PCR-based detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle in South Korea using fecal samples

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ABSTRACT. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of bovine paratuberculosis (PTB). The first step in the control of PTB is the identification and isolation of sub-clinical fecal shedders from the herd. In the current study, real-time and nested PCR targeting MAP-specific genetic elements (*IS900* and *ISMAP02*) DNA isolated from fecal samples were used to detect MAP infection in cattle. Of the 1,562 fecal samples obtained from 37 herds, regardless of diarrhea, 35 samples tested positive in both *IS900*-targeted real-time and *ISMAP02*-targeted nested PCR. At the herd level, 12 of the 37 herds were found to be positive for MAP. Detection rates of the PCR tests were similar to those reported for ELISA-based methods. These results suggest that PCR can be used to detect sub-clinical fecal shedders of MAP.
KEY WORDS: diagnosis, Korea, *Mycobacterium avium* subsp. *paratuberculosis*, nested PCR, real-time PCR

Mycoabcterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis (PTB) or Johne's disease (JD), which is characterized by chronic and debilitating enteritis in ruminants. MAP has a global distribution and causes serious economic losses to the cattle industry [12].

Clinical onset of PTB is noted after 2 or more years of the initial infection, which usually occurs shortly after birth [7]. Additionally, the bacteria are shed in the feces of infected animals that show no clinical signs. These 'sub-clinical' fecal shedders contaminate various components of the cattle sheds, such as feed, water, bedding and other materials. Contamination of cattle shed components results in spreading of the infection to other animals through the fecal–oral route. Specifically, young animals that are highly susceptible to MAP are easily infected as they ingest the bacteria via milk, colostrum and the surface of the teats, which get contaminated through the cattle shed components [6, 17]. Therefore, identification and isolation of sub-clinical fecal shedders are the most effective strategy to control PTB.

Culture-based methods are the 'gold standard' for diagnosis of MAP infection. However, these methods are time-consuming and have relatively low sensitivity. Recently, molecular biological methods, such as PCR, have been developed for the detection of MAP infection in animals [5]. Several target sequences unique to MAP have been identified for PCR-based detection. The insertion sequence *IS900* is the most selected target element, because it is present in

multiple copies and thought to be unique to MAP [3]. However, recent studies have reported the presence of *IS900*-like sequences in non-MAP mycobacteria, which could lower the specificity of *IS900*-based assays. Therefore, a new target sequence, *ISMAP02*, which is also present in multiple copies and is specific to MAP, has been identified for real-time or nested PCR-based assays. *ISMAP02*-targeted nested PCR is usually combined with *IS900*-targeted real-time PCR to improve detection rates [4, 7, 18].

PCR-based diagnostics have been used in many countries, and the methods have been improved constantly to overcome the limitations of other diagnostic methods, such as the culture-based method [1, 2]. However, MAP detection from cattle feces using a PCR-based assay has not been reported in South Korea.

In the present study, a PCR-based assay targeting both *IS900* and *ISMAP02* was used to detect MAP infection in the fecal samples collected from South Korean cattle herds.

Fecal samples from 982 cattle in 25 beef herds and 580 cattle in 12 dairy herds, regardless of diarrhea, were collected between September 2013 and August 2014. The samples were collected individually from the rectum and transported immediately to the laboratory in a container maintained at 4°C. DNA was extracted using the mGITC/SC method [14] and was used for downstream analyses—real-time and nested PCR targeting *IS900* and *ISMAP02* sequences. The real-time PCR was performed using the reaction conditions described previously [14]. Nested PCR was performed using primer sequences described previously with modifications [18]. Briefly, the 20-μl PCR mixture comprised 1 μl of template DNA, 10.5 μl of nuclease-free water, 2 μl of i-Taq^{TM} 10X PCR buffer (Intron biotechnology, Seoul, Korea), 2.5 mM of MgCl$_2$, 0.25 mM of deoxyribonucleotide triphosphates, 500 nM of each primer and 2.5 U of i-Taq^{TM} DNA polymerase. The PCR conditions were as follows: ini-

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Table 1. Number of samples infected with *Mycobacterium avium* subsp. *paratuberculosis* in beef and dairy cattle herds

Species	Herd ID	Sampling	No. of samples	No. of samples positive (percentage)		
				PCR		
				IS900	ISMAP02	Both
Beef	GY2	09/2013	6	1 (16.7)	0 (0)	0 (0)
	GY3	09/2013	16	0 (0)	0 (0)	0 (0)
	GW2	09/2013	52	4 (7.7)	1 (1.9)	1 (1.9)
	JJ1	11/2013	13	0 (0)	0 (0)	0 (0)
	JJ2	11/2013	11	2 (18.2)	0 (0)	0 (0)
	JJ3	11/2013	13	0 (0)	0 (0)	0 (0)
	JJ4	11/2013	13	6 (46.2)	4 (30.8)	4 (30.8)
	JJ5	11/2013	7	0 (0)	0 (0)	0 (0)
	JJ6	11/2013	7	2 (28.6)	1 (14.3)	1 (14.3)
	JJ7	11/2013	13	0 (0)	0 (0)	0 (0)
	JJ8	11/2013	16	0 (0)	0 (0)	0 (0)
	JJ9	11/2013	11	0 (0)	0 (0)	0 (0)
	GN1	03/2014	21	0 (0)	0 (0)	0 (0)
	GN2	03/2014	25	0 (0)	0 (0)	0 (0)
	CB1	03/2014	25	3 (12.0)	4 (16.0)	3 (12.0)
	CB2	03/2014	18	1 (5.6)	0 (0)	0 (0)
	CB3	03/2014	20	1 (5.0)	2 (10.0)	1 (5.0)
	CB4	03/2014	10	2 (20.0)	1 (10.0)	0 (0)
	CB5	03/2014	10	0 (0)	0 (0)	0 (0)
	CB6	03/2014	5	1 (20.0)	1 (20.0)	1 (20.0)
	CB7	03/2014	5	1 (20.0)	0 (0)	0 (0)
	CB8	03/2014	8	0 (0)	0 (0)	0 (0)
	CB9	03/2014	7	0 (0)	0 (0)	0 (0)
	CB10	03/2014	7	0 (0)	0 (0)	0 (0)
	GW3	03/2014	643	36 (5.6)	22 (3.4)	13 (2.0)
	Subtotal		982	60 (6.1)	36 (3.7)	24 (2.4)
Dairy	GY1	09/2013	18	0 (0)	0 (0)	0 (0)
	GY4	09/2013	10	1 (10.0)	1 (10.0)	1 (10.0)
	GY5	09/2013	24	0 (0)	0 (0)	0 (0)
	GY6	09/2013	15	0 (0)	0 (0)	0 (0)
	GY7	09/2013	29	0 (0)	0 (0)	0 (0)
	GW1	09/2013	60	3 (5.0)	3 (5.0)	3 (5.0)
	GY8	03/2014	40	2 (5.0)	1 (2.5)	1 (2.5)
	GY9	03/2014	20	0 (0)	0 (0)	0 (0)
	GY10	03/2014	20	0 (0)	0 (0)	0 (0)
	GY11	03/2014	20	1 (5.0)	2 (10.0)	1 (5.0)
	CN1	04/2014	284	6 (2.1)	10 (3.5)	5 (1.8)
	GW4	08/2014	40	0 (0)	0 (0)	0 (0)
	Subtotal		580	13 (2.9)	17 (2.9)	11 (1.9)
Total			1,562	73 (4.7)	53 (3.4)	35 (2.2)

tial denaturation at 94°C for 5 min, 35 cycles at 94°C for 15 sec, 58°C for 15 sec and 72°C for 20 sec, followed by a final extension at 72°C for 7 min. The amplicons (1 μl) from the first PCR were used as the template in the nested PCR, which was performed using similar reaction conditions (30 cycles). After electrophoresis on a 2% agarose gel, presence of 117 bp size bands was considered as a positive result. To minimize the risk of carry-over contamination in the nested PCR, reagent preparation at each step and PCR amplification were performed using different sets of the pipetting system and

on different work benches with a separate air-conditioning. Tips with filters were used to protect the PCR samples from aerosol contamination. Samples that showed positive signals in both *IS900*-targeted real-time PCR and *ISMAP02*-targeted nested PCR were considered as positive samples.

The PCR results are summarized in Table 1. *IS900* was detected in 73 samples, accounting for 4.7% of the total samples, whereas *ISMAP02* was detected in 53 samples, accounting for 3.4% of the total samples. Thirty-five samples that showed a positive result for both target sequences were

Table 2.   Age distribution of the number of beef and dairy cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*

Species	No. of positive samples/tested (%)				
	Age distribution				
	≤2	3~4	5~6	≥7	unknown
Beef	6/326 (1.8)	7/269 (2.6)	2/113 (1.8)	6/187 (3.2)	3/87 (3.4)
Dairy	2/87 (2.3)	2/154 (1.3)	2/105 (1.9)	1/64 (1.6)	4/170 (2.4)
Total	8/413 (1.9)	9/423 (2.1)	4/218 (1.8)	7/251 (2.8)	7/257 (2.7)

considered as definite MAP positives (2.2%). Thirty-eight (2.4%) samples were detected positive only for *IS900*; whereas 18 (1.2%) samples were detected positive only for *ISMAP02*. Among the 37 herds, 12 (32.4%) were detected positive for MAP infection.

Age distribution of the positive samples was analyzed statistically (Table 2). In both beef and dairy cattle, the number of positive samples showed no notable difference with respect to age. However, the number of positive samples (3.2%) noted in beef cattle aged more than 7 years was higher than that in other age groups. These samples were obtained from 2 herds, which had a higher proportion of old cattle than the other herds (23.1% and 23.7% of collected samples). Population of cattle aged more than 7 years was 9.5% in other herds.

In a previous study, seroprevalence of MAP in South Korean cattle was reported in about 7.1% samples analyzed by ELISA followed by the Bayesian approach [13, 15]. In addition, the seroprevalence of MAP in the Gyeongnam province in South Korea was estimated to be 3.3% [10]. The present investigation also revealed a similar result at the individual level (2.2%). Although the prevalence of MAP at the cattle herd level has not been reported earlier in South Korea, the prevalence found in the present study was similar to that in the Korean black goat [11]. Cattle type-specific statistical analysis showed 41.7% positive samples in dairy herd, which was similar to that reported in a previous study conducted in Gyeongnam, whereas 28.0% positive samples were noted in beef herd, which was much higher than a previous report [10].

A weak correlation was noted between the results of two PCR assays. The number of single positives for *IS900* noted in real-time PCR was similar to the number of positives for both the sequences. This could be because the sensitivity of *IS900*-targeted real-time PCR was higher than the *ISMAP02*-targeted nested PCR. Theoretical sensitivity of *IS900* is higher than that of *ISMAP02*, because 15~20 copies of the *IS900* elements and only 6 copies of the *ISMAP02* elements are present in MAP [18, 21]. Experimental sensitivity of *IS900*-targeted real-time PCR and *ISMAP02*-targeted nested PCR can be correlated to their theoretical sensitivity. Detection limit of *IS900*-targeted real-time PCR is 10 fg of DNA extracted from pure cultured cells, whereas that of the *ISMAP02*-targeted nested PCR is 100 fg [18]. Our previous study reported that the detection limit of *IS900*-targeted real-time PCR was 4.6 fg of DNA extracted from pure cultured cells by mGITC/SC method [14]. Therefore, single positive

of *IS900*-targeted real-time PCR may indicate the presence of MAP at a very low level. Although *IS900*-targeted PCR has relatively higher sensitivity, it lacks specificity, which is a notable problem because of the presence of *IS900*-like sequences [8, 16, 19]. This problem can be solved by designing primers that are not complementary to all the known *IS900*-like sequences. However, confirming MAP-specificity in every *IS900* single positive would be time-consuming and cumbersome [7].

Some samples were positive only for *ISMAP02* and can be assumed as true positives, because *ISMAP02* is known to be MAP-specific [18]. Moreover, various PCR inhibitors obtained from fecal samples may lower the sensitivity of *IS900*-targeted real-time PCR when low amounts of target DNA is used for the assay [20]. Although a large amount of bacteria is present in feces, PCR inhibitors sometimes can lead to false-negative results [18]. However, the possibility of non-specific bands similar in size should be considered during electrophoresis of samples after nested PCR. Additionally, there may be a potential risk in resulting false-positive data in attempting to increase the sensitivity via the inclusion of nested PCR [4]. Consequently, the samples showing a positive result for both the sequences could be confirmed as definite positive, but samples showing a positive result for a single sequence should be investigated further. Therefore, developing a comprehensive system that allows further investigation of the false-positive results is crucial. Such a system may utilize repeated analysis of samples for validation by PCR or may include other types of tests, such as ELISA.

Analysis of the age-wise distribution of MAP-infection showed that infection rates were similar in all age groups. However, in the group with age less than 2 years, no calves or heifers less than one-year-old showed a positive result. This phenomenon is similar to the pathogenesis of MAP infection, indicating fecal shedding without clinical signs. In fact, all the cattle diagnosed as positive for MAP had no clinical signs, such as diarrhea. This suggests that, the PCR method is considerable when the sub-clinical fecal shedders of infection are to be diagnosed.

Molecular biological diagnostic methods, such as PCR, are used as supplementary detection methods, because they cannot determine the number of viable bacteria [9]. In case of MAP, however, the PCR-based method is worth considering, because it provides higher sensitivity and specificity than other detection methods.

In this study, we detected MAP from fecal samples of

cattle showing no clinical signs using an improved DNA extraction method and PCR analysis. Our findings suggest that detection of MAP-specific genetic elements using PCR can be used for early diagnosis, which will in turn help control MAP infection because identification and isolation of fecal shedders from the herd are crucial for preventing the spread of infection.

REFERENCES

1. Alinovi, C. A., Ward, M. P., Lin, T. L., Moore, G. E. and Wu, C. C. 2009. Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* ssp. *paratuberculosis*. *Vet. Microbiol.* **136**: 177–179. [Medline] [CrossRef]
2. Bögli-Stuber, K., Kohler, C., Seitert, G., Glanemann, B., Antognoli, M. C., Salman, M. D., Wittenbrink, M. M., Wittwer, M., Wassenaar, T., Jemmi, T. and Bissig-Choisat, B. 2005. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss dairy cattle by real-time PCR and culture: a comparison of the two assays. *J. Appl. Microbiol.* **99**: 587–597. [Medline] [CrossRef]
3. Bull, T. J., Hermon-Taylor, J., Pavlik, I., El-Zaatari, F. and Tizard, M. 2000. Characterization of IS900 loci in *Mycobacterium avium* subsp. *paratuberculosis* and development of multiplex PCR typing. *Microbiology* **146**: 2185–2197. [Medline] [CrossRef]
4. Douarre, P. E., Cashman, W., Buckley, J., Coffey, A. and O'Mahony, J. M. 2010. Isolation and detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from cattle in Ireland using both traditional culture and molecular based methods. *Gut Pathog.* **2**: 11. [Medline] [CrossRef]
5. Fang, Y., Wu, W. H., Pepper, J. L., Larsen, J. L., Marras, S. A., Nelson, E. A., Epperson, W. B. and Christopher-Hennings, J. 2002. 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.* **40**: 287–291. [Medline] [CrossRef]
6. Giese, S. B. and Ahrens, P. 2000. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. *Vet. Microbiol.* **77**: 291–297. [Medline] [CrossRef]
7. Irenge, L. M., Walravens, K., Govaerts, M., Godfroid, J., Rosseels, V., Huygen, K. and Gala, J. L. 2009. Development and validation of a triplex real-time PCR for rapid detection and specific identification of *M. avium* subsp. *paratuberculosis* in faecal samples. *Vet. Microbiol.* **136**: 166–172. [Medline] [CrossRef]
8. Kim, S. G., Shin, S. J., Jacobson, R. H., Miller, L. J., Harpending, P. R., Stehman, S. M., Rossiter, C. A. and Lein, D. A. 2002. Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis*. *J. Vet. Diagn. Invest.* **14**: 126–131. [Medline] [CrossRef]
9. Kralik, P., Nocker, A. and Pavlik, I. 2010. *Mycobacterium avium* subsp. *paratuberculosis* viability determination using F57 quantitative PCR in combination with propidium monoazide treatment. *Int. J. Food Microbiol.* **141** Suppl 1: S80–S86. [Medline] [CrossRef]
10. Lee, K. W. and Jung, B. Y. 2009. Seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* in cattle in Korea. *Vet. Rec.* **165**: 661–662. [Medline] [CrossRef]
11. Lee, K. W., Jung, B. Y., Moon, O. K., Yang, D. K., Lee, S. H., Kim, J. Y. and Kweon, C. H. 2006. Seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Korean black goats (*Capra hircus aegagrus*). *J. Vet. Med. Sci.* **68**: 1379–1381.
12. Ott, S. L., Wells, S. J. and Wagner, B. A. 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. *Prev. Vet. Med.* **40**: 179–192. [Medline] [CrossRef]
13. Pak, S. I., Kim, D. and Salman, M. 2003. Estimation of paratuberculosis prevalence in dairy cattle in a province of Korea using an enzyme-linked immunosorbent assay: application of Bayesian approach. *J. Vet. Sci.* **4**: 51–56. [Medline]
14. Park, H. T., Shin, M. K., Sung, K. Y., Park, H. E., Cho, Y. I. and Yoo, H. S. 2014. Effective DNA extraction method to improve detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. *Korean J. Vet. Res.* **54**: 55–57. [CrossRef]
15. Park, K. T., Ahn, J., Davis, W. C., Koo, H. C., Kwon, N. H., Jung, W. K., Kim, J. M., Hong, S. K. and Park, Y. H. 2006. Analysis of the seroprevalence of bovine paratuberculosis and the application of modified absorbed ELISA to field sample testing in Korea. *J. Vet. Sci.* **7**: 349–354. [Medline] [CrossRef]
16. Rajeev, S., Zhang, Y., Sreevatsan, S., Motiwala, A. S. and Byrum, B. 2005. Evaluation of multiple genomic targets for identification and confirmation of *Mycobacterium avium* subsp. *paratuberculosis* isolates using real-time PCR. *Vet. Microbiol.* **105**: 215–221. [Medline] [CrossRef]
17. Slana, I., Liapi, M., Moravkova, M., Kralova, A. and Pavlik, I. 2009. *Mycobacterium avium* subsp. *paratuberculosis* in cow bulk tank milk in Cyprus detected by culture and quantitative IS900 and F57 real-time PCR. *Prev. Vet. Med.* **89**: 223–226. [Medline] [CrossRef]
18. Stabel, J. R. and Bannantine, J. P. 2005. 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.* **43**: 4744–4750. [Medline] [CrossRef]
19. Tasara, T., Hoelzle, L. E. and Stephan, R. 2005. Development and evaluation of a *Mycobacterium avium* subspecies *paratuberculosis* (MAP) specific multiplex PCR assay. *Int. J. Food Microbiol.* **104**: 279–287. [Medline] [CrossRef]
20. Thornton, C. G. and Passen, S. 2004. Inhibition of PCR amplification by phytic acid, and treatment of bovine fecal specimens with phytase to reduce inhibition. *J. Microbiol. Methods* **59**: 43–52. [Medline] [CrossRef]
21. Whipple, D., Kapke, P. and Vary, C. 1990. Identification of restriction fragment length polymorphisms in DNA from *Mycobacterium paratuberculosis*. *J. Clin. Microbiol.* **28**: 2561–2564. [Medline]