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Multiplex Quantitative Real-time Polymerase Chain Reaction Assay for Rapid Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Fecal Samples

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Abstract : *Mycobacterium avium* subsp. *paratuberculosis* (MAP) causes paratuberculosis or Johne's disease, an intestinal granulomatous infection in domestic and wild animals. The study aimed to develop and evaluate a panel of multiplex quantitative real-time polymerase chain reaction (mqPCR) assay for simultaneous detection of three MAP-specific genes (IS*900*, F57 and ISMAP*02* genes). The analytic sensitivity (i.e., limit of detection, expressed as cells per 1 ml) was 150 for IS*900*, 1500 for F57, and 50 for ISMAP*02*. The specificity of the method was determined by testing 152 bovine fecal samples. Based on the test, it showed that the assay simultaneously detected the target genes in short period of time and at lower cost compared to laboratory routine tests. The test agreement between the assay and routine test was 94%. The discrepancy in the results was due to samples that were tested positive by the panel but negative by the routine tests, suggesting that the assay has higher sensitivity than the routine tests. In conclusion, the mqPCR assay could be a rapid and accurate testing tool for investigating paratuberculosis or Johne's disease cases in domestic and wild animals.

Key words **:** *Mycobacterium avium* subsp. *paratuberculosis*, multiplex quantitative real-time polymerase chain reaction, diagnosis.

Introduction

Since 1895, *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is known as a pathogen that causes paratuberculosis or Johne's disease, an intestinal granulomatous infection in domestic (cattle, sheep, goats, and camelid) and wild ruminants (cervidae and bovidae) as well as wild Canidae, Mustelidae, and Herpestidae (10,12,15,16,23,24). Recent studies showed the potential etiological role of MAP in the pathogenesis of Crohn's disease in human and the zoonotic potential of the organism (1,11,14). The disease in animals spreads by ingestion of the organism from the feces of infected animals or contaminated food and surface water (23). The infected animal served as the carrier of the disease that can be easily transmitted to other animals. Excretion of the organism may occur intermittently for a prolonged period before the onset of clinical disease. Therefore, early detection and management strategy planning for the infected animals is important to reduce the spread of the disease in domestic and wild animal populations.

Laboratory techniques that have been commonly used to diagnose paratuberculosis are fecal examination, bacterial culture, enzyme-linked immunosorbent assay (ELISA), complement fixation (CF) test, agar gel immunodiffusion (AGID), and polymerase chain reaction (PCR) (3,5-7). These traditional methods are laborious, expensive, and/or slow in turnaround. Some of them can also be less sensitive depending on the specimen quality, timing of sampling or prior antimicrobial therapy. Recent studies suggested the use of a multiplex quantitative real-time PCR panel (mqPCR) as an alternative diagnostic method when investigating multi-factorial diseases because it gives a good diagnostic performance with simultaneous detection of multiple targets and rapid results compared to conventional diagnostic methods (2,22).

The objective of this study was to develop and evaluate a quantitative real-time PCR-based panel which can simultaneously detect three (3) MAP-specific genes (IS*900*, F57 and ISMAP*02* genes). Its diagnostic performance was evaluated on clinical specimens in comparison to the various laboratory test methods that were routinely used for MAP detection.

Materials and Methods

Biological materials

The MAP strain purchased from the American Type Culture Collection (ATCC19698) and 152 fecal samples collected from 10 cattle herds were used for assessing analytic sensitivity and for evaluating diagnostic performance of the assay.

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Table 1. Oligonucleotide sequences of primers and probes used for multiplex real-time polymerase chain reaction assay

Target gene	Primer/probe	Sequences (5' to 3')	Annealing temperature (°C)	Product size (bp)	Reference
IS900	IS900(Q)-F	GATGGCCGAAGGAGATTG	60	145	20
	IS900(Q)-R	CACAACCACCTCCGTAACC			
	IS900(Q)-probe	FAM-ATTGGATCGCTGTGTAAGGACACGT-BHQ1			
F57	F57(Q)-F	GCCCATTTCATCGATACCC	60	147	20
	F57(Q)-R	GTACCGAATGTTGTTGTCAC			
	F57(Q)-probe	Joe-CAATTCTCAGCTGCAACTCGAACACAC-BHQ1			
ISMAP02	ISMAP02-for	CGCCAGGAACGCAAACAT	60	96	13
	ISMAP02-rev	GTGCAGGGTCGCTCTGATG			
	ISMAP02-probe	Cy5-ACTCCGCATCCAACAACTCACGCTG-BHQ2			

All fecal samples were collected from diarrheic cattle. Of 152 fecal samples, 24 were positive for MAP in culture or MAP-specific gel-based PCR targeting ISMAP02 gene and the remaining 128 samples have negative result. Among 128 samples, 6 samples were positive for MAP antibody-targeting ELISA test, but negative for the PCR.

Primer and probe

The composition of the multiplex quantitative real-time PCR (mqPCR) assay is summarized in Table 1. The assay included 3 primer/probe sets for the target genes (i.e, IS*900*, F57 and ISMAP*02*), 1 primer/probe set for internal control (IC), and reference dye. Sequences of primers and probes used in the assay were adopted separately from published information (13,20). All the primers and probes were synthesized and purchased from Bioneer (Daejeon, Korea). To minimize interference among reporting dyes for the probe, the assay comprised dyes in a combination of FAM, VIC, Cy5 for the genes, TAMRA for IC, and ROX reference dye.

Internal control (IC)

A 65-bp oligonucleotide IC was designed to monitor false negative result due to failure of the PCR process causing the presence of inhibitory substances in reactions. The IC contained a non-specific 16S ribosomal RNA gene sequence flanked by the same primer sequence for *M. bovis* (i.e., uvrC gene). The sequence for the IC was TCTAATTTTT TCATATCGCT AATGCTCTTG TACACACCGC CCAGTTCATT GTAGCAAAGG CCTGA. For every reaction, 0.001 μM of IC was added resulted to positive signal with Ct values of 36 to 38 if PCR inhibitory substances were not present in the reaction.

Nucleic acid extraction

Bacterial nucleic acids were extracted using mGITC/SC method as described previously (18). Briefly, one ml of GITC L6 lysis buffer [5.25 < GuSCN, 50 mM Tris-HCl (pH 6.4), 20 mM EDTA, 1.3% Triton X-100, distilled water] was added to the fecal suspension in a 2 ml tube, vortexed for 30 sec, and then incubated at 95°C for 15 min. The tube was vortexed again and centrifuged at 13,000 g for 2 min. The supernatant (300 μL) was transferred into a new 1.5 ml tube containing 700 μL of L6 lysis buffer and incubated at 70°C for 5 min, after which 250 μL of 100% ethanol were added; the mixture was then incubated at 56°C for 5 min. After incubation, the mixture was passed through a mini spin column fitted with a silica membrane (Epoch Biolab, Sugerland, TX, USA), washed with 700 μL of L2 washing buffer (5.25M GuSCN, 50 mM Tris-HCl, distilled water) and washed again with 700 μL of 70% ethanol twice. Finally, DNA was eluted with 40 μL of nuclease-free water.

PCR condition

The panel was optimized using a multiplex PCR kit (Applied Biosystems, Austin, TX, USA) by following the manufacturer's recommended protocols in a reaction volume of 25 μl. ABI7500 Fast Real-Time PCR system (Applied Biosystems) was used for PCR amplification. Each reaction contained 0.4 μM of each primer, 0.2 μM of each probe, and 5 μl (for PCR B and C) of template. The cycling conditions were as follows: a) a 15-min initial activation step at 95°C; and b) 40 cycles of 90 sec at 60°C. Samples with a threshold cycle (Ct) of 37 or lower were considered positive for the corresponding gene.

Analytic sensitivity and diagnostic performance

To assess analytic sensitivity of the assay, a bacterial suspension with known colony-forming units (CFU) was used. Briefly, MAP (ATCC19968) was cultured in modified Her-

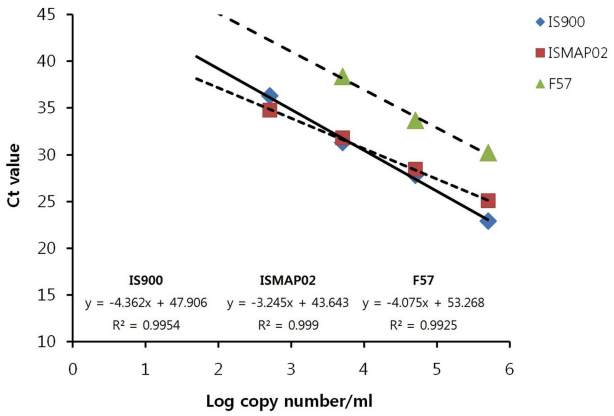

Fig 1. Multiple detection of MAP-specific gene sequences. A series of 10-fold dilutions of cultured MAP strain from 5×10^1 to 10^5 was prepared and used for assessing analytic sensitivity of the assay. The Y-axis indicates the Ct value of targets using each dilution of the strain. Each regression line was constructed based on duplicate measurement.

Table 2. Comparative performance of multiplex real-time polymerase chain reaction (PCR) panel and conventional diagnostic assays

		Multiplex real-time PCR		% overall agreement (κ value)
		Positive	Negative	
Routine tests (culture or gel-based PCR)	Positive	24	0	94% (0.807)
	Negative	9	119	

rold's egg yolk medium (HEYM) supplemented with 2 mg/L of mycobactin J (ID-Vet; Montpellier, France) and three antibiotics (nalidixic acid, vancomycin, and amphotericin B) conditioned at 37°C for 8 weeks. Colonies were harvested by swab and transferred into 5 ml of phosphate buffered saline (PBS) supplemented with 0.25% Tween 80, and then vortexed for 1 min to minimize the clumping of cells. Finally, cell concentrations from 5×10^1 to 10^5 spiked into 250 mg of feces were used for analytic sensitivity testing.

The mqPCR assay was performed on 152 fecal samples. Samples with discrepancy between the mqPCR and routine test methods were re-evaluated in two ways. First, PCR amplicons were obtained from each sample using singleplex qPCR with only the primer set in question and examined by electrophoretic analyses to verify that the PCR amplified the target gene with the expected molecular size. Second, PCR products with expected molecular sizes were sequenced for intended target genes. After sequencing, the deduced sequence was compared with sequences available in GenBank database to identify corresponding pathogens.

Results

Analytic sensitivity of the panel

The analytic sensitivity (i.e., detection limit) of the mqPCR assay was estimated using serially-diluted bacterial suspensions with known CFU per 1 ml. The estimated detection limit (cells/ml) of the assay for each target gene was: 150 for IS*900*; 50 for ISMAP*02*; and 1500 for F57. Standard curves generated by the assay using 10-fold serial dilutions of each target genes showed correlation coefficients (R_2) ranging from 0.992 to 0.999 and slopes of 3.246-4.362 (Fig 1), indicating good linearity of the PCR reaction.

Diagnostic performance of the assay in comparison to other tests

Comparative test results on the 152 fecal samples between the mqPCR assay and other laboratory tests are summarized in Table 2. The mqPCR assay detected 33 samples that were 9 higher than the number of positive samples identified by the conventional laboratory tests for each agent. Notably, all 6 samples that were positive for MAP antibody, but negative for culture or gel-based PCR were positive in the mqPCR assay, especially an assay targeting ISMAP*02* gene sequence. Overall test agreement between the mqPCR assay and conventional tests was 94% ($\kappa = 0.807$).

Discussion

In this study, mqPCR assay targeting MAP-specific gene sequences (IS*900*, F57, and ISMAP*02*) was designed and evaluated for simple and rapid detection of MAP-positive individuals in clinical laboratories. Overall analytic and diagnostic performance of the mqPCR assay showed good analytic sensitivity and test agreement (94%). In particular, the mqPCR assay detected 9 additional positive samples than conventional tests which includes 6 samples which were MAP antibody positive, but negative for MAP antigen in antigen-targeted test. These observations suggest that the mqPCR assay could be a tool to get more accurate diagnostic data in a short turnaround time.

Traditionally, diagnostic assays for the detection of MAP in fecal samples have utilized the IS*900* gene sequence as the target because of its multicopy nature, raising analytic sensitivity of the test (9,17,21). However, there are some debates on whether the IS*900* is indeed unique to MAP (4,8). Two studies that demonstrated the presence of IS*900* in environmental mycobacteria, *M. cookii* and *M. scrofulaceum*, suggesteding that the use of the IS*900* sequence alone may yield false-positive results. To deal with the issue on IS*900*, the mqPCR assay targeting more than 2 genes have been described (13,19,20); however, the assay focused on the gene with low copy number (24) or the exact detection limit was not reported (13,20). In this study, the mqPCR assay included IS900 along with two additional MAP-specific elements, F57 and ISMAP*02* and the assay was optimized to test three target genes in a single reaction with high analytic sensitivity. In particular, ISMAP*02* presents six copies in a single genome of MAP (21), making it more sensitive and specific target for the detection of MAP. The analytic sensitivity testing in this study also showed that the subset for ISMAP*02* had the most sensitive result (50 cells/ml) followed by the subset for IS*900* (150 cells/ml).

In conclusion, the mqPCR assay in this study showed higher diagnostic performance than conventional diagnostic tests such as culture or gel-based PCR. Likewise, this assay could be an alternative or additional method for the rapid detection of MAP in fecal samples from suspected animals.

Acknowledgements

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References

1. Atreya R, Büite M, Geriach GF, Goethe R, Hornef MW, Köhler H, Meens J, Möbius P, Roeb E, Weiss S, ZooMAP Consortium. Facts, myths, and hypotheses on the zoonotic

nature of Mycobacterium avium subspecies paratuberculosis. Int J Med Microbiol 2014; 304: 858-867.
2. Cho YI, Kim WI, Liu SL, Kinyon JM, Yoon KJ. Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in feces. J Vet Diagn Invest 2010; 22: 509-517.
3. Collins MT, Kenefick KB, Sockett DC, Lambrecht RS, McDonald J, Jorgensen JB. Enhanced radiometric detection of Mycobacterium paratuberculosis by using filter-concentrated bovine fecal specimens. J Clin Microbiol 1990; 28: 2514-2519.
4. Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ, Kluver P. Mycobacteria distinct from Mycobacterium avium subsp. paratuberculosis isolated from the feces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: implication for diagnosis. Mol Cell Probes 1999; 13: 431-442.
5. Cox JC, Drane DP, Jones SL, Ridge R, Milner AR. Development and evaluation of a rapid absorbed enzyme immuno-assay test for the diagnosis of Johne's disease in cattle. Aust Vet J 1991; 68: 157-160.
6. Eamens GJ, Whittington RJ, Marsh IB, Turner MJ, Saunders V, Kemsley PD, Rayward D. Comparative sensitivity of various fecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease. Vet Microbiol 2000; 77(3-4): 357-367.
7. Ellingson JLE, Bolin CA, Stabel JR. Identification of a gene unique to Mycobacterium avium subspecies paratuberculosis and application to diagnosis of paratuberculosis. Mol Cell Probes 1998; 12: 133-142.
8. Englund S, Bolske G, Johansson KE. An IS900-like sequence found in a Mycobacterium sp. other than Mycobacterium avium subsp. paratuberculosis. FEMS Microbiol Lett 2002; 209: 267-271.
9. Fang Y, Wu WH, Pepper JL, Larsen JL, Marras SA, Nelson EA, Epperson WB, Christopher-Hennings J. Comparison of real-time quantitative PCR with molecular beacons to nested PCR and culture methods for detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples. J Clin Microbiol 2002; 40: 287-291.
10. Fodstad FH, Gunnarsson E. Post-mortem examination in the diagnosis of Johne's disease in goats. Acta Vet Scand 1979; 20: 157-167.
11. Greenstein RJ. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. Lancet Infect Dis 2003; 3: 507-514.
12. Greig A, Stevenson K, Henderson D, Perez V, Hugues V, Pavlik I, Hines ME 2nd, McKendrick I, Sharp JM. Epidemiological study of paratuberculosis in wild rabbits in Scotland. J Clin Microbiol 1999; 37: 1746-1751.
13. Irenge LM, Walravens K, Govaerts M, Godfroid J, Rosseels V, Huygen K, Gala J. Development and validation of a triplex real-time PCR for rapid detection and specific identification of M. avium sub sp. paratuberculosis in fecal samples. Vet Microbiol 2009; 136: 166-172.
14. Liverani E, Scaioli E, Cardamone C, Dal Monte P, Belluzzi A. Mycobacterium avium subspecies paratuberculosis in the etiology of Crohn's disease, cause or epiphenomenon? World J Gastroenterol 2014; 20: 13060-13070.
15. Matos AC, Figueira L, Martins MH, Loureiro F, Pinto ML, Matos M, Coelho AC. Survey of Mycobacterium avium subspecies paratuberculosis in road-killed wild carnivores in Portugal. J Zoo Wildl Med 2014; 45: 775-781.
16. Matos AC, Figueira L, Martins MH, Matos M, Alvares S, Pinto ML, Coelho AC. Disseminated Mycobacterium avium subsp. paratuberculosis infection in two wild Eurasian otters (Lutra lutra L.) from Portugal. J Zoo Wildl Med 2013; 44: 193-195.
17. O'Mahony J, Hill C. Rapid real-time PCR assay for detection and quantification of Mycobacterium avium subsp. paratuberculosis DNA in artificially contaminated milk. Appl Environ Microbiol 2004; 70: 4561-4568.
18. Park HT, Shin MK, Sung KY, Park HE, Cho YI, Yoo HS. Effective DNA extraction method to improve detection of Mycobacterium avium subsp. paratuberculosis in bovine feces. Korean J Vet Res 2014; 54: 55-57.
19. Schönenbrücher H, Abdulmawjood A, Failing K, Bülte M. New triplex real-time PCR assay for detection of Mycobacterium avium subsp. paratuberculosis in bovine feces. Appl Environ Microbiol 2008; 74: 2751-2758.
20. Slana I, Kralik P, Kralova A, Pavlik I. On-farm spread of Mycobacterium avium subsp. paratuberculosis in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. Int J Food Micrbiol 2008; 128: 250-257.
21. Stabel JR, Bannantine JP. Development of a nested PCR method targeting a unique multicopy element, ISMAP02 for detection of Mycobacterium avium subsp. paratuberculosis in fecal samples. J Clin Microbiol 2005; 43: 4744-4750.
22. Thonur L, Maley M, Gilray J, Crook T, Laming E, Turnbull D, Nath M, Willoughby K. One-step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine herpesvirus 1 and bovine parainfluenza virus 3. BMC Vet Res 2012; 8: 37.
23. Thorel MF, Huchzermeyer H, Weiss R, Fontaine JJ. Mycobacterium avium infections in animals. Literature review. Vet Res 1997; 28: 439-447.
24. Thorel MF, Krichevsky M, Vincent Levy-Frebault V. Numerical taxonomy of mycobactin-dependent mycobacteria, amended description of Mycobacterium avium subsp. avium subsp. nov., Mycobacterium avium subsp. paratuberculosis subsp. nov., and Mycobacterium avium subsp. silvaticum subsp. nov. Int J Syst Bacteriol 1990; 40: 254-260.

분변 시료에서 *Mycobacterium avium* subsp. *paratuberculosis* 의 빠른 검출을 위한 다중 실시간 중합효소연쇄반응기법의 개발

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요 약 : *Mycobacterium avium* subsp. *paratuberculosis* (MAP)는 가축 및 야생동물에서 장 내 육아종성 감염증을 유발한다. 이 연구의 목적은 MAP 특이유전자 3개(IS*900*, F57 및 ISMAP*02*)의 빠른 검사를 위한 다중 실시간 중합효소연쇄반응기법을 개발하고 평가하는데 있다. 평가 결과 분석 민감도는 IS*900*이 150 cells/ml, F57이 1500 cells/ml, ISMAP*02*가 50 cells/ml로 확인되었다. 152개 소 분변시료를 대상으로 실시한 검사 결과 개발한 기법이 기존 검사방법보다 빠르고 적은 비용으로 동시검사가 가능한 것으로 확인되었다. 검사결과의 일치도는 94%로 나타났다. 불일치 결과는 개발한 기법이 양성으로 확인하였으나, 기존 검사에서는 음성으로 나타난 것 때문으로 확인되어, 개발한 기법이 더 높은 민감도를 갖는 것으로 나타났다. 개발한 다중 실시간 중합효소연쇄반응기법은 가축 및 야생동물에서 파라결핵 또는 요네병의 조사를 위한 빠르고 정확한 검사기법이 될 것이다.

주요어 : *Mycobacterium avium* subsp. *paratuberculosis*, 다중 실시간 중합효소연쇄반응기법, 진단