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Research article

Direct DNA extraction to detect *Mycobacterium bovis* from the lungs of buffaloes positive to intradermal tuberculin testing

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Abstract

Bovine tuberculosis (bovine TB), caused by *Mycobacterium bovis*, is an important significant zoonotic disease. The infection course is usually chronic, which directly affects animal health and production. Bacterial culture, which is time-consuming, is the gold standard of diagnosis. Nowadays, rapid molecular techniques such as polymerase chain reaction (PCR) are used for the rapid identification of bovine TB. However, a convenient and rapid DNA extraction method to detect mycobacterial organisms directly from tissue samples is understudied. In this study, tissue samples were collected from buffaloes that were positive to a single intradermal test. Three different direct DNA extraction methods were undertaken to find a simple procedure for rapid detection of *M. bovis* from tissue: 1) a tissue genomic extraction kit; 2) a combination of enzymatic (lysozyme) extraction and the tissue genomic extraction kit; and 3) boiling the tissue for 30 min before using a combination of lysozyme extraction and the tissue genomic extraction kit. The DNA samples were then used to identify *M. bovis* using PCR. The greatest yield of DNA concentration was obtained from the combination of enzymatic extraction and the tissue genomic extraction kit. In addition, this method also provided the highest percentage of positive results for *M. bovis*.

Introduction

Tuberculosis, commonly known as TB, is one of the most life-threatening infectious diseases and is distributed worldwide, especially in developing countries (World Health Organization, 2018). Bovine TB is considered as an important zoonotic disease transmitted from an infected cow to humans through inhalation and ingestion of contaminated or unpasteurized milk or milk products (The Center for Food Security & Public Health, 2009). The most common cause of TB in humans is *Mycobacterium tuberculosis*; however, *M. bovis* is a main cause of bovine TB (World Health Organization, 2018). These two mycobacterial species are members of the *M. tuberculosis* complex (MTC), for which infection can occur in a wide range of domestic animals, wildlife animals and humans (Wayne and Kubica, 1986). A routine technique that has been used to diagnose TB in cattle is a single intradermal tuberculin test (IDTT). Although the specificity and sensitivity of the IDTT is generally moderate to high (Francis et al., 1978; Wood et al., 1991), a false-positive reaction and a time-consuming retest within 60–90 d are two disadvantages of the IDTT. The interferon gamma (IFN-γ) assay is another ante-mortem diagnostic procedure to confirm bovine TB (Liébana et al., 1995). However, the gold standard method to diagnose bovine TB infection is a culture of causative mycobacterium on a specific medium. This subculture technique requires 8–12 wk for the growth of the isolates and an additional 2–3 wk for biochemical testing to identify the isolates. Moreover, the sensitivity of culture is not absolutely correct and false negative culture results may occur because it requires viable organisms. Unsuitable handling of tissues will cause some

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false negatives by this technique (Wards et al., 1995). In the last two decades, rapid molecular techniques such as PCR have been used for the rapid identification of *M. bovis* in bovine tissue specimens (Liébana et al., 1995; Wards et al., 1995; Mishra et al., 2005; Taylor et al., 2007; Nahar et al., 2011; Thacker et al., 2011; Selim et al., 2014) and identification of the *M. tuberculosis* complex in animal tissues (Wilbur et al., 2012; Costa et al., 2013; Araujo et al., 2014; Fell et al., 2016). In Thailand, information of mycobacterial DNA extraction from animal tissues is limited; however, there have been some reports of the extraction of MTC and *M. bovis* DNA from human sputum using sonification (Anek-vorapong et al., 2010; Kaewphinit et al., 2013) and a commercial genomic DNA extraction kit (Tharincharoen et al., 2016). Some studies have compared procedures of DNA extraction such as boiling, boiling and freezing and using a commercial kit with lysis reagent (Afghani and Stutman, 1996; Aldous et al., 2005; Ikuta et al., 2016). However, the DNA extraction protocols and types of clinical samples used differ according to the procedure, making it difficult to compare these techniques. In addition, those studies have been performed using mycobacterial isolates rather than clinical specimens. In the current study, three different DNA extraction methods were described for the simple procedure on direct DNA extraction from buffalo lung tissue as a rapid and direct detection of *M. bovis*.

Materials and Methods

This study was approved by the Committee for Ethics in Procedures on Animals for Scientific Purposes of Kasetsart University, Bangkok, Thailand (animal ethics permission number ACKU-VET-033).

Lung samples

The lung samples in this study were from buffaloes that had been tested positive to IDTT and were to be slaughtered under the guidelines of the Department of Livestock Development, Thailand. In total, 83 lung samples from positive-IDTT buffaloes consisting of 27 specimens with visible lesions (VL) and 56 with nonvisible lesions (NVL). Approximately, 5 g of the lung samples were dissected and cut into small pieces, and any fat or connective tissues were removed. The samples were then homogenized with 3 mL phosphate-buffered saline in a sterile grinder. The homogenates were decontaminated by the addition of 3 mL of a solution of 3% NaOH for 10 min. The mixture was neutralized with 2 N HCl using phenol red as the pH indicator (Quinn et al., 1993). The neutralized suspensions were kept at -20 °C for further DNA extraction.

DNA extraction

Three different DNA extraction protocols were applied for all lung samples as follows*:*

Protocol 1 (Kit)*:* The neutralized suspensions of lung tissue were directly extracted using a tissue genomic extraction kit (FavorPrep^{TM} Tissue Genomic DNA Extraction Mini Kit; Favorgen Biotech Corporation; Changzhi, Taiwan). The procedure followed the manufacturer's instructions. Briefly, the tissue was dissociated by adding 200 μL of FATG1 buffer into 200 μL of homogenized tissue suspensions. For cell-lysis, 20 μL of proteinase K (10 mg/mL) was added and incubated at 60˚C until tissue was lysed completely. Then, 200 μL of FATG2 buffer was added and incubated at 70˚C for 10 min. DNA binding was performed by adding 200 μL of ethanol and washing twice before elution.

Protocol 2 (Lys+Kit): The neutralized suspensions of lung tissue were extracted with a combined enzymatic (lysozyme) and tissue genomic extraction kit (Lys+Kit). The 20 μL of lysozyme (50 mg/mL) were added into 200 μL of homogenized tissue suspensions. The suspensions were then incubated at 37˚C for 10 min and followed by using the tissue genomic extraction kit as mentioned in protocol 1.

Protocol 3 (Boil+Lys+Kit): The neutralized suspensions of lung tissue were boiled for 30 min and followed by the combined lysozyme extraction and using the tissue genomic extraction kit exactly as mentioned in protocol 2.

DNA analysis and amplification using polymerase chain reaction

DNA concentrations and purities were estimated using Nano Drop® (Thermo Scientific; Waltham, MA, USA). DNA samples extracted using the three protocols were amplified using PCR under uniform conditions. The 50 μL of PCR volume consisted of Dreamtaq DNA polymerase (Thermo Scientific; Vilnius, Lithuania), 1 mM each of dATP, dCTP, dGTP and dTTP and 1 μL each of primers. The mixture was subjected to 35 cycles of denaturation at 94˚C for 30 s, annealing at 65˚C for 30 s and an extension at 72˚C for 30 s. *M. bovis* specific primers (JB21-22) were described by Rodriguez et al. (1995). A band of amplified DNA fragment at 495 bp was detected in 1.5% agarose gel electrophoresis. The positive control sample was derived from the lung tissue of culled dairy cattle, which had been tested positive to a single intradermal test, provided by the Department of Livestock Development. No template was used as the negative control. The tissue sample was cultured on Lowenstein-Jensen medium and the colony was subjected to biochemical tests for *M. bovis* identification and the PCR product was sequenced to confirm for *M. bovis*.

Statistical analysis

Data were explored for a normal distribution using the Shapiro-Wilk test (Patrie and Watson, 1999). Normally distributed data were subjected to differences of the means of DNA concentrations among the three DNA extraction protocols using analysis of variance, followed by multiple comparisons using the Scheffe test (Patrie and Watson, 1999). Differences were determined to be significant when $p < 0.05$.

Results

The DNA concentrations and purity are presented in Table 1. The DNA extraction protocols showed significant differences in DNA concentrations and purity. DNA extraction protocol 1 (Kit) and protocol 2 (Lys+Kit) provided similar DNA concentrations and purity, while DNA extraction protocol 3 (Boil+Lys+Kit) produced a lower DNA concentration and purity than either protocol 1 or 2.

The results of using PCR to detect *M. bovis* are also shown in Table 1. Of the 83 lung samples, the highest number of PCR positive results was observed for protocol 2, followed by using the kit (protocol 1) and protocol 3. For the total of 27 VL samples, 22 samples (81.48%) provided positive PCR results while there were 19 samples (33.92%) from the total of 56 NVL samples. Protocol 1 produced 44.44% (4 out of 9 samples) positive PCR samples from the VL specimens while protocols 2 and 3 produced 60% (15 out of 25 samples) and 42.85% (3 out of 7 samples), respectively. A typical result of agarose electrophoresis to detect the 495-bp PCR products after amplification of direct extracted DNA from lung samples is shown in Fig. 1.

Table 1 Comparison of DNA concentrations and purity and the positive polymerase chain reaction (PCR) results obtained from three protocols used for extraction of DNA from 83 buffalo lung tissue samples

Protocol	DNA concentration (ng/µL)	Purity	Number of positive PCR results (%)
Protocol 1 (Kit)	9.97 ± 7.6^{a}	2.24 ± 1.49^{a}	9 (10.84)
Protocol 2 (Lys+Kit)	10.43 ± 11.35^{a}	1.98 ± 1.82^{a}	25 (30.12)
Protocol 3 (Boil+Lys+Kit)	2.06 ± 3.50^{b}	1.29 ± 1.79^{b}	7 (8.43)

^{a,b} different lowercase superscripts in the same column indicate that the means differ significantly at $p < 0.05$.

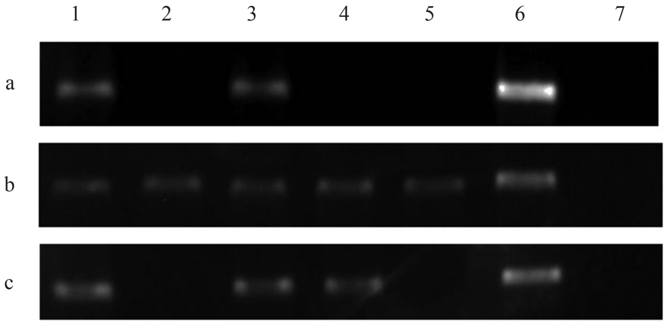

Fig. 1 PCR products of direct DNA extract from buffalo lungs. a = Protocol 1; b = Protocol 2; c = protocol 3. Lane 1–5 = DNA samples; lane 6 = positive control; lane 7 = negative control

Discussion

In the present study, all lungs were collected from buffaloes that had been tested positive to IDTT; there were no lung samples from buffaloes negative to IDTT, which was a limitation of this study as in the field, following buffalo testing for TB using IDTT, only those producing a positive result were then slaughtered.

Tuberculous lesions do not always produce mycobacterial agents because the pathogens are not evenly distributed in the tissue sample (Shitaye et al., 2006) or lesions can be caused by other intracellular organisms. Moreover, tuberculous lesions generally reveal strong fibrosis and calcification resulting in difficulty to get into the mycobacterial DNA (Liebana et al., 1995). In order to overcome the limitations in detecting mycobacterial organism in tissue samples, several methods lyse mycobacterial cells and DNA extraction. These methods include mechanical disruption, enzymatic extraction and the combined methods, with the protocols in this study performing some necessary steps as suggested to increase the success rate of mycobacterial DNA extraction from clinical samples (Amita et al., 2002). A physical treatment was undertaken to weaken the cell walls by freezing at -20˚C or boiling for 10 min prior to cell lysis. Then, chemical agents such as lysozyme were used to lyse the mycobacterial cell walls. Proteinase K was used in the DNA purification step to remove proteins that acted as inhibitors in the samples. Precipitation of DNA using ethanol helped to remove inhibitors from the target DNA. Though heating could provide a high DNA yield because of the weakening of linkages between the lipid contents of mycobacterial cell walls (Cormican et al., 1992; Amita et al., 2002), the boiling step in the current study (protocol 3) resulted in low DNA concentration results, which differed from another study (Wards et al., 1995). This finding could have been due to the differences in the sample types (bacterial isolates versus clinical tissue). Boiling is not recommended for extracting the DNA from clinical specimens because it provides less target DNA (Amita et al., 2002; Taylor et al., 2007), and is applied to inactivate viable mycobacteria rather than to lyse bacterial cell walls (Liébana et al., 1995; Aldous et al., 2005). A strong protective barrier of lipophilic molecules and polysaccharides that makes up mycobacterial cell walls compared with other bacterial cell walls is more likely to be resistant to lysis in conventional DNA extraction methods. Lysozyme is a commonly used enzyme in the digestion process for the complete lysis of mycobacterial cell walls. Moreover, an improvement in cell wall disruption can be achieved by a combination of suitable chemical and enzymatic digestion or by strong mechanical disruption such as bead beating in a specific enzyme and detergent to obtain greater amounts of mycobacterial DNA (Amita et al., 2002; Amaro et al., 2008). Regarding tissue lesions, the results of the current study revealed that using VL specimens provided a great opportunity to increase the positive result of mycobacterial detection from tissues. This outcome was similar to that in other studies of MTC detection from animal tissues which presented higher detection of MTC DNA in VL samples than NVL samples (Thacker et al., 2011; Araujo et al., 2014; Selim et al., 2014). The important points to be considered when selecting DNA extraction methods for mycobacterial species using PCR assay were the cost-effectiveness and amount of time. Currently, protocol 1 (Kit) had an evaluated cost per extraction of USD 3.10, while for protocol 2 (Lys+ Kit) or protocol 3 (Boil+Lys+kit) the equivalent cost was USD 3.75. The time required to complete each extraction was less than 60 min for protocol 1 and 70 and 100 min for protocols 2 and 3, respectively. Though protocol 2 required a slightly higher cost and longer time, it was technically simple which resulted in better performance and results.

In conclusion, there were some methodological differences between this study and others; however, all three extraction techniques could be used for PCR amplification. Nevertheless, the combination of lysozyme lysis and using a tissue genomic extraction kit was the most effective method for direct *M. bovis* detection from clinical tissues because it produced high DNA yields and high positive PCR results. Specimens with TB-like lesions are also suggested for using with this method to enhance the detection proficiency. This technique accompanied with PCR should enable specimens to be screened for *M. bovis* within 2 d, which is a distinct time advantage over the conventional method (extraction from bacterial isolates), even though it was difficult to acquire pure DNA because the homogenized tissue may contain various inhibitors of PCR amplification (Wards et al., 1995). However, the purity ratio (ratio of the absorbance at 260 and 280 nm) was close to the generally accepted value of pure DNA with pure preparation of DNA having A260:A280 values of approximately 1.8 (Glasel, 1995).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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