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RESEARCH ARTICLE

Prospective comparison of Classical and Modern Molecular mycobacterial detection techniques in cattle infected with *M. avium* subspecies *paratuberculosis*

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ABSTRACT

Background: "Classical" mycobacterial identification is difficult to perform, time consuming, inefficient and often insensitive. Modern techniques, incorporating molecular methods, may be more rapid, less expensive, more sensitive and more accurate. We prospectively compared "classical" and modern mycobacterial detection techniques.

Methods: A plaque/qPCR study has been published on the blood of a herd of Johne disease (cattle infected with *M. avium* subspecies *paratuberculosis* (MAP.)) Simultaneously aliquots were submitted for "classical" mycobacterial identification. Plaque/qPCR could be completed in <3days. "Classical" cultures were followed for a minimum of 2 years.

Results: Plaque/qPCR MAP positivity were observed in 74% of Johne and 34% of Control animals. "Classical" detection on appropriate culture medium were seen in 19% in Johne disease and 18% Controls.

Conclusions: "Classical" mycobacterial detections methodology has demonstrably been supplanted by modern molecular methods.

Abbreviations used:

MAP. *M. avium* subspecies *paratuberculosis*.
JD. Johne Disease.
HEYM. Herrold egg yolk medium
MGIT®: Mycobacterial Growth Indicator Tubes
IFNγ: Interferon γ
OD: Optical density

Introduction

The identification and culture of mycobacteria has a long and storied history. In 1874, in lepromatous patients, Hansen microscopically identified bacilli [1,2] In 1882 Koch identified and cultured the tuberculosis bacillus [3,4], for which he received the 1905 Nobel prize in Medicine (http://nobelprizes.com/nobel/medicine/1905a.html). Specific staining of the mycolic acid rich cell wall of mycobacteria[5,6] enhanced detection of mycobacteria in 1882 by Ziehl [7] and in 1883 by Neelsen.[8] Over the ensuing 150 years a multitude of efforts occurred to improve mycobacterial detection and culture evolved. In the 1930's the agar-based slopes of Lowenstein [9,10] and Jensen [11,12] became the norm. This led to the evolution of semi-solid agar in 1955 [13] followed by the introduction in of pure liquid culture media by Middlebrook in 1958.[14] The introduction of radiometric ^{14}C led to a detection system (Bactec 460®) of unsurpassed sensitivity of mycobacterial growth rates.[15] Regrettably, because the Bactec460 system is only semi-automatic and onerous regulations about disposing of the ^{14}C,with its half-life of 5700 years, the Bactec460 is no longer produced or supported. The present fluorometric Bactec 960® is the most prevalent extant mycobacterial growth detection system. In our experience it is less sensitive than the radiometric Bactec460.[16-19] All of these detection systems were invariably time-consuming process, taking weeks, months or even years.[20]

A potentially major improvement in the detection of viable mycobacteria uses mycobacteriophage to infect the viable mycobacteria [21-23] and RT PCR to identify the specific DNA of that mycobacterium. The use of mycobacteriophage based assays has multiple advantages to the aforementioned "Classical method", including improved detection sensitivity and speed.[21,23-26]

We previously published a phage/qPCR study on blood from a herd of cattle naturally infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) known as Johne disease (JD) and Control animals. [27] The phage component of the assay was completed within 36 hours of samples arriving in the laboratory. MAP was detected in 72% of the JD and, unsuspectedly, in 36% of the controls. [27] Simultaneously to performing the phage/qPCR assay, samples were submitted for standard "Classical" mycobacterial culture (*vide supra,* specifically [28] & MGIT®.)

The purpose of this manuscript is to report on the comparison of the "Classical method" of mycobacterial culture with our published phage/qPCR data. [27] The "Classical method" cultures were studied for a minimum of two years following inoculation.

Methods

Except for the subculture from Pozzato medium and MGIT tubes when indicated, and cultured onto HEYM slopes, all Methods have been previously published in detail.[27]

In brief:
From March 15, 2019, until January 21, 2020, we received a total of 150 blood samples from USDA herds in Ames Iowa. Of the total 49 animals, 32 had Johne and 17 were Control. Conventional blood and stool tests for MAP and/or its signal was compared with a MAP phage/qPCR assay.[27]

White blood cells were isolated from cattle whole blood using a Ficoll gradient (Becton-Dickinson: Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate (8 ml), Cat #362761), lysed and 25% of the lysate was then cultured using both Pozzato[28] and 7H-9 media in MGIT® (Becton Dickinson) both of which contained PANTA and *Mycobactin J*.[29] Occasionally so many plaques occurred, the appearance of the Petri dish appeared as a spurious "clear" (Figure 1B.) When this "clearance" occurred when plating 0.5 ml. of the lysate, we began to simultaneously plate 0.2 ml of the lysate (Figure 1C.) Some Control animals were appropriately negative in the plaques assay (Figure 2.) Idiosyncratically occasionally Control animals were positive in the plaque assay as previously reported [27] (Figure 3.)

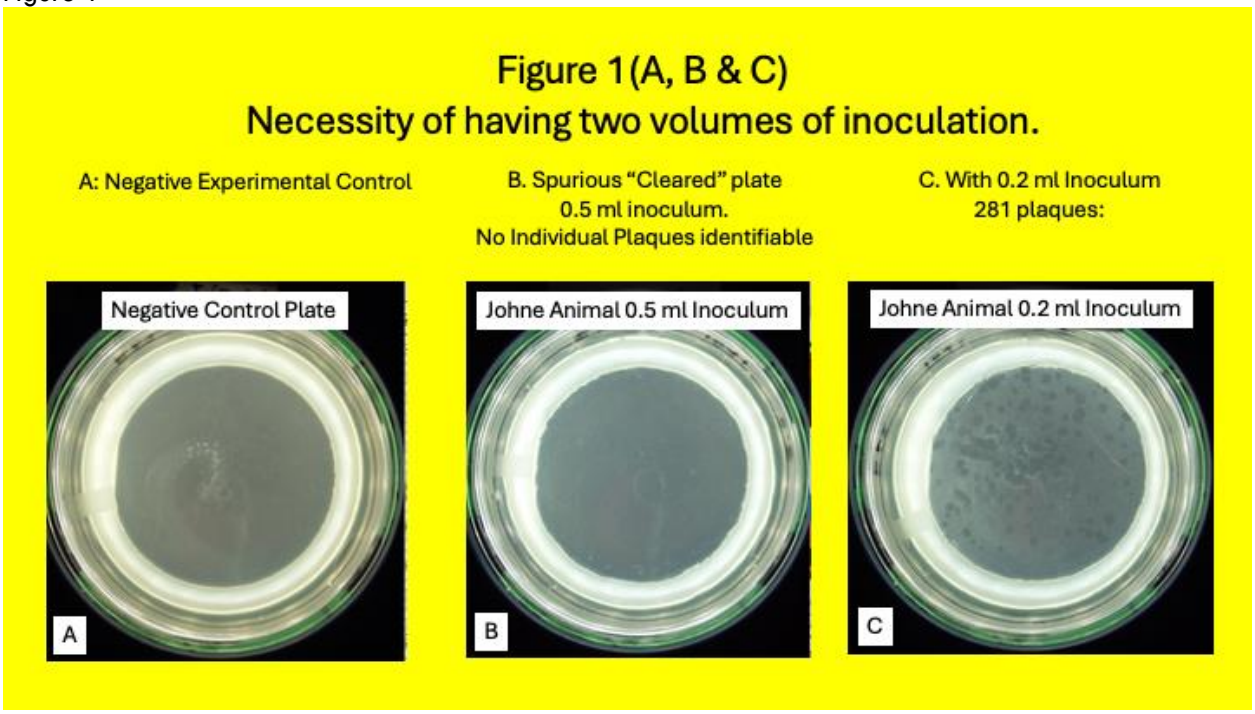
Every isolate from the 150 received samples were submitted to MGIT (7H9 based) and Pozzato culture. Both contained PANTA. Cultures were followed with serial Optical Densities (OD's.) When turbidity was detected, cultures were subcultured onto HEYM. Slopes were placed in incubators at 37°C and regularly observed.

When evidence of growth was detected (by increase in turbidity, increasing Optical Densities or Positivity on Bactec960® MGIT® tubes,) samples were sub-cultured onto Herrold's egg yolk slopes (Herrold's egg yolk agar with amphotericin, nalidixic acid, vancomycin, with mycobactin J. SKU 222232: GTIN 00382902222323 BD. New Jersey) Visually identified colonies, detected from 13 to 28 months following inoculation) were sub-cultured in 7H-9 to permit nucleic acid identification in the future. All cultures were supplemented with *Mycobactin J*.[30] All flasks were incubated at 37°C. Identified colonies were harvested and subjected to Ziehl [7]-Neelsen [8] staining

Results:

A total of 150 samples were received and assayed from 17 Control and 32 Johne animals.

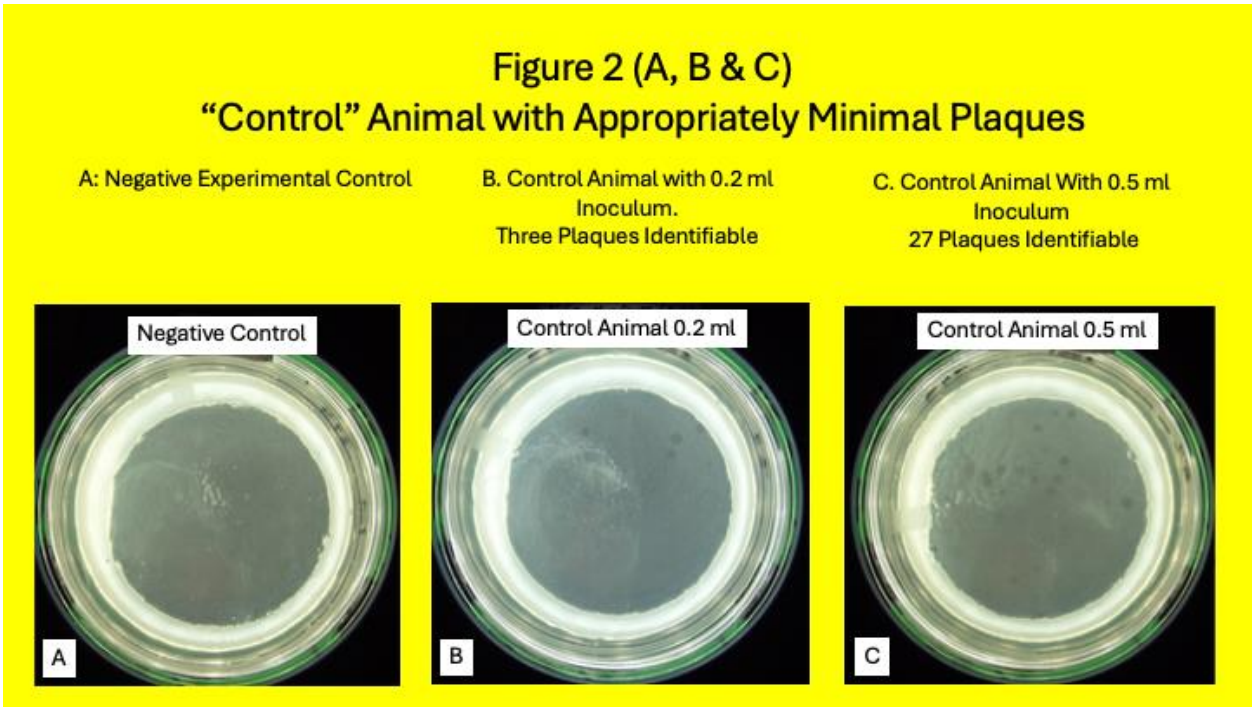
Figure 1



Legend to Figure 1. Johne Animal:

Shown in Figure 1 is a phage assay from a Johne animal. Figure 1 A is the negative experimental control plate. No plaques are seen. Figure 1B (a 0.5 ml inoculum) shows no identifiable plaques. However, the plate had been "cleared," because of the overwhelming number of colonies. Figure 1C a 0.2 ml inoculum has 281 identifiable plaques. Because of data like these, we routinely inoculated two aliquots (0.5 and 0.2 ml) from the lysis of the post Ficoll buffy coat.

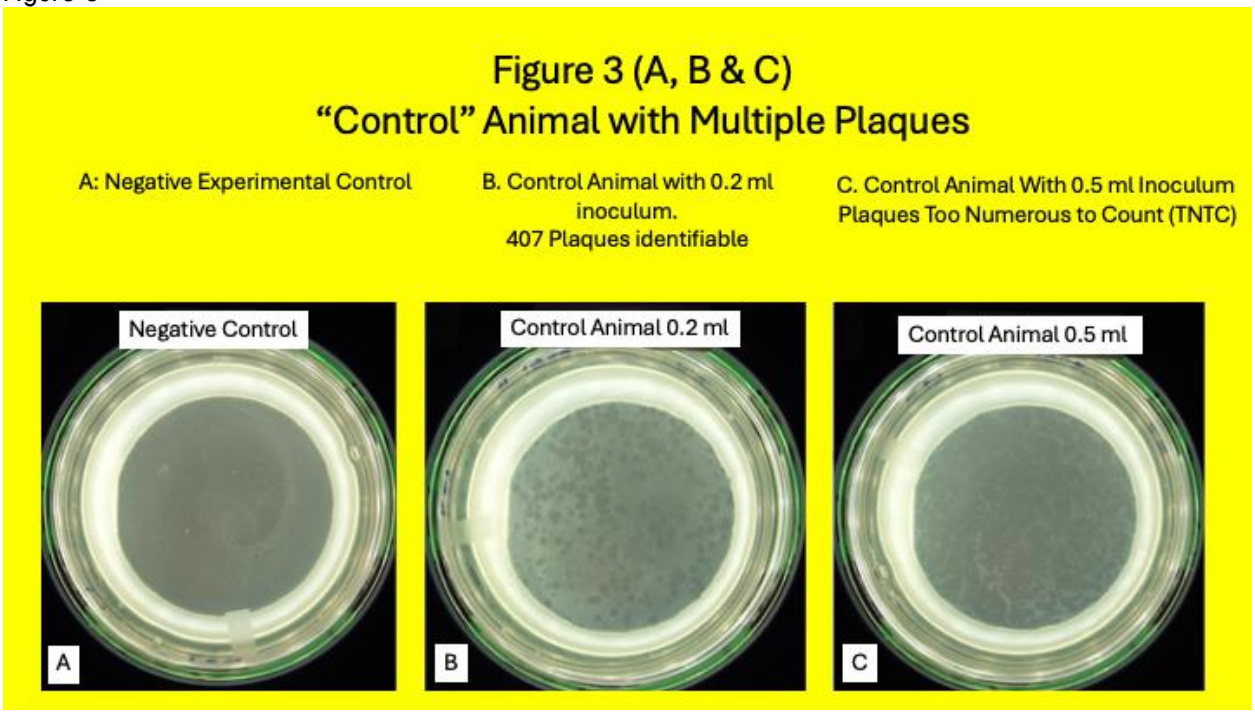
Figure 2



Legend to Figure 2. Control Animal.

Control animal where the experiment is read as "Negative." A is the negative experimental Control. No plaques are seen. B. With 0.2 ml inoculum three plaques are seen. C. With 0.5 ml inoculum 27 plaques are seen. This is considered to be an appropriately "Negative" experiment.

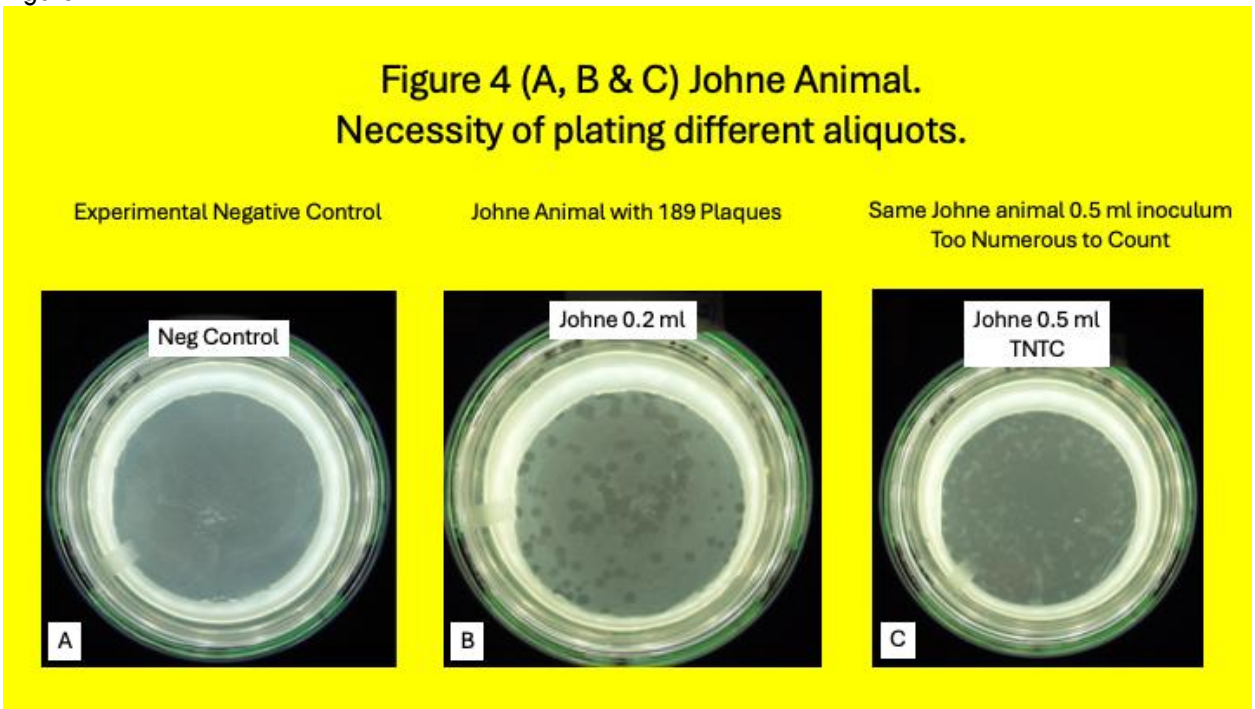
Figure 3



Legend to Figure 3. Control Animal.

In these "Control" animal samples, A: is the Negative experimental control. B: With a 0.2 ml inoculation 407 plaques are seen. C: With 0.5 ml inoculum the number of plaques are TNTC (Too Numerous To Count). In the subsequent qPCR stage of the experiment the DNA was positive for MAP. PCR on the cultured colonies with probes specific for MAP (IS900) was positive and was negative for IS901 (*M. segmatis*) and IS6110 (*M. tb* specific.)

Figure 4



Legend to Figure 4. Johne Animal.

4A: is the negative experimental control plate. No plaques are seen. 4B: With 0.2 ml inoculum 189 plaques are identified. 4C; With 0.5 ml inoculum the plaque count is TNTC. Observe the necessity of plating different volumes of the lysate.

Samples were subcultured onto HEYM and followed for a minimum of 2 years. Colonies were seen HEYM slopes in 18% (3/17) Control animals (Table 1& Figure 5.) Of these Three Control animals where identifiable colonies grew, MAP plaque IS 900 qPCR was positive in one. (Table 1 & [27].)

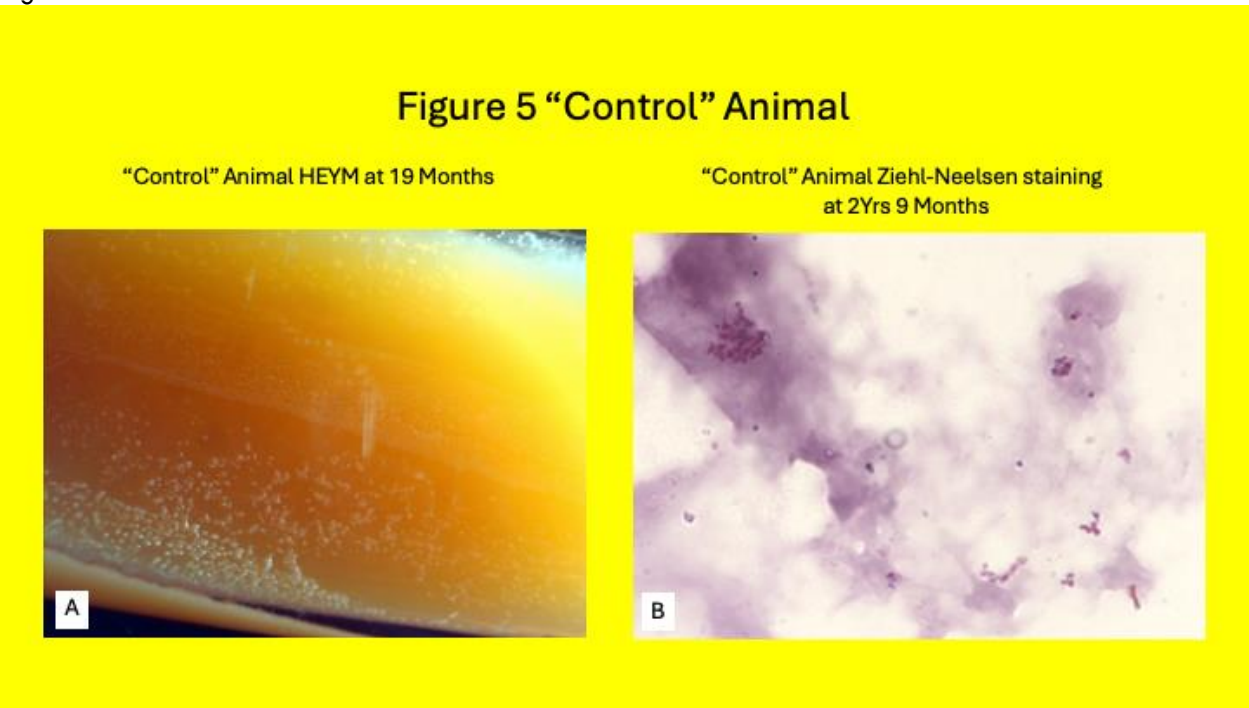
Table 1

Table 1: Control Animals: HEYM Culture +/-& Plaque # & qPCR (IS900) +/-[27]					
Control Animal #	HEYM Colonies Identified	Negative	Plaque #[27]	IS 900 DNA qPCR Positive[27]	IS 900 DNA qPCR Negative[27]
1c		-	11	+	
2c	+		1000	+	
3c		-	3		-
4c		-	238	+	
5c		-	688	+	
6c		-	29	+	
7c		-	10		-
8c		-	492	+	
9c	+		1		-
10c		-	151		-
11c	+		47		-
12c		-	4		-
13c		-	**1**		**-**
14c		-	8		-
15c		-	0		-
16c		-	4		-
17c			6		-
% positive (# positive/total tested)	18% (3/17)				

Legend to Table 1:

Shown are the three cases of HEYM positive "Control" animals compared with their previously published plaque numbers and their IS 900 qPCR positivity.[27]

Figure 5



Legend to Figure 5. Control Animal
5A: Herrolds egg yolk medium (HEYM) inoculum. Positive colonies seen at 19 months. 5B: Ziehl-Neelsen staining at two years nine months following inoculation onto HEYM. Note the clumps of alcohol-acid positive colonies.

Colonies were seen HEYM slopes in 19% (6/32) Johne animals (Table 2 & Figure 6.) Of the six Johne animals with identifiable colonies on HEYM slopes, four were plaque IS900 DNA positive (Table 2 & [27].)

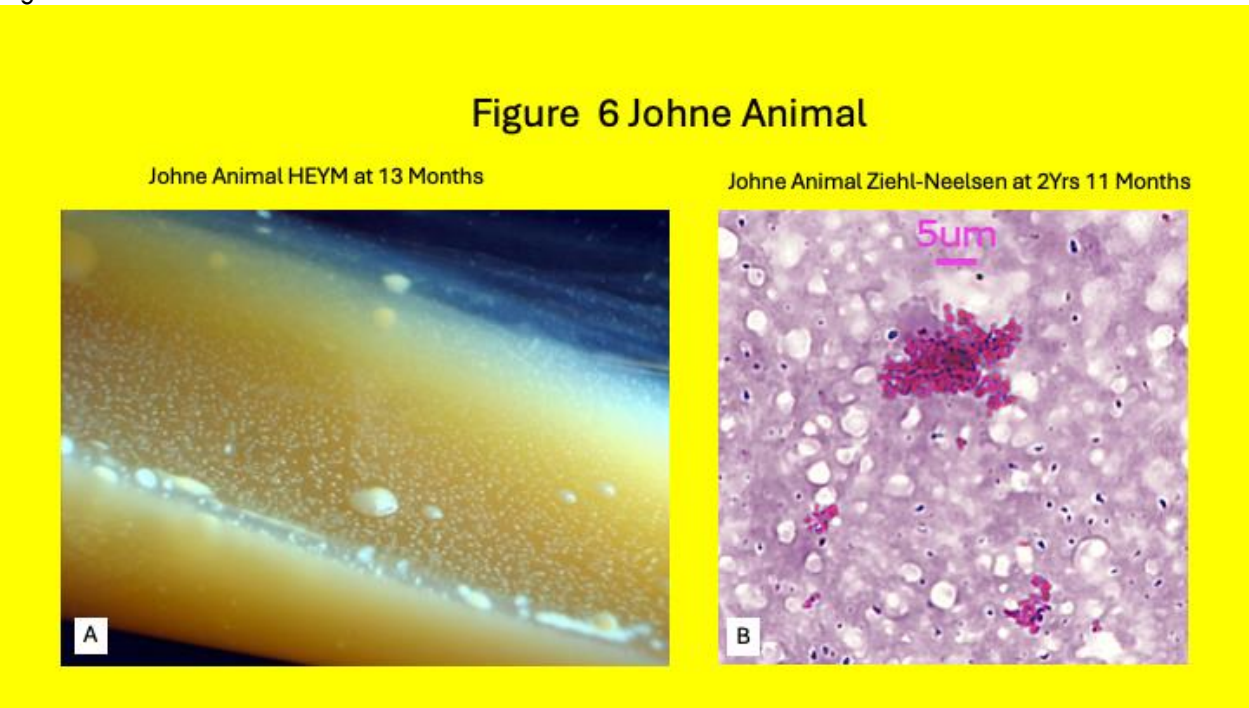
Table 2

Table 2: Johne HEYM Culture +/- & IS900 DNA qPCR +/-[27]					
Johne Animal Chart #	HEYM Culture +	Culture -	Plaque #[27]	IS 900 DNA qPCR Positive[27]	IS 900 DNA qPCR Negative[27]
1j		-	27	+	
2j		-	312	+	
3j		-	139		-
4j		-	1000est	+	
5j		-	25		-
6j		-	30	+	
7j		-	189	+	
8j		-	98	+	
9j		-	14	+	
10j		-	5	+	
11j		-	8		-
12j		-	545		-
13j		-	129	+	
14j	+		78	+	
15j		-	38**cal**	+	
16j		-	59		-
17j		-	26	+	
18j	+		214	+	
19j		-	48		-
20j	+		449		-
21j	+		91	+	
22j		-	135		-
23j		-	308	+	
24j	+		321	+	
25j		-	97	+	
26j		-	145	+	
27j		-	1000est	+	
28j	+		359		-
29j		-	229	+	
30j		-	17	+	
31j		-	64	+	
32j		-	5	+	
% positive (# positive/total tested)	19% (6/32)				

Legend to Table 2.
Shown are the six cases of HEYM positive Johne animals compared with their previously published plaque numbers and their IS 900 qPCR positivity.[27]

Shown in Figure 6A are the HEYM + from a Johne animal the Ziehl-Neelsen + staining on that animal (6B.)

Figure 6



Legend to Figure 6. Johne Animal

6A: HEYM inoculum with positive colonies seen at 13 months. 6B: Positive Ziehl-Neelsen staining at 2 years 11 months after inoculating onto HEYM. Note the clumps of alcohol-acid positive colonies.

IFNγ was positive in one Control animal with HEYM positive colonies, and negative for both ELISA as well as fecal IS900 PCR (Table 3 &[27].)

Table 3

Table 3: Control: Culture HEYM +/- & Serum studies & Fecal Culture +/- [27]					
Animal #	HEYM Culture Positive	ELISA +/-[27]	IFNγ +/-[27]	Fecal PCR +/-[27]	Plaque IS900 qPCR +/-[27]
1c		-	-	-	+
2c	+	-	-	-	+
3c		-	-	-	-
4c		-	-	-	+
5c		-	-	-	+
6c		-	-	-	+
7c		-	-	-	-
8c		-	-	-	+
9c	+	-	-	-	-
10c		-	-	-	-
11c	+	-	+	-	-
12c		-	-	-	-
13c		-	-	-	-
14c		-	-	-	-
15c		-	-	-	-
16c		-	+	-	-
17c		-	-	-	-
% positive (# positive/total tested)	18% (3/17)	0% (0/17)	12% (2/17)	0% (0/17)	35% (6/17)

Legend to Table 3

Identified are the three "Control" animals that were HEYM culture positive compared with their previously published serum and fecal culture data.[27]

In the six Johne animals with visible HEYM slope colonies, ELISA was positive in two, IFNγ was positive in five and all six of IS900 fecal PCR (Table 4 &[27].)

Table 4

Table 4: Johne: HEYM +/- & Serum data[27]					
Animal #	HEYM Culture +	ELISA +/-[27]	IFNγ +/-[27]	Fecal IS 900 PCR +/-[27]	Phage / qPCR +/-[27]
1j		-	+	+	+
2j		-	+	-	+
3j		-	+	+	-
4j		-	-	-	+
5j		-	-	+	-
6j		-	-	-	+
7j		-	+	-	+
8j		-	+	-	+
9j		-	+	+	+
10j		+	+	+	+
11j		-	+	+	-
12j		-	+	+	-
13j		-	+	-	+
14j	+	-	+	+	+
15j		-	-	+	+
16j		-	+	+	-
17j		-	+	+	+
18j	+	-	+	+	+
19j		+	-	+	-
20j	+	-	+	+	-
21j	+	+	+	+	+
22j		+	-	+	-
23j		-	+	+	+
24j	+	-	+	+	+
25j		-	+	-	+
26j		+	-	+	+
27j		-	-	+	+
28j	+	+	-	+	-
29j		+	-	-	+
30j		+	+	+	+
31j		+	+	+	+
32j		+	+	+	+
% positive (# positive/total tested)	19% (6/32)	31% (10/32)	69% (22/32)	75% (24/32)	72% (23/32)

Legend to Table 4

Identified are the six Johne animals that were HEYM culture positive compared with their previously published serum and fecal culture data.[27]

Discussion:

The evolution in the detection and identification of mycobacteria has been progressive over more than 130 years. A time consuming and insensitive system has increasingly and rapidly incorporated modern, rapid and mores sensitive techniques. In this study we have had an unparalled opportunity to compare classical and modern techniques.

We clearly show that the modern, rapid techniques are more sensitive and specific than the classical methods. Additionally, the modern techniques increased sensitivity show that putatively negative Control animals may be infected. The majority of the infected control animals were without clinical symptoms. The implications of this asymptomatic colonization/cryptic infection have far reaching implications. It signifies that animals that are colonized but not clinically symptomatic can be identified early in the course of their exposure, before they are symptomatic and possibly amenable to early and possible less intrusive interventions. For example, it is well described that elevated vitamin D levels are associated with improved outcomes in many mycobacterial diseases.[31-33]

In the Johne animals, all that were positive on HEYM had also been positive on fecal IS900 PCR. However, 17 animals that were fecal IS900 positive were not positive on HEYM (Table 4): again, attesting on the lack of diagnostic sensitivity of Classical mycobacterial detection methods. In contrast, none of the Control animals were fecal PCR positive, whereas 5/17 Control animals were positive, attesting the sensitivity of the plaque/qPCR assay over Classical mycobacterial detection methods.

One of the most powerful aspects of the plaque/qPCR methodology is that it can only detect living mycobacteria. A viable mycobacterium is necessary for

the mycobacteriophage to infect, replicate and burst releasing the DNA of the infected mycobacterium to be identified during the subsequent qPCR process. Thus, the concern that PCR is only detecting the DNA of dead mycobacteria DNA in milk will no longer be acceptable.

This technology has the potential to address a century long mycobacteriologist dilemma. [34] Although Leprosy has been considered to be a mycobacterial infection since 1882 [1], it has never fulfilled Kochs postulates.[3] As an obligate intracellular parasite, because of massive genomic deletion, [35] it cannot be grown in pure culture and therefore the second and fourth postulates cannot be met. The diagnosis of Leprosy is clinical. It is relatively simple with lepromatous leprosy but is clinical for tuberculoid leprosy [36,37], where the organism is not detectable. Multiple serum diagnostic tests to confirm an *M. Leprae* infection are proposed.[38-40] However, the phage/qPCR technology discussed in this manuscript has the potential to identify living *M. lepra* and render null and void Koch's unmet postulates. [3]

Conclusions:

Clinically useful detection of viable MAP in blood is feasible within 48 hours using a rapid phage/qPCR assay. In contrast, although of intellectual and academic interest, attempting to culture mycobacteria from blood has no role in clinical practice. In a miniscule number of cases, it may be of use in mycobacterial nucleic acid analyses.

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