Studies of Johne's Disease in Canada.
III. Diagnostic Complement-Fixation Tests

by Christine E. Rice, H. Konst and A. N. Smith[1]

TWORT AND INGRAM (1) and Bang and Andersen (2) in 1913 presented encouraging results with the complement-fixation test with Johne's bacillus antigen. Later, Hagan and Zeissig (3) reported that the test was of definite value in confirming the aetiology of suspected clinical disease but less useful in demonstrating the presence of preclinical infection. More recently Sigurdsson in Iceland (4, 5), Hole in England (6-8) and van der Schaaf in the Netherlands (9) have utilized the complement-fixation test extensively for diagnostic purposes. It seems to be the general conclusion of these and other investigators that although such tests may be expected to detect the great majority of cattle with well-established lesions, a certain number will be missed. In his post-mortem examinations of such serologically negative but diseased cattle, van der Schaaf (9) noted that the pathological lesions were usually small in size and number, whereas in cattle with positive complement-fixation tests the lesions were both extensive and numerous. Certain cattle apparently fail to develop circulating antibodies for some time after infection has become established and may indeed remain serologically negative into the period when organisms are being shed in their faeces. This failure to detect many cases of minimal infection together with the difficulties involved, apparently accounts for the rather limited application of this serological method as an aid in the diagnosis and control of Johne's disease.

Since the allergic state often becomes established in cattle infected with *Mycobacterium paratuberculosis* (Johne's bacillus) some weeks or months before serological activity is demonstrable, the intradermal johnin test may be of greater diagnostic value in early disease than the complement-fixation test. Later in the infection both tests are commonly positive, but as the disease progresses in cattle, the skin may lose its reactivity to johnin whereas antibody detectable by complement-fixation tests may persist in the blood up to and through the terminal stages. On the other hand, Sigurdsson (4) has noted that in sheep with Johne's disease, the complement-fixation test tends to become positive earlier than the johnin test and usually continues to be so after most animals have become non-reactive to the skin test.

The intradermal test with johnin P.P.D. has been applied in Canada as an aid in the diagnosis of Johne's disease for over twenty years (10); prior to that crude johnin was utilized. In January 1954, the complement-fixation test with Johne's bacillus antigen was introduced as a supplementary diagnostic method. Attempts have also been made to secure bowel specimens for microscopic examination from animals that have given positive or questionable reactions in either or both tests when these are slaughtered.

MATERIALS AND METHODS

Complement-fixation Methods

Antigens: All sera have been tested for complement-fixing activity with two Johne's bacillus antigens, *Antigen I*,

1. Animal Pathology Laboratories, Health of Animals Division, Canada Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec.

a suspension of *Myco. paratuberculosis* organisms killed by heating at 68°C for 48 hours, and *Antigen II*, a suspension of unheated bacilli killed by treatment with 2% phenol. In each case the organisms were washed three times, ground in a glass grinder with 0.85% salt solution, and shaken for two hours with glass beads. All preparations settled to some degree on standing and on further dilution with saline. A short preliminary extraction of the organisms with acetone did not appreciably improve the stability of the suspension. Each antigen was pretested in serial dilution for anticomplementary properties and for fixability with negative and positive cattle sera.

Antigen II was replaced subsequently by a third form of antigen prepared by extraction with strong phenol of organisms disintegrated by sonic vibration, followed by precipitation with alcohol and ether (11). *Antigen III* being soluble, was less likely than the particulate antigens to fix complement non-specifically with unstable test sera.

Sera: Prior to testing, all sera were inactivated for 30 min. at 56°C. Those found in preliminary tests to have anticomplementary or non-specific properties were heated for 20 min. at 60°C, before retest.

Complement: Individual guinea-pig sera of satisfactory haemolytic titre were pooled, dispensed in 3 to 5 ml. amounts and stored in the frozen state for periods up to 6 weeks. When required, a sample was thawed and the 50% haemolytic "unit" determined (12). Complement dilutions were prepared to contain three 50% haemolytic units in 0.1 ml.

Sensitized Sheep-Erythrocyte Suspension: Anti-sheep red cell haemolysin was prepared in rabbits and carefully standardized (12). Well washed sheep red cells were suspended in saline to a 5% concentration, and mixed with an equal volume of suitably diluted haemolysin. To insure uniformity a spectrophotometer was used in adjusting the density of the sheep red cell suspension.

Technique of Complement-fixation Test: The heat-inactivated sera were diluted 1:2, 1:5 and 1:10 in 0.85% salt solution, pipetted in 0.1 ml. amounts in triplicate into 11 x 75 mm. tubes, and 0.1 ml. of the 3-unit dilution of complement added. The three sets received respectively 0.1 ml. of antigen I, of antigen II *or* III, or of salt solution (serum control.) After shaking, the racks of tubes were placed in the refrigerator for 18 hours, then removed and 0.2 ml. of the sensitized sheep-red-cell suspension added to each tube. A period of 30 min. at 37°C was allowed for haemolysis. The tubes were centrifuged at low speed to sediment the cells, and the % haemolysis in the supernatant fluid read by comparison with colour standards prepared from the day's reagents (12). Positive and negative cattle sera as well as the customary complement and antigen controls were included in each test.

When complete or almost complete fixation of complement (0 to 20% haemolysis) was recorded with the 1:10 dilution of test serum, a retest was made with 1:10, 1:20, 1:50 and 1:100 or higher serum dilutions.

The serum titre was expressed as the reciprocal of the highest dilution that showed 50% haemolysis or less in the presence of antigen. A titre of 20 or higher was reported as "positive", titres of 5 and 10 as "questionable" serological reactions, providing the corresponding serum controls in each case were completely haemolysed.

RESULTS

A total of 3481 cattle sera were tested during the four-year period following January 1, 1954. These cattle fell into three general groups, (1) cattle from 37 herds in which the presence of Johne's disease was suspected or previously proven, (2) cattle imported from Great Britain and (3) cattle for export to other countries requiring certificates that a test for Johne's disease had been made just prior to shipment. The num-

TABLE I

Number of complement-fixation tests for Johne's disease in cattle made between January 1, 1954 and December 31, 1957.

Group of Cattle	Number of Sera Tested				
	1954	1955	1956	1957	Total
Imported	499	353	380	321	1553
Export	0	27	201	189	417
Diagnostic.....	28	313	378	792	1511
Total....	527	693	959	1302	3481

bers of sera tested during the four-year period are given in Table I. The results with the first or diagnostic group of 1511 sera from cattle in the suspected herds will be reviewed in some detail in this paper.

Reactions reported as positive were observed in sera of one or more animals in nine of the 37 herds; tests in the other 28 herds were negative. Periodic serological and intradermal tests in these nine herds were made and are being continued in an attempt to eradicate the disease through detection of early cases. In return for this service the owners have signed an agreement not to sell any of their animals except for slaughter. The data on six of the nine herds will be presented below.

Herd D-1

In this herd, a total of 47 reactors to the intradermal test had been discovered in four tests performed during the year prior to the first serological examination. All of these cattle had been slaughtered and the premises cleaned as thoroughly as possible. Thus the 162 animals bled August 30, 1955, had all been negative in the preceeding intradermal tests, including the last one conducted about seven weeks earlier. Five that showed a definite reaction in the complement-fixation test with Johne's bacillus antigen were slaugh-

TABLE II

Summary of results in thirteen animals in herd D-1 in which microscopic as well as serological and intradermal tests were made.

No. of Animal	Age yr.	Sex.	Results of C.F. and J.I. tests				Microscopic Findings
			Date	C.F.*	J.I.	Date of Slaughter	
26	4	F	Aug. 8/55	P(20)	x	Sept. 26/55	No acid-fast bacilli
27	4	F	"	P(20)	x	"	No " " "
59	2	S	"	P(100)	x	"	Many " " "
72	2	S	"	P(100)	x	"	Many " " "
78	2	S	"	P(20)	x	"	Many " " "
17	5	F	Nov. 28/55	Neg.	Pos.	Dec. 7/55	No " " "
64	2		"	Neg.	Pos.	"	Few " " "
71	2	S	"	P(20)	Pos.	"	Many " " "
109	1½	S	"	Q(5)	Pos.	"	Many " " "
14	6	F	Feb. 14/56	Neg.	Pos.	Feb. 28/56	No " "
46	4	F	"	Neg.	Pos.	"	Few " " "
58	3	F	"	Q(5)	Pos.	"	Few " " "
59	2	F	"	Neg.	Pos.	"	Moderate " "

x = not tested intradermally on this date.
* = trace reactions reported as negative.

tered and bowel specimens taken. Microscopic examination revealed acid-fast bacilli in three of the five (Table II).

On November 2, the 137 animals remaining in the herd, were bled and a fifth johnin test made. Ten reacted to johnin. One of the ten gave a positive complement-fixation test. All others were negative in both tests. When the ten johnin reactors were slaughtered specimens were taken from four; three of these specimens showed acid-fast bacilli. On February 14, 122 animals were bled and johnin tested. Two were positive serologically, four in the intradermal test. The four serologically-negative johnin reactors were slaughtered; acid-fast bacilli were seen in tissue specimens from three. Unfortunately no tissue specimens were obtained from the two serologically positive cattle.

Herd D-2

The first animal in this herd had a complement-fixation titre of 50, and acid-fast bacilli were observed on microscopic examination of tissues taken at slaughter. The entire herd of 137 cattle was bled and johnin tests made. One animal was positive in both tests, another was serologically-positive only, and a third reacted only in the intradermal test. These three cattle were sold for slaughter but unfortunately the collection of specimens was overlooked. Subsequently two of the serologically-negative animals, one of which appeared to be a clinical case, were slaughtered; microscopic findings on both were negative.

A second herd test was made two months later when two more serologically-positive animals were found but no additional reactors in the johnin test. One of the serologically-positive animals was a six months' old calf whose dam had been negative in all tests. A few weeks later, two of the negative cattle began to show symptoms suggestive of early Johne's disease and were slaughtered; bowel specimens were negative on microscopic examination. When a third herd test was conducted after about eight months, serological reactions were obtained with sera of two of the 87 cattle examined; both were non-reactive in the intradermal test. Conversely, eight of the serologically-negative cattle were classified as reactors in the johnin test. No tissue specimens were submitted.

Herd D-3

Following a positive serological test in one animal, the owner requested that the herd of 62 cattle be tested. Two additional animals were found to be serologically positive and six questionable. One of the serologically-positive animals was also a reactor in the johnin test; 54 cattle were negative in both tests. When the two serologically-positive cattle were slaughtered, intestinal lesions resembling those in Johne's disease were seen in one but not in the other. No specimens were taken for microscopic examination.

Herd D-4

A first blood specimen from this herd, taken from an emaciated animal with recurrent diarrhoea, had a titre of 100. The animal died some days later. Four other cows had died during the preceeding year with symptoms suggestive of Johne's disease. When a herd test was made, three cattle proved serologically positive; two of these gave questionable reactions in the johnin test, the third was negative. Conversely, one of the serologically-negative animals showed a positive and two a questionable reaction to johnin. The positive reactor was isolated but retained in the herd. Two serologically-positive cattle were slaughtered but tissue specimens taken from only one. This proved positive on microscopic examination. Two johnin reactors, one of which was the previous reactor mentioned above, were found on herd retest about five

months later. No serological reactors were detected at this time.

Herd D-5

To date only one test has been made on this herd of 82 cattle. One animal was serologically positive, two questionable. Five were strongly reactive in the intradermal test, four were moderately reactive. Only one reacted in both tests. This animal showed no clinical symptoms, but at post mortem the appearance of the intestine was highly suggestive of early Johne's disease and numerous acid-fast bacilli were seen in tissue smears. Two of the serologically negative, johnin reactors were slaughtered and many acid-fast bacilli likewise demonstrated in smears.

Herd D-6

One animal in this herd which was showing symptoms of Johne's disease, was bled August 29, 1957 and an intracutaneous test made. It died two days later before the intradermal test was read. At post mortem, definite evidence of Johne's disease was found and acid-fast bacilli seen on microscopic examination. The herd of 37 animals was tested September 18. One animal gave a slightly questionable reaction in the complement-fixation test; 36 were negative to both tests. Periodic testing will be continued in this herd.

DISCUSSION

A total of 96 cattle from these nine suspected herds showed positive or questionable reactions in the serological or intradermal tests; 16 of these reacted in both tests. Forty-six serologically-positive or questionable animals were negative in intradermal tests, whereas 34 cattle classified as reactors or suspicious reactors to johnin P.P.D. were negative serologically. Acid-fast bacilli were seen in direct smears of tissues taken at post mortem from 19 of the serologically-positive animals, only four of which were showing clinical symptoms. Ten serologically-negative cattle were likewise positive for acid-fast bacilli on microscopic examination; one of the ten had been a far-advanced clinical case. Thus among these 24 microscopically-positive, subclinical cases of Johne's disease, 15 were detected by the complement-fixation but nine were missed.

Microscopic examinations were made on tissues of only 21 of the cattle tested both serologically and intradermally. Acid-fast bacilli were seen in smears from 16 of these 21. Seven of the 16 reacted in both the complement-fixation and allergic tests, 3 in the former test only, four in the latter only, and two in neither. Thus the complement-fixation test if used alone would have missed six of these cases, the intradermal test alone would have missed five of them. On the other hand the microscopic findings were negative in two cattle that reacted in the complement-fixation test only and in one that reacted in the intradermal test only. This should not, however, be considered a reflection on the specificity of these tests since these particular animals were from herds in which clinical or bacteriologically-proven cases of Johne's disease were concurrently present. It seems more likely that the tissue specimens received were inadequate.

SUMMARY

The results of complement-fixation tests with Johne's bacillus antigen in 39 herds of cattle in which Johne's disease was suspected are described; serological reactors were found in only nine of these herds. These data were compared with the results of intradermal tests with johnin P.P.D. and of microscopic examinations of tissue specimens for acid-fast bacilli. In general the intradermal test appeared to become positive earlier in the disease than the serologcal test. Although a small number of cases were missed by both methods, the parallel use of the two tests in-

creased the proportion of non-clinical cases detected.

RESUME

Les auteurs décrivent les résultats de l''épreuve de la faxation du complément faite avec l'antigène du bacille de Johne dans 39 troupeaux de bovins suspects d'infection; des réacteurs à l'épreuve sérologique ont ét trouvés dans neuf de ces troupeaux. Ces données ont été comparées avec les résultats de l'intradermoréaction avec la johnine P.P.D. et la recherche microscopique de bacilles acido-résistants dans des échantillons tissulaires. En généal, la réaction positive à l'épreuve intradermique semble apparaître plus tôt que celle de l'épreuve sérologique. Quoiqu'un petit nombre de malades n'ont pas été dépistés par ces méthodes, l'emploi conjoint de ces deux épreuves a augmenté la proportion de cas non-cliniques dépistés dans ces troupeaux.

ACKNOWLEDGEMENTS

The technical assistance of Miss Louise Duhamel, Miss Marie Schingh and Mr. E. Sally is gratefully acknowledged.

REFERENCES

1. TWORT, F. W. and INGRAM, G. L. Y. Johne's Disease. Ballière, Tindall and Cox. 1913.

2. BANG O. and ANDERSEN, C. W. Einige Untersuchungen über komplementbindende Antistoffe bei experimenteller und spontaner Tuberkulose sowie bei paratuberkulöser Darmentzündung, Centrabl. f. Bakt. Abt. I Orig. 69:517-519, 1913.

3. HAGAN, W. A. and Zeissig. A. J. Studies on control of Johnes' disease. J. Amer. Vet. Med. Assoc. 82: 391-404, 1933.

4. SIGURDSSON, B. Paratuberculosis (Johne's disease) of sheep in Iceland. Immunological studies and observations on its mode of spread. Brit. Vet. J. 110:307-322, 1954.

5. SIGURDSSON, B. VIGFUSSON, H. and THEODORS, S. The serological diagnosis of Johne's disease of sheep. J. Comp. Path. Therap. 55:268-276, 1945.

6. HOLE, N. H. Johne's Disease. I. Present day diagnosis and a preliminary note on an investigation into the value of a serological method. Vet. Rec. 64:601-603, 1952.

7. HOLE, N.H. Johne's Disease. The theory behind herd complement-fixation testing and its proper usage in the field experiment in progress. Brit. Vet. J. 112:272-278, 1956.

8. HOLE, N. H. The diagnosis of Johne's disease. In: Control of Johne's Disease. Project No. 207, European Productivity Agency. Organiz. Europ. Econ. Co-Op. Paris, pp. 91-120, 1956.

9. van der Schaaf, A. Johne's disease in bovines. The disease in practice and its control. In: Control of Johne's Disease. Project 207, European Productivity Agency. Organiz. Europ. Econ. Co-Op. Paris. pp. 143-158, 1956.

10. KONST, H. and MCINTOSH, C. W. Studies of Johne's Disease in Canada. I. Early intradermal testing with johnin P.P.D. Can. J. Comp. Med. (in press).

11. ANNAU, E. A purified complement-fixing antigen from **Mycobacterium Johne.** Nature 180:1206-1207, 1958.

12. WADSWORTH, A. Standard Methods of the Division of Laboratories and Research, New York State Department of Health, Albany N.Y. Williams and Wilkins, Baltimore, Md., 3rd Ed. pp. 367-387, 1947.

COMING EVENTS

10TH ANNUAL CONVENTION — CANADIAN VETERINARY MEDICAL ASSOCIATION ROYAL ALEXANDRA HOTEL — WINNIPEG — JULY 21-23, 1958

CONGRES DU COLLEGE DES MEDECINS VETERINAIRES DE LA PROVINCE DE QUEBEC AOUT 8-9—ECOLE DE GENIE FORESTIER (CAMPUS DE L'UNIVERSITE LAVAL)

50TH ANNUAL MEETING — VETERINARY ASSOCIATION OF SASKATCHEWAN AUGUST 28-30 — HOTEL SASKATCHEWAN — REGINA.