

Abstracts concerning *M. paratuberculosis*
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Evaluation of In-house Produced Herrold's Egg Yolk Agar with Commercially Prepared Formulations with and Without Nalidixic Acid and Vancomycin

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In February 2000, Becton Dickinson Biosciences initiated a study to evaluate a potential new product, Herrold's Egg Yolk Agar with Mycobactin J and amphotericin B, nalidixic acid, and vancomycin (BBL HEYA-MANV). The study involved laboratories which produced their own similar product, and compared the ability of the two media to support the growth of *Mycobacterium avium* subsp. *paratuberculosis* while suppressing contaminating overgrowth of fungi or bacteria. Fecal samples which the laboratories received for Johne's culture were processed according to the laboratory's routine procedure, inoculated onto both media, and evaluated for growth of *M. avium* subsp. *paratuberculosis* and contaminated at weekly intervals. Additionally, our laboratory inoculated tubes of BBL's Herrold's Egg Yolk Agar with Mycobactin J and amphotericin B (BBL HEYA), for purposes of comparing results to the two media listed above. Our hypothesis was that the additional antibiotics (nalidixic acid and vancomycin) in the media could suppress growth of *M. avium* subsp. *paratuberculosis*. The proportion of tubes with contaminating overgrowth was compared among media using the Pearson chi square test. Mean colony counts for the tubes with *M. avium* subsp. *paratuberculosis* growth were compared among media using the Kruskal-Wallis test.

One hundred sixty two fecal samples were inoculated onto the three types of HEY agar. Growth of *M. avium* subsp. *paratuberculosis* was detected on all media as early as 5 weeks. BBL media without amphotericin B and vancomycin had much higher levels of contaminating overgrowth than did the BEL HEYA-MANV or the in-house produced media (31/486 tubes, vs. 0/486 for both in-house and BBL HEYA_MANV; P <0.001). *M. avium* subsp. *paratuberculosis* were recovered from 20 (4.1%) of the samples, with growth detected on all 3 media. Thus, media type does not appear to affect the sensitivity or specificity of *M. avium* subsp. *paratuberculosis* culture. Mean (SE) colony count among tubes with *M. avium* subsp. *paratuberculosis* growth were 22.8 (6.3), 25.4 (6.8), and 31.0 (7.3) for in-house, BBL HEYA-MANV, and BBL HEYA media, respectively. Differences in mean colony counts were not detected, although statistical power to detect differences was limited.

A Rapid Serologic Test for the Detection of Antibodies to *Mycobacterium avium* subsp. *paratuberculosis* with Applications for Bovine Practitioners

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A prototype ELISA test has been developed on the IDEXX SNAP® device platform to detect antibodies to *Mycobacterium avium* subsp. *paratuberculosis* (Mpt.), the causative organism of Johne's disease in ruminants. Initial validation studies have been completed utilizing bovine serum as the specimen type. This test system has potential applications as an animal-side or in-clinic assay for diagnosing Johne's disease in symptomatic or suspicious animals. The SNAP® test platform has demonstrated efficacious utility as an in-clinic test system for the diagnosis of a variety of infectious diseases in dogs and cats. This new serologic test for Johne's disease is formatted to provide the same utility to large animal veterinarians who require a rapid test result. Total assay time is seventeen minutes after a five minute *M. phlei* pre-treatment. Results are interpreted visually, and may also be quantified with a densitometer.

The performance of this new M. pt. SNAP® ELISA was evaluated by testing populations of dairy cattle (n = 406) with the prototype test system and with an USDA-licensed microtitre plate antibody test kit. Quantitative data were measured on the SNAP® test platform by taking densitometric readings of the diagnostic spot at the completion of the test protocol. These values were compared to the S/P ratios yielded by the microtitre-plate technique. Regression analysis of these data shows a significant correlation of test results between the two techniques utilized, ($r^2 = 0.82$; $p < 0.0001$). 96.1% of sera tested (390 of 406) yielded an agreement in serologic status as determined by the two test methods evaluated.

Specimens tested were collected from seven different dairy herds of varying geographic origin. Herd status was investigated with regard to previous diagnostic test histories, (serology and culture); differential observations of herd veterinarians, (evidence of symptomatic animals); and with regard to the introduction of replacement cattle into the herds, (open vs. closed herd). Three of the seven herds tested (n = 178) had a history of diagnosed *M. avium* subsp. *paratuberculosis* cases within each of the herds. Two of the seven herds (n = 133) had no previous evidence of paratuberculosis, and are presumed to be negative for antibodies to Mpt. Each of these two herds did have a prior test history for Mpt. detection by culture and serology. One of the two presumed negative herds was closed to replacement animals. The final two herds tested (n = 95) were of unknown status, and had no previous test history for Johne's disease. The percent agreement in final test dispositions between the two assay methods utilized (for these three herd groupings) were as follows: known infected herds = 93.3%; presumed negative herds = 99.2%, herds of unknown status = 96.8%. This study has served as a basis in comparing two techniques for the detection of antibodies to *M. avium* subsp. *paratuberculosis*. The relative sensitivity and specificity of a new prototype lateral flow test was measured against an established USDA-approved diagnostic product. These data provide evidence that the performance of the prototype Mpt. SNAP® test for detecting antibodies to *M. avium* subsp. *paratuberculosis* correlates significantly to the assay performance of the USDA-licensed IDEXX Mpt. Antibody Test Kit which is manufactured in a microtitre-plate format. Further, the prototype SNAP® test yields specimen dispositions which are consistent with the known source-herd histories for the bovine populations studied.

A New Liquid Culture Method, the Trek ESP Culture System II, for the Rapid Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Bovine Fecal Samples

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Solid medium culture has been the standard procedure for detection of *M. avium* subsp. *paratuberculosis* in bovine fecal samples. However, because of the long generation time of the bacterium, it is a slow and labor intensive procedure requiring up to 12 weeks of incubation. The purpose of this study was to develop a rapid detection procedure for *M. avium* subsp. *paratuberculosis* in bovine fecal samples using a liquid culture method, the Trek ESP Culture System II (ESP), in conjunction with the Cornell double incubation decontamination process.

Bovine fecal samples, a total of 85 including 30 known positive samples and 55 unknown samples, were decontaminated by the Cornell double incubation process prior to culture in ESP MYCO bottles and on Herrold's egg yolk (HEY) agar slants. Of 30 known bovine fecal samples, 10 heavy shedders (>300 CFU/g), 10 medium shedders (31-300 CFU/g) and 10 low shedders (1-30 CFU/g) were included. Of 55 unknown samples, 25 were NVSL were check samples and 30 were field samples. All bottles flagged as positive in ESP and all suspect colonies on HEY agar slants were confirmed as *M. avium* subsp. *paratuberculosis* by acid fast staining and PCR.

Of 85 bovine fecal samples, 59 (69.4%) were positive for *M. avium* subsp. *paratuberculosis* by the ESP method while 51 (60%) were positive by the standard solid medium culture method (HEY). Both systems were able to detect 100% of heavy and medium shedders; however, the ESP detected 8 more low shedders than the standard HEY agar method.

Mycobacterium avium subsp. *paratuberculosis* was detected by the ESP with a mean time to detection of 14.97 days for heavy shedders, 22.79 days for medium shedders, and 34.5 days for low shedders. In contrast, by the standard solid medium culture on HEY agar, *M. avium* subsp. *paratuberculosis* was isolated with a mean time to detection of 39.71 days for heavy shedders, 41.9 days for medium shedders, and 48 days for low shedders. Two samples (2.4%) from each method were contaminated with fungus and other microorganisms.

In conclusion, the ESP, a liquid medium based detection system, was able to detect *M. avium* subsp. *paratuberculosis* in bovine feces 2 to 3 weeks earlier than the standard HEY culture method with greater sensitivity and identical contamination rate.

Paratuberculosis in Dairy Cattle: Classification of Cattle by Colony Counts of *Mycobacterium avium* subsp. *paratuberculosis* in Fecal Samples

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The extent of fecal shedding by cattle infected with *Mycobacterium avium* subsp. *paratuberculosis* is important to determine, since animals shedding more organisms represent the greatest risk to spread the disease and to contaminate the environment. Generally, cattle shedding more organisms (high shedders) represent later stages of infection which also tend to have higher antibody titers and therefore detectable by ELISA. Cattle shedding few detectable organisms (low shedders) by culture represent earlier stages of infection and have lower antibody levels. Awareness of the relative expected proportion of cattle in each shedding category provides more information to the producer, herd veterinarian and regulatory officials about the extent and distribution of infected cattle in herds.

The primary objective of this paper is to describe the relative proportion of cattle in one of three categories: high shedders, mid-shedders and low shedders. High shedders are those animals with 70 or more colonies of *M. avium* subsp. *paratuberculosis* per culture tube for a total of more than 280 colonies in four tubes. Mid-shedders have less than 70 colonies per tube but more than 10 colonies of *M. avium* subsp. *paratuberculosis* per tube with a range of 40 colonies to 279 colonies in four tubes. Low shedders have less than 10 colonies per tube or less than 40 total colonies in four tubes of Herrold's egg yolk media all with mycobactin J.

Three populations of Johne's infected cattle including ten dairy herds cultured every six months for four years, twenty-six herds cultured the first time and thirty Johne's infected dairy herds cultured once served as the basis for this study. In the last group of herds, 10 herds had more than 10% fecal culture positive, ten herds had less than 10% culture positive and ten herds were cultured the first time. In each category of infection among the 66 dairy herds in which fecal cultures were done, the relative proportion of infected cattle as detected by fecal culture was similar: 20-30% high shedders, about 10% mid-shedders and 60-70% low shedders. Herds cultured the first time tend to have a higher number of high shedders, while herds tested several times tended to have more low shedders.

If you use a different test, you will get a different answer

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Objective: To test the level of agreement among four serological tests for Johne's disease.

Methods: The IDEXX ELISA for antibody to *M. paratuberculosis* was arbitrarily selected as the basis for comparison. One hundred bovine sera from cattle of unknown infection status submitted to our laboratory for Johne's disease serology were selected to fulfill the following IDEXX ELISA s/p criteria based on the original test result (IDEXX-1): 15 sera with s/p <0.10 (negative); 10 sera with s/p = 0.10 to 0.24 (suspect); 25 sera with s/p = 0.25 to 0.39 (low positive); 25 sera with s/p = 0.40 to 0.99 (positive); and 25 sera with s/p >1.00 (strong positive). These sera were stored at -20EC, thawed, and tested by four USDA-licensed tests for serum antibody to *M. paratuberculosis*, including a repeat of the IDEXX ELISA (IDEXX-2) using a different kit lot number from the one used to test the original sample. Other tests used were Parachek® (Biocore Animal Health, Inc.) TipTest® and RJT® (also known as the AGID, ImmuCell, Inc.). All tests were interpreted by manufacturer's guidelines. For the subjectively interpreted assays, TipTest® and RJT®, all visible reactions, even if weak, were classified as positive.

Results: Table 1. Kappa and agreement between pairs of serological tests.

		Kappa values ¹			
	IDEXX-1	IDEXX-2	Parachek®	TipTest®	RJT® ²
IDEXX-1	-	.80	.39	.26	.12
IDEXX-2	93%	-	.38	.27	.09
Parachek®	67%	62%	-	.43	.34
TipTest®	65%	62%	71%	-	.25
RJT®	41%	41%	71%	61%	-
		Percentage of test results in agreement			

¹Chi square analysis: all tests except IDEXX-2 had results significantly different from IDEXX-1 p<0.01.

²RJT® is only recommended for use on animals with clinical signs of paratuberculosis.

Table 2. Agreement between IDEXX-1 and Parachek® or TipTest® by s/p range.

		Parachek®			TipTest®		
		Neg	Pos	Agreement	Ne g	Pos	Agreement
IDEXX-1	Neg+suspect	25	0	100%	17	8	68%
	Low-pos	18	7	28%	14	11	44%
	Positive	12	13	52%	11	14	56%
	Strong-pos	3	22	88%	5	20	80%

When analyzed by level of s/p value, highest levels of agreement among tests were found for sera classified at the extremes: negative or strong positive. Five times a serum classified as "suspect" (s/p = 0.10 to 0.24) was positive on retest and one time the reverse occurred. Regression analysis of all 100 s/p results for IDEXX-1 and IDEXX-2 showed a high correlation ($r^2 = 0.96$).

Conclusion: For 6% of sera a change in interpretation (pos/neg) occurred between repeated IDEXX ELISAs. For 29%-59% of bovine sera interpretations were different using different commercial kits. This was least common among sera with very low or very high "titers" of serum antibody to *M. paratuberculosis*. This may be due to differences in antigens used in the kits or other factors. Regardless, the study shows that if laboratories use different commercial kits for Johne's disease serology, diagnostic results will differ. While the basis for this difference is unknown, it merits detailed

investigation.

A Fast and Sensitive Diagnostic Assay for the Detection of *Mycobacterium paratuberculosis* in Bovine Feces

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Johne's disease is a chronic, debilitating enteritis of cattle, sheep, goats and other ruminants caused by the organism *Mycobacterium paratuberculosis*. The standard diagnostic assay for Johne's disease is cultivation of *M. paratuberculosis* from fecal specimens. This procedure requires a large amount of incubator space and may take months to produce visible colonies. We have developed an assay to determine the presence of *M. paratuberculosis* in feces that requires no cultivation and can be performed in 2 days. This assay, *Mycobacterium paratuberculosis* DNA Test Kit, relies on the amplification of *M. paratuberculosis* from fecal DNA preps. DNA is extensively purified from fecal organisms, amplified using first-time PCR and detected in a dot-blot format with enzyme conjugates using microarray hybridization conditions.

The specificity of the assay was demonstrated using two groups of fecal specimens. One group was comprised of field fecal specimens from two NVSL check sets in which the presence or absence of *M. paratuberculosis* had previously been determined using cultivation techniques; the other group consisted of fecal samples from a certified negative *M. paratuberculosis* herd. The results indicated that there was a 100% correlation between culture negative data and the lack of a signal with the PCR based assay for negative fecal specimens within the NVSL check sets and all the specimens collected from certified *M. paratuberculosis* negative cattle. With regard to the culture positive specimens, there was a 70% (21/30) correlation between culture positive data and positive PCR results. Since the culture positive specimens that failed to generate a positive PCR signal contained a low number of *M. paratuberculosis* organisms, the assay's sensitivity was reexamined in terms of colony forming units (CFU) per sample as originally determined by NVSL. With fecal specimens that contained 0-8 CFU there was a 22.2% agreement between culture and PCR results. Fecal specimens that contained 9-28 CFU had 81.2% agreement between the two diagnostic methods. Similarly there was a perfect correlation between culture results and PCR results on samples that contained greater than 29 CFU. The low level of agreement with samples that contained 0-8 CFU may represent the detection limit of the PCR assay; however, personnel from many other diagnostic labs were unable to recover *M. paratuberculosis* from these samples. Therefore, the inconsistent recovery of the organism using these samples most likely represents organisms partitioning in the feces during shedding. This is further supported by a lack of correlation between CFU and PCR signal intensities and the reported clumping of these organisms when propagated in liquid culture.

Application of Molecular-based Techniques in the Accurate Detection of *Mycobacterium avium* subsp. *paratuberculosis*

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Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is the causative agent of paratuberculosis or Johne's disease, a chronic progressive disease affecting the intestinal tract of ruminants. The disease results in a wasting syndrome of infected dairy and beef cattle with tremendous economic loss to the agricultural industry. The diagnosis of Johne's disease and the identification of *MAP* are extremely difficult due to the slow growth of *MAP* in the laboratory and the poor sensitivity of laboratory tests, especially during the subclinical stages of disease. Prompt and accurate diagnosis of infection is central to effective management of livestock enterprises.

Although *MAP* shares 99-99.5% DNA homology with other related mycobacteria belonging to the *Mycobacterium avium* complex (MAC), the insertion sequence IS900 differentiates *MAP* from these close phylogenetic neighbors and has been used for the basis of molecular diagnostic testing. Insertion sequences similar to the IS900 in other MAC strains have been a source of false-positives in commercial PCR-based assays that target IS900. Analysis of the alignment of these similar IS elements reveal a 4 base pair deletion in each element (at bases 121 and 125) which is not present in the IS900 sequence. We have used this unique region to design primers that have an increased specificity for the IS900 sequence, resulting in a decrease in the rate of false positives. Our testing strategy also relies on size characterization of the amplification product providing an additional level of confidence for specificity. PCR detection methods which employ primers that do not take into consideration the potential for cross-amplification of portions of other insertion sequences, or that are unable to distinguish nonspecific amplification products of related sequences, have suffered from high rates of false-positives. Primer design as well as size characterization of the PCR product are both critical in discerning IS900 from other related IS elements in mycobacteria, and greatly increase the specificity of the diagnostic probe for IS900.

Another insertion sequence designated IS1311 has been identified in *MAP* and *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *silvaticum*. According to restriction enzyme digestion patterns of IS1311, it has been recently reported that *MAP* can be further classified into "sheep" and "cattle" strains, and can be differentiated from *Mycobacterium avium-avium* and *Mycobacterium avium-silvaticum*. Five point mutations differentiate the IS1311 sequence of *MAP* from *M. avium-silvaticum* and *M. avium-avium*. The IS1311 test has a sensitivity equal to that of the IS900 test and has been shown to be useful on a range of crude and purified DNA preparations from a variety of sources. We have used the IS1311 test to evaluate *MAP* strains isolated from a wide range of hosts besides cattle and sheep. Most of these strains have resulted in a pattern similar to cattle strains; however, *MAP* strains isolated from bison gave a unique restriction pattern, suggesting evolutionary divergence of *MAP* in unique hosts. Testing for IS900 and IS1311 is simple and rapid, and when performed together can provide accurate and confirming diagnosis of *MAP* infection, thereby offering herd managers and animal health programs useful information for the control and management of paratuberculosis.

Molecular Cloning and Characterization of *Mycobacterium avium* subspecies *paratuberculosis* Antigen 85 Complex Gene Family, 85A, 85B and 85C

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Mycobacterium avium subspecies *paratuberculosis* (*M. paratuberculosis*) is the etiologic agent of paratuberculosis (Johne's disease), a chronic granulomatous enteritis in cattle, sheep and goats. Currently, there is an urgent need for an effective recombinant vaccine to control the disease and an improved diagnostic test for reliable detection of *M. paratuberculosis* inactivation. The dominant exported proteins and protective antigens of other *Mycobacterium* spp. have been identified to be the antigen (Ag85) complex. In this study, we have cloned and sequenced the genes (85A, B and C) from *M. paratuberculosis* and expressed the proteins in *E. coli*. Ag85A, B, and C have been implicated in disease pathogenesis through its fibronectin-binding capacities. A carboxylesterase domain was found within the amino acid sequences of these proteins. The function of 85A, B and C is to act as a mycolyltransferase involved in the final stages of mycobacterial cell wall assembly. These secreted proteins or genes may be useful as antigens for serologic diagnosis and/or recombinant and DNA vaccine development.

Development of Quantitative PCR-based on the ABI 7700 System (TaqMan) for *Mycobacterium avium* subsp. *paratuberculosis*

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There have been numerous reports for PCR-based diagnostic methods to detect *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease. The result of conventional PCR tests has been only qualitative, either positive or negative; therefore, the result does not present any quantitative information about the number of the agents in the specimen. We have developed a quantitative PCR method using the ABI system (TaqMan) to measure the number of *M. avium* subsp. *paratuberculosis* present in test samples. The sensitivity of the method was 10 CFU for *M. avium* subsp. *paratuberculosis* ATCC 19698. The specificity of the method was tested for 14 Mycobacterial species (*M. abscessus*, *M. asiaticum*, *M. avium* subsp. *avium*, *M. bovis*, *M. fortuitum* subsp. *fortuitum*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. phlei*, *M. scrofulaceum*, *M. simiae*, *M. smegmatis*, *M. terrae*, *M. ulcerans*) and 9 non_Mycobacterial species (*Borrelia burgdorferi*, *Chlamydia psittaci*, *Ehrlichia canis*, *E. equi*, *E. risticii*, *Escherichia coli*, *E. coli* O157:H7, *Streptococcus equi*, *S. zooepidemicus*). Even at high level of cell numbers (10^5 CFU), most of the organisms tested negative except *M. marinum* and *M. scrofulaceum*. The finding with *M. scrofulaceum* was consistent with a recent report by Austrian investigators who found some isolates closely related to *M. scrofulaceum* carry 70% to 79% homology with *M. paratuberculosis* in the region of IS900.

Using this TaqMan-based quantitative PCR method with the Trek ESP System II for bovine clinical fecal samples, we were able to confirm that most of the positive samples contained 10^5 to 10^6 CFU/ml of *M. avium* subsp. *paratuberculosis*. The quantitative PCR was also useful in the study of growth characteristics of three groups of *M. avium* subsp. *paratuberculosis* strains classified by shedding levels, heavy, medium, and low based on CFUs on HEY slants.

Comparison of a Novel and Traditional Processing Method with Several Media Formulations to Detect *Mycobacterium avium* subsp. *paratuberculosis* in Bison Tissues

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Isolation of *Mycobacterium avium* subsp. *paratuberculosis* (Map), the causative agent of Johne's disease in ruminants remains the gold standard for diagnosis of infection. Traditional culture methods have relied on decontamination with hexadecylpyridium chloride (HPC) and Herrold's egg yolk media (HEYM). This study evaluated a novel decontamination method that relies on the combination of the zwitterionic detergent C18-carboxpropylbetaine (CB-18) and lytic enzymes (CB-18/LE) and compared the results with the traditional method using a variety of Bison tissues.

Tissues harvested from 59 bison at slaughter included ileum, one section of mesenteric lymph node from each of three levels (upper, middle and lower) of the mesenteric lymph node chain and the ileo-cecal-colic lymph node in most animals. In total, 270 bison tissues suspected of being infected with Map were processed using both decontamination methods. HPC processed specimens were inoculated onto four different media formulations: HEYM with pyruvate (HEYMP) HEYM without pyruvate (HEYMNP), Lowenstein-Jensen (L-J) media and Middlebrook 7H10 media supplemented with egg yolk (7H10EY). CB-18/LE processed specimens were inoculated into BACTEC 12B liquid media supplemented with 1% egg yolk and PANTA, as well as inoculated in duplicate onto 7H10EY and HEYMNP media slants (both solid media formulations used in conjunction with CB-18/LE processing included PANTA to control contamination).

Preliminary results indicated tissues from 46 (88%) of the 59 bison and 149 of 270 (55%) tissues were classified as positive. Of the 149 tissues processed by CB-18/LE on three media formulations (I2B, 7H10EY and HEYMNP) identified 98% (142) of the tissues positive compared to 57% (85) when processed by HPC and four media formulations (HEYMP, HEYMNP, L-J and 7H10EY). Similar results were obtained when bison were classified as positive according to processing method, 44 (95.7%) were positive with CB-18/LE while only 28 (60.9%) were positive when processed by HPC.

These results indicate both tissue processing method and media formulation greatly influence the recovery of *M. avium* subsp. *paratuberculosis* from bison suspected of Johne's disease. Overall, bison isolates of Map grew better when processed with CB-18/LE. The most sensitive detection method was the combination of CB-18/LE processing and BACTEC 12B liquid culture compared to the traditional method of HPC/HEYM analysis.

Molecular Epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*

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A commercially available kit consisting of twenty 10-mer random primers was evaluated to allow selection of a suitable primer that would permit identification and subtyping of *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) and *Mycobacterium avium* subsp. *avium* (*M. avium*) by Randomly Amplified Polymorphic DNA (RAPD). A primer OPE-20 (5-AAC-GGT-GAC-C-3) was identified to be the most suitable primer when tested with 4 ATCC reference strains of *M. paratuberculosis* and 8 well characterized field strains each of *M. paratuberculosis* and *M. avium*. Primer OPE-20 was further tested for its ability to identify and subtype 200 field isolates of *M. paratuberculosis*. The fingerprint patterns of *M. paratuberculosis* (n=212) consisted of 5 unique common fragments (620-, 450-, 310-, 230-, 180-bp) and 9 variable fragments resulting in 6 distinct genotypes (Table I).

Table 1. DNA fingerprint profiles of *M. paratuberculosis* and *M. avium* using primer OPE-20

Organism	Amplified DNA fragments (bp)			
	Primary	Variable	Genotype	Frequency (%)
<i>M. paratuberculosis</i> (n=212)	620,450,310,230, 180	1300, 1000, 850, 580	mp1	7.5
		1300, 1000, 850, 420	mp2	15.5
		1300, 1100, 1000, 850, 730	mp3	34.5
		1000, 850	mp4	23.5
		850, 210	mp5	11.5
		350	mp6	7.5
<i>M. avium</i> (n=8)	620	650, 380, 310	ma1	12.5
		1120, 1000, 850, 750, 650, 380	ma2	37.5
		1300, 1120, 1000, 980, 910, 850, 650, 480, 380, 240, 220	ma3	12.5
		1300, 1120, 1000, 850, 750, 650, 480, 380, 240, 220	ma4	12.5
		1120, 910, 800, 750, 460, 380	ma5	12.5
		1120, 460, 380	ma6	12.5

The DNA fingerprints of *M. avium* (n=8) consisted of a single common fragment of 620-bp, and 15 variable fragments resulting in 6 different genotypes. Cattle, human and goat isolates of *M. paratuberculosis* were genetically similar, but a sheep isolate had a different RAPD profile as compared to RAPD profiles from other species. RAPD was observed to be a rapid, reproducible and reliable technique for identification and subtyping of *Mycobacterium avium* subsp. *paratuberculosis*.

IS900-PCR Assay for *Mycobacterium avium* subsp. *paratuberculosis* from Quarter Milk and Bulk Tank Milk Samples Allows Detection of Herds with Johne's Disease

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A total of 180 cows from 4 herds with known history of Johne's disease were examined for *M. paratuberculosis*. Pooled quarter milk (50 ml), serum (1 ml) and fecal samples (10 g) were collected for analysis. Bulk tank samples (200 ml) were also collected and split into 4 samples (50 ml). Quarter milk and bulk tank milk samples were tested by IS900-PCR assay and cultured also for *M. paratuberculosis* on Herrold's egg yolk medium slants supplemented with mycobactin. Serum samples were evaluated for *M. paratuberculosis* by a Kinetics based ELISA (KELA). Fecal samples were cultured for *M. paratuberculosis* on Herrold's egg yolk medium slants according to the Cornell method. The IS900-PCR assay was optimized to detect *M. paratuberculosis* directly from milk samples. The results of the study are summarized in Tables 1 and 2.

Table 1. Detection of *M. paratuberculosis* by fecal culture, KELA, IS900 PCR and milk culture.

Samples	Herd			
	A (n=91)	B (n=38)	C (n=29)	D (n=32)
Feces	19 (21%)	6 (16%)	1 (3%)	1 (3%)
KELA	15 (17%)	5 (13%)	6 (20%)	7 (22%)
Pooled Quarter Milk (IS900-PCR assay)	28 (31%)	16 (42%)	11 (38%)	13 (40%)
Pooled Quarter Milk (Culture)	9 (10%)	0	0	0

Table 2. Number of samples positive for *M. paratuberculosis* by IS900 PCR assay and culture

Bulk Tank Milk	Herd			
	A	B	C	D
IS900-PCR assay	4/4	3/4	1/4	2/4
Culture	1/4	0/4	0/4	0/4

It was observed that 68 of 180 (36%) pooled quarter milk samples were positive for *M. paratuberculosis* by the IS900-PCR assay, while fecal culture, KELA and milk culture detected *M. paratuberculosis* in 27/180 (14%), 33/180 (17%) and 9/180 (5%) samples, respectively. Fifteen cows (8%) were positive by both ELISA and fecal culture, and 16 (8%) were positive by both IS900-PCR from milk and fecal culture. IS900-PCR assay allowed detection of *M. paratuberculosis* from 10/16 (63%) samples of bulk tank milk, while milk culture detected *M. paratuberculosis* in 1 of 16 (6%) samples. Preliminary results suggest that IS900-PCR assay can be applied to screen herds using bulk tank milk samples and quarter milk samples to detect

cows with Johne's disease. The IS900-PCR assay needs to be further evaluated on both Johne's positive and negative herds.

Evaluation of IS900-PCR Assay for Detection of *Mycobacterium avium* subsp. *paratuberculosis* Directly From Raw Milk

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Johne's disease which is caused by *Mycobacterium avium* subsp. *paratuberculosis* results in losses exceeding \$1.5 billion/year. Current detection methods for Johne's disease rely on culture and ELISA-based assays. However, both of these methods have poor sensitivity. DNA probes based on the insertion sequence 900 (IS900) of *M. paratuberculosis* offer an alternative diagnostic test that is rapid and specific. The objective of this study was to standardize the IS900-PCR assay for detection of *M. paratuberculosis* directly from raw milk.

Raw milk and Middlebrook's 7H9 (M7H9) broth samples were inoculated with 1 ml of a suspension containing 10⁸-0 CFU/ml (Table 1) of each of 4 American Type Culture Collection (ATCC) strains of *M. paratuberculosis* (ATCC 19698, ATCC 43544, ATCC 43545, ATCC 43015). Milk and M7H9 samples were centrifuged at 1950 x g for 30 min, and pellets obtained were divided equally. One half of the pellet was used for IS900-PCR. The other half was decontaminated in HPC (0.75%) and cultured (150FI) on 2 slants each of Herrold's egg yolk medium (HEYM). All recovery and detection experiments were repeated 6 times.

Table 1. Comparison of culture and IS900-PCR for detection of *M. paratuberculosis*.

Milk (n=6 replicates, 2 HEYM slants per replicate)				M7H9 (n=6 replicates, 2 HEYM slants per replicate)*			
CFU/ml	+ve by IS900 PCR	Counts (Avg CFU/ml)	Culture +ve	CFU/ml	+ve by IS900 PCR	Counts (Avg CFU/ml)	Culture +ve
10 ⁸	24/24	TNTC [#]	48/48	10 ⁸	24/24	TNTC	48/48
10 ⁶	24/24	TNTC	48/48	10 ⁶	24/24	TNTC	48/48
10 ⁴	24/24	TNTC	48/48	10 ⁴	24/24	TNTC	48/48
10 ²	24/24	53	40/48	10 ²	24/24	75	44/48
10	12/24	2	10/48	10	24/24	7	20/48
1	0/24	-	0/48	1	0/24	-	0/48
0	0/24	-	0/48	0	0/24	-	0/48

*6 replicates each of 4 ATCC reference strains of *M. paratuberculosis*: ATCC19698-Bovine, ATCC 43544-Ben, ATCC 43545-Dominic, ATCC 43015-Linda.

* Too numerous to count

Under experimental conditions, IS900-PCR assay could detect counts as low as 10 CFU/ml - 100 CFU/ml of *M. paratuberculosis* (Table 1). Further, IS900-PCR detected *M. paratuberculosis* in 5/8 farm bulk tank milk samples from herds with known clinical history of Johne's disease. Two out of the 5 samples positive by IS900-PCR were also positive by culture for *M. paratuberculosis*. The results of this experimental study are currently being validated by testing bulk tank samples from several herds in Pennsylvania.

Evaluation of the Svanovir™ ELISA for Bovine Leukosis, Bovine Viral Diarrhea and *M. paratuberculosis*

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Three novel serologic tests (SVANOVA Biotech, Uppsala, Sweden) were evaluated: an indirect ELISA for Bovine Leukosis Virus (BLV), an indirect ELISA for Bovine Viral Diarrhea (BVD), and an indirect ELISA for *M. paratuberculosis* (Johne's disease).

Each test was compared to industry standards in North America: IDEXX ELISA for BLV and Johne's (IDEXX, Westbrook, Maine USA) and serum neutralization for BVD. Where appropriate, sensitivity and specificity values or Kappa scores were calculated. Serum samples were collected as part of a seroprevalence survey for production limiting diseases in Eastern Canada. Thirty lactating cows in each of 30 herds in Nova Scotia, New Brunswick and Prince Edward Island were sampled. Both herd and cow selection was by formal random sampling. From this serum bank, 528 cows were selected for the comparative BLV testing, 498 cows for *M. paratuberculosis*, and 218 unvaccinated animals, greater than 6 m of age, for BVD testing.

Table 1 is a contingency table of the BLV results. Serologic diagnosis of infection with BLV is very accurate, therefore, sensitivity and specificity could be calculated. There were no false positives or false negatives, therefore, the test scored 100% on both these parameters.

	IDEXX +ve	IDEXX -ve	Total
SVANOVA +ve	225	0	225
SVANOVA -ve	0	303	303
Total	225	303	528

Table 2 contains the BVD results. SN is considered the gold standard for BVD. When compared to a SN dilution \$1:256, the sensitivity and specificity of the ELISA was 94% and 96%, respectively.

	ISN 1:256	SN<1:256	Total
SVANOVA +ve	33	7	40
SVANOVA -ve	2	176	178
Total	35	183	218

Table 3 contains the Johne's results. The reported sensitivity and specificity of the IDEXX method is 47.3% and 99%, respectively. There was a relatively low agreement, beyond what

	IDEXX +ve	IDEXX -ve	Total
SVANOVA +ve	22	83	10
SVANOVA -ve	5	388	39
Total	27	471	498

is expected due to chance alone, between tests (Kappa 0.27). It is interesting to note, however, that the SVANOVA test identified many more animals as positive. This may be an improvement on the high false negative rate of the IDEXX test.

The SVANOVA ELISA for BLV and BVD appear to be equivalent to the current industry standards. The SVANOVA ELISA for *M. paratuberculosis* shows some promise of being an improvement on the current testing methods utilized in North America. Additional evaluation of this test is necessary to truly discern its capabilities.

