Korean Journal
of Veterinary Service

Kor J Vet Serv (2007) 30(2) : 233-241

Detection of *Mycobacterium bovis* in the lymph node of tuberculin positive cattle by guanidium isothiocyanate/silica DNA extraction and polymerase chain reaction

Yun-Sang Cho*, Suk-Chan Jung, Han-Sang Yoo[1], Jong-Man Kim

Bacteriology and Parasitology Division, Animal Disease Research Department, National Veterinary Research and Quarantine Service, Anyang, Gyeonggi 430-824,
[2] *Laboratory of Infectious Diseases, College of Veterinary Medicine and the School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea*
(Received 13 April 2007, accepted in revised from 12 June 2007)

Abstract

Tuberculin positive cattle without gross tubercle lesions should be confirmed by the bacteriological examination to determine the state of the infection. To overcome the time-consuming and laborious identification by culture and biochemical tests, polymerase chain reaction (PCR) has been used to identify *Mycobacterium bovis*. Due to various lipids in the cell wall of *Mycobacterium* spp, novel methods of DNA extraction from *Mycobacterium* spp have been developed. In this study, a newly developed guanidium isothiocyanate/silica DNA extraction method was directly applied to specimens from the tuberculin positive cattle. DNAs were directly extracted from the lymph nodes and the major polymorphic tandem repeat (MPTR) and mycobacterial protein of BCG 70 (MPB70) were amplified using PCR. The DNA extraction method using guanidium isothiocyanate/silica was efficient and safe, and the MPTR and MPB70 primers were specific to *M bovis*. Therefore, MPTR and MPB70 PCRs will be useful for the detection of *M bovis* in the lymph node from skin-test positive cattle.

Key words: MPTR, MPB70, Guanidium isothiocyanate/silica, DNA extraction, *Mycobacterium bovis*

* Corresponding author
Phone: +82-31-467-1769. Fax: +82-31-467-1778
E-mail: choys@nvrqs.go.kr

Introduction

Tuberculosis in both humans and animals is a worldwide problem of an enormous scale [1-7]. In many countries, the efforts to control or eradicate bovine tuberculosis have been hindered by the lack of an unequivocal and objective diagnostic test for the disease. Current diagnosis of bovine tuberculosis is accomplished by the caudal fold skin test that is similar to the human manteux test[8]. Even though the tuberculin test has been used worldwide for the control of bovine tuberculosis, the conventional diagnosis of this disease using bacteriological and immunological tests has been insufficient for the field environment because of the lack of simplicity as well as the low specificity and sensitivity of the tests [9]. Bacteriological identification of *Mycobacterium bovis* includes the isolation and culture of the bacilli and time-consuming biochemical assays [6].

The advent of the polymerase chain reaction (PCR) and the discovery of a repetitive DNA sequence that is specific to the *M tuberculosis* complex, including *M tuberculosis*, *M bovis*, *M bovis* BCG, *M microti* and *M africanum*, offer a fast, sensitive and specific diagnosis of the *Mycobacterium* spp [10]. Primers for the major polymorphic tandem repeat (MPTR) and the mycobacterial protein of BCG 70 (MPB 70), were used to detect *M bovis* by PCR. The MPTR primers have been used for the differentiation of the *M tuberculosis* complex by Frothingham [11]. MPB70, which is highly species-specific protein of *M bovis*, was purified and characterized by Nagai et al[12]. The MPB 70 protein is a potent and specific T-cell antigen in *M bovis* and *M bovis* BCG [13-15] and Harboe et al [16] used this protein as the specific antigen in an ELISA to diagnose tuberculosis.

To remove any contaminating bacteria from the lymph node of tuberculin skin test positive cattle, the decontaminants NaOH-sodium citrate-N-acetyl-L-cysteine (NALC), sodium hypochlorite, benzalkonium chloride, and hexadecylpyridinium chloride have been used[17-19]. We used hexadecylpyridinium chloride because it is an equivalent decontaminant but has a less detrimental effect to the *Mycobacterium* spp[20]. To extract DNA from the *Mycobacterium* spp, conventional methods such as heating, chloroform/phenol extraction, sonication, glass beads, hexadecyl trimethyl ammonium bromide (CTAB), and sodium iodide/sodium-N-lauryl sarcosine have been used[1,3-8,17,21-23,25-27]. Recently, guanidium isothiocyanate (GuSCN)/silica was developed for DNA extraction from *Mycobacterium* spp[28-30]. The conventional methods were not suitable for the clinical samples of bovine tuberculosis because they were laborious and time-consuming[30], and the efficiency of DNA preparation by some conventional methods, especially heating and chloroform/phenol extraction, was poor for subsequent PCR of tuberculosis clinical specimens[31]. In addition, the conventional methods are also limited by the lipid content of the *M bovis* cell wall[6,21]. In contrast, GuSCN/silica DNA extraction was effective for Gram-negative bacteria

from human blood and urine[28], and was also effective for *M tuberculosis*[5,30,32].

In this study, GuSCN/silica DNA extraction was directly applied to the DNA from the lymph nodes of tuberculin skin-test positive cattle, of which the major infectious agent is *M bovis*. We amplified MPTR and MPB70 using PCR to overcome the slow isolation and identification of *M bovis* by bacteriological and biochemical tests.

Materials and Methods

Bacterial strains

M bovis AN5 (ATCC 35726), *M avium* NVDL 1414 (ATCC 35716), *M avium* P18 (ATCC 12227), and *M phlei* (ATCC 11758) were used in this study. After each strain was cultured at 37°C for 6 weeks in Sauton broth (Asparagine 4.8 g, Citric acid 2.4 g, $MgSO_4$ 0.6 g, K_2HPO_4 0.6 g, Ferric ammonium citrate 0.06 g, Glycerine 72 ㎖, $ZnSO_4$ 0.0096 g, $CuSO_4$ 0.0012 g, Ammonia water 2.7 ㎖, DW 1200 ㎖, pH 7.0 − 7.2), the culture was transferred into an eppendorf tube for the extraction of DNA.

Clinical specimens

Lymph nodes of three skin test positive reactors at necropsy were sampled and aseptically homogenised. 5 ㎖ sterile saline was added to the minced specimens. The diluted homogenate was transferred into a 50 ㎖ sterile conical tube, pretreated with 0.75% HPC (hexadecylpyridium chloride: Fluka 52349) solution and then vortexed for 20 sec. After the homogenate was allowed to settle at RT for 30 min, the supernatant from each sample was precisely collected by pipette and then centrifuged at 2,500 g for 15 min. The resulting supernatant was discarded and then 1 ㎖ of 0.2% bovine serum albumin (BSA) was added to each pellet for DNA extraction.

DNA extraction by GuSCN/silica

A modified GuSCN/silica method was applied to the *Mycobacterium* spp control strains and the clinical specimens. This procedure was a modification of the basic 1−h procedure (protocol Y)[28,29].

Briefly, we first prepared the cell lysis buffer (100 ㎖ of 0.1 M Tris−HCl (pH 6.4), 120g GuSCN, 22 ㎖ 0.2 M EDTA (pH 8.0), 2.6 g Triton X−100) and the silica dioxide solution (60 g SiO_2 was resuspended in 500 ㎖ distilled water and placed at RT for 24 hrs. Then, 430 ㎖ of the supernatants was carefully removed and discarded. Distilled water was added to the precipitates to a final volume of 500 ㎖, and then the mixture was shaken and allowed to settle at RT for 5 hrs. After that, 440 ㎖ of the supernatant were discarded carefully and the pH of the precipitates was adjusted to 2.0 by HCl and then autoclaved.). Then, 900 ℓ of the cell lysis buffer and 40 ℓ of silica dioxide solution and 50 ℓ of the test sample were mixed in a 1.5 ㎖ tube and incubated at RT for 10 min. The mixture was centrifuged at 12,000 g, 15 sec and the supernatant was carefully removed and discarded. 1 ㎖ of the washing solution (GuSCN 120 g, 0.1 M

Tris-HCl (pH 6.4) 100 mℓ) was added to each resultant precipitate and washed twice. Then each pellet was washed twice with 70% ethanol, followed by one acetone wash and dried at 56℃. The silica-DNA pellet was then resuspended in 50 ℓ of TE buffer, incubated at 56 ℃ for 10 min, and centrifuged at 12,000 g, 2 min. The supernatant contained the DNA used for PCR.

Polymerase chain reaction

Each reaction volume of the MPTR and MPB70 PCRs was 50 ℓ. In the MPTR PCR, the mixture consisted of 1 mM Tris-HCl (pH 8.3), 5 mM KCl, 0.001 % gelatin, mM $MgCl_2$, 200 M dNTP, 20 nM of each primer, 1.25 U *Taq* polymerase, 10 ng template DNA, and in the MPB70 PCR, the mixture consisted of 5 mM KCl, 1 mM Tris-HCl (pH 8.0), 0.01 % gelatin, 1 mM $MgCl_2$, 200 M dNTP, 7.5 % dimethyl sulfoxide, 2.5 U *Taq* DNA polymerase, 20 pmole of each primer, 10 ng template DNA. The MPTR and MPB 70 primers amplified a 343 and 678 bp product, respectively. The MPTR primers were 5'-GGTTACCACTTCGATGCGTCTG CG-3' (forward primer) and 5'-AGCC-GCCGAAACCCATC-3' (reverse primer). The MPB70 primers were 5'-AAAGAA-TTCGGACGGCTCCGAAGAAATC-3' (forward primer) and 5'-CCCGGATCC-TTACGCCGGAGGCATTAGCAC-3' (reverse primer)[11,14]. The conditions for the MPTR PCR were as follows: pre-denaturation at 95°C, 3 min, 30 thermal cycles of 94°C for 0.5 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 min for the final extension. The MPB70 PCR was initiated by a predenaturation phase of 95°C for 5 min, and followed by 30 thermal cycles of 95°C for 1 min, 55°C for 2 min, 72°C for 1 min, and 72°C for 5 min for the final extension. Each amplified product was electrophoresed on a 1 % agarose gel containing 0.5 g/mℓ ethidium bromide and examined under the ultraviolet light.

Results

DNA extraction

DNA from the standard *Mycobacterium* spp and clinical specimens were extracted using the GuSCN/silica method, which took about 90 min per sample. GuSCN/silica extraction was more efficient than a phenol extraction method for extracting mycobacterial DNA, and it was also safer because phenol and chloroform were not required and the risk of accidential infection was reduced.

Polymerase chain reaction using the MPTR primers

PCR using the MPTR primers was specific for the detection of *M bovis* AN5 compared with *M avium, M paratuberculosis,* and *M phlei* (Fig 1). A specific 343 bp PCR product was observed for *M bovis*, but no PCR product was detected for the other mycobacterial standard strains (Fig 1). The size of the PCR product was the same as that detected by Frothingham[11]. The *M bovis* infection in the three clinical specimens, which were the lymph nodes with granulomatous lesions, was confirmed by the

MPTR PCR of the DNA extracted by GuSCN/silica.

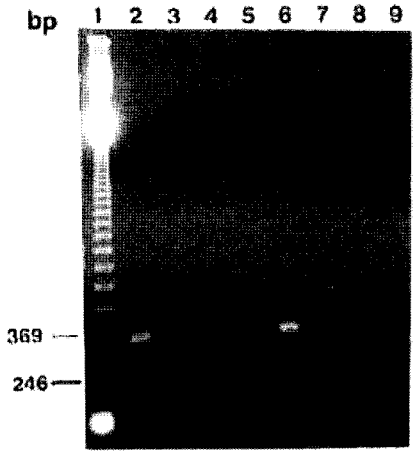

Fig 1. Agarose gel electrophoresis of the PCR products amplified from the DNA of the *Mycobacterium* spp and tuberculin positive reactors using the major polymorphic tandem repeat (MPTR) primers.
Lane 1. 123 bp ladder, lane 2. *M bovis* AN5, lane 3. *M avium* NVDL 1414, lane 4. *M avium* P18, lane 5. *M phlei*, lane 6. DNA extracted from pulmonary lymph node 1, lane 7. DNA extracted from mesenteric lymph node, lane 8. DNA extracted from pulmonary lymph node 2, lane 9. Negative control (the same conditions as other lanes without template DNA). The amplified products were con-firmed as 343 base pairs (bp).

Polymerase chain reaction using the MPB70 primers

The MPB70 PCR was specific for the detection of *M bovis* AN5 compared with *M avium* and *M phlei*. The size of the PCR product was 678 bp (Fig 2), which was identical to the anticipated product as determined by NCBI. The MPB70 PCR was then applied to the DNA from the pulmonary and mesenteric lymph nodes from the same tuberculin positive reactors that were used in the MPTR PCR. We found the same sized PCR product in the clinical specimens as the *M bovis* standard strains (Fig 2).

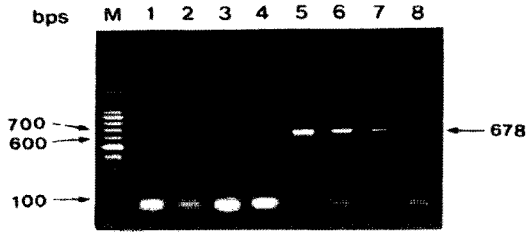

Fig 2. Agarose gel electrophoresis of the PCR products amplified from DNA of the *Mycobacterium* spp and the tuberculin positive reactors using the mycobacterial protein of BCG 70 (MPB70) primers.
M. 100 bp ladder, lane 1. *M bovis* AN5, lane 2. *M avium* NVDL 1414, lane 3. *M avaium* P18, lane 4. *M phlei*, lane 5. DNA extracted from pulmonary lymph node 1, lane 6. DNA extracted from mesenteric lymph node, lane 7. DNA extracted from pulmonary lymph node 2, lane 8. Negative control (the same conditions as other lanes without template DNA). The amplified products were confirmed as 678 base pairs (bp).

Discussion

We have applied the GuSCN/silica DNA extraction method to *M bovis* and lymph nodes of tuberculin positive cattle, which was introduced by Boom et al[28,29] and applied to *M tuberculosis* by Choi et al[7,8,30,33]. In the GuSCN/silica extraction, only DNA was absorbed to the silica and the impurities were completely removed during the washing steps. Therefore, this extraction method would overcome false positive reactions

caused by impurities, such as phenol and chloroform, in PCR [30,33].

Mycobacterium spp isolated from the specimens using a selective medium were identified at the species level by bacteriological and biochemical tests, such as Ziehl-Nielsen staining, niacin production, nitrate reduction, Tween 80 hydrolysis, catalase production, and the urease test. The Lowenstein-Jensen medium, Herrold Egg Yolk medium, 7H9 medium, 7H11 medium, Middlebrook medium, Ogawa medium, and BM medium have been used as the selective media[34]. However, the culture of *Mycobacterium* spp takes a long time according to its pathogenicity and usually *M bovis* takes 6 to 8 weeks for the visible growth on the surface of selective media. The biochemical tests also require a long time approximately 3 to 4 weeks. Therefore, the identification of *M bovis* by the bacteriological and biochemical tests is both time consuming and labor intensive. Even though some specimens contain a small number of pathogens that cannot be isolated and cultured, PCR has been used to simultaneously and rapidly confirm and identify the causative microorganism [34] using the insertion[10,36,37] and rRNA sequences[35]. When the PCR was applied to *M bovis, M avium* and *M phlei* in this study using the primers specific to the MPTR gene[11], which can differentiate between the *M tuberculosis* complex (*M tuberculosis, M bovis, M microti*, and *M africanum*), the specific gene of *M bovis* was amplified without any non-specific reactions. In addition, the primers specific to the MPB70 gene, known as *M bovis* specific protein[13,16] were applied to the same bacteria standards as the MPTR PCR in this study. *M bovis* was specifically amp-lified because no products were amplified for *M avium,* and *M phlei* in the MPB70 PCR. In addition, the novel extraction method in this study was more efficient and safer than the conventional methods. When the established MPTR and MPB70 PCRs were directly applied to the DNA from the lymph nodes of tuberculin positive reactors, the PCR product confirmed the tuberculin positive status. Therefore, the MPTR and MPB70 PCR with GuSCN/silica DNA extraction would be useful for the rapid and specific identification of *M bovis* directly from the lymph node.

Acknowledgments : This study was funded by the National Veterinary Research and Quarantine Service (NVRQS), Anyang, Republic of Korea.

References

1. Bakshi CS, Shah DH, Verma R, et al. 2005. Rapid differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis* based on a 12.7-kb fragment by a single tube multiplex-PCR. *Vet Microbiol* 109 : 211-216.
2. Collins DM, Stephens DM. 1991. Identification of an insertion sequence, IS1081, in *Mycobacterium bovis. FEMS Microbiol Lett* 83 : 11-16.
3. Cornejo BJ, Sahagun-Ruiz A, Suarez-Guemes F, et al. 1998. Comparison of C_{18}-carboxypropylbetaine and glass bead DNA extraction methods for detection of

Mycobacterium bovis in bovine milk samples and analysis of samples by PCR. *Appl Environ Microbiol* 64 : 3099-3101.

4. Liebana E, Aranaz A, Mateos A, et al. 1995. Simple and Rapid Detection of *Mycobacterium tuberculosis* Complex Organisms in Bovine Tissue Samples by PCR. *J Clin Microbiol* 33 : 33-36.
5. Mishra A, Singhal A, Chauhan DS, et al. 2005. Direct detection and identification of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in bovine samples by a novel nested PCR assay: Correlation with conventional techniques. *J Clin Microbiol* 43 : 5670-5678.
6. Portillo PD, Murillo LA, Patarroyo ME. 1991. Amplification of a species-specific DNA fragment of *Mycobacterium tuberculosis* and its possible use in diagnosis. *J Clin Microbiol* 29 : 2163-2168.
7. Sreevatsan S, Bookout JB, Ringpis F, et al. 2000. A Multiplex approach to molecular detection of *Brucella abortus* and/or *Mycobacterium bovis* infection in cattle. *J Clin Microbiol* 38 : 2602-2610.
8. Radford AJ, Wood PR, Billman-Jacobe H, et al. 1990. Epitope mapping of the *Mycobacterium bovis* secretory protein MPB70 using over-lapping peptide analysis. *J Gen Microbiol* 136 : 265-272.
9. Pao CC, Yen TSB, You JB, et al. 1990. Detection and identification of *Mycobacterium tuberculosis* by DNA amplification. *J Clin Microbiol* 28 : 1877-1880.
10. Hermans PWM, van Soolingen D, Dale JW, et al. 1990. Insertion element *IS986* from *Mycobacterium tuber-culosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *J Clin Microbiol* 28 : 2501-2508.
11. Frothingham R. 1995. Differentiation of stains in *Mycobacterium tuberculo-sis* complex by DNA sequence poly-morphisms, including rapid identifi-cation of *M. bovis* BCG. *J Clin Micro-biol* 33 : 840-844.
12. Noordhoek GT, Kolk AHJ, Bjune G, et al. 1994. Sensitivity and Specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven labo-ratories. *J Clin Microbiol* 32 : 277-284.
13. Harboe M, Nagai S, Patarroyo ME, et al. 1986. Properties of proteins MPB 64, MPB70, and MPB80 of *Mycobac-terium bovis* BCG. *Infect Immun* 52 : 293-302.
14. Hewinson RG, Russell WP. 1993. Processing and secretion by *Es-cherichia coli* of a recombinant from of the immunogenic protein MPB70 of *Mycobacterium bovis*. *J Gen Microbiol* 139 : 1253-1259.
15. Pollock JM, Douglas AJ, Mackie DP, et al. 1994. Identification of bovine T-cell epitopes for three *Myco-bacterium bovis* antigens: MPB70, 19,000 MW and MPB57. *Immunology* 82 : 9-15.
16. Harboe M, Wiker HG, Duncan JR, et al. 1990. Protein G-based enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuber-culosis. *J Clin Microbiol* 28 : 913-921.

17. Merkal RS, Richards WD. 1972. Inhibition of fungal growth in the cultural isolation of mycobacteria. *Appl Microbiol* 24 : 205-207.
18. Merkal RS, Thurston JR. 1968. Susceptibilities of mycobacterial and nocardial species to benzalkonium chloride. *Am J Vet Res* 24 : 759-761.
19. Thoen CO, Richards WD, Jarnagin JL. 1974. Comparison of six methods for isolating mycobacteria from swine lymph nodes. *Appl Microbiol* 27 : 448-451.
20. Corner LA, Trajstman AC. 1988. An evaluation of 1-hexadecylpyridium chloride as a decontaminant in the primary isolation of *Mycobacterium bovis* from bovine lesions. *Vet Microbiol* 18 : 127-134.
21. Eisenach KD, Sifford MD, Cave MD, et al. 1991. Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction. *Am Rev Respir Dis* 144 : 1160-1163.
22. Fifis T, Costopoulos C, Radford AJ, et al. 1991. Purification and characterization of major antigens from a *Mycobacterium bovis* culture filtrate. *Infect Immun* 59 : 800-807.
23. Del Portillo P, Thomas MC, Martinez E, et al. 1996. Multiprimer PCR system for differential identification of *Mycobacteria* in clinical samples. *J Clin Microbiol* 34 : 324-328.
24. Pierre C, Lecossier D, Boussougant Y, et al. 1991. Use of a reamplification protocol improves sensitivity of detection of *Mycobacterium tuberculosis* in clinical samples by amplification of DNA. *J Clin Microbiol* 29 : 712-717.
25. Romero RE, Garzon DL, Mejia GA, et al. 1999. Identification of *Mycobacterium bovis* in bovine clinical samples by PCR species-specific primers. *Can J Vet Res* 63 : 101- 106.
26. Roring S, Hughes MS, Skuce RA, et al. 2000. Simultaneous detection and strain differentiation of *Mycobacterium bovis* directly from bovine tissue specimens by spoligotyping. *Vet Microbiol* 74 : 227-236.
27. Wards BJ, Collins DM, De Lisle GW. 1995. Detection of *Mycobacterium bovis* in tissues by polymerase chain reaction. *Vet Microbiol* 43 : 227-240.
28. Boom R, Sol CJA, Salimans MMM, et al. 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 28 : 495-503.
29. Boom R, Sol CJA, Heijtink R, et al. 1991. Rapid purification of hepatitis B virus DNA from serum. *J Clin Microbiol* 29 : 1804-1811.
30. Choi CS, Lee KO, Lee KP. 1994. Rapid and simple method for extraction of Mycobacteria species DNA for polymerase chain reaction. *J Korean Soc Microbiol* 29 : 147-152.
31. Buck GE, O'hara LC, Summer S, et al. 1992. Rapid, Simple method for treating clinical specimens containing *Mycobacterium tuberculosis* to remove DNA for polymerase chain reaction. *J Clin Microbiol* 30 : 1331-1334.
32. Nagai S, Matsumoto J, Nagasuga T. 1981. Specific skin-reactive protein from culture filtrate of *Mycobacterium bovis* BCG. *Infect Immun* 31 : 1152-1160.
33. Choi CS, Lee KO, Lee KP. 1994.

Amplification of *Mycobacterium tuberculosis* DNA in blood of patients with granulomatous lesions by polymerase chain reaction. *J Korean Soc Microbiol* 29 : 269-274.

34. Bai GH, Hwang HS, Shon BW. 1994. Detection of *Mycobacterium bovis* in the pathological lesions of slaughtered dairy cattles using modified culture medium and polymerase chain reaction. *Kor J Vet Publ Hlth* 18 : 183-190.
35. Bai GH. 1992. Rapid identification of *Mycobacterium avium* and *Mycobacterium intracellulare* by the amplification of rRNA sequences. *J Korean Soc Microbiol* 27 : 443-448.
36. Hermans PWM, van Soolingen D, Bik EM, et al. 1991. Insertion elements IS987 from *Mycobacterium bovis* BCG is located in a hot-spot integration for insertion elements in *Mycobacterium tuberculosis* complex strains. *Infect Immun* 59 : 2695- 2705.
37. Thierry D, Cave MD, Eisenach KD, et al. 1990. IS6110, an IS-like element of *Mycobacterium tuberculosis* complex. *Nucleic Acids Res* 18(1) : 188.