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 present 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.

**KEYWORDS:** diagnosis, Korea, *Mycobacterium avium* subsp. *paratuberculosis*, nested PCR, real-time PCR

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*Mycobacterium 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 nuclelease-free water, 2 µl of i-Taq™ 10X PCR buffer (Intron biotechnology, Seoul, Korea), 2.5 mM of MgCl₂, 0.25 mM of deoxyribonucleotide triphosphates, 500 nM of each primer and 2.5 U of i-Taq™ DNA polymerase. The PCR conditions were as follows: ini-
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

| Species | Herd ID | Sampling | No. of samples | No. of samples positive (percentage) | PCR |
|---------|---------|----------|----------------|-------------------------------------|-----|
| 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)             |     |
|         | GW8     | 03/2014  | 40             | 2 (5.0) 1 (2.5) 1 (2.5)             |     |
|         | GW9     | 03/2014  | 20             | 0 (0) 0 (0) 0 (0)                   |     |
|         | GW10    | 03/2014  | 20             | 0 (0) 0 (0) 0 (0)                   |     |
|         | GW11    | 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)          |     |
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 earlier 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 positive singles 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.

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) |
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.

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