Investigating sheep abortions: New PCR options for diagnosis

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Introduction

Ovine abortion is dominated by Campylobacter fetus fetus, Toxoplasma gondii and Salmonella Brandenburg infection in New Zealand (West 2002).

Investigation of the cause of abortions has relied on a combination of; necropsy of the foetus, histopathological examination of foetal tissues and placenta, along with bacterial culture of foetal stomach contents or tissue in combination with serological tests. Serology tests are primarily the latex agglutination test (LAT) for T. gondii but have included Neospora caninum immunofluorescent antibody testing (IFAT) when this was suspected to be abortifactent (West et al. 2006), and sometimes leptospira microscopic agglutination tests (MAT).

Histopathology is a very useful test as bacteria and toxoplasma can be seen directly and evidence of inflammation (fibrin and inflammatory cells) visualised. Inflammation can include thrombosis and neutrophilia of various tissues with or without suppuration, mineralisation and necrosis in the placenta, confirming an inflammatory process had occurred and an infectious agent was the most likely cause of the abortion.

This study describes the application of a number of recently developed PCR assays to the diagnosis of ovine abortion in New Zealand.

Materials and methods

In 2015, sheep abortion cases sent to Gribbles laboratories in Palmerston North, Christchurch and Dunedin for routine diagnostic testing were further analysed. If the cases included foetal stomach contents, PCR testing and microbiology was undertaken as well. If cases included fixed tissues and histopathology was requested this was undertaken. If a foetal serum or thoracic fluid sample was included, latex agglutination testing (LAT) for antibodies to Toxoplasma gondii was undertaken.

Microbiology

Culture of foetal stomach contents for Acinetobacter wolffii, Bacillus licheniformis, Campylobacter fetus fetus and jejuni, Corynebacterium pseudotuberculosis, Escherichia coli, Listeria ivanovii, Listeria monocytogenes, Salmonella spp., Streptococcus uberis, and Yersinia pseudotuberculosis was undertaken using standard methods. Fungal culture for Aspergillus fumigatus, and Mortierella wolffii were also undertaken at the Palmerston North laboratory. For culture a sterile swab was suspended in the stomach content, then used to inoculate the plate before being streaked by a heat sterilised loop. If present, bacteria and fungi were identified on the basis of growth on the various media, morphology of colonies, biochemical tests and Gram-staining characteristics.

Histopathology

All available fixed tissue samples were trimmed into cassettes, embedded in paraffin, sectioned at ~5μm, and stained with haemotoxylin and eosin for histological examination. Special stains were applied on an as-needed basis to aid in the detection of infectious organisms. Stains could include Gram, Warthin Starry, or Giemsa for bacteria.
PCR

Real time PCR assays for each of the infectious agents; *Aspergillus fumigatus*, *Bacillus licheniformis*, *Campylobacter fetus fetus*, *C. jejuni*, pathogenic Leptospira, *Listeria monocytogenes* and *L. ivanovii*, pestivirus (combination bovine viral diarrhoea virus (BVDv) and hairy shaker disease (HSD)), *Neospora caninum*, pathogenic leptospira, *Salmonella* spp., and *Toxoplasma gondii*, were performed based on methods previously reported in the literature. For the *Mortierella wolfii* and *Helicobacter* spp. (Flexispira) PCRs, primers and probes were designed in-house based on the alignment of published gene sequences available in the GenBank database. Positive samples were confirmed by conventional PCR and nucleotide sequencing of the PCR products.

Results

A total of 179 samples were collected and tested by PCR. Culture was undertaken on 162, toxoplasma serology on 79, and histopathology on 54. When results from all the tests were combined a diagnosis was reached in 155 cases, consistent with a diagnostic rate of 87%. PCR detected a pathogen in 135 cases consistent with a diagnostic rate of 75%. PCR results are listed in Table 1.

The most common pathogen detected by PCR were *Salmonella* species (32%), then *C. fetus fetus* (14%) and *T. gondii* (13%). Smaller numbers of *C. jejuni* (8%), *Helicobacter* species (7%), *L. ivanovii* (8%) and *L. monocytogenes* (7%) were identified. Pestivirus (border disease virus) was found in 2% of samples. *N. caninum*, Leptospira, *A. fumigatus*, *M. wolfii* and *B. licheniformis* were not detected in any samples.

Toxoplasma

Titres to *T. gondii* were detected in 33 out of 79 (41%) foetal serum samples tested by LAT (range 1/16 to 1/1024). PCR detected *T. gondii* organisms in the stomach contents of 23 of 179 (13%) samples tested.

Microbiology

Microbiology results are shown in Table 2. When the microbiology isolate was compared with PCR results on the same foetal stomach contents there was 100% concurrence for *C. fetus fetus*, *L. ivanovii* and *L. monocytogenes*. Five isolates of *C. jejuni* were identified by culture but only four of those samples tested positive by PCR. *Y. pseudotuberculosis* was identified by culture in two samples; PCR identified *C. jejuni* and *Salmonella* in these same samples.

*Streptococcus uberis* were isolated from two foeti. In one of these Helicobacter was detected by PCR. *Acinetobacter wolfii* were isolated from two foeti. Both also had *T. gondii* titres and *C. fetus fetus* was detected in one by PCR.

Twenty two mixed growths were recorded by microbiology. Toxoplasma titres or *T. gondii* detected by PCR were found in nine of them, *Salmonella* spp. were detected by PCR in two; *C. fetus fetus* in one, *L. monocytogenes* in one, *L. ivanovii* in one, and HSD virus found in one.
No bacteria were grown in 62 stomach content samples. Of these samples, 27 were also negative on PCR and 22 samples were negative for pathogens or pathology by all test methods.

Thirty five (56%) of the microbiology negative samples had one or more pathogens detected by PCR including; twelve *T. gondii*, eight *Helicobacter spp.*, one HSD virus, three *C. fetus fetus*, one *C. jejuni*, five *L. monocytogenes*, and eight Salmonella spp. Of the microbiology negative samples, five had lesions detected by histopathology, one of these samples was negative by all other test methods. Thus in 65% of samples classified as negative by microbiology, a diagnosis was confirmed by another test method.

**Discussion**

PCR testing of foetal stomach contents provides an additional means of increasing the diagnostic rate of ovine abortions both singularly, and in conjunction with more commonly applied disciplines for abortion diagnosis. Complete agreement was found between PCR testing and microbiology for the pathogens *C. fetus fetus*, *L. ivanovii* and *L. monocytogenes*.

When microbiology was unsuccessful in growing a pathogen, PCR detected a pathogen in 56% of the samples providing a useful adjunct test for apparently sterile samples.

Stomach contents were used for sample testing for microbiology and PCR as this is a convenient sample to collect aseptically from aborted foetuses. Foetal stomach contents is largely derived from amniotic fluid (Marques et al. 2012a), and amniotic fluid is generated from maternal plasma, hence if a pathogen is in maternal circulation it can reach the amnion. Bacteria and fungi can enter and multiply in the placenta before entering the amnion (Anderson 2012). During development of the foetus and placenta, maternal fluids pass across the placenta into the foetus before entering the amniotic fluid (Silberstein et al. 2015) and are cycled through the foetus by swallowing and excretion (Underwood et al. 2005). Foetal stomach content is an ideal sample for culture and PCR testing to detect pathogens affecting the foetus.

The response of the foetus to infection depends on the age of the foetus. In the first and second trimesters, the foetus has no immune system so infectious agents kill foetal cells directly. In the third trimester an immune response is possible and the foetus may repel the infection, or the products of inflammation may overwhelm the foetus.

*T gondii* were detected in high numbers in amniotic fluid from 35 days post infection in experimentally infected sheep (Marques et al. 2012b), so stomach contents were chosen as the preferred test sample. In the same study seroconversion could be detected at 21 days post infection. A comparison of LAT toxoplasma testing with PCR found that of the 33 LAT positive cases, 20 had significant titres (>1/64) and PCR found *T. gondii* genetic material in five of them. Toxoplasma titres >1/64 may indicate exposure and seroconversion but not necessarily the cause of abortion (Hill 2015). Of the 13 LAT positive cases with titre <1/64, PCR detected *T. gondii* in three cases. The significance of low foetal titres <1/64 is unclear, they may represent non-specific binding or presence of low levels of cross reacting antibodies to other pathogens (Marques et al. 2012). Of the 46 LAT negative cases, PCR detected *T. gondii* genetic material in the stomach contents of six. Toxoplasma PCR negative and serology positive foetuses may also be found if the foetus is immunocompetent and has resolved the infection. It is unclear whether PCR
testing of stomach contents as the primary sample is sufficient to diagnose toxoplasma infection. Serology along with histopathology remain important adjunct tests. Further work is in progress to compare T. gondii PCR detection rates in stomach contents with other foetal sample types.

Helicobacter spp. infection with flexispira-like organisms has been associated with multiple abortion outbreaks in successive seasons in the lower South Island. Aborted lambs had enlarged livers, some containing pale foci resembling C. fetus fetus infection, although, no Campylobacter were isolated. Flexispira rappini (subsequently called Helicobacter spp. with flexispira morphology) were eventually identified (Gill et al. 2016) as the cause.

A lack of isolation or identification of A fumigatus, B licheniformis or M wolfii in the aborted foeti confirmed they were not a factor in these ovine abortion. Neospora caninum was not detected in any of the samples and was not a factor in any of the abortions. Earlier work in New Zealand investigated a role for Neospora (West et al. 2006) but a definitive association has not been established (Howe et al. 2012). No leptospires were detected in this study, although recent work suggests they may play a role in foetal loss (Ridler et al. 2015).

With all three disciplines combined a diagnosis was made in 87% of the cases representing a high diagnostic rate and giving confidence to the probability of making a diagnosis when investigating ovine abortions.

Use of PCR technology increases the ability to search for pathogens implicated in ovine abortion and significantly improves the diagnostic rate but needs to be used in combination with other testing methods to maximise the chance of making a diagnosis. Serological tests alone are not recommended as the only method of diagnosing ovine abortion (Dempster et al. 2011). A full ovine abortion diagnostic workup can involve a combination of microbiology, serology, histopathology and now PCR testing to increase the chances of confirming the cause of the abortion.

Acknowledgments

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WEST DM, POMROY WE, COLLETT MG, HILL FI, RIDLER AL, KENYON PR, MORRIS ST, PATTISON RS. A possible role for Neospora caninum in ovine abortion in New Zealand. Small Ruminant Research 62, 135–8, 2006
<table>
<thead>
<tr>
<th>Organism</th>
<th>Number positive</th>
<th>%</th>
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<tbody>
<tr>
<td>Salmonella spp.</td>
<td>58</td>
<td>32</td>
</tr>
<tr>
<td>C fetus fetus</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>T gondii</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>C jejuni</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>L ivanovii</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Helicobacter</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>L monocytogenes</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Pestivirus (BDV)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>N caninum</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leptospires</td>
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<td>0</td>
</tr>
<tr>
<td>A fumigatus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B licheniformis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M wolfi</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. PCR results for identification of abortifacent pathogens from foetal lamb stomach contents

<table>
<thead>
<tr>
<th>Organism</th>
<th>Culture positive</th>
<th>PCR positive</th>
<th>Other PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. fetus fetus</td>
<td>18</td>
<td>18</td>
<td>3 Salmonella spp; 2 L. ivanovii; 1 L. monocytogenes; 2 C. jejuni</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>L. ivanovii</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Y. pseudotuberculosis</td>
<td>2</td>
<td>Not looked for</td>
<td>C. jejuni Salmonella positive</td>
</tr>
</tbody>
</table>

Table 2. Microbiology results compared with PCR