

BRIEF COMMUNICATIONS

Histochemical and immunohistochemical evidence of a bacterium associated with lesions of epizootic bovine abortion

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Abstract. Epizootic bovine abortion (EBA), a tick-transmitted disease of pregnant cattle grazing foothill pastures, is a major cause of reproductive failure in California and adjacent states. Affected fetuses develop a chronic disease, resulting in late-term abortion or premature calving. Despite investigations spanning 50 years, to the authors' knowledge, the etiologic agent of EBA has not yet been isolated from affected fetuses or the tick vector. The diagnosis of EBA is based on gross and microscopic lesions. Recently, documentation that the etiologic agent is susceptible to antibiotics and identification of a unique 16S deltaproteobacterial rDNA gene sequence in 90% of thymus tissues from aborted fetuses have supported the role of a bacterial infection as the cause of EBA. To determine whether bacteria could be detected in the tissues, histochemical staining and immunohistochemical procedures were used on formalin-fixed, paraffin-embedded tissues. Use of a modified Steiner silver stain revealed small numbers of intracytoplasmic bacterial rods in 37 of 42 thymic samples from EBA-affected fetuses. Improved detection was achieved by use of immunohistochemical staining with serum from EBA-affected fetuses that resulted in detection of numerous bacterial rods in the cytoplasm of histiocytic cells in the thymus from all 42 EBA-affected fetuses. Immunohistochemical examination of additional tissues from 21 field and experimental EBA cases revealed positively stained intracytoplasmic bacterial rods in many organs with inflammatory lesions. Use of the modified Steiner stain and immunohistochemical staining of tissues from negative-control fetuses failed to reveal organisms. To the authors' knowledge, this is the first report to document morphologic evidence of a bacterium associated with the lesions of EBA.

Key words: Bovine abortion, deltaproteobacterium, epizootic bovine abortion.

Epizootic bovine abortion (EBA), also known as foothill abortion, is an infectious fetal disease associated with abortion and/or premature birth in cattle grazing foothill and mountain pastures in California, Nevada, and Oregon.^{1,2} The name of the disease is epidemiologically misleading as the disease is endemic, being confined to areas where the transmitting tick, *Ornithodoros coriaceus*, is present, and possibly, is further confined to locations where the ticks can acquire the agent from a still unidentified intermediate host. The disease is observed in heifers or cows brought into endemic areas for the first time during or after the breeding season. Abortions, either sporadic or as an outbreak, occur in the last trimester. Premature,

weak calves may also be born as part of an EBA outbreak. The diagnosis of EBA in an aborted fetus is based on the identification of characteristic gross and microscopic lesions that develop progressively over a period of 3 months or longer.^{4–7} Aborted fetuses usually are not autolyzed. Typical lesions include petechiae in the conjunctiva and oral mucosa, lymphadenopathy, splenomegaly, ascites, and hepatopathy. The thymus may be reduced in size, with interlobular or widespread hemorrhage and edema in the cranial portion. Histologic examination of fetal tissues, particularly the lymphoid organs, is required to confirm a diagnosis of EBA.^{5,6} Thymic lesions, which are unique in cases of EBA, develop late in the course of the disease and consist of a loss of cortical thymocytes and infiltration of the medullary region with macrophages (Fig. 1A). The thymic interlobular septa are distended with fibrin, hemorrhage, and cellular infiltrates consisting of macrophages and other mixed inflammatory cells. The gross enlargement of the lymph nodes is associated with lymphoid hyperplasia and widespread macrophage infiltration in the sinuses and medulla. There is lymphoid hyperplasia and infiltration in the spleen late in the course of the disease. Follow-

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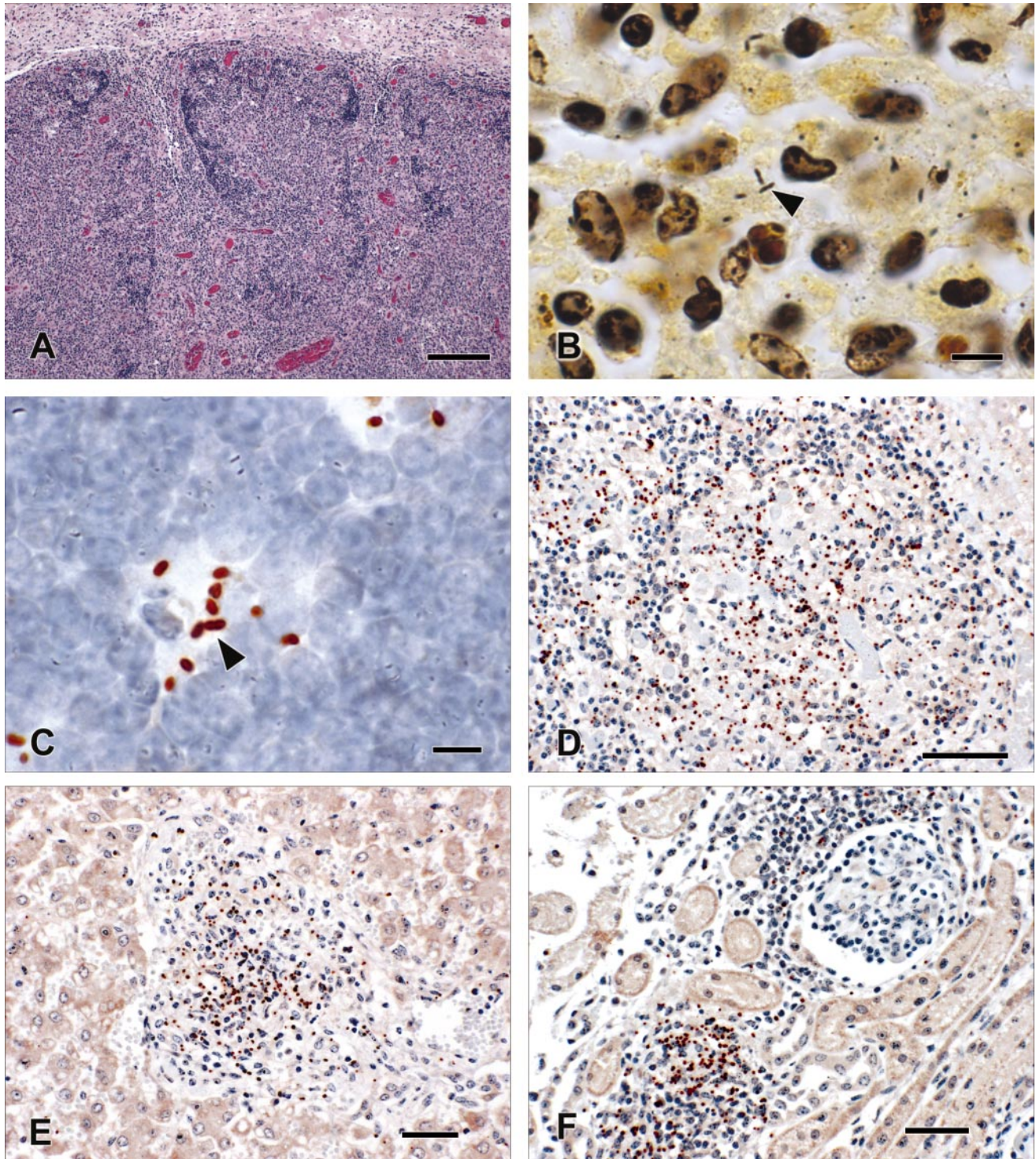


Figure 1. Sections of fetal tissues from field cases (**A, D, E, F**) and experimentally induced cases (**B, C**) of epidemic bovine abortion (EBA). **A**, Fetal thymus, field case. Notice characteristic thymositis with depletion of cortical thymocytes and reduction in the size of thymic lobules. The interlobular septa are dilated by a histiocytic and mixed mononuclear cell infiltrate with edema, HE. Bar = 200 μm . **B**, Fetal thymus, experimental case. Histochemical staining indicates small numbers of bacterial rods (arrowhead), Steiner stain. Bar = 5 μm . **C**, Fetal thymus, experimental case. Immunohistochemical (EBA-IHC) staining indicates clusters of bacterial rods (arrowhead), EBA IHC stain. Bar = 5 μm . **D**, Cortical region of fetal thymus, field case. Notice widespread staining of bacterial rods, EBA IHC. Bar = 100 μm . **E**, Section of fetal liver, field case. Notice lymphohistiocytic portal infiltrate with numerous bacterial rods. EBA IHC. Bar = 50 μm . **F**, Section of fetal kidney, field case. Notice interstitial infiltrate of lymphohistiocytic cells with bacterial rods. EBA IHC. Bar = 50 μm .

ing the proliferative response, foci of acute necrosis may develop in lymphoid organs, and acute vasculitis, which appears to be immune mediated, may be present in many organs. There are widespread inflammatory lesions in most organs, and the fetal immunoglobulin concentration is usually high.

The search for the etiologic agent of EBA has a long history since the first description of the disease in 1956.³ In the 1960s, Schmidtman et al. documented that the geographic distribution of the argasid tick, *Ornithodoros coriaceus*, coincided with the known areas where EBA had been reported.¹⁰ Experimental feeding of this tick on susceptible pregnant heifers was capable of reproducing the disease.¹¹ Pooled tissue extracts taken from EBA-affected fetuses and injected into pregnant cows also reproduced the disease.⁸ More recently, thymic aliquots from EBA-affected fetuses have been used in a series of experiments to reproduce the disease.¹² Although an infective agent has been presumed to be the cause of EBA on the basis of fetal pathologic changes and results of experimental inoculations, many attempts over the past 40 years have not resulted in isolation of an etiologic agent that has been confirmed to cause EBA. Various agents, including chlamydiae, spirochetes, and viruses, have been incriminated, but none of these agents reliably reproduced EBA.^{1,7} Evidence of bacterial involvement in EBA was supported by the documentation that antibiotic treatment of pregnant heifers could prevent development of EBA in their fetuses.¹² With evidence of antibiotic susceptibility of the EBA agent, molecular techniques were used to identify bacterial DNA in tissues from EBA-affected fetuses. A suppression-hybridization polymerase chain reaction technique was used to identify a 767-base pair fragment of the 16S rDNA gene of a previously undescribed deltaproteobacterium in thymus and other tissues from EBA-affected fetuses.⁹ Various microbiological techniques have been unsuccessful in propagating this bacterium in culture, and as a result, the disease has not been reproduced by pure cultures of this putative causative agent.¹²

The study reported here is a retrospective histochemical and immunohistochemical examination of selected tissues from fetuses with naturally acquired and experimentally induced EBA in an effort to identify a possible bacterial agent. Seventy bovine abortion cases were selected from California Animal Health and Food Safety laboratory case material for this retrospective study. Cases were selected on the basis of the final diagnosis established after a complete necropsy. The 42 EBA cases included 21 naturally acquired EBA cases and 21 experimentally induced EBA cases. Samples from 28 fetuses with no gross or microscopic evidence of EBA served as negative controls. The diagnosis for the non-EBA-affected fetuses included

neosporosis (11 cases), leptospirosis (5 cases), brucellosis (4 cases), listeriosis (1 case), other infectious causes (3 cases), undetermined infectious causes (2 cases), and noninfected control fetuses (2 cases). Samples of brain, lung, heart, liver, kidney, adrenal gland, spleen, thymus, lymph node, skeletal muscle, abomasum, small intestine, colon, and placenta were fixed in buffered 10% formalin. Formalin-fixed tissues were paraffin embedded, sectioned at 3- μ m thickness, and stained with hematoxylin and eosin. Standard laboratory procedures to detect bacterial, fungal, and viral bovine abortion agents were applied to samples from the fetuses. Serologic testing of fetal fluid for infectious bovine rhinotracheitis (IBR), bovine viral diarrhea virus (BVDV), and leptospirosis, and of the dam for IBR, BVDV, brucellosis, and leptospirosis was performed.

Previous attempts to detect an etiologic agent in the tissues of EBA-affected fetuses using various histochemical stains had been unrewarding. In 3 of the experimental EBA cases, an extensive number of media, environmental conditions, and incubation times were used in an attempt to recover the suspected agent. Specific methods have been described.¹² On the basis of the 16S ribosomal data, the EBA agent is not closely related phylogenetically to other known veterinary pathogens, although it does have some homology with *Lawsonia intracellularis*.⁹ This information led us to use staining techniques for *L. intracellularis* in an attempt to detect the EBA agent. A Steiner silver stain was modified using *L. intracellularis*-infected porcine colon as the positive endpoint for enhanced silver deposition. Thymus was selected for staining because of documented infectivity of this tissue in experimental inoculation of pregnant heifers.¹²

An EBA immunohistochemical (EBA IHC) stain was developed to detect the proposed bacteria in formalin-fixed, paraffin-embedded tissue. The EBA IHC stain used sera obtained from field cases of EBA as the primary antibody at a 1:100 dilution in an avidin-biotin-complex procedure, with biotinylated goat anti-bovine IgG^a as the secondary antibody and peroxidase-conjugated streptavidin^b as the tertiary reagent. The chromogen used was aminoethylcarbazole (AEC).^c Prior to application of the primary antibody, the sections were autoclaved at 120°C for 10 minutes in an antigen-retrieval citra solution.^d Tissues stained included thymus, lymph node, spleen, lung, liver, kidney, heart, and intestine from field cases and experimentally induced EBA cases. On duplicate tissue sections, control sera obtained from congenitally *Neospora*-infected fetuses with no evidence of EBA was substituted for the primary antibody at a 1:100 dilution in the IHC procedure as a negative control.

Sections of thymus from 70 fetuses, 42 confirmed

Table 1. Immunohistochemical staining results in 21 cases of epidemic bovine abortion.

Case No.	Thymus	Lymph node	Spleen	Lung	Heart	Liver	Kidney	Ileum	Skeletal muscle	Brain
1	+++	+++	+	+	++	++	++	++	+	++
2	++	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+
4	+++	+++	++	++	++	+++	+	+++	++	+
5	+++	+++	+++	++	+	+++	++	++	+	+
6	+	++	+	+	+	+	+	+	+	+
7	+++	++	+	+	+	+	+	+	+	+
8	+++	+++	++	++	+	++	+	+	+	+
9	+++	++	+++	++	+	++	+	+	+	+
10	+++	++	++	+	+	+	+	+	+	+
11	+++	+++	+++	+	+	++	+	+++	+	+
12	+++	+++	+++	+	++	+++	+++	+++	+	+
13	+++	+++	++	+	+	+	+	+	+	++
14	+++	+++	++	+	+	+	+	+++	+	+
15	+++	+++	+++	+	++	+++	++	+++	+	++
16	+	++	++	++	++	++	++	++	Neg.	Neg.
17	+++	+++	+++	++	+	+++	+	+++	++	+
18	+++	+	++	+	+	+	++	++	+	+
19	+++	++	+++	++	+	+	+	++	+	+
20	+++	++	++	+	+	+	+	+++	+	+
21	+	++	++	Neg	+	+	+	++	+	Neg.

Nos.: 1–15 are field cases, and 16–21 are experimentally induced cases. Tissues examined and results recorded are thymus, lymph node, spleen, heart, lung, liver, kidney, skeletal muscle, ileum, and brain. Subjective grading scheme is based on a 1-mm-diameter field: + = small numbers (<10), ++ = moderate numbers between 10 and 200), +++ = large numbers (>200), Neg = no positively stained bacterial rods observed in the entire section of tissue.

EBA cases, and 28 control fetuses were treated with the Steiner stain and the EBA IHC stain. The Steiner staining revealed rods, 1.5 to 3 μm long, in the cytoplasm of histiocytic cells, most frequently in the medullary region of the thymus of 37 of the 42 samples stained (Fig. 1B). The 28 non-EBA-affected thymuses had a negative reaction. The EBA IHC staining of the same samples revealed similar rods in the thymus of all 42 EBA fetuses and in none of the 28 non-EBA-affected fetuses (Fig. 1C). The rods observed with the EBA IHC stain appeared thicker than those observed with the Steiner stain, but were of approximately equal length. The difference in diameter of the rods observed by use of the 2 methods may be the result of differences in stain deposition. Rods seen by the Steiner method were found in locations similar to those seen by use of the EBA IHC method. Using the Steiner stain, the rods were more difficult to identify and were fewer in number, compared with the EBA IHC staining of the same thymic sample. Relative numbers of rods observed were comparable between the 2 staining methods; a thymus sample with low numbers that were identified by use of the EBA IHC stain had rare Steiner stain-positive rods, and a thymus with large numbers of EBA IHC-stained rods had moderate numbers of Steiner stain-positive rods.

Comparison of the Steiner and the EBA IHC staining results for the thymic samples indicated consistent

staining of bacteria using the EBA IHC method. Using that method, the rods were more clearly visible. On the basis of these results, the EBA IHC stain was chosen to evaluate tissue distribution of the bacterium in 10 organs from 38 fetuses. Tissue from each fetus included thymus, lymph node, spleen, lung, heart, liver, kidney, ileum, skeletal muscle, and brain. Twenty-one fetuses in which a diagnosis of EBA had been established, including 15 naturally acquired field cases and 6 experimentally induced cases, were selected (Table 1). Seventeen fetuses without lesions suggestive of EBA served as negative controls.

The results of the EBA IHC staining of samples from the EBA-affected fetuses detected plump, intracytoplasmic rod-shaped bacteria, 1.5 to 3- μm long in all 21 EBA-affected fetuses. The pattern of positive staining was widespread in the thymus (Fig. 1D), spleen, lymph node, and the Peyer's patches of the ileum. The staining was restricted to foci of inflammation in the lung, heart, liver (Fig. 1E), kidney (Fig. 1F), skeletal muscle, and brain. The 17 non-EBA-affected, negative-control fetuses were uniformly EBA negative for all tissues. In the 15 EBA-affected field cases, all 10 tissues had positive staining reaction. The only tissues that stained negatively with the EBA IHC method were from 2 experimentally infected EBA fetuses that also did not have inflammation in the respective tissue sections examined. One fetus had neg-

ative staining in the brain and skeletal muscle samples, and in the other fetus, the brain and lung sections did not stain positive.

The numbers of positively stained rods was variable among the EBA-affected fetuses and among the tissues. Frequently, large numbers of widely distributed positively stained rods were identified in the lymphoid organs including the thymus, lymph node, spleen, and Peyer's patches of the ileum (Table 1). In the thymus, the greatest numbers were seen in the expanded medullary and interlobular regions. In the lymph node, the positively stained rods were widely distributed throughout the cortex and medullary cords, with a few single-to-multiple rods in the cytoplasm of cells within the sinuses. In the spleen, the positively stained rods were widely dispersed in the cytoplasm of histiocytic cells, in the red and white pulp. Bacteria were not detected within any focus of necrosis that was occasionally present in spleen or lymph node sections from some EBA cases. In the sections of ileum, the bacterial rods were present in follicles of the Peyer's patches and in the lamina propria of the overlying villi.

The staining results for the nonlymphoid organs including brain, lung, heart, liver, kidney, and skeletal muscle detected bacterial rods that were focally distributed and confined to sites of histiocytic inflammatory infiltrate. The numbers of bacterial rods were variable. In the brain, small numbers of bacterial rods were seen in the meninges or in rare, scattered, histiocytic foci in the brain. In the lung, bacteria were confined to a focal histiocytic infiltrate in the interlobular septa or in the interstitium. In the liver, small-to-large numbers of stained bacteria with a histiocytic periportal infiltrate were identified in portal regions (Fig. 1E). Positively stained bacterial rods in the kidney sections were confined to scattered histiocytic interstitial infiltrates (Fig. 1F). In the heart and skeletal muscle sections, numbers of bacteria were usually low and confined to scattered foci of inflammation.

On the basis of diagnostic submissions to the California Animal Health and Food Safety Laboratory, EBA is the most common, identified cause of abortion in California beef cattle. In the absence of an identified etiologic agent, diagnosis of EBA has relied on the presence of characteristic macroscopic and microscopic fetal changes. Previous research using affected fetal tissue as the inoculum in pregnant cows has reproduced the disease and offered proof that an antibiotic-susceptible infective agent was the cause.¹² However, the etiologic agent has not been isolated in culture. There is convincing evidence that a unique and previously undescribed deltaproteobacterium is involved on the basis of consistent demonstration of a specific 16S rDNA partial gene sequence in EBA-affected fetal

tissues. The information presented here offers additional morphologic evidence that a bacterium is involved on the basis of the detection of bacterial rods in the lymphoid tissues and in association with lesions of EBA.

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Sources and manufacturers

- a. Kirkegaard and Perry Laboratories, Inc, Gaithersburg, MD.
- b. Jackson ImmunoResearch Laboratories, Inc, West Grove, PA.
- c. DakoCytomation USA, Carpinteria, CA.
- d. BioGenex, San Ramon, CA.

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