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Note

Comparison of three DNA extraction methods for molecular confirmation of *Mycobacterium avium* subspecies *paratuberculosis* from the VersaTrek™ liquid cultures of bovine fecal samples

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ABSTRACT

We evaluated three DNA extraction methods for confirmation of *Mycobacterium avium* subspecies *paratuberculosis* from liquid cultures of bovine feces. Use of DNA Extract All Reagents Kit™ resulted in efficient extraction of amplifiable DNA from higher proportion (96.29%) of known positive samples compared to Chelex-100 resin (25.92%) and polyethylene glycol (0%).

Johne's disease (paratuberculosis) is a chronic granulomatous enteric disease of cattle and several other domestic and wild animals, clinically manifested as loss of weight, intermittent or chronic diarrhea and death (Harris and Barletta, 2001; Tiwari et al., 2006). *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a fastidious acid-fast bacterium, is the etiological agent of Johne's disease (Harris and Barletta, 2001). The disease is distributed worldwide and is economically significant to livestock industry due to loss of production, culling, mortality and test costs (Garcia and Shalloo, 2015; Windsor, 2015). Laboratory diagnosis of Johne's disease is commonly achieved either by detecting the causative agent in fecal or tissue samples or indirectly by demonstrating the presence of pathogen-specific antibodies in serum or milk samples (Tiwari et al., 2006; Collins, 2011). Culture of MAP from diagnostic specimens such as feces and tissue samples has been considered the "gold standard" for the diagnosis of Johne's disease. Several liquid and solid microbiological media and culture systems have been used for cultivation of MAP in the laboratory (Stich et al., 2004; Gumber and Whittington, 2007; Pozzato et al., 2011; Whittington et al., 2013). The liquid culturing systems facilitate faster diagnosis of Johne's disease in about 6 weeks compared to > 16 weeks needed with use of solid media (Whittington et al., 1998). In addition, liquid culture systems allow monitoring of bacterial growth based on production of $^{14}CO_2$ (Bactec 460), changes in the pressure (VersaTREK system), or consumption of oxygen (Bactec MGIT [mycobacterial growth indicator tube] 960 para TB system) in the culture bottles. However, presence of MAP in liquid cultures requires further confirmation which can be achieved by PCR in conjunction with acid-fast staining (Whittington et al., 1998; Kim et al., 2004).

The complex lipid-rich cell wall of MAP in addition to presence of PCR inhibitors in the egg yolk-rich dye containing culture medium renders isolation of high quality DNA in sufficient quantities difficult for sensitive detection of MAP by PCR (Whittington et al., 1998; Okwumabua et al., 2010). Several methods of DNA isolation from MAP cultures including boiling, ethanol precipitation, phenol-chloroform method, enzymatic cell lysis and mechanical cell disruption such as bead beating have been attempted with varying degrees of success (Whittington et al., 1998; Sweeney et al., 2006; Okwumabua et al., 2010; Kawaji et al., 2014; Plain et al., 2015). An efficient and cost effective method for extraction of DNA from MAP cultures is highly desirable.

In the present study, we evaluated three DNA extraction methods for isolation of DNA from MAP cultures; Chelex 100 resin 6% w/v (InstaGene Matrix, Bio-Rad Laboratories, Inc., Hercules, CA), alkaline polyethylene glycol (DNAzol Direct, Molecular Research Center, Inc., Cincinnati, OH) and DNA Extract All Reagents Kit™ (Life Technologies, Carlsbad, CA). These methods were chosen as they were rapid and cost effective involving incubation of samples with reagents at elevated temperature for short duration without the need for any expensive equipment. Fecal samples from the 2015-, and 2016 Johne's disease proficiency test panels obtained from the National Veterinary Services Laboratories and diagnostic specimens submitted to the Pennsylvania Veterinary Laboratory were used in the study. Fecal samples were

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Table 1
Efficacies of amplifiable DNA extraction, as assessed by the IS*900* PCR, from *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cultures of fecal samples using three methods.

Samples source	Shedding status[a] (No. of samples)	Recovery of acid-fast bacilli	Chelex 100 resin	Alkaline polyethylene glycol	DNA extract all reagent kit
NVSL Johne's disease proficiency test panels	Negative (13)	0/13	0/13	0/13	0/13
	Low (4)	3/4	0/4	0/4	3/4
	Moderate (7)	7/7	2/7	0/7	7/7
	High (16)	16/16	5/16	0/16	16/16
No. of positives identified/total positives		26/27 (96.29%)	7/27 (25.92%)	0/27 (00.00%)	26/27 (96.29%)

[a] Shedding status provided by the National Veterinary Services Laboratories (NVSL).

cultured in para-JEM medium using a VersaTREK Automated Microbial Detection System (TREK Diagnostic Systems, Oakwood Village, OH) as described previously (Kim et al., 2004). Briefly, fecal sample was decontaminated by incubation in 0.9% cetylpyridinium chloride for 18 to 24 h followed by treatment with antibiotic solution containing amphotericin B, vancomycin, and nalidixic acid for 24 h. The sample was then inoculated into para-JEM culture bottle containing para-JEM EYS (egg yolk supplement), para-JEM AS (antibiotic supplement) and para-JEM GS (growth supplement) and incubated in the VersaTREK™ Automated Microbial Detection System for 42 d. After incubation, smears of cultures were subjected to acid-fast staining and examined under a microscope to assess the presence of acid-fast bacilli (AFB).

DNA from MAP cultures was isolated using Chelex 100 resin, alkaline polyethylene glycol or DNA Extract All Reagents Kit™ following manufacturers' recommendations. Briefly, 300 µl culture sample was centrifuged at 20,000 ×*g* for 3 min and the resulting culture pellet was washed once with 1 ml sterile PBS and used as starting material for DNA extraction in all protocols. For extraction using Chelex 100 resin, the culture pellet was suspended in 200 µl of the reagent and incubated at 56 °C for 30 min. After incubation, the sample was briefly vortexed and incubated on a heat block at 100 °C for 8 min. The sample was briefly vortexed before centrifugation at 20,000 ×*g* for 10 min and 2 µl of the resulting supernatant was used as template in PCR analysis. For extraction using alkaline polyethylene glycol, the culture pellet was suspended in 100 µl of the reagent and incubated at 90 °C for 15 min. After incubation, the sample was vortexed at high speed for 10 s and centrifuged at 20,000 ×*g* for 3 min and 2 µl of the resulting supernatant was used as template in PCR analysis. For extraction using DNA Extract All Reagent kit, the culture pellet was suspended in 50 µl of Lysis Solution and incubated at 95 °C for 5 min. After incubation, the sample was allowed to cool at room temperature for 1 to 2 min and 50 µl of DNA stabilizing solution was added and thoroughly mixed. The sample was centrifuged at 20,000 ×*g* for 10 min and 2 µl of the resulting supernatant was used as template for PCR analysis.

DNA extracted from cultures were analyzed by a MAP-specific genetic element, Insertion Sequence 900 (IS*900*) PCR as described previously with modifications (Vary et al., 1990; Secott et al., 1999; Tewari et al., 2014). The forward primer, 5-CCGCTAATTGAGAGATGCGAT TGG-3 and the reverse primer, 5-AATCAACTCCAGCAGCGCGGCCTCG-3 amplify a 229-bp MAP-specific sequence. SYBR Green I (iQ SYBR Green Supermix™, Bio-Rad Laboratories, Inc., Hercules, CA), a fluorescent DNA binding dye, was used to monitor target amplification and the specificity of the amplified target was verified by melting curve analysis following PCR. Each 25 µl reaction contained 1× iQ SYBR Green Supermix, 0.5 µM of each primer and 2 µl of DNA template. PCR amplifications were performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) with the following conditions: 1 cycle of initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 30 s, annealing/extension at 63 °C for 45 s with fluorescence acquisition at the end of annealing/extension step. The conditions for melting curve analysis of the amplified products included denaturation at 95 °C for 10 min, renaturation at 55 °C for 15 min followed by gradual increase of temperature from 55 °C to 95 °C by 0.5 °C increment, each lasting 10 s. Melt-curve analysis was performed to identify the PCR amplification products per instrument-specific instructions. Melting temperature (T_m) of the PCR amplification products was determined by analyzing the graph of the negative first derivative of the change in fluorescence plotted as function of temperature ($-dF/dT$). MAP-specific IS*900* amplicons had a T_m of 88.5 °C. A MAP DNA positive amplification control, a positive extraction control (a known MAP positive fecal culture) and a no template control were included with each PCR run for the analysis of laboratory derived diagnostic specimens.

Efficacies of DNA extraction using the three methods, as assessed by the detection of MAP-specific genetic element IS*900*, for 27 known positive and 13 known negative fecal samples from the Johne's disease proficiency test panels are presented in Table 1. After six weeks of culture, 26 out of 27 positive fecal samples yielded AFB. Of the 27 positive fecal samples tested, MAP IS*900* was detected in 7 (25.92%) and 26 (96.29%) samples following DNA extraction using Chelex 100 resin and DNA Extract All Reagents kit™, respectively. None of the cultures from positive fecal samples were PCR positive following DNA extraction using alkaline polyethylene glycol. One sample with low load of MAP that was AFB negative was also PCR negative for the all three DNA extraction methods examined at six weeks of incubation. The results indicated that DNA Extract All Reagent Kit™ performed superior to other reagents evaluated for molecular confirmation of MAP from the VersaTrek liquid cultures of fecal samples.

We further evaluated DNA Extract All Reagent Kit™ using 403 diagnostic fecal samples submitted to the Pennsylvania Veterinary Laboratory. The majority (> 95%) of the fecal samples tested were of bovine origin. Of the 403 diagnostic fecal samples cultured, 92 cultures yielded AFB and 83 cultures were positive for MAP genetic element IS*900* by PCR analysis following DNA extraction using DNA Extract All Reagent Kit™ (Fig. 1). Eleven cultures were positive for AFB but PCR negative and two samples were negative for AFB but PCR positive. Overall, there was a high degree of agreement (Kappa = 0.905) between the detection of acid-fast organisms and positive PCR results. Recovery of AFB in the absence of detectable MAP DNA may indicate the presence of *Mycobacteria* other than MAP or potential PCR inhibition. Absence of detectable MAP IS*900* in about 5% AFB positive liquid cultures of fecal specimens was attributed to the presence of mycobacteria other than MAP in a previous study (Kim et al., 2004).

DNA extraction using Chelex 100 chelating resin (a divalent cation-binding resin) involves cell lysis step by boiling in the presence of the Chelex matrix which binds to cell lysis products that may interfere with the PCR amplification. Chelex 100 resin has been used for extraction of DNA from various biological materials such as genomic DNA from blood and bacterial and viral DNA from clinical specimens (Walsh et al., 1991; Tomasek et al., 2008; Thomas et al., 2017). A previous study used Chelex resin for extraction of DNA from MAP cultured in the Middlebrook 7H9 broth containing oleic acid, albumin, dextrose and catalase (OADC) Growth Supplement (Ravva and Stanker, 2005). However, a recent study (Kawaji et al., 2014) reported that Chelex resin is