CASE REPORT

Post-viraemic detection of bovine ephemeral fever virus by use of autogenous lymphoid tissue-derived bovine primary cell cultures

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Background The potential tissue replication sites and specific cell types that support in vivo virus survival beyond the acute phase of bovine ephemeral fever virus (BEFV) infection have not been fully defined in cattle. To clarify the knowledge gap, tissue specimens were tested after collection from an adult steer necropsied 1 week after acute BEF.

Case report Significant necropsy findings included fibrinoproliferative synovitis in the stifle joints and fibrin clot-laden fluid in serous body cavities. Moderate numbers of infiltrating neutrophils were demonstrated in sections of the prefemoral lymph nodes and haemal node, and lymphoid hyperplasia in the spleen, haemal node and prefemoral lymph nodes. Viral RNA was detected by qRT-PCR in fresh spleen, haemal node, prefemoral lymph node, synovial fluid and in several spleen-derived cell cultures. BEFV was isolated from autogenously derived splenic primary cell cultures. BEFV was reportedly isolated from the bone marrow of three experimentally infected cattle before the onset of detectable viraemia.

Conclusions BEFV has preferential tropism for bovine lymphoid tissues and the spleen and haemal node may be potential sites for post-viraemic virus replication.

Keywords bovine ephemeral fever virus; cattle; haemal node; lymphoid tissue; spleen; tropism

Abbreviations Ct, cycle threshold; IHC, immunohistochemistry; mAb, monoclonal antibody; qRT-PCR, quantitative reverse transcription-PCR; RES, reticuloendothelial system

The BEFV-affected Brahman steer found sick and recumbent in the paddocks at Berrimah Research Farm, Department of Primary Industry and Fisheries, Northern Territory, Australia, belonged to a sentinel herd of 24 animals that were being used for arbovirus monitoring and surveillance. A confirmatory BEF diagnosis was made on the basis of a positive qRT-PCR result (cycle threshold (Ct) value ≤ 45.0 = positive; Ct value > 45.0 = negative) on jugular blood samples.

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The largely intracytoplasmic viral antigens were seen in different cells, including but not limited to neutrophils, macrophages, spindle-shaped cells of peripheral nerve tissue. Although these findings have elucidated the tissue tropism of the virus in cattle, the specific cell types that support virus replication remain to be definitively identified. The present report underscores the role of lymphoid tissues as potential sites of virus replication after biologically viable BEFV was detected in the spleen and haemal node of a Brahman steer 6 days after cessation of viraemia.

**Case report**

During necropsy, the carcase was noted to be in fair nutritional condition and the peritoneal, thoracic and pericardial cavities contained...
unremarkable amounts of serous fluid that contained small flakes of clotted fibrin. In addition, the synovial membranes of the left and right stifle joints were multifocally congested, moderately proliferative and the joints contained copious amounts of synovial fluid that contained small flecks of clotted fibrin. The spleen and a few abdominal and thoracic haemal nodes were markedly congested, presumably as a consequence of barbiturate euthanasia. A few petechial haemorrhages were present in the left fibular nerve and a focally extensive pale area of presumed compression myopathy was seen in the right gluteal muscle.

**Specimens**

Samples of pericardial, thoracic and peritoneal fluids and fresh and formalin-fixed lymphoid tissues (spleen, haemal nodes, prefemoral lymph nodes), plus several non-lymphoid tissues were collected.

Samples of peripheral blood collected over the course of the 1-week illness, fresh tissues and pericardial, thoracic, peritoneal and synovial fluids taken during necropsy examination were tested by qRT-PCR using a recently described protocol,9 based on a previously standardised method.10

Aseptically collected fresh spleen and haemal node samples for virus isolation were placed into sterile Gibco® Medium 199 (Life Technologies, NY, USA).

**Histopathology and immunoperoxidase staining**

Tissues fixed in 10% buffered formalin were processed for histopathology and immunohistochemistry (IHC).

The formalin-fixed tissue samples were paraffin-embedded, stained with haematoxylin and eosin and evaluated by light microscopy (Olympus-BX41, Model U-MDOB3; Tokyo, Japan). In addition, 4-μm sections of the spleen, haemal node and other tissues were also tested.9

The immunoperoxidase assay for demonstrating BEFV antigens was based on a Dako EnVision + System-HRP kit (Dako Australia Pty Ltd, VIC, Aust) with a few modifications and used the mouse monoclonal antibody (mAb) DB5 as the primary antibody probe.9 DB5 was initially developed by Cybinski et al.11 and subsequently commercialised by TropBio Pty Ltd (Townsville, QLD, Aust). It targets epitopes located within the G1 site of the viral envelope glycoprotein11,12 and has been previously used to characterise bovine-derived BEFV.11,12 The immunohistochemically and immunocytochemically stained sections were evaluated for viral antigens under the low- and high-power objectives of a light microscope (Olympus-BX41, Model U-MDOB3) and photomicrographs taken using Olympus Labsens imaging software.

Other than marked congestion consistent with an artefact of barbiturate euthanasia, the lymphoid follicles in the spleen, haemal node and prefemoral lymph nodes were characterised by massive, pale germinal centres consistent with moderate to intense immune activation. In addition, large areas of the haemal node and the medullary regions of the lymph nodes were infiltrated by numerous neutrophils, consistent with moderately acute haemadenitis and lymphadenitis, respectively.

Immunohistochemically stained sections of spleen (Figure 1), haemal node (Figure 2), and lung (Figure 3) showed positive intracytoplasmic staining within cells that were morphologically consistent with macrophages. In the stained slides of spleen and haemal node-derived cell cultures, weak staining suspected to be viral antigen was also seen within round-shaped cells of histiocytic morphology (data not included).

To examine virus morphology, ultrathin sections of spleen and haemal node were examined by transmission electron microscopy at Charles Darwin University, Darwin. Within an ultrasection of haemal node, characteristic bullet-shaped (rhabdovirus) virus particles, measuring approximately 75 × 140 nm, were observed budding into
smooth-sided vesicles within the cytoplasm of nucleated cells of presumed histiocytic morphology (data not shown).

qRT-PCR on antemortem blood and postmortem samples

The qRT-PCR test was positive for viral RNA on day 1 in peripheral blood but negative (Ct value > 45.0) for peripheral blood samples taken on days 2–7.

qRT-PCR was positive for viral RNA for the postmortem samples of spleen, haemal node, left and right prefemoral lymph nodes and synovial fluid taken from the left and right stifle joints.

qRT-PCR was negative for specimens of synovial fluid from the left and right carpal joints, pericardial, thoracic and peritoneal fluids, and liver, lung, kidney, urinary bladder, synovial membranes (left and right stifle joints, and left and right carpal joints), tongue, skeletal muscle (left and right semitendinosus, gluteus medius, biceps femoris, biceps branchii, longissimus dorsi, extensor carpi radialis, extensor carpi ulnaris, supraspinatus), left and right gluteal nerves, left and right fibular nerves, nerve tissue from the left and right brachial plexuses, spinal cord (cervical, thoracic and lumbar) and brain (cerebrum, cerebellum, mid-brain, thalamus and medulla oblongata).

Cell isolation and culture

The aseptically collected spleen and haemal node were separately processed according to an in-house protocol standardised at the Bertramah Veterinary Laboratories. Autogenous primary bovine cell cultures derived from both organs were established and periodically tested by qRT-PCR to assess the status of BEFV infection. Briefly, the spleen and haemal node were separately macerated and mechanically agitated to yield fine, cell-rich slurries, which were then mixed with a small amount of 0.12% trypsin (SAFC, Sigma-Aldrich, St Louis, MO, USA) and homogenised by additional mechanical agitation. The resulting cell-rich homogenate was mixed with Gibco Medium 199 supplemented with bovine pituitary-derived fibroblast growth factor (Sigma-Aldrich) to a final concentration of 3.2 ng/mL.

Next, 10 mL of the cell suspension was dispensed into 25-cm² tissue culture flasks, yielding a total of 16 spleen and 4 haemal node-derived level 0 passages (P0) that were incubated at 37°C. After 24 h of incubation, the flasks were washed with unsupplemented Medium 199, replenished with fresh medium supplemented with bovine pituitary-derived fibroblast growth factor at 10 ng/mL (Complete Medium 199) and then further incubated at 37°C. At days 8, 15 and 21 of incubation, small aliquots of P0 tissue culture medium were tested for BEFV by qRT-PCR. Results are shown in Table 1.

On day 15, 4/16 splenic cultures with confluent cell monolayers were passaged and level 1 (P1) cultures established. Briefly, the culture medium was removed and the cells washed with 10 mL of phosphate-buffered saline after which they were treated with 1 mL of 0.12% trypsin. The monolayers were then removed and resuspended in Complete Medium 199 held in 25-cm² flasks or chamber slides that were then incubated at 37°C. After 24 h, the medium was removed from one flask, an aliquot of the supernatant tested for BEFV by qRT-PCR, the cells pelleted by centrifugation (and the medium on the chamber slides decanted), fixed for 1 h in 10% buffered formalin and then processed for BEFV immunocytochemistry.

For the immunocytochemical evaluation of the autogenously derived splenic and haemal node cell cultures, processed chamber slides and 4-μm sections of paraffin-embedded cell pellets were also tested using the same protocol.

After 21 days of incubation, aliquots of the spleen and haemal node tissue culture supernatants from all cultures were again tested by qRT-PCR (Table 1). After incubation of each passage level, adherent multiplying cell colonies were found in all cultures, with the majority of cells having a typical spindle-shaped morphology consistent with fibroblasts (data not shown).

Table 1. Fractions of the different passage levels of spleen and haemal node-derived cell cultures that were positive for bovine ephemeral fever virus (BEFV) RNA by qRT-PCR assay (Ct value ≤45.0 = positive)

<table>
<thead>
<tr>
<th>Passage level (P) culture</th>
<th>No. of bovine primary cell cultures that tested positive for BEFV by qRT-PCR</th>
</tr>
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<tbody>
<tr>
<td>P0/day 8</td>
<td>8/16</td>
</tr>
<tr>
<td>P0/day 15</td>
<td>2/16</td>
</tr>
<tr>
<td>P0/day 21</td>
<td>2/12</td>
</tr>
<tr>
<td>P1/day 5</td>
<td>0/4</td>
</tr>
<tr>
<td>P1/day 6</td>
<td>0/1</td>
</tr>
<tr>
<td>P1/day 7</td>
<td>1/11</td>
</tr>
<tr>
<td>NT, not tested; qRT-PCR, quantitative reverse transcription-PCR.</td>
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</tbody>
</table>

Discussion

The most significant finding in the present study was the detection of apparently biologically viable BEFV in bovine lymphoid tissue specimens from an adult steer 1 week after cessation of viraemia. To the authors’ knowledge, this is the first time BEFV has been isolated and viral antigens definitively demonstrated in bovine lymphoid tissues. The aseptically collected spleen and haemal node were separately processed according to an in-house protocol standardised at the Bertramah Veterinary Laboratories. Autogenous primary bovine cell cultures derived from both organs were established and periodically tested by qRT-PCR to assess the status of BEFV infection. Briefly, the spleen and haemal node were separately macerated and mechanically agitated to yield fine, cell-rich slurries, which were then mixed with a small amount of 0.12% trypsin (SAFC, Sigma-Aldrich, St Louis, MO, USA) and homogenised by additional mechanical agitation. The resulting cell-rich homogenate was mixed with Gibco Medium 199 supplemented with bovine pituitary-derived fibroblast growth factor (Sigma-Aldrich) to a final concentration of 3.2 ng/mL.

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tissues 1 week after cessation of viraemia. A confirmatory BEF diagnosis was initially made on the basis of a positive qRT-PCR test result on peripheral blood of the steer that had recently manifested BEF-characteristic clinical signs including but not limited to anorexia, fever and recumbency. Hallmark necropsy findings, including copious amounts of synovial fluids as well as proliferative fibrinous synovitis in the stifle joints and unremarkable amounts of fibrin clot-containing serous fluids in the peritoneal, thoracic and pericardial cavities, were also present.1,5 1 week after viraemia had subsided. Although peripheral blood samples from days 2–7 were negative for viral RNA, lymphoid tissues (spleen, haemal node, prefemoral lymph node) and synovial fluid from the stifle joints were positive for BEFV by qRT-PCR. These findings suggest that post-viraemic virus replication most likely occurs within lymphoid tissues and possibly within cellular components of synovial fluid such as inflammatory cells. As viral RNA was detected in different passage levels of autogenously derived splenic cell cultures, and characteristic bullet-shaped rhabdovirus virions were confirmed in an ultrathin haemal node section, our results confirmed the tropism of BEFV for lymphoid tissues. Although we recently reported the detection of BEFV antigens and RNA in post-viraemic cattle samples,9 we now provide additional evidence on detection of apparently biologically viable BEFV in lymphoid tissues, based on data obtained using a combination of qRT-PCR, IHC, virus isolation, and electron microscopy. Previously, BEFV was detected in bone marrow before detection of viraemia8 and because bone marrow, spleen, haemal node and lymph nodes are part of the reticuloendothelial system (RES), it is possible that the tissues of the RES support transient replication of BEFV. In the present study, IHC showed most of the viral antigens to be intracytoplasmic within probable macrophages but not lymphocytes. Previously, BEFV was shown to be associated with neutrophils,4,6,7 which, similar to macrophages, play a phagocytic role during infection. In the present case, numerous neutrophils had infiltrated the prefemoral lymph nodes, haemal node and, to a lesser extent, the synovial membranes of the stifle joints 1 week after cessation of viraemia in a BEFV-affected steer. All the lymphoid tissues in question and the synovial fluid samples taken from the stifle joints were positive for viral RNA by qRT-PCR. By the time necropsy examination was done, the animal had already seroconverted (data not shown), suggesting that the innate adaptive immune response transition was already underway. As such, we suggest that by the time necropsy examination was done, antigen processing and presentation mediated by macrophages and other specialised antigen-processing cells such as dendritic cells were already in progress as part of the broader anti-BEFV adaptive immune defences. The majority of the antigen-laden cells detected by IHC were morphologically consistent with macrophages, suggesting that phagocytosis is the likely mode of virus uptake by these cells. Such virus uptake by macrophages may be arguably followed by virus destruction, together with antigen processing/presentation, with the possibility of concurrent intrahistiocytic virus survival and transient replication. Testing of both the parent (P0) and P1 splenic cultures by qRT-PCR was positive for viral RNA, suggesting that BEFV may continue to replicate within lymphoid tissues after cessation of viraemia. Moreover, of the numerous P1 cultures that were initially BEFV-negative, one cell culture eventually tested positive for viral RNA on day 7 of incubation, suggesting that virus replication had been in progress and finally attained levels detectable by qRT-PCR. Currently, however, the precise period during which the virus may stay viable in vivo within bovine lymphoid tissues is unknown and needs to be empirically defined. What is clear is the apparent tropism of BEFV for bovine lymphoid tissues. In particular, the spleen and haemal node appear to be important sites of transient virus replication during the post-viraemic period of BEFV infection. The presence of the virus in these organs/tissues 1 week after cessation of viraemia may contribute to the mechanisms responsible for generating the solid immunity typically seen after infection with BEFV.

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References


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