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Abstract

Abattoir surveillance for bovine tuberculosis, which consists of identifying and submitting granulomas for histopathology and mycobacterial culture was the primary means for detecting new cases in the United States. Mycobacterial culture is expensive, labor intensive and identifies cases weeks after slaughter, hampering trace back efforts. To address this inefficiency, the United States Department of Agriculture replaced culture with real-time PCR for screening granulomas. The objectives of this paper were to describe the development and validation of this PCR as well as the performance of the assay during the first year of implementation. Using archived culture and histologically positive tissue, the sensitivity was 0.96 (95% CI: 0.89, 0.99) for the *Mycobacterium tuberculosis* complex primer-probe set and 0.89 (95% CI: 0.80, 0.95) for the *Mycobacterium bovis* specific primer-probe set. Specificity, estimated during by side by side testing was 0.998 (95% CI: 0.994, 1.000). After implementation, 6124 samples over 54 weeks were tested and all 36 histopathology positive samples were detected including 2 additional cases initially misclassified by histopathology. It appeared that specificity may have declined during post validation testing with 47/6086 signaling positive but not confirmed by either histopathology or culture. While PCR implementation has significantly improved the efficiency of the US slaughter surveillance program, careful attention must be paid to prevent and address cross contamination in the laboratory.

Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis,* is an important zoonotic disease that impacts international trade. Many countries spend significant resources eradicating, controlling or conducting surveillance for bTB in livestock and wildlife species. In the United States, the primary method to detect new bTB cases is abattoir surveillance where suspicious granulomas identified during inspection are submitted to the United States Department of Agriculture's (USDA) National Veterinary Services Laboratories (NVSL) in Ames, IA for examination.(Naugle et al. 2014) Historically, submitted granulomas were tested with histopathology and a subset of approximately 40% were also parallel tested using mycobacterial culture (**Fig. 1**). Mycobacterial culture was used to obtain isolates for genotyping and improve sensitivity by identifying cases not found on histopathology. Unfortunately, bTB cases identified by culture but not detected by histopathology would be found weeks after the carcass was sampled making tracing the animal back to the herd of origin significantly more expensive and challenging.

Direct PCR on fresh or borate preserved tissue has the potential to offer a parallel test to histopathology, and greatly reduce the labor, expense, and turnaround time required for mycobacterial culture. Published literature contains numerous PCR methods for the *Mycobacterium tuberculosis* complex (MTBC), including *M. bovis,* starting in 1990 with the exploitation of IS*6110* and then IS*1081* in 1991. (Collins & Stephens 1991; Eisenach et al. 1990) After 15 years of use in laboratories, two published meta-analyses reviewed in-house PCR methods analyzing sputum samples for human TB diagnosis, and these meta-analyses highlight variability in methods and in sensitivity and specificity for a relatively homogeneous specimen.(Flores et al. 2005; Greco et al. 2009) Variation from laboratory to laboratory

significantly impacts the usefulness and reliability of an assay, especially for a disease that requires regulatory guidelines. The development of real-time PCR technology, which provides a quantification of the nucleic acid target, was an important advancement in PCR.(Wittwer et al. 1997) Several researchers published primer-probe combinations for the IS*6110* and IS*1081* insertion elements.(Broccolo et al. 2003; Pan et al. 2013; Taylor et al. 2007; Thacker et al. 2011) In 2009, the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE) guidelines outlining documentation of real time PCR protocols were published.(Bustin et al. 2009).

Veterinary researchers and diagnosticians have recognized the advantages of using PCR to detect *M. bovis* in tissues, but the variation in tissue matrices adds complexity not generally seen with a sputum sample. Extraction methods must be able to deal with the diverse tissues matrices at a reasonable cost and be scalable to the daily influx of specimens. Previous studies investigated several methods including sequence capture, immunomagnetic methods, bead disruption, proteinase K digestion and others with moderate to successful results, some amenable to higher throughput testing.(Garbaccio & Cataldi 2010; Stewart et al. 2013; Taylor et al. 2007; Taylor et al. 2001). Commercialized PCR reagents have been developed and marketed such as: LSI VetMAX targeting all MTBC organisms (Thermo Fisher Scientific Waltham, MA, USA), and BoviMAN (Sliverline Bio, Valdivia, Chile), which targets *M. bovis* and was adopted as an official test by Servicio Agricola y Ganadero (SAG) the Animal Health Agency of Chile. However, a complete system, including extraction and control reagents, is not currently available. Furthermore, to the authors' knowledge, no country has yet published complete methods, workflow and performance of an in-house direct real-time PCR while being used in a national slaughter surveillance program.

The objectives of this paper were to describe the development and validation of an optimized extraction method, various probe-primer combinations and the manufacturing of controls used to monitor the performance of the assay. Finally, we report on the overall performance of the assay in the first year of national program use.

Materials and Methods

Development of primer-probe sets

To develop primer-probe sets, IS*1081* and IS*6110* transposase sequences representing *Mycobacterium africanum, M. bovis, Mycobacterium canetti, and Mycobacterium tuberculosis* species were obtained from NCBI database and aligned using Geneious v. 6.0.3 (Biomatters, Auckland, New Zealand) to identify conserved regions. To identify the most optimal primer-probe combinations, regions were selected throughout the transposases (**Fig S1**). The design feature available in Geneious was used to construct primers and probe, adjusting the Tm range to 64-68°C (66°C optimum) for the primers and 70-74°C (72°C optimum) for the probe. The best 3 combinations were selected for each insertion element, based on no dimer formation, no self-complementarity, and to hold amplicon size below 150bp. This study also included a previously published primer-probe set, extRD9, which targets a single copy region of the MTBC (Halse et al. 2011).

To develop primer-probe sets specific for *M. bovis*, differences between other *Mycobacterium tuberculosis* complex (MTBC) and *M. bovis* genomes were analysed using WebACT, the web-based version of Artemis Comparison Tool (www.webact.org). Large rearrangements were identified and several real-time PCR primer-probe sets were designed following parameters described above using MacVector (MacVector, Inc., Cary, North Carolina, USA) based on the

sequence of *M. bovis* (NCBI reference sequence NC_002945). Each of these primer sets were evaluated against publically available sequences of MTBC isolates. Two sites, Locus 2 (L2) and Locus 3 (L3), were identified to be specific for *M. bovis*. This study also included a previously published primer-probe set, targeting the lpqT locus, specific to *M. bovis*.(Reddington et al. 2011) Sequences and genome location of primer probe sets are contained in **Table S1**.

All primer-probe sets were manufactured at Integrated DNA Technologies (Coralville, Iowa, USA). All probe oligonucleotides incorporate a 5' FAM reporter, with the exception of extRD9 which signals with a 5' CY5 reporter. All probe oligonucleotides incorporated an internal fluorescent quencher (zen) and a 3' non-fluorescent quencher, both recommended by the manufacturer (Table S1). All PCR reactions reported in this study were performed in 20 µl reaction volumes using 10 µl Taqman Fast-Advanced PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1 µl 20x primer-probe mix (final concentration: primer, 500 nM; probe, 250nM), 4 µl Milli-Q pure water, and 5 µl of the purified DNA template. The reaction mixture was initially incubated at 50°C for 2 minutes, then 95°C for 10 minutes. Amplification occurred in 40 cycles: denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 60 seconds. The PCR reaction was performed on either a ViiA7 or 7500 Fast real-time PCR system (Thermo Fisher Scientific).

Analytical sensitivity and specificity

Genomic DNA from selected *Mycobacterium* species (**Table 1**) was quantified using a Qubit® (Thermo Fisher Scientific). Ten-fold serial dilutions of DNA were made using 1x TE pH8.0 to reach the following concentration range: 1 ng/5µl to 0.1 fg/5 µl and were used to evaluate the analytical sensitivity and efficiency of the 10 primer-probe sets. These DNA dilutions were also used to test a cross-reactivity to *Mycobacterium fortuitum* with the primer-probe sets targeting

IS*1081* and IS*6110*. Results were analysed by calculating % efficiency from the slope of the standard curve for each primer-probe set.

Tissue extraction

The extraction process was separated into 2 parts, DNA isolation and then DNA purification. *M. bovis* culture-positive, granulomatous tissue was obtained from 5 animals, dissected into 27, 300 mg portions, and stored at -20°C. Using a generalized randomized complete block design, 3 isolation methods and 3 purification methods were evaluated using 3 aliquots from each of the 5 animals. DNA purifications for a given DNA isolation method were performed on the same day, and analyzed on the same PCR plate. (Note: the fourth DNA isolation method was adopted after some inhibition issues were identified.).

DNA isolation methods:

1) NaOH: tissues were incubated in 50 mM sodium hydroxide (NaOH) at 95°C for 30 minutes, bead-disrupted using an equal mixture of 0.1 mm and 1.0 mm silicon beads (Bio Spec Products, Inc., United Kingdom) for 2 minutes, and then centrifuged 10 minutes at 13K x g at 20°C.
2) Phenol/chloroform (PC): tissues were added to vials containing equal volumes TE and phenol/chloroform (approximately 400 µl each), bead-disrupted and centrifuged as above.
3) TE: tissues were incubated with 400 µl 1x TE at 95°C for 30 minutes, bead-disrupted and centrifuged as above.
4) TE/ PC: tissues were incubated with 400 µl 1x TE at 95°C for 30 minutes and bead-disrupted; tubes were centrifuged as above; approximately 400 µl of the aqueous liquid was removed and purified with phase separation using an equal volume of phenol/chloroform and centrifuged as above.

Aqueous eluants from 1-3 were used to test three purification methods:

1) Method A: a commercial kit and protocol was followed (MagMAX™ Total Nucleic Acid Isolation Kit, 96 well plate format, Thermo Fisher Scientific).
2) Method B: a customized technology for high-throughput preparations; briefly, up to 400 µl of the aqueous portion of the phase-separation extraction was removed and mixed with 1.2 ml DNA Binding Buffer (Zymo Research, Irvine, California, USA); this solution was loaded onto the appropriate well either spin columns or 96-well spin plates, depending on the number of samples; rinsed with pre-wash and wash buffers, and eluted with 100 µl buffer. (ZR Fecal DNA Miniprep, Spin columns or Zymo-Spin™ I-96 Plate (Deep-well) formats, Zymo Research).
3) Method C: a traditional nucleic acid precipitation (400 µl of aqueous liquid was combined with 1/10th volume 3M sodium acetate and 2 times volume ice-cold ethanol).

To evaluate the performance of the initial three DNA isolation and the three purification methods, a linear model was fit using data from 4 of the 5 animals. One animal was eliminated from the analysis because not all methods produced a Cτ value. Methods were compared to each other using mean differences in Cτ values and 95% confidence intervals were calculated. A result was considered statistically significant if the 95% CI did not span zero. DNA isolation method 4 (TE/PC), developed later, was not included in these analyses.

Extraction and PCR controls

To monitor the extraction and PCR efficiency, 2.5 µl of a commercially available control was added to each disruption tube (*E. coli* hosting a plasmid containing a unique sequence, DNA Extraction Control 670 (DEC670), Bioline, London, United Kingdom). One microliter of DEC670 primer-probe mix (to achieve a Cτ value of 32-34) as added to the PCR mix and the

amount of water was adjusted to maintain a 20 µl reaction volume. To establish an inhibition cutoff value, standard deviations were calculated for 15 PCR runs and the acceptable range for DEC670 was set at 3 standard deviations from the mean.

In addition to the commercially available control, three tissue controls were developed; a negative control, and two tissue positive controls containing either H37Ra or BCG. The negative control was produced by homogenizing approximately 300 g of bovine liver in 200 ml PBS. To produce the positive controls, cultures of *M. bovis* BCG (ATCC35734) and *M. tuberculosis* H37Ra (ATCC25177) were grown in 7H9 broth containing 0.8% Tween-80 at 37°C with 10% CO_2 for 3 - 4 weeks. Using a spectrophotometer, the optical density was measured at 600 nm. Cells per ml was calculated using a modified extinction coefficient: 1 O.D. ~ 3 x 10^6 cfu/ ml.(Larsen et al. 2007) Two ml of a type culture was added to 200 ml of the homogenized liver, (final concentrations of bacteria per ml homogenate: H37Ra = 0.5 x 10^2 to 1.0 x 10^2 cfu/ml, BCG = 0.5 x 10^3 to 1.0 x 10^3 cfu/ml). One ml aliquots were stored at -20°C, thawed once and kept refrigerated for up to 5 days before discarding. Approximately 100 µl of the controls were added directly to the prepared disruption tubes. The negative control and the H37Ra tissue control were added between every 5th test sample for the first 20 samples and after that, every 10th sample. The BCG tissue control was used one time at the end of the run. Performance of the positive tissue controls was evaluated by measuring the mean and standard deviation of the Cτ values between replicate controls on the same plate and then comparing those against previous runs. These controls are available through NVSL's reagent catalog www.aphis.usda.gov/nvsl .

Diagnostic sensitivity evaluation using archived tissues

Initially, 26 tissues archived at -20ºC, (24 *M. bovis* culture positive, 2 *M. tuberculosis* positive and 2 culture negative tissues) were blinded and tested with DNA isolation method 2 (PC), DNA purification method B and MTBC primer-probe IS*1081*-3. Once method 2 was shown to be problematic, those same tissues, along with 54 additional archived tissues were used to estimate the sensitivities of both IS*1081*-3 and L3 primer-probe sets using DNA isolation method 4-TE+PC, DNA purification method B. Inhibited samples were excluded from the analysis. Sensitivity was calculated and 95% confidence intervals were obtained using the Clopper Pearson method.(Agresti 2002)

Diagnostic specificity during side by side testing with slaughter surveillance

To characterize specificity, and develop a standardized laboratory workflow, the PCR was implemented while continuing standard histopathology and culture testing during 4 months of routine slaughter surveillance testing. Routine slaughter surveillance was defined as granulomas identified during regular slaughter from animals with no known previous bTB exposure or antemortem bTB test results. Briefly, granulomas identified during carcass inspection were split with ½ the lesion placed in 10% buffered formalin for histopathology and ½ the lesion was placed in sodium borate for culture. Histopathology was conducted the day after the samples arrived, and tissue submitted in formalin for histopathology were routinely processed, sectioned and stained with hematoxylin and eosin stains. If inflammatory lesions were identified microscopically, additional histochemical stains were performed on the formalin fixed paraffin embedded tissue and mycobacterial cultures were started on the tissue submitted in sodium borate to help identify the underlying etiology of the inflammatory lesions. Samples were diagnosed as ‘mycobacteriosis compatible” based on histopathology when there were

granulomatous lesions that contained acid-fast bacilli, and both the lesion characteristics and the bacteria morphology were consistent with an *M. bovis* infection.(Waters et al. 2011) If the diagnosis was mycobacteriosis compatible, the Pathology section would also perform conventional PCRs targeting IS*6110* for MTBC, 16S for *M. avium*, and IS*900* for *M. paratuberculosis* on sections from the paraffin block.(Miller et al. 1997; Waters et al. 2011)

Mycobacterial culture was performed by homogenizing the tissue, decontaminating with NaOH and inoculating on to in-house modified 7H11 Middlebrooks solid media and BACTEC™ MGIT™ 960 liquid media.(Robbe-Austerman et al. 2013) Acid fast stains were conducted on all signal positive media and suspicious colonies. If acid fast positive, DNA hybridization probes specific to the MTBC were performed (AccuProbe *Mycobacterium tuberculosis* complex culture identification test, Hologic, Sunnyvale, CA). If results were negative, cultures were reported out as *Mycobacterium* species – not *Mycobacterium tuberculosis* complex. If results were positive, whole genome sequencing was conducted to determine the species and genotype the isolate.

Direct PCR was performed on all sodium borate submitted samples following this workflow: processing technicians sampled and heat inactivated the tissue, molecular technicians conducted the bead disruption, extraction and PCR, and microbiologists analyzed the PCR run the next morning. A run was considered valid if all the controls performed as expected and fewer than 1 in 10 samples were inhibited. Validated PCR results were provided to the pathologists after histopathology was completed but prior to the report being released. Discrepant test results were reviewed and the pathologists determined the final diagnosis. Once at least 1000 samples were tested successfully, officials at the United States Department of Agriculture Animal Plant Health Inspection Service (USDA-APHIS) evaluated the direct PCR assay workflow and results, and approved its use in the National slaughter surveillance program.

To analyze these data, a true *M. bovis* case was defined as an animal that had both a mycobacteriosis compatible histopathology result and a positive *M. bovis* culture. All other samples were considered truly negative. Procedures allowed for specimens to be sampled multiple times during the same PCR run at the discretion of the technician, and a sample was considered PCR positive if any one subsample was positive. All direct PCR results with a determined Cτ value were considered test positive.

Post validation performance

Once test implementation was approved by USDA-APHIS, the workflow outlined in **Fig. 1** was implemented. All borate samples were tested with the direct PCR independently and in parallel with histopathology. If the results were PCR negative and the histopathology diagnosis was anything other than mycobacteriosis compatible, no further testing was done. PCR signal positive, mycobacteriosis compatible samples, and any discrepant results were cultured. Histopathology results from discrepant cases were reviewed by the pathologists to re-access and confirm their diagnosis. If the Cτ value was less than 35, an *M. bovis* specific PCR was conducted. To assist with trouble shooting the direct PCR assay, if the PCR results had a Cτ value greater than 35, a *M. bovis* specific PCR was generally not done, and the assay for that sample was repeated. Data was collected from 2014-04-21 to 2015-05-06. The proportion of histopathology positive and negative samples that tested positive and negative by PCR, respectively, was estimated.

In addition to routine slaughter surveillance, animal health officials requested direct PCR testing on cattle from known infected herds with gross lesions identified at necropsy or slaughter. A total of 341 cattle from 3 affected premises were tested with the direct PCR in parallel with either histopathology, culture, or both histopathology and culture as described above. For this

dataset, true positive cases were defined as either having a histopathologic diagnosis of mycobacteriosis compatible or *M. bovis* culture positive results. Unlike the post validation slaughter surveillance where direct PCR testing influenced culture results, these samples were cultured independently of direct PCR results; consequently, sensitivity and specificity could be calculated.

Results and discussion

Development of primer-probe sets, analytical sensitivity and specificity

The ten primer-probe sets ranged from 82-103% efficiency during the initial evaluation (**Table S2**). All sets detected *M. bovis* DNA, and those designed to detect only *M. bovis* DNA did not cross-react with other MTBC DNA (**Table 1A**). Primer-probes designed against IS*1081* and IS*6110* did, however, cross-react with high concentrations of *M. fortuitum* DNA although at significantly higher Cτ values than comparable amounts of MTBC DNA. (**Table 1B**). At 100 pg, these primer-probe sets did not detect *M. fortuitum* DNA, but still detected *M. bovis* DNA with Cτ values ranging from 19.52 to 21.89. While this cross-reactivity was interesting, *M. fortuitum* DNA would not be expected to be found in diagnostic specimens at those levels, so the cross-reactivity was not likely to be clinically relevant.

Because laboratory contamination with amplicons or even DNA is a well-known reoccurring problem in clinical laboratories, it was prudent to identify and maintain multiple primer-probe sets.(Mandal et al. 2012) Primer-probe sets targeting the multi-copy insertion sequences consistently generated lower Cτ values than those targeting single copy locus (**Table S2**). While no cross reactions occurred using the extRD9 primer-probe set, the analytical sensitivity improvements gained by using a MTBC primer-probe sets to IS*1081* or IS*6110* was compelling.

Therefore, these were selected for the initial screening of borate submitted samples. Positive results were followed with a *M. bovis* specific probe-primer set if *M. bovis* was suspected or with extRD9 if another MTBC organism was expected. Consequently, the primer-probe sets 1081-3, 6110-2, extRD9, L2, L3, and lpqT were evaluated against tissue extracts reported below (**Fig. 2B**).

Tissue extraction

DNA isolation method 1 (NaOH) clearly underperformed when combined with purification method A, failing to consistently identify 4 of the 5 culture positive animals (**Fig. 2A, Table S3**). Sample 07315 failed to produce Cτ values for all three subsamples in a majority of the testing, and was excluded from comparative analysis of the methods. No significant differences were identified between method 2 (phenol-chloroform), or method 3 (TE) (**Table S4 A, B**). DNA purification method B was the only method to produce statistically significant lower mean Cτ values (**Table S4 C, D**). Demonstrating statistical significance with small sample sizes is difficult, however, these small studies can provide rapid guidance when testing diverse methods. Other criteria also influence choices of methodology, for example DNA isolation method 3 required a 30 minute incubation step not required in method 2. All samples purified by method C, ethanol precipitation, showed negative test results. This may be due to high quantities of DNA purified, which likely overwhelmed the PCR reactions. The spin filter (method B) and magnetic beads (method A) inherently normalize DNA amounts in the final eluent. Because method C would probably require an additional DNA quantification step to normalize the amount of DNA added to the PCR reaction, this method was not investigated further. Because < 24 hour

turnaround time was desired, the more rapid, DNA isolation method 2 was initially chosen and combined with purification method B.

Sensitivity

During the first week of slaughter surveillance side by side testing, all 5 PCR runs contained inhibited samples (no Cτ value detected for both the DEC670 and MTBC primer-probe sets) with an overall rate of 22%, a problem not seen during development. A decision was made to combine methods 2 and 3 into method 4, which solved the inhibition problem. The sensitivity evaluation was repeated with 80 archived tissues (the original 26 in addition to another 54). (**Files S1, S2**) It did not appear that the sensitivity was negatively impacted by combining methods 2 and 3. The sensitivity for the 1081-3 primer-probe set was 0.96 (95% *CI*: 0.89, 0.99) and for the L3 primer-probe set was 0.89 (95% *CI*: 0.80, 0.95). (**Table 2A and 2B**)

Specificity

During the routine slaughter surveillance side-by-side testing, 1742 tissues were tested using the 1081-3 primer-probe set (**File S3**). Of those, 1736 tested negative by both PCR and histopathology. PCR correctly identified all three positive samples and misidentified three samples as positive for a specificity of 0.998 (95% *CI*: 0.994, 1.000). (**Table 2C**) This supported initial thoughts that the cross reactivity with *M. fortuitum* was not clinically relevant. Because the L3 *M. bovis* specific probe-primer set was used only to identify if the positive results from the MTBC primer-probe sets were due to *M. bovis*, diagnostic specificity was not done for this primer-probe set.

Post validation performance

A total of 273 PCR runs were performed during 54 weeks, 251 of which were deemed as valid, and 15 as invalid. The most common reason for determining a failed run was an excessive number of inhibited controls or samples, often complicated with false positive signals in samples and negative controls. Of the 6162 samples tested during this time period, 38 were PCR MTBC positive and confirmed by both histopathology and culture, and 47 were PCR positive but were not confirmed by either histopathology or culture (**Table 2D, File S4**). While the PCR detected all the mycobacteriosis compatible cases identified by histopathology (that were not caused by atypical mycobacteria) during this time period, there were slightly more false positive samples than expected. Nearly all of the samples with false positive signals were retested the next day while they were prepared for culture and in all but 2 cases, the false positive signals were not repeated. Despite this problem, the PCR did identify 2 cases that were initially missed by the histopathology, and corrected upon review of the slides. Of importance, one of those cases had an initial diagnosis of coccidiomycosis which, under the old system, would not have been cultured. These results reinforced the importance of performing two independent tests during routine diagnostic testing.

Because the PCR influenced how histopathology and culture were conducted, a true sensitivity and specificity cannot be calculated from this sample set; however, estimations should be calculated to evaluate post validation performance. The proportion of probable negative samples that tested PCR negative was 0.992 (95% *CI*: 0.990, 0.994) and the proportion of probable positive samples that tested PCR positive was 1.00 (95% *CI*: 0.91, 1.00).

All three controls used were designed to have Cτ values between 32 - 35, a value that is about as high as possible and yet still consistently test positive during a normal run. The BCG control was developed to consistently be 2-3 Cτ values lower than the H37Ra control because it needed to be

reliably positive when testing samples with the less sensitive *M. bovis* specific primer probe sets. Since most PCR runs had greater than 5 negative and H37Ra controls, within plate and day to day precision can be monitored over time. **Table 3** shows the mean Cτ values and their standard deviations during post validation performance for all control samples (**File S4**). Interestingly, the variability between the controls was fairly consistent, but rather high (1.3 – 1.8). Several unsuccessful attempts were made to improve this variability, such as increasing or decreasing tissue concentrations and volumes. **Fig. 3** shows a box-and-whisker plot of the confirmed positive (both 1081-3 and L3), the false positive samples and the spiked controls. Nearly all of the false positive samples had higher Cτ values than the controls, and the controls generally had higher Cτ values than positive samples. This assisted in predicting the false positive samples.

The apparent reduction in specificity from the side by side testing (3/1739) to the post validation testing (49/6124), suggests laboratory cross contamination, especially since the vast majority were not confirmed when re-tested.(Borst et al. 2004) This is not unexpected with an open DNA isolation and purification system containing a large number of positive controls that challenge the system. Potential improvements to reduce this risk would depend on the cause of the contamination. If contamination is due to within plate cross contamination, centrifugation of spin plates could be replaced with a vacuum apparatus, and manual pipetting could be replaced by a liquid handling system. If contamination is due to amplicons or carryover from previous runs, strict work flow guidelines and environmental controls must be evaluated and potentially altered.(Aslanzadeh 2004; Lo & Chan 2006) Parallel testing with histopathology has prevented this false positive rate from negatively impacting the program, however, it is critical to keep cross contamination at a minimum.

Performance of the PCR on samples from known infected herds

While the goal of the PCR was to replace mycobacterial culture screening for routine slaughter surveillance, State and Federal animal health officials also request its use during the surveillance of bTB affected herds to assist with ante mortem test evaluation (**File S5**). This was an opportunity to conduct a more robust analysis of sensitivity. Of the 341 samples tested in parallel, one was eliminated due to inhibition, 307 were confirmed positive and 33 negative by histopathology or culture. PCR detected 297 of the 307 using the 1081-3 primer-probe set for a sensitivity of 0.97 (95% CI: 0.94, 0.99) (**Table 2E**). During follow-up testing for *M. bovis*, the L3 primer-probe set correctly identified 289 of the 303 positive samples tested for a sensitivity of 0.95 (95% CI: 0.92, 0.97) (**Table 2F**). The 97% sensitivity of the assay in cattle from known infected herds was similar to the initial sensitivity estimate of 0.96 (95% *CI*: 0.89, 0.99) using archived samples.

Interestingly, testing tissue samples from affected herds (both archived and diagnostic cases) had a higher rate of false negatives than routine slaughter-surveillance, of which there were no apparent misses. This most likely was caused by sampling. For routine slaughter surveillance, granulomas must be developed well enough to be identified during the inspection process. In contrast, animals from infected herds go through an enhanced inspection process and often the slightest abnormality is submitted for testing. During culture, up to 50 g of tissue was processed and concentrated verses a 300 mg portion used in the direct PCR. This puts tissues in the early stages of granuloma formation with very few bacteria more susceptible for false negative results.

Conclusion

This paper describes the validation and implementation of direct PCR in the USDA bTB slaughter surveillance program. This assay has allowed the USDA to efficiently test all samples submitted in parallel with histopathology, improving accuracy by reducing laboratory error. Limitations of the PCR assay include the need for highly trained staff, strict workflow procedures, environmental controls to prevent cross contamination, and the small sample volume requiring careful dissections of visible lesions. Despite these limitations, the assay appears to be highly sensitive and specific. During the year of post validation slaughter surveillance testing, no histologic positive samples were missed by the PCR, and 2 additional cases were detected preventing erroneous results from being released.

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482

Table 1 (on next page)

Analytical specificity of *M. tuberculosis* complex and *M. bovis* primer-probe sets

A) Cycle threshold results from each primer-probe set against DNA extractions from selected *Mycobacterium* species. **B)** Cycle threshold results from the primer-probe sets targeting IS*1081* and IS*6110* elements against serial dilutions of *M. fortuitum* genomic DNA as compared to 100 pg BCG genomic DNA.

A

Isolate	ATCC ID	Cycle threshold number								
		1081_1	1081_2	1081_3	6110_1	6110_2	lpqT	L2	L3	extRD3
M. bovis BCG	35734	23.59	23.25	22.24	26.33	23.97	27.54	28.66	28.3	25.58
M. tuberculosis H37Ra	25177	14.62	14.3	14.4	12.87	11.97	U	U	U	17.65
M. microti	11152	15.63	14.81	15.26	13.87	12.97	U	U	U	18.39
M. africanum	25420	14.996	14.29	14.65	14.75	13.83	U	U	U	17.98
M. kansasii	12478	U*	U	U	U	U	U	U	U	U
M. avium subsp. *avium*	25291	U	U	U	U	U	U	U	U	U
M. avium subsp. *paratuberculosis*	19698	U	U	U	U	U	U	U	U	U
M. fortuitum	6841	33.68	35.005	33.35	33.16	31.41	U	U	U	U
M. terrae	15755	U	U	U	U	U	U	U	U	U

*U = undetermined Cycle threshold

B

Isolate	Cycle threshold number			
	1081-1	1081-3	6110-1	6110-2
M. bovis BCG (100pg)	19.75	19.52	21.89	21.13
M. fortuitum (100ng)	37.6	34.87	33.6	32.78
M. fortuitum (10ng)	36.39	36.98	34.56	33.95
M. fortuitum (1ng)	36.84	U	U	U
M. fortuitum (100pg)	U	U	U	U

*U = undetermined Cycle threshold

Table 2 (on next page)

Comparison of direct real-time PCR to histology and / or culture.

A) Eighty archived tissues previously tested with histology and culture were compared to PCR using the *M. tuberculosis* complex 1081-3 primer-probe set and **B)** the *M. bovis* L3 primer-probe set of which 5 were inhibited and removed. **C)** Routine slaughter-surveillance samples compared with the traditional workflow of histology and culture to the IS1081-3 PCR (side by side testing), primarily to evaluate specificity. **D)** Routine slaughter surveillance, post validation, comparing IS1081-3 PCR to histology (followed with culture when indicated). **E)** Lesioned tissue from cattle in known bTB affected herds that compared histology and /or culture to the 1081-3 primer-probe set and **F)** the L3 primer-probe set (5 samples not tested).

1

A Archived lesioned tissues

Histology and culture

		bTB Positive	bTB Negative	
IS1081-3 PCR	Positive	74	0	74
	Negative	3	3	6
		77	3	80

B Archived lesioned tissues

Histology and culture

		bTB Positive	bTB Negative	
L3 PCR	Positive	65	0	65
	Negative	8	2	10
		73	2	75

C Slaughter surveillance, side by side testing

Histology and culture

		bTB Positive	bTB Negative	
IS1081-3 PCR	Positive	3	3	6
	Negative	0	1736	1736
		3	1739	1742

D Slaughter surveillance, post validation

Histology (confirmed by culture)

		bTB Positive	bTB Negative	
IS1081-3 PCR	Positive	38	47	85
	Negative	0	6077	6077
		38	6124	6162

E Lesioned tissue from bTB affected herds

Histology or culture

		bTB Positive	bTB Negative	
IS1081-3 PCR	Positive	297	1	298
	Negative	9	32	41
		306	33	339

F Lesioned tissue from bTB affected herds

Histology or culture

		bTB Positive	bTB Negative	
L3 PCR	Positive	289	0	289
	Negative	14	31	45
		303	31	334

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Table 3(on next page)

In-house PCR tissue control performance

The real-time PCR performance of in-house PCR tissue controls using a commercial exogenous control and the *M. tuberculosis* complex primer-probe set 1081-3 during the 54 weeks of post validation slaughter surveillance.

1

PCR Primer-probe	Sample	Total # tested	Mean (Cτ)	Standard deviation
DEC670	Negative Control	1233	34.4	1.8
DEC670	H37Ra Control	1233	34.5	1.6
1081-3	H37Ra Control	1233	33.0	1.4
DEC670	BCG Control	251	34.6	1.6
1081-3	BCG Control	251	31.5	1.5

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Figure 1(on next page)

Diagnostic testing work flow before and after the implementation of direct PCR for bTB slaughter surveillance

Prior to the implementation of direct PCR, parallel testing by mycobacterial culture was only able to be conduted on approximately 40% of submitted samples and bTB cases misclassified by histology were detected weeks after the lesion was submitted. After direct PCR implementation, 100 % of cases were tested in parallel with histology and PCR prior to release of histology results and only test positive or discrepant cases were subsequently tested by mycobacterial culture.

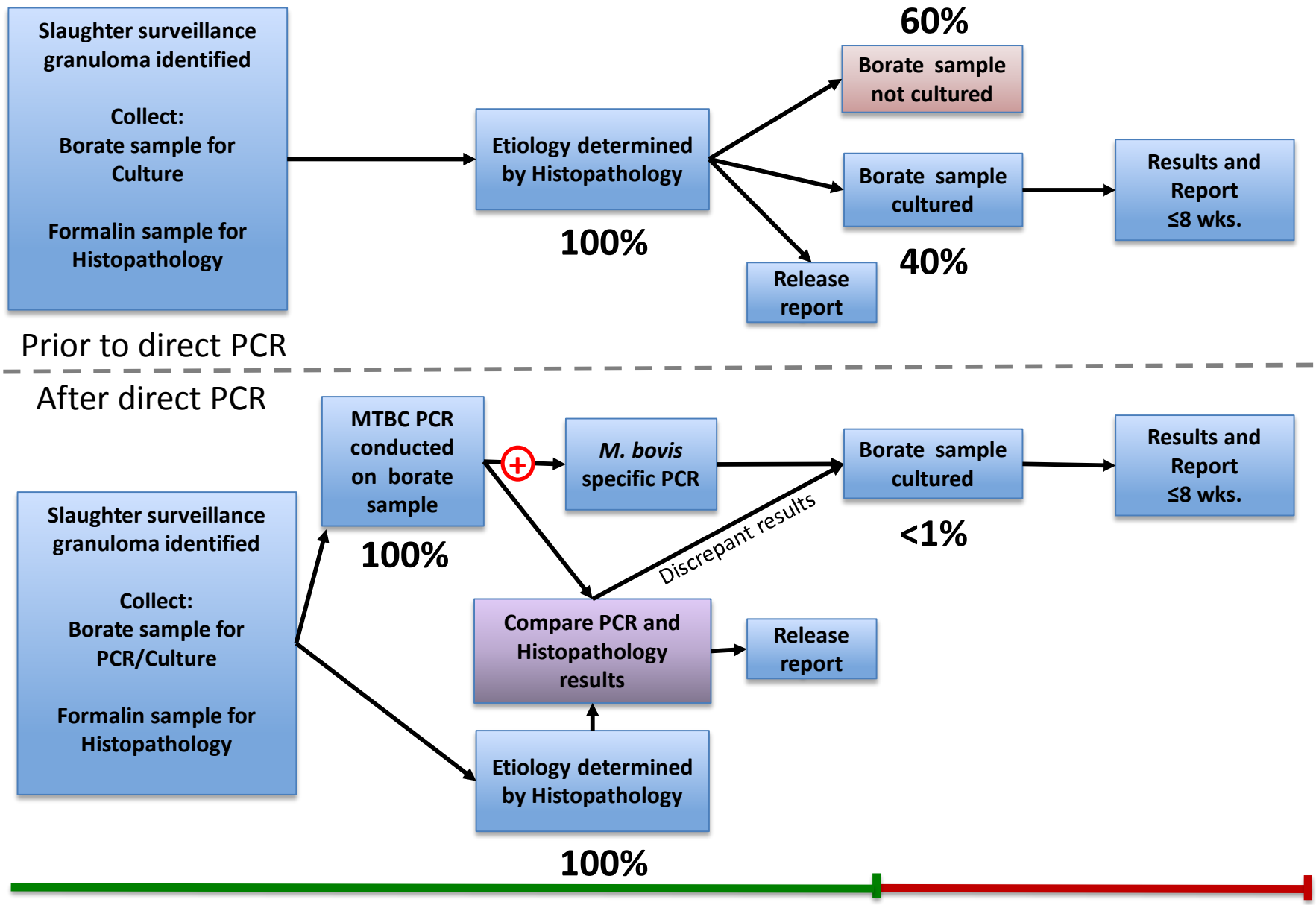

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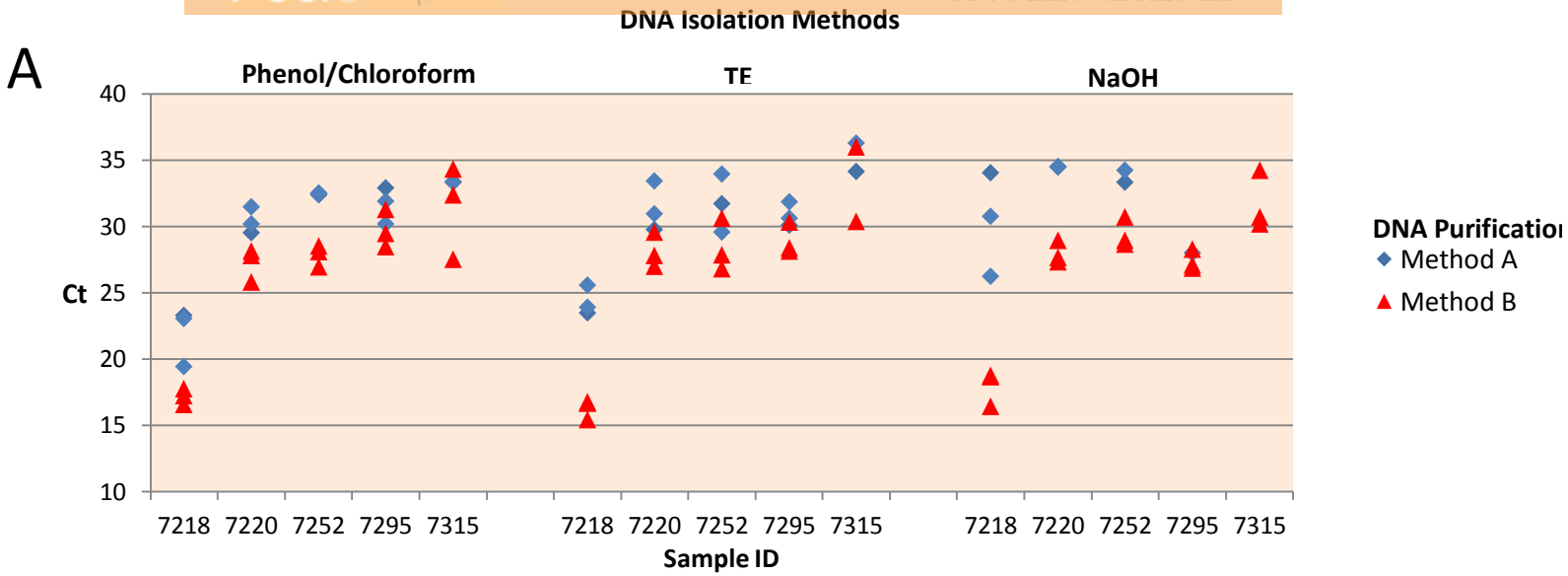
Figure 2(on next page)

Comparison of DNA extraction, purification methods and primer-probe sets

A) Comparison of purification methods based on DNA isolation technique. Five samples were subsampled three times and extracted by phenol/chloroform, TE, or NaOH; aqueous extracts were further purified by either Method A or Method B; eluants were analyzed by PCR using the 1081-3 primer-probe set. **B)** Evaluation of the performance of selected primer-probe sets based on DNA isolation method 3 and purification method B (negative results not displayed).



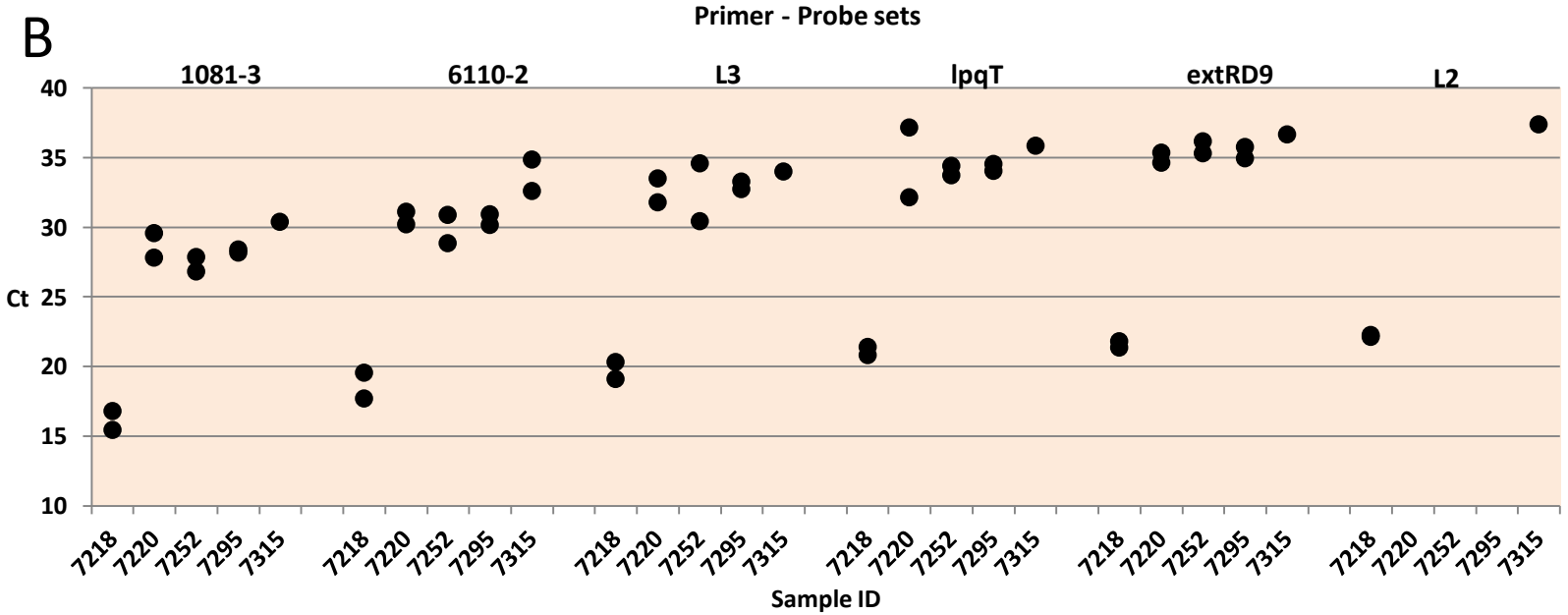


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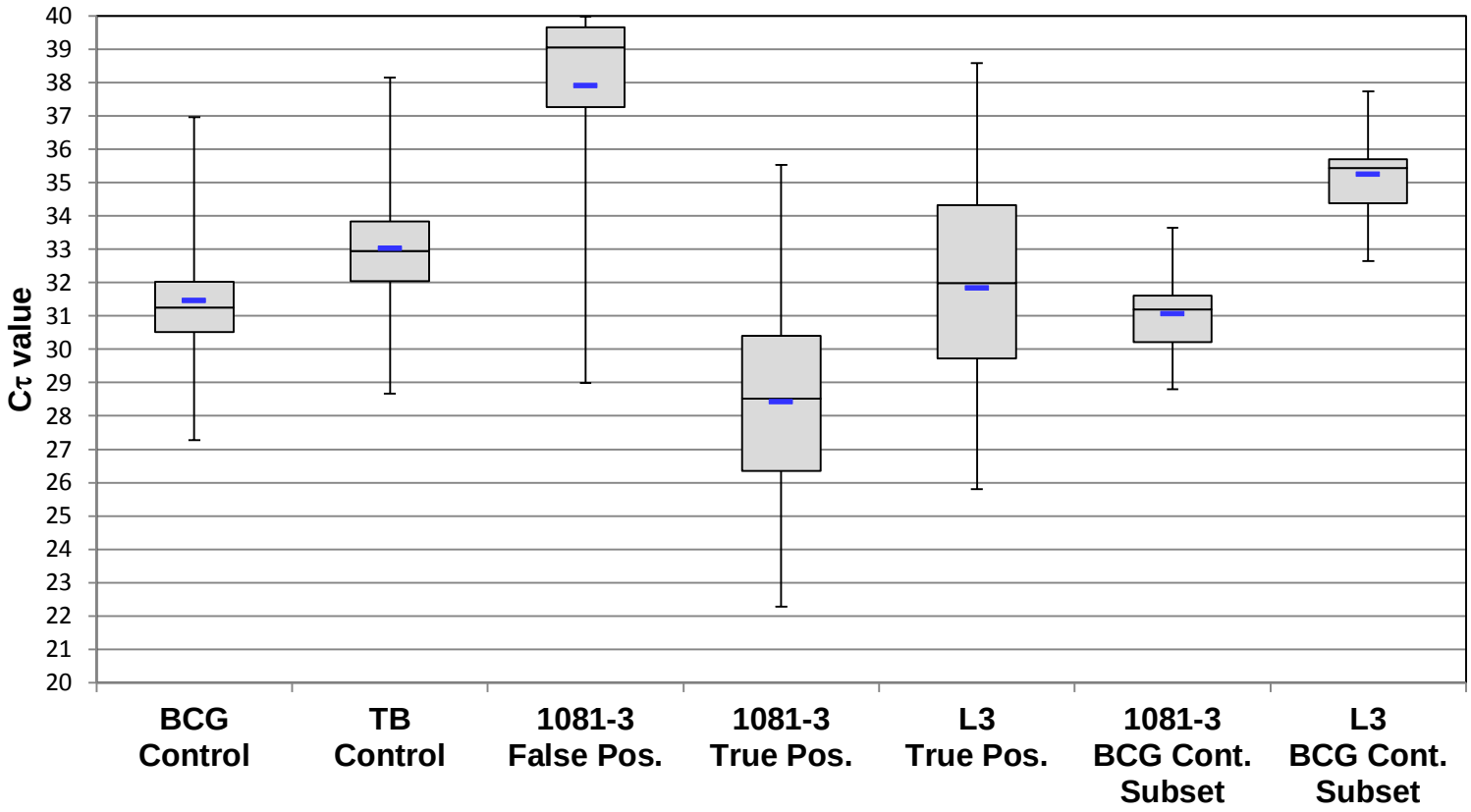
Figure 3(on next page)

Cycle threshold results of controls, false positive and positive cases

Box whisker plot of Cτ results during 1 year post validation. The first two plots shows all BCG and the H37Ra (TB) control results using 1081-3. The next two plots show the false positive and histology and culture confirmed bTB cases using 1081-3. The last three plots contain the subset of confirmed bTB samples using L-3 and the corresponding controls.



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