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Rapid Identification of *Mycoplasma bovis* Strains from Bovine Bronchoalveolar Lavage Fluid with Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry after Enrichment Procedure

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Filip Boyen and Bart Pardon contributed equally to this work. They jointly supervised this work; however, as most of the experiments were performed in the laboratory of bacteriology, Filip Boyen (bacteriologist) was put as the last author.

ABSTRACT *Mycoplasma bovis* is a leading cause of pneumonia in modern calf rearing. Fast identification is essential to ensure appropriate antimicrobial therapy. Therefore, the objective of this study was to develop a protocol to identify *M. bovis* from bronchoalveolar lavage fluid (BALf) with matrix-assisted laser desorption ionization–time of flight mass spectrometry MALDI-TOF MS and to determine the diagnostic accuracy in comparison with other techniques. BALf was obtained from 104 cattle, and the presence of *M. bovis* was determined in the following three ways: (i) rapid identification of *M. bovis* with MALDI-TOF MS (RIMM) (BALf was enriched and after 24, 48, and 72 h of incubation and was analyzed using MALDI-TOF MS), (ii) triplex real-time PCR for *M. bovis*, *Mycoplasma bovirhinis*, and *Mycoplasma dispar*, and (iii) 10-day incubation on selective-indicative agar. The diagnostic accuracy of the three tests was determined with Bayesian latent class modeling (BLCM). After 24 h of enrichment, *M. bovis* was identified with MALDI-TOF MS in 3 out of 104 BALf samples. After 48 and 72 h of enrichment, 32/104 and 38/100 samples, respectively, were *M. bovis* positive. Lipase-positive *Mycoplasma*-like colonies were seen in 28 of 104 samples. Real-time PCR resulted in 28/104 positive and 12/104 doubtful results for *M. bovis*. The BLCM showed a sensitivity (Se) and specificity (Sp) of 86.6% (95% credible interval [CI], 69.4% to 97.6%) and 86.4% (CI, 76.1 to 93.8) for RIMM. For real-time PCR, Se was 94.8% (CI, 89.9 to 97.9) and Sp was 88.9% (CI, 78.0 to 97.4). For selective-indicative agar, Se and Sp were 70.5% (CI, 52.1 to 87.1) and 93.9% (CI, 85.9 to 98.4), respectively. These results suggest that rapid identification of *M. bovis* with MALDI-TOF MS after an enrichment procedure is a promising test for routine diagnostics in veterinary laboratories.

KEYWORDS Bayesian latent class model, lipase activity, *Mycoplasma bovirhinis*, *Mycoplasma bovis*, *Mycoplasma dispar*

Mycoplasma bovis is one of the primary pathogens causing severe pneumonia in cattle and is also associated with arthritis, otitis, mastitis, and reproductive disorders (1, 2). Bovine respiratory disease (BRD) is the leading cause of antimicrobial use in calves (3, 4), and *M. bovis* is involved in approximately 20% to 30% of pneumonia outbreaks in conventional dairy and beef calves, and almost 100% of veal calf herds have been in contact with this bacterium (5–7). Rapid diagnosis of *M. bovis* is of great importance to rationally use antimicrobials and limit economic losses, since *M. bovis* is

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inherently resistant against widely used antimicrobial agents and is difficult to eradicate once it is chronically present (1, 8). In contrast to other *Mycoplasma* species, *M. bovis* can be cultured quite well, although it easily takes 5 to 10 days before culture results become available. Also, to obtain definite *Mycoplasma* spp. identification, other techniques, such as biochemical characterization or PCR, are needed (9, 10). This is of great importance, as *M. bovis* is generally recognized as a primary pathogen. However, the pathogenic significance of other *Mycoplasma* species, such as *M. arginini*, *M. bovirhinis*, and *M. dispar*, are more controversial, as they are isolated from both healthy and pneumonic lungs (11–14), and incorrect identification could lead to unnecessary antimicrobial use. Selective-indicative agar using lipase activity as an *M. bovis*-specific feature has been described to distinguish *M. bovis* from other bovine *Mycoplasma* spp. (15, 16). Unfortunately, its diagnostic performance is currently not known. PCR is the preferred method for final identification of *Mycoplasma* species. We currently are observing a shift toward PCR identification directly on the specimen, such as bronchoalveolar lavage fluid (BALf), in the case of pneumonia. Even though this is more rapid, due to logistic reasons, laboratories usually collect samples to perform in a weekly or twice weekly analysis, whereupon diagnostic results still take several days.

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) is widely used as a rapid, low cost, culture-based diagnostic tool for the identification of bacteria, including *Mycoplasma* spp. (17). Nevertheless, prior isolation of *M. bovis* on specific solid medium is still necessary, and final identification can take up to 10 days (10, 18, 19). To reduce sample turnaround time, at present, there is great interest in the identification of bacteria by MALDI-TOF MS directly from the sample or after a short enrichment period in liquid broth as is already done for urine, blood, milk, and BALf specimens (20–23). However, such a technique is currently not available for *Mycoplasma* spp., presumably because of difficulties such as their fastidious growth and overgrowth by other bacteria.

Therefore, the objective of this study was to develop a protocol to identify *M. bovis* directly from BALf after an enrichment procedure with MALDI-TOF MS. The accuracy of this diagnostic test was compared with real-time PCR and biochemical characterization (lipase activity) on solid medium in a Bayesian latent class model (BLCM).

MATERIALS AND METHODS

Development of a protocol for fast MALDI-TOF detection of *M. bovis* in BALf. (i) Determination of an enrichment procedure and antimicrobial concentrations. *M. bovis* concentration in BALf usually ranges from 1.8×10^3 to 1.03×10^8 CFU/ml (24), whereas a minimum concentration of 1.0×10^8 CFU/ml is necessary to obtain interpretable spectra with MALDI-TOF MS starting from mycoplasma grown in broth (18, 19). Therefore, an enrichment procedure seemed necessary to identify *M. bovis* directly from BALf. We explored different broths as described earlier (19) and experimented additionally with different antimicrobials, since overgrowth of *M. bovis* by other bacteria, such as (fecal) contaminants (e.g., *Enterobacteriaceae*), commensals (e.g., *Streptococcus*, *Staphylococcus*, and *Enterococcus*) or pathogenic bacteria (e.g., *Pasteurellaceae*) in liquid medium is very likely (13, 25).

Starting from a fresh culture, three *M. bovis* strains obtained from clinical field samples (Mb144, K6, and K7; passaged 3 to 5 times) were cultured in modified basic pleuropneumonialike organism (PPLO)-broth (255420; BD Difco, Berkshire, United Kingdom) with inactivated horse serum (25%; Gibco, Ireland), and technical yeast extract (0.7%; Bacto, Belgium) supplemented with sodium pyruvate (0.5%; Sigma-Aldrich, Germany), ampicillin sodium salt (520 μg/ml; Sigma-Aldrich, Germany) (19), and colistin sulfate (VMD, Belgium) at a concentration of 967 IU/ml, similar to what was described previously (26, 27). Then, 1 ml of the *M. bovis* culture with either meropenem (USP reference standard, Sigma-Aldrich, Germany) or vancomycin (vancomycin hydrochloride from *Streptomyces orientalis*; Sigma-Aldrich, Germany) in final concentrations of 32, 16, 8, 4, 2, 1, 0.5, and 0 μg/ml was prepared in Eppendorf tubes at a final *M. bovis* concentration of 1 to 3×10^4 CFU/ml (28). After 48 h of incubation (37°C, 5% CO_2), protein extraction was performed as described before (18). Antimicrobial concentrations where the MALDI-TOF MS identification score (ID-score) for *M. bovis* was $\geq$1.7 were considered to not inhibit successful identification (18, 29). The highest antimicrobial concentration that did not inhibit successful *M. bovis* identification with MALDI-TOF MS was chosen for the rapid identification protocol.

(ii) Identification and enrichment protocol with MALDI-TOF MS from BALf. The final protocol is presented in the next paragraphs as part of the diagnostic test study and will be referred to as "rapid identification of *M. bovis* with MALDI-TOF MS" (RIMM).

Diagnostic test study. (i) Study population and sampling method. A prospective diagnostic test accuracy assay was performed. To detect a difference in sensitivity of 0.90 to 0.70 with 80% power, a minimum of 103 samples were needed (30). Therefore, a convenience sample of 104 BALf specimens was

collected for diagnostic purposes as described before (31). Samples were taken from 3-week-old to 4-year-old cows originating from 10 different farms (5 beef [A to D and F], 3 dairy [E, G, and H], and 2 veal [I and J]) in Flanders, Belgium, from January 2019 to May 2019. Subsequently, samples were stored at 4°C for 3 to 20 hours before culture-based methods (index tests: RIMM and selective-indicative agar) were performed. All samples were stored (–20°C) before the reference test (real-time PCR) was performed blindly. All procedures were approved by the local ethical committee under approval number EC2019-1.

(ii) Rapid identification of *M. bovis* with MALDI-TOF MS (RIMM). BALf was vigorously vortexed, and 4 ml was inoculated in 8 ml modified basic PPLO broth as described above, supplemented with 32 μg/ml vancomycin and 32 μg/ml meropenem. After 24, 48, and 72 h of incubation (37°C, 5% CO_2), protein extraction was performed on 1 ml of the enriched BALf culture as described before, and 1 μl of lysate was spotted in triplicate on target polished steel barcode (BC) plates (18, 19). Spotted samples were air dried and covered with 1 μl alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany). All samples were processed with an Autoflex III Smartbeam MALDI-TOF MS instrument using FlexControl and MBT Compass software (Bruker Daltonics, Bremen, Germany). External calibration and validation were performed by adding bacterial test standard as described by the manufacturer (Bruker Daltonics, Bremen, Germany). Negative controls were performed by adding 1 μl of the matrix only. The standard library (MBT Compass server version 4.1.90 PYTH) was extended with four in-house main spectrum profiles (MSPs) for *M. bovis* as outlined before (19) and extra MSPs of *M. bovirhinis* (NCTC 10118), *Mycoplasma ovipneumoniae* (NCTC 10151), and *M. dispar* (NCTC 10125). Identification of *Mycoplasma* spp. was considered reliable at the species level when logarithmic score values were $\geq$1.7 as proposed in previous studies (18, 29), whereas identification of nonmycoplasmal bacteria was considered reliable at the species level when score values were $\geq$2.0 and at the genus level when score values were $\geq$1.7 and $<$2.0 (20).

(iii) Selective-indicative agar. One hundred μl of 10-fold dilutions of BALf was inoculated on an in-house modified PPLO agar containing Difco PPLO agar (product number 241210) enriched with 25% inactivated horse serum (Gibco), 0.7% technical yeast extract (Bacto), 0.5% D-(+)-glucose monohydrate (Sigma-Aldrich), 520 μg/ml ampicillin sodium salt (Sigma-Aldrich), 967 IU/ml colistin sulfate (VMD), and 0.1% Tween 80 (polysorbate 80; Sigma-Aldrich). Tween 80 was added for observing lipase activity as an indicator for *M. bovis* (15, 16). After 1 to 10 days of incubation (37°C, 5% CO_2), presumptive *Mycoplasma* colonies (fried egg morphology) were identified as *M. bovis* based on the presence of lipase activity, observed as an "oil-like" film surrounding the colonies, and were counted.

(iv) Triplex real-time PCR. A previously described triplex real-time PCR was chosen as the reference test, as this method showed results comparable to other PCR methods used for routine diagnostics to identify *M. bovis* from BALf (32, 33). The limit of detection was determined at 30 CFU/ml for *M. bovis* and *M. bovirhinis* and 300 CFU/ml for *M. dispar* as described previously (32). BALf samples were thawed before DNA extraction. After vortexing, 200 μl of sample was used for DNA extraction with the MagNA Pure 96 Instrument (Roche) using the MagNA Pure 96 DNA and viral NA small volume kit (Roche) for DNA extraction. Then, 5 μl extracted DNA was used for the triplex real-time PCR detecting *M. bovis*, *M. dispar*, and *M. bovirhinis* as described before (32). Fresh *M. bovis*, *M. dispar*, and *M. bovirhinis* cultures from in-house reference strains were used as an internal control to monitor DNA extraction, as well as inhibition of the PCRs. Threshold cycle (C_T) values were interpreted as positive ($<$35), doubtful (35 to 40), or negative ($>$40) as previously described (33).

(v) Conventional bacterial culture for nonmycoplasmal bacteria. An essential part of the protocol was to avoid overgrowth of *M. bovis* by other pathogens. Therefore, to quantify other fast-growing nonmycoplasmal pathogens and the contamination present in the BALf samples, 100 μl of 10-fold dilutions of BALf was cultured on Columbia agar supplemented with 5% sheep blood (blood agar; Oxoid, UK). Nonmycoplasmal bacteria were identified with MALDI-TOF MS after 1 day of incubation (37°C, 5% CO_2). Additionally, after 72 h of enrichment in the BALf in modified PPLO medium, 50 μl was cultured on blood agar for 24 h (37°C, 5% CO2) to check for the residual presence of nonmycoplasmal species.

Statistical analysis. (i) Crosstabulation. First, the diagnostic accuracy (sensitivity and specificity) of both the RIMM and the selective-indicative agar (index tests) was determined with real-time PCR as the reference test (WinEpi, Zaragoza, Spain). BALf was considered positive for *M. bovis* when the ID-score of MALDI-TOF MS was $\geq$1.7 (direct identification) after 72 h of incubation, when the C_T score was $<$40 (real-time PCR), or when *Mycoplasma*-like colonies showed lipase activity (bacterial culture). For 4 BALf samples, no results for 72 h of incubation were obtained (due to a practical problem), and for these samples, results from 48 h were used to compare with real-time PCR and the selective-indicative agar.

(ii) Bayesian latent class modeling. *(a) Definition of outcome tested.* The sensitivity and specificity of the real-time PCR are not 100% (32, 33). Therefore, and also because of issues with clinical interpretation (detection of a small amount of nonviable pathogens), this test cannot truly be considered a gold standard. To account for this issue, a second statistical analysis was performed to determine the diagnostic accuracy of the three methods. Bayesian latent class models create their own probabilistic definition of the outcome studied, depending on what the tests actually detect. In this study, PCR detects DNA from either living or dead bacteria. The selective-indicative agar detects culturable bacteria with lipase activity. Culture enrichment combined with MALDI-TOF MS detects protein spectra from culturable bacteria. Therefore, in our judgement, the three tests detect three distinguishing parameters, and a model for three independent tests was built. In addition, the model for dependent tests (with both culture-based methods being dependent of each other) was built to compare independent and dependent outcomes.

(b) Model development. In order to assess the accuracy of the three tests—(i) real-time PCR (detection of DNA), (ii) RIMM (detection of proteins), and (iii) selective-indicative agar (detection of lipase activi-

ty)—to detect the presence of *M. bovis*, a latent class model (1 population, 3 tests) was considered, with each test regarded as independent from the others. The unknown parameters of interest were sensitivity and specificity of the three diagnostic tests and the prevalence of *M. bovis* in the study population. Once the likelihood of the process generating the data observation is described, which in this case is a multinomial probability distribution, the estimation of posterior densities can be obtained using the Bayes theorem, which links the likelihood with the posterior distribution (inference). At this stage, prior information on any parameter in the likelihood can be added to obtain posterior densities of the different parameters using a Markov chain Monte-Carlo algorithm (Gibbs sampling). The prior information is a way to narrow parameter uncertainty when previous scientific information is available. In terms of prevalence and the Se/Sp of tests, the priors are modeled using beta distributions that are naturally bound from 0 to 1. Priors can be uninformative (any value has same probability of happening) or informative (some values are more or less probable) (34).

(c) Prior distribution determination process. Prior information was derived from the available literature and expert opinion. For both RIMM and the selective-indicative agar, no literature information was available. Previous work on *M. bovis* real-time PCR estimated an Se of 95.2% (95% confidence interval [CI95], 76.1% to 99.9%) and Sp of 73.9% (CI95, 51.6% to 89.8%) (32). Also, for the prevalence of *M. bovis* in the population, prior information was available (7, 35). This literature information was combined with the best guesses of experts in the field (first author and senior authors). The 5th percentiles were guessed at 90%, 95%, and 50% for Se, Sp, and prevalence, respectively. These values were used to determine the beta distribution parameters of the corresponding prior distribution using a free online beta distribution calculator (Epitools, Sergeant, ESG, Ausvet Animal Health Services and Australian Biosecurity calculator; available at https://epitools.ausvet.com.au/), resulting in beta (99.7, 6.19), beta (1), and beta (6.28, 13.32) for Se, Sp, and prevalence, respectively.

In total, the three following models were run: the first model with all prior information on all parameters set at uninformative (beta 1,1), the second model with informative priors on the Se and Sp of real-time PCR, and the third model with informative priors on the *M. bovis* prevalence and real-time PCR.

The parameters of interest were determined based on a sample from the posterior distribution using Gibbs sampling with WinBUGS statistical freeware version 1.4.3. (MRC Biostastics Unit, Cambridge, UK). Estimation of posterior densities and model assessment was done using recommended techniques (36). A total of 100,000 iterations were used for each model after a burn-in of 5,000 iterations. Three chains were run with different initial values. The posterior median and 2.5 to 97.5 credible intervals (CI) were extracted for each parameter. Model convergence was checked by visual inspection of density and Gelman-Rubin plots. Plots of chain autocorrelation were inspected to investigate the need for thinning of chains.

For smaller data sets, informative prior elicitation can be a process that could potentially have an impact on posterior density. Therefore, a sensitivity analysis was performed, running alternative models with very different prior specifications to the main model. It was determined whether posterior estimates of these alternative models were included in the 95% CI of the main model (34).

RESULTS

Development of a protocol for fast MALDI-TOF detection of *M. bovis* in BALf. Determination of an enrichment procedure and antimicrobial concentrations. After 48 h of incubation, *M. bovis* strains were identified with MALDI-TOF MS as *M. bovis* (ID-score, $\geq$2.0) after protein extraction for all tested antimicrobial concentrations. Therefore, a concentration of 32 μg/ml for both meropenem and vancomycin was selected for further testing.

Diagnostic test study. Triplex real-time PCR resulted in 26.9% (28/104) positive BALf samples (C_T, $\leq$35), 11.5% (12/104) were doubtful (35 $<$ C_T $<$ 40), and 61.5% (64/104) were negative (C_T, $\geq$40 or no detection) for *M. bovis*. For *M. bovirhinis*, 79.8% (83/104) were positive, 11.5% (12/104) were doubtful, and 8.7% (9/104) were negative, and for *M. dispar*, these figures were 92.3% (96/104), 3.8% (4/104), and 3.8% (4/104), respectively (see Table S1 in the supplemental material).

After 24 h of enrichment, with the RIMM method, 2.9% (3/104) of the BALf samples were positive for *M. bovis*, and after 48 and 72 h, 30.7% (32/104) and 38.0% (38/100), respectively, were positive. For 4 samples, no results were obtained after 72 h of incubation, because of a practical problem. ID-scores for *M. bovis* ranged from 1.74 to 2.65 and are shown in Table S1. *M. bovirhinis* was identified after 24 h (BALf sample no. 18, 23, 28, 60, and 95), 48 h (BALf sample no. 26, 28, 29, 32, 35, 38, 43, 60, 64, and 102), and 72 h (BALf sample no. 60, 63, 66, and 88) with ID-scores of 1.70 to 1.88. In BALf sample no. 24, *M. ovipneumoniae* was identified after 48 and 72 h of incubation (ID-score, 1.78 and 1.72, respectively). *M. dispar* was not detected with the RIMM method in any sample throughout the experiment.

TABLE 1 2 × 2 contingency table for direct MALDI-TOF MS identification as index test compared to real-time PCR as reference test for identification of *Mycoplasma bovis* from BALf samples ($n = 104$)

Test	Result category	Reference test results (real-time PCR) (%) (no.)		Total no. of tests
		Positive	Negative	
MALDI-TOF MS	Positive	75 (30/40)	14 (9/64)	39
	Negative	25 (10/40)	86 (55/64)	65
	Total no.	40	64	104
Selective-indicative agar	Positive	60 (24/40)	6 (4/64)	28
	Negative	40 (16/40)	94 (60/64)	76
	Total no.	40	64	104

Out of 104 samples, 28 (26.9%) showed lipase-positive *Mycoplasma*-like colonies on the selective-indicative agar (Table S1), ranging from 1.0×10^1 to 4.6×10^4 CFU/ml BALf (mean, 3.8×10^3; median, 3.3×10^2). Of those 104 BALf samples, 18% (19/104) did not show additional bacterial growth on blood agar plates, whereas 81.7% (85/104) did, among which were multiple pathogens and commensals (Table S1). Despite the presence of these nonmycoplasmal bacteria in the original BALf samples, the selective enrichment phase did not allow nonmycoplasmal bacterial growth to an extent that was detected by MALDI-TOF MS.

Residual contamination was checked after 72 h of enrichment. There was no microbial growth on blood agar in 70% of the BALf samples after 24 h of incubation (70/100). For the samples with microbial growth on blood agar, MALDI-TOF MS identified *Candida* spp. (4/30), *Aspergillus fumigatus* (2/30), *Stenotrophomonas maltophilia* (1/30), and *Staphylococcus* spp. (1/30). In 23 out of 30 blood agars showing microbial growth, no identification was possible with MALDI-TOF MS, but their macroscopic appearance suggested mainly fungal contaminants.

The 2 × 2 contingency tables of the RIMM method and the selective-indicative agar compared with real-time PCR as the reference test are shown in Table 1. The sensitivity (Se) and specificity (Sp) for the RIMM compared to real-time PCR were 75.0% (CI95, 61.6% to 88.4%) and 85.9% (CI95, 77.4% to 94.5%), respectively. The selective-indicative agar showed a 60.0% (CI95, 44.8% to 75.2%) Se and a 93.8% (CI95, 87.8% to 99.7%) Sp compared to the real-time PCR test.

All latent class models converged, and the prior distributions and posterior summary statistics of each model are shown in Table 2. Parameters were particularly stable between models, and the results obtained from the sensitivity analysis were highly robust to changes in the prior distributions. Model 3 included the most prior informa-

TABLE 2 Posterior median and 95% CI of three independent Bayesian latent class models for the prevalence of *M. bovis*, sensitivity and specificity of the RIMM, triplex real-time PCR and selective-indicative agar used to diagnose *M. bovis* from bronchoalveolar lavage fluid samples

Parameter[a]	Model 1[b]		Model 2[c]		Model 3[d]	
	Prior densities	Posterior densities (95% CI)	Prior densities	Posterior densities (95% CI)	Prior densities	Posterior densities (95% CI)
Se_{pcr}	Beta (1)	93.5 (77.2–99.7)	Beta (99.7, 6.19)	94.8 (89.8–97.8)	Beta (99.7, 6.19)	94.8 (89.9–97.9)
Sp_{pcr}	Beta (1)	89.2 (78.1–97.8)	Beta (1)	89.1 (78.1–97.7)	Beta (1)	88.9 (78.0–97.4)
Se_{rimm}	Beta (1)	86.0 (68.3–97.5)	Beta (1)	86.3 (68.8–97.5)	Beta (1)	86.6 (69.4–97.6)
Sp_{rimm}	Beta (1)	86.8 (76.3–94.9)	Beta (1)	86.5 (76.2–93.9)	Beta (1)	86.4 (76.1–93.8)
Se_{sia}	Beta (1)	69.5 (50.8–86.7)	Beta (1)	70.2 (51.8–86.9)	Beta (1)	70.5 (52.1–87.1)
Sp_{sia}	Beta (1)	94.1 (86.0–98.8)	Beta (1)	94.0 (86.0–98.5)	Beta (1)	93.9 (85.9–98.4)
Prev	Beta (1)	33.7 (23.2–45.9)	Beta (1)	33.3 (23.1–44.6)	Beta (6.28, 13.32)	32.6 (23.5–42.6)

[a]pcr, triplex real-time PCR; Prev, prevalence of *M. bovis*; rimm, rapid identification of *M. bovis* with MALDI-TOF MS method; Se, sensitivity; sia, selective-indicative agar; Sp, specificity.
[b]Model 1, no informative priors.
[c]Model 2, informative priors on prevalence and Se_{pcr} (mode, 95%; 5th percentile, 90%) and Sp_{pcr} (mode, 74%; 5th percentile, 95%) (30).
[d]Model 3, informative prior on Se_{pcr}, Sp_{pcr}, and prevalence of *M. bovis* in BALf (mode. 30%; 5th percentile; 50%) (33).

tion and is therefore expected to be the most accurate. The prevalence of *M. bovis* was 32.6% (CI, 23.5% to 42.6%), which was comparable to the prior information added to the third model. In independent model 3, RIMM showed an Se and Sp of 86.6% (CI, 69.4% to 97.6%) and 86.4% (CI, 76.1% to 93.8%), respectively. Real-time PCR had an Se of 94.8% (CI, 89.9% to 97.9%) and an Sp of 88.9% (CI, 78.0% to 97.4%). The selective-indicative agar had an Se and Sp of 70.5% (CI, 52.1% to 87.1%) and 93.9% (CI, 85.9% to 98.4%), respectively. No significant differences between the independent and dependent models were observed.

DISCUSSION

The objective of the present study was to achieve a proof of concept for rapid identification of *M. bovis* from bovine BALf samples after enrichment with MALDI-TOF MS and to compare these with another culture-based method (selective-indicative agar) and a DNA-based reference test (real-time PCR). In this study, we identified *M. bovis* from the majority of BALf samples with RIMM within 2 days after incubation, and even more after 3 days. The current protocol reduced the relevant growth of nonmycoplasmal bacteria and nonpathogenic *Mycoplasma* spp. present in BALf, resulting in high sensitivity (86.6%; CI, 69.4 to 97.6) and specificity (86.4%; CI, 76.1 to 93.8) of this diagnostic test as determined by BLCM. Prior information of the BLMC was extracted from peer-reviewed journals, and the sensitivity analysis for robustness of all models was verified. These methods together make the possibility of bias due to the best guesses of the experts in the field less likely.

False negatives of the RIMM method compared to real-time PCR can be explained by the viability of *M. bovis*. PCR detects DNA, while for culture-based methods, such as MALDI-TOF MS, bacteria need to be alive. Some of the sampled calves might have been treated with antimicrobials before BALf samples were obtained, resulting in nonviable *M. bovis* in the lungs, so only DNA could be detected. This would suggest that no active *M. bovis* infection is currently present, and the clinical relevance of positive results from the real-time PCR might be questioned, in contrast to culture-based methods (RIMM and selective-indicative agar). In addition, cross-reaction with *Mycoplasma agalactiae* has been described for the currently used real-time PCR (32), while MALDI-TOF MS has been described to accurately distinguish *M. bovis* and *M. agalactiae* (17, 18). Both previous arguments therefore more likely result in a false-positive PCR result rather than a false-negative culture-based result, and therefore the current specificity of the RIMM might be underestimated.

Disagreement between a negative result for *M. bovis* with real-time PCR but a positive result with the RIMM method might be explained by the fact that the BALf volume used for RIMM was 4 ml, while the volume was only 200 μl for the real-time PCR reference test and 100 μl for biochemical identification (lipase activity) on agar. BALf contains mucus clumps and cells, which could lead to a heterogenic suspension of *M. bovis*. Although samples were vortexed vigorously, it is possible that certain parts of the BALf did not end up in the aliquot for real-time PCR. This could have caused a higher chance of isolation using the RIMM method compared with detection using real-time PCR or selective-indicative agar. The ability to process larger volumes with the RIMM method from BALf without extra labor is an advantage over both other tests (real-time PCR and selective-indicative agar) and might even increase the sensitivity of culture-based methods. Considering (i) that most clinical samples in this study contained 10^2 to 10^3 CFU/ml, (ii) the generation time of *M. bovis* (2 h in exponential phase) (37), and (iii) that the detection limit of the MALDI-TOF MS is 10^8 CFU/ml (18), detection of *M. bovis* from BALf after 48 h of incubation can be expected and is in line with the observations. For samples in which *M. bovis* was detected after 72 h of incubation at the earliest, for example, the presence of other pathogens, mucus composition, number of inflammatory cells, or other antimicrobial substances might have influenced the *M. bovis* growth rate or MALDI-TOF MS identification efficacy.

Various antimicrobials were added to the modified PPLO broth, as high antimicrobial resistance levels against different antimicrobials were observed in bacteria isolated

from cattle (38–40). Meropenem was considered due to its broad spectrum and strong activity against Gram-negative bacteria, although this would probably not suppress all bacterial growth in BALf, as for example, methicillin-resistant *Staphylococcus aureus* (MRSA) shows resistance against meropenem in humans but is also a common pathogen in calves (41). Therefore, vancomycin was used as well. Until now, only very low levels to no resistance is detected against this antibiotic in MRSA and *Enterococcus* strains obtained from cattle (42, 43). It should be kept in mind that critically important antibiotics, even though used under laboratory circumstances, should be properly disposed of after use.

Even though the current method was able to suppress nonmycoplasmal bacterial growth, there is still room for improvement for the reduction of yeast and fungal growth. In five of the BALf samples with a false-negative MALDI-TOF MS result compared to real-time PCR, fungal growth was observed, and this might have caused interference with identification of *M. bovis*, as fungal pigments can suppress the desorption process (44) or may lead to the presence of interfering peaks. Adding an antimycotic drug, such as amphotericin B, might help increase the sensitivity of the rapid MALDI-TOF MS identification method (45).

The real-time PCR cannot be seen as the gold standard technique for several reasons. First, sensitivity and specificity are not 100% (32). Second, studies concluded that the sensitivity of culture was sometimes higher than that of real-time PCR assays (24, 33), and in this study 11.5% of the real-time PCR results were doubtful and therefore difficult to compare to the culture-based methods where culture was either positive or negative. Therefore, a third test was included in this study to perform a BLCM, as is recommended when no gold standard is available (36).

The selective-indicative agar using lipase activity to identify *M. bovis* is not widely used and is therefore somewhat controversial. Though national laboratories in Belgium have used this method for years, this study is the first to show its diagnostic accuracy. This method resulted in a moderate sensitivity (70.5%) on the one hand, but on the other hand, a specificity (93.8%) that was even higher than real-time PCR (88.9%). It has been described that not all *M. bovis* strains show lipase activity, which could explain false negatives, whereas some other *Mycoplasma* spp. do possess this characteristic, which could result in false positives (46). Nevertheless, current results show that this medium might be helpful in *M. bovis* screenings where low cost is necessary and less experienced staff is located. It could also be helpful in choosing relevant colonies on agar plates for subsequent identification methods, such as real-time PCR. In addition, it cannot be ruled out that the use of other selective-indicative agar media, commercially available or not, could have resulted in different sensitivity or specificity data.

Other *Mycoplasma* spp. were identified as well in the BALf. Real-time PCR showed that at least 79.8% of the BALf samples were positive for *M. bovirhinis*, and 92.3% were positive for *M. dispar*. However, only 15.4% were positive for *M. bovirhinis* using the RIMM method with MALDI-TOF MS after 72 h of enrichment. Mixtures of *M. bovis*, *M. bovirhinis*, and *M. dispar* in BALf of cattle are common (11). Real-time PCR might have overestimated the prevalence of *M. bovirhinis* and *M. dispar*, because *M. bovirhinis* PCR cross-reacts with *M. canis*, and *M. dispar* PCR cross-reacts with *Acholeplasma axanthum* and *Mycoplasma alkalescens* (32). However, cross-reaction can probably not explain the large difference observed between the RIMM method and the real-time PCR. The enrichment medium used in this study seemed to preselect for *M. bovis* growth. *M. bovirhinis* and *M. dispar* are glucose fermenting, while in our medium only pyruvate was added as a carbon source (47). The latter is a great advantage in the diagnosis of *M. bovis*, as *M. bovirhinis* and *M. bovis* can both be identified with MALDI-TOF MS after 2 days of incubation (17). We did, however, observe a shift in identification of *M. bovirhinis*, where two samples were positive after 24 h and negative after 48 h. As the medium was not optimal for *M. bovirhinis*, one reason for this disparity could be that the concentration of (viable) *M. bovirhinis* balanced around the detection limit of the MALDI-TOF MS. Another reason might be that after 24 h, the concentration of *M. bovis* became higher than the concentration of *M. bovirhinis*, as Pereyre et al. (18) confirmed

that *M. bovis* was the only pathogen recognized by MALDI-TOF MS when samples contained 2 to 3 *Mycoplasma* species. Irrelevant *M. dispar* growth is less of a concern in culture-based methods, as this is a fastidious grower and is more difficult to isolate (47). Identification of *M. ovipneumoniae* from bovine BALf was unexpected. However, a recent study showed this species to be abundantly present in bovine BALf as well (48). The clinical relevance in cattle is unknown, although *M. ovipneumoniae* infections can have serious consequences in small ruminants, such as pneumonia, decreases in lamb production, and decreases in average daily gain (49, 50). Even though the MALDI-TOF MS specificity for accurate *M. ovipneumoniae* detection is not described, Spergser et al. (17) tested 19 *M. ovipneumoniae* clinical isolates against their own in-house library, which resulted in 100% identification with a score value of $\geq$1.7 (17).

MALDI-TOF MS is already proven to be of assistance for the identification of human and veterinary mycoplasmas from culture (17, 18, 29). In the future, there might be a great opportunity to develop a rapid and specific diagnostic tool to identify other pathogenic *Mycoplasma* spp. from BALf as well (for example, *M. ovipneumoniae* from small ruminants or *Mycoplasma pneumoniae* from humans) and accelerate turnover time in pneumonia diagnostics.

The current study shows that identification of *M. bovis* from BALf with the RIMM method is possible within 48 to 72 h after sampling. Compared to real-time PCR, RIMM is probably less expensive, the clinical relevance might be higher, and when desired, it holds the opportunity to perform additional susceptibility testing and strain typing (51, 52). Therefore, rapid identification of *M. bovis* with MALDI-TOF MS is a promising method for diagnosis of *M. bovis* in veterinary laboratories. However, it is necessary for laboratories using this approach for the detection of *M. bovis* from clinical samples to fully validate or comprehensively qualify this method. The validation parameters should include accuracy, precision, linearity and range of measurement, specificity, limit of detection, limit of quantitation, and robustness.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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