YERSINIA ENTEROCOLITICA: AN UNLIKELY CAUSE OF POSITIVE BRUCELLOSIS TESTS IN GREATER YELLOWSTONE ECOSYSTEM BISON (BISON BISON)

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ABSTRACT: Yersinia enterocolitica serotype O:9 has identical O-antigens to those of Brucella abortus and has apparently caused false-positive reactions in numerous brucellosis serologic tests in elk (Cervus canadensis) from southwest Montana. We investigated whether a similar phenomenon was occurring in brucellosis antibody–positive bison (Bison bison) using Y. enterocolitica culturing techniques and multiplex PCR of four diagnostic loci. Feces from 53 Yellowstone bison culled from the population and 113 free-roaming bison from throughout the Greater Yellowstone Ecosystem (GYE) were tested. Yersinia enterocolitica O:9 was not detected in any of the 53 bison samples collected at slaughter facilities or in any of the 113 fecal samples from free-ranging bison. One other Y. enterocolitica serotype was isolated; however, it is not known to cause cross-reaction on B. abortus serologic assays because it lacks the perosamine synthetase gene and thus the O-antigens. These findings suggest that Y. enterocolitica O:9 cross-reactivity with B. abortus antigens is unlikely to have been a cause of false-positive serology tests in GYE bison and that Y. enterocolitica prevalence was low in bison in the GYE during this study.

Key words: Bison, brucellosis, elk, Greater Yellowstone Ecosystem, perosamine synthetase, Yellowstone, Yersinia enterocolitica O:9.

INTRODUCTION

Yersinia enterocolitica is an enteric, gram-negative, pathogenic bacterium found in domestic animals, wildlife, and humans (Bottone, 1997). Yersinia enterocolitica serotype O:9 and Brucella abortus, the etiologic agent of brucellosis in elk (Cervus canadensis), bison (Bison bison), cattle (Bos taurus), and certain other ungulates, possess an identical O-antigen structure encoded by the gene perosamine synthetase (Godfroid et al., 2002). This genetic similarity is known to cause cross-reactivity between the two bacteria during serologic testing. As a result, infection with Y. enterocolitica serotype O:9 can lead to false-positive Brucella serology tests, making identification of Brucella-positive animals difficult in livestock and wildlife populations including cattle, bison, and elk (Weynants et al., 1996; Munoz et al., 2005; Atkinson et al., 2007).

In 2004–2005, staff from Montana Fish, Wildlife, and Parks detected high numbers of B. abortus antibody–positive elk (11.4%) in the Pioneer Mountains on the northwest perimeter of the Greater Yellowstone Ecosystem (GYE), ~100 km northwest of Yellowstone National Park where B. abortus occurs in bison and some elk (e.g., Beja-Pereira et al., 2009). In the Madison Valley Elk Management Unit, approximately 20 km east of the Pioneer Mountains, antibody prevalence in sampled elk rose to 6.9% in 2004–2005 and to 17.5% in 2005–2006 samples (Atkinson et al., 2007). Before 2004–2005, annual estimates of antibody prevalence were consistently less than 2% in both areas. The rise in antibody prevalence prompted further investigation to determine whether Y. enterocolitica O:9
cross-reactivity on *B. abortus* serology testing was causing artificially high *Brucella* antibody prevalence estimates in elk.

Using *Y. enterocolitica*–specific, antibody-based, Western immunoblot tests, Atkinson et al. (2007) found antibodies to *Y. enterocolitica* in all of the *Brucella* antibody-positive samples from the two areas. With the use of the *Y. enterocolitica* Western immunoblot, *Brucella* antibody prevalence estimates were revised to 1.93% in the Madison Elk Management Unit and 0.0% in the Pioneer Mountains. The authors concluded that most *B. abortus* antibody–positive elk from the initial *Brucella* serology (based on the O:9 antigen) could represent false-positives caused by cross-reactivity between *Y. enterocolitica* serotype O:9 and *B. abortus* O:9. However, we note that Western blotting is not a well-validated test, and its sensitivity and specificity have not been evaluated against the gold standard: cultivation of *Y. enterocolitica*.

False-positive brucellosis tests as reported by Atkinson et al. (2007) could have severe social and political repercussions if the same error rate were discovered for Yellowstone bison, where antibody-positive individuals are sometimes culled when captured at the park boundary and antibody-negative animals are afforded release back onto the conservation areas. Because the incidence of bison infected with *B. abortus* in the GYE has fluctuated between 40% and 60% during the past 20 yr (Treanor et al., 2007), we investigated whether seemingly antibody-positive bison were actually infected with *Y. enterocolitica* O:9, producing false-positive reactions in *B. abortus* serologic assays.

**MATERIALS AND METHODS**

**Sample collection**

Fecal and tissue (lymph node) samples were collected from 53 GYE bison during slaughter (February through April 2008) at private livestock facilities. Thirty-five of these (66%) had detectable antibodies for brucellosis. Seven of the 35 (20%) were also culture-positive for *B. abortus*. Fecal samples alone were collected from 113 free-ranging bison from the GYE, including 60 Yellowstone bison sampled in July and August 2008 and 53 Jackson-area bison sampled from near Grand Teton National Park (Moose, Wyoming, USA) in March through August 2008. Frozen tissue and fecal samples from slaughtered bison were sent to the National Veterinary Science Lab (NVSL; Ames, Iowa, USA) for *Brucella* culture. After thawing, each sample was individually homogenized, and aliquots of each homogenate were smeared on *Brucella*-selective media. Plates were incubated at 37°C for up to 10 days. Species and biovar identification was performed according to standard procedures (Alton et al., 1988) by NVSL staff.

Fecal samples collected from free-ranging bison were gathered within 20 min of defecation after the individuals had moved away. Samples were immediately placed on wet ice and transported to a 10°C refrigerator or −20°C freezer within a few hours of collection. Most samples were stored frozen at −20°C (for <2 mo) before processing for cultivation, with the exception of 28 samples that were held at 10°C and never frozen before cultivation. Earlier research with meat samples (De Zutter et al., 1994), and our own experiments holding feces spiked with *Y. enterocolitica* at −20°C before cultivation (data not shown), showed that storage at 10°C or −20°C for <2 mo did not affect the cultivation results. One hundred and thirty-eight fecal samples were sent to Wyoming Game and Fish Department Wildlife Disease Laboratory (Laramie, Wyoming, USA), and 28 samples were sent to Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, Colorado, USA).

**Enrichment and culture to detect *Yersinia***

To enrich for cultivation of *Y. enterocolitica*, 1 g of feces was placed in 9 ml of 0.1% peptone water and homogenized in a Stomacher® laboratory blender (Metrohm USA, Riverview, Florida, USA) for 30 sec, as described by Bharduri et al. (2005). One milliliter of the homogenized mixture was added to 9 ml of Irgasan broth, mixed by inverting, and incubated at room temperature for 48 hr, as previously described (De Zutter et al., 1994). After incubation in enrichment broth, tubes were inverted several times and centrifuged for 1 min at 430 × G to remove large particulates. The remaining suspension was then plated on Cefsulodin-Irgasan Novobiocin/*Yersinia*-selective agar (CIN/YSA).
plates, as described by Bhaduri (2005), with the addition of 0.028 g of CaCl\(_2\) per liter of agar medium as described by Li et al. (1998). The plates were incubated for 24 hr at 28°C, then checked for colony growth. Cultures were restreaked on CIN/YSA agar if individual colonies were not obtained on the original plates.

Colonies were identified as putative \textit{Y. enterocolitica} based on morphology, with colonies of 1–2 mm diameter, having a deep-red center surrounded by a clear, colorless edge scored as positive as described by Weagant and Feng (2007). Single colonies demonstrating these characteristics were selected for DNA extraction, followed by PCR analysis for confirmation of identity.

**Molecular methods for \textit{Y. enterocolitica} confirmation**

DNA was extracted from the isolated bacterial colonies described above using the QIAamp\textsuperscript{®} DNA Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer’s instructions. We conducted multiplex PCR to confirm the preliminary identification of \textit{Y. enterocolitica} from cultures in 20-μl reaction mixtures consisting of 1× Promega Master Mix (Promega Corporation, Madison, Wisconsin, USA), 2.5 μl of DNA template, and 0.25 μM each of forward and reverse primer. The primers were designed to target four separate diagnostic gene fragments for the \textit{ail}, \textit{yst}, and \textit{virF} genes (356, 134, and 231 base pairs [bp], respectively), as previously described (Harnett et al., 1996), and a 312-bp fragment of the perosamine synthetase gene (\textit{per}) as described by Lübeck et al. (2003). Thermal cycler conditions for respective primers were identical to those in Harnett et al. (1996) and Lübeck et al. (2003).

Amplified products were separated on a 1.5% agarose gel in 1× Tris-Borate–ethylene-diaminetetraacetic acid buffer. The amplified bands were visualized in the presence of ethidium bromide under ultraviolet transillumination and digitally captured using Quantity One\textsuperscript{®} software (BioRad, Hercules, California, USA). The size and number of amplified fragments were used to determine the presence or absence of \textit{Y. enterocolitica} as described by Harnett et al. (1996) and Lübeck et al. (2003). Isolates identified as \textit{Y. enterocolitica} by PCR were submitted for confirmation via serotyping to the Division of Foodborne, Bacterial, and Mycotic Diseases at the National Center for Zoonotic, Vector-Borne, and Enteric Diseases, Centers for Disease Control (Atlanta, Georgia, USA).

**RESULTS AND DISCUSSION**

When staff from Montana Fish, Wildlife, and Parks reported on possible false-positive brucellosis tests in several of their southern elk herds assumed to be caused by \textit{Y. enterocolitica} O:9, we questioned whether the same phenomenon could be occurring in GYE bison. In 2008, we performed cultivation assays on 166 fecal samples, including from 35 slaughtered Yellowstone bison that were antibody-positive for brucellosis. Seven of these 35 antibody-positive animals (20%) were also culture-positive for \textit{B. abortus}.

\textit{Yersinia enterocolitica} O:9 was not isolated from any of the 166 Yellowstone and Jackson bison sampled in winter and summer. This suggests that \textit{Y. enterocolitica} O:9 cross-reactivity with \textit{B. abortus} antigens was unlikely to cause false-positive serology tests in GYE bison during this study, and that \textit{Y. enterocolitica} prevalence was apparently low in bison from this area. Possible explanations for this observation include that some bacterial infections likely infect primarily cervids and not bovids or vice versa. It is also possible that elk and bison immune systems have different efficiencies for clearing \textit{Yersinia}. Finally, elk more often encounter livestock outside the park and could be occasionally infected or reinfected from livestock, whereas bison are not allowed to leave the confines of the park to overlap with cattle or other livestock. Ultimately, the explanation for this observation can only be resolved through additional research into this phenomenon.

Two isolates of \textit{Y. enterocolitica} biotype O:2,3 were identified by the Centers for Disease Control, but those biotypes are not known to cross-react with \textit{B. abortus} serology tests because of a lack of the O-antigen structure encoded by the perosamine synthetase gene. Both of these isolates were obtained from samples collected at Antelope Flats in Grand Teton National Park. \textit{Yersinia enterocolitica} O:9 has been shown to be shed at high levels from cattle.

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during the first month following infection, with continued shedding for months until slaughter (Garin-Bastuji, 1999). The absence of *Y. enterocolitica* O:9 in feces from GYE bison samples indicated that the bison sampled were not infected at a detectable level with *Y. enterocolitica* O:9 and that cross-reactivity by *Y. enterocolitica* O:9 on *B. abortus* serology tests should not be of concern to bison managers in the GYE.

Any decision to remove animals from the population based on brucellosis serology results is controversial. It is, therefore, important for wildlife managers to understand the probability of false-positive data, and the implications of the negative *Y. enterocolitica* O:9 culture results reported herein are important to the management and conservation of bison. There remains a possibility that, although *Y. enterocolitica* O:9 was not cultured in the fecal samples we collected, the organism may still be present in the ecosystem. More detailed sampling and testing of intestinal tissues and perhaps gut-associated lymphoid tissue would be needed to completely rule out the presence of this organism in these populations of bison.

Another important implication of these findings is in the management of herd and human health. *Yersinia enterocolitica* has increasingly been implicated in human diarrheal disease and may approach the level of *Salmonella* spp. infection in northern climates (Burnens et al., 1996). Although most cases of yersiniosis are linked to pork (Ostroff et al., 1995), the substantial rate of seroconversion in Montana elk raises questions about its prevalence in Montana wildlife and the potential transfer to humans through contact, such as in the hunting and processing of elk.

We recommend that future studies include testing for *Y. enterocolitica* in different seasons, during the course of multiple years and to use both cultivation and quantitative PCR to complement serology and Western blots. Testing bison in different seasons and years might detect *Y. enterocolitica* O:9 appearing only during outbreaks or epizootics. Quantitative PCR-based tests should provide more sensitivity for the reliable detection of low numbers of *Y. enterocolitica* in intestinal tissue, gut-associated lymphoid tissue, or feces. Although one disadvantage of DNA-based PCR assays is that they might detect DNA from nonviable pathogens, positive results in these tests would still provide clues as to whether *Y. enterocolitica* O:9 is present in GYE wildlife.

Given the recent high incidence of apparently false-positive brucellosis tests in elk populations on the northwest side of the GYE, careful distinction between true brucellosis infection and false-positive brucellosis tests due to *Y. enterocolitica* O:9 is important. In summary, our study indicated that *Y. enterocolitica* O:9 was absent or at low prevalence in the GYE and that cross-reactivity with *B. abortus* antigens was unlikely to have caused false-positive serology tests in Yellowstone and Jackson bison populations during this study.

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**LITERATURE CITED**


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