Bovine Paratuberculosis I. A Herd Study Using Complement Fixation and Intradermal Tests

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ABSTRACT

A dairy herd (102 cattle) which had been enrolled under a paratuberculosis control program for two years utilizing a complement fixation test (carbohydrate antigen) and intradermal skin test (johnin PPD) was subjected to two further herd tests and followed to slaughter to determine infection status by culture and histology. Mycobacterium paratuberculosis infection was demonstrated in 37 of the animals of which only five were considered reactors on the basis of the last two herd tests applied. Cultural and histopathological evaluation indicated the testing procedures had eliminated heavily infected animals. The limitations of these testing procedures under free stall housing conditions are discussed.

RÉSUMÉ

Cette étude portait sur un troupeau de 102 vaches laitières où on tentait depuis deux ans d'éliminer la paratuberculose en se servant de l'épreuve de la déviation du complément, dans laquelle on utilisait un antigène glucidique, et de l'épreuve intra-dermique à la johnine DPP. Au bout de cette période, on soumit le troupeau à deux épreuves additionnelles et on le fit abattre afin d'en déterminer le taux réel d'infection, cette fois à l'aide d'études bactériologiques et histologiques. On démontra ainsi que 37 sujets, dont seulement cinq avaient réagi de façon positive aux deux épreuves additionnelles, recelaient une infection à Mycobacterium paratuberculosis. La bactériologie et l'histopathologie démontrèrent toutefois que les autres épreuves avaient permis de déceler les sujets les plus gravement atteints. Les auteurs commentent les limites de l'épreuve intra-dermique à la johnine et de celle de la déviation du complément, dans les troupeaux où on utilise la stabulation libre.

INTRODUCTION

Since the beginning of this century paratuberculosis has been recognized as a significant cause of economic loss to the cattle industry. A number of different diagnostic tests have been developed to identify animals with paratuberculosis, although none of them has yet been shown suitable for the effective control of the disease (7). Many tests have not been adequately evaluated under field conditions. An appreciation of the wide variation in immunological responses and clinical signs between animals infected with *Mycobacterium paratuberculosis* is essential for the development of an effective control program for bovine paratuberculosis.

In a series of papers the usefulness and limitations of a number of different diagnostic procedures for paratuberculosis will

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be examined. This paper describes a herd study utilizing the complement fixation and intradermal tests.

MATERIALS AND METHODS

HERD HISTORY

In May 1974 a case of paratuberculosis was diagnosed in a six year old cow, from a dairy herd in the province of Quebec. The herd consisted of approximately 70 cows and 30 replacements. Approximately 75% of the herd were Jerseys; the remainder were Holsteins, Ayrshires and crossbred animals. The herd was housed in a free stall barn in which there was heavy fecal contamination of the environment. Calves were separated from the dams at birth and had no contact with the adult stock until their first lactation. In May 1974 a control program was instituted at the request of the owner. Adult animals were skin tested with johnin PPD and the complement fixation test was conducted on serum. Animals which reacted to either test and animals which had clinical signs of paratuberculosis were sent to slaughter. Clinical signs included diarrhea, weight loss and poor production. The herd was tested eight times between May 1974 and May 1976. The herd was also tested in August 1976 and in January 1977. Starting in June 1976 the entire herd was disposed of by slaughter over a nine month period. All but three animals were examined to establish their infection status.

INTRADERMAL SKIN TEST

A tenth of a mL of johnin PPD[1] was injected intradermally into the caudal fold. Double skin thicknesses were measured at the time of injection and at 48 and 72, or 72 and 96 hours later. An increase of three mm or greater was considered a positive reaction.

COMPLEMENT FIXATION TEST

The manual tube complement fixation (CF) test using Annau's carbohydrate antigen (1) was conducted as described by Rice *et al* (11). The test was performed in 12 x 75 mm test tubes. Each reagent, test serum, antigen, complement, amboceptor and sheep red blood cells (SRBC), was used in a volume of 0.1 mL, for a total of 0.5 mL. The test is based on a 50% hemolytic unit of complement and three corrected (1.5x) 50% units are used in the test. The primary incubation period for fixation of complement was 16-18 hours at 4-9°C and the secondary incubation after the addition of the 2.5% maximally sensitized SRBC was for 30 minutes in a water bath at 37°C. The amount of fixation was read by visual comparison with a standard. The test sera were heat inactivated for 30 minutes at 60°C before titration. Doubling dilutions from 1/5 to 1/160 were utilized. An end titer of 1/10 or greater was considered positive for the purposes of this study.

POSTMORTEM EXAMINATION

All animals were slaughtered by April 1977. Fresh and formalin fixed tissues were taken from the ileum adjacent to the ileocaecal valve and 30 cm anterior to the valve. Mesenteric lymph nodes adjacent to these two sites of the ileum were also taken. The uteri from pregnant animals were removed intact from the carcass and taken to the laboratory for the collection of tissues under aseptic conditions. Fresh and formalin fixed tissues were taken from the cotyledon, fetal spleen, liver and small intestine. Fetal stomach content was collected for culture.

HISTOPATHOLOGY

Sections (5 μ) were prepared and stained with haematoxylin and eosin and by Kinyoun's acid fast method. Selected tissues were also stained with phenol auramine 0 stain for examination by ultraviolet microscopy.

BACTERIOLOGY

One gram of tissue was homogenized with 20 mL of sterile distilled water by a Colworth stomacher[2]. The tissue suspension was then filtered through sterile cheese cloth into a sterile 50 mL tube containing 20 mL of 0.4% alkylbenzyldime-

[1]Animal Diseases Research Institute, Nepean, Ontario.

[2]VAC International, London, England.

thyl-ammonium chloride[3]. After 24 hours decontamination, 0.2 mL of sediment was inoculated onto each of three tubes of modified Herrold's egg yolk medium (10) with mycobactin and one tube without mycobactin. The mycobactin[4] was prepared from *Mycobacterium phlei* according to the method described by Francis (5). After 13 weeks incubation at 37°C, cultures were examined for *M. paratuberculosis*.

OTHER TESTS

This herd was also examined with the lymphocyte transformation test and by fecal culture. The results of these studies are presented elsewhere (3, 4).

RESULTS

The results of the first eight herd tests are summarized in Table I. Only two of five animals necropsied were confirmed as having paratuberculosis, the diagnosis of the remaining 66 animals can only be presumptive. Thirty-five animals identified in these eight tests were reared on the farm, 29 were purchased as adults and the origin of seven animals could not be determined.

The infection status of the animals slaughtered since August 1976 is shown in Table II. Animals were classified as infected on the basis of bacterial isolation of *M. paratuberculosis* from tissues and/or histopathological lesions consistent with paratuberculosis. Acid fast organisms had to be demonstrated in a lesion consistent with bovine paratuberculosis for an animal to be classified as "positive" by histopathology. Sixteen animals were culturally positive but had no demonstrable lesions of paratuberculosis. One animal was histologically positive but culturally negative.

Tissues from 31 fetuses of varying gestational length were examined. Nineteen of them were from infected dams. *Mycobacterium paratuberculosis* was isolated from only one fetus in which no histopathological lesions were demonstrated. The dam of this fetus was culturally and histopathologically positive for paratuberculosis.

The results of the last two herd tests are presented in Tables III and IV. The last test examined 26 animals for the first time. With the exception of three cows, these 26 animals were between the ages of six months and two years. Eighteen animals tested in August 1976 were slaughtered prior to the last test and 11 of them were infected. These animals included the one 1/10, and three 1/5 CF reactors from the August test, as well as the one skin test reactor. It should be noted that three of the four noninfected animals classified as skin test reactors (false positives) at the final reading were negative 24 hours earlier.

The age distribution of all but three animals present in the herd since August 1976, is shown in Table V.

DISCUSSION

This study emphasizes a number of important points in the control of bovine paratuberculosis. Elimination of *M. paratuberculosis* infection was not possible in this herd using only the CF and intradermal tests to identify infected animals. The free stall barn with heavy fecal contamination of the environment would appear to be conducive to maintaining the infection in the herd, in spite of the removal of many animals with presumptive paratuberculosis by the testing procedures. Further studies are required to determine whether selective culling and improved management can lead to control of the disease. The study of Larsen and Merkal (8) demonstrated the importance of management practices in the control of bovine paratuberculosis.

The accuracy of the eight herd tests conducted prior to August 1976, in identifying infected animals cannot be determined because the infection status of the reactors is unknown. An essential part of any control program for paratuberculosis must be a postmortem examination of reactor animals to check the accuracy of diagnostic tests. One can assume from the amount of infection present in the herd in August 1976 that many infected animals

[3]Matheson, Coleman and Bell, Norwood, Ohio.

[4]Supplied by Dr. R.S. Merkal, USDA, Ames, Iowa and Dr. J.A.J. Carrière, ADRI, Nepean, Ontario.

TABLE I. Summary of Eight Herd Tests for Paratuberculosis Conducted between May 1974 and May 1976

Date of Test	Shipped to Slaughter					Died on Farm				
	CF and ID	CF	ID	NR	Total[a]	CF and ID	CF	ID	NR	Total
May 1974	2	2	3	2	9	—	—	1	2	3[c]
Oct. 1974	—	2	1	4	7	—	—	—	—	—
Jan. 1975	1	3	6	1	11	—	—	—	—	—
June 1975	—	—	16	6	22	2	—	2	1	5[d]
Sept. 1975	—	1	—	—	1	—	—	—	—	—
Dec. 1975	—	—	3	1	4	—	—	—	1	1[d]
March 1976	1	3	1	—	5	—	—	—	—	—
May 1976	—	—	1[b]	—	1	—	—	—	2	2[e]
Total	4	11	31	14	60	2	—	3	6	11

CF = complement fixation reactors
ID = intradermal reactors
NR = animals removed from herd on basis of clinical signs of paratuberculosis
[a] None of animals sent to slaughter prior to May 1976 examined for infection status
[b] Confirmed paratuberculosis
[c] Two animals necropsied, 1 confirmed paratuberculosis
[d] One animal necropsied, 0 confirmed paratuberculosis
[e] Two animals necropsied, 0 confirmed paratuberculosis

TABLE II. Summary of Histopathological and Cultural Studies for Paratuberculosis in Cattle Slaughtered since August 1976

Category	Number of Animals
Histologically and culturally + ve	20
Histologically + ve, culturally − ve	1
Culturally + ve, histologically − ve	16
Not proven infected	65
Total	102

Note: 19/21 histopathologically and 31/36 culturally positive animals reared on farm. Twenty-five percent of animals sent to slaughter were purchased as adults of which five were infected

did not respond to the testing procedures. One animal culled following the August 1976 test on the basis of clinical signs was found not to be infected at postmortem. This animal emphasizes the nonspecific nature of the clinical signs of paratuberculosis.

Thirty-six percent of the herd was still infected after it had been examined eight times with the CF and intradermal tests. The absence of histopathological lesions in 16 of these infected animals indicates that they contained very few bacilli. Similarly, very few organisms were seen in the sections of some animals with histopathological lesions of paratuberculosis. Variations in the degree of infection is an important characteristic of a number of chronic infectious diseases, including paratuberculosis. The testing and culling procedure used in this herd appears to have identified and removed the more heavily infected animals. No estimate of the economic losses caused by *M. paratuberculosis* was attempted because of deficiencies in herd management.

The CF test identified only three of the 37 infected animals (CF titers of 1/10 or greater); however, no false positive reactions occurred. Three infected animals had a titer of 1/5, while none of the noninfected animals reacted at this dilution. The CF test using the carbohydrate antigen (1) identifies animals with a large bacterial load, yet does not detect minimally infected animals. This concept is further examined in the second paper in this series (4).

The intradermal test identified only two of the 37 infected animals but also had four false positive reactions. Three of these false positive reactors were negative at the 72 hour reading but positive at 96 hours. This suggests that sequential skin test results should be considered when classifying animals as reactors. The large percentage of false negative reactions may be due to either the lack of sensitivity of the test or an absence of delayed type hypersensitivity to johnin PPD in many animals infected with *M. paratuberculosis*. Specific immunological reactivity to johnin PPD has been demonstrated in minimally infected animals by the lymphocyte transformation test (2).

TABLE III. Intradermal and Complement Fixation Test for Paratuberculosis Conducted in August 1976

A: Intradermal Test	Time Examined	Increase in Skin Thickness in mm			
		0	1	2	> 3
Infected[a]	48 hours	20[b]	11	2	0
"	72 hours	17	16	0	0
Noninfected[a]	48 hours	21	12	3	0
"	72 hours	15	14	6	1
B: Complement Fixation Test		**Reciprocal Titer**			
		0	**5**	**10**	**20**
Infected[a]		29[b]	3	1	0
Noninfected[a]		36	0	0	0

[a] Infection status as determined by culture of tissues for *M. paratuberculosis* and histopathology
[b] Number of animals

TABLE IV. Intradermal and Complement Fixation Test for Paratuberculosis Conducted in January 1977

A: Intradermal Test	Time Examined	Increase in Skin Thickness in mm			
		0	1	2	> 3
Infected[a]	72 hours	10[b]	11	1	2
"	96 hours	5	11	6	2
Noninfected[a]	72 hours	24	22	6	1
"	96 hours	25	21	4	3
B: Complement Fixation Test		**Reciprocal Titer**			
		0	**0**	**10**	**20**
Infected[a]		22[b]	0	1	1
Noninfected[a]		53	0	0	0

[a,b] See Table III

TABLE V. Age Distribution and *M. paratuberculosis* Infection Status of Animals Sent to Slaughter since August 1976

Age	Infected[a]	Noninfected[a]	Total
< 2 years	1	27	28
2–3 years	13	6	19
3–4 "	10	9	19
> 4 "	12	18	30
Unknown[b]	1	5	6
Total	37	65	102

[a] See Table III
[b] Includes four adults not reared on farm

The small number of infected animals less than two years old reflects the adequate segregation of the young stock from the adult milking cows. This observation suggests that many animals were becoming infected as adults. Twenty-nine of 71 animals culled as a result of eight herd tests for paratuberculosis or which died on the farm were purchased as adults and not reared on the farm. Similarly five purchased adults were found to be infected at postmortem (Table II). The infectious status of the purchased animals on entry to the herd was not determined. The studies of Hagan (6) and Larsen *et al* (9), which indicate that adult animals are highly resistant to infection with *M. paratuberculosis*, were carried out under highly artificial conditions. Our study indicates that adults can become infected but does not determine whether such animals will develop clinical disease.

The isolation of *M. paratuberculosis* from only one of 19 fetuses from infected dams demonstrated that congenital infection is not common in minimally infected animals. A higher percentage of fetuses with congenital infection would be expected from more heavily infected dams (12). The majority of the heavily infected animals would have been removed from the herd by reacting to the CF test.

Further studies are required to deter-

mine whether or not a reduction in the economic losses due to bovine paratuberculosis can be achieved by use of the complement fixation test with the carbohydrate antigen plus improved management. The intradermal skin test as it is currently used is contraindicated as a routine diagnostic test for paratuberculosis. Previous and concurrent histological and cultural studies of skin test reactors (unpublished data) have confirmed the high percentage of false positives (up to 90 percent) in as many as ten percent of the adult cattle on a single herd test.

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REFERENCES

1. ANNAU, E. A purified complement-fixing antigen from **Mycobacterium johnei**. Nature 81: 1206-1207. 1958.
2. BUERGELT, C.D., C.E. HALL, R.S. MERKAL, R.H. WHITLOCK and J.R. DUNCAN. Lymphocyte transformation: An aid in the diagnosis of paratuberculosis. Am. J. vet. Res. 38: 1709-1715. 1977.
3. DE LISLE, G.W. Johne's disease. A study of the immunological responses of cattle infected with **Mycobacterium paratuberculosis**. Ph.D. Thesis, Cornell University, Ithaca, New York, 1979.
4. DE LISLE, G.W., B. SAMAGH and J.R. DUNCAN. Bovine paratuberculosis II. A comparison of fecal culture and the antibody response. Can. J. comp. Med. 44: 183-191. 1980.
5. FRANCIS, J., H.M. MACTURK, J. MADINAVEITA and G.A. SNOW. Mycobactin, a growth factor of **Mycobacterium johnei** I. Isolation from **Mycobacterium phlei**. Biochem. J. 55: 596-607. 1953.
6. HAGAN, W.H. Age as a factor in susceptibility to Johne's disease. Cornell Vet. 28: 34-40. 1938.
7. JULIAN, R.J. A short review and some observations on Johne's disease with recommendations for control. Can. vet. J. 16: 33-45. 1975.
8. LARSEN, A.B. and R.S. MERKAL. The effect of management on the incidence of clinical Johne's disease. J. Am. vet. med. Ass. 152: 1771-1773. 1968.
9. LARSEN, A.B., R.S. MERKAL and R.C. CUTLIP. Age of cattle as related to infection with **Mycobacterium paratuberculosis**. Am. J. vet. Res. 36: 255-257. 1975.
10. MERKAL, R.S., K.E. KOPECKY, A.B. LARSEN and J.D. THURSTON. Improvement in the technique for primary cultivation of **Mycobacterium paratuberculosis**. Am. J. vet. Res. 25: 1290-1294. 1964.
11. RICE, C.E., H. KONST and A.N. SMITH. Studies of Johne's disease in Canada III. Diagnostic complement-fixation tests. Can. J. comp. Med. 22: 249-254. 1958.
12. SCHAAF, J. and W. BEERWERTH. Die Bedentung der Generalisation der Paratuberkulose, der Ausscheidung des Erregers mit der Milch und der kongenitalen Uebertragung fuer die Bekaempfung der Seuche. Rindertuberk. und Brucell 9: 115-124. 1960.