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Development and Evaluation of a Novel Multicopy-Element-Targeting Triplex PCR for Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Feces

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The enteropathy called paratuberculosis (PTB), which mainly affects ruminants and has a worldwide distribution, is caused by *Mycobacterium avium* subsp. *paratuberculosis*. This disease significantly reduces the cost-effectiveness of ruminant farms, and therefore, reliable and rapid detection methods are needed to control the spread of the bacterium in livestock and in the environment. The aim of this study was to identify a specific and sensitive combination of DNA extraction and amplification to detect *M. avium* subsp. *paratuberculosis* in feces. Negative bovine fecal samples were inoculated with increasing concentrations of two different bacterial strains (field and reference) to compare the performance of four extraction and five amplification protocols. The best results were obtained using the JohnePrep and MagMax extraction kits combined with an in-house triplex real-time PCR designed to detect IS*900*, IS*Map02* (an insertion sequence of *M. avium* subsp. *paratuberculosis* present in 6 copies per genome), and an internal amplification control DNA simultaneously. These combinations detected 10 *M. avium* subsp. *paratuberculosis* cells/g of spiked feces. The triplex PCR detected 1 fg of genomic DNA extracted from the reference strain K10. The performance of the robotized version of the MagMax extraction kit combined with the IS*900* and IS*Map02* PCR was further evaluated using 615 archival fecal samples from the first sampling of nine Friesian cattle herds included in a PTB control program and followed up for at least 4 years. The analysis of the results obtained in this survey demonstrated that the diagnostic method was highly specific and sensitive for the detection of *M. avium* subsp. *paratuberculosis* in fecal samples from cattle and a very valuable tool to be used in PTB control programs.

Paratuberculosis (PTB) is an infectious enteropathy with worldwide distribution that mainly affects ruminants and is caused by *Mycobacterium avium* subsp. *paratuberculosis*. It significantly reduces the cost-effectiveness of cattle farms due to reduced milk production and early replacement of infected animals (1–4). Recent studies seem to confirm an association between Crohn's disease and the etiological agent of PTB (5), and others have demonstrated the presence of viable *M. avium* subsp. *paratuberculosis* cells in milk, dairy products, water, and meat (6, 7). Therefore, reliable and rapid diagnostic methods are needed to detect infected animals that contribute to the maintenance and spread of this pathogen both in livestock and in the environment.

Enzyme-linked immunosorbent assay (ELISA) and fecal culture have been the methods most commonly used in the diagnosis of PTB. Compared to fecal culture, the sensitivity of ELISA is often under 30% (8). On the other hand, traditional fecal culture, though considered the gold standard test, is slow and expensive and usually shows low sensitivity as well. Animals shedding the microorganism in their feces can advantageously be identified by means of real-time PCR. However, the presence of low and variable numbers of bacteria in feces and the copurification of PCR inhibitors during DNA extraction are factors that can affect the sensitivity of PCR methods. New and more sensitive extraction methods have been developed to avoid these issues. Commercially available kits include specific reagents to remove PCR inhibitors or have greatly improved their DNA capture technology by using DNA-binding magnetic beads or DNA filters. A good strategy to rule out false-negative results due to PCR inhibition is the inclusion of an internal amplification control (IAC) in the reaction mixture. In addition to sensitivity problems, some reports have questioned the specificity of IS*900* (9), the preferred target for PCR detection, due to the high number of copies per *M. avium* subsp. *paratuberculosis* cell. Other *M. avium* subsp. *paratuberculosis*-specific elements, such as F57 (10), locus 251 (11), or HspX (12), have been employed as substitutes or for confirmation. These single-copy targets are very interesting for quantification assays, but multicopy elements are presumably better for detection approaches because they increase the chance to detect the pathogen. IS*Mav2* and IS*Map04* are insertion sequences of *M. avium* subsp. *paratuberculosis* present in three and four copies, respectively, in the genome of strain K10 (sequenced reference strain) (13). A multicopy element called IS*Map02* with six repeats inserted in the genome of *M. avium* subsp. *paratuberculosis* seems to be an adequate candidate to complement IS*900*-based PCR detection because no homologues have been described in other mycobacteria (13).

The aim of the present study was to identify the best combinations of several available fecal DNA extraction and real-time amplification methods for a sensitive and specific PCR diagnosis of bovine PTB. In the first part of the study, four DNA extraction methods, a quantitative real-time PCR kit, and four real-time PCR methods were compared using a set of artificially inoculated fecal samples. The genetic targets used for amplification were F57,

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IS*900*, IS*Mav2*, and IS*Map02*. Then, the best-performing combination of DNA extraction and real-time PCR was chosen to test archival fecal samples obtained from 615 animals from nine Friesian cattle herds included in a PTB control program.

MATERIALS AND METHODS

Comparison of DNA extraction and amplification methods using spiked fecal samples. (i) Preparation of spiked feces. Feces from a PTB-free cow, confirmed by previous results of fecal and tissue culture, serology, histopathology, and fecal and tissue PCR, were collected. A low-passage-number field strain (St 764) isolated from bovine feces and the reference strain K10 were used to inoculate the negative feces. These strains represent pulsed-field gel electrophoresis (PFGE) (using SnaBI-SpeI restriction endonucleases) profiles 1-1 and 2-1 and multilocus short-sequence-repeat (G and GGT residues) profiles 14_5 and 7_4, respectively. These profiles cluster into the cattle type (also called type II) group of *M. avium* subsp. *paratuberculosis* organisms and are dominant in our area (14, 15). Glycerol stocks of the strains were grown in Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton, Dickinson and Company, MD, USA) and mycobactin J (Allied Monitor, Inc., Fayette, MO, USA). When sufficient growth was obtained, cells were harvested by centrifugation at 2,800 × *g*. Pellets were washed twice in phosphate-buffered saline (PBS) and finally resuspended in 5 ml of PBS. Bacterial suspensions were mechanically declumped by making the liquid flow up and down through a needle (26 gauge, 3/8 in) several times. The cell numbers in the inocula of both strains were estimated by visual direct count in a Neubauer chamber and the concentration adjusted to 10^8 cells/ml. Ten-fold serial dilutions of this suspension were carried out to a final inoculum containing 100 cells per ml. Suspensions were flowed up and down through the needle in all steps to avoid reclumping of cells. One-gram fecal aliquots were prepared and spiked with 0.1 ml of the appropriate inoculum to obtain samples ranging from 10 to 10^7 cells/g of feces in triplicate for both strains. Spiked samples were frozen at −20°C until used. Amounts of 0.1 ml of each dilution were also plated onto 4 solidified-agar–Middlebrook 7H9 (agar-7H9) flasks to assess the CFU counts in the original inocula.

(ii) DNA extraction methods. Four different DNA extraction procedures were used, including a modified QIAamp DNA stool minikit (Qiagen), the JohnePrep kit (Kyoritsu Seiyaku, Shimadzu Corp., Kyoto, Japan), the Adiapure kit (Adiagene, Saint Brieuc, France), and the manual version of the MagMax total nucleic acid isolation kit (Ambion, Applied Biosystems, Foster City, CA, USA). Negative controls were included in the first, middle, and last position of all extractions carried out. All DNA extraction kits were used as indicated by the manufacturer, with the exception of the modified QIAamp DNA stool minikit (Qiagen), which was used as described previously (16). Aliquots of DNA samples from all extraction methods were prepared and stored at −20°C until used in downstream PCR assays.

(iii) Real-time PCR methods. The ParaTB Kuanti-VK quantitative PCR (Vacunek SL, Derio, Spain), TaqMan MAP (*M. avium* subsp. *paratuberculosis*) (Applied Biosystems), and Adiavet PTB (Adiagene) real-time PCR kits and two in-house triplex real-time PCR methods were employed. The three replicate purified DNA samples derived from all extraction methods were submitted to PCR amplification with the ParaTB Kuanti-VK kit and both in-house PCR systems, while TaqMan MAP and Adiavet PTB PCR kits were only used with extracts obtained with the corresponding extraction kit (MagMax and Adiapure, respectively). All PCR systems included an internal amplification control (IAC) and ROX dye as a passive reference reporter. Samples were always tested in duplicate for every combination. All plates included two negative controls, in the first and last well. A standard amplification program, consisting of one denaturation and polymerase activation cycle of 10 min at 95°C, 45 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min, was performed in a 7500 real-time PCR instrument (Applied Biosystems) for every PCR method. The results were analyzed using the 7500 system SDS software, version 1.4 (Applied Biosystems). Threshold cycle (C_T) values and baseline values were automatically determined by the software and visually confirmed by checking amplification plots.

(a) ParaTB Kuanti-VK quantitative PCR kit. The ParaTB Kuanti-VK quantitative PCR kit was tested for its ability to detect *M. avium* subsp. *paratuberculosis*, but it also served as a reliable indicator of the efficiency of the DNA extraction protocols. The targeted genomic sequence is the single-copy gene F57. Five-microliter amounts of DNA samples were added to 45 μl of PCR premix in duplicate. The dyes for the target and IAC probes were 6-carboxyfluorescein–Black Hole Quencher-1 (6-FAM–BHQ-1) and JOE–BHQ-1, respectively. Serial dilutions of a standard control provided with the kit were performed using 10 mM Tris-HCl, pH 8. Standard controls with final concentrations ranging from 10^7 to 10 copies of F57 were loaded in triplicate in every plate in order to quantify the number of *M. avium* subsp. *paratuberculosis* genomes in each sample. Only results obtained in plates with standard curves showing a slope between −3.4 and −3.67 and R^2 values above 0.998 were considered. In order to estimate the number of genome copies quantified in 1 g of feces for each sample, the following mathematical operations were done, assuming an ideal homogeneous distribution of *M. avium* subsp. *paratuberculosis* in spiked samples. The quantification results were divided by 5 (5 μl of DNA extract were loaded into each PCR mixture) and multiplied by the total volume used for elution (200 μl for the modified QIAamp DNA stool kit, 50 μl for JohnePrep, 100 μl for Adiapure, and 50 μl for MagMax) to assess the number of F57 copies estimated for the whole volume of DNA extract. The extracts represented different volumes of resuspended fecal material according to each DNA extraction protocol (for the modified QIAamp DNA stool kit, each DNA extract represented 1.3 ml of a mixture containing 1 g of feces resuspended in 5 ml; for the JohnePrep, extracts represented 1 ml of a mixture containing 1 g of feces resuspended in 20 ml; for the Adiapure, extracts represented 0.3 ml of a mixture containing 1 g of feces resuspended in 20 ml; and for the MagMax, extracts represented 0.175 ml of a mixture containing 1 g of feces resuspended in 3.33 ml. Thus, the number of copies calculated for DNA extracts was multiplied by the corresponding volume used to resuspend 1 g of feces and divided by the starting volume used in each case.

(b) F57 and ISMav2 real-time PCR system. The F57 and IS*Mav2* triplex real-time PCR was performed under conditions previously described by Schonenbrucher et al. (16). Locked nucleic acid (LNA; Sigma-Aldrich Co. Ltd., Haverhill, United Kingdom) and minor groove binder (MGB; Applied Biosystems) probes were used for this assay. The reaction mixtures included 1× TaqMan universal PCR master mix without AmpErase UNG (uracil-DNA glycosylase) (Applied Biosystems), 0.3 μM F57 forward and reverse primers, 0.2 μM IS*Mav2* forward and reverse primers, 0.25 μM F57 LNA probe (JOE-cCt gAc Cac CctT-BHQ-1; uppercase letters represent LNA bases), 0.25 μM F57 IAC MGB probe (NED-CGA GTT ACA TGA TCC C-MGB), 0.25 μM IS*Mav2* LNA probe (FAM-cGc tGa GtT cCt TaG-BHQ-1), 175 copies of IAC DNA, and 5 μl of DNA extract in a total volume of 50 μl.

(c) IS900 and ISMap02 real-time PCR system. The IS*900* and IS*Map02* triplex real-time PCR combined the detection of IS*900* using previously described primers and probe (17) and the detection of IS*Map02* with primers and probe that were designed for this study or previously reported (18), assisted by Primer Express software, version 3 (Applied Biosystems) (Table 1). The IS*Map02* consensus sequence was determined by the alignment of all IS*Map02* sequences available in public DNA sequence databases. Oligonucleotides were designed and checked by the BLAST procedure (National Center for Biotechnology Information) in order to avoid possible homologies with other sequences. DNAs of different *M. avium* subsp. *paratuberculosis* strains and DNAs of other mycobacteria and other nonmycobacterial species were submitted to PCR using these probes and primers to assess the specificity of the assay (Table 2).

An IAC was designed and constructed to be coamplified with IS*Map02* primers. Briefly, two complementary single-stranded DNA molecules containing the sequences for IS*Map02* forward and reverse primers in

TABLE 1 Sequences of primers and probes used in the IS*900* and IS*Map02* real-time PCR system

Primer or probe	Sequence (5′–3′)	Reference
IS*900*-F	CCGCTAATTGAGAGATGCGATT	17
IS*900*-R	CCAGACAGGTTGTGCCACAA	
IS*900* probe	6-FAM-ACCTCCGTAACCGTCAT TGTCCAGATCA-BHQ-1	
IS*Map02*-F	CGGCTGGACACGGAATG	18
IS*Map02*-R	CATGAGCGACAGTATCTTTCGAA	
IS*Map02* probe	JOE-ATCCGTCCCAGTGGCGGAG TCAC-BHQ-1	
IAC probe	Cy5-CTCTGACACATGCAGCTCC CGGAGAC-BHQ2	

their 5′ ends were synthesized (Sigma-Aldrich) and annealed. This double-stranded molecule was amplified under standard PCR conditions using IS*Map02* primers. After visualization in a 3% agarose gel stained with GelRed (Biotium, Inc., Hayward, CA, USA), the amplicon was purified using a QIAquick gel extraction kit (Qiagen). The purified product was then cloned into a pCR4-TOPO plasmid vector using the TOPO TA cloning kit (Invitrogen, Ltd., Paisley, United Kingdom). The plasmid was purified from transformed *E. coli* cells with an illustra plasmidPrep minispin kit (GE Healthcare Europe GmbH, Barcelona, Spain) and linearized using SnaBI endonuclease (New England BioLabs, Inc., Beverly, MA, USA). After repurification of the digested plasmid, the DNA concentration was measured. The appropriate amount of plasmid yielding C_T values between 33 and 36 in noninhibited triplex PCR assays was calculated by using serial dilutions of the purified plasmid.

The final PCR mixture contained 1× TaqMan universal PCR master mix without AmpErase UNG (Applied Biosystems), 0.4 μM each IS*Map02* primers, 0.3 μM each IS*900* primers, 0.2 μM each IS*Map02*, IAC, and IS*900* probes, 20 copies of IAC DNA, and 5 μl of extracted DNA in a total volume of 50 μl. The sequences of primers and probes used in this PCR are shown in Table 1.

The analytical sensitivity of this PCR was additionally assessed using 10-fold serial dilutions of a solution containing 10 ng/μl of DNA extracted from the K10 reference strain as measured on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The DNA concentration was used to calculate the number of *M. avium* subsp. *paratuberculosis* genomes present by the following formula, taking into account that one genome of *M. avium* subsp. *paratuberculosis* K10 is 4,829,781 bp in length and that it contains 17 copies of IS*900* and 6 copies of IS*Map02* (13): DNA molecule copy number = (ng DNA × 6,023E+23)/[(bp length × 1E+09) × 660].

(d) TaqMan MAP real-time PCR kit. The TaqMan MAP real-time PCR assay targets a unique sequence element in the *M. avium* subsp. *paratuberculosis* genome other than IS*900*, but it is not specified in the kit. The reaction mixture consisted of a 25-μl volume containing 1× qPCR master mix, 1× MAP primer probe mix, and 2.5 μl of DNA extract. The TaqMan reporter dyes and quenchers are FAM–BHQ-1 for the target and CAL Fluor orange 560–BHQ-1 for the IAC (Xeno DNA control).

(e) Adiavet PTB real-time PCR kit. The Adiavet PTB real-time PCR kit (previous version; Adiagen) amplifies a region of the IS*900* insertion element. Two microliters of purified DNA was added to 23 μl of ready-to-use PCR premix. The target and IAC probes are labeled with FAM and VIC dyes.

(iv) Culture of spiked feces. Frozen replicates of previously prepared 1-g fecal aliquots of all levels of inoculation were decontaminated with 0.75% hexadecyl pyridinium chloride (wt/vol) and cultured on Herrold's egg yolk (HEY) and agar-7H9 flasks as described elsewhere (19). The flasks were incubated at 37°C for 7 months.

(v) Analysis of PCR results. In order to obtain more robust results for both DNA extraction and amplification procedures, duplicate PCR results corresponding to the three DNA extraction replicates were grouped, making a total of 6 DNA extraction-amplification replicates. The mean C_T values and standard deviations corresponding to each concentration of inoculated *M. avium* subsp. *paratuberculosis*/g of feces were calculated for every DNA extraction and PCR system combination. In the case of the ParaTB Kuanti-VK quantitative PCR, these results and the linear regression curves were plotted and analyzed with a 95% confidence interval using the SigmaPlot software, version 10.0 (Systat Software, Inc.).

Evaluation of the chosen combination using samples from naturally infected cattle. To assess the performance of the combination of molecular methods chosen (i.e., the MagMax kit for DNA extraction and the IS*900* and IS*Map02* PCR for amplification), archived fecal samples were used. This DNA extraction method was chosen because, in the study with spiked feces, it was determined to be one of the most sensitive protocols (the PCR system was selected for the same reason) and because an automated version was available. Six hundred fifteen bovine fecal samples collected between 2006 and 2007 in a PTB control program were used for this part of the study. The specimens were from the first sampling of two control or "test and cull" herds (TC group; 319 samples) and 7 vaccinated herds (VH group; 296 samples). Animals that were in the vaccination group were vaccinated 3 days after the month zero (M0) sampling (beginning of the project). All herds had previous records of PTB that showed annual incidences of clinical PTB between 2 and 5% (animals with clinical symptoms of PTB confirmed after necropsy). These herds were analyzed using conventional ELISA (Pourquier, Montpelier, France), IS*900* PCR (the first version of Adiavet [Adiagene]), and culture (HEY and Lowenstein-Jensen media made in-house) before the beginning of the project (M0) and were followed up annually using the same methods for at least 4 more years, except for one of the vaccinated herds. Only stored (−20°C) samples obtained during the first sampling (M0) of the control program were resubmitted to DNA extraction and amplification using the combination of methods chosen. Follow-up sensitivities and specificities of the IS*900* and IS*Map02* PCR were estimated for both the TC and VH group, comparing the results obtained for M0 samples with a reference result that combined all results of conventional methods for all samplings. Thus, the reference result was considered positive when the animal was positive to at least one of these tests in any of the samplings carried out and negative if all tests performed during the 4-year follow-up were negative. Complete necropsy results were also available for 36 and 33 animals from the TC and VH groups, respectively. This information, coupled with the data obtained during the follow-up, provided a final thorough classification of these 69 animals.

In order to make the results more informative, triplex real-time PCR, ELISA, conventional fecal PCR, culture, and histopathology results were given a number according to the following categories. (i) for IS*900* and IS*Map02* real-time PCR (mean C_T values of IS*Map02* and IS*900*), the numbers used were 3 (heavy shedder) for C_T values lower than or equal to 31, 2 (intermediate shedder) when C_T values were between 31 and 35, 1 (low shedder) if C_T values were higher than 35, and 0 (nonshedder) when these values were undetermined (amplification curve not crossing the threshold line or absent). (ii) For ELISA, the numbers used were 3 (highly reactive) when the sample-to-positive percentage (S/P%) was higher than or equal to 180, 2 (intermediate-highly reactive) when S/P% was lower than 180 and higher than or equal to 70, 1 (intermediate or inconclusive) when S/P% was lower than 70 and higher than or equal to 60, and finally) 0 (low/nonreactive) if S/P% was lower than 60. {S/P% = [(sample OD − mean NC OD)/(mean PC OD − mean NC OD)] × 100], where OD is the optical density of serum or plasma at 450 nm [ELISA], NC is negative control, and PC is positive control.} (iii) For conventional PCR, the numbers used were 1 to indicate a positive PCR result (amplicon present) and 0 to indicate a negative PCR result (amplicon absent), and the results could not be further classified. (iv) For fecal/tissue culture, the numbers used were 3 (heavy shedder or high bacterial burden) when 50 or more colonies were counted per tube, 2 (intermediate shedder or intermediate bacterial burden) when the colony counts were between 50 and 10, 1 (low

TABLE 2 Bacterial strains used to assess the specificity of primers and probe designed for IS*Map02*

Strain(s)	Type and/or origin	IS*Map02* PCR result
Mycobacterium avium subsp. *paratuberculosis* strains		
ATCC 19698	Cattle-type collection isolate	+
K10, ATCC BAA-968	Cattle-type collection isolate	+
316F	Cattle-type collection isolate	+
St 764	Cattle-type field isolate from bovine feces	+
St 681	Cattle-type field isolate from wild boar tissues	+
St 22G	Sheep-type field isolate from sheep tissues	+
St ovicap18	Sheep-type field isolate from caprine tissues	+
St 332	Bison-type field isolate from caprine tissues	+
Mycobacterium avium subsp. *avium* ATCC 25291	Collection isolate	−
Mycobacterium avium subsp. *avium* St18	Collection isolate	−
Mycobacterium avium subsp. *hominissuis* St 104	Collection isolate	−
Mycobacterium avium subsp. *silvaticum* ATCC 49884	Collection isolate	−
Mycobacterium asiaticum St 336	Field isolate from caprine feces	−
Mycobacterium tuberculosis St H37Ra, ATCC 25177	Collection isolate	−
Mycobacterium bovis BCG, ATCC 19015	Collection isolate	−
Mycobacterium bovis St 1403	Field isolate from wild boar tissues	−
Mycobacterium caprae St 1491	Field isolate from goat tissues	−
Mycobacterium microti ATCC 11152	Collection isolate	−
Mycobacterium chimaera FI1069T	Collection isolate	−
Mycobacterium marinum CECT 7091T	Collection isolate	−
Mycobacterium chelonae St 362	Field isolate from bovine tissues	−
Mycobacterium flavescens St 9H	Field isolate from llama tissues	−
Mycobacterium intracellulare GM51	Field isolate from human blood	−
Mycobacterium scrofulaceum SN	Field isolate from llama feces	−
Mycobacterium vaccae ATCC 15483	Collection isolate	−
Mycobacterium phlei ATCC 11758	Collection isolate	−
Mycobacterium fortuitum ISCIII	Field isolate	−
Mycobacterium smegmatis St mc2155 ATCC 700084	Collection isolate	−
Mycobacterium kansasii CECT 3030T	Collection isolate	−
Mycobacterium gordonae CECT 3029	Collection isolate	−
Mycobacterium genavense ATCC 51234	Collection isolate	−
Salmonella enterica serovar Typhimurium, *S. enterica* serovar Enteritidis	Field isolates	−
Listeria monocytogenes	Field isolate	−
Corynebacterium pseudotuberculosis	Field isolate	−
Nocardia cyriacigeorgica, *N. nova*, *N. farcinica*	Field isolates	−
Rhodococcus equi ATCC 33701	Collection isolate	−
Yersinia enterocolitica, *Y. pseudotuberculosis*	Field isolates	−
Yersinia ruckeri ATCC 29473	Collection isolate	−
Neospora caninum	Field isolates	−
Actinomyces spp	Field isolates	−
Clostridium spp	Field isolates	−
Staphylococcus pseudointermedius	Field isolates	−
Mannheimia haemolytica	Field isolates	−
Leuconostoc mesenteroides	Field isolates	−
Pseudomonas spp.	Field isolates	−
Campylobacter jejuni ATCC 33560	Collection isolate	−
Escherichia coli ATCC 35150, O1576:H7	Collection isolate	−
Ehrlichia canis	Field isolates	−

shedder or low bacterial burden) if there were less than 10 colonies per tube, and finally, 0 (nonshedder or culture negative) if culture did not yield any colonies. The ileocecal lymph node, ileocecal valve, and jejunum were cultured separately for tissue culture. (v) For histopathological findings (20), the numbers used were 2 for patent forms (PF), encompassing diffuse multibacillary, diffuse intermediate, diffuse paucibacillary lesions, and multifocal lesions (21), 1 for latent forms (LF) or focal lesions, and 0 for apparently free (AF) of PTB (i.e., when no PTB-compatible lesions were observed).

An automated version adapted to the BioSprint 96 workstation (Qiagen) was used for DNA extraction. Briefly, 0.3 g of sample was mixed with 1 ml of PBS and vigorously vortex mixed for 3 min. The mixture was centrifuged at 100 × *g* for 1 min to discard the heaviest fecal material. A 175-μl amount of the liquid phase was transferred into tubes containing 235 μl of lysis/binding solution and zirconia beads. The tubes were bead beaten at 30 Hz for 10 min in a TissueLyser (Qiagen). After centrifugation at 16,000 × *g* for 3 min, 300 μl of supernatant was transferred into 1.5-ml microcentrifuge tubes. Samples were further clarified by centrifugation at

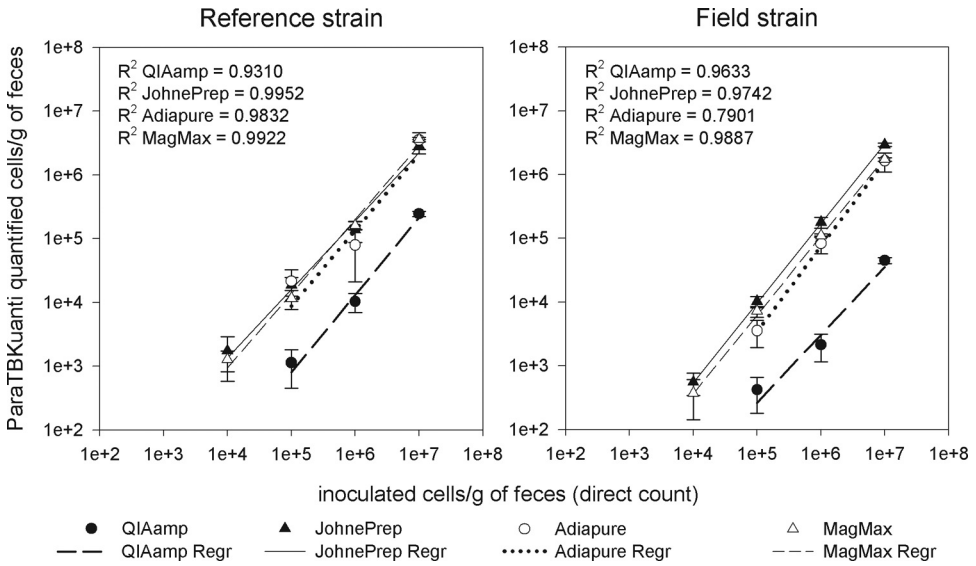

FIG 1 ParaTB Kuanti-VK results. Mean *M. avium* subsp. *paratuberculosis* genome copy number was quantified in extracts obtained with different kits at different inoculation levels for both field and reference strains. Standard deviations (error bars) and linear regression lines (Regr) have a 95% confidence level.

16,000 × *g* for 6 min, and 115 μl of supernatant was transferred into sample plate wells containing 20 μl of magnetic bead mix. Immediately, 65 μl of 100% isopropanol was added and the mixture homogenized. The wash solution plates in duplicate (150 μl of washing reagents per well), elution plate (90 μl of elution buffer per well), and sample plate were prepared according to the instructions of the manufacturer and loaded into the BioSprint workstation. The AM1840 DW 50v2 protocol (http://www.lifetechnologies.com/content/dam/LifeTech/Documents/XLS/Guide.xls) was run, and the elution plate containing DNA extracts was stored at −20°C until used. Three microliters of DNA in 25-μl reaction mixtures was used for IS*900* and IS*Map02* real-time amplification under the same conditions as described above. Three DNA extraction negative controls (distributed in the first, middle, and last positions of the processing plate) and two no-template PCR negative controls were included in each PCR plate. Two PCR positive controls obtained by diluting DNA extracted from the K10 strain and yielding C_T values of 32 to 34 were loaded in each plate and used as a reference for performance. This time, only samples yielding C_T values of below 40 for both the IS*900* and IS*Map02* probes were considered positive.

RESULTS

Comparison of DNA extraction and amplification methods using spiked fecal samples. The CFU/ml concentrations of stock inocula containing 10^8 cells/ml according to Neubauer counts were 1.24×10^7 (standard deviation, 3.33×10^6) in the case of the field strain and 2.24×10^6 (standard deviation, 4.98×10^5) in the case of the reference strain, as assessed by agar-7H9 plate counts. A difference of 1 to 2 logs was observed between the Neubauer and plate counts.

(i) ParaTB Kuanti-VK quantitative PCR kit. The quantification results for the ParaTB Kuanti-VK quantitative PCR kit are summarized in Fig. 1. With reference to the extraction protocols, the JohnePrep and MagMax extraction kits showed the best values for correlation between inoculated and quantified cells/g of feces. The coefficients of correlation (R^2) obtained from linear regression curves plotted for both field and reference strains were 0.9952 and 0.9742 for the JohnePrep kit and 0.9922 and 0.9887 for the MagMax kit. According to the standard deviations of replicates and by omitting samples with the lowest bacterial burdens, these kits also provided better reproducibility than the other two kits. Regarding the detection/quantification limits using these DNA extracts, the lowest bacterial concentrations at which the PCR quantified copies of F57 were 10^4 cells/g of feces or 1.24×10^3 CFU/g of feces for the field strain and 2.24×10^2 CFU/g of feces for the reference strain, depending on the method used as the reference to assess the bacterial burdens of inocula used to spike negative feces (Neubauer direct counts or CFU plate counts). Overall, the ParaTB Kuanti-VK kit's quantification of *M. avium* subsp. *paratuberculosis* genome copies was 1 log below the number of bacteria inoculated/g of feces (considering Neubauer direct counts as the reference) when DNA extracted with JohnePrep, Adiapure, and MagMax kits was tested and at least 2 logs below when the tested DNA was purified with the QIAamp stool kit.

(ii) F57 and IS*Mav2* real-time PCR system. The results obtained using the F57 and IS*Mav2* real-time PCR are shown in Table 3. Some amplification was detected in extracts obtained with JohnePrep and MagMax kits from feces inoculated with 10^3 cells/g, but the detection limit could only be consistently established at 10^4 *M. avium* subsp. *paratuberculosis* cells/g of feces according to the bacterial concentrations assessed by Neubauer counts. The DNA samples obtained with the JohnePrep and MagMax kits yielded the lowest mean C_T values at all inoculation levels. Although the difference was not large, the mean C_T values obtained for the F57 probe were lower than those obtained for the IS*Mav2* probe.

(iii) IS*900* and IS*Map02* real-time PCR system. According to the results for all bacterial isolates tested (Table 2), the primers and probe designed for the IS*Map02* sequence were 100% specific. Only *M. avium* subsp. *paratuberculosis* strains yielded positive results regardless of their origin or genotype. No amplification was obtained with the remaining species and subspecies tested. No cross-hybridization with other oligonucleotides was detected in the triplex PCR mixture containing all the ingredients.

Regarding the analytical detection limit and according to the

TABLE 3 Mean C_T values and standard deviations calculated for all inoculation levels and extraction methods with both field and reference strains using the F57 and IS*Mav2* triplex real-time PCR of Schonenbrucher et al.

Extraction method	Inoculum (cells/g)	Value for indicated probe ($n = 6$ replicates) in:							
		Field strain				Reference strain			
		F57 LNA probe		IS*Mav2* probe		F57 LNA probe		IS*Mav2* probe	
		Mean C_T value (SD)	PR[a]	Mean C_T value (SD)	PR	Mean C_T value (SD)	PR	Mean C_T value (SD)	PR
QIAamp stool	10^1		0		0		0		0
	10^2		0		0		0		0
	10^3		0		0		0		0
	10^4	40.60 (0)	1		0		0		0
	10^5	37.41 (0.64)	6	39.67 (0.65)	6	40.41 (0.89)	5	38.85 (0.92)	4
	10^6	33.95 (0.93)	6	34.29 (0.93)	6	35.96 (0.83)	6	36.31 (0.77)	6
	10^7	29.06 (0.11)	6	29.12 (0.28)	6	31.19 (0.28)	6	31.65 (0.63)	6
JohnePrep	10^1		0		0		0		0
	10^2		0		0		0		0
	10^3	38.48 (0)	1	41.85 (0)	1		0		0
	10^4	37.20 (0.85)	5	40.30 (1.08)	6	38.92 (0.79)	5	41.50 (1.27)	6
	10^5	33.33 (0.73)	6	34.95 (1.05)	6	34.39 (0.67)	6	35.19 (0.39)	6
	10^6	28.81 (0.50)	6	29.79 (0.57)	6	29.73 (0.82)	6	31.76 (0.80)	6
	10^7	24.58 (0.32)	6	25.83 (0.48)	6	25.17 (0.30)	6	25.98 (0.47)	6
Adiapure	10^1		0		0		0		0
	10^2		0		0		0		0
	10^3		0		0		0		0
	10^4	42.35 (0.14)	2		0	43.53 (0)	1		0
	10^5	36.92 (0.41)	6	38.32 (0.58)	6	36.40 (0.79)	6	37.68 (1.53)	6
	10^6	32.70 (0.41)	6	33.74 (0.33)	6	34.36 (1.24)	6	35.34 (1.44)	6
	10^7	28.18 (0.69)	6	29.06 (0.78)	6	28.56 (0.52)	6	28.77 (0.43)	6
MagMax	10^1		0		0		0		0
	10^2		0		0		0		0
	10^3	44.02 (0)	1		0		0		0
	10^4	37.31 (0.07)	6	38.11 (0.54)	6	39.58 (0.43)	6	39.95 (0.06)	6
	10^5	32.38 (0.24)	6	33.18 (0.25)	6	34.70 (0.26)	6	35.60 (0.26)	6
	10^6	28.27 (0.10)	6	28.92 (0.26)	6	30.76 (0.30)	6	31.78 (0.34)	6
	10^7	24.25 (0.13)	6	24.85 (0.22)	6	25.77 (0.32)	6	26.84 (0.20)	6

[a] PR, number of positive replicates.

results of the serially diluted DNA of the K10 strain, the minimum DNA amount needed for detection by both the IS*900* and the IS*Map02* probe was 1 fg (Table 4, footnote *a*). This is equivalent to 0.07 to 0.19 organisms or 1.13 copies of IS*Map02* and 3.21 copies of IS*900*.

The mean C_T values with standard deviations for all 4 extraction methods and both strains using this PCR are shown in Table 4. Although amplification could be detected in feces inoculated with 10 *M. avium* subsp. *paratuberculosis* cells/g and extracted with the JohnePrep and MagMax kits according to concentrations calculated on the basis of direct counts, the PCR detection limit was consistently established at 100 cells/g. The lowest C_T values were obtained with the JohnePrep and MagMax kits. In general, IS*900* was detected before IS*Map02*, but the difference was very small.

(iv) TaqMan MAP and Adiavet PTB kits. The TaqMan MAP and Adiavet PTB PCR assays were performed only with DNA extracted using the extraction kit recommended and manufactured by the same companies. In Table 5, the mean C_T values and standard deviations calculated for every bacterial concentration for both kits and both field and reference strains are shown. The MagMax extraction kit combined with the TaqMan MAP PCR detected *M. avium* subsp. *paratuberculosis* 1 level of inoculation earlier than the Adiapure and Adiavet PCR combination did. In the case of the feces spiked with the field strain, the first combination detected the mycobacteria in one of the six PCR replicates containing 100 *M. avium* subsp. *paratuberculosis* cells/g of feces. The detection limit for the same combination applied to the feces spiked with the reference strain was 1,000 *M. avium* subsp. *paratuberculosis* cells/g. The C_T values obtained with this combination were always lower than those obtained with the Adiapure and Adiavet combination.

(v) Culture of spiked feces. When the number of bacteria inoculated into feces was calculated according to direct visual counts, only samples inoculated with 10^5 (field strain) and 10^6 (reference strain) cells or more yielded visible colonies on HEY and agar-7H9. According to CFU counts of plated inocula, these values indicate that the detection limit of the culture method employed was approximately 10^4 spiked CFU of *M. avium* subsp. *paratuberculosis*/g of feces.

Evaluation of the chosen combination using samples from naturally infected cattle. The combination of MagMax extraction

TABLE 4 Mean C_T values and standard deviations calculated for all inoculation levels and extraction methods with both field and reference strains using the IS*900* and IS*Map02* triplex real-time PCR[a]

Extraction method	Inoculum (cells/g)	Value for indicated probe ($n = 6$ replicates) in:							
		Field strain				Reference strain			
		IS*900* probe		IS*Map02* probe		IS*900* probe		IS*Map02* probe	
		Mean C_T value (SD)	PR[b]	Mean C_T value (SD)	PR	Mean C_T value (SD)	PR	Mean C_T value (SD)	PR
QIAamp stool	10^1		0		0		0		0
	10^2		0		0		0		0
	10^3		0		0		0		0
	10^4	38.67 (1.55)	6	38.24 (0.57)	6	39.99 (0.94)	6	38.70 (1.12)	5
	10^5	35.06 (0.97)	6	35.79 (0.61)	6	35.01 (0.38)	6	35.24 (0.26)	6
	10^6	30.94 (0.76)	6	31.14 (0.87)	6	32.09 (0.70)	6	32.55 (0.57)	6
	10^7	25.54 (0.26)	6	26.01 (0.19)	6	27.24 (0.14)	6	27.82 (0.03)	6
JohnePrep	10^1	40.28 (1.81)	3	38.92 (0)	1	41.98 (0)	1		0
	10^2	38.57 (1.45)	6	37.63 (1.21)	6	38.56 (2.05)	5	39.00 (1.92)	4
	10^3	37.21 (0.14)	6	36.55 (0.87)	6	37.62 (0.96)	6	38.57 (0.63)	6
	10^4	35.20 (0.23)	6	35.26 (0.32)	6	36.56 (1.18)	6	35.67 (0.92)	6
	10^5	30.22 (0.35)	6	31.25 (0.29)	6	32.72 (0.30)	6	32.94 (0.06)	6
	10^6	25.86 (0.30)	6	26.99 (0.32)	6	26.13 (0.77)	6	26.44 (0.63)	6
	10^7	21.47 (0.12)	6	22.70 (0.16)	6	21.53 (0.34)	6	22.01 (0.26)	6
Adiapure	10^1		0		0		0		0
	10^2		0		0		0		0
	10^3		0		0		0		0
	10^4	37.37 (0.52)	6	37.88 (0.27)	6	37.95 (1.65)	6	37.06 (0.69)	6
	10^5	34.38 (0.87)	6	34.79 (1.31)	6	34.31 (1.81)	6	34.96 (1.59)	6
	10^6	29.43 (0.25)	6	30.20 (0.43)	6	32.06 (1.24)	6	32.54 (1.22)	6
	10^7	25.13 (0.56)	6	26.04 (0.49)	6	25.26 (0.76)	6	26.08 (0.61)	6
MagMax	10^1	41.38 (2.44)	2	38.75 (0)	1		0		0
	10^2	38.52 (0.62)	5	38.03 (0.29)	5	39.83 (1.38)	6	38.06 (0.82)	4
	10^3	37.43 (0.38)	6	37.09 (0.71)	6	38.41 (1.24)	6	38.29 (0.69)	6
	10^4	34.49 (0.12)	6	34.95 0.61)	6	37.11 (0.51)	6	36.63 (0.79)	6
	10^5	29.80 (0.34)	6	30.21 (0.14)	6	31.20 (0.12)	6	31.41 (0.17)	6
	10^6	25.47 (0.04)	6	26.26 (0.05)	6	27.13 (0.31)	6	27.70 (0.26)	6
	10^7	21.42 (0.14)	6	22.35 (0.09)	6	22.05 (0.11)	6	22.66 (0.08)	6

[a] The minimum detectable limits assessed by serial dilutions of genomic DNA (gDNA) purified from the K10 reference strain were as follows ($n = 6$ replicates). The minimum detectable amount of gDNA was 1 fg for both the IS*900* and the IS*Map02* probe. For the IS*900* probe, the mean C_T value (standard deviation) was 38.20 (1.16), the number of positive replicates was 5, the number of *M. avium* subsp. *paratuberculosis* genomes detected was 0.19, and the number of IS*900* copies detected was 3.21. For the IS*Map02* probe, the mean C_T value (standard deviation) was 37.56 (0.62), the number of positive replicates was 2, the number of *M. avium* subsp. *paratuberculosis* genomes detected was 0.07, and the number of IS*Map02* copies was 1.13.

[b] PR, number of positive replicates.

and the IS*900* and IS*Map02* PCR detected 152 positive fecal samples, 87 in TC herds and 65 in VH herds. These results yielded a positive proportion of 27.27% among TC herds, 21.96% in the VH group, and 24.72% when both groups of animals were considered. The previously obtained prevalence values, where an animal was considered positive when it was positive for at least one of the techniques used (ELISA, conventional PCR, and culture), were 15.12% as a whole and 12.23% and 18.24% for each group, respectively.

Categorized and grouped results, together with follow-up sensitivity and specificity estimations for each group, are shown in Table 6. In the case of animals that were not vaccinated after M0 (TC group), 66.7% of PCR positives fell within IS*900* and IS*Map02* PCR category 1 (low shedder), 16.1% within category 2 (intermediate shedder), and 17.2% within category 3 (heavy shedder). Similarly, these figures were 63.1%, 29.2%, and 7.7% in the case of animals that underwent vaccination after M0 sampling (VH). More than 85% and 91% of animals in category 0 from the TC and VH herds, respectively, remained PTB negative in subsequent samplings. In the VH group, this proportion does not include 3% of animals that were ELISA positive in M0 and negative in the rest of the samplings. At least 43.1% (TC group) and 58.5% (VH group) of animals that fell into category 1 could be confirmed as PTB positive for any of the samplings carried out. With reference to animals included in category 2, 71.4% belonging to the TC group and 84.2% belonging to the VH group received a final classification of PTB positive. The latter percentages do not include three cows that could not be deemed PTB positive because no sample could be collected after they were slaughtered, but they did show typical PTB symptoms, so this percentage could rise to 92.7%. Correspondingly, the percentage for VH herds does not include an extra 10.5% of animals that were positive by conventional PCR and culture at M0 but were PTB negative in successive samplings.

TABLE 5 Mean C_T values and standard deviations obtained using the DNA extraction and real-time amplification kit combinations Adiapure and Adiavet and MagMax and TaqMan MAP

Inoculum (cells/g)	Value ($n = 6$ replicates) for:							
	Field strain				Reference strain			
	Adiapure + Adiavet		MagMax + TaqMan MAP		Adiapure + Adiavet		MagMax + TaqMan MAP	
	Mean C_T value (SD)	PR[a]	Mean C_T value (SD)	PR	Mean C_T value (SD)	PR	Mean C_T value (SD)	PR
10^1		0		0		0		
10^2		0	37.13 (0)	1		0		
10^3		0	36.75 (0.33)	6		0	38.42 (0)	1
10^4	39.17 (1.63)	6	35.02 (1.21)	6	41.62 (1.12)	6	37.64 (2.32)	6
10^5	35.57 (1.45)	6	29.96 (0.26)	6	36.74 (1.18)	6	32.69 (0.26)	6
10^6	30.19 (0.21)	6	26.15 (0.06)	6	34.55 (1.40)	6	29.02 (0.37)	6
10^7	25.89 (0.67)	6	22.11 (0.02)	6	27.11 (0.61)	6	23.86 (0.20)	6

[a] PR, number of positive replicates.

Table 7 summarizes case by case the whole result panel of the 69 animals necropsied. All PTB-negative cases were in the 0 (negative) category of the IS*900* and IS*Map02* PCR, except for one particular animal in the vaccinated group (27vh). Eight of the TC animals in this category (11tc to 18tc), although negative also for all conventional tests in M0, turned out to be positive to at least one of these tests in M12. Four of them (11tc to 13tc and 18tc) were only positive after necropsy, carried out between M0 and M12. In the vaccinated group, nine animals (13vh to 21vh) that fell into category 0 were later confirmed to be PTB positive. All animals included in categories 1 to 3 received a final classification of PTB positive, except for the above-mentioned particular case (27vh).

TABLE 6 Number of animals from control herds and vaccinated herds included in each shedder category according to IS*900* and IS*Map02* PCR results, grouped according to their combined results from conventional methods

Group and results (shedder category, C_T value) in IS*900* and IS*Map02* PCR[a]	No. (%) of animals ($n = 615$) with result using conventional methods[b]		
	Negative	Positive	Total
TC			
0, >40	196 (61.44)	36 (11.29)	232 (72.73)
1, >35	31 (9.72)	27 (8.46)	58 (18.18)
2, 31–≤35	4 (1.25)	10 (3.13)	14 (4.39)
3, ≤31	0	15 (4.7)	15 (4.7)
All positives (1 to 3)	35 (10.97)	52 (16.3)	87 (27.27)
Total	231 (72.41)	88 (27.59)	319
VH			
0, >40	209 (70.61)	22 (7.43)	231 (78.04)
1, >35	12 (4.05)	29 (9.8)	41 (13.85)
2, 31–≤35	1 (0.34)	18 (6.08)	19 (6.42)
3, ≤31	0	5 (1.69)	5 (1.69)
All positives (1 to 3)	13 (4.39)	52 (17.57)	65 (21.96)
Total	222 (75)	74 (25)	296

[a] IS*900* and IS*Map02* real-time PCR results are from month zero. TC, test and cull control group; VH, vaccinated herd; 0, nonshedder; 1, low shedder; 2, intermediate shedder; 3, heavy shedder.

[b] The conventional methods used were conventional PCR, fecal culture, and ELISA, performed during 5 years of sampling; animals were considered positive when positive to at least one of the conventional methods in any of the 5 annual samplings, and the rest were considered negative. Conventional method results were used as the reference to estimate the follow-up sensitivity and specificity of the triplex PCR. Follow-up specificity was 84.85% for the TC group and 94.14% for the VH group. Follow-up sensitivity was 59.09% for the TC group and 70.27% for the VH group.

DISCUSSION

Comparison of DNA extraction and amplification methods using spiked fecal samples. The difference observed between Neubauer and plating counts of the inocula prepared to spike the fecal samples could be due to several issues, such as inaccuracy of direct visual counts in a Neubauer chamber, the presence of dead or dormant cells that did not grow, or persistence of cell clumps that only yield one CFU. This finding is in agreement with previous reports (22) showing significant differences between distinct quantification techniques. A direct counting method able to distinguish between dead or inactive and viable or active cells could yield a greater agreement between methods, but dead or dormant cells will still pose a problem if the DNA they harbor is intact.

With reference to the DNA extraction methods compared, gross differences were observed between them. According to the amplification results of all PCR methods used (all PCR methods were in agreement), the MagMax and JohnePrep DNA extraction methods showed the highest sensitivity for the purification of *M. avium* subsp. *paratuberculosis* DNA from bovine feces. In the present study, the DNA concentration and purity of extracts were not measured because we think this will not lead to the identification of the best methods for the DNA purification of this pathogen from fecal specimens. Though such data are valuable for other purposes, they would only provide information about the amount and purity of total DNA purified using different methods. This total DNA includes DNA from the host and from other microorganisms that are irrelevant to the purpose of this study. Apart from the information obtained from all PCR primer and probe sets, a quantitative PCR targeting a single-copy gene of *M. avium* subsp. *paratuberculosis* (ParaTB Kuanti-VK kit) was used to assess the ability of each DNA extraction method to purify *M. avium* subsp. *paratuberculosis* DNA from feces. Additionally, this kit includes an internal amplification control to avoid false-negative results and obtain some information on the purity of the extracted DNA. As shown by the results obtained using the ParaTB Kuanti-VK quantitative PCR, the JohnePrep and MagMax kits were the extraction methods that performed best. This observation is supported by the high R^2 values obtained from linear regression curve analyses

TABLE 7 Summary of all the results obtained in the case of animals from TC and VH herds that were followed up until they were slaughtered and necropsied

Group, animal(s)[a]	Score in indicated analysis at[b]:																		
	M0				M12			M24			M36			M48			Necropsy			
	PCR	E	P	C	E	P	C	E	P	C	E	P	C	E	P	C	HP	FC	TC	PTB status
Test and cull																				
1tc	0	0	0	0	0	0	0	0	0	0							0	0	0	No PTB
2tc	0	0	0	0	0	0	0										0	0	0	No PTB
3tc to 6tc	0	0	0	0													0	ND	0	No PTB
7tc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	ND	3	PTB
8tc	0	0	0	0	0	0	0	0	0	0							1	0	0	PTB
9tc	0	0	0	0	0	0	0										0	0	0.3	PTB
10tc	0	0	0	0	0	0	0										2	2	3	PTB
11tc	0	0	0	0													1	0	0	PTB
12tc, 13tc	0	0	0	0													0	0	0.7–1.3	PTB
14tc, 15tc	0	0	0	0	2–3	0	0	3	1	1–2							1–2	1–2	2.3–2.7	PTB
16tc, 17tc	0	0	0	0	3	1	1–2										2	1–3	2.5–2.7	PTB
18tc	0	0	0	0													2	AC	3	PTB
19tc	1	0	0	0	2	1	1	3	1	1							2	2	2.3	PTB
20tc	1	0	0	0	3	1	3										2	3	2.7	PTB
21tc	1	0	0	0	3	0	0										2	3	3	PTB
22tc	1	0	1	0	3	0	0										2	1	2.3	PTB
23tc	1	1	0	0	3	0	0										0	2	1	PTB
24tc	1	0	0	0	0	0	0	2	1	1							2	0	2	PTB
25tc to 27tc	1	0	0	0	0	0	0										0	0	0.3–1	PTB
28tc, 29tc	2	1–2	0	0	2–3	1	1–2										2	3	2–3	PTB
30tc	2	0	0	0	3	1	1										2	3	3	PTB
31tc	2	0	0	0	0	0	0										0	0	1.3	PTB
32tc to 36tc	3	2–3	0–1	1–2													2	1–2	2.7–3	PTB
Vaccinated																				
1vh	0	0	0	0	NC	0	0	NC	0	0	NC	0	0				0	0	0	No PTB
2vh to 4vh	0	0	0	0	NC	0	0	NC	0	0							0	0	0	No PTB
5vh, 6vh	0	0	0	0	NC	0	0										0	0	0	No PTB
7vh to 11vh	0	0	0	0													0	0	0	No PTB
12vh	0	0	1	0													0	0	0	No PTB
13vh	0	0	0	0	NC	0	0	NC	0	0							0	0	0.3	PTB
14vh	0	0	0	0	NC	0	0										1	0	0.7	PTB
15vh	0	0	1	0	NC	0	0										0	0	1.3	PTB
16vh	0	0	0	0													1	0	0	PTB
17vh, 18vh	0	0	0	0													0	0	0.7–1	PTB
19vh, 20vh	0	0	0	0													2	1–2	3	PTB
21vh	0	0	0	0	NC	0	0										2	3	3	PTB
22vh	1	2	0	0	NC	1	1										2	1	2.7	PTB
23vh	1	3	0	0	NC	0	0	NC	1	2							2	3	3	PTB
24vh	1	0	0	0	NC	1	0	NC	0	0	NC	0	0	NC	0	0	2	ND	3	PTB
25vh	1	2	0	0													0	0	0.7	PTB
26vh	1	0	0	0													1	0	0	PTB
27vh	1	0	0	0													0	0	0	No PTB
28vh	2	3	0	0	NC	1	1										2	2	3	PTB
29vh, 30vh	2	3	0	0–2													2	0–1	2–3	PTB
31vh	2	0	1	0	NC	0	0	NC	1	2							2	0	3	PTB
32vh	2	0	0	0	NC	0	1										0	0	0.3	PTB
33vh	3	3	1	2													2	0	3	PTB

[a] tc, control or test and cull herds; vh, vaccinated herds.

[b] M0 to M48, month 0 to month 48 sampling; PCR, IS*900* and IS*Map02* triplex real-time PCR (3, heavy shedder; 2, intermediate shedder; 1, low shedder; 0, nonshedder); E, ELISA (3, highly reactive; 2, intermediate-highly reactive; 1, intermediate or inconclusive; 0, low/nonreactive); P, conventional PCR (1, positive; 0, negative). C, culture; HP, histopathology (2, patent forms; 1, latent forms; 0, apparently free of PTB); FC or TC, fecal culture or tissue culture (3, heavy shedder/high burden; 2, intermediate shedder/burden; 1, low shedder/burden; 0 nonshedder/culture negative); ND, not done; AC, all tubes contaminated; NC, not considered because the animal(s) was vaccinated.

(Fig. 1) and is particularly relevant when taking into account that these results are from DNAs extracted from spiked fecal samples and not from pure genomic DNA. Other authors have previously demonstrated the good performance of the JohnePrep kit using ovine fecal samples (23). In a recent study, the MagMax kit also exhibited efficient extraction results (24). Conversely, in our study, the modified QIAamp stool kit did not perform as well as previously reported by other authors (16, 24). The modified protocol of Schonenbrucher et al. was followed (16). A possible explanation could be the different nature of the negative feces used to inoculate *M. avium* subsp. *paratuberculosis* in each study or slight differences in the handling of samples and reagents by different operators during the extraction process.

Although the ParaTB Kuanti-VK kit did not show the highest sensitivity among the amplification methods tested, its sensitivity was not lower than those of other systems. Overall, the ParaTB Kuanti-VK kit quantification of *M. avium* subsp. *paratuberculosis* genome copies/g of feces was 1 log below the number of bacteria inoculated when a direct count of cells in the inocula used to spike feces was considered the reference. This PCR is able to correctly identify at the logarithm level the CFU of *M. avium* subsp. *paratuberculosis* present in a sample when working with pure cultures (22). If the origin of the DNA type tested in the present work (inoculated bovine fecal samples) is taken into account, these quantification results can be considered to display a highly acceptable accuracy.

The F57 and IS*Mav2* PCR system was able to detect 10^3 to 10^4 cells/g of feces according to Neubauer counts, but its detection level was 10^2 to 10^3 CFU/g of feces according to plate counts. This is partly in agreement with the findings reported in the original study using this amplification system when spiked bovine fecal samples were tested (16), but it seems that this PCR did not show the same efficiency as previously. In that study, different PCR master mixes, oligonucleotide vendors, and probe chemistries were compared as well. In our study, only one of the possible combinations was used, with slight differences in probe dye formulation. As stated in Materials and Methods, we used TaqMan universal PCR master mix without AmpErase UNG (Applied Biosystems), forward and reverse primers for each target (Sigma-Aldrich), an F57 LNA probe (JOE–BHQ-1; Sigma-Aldrich), an IAC MGB probe (NED-MGB), and an IS*Mav2* LNA probe (FAM–BHQ-1). It is likely that our ingredient combination performed slightly worse than that used in the previous work. This difference could also explain why the F57 (one copy per *M. avium* subsp. *paratuberculosis* genome) probe exhibited lower C_T values than the IS*Mav2* (at least three copies per genome) probe.

The IS*900* and IS*Map02* PCR showed the lowest detection limit (up to 10 *M. avium* subsp. *paratuberculosis* cells as counted in a Neubauer chamber or 1 CFU as assessed by CFU plate counts) when combined with the MagMax and JohnePrep DNA purification methods. These could be consistently established at 100 inoculated *M. avium* subsp. *paratuberculosis* cells/g of feces. When the sensitivity of this system was analyzed using serial dilutions of genomic DNA extracted from the K10 reference strain, the minimum detectable limit was 1 fg. In other terms, the equivalent of less than one *M. avium* subsp. *paratuberculosis* organism can be detected due to the multicopy nature of the genetic targets used. The analytical sensitivity of this test was similar to or higher than those of previously published PCR methods for the detection of *M. avium* subsp. *paratuberculosis*. The mean C_T values for samples with the lowest bacterial burden using the IS*Map02* probe were slightly lower than those using the IS*900* probe. It could seem striking, but if the number of positive replicates and the remaining results are analyzed in minute detail, it is readily regarded as an effect of a higher amplification efficiency of the IS*Map02* component of the system than of the IS*900* component. It must be taken into consideration that the IS*900* component of the assay is a much older design (17) that was not changed because it worked well in the singleplex format. As far as we know, this PCR system is the first triplex real-time PCR that combines the detection of these two multicopy elements and an IAC for the detection of *M. avium* subsp. *paratuberculosis* (18). In the most recent study mentioned earlier (24), both IS*900* and IS*Map02* were used to detect *M. avium* subsp. *paratuberculosis*, but separate real-time PCRs were used to amplify these targets and no control was included to monitor amplification reaction inhibition. In addition, a conventional nested preamplification of IS*Map02* was carried out, followed by a real-time amplification of the products obtained in the first round. As the authors stated, a nested PCR system is more likely to suffer from cross-contamination events and yield false-positive results.

During the field evaluation part of the present work, several samples yielded repeated IS*900*-positive results that could not be confirmed as *M. avium* subsp. *paratuberculosis* positive by the detection of IS*Map02*. Some cases were considered to be due to a lack of sensitivity of IS*Map02* compared to that of IS*900*, given the late C_T values recorded for IS*900*. However, four of these results were deemed to be due to a lack of specificity of this part of the PCR because the C_T values for IS*900* were lower, and with such values, we normally expect positive C_T readings for IS*Map02* as well. This fact highlights the usefulness of targeting not only IS*900* but also other sequences pertaining to *M. avium* subsp. *paratuberculosis*.

Between the two DNA extraction kits for which there were proprietary amplification kits, the MagMax yielded the best results, in combination with the TaqMan MAP PCR. This combination displayed good sensitivity and performed better than some in-house protocols. However, it should be noted that the Adiavet PCR kit studied here is not the latest version developed by Adiagene, and as far as we know, there is a more recent version available. Collectively, it can be concluded that there are interesting ready-to-use commercial tools available for the quantification (ParaTB Kuanti-VK) and detection of *M. avium* subsp. *paratuberculosis*.

Culture of spiked feces. The detection limit in spiked feces observed in the present study seems to be quite high. This lack of sensitivity is likely to be due to loss of cell viability, the dilution effect during preparation of artificially inoculated samples, and the use of a relatively insensitive culture protocol. Strains were grown in 7H9, resuspended in PBS, and inoculated directly into the fecal material. Spiked samples were then frozen and stored at −20°C until they were processed for culture. They were blended with hexadecyl pyridinium chloride (HPC) (1 g of feces in 19 ml of 7.6 g/liter HPC), and only half of this mixture (10 ml) was subjected to decontamination (18 h) and concentration. No centrifugation steps were carried out for bacterial concentration; the material to inoculate the tubes was obtained by sedimentation during incubation with HPC. The CFU numbers in the inocula indicated by the plating method were lower than those estimated by the direct counting method. This difference is responsible for a reduction of at least 1 to 2 logs in culturable *M. avium* subsp. *paratuberculosis* organisms spiked in the fecal samples.

Evaluation of the chosen combination using samples from naturally infected cattle. At first sight, it seems that the selected combination of DNA extraction and amplification methods is unspecific. But if the results are analyzed in depth, it is clear that, more than a lack of specificity of the selected detection system, it could be that older conventional methods failed to detect many *M. avium* subsp. *paratuberculosis*-infected animals, thus showing very low sensitivities. The estimations given in Table 6 for sensitivity and specificity could be misleading because, as explained above, the results obtained with this method were compared to those of a combined reference method that considered as positive any positive result from the rest of the tests used during all samplings. The vast majority (more than 85%) of animals included in IS*900* and IS*Map02* PCR category 0 remained PTB negative during the following years. Most of the animals yielding a positive PCR result fell within category 1, the low shedder category (IS*900* and IS*Map02* PCR). Considering the TC and VH herds as a whole, almost 50% of cows that fell into category 1 were confirmed as PTB cases during the follow-up, but this proportion could still be much higher because we missed relevant information on the final PTB status of at least 24% of the animals in category 1 from TC herds. It could additionally be higher since 12.2% of the animals in this category from the VH group were ELISA positive (intermediate-highly reactive) at the M0 sampling, although they became negative in subsequent samplings. This could be attributed to the beneficial effect of vaccination against the progression of PTB and is applicable to all follow-up data obtained from VH herds. In the case of animals included in category 2, if the three TC group cattle (3 of 14) with suspected PTB that were slaughtered and could not be tested and the two VH group cows (2 of 19) that were positive to fecal culture and conventional PCR at M0 are considered PTB positive, only one cow per group yielded negative results in the remaining samplings. The negative results obtained during the following 4 years for the cow belonging to the VH herd could be attributed to the vaccine, as suggested above, but the case in the TC group seems more difficult to explain reliably. Reasons that could account for this apparent lack of specificity, other than an unlikely identification error when samples were collected, stored, or processed, include potential cross-contamination during the processing of samples (although controls were always included) and the possibility of its being an atypical case.

Other researchers have shown higher positive-proportion values than those expected according to previous results or those obtained by the use of conventional tests and have attributed this effect to the low sensitivity of such diagnostic methods (23). It is also accepted that cells of this persistent pathogen can be ingested and shed passively or be considered "pass-through" bacteria that go through the gut of the host and are shed for some days after being ingested (23, 25, 26). As shown by other authors, most *M. avium* subsp. *paratuberculosis*-infected or carrier cattle seem to show only focal lesions according to careful anatomopathological processing after necropsy (20), probably because the majority of *M. avium* subsp. *paratuberculosis*-infected animals fall in the latent form (LF) category and are more or less resistant and able to contain the propagation of bacteria in their tissues, especially when the bacterial load of the contact was low.

The specific and sensitive performance of the detection method used was further confirmed by the data for the 69 animals that could be followed up throughout their life until they were slaughtered and necropsied (Table 7). All animals that were considered positive by this system were sooner or later confirmed as positive, except for one case (27v). This particular animal fell within category 1 in M0 sampling. It was vaccinated after being sampled at M0 and was slaughtered between M0 and M12. This positive result is more likely to be a true positive rather than an unspecific result, because the rest of the results suggest that infected animals can be detected by this method but not always by the ELISA, conventional PCR, and culture methods used and can only be confirmed after several samplings or at necropsy. Additionally, vaccination against *M. avium* subsp. *paratuberculosis* has been shown to reduce the bacterial load in tissues and feces and the lesions produced by this bacterium (27–29). There were mainly 8 TC and 9 VH cases that could slightly compromise the high sensitivity attributed to this molecular test. But these were only confirmed to be PTB positive in subsequent samplings or after necropsy. Moreover, the majority of cases that were only confirmed after necropsy exhibited low and/or 0 culture and histopathology scoring.

The overall comparison of sensitivity and specificity suggest a better performance of the test in vaccinated herds than in control herds. This fact leads us to think that the problem in control herds is more a lack of detection of infected animals that later were shown to be positive than a lack of specificity in vaccinated herds. A reliable explanation could be that, during follow-up samplings, animals undergoing a vaccination strategy display a more constant immune status and shedding pattern as an effect of vaccine administration when compared to the more variable immune status and intermittent shedding in animals from control herds. This deserves further research that lies outside the scope of the present report.

The results obtained with this detection method showed very high sensitivity and specificity in a blind ring-trial carried out during the ParaTBTools International Specific Targeted Research Project (FOOD-2004–T5.4.6.10, final report, Dowe Bakker, unpublished data). Fourteen negative and 22 positive fecal specimens were tested by each partner laboratory. All negative samples were negative to this detection method, and 21 of 22 were identified as positive. The remaining sample was considered inconclusive because the C_T values obtained were above 40.

When taking all information into consideration, it must be noted that fecal real-time PCR results should be analyzed with caution because it might be that not all animals identified as *M. avium* subsp. *paratuberculosis* shedders will develop clinical signs of PTB, especially those in which *M. avium* subsp. *paratuberculosis* has been detected at very low concentrations. A positive result will always indicate the presence of the pathogen in the sample, the animal, the herd, or the area tested but not necessarily the disease status of an animal. In conclusion, we present a reliable, specific, and sensitive combination of DNA extraction and amplification to detect *M. avium* subsp. *paratuberculosis* in feces. In addition, the classification of animals in different shedder categories according to their fecal PCR result that we have used in this work, though not being important to confirm PTB of suspicious cases, can provide researchers with a very interesting tool to be used in PTB control programs, especially when the control strategy includes vaccination.

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