of wild-type virus
Subculture of healthy cells with infected culture supernatants: After initial 2-3 blind passages, CPE similarly developed as characteristic cell rounding and detachment as with the vaccine virus. There was an increase in cytoplasmic granularity and presence of refractile bodies by 2-3 days post-inoculation. No cytolysis or distinct plaque formation could be observed. However, infection with the wild-type virus caused more and pronounced CPE, with ~50-60% detachment of the monolayers by about 6-7 days post-infection. Haematoxylin and eosin stained infected cells showed small eosinophilic intracytoplasmic inclusion.

Co-culture of healthy culture cells with infected cells: CPE was readily noticed by 2-3 dpi in the first passage, and 70-80% cytopathy occurred by 5 dpi. The refractile bodies in culture cells increased in number and were also noticeably larger, and so were the inclusions observed on staining (Figure 1: E, F). However, the toxicity of the flask increased with every subsequent 3-4 passage due to the accumulation of the dead cells and toxic wastes.

Detection of ORFV antigen in OA3.Ts: Viral antigen could also be demonstrated in the cytoplasm of the infected cells by immunofluorescence as dense aggregates of immunopositive reaction. These aggregates appeared dispersed in the cytoplasm, but mostly along the peripheral margin of the nucleus. No signal was detected from the nucleus. In addition, there was no signal detection in uninfected cell controls or with negative sheep sera (data not shown). Immunofluorescent detection of viral antigen was done at various time intervals of cells with both vaccine and field virus (BAG17) as shown in Figure 2. With the

Fig 1: Primary and continuous (OA3.TS) lamb testis culture cells showing characteristic cytopathic effect (CPE) when infected with vaccine and field isolates of orf virus (ORFV). A: Confluent OA3.Ts cells, H&E X 400; B: OA3.Ts cells infected with orf virus from clinical samples showing characteristic CPE and cell detachment, unstained X 400; C: OA3.Ts cells infected with orf vaccine virus showing eosinophilic cytoplasmic inclusions, H&E X 1000; D: Orf vaccine virus infected primary lamb testis cells showing eosinophilic cytoplasmic inclusions, MGG X 400; E-F: OA3.Ts cells infected with field orf virus isolates using infected cells as inoculum (E: unstained and F: H&E X 400).
vaccine virus, immunofluorescence of viral antigen was feebly recognized in some culture cells after 24 hrs of infection, while with field isolate, the first viral antigens were faintly recognizable by 12 hrs of infection. The intensity of the fluorescent signal and number of infected cells increased at 24 and 48 hrs after infection. Vivid, fluorescent, inclusion-like patches were detected in the cytoplasm at 72 hrs (3 dpi). Similarly, in cells infected by the co-culture method, fluorescent signal could be seen from 12 hrs, which increased progressively with presence of very dense signal aggregates at 48-72 hrs post infection.

Apart from detection of viral antigen by immunofluorescence, ORFV infection of culture cells were confirmed by semi-nested PCR with amplification of a DNA product size of 594 bp with the first set, and 235 bp with the second set of primers.

The present study shows that OA3.Ts can be used to propagate ORFV from clinical samples. Babiuk et al. (2007), found that growth curve of OA3.Ts cells exhibited a decline in the exponential growth phase of cells from passage 17-36, but viability remained greater than 97% at all passage levels, which however decreased by 35% after 168 hours of seeding and subsequently continued to decrease further. They also reported the heterogeneity of the cells, from being more elongated, ordered and tightly packed to form monolayers at initial passages, that later became larger, less elongated, vacuolated and swollen, representing signs of stress at higher passage levels up to 36 passage. These findings were similarly observed in the present study, although growth kinetics was not quantitatively determined at subsequent passages, and the cultures were all maintained at a relatively lower passage level (up to 17 passages), the status of the cells at yet higher passage levels could not be ascertained. Nevertheless, it was observed in the present study that the morphological alterations in the cells translated to a similar confluency but less absolute cell count for the higher passages.

The number of blind passages reported in the literature that are required for Parapoxvirus isolation and recognition of cytopathic effects vary with different culture cells. The variability observed in the present study for vaccine and field virus isolates might be due to the initial adaptation of the virus to in vitro conditions, the availability of appropriate growth factors, the growth phase of the cells expressing appropriate surface receptors for virus attachment, etc. The absence of CPE when inoculum was filtered through 200 nm pore membranes was supported by the observations of Kuroda et al. (1999) who found that while using different pore sized filters for Parapoxvirus, most viruses passed through a pore size of 450 nm, some passed through 200 nm pores, while none through 100 nm filters. This is obvious, with the size of the average Parapoxvirus ranging from 200-260 nm x 130-160 nm, only few infective virions in specific orientations could possibly pass through.

CPE associated with ORFV are very much characteristic and differ from other poxviruses. The observed CPE were typical and similar lesions in cell cultures have been described in the literature (Pye, 1990; Li et al., 2012; Ali et al., 2013; Oem et al., 2013). Although observation of CPE was done in limited time intervals on subsequent days, it generally concurred with the observations of Pospischil and Bachmann (1980). In an experiment on ORFV infected bovine and ovine embryonic lung cells, Pospischil and Bachmann (1980) studying infected cells at regular intervals at 6, 9, 12, 14, 18, 24, 36 and 48 hpi observed that characteristic CPE began around 12 hpi that progressed rapidly through 48 hpi to destroy most cells. Traditional endpoint dilution of virus, and a TaqMan® PCR analysis of the viral DNA at 24, 36 and 48 hpi does illustrate an exponential growth of ORFV in organotypic lamb kidney cultures (Gallina et al., 2006).

Staining the affected cells revealed distinct eosinophilic inclusions with varying sizes in the cytoplasm that were either solitary or multiple, with a clear demarcating halo around them. Similar intracytoplasmic inclusions on hematoxylin and eosin stained preparations could be demonstrated in foetal bovine lung (FBL) cells infected with Parapoxvirus (Kuroda et al., 1999).

In the present study, the detection of viral antigens in cell cultures were analogous to the observations of Pospischil and Bachmann (1980), who however studied ORFV antigen at more elaborate time intervals in infected bovine and ovine embryonic lung cells (BEL/OEL) at 6, 9, 12, 14, 18, 24, 36 and 48 hpi using immunofluorescence. They observed specific cytoplasmic fluorescence at 10 hpi that intensified gradually up to 20 hpi when about 70 to 80% of the cells were positive. Further, they observed that the early fluorescence appeared granular, which became brightly fluorescing inclusion-like patches in the cytoplasm at later stages, while no nuclear fluorescence was demonstrable in either cell types. They also confirmed the virus associated CPE by TEM examination of BEL cultures showing mature virions at 12 hpi, and as early as 16 hpi in OEL infected cells. Similarly, Li et al. (2012) could demonstrate ORFV antigen targeted with monoclonal antibodies in infected primary ovine foetal turbinate starting from 12 hpi. The detection of ORFV antigen with specific monoclonal antibodies may also be dependent on the target epitope/protein, as many of the viral proteins are synthesised at varying time intervals post infection. Surface proteins may be lately synthesised and therefore may be detected lately. The exact reason for the difference in the time and passage involved in the observation of CPE associated with the vaccine virus and the field viruses could not be ascertained. However, it may be presumed that the vaccine virus has been
already adapted with repeated passages in primary lamb testes cells and therefore could not readily adapt to OA3.Ts cells. Additionally, the vaccine virus after repeated passage in culture cells may also be associated with some degree of attenuation. The field viruses on the other hand, were wild type virulent strains, and therefore readily express their infectivity and show earlier and pronounced CPE. The infective dose may also contribute to the CPE, as field scab samples may contain a higher infective concentration of the virus (i.e. multiplicity of infection; MOI) than its counterpart.

Initial subculture with infected supernatant fluid required more blind passages before noticeable CPE could be observed. Besides, few cells produced inclusions when observed in stained cells. It was presumed initially that the virus did not efficiently release itself from the infected cells into the culture supernatant leading to a lower multiplicity of infection (MOI). When the simultaneous seeding of 30-40% confluent healthy cells was adopted with infected cells as a co-culture, it ensured that cells were in a very active phase of growth and readily provided infective virions with new replicative cells. The virus titre was not estimated to calculate the MOI; however, this procedure apparently increased the infectivity. Literature on similar studies is apparently lacking.

ORFV could be successfully isolated in OA3.Ts foetal lamb testes cells and can be used for direct isolation of the virus from clinical samples. The present study indicates that a higher MOI can probably be achieved with inoculating infected cells with healthy ones during propagation of ORFV as demonstrated with the exaggerated CPE exhibition than cell infected with infected supernatant alone. However, the exact mechanism needs to be evaluated further with quantification of virus titres.

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