STUDIES ON MYCOBACTERIUM JOHNEI INFECTION IN CATTLE AND MICE

BY

N. J. L. GILMOUR

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

October 1960

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

INDEX

INTRODUCTION

Johne's disease is a specific infectious disease of the order Ruminantia. The clinical syndrome is one of chronic wasting and diarrhoea. Once clinical signs of the disease manifest themselves death almost invariably occurs after a variable period. The disease is widespread among cattle in this country. Sheep are also affected but little is known of the incidence of infection in sheep. Although the syndrome is distinct some of the signs of the disease e.g. loss of condition, dehydration, loss of pigmentation of the coat, diarrhoea and lowered milk yield, are common to several diseases of cattle. Confirmation of diagnosis on clinical grounds must be obtained from the microscopical examination of faecal smears or by serological techniques.

It is generally accepted that only one third of cases of Johne's disease can be diagnosed on a single faeces examination by the finding of clumps of acid-fast bacteria in the faeces and that faecal smear examination is of no value in detecting infected animals in the pre-clinical stage of the disease. Because of the time taken for the isolation and identification of M. johnei by cultural techniques, examination of the faeces by this method is of little value in routine field diagnosis.

As control of the disease on a herd or nation-wide basis must rest on the early detection of infection improvements in diagnostic methods are necessary. It had been noted that single acid-fast bacilli were a common finding in faecal smears both alone and in association with clumps of acid-fast bacteria. In the past, the significance of these in smears has been discounted, mainly on the grounds that saprophytic acid-fast bacteria were a common finding in faeces. This study was undertaken to test the hypothesis that the finding of single acid-fast bacilli on

microscopical examination of faecal smears was of significance in the diagnosis of M. johnei infection, and that pre-clinical infection could be detected by this means.

The complement fixation test for M. johnei antibodies in the serum has been widely used for the confirmation of the presence of the clinical signs of Johne's disease and, on a lesser scale, for the control of Johne's disease on a herd basis. Unfortunately false positive results were common, especially when the test was applied to apparently normal animals. However, it was seldom that a detailed post-mortem examination of these normal cattle was carried out immediately following a positive test. It was thought that the post-mortem techniques used in the present studies might clarify the position regarding the accuracy of this test.

In view of the fact that no laboratory animal has been found to be uniformly susceptible to experimental M. johnei infection, bacteriological and immunological studies have had to be carried out on cattle and sheep which are expensive to maintain. This study was extended to investigate again the mouse as an experimental animal with special reference to the behaviour of different strains of M. johnei in that animal.

REVIEW OF THE LITERATURE

The first reports of the occurrence of a syndrome indistinguishable from that of Johne's disease were those of Farrow (1831) and Hale (1831). The disease they described under the names "scantering" and "shooting" had been known to exist for a long time before their reports.

Johne and Frothingham (1895) working at Dresden, investigated the condition and described changes they found in the alimentary tract. They were of the opinion that they were dealing with an unusual form of intestinal tuberculosis.

Bang (1906) differentiated the disease from tuberculosis. As a result of the administration of 300 gm. of infected material he reproduced the disease in two calves.

McFadyean (1906, 1907) gave an account of the disease as it occurred in England. He concluded that the majority of fatal cases of diarrhoea in cattle were due to Johne's disease.

Attempts to isolate the causal organism, Mycobacterium johnei, on laboratory media were unsuccessful prior to 1910 when Twort and Ingram obtained growth on Dorset's egg medium containing dead human tubercle bacilli and glycerol (Twort, 1910; Twort and Ingram, 1912).

Johne's disease is the cause of considerable economic loss. An estimate of £4 million per annum was the figure arrived at by Rankin (1958b). This accounts for the loss due to clinical disease in cattle. It disregards losses due to infertility and poor milk production and the loss due to the disease in sheep.

Susceptible species

There have been many reports of the disease in cattle and sheep (Report 1956b). Although it is experimentally susceptible, there are few

references to its occurrence in the goat. McFadyean and Sheather (1916) described the disease in goats and reported that concurrent parasitism was a common finding. Buffaloes (Burggraaf, 1931) and camels (Strogov, 1957) were susceptible. Cases in exotic species kept in zoological collections have occurred. Appleby and Head (1954) reported a suspected case in a llama. Johne's disease has occurred in park-land deer (McFadyean, 1907).

M. johnei has been recovered from pigs (Eveleth and Gifford, 1943) and from a horse (Williams Smith, 1954) but there was no evidence that the presence of the organism was connected with disease in these cases.

Distribution

Although no nation-wide surveys have been carried out Johne's disease would appear to exist in all areas of Britain, but the incidence in native Irish cattle is low (Rankin, 1958b; Pearson, 1953).

Reports of the occurrence of the disease in cattle have come from all over the world:- U.S.S.R. (Posokhin, 1940), India (Cooper et al., 1931), Australia (Albiston et al., 1936), Tasmania (Phillip, 1942), New Zealand (Stephens-Gill, 1937), Uruguay (Cassamagnaghi, 1947), Argentine (Rosenbusch, 1937), Canada (McIntosh, 1939), U.S.A. (de Fosset, 1936), S. Africa (Robinson and de Kock, 1939) and the Belgian Congo (Déom and Mortelmans, 1955).

Johne's disease in sheep was first described in Bosnia in 1908 (Vukovic, 1908). It is known to occur in Britain, Germany, France, U.S.S.R., India, New Zealand, U.S.A. and Iceland.

Incidence

Surveys of the incidence of clinical disease have been carried out by Withers (1959) who found that the rate varied from 0.14 per cent. of adult

cattle at risk in a sample of farms in Ayrshire and Lanarkshire to 0.91 per cent. in an area embracing Berkshire, Buckinghamshire, Northamptonshire, Oxfordshire, Warwickshire and Gloucestershire. These figures represented only animals disposed of for obvious clinical disease but did not include animals culled because of lowered milk yield in the early stages of the disease.

In comparison, cultural examination of mesenteric lymph nodes of apparently normal cattle slaughtered at various abattoirs revealed an incidence of up to 17 per cent. infection (Rankin, 1954; Williams Smith, 1954; Taylor, 1949). In imported Irish cattle the incidence was 0.8 per cent. (Rankin, 1954). From these figures, it is clear that all animals which harbour M. johnei in their tissues did not develop clinical disease.

The incidence of infection or disease in sheep is not known, though it would appear to be irregular in frequency but widespread in distribution. Both hill and low ground flocks are affected. The incidence in infected flocks varied from 5 - 15 per cent. (McEwen, 1939; Michael, 1946; Stamp, 1956). Heath (1955) found clumps of acid-fast bacilli in intestinal scrapings from 21.5 per cent. of 214 sheep examined at a Cumberland abattoir, while M. johnei was recovered from 1 of 200 sheep slaughtered in the Huntingdon-Cambridge area (Williams Smith, 1954).

Johne's disease was unknown in sheep in Iceland until 1938. Circumstantial evidence indicated that it was introduced in 1933 by 5 of 20 rams imported from Germany. Losses for the period 1941 to 1956 were estimated to be of the order of 100,000 sheep (Sigurdsson, 1956). The annual loss on infected farms was reported by him to be 8 - 10 per cent. The first reports of the disease in Icelandic cattle were made in 1945 (Gislason, 1956). He noted that the distribution of the disease in cattle

was closely related to that of heavily infected sheep stocks.

The clinical syndrome of Johne's disease

Doyle and Spears (1951) reported that in cattle the majority of clinical cases occurred between 2½ and 6 years of age although all except very young animals could be affected. Hole (1956b) described a case in a 13 year old cow which had been recognised as a carrier for a period of years.

Withers (1959) found a statistically significant higher incidence of Johne's disease in Jersey cattle, thus confirming the often recorded impression that the Channel Islands breeds were hypersusceptible.

Hole (1956b) reported that the appearance of clinical evidence of Johne's disease was, in many instances, associated with changes in management or movement from one farm to another. He noted a high incidence following the second and third pregnancies and that the time elapsing from calving to the onset of symptoms was variable.

Rankin (1957) noted that the character of the diarrhoea was variable. He described the consistency of the diarrhoeic faeces as paint-like, gross particles of undigested food being uncommon. Submandibular oedema was a feature of Johne's disease and might be the first obvious abnormality but tended to disappear with the occurrence of diarrhoea (Rankin, 1958b). Rankin (loc. cit.) stated that, in the lactating cow, the milk yield fell progressively till lactation ceased. Examination of milk records of cows suffering from Johne's disease showed that the yield in the lactation prior to that in which clinical signs manifested themselves was below that anticipated.

A small number of cases of recovery after the exhibition of clinical

signs of the disease have been recorded (Doyle and Spears, 1951; Hagan and Zeissig, 1933).

Few specific changes in the haematology or in the biochemistry of blood, urine or milk have been reported. The erythrocyte sedimentation rate was unaltered according to Rankin (1955b). Stewart, McCallum and Taylor (1945) reported a depression of serum calcium and magnesium levels in Johne's disease in cattle and sheep.

Clinical signs in sheep

The signs of Johne's disease in sheep are essentially the same as those in cattle. In sheep, however, there has been noted a tendency for some cases to exhibit prolonged unthriftiness with eventual recovery (Howarth, 1932, 1937; Brotherston, 1959).

Stamp (1956) stated that diarrhoea was not a feature of Johne's disease in sheep. Levi (1948b) noted that the faeces of experimentally infected goats become paste-like.

Other features noted include loosening of the wool (Stamp and Watt, 1954) and submandibular oedema (Johnstone, 1933). McEwen (1939) recorded a higher incidence of the disease associated with the lambing season.

Epidemiology

Surveys conducted by Withers (1959) have shown that there was a variation in the incidence of clinical disease from farm to farm. The mean incidence rate in infected herds was 3 to 5 per cent. while the annual loss in some herds might be as high as 13 per cent.

Taylor (1952) showed that the absence of clinical disease in a herd did not mean that M. johnei infection was not present. He found an

incidence of 6 per cent. M. johnei infection in cattle disposed of from an estate where there was no Johne's disease problem.

Both Taylor (1953) and Rankin (1957) have reported that M. johnei was present in the faeces of artificially infected calves soon after infection and many months before clinical symptoms were apparent. This was also the finding in cases in which clinical signs did not develop.

Calves placed in contact with non-clinically affected excretor calves become infected and develop clinical disease in a proportion of cases (Rankin, 1957).

M. johnei has been recovered from the milk and mammary glands of clinical cases of Johne's disease (Alexejeff-Goloff, 1929; Doyle, 1954).

Congenital infection has been reported to have occurred (Lawrence, 1956; Pearson and McClelland, 1955; Doyle, 1958), although what proportion of calves from clinically affected dams is infected is not known.

It has been shown that younger calves are more likely to develop clinical disease following experimental infection than those inoculated at a later date (Taylor, 1953). Rankin (1957) failed to produce disease in adult cattle inoculated with 100 mgm. of M. johnei intravenously. Hagan (1938) reported that calves born into an infected herd were more susceptible than calves or yearlings introduced into that herd.

Bacteriology

Mycobacterium johnei (syn. M. paratuberculosis) belongs to the Order Actinomycetales family Mycobacteriaceae genus Mycobacterium (Bergey, 1948).

M. johnei is non-motile, Gram positive and acid- and alcohol-fast when stained by the Ziehl-Neelsen method. It is a slender rod with parallel sides and rounded ends, measuring 0.5 x 1-2μ. Occasionally longer forms occur. These may show irregular staining. Plumper and longer forms are

found on laboratory media, especially when the medium is not completely suitable.

In primary culture, M. johnei has been found to be difficult to isolate and has only been grown on media containing killed or extracts of killed acid-fast bacteria (Boquet, 1925). It failed to grow in the absence of this growth factor.

After 4 - 6 weeks' incubation at 37°C on solid media colonies are minute, raised, dull-white and circular with a thin, irregular margin. Older colonies become more raised, radially striated or irregularly folded and yellowish-white in colour (personal observations).

On glycerol-broth containing the growth factor, M. johnei grows as a thin surface pellicle which becomes thickened and folded (Bergey, 1948).

Twort and Ingram (1913) attempted isolation of M. johnei on media containing extracts of tissues of normal cattle alone or in combination with glycerine, cholesterine, sugars and blood. Extracts of infected intestine were also ineffective in promoting growth. Taking into account the obvious relationship that M. johnei has with the tubercle bacillus, they hypothesised that both would require the same metabolites but that M. johnei had lost the ability to synthesise these in vitro. This led them to incorporate heat-killed, human-type tubercle bacilli into Dorset's egg medium. Colonies of M. johnei were obtained after 4 weeks' incubation at 37°C. Sub-cultures from this primary growth failed to grow on unmodified Dorset's egg medium but grew on that medium fortified with dead tubercle bacilli. Other acid-fast bacilli were tested for growth-promoting activity. The authors concluded that the best results were obtained by the addition of M. phlei. M'Fadyean, Sheather and Edwards (1912) found that the avian tubercle bacillus was the most active source

of growth factor.

The growth factor has been isolated and its chemical composition determined (Francis, McTurk, Medinavietia and Snow, 1953). The incorporation of the purified product in media used for the isolation of M. johnei would appear to have little advantage over heat-killed or crude extracts of M. phlei.

Twort and Ingram found that their original medium was improved by the addition of 4 per cent. glycerol.

Dunkin (1928) modified Twort and Ingram's egg medium by the incorporation of liver extract and 0.25 per cent. gentian violet. Minett (1942) compared the growth of M. johnei on primary culture and on subculture on 27 media. He concluded that the most satisfactory were those containing whole egg and 1 per cent. heat-killed M. phlei. The addition of potassium tellurite or penicillin did not facilitate the isolation of M. johnei.

Taylor (1950) found that media containing more than 50 per cent. egg-yolk were superior to those containing whole egg. Because of its ease of preparation, firm surface and consistency from batch to batch, Finlayson's (1946) medium modified by the addition of heat-killed M. phlei was preferred. This medium supported the growth of pigmented sheep strains which, hitherto, had been grown on laboratory media (Taylor, 1951).

Williams Smith (1953) modified Dubos' (1947) liquid and agar media by the addition of an alcoholic extract of M. phlei. This medium permitted the growth of both pigmented ovine and the classical bovine strains of M. johnei.

Satellitism between subsurface colonies of staphylococci and M. phlei and M. johnei on modified Dubos' oleic acid agar medium was reported by

Stuart (1954).

The isolation of M. johnei from natural material

The fact that the isolation of M. johnei is complicated by the occurrence at the sites from which it is usual to culture M. johnei - alimentary tract and faeces - of non-pathogenic bacteria and fungi, has led many workers to devise methods of controlling these contaminants.

Minett (1942) reviewed the techniques commonly employed in the isolation of the tubercle bacillus and their adaptation to the isolation of M. johnei. He concluded that exposure of the material to 20 per cent. antiformin for 30 minutes was the method of choice.

Taylor (1950) adapted the oxalic acid method of Corper and Uyei (1929-30) for the preliminary treatment of tissue and found it as effective in controlling contaminants, less laborious and less lethal to M. johnei than antiformin. However, he concluded that antiformin was the agent of choice in the treatment of faeces but that there was no completely reliable method, especially if M. johnei was present in small numbers.

Cameron (1956) compared the results of the use of antiformin with the control of contamination obtained by a mixture of 10 per cent. oxalic acid and 0.02 per cent. malachite green and found his method to be effective in the control of faecal contaminants.

Taylor (1950) found that penicillin (100 units per ml. of faecal suspension) was ineffective in controlling contamination even when used in conjunction with other methods. Williams Smith (1953) came to the same conclusion but found that contamination in faecal samples was

lowered by the addition of penicillin to his modification of Dubos' agar and liquid media.

Media containing gentian violet were used by Levi (1948a) to control contamination when culturing faeces. Taylor (1950) used congo red at a concentration of 0.01 per cent. in his modification of Finlayson's medium. While the dye at this concentration was not inhibitory to micro-organisms it stained the colonies of M. johnei so making them more easily seen.

Heating at 64°C for 5 minutes was lethal to M. johnei (Dunkin, 1938).

Vishnevskii et al. (1940) reported that M. johnei survived in bovine faeces and soil for 11 months but not longer than 7 days in urine. Exposure to 10.20 per cent. chlorinated lime, 5 per cent. formalin and 5 per cent. lysol for 10 minutes killed M. johnei. Lovell, Levi and Francis (1944) recovered M. johnei from faeces after 8 months' exposure to atmospheric conditions.

Pathology

The lesions of Johne's disease are found in the wall of the alimentary tract.

Rankin (1958b) found that the most common site of macroscopic changes was the mucous membrane of the terminal few feet of the ileum but that in a small percentage of cases lesions may be found in the caecum, colon, rectum and in the mucosa of the small intestine from the pylorus to the ileo-caecal valve. He found that there was no correlation between the severity of the symptoms and the extent of the lesions in the mucosa. Specific changes were found in the mesenteric lymph nodes

but little reliance for diagnostic purposes could be put on their appearance due to the wide variation in the size of these nodes in the normal animal.

Rankin considered the pathognomonic lesions of the intestinal mucosa to be thickening and corrugation. These corrugations could not be obliterated by gentle stretching. Haemorrhage and ulceration were absent.

Severe microscopic lesions could be present with no macroscopic changes (Rankin, 1958b).

There have been few descriptions of the macroscopic lesions of pre-clinical cases. Hallman and Witter (1933) reported the finding of patchy congestion of the mucosa, petechial haemorrhages and slightly raised glistening areas a few millimetres across. They were of the opinion that extension and fusion of these lesions would produce thickening and corrugation.

Histology

(a) Cattle

McFadyean (1918) described the microscopic changes occurring in Johne's disease. The lesions in the alimentary tract were produced by an infiltration into the mucosa of endothelioid cells and multinucleate giant cells. Initially these cells occurred in isolated aggregations (cf. early tubercle formation) in which case there was little alteration in the villous structure. More advanced cases showed a confluent infiltration with endothelioid cells and a consequent obliteration of the normal architecture of the mucosa. This process extended to involve the submucosa and, in advanced cases, foci of endothelioid cells could

be found in the muscular and serous coats.

Despite the similarity between the early lesions of Johne's disease and early tuberculous lesions, encapsulation, necrosis, caseation and calcification have not been described in Johne's disease of cattle.

The cellular changes in the lymph nodes draining the intestines were similar to those in the mucosa. The lesions were found in the subcapsular area of the cortex of the nodes (McFadyean, 1918).

In the typical advanced case, sections or smears stained by Ziehl-Neelsen's method reveal the presence of acid-fast bacilli in relation to the areas of endothelioid cell infiltration. Hallman and Witter (1933) were of the opinion that the bacilli were always intracellular and that the endothelioid cells were macrophages, contrary to McFadyean's hypothesis that the endothelioid cells were derived from plasma cells.

Lesions have been reported to occur in the liver (Hallman and Witter, 1933; Matthews, 1930) and in the gall bladder, portal lymph nodes and spleen (Borodenok, 1941). There were no indications that lesions in these sites played any part in the pathogenesis of the disease.

M. johnei has been recovered from the mammary gland, uterus, cotyledons and foetuses of cattle suffering from clinical Johne's disease (Doyle, 1954; Lawrence, 1956) but no lesions at these sites have been described.

There has been no report of work done to elucidate the mechanism of production of the symptoms of Johne's disease.

(b) Sheep

There are few descriptions of the pathology of Johne's disease in

the sheep. Stockman (1911) described congestion of the mucosa of the abomasum and thickening of the small intestine. Greatly enlarged mesenteric nodes have been noted (Howarth, 1937). The fat depôts were depleted, the normal whitish fat being replaced by gelatinous, opaque, myxoedematous tissue (McEwen, 1939). As in cattle, thickening was most marked in the ileum. McEwen recorded the finding in smears of very large numbers of acid-fast bacilli in some cases.

Stamp and Watt (1954) described four types of lesions, classified on gross and microscopical findings, but indicated that there was some degree of overlap between the last three groups. In Group 1, the intestinal mucosa was a bright chrome yellow. From this type a pigmented strain of M. johnei could be isolated. The histological picture was similar to that seen in cattle, differing only in the fact that enormous numbers of acid-fast bacilli were present. Group 2 - pigmentation was absent. Thickening of the mucosa was not marked. The epithelioid infiltration was more focal, with leucocytic infiltration at the periphery of the foci. M. johnei might or might not be numerous in the lesions. There was no thickening of the bowel wall in Group 3. On handling, the mucous membrane was friable and cracked on being bent over the finger. Group 4 - the lymphatic vessels of the abdominal cavity were thickened and contained calcified nodules. The lymph nodes were enlarged and showed nodule formation. Histologically Groups 3 and 4 revealed stages towards frank tubercle formation. Giant cells were numerous. In Group 4, necrosis, caseation, calcification and encapsulation occurred.

McFadyean and Sheather (1916) and Pande (1942) noted the presence of caseation in the mesenteric nodes of naturally occurring Johne's disease in goats. Levi (1948b) found this to be a feature of the

disease in artificially infected goats. Otherwise there were no salient differences from the disease as it occurred in cattle.

The experimental infection of cattle, sheep and goats with M. johnei

Bang (1906) was responsible for the first successful transmission of Johne's disease to calves, two of which were infected with 300 gms. of infected node and mucosa given intravenously and intraperitoneally.

Following the isolation of M. johnei in pure culture, Twort and Ingram (1913) reproduced lesions in calves, sheep and goats.

From their experiments and those of McFadyean and Sheather (1916), Taylor (1953) and Rankin (1957) there were indications that cattle of 9 - 12 months of age or older were refractory to the reproduction of clinical disease. The same applied to experimental contact infection (Hagan, 1938), although Rankin has shown little difference between the susceptibility of calves placed in contact with known M. johnei excretors at 1, 3 and 6 months of age. From the results of monthly cultural examination of the faeces of these calves it appeared that positive cultural results depended on establishment of infection in the host and was not the result of the passage of M. johnei through the intestines following ingestion from the environment.

Rankin (1957) found the $I.D._{50}$ for intravenous infection in calves to be 5 mgm. wet weight of pure culture.

McEwen and Samuel (1958) produced clinical disease in 2 and infection in 14 of 16 sheep inoculated intravenously with 2 mgm. of a culture of M. johnei. Hundred-fold dilutions of the original inoculum produced infection in 12, 12 and 5 of three groups of 16 sheep respectively. The distribution of M. johnei in the non-clinically

affected sheep led them to conclude that the most susceptible tissue was the intestine, and that it was probably the site of multiplication in the host.

Watt (1954) described the post-mortem findings, histology and bacteriology of a naturally occurring case of disease in a bullock, due to the pigmented ovine strain. He successfully produced disease in sheep with this strain.

Levi (1948b) found goats susceptible to oral dosage with pure culture and intestinal scrapings. A feature of post-mortem findings was the presence of caseation and calcification. It was established that M. johnei was present in the faeces both in the incubative and clinical phases of the infection.

The pathogenicity of M. johnei for laboratory animals

Many of the experiments designed to reproduce the lesions of Johne's disease in the alimentary tract of laboratory animals have failed.

Intraperitoneal injections of M. johnei usually resulted in nodules in the peritoneum and the presence of M. johnei in the liver and spleen (Twort and Craig, 1913; Boquet, 1925; Hagan and Mansfield, 1930; Mohler, 1939; Sahai, 1940, 1941; Taylor, 1940, 1951; Glover, 1941; Johnson and Cox, 1942; Verlinde and Bekker, 1945). Pathological changes were enhanced when the inoculum of M. johnei was suspended in an adjuvant of light mineral oil. The lesions produced did not differ from those produced by the injection of killed M. johnei and saprophytic acid-fast bacteria (Hagan and Levine, 1932).

Francis (1943), using 13 - 14 day old mice injected with pure cultures on two occasions at 7 day intervals intraperitoneally, was

able to reproduce progressive infections involving the liver, spleen and later the intestines. The cellular reactions in his mice were similar to those in the naturally occurring disease in cattle. Slight infections were obtained in hamsters. Rabbits and guinea-pigs were refractory.

Lominski, Cameron and Roberts (1956) reported the results of the inoculation of 8 strains of M. johnei into W-Swiss mice aged from 7 days to 3 months. The dose used varied between 0.36 mgm. and 2.64 mgm. The time taken for infection to develop varied inversely with the doses, being from 7 to 19 months. Suspending the inoculum in 1.75 per cent. mucin suspension or liquid-treated serum did not increase its pathogenicity. Lesions in the alimentary tract similar to those occurring in cattle were described. About 20 per cent. of the mice treated died from no apparent cause other than extensive intestinal lesions of endothelial cell infiltration. During the course of the infection M. johnei was excreted in the faeces in increasing numbers.

Ford (1957) attempted viable counts on tissues of mice which had been injected intraperitoneally before weaning age with 2 or 4 mgms. moist weight of M. johnei. Pools of livers and spleens were examined at intervals after infection. Irregular results were obtained but in no case was M. johnei recovered later than 50 weeks after inoculation. He found that the administration of cortisone appeared to accelerate the clearance of M. johnei from the tissues.

The age of initial infection had some influence on the success of experimental infection in rabbits (Rankin, 1958c). In 6 adult rabbits injected intravenously with 2.5 mgm. wet weight M. johnei there were no lesions after 2 years, while of 8 rabbits inoculated at

2 weeks of age, intestinal lesions were produced in 5 rabbits, 3 of which were clinically affected.

Splenectomised cats fed infected mucosa developed thickening of the intestinal wall in which epithelioid cells containing acid-fast bacilli were found at post-mortem examination 14 days after dosing (Johnson and Pratt, 1944).

Proliferative lesions containing giant cells and evidence of bacterial multiplication on the chorio-allantoic membranes of chick embryos have been reported following the inoculation of a strain of M. johnei which grew independently of the growth factor. Slight cellular reactions with no evidence of multiplication of the inoculum followed the injection of phlei-dependent strains (Stavitsky and Beck, 1946).

Diagnosis

The microscopical examination of the faeces

Many workers have reported that in smears of faeces stained by the Ziehl-Neelsen method the characteristic clumps of small acid-fast bacilli might be found in the faeces of clinical cases of the disease. In about 30 per cent. of clinical cases could diagnosis be confirmed at any one examination by the finding of these clumps of acid-fast bacteria (Hole, 1953; Doyle, 1956; Rankin, 1958b). The microscopical examination of faeces from clinically normal animals was stated to be of little value (Hole, loc. cit.).

Because of the difficulty of isolating M. johnei from faeces and because of the time taken before a result was obtained, cultural examination of the faeces was considered to be of little value in routine field diagnosis (Levi, 1948a).

Doyle (1956) discounted the value of smears from the rectal mucosa as a diagnostic aid since these were positive in only a small proportion of advanced clinical cases in which the lesions had extended to the rectal wall. The examination of faecal smears from clinically suspicious sheep enabled a positive diagnosis to be made in about 55 per cent. of cases (Williamson and Salisbury, 1952; Chandler, 1958; Armstrong, 1956).

Allergic Tests

Allergens have been used subcutaneously, intravenously, intraocularly and intradermally but because of convenience the last mentioned has been most commonly employed. It has been found that neither johnin nor avian tuberculin could be relied upon for the detection of M. johnei infection or the confirmation of diagnosis in clinical Johne's disease in cattle (McFadyean, Sheather and Edwards, 1916; Rinjard, 1934; Jansen, 1948).

Rankin (1957) found that cattle infected with M. johnei gave an allergic response to the intradermal inoculation of johnin or avian tuberculin at some stage of the infection. Sensitivity tended to wane and persisted at a low level as the disease progressed. He found, too, that the anergic state was most likely to occur when intestinal lesions were extensive and when complement fixing antibodies were demonstrable.

Doyle (1956) stated that good allergic reactions were usual following experimental infection but in natural infections allergic reactions were variable and unreliable and that repeated application of allergic tests in infected herds rarely, if ever, succeeded in

eradicating the disease.

Similar inconsistencies made skin sensitivity tests in sheep of no value (McEwen and Samuel, 1958).

Serology

Agglutination Tests

It has been shown that the direct agglutination test was of little diagnostic significance due to the high titres obtained in non-infected cattle (Twort and Ingram, 1913).

Haemagglutination Tests

Larsen, Porter and Vardaman (1953) used a modification of the Middlebrook-Dubos haemagglutination reaction. They obtained what they considered positive results in 25 of 67 cattle from farms where Johne's disease was known to exist. No post-mortem examination of these animals was made.

McEwen and Samuel (1958) tested sera from experimentally infected sheep with the haemolytic modification of the haemagglutination test of Middlebrook. They found that the test was not of value in detecting infected sheep.

Complement Fixation Tests (C.F.T.)

Twort and Ingram (1913) obtained positive results in 5 of 7 naturally occurring cases and one slightly positive result in 5 pre-clinical artificially infected calves. Bang and Anderson (1913) were of the opinion that the C.F. test was efficient in diagnosing Johne's disease in the absence of tuberculosis.

Such encouraging results were ignored till Hole (1952a and b, 1953), having realised the opportunity arising from the imminent

eradication of tuberculosis, employed his modification of the test on a large scale. The test was extremely valuable in confirming clinical Johne's disease but was of less significance in pre-clinical cases. In 1,510 cases in which acid-fast bacilli were demonstrated, 87 per cent. were positive to the C.F. test, 8 per cent. suspicious and 5 per cent. negative. In 520 negative cases, 29 per cent. were positive to the test (Report, 1956a).

Rankin (1958a) described the test as applied to experimentally infected cattle and noted that the titre could be related to the severity of lesions in the alimentary tract. Pre-clinical cases generally failed to react to the test. Between 10 and 20 per cent. of C.F. tests carried out on adult cattle in herds from which M. johnei had not been isolated over a number of years gave positive results.

Chandler (1956b), using a micro-complement fixation test, obtained results comparable to those of Hole and concluded that the test was a valuable confirmatory one in clinically suspicious cases.

The eradication of Johne's disease from a herd by the elimination of reactors to the C.F. test has been reported (Hole, 1956a).

Sigurdsson et al. (1945) developed a C.F. test utilising as antigen an extract of infected intestinal mucosa from sheep. They noted that the test became positive at an earlier stage of infection than did allergic reactions. Positive tests were obtained in the terminal stage of the disease; non-specific positive results were rare.

Rankin (1957) found that C.F. antibodies were transferred from dam to calf via the colostrum. Whether or not these had a protective effect in the calf was not shown.

Post-mortem Diagnosis

There was no difficulty in confirming the presence of Johne's disease when the typical lesions and acid-fast bacilli were found, but their absence did not exclude the possibility of the presence of microscopic lesions (Rankin, 1958b). The value of histological examination of suspected tissues has been recognised (Hallman and Witter, 1933; Rankin, 1957). Cellular changes may occur without demonstrable acid-fast bacilli (personal observation). The use of fluorescent microscopy for detecting small numbers of acid-fast bacilli in sections and a comparison of the method with cultural results have been reported by Harding (1957) and Polyakova (1957).

Therapy

Early attempts at the treatment of Johne's disease were aimed at the alleviation of clinical signs. Intestinal astringents e.g. ferrous sulphate, copper sulphate, sulphuric acid, tannoform , and opium, produced only a temporary remission of symptoms (McFadyean, Sheather and Edwards, 1915). Sheather (1927) used formalin intravenously. Chaulmoogra oil, used extensively in the treatment of leprosy, was tried unsuccessfully (Downham, 1937; Gunning, 1933).

In vitro studies have shown that M. johnei was sensitive to streptomycin, viomycin and isonicotinic acid hydrazide although the growth of strains tested by Williams Smith (1954) was not inhibited by the last named. M. johnei was found to be resistant to 4:4 diamino-diphenyl sulphone, aminosalicylic acid, promin, stilbamide, pentamide, several thiosemicarbazones, penicillin, chloramphenicol and terramycin (Larsen, Vardaman and Groth, 1950; Larsen and

Vardaman, 1952; Rankin, 1953).

Isonicotinic acid hydrazide alone and in combination with streptomycin failed to prevent the establishment of experimental infection in calves (Rankin, 1955a).

The antibiotics and chemotherapeutic agents which are active against M. johnei in vitro were not of value in the therapy of clinical cases (Rankin, 1953; Larsen and Vardaman, 1953).

Vaccination

As a result of the finding that durable immunity was produced against bovine tuberculosis in cattle vaccinated with an attenuated strain of equine origin suspended in adjuvant as opposed to only transient protection afforded when the vaccine was administered in a soluble base (Vallée, 1924) and, appreciating that the subcutaneous inoculation of M. johnei did not cause progressive infection, Vallée and Rinjard (1926) used a culture of unattenuated living M. johnei suspended in liquid paraffin, olive oil and pumice. Reporting on the field use of this vaccine in 35,000 cattle, Vallée, Rinjard and Vallée (1934) stated that no ill-effects had been noted and that a high degree of protection had been obtained.

Five calves and 18 goats given 30 mgms. of viable M. johnei in liquid paraffin subcutaneously showed no evidence of infection at post-mortem examination $2\frac{1}{2}$ years later (Doyle, 1945).

Using a dose of 5 mg. of M. johnei in adjuvant, a field trial was begun in selected herds in Britain. Initially, vaccination was confined to calves under one month of age, but subsequently some cattle were revaccinated at intervals of 16 - 24 months. Results

for the period 1941-57 showed that the incidence of clinical disease was reduced from 3 - 4 animals per herd to 0.3 animals per herd (Spears, 1959). A further field trial in which only half the calves are to be vaccinated has been initiated.

The objection to the widespread use of vaccination in this country was that the allergy produced by vaccination rendered interpretation of the comparative intradermal tuberculin test difficult (Herbert, Doyle and Paterson, 1959). Vaccinated goats with superimposed tuberculous infection and goats which had received vaccine only reacted to the comparative tuberculin test in such a way that it was not always possible to differentiate the two groups (Doyle, 1953). Similar difficulty in interpretation was reported in vaccinated calves artificially infected with tuberculosis by Ritchie, Robertson and Muir (1952). They found that, in cattle free from infection with M. johnei, vaccination produced sensitivity to both avian and mammalian tuberculins, the avian reading usually being the greater.

Sigurdsson (1956) used heat-killed M. johnei suspended in mineral oil to immunise Icelandic sheep. Five mg. of killed organisms produced a prolonged serological response but did not produce too severe a local reaction. On infected properties half the lambs were vaccinated and the remainder left as controls. He found the incidence of confirmed cases of Johne's disease in 3,273 vaccinated sheep was 1.3 per cent. while in 3,184 control sheep it was 21.1 per cent. In the unvaccinated sheep the highest incidence was during their second and third years.

Hygiene and Management

Little experimental work has been done to elucidate the importance of such factors as nutrition and management in the epidemiology of Johne's disease. Using artificially infected goats, Hirch and Lawrence (1954) observed no difference in cultural results and pathology between these kept on a calcium deficient diet and those on a diet containing adequate calcium. It was noted, however, that calcification of lymph node lesions was a feature in the group receiving adequate dietary calcium.

Jansen (1948) observed that poorly drained pastures and soils deficient in phosphoric acid and calcium carbonate appeared to contribute to a high incidence of clinical disease. He also observed that in Holland infection was most common in regions with soils low in calcium and of low pH. A similar occurrence was observed by Smythe (1935) who noted the absence of clinical disease on the Cornish coast, where the soil contained up to 25 per cent. calcium carbonate and a high incidence in inland areas where soil calcium levels were low.

P A R T I

INVESTIGATIONS INTO JOHNE'S DISEASE IN CATTLE

Introduction

The diagnosis of Johne's disease

This study was carried out to attempt to elucidate some of the problems associated with the accurate diagnosis of Johne's disease with special reference to the significance of the finding of singly disposed acid-fast bacilli in the faeces of clinically suspicious and of apparently healthy cattle.

Section 1

The diagnosis of Johne's disease in wasting cattle

Introduction

The diagnosis of Johne's disease rests on clinical findings with confirmatory evidence obtained by microscopical examination of the faeces or by the use of the complement fixation test. It was decided to investigate the significance of isolated acid-fast bacilli in the faeces by correlating their presence there with the results of a detailed post-mortem examination of infected and non-infected cattle.

Review of the Literature

At present it is accepted that only one third of clinical cases of the disease can be confirmed at one examination by the finding of the pathognomonic clumps of acid-fast bacteria in the faeces (Rankin, 1958b). Surveys of the incidence of acid-fasts in faeces samples from clinically suspect cattle have revealed that 25 - 30 per cent. of samples showed clumps of acid-fast bacteria morphologically resembling M. johnei, while 15 per cent. of samples were classified as suspicious because of the finding of isolated acid-fast bacilli (Doyle and Spears, 1951; Soltys, 1951). There was no attempted correlation with post-mortem findings in these surveys, nor was it probable that all the samples came from cases of Johne's disease.

The significance of the finding of single acid-fast bacilli resembling M. johnei has long been in dispute. Hole (1956b) expressed doubt as to whether the presence of single M. johnei in the tissues in the absence of a demonstrable tissue reaction was evidence of disease, thereby implying that the finding of single bacilli in the faeces was of no value.

It has been stated that saprophytic acid-fast bacilli found in the faeces are indistinguishable from M. johnei (Chandler, 1958; Levi, 1948a).

A positive diagnosis was warranted only when clumps of acid-fast bacilli were found in the faeces (Anon, 1956; Levi, 1948; Doyle, 1956).

Levi (1948a) correlated microscopical and cultural findings in faeces samples from clinically-suspicious cases of Johne's disease and concluded that the finding of isolated acid-fast bacilli did not justify a diagnosis of Johne's disease. Taking into account the difficulty of isolating M. johnei from faeces, it is significant that M. johnei was isolated from 50 per cent. of samples in which isolated acid-fast bacilli were found microscopically.

Rankin (1958a) has reviewed and augmented the present knowledge of the complement fixation test in the diagnosis of Johne's disease in cattle. His summary indicated that the test was satisfactory for confirmation of clinical disease but in non-infected cases the test was non-specific in that 10 per cent. of positive reactions were obtained in two herds which he considered likely to be free from infection with M. johnei. The test failed to detect pre-clinically infected animals. It was thought that examination of the complement fixation test in the light of the results of extensive post-mortem examinations might explain these apparently anomalous results.

Materials and Methods

Cattle. The material examined was obtained from cattle admitted to Glasgow University Veterinary Hospital. These were from two main sources, cattle which had been culled from herds in Southwest

Scotland because of productive inefficiency, and confirmed clinical cases of Johne's disease from farms where epidemiological investigations were being carried out. All were in poor clinical condition i.e. in a condition in which it would be justified to suspect the presence of Johne's disease.

They were kept in the hospital for periods of between two days and one month depending on their clinical condition. All were housed in stalls or loose boxes and fed a diet of concentrates, hay and water. On admission a complete clinical examination of each case was undertaken. Further clinical observations were made daily, special attention being paid to the character of the faeces. Blood samples were collected for routine haematology and for the complement fixation test for Johne's disease. Faeces samples were collected at frequent intervals. An extensive post-mortem examination was carried out on each case and the undernoted procedure with reference to the present investigation adopted.

The small and large intestines were removed from the abdomen. The ileo-caecal lymph nodes were collected in sterile universal containers. The intestines were separated from their mesenteric attachments and their contents expelled. The intestinal wall was opened to expose the mucous surface which was examined for abnormalities. Samples of mucous membrane were removed for cultural examination. Tissues from the mesenteric nodes and from the mucosa were fixed in corrosive formol for histological examination.

The Complement Fixation Test

The test used was that described by Hole (1952a and b), using

antigen and standards supplied by him. Sera which gave 25 per cent. haemolysis or less were regarded as positive to the test. The test was carried out by Mr. M. P. Cunningham, M.R.C.V.S., of Glasgow University Veterinary Hospital, who also provided any histological results used in this thesis.

The Microscopical Examination of Faeces

Samples of rectal faeces were collected in waxed cartons and smears made on the day of collection. The faeces were prepared for microscopical examination by the method described by Cunningham and Gilmour (1959). Approximately 0.5 gm. of faeces was transferred to a glass slide and sufficient sterile distilled water added to reduce the faeces to a semi-fluid state. A straight edge was produced by aligning the faeces along the interface between the slide and another held lengthwise at right angles to it. The smears were dried slowly. Excess faecal débris was removed, care being taken to leave the edge for examination intact. Staining was carried out by the Ziehl-Neelsen method:- Ziehl-Neelsen carbol fuchsin for 10 minutes with heating to steaming twice within that period; decolourisation with acid-alcohol (70 per cent. alcohol containing 3 per cent. hydrochloric acid) for 30 minutes; washing in tap water followed by counterstaining for ½ minute with polychromatic methylene blue; the slides were washed in tap water and blotted dry.

It was found that decolourising with acid-alcohol for 30 minutes was effective in reducing the amount of acid-fast débris in the preparation. Pure cultures of M. johnei retained their acid-fastness for much longer, while saprophytic acid-fast bacilli were less

resistant to the effect of acid-alcohol (unpublished observations).

Every field in 3 cms. of straight edge was examined under the 1/12" oil-immersion objective. The number of clumps (3 or more acid-fast bacilli in close proximity) of acid-fast bacilli and single, isolated, acid-fast bacilli measuring 0.5μ x 1-2μ with rounded ends and parallel or slightly bulging sides and staining a bright metallic red was noted. Acid-fast objects not fulfilling these criteria were ignored. By examining an approximately constant number of microscope fields per sample (CHCA 500), it was possible to obtain a rough comparison between the number of acid-fast bacteria in the faeces from time to time.

The isolation of M. johnei from tissues

Tissues for cultural examination were collected in sterile universal bottles. Whenever possible the material was cultured on the same day as the animal was killed: otherwise it was stored at -10°C till required.

A pooled sample of ileo-caecal lymph nodes and three or four pieces of intestinal mucosa taken from the terminal ileum or from areas where lesions were present was examined. The lymph nodes were freed from fat, flamed in a bunsen flame for a few seconds and macerated in a sterile petri dish. The mucous membrane was washed in water to remove gross débris and separated from the muscular layers. About 2 gm. of the macerated material and mucosal scrapings were transferred to a sterile Griffith tube and ground in 10 ml. of 0.85 per cent. sterile saline solution. The resulting suspension was transferred to a sterile universal container, an equal volume of

10 per cent. oxalic acid solution added (Corper and Uyei, 1929), and the container stood in a water bath at 37°C for 30 minutes. The bottle was shaken frequently during incubation.

The suspension was centrifuged at 3,000 r.p.m. for 30 minutes. The supernatant fluid was discarded. Several platinum-loopfuls of the sediment were spread thinly over the surface of three slopes of Lowenstein-Jensen medium modified with 1 per cent. w/v heat-killed M. phlei (Cameron, 1956). The cultures were incubated aerobically at 37°C for twelve weeks, examined, and incubated for a further four weeks when they were re-examined and discarded. The slopes were examined for small, raised, greyish-white, rough colonies of acid-fast bacilli. When such colonies were seen they were subcultured onto Lowenstein-Jensen medium and 4 per cent. glycerol agar which were incubated at 37°C for twelve weeks and examined frequently. Colonies of acid-fast bacilli which morphologically resembled M. johnei and which required the growth factor provided by M. phlei were regarded as those of M. johnei. Williams Smith (1954) has discussed the identification of M. johnei on cultural grounds alone. He concluded it was justified, as Taylor (1952) produced clinical disease in calves following the inoculation of a random selection of strains recovered during abattoir surveys.

Biological tests and the examination of cultural characteristics of strains of acid-fast bacteria which did not require the growth factor were carried out and will be reported on in full in Part III.

The isolation of M. johnei from faeces

Two gm. of freshly-collected rectal faeces were shaken up with

20 ml. of 0.85 per cent. sterile saline solution and filtered through two layers of sterile muslin. The filtrate was centrifuged at 3,000 r.p.m. for 30 minutes, the supernatant discarded, the sediment transferred to a sterile Griffith tube and ground with 10 ml. of 0.85 per cent. sterile saline solution. This suspension was allowed to stand for 20 minutes and 10 ml. of the supernatant layer of the suspension transferred to a universal container. An equal volume of 10 per cent. oxalic acid containing 0.02 per cent. malachite green was added (Cameron, 1956). The mixture was incubated in a water bath at 37°C for 30 minutes and centrifuged thereafter at 3,000 r.p.m. for 30 minutes. The sediment was sown onto the surface of six slopes of modified Lowenstein-Jensen medium and incubated aerobically at 37°C for six months.

Statistical Analysis of Results

To determine if there was a significant association between variables the significance of X^2 was determined by consulting Fisher's[*] tables for n = 1 where "n" is the number of degree of freedom. If X^2 was equal to or greater than 3.841 the association was significant.

Results

The results which are presented in Tables 1 - 5 are described under the following headings.

---

[*] Ref. Statistical Methods for Research Workers. R. A. Fisher. 10th Ed. Oliver & Boyd, Edinburgh. 1948.

1. Case number.

2. Breed. Where cross-bred animals were encountered the case was assigned to the predominant breed of the animal.

3. Age. The age of each animal was estimated by its dental formula up to four years of age. If older than four years the age was estimated by reference to the horn structure, if any. In some cases the age was obtained from the history of the case. An animal was classified as aged if over four years and if no indication of its age was available.

4. Diarrhoea. The number of days on which the presence of diarrhoea was noted is given in the numerator. The number of days on which observations of the faeces were made is shown in the denominator.

5. Examination of faeces smears. The number of smears in which acid-fast bacteria were seen is given in the numerator. The number of smears examined is given in the denominator.

6. Complement fixation test results. These are listed as either positive or negative according to the interpretation of the test already stated.

7. Gross lesions. These are listed as present or absent depending on the finding or failure to find the specific changes in the intestinal wall with the naked eye.

8. Faeces culture results. These were classified as positive if M. johnei was recovered, negative if M. johnei not recovered, and contaminated if all the tubes inoculated were contaminated.

The abbreviations used in Tables 1 - 5 are shown on page 48.

TABLE 1

Cases in which M. johnei was recovered from the ileo-caecal nodes and which had clumps of acid-fast bacilli in the faeces (25 cases)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
U2	A	4	13/21	11/20	+	+	
R13	A	2	1/1	1/1	+	+	C
R4	A	aged	3/4	3/3	+	-	
C21	A.A.	aged	0/4	4/4	+	+	A.T.
C20	A	3	7/8	5/8	+	+	+
C5	A	2½	-/1	1/1	-	-	
C3	A	2½	3/5	6/6	+	+	
C2	A	aged	8/17	15/18	+	+	
158	A	4½	12/12	11/12	+	+	+
148	B.F.	2½	-/2	2/2	+	+	
142	A	4	2/2	1/1	+	+	+
127	H	2½	1/1	1/1	+	-	+
126	A	6	3/3	2/2	+	+	+
121	A	aged	0/2	2/2	+	+	+
107	A	aged	2/2	2/2	+	+	+
106	A	aged	3/3	3/3	+	-	+
96	A	aged	1/1	1/1	+	+	
81	A	aged	2/2	2/2	+	+	+
80	A	aged	1/1	1/1	+	+	
78	A	aged	2/2	2/2	+	+	
65	S	aged	1/1	1/1	+	+	
35	G	13	1/11	9/11	+	+	
31	G	3½	1/1	1/1	+	+	
17	A	aged	2/12	12/12	+	+	
23	G	aged	3/7	7/7	+	+	

TABLE 2

Cases in which M. johnei was recovered from the ileo-caecal nodes and which had single acid-fast bacilli in the faeces (30 cases)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
W11	W	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
R16	A	3	$\frac{-}{1}$	$\frac{1}{1}$		+	
G30	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
G22	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
G11	A	aged	$\frac{-}{1}$	$\frac{1}{1}$	+	+	
C17	A	3	$\frac{1}{1}$	$\frac{1}{1}$	+	+	+
C12	A	2	$\frac{-}{1}$	$\frac{1}{1}$		-	
C11	Gu.	aged	$\frac{-}{3}$	$\frac{2}{3}$	+	-	
C10	A	2	$\frac{2}{2}$	$\frac{2}{2}$	-	+	
C4	S	aged	$\frac{1}{1}$	$\frac{2}{2}$		-	
C1	J	aged	$\frac{-}{5}$	$\frac{1}{1}$	-	-	
155	A	aged	$\frac{1}{2}$	$\frac{2}{2}$	+	+	
153	A	aged	$\frac{1}{3}$	$\frac{1}{3}$	+	-	
147	G	aged	$\frac{1}{1}$	$\frac{1}{1}$	+	+	
131	A	3	$\frac{4}{4}$	$\frac{2}{2}$	+	+	+
125	S	aged	$\frac{-}{1}$	$\frac{1}{1}$	+	-	-
111	A	aged	$\frac{-}{1}$	$\frac{1}{1}$	-	-	-
105	A	aged	$\frac{2}{2}$	$\frac{1}{2}$	+	-	-
101	A	aged	$\frac{2}{2}$	$\frac{2}{2}$	+	+	-
94	A	aged	$\frac{-}{2}$	$\frac{1}{2}$	-	-	
89	J	aged	$\frac{1}{2}$	$\frac{1}{2}$	+	+	
87	A	aged	$\frac{-}{2}$	$\frac{2}{2}$	-	-	

TABLE 2 (contd.)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
79	A	aged	$\frac{2}{2}$	$\frac{2}{2}$	+	+	
67	A	aged	$\frac{=}{1}$	$\frac{1}{1}$	-	-	
66	A	aged	$\frac{=}{1}$	$\frac{1}{1}$	-	-	
60	A	aged	$\frac{1}{1}$	$\frac{1}{1}$		+	
59	A	aged	$\frac{1}{1}$	$\frac{1}{1}$		+	
54	A	aged	$\frac{1}{1}$	$\frac{1}{1}$	+	+	
42	A	aged	$\frac{5}{7}$	$\frac{1}{7}$	+	+	
32	A	aged	$\frac{7}{7}$	$\frac{3}{7}$	+	+	

TABLE 3

Cases in which M. johnei was recovered from the ileo-caecal nodes and which had no acid-fast bacilli in the faeces (11 cases)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesioms	Faeces culture
G20	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G19	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G18	A	2	$\frac{-}{1}$	$\frac{-}{1}$		-	
C18	A	3	$\frac{1}{1}$	$\frac{-}{1}$	+	-	+
139	A	aged	$\frac{2}{2}$	$\frac{-}{2}$	+	$\frac{+}{-}$	-
130	A	2	$\frac{2}{3}$	$\frac{-}{1}$	-	-	
129	A	aged	$\frac{1}{4}$	$\frac{-}{3}$	+	-	
112	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	+	-	-
91	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
75	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	-	-	
27	A	aged	$\frac{1}{1}$	$\frac{-}{1}$	+	+	

TABLE 4

Cases in which M. johnei was not recovered from the ileo-caecal nodes but which had single acid-fast bacilli in the faeces
(19 cases)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
W12	W	$2\frac{1}{2}$	$\frac{-}{1}$	$\frac{1}{1}$		-	
U10	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
U6	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
U7	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
U5	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
G28	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
G27	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	-
G24	A	aged	$\frac{-}{1}$	$\frac{1}{1}$	+	-	-
122	A	2	$\frac{-}{1}$	$\frac{1}{1}$	-	-	
103	A	aged	$\frac{2}{2}$	$\frac{2}{2}$	-	-	
95	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
92	A	aged	$\frac{-}{2}$	$\frac{1}{3}$	-	-	
86	A	aged	$\frac{1}{1}$	$\frac{1}{1}$	-	-	
71	A	aged	$\frac{2}{3}$	$\frac{2}{3}$	-	-	
68	A	aged	$\frac{1}{1}$	$\frac{1}{1}$	+	-	
64	A	aged	$\frac{-}{1}$	$\frac{1}{1}$		-	
49	A	aged	$\frac{-}{1}$	$\frac{1}{1}$	+	+	
48	A	aged	$\frac{-}{2}$	$\frac{1}{2}$	+	-	
1	A	aged	$\frac{3}{11}$	$\frac{3}{11}$	+	-	

TABLE 5

Cases in which M. johnei was not recovered from the ileo-caecal nodes and in which no acid-fast bacilli were found in the faeces
(122 cases)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
W13	W	$2\frac{1}{2}$	$\frac{-}{1}$	$\frac{-}{1}$		-	
G25	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G26	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G29	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G31	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G32	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
156	B.F.	$11\frac{1}{2}$	$\frac{-}{1}$	$\frac{-}{1}$		-	
157	A.A.	aged	$\frac{-}{1}$	$\frac{-}{1}$	-	-	
C6	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	+	-	
C7	A	$2\frac{1}{2}$	$\frac{-}{1}$	$\frac{-}{1}$		-	
C13	A	$2\frac{1}{2}$	$\frac{1}{1}$	$\frac{-}{1}$		-	
C14	A	aged	$\frac{1}{3}$	$\frac{-}{3}$		-	-
C15	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	-
C16	A	aged	$\frac{-}{3}$	$\frac{-}{3}$		-	
C19	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	-	-	-
G13	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	+	-	
G12	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	+	-	
G14	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	+	-	
G15	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	+	-	
G16	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G23	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G21	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	

TABLE 5 (contd.)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
G17	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G33	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G34	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G35	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
G36	A	aged	$\frac{-}{2}$	$\frac{-}{2}$		-	
149	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
151	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	+	-	
152	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	+	-	
63	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
33	A	aged	$\frac{-}{5}$	$\frac{-}{5}$	-	-	
30	A	aged	$\frac{-}{8}$	$\frac{-}{8}$	-	-	
29	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	-	-	
28	A	aged	$\frac{4}{4}$	$\frac{-}{4}$	+	-	
46	A	aged	$\frac{1}{2}$	$\frac{-}{2}$	-	-	
45	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	-	-	
44	A	aged	$\frac{2}{4}$	$\frac{-}{4}$	-	-	
51	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	-	-	
50	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	+	-	
61	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
62	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
59	B.F.	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
58	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
57	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	-	-	
56	A	6	$\frac{-}{1}$	$\frac{-}{1}$	-	-	

TABLE 5 (contd.)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
55	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	-	-	
53	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	+	-	
52	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	-	-	
43	A	aged	$\frac{-}{6}$	$\frac{-}{6}$	+	-	
41	A	7	$\frac{-}{1}$	$\frac{-}{1}$	+	-	
37	B.F.	aged	$\frac{1}{7}$	$\frac{-}{7}$	-	-	
38	A	aged	$\frac{-}{7}$	$\frac{-}{7}$	+	-	
36	A	aged	$\frac{-}{7}$	$\frac{-}{7}$	-	-	
34	A	aged	$\frac{8}{8}$	$\frac{-}{8}$	-	-	
97	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
74	A	aged	$\frac{-}{2}$	$\frac{-}{2}$		-	
73	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
76	A	aged	$\frac{-}{3}$	$\frac{-}{3}$	-	-	
77	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	-	-	
82	A	aged	$\frac{1}{2}$	$\frac{-}{2}$	-	-	
83	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	+	-	
84	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	-	-	
85	A	aged	$\frac{-}{2}$	$\frac{-}{2}$	-	-	
88	A	aged	$\frac{-}{1}$	$\frac{-}{2}$		-	
90	A	aged	$\frac{3}{3}$	$\frac{-}{3}$	-	-	
93	A	aged	$\frac{-}{3}$	$\frac{-}{3}$	-	-	
98	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
99	A	aged	$\frac{1}{1}$	$\frac{-}{1}$		-	
100	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	

TABLE 5 (contd.)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
102	A	aged	++	++	+	-	
104	A	aged	++	++	-	-	
108	G	aged	++	++	-	-	-
109	A.A.	aged	++	++	-	-	-
110	A	aged	+	++	+	-	-
113	A	aged	++	++	-	-	-
114	A	aged	++	++	-	-	-
115	A	aged	++	++	-	-	-
70	A	aged	++	++	-	-	
69	A	3	++	++	-	-	
145	A	aged	++	++	+	-	
146	A	aged	++	++	+	-	
116	A	aged	++	++	-	-	
117	A	aged	++	++	+	-	
118	A	aged	++	++	+	-	
119	A	8	++	++	+	-	-
120	A	aged	++	++		-	-
123	A	$2\frac{1}{2}$	++	++	+	-	-
124	A	aged	++	++	+	-	-
128	A	$2\frac{1}{2}$	++	++	-	-	-
133	A.A.	aged	++	++	-	-	-
134	A	aged	++	++	-	-	
135	A	aged	++	++	-	-	
136	A	aged	++	++	-	-	

TABLE 5 (contd.)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
137	A	3	$\frac{-}{1}$	$\frac{-}{2}$	+	-	
138	A	$3\frac{1}{4}$	$\frac{-}{1}$	$\frac{-}{2}$	+	-	
140	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	-
143	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
141	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
144	B.F.	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	-
200	G	$3\frac{1}{4}$	$\frac{1}{1}$	$\frac{-}{1}$		+	-
201	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
202	A	5	$\frac{-}{1}$	$\frac{-}{1}$		-	
R6	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
R5	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
R3	A.A.	aged	$\frac{2}{2}$	$\frac{-}{2}$		-	
R2	S	aged	$\frac{2}{8}$	$\frac{-}{8}$	-	-	
R1	A	13	$\frac{1}{3}$	$\frac{-}{3}$		-	
203	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
204	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
U3	A	2	$\frac{-}{1}$	$\frac{-}{1}$	-	-	
U8	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
U9	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
U11	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
U12	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
U13	B.F.	$2\frac{1}{2}$	$\frac{-}{1}$	$\frac{-}{2}$		-	-
U92	A	aged	$\frac{-}{1}$	$\frac{-}{1}$	+	+	
U93	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	

TABLE 5 (contd.)

Case no.	Breed	Age in years	Diarrhoea	Faeces exam.	C.F.T.	Gross lesions	Faeces culture
U94	B.F.	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
W14	W	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	
W15	W	aged	$\frac{-}{2}$	$\frac{-}{2}$	+	-	-
205	A	aged	$\frac{-}{1}$	$\frac{-}{1}$		-	

Abbreviations used in Tables 1 - 5

+ = present or positive

- = absent or negative

no result = examination or test not done

A.T. = avian tubercle bacilli recovered

C = contaminated

A = Ayrshire

Gu. = Guernsey

S = Shorthorn

B.F. = British Friesian

H = Highland

G = Galloway

A.A. = Aberdeen-Angus

J = Jersey

W = Wild White

In Tables 1 - 5 are presented data obtained from ante- and post-mortem examinations of material from 207 wasting cattle. The results are analysed in Tables 6 - 19.

The isolation of M. johnei from the tissues of wasting cattle

Table 6. The isolation of M. johnei from wasting cattle

Total number of cattle examined	= 207
Number of cases from which M. johnei isolated	= 66 (31.8%)
Number of cases from which M. johnei not isolated	= 141 (68.2%)

From Table 6 it will be seen that M. johnei was recovered from the pooled samples of ileo-caecal node and terminal ileal mucosa in 66 (31.8%) of 207 cattle examined. M. tuberculosis var. avium was isolated from the tissues of one case; an incidence of 0.48 per cent.

The microscopical examination of faecal smears from 66 cases from whose tissues M. johnei was isolated

Table 7. The microscopical examination of faecal smears from 66 cases from whose tissues M. johnei was isolated

No. of cases positive on culture	= 66
No. of cases in which clumps of acid-fast bacilli seen in faeces	= 25 (37.7%)
No. of cases in which only single acid-fast bacilli seen in faeces	= 30 (45.4%)
No. of cases in which no acid-fast bacilli seen in faeces	= 11 (16.9%)

From Table 7 it will be seen that in 25 of the 66 culturally positive cases clumps of acid-fast bacilli were found on at least one

occasion on microscopical examination of the faeces (Table 1). There were 30 cases from the tissues of which M. johnei was cultured. On examination of the faeces of these, there were found only isolated acid-fast bacilli morphologically resembling M. johnei and at no time clumps of acid-fast bacilli. These cases are listed in Table 2. Of the 66 cases which yielded isolates of M. johnei from their tissues there were 11 in the faeces of which no acid-fast bacilli could be found on repeated examination.

The microscopical examination of faecal smears from 141 cases from the tissues of which M. johnei was not isolated

Table 8. The microscopical examination of faecal smears from 141 cases from the tissues of which M. johnei was not isolated

No. of cases negative on culture	= 141
No. of cases in which no acid-fast bacilli seen in the faeces	= 122
No. of cases in which acid-fast bacilli seen in the faeces	= 19

The results of microscopical examination of faecal smears are given in Table 8. M. johnei was not isolated from 141 of the 207 wasting cattle examined. Shown in Table 4 are the 19 cases from which M. johnei was not obtained in culture but in whose faeces acid-fast bacilli (in all instances, isolated ones) were found on microscopical examination.

One hundred and twenty-two cases were negative on cultural examination and on microscopical examination of the faeces (Table 5). Some of the conditions to which the clinical status of these animals might be attributed included chronic mastitis, chronic pneumonia, mucosal

disease, traumatic gastritis and peritonitis, nephritis, fascioliasis and lameness.

The significance of the finding of single acid-fast bacilli resembling M. johnei on microscopical examination of faeces from clinically suspicious cattle

Table 9. The correlation between the finding of single acid-fast bacilli in the faeces and the recovery of M. johnei from the tissues of clinically suspicious cattle

No. of cases from which M. johnei was isolated	= 41
No. of cases from which M. johnei was not isolated	= 141
No. of cases in whose faeces single acid-fast bacilli present	= 49
No. of cases in whose faeces single acid-fast bacilli not present	= 133

Faeces result	Culture result	
	+	-
+	30	19
-	11	122

$X^2 = 57.51$

As is shown in Table 9, M. johnei was recovered from the pooled ileo-caecal lymph node and intestinal mucosa of 41 cases and was not recovered from the remaining 141 cases. Single acid-fast bacilli were found in at least one of successive faecal examinations in 49 animals and were not found in 133. If clumps of three or more acid-fast bacilli were found on any occasion the case was not used in

this investigation. Of the 41 culturally positive cases, 30 were faeces positive and 11 negative, while in 141 culturally negative cases 19 were faeces positive and 122 faeces negative.

As the value obtained for X^2 was greater than 3.841, the critical value of X^2 for one degree of freedom, it was concluded that there was a significant relationship between the variables i.e. the result of faeces examination and cultural examination. Therefore, in the cattle examined, the finding of single acid-fast bacilli in the faeces indicated the presence of M. johnei infection.

The microscopical examination of faeces as a diagnostic method in wasting cattle

Table 10. The examination of the significance of the presence of acid-fast bacilli in the faeces of clinically suspicious cattle

No. of cases from which M. johnei isolated on culture	= 66
No. of cases from which M. johnei not isolated on culture	= 141
No. of cases in which faeces test positive	= 74
No. of cases in which faeces test negative	= 133

Faeces test	Culture result	
	+	-
+	55	19
-	11	122

$X^2 = 141.4$

As a result of the finding that single acid-fast bacilli in the faeces of clinically suspicious cattle were of diagnostic significance in the detection of M. johnei infection, examination of the faeces was regarded as positive if acid-fast bacilli, either singly or in clumps, were found in the faeces. Examinations were made on 207 cattle. The findings are shown in Table 10. M. johnei was isolated in culture of the tissues of 66 cases, of which 55 were faeces positive and 11 negative. Nineteen of the 141 culturally negative cases were faeces positive.

As the value obtained for X^2 was greater than 3.841, the critical value of X^2 for one degree of freedom, it was concluded that the finding of acid-fast bacilli singly or in clumps, on microscopical examination of the faeces was diagnostic of M. johnei infection in the wasting cattle examined.

The accuracy of the microscopical examination of faeces as a diagnostic test for Johne's disease

Table 11. Microscopical examination of faeces smears from 207 wasting cattle

Microscopic examination of faeces	Culture +ve				Culture -ve		
	Faeces +ve (clumps)	Faeces +ve (singles)	Faeces -ve	Total	Faeces +ve	Faeces -ve	Total
No. of examinations	124	54	16	194	35	217	252
No. +ve	106 (85%)	40 (71.4%)	-	146 (75.2%)	23 (66%)	-	23 (9%)

The results of the microscopical examination of faeces are shown in Table 11. Of 194 examinations on the faeces of 66 M. johnei

infected cattle, 146 were positive i.e. either clumps or isolated acid-fast bacilli were found on microscopical examination of faecal smears. Of 252 samples from non-infected wasting cattle, 23 (9%) were positive.

It was therefore possible to diagnose M. johnei infection in 75.2% of the smears from infected wasting cattle. Single acid-fast bacilli were found in 9% of smears from culturally negative cattle. This is a measure of the non-specificity of the test under the circumstances described.

The complement fixation test as a diagnostic test in cattle suspected on clinical grounds of having Johne's disease

Table 12. The complement fixation test on sera from 130 wasting cattle

C.F.T.	Culture +ve				Culture -ve		
	Faeces +ve (clumps)	Faeces +ve (singles)	Faeces -ve	Total	Faeces +ve	Faeces -ve	Total
+ve	24 (96%)	15 (68.5%)	5 (72%)	44 (81.5%)	5 (50%)	27 (40.9%)	32 (42.1%)
-ve	1 (4%)	7 (31.5%)	2 (28%)	10 (18.5%)	5 (50%)	39 (59.1%)	44 (59.9%)

Table 12a. The significance of the C.F.T. as a diagnostic test in clinically suspicious cattle (130 cases)

No. of cases from which M. johnei recovered in culture	= 54
No. of cases from which M. johnei not recovered in culture	= 76
No. of cases in which C.F.T. +ve	= 76
No. of cases in which C.F.T. -ve	= 54

C.F.T. result	Culture result	
	+	-
+	44	32
-	10	44

$X^2 = 20.15$

The findings are given in Tables 12 and 12a. Of 54 cases from which M. johnei was recovered in culture 44 were positive to the test and 10 were negative. Of 76 culturally negative cases 32 were positive to the test while 44 were negative. As the value for X^2 (20.15) was greater than 3.841, the critical value for one degree of freedom it is concluded that there is a significant relationship between the complement fixation test results and the recovery of M. johnei from the tissues of the wasting cattle examined.

Forty-four (81.5%) of the 54 culturally positive cases were positive to the complement fixation test, while of 25 cases which had clumps of acid-fast bacilli in their faeces 24 (96%) gave a positive C.F.T. The test is therefore an accurate one for determining the presence of M. johnei infection in wasting cattle. On the other hand, the test was positive in 32 (42.1%) of 76 wasting cattle from which M. johnei was not recovered.

The age of cattle exhibiting wasting due to M. johnei infection

Table 13. The incidence of M. johnei infection in relation to the age of wasting cattle

Age	Culture +ve				Culture -ve		
	Faeces +ve (clumps)	Faeces +ve (singles)	Faeces -ve	Total	Faeces +ve	Faeces -ve	Total
2 - 3	5 (20%)	2 (6.7%)	2 (18%)	9 (13.6%)	2 (10.5%)	9 (7.4%)	11 (7.8%)
3 - 4	2 (8%)	3 (10%)	1 (9%)	6 (9.1%)	-	2 (1.6%)	2 (1.4%)
over 4 (aged)	18 (72%)	25 (83.3%)	8 (73%)	51 (77.3%)	17 (89.5%)	111 (91%)	128 (90.8%)

The distribution of M. johnei infection in relation to age is shown in Table 13. With reference to the cattle from which M. johnei was recovered in culture, 51 (77.3%) were over four years of age, six were between three and four and nine were between two and three years of age. Of the cattle which were culturally negative, 128 (90.8%) were over four years of age, two were between three and four and 11 were between two and three years of age.

To see whether there was a significant relationship between age and the presence or absence of M. johnei infection as determined by cultural methods, the incidence of infection in cattle over and under four years of age was compared statistically.

Table 14. Statistical comparison of the incidence of M. johnei infection in cattle under and over 4 years of age

	Culture result	
	M. johnei recovered	M. johnei not recovered
over 4 years old	51	128
under 4 years old	15	13

$X^2 = 7.01$

Since X^2 = 7.01 and is greater than 3.841, the critical significance value when n = 1, it is concluded that there is a significant relationship between the variables. For the wasting cattle examined, M. johnei infection was more likely to occur in cattle under four years of age. Wasting cattle over four years of age were more likely to be free from M. johnei infection.

The incidence of diarrhoea in wasting cattle infected with M. johnei and a comparison of the incidence with that in non-infected cattle wasting due to other causes

Table 15. The incidence of diarrhoea in relation to M. johnei infection in wasting cattle

Diarrhoea	Culture +ve				Culture -ve		
	Faeces +ve (clumps)	Faeces +ve (singles)	Faeces -ve	Total	Faeces +ve	Faeces -ve	Total
Present	21 (84%)	16 (53.3%)	5 (45.5%)	42 (63.6%)	5 (26.3%)	15 (4.5%)	20 (14.5%)
Absent	4 (16%)	14 (46.7%)	6 (64.6%)	24 (36.4%)	14 (73.7%)	107 (95.5%)	121 (85.5%)

The results are shown in Table 15. Of 66 wasting cattle from which M. johnei was isolated in culture, diarrhoea was present on at least one occasion in 42 (63.6%) cases. Where clumps of acid-fast bacilli were found in the faeces on microscopical examination of faecal smears, diarrhoea was noted at one or more observations in 21 (84%) of these cases: diarrhoea was present in about 50 per cent. of those whose faeces showed only single acid-fast bacilli on microscopical examination of smears. Of 141 wasting cattle from which M. johnei was not

isolated in culture diarrhoea was present on at least one observation of the varying number made in 20 (14.5%) cases.

Table 16. Statistical comparison of the incidence of M. johnei infection and the incidence of diarrhoea in 207 wasting cattle

		Culture result	
		M. johnei recovered	M. johnei not recovered
Diarrhoea	Present	42	20
	Absent	24	121

$X^2 = 50.82$

Since the value for X^2 was significant for n = 1, it is concluded that, for the cattle examined, diarrhoea was more likely to occur in the M. johnei infected cattle than in cattle from which M. johnei was not isolated in culture.

The presence of macroscopic lesions of Johne's disease in the alimentary tract of 207 wasting cattle

Table 17. The incidence of macroscopic lesions in the intestines of wasting cattle

Macroscopic lesions	Culture +ve				Culture -ve		
	Faeces +ve (clumps)	Faeces +ve (singles)	Faeces* -ve	Total	Faeces +ve	Faeces -ve	Total
Present	21 (84%)	15 (50%)	1 (11%)	37 (56.9%)	1 (5.5%)	2 (1.5%)	3 (2.2%)
Absent	4 (16%)	15 (50%)	9 (89%)	28 (43.1%)	18 (94.5%)	120 (98.4%)	138 (97.8%)

* The changes in the intestinal wall in one case in this series were described as "doubtful".

The findings are tabulated in Table 17. Macroscopic lesions of Johne's disease were present in the alimentary tract of 37 (56.9%) of the wasting cattle from which M. johnei was isolated in culture. In those cases where clumps of acid-fast bacilli, single acid-fast bacilli and no acid-fast bacilli were found on microscopical examination of the faeces, 84%, 50% and 11% respectively had visible lesions of the intestinal wall. Of 141 cattle from which M. johnei was not isolated in culture of the ileo-caecal nodes and intestines, macroscopic lesions which were thought to be typical of those of Johne's disease were found on three occasions.

The recovery of M. johnei from foetuses

Table 18. The recovery of M. johnei in culture from foetuses from clinical cases of Johne's disease

Isolation from foetuses	Dam's faeces +ve clumps	Dam's faeces +ve singles	Total
No. examined	7	2	9
No. +ve	3	1	4

The results are shown in Table 18. The foetuses were from nine pregnant cows which were clinical cases of Johne's disease. M. johnei was isolated from four foetuses, three of which were from dams whose faeces contained clumps of acid-fast bacilli and one of which was from a dam in the faeces of which there were only single acid-fast bacilli as determined by microscopical examination of smears.

The isolation of M. johnei from faeces

Table 19. The isolation of M. johnei from faeces

Faeces culture	Culture +ve				Culture -ve		
	Faeces +ve (clumps)	Faeces +ve (singles)	Faeces -ve	Total	Faeces +ve	Faeces -ve	Total
No. of samples	11	6	2	19		21	21
No. +ve	9	2	1	12		-	-

The results are given in Table 19. M. johnei was not cultured from the faeces of 21 cattle whose tissue failed to yield M. johnei in culture. Of 19 culturally positive cattle, M. johnei was isolated in culture from the faeces of 12 cattle. Of 11 samples in which there were clumps of acid-fast bacilli on microscopical examination nine were positive culturally; of six samples containing single acid-fast bacilli two were positive. M. johnei was recovered in culture from one of two samples from culturally positive cattle in the faeces of which no acid-fast bacilli were seen.

Discussion

The fact that M. johnei was cultured from 66 (31 per cent.) of 207 adult wasting cattle does not indicate that the infection rate is 31 per cent., since a number of the cattle examined were specifically chosen because of their history of coming from M. johnei infected herds. Nevertheless, there was every indication that Johne's disease is an important cause of wasting and therefore of economic loss among adult cattle in Southwest Scotland.

Due to the fact that the period of observation of each case varied, it was not possible to examine a constant number of samples from each but, from the limited numbers of histological reports available, there are adequate grounds for believing that a fairly accurate classification on the results of the faeces examination was possible, i.e. the separation of those cases in which clumps of acid-fast bacilli were noted on at least one examination of faeces from those in which only single acid-fast bacilli but never clumps were found on one or more occasions.

In 25 of the 66 cattle from which M. johnei was isolated in culture, clumps of acid-fast bacilli were found on microscopical examination of faecal smears on at least one occasion. Histological examination of the intestinal mucosa confirmed the presence of Johne's disease in every instance. Microscopical examination of Ziehl-Neelsen stained sections revealed the presence of micro-colonies of M. johnei in these lesions (Cunningham, 1958). Severe wasting and diarrhoea was a feature of most of the cases in this series. Several quickly became recumbent and were destroyed. No case of improvement other than of a purely temporary nature was

noted and it is considered that every case in this series would have had a fatal termination.

There were 30 cattle from the tissues of which M. johnei was cultured and examination of faeces showed the presence only of singly disposed acid-fast bacilli morphologically resembling M. johnei. Histological examination of the intestines showed that isolated intracellular acid-fast bacilli were present in the lesions and that micro-colonies of acid-fast bacilli were an exceptional finding (Cunningham, 1958). Some cases in this series (Table 2) were as severely affected clinically as those described in Table 1, while others were exhibiting sufficient wasting to be included in this investigation but no other specific signs of Johne's disease. The fact that some of the latter might have recovered cannot be ignored.

M. johnei was not isolated from 141 of the 207 cattle examined. This does not prove that they were in fact free from M. johnei infection. The sampling and cultural techniques used have known inadequacies, whereby small numbers of M. johnei may be missed. The sites chosen for cultural examination, namely the ileo-caecal lymph nodes and ileal mucosa were those thought most likely to be infected in connection with significant lesions of the disease, although a wider coverage of the mesenteric nodes and intestinal mucosa might have been of advantage. The recovery of M. johnei from the liver, spleen and retropharyngeal nodes and from no other site as reported by Williams Smith (1958), is of unknown significance in the pathogenesis of Johne's disease.

It was decided to use as the test of the accuracy of the diagnostic techniques the recovery of M. johnei from the tissues.

While this might mean that a number of what were regarded as positive cases were not wasting due to M. johnei infection but were symptomless carriers of M. johnei and wasting due to some undetermined cause, it would result in the efficiency of the techniques being assessed by the most rigorous criterion i.e. the ability to detect M. johnei infection, not Johne's disease.

The significance of the finding of single acid-fast bacilli in the faeces has been discounted by most workers, although, in examining faeces samples from clinically suspicious cattle, some authors (Doyle and Spears, 1951; Soltys, 1951) classified those samples in which they found single acid-fast bacilli as "suspicious". This would imply that the finding of such bacilli might be of significance. The results presented here indicated that the finding of single acid-fast bacilli was indicative of the presence of Johne's disease in the wasting adult cattle examined. At the time of sampling, all the cattle examined were maintained under the same conditions. They were in stalls or individual loose-boxes and were fed on hay, concentrates and water. It may well be that saprophytic acid-fast bacilli occur sporadically in the faeces of cattle kept under circumstances and on diets different from those described above. Rankin (1957) suggested that prolonged examination of faecal smears containing single acid-fast bacilli would, in some cases, reveal clumps of acid-fast bacilli. Prolonged examination of any one smear was not carried out in the studies described here, but examination of a series of samples from the same animal did not confirm this hypothesis. It is presumed that single acid-fast bacilli appear in the faeces as a result of a shedding of infected cells from the mucosa

into the intestinal lumen. Histological examination of the mucosa supported this theory. When single intracellular bacilli were found in the lesions and single acid-fast bacilli in faecal smears, the finding of clumps of acid-fast bacilli in either was very uncommon. It has been considered in the past that only one third of clinical cases of Johne's disease could be diagnosed on faeces smear examination at any one examination. Of 194 examinations on the faeces of 66 M. johnei infected cattle, 146 (75.2 per cent.) were positive (i.e. either clumps or isolated acid-fast bacilli were found on microscopical examination of faecal smears). Of 252 faeces samples from 141 M. johnei-free wasting adult cattle, 23 (9 per cent.) were positive. In all instances only single acid-fast bacilli were found. This latter figure is a measure of the non-specificity of the test assuming the accuracy of the post-mortem cultural examination to be high. However, it is possible that cultural examination failed to detect infection in some cases. The results of histological examination might have been useful in this context. The figures presented here show the usefulness of the examination of the faeces from the cases clinically suspicious of Johne's disease examined, using the methods described.

Unfortunately, complement fixation test results were not available for all the cases in the series of 207 but the results obtained in those examined indicated that, in infected animals, the test was 82 per cent. accurate. Where animals with clumps in their faeces were concerned, 96 per cent. gave a positive reaction to the test. Of the 22 cases which had single acid-fast bacilli in the faeces and less advanced and, in some cases, purely focal lesions 15 (68.5 per cent.) were positive to the test. The figure of 82 per

cent. accuracy in confirming the presence of Johne's disease is of the same order as those given by Rankin (1958a) and Hole (1956b). The decreasing percentage of positive results with decreasing extent of severe lesions are compatible with Rankin's observation that a high C.F. titre was associated with extensive, advanced intestinal lesions. Forty-two per cent. of sera from cattle from which M. johnei was not isolated were positive according to the interpretation of the test used in this laboratory. This may indicate that a proportion of the M. johnei-free cattle came from infected herds and that the positive titres were due to past experience of M. johnei or that positive titres can result from infection with other acid-fast bacteria. The validity of the interpretation of the complement fixation test used in these experiments i.e. 0 - 25 per cent. haemolysis classified as positive, 25 - 100 per cent. haemolysis classed as negative, and the abandonment of the intermediate classifications of weak positive and doubtful, may be open to criticism. It was thought to be justified, however, because of the difficulty of relating these intermediate reactions to the recovery of M. johnei from the tissues, the severity of lesions or previous or subsequent tests in the same animal. In this work, the complement fixation test was performed on sera taken immediately prior to post-mortem examination, thus meeting a criticism that in previous attempts at evaluation of the test there had often been considerable lapses of time between the test and post-mortem examination, during which time recovery from infection with M. johnei might have occurred.

Due to the disparity between the numbers of each breed examined

and the preponderance of Ayrshire cattle in the series, no comment can be made on breed susceptibility. All the common breeds found in Southwest Scotland were represented.

As the majority of cases were received without a history it was necessary to estimate the age of each case by means of its dental formula up to three and a half to four years of age and by its horn structure beyond that. Many of the animals were polled. These were classified as aged if over four years old. Comparing the ages of cattle which were thought to be wasting due to Johne's disease with the ages of cattle thought to be free from M. johnei infection there was a significant tendency for young adult cattle up to four years old to be in the M. johnei infected group, while the converse was true for cattle over four years old. Wasting in aged cattle was more likely to be due to a cause other than Johne's disease. The most common conditions found in the older cattle were chronic mastitis and chronic pneumonia. The age distribution of Johne's disease found in the cattle examined confirms the field observation that the appearance of clinical symptoms of Johne's disease is often associated with the second parturition which normally takes place at about three and a half to four years of age.

Forty-two (63.6 per cent.) of the 66 cattle from which M. johnei was isolated in culture exhibited diarrhoea on at least one occasion during the period of observation in the hospital. It was found that little stress as a diagnostic aid could be placed on the colour of the faeces or the presence of bubbles in the diarrhoea. Only 20 (14.2 per cent.) of 141 cattle which were negative for M. johnei on culture had diarrhoea during the period of observation. The most

common cause of diarrhoea in this group was mucosal disease. These results indicate that Johne's disease was the most important cause of diarrhoea in the cattle examined.

Where there was severe thickening of the walls of both the small and large intestines, it might have been possible to have made a tentative diagnosis of the presence of Johne's disease without opening the alimentary tract and inspecting the mucosa, but in most cases it was impossible to differentiate the thickening which occurred in Johne's disease from the thickening which was a feature of many unopened intestines free from Johne's disease. The criterion for determining whether the lesions in the intestinal wall were those of Johne's disease was the finding of thickening of the mucous membrane with permanent corrugation and a roughening and fissuring of the mucosa which can best be described as having a granular appearance. These corrugations have been likened to the convolutions on the brain surface (Rankin, 1958b) and cannot be obliterated on gentle stretching. Using this criterion, what were considered to be typical lesions were seen in the intestines of 56 per cent. of cases from which M. johnei was isolated. Histological lesions may be present in the absence of macroscopic lesions (Rankin, 1958b) and in some cases the microscopic lesions may be extensive (Cunningham, 1958). The fact that in cattle from which M. johnei was isolated in culture and which had clumps of acid-fast bacilli in their faeces 84 per cent. had lesions visible to the naked eye, while in cattle which were culturally positive but only had single acid-fast bacilli in the faeces only 50 per cent. had macroscopic lesions, shows that the latter group, taken as a whole, may be in a

less advanced state of the disease. In the absence of histological results the significance of the finding of lesions in cattle from which M. johnei was not recovered cannot be discussed satisfactorily. However, there is no reason to believe that lesions may not be present in the absence of M. johnei. This was found to be the case in sheep (Stamp and Watt, 1954). The difficulty of isolating in culture some sheep strains of M. johnei makes proper evaluation of that finding difficult.

Of nine foetuses from dams affected with Johne's disease, M. johnei was recovered in culture from four. It is of interest that M. johnei was isolated from a foetus whose dam had only single acid-fast bacilli in its faeces and intestinal mucosa and was therefore probably in a less advanced stage of the disease bacteriologically than those which had clumps of acid-fast bacilli in their tissues. Congenital infection is potentially an important factor in the epidemiology of Johne's disease. The consequences in later life of congenital infection are not known. At the present state of knowledge, it would seem to be unwise for the progeny of cattle in the clinical stage of the disease to be kept in a herd.

M. johnei was isolated in culture from 12 of 19 faeces samples from infected cattle. Of 11 samples in which clumps of acid-fast bacilli were seen microscopically, M. johnei was recovered in nine, but in six samples which had single acid-fast bacilli on microscopical examination, only two were culturally positive.. This indicates either that the techniques used were not sensitive enough to recover small numbers of acid-fast bacilli from the faeces or that many of the single acid-fast bacilli seen in faeces samples were not viable. It is

noteworthy that no saprophytic acid-fast bacilli were isolated from any of the faeces samples or from the tissues of the 207 cattle examined. Avian tubercle bacilli were isolated on two occasions. This finding will be reported on in full and discussed in Part III.

It is postulated that there are several ways in which an animal reacts to M. johnei infection, depending on the infecting dose, the virulence of the infecting strain (about which nothing is known), the age at which it first experiences infection and its resistance to infection. These varying reactions may be classified into three loosely defined groups:-

i. Infection may be followed by the production of minimal lesions and subsequent recovery. This process may be repeated many times in a resistant animal in an infected environment.

ii. Infection with M. johnei and a host reaction as shown by the production of more severe lesions which persist. This group may contain the healthy "carrier" animals which are constant excretors. Depending on the individuals resistance recovery may follow after a time or the infection may progress to iii.

iii. Massive multiplication of M. johnei with the production of severe lesions and progressive fatal clinical disease.

Group ii may be represented by the unthrifty animal which is on the borderline of being classified as wasting and which is often seen in infected herds. Cases which have recovered from a group ii type infection may be those non-infected cases which have complement fixing antibodies in their sera.

Conclusions

In the 207 wasting adult cattle examined:-

(i) Johne's disease was an important cause of the culling of these animals from herds.

(ii) The finding of single acid-fast bacilli on microscopical examination of faeces smears was diagnostic of M. johnei infection. Using the techniques described, Johne's disease could be diagnosed with a fairly high degree of accuracy. False positive results occurred in what were considered M. johnei-free cattle.

(iii) In infected cattle, the complement fixation test was of comparable accuracy to the faeces smear test but in non-infected cattle a higher percentage of non-specific results were obtained.

(iv) Johne's disease was more likely to be the cause of wasting in cattle under four years of age than in cattle over four years of age.

(v) The presence of diarrhoea was more likely to be due to Johne's disease than to any other cause.

(vi) Macroscopic lesions of Johne's disease may be absent in the presence of microscopical lesions and bacteriological infection.

(vii) Intrauterine infection of the foetus was noted.

(viii) The isolation of M. johnei from faeces was not a reliable diagnostic method in a limited series attempted.

Section 2

The incidence of M. johnei in the mesenteric lymph nodes of apparently normal cattle and the investigation into the accuracy of the in vivo diagnosis of M. johnei infection in the cattle examined

Introduction

The reasons for undertaking this study were to determine the level of infection in apparently normal cattle in Southwest Scotland where there is reputed to be much clinical disease and to correlate the finding of single acid-fast bacilli in the faeces with the presence or absence of M. johnei infection.

Review of the Literature

The only figures available for the estimated mortality due to Johne's disease in Southwest Scotland are given by Withers (1959) who found the incidence of Johne's disease in 1954/5 and 1955/6 to be 0.14 per cent. and 0.15 per cent. of cattle at risk. This survey covered selected farms in Ayrshire and Lanarkshire. Surveys carried out in England showed an infection rate of from 6-17 per cent. in the mesenteric lymph nodes of apparently normal cattle (Taylor, 1952; Rankin, 1954). In contrast, Williams Smith (1958) recovered M. johnei from the mesenteric lymph nodes of two of 100 cattle slaughtered in Essex although this incidence rose to 7 per cent. if recoveries from retropharyngeal lymph nodes were included.

Materials and Methods

Female cattle of the dairy breeds being slaughtered at a local abattoir were examined. All were in good or very good physical condition. The following procedure for collecting data and samples was adopted. The age and breed of each case were noted. A blood sample for the complement fixation test was collected from the jugular vein at slaughter. The large and small intestines as

removed from the abdomen in the course of normal slaughterhouse procedure were put in a sack, labelled and removed to the Veterinary Hospital where they were examined within one hour of slaughter. The faeces samples for microscopical examination were taken from the rectum. The techniques for pathological examination and the complement fixation test were as described in Part I, Section 1. The procedure for the recovery of M. johnei in culture was also as previously described, apart from the fact that, in addition to samples of the ileo-caecal nodes, portions from the mesenteric chain were pooled for cultural examination. The strains isolated were tested for their dependance on the phlei growth factor.

Results

The results are shown in Tables 20 and 21.

Table 20. The correlation between the finding of single acid-fast bacilli in the faeces and the recovery of M. johnei from the mesenteric lymph nodes of 100 apparently normal cattle

No. of cases from which M. johnei was recovered	= 3
No. of cases from which M. johnei was not recovered	= 97
No. of cases in which single acid bacilli found	= 17
No. of cases in which single acid bacilli not found	= 83

	Culture of mesenteric nodes	
Macroscopic examination of faeces	M. johnei recovered	M. johnei not recovered
+ve	1	16
-ve	2	81

Table 21. The correlation between the presence of C.F. antibodies in the sera and the isolation of M. johnei from the mesenteric lymph nodes of 100 apparently normal cattle

No. of cases C.F. positive = 33
No. of cases C.F. negative = 67

	Culture of mesenteric nodes	
C.F. test	M. johnei recovered	M. johnei not recovered
+	2	31
-	1	66

M. johnei was isolated from the mesenteric lymph nodes of three (3 per cent.) of the 100 cases examined. No macroscopic lesions were found. The results of histological examinations are not yet available.

Single acid-fast bacilli morphologically resembling M. johnei were found in the faeces of one of these three. M. johnei was not recovered in culture from any of the 100 faeces samples. Of the 97 cases from the tissues of which M. johnei was not recovered, single acid-fast bacilli were found in the faeces of 16 (16.4%) on microscopical examination. Inspection of the results showed that there was no correlation between the finding of acid-fast bacilli in the faeces and the presence of M. johnei infection (Table 20).

In the three cases from which M. johnei was isolated in culture, the complement fixation test was positive twice but it was also positive

in 31 (32 per cent.) of the 97 cows which were negative on culture (Table 21).

Discussion

The incidence of M. johnei infection is lower than might be anticipated from an area where Johne's disease is considered to be prevalent. Two factors may contribute to this; firstly, the smallness of the sample with an undue proportion of cattle from a relatively Johne's disease-free area and, secondly, infected cattle may have been missed due to the fact that only some of the mesenteric lymph nodes and intestinal mucosa were cultured.

While the incidence is lower than that obtained by Taylor (1952) and Rankin (1954), it is similar to that obtained by Williams Smith (1958). The last mentioned does not comment on the incidence of clinical disease in the area covered by the survey. The epidemiological significance of the presence, as reported by Williams Smith, of M. johnei in retropharyngeal lymph nodes is not clear. It may indicate that the animal was from an infected environment, but not that infection was present or ever likely to be present in the alimentary tract. The low incidence may be a true reflection of the incidence of infection in that area or may be due to the small number sampled, a criticism which applies to the present survey.

Because of the number of cases in this series in which acid-fast bacilli were seen on microscopical examination of the faeces from animals which were, as far as could be determined by the methods employed, free from M. johnei infection, it must be concluded that these acid-fasts were not M. johnei. They may be saprophytic

acid-fast bacilli and normal inhabitants of the bovine alimentary tract.

This is in contradistinction to the findings in Part I, Section 1, in which it was shown that the finding of single acid-fast bacilli in the faeces of wasting cattle was of significance in the diagnosis of Johne's disease.

The complement fixation test proved to be of no value in detecting M. johnei infection in these apparently normal cattle. The test was positive in two of the three infected cattle but considering the number of positives in the whole series this may be due to chance, and is not significant.

The incidence of 32 per cent. reactions to the complement fixation test in M. johnei-free cattle is a measure of the non-specificity of the test as applied to the apparently healthy cattle examined. This agrees in general with the figures of Rankin (1958a) who found that, over a number of tests, 8 - 36 per cent. of normal cattle in an infected herd reacted, while 8 - 22 per cent. of cattle in a Johne's disease-free herd reacted. The former figures and those obtained in the present work may be explained by the reaction of cattle to the test as a result of a transient experience of M. johnei but this does not account for the reactions in the latter case. Cross-reactions with other unspecified acid-fast bacilli may be implicated. Positive complement fixation test results from non-infected cattle have been reported by Chandler (1955).

Conclusions

The finding of single acid-fast bacilli in faeces smears from the apparently normal cattle examined could not be correlated with

M. johnei infection. The complement fixation test did not detect M. johnei infection in the same cattle. In the cattle free from M. johnei infection, the complement fixation test was positive in many cases.

S e c t i o n 3

The microscopical examination of faeces from cattle thought to be free from M. johnei infection

Introduction

The object of this study was to determine whether or not single acid-fast bacilli could be found in the faeces of cattle thought to be free from M. johnei infection.

Results obtained in the examination of the correlation between the finding of single acid-fast bacilli in the faeces and the recovery of M. johnei from the mesenteric lymph nodes indicated that acid-fast bacilli were present in the faeces of a number of cattle which were, within the limits of the techniques used, free from M. johnei infection. The possible identity and significance of these acid-fast bacilli has been discussed.

Materials and Methods

At the Agricultural Research Council Field Station, Compton, there are three dairy herds, in two of which there has been no clinical Johne's disease for many years. M. johnei has not been recovered from over 200 cattle slaughtered immediately after disposal from the disease free herds. It was considered that if acid-fast bacilli were found in the faeces of cattle in these herds it would be improbable that they were M. johnei.

The third herd has had two clinical cases of Johne's disease during the period 1953-58. M. johnei has been recovered from the tissues of 10 per cent. of cattle disposed of from this herd (Rankin, 1958a).

Samples of faeces from cattle in the three herds and in the experimental herd at Compton were collected and identified by the allocation of random numbers so that their identity and source

were unknown to the examiner.

Faeces samples which showed single acid-fast bacilli on microscopical examination were cultured by Dr. J. Deans Rankin.

The technique for the preparation and examination of the faeces was as described on page 31.

Results

The results are shown in Table 22.

Table 22. The distribution of faeces samples containing single acid-fast bacilli in infected and non-infected herds

	Faeces samples	
	+ve	-ve
Occurrence in infected herd	6	49
Occurrence in non-infected herds	1	132

Statistical analysis of this distribution shows that it was highly significant $X^2 = 11.198$ $P = .001$.

Single acid-fast bacilli were found in six samples of 55 examined from cattle in the herds in which M. johnei was known to exist, but in only one sample from 133 cattle in the two herds thought to be free from M. johnei infection. On microscopical examination of this one smear there was found a single acid-fast bacillus with bi-polar staining. It was not regarded as typical.

M. johnei was not isolated in culture from any of the positive faeces samples nor from an additional twelve potentially positive samples which were microscopically negative.

Discussion

The finding of single acid-fast bacilli in the faeces of cattle from the infected herd does not prove that they were M. johnei. Indeed, the failure to isolate M. johnei in culture from these samples might seem to indicate that they were not. However, the isolation of M. johnei from faeces samples has proved difficult (Levi, 1948a). What is of significance is that single acid-fast bacilli were not found (apart from one dubious bacillus) in the faeces of cattle probably free from M. johnei infection. While it is not disputed that saprophytic or otherwise unidentifiable acid-fast bacilli may occur from time to time, it is postulated that in examining faeces from cattle in a herd of known status regarding M. johnei infection it may be possible to identify infected animals. What proportion of infected animals would be diagnosed and the prognosis in animals so diagnosed is not known. Further investigations are indicated.

Conclusions

In the herd thought to be free from M. johnei infection, no acid-fast bacilli morphologically resembling M. johnei were found in the faeces, but acid-fast bacilli morphologically resembling M. johnei were found in the faeces of a number of cattle in herds in which M. johnei infection exists.

Section 4

A survey of the incidence of M. johnei infection in calves from Southwest Scotland

Introduction

This survey was designed to assess the level of infection and to see if the result was of significance in the epidemiology of Johne's disease. Any calves found to be infected must have acquired the infection congenitally or during the first few days of life.

Review of the Literature

Alexejeff-Goloff (1929) was the first to report the recovery of M. johnei from a bovine foetus. Circumstantial evidence of congenital infection in a three year old bull whose dam was a clinical case was presented by Dunkin (1935). Conclusive evidence of foetal infection was given by Pearson and McClelland (1955). Lawrence (1956) examined foetuses from 24 infected dams, 19 of which were clinically infected, and recovered M. johnei from five of them. Doyle (1958) examined foetuses from 24 clinical cases and isolated M. johnei in nine instances.

In abattoir surveys in England the incidence of infection with M. johnei in adult cattle has been found to be from 6 - 17 per cent. (Rankin, 1954; Taylor, 1952; Williams Smith, 1954). Chandler (1959) recovered M. johnei from two (0.44 per cent.) of 447 calves of up to two months of age slaughtered at Birmingham abattoir. Only one ileo-caecal node from each calf was examined.

Materials and Methods

The calves used in this survey were bought at a local market in groups of 40 and slaughtered later the same day. All were healthy bull calves of the dairy breeds and were considered to be

under one week old.

The ileo-caecal lymph nodes and four samples of the mesenteric lymph nodes were taken from each calf and stored in universal containers at 0°C. till cultured. The method for the isolation of M. johnei and the criteria for its identification were as described in Part I, Section 1.

Seventy-five day-old bull calves of the dairy breeds bought at one week old and kept in isolation in individual metal huts till they were three to four months of age were examined by the same methods.

Results

In 158 week-old calves examined, M. johnei was recovered from one (0.63 per cent.). M. johnei was recovered in culture from two (2.66 per cent.) of 75 three to four month old calves. M. johnei was isolated from the tissues of three (1.3 per cent.) of the 223 calves examined.

Discussion

Due to the lethal effect of the oxalic acid treatment of the tissues on M. johnei and the impracticability of examining all the potentially infected sites, the true incidence may be greater than that obtained. As Williams Smith (1958) recovered M. johnei from the retropharyngeal lymph nodes of apparently normal cattle, the failure to examine this site and the intestinal mucosa, which may be the primary focus, may have been a source of error. Since it is improbable that ingestion of M. johnei during the first week of life would establish sufficient infection in the intestines to infect the

mesenteric lymph nodes, the result obtained may be a true estimation of the rate of congenital infection.

Since the infection rate in calves under one week old was found to be under one per cent. and the incidence of infection in normal adult cattle as much as 17 per cent., many cattle must become infected subsequent to the first week of life. There are several sources of infection, the principal one being contact with clinically or preclinically infected cattle (Rankin, 1957). It is obvious that the practice of rearing calves in communal pens plays an important part in the dissemination of infection. As it has been shown that, experimentally at least, young calves are more susceptible (Taylor, 1953), some measure of control would be achieved if calves were reared in individual pens for as long as possible.

Conclusion

The incidence of *M. johnei* infection in the mesenteric lymph nodes of the calves under one week old and in the calves kept isolated till four months of age was of a low order.

P A R T II

EXPERIMENTS INVOLVING M. JOHNEI INFECTION OF MICE

Section 1

The experimental infection of mice with M. johnei and M. tuberculosis var. avium

Introduction

The object of this experiment was to reproduce the lesions of Johne's disease in the alimentary tract of mice. In view of the paucity of reports of successful infection of laboratory animals, it was felt that further investigations might be of value.

Review of the Literature

The use of laboratory animals in research on Johne's disease has been limited by their relative insusceptibility to progressive infection. Francis (1943) and Lominski et al. (1956) were successful in establishing in mice intestinal lesions which they considered histologically similar to those in cattle. Levi's (1950) findings in voles were essentially similar to those described by Francis in the mouse, but the dose required to establish infection was much smaller (0.0001 mg.). Lominski et al. (1956) using mice, and Rankin (1958c) using rabbits, are the only workers to report the reproduction of a wasting syndrome in laboratory animals.

Failure to infect laboratory animals or the production of minor lesions have been reported by many workers including Twort and Craig (1913), Boquet (1925), Hagan and Mansfield (1930), Mohler (1939), Sahai (1940, 1941), Taylor (1940, 1951), Glover (1941), Johnson and Cox (1942) and Verlinde and Bekker (1945). Some of these workers' results might be attributed to failure to keep the experimental animals under observation for a sufficient length of time.

Materials and Methods

The mice used were newly weaned male mice, W Swiss strain.

They were housed in metal boxes, five to a box, and fed a balanced ration in pellet form and water ad lib. Infection was by the intraperitoneal route.

The strain of M. johnei used was obtained from the A.R.C. Field Station, Compton. This strain was chosen because of its known pathogenicity for cattle. At Compton successive isolations of this strain are being constantly obtained from experimentally-produced clinical cases of Johne's disease in calves or from the faeces of experimentally infected calves before the appearance of clinical signs. The M. johnei for inoculation was received as a first subculture from a primary isolate and had been incubated for six weeks. The inoculum was prepared by scraping the growth, which was confluent, off the surface of the medium with a sterile platinum loop, weighing it on sterile filter paper and suspending it in an appropriate volume of isotonic sodium chloride solution using a Griffith tube.

Experiment A. Twelve mice were inoculated intraperitoneally with one mgm. wet weight of M. johnei in 0.5 ml. of isotonic saline solution.

Experiment B. One month after the start of Experiment A another batch of slopes was received from Compton. The harvest of M. johnei was used to inoculate 30 newly weaned mice intraperitoneally with two mgm. wet weight of M. johnei prepared as already described.

The technique for obtaining counts of viable M. johnei in mouse tissues was that described by Brotherston (1959). Livers and mesenteric lymph nodes were removed aseptically and ground,

using Griffith tubes, with known volumes of sterile distilled water. Tenfold increasing dilutions of the suspensions were made in Williams Smith's (1953) liquid medium. One tenth ml. volumes from each dilution were measured using serological one ml. pipettes and sown onto the dried surface of duplicate slopes of Williams Smith's medium (1953). The slopes were stoppered with cotton-wool plugs, allowed to remain horizontal at room temperature overnight to allow the inoculum to be absorbed, stoppered with screw caps, and incubated at 37°C. for eight weeks. The small intestine was opened and the mucous membrane examined for gross lesions. The intestines were washed in two changes of sterile distilled water and suspended in an excess of 5 per cent. oxalic acid solution using a homogeniser. The resulting suspensions were filtered through a layer of sterile muslin and incubated at 37°C. for 30 minutes. An equal volume of sterile distilled water was added to the filtrate. This was centrifuged at 3,000 r.p.m. for 30 minutes, the supernate discarded, and the sediment suspended in 1 ml. of Williams Smith's liquid medium. Dilutions increasing by tenfold steps were prepared from the suspension. Volumes of 0.1 ml. of aliquots from the dilutions of the suspensions were sown as before.

Counts of viable M. johnei per ml. of tissue suspension were obtained by multiplying the mean number of colonies on the duplicate slopes at the dilution whereby counts of 10 - 100 colonies were obtained by the appropriate dilution factor and by 10 since 0.1 ml. volumes were inoculated.

Tissues for histological examination were fixed in corrosive-formol solution. Sections were stained with haemotoxylin and eosin

and by the Ziehl-Neelsen method.

Results

Experiment A

Six mice which died shortly after injection or were not discovered soon after death and so could not be examined because of putrefaction or cannabalism were not examined.

The results of viable counts on the livers, mesenteric lymph nodes and small intestines of six mice are shown in Table 23.

Table 23. Viable counts per ml. of tissue suspensions 15 and 17 months after the i/p inoculation of M. johnei into three-week old mice

	Killed at 15 months				17 months	
Mouse no.	1	2	3	4	5	6
Liver	-	720×10^6	-	50×10^4		
Mesenteric lymph node	115×10^4	720×10^6	245×10^4	460×10^4	50×10^4	75×10^4
Small intestine	5000	720×10^6	80×10^4	35×10^4		

No result = not examined

- = no growth obtained

Cultures from all the mice contained small, raised, wrinkled, greyish-white colonies with irregular margins. Smears from these colonies stained by the Ziehl-Neelsen method were found to contain short acid-fast bacilli. The colonial morphology and staining reaction were those of M. johnei.

Counts of more than 20×10^6 viable units/ml. of the liver,

mesenteric lymph node and small intestine suspensions were obtained. The growths obtained from the highest dilution of these tissues made were almost confluent. It was not possible to count accurately more than 200 colonies on a slope. M. johnei was recovered from all the tissue examined in Mouse 4, and from the mesenteric lymph nodes and small intestines only in Mouse 1 and Mouse 3. Only the mesenteric lymph nodes of Mice 5 and 6 were examined. M. johnei was isolated from both of these mice.

At post-mortem examination of the mice 15 and 17 months after inoculation, no gross abnormalities were detected in any of the viscera. As material for histological examination was taken before it was known that Experiment B mice had not in fact received M. johnei, tissues from only two mice (Nos. 5 and 6) from Experiment A were examined. No lesions or acid-fast bacilli were found, although M. johnei was recovered from the small intestinal wall of both.

Experiment B

Thirteen mice which died shortly after injection or which were decomposed before discovery were not examined. Seven mice died between seven and twelve months after inoculation. No gross lesions which could be attributed to the inoculum were found. Microscopical examination of Ziehl-Neelsen stained smears of the intestinal mucosae, faeces, livers and spleens revealed small numbers of long, slender acid-fast bacilli in every case. Examination of sections of intestinal walls, livers and spleens showed that there were present, in three of the five mice examined histologically, lesions consisting of endothelioid cell infiltrates. The lesions in the intestinal wall

were confined to the areas of lymphoid tissue.

Ten mice were examined 15 - 17 months after inoculation. The results of counts of viable M. johnei in the livers, mesenteric lymph nodes and small intestines of those killed at 15 months and counts from the mesenteric lymph nodes only, of those killed at 17 months after inoculation are given in Table 24.

Table 24. Viable counts per ml. of tissue suspensions 15 and 17 months after i/p inoculation of two mgm. avian tubercle bacilli into three-week old mice

	Killed at 15 months					17 months				
Mouse no.	1	2	3	4	5	6	7	8	9	10
Liver	$115x10^4$	$130x10^5$	X	X	$460x10^4$					
Mesenteric lymph node	$60x10^4$	$110x10^5$	X	X	$590x10^4$	X	X	X	X	X
Small intestine	$45x10^4$	$415x10^5$	$80x10^4$	$165x10^5$	$190x10^5$					

No result = not examined

X = confluent growth at highest dilutions made

Colonies recovered from these mice were smooth, white, slightly raised, glistening, round and having sharply defined margins. When examined by transmitted light, the colonies appeared to have dense centres and more translucent peripheries. Older colonies had heaped-up centres on which dome-shaped daughter colonies appeared. Ziehl-Neelsen stained smears made from these colonies were found, on microscopical examination, to contain long, slender acid-fast bacilli not resembling typical M. johnei. Morphologically the colonies and bacteria resembled M. tuberculosis var. avium. Biological and

cultural tests to be described later confirmed this.

M. tuberculosis var. avium was recovered from the livers, mesenteric lymph nodes and small intestines of Mice 1 - 5 and from the mesenteric lymph nodes, which were the only tissues examined, in Mice 6 - 10. In the case of the livers and mesenteric lymph nodes of Mice 3 - 4 no counts were obtained because confluent growths were obtained at the highest dilutions of the inocula.

No lesions were visible on naked eye examination of the viscera. The distribution and relative severity of the microscopic lesions are shown in Table 25.

Table 25. The distribution of lesions in the tissues of mice killed 17 months after the i/p inoculation of two mgm. of avian tubercle bacilli into three-week old mice

Mouse no.	6	7	8	9	10
Liver	++++	-	-	++++	+++
Peyer's patches	++	-	+	+++	++
Mesenteric lymph node		+			

No result = not examined

+ to ++++ = severity of lesions

- = no growth obtained

The lesions in the haematoxylin and eosin stained sections of the livers are composed of aggregations of large pale cells with round or oval nuclei and abundant eosinophilic cytoplasm. These aggregations have at their peripheries a zone of smaller cells with densely staining nuclei. In Mouse 10 the endothelioid cell infiltration is focal in contradistinction to Mouse 6 where the

infiltration is more diffuse (Figs. 1—4). The lesions in the other tissues are of the same cellular composition. In the intestinal walls the cellular changes are confined to the lymphoid tissue in the submucosa. No abnormalities were noted in the villi. Pleomorphic, slender, long, acid-fast bacilli were found in association with the lesions on microscopical examination of Ziehl-Neelsen stained sections.

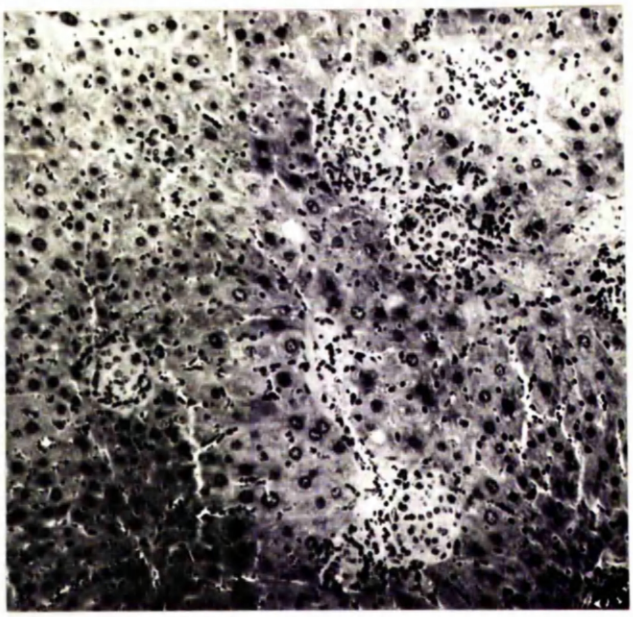

Fig. 1. Section of liver of mouse killed 17 months after inoculation of avian tubercle bacilli. There are focal aggregations of macrophages. H and E x 150.

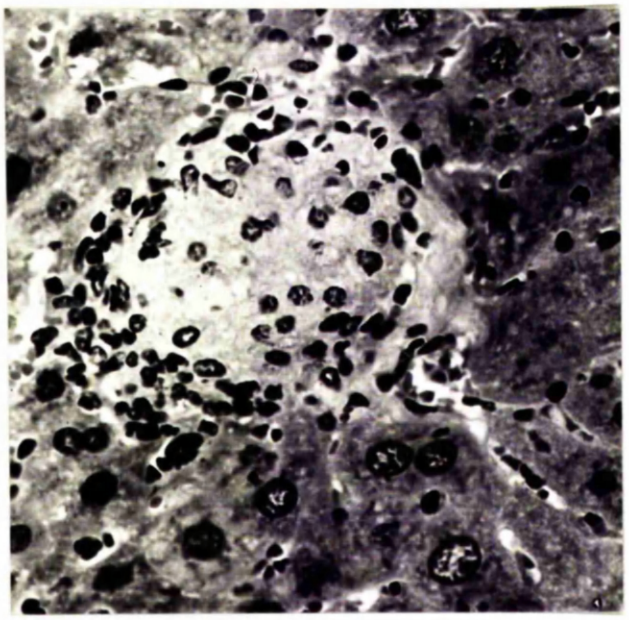

Fig. 2. Section of liver of the same mouse as in Fig. 1. There is a group of large pale macrophages which contain acid-fast bacilli. H and E x 520.

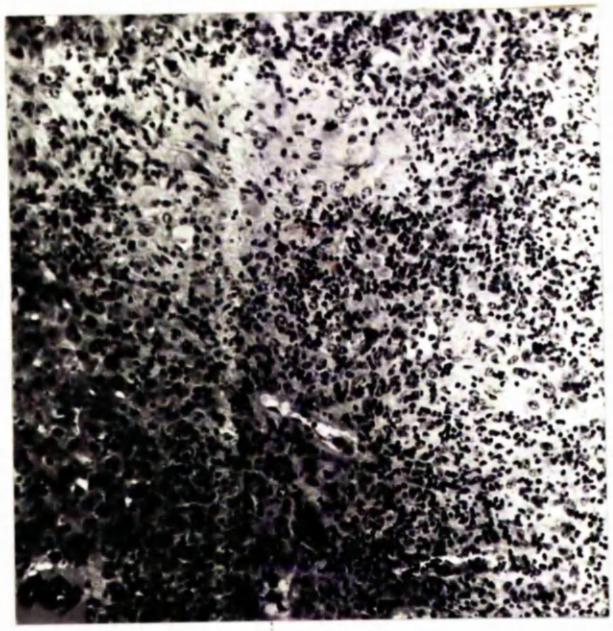

Fig. 3. Section of the liver of a mouse killed 17 months after inoculation of avian tubercle bacilli. There is a diffuse infiltration of macrophages. c.f. Fig. 1. H and E x 150.

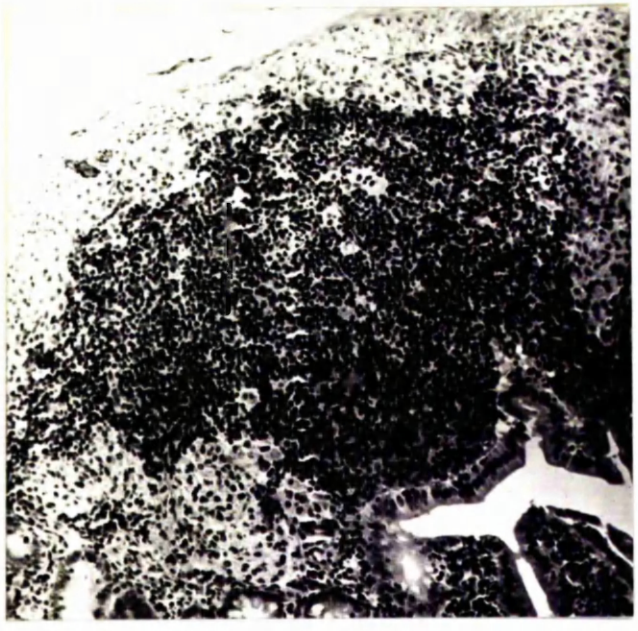

Fig. 4. Section of small intestine of the same mouse as in Fig. 1. There are lesions at the periphery of a Peyer's Patch. H and E x 150

Discussion

There was no evidence of a progressive multiplication of M. johnei in the mouse tissues. The most significant finding was the evidence of the ability of M. johnei to persist in the tissues for at least 17 months. M. johnei was not recovered from two of the four livers examined. The viable counts per ml. of tissue suspensions of the two infected livers were equal to or less than the counts from the corresponding mesenteric lymph node suspensions. As counts were also obtained from the small intestines in this instance, this distribution of M. johnei may indicate that, even in what must be regarded as a relatively resistant host, M. johnei shows a predilection for an an ability to survive or multiply very slowly in the intestinal wall and regional lymph nodes.

In Experiment B, it is probable that multiplication of the inoculum of avian tubercle bacilli occurred in Mice 3, 4, and 6 - 10 inclusive, from which confluent growths were obtained from the highest dilutions of the tissue suspensions examined. The viable counts of M. tuberculosis var. avium tended to be higher than those of M. johnei in Experiment A.

It is presumed that the confluent growth on the slopes which were used to provide the inoculum for Experiment B was either a pure culture of the avian tubercle bacillus or a culture of M. johnei contaminated with the avian tubercle bacillus which, being more pathogenic, became the predominant organism in the mice.

It is relevant to note here that M. tuberculosis var. avium was isolated from the faeces of a clinical case of Johne's disease in a cow in the series described in Part I, Section 1.

The lesions resulting from the avian tubercle infection in mice closely resemble those described by Francis (1943) following the intravenous and intraperitoneal administration of M. johnei to mice, and differ only in degree from those described in the intestinal wall of M. johnei infected mice by Lominski et al. (1956). Similar cellular reactions have been noted following the injection of viable M. johnei and avian tubercle bacilli into voles (Levi, 1950) and as a result of the inoculation of killed M. phlei and M. johnei into rodents (Hagan and Levine, 1932).

It is clear, therefore, that the lesions resulting from the inoculation of killed or viable acid-fast bacilli into mice are not specific for the organism injected. While it is realised that infection in a laboratory animal does not necessarily follow the same course as that in the natural host, the use of mice as an experimental animal in M. johnei infection depends on its ability to reproduce intestinal lesions following infection.

Conclusions

M. johnei persisted in the viscera of mice for up to 17 months after intraperitoneal inoculation. Infection of mice with the avian tubercle bacillus caused an infection of like duration. Large numbers of organisms were recovered for the livers, mesenteric lymph nodes and small intestines of the mice infected with the avian tubercle bacillus. Histological examination of these tissues showed an endothelial cell reaction of varying degree.

Section 2

A comparison of the behaviour of three strains of M. johnei in mice

Introduction

This experiment was carried out to see if any difference could be detected in the ability of three strains to persist or multiply in mouse tissues. While these properties would not be indicative of their virulence for cattle or sheep, this information would be of value in the classification of strains of M. johnei. While M. johnei is known to survive in mouse tissues for up to 20 months (Lominski et al., 1956) a much shorter period of observation would be necessary to be of value as a routine for classification.

Mammalian tubercle bacilli show a wide variation of behaviour in animal tissues - from extreme virulence to the inability to multiply (Dubos, 1955).

Review of the Literature

Lominski et al. (1956) did not report any variation in the pathogenicity for mice of eight strains of M. johnei but, since there were variations in the dose, route of administration and numbers of mice examined, only marked differences would have been evident. In discussing the failure of previous workers to produce intestinal lesions in mice, they postulated that one of the factors concerned might be a variation in virulence of the strains of M. johnei used.

Stavitsky and Beck (1946) reported that a strain of M. johnei which grew in the absence of the phlei growth factor had the ability to multiply in chick embryos and that exacting strains did not survive nine days in the embryos. The identity of this non-exacting strain is questionable. Although strains of M. johnei can be trained to grow in the absence of the growth factor, dependance on the growth

factor is the most important *in vitro* characteristic of *M. johnei*.

Materials and Methods

The following strains of *M. johnei* were used:-

Strain VB/4/7. This was a strain of bovine origin which had undergone seven subcultures on Williams Smith's medium. It still required the phlei factor for growth.

Strain B/S1/3 was isolated from a clinical case of Johne's disease in a sheep which had been infected experimentally with an isolate from a clinically affected cow.

Strain S/S1/3 was isolated from a clinical case of Johne's disease in a sheep inoculated with a sheep strain of *M. johnei*. It was non-pigmented.

The Inocula. The organisms were harvested by scraping off the growth from six-week old cultures on Williams Smith's solid medium. The organisms were triturated in Williams Smith's liquid medium using a Griffith tube. The suspension was allowed to stand for 30 minutes to allow the larger aggregates of bacilli to deposit. The supernatant suspension was pipetted off and standardised to the opacity of Brown's tube 1. Viable counts were obtained by sowing known volumes of tenfold dilutions of the standardised suspension onto the dried surface of slopes of Williams Smith's solid medium.

Mice. The mice used in this experiment were W Swiss, Moredun stock strain.

Dose. One tenth ml. volumes of a Brown's tube 1 suspension of each strain were inoculated intravenously by a tail vein.

Viable counts of the strains of *M. johnei* in mice

Pools of tissues from two groups of five mice per strain were

examined at one and four months after infection. Groups of five mice were maintained in metal cages. Water and a balanced, pelleted ration were available ad. lib. The mice were killed using ether. The spleens and mesenteric lymph nodes from each group of five mice were removed aseptically and each pool weighed. Twenty per cent. weight/volume suspensions were made in sterile distilled water using a micro-homogeniser. Two 1.0 ml. aliquots of this suspension were diluted in Williams Smith's liquid medium in tenfold steps. One tenth of a millilitre of each dilution was sown onto the dried surface of Williams Smith's solid medium slopes in McCartney bottles. The slopes, stoppered with cotton wool plugs, were allowed to lie horizontally overnight at room temperature. They were then sealed with screw-caps having rubber washers. Incubation was at 37°C. for eight weeks.

The small and large intestines were opened, washed in two changes of sterile distilled water and homogenised in five ml. of sterile distilled water per pool of five intestines. Five millilitres of 10 per cent. oxalic acid solution were added and the mixture incubated at 37°C. for 30 minutes. A further five ml. of sterile distilled water were added. Two one ml. aliquots were removed, diluted by tenfold stages and inoculated onto slopes as described above.

Counts of the colonies on each slope were made after eight weeks' incubation under aerobic conditions.

The calculation of the viable units in the suspensions of tissue pools

Counts of the number of colonies were made at each dilution and

the mean for the two aliquots found. The count used for the final calculation of viable units was that obtained at the dilution giving colony counts of between 10 and 100.

The count of viable units per ml. of tissue suspension was obtained by multiplying the mean number of colonies 'n', by the dilution factor and by 10 since 0.1 ml. volumes were inoculated onto each slope.

Viable units/ml. of suspension = n x dilution of slopes counted x 10.

Results

Viable counts on the inocula prepared from the three strains showed that the suspensions each contained 10^{10} viable units.

The viable counts of M. johnei in the pools of mouse tissues are shown in Tables 26 and 27. The counts are expressed as the number of viable units per millilitre of suspension of the pooled tissues from five mice. Viable unit counts are given for the spleen, mesenteric lymph nodes, small and large intestines from two groups of mice for each of three strains of M. johnei tested. The results in Table 26 were obtained from counts made on mice examined one month after inoculation and those in Table 27 were obtained from counts made on mice examined four months after inoculation.

In the mice killed one month after inoculation no counts of spleen pools were obtained due to there being confluent growths at the highest dilutions made (10^{-3}). At the next examination 10^{-4} dilutions were made and satisfactory counts obtained.

At the four-month examination of Pool A of mesenteric lymph nodes of mice inoculated with strain VB4/7 and of Pool A of the small

intestines of the same mice, no counts were obtained due to all the tubes inoculated being overgrown with contaminants (Table 27).

Table 26. Viable counts/ml. of tissue suspension of M. johnei in mice one month after i/v inoculation

		Strain inoculated		
		VB4/7	$B/S_1/3$	$S/S_1/3$
Spleen	Pool A	All confluent growths at highest (thousandfold) dilutions made		
	Pool B			
Mesenteric lymph node	Pool A	170,000	680,000	250,000
	Pool B	215,000	520,000	230,000
Small intestine	Pool A	300	1,050	700
	Pool B	300	450	900
Large intestine	Pool A	400	1,250	800
	Pool B	400	850	400

Table 27/

Table 27. Viable counts/ml. of tissue suspension of M. johnei in mice four months after i/v inoculation

		Strain inoculated		
		VB4/7	$B/S_1/3$	$S/S_1/3$
Spleen	Pool A	$12,400 \times 10^3$	$16,000 \times 10^3$	$16,950 \times 10^3$
	Pool B	$11,300 \times 10^3$	$13,000 \times 10^3$	$18,600 \times 10^3$
Mesenteric lymph node	Pool A	Contaminated	$1,355 \times 10^3$	565×10^3
	Pool B	$1,475 \times 10^3$	$1,970 \times 10^3$	$1,665 \times 10^3$
Small intestine	Pool A	Contaminated	5,600	4,350
	Pool B	3,850	12,500	3,650
Large intestine	Pool A	3,300	7,650	1,950
	Pool B	5,850	4,100	4,600

The counts of M. johnei obtained from the spleens were in the order of 11 - 16 million per ml. of tissue suspension; those from the mesenteric lymph nodes were in the range 170×10^3 - $1,970 \times 10^3$, and those for the small and large intestines ranged from 300 viable M. johnei per ml. of homogenate to 12,500/ml.

Discussion

By using the methods described it was possible to compare the

behaviour of the strains of M. johnei in duplicate at each examination and also to compare the counts obtained one and four months after inoculation.

Comparing the viable counts in the mesenteric nodes, large and small intestines for the three strains there were higher counts in the tissues from mice inoculated with strain $S/S_1/3$ than in tissues from mice inoculated with strain VB4/7. The counts for strain $B/S_1/3$ were higher still. However these differences were within the range of experimental error. Although the inocula were standardised to a given opacity and there were no differences in viable counts on them, there would be slight variations in the numbers of bacteria in the clumps of organisms in each suspension, variations which might not be detected by viable count techniques but which might have a bearing on the survival of the inocula in vivo.

There were no significant differences between the counts of the strains four months after inoculation. While one strain, VB4/7, was a much quicker growing one on laboratory media than the other two, there was no apparent difference in its behaviour from the other two strains.

Comparison between the counts obtained from the two pools of tissues from five mice showed good correlation, with a few exceptions. Differences in the susceptibility of individual mice were probably not an important factor. Any differences present might account for the variation between the pools.

No conclusive evidence of multiplication of the inocula was obtained by comparing counts on the inocula themselves with counts from the tissues at one and four months later. In no instance were the counts at the second examination less than those at the first. There was a proportionately greater increase in the counts from the small and large intestines

compared with that in the mesenteric lymph node in the case of strain VB4/7 over the two examinations. In the case of strain B/S1/3, the mesenteric node counts doubled while there was a twelve-fold increase in the counts from the small intestine pools. This might indicate that, even in the non-susceptible host, the intestinal wall is the predilection site for the survival or even multiplication of M. johnei.

It is not known whether the inocula survived in the mouse tissues without multiplying or whether the numbers of bacteria remained relatively static due to a balance between destruction by the defence mechanisms of the host and very slow reproduction by the bacteria.

The results given here, especially with reference to strain VB4/7, suggest that a low grade multiplication occurred but the differences in counts were not sufficient to be conclusive.

In this experiment the dose used may have been too large to make any differences in the ability of the various strains to survive obvious, but in the first instance it was necessary to ensure that all mice were infected so that variations between mice in the pools would be minimised.

Conclusion

No differences could be detected in the behaviour in mice of three strains of M. johnei of widely differing origins.

P A R T III

THE IDENTIFICATION OF STRAINS OF MYCOBACTERIA ISOLATED FROM CATTLE AND MICE DURING THE STUDIES DESCRIBED IN PARTS I AND II

Introduction

The undernoted strains were examined because they did not resemble *M. johnei* in colonial morphology and did not require the phlei growth factor on subculture from the primary isolate. The identification of these strains rested on their pathogenicity for laboratory animals and their cultural characteristics.

Materials and Methods

The strains examined and their sources and characteristics on primary isolation are described below.

Strain C21. This strain was isolated from the faeces of a clinical case of Johne's disease. Colonies of acid-fast bacteria requiring the growth factor were isolated from the ileo-caecal lymph nodes of this animal at post-mortem examination. Histological examination revealed extensive lesions of Johne's disease in the intestinal wall. There were typical clumps of acid-fast bacteria in the faeces. The small number of minute colonies recovered from the faeces on primary isolation on the medium used, modified Lowenstein-Jensen, did not appear to be atypical, but on sub-culture growth of the strain was obtained on Lowenstein-Jensen medium prepared without the growth factor.

Strain W13. This strain was recovered from the ileo-caecal lymph node of a two and a half year old heifer. There was no evidence of Johne's disease being present. Primary growth on modified Lowenstein-Jensen medium gave colonies resembling those of *M. tuberculosis var. avium*.

Strain M. Strain M was recovered from a mouse in Experiment A (Part II, Section 1). On Williams Smith's solid medium it had the

colonial form of M. johnei.

Strain G. This strain was a typical example of the strains recovered from mice in Experiment B (Part II, Section 1). It had the morphology in culture of the avian tubercle bacillus.

In the examinations carried out on each of the above strains, single colonies were removed from the surfaces of the slopes having primary growths or subcultures of the strains to be tested. Each colony was suspended, using a Griffith tube, in Williams Smith's liquid medium. For tests involving cultural techniques, tenfold increasing dilutions of the suspensions were made in the same menstruum. Aliquots of these dilutions were sown onto the appropriate media. For animal inoculations the suspensions were prepared as above and standardised to correspond in opacity to Tube 1 of Brown's scale.

To test a strain's ability to grow in the absence of the phlei growth factor, strains were seeded onto slopes of Williams Smith's solid medium containing the growth factor and with the growth factor omitted. Incubation was at 37°C. under aerobic conditions.

The strains to be tested were inoculated onto nutrient agar slopes containing 4 per cent. glycerol to test their ability to grow on simple media.

To determine the optimum growth temperature for the strains, each strain was inoculated onto Williams Smith's solid medium and 4 per cent. glycerol agar and incubated at 26°C., 37°C. and 43°C. The cultures were examined daily and the time taken for visible growth to appear noted.

For biological tests nine-month old fowls reared in isolation,

adult guinea-pigs and three-day old Ayrshire bull calves from healthy dams were used.

Half a milligram of strains G and M were inoculated intramuscularly into one fowl respectively. The same dose of strain C21 was inoculated into another fowl intravenously. The fowls were observed daily. Another fowl from the same source as those used was kept under the same conditions. It remained healthy and at post-mortem examination no abnormalities were detected.

Suspensions of strains C21, G and M, containing 0.5 mgm. of organisms, were given to pairs of guinea-pigs, one of each pair receiving the inoculum intraperitoneally, the other receiving it subcutaneously at the inside of the thigh. Four weeks after inoculation the guinea-pigs were tested intradermally with 0.1 ml. P.P.D. Weybridge avian and mammalian tuberculins. The tests were read 48 and 72 hours later.

Two three-day old bull calves were given 10 mg. wet weight of strain M intravenously. This inoculum was prepared and inoculated simultaneously with the inoculum used to infect the mice in Experiment A (Part II, Section 1). One calf died three weeks after inoculation due to intercurrent omphalophlebitis. The remaining calf was housed in isolation till it was slaughtered at 18 months of age. It had remained healthy. Post-mortem examination was carried out using the techniques described in Part I, Section 1.

Results

The results of tests carried out on the four strains of bacteria are shown in Table 28.

Table 28. The results of cultural and biological tests on four strains of acid-fast bacilli encountered during these studies

Strain	Growth in absence of growth factor	Opt. temp. for growth			Growth on glycerol agar	Pathogenicity for exptl. animals		
		26°C	37°C	43°C		guinea-pig	fowl	calf
W13	+				+	-		
C21	+	-	14^x	10^x	+	-	+	
G	+	-	14^x	10^x	+	-	+	
M	-	-	21^x	-	-	-	-	+

+ = growth present or pathogenic

- = no growth or non-pathogenic

14^x = growth visible in days

no result = not done

Strain C21. Strain C21 grew well on Williams Smith's solid medium which had been prepared without the growth factor, colonies being visible after 14 days' incubation at 37°C. and 10 days' incubation at 43°C. The colonies were round, raised, non-pigmented, 0.5 - 1 mm. in diameter with discrete edges, and had dense centres with less dense peripheries. Growth was obtained on glycerol agar medium. At post-mortem examination of the guinea-pigs eight weeks after inoculation with this strain, the only abnormality detected was the presence of pea-sized abscesses at the sites of inoculation. In Ziehl-Neelsen stained smears of pus from these abscesses a few long slender acid-fast bacilli were found on microscopical examination. Comparative tuberculin tests were carried out on the

guinea-pigs one month after infection. There was a marked sensitivity to the intradermal injection of avian tuberculin and only a slight reaction to mammalian tuberculin. The fowl which received this strain died in an emaciated condition four weeks after inoculation. At post-mortem examination the typical nodules of avian tuberculosis were found in the liver, spleen and peritoneum. Smears from these nodules were found to contain numerous pleomorphic acid-fast bacilli. An organism with all the characteristics of the avian tubercle bacillus was isolated from the liver of this fowl.

It was considered that strain C21 fulfilled the criteria for its identification as M. tuberculosis var. avium.

Strain W13. On primary isolation this strain had the colonial morphology of the avian tubercle bacillus. It grew within three weeks in the absence of the phlei growth factor and on glycerol agar at 37°C. It was not pathogenic for the guinea-pigs inoculated but sensitised them to intradermal P.P.D. avian tuberculin. At post-mortem examination of the guinea-pigs no evidence of a generalised infection was present as would have been the case if the inoculum had contained mammalian tubercle bacilli. No fowl pathogenicity tests were carried out. This strain can tentatively be described as M. tuberculosis var. avium.

Strain M. No growth of this strain was obtained after three months' incubation on glycerol agar medium and on Williams Smith's solid medium prepared without the growth factor. Visible colonies were obtained after 21 days' incubation at 37°C. on media containing the growth factor, but no growth was visible after incubation at 26°C.

or 43°C. The fowl which received this strain was killed twelve weeks after inoculation. No abnormalities were detected at post-mortem examination. Cultures of the spleen and liver on Williams Smith's solid medium remained sterile after three months' incubation at 37°C. This strain sensitised guinea-pigs to P.P.D. avian tuberculin intradermally but not to mammalian tuberculin, when tested four weeks after inoculation. The guinea-pigs remained healthy. No abnormalities were detected at post-mortem examination twelve weeks after inoculation. At post-mortem of the remaining one of two calves injected intravenously with strain M eighteen months previously, no macroscopic lesions of Johne's disease were detected. However, an organism having all the characteristics of M. johnei was recovered from the mesenteric lymph nodes. Histological examination of the ileal mucous membrane showed an endothelioid and giant cell reaction. Acid-fast bacilli were found within these giant cells on examination of Ziehl-Neelsen stained sections. It is concluded this calf was infected with M. johnei and that these lesions were the result of the inoculation of Strain M.

Although clinical disease was not produced as a result of the administration of the strain to the calf, the findings described in it, and the strain's rigid requirement of the growth factor provided by M. phlei are thought sufficient to identify strain M as being M. johnei.

Strain G. The cultural and biological findings in relation to this strain were exactly as those described above for strain C21. It was therefore identified as a strain of M. tuberculosis var. avium.

Discussion

A point of some importance is illustrated by the isolation of

strain C21 from the faeces of a cow. It was not possible to distinguish the colonies obtained on primary isolation from those of M. johnei. The medium used was modified Lowenstein-Jensen. Its identity was only suspected when, on routine sub-culture onto media without the growth factor, growth was obtained. These findings emphasise the importance of all isolates provisionally identified as M. johnei being sub-cultured and examined for dependence on the growth factor and inability to grow on simple media. The use of the transparent Williams Smith's medium has many advantages, the main advantages being that early growth is much more easily detected than when opaque media are used, and that colonial morphology can be studied conveniently by transmitted light.

DISCUSSION

The significance of these studies on M. johnei infection in cattle and mice is discussed below.

The main problem associated with Johne's disease is one of diagnosis, especially diagnosis of infection before the appearance of clinical signs of the disease. The studies described here have failed to elucidate this problem, but the accuracy of the diagnosis of clinical disease in the cattle examined has been improved by the use of the methods described. The finding of single acid-fast bacilli on microscopical examination of faecal smears from clinically suspicious, wasting cattle could be correlated with M. johnei infection in these cattle, thus increasing the accuracy of the examination of faeces as a diagnostic test for Johne's disease. Early infection with M. johnei could not be diagnosed by examination of faeces from apparently normal cattle. The complement fixation test was found to be a valuable one for confirming the presence of clinical disease. When this test was applied to apparently normal cattle a high proportion of false positive results were obtained from cattle from which M. johnei could not be isolated by the methods described. At the present state of knowledge, the use of this test to attempt to detect early infection is not advisable.

Due to the method of sampling used, no accurate information on the incidence of clinical Johne's disease in Southwest Scotland was obtained but the results showed that Johne's disease was an important cause of the culling of cattle from dairy herds in that area. In the cattle examined, cattle under four years of age were more likely to be

wasting due to Johne's disease than cattle over that age. Johne's disease was the predominant cause of diarrhoea in the cattle exhibiting this sign.

Of importance in the epidemiology of Johne's disease was the fact that, while congenital infection was shown to occur, the incidence of M. johnei infection in young calves was found to be low. This would indicate that control measures such as vaccination or segregation of cattle on heavily infected premises should be carried out at an early age when the infection rate is low and before dissemination of infection from congenitally infected calves has taken place.

A review of the literature and the results of the experiments described in Part II with reference to the non-specificity of the lesions produced, the insusceptibility of the strains of mice used, and the identical behaviour of three strains of M. johnei in mice, indicated that the mouse was of limited value as an experimental animal in research into M. johnei infection. A more susceptible laboratory animal or a more susceptible strain of mice should be sought.

The importance of cultural and biological tests in the identification of cultures of acid-fast bacilli suspected of being M. johnei is discussed in Part III. M. tuberculosis var. avium was isolated from the faeces of a cow suffering from Johne's disease.

ACKNOWLEDGMENTS

I wish to thank Professor W. L. Weipers for encouragement throughout these studies, Drs. W. I. M. McIntyre and W. H. F. Jarrett for many helpful discussions, Mr. M. P. Cunningham who made available results used in this work, and Mr. Crawford Fergusson for invaluable technical assistance. Thanks are also due to Dr. J. T. Stamp and Mr. J. G. Brotherston for advice and permission to use the results of work undertaken at Moredun Institute.

REFERENCES

Albiston, H. E., and Talbot, R. J. de C. (1936). Aust. vet. J., 12, 125.

Alexejeff-Goloff, N. A. (1929). Z. InfektKr. Haustiere, 36, 313; abs. in J. comp. Path., 48, 8.

Anon. (1956). 'Control of Johne's Disease', Proj. 207, European Productivity Agency, Paris, 1956. p.37.

Appleby, E. C., and Head, K. W. (1954). J. comp. Path., 64, 52.

Armstrong, M. C. (1956). N.Z. vet. J., 4, 56.

Bang, B. (1906). Berl. tierarztl. Wschr., 42, 759.

Bang, O., and Andersen, C. W. (1913). Zbl. Bakt. Abt. I Orig., 69, 517.

Bergey, D. H. (1948). 'Manual of Determinative Bacteriology', 6th Ed., Baillière, Tindall & Cox, London. p. 881.

Boquet, A. (1925). C. R. soc. Biol. Paris, 93, 219.

Borodenok, A. I. (1941). Veterinariya, Moscow, 3, 62; abs. in Vet. Bull., 12, 570.

Burggraaf, H. (1931). Ned.-Indisch. Blad. v. Diergeneesk., 43, 359.

Brotherston, J. G. (1959). Personal communication.

Cameron, J. (1956). J. Path. Bact., 71, 223.

Cassamagnaghi, A. and A. (1947). Ann. Fact. Vet. Montevideo, 5, 83.

Cauchemez, L., and Sergent, G. (1934). Bull. Acad. vet. Fr., 7, 372.

Chandler, R. L. (1955). N.Z. vet. J., 3, 145.

Chandler, R. L. (1956). Ibid., 4, 121.

Chandler, R. L. (1958). Brit. vet. J., 114, 30.

Chandler, R. L. (1959). Vet. Rec., 71, 147.

Cooper, H., and Scrinivasan, M. K. (1931). Ind. J. vet. Sci., 1, 215.

Corper, H. J., and Uyei, N. (1929-30). J. Lab. clin. Med., 15, 348.

Cunningham, M. P. (1958). Personal communication.

Cunningham, M. P., and Gilmour N. J. L. (1959). Vet. Rec., 71, 47.

Déom, J., and Mortelmans, J. (1954). Bull. Epiz. Dis. Afr., 2, 11; abs. in Vet. Bull., 25, 541.

Downham, K. D. (1933). Vet. Rec., 13, 39.

Doyle, T. M. (1945). Ibid., 57, 385.

Doyle, T. M. (1953). Ibid., 65, 363.

Doyle, T. M. (1954). Brit. vet. J., 110, 215.

Doyle, T. M. (1956). Vet. Rec., 68, 869.

Doyle, T. M. (1958). Ibid., 70, 238.

Doyle, T. M. (1959). 'Diseases due to Bacteria', ed. by A. W. Stableforth and I. A. Galloway, Butterworth's Scientific Publications, London, 1959. p. 319.

Doyle, T. M., and Spears, H. N. (1951). Vet. Rec., 63, 355.

Dubos, R. J. (1955). 'Mechanisms of Microbial Pathogenicity', ed. by J. W. Howie and A. J. O'Hea, Cambridge U.P., 1955. p.103.

Dubos, R. J., and Middlebrook, G. (1947). Amer. Rev. Tuberc., 56, 334.

Dunkin, G. W. (1928). J. comp. Path., 41, 94.

Dunkin, G. W. (1935). Ibid., 48, 36.

Dunkin, G. W. (1938). Rep. 1st Imp. Vet. Conference, London. 68.

Eveleth, D. F., and Gifford, Rebecca (1943). J. Amer. vet. med. Ass., 102, 27.

Farrow, G. (1831). Veterinarian, 4, 316.

Finlayson, M. K. (1946). J. Path. Bact., 58, 88.

Ford, E. J. H. (1957). Ibid., 73, 363.

Fosset, A. J. de (1936). J. Amer. vet. med. Ass., 88, 352.

Francis, J. (1943). J. comp. Path., 53, 140.

Francis, J., McTurk, H. M., Madinaveitia, J., and Snow, G. A. (1953). Bioch. J., 55, 596.

Gislason, G. (1956). 'Control of Johne's disease', Proj. 207, European Productivity Agency, Paris. p. 121.

Glover, R. E. (1941). Brit. vet. J., 97, 3.

Gunning, O. V. (1933). Vet. Rec., 13, 40.

Hagan, W. A. (1938). Cornell Vet., 28, 34.

Hagan, W. A., and Levine, P. (1932). J. Amer. vet. med. Ass., 81, 723.

Hagan, W. A., and Mansfield, H. L. (1930). Ibid., 76, 182.

Hagan, W. A., and Zeissig, A. (1933). Ibid., 82, 391.

Hales, -. (1831). Veterinarian, 4, 437.

Hallman, E. T., and Witter, J. F. (1933). J. Amer. vet. med. Ass., 83, 159.

Harding, H. P. (1957). J. comp. Path., 67, 180.

Heath, G. B. S. (1955). Vet. Rec., 67, 980.

Hebert, C. N., Doyle, T. M., and Paterson, A. B. (1959). Ibid., 71, 108.

Hirch, Ann., and Lawrence, W. E. (1954). J. comp. Path., 64, 102.

Hole, N. H. (1952a). Proc. roy. Soc. Med., 45, 481.

Hole, N. H. (1952b). Vet. Rec., 64, 601.

Hole, N. H. (1953). Proc. 15th Int. Vet. Cong., 1, 173.

Hole, N. H. (1956a). Brit. vet. J., 112, 272.

Hole, N. H. (1956b). 'Control of Johne's Disease', Proj. 207, European Productivity Agency, Paris, 1956. p. 91.

Houthuis, M. J. J. (1932). Vet. Rec., 12, 1488.

Howarth, J. A. (1932). J. Amer. vet. med. Ass., 81, 383.

Howarth, J. A. (1937). Cornell Vet., 27, 223.

Jansen, J. (1948). J. Amer. vet. med. Ass., 112, 52.

Johne, H. A., and Frothingham, L. (1895). Dtsch. Z. Tiermed. Path., 21, 438.

Johnson, H. W., and Cox, B. F. (1942). Am. J. vet. Res., 3, 131.

Johnson, E. P., and Pratt, E. D. (1944). Ibid., 5, 117.

Johnstone, D. (1933). Vet. Rec., 13, 1388.

Larsen, A. B., Porter, D. A., and Vardaman, T. H. (1953). Am. J. vet. Res., 14, 362.

Larsen, A. B., and Vardaman, T. H. (1952). Ibid., 13, 466.

Larsen, A. B., and Vardaman, T. H. (1953). J. Amer. vet. med. Ass., 122, 309.

Larsen, A. B., Vardaman, T. H., and Groth, A. H. (1950). Am. J. vet. Res., 11, 374.

Lawrence, W. E. (1956). Vet. Rec., 68, 312.

Levi, M. L. (1948a). Ibid., 60, 336.

Levi, M. L. (1948b). J. comp. Path., 58, 38.

Levi, M. L. (1950). Ibid., 60, 10.

Lominski, I. R. W., Cameron, J., and Roberts, G. B. S. (1956). J. Path. Bact., 71, 211.

Lovell, R., Levi, M. L., and Francis, J. (1944). J. comp. Path., 54, 120.

McEwen, A. D. (1939). Ibid., 52, 69.

McEwen, A. D., and Samuel, I. M. (1958). Ibid., 68, 45.

M'Fadyean, Sir J. (1906). J. roy. Agr. Soc., England, 67, 235.

M'Fadyean, Sir J. (1907). J. comp. Path., 20, 48.

M'Fadyean, Sir J. (1918). Ibid., 31, 73.

M'Fadyean, Sir J., and Sheather, A. L. (1916). Ibid., 29, 201.

M'Fadyean, Sir J., Sheather, A. L., and Edwards, J. T. (1912). Ibid., 25, 217.

M'Fadyean, Sir J., Sheather, A. L., and Edwards, J. T. (1915). Ibid., 28, 196.

McIntosh, C. W. (1939). Canad. J. comp. Med., 3, 182.

Matthews, F. P. (1930). J. Amer. vet. med. Ass., 76, 248.

Michael, D. T. (1946). Vet. Rec., 58, 297.

Minett, F. C. (1942). J. Path. Bact., 54, 209.

Mohler, W. M. (1939). J. Amer. vet. med. Ass., 94, 500.

Pande, P. G. (1942). Ind. J. vet. Sci., 12, 160.

Pearson, J. K. L. (1953). Irish vet. J. Nov. cited by Rankin, J. D., (1957). Thesis University of Reading.

Pearson, J. K. L., and McClelland, T. G. (1955). Vet. Rec., 67, 615.

Phillip, R. C. T. (1942). Tasmanian J. Agric., 13, 138.

Polyakova, O. A. (1957). Veterinariya, Moscow, 34, 62; abs. in Vet. Bull., 28, 284.

Posokhin, E. G. (1940). Sovyet. Vet. 11/12, 11; abs. in Vet. Bull., 12, 569.

Rankin, J. D. (1953). Vet. Rec., 65, 649.

Rankin, J. D. (1954). Ibid., 66, 550.

Rankin, J. D. (1955a). Ibid., 67, 1105.

Rankin, J. D. (1955b). Brit. vet. J., 111, 480.

Rankin, J. D. (1957). Thesis University of Reading.

Rankin, J. D. (1958a). Vet. Rec., 70, 383.

Rankin, J. D. (1958b). Ibid., 70, 693.

Rankin, J. D. (1958c). J. Path. Bact., 75, 363.

Report (1956a). Report of Animal Health Services, H.M.S.O. London, 1958. p. 35.

Report (1956b). 'Control of Johne's Disease', Proj. 207, O.E.E.C. Paris.

Rinjard, P. (1934). Bull. Off. Int. Epiz., 8, 326.

Ritchie, J. N., Robertson, A., and Muir, R. O. (1952). Cited in Vet. Rec., 71, 1156.

Robinson, E. M., and De Kock, G. (1939). Farming S. Africa, 14, 308.

Rosenbusch, F. (1937). J. agron. vet. Univ. B. Aires, 15; abs. in Vet. Bull., 13, 119.

Sahai, L. (1940). Vet. J., 96, 407.

Sahai, L. (1941). Ibid., 97, 101.

Sheather, A. L. (1927). J. comp. Path., 40, 224.

Sigurdsson, B. (1945). J. Immun., 51, 279.

Sigurdsson, B. (1956). 'Control of Johne's Disease', Proj. 207, European Productivity Agency, Paris. p. 169.

Smith, H. Williams, (1953). J. Path. Bact., 66, 375.

Smith, H. Williams (1954). J. Path. Bact., 68, 367.

Smith, H. Williams (1958). Ibid., 76, 201.

Smythe, R. H. (1935). Vet. Rec., 15, 85.

Soltys, M. A. (1951). Ibid., 63, 492.

Spears, H. N. (1959). Ibid., 71, 1154.

Stamp, J. T. (1956). 'Control of Johne's Disease', Proj. 207, European Productivity Agency, Paris. p. 189.

Stamp, J. T., and Watt, J. A. A. (1954). J. comp. Path., 64, 26.

Stavitsky, A. B., and Beck, J. D. (1946). Am. J. vet. Res., 7, 470.

Stephens, H. E., and Gill, D. A. (1937). N.Z. J. Agric., 54, 1.

Stewart, J., McCallum, J. M., and Taylor, A. W. (1945). J. comp. Path., 55, 45.

Stockman, S. (1911). Ibid., 24, 66.

Strogov, A. K. (1957). Trud. vses. Inst. eksp. Vet., 20, 120; abs. in Vet. Bull., 28, 484.

Stuart, P. (1954). Vet. Rec., 66, 305.

Taylor, A. W. (1940). Vet. J., 96, 415.

Taylor, A. W. (1949). Vet. Rec., 61, 539.

Taylor, A. W. (1950). J. Path. Bact., 62, 647.

Taylor, A. W. (1951). Ibid., 63, 333.

Taylor, A. W. (1952). Vet. Rec., 64, 603.

Taylor, A. W. (1953). J. comp. Path., 63, 368.

Twort, C. C., and Craig, T. (1913). Zbl. Bact. Orig., 68, 455.

Twort, F. W. (1910). Proc. roy. Soc. B., 83, 156.

Twort, F. W., and Ingram, G. L. Y. (1912). Ibid., 84, 517.

Twort, F. W., and Ingram, G. L. Y. (1913). 'A monograph on Johne's Disease', Baillière, Tindall & Cox, London, 1913.

Vallée, H. (1924). C. R. Acad. Sci. Paris, 178, 152.

Vallée, H., and Rinjard, P. (1926). Rev. gén. Méd. vét., 35, 1.

Vallée, H., Rinjard, P., and Vallée, M. (1934). Rev. gén. Méd. vét., 43, 77.

Verlinde, J. D., and Bekker, J. H. (1945). Tijdsch. Diergeneesk., 70, 329; abs. Vet. Bull., 17, 403.

Vishnevskii, P. P., et al. (1940). Sovyet, Vet. 11/12, 89; abs. in Vet. Bull., 13, 5.

Vukovic, -. (1908). Quoted by Twort and Ingram (1913) loc. cit., p. 12.

Watt, J. A. A. (1954). Vet. Rec., 66, 387.

Williamson, G. T. and Salisbury, R. M. (1952-53). N.Z. vet. J., 1, 15.

Wilson, G. S., and Miles, A. A. (1955). Principles of Bacteriology Immunity, 4th Ed. Arnold, London, 1955, 1, 500.

Withers, F. W. (1959). Vet. Rec., 71, 1150.