JOURNAL OF CLINICAL MICROBIOLOGY, June 1984, p. 896–898
0095-1137/84/060896-03$02.00/0

Vol. 19, No. 6

Differentiation of *Brucella ovis* from *Brucella abortus* by Gas-Liquid Chromatographic Analysis of Cellular Fatty Acids

P. J. COLOE,[1]* A. J. SINCLAIR,[1] J. F. SLATTERY,[1] AND D. BURKE[2]

Victorian Department of Agriculture, Veterinary Research Institute, Parkville,[1] and State Chemistry Laboratory, Melbourne,[2] Victoria, Australia

Received 6 January 1984/Accepted 23 January 1984

The cellular fatty acid composition of *Brucella ovis* and *Brucella abortus* strains was determined by gas-liquid chromatography. Both species were characterized by the presence of fatty acids 16:0, 17:0, 17:0 cyclopropane, 18:0, 18:1, and 19:0 cyclopropane; *B. ovis* also contained some 15:0. There were differences in the relative proportions of the fatty acids present, and it was possible to differentiate *B. ovis* from *B. abortus* on the basis of the absence of 15:0, lower concentrations of 17:0 and 18:1, and higher concentrations of 19:0 cyclopropane in *B. abortus*. The data indicate that analysis of cellular fatty acid composition by gas-liquid chromatography can be used for the identification of *B. ovis* and its differentiation from *B. abortus*.

Brucella ovis infection in sheep is a serious economic problem in many parts of the world. *B. ovis* infection has been reported in New Zealand (4), the United States (10), the United Kingdom (3), South Africa (19), Rumania (18), Argentina (15), and Australia (9).

B. ovis is a slow-growing bacterium, taking up to 3 days to be visible on solid media. It is relatively unreactive biochemically, and so identification is a time-consuming process involving specialized biochemical tests and dye tolerance tests (1). Also, because *B. ovis* is rough, serological techniques cannot be used to determine the generic identity of isolates. Care must be taken to differentiate *B. ovis* from *Alcaligenes* spp. and organisms of Group IVe (8) as well as from other *Brucella* spp.

There have been a number of attempts to identify specific components of *Brucella* spp. that may be useful to discuss (5, 8, 16, 17). Thiele and co-workers (16), working with *Brucella abortus* and *Brucella melitensis*, showed that palmitic acid, a C18 monoenoic acid (*cis*-vaccenic acid), and a C19 cyclopropane fatty acid (lactobacillic acid, 19:0 cyclopropane [19:0 cyc]) could be detected in various lipid fractions of these *Brucella* spp. On the basis of lipid studies, Thiele and Schwinn (17) found that *Brucella* spp. differed significantly from *Bordetella* spp. and concluded that it was not justifiable to include the two in the one family *Brucellaceae*.

Subsequently, it was shown (8) that the major cell wall fatty acids of *Brucella* spp. were 19:0 cyc, 16:0, and 18:1. This work was further extended (5) to differentiate *Brucella suis* and *Brucella canis* on the basis that *B. canis* contained no lactobacillic acid.

Since analysis of cellular fatty acids has been used to differentiate closely related bacteria, we have applied the technique to the rapid identification of *B. ovis*. *B. ovis* and *B. abortus* are the only *Brucella* spp. that infect sheep in Australia, and since there is a national *B. abortus* eradication program under way, it is essential that a specific identification technique be available to identify to species level *Brucella* isolates from sheep to ensure that no undetected reservoirs of *B. abortus* remain.

* Corresponding author.

MATERIALS AND METHODS

Strains of *B. ovis* (n = 20) and *B. abortus* (n = 7) were isolated from clinical specimens submitted to the Veterinary Research Institute, Parkville, Victoria, Australia. Reference strains of *B. abortus* (n = 7) and *B. ovis* (n = 1) were obtained from the Brucella Reference Laboratory, National Biological Standards Laboratory, Canberra, Australia. The morphological and cultural characteristics of each strain were determined (1). Criteria for identification of isolates included colonial characteristics, CO_2 requirement for growth, carbohydrate utilization, H_2S production, and tolerance of basic fuchsin, thionin, and crystal violet dyes.

For fatty acid analysis, strains were cultured on plates of horse blood agar for 5 days at 37°C in an atmosphere of 5% CO_2 in air. After incubation, the bacterial cells from one blood agar plate were removed and processed to prepare fatty acid methyl esters. The derivatization procedure was based on the method of Moss (12). The cells were suspended in 0.5 ml of distilled water and then heated for 30 min at 100°C in 3 ml of 5% NaOH in 50% methanol. The saponified material was cooled and acidified by the addition of 1 ml of 6 N HCl. A 3-ml portion of 14% BF_3CH_3OH or 10% BCl_3CH_3OH was added, and the mixture was heated for 5 min at 85°C. The esterified components were extracted with 8 ml of petroleum spirit-ether (1:1). The extracting solvent was removed, evaporated almost to dryness with dry N_2, and resuspended in 1 ml of petroleum spirit. One microliter of this material was injected into a gas-liquid chromatograph.

The fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) with a Packard model 427 gas chromatograph, equipped with either a wall-coated open tubular glass capillary column (40 m by 0.5 mm inner diameter) coated with OV-275 or a 2-m, packed glass column of SP2100. The chromatograph was fitted with a flame ionization detector. Tentative identification of the bacterial fatty acids was made by comparison of GLC retention times with those of purified fatty acid standards (Supelco, Inc., Bellefonte, Pa.).

To assist in the confirmation of the major peak from each species, representative samples of fatty acid methyl esters were separated into saturated and unsaturated fractions by silver nitrate thin-layer chromatography (14).

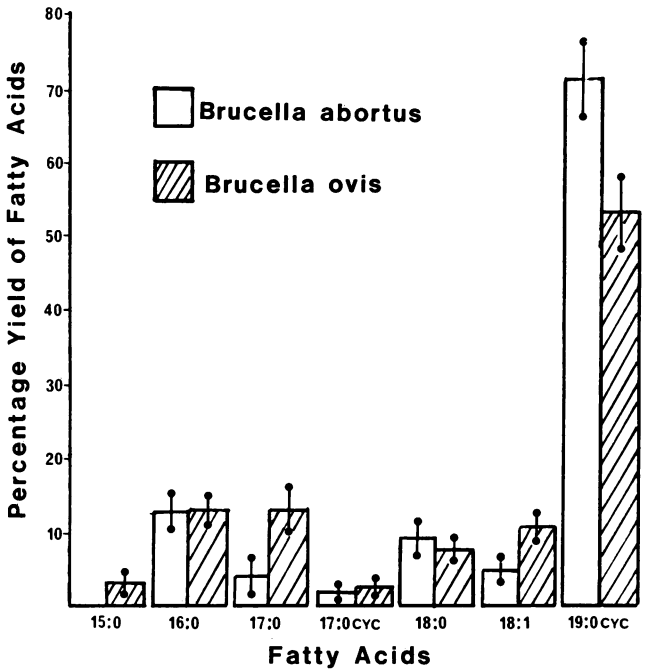

FIG. 1. Cellular fatty acid composition of *B. ovis* and *B. abortus*. Fatty acids are expressed as the mean plus standard deviation of the total fatty acids of *B. ovis* and *B. abortus* as detected by GLC. For the fatty acid identification, the number to the left of the colon refers to the number of carbon atoms and the number to the right refers to the number of double bonds.

RESULTS

The cellular fatty acids of *B. ovis* and *B. abortus* strains are shown in Fig. 1. All *B. ovis* strains contained fatty acids 15:0, 16:0, 17:0, 17:0 cyc, 18:0, 18:1, and 19:0 cyc. *B. abortus* did not contain any 15:0 but did contain 16:0, 17:0, 17:0 cyc, 18:0, 18:1, and 19:0 cyc. There were, however, differences in the relative concentrations of the fatty acids between the two species. Typical GLC profiles of the fatty acids of *B. ovis* and *B. abortus* are shown in Fig. 2. The major fatty acid of each species was 19:0 cyc (lactobacillic acid, *cis*-11,12-methylene octadecanoic acid). The 19:0 cyc was identified tentatively by comparison of the GLC retention time with a reference standard. Silver nitrate thin-layer chromatography showed that the compound eluted with the saturated fatty acid methyl esters, and it was confirmed as lactobacillic acid by gas chromatography-mass spectrometry techniques (11). Other long-chain fatty acids were detected in both species (Fig. 2) but at concentrations of <1%.

The cellular fatty acid composition did not appear to be influenced by the growth media, since analysis performed on isolates of *B. ovis* grown on chocolate agar and blood agar did not reveal any significant differences in fatty acid composition. However, differences were observed in both *B. ovis* and *B. abortus* when the incubation period of the cultures was varied. Two strains, *B. ovis* 29 and *B. abortus* 28, were analyzed after 3, 4, 5, and 6 days. Their fatty acid composition was essentially the same at 4, 5, and 6 days, but at 3 days there was relatively more 18:1 and less 19:0 cyc.

When the results were expressed as a ratio of 19:0 cyc to the sum of 15:0 + 17:0 + 18:1 [(19:0 cyc)/(15:0 + 17:0 + 18:1)], the figures for *B. ovis* ranged from 1.3 to 3.4, whereas the values for *B. abortus* ranged from 5.3 to 14.4.

The fatty acids of all available biotypes of *B. abortus* (strains 1, 2, 3, 6, 7, 9, and 19) were also examined, however, no obvious differences between the biotypes could be detected.

DISCUSSION

Although *B. ovis* and *B. abortus* contain similar fatty acids, it was possible to differentiate *B. ovis* from *B. abortus* on the basis of the presence in *B. ovis* of 15:0, higher concentrations of 17:0 and 18:1, and lower concentrations of 19:0 cyc. The differences between *B. ovis* and *B. abortus* are clearly illustrated in Fig. 1 and 2. When the fatty acids are expressed as (19:0 cyc)/(15:0 + 17:0 + 18:1), *B. ovis* is <3.5 and *B. abortus* is >5. The percentages of fatty acids reported here differ from those reported for *B. abortus* by Dees and co-workers (8). They found higher amounts of 18:1, slightly higher amounts of 18:0, and much lower amounts of 19:0 cyc in *B. abortus* than we report, but they did not detect any 15:0

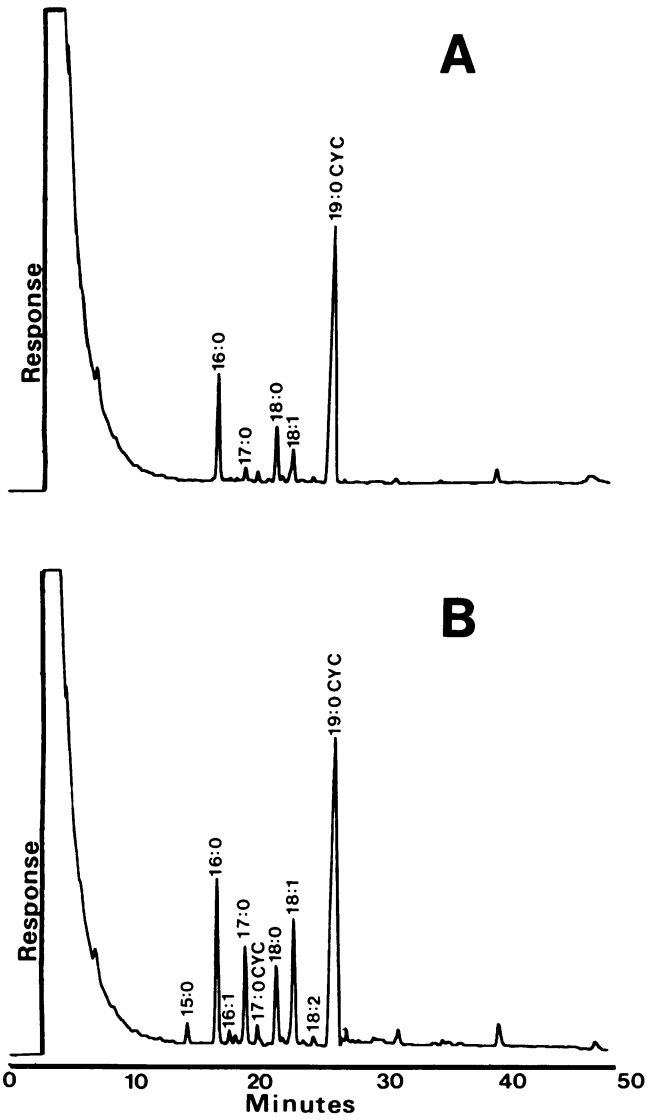

FIG. 2. Gas-liquid chromatogram of cellular fatty acid methyl esters of whole cells of *B. abortus* (A) and *B. ovis* (B). Analysis was made on a glass capillary column coated with OV-275.

in *B. abortus*. The difference between our work and that of Dees et al. (8) is likely to be because the specimens they analyzed were from 24-h cultures, whereas those described in this paper were from 5-day-old cultures.

When *B. ovis* was first isolated and described (3, 4), there was extensive discussion about the source of the organism and whether it should be included in the *Brucella* spp. Our results show that all strains of *B. ovis* have a fatty acid composition similar to *B. abortus*. Also, *B. ovis* cellular fatty acids differ significantly from those of the Group IVe class, *Alcaligenes denitrificans, Bordetella bronchiseptica* (8), and other closely related genera (6, 7, 13). This information supports the classification of *B. ovis* within the *Brucella* spp.

We have applied the described technique to >60 additional field isolates of *B. ovis* and *B. abortus*, and in all instances, the correct identification was made at least 1 week faster compared with established biochemical methods. The technique cannot distinguish individual biotypes of *B. abortus*. However, since these biotypes have differing protein antigenic determinants, it is likely that GLC analysis of fatty acids could be used in conjunction with polyacrylamide electrophoresis of cellular proteins (2) for identification and typing of all *Brucella* spp.

ACKNOWLEDGMENTS

We acknowledge R. Crighton, National Biological Standards Laboratory, Canberra, Australia, for supplying the reference *Brucella* strains.

LITERATURE CITED

1. **Alton, G. G., L. M. Jones, and D. E. Pietz.** 1975. Laboratory techniques in brucellosis, 2nd ed. W.H.O. Monogr. Ser. **55**:39–58.
2. **Barenkamp, S. J., R. S. Munson, and D. M. Granoff.** 1981. Subtyping isolates of *Haemophilus influenza* type b by outer membrane protein profiles. J. Infect. Dis. **143**:668–676.
3. **Buddle, M. B.** 1956. Studies on *Brucella ovis* (N. sp.), a cause of genital disease in New Zealand and Australia. J. Hyg. (Cambridge) **54**:351–364.
4. **Buddle, M. B., and B. W. Boyes.** 1953. A *Brucella* mutant causing genital disease of sheep in New Zealand. Aust. Vet. J. **39**:145–153.
5. **Dees, S. B., D. G. Hollis, R. E. Weaver, and C. W. Moss.** 1981 Cellular fatty acids of *Brucella canis* and *Brucella suis*. J. Clin. Microbiol. **14**:111–112.
6. **Dees, S. B., and C. W. Moss.** 1975. Cellular fatty acids of *Alcaligenes* and *Pseudomonas* species isolated from clinical specimens. J. Clin. Microbiol. **1**:414–419.
7. **Dees, S. B., and C. W. Moss.** 1978. Identification of *Achromobacter* species by cellular fatty acids and by production of keto acids. J. Clin. Microbiol. **8**:61–66.
8. **Dees, S., S. Thanabalasundrum, C. W. Moss, D. G. Hollis, and R. E. Weaver.** 1980. Cellular fatty acid composition of group IVe, a nonsaccharolytic organism from clinical sources, J. Clin. Microbiol. **11**:664–668.
9. **Hughes, K. L., and P. D. Claxton.** 1968. *Brucella ovis* infection. Aust. Vet. J. **44**:41–46.
10. **Kennedy, P. C., L. M. Frazier, and B. McGowan.** 1956. Epididymitis in rams: pathology and bacteriology. Cornell Vet. **46**:303–319.
11. **McCloskey, J. A., and J. H. Law.** 1967. Ring location in cyclopropane fatty acid esters by a mass spectrometric method. Lipids **2**:225–230.
12. **Moss, C. W.** 1979. Analysis of cellular fatty acids of bacteria by gas-liquid chromatography, p. 117–122. *In* G. L. Jones and G. A. Herbert (ed.), "Legionnaires' ": the disease, the bacterium and methodology. Center for Disease Control, Atlanta, Ga.
13. **Moss, C. W., and S. B. Dees.** 1978. Cellular fatty acids of *Flavobacterium meningosepticum* and *Flavobacterium* species group IIb. J. Clin. Microbiol. **8**:772–774.
14. **Sinclair, A. J., J. G. McLean, and E. A. Monger.** 1979. Metabolism of linoleic acid in the cat. Lipids **14**:932–936.
15. **Szyfres, B., and R. Chappel.** 1963. Bacteriological diagnosis of infectious epididymitis of sheep in Argentina. Rev. Vet. Venez. **15**:328–334. In: Vet. Bull. (Weybridge) **34**:641 (1964).
16. **Thiele, O. W., C. Lacave, and J. Asselineau.** 1969. On the fatty acids of *Brucella abortus* and *Brucella melitensis*. Eur. J. Biochem. **7**:393–396.
17. **Thiele, O. W., and G. Schwinn.** 1973. The free lipids of *Brucella melitensis* and *Brucella pertussis*. Eur. J. Biochem. **34**:333–344.
18. **Tudoriu, C. D., M. Andrei, S. Cambir, P. Moldovanu, and C. Ionica.** 1959. Studiu epizootologic asupra epididimitei infectioase a berbecilor. (Epidemiology of infectious epididymitis in rams). Lucr. Stünt. Inst. Patol. Ig. Anim. **9**:59–69. In: Vet. Bull. (Weybridge) **30**:302 (1960).
19. **Van Rensburg, S. W. J., K. M. van Heerden, D. J. Le Roux, and A. J. Snyders.** 1958. Infectious infertility in sheep. J. S. Afr. Vet. Med. Assoc. **29**:223–233.