Investigation of a bluetongue disease epizootic caused by bluetongue virus serotype 17 in sheep in Wyoming

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Objective—To characterize a 2007 bluetongue disease (BT) epizootic caused by bluetongue virus (BTV) serotype 17 in sheep in the Big Horn Basin of Wyoming.

Design—Cross-sectional study.

Animals—1,359 sheep from ranches in Wyoming and Montana.

Procedures—Information on clinical signs and history of BT in sheep was obtained from ranchers and attending veterinarians. At 3 to 6 months after the 2007 BT epizootic, blood samples were collected from rams, ewes, and lambs within and outside the Big Horn Basin; blood samples were also collected from lambs born in the spring of 2008. Sera were tested for anti-BTV antibodies by use of a competitive ELISA to determine the seroprevalence of BTV in sheep and to measure antibody titers. Virus isolation and reverse transcriptase PCR assays were used to determine long-term presence of the infectious virus or viral genetic material in RBCs of sheep.

Results—The percentage of sheep seropositive for BTV closely matched morbidity of sheep within flocks, indicating few subclinical infections. Flocks separated by as little as 1 mile had substantial variation in infection rate. Rams were infected at a higher rate than ewes. There was no evidence of BTV successfully overwintering in the area.

Conclusions and Clinical Relevance—This epizootic appears to be a new intrusion of BTV into a naive population of sheep previously protected geographically by the mountains surrounding the Big Horn Basin. Rams may have a higher infection rate as a result of increased vector biting opportunity because of the large surface area of the scrotum. (J Am Vet Med Assoc 2010;237:955-959)

Bluetongue virus causes vasculitis resulting in edema and hemorrhage. Clinical disease in sheep is characterized by facial and pulmonary edema, hyperemia and ulceration of the oral mucosa, saliva­tion, profuse nasal discharge, lameness, and reluctance to walk. Secondary pneumonia is common and is often the cause of death. Pronghorn and deer often develop a rapidly fatal hemorrhagic illness. Cattle and goats are susceptible to BTV but typically have mild or inapparent infections. In studies on sheep experi-

<table>
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<th>ABBREVIATIONS</th>
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<tr>
<td>BT</td>
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<td>BTV</td>
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<td>BTV-17</td>
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<td>RT-PCR</td>
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mentally infected with BTV, viremia has been detected from 11 to 54 days after infection, and BTV RNA has been detected only up to 100 days after infection. Sheep surviving infection with BTV acquire serotype-specific protective immunity via serum antibodies that is estimated to last at least 2 years.20

A 2007 BT epizootic caused by BTV-17 was first reported in Montana sheep flocks; BT was subsequently reported in Wyoming in pronghorn, white-tailed deer, and mule deer in early September and in sheep from the Big Horn Basin in late September.21 Quarantines restricting the movement of sheep were placed on 16 eastern Montana counties and 3 Wyoming ranches until the first frost. Weather data indicated that the summer of 2007 was warmer than average and that intermittent warm days continued into late November. The Big Horn Basin of Wyoming is a dry valley (elevation, 1,280 to 1,340 m [4,200 to 4,400 feet]) in north central Wyoming surrounded by mountains. The Big Horn River flows through the basin and supplies water for irrigation to grow a variety of crops. Lambing season for most sheep flocks is from late March to early May. Some ranches in the area move their ewe and lamb flocks to pastures in the Big Horn Mountains for the summer, where they are tended by sheep herders, but the rams are kept at the ranch all summer. Ranchers and veterinarians in the Big Horn Basin reported that they had not previously seen BT, and the disease severity was much greater than previous outbreaks in other areas of the state.22 Previous BT epizootics caused by BTV-17 have occurred in northeast Wyoming, affecting sheep and wildlife, and a mild BT epizootic occurred in sheep flocks in the plains east of the Big Horn Mountains in 2005. Bluetongue virus serotype 11 has been found to be endemic in southern Wyoming and northern Colorado.23,24

The purpose of the study presented here was to characterize the 2007 BT epizootic in sheep in the Big Horn Basin of Wyoming and to gather information to help ranchers make future management decisions. We hypothesized that BT was endemic throughout Wyoming and that this BT epizootic resulted from favorable weather conditions for vector populations and virus amplification. We also hypothesized that most of the sheep in the affected flocks had been exposed to the virus and would be immune to new infection. Serum samples were tested for antibodies against BTV by use of a competitive ELISA to determine the seroprevalence of BTV in sheep and to measure BTV-specific antibody titers. Virus isolation and RT-PCR assays were used to determine long-term presence of the infectious virus or viral genetic material in RBCs of sheep.

**Materials and Methods**

**Site selection**—Blood samples were collected from sheep at 3 ranches (ranches A, B, and C) in the Big Horn Basin (Figure 1) in February and March 2008 when the flocks were gathered for shearing. These ranches had experienced substantial morbidity and mortality rates from BT in 2007. When possible, blood samples were collected from replacement lambs born in the spring of 2007 as well as adult rams and ewes. The yearling lambs were considered the sentinel population for the study to give a true indication of the infection rate. These lambs were 4 to 5 months old at the time of the outbreak and would have been past the period of passive protection from maternal antibodies.

Two of these ranches (A and B) were managed as range flocks with the ewes and their lambs moved to mountain pastures from July to September, but the rams were kept at the ranch year-round. In addition, ranch A had a number of goats commingled with the sheep flock that were also tested. The third ranch in the Big Horn Basin (C) was managed as a farm flock with ewes, lambs, and rams at the ranch year-round. Ewes and lambs from ranch A were randomly selected from the whole flock by separating out alternate groups into a catch pen as the flock was moved through a gate. Sheep were similarly selected from ranch B, but the ewe and lamb flock had the sheep identified as having had BT removed.

Sheep were also tested from ranches outside of the Big Horn Basin in areas with and without histories of previous BT. These included ranch D on the plains east of the Big Horn Mountains that had a mild BT epizootic in 2005, ranch E in the black hills of northeastern Wyoming in an area with known endemic history of BT in sheep and wildlife, and ranch F in western Wyoming that had no history of bluetongue-like disease (Figure 1). These ranches were selected for comparison to ranches within the Big Horn Basin for number of sheep seropositive for BTV and viral titers.

Lambs born in the spring of 2008 were tested from 3 farm flocks kept in the Big Horn Basin all summer and from a flock in Miles City, Mont, (Figure 1) pastured in the area affected by BT in 2007. Blood samples from these lambs were collected in October and tested for serum antibodies against BTV as evidence of the virus successfully overwintering and circulating within the outbreak area in the summer of 2008.

**Blood sample collection**—Blood sample collection from sheep in this study was done under a protocol reviewed and approved by the University of Wyoming Institutional Animal Care and Use Committee and with
the written approval and assistance of the sheep owners. Blood samples were collected by jugular venipuncture into plain and EDTA vacuum tubes. Information on clinical signs and history of BT in sheep was recorded from all ranchers and the attending veterinarian. All flocks were made up of white-faced breed ewes and rams except for a few black-faced breed rams.

Serologic detection of antibodies against BT—Blood samples were tested in triplicate for antibodies against BTW with a previously published competitive ELISA. Briefly, polyclonal rabbit anti-BTV serum was used to coat the wells of microtiter plates and to capture baculovirus expressed BTV viral protein 7 antigen. Mouse monoclonal antibody specific for viral protein 7 of BTV diluted 1:50 and test serum samples diluted 1:5 were allowed to compete for binding to the captured viral protein 7 antigen. This was followed by reaction with biotinylated goat anti-mouse antibody and peroxidase-conjugated streptavidin. Optical density measurement of the color change following addition of ortho-phenylene diamine allowed calculation of the percent inhibition of the mouse monoclonal antibody binding to the captured antigen. Sera were scored positive for antibodies against BTV if the optical density was ≤ 35% of the negative control. Thirty blood samples were also assayed by the Wyoming State Veterinary Laboratory in Laramie, Wyo, by use of a commercial competitive ELISA BTV kit and gave identical results.

Relative serum BTV-specific antibody titers were determined for blood samples from 16 seropositive sheep from ranches A, B, and D and for blood samples from 20 seropositive sheep from ranch E. Serial 2-fold dilutions were made of each serum sample and assayed in duplicate by use of the competitive ELISA. Titer units were calculated as the reciprocal of the serum dilution that was positive on the competitive ELISA. Mean titers were calculated for each ranch, and the differences among mean values were analyzed.

Virus serum neutralization—Virus neutralization was used to determine the serotype of BTV for ranch B. A 1:10 dilution of serum samples from 8 seropositive and 8 seronegative lambs was used to test for virus neutralization of BTV serotypes 11 and 17, and epizootic hemorrhagic disease virus serotype 2 by use of a previously described protocol.

Virus isolation—All samples were tested by virus isolation in duplicate and on 2 cell lines. Briefly, 25 µL of RBCs oruffy coat diluted in 75 µL of media was added to 100 µL of cattle pulmonary artery endothelial or Vero cells (2.5 x 10⁵ cells/mL) in 96-well plates. All cells were incubated for 7 days at 37°C with 5% to 6% CO₂. Cells were checked for cytopathic effect after 48 hours and at 7 days. The RNA was extracted from cells that had cells with a cytopathic effect, and homogenate from these wells was passed onto fresh cells.

PCR assay—Total RNA was extracted from cell cultures that had a cytopathic effect and from all original sheep RBCs with a kit and eluted in 30 µL of nuclease-free water by following the manufacturer’s protocol. Extracts were tested for BTV by use of a previously published infrared RT-PCR method, and PCR products were separated by 3% agarose gel electrophoresis under UV light with nucleic acid stain. Blood samples were also analyzed by use of an improved RNA extraction and a real-time RT-PCR as previously described.

Statistical analysis—Confidence intervals for serologic testing results were calculated by use of a software program on the basis of a 90% confidence level and a 5% margin of error. Difference in serum BTV-specific antibody titers of sheep among ranches was determined by use of a 2-tailed Student t test. All values of P < 0.05 were considered significant.

Results

Ranch A had the highest morbidity (500/1,404 [36%]), case fatality (175/500 [35%]), and total mortality (175/1,404 [12%]) rates, as determined on the basis of ranch records. Disease, with signs of lameness, lethargy, and lack of appetite, was first noticed in the rams pastured at the ranch near irrigated land but was not recognized as being caused by BT infection. The ewe and lamb flock returned from mountain pastures in September and began to have signs of illness within 10 to 14 days, with sheep continuing to be newly affected into early November. Signs of lameness and reluctance to stand were quickly followed by swollen lips, tongue, and face, with salivation and nasal discharge. Sick sheep were treated with an NSAID (flunixin meglumine) and antimicrobial (oxytetracycline) and were tube fed water. For ranch A, 93% (14/15) of tested rams were seropositive for BTV, while 27% (49/182) and 40% (27/68) of tested ewes and lambs, respectively, were seropositive (Table 1). Mean ± SEM serum BTV-specific antibody titers for the ranch A rams, ewes, and lambs were 583 ± 170, 660 ± 245, and 480 ± 269, respectively. Four of 6 goats tested from this ranch were seropositive for BTV and 1 of 3 suckling kids was seropositive.

Ranch B is roughly 24 km (15 miles) from ranch A and also had rams becoming ill in August before the outbreak was recognized and diagnosed as BT. Lameness and depression were noted, and 4 of 56 rams died. Rams were pastured between 2 branches of a creek and near irrigated pastures. Ewes and lambs became ill after returning to the ranch in September, and mountain pasture. This ewe flock was pastured approximately 16 km (10 miles) from the ram pasture and had less exposure to small creeks and irrigated fields. The rancher recorded clinical disease in 14% (233/1,679) of the ewe and lamb flock with a 3% (7/233) case fatality rate. Sick sheep were removed from the flock and treated by the sheep herders with an NSAID (flunixin meglumine), an antimicrobial (oxytetracycline), electrolytes, citrus fruit rubbed on oral lesions, and silage offered as a soft food source. For ranch B, 90% (18/20) of tested rams were seropositive for BTV, while 4% (8/201) and 5% (8/170) of tested ewes and lambs, respectively, were seropositive (Table 1). Virus serum neutralization was used to confirm virus serotype; sera from 9 of 10 sheep seropositive for BTV neutralized BTV-17 but not BTV serotype 11 or epizootic hemorrhagic disease virus serotype 2. Mean ± SEM serum BTV-specific antibody titers for the ranch B
rams, ewes, and lambs were 610 ± 168, 853 ± 213, and 1,120 ± 160, respectively.

Limited testing was done on ranch C to compare infection rates between ewes and rams when both spent the summer in ranch pastures within 1 mile of each other. All rams were tested, but only a pen of nonpregnant, poor-doing, or late-to-lamb mature ewes was tested. Of these sheep, 100% (9/9) of the rams were seropositive for BTV and 86% (18/21) of the ewes were seropositive (Table 1).

For ranch D, located east of the Big Horn Mountains (an area with BT in 2005), 34% (40/119) of mature ewes were seropositive for BTV and had a mean ± SEM serum BTV-specific antibody titer of 176 ± 82 (Table 1). For ranch E, located within the Black Hills region of Wyoming, 14% (4/29) and 16% (17/109) of the rams and ewes, respectively, were seropositive for BTV. Mean ± SEM serum BTV-specific antibody titers for the rams and ewes of ranch E were 123 ± 75 and 302 ± 57, respectively. On ranch F, no ewes or yearling lambs were seropositive for BTV; none of the 2008 spring-born lambs were seropositive for BTV in the fall of 2008. Testing of blood samples yielded negative results for BTV by virus isolation and for viral RNA detection by RT-PCR assay.

**Discussion**

Woolgrowers suffer substantial economic losses because of BTV and the resulting quarantine that prevents movement of sheep at the time lambs are usually sent to market. Ranchers had specific questions that included whether the disease was likely to recur the next summer, whether their flocks would be immune to new infection, and whether vector control programs at the premise or animal level could help to limit the spread of disease. The current knowledge of BTV is limited, in part, because it is difficult to reproduce the disease experimentally. Most of the current knowledge of BTV infection and vector spread is based on information from experimental infections with small numbers of animals and on assumptions as to the mobility of the vector. This outbreak was a chance to collect data from a large number of sheep that had been infected in a natural epizootic and to give the woolgrowers information to help manage future outbreaks.

We had hypothesized that BTV was endemic throughout Wyoming and expected to find sheep seropositive for BTV from all ranches tested. Mature sheep were expected to have a higher seropositivity as a result of multiple years of possible exposure. We found, however, that among lambs born in 2007, there was the same number of seropositive sheep as in other age groups, suggesting an introduction into a naive population. This could also have contributed to the severity of disease, although the possibility of a more virulent form of the virus cannot be ruled out as a contributing factor and would require further studies to identify.

We had also hypothesized that there were a large number of subclinical infections in the affected flocks and thus most of the sheep would be seropositive for BTV and resistant to disease the following summer. However, even for ranch A, which had the highest morbidity and mortality rates, the flock was overwintering and circulating in the summer after the 2007 epizootic. A notable finding in this outbreak was the apparent viremic movement of sheep at the time lambs were usually sent to market. Ranchers had specific questions that included whether the disease was likely to recur the next summer, whether their flocks would be immune to new infection, and whether vector control programs at the premise or animal level could help to limit the spread of disease. The current knowledge of BTV is limited, in part, because it is difficult to reproduce the disease experimentally. Most of the current knowledge of BTV infection and vector spread is based on information from experimental infections with small numbers of animals and on assumptions as to the mobility of the vector. This outbreak was a chance to collect data from a large number of sheep that had been infected in a natural epizootic and to give the woolgrowers information to help manage future outbreaks.

**Table 1—Seroprevalence of BTV in 1,359 sheep from ranches A, B, and C that were outside of the Big Horn Basin.**

<table>
<thead>
<tr>
<th>Ranch or flock (location)</th>
<th>Group</th>
<th>No. of sheep in flock or pen</th>
<th>No. of sheep tested</th>
<th>% seropositive (CI)*</th>
<th>Mean ± SEM antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Big Horn Basin)</td>
<td>Rams</td>
<td>15</td>
<td>15</td>
<td>93</td>
<td>583 ± 170</td>
</tr>
<tr>
<td></td>
<td>Ewes</td>
<td>50</td>
<td>182</td>
<td>27 (22-32)</td>
<td>660 ± 245</td>
</tr>
<tr>
<td></td>
<td>Lambs</td>
<td>130</td>
<td>68</td>
<td>40 (33-46)</td>
<td>400 ± 209</td>
</tr>
<tr>
<td>B (Big Horn Basin)</td>
<td>Rams</td>
<td>56</td>
<td>20</td>
<td>90 (81-99)</td>
<td>610 ± 168</td>
</tr>
<tr>
<td></td>
<td>Ewes</td>
<td>1,500</td>
<td>201</td>
<td>4 (3-6)</td>
<td>853 ± 213</td>
</tr>
<tr>
<td></td>
<td>Lambs</td>
<td>510</td>
<td>170</td>
<td>5 (4-7)</td>
<td>1,120 ± 180</td>
</tr>
<tr>
<td>C (Big Horn Basin)</td>
<td>Rams</td>
<td>9</td>
<td>9</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Ewes</td>
<td>50</td>
<td>21</td>
<td>86 (76-95)</td>
<td>ND</td>
</tr>
<tr>
<td>D (east of Big Horn Mountains, Wyo)</td>
<td>Rams</td>
<td>350</td>
<td>119</td>
<td>34 (28-38)</td>
<td>178 ± 824</td>
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<tr>
<td></td>
<td>Ewes</td>
<td>29</td>
<td>29</td>
<td>14</td>
<td>123 ± 786</td>
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<tr>
<td>E (Black Hills, Wyo)</td>
<td>Rams</td>
<td>106</td>
<td>105</td>
<td>16</td>
<td>302 ± 57</td>
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<tr>
<td></td>
<td>Ewes</td>
<td>1,000</td>
<td>102</td>
<td>0 (0-1.5)</td>
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<tr>
<td>F (western Wyoming)</td>
<td>Rams</td>
<td>1,000</td>
<td>125</td>
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<tr>
<td></td>
<td>Ewes</td>
<td>1,120</td>
<td>125</td>
<td>0 (0-1.8)</td>
<td>ND</td>
</tr>
<tr>
<td>Flock 1 (Big Horn Basin)</td>
<td>Lambs</td>
<td>37</td>
<td>35</td>
<td>0 (0-0.7)</td>
<td>ND</td>
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<tr>
<td>Flock 2 (Big Horn Basin)</td>
<td>Lambs</td>
<td>80</td>
<td>49</td>
<td>0 (0-1.8)</td>
<td>ND</td>
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<tr>
<td>Flock 3 (Big Horn Basin)</td>
<td>Lambs</td>
<td>60</td>
<td>20</td>
<td>0 (0-3)</td>
<td>ND</td>
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<tr>
<td>M (Miles City, Mont)</td>
<td>Lambs</td>
<td>94</td>
<td>94</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Confidence interval was calculated on the basis of a 95% confidence level and a 5% margin of error. Serum BTV-specific antibody titers are the reciprocal of the highest dilution of serum that yielded a positive result by use of the competitive ELISA. Mean serum BTV-specific antibody titer was significantly (P < 0.05) lower than the mean titer for all sheep on ranches A and B. Ewes tested were from a pen of sick or late-to-lamb ewes. Ewes were from a flock with a history of BT in 2005. §Ewes tested were from a pen of sick or late-to-lamb ewes. Ewes were from a flock with a history of BT in 2005.

CI = Confidence interval. NO = Not determined.
tured had cooler temperatures less favorable for the vector and rapid virus amplification. The mountains surrounding the Big Horn Basin have likely been a natural barrier to infected vectors from endemic regions of the state. The introduction of BTV into the Big Horn Basin causing the 2007 outbreak could have occurred by the movement of infected animals (domestic or wildlife), or vectors with the help of weather events or manmade transportation.

As a disease borne by a flying insect, there is controversy as to the distance the vector for BTV is likely to travel and therefore the utility of animal and ranch-level vector control programs. With this outbreak, there were significant differences in the infection rate in flocks separated by only a few miles, suggesting that effective repellents applied to the animals or implementing site level vector control strategies might be of use in a BT outbreak. Further research to identify effective repellents for application to the animal and local vector control is needed.

An unexpected finding was the near 100% infection rate in the rams. The location of the pastures and earlier exposure to the virus are a likely cause. However, ram infection rates were still significantly higher than that of ewes kept in pastures separated by < 1 mile throughout the summer. There are no published reports on differences in susceptibility to infection in males versus females and a possible explanation for the higher rates in rams is increased vector biting opportunity because of the large scrotal surface. In the study reported here, all blood samples had negative results for BTV isolation and detection of BTV RNA.

References