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Update on paratuberculosis: 2. Pathology and diagnosis

This is the second in the series of papers adapted from the Johne's Information Centre website (<http://johne.org>), updated by MICHAEL T. COLLINS, President, International Association for Paratuberculosis.

Images: Courtesy of the Central Veterinary Research Laboratory, Abbotstown.



Cow with Johne's disease: emaciation, bottle jaw, diarrhoea.

Introduction

As stated in the first paper in this series (Collins, 2003), although clinical signs of paratuberculosis typically do not appear until infected animals are adult, the infection by *Mycobacterium paratuberculosis* begins in young animals. Infected cows are the primary source of infection for calves; the vast majority of infections are acquired by ingestion, in most cases through drinking contaminated milk. Tissue invasion is most likely to occur in the terminal ileum. The wall of the ileum contains a large number of aggregated lymphoid nodules (Peyer's patches), with the highest concentration in the terminal ileum and it is for this anatomical reason that tissue invasion is most prevalent here. Overlying the Peyer's patches are specialised M cells to which bacteria adhere selectively prior to

penetration into mucosa, where they are engulfed by macrophages, phagocytic cells that ingest and destroy most species of microbial invader. For reasons that are only partially understood, mycobacteria are able to resist killing by macrophages; they survive within the macrophages and they replicate, causing inflammatory changes that impair intestinal function leading to diarrhoea and loss of body condition.

Pathology

Early stages of infection

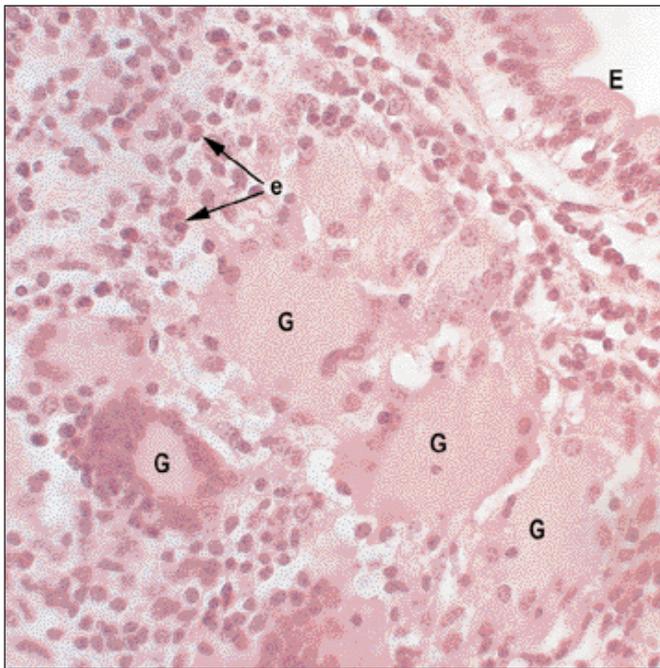
The immune system of the calf reacts to invasion by *M. paratuberculosis* by recruiting more macrophages and lymphocytes to the site of infection. The lymphocytes release a variety of chemical signals, called cytokines, in an attempt to increase the bacterial killing power of the macrophages and to recruit more cells to fight off the infection. The cytokine gamma-interferon is most likely to be detected at this time. Macrophages fuse together to form large multinucleated cells, called giant cells, in an apparent attempt to kill the mycobacteria.

Mid-stage of infection

Infiltration of infected tissues with millions of lymphocytes and macrophages progresses over years leading to visible thickening

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Photomicrograph of a section of intestine from a cow with Johne's disease.
E: intestinal epithelium; G: giant cells; e: eosinophils.

of the intestine. The mucosal surface becomes corrugated and granular in appearance. The ileum is no longer thin and pliable, and can not be easily stretched. The serosal surface of the ileum may be tracked with thickened and dilated lymphatics. These clearly visible tube-like vessels have thickened walls and are congested with lymphocytes, a phenomenon called cording. In infected cattle, lymph transports macrophages containing *M. paratuberculosis* to the local lymph nodes where they are trapped. Just as in Peyer's patches, the mycobacteria are undisturbed by the abundant macrophages in the lymph nodes and continue to multiply. As immune cells are recruited to fight the infection the lymph nodes become enlarged and pale in colour.

Most of the characteristic pathology of Johne's disease is limited to the intestinal tract and local lymph nodes. A particular finding in some cattle with Johne's disease is calcified plaques in the wall of the aorta.

Terminal stage of infection

In the last phases of this infection, the animal begins to produce circulating antibody against *M. paratuberculosis*, an antibody that does not provide protection against the multiplication of the mycobacteria. In fact, detection of antibody to *M. paratuberculosis* is an indicator that clinical signs of disease and death from the infection will soon follow. It is not known how often spontaneous recovery from infection occurs, if it occurs at all. It appears that most infections progress slowly and inevitably until the animal dies as a consequence of the pathological

changes in the intestine. The course of the infection may take so long that some affected animals are culled for other reasons or die from other causes.

When the ileum becomes thickened by granulomatous inflammation, the animal will develop a protein-losing enteropathy. It will have diarrhoea, impaired absorption of protein, and depletion of the body protein. In particular, there may be marked hypoalbuminaemia, which, in turn, impairs the ability of the animal to retain fluids within the vasculature leading to oedema, which may be most noticeable in the submandibular region as "bottle jaw".

During the final stage of this disease, *M. paratuberculosis* may disseminate beyond the ileum and associated lymph nodes. For instance, the organism can be cultured from tissue samples taken from organs such as liver, spleen, mammary gland and uterus of cattle in the late stage Johne's disease. During this late bacteraemic stage, tissue response to these bacteria is minimal and it is thought that the animal's immune system has essentially shut down and is no longer able to respond to the infection. At this stage of disease diagnostic tests usually detect high numbers of *M. paratuberculosis* in faeces, high concentrations of serum antibodies and little to no evidence of the gamma-interferon response

Lesions in infected sheep and goats

A range of pathology can be seen in infected sheep and goats. The likely sites of infection are the terminal ileum and mesenteric lymph nodes although neighbouring portions of the gastrointestinal tract (colon, jejunum) are frequently affected as well.

Grossly, there can be a complete absence of lesions - at necropsy, the intestine may appear entirely normal. In cases at the other extreme, the affected small intestine may appear to be enlarged and corrugated due to a massive invasion of the mucosa by inflammatory cells in response to invasion of the ileum by *M. paratuberculosis*. The local lymph nodes are enlarged and oedematous. There are cases with lesions that fall between these two extremes.

When the tissues are examined microscopically, again the animal may present a number of pathological pictures for both clinically and subclinically affected sheep. The tissues may appear normal in virtually every site. In another case, florid signs of infection such as numerous macrophages and giant cells packed with acid-fast rods may be present. Other cases may fall between these two extremes. The most likely site of infection is the terminal ileum, while other portions of the gastrointestinal tract (colon, jejunum) are frequently affected as well.

Diagnosis

Diagnosis of Johne's disease in cattle showing clinical signs (diarrhoea and weight loss) is not difficult. However, diagnosis of the infection in cattle that are clinically normal is challenging. Proper test selection, application, and interpretation are vital.

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TABLE 1: Sensitivity on a set of 142 repository serum samples from cows with subclinical Johne's disease

	CULTURE METHODS		BLOOD TEST METHODS		
	Traditional	Automated (BACTEC)	AGID (ImmuCell)	ELISA (Biocor)	ELISA (IDEXX)
Sensitivity	41.5%	51.4%	26.8%	42.3%	45.8%

How many types of tests are there for Johne's disease?

There are two types of tests for Johne's disease in routine use today:

- tests on manure samples that find *Mycobacterium paratuberculosis* (faecal culture) or its DNA (PCR test);
- tests that measure antibodies in blood.

To test manure samples, most laboratories use traditional culture methods. In addition, a few laboratories use a commercial, automated culture system called the BACTEC system. This system, adapted from technology used to diagnose tuberculosis in humans, involves both a commercial culture medium and a machine that "reads" the cultures.

Standard bacterial culture takes at least 16 weeks to complete because of the extremely slow growth rate of *M. paratuberculosis*. A problem with this test is that strains of *M. paratuberculosis* from sheep and perhaps other non-bovine species may fail to grow on standard culture media. BACTEC culture is a radioisotope-based detection method. The main advantages of this method are that it can detect low numbers of *M. paratuberculosis*, and it can detect the bacterium faster than standard culture methods (eight weeks compared to 16 weeks). In addition, it can grow *M. paratuberculosis* from a wide variety of animal species, including sheep.

Detection of serum antibody to *M. paratuberculosis* is good evidence the animal is infected. Moreover, for those assays producing quantitative results, the magnitude of the test results (a measure of the level of serum antibody) is directly related to the probability that the animal is infected. There are three techniques for detection of serum antibodies in common use today: complement fixation (CF), agar-gel immunodiffusion (AGID), and enzyme-linked immunosorbent assay (ELISA). Fundamentally, all three tests are measuring the same thing, antibodies to *M. paratuberculosis*, but they use different types of technology and different reagents.

There is a third category: tests for cellular immunity. Cell-mediated immune responses are considered to be the first and most important response of animals to infection with mycobacteria. A long-standing way of measuring cell-mediated immune responses is skin testing, a technique that has been valuable for diagnosing tuberculosis in humans and animals. Early studies indicated skin testing did not work well for diagnosis of Johne's disease; however, investigators who have re-evaluated this question suggest that skin testing may indeed be valuable. More studies are needed to substantiate this

finding.

A newer, more sophisticated, laboratory test for cellular immunity to mycobacteria is done on blood samples and measures gamma-interferon released from white blood cells of infected animals. The assay is now available as a diagnostic kit for cattle. Reports from scientific meetings indicate that this test will be a useful addition to the laboratory tools for diagnosis of *M. paratuberculosis* infections in a variety of species.

How are tests for Johne's disease compared for accuracy?

There are two measures of accuracy most often used to compare diagnostic tests: *sensitivity* and *specificity*.

Test specificity is a measure of the percentage of times a test result is negative for non-infected animals (how well the test correctly identifies uninfected animals). Available blood tests for Johne's disease have a high specificity: 97% to 99% and culture-based tests are considered 100% specific (i.e., no false-positive tests). In general terms, this means that 97 to 99% of the time when a blood test is positive, the diagnosis of Johne's disease is right.

Barring rare laboratory contamination or some on-farm mistake in sample collection, a positive faecal culture correctly diagnoses Johne's disease 100% of the time: i.e., culture is 100% specific - no false-positives. Some experts might argue with this, but it is basically true. Exceptions mostly occur in very heavily infected herds.

Test sensitivity is a measure of the percentage of times a test result is positive for infected cattle (how well the test correctly identifies infected animals). Subtracting test sensitivity from 100% gives you the percentage of infected cattle missed by the test (false-negative result).

Maximising test sensitivity is the biggest challenge for Johne's disease tests.

How do tests for Johne's disease compare for accuracy?

Evaluating tests for Johne's disease takes a long time and is quite expensive because of the chronic nature of the infection. It makes sense to pool resources and to compare as many tests as possible on the same cows.

In the mid-1990s the University of Wisconsin, School of Veterinary Medicine created a repository of samples with which to evaluate diagnostic tests for Johne's disease. Manure and blood samples were collected from over 170 cows that were clinically normal but infected with *M. paratuberculosis* and from

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196 cows in Wisconsin certified-free dairy herds. This was later supplemented with serum samples from over 500 additional US cattle free of the infection and 2,700 Dutch cattle in certified-free herds. The serum and manure samples from these cows were saved in a repository so that tests developed in the future could also be evaluated. These samples have been shared with commercial and academic researchers around the world. Although the supply of material is almost exhausted, these same samples have been used to evaluate some of the newer tests that came on the US market in the last few years. The findings are reported here.

Table 1 shows test sensitivity on a set of 142 repository serum samples from cows with subclinical Johne's disease.

Both ELISA sensitivities were essentially the same, twice as high as the AGID and the automated culture method was more sensitive (gave a positive result for a truly infected cow) than the traditional culture method.

Figure 1 shows the pattern of results for the two most sensitive tests applied to 142 cows. Fifty-one (35.9%) of the cows were positive on both the BACTEC culture method and the IDEXX ELISA, 22 cows (15.5%) were positive on BACTEC only, 14 cows (9.9%) were positive on the IDEXX ELISA only, and 55 cows (38.7%) were negative on both tests.

If you used both tests at the same time on all cows and consider cows to be infected if they test positive on either test, you would correctly diagnose Johne's disease in 87 (61.3%) of the cows. This figure of 61.3% represents the maximum sensitivity you can get using the two most sensitive tests available at the same time. The infected cows missed by both tests (false-negative results) were simply at an early stage of infection when they are not yet producing the element the test sought to detect. I call these "currently undiagnosable" - these cows would be detected later when they begin to produce the antibody or to excrete the organism.

If you consider only the 87 cows that were positive on either the BACTEC faecal culture or ELISA, 65 (74.7%) of the

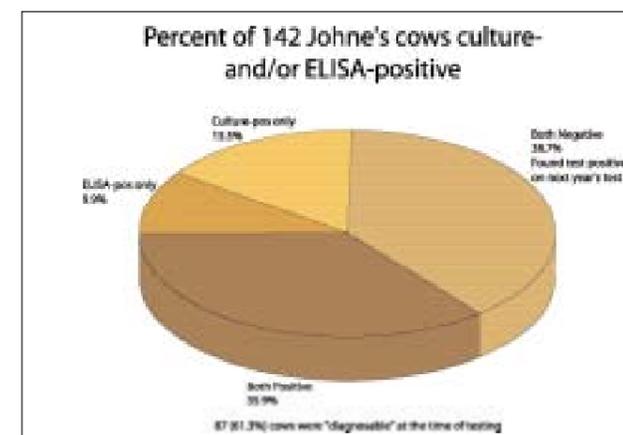


FIGURE 1: The pattern of results for the two most sensitive tests applied to 142 cows.

"diagnosable" cases of Johne's disease were ELISA-positive and 51 of them (58.6%) were also faecal culture-positive.

Faecal culture is often called a confirmatory test. Many people misunderstand what this means. If an ELISA-positive cow also tests faecal culture-positive you can be certain the cow has Johne's disease: the diagnosis by ELISA was confirmed by culture. However, the data shown in the pie chart also illustrate an important lesson. If a faecal culture done on an ELISA-positive cows is negative, it does NOT necessarily mean the cow does NOT have Johne's disease. It may mean either that the animal was not shedding the organism that day or that the 3 gram sample collected for testing from the pounds of manure produced that day did not happen to contain it. On these 87 diagnosable cases of Johne's disease the culture was false-negative for 14 (16.1%) of the infected cows on the day of sampling.

Is the most sensitive test for Johne's disease the best test?

Sensitivity is important, but there are many other factors to consider. Two factors are the cost of the test and the turnaround time for test results. Faecal culture, for example, is two to three times as expensive as the ELISA and results for culture are not available for two to four months as compared to just a few days for ELISA. Selection of a test will be influenced by why the test is being done and by what you plan to do with the test results.

In general, tests for Johne's disease have a high specificity: low rates of false-positive results. To avoid making management decisions based on false-positive blood tests, faecal culture can be completed as a "confirmatory" test for blood test-positive animals. Test sensitivity (percentage of infected animals that test positive) varies among tests, but due to the biology of this slowly progressing chronic disease is often less than 50%. In the very early stages of *M. paratuberculosis* infection, before animals start shedding the bacterium in faeces or begin an immune response to the infection, all animals will be test-negative. As the infection progresses, most tests will eventually become positive as the animal begins to produce the signals of infection detected by the tests (e.g., antibody in blood, gamma-interferon in blood, the organism in manure). Tests have maximum sensitivity when used for animals with clinical signs of the infection: diarrhoea and/or weight loss. Unfortunately, exceptions to these generalisations are common since individual animals don't "read the book" and follow the disease pattern exactly. For this reason, when confidence in the ABSENCE of *M. paratuberculosis* infections is desired, use of two or more different types of tests at the same time is recommended for the best available information.

Reference

Collins, M.T. (2003). Update on paratuberculosis: 1. Epidemiology of Johne's disease and the biology of *Mycobacterium paratuberculosis*. *Irish Veterinary Journal* 56: 565-574.