Detection and Confirmation of *Mycobacterium avium* subsp. *paratuberculosis* in Direct Quantitative PCR Positive Fecal Samples by the Manual Fluorescent MGIT Culture System

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ABSTRACT. An efficient protocol for the manual fluorescent MGIT culture system combined with rapid confirmation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) growth in the broth culture was established and evaluated for the detection of viable MAP in direct quantitative PCR (QPCR) positive bovine feces. Manually detected fluorescence emissions from MGIT tubes were analyzed objectively using an open source software, ImageJ. For molecular confirmation of MAP growth, DNA samples harvested by simply boiling the broth, an inexpensive and time- and labor-saving DNA preparation method, yielded adequate results. The sheep strain of MAP required longer incubation time relative to the cattle strain, suggesting that the MGIT system may not support well the growth of ovine isolates as described previously. Of 61 direct QPCR positive bovine feces, the recovery rate of MAP in the MGIT system (62.3%) was significantly higher (*P*<0.05) than that using 7H10 agar-based slants (44.3%). The time to obtain a final result for fecal culture by the MGIT system was several weeks earlier compared to solid media. In MGIT culture positive samples, the time to detect fluorescence was correlated with the DNA quantity detected in fecal QPCR. As a positive result in the direct fecal QPCR test does not mean fecal excretion of viable MAP, bacterial isolation by fecal culture could be conducted to verify the QPCR result. For this purpose, the manual MGIT system is a sensitive and rapid culture method at least for bovine samples.
KEY WORDS: fecal culture, ImageJ, MGIT, paratuberculosis, real-time quantitative PCR.

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Johne's disease (JD) is prevalent worldwide in domestic ruminant animals and causes significant economic damage to livestock farming enterprises. Treatment of JD is not considered a practical option, and therefore, test-based culling of infected animals and preventing transmission are currently accepted as the most effective and rewarding strategies to control the disease [10, 18]. In Japan, the prevalence of JD has been kept at a low level by conducting nationwide active surveillance and culling infected animals with compensation since 1997.

Infected animals become infectious primarily by starting to shed *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the etiological agent of JD, in their feces. These animals can shed approximately 10^8 organisms per gram of feces [2, 22]. Isolation of MAP from shedding animals by fecal culture is one of the most frequently used ante-mortem diagnostic tests for JD. To circumvent the dependence on time consuming culture methods, employment of a real-time quantitative PCR (QPCR)-based test for detection and quantification of MAP DNA in feces has been considered in Japan. It has been shown that a direct fecal QPCR test has similar sensitivity compared to fecal culture and provides immediate information to estimate the risk of transmission by quantification of MAP DNA in feces [8, 9]. However, the QPCR assay also detected many culture negative fecal samples from animals exposed to MAP, and it was not possible to distinguish live MAP from dead MAP [8]. To validate the QPCR assay for the detection of 'live' MAP shedders, employment of a practical rapid and sufficiently sensitive fecal culture method is still required.

For cultivation of MAP from clinical samples, solid media, such as Herrold's egg yolk medium (HEYM) or Middlebrook 7H10 agar-based slants, are commonly used in Japan. However, many reports from abroad have suggested that liquid culture methods have advantages over solid media in terms of higher sensitivity [3, 12, 21] and shorter incubation time [4]. Among the commercially available liquid culture media, adequate performance of the MGIT culture system designed specifically for recovery of MAP (MGIT Para TB medium) has been shown for bovine samples [5, 6, 20]. MGIT medium, which is non-radioactive, replaced the BACTEC 460 system which involved use of radioactive media. MGIT media can be monitored manually using a simple UV light reader for fluorescence detection where an automated MGIT instrumentation is not available, but there are conflicting reports on the performance of the manual method. In one study, manual and automated MGIT systems

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Convert the image file to '8 bit' grayscale
Save as a TIFF file

↓

Create a circular selection (shown in a dotted circle)

↓

Drag it over the first MGIT tube
Measure the integrated density of the spot
(Press "m" (Analyse/Measure))

↓
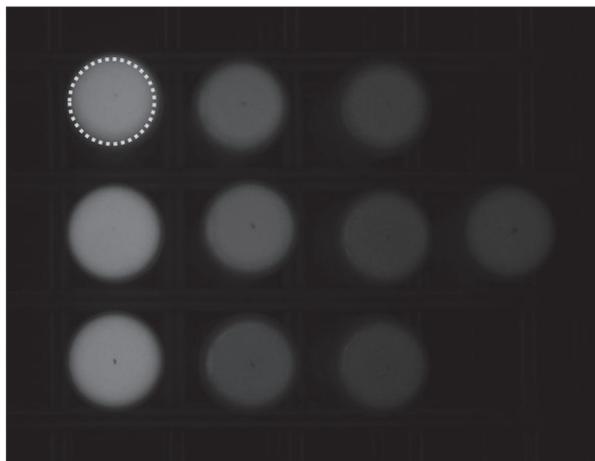
Repeat for the other tubes

Fig. 1.   Image analysis by ImageJ 1.46p.

showed similar detection times and recovery rates of my-cobacteria, although a significant number of false-positive results were observed in the manual MGIT system [16]. In a second study, manual reading under a UV transilluminator required a longer incubation period to be positive and had a lower rate of detection of MAP compared to the results obtained by the automated MGIT system [7].

In order to introduce a liquid culture system to Japan for diagnosis of JD, an efficient protocol for the manual MGIT system combined with rapid confirmation of MAP growth was established in this study. The system was evaluated for the detection of viable MAP in direct QPCR positive bovine feces by comparison with fecal culture on solid medium.

MATERIALS AND METHODS

Culture media: A 7H10 agar-based plate containing Difco™ Middlebrook 7H10 Agar (Becton, Dickinson and Co., Sparks, MD, U.S.A.) supplemented with 10% BBL™ Middlebrook OADC Enrichment (Becton, Dickinson and Co.), 7.5% egg yolk, 2 μg/ml of mycobactin J (Kyoritsu Seiyaku, Tokyo, Japan), 0.5% v/v glycerol and 50 μg/ml of amphotericin B was used for bacterial culture.

For fecal culture, Middlebrook 7H10 agar-based slants

were also prepared. Ingredients were the same as the plate described above with the addition of 0.005% malachite green, 50 μg/ml vancomycin and 50 μg/ml nalidixic acid.

The manual fluorescent MGIT system was conducted as follows. An MGIT Para TB medium tube (Becton, Dickinson and Co.) was prepared and handled as recommended by the manufacturer with the exception that vancomycin was omitted for culturing the S strain of MAP. Cultures were incubated at 37°C for at least 49 days as prescribed by the manufacturer and monitored manually at least twice a week using a homemade device to capture an image of fluorescence from MGIT tubes. Digital images were analyzed with Java-based open source software, ImageJ 1.46p, freely downloadable from the US National Institute of Health website (http://imagej.nih.gov/ij/). Using a built-in method for dot blot analysis, the integrated density of each MGIT tube was measured according to the guidelines (also summarized in Fig. 1). Positive signals were defined as an integrated density over 30,000.

DNA extraction from MGIT medium: Three DNA extraction methods were compared in this study for molecular confirmation of MAP growth in the MGIT medium. After 7 weeks culture of MAP strains (K10 and Telford as described below) and a fecal sample from experimentally infected cattle in the MGIT medium, tenfold serial dilutions of the culture broth were prepared in control MGIT medium, and 170 μl of each dilution was processed for DNA extraction described as follows (also summarized in Table 1). The DNA harvesting process was conducted in duplicate.

Johne-Spin; DNA extraction using a Johne-Spin (FAS-MAC, Atsugi, Japan) employed mechanical disruption of cells with bead beating followed by DNA purification using a spin column. Briefly, 170 μl of MGIT medium was transferred to a tube containing zirconia beads and centrifuged at 20,000 × g for 5 min. After the supernatant was carefully removed, 600 μl of lysis buffer and 6 μl of RNase A solution were added to the pellet with microbeads. Samples were agitated at 4,600 rpm for 3 min using a homogeniser (Micro Smash™ MS-100, TOMY SEIKO Co., Ltd., Tokyo, Japan). Following centrifugation at 13,000 × g for 1 min, 75 μl of extraction buffer was added to the homogenized sample. After centrifugation at 20,000 × g for 10 min, 500 μl of the supernatant was transferred to a new 1.5 ml tube containing 400 μl of binding buffer and then mixed by inverting. All the mixture was transferred to a spin column and centrifuged at 13,000 × g for 1 min. The column was washed once with 600 μl of washing buffer by centrifugation at 13,000 × g for 1 min and then placed onto a new 1.5 ml tube. Fifty microliter of elution buffer was added onto the center of the membrane, and the column was stood for 3 min at room temperature. DNA samples were eluted by centrifugation the column at 13,000 × g for 1 min.

InstaGene; DNA extraction using an InstaGene™ Matrix (Bio-Rad, Hercules, CA, U.S.A.) was conducted according to the manufacturer's instructions. Briefly, 170 μl of MGIT medium was transferred to a fresh microfuge tube. After centrifugation at 13,000 rpm for 5 min, 200 μl of InstaGene™ Matrix was added to the pellet and incubated at 56°C

Table 1.   Methods for DNA extraction from MGIT culture tested in this study

	Johne-Spin	InstaGene	Boil
Protocol	170 μl MGIT culture ↓ Beads tube 15,000 rpm, 5 min ↓ Bead beating, 3 min ↓ 15,000 rpm, 10 min ↓ DNA purification using a spin column (total 6 min)	170 μl MGIT culture ↓ 13, 000 rpm, 5 min ↓ Resuspend the pellet in Insta-Gene Matrix ↓ 56°C, 15 min ↓ 100°C, 8 min ↓ 12,000 rpm, 2 min Harvest the supernatant	170 μl MGIT culture ↓ 100°C, 8 min ↓ 13,000 rpm, 5 min Harvest the supernatant
Approx. time/sample	25 min	30 min	13 min
Price (yen)/sample [a]	892.5	150	0

a) The labor/time costs were excluded.

for 15 min. The tube was placed in a boiling waterbath for 8 min and then centrifuged at 12,000 rpm for 2 min. After centrifugation, the supernatant was used for the QPCR assay.

Boiling; 170 μl of MGIT medium was transferred to a microcentrifuge tube and placed in a boiling waterbath for 8 min. After centrifugation at 13,000 rpm for 5 min, the supernatant was used for the QPCR assay.

To evaluate the ability of each extraction method to eliminate PCR inhibitors in the MGIT components, 2.5 μl of each template DNA harvested from control media was mixed with 1 pg of MAP genomic DNA and tested by QPCR. As a negative control for this experiment, 2.5 μl of TE buffer was used.

Molecular confirmation by QPCR: The QPCR assay to detect MAP IS*900* was as described previously [8] with small modifications. A reaction volume of 25 μl contained 2.5 μl of template DNA, 12.5 μl of 2× QuantiTect SYBR Green PCR Master Mix (QIAGEN, Tokyo, Japan), 0.125 μl of 100 pmol of forward (MP10-1) and reverse (MP11-1) primers and 0.125 μl of 5,000 U/ml Uracil-DNA Glycosylase (UDG, New England BioLabs, Ipswich, MA, U.S.A.) in nuclease free water. The reaction conditions were arranged as follows; an initial incubation step at 37°C for 10 min to control carry-over contamination by UDG, one cycle of 15 min at 95°C and 45 cycles of PCR amplification at 95°C for 30 sec and 68°C for 60 sec followed by the dissociation curve analysis. For quantification, a standard curve was created as described previously [8]. All DNA templates were tested by the QPCR assay in duplicate.

MAP strains and preparation of bacterial suspensions: Two genotypically-distinct type strains of MAP were used in this study; cattle (C) strain K-10 (BAA-968) and sheep (S) strain Telford 9.2 (generous gift from Dr. R. Whittington). MAP strains were grown on Middlebrook 7H10 agar plates for 6 weeks at 37°C in air supplemented with 5% CO_2. Bacterial cells were harvested and resuspended in PBS. The total number of MAP cells in each suspension was estimated by the DNA quantity (one genome of MAP contains 5 fg DNA) detected by a QPCR assay described previously [8].

The stock suspension was diluted in serial one hundred-fold steps in PBS to provide bacterial suspensions ranging from 10^8 to 10^2 cells/ml, and 100 μl of each dilution was inoculated into each of 2 MGIT Para TB tubes to yield final concentrations ranging from 10^7 to 10^1 cells/tube. The tubes were incubated at 37°C, and the fluorescence emission was monitored twice a week. DNA templates were prepared from the MGIT cultures using the 'Boiling' method described above and tested by QPCR twice a week for the C strain of MAP (K-10) up to 5 weeks and once or twice a week for the S strain (Telford 9.2) up to 7 weeks.

Fecal samples and sample preparation for fecal culture: Fecal samples collected from naturally and experimentally infected cattle were tested by a direct QPCR assay described previously [8] using Johne-Spin (FASMAC) for DNA extraction from feces [11]. A total of 61 QPCR positive feces were processed for fecal culture. Sample preparation for fecal culture was undertaken by the NADC method of Stabel [17] with some modifications. Briefly, 1 g of feces added to 20 ml of sterile saline was shaken vigorously for 30 min and then allowed to settle for 30 min at room temperature. Five ml of the surface fluid was transferred to a new 50 ml tube containing 20 ml of 0.75% hexadecylpyridinium chloride made up in half-strength brain heart infusion broth and incubated at 37°C overnight. After centrifugation at 900 × g for 30 min, the supernatant was discarded, and the pellet was resuspended in 1 ml of a solution of antibiotics (50 μg/ml vancomycin, 50 μg/ml nalidixic acid and 50 μg/ml amphotericin B). After incubation at 37°C for 48 hr, 0.1 ml aliquot of the prepared fecal sediment was inoculated into 2 Middlebrook 7H10 solid medium slants and 2 MGIT Para TB liquid medium culture tubes. Cultures in MGIT medium were incubated at 37°C for 12 weeks (84 days), and the fluorescence emission was monitored at least once a week. For confirmation of MAP growth, DNA templates were prepared using the 'Boiling' method described above and tested by QPCR. In case MAP was identified only from MGIT culture, MGIT positive tubes were subcultured onto solid culture slants to confirm bacterial growth on solid media.

(a)

(b)

(c)
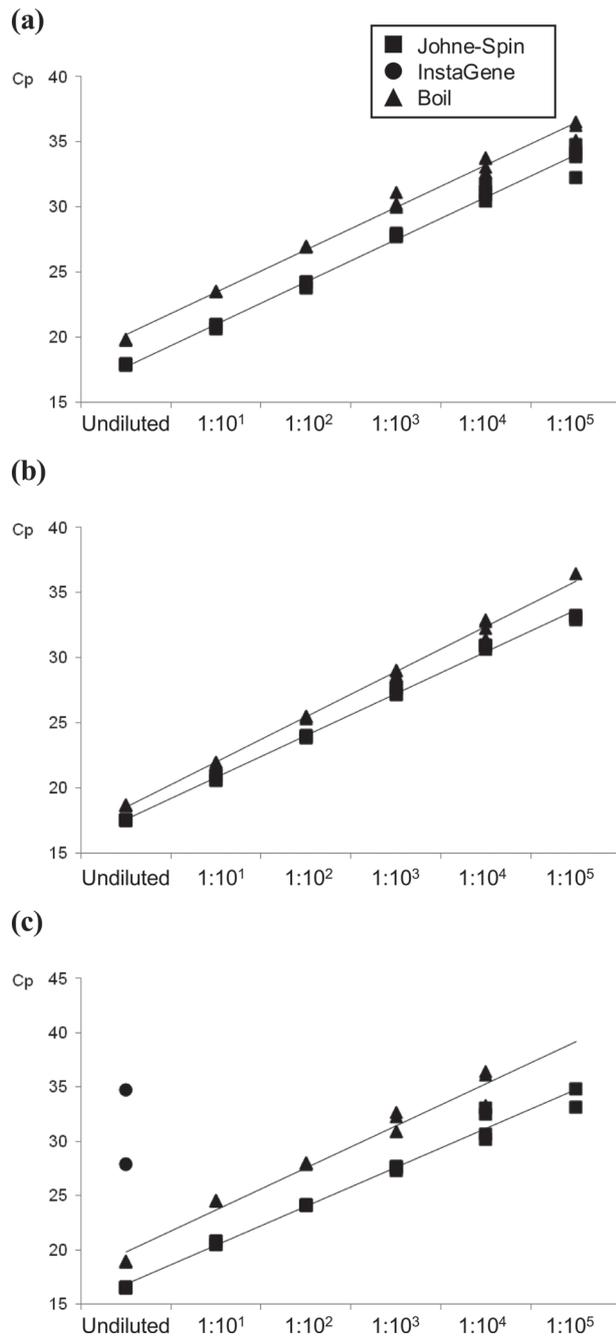
Fig. 2.   Comparison of three DNA extraction methods for detection of MAP in MGIT culture by QPCR. (a) C-strain (K10), (b) S-strain (Telford) and (c) positive feces from experimentally inoculated cattle.

Statistical analysis: Mean Cp values between each DNA harvesting method and control (TE buffer) were compared by a *t*-test. A McNemar's test was used to compare the results for the isolation of MAP between solid and liquid culture media, and a kappa value was calculated to assess the agreement of the results (http://graphpad.com/quickcalcs/).
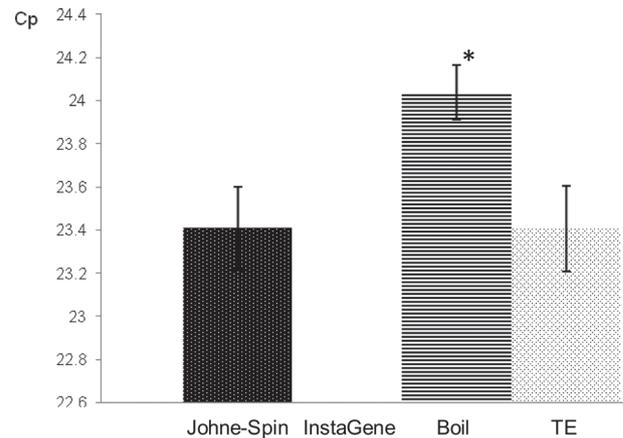
Fig. 3.   Inhibition of PCR analytical sensitivity for amplification of 1 pg of MAP genomic DNA. *Indicates the mean Cp was significantly different ($P<0.01$) compared to that in control (TE buffer).

Differences in DNA quantities detected by the direct fecal QPCR assay for the result of fecal culture were evaluated by a Kruskal-Wallis test with multiple comparisons using a Dunn's post hoc test. The relationship between the DNA quantity detected in the direct fecal QPCR test and the time for detection in the manual fluorescent MGIT Para TB system was assessed by nonlinear regression among 38 samples that were positive in MGIT culture.

RESULTS

Comparison of DNA harvesting methods for QPCR confirmation of MAP growth: Three DNA extraction methods summarized in Table 1 were compared for molecular confirmation of MAP in MGIT culture. The crossing point (Cp) values in QPCR for each DNA sample harvested from MGIT culture broth of MAP strains and a positive fecal sample by the 3 DNA extraction methods are shown in Fig. 2. Compared to the mean Cp values for DNA extracted by Johne-Spin, they were delayed by 2.43 (K10), 1.52 (Telford) and 3.48 (positive feces) cycles for DNA samples harvested by the boil method (Fig. 2). Except for DNA samples from undiluted MGIT culture of positive feces, QPCR results were all negative when DNA was harvested using InstaGene.

The mean Cp for amplification of 1 pg of MAP genomic DNA mixed with DNA template extracted from control MGIT broth by Johne-Spin was similar to that of MAP DNA with TE buffer, while it was significantly delayed by an average of 0.63 cycles when DNA harvested by the boil method was added (Fig. 3). One pg of MAP DNA was not amplified within 45 cycles when it was mixed with a DNA sample prepared using InstaGene.

Growth of C and S strains of MAP in the manual MGIT system: Growth and detection of MAP in the manual MGIT system combined with the boil method for DNA harvesting followed by QPCR were evaluated using pure cultures of C and S strains (Fig. 4). Growth of C strain in the MGIT medium occurred quickly, and fluorescence was detected after
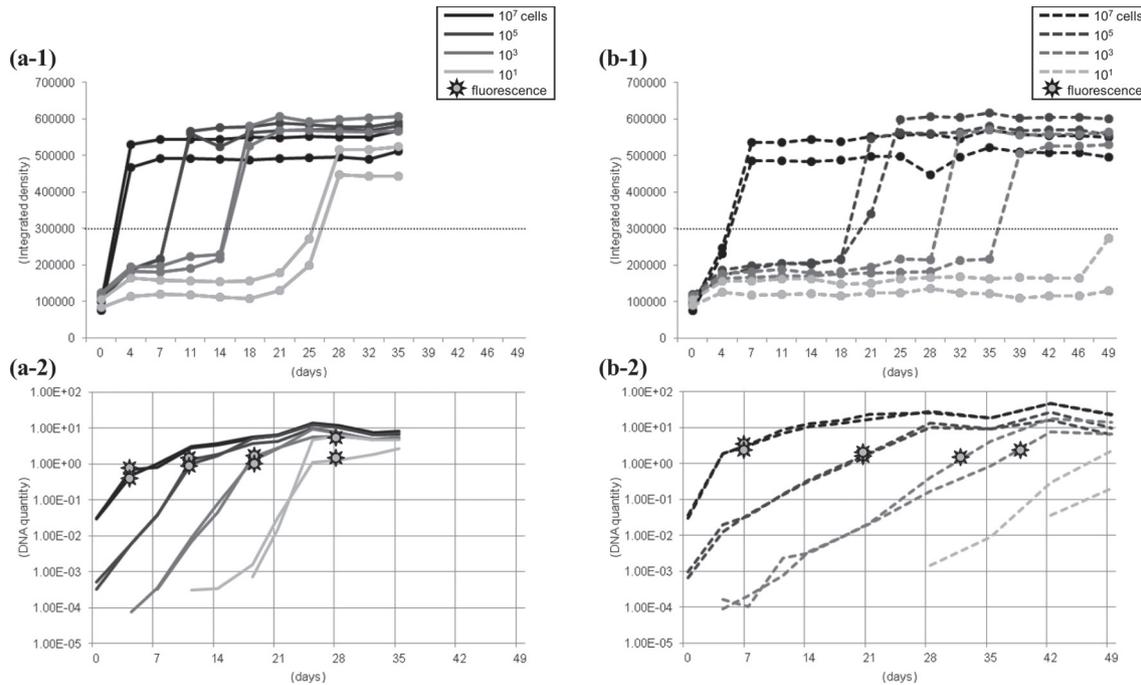
Fig. 4.   Growth and detection of C (a) and S (b) strains of MAP in the manual MGIT system combined with the boil method for DNA harvesting followed by QPCR. (a-1 and b-1) fluorescence, (a-2 and b-2) DNA quantity.

Table 2.   Fecal culture by MGIT Para TB compared to solid media for the 61 direct fecal QPCR positive samples

		MGIT		Total
		No. +ve	No. -ve	
		(median DNA quantity (*p*g) in direct fecal QPCR*)		
7H10	No. +ve	22 (5.72E–02 [a])	5 (8.31E–04 [b])	27
	No. −ve	16 (1.30E–03 [b])	18 (5.69E–04 [b])	34
Total		38	23	61

Kappa value=0.33, McNemar's χ^2=4.76, $P<0.05$. * Different letters indicate differences in a Kruskal-Wallis test with post hoc multiple comparisons ($P<0.05$).

4 weeks of incubation when 10^1 cells of the C strain were inoculated (a-1). The S strain required more time to grow particularly in the lower inoculum size (b-1). When 10^1 cells of the S strain were inoculated, the integrated density did not reach the positive level within 49 days of incubation (b-1), although MAP DNA was increasing in the culture broth at that time (b-2). MAP DNA was detected by QPCR about 2 weeks and more than 3 weeks before detection of the fluorescence for culturing C and S strains of MAP, respectively (a-2 and b-2). Regardless of the starting quantity of MAP or the inoculum strain, approximately 2 pg of MAP DNA was detected in 2.5 μl of the DNA sample at the time of fluorescence positive: it can be estimated that there were at least 1.6×10^5 cells/ml in the MGIT medium.

Detection of 'live' MAP in QPCR positive bovine feces by the manual MGIT system: A total of 61 direct fecal QPCR positive samples were cultured in the MGIT system and on 7H10 slopes (Table 2). MAP growth was confirmed in 38 fecal samples (62.3%) cultured using the MGIT system, while MAP was isolated from 27 feces (44.3%) by 7H10 solid slants. The sensitivity of fecal culture in the MGIT system was significantly higher than that on 7H10 slants (McNemar's χ^2=4.76, $P<0.05$), although there were 5 samples that the result of fecal culture was positive only by the solid media (Table 2). In total, MAP was isolated from 43 of 61 fecal QPCR positive feces (70.5%). MAP DNA quantities contained in the fecal samples that were culture positive in both liquid and solid media were significantly higher than those in either liquid or solid culture positive feces and culture negative feces (Table 2). In all the fecal samples from which MAP was isolated only by the MGIT system (n=16), MAP growth was confirmed on the 7H10 solid media after
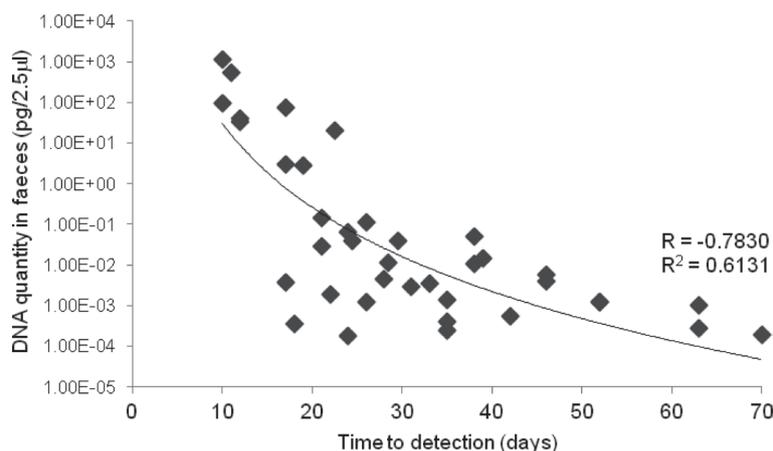
Fig. 5.   The relationship between the DNA quantity in feces and the time to detection
by MGIT culture (n=38).

subculturing the MGIT culture broth. The average incuba-
tion time for positive samples in the MGIT system was 30.5
days, although 5 of them required more than 50 days of
incubation to detect fluorescence. It took 2.5 to 5 months to
confirm MAP colonies on the solid media.

Among 38 samples that were positive in the MGIT sys-
tem, there was a significant relationship between the DNA
quantity detected in the direct fecal QPCR assay and the time
to detect fluorescence in MGIT cultures (Fig. 5).

DISCUSSION

In this study, an efficient protocol for the MGIT culture
system using manual fluorescence detection combined with
rapid confirmation of MAP growth in the broth culture was
established. The system was evaluated for the detection of
viable MAP in direct QPCR positive bovine feces by com-
parison with a conventional fecal culture method on solid
medium.

As manually or automatically detected fluorescence
in the MGIT culture tube does not prove growth of MAP,
subculturing on solid media for demonstration of mycobac-
tin dependency or molecular confirmation is necessary for
identification of MAP in the sample. For rapid confirmation,
harvesting DNA from broth culture followed by detection of
MAP specific genes by PCR is commonly applied. However,
DNA sample preparation from liquid media often requires
removal of egg yolk which inhibits PCR amplification. It
has been shown that direct addition of unprocessed MGIT
culture broth to the PCR reaction mixture resulted in an ex-
tended Cp value and a low sensitivity in the QPCR analysis
[19]. Simple methods for DNA extraction from broth cul-
tures using ethanol [23] or by boiling [19] have already been
reported, although primers and/or the target DNA of the PCR
procedure was different from the present study. In this study,
three methods of harvesting DNA from MGIT culture media
were compared, and the effect of residual PCR inhibitors
was seen for the boil method as delayed Cp values in the

QPCR assay (Fig. 3). Such effect was not observed in the
DNA samples harvested using Johne-Spin, suggesting that
inhibitors of the PCR reaction in the broth culture could be
removed. For detection of MAP in MGIT culture, the DNA
sample extracted by Johne-Spin always had lower Cp val-
ues compared with the boil method, suggesting that those
samples had a greater quantity of target DNA and/or less
PCR inhibitor (Fig. 2). The QPCR results also suggested that
InstaGene was unsuitable for DNA extraction from MGIT
cultures.

Although the quality and quantity of the DNA sample
prepared using Johne-Spin seemed to be adequate, there are
still sufficient reasons to employ the boil method for DNA
harvesting from broth culture. The boil method was an inex-
pensive and time- and labor-saving DNA preparation method
for confirmation of MAP growth in the MGIT medium by
QPCR [19]. In this study, DNA samples prepared by the boil
method were positive in QPCR few weeks before detection
of fluorescence, suggesting that the boil method can be use-
ful at least at the time when cultures become fluorescence
positive, where massive amounts of MAP DNA should be
present in the MGIT tube.

One advantage of employing the MGIT system is that
MGIT culture tubes can be read manually using a UV trans-
illuminator, which most laboratories already have. An auto-
mated MGIT reader, the BACTEC MGIT 960 Mycobacterial
Detection System, is available from the manufacture, but
the price of this instrument would be prohibitive for many
of the veterinary and clinical laboratories in Japan. It has
been demonstrated that detection time and recovery rates
for mycobacteria were comparable between the manual and
automated MGIT systems [16]. Another study confirmed
that no statistical difference in recorded detection time was
observed in the manual reading of MGIT tubes by three
individual readers [6]. However, a contradictory result has
also been reported, which is that the manual MGIT system
was less sensitive and time consuming compared to the
automated system [7]. In this study, manually detected fluo-

rescence emissions from MIGT cultures were measured and analyzed objectively by use of ImageJ. The time to detection data for the C strain of MAP (K-10) recorded by the manual MGIT system in the present study seemed to be comparable to the universal standard curve for quantification of bovine and human isolates of MAP in the automated MGIT system demonstrated by Shin *et al.* [15].

Vancomycin was not included in the MGIT medium for culturing the S strain in this study due to its negative effect on growth [7]. The detection time for culturing a laboratory adapted S strain (Telford 9.2) in the MGIT system was improved; the time to detection for 1.0×10^3 cells of the S stain was less than 40 days in the present study, while it took 63 days to detect the growth of 2.3×10^3 cells in the MGIT system with vancomycin [7]. Nevertheless, the S strain of MAP in the MGIT system required a prolonged incubation relative to the C strain. Similar results that S-type strains isolated from sheep and goats tended to grow slower than the C-type (strain K-10) in the MGIT system have been reported [1]. Interestingly, C- and bison (B)-types of MAP strains isolated from sheep also required longer incubation times compared to those from cattle, goats and wildlife animals [1]. The correlation between genotype and growth in the MGIT system for the ovine isolates is still unclear.

The manual MGIT system showed significantly higher sensitivity for isolation of MAP from bovine feces compared to 7H10 agar-based slants. All the MAP isolates detected by the liquid culture alone grew on the solid media after subculturing the MGIT culture broth, suggesting a role for the MGIT culture system as an enrichment medium. However, in some feces, the result of fecal culture was positive only by the solid media. A contradictory result between the culture methods was observed in bovine feces which contained lower numbers of MAP, explained by non-homogeneous distribution or clumping of the bacteria. The isolation rate of 'live' MAP would be improved by using both liquid and solid cultures when the result of fecal QPCR suggests a low MAP DNA content in the fecal sample. Alternatively, employment of more replicates of the MGIT tubes would also increase the sensitivity of fecal culture. In this study, 'live' MAP was recovered from 70% of the direct fecal QPCR positive samples when both MGIT Para TB medium and 7H10 agar slants were used.

As expected, the time to obtain a final result of fecal culture by the MGIT system was several weeks earlier than the time to confirm MAP colonies on solid media. According to the protocol provided by the manufacturer, the incubation time to declare the positive/negative result is 49 days using an automated MGIT instrument. However, longer incubation time (>49 days) was required in some fecal samples in this study. The maximum incubation time in culture positive feces was 70 days (10 weeks), suggesting that reporting the fecal sample as culture negative would be possible at >70 days of incubation at least in the manual MGIT Para TB system. This should be confirmed in another study using more fecal samples which contain low numbers of MAP.

In MGIT culture positive samples, the time to detect fluorescence in the MGIT system was correlated with the DNA quantity detected in the direct fecal QPCR assay ($r= -0.78$). A similar correlation coefficient ($r= -0.70$) was obtained previously using BACTEC radiometric culture and the fecal QPCR in ovine feces [8]. Instead of counting the number of colonies on solid media, the starting number of viable organisms can be determined from the time to detection data in liquid media [14, 15]. It has been shown that the estimation of initial bacillary load by using the time to detection in the MGIT system was accurate and highly reproducible in pure cultures of MAP strains [15]. However, the time to detection in fecal culture with MGIT may be influenced by other factors: contribution to the time to signal positive by growth of irrelevant microbes in feces and potential loss of viable MAP through the decontamination procedure [13].

It has been suggested that quantification of MAP DNA in feces by QPCR possibly provides immediate information to estimate the risk of transmission from infected animals [8, 9]. However, a PCR-based test detects the entire target DNA regardless of the bacterial viability, and therefore, the number of viable MAP in a sample cannot be estimated from the DNA quantity detected in the QPCR test. A follow-up test by fecal culture needs to be conducted, especially when a relatively small amount of MAP DNA is detected by fecal QPCR, to confirm if the animal is shedding 'live' MAP and serves as a source of infection. For this purpose, the sensitivity of fecal culture is critical. As suggested previously by Whittington [21], our study also confirmed that the sensitivity of fecal culture would drop, if solid medium alone was used for primary culture.

In conclusion, rapid confirmation of MAP growth in the MGIT system would be possible by the boil method of DNA harvesting followed by the QPCR assay. For detection of MAP shedders in animals which were positive in the direct fecal QPCR test, the manual MGIT system showed significantly higher sensitivity compared with 7H10 agar-based slants.

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