Isolation of Two *Brucella abortus* Biotypes from Tissues of a Naturally Infected Cow

ROBERT L. JONES,1,4* BILLY L. DEYOE,2 MARGARET E. MEYER,3 GERALD M. BUENING,1 AND WILLIAM H. FALES1

Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 652111; National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa 500102; and Department of Epidemiology and Preventive Medicine, School of Veterinary Medicine, University of California, Davis, California 956163

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*Brucella abortus* biotypes 1 and 2 were recovered from various tissues of a naturally infected cow. This result indicates a need for biotyping multiple isolates of *B. abortus* from several affected animals in a herd before the biotype of the infecting strain can be definitively established.

The biotyping of *Brucella abortus* isolates is generally recommended as a valuable epizootiological tool (1, 3, 4). Occasional isolation of multiple biotypes from a herd has been reported (9), which might diminish the value of biotyping as an indicator of the source and spread of infection. The concurrent identification of multiple biotypes of *B. abortus* from a milk sample from a naturally infected cow has been reported only once (10).

We isolated two strains of *B. abortus* from the tissues of a cow which were identified as biotypes 1 and 2. Because such a finding has not been reported, this study was conducted to substantiate the preliminary findings by characterizing multiple isolates from several tissues.

MATERIALS AND METHODS

The following tissues had been collected at slaughter from an adult purebred Angus cow: two retropharyngeal and two supramammary lymph nodes, and two sections of the udder. The tissues were stored at −60°C before being cultured. Tryptose agar (Difco Laboratories, Detroit, Mich.) (5% bovine serum supplement) plates were inoculated by direct impression from the freshly and aseptically cut surfaces of the tissues. All cultures were incubated at 37°C in a humidified atmosphere of air (7% CO2 supplement). Discrete colonies were selected after 4 days of incubation and transferred to tryptose agar (5% bovine serum supplement) slants to maintain pure cultures while the isolates were tentatively being identified as *Brucella*. Duplicate second subcultures were numbered, using a random digit table. These duplicate cultures were sent to two investigators (B. L. Deyoe and M. E. Meyer) for independent biotyping by standard methods (1, 2). The biotype determinations from the two laboratories agreed for each isolate. Oxidative metabolic rates were determined as described by Meyer (7). Reference strains which were included for comparison were *B. abortus* strains 19 and 2308.

RESULTS

Five of the six tissue specimens yielded numerous colonies of *B. abortus* on the primary impression-inoculated culture plates. Six colonies were selected from each culture plate, subcultured twice, and identified. All of the selected isolates were confirmed to be *B. abortus*. Specifically, each isolate produced a typical oxidative metabolic profile of carbohydrate and amino acid substrates (data not shown), agglutinated with monospecific anti-A serum, and was lysed by *Brucella* phage (Tb strain, RTD and 104 RTD). In addition, each isolate required CO2, produced H2S, reduced nitrates, did not ferment glucose, hydrolyzed urea in 2 to 4 h, produced catalase, grew in the presence of erythritol (1 mg/ml), and was inhibited by thionin (1:200,000). Characteristics of the isolates which differed and determined the biotype are shown in Table 1.

Two predominant reaction patterns to conventional biotyping tests were found. Twenty-two isolates, recovered from the udder and supramammary lymph nodes, were identified as biotype 2. Growth by the biotype 2 isolates was inhibited by the aniline dyes at the lowest concentrations tested (1:200,000). Eight isolates were identified as biotype 1 by growth in the presence of basic fuchsin at a concentration of 1:50,000. Six of these isolates were recovered from the retropharyngeal lymph node and one each from a section of udder and a supramammary lymph node. The single biotype 1 isolate from supramammary lymph node tissue grew poorly in the presence of a 1:50,000 concentration of basic fuchsin and differed from the other

† Present address: Department of Microbiology, Colorado State University, Fort Collins, CO 80523.
biotype 1 isolates in this study by being inhibited by thionin blue.

**DISCUSSION**

The results established that a naturally infected cow can be concurrently infected with *B. abortus* biotypes 1 and 2. It has been reported (10) that a culture of a milk sample from a naturally infected cow yielded a large number of biotype 2 colonies and a few dye-resistant colonies identified as biotype 1. However, those isolates were characterized only by aniline dye sensitivity at one concentration (1:30,000) and by penicillin resistance. Because dye sensitivity at a 1:50,000 concentration is currently used for biotype 1 and 2 differentiation, the classification of those strains cannot be considered definitive. Experimental studies have shown that two biotypes of *B. abortus* can coexist in tissues of the same cow for extended periods of time. Manthei and Carter (5) experimentally infected 25 cows by simultaneous conjunctival exposure to equal amounts of two distinguishable strains of *B. abortus*, identified as strains 2308 and 2016. Subsequent bacteriological procedures revealed that 12 cows were infected with each of the two strains, whereas infection with both strains could be demonstrated only in one cow. This cow persistently excreted one strain of *B. abortus* from one quarter of the udder, and the other strain was persistently excreted from a different quarter over a period of seven lactations. Characterization of strains 2308 and 2016 by current classification procedures has revealed that they are properly classified as biotype 1 and biotype 2, respectively (B. L. Deyoe, unpublished data). Our recovery and characterization of two biotypes of *B. abortus* from multiple tissues allows one to conclude that infection with two biotypes can occur as a result of natural infection as well as by experimental infection.

The spontaneous mutation rate of *B. abortus* for conventional characteristics is reported to occur at the rates of $3 \times 10^{-10}$ per cell division for CO$_2$ requirement (6) and $2.2 \times 10^{-10}$ per cell division for basic fuchsin resistance (10). These mutation rates indicate that the phenotypic markers are quite stable. It is unlikely that spontaneous mutation occurred during the isolation and identification procedure, because the isolates were only subcultured twice.

Biotype classification of *B. abortus* is determined by quantitative phenotypic differences in growth requirements and sensitivity to aniline dyes. It has been suggested that these differences are due to a spectrum of cell wall permeabilities and can be arranged in an order reflecting the evolution of the species (8). Meyer (8) suggested that biotypes 1 and 2 are closely related, and proposed that biotype 1 may have evolved from biotype 2.

The biotype 2 isolates identified in this study did not obligately require serum for growth; however, serum notably enhanced the growth rates. Biotyp e 2 is generally considered to be serum dependent (2), but, due to the extreme sensitivity to growth inhibition by the aniline dyes, these isolates were still considered to be biotype 2. The characteristics of the biotype 1 isolate from the supramammary lymph node showed a lack of homogeneity with the other biotype 1 isolates. It was sensitive to thionin blue and slightly more sensitive to basic fuchsin. These findings suggested a quantitative gradation of differences in these isolates which could be explained by the evolution of one biotype to another.

Although it is interesting to speculate that the two biotypes isolated in this study had a common origin within the cow, there is inadequate evidence to defend such a conclusion. Without knowledge of the exposure history of the cow, it is impossible to rule out concurrent exposure with both biotypes or superinfection with a second biotype. The cow was from a herd with a prevalence of *Brucella* serological reactors of greater than 50%. Therefore, great potential for repeated exposure existed.

Recently, the epizootiological significance of the biotype of *B. abortus* has been questioned (9). It appears that obtaining only a single isolate
from an animal or herd is of limited value for epizootiological studies. Comprehensive evaluation of several affected animals and the culturing of several tissues from each one are necessary before the biotype or biotypes of B. abortus infecting a herd can be conclusively identified. Unfortunately, the herdmates of the cow in this study had been sent to slaughter, so further studies could not be performed.

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LITERATURE CITED