Mycoplasma detection by triplex real-time PCR in bronchoalveolar lavage fluid from bovine respiratory disease complex cases

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Abstract

Background: In this study we evaluated the RespoCheck Mycoplasma triplex real-time PCR for the detection in bronchoalveolar lavage fluid (BALF) of Mycoplasma (M.) dispar, M. bovis and M. bovirhinis, all three associated with bovine respiratory disease (BRD). Primers and probes of the RespoCheck Mycoplasma triplex real-time PCR are based on the V3/V4 region of the 16S rRNA gene of the three Mycoplasma species.

Results: The analytical sensitivity of the RespoCheck triplex real-time PCR was, as determined by spiking experiments of the Mycoplasma strains in Phosphate Buffered Saline, 300 colony forming units (cfu)/mL for M. dispar, and 30 cfu/mL for M. bovis or M. bovirhinis. The analytical sensitivity of the RespoCheck Mycoplasma triplex real-time PCR was, as determined on purified DNA, 10 fg DNA per assay for M. dispar and 100 fg for M. bovis and M. bovirhinis. The analytical specificity of the RespoCheck Mycoplasma triplex real-time PCR was, as determined by testing Mycoplasmas strains (n = 17) and other bacterial strains (n = 107), 100, 98.2 and 99.1% for M. bovis, M. dispar and M. bovirhinis respectively. The RespoCheck Mycoplasma triplex real-time PCR was compared with the PCR/DGGE analysis for M. bovis, M. dispar and M. bovirhinis respectively by testing 44 BALF samples from calves.

Conclusion: In conclusion, the RespoCheck PCR assay can be a valuable tool for timely and accurate detection of three Mycoplasma species associated with in bovine respiratory disease.

Keywords: Bovine Mycoplasma, M. dispar, M. bovis, M. bovirhinis, Triplex PCR, RespoCheck, Bovine respiratory disease

Background

Bovine respiratory disease complex (BRDC) is a global problem causing severe economic losses to the cattle farming industry through mortality, loss of production, and treatment costs [1, 2]. It has a complex etiology that involves various pathogens, host factors, and environmental factors. Viruses such as bovine herpes 1 virus (BoHV-1), parainfluenza virus 3 (PIV-3), bovine respiratory syncytial Virus (BRSV), respiratory bovine coronavirus (BoCoV) and bovine viral diarrhoea virus (BVDV) in conjunction with stress factors have been implicated as causes of respiratory tract infections of cattle by immunosuppression and damage to the respiratory epithelium [3]. A primary viral infection can be followed by an opportunistic secondary infection with bacteria like Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, or Trueperella pyogenes [2, 4, 5], but these bacteria could also act as primary pathogen. In addition it has become increasingly clear that Mycoplasmas are important contributors to BRD, either as primary pathogens or in co-infection [2, 6–9]. M. bovis is the best known Mycoplasma species causing respiratory disease [4, 7], but also M. dispar and M. bovirhinis have been associated with BRD [2, 9–11]. M. bovis has not only been identified as a primary or opportunistic pathogen in BRD in beef cattle worldwide, but it has also been implicated in other clinical manifestations in cattle, such as mastitis, otitis, arthritis, and reproductive disorders [7]. M. bovirhinis and M. dispar are regularly isolated from the nasal cavity of cattle with respiratory disease and are...
usually regarded as an opportunistic pathogen in respiratory diseases [7, 12].

Bacteriological, serological and histopathological examinations are important tools to detect particular animal-carriers of Mycoplasma [13], however, these assays are time-consuming, insensitive and can give false positive results. Bronchoalveolar lavage fluid (BALF) from calves with BRD may contain various potential pathogens, but additional antibiotic use in the affected herds can inhibit cultivation and thereby can cause false-negative test results. In BRD, differential diagnosis of these pathogens with rapid turnaround time procedure is essential to implement appropriate treatment and intervention measures in a timely manner. Rapid detection of these pathogens at the early stage of outbreak can contribute substantially to minimize the spread of infection and increase treatment efficiency. Today quick, highly sensitive and species-specific PCRs are used in the diagnosis of Mycoplasma-associated diseases for M. dispar [14, 15], M. bovis [4, 16] and M. bovirhinis [17] in BALF or nasal swabs. Combining a 16S Ribosomal DNA PCR with denaturing gradient gel electrophoresis fingerprinting (PCR/DGGE) enabled the simultaneous detection of mixed Mycoplasma populations, however information about the detection limit in clinical samples is limited [18]. Additionally, a DNA microarray assay was developed for the parallel detection of 37 Mycoplasma species [19], in which species-specific probes derived from the 23S rRNA and tuf genes were used for species differentiation.

Multiplex real-time PCR could be a promising and practical approach to speed up the differential diagnosis from 1 to 2 weeks for traditional culture to 24 h, with limited expenses. This will make diagnostic testing more accessible for veterinary practitioners and thereby improve BRD diagnosis. This report describes the RespoCheck triplex PCR developed by Central Veterinary Institute (CVI, Lelystad, The Netherlands) for detection of three Mycoplasma species.

Methods

Strains and growth conditions

M. bovis (ATCC 25025) and M. bovirhinis (ATCC 5189985) were purchased from the ATCC (United Kingdom (U.K.), Guernsey, Ireland, Jersey and Liechtenstein) and cultured in Heart Infusion Broth Medium (Difco, Detroit, Mich.). All isolates were grown at 37 °C and 5% CO₂ for seven days in a modified standard mycoplasma broth medium [20] containing 19 g of Heart Infusion Broth, 50 mL of liquid yeast extract (10% vol/vol); Oxoid, London, United Kingdom), 2 × 10⁶ U of penicillin G (Hoechst, Frankfurt, Germany), and 200 mL of heat-inactivated (56 °C, 30 min) horse serum per liter. Stocks of each isolate were prepared by freezing 1 mL portions of a 10 mL logarithmic-phase broth culture with 15% glycerol at −80 °C. Cultures were titrated on Heart Infusion Agar and were shown to contain 7 × 10⁶ cfu/mL for M. bovis and 4 × 10⁵ cfu/mL for M. bovirhinis. M. dispar (NCTC 10125) was provided by Helena Windsor (Mycoplasma Experience LTD, Bletchingley, UK) with a titre of 1.6 × 10⁷ cfu/mL. In addition, DNA from 14 Mycoplasma strains (Table 1) were provided by Prof. Konrad Sachse (Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Bundesforschungsinstitut für Tiergesundheit, Jena, Germany).

Hundred and seven bacterial isolates, representing 39 different species, were used to evaluate of specificity of the RespoCheck Mycoplasma triplex real-time PCR assay (Table 2). These included isolates associated with BRD and isolates associated with other bovine diseases. Prior to testing by PCR, the identity of the isolates was confirmed using MALDI-TOF mass spectrometry (MS Bruker MALDI Biotyper Microflex, version 3.1 with the reference database version 3.1.66 Bruker Daltonics GmbH, Germany).

Field samples and isolation of DNA

Calves (n = 44) with or without BRD (increased respiratory rate and/or dyspnoea) were sampled for diagnostic purposes. Sampling of the calves was granted an exemption from requiring ethics approval by the institutional Animal Experiment Commission “Dier Experimenten Commissie (DEC) Lelystad (2013111.b)” because sampling was performed for diagnostic purposes. BALF samples were obtained as described [21]. Approximately 35–75 mL BAL was obtained from each calf after instillation of 100 mL PBS with 10% Fetal Calf serum (FCS). Foam, large purulent exudates and blood clots were removed from the BALF samples under aseptic conditions. BALF (25 mL) was centrifuged (4600×g, 10 min, 4 °C). Sediment was resuspended in 0.5 mL Dulbecco’s minimal essential medium (DMEM) with 5% FCS, carefully added to 1 mL freeze medium (DMEM, 50% FCS and 20% DMSO) and frozen at −80 °C. The BALF supernatants were also stored at −80 °C.

For testing the influence of centrifugation of BALF samples (4600×g, 10 min, 4 °C) on the PCR results we tested three variants of BALF samples: without centrifugation, supernatant and pellet obtained after centrifugation (50 times concentrated). DNA was extracted from 200 μL aliquots of BALF samples. We used the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science), with the Total NA External lysis” protocol (Version 2.11). With the MagNA Pure LC Total Nucleic Acid Isolation Kit 32 samples can processed per run. In all runs a positive control (a mix of 1.4 × 10⁶ cfu/mL M. bovis, 0.5 × 10⁷ cfu/mL M. dispar
and 1.3 × 10^5 cfu/mL *M. bovirhinis* and a negative water control (NTC) was included.

**RespoCheck primers and probes**

To enable testing of testing for BRD associated pathogens in a routine setting, real-time PCRs for detection of viral, bacterial and mycoplasma pathogens in bronchoalveolar lavage fluid (BALF) of calves have been set up by the Central Veterinary Institute (Lelystad, The Netherlands) under the name RespoCheck. Primers and probes specific for the bacterial 16S, V3 and V4 regions were based on the Full length, bacterial 16S sequences (50,000 in July 2012) were used from the **nuccore** database at the National Center for Biotechnology Information (NCBI, USA, http://www.ncbi.nlm.nih.gov/nuccore). For *M. bovirhinis* and *M. dispar* the nearly full length 16S sequences were used. These sequences and their taxonomic information were used to build an Insignia-based database [22] from which pathogen-specific sequence regions were extracted with special interest for the V3 and V4 region because these sequences are often targeted for metagenomic next-generation sequencing (NGS) [23]. Using the identified regions, primers and probes were designed with AlleleID 7.8. (Premier Biosoft, palo Alto, USA). The resulting triplex PCR was designated RespoCheck *Mycoplasma* triplex real-time PCR

The specificity of the *Mycoplasma* primers and probes was also verified against V3-V4 partial sequences of *M. flocculare, M. ovipneumonia* and *M. hyopneumonia.*

**RespoCheck triplex and single real-time PCR**

The QuantiFast triplex Kit Real Time-PCR kit (Qiagen) was used for the RespoCheck *Mycoplasma* triplex real-time PCR. The assays were conducted in a 20 μL reaction mix containing 5 μL of the nucleic acid sample, 250 nM of each primer, 100 nM of each MGB probe, 1× QuantiFast triplex Real Time-PCR Master Mix and sterile deionised water. All reactions were conducted with an ABI-7500 with the following cycling parameters: 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 60 s. The machine was set to acquire fluorescence on the FAM, VIC, and NED channels for respectively *M. bovis, M. dispar* and *M. bovirhinis* All primers and probes were obtained from Life Technologies Europe BV (Bleiswijk, the Netherlands). The final results were analysed using ABI-7500 software (Version 1.4). Samples with a Ct of 40 cycles or less were considered to be positive.

**Evaluation of the analytical sensitivity and the analytical specificity**

The analytical sensitivity of the RespoCheck triplex PCR was defined as the ability to detect the lowest concentration of *M. bovis, M. dispar* and *M. bovirhinis* expressed as a concentration (cfu/mL) [24]. The analytical

| Species (Type strain) | ID      | *M. bovis* | *M. dispar* | *M. bovirhinis* |
|-----------------------|---------|------------|-------------|----------------|
| *M. agalactiae* (PG2) | R 41^b  | 20.9       | -           | -              |
| *M. alkalescens* PG 31/D 12 | R 18^b | -          | 35.7^a      | -              |
| *M. bovis* PG45       | R 9^b   | 19.4       | -           | -              |
| *M. bovirhinis* PG43  | R 12^b  | -          | -           | 25.8           |
| *M. bovigenitalium* PG11 | R 8^b  | -          | -           | -              |
| *M. californicum* ST-6 | R 26^b | -          | -           | -              |
| *M. canadense* 275C   | R 22^b  | -          | -           | -              |
| *M. canis*, PG14      | R 74^b  | -          | -           | 20.0^a         |
| *M. dispar* 462/2      | R 11^b  | 18.7       | -           | -              |
| *M. leachii* PG50     | R 23^b  | -          | -           | -              |
| (former *M. bovine group VII*) |         |            |             |                |
| *M. mycoides* subsp. *Mycoides* PG1 | R 84^b | -          | -           | -              |
| (former Small Colony Type) |         |            |             |                |
| *Acholeplasma* axanthum 5743 | R 17^b | -          | 33.1^a      | -              |
| *A. laidlawii* PG8    | R 10^b  | -          | -           | -              |
| *A. oculi* 19-L       | R 62^b  | -          | -           | -              |
| *M. bovis* ATCC 25025 | ATCC   | 23.9       | -           | -              |
| *M. dispar* (NCTC 10125) | ATCC 27140 | -          | 20.3        | -              |
| *M. bovirhinis* ATCC 5189985 | ATCC | -          | 20.9        |                |

^aCross-reactions in the RespoCheck triplex Mycoplasma PCR

^bID Friedrich-Loeffler-Institut
Table 2 Bacterial strains \((n = 107)\), that were used as reference material

| Identification (number of isolates tested) | CCUG identification\(^c\) | Source |
|-------------------------------------------|--------------------------|--------|
| Acidovorax spp. \((3)\)                  | NA                       | CVI collection\(^a\) |
| Actinomyces                                | NA                       | CVI collection\(^a\) |
| Aerococcus viridans                        | NA                       | CVI collection\(^b\) |
| Bibersteinia trehalosi                     | Pasteurella trehalosi    | CCUG 37711 |
| Bibersteinia trehalosi 20 AA III 3 E3      | NA                       | CVI collection\(^a\) |
| Bibersteinia trehalosi 21 AA III 3 E4      | NA                       | CVI collection\(^a\) |
| Brucella abortus                           | NA                       | CVI collection\(^b\) |
| Cornamonas kersteií                       | NA                       | CVI collection\(^a\) |
| Corynebacterium bovis \((2)\)              | NA                       | CVI collection\(^b\) |
| Corynebacterium pseudotuberculosis         | NA                       | CVI collection\(^b\) |
| Escherichia coli                           | NA                       | CVI collection\(^b\) |
| Gallibacterium anatis \((5)\)              | NA                       | CVI collection\(^a\) |
| Hafnia alvei                               | NA                       | CVI collection\(^a\) |
| Histophilus somni                          | NA                       | ATCC 22132\(^e\) |
| Histophilus somni \((4)\)                  | NA                       | CVI collection\(^a\) |
| Klebsiella oxytoca                         | NA                       | CVI collection\(^b\) |
| Klebsiella pneumonialae                    | NA                       | CVI collection\(^b\) |
| Lactobacillus mucosae                      | NA                       | CVI collection\(^a\) |
| Lactococcus garvieae                       | NA                       | CVI collection\(^b\) |
| Lactococcus lactis                         | NA                       | CVI collection\(^b\) |
| Listeria monocytogenes                     | NA                       | CVI collection\(^b\) |
| Mannheimia haemolytica                     | Mannheimia haemolytica   | ATCC 14003 |
| Mannheimia haemolytica                     | Mannheimia haemolytica   | ATCC 14003 |
| Mannheimia haemolytica                     | Mannheimia granulomatis  | CCUG 38457-T |
| Mannheimia granulomatis 25 AA III 3 E8     | NA                       | CCUG 45422-T |
| Mannheimia haemolytica                     | Mannheimia ruminalis     | CCUG 38470-T |
| Mannheimia haemolytica \((5)\)              | NA                       | CVI collection\(^a\) |
| Mannheimia haemolytica 3 AA III 2 H2       | NA                       | CVI collection\(^a\) |
| Mannheimia varigena                        | Mannheimia varigena      | CCUG 38462-T |
| Mannheimia varigena 19 AA III 3 E2         | NA                       | CVI collection\(^a\) |
| Mannheimia varigena 24 AA III 3 E7         | NA                       | CVI collection\(^a\) |
| Micrococcus luteus                         | NA                       | CVI collection\(^b\) |
| Moraxella bovis                            | NA                       | CVI collection\(^b\) |
| Moraxelle lacunata \((2)\)                 | NA                       | CVI collection\(^a\) |
| Mycobacterium avium subsp. paratuberculosis| NA                       | CVI collection\(^b\) |
| Mycobacterium bovis                        | NA                       | CVI collection\(^b\) |
| Mycobacterium tuberculosis                | NA                       | CVI collection\(^b\) |
| Neisseria zoodegmatií                      | NA                       | CVI collection\(^a\) |
| Pantoæ agglomerans Erwina herbicola \((n = 13)\) | NA                       | CVI collection\(^a\) |
| Pasteurella multicida                      | NA                       | Bisgaard Taxon 13 |
| Pasteurella multicida                      | NA                       | ATCC 15743\(^a\) |
| Pasteurella multicida                      | NA                       | CVI collection\(^a\) |
| Pasteurella multicida                      | NA                       | CCUG 16497\(d\) |
sensitivity of the single and triplex PCRs for *M. bovis*, *M. dispar* and *M. bovirhinis* was determined with DNA isolated from 200 μL culture (*M. bovis*, *M. dispar* and *M. bovirhinis* strain) in a volume of 200 μL elution buffer at a final DNA concentration of 10 ng/μL. This DNA preparation was tested in seven 10-fold serial dilutions (5 μL per assay) in PBS, resulting in a range with 10 ng down to 1 fg *Mycoplasma* DNA per assay. The Ct was determined for each sample by single and RespoCheck triplex real-time PCR with a threshold of 50% of the Delta Rn value (log). The threshold was manually set at 0.04 in the linear phase of the amplification plot, whereby the Slope and Correlation Coefficient values were 3.22 and 99.99% respectively.

The analytical sensitivity of the *M. bovis*, *M. dispar* and *M. bovirhinis* single and RespoCheck triplex real-time PCR, was also determined by testing a mixture of *M. bovis* (3 × 10^5 cfu/mL), *M. dispar* (3 × 10^6 cfu/mL) and *M. bovirhinis* (3 × 10^5 cfu/mL) in seven 10-fold serial dilutions in BALF of specific pathogen free (SPF) calves of 3–4 weeks old. Dilution resulted in a series of *M. bovis*, *M. dispar* and *M. bovirhinis* spiked BALF samples, ranging from 3 × 10^6 cfu/mL down to 0.3 cfu/mL. Total DNA was isolated from each 200 μL sample with the MAGNA pure isolation kit and the Ct was determined for each sample (5 μL) by both the single and RespoCheck triplex PCR assays. The slope of the curve, the efficiency and the detection limit (for DNA ng/μL; for cells cfu/mL) for each PCR was determined. To determine the analytical specificity of the designed RespoCheck triplex PCR, 17 *Mycoplasma* isolates and 107 bacterial strains (Table 2) were tested.

**Diagnostic sensitivity and specificity in BALF samples from calves.**

For determining the diagnostic specificity, BALF samples were analysed with the PCR/DGGE method by the...
Animal and Plant Health Agency (APHA, Mycoplasma Team, Addlestone Surrey, UK) as earlier described [18, 25]. To determine the analytical sensitivity of the PCR/DGGE analysis, four 10-fold serial dilutions of M. bovis \((7 \times 10^4 \text{ cfu/mL})\), M. dispar \((16 \times 10^4 \text{ cfu/mL})\), and M. bovirhinis \((0.5 \times 10^4 \text{ cfu/mL})\), were prepared in PBS. Samples were sent to the APHA and analysed using the PCR/DGGE method.

**Sequencing amplicons**
16S rDNA PCR-sequencing was used for confirmation of the results of RespoCheck Mycoplasma triplex real-time PCR. 16S rDNA of the DGGE positive /PCR positive \((n = 5)\) and DGGE negative /PCR positive \((n = 5)\) was amplified using the specific Mycoplasma primers of the RespoCheck Mycoplasma triplex real-time PCR. DNA was sequenced by BaseClear (Leiden, the Netherlands) by an automated DNA sequencer. The nucleotide sequences were compared with GenBank sequences using the Basic Local-Alignment Search Tool (BLAST) of the NCBI-NIH for homology [26]. Pairwise sequence alignments were performed using the Clustal algorithm implemented in the program DNA star (DNASTAR Inc., Madison, WI).

**Analyses of sensitivity and specificity**
The analytical sensitivity of the RespoCheck triplex PCR was determined by its ability to detect a low concentration of M. bovis, M. dispar and M. bovirhinis and therefore expressed as a concentration (ng/assay and cfu/mL) [24]. The analytical specificity of the assay was calculated for each target microorganism using the following definition for specificity as the percentage of true negative samples/ the number of true negative samples and the number of false positive samples [27].

Calculation of diagnostic sensitivity, specificity and Cohen’s Kappa Coefficient was performed as described [28]. We therefore used the results of the PCR/DGGE analysis as reference standard.

**Statistical analyses**
Differences in PCR results were analysed for statistical significance by the non-parametric Mann–Whitney U test in the GraphPad Prism version 5.0 software, with \(P < 0.05\) considered significant.

**Results**
**Analytical sensitivity and linear detection range of the RespoCheck triplex**
The linearity of quantification of the RespoCheck triplex Mycoplasma real-time PCR was established through a linear regression plot by plotting the Ct-values against the values of log10 DNA concentration tested per reaction. The M. dispar single and RespoCheck triplex real-time PCR showed a linear detection range from 10 ng to 10 fg DNA per assay with a linear correlation \((R^2)\) value of 0.999 (Table 3; Fig. 1.). The M. bovis and M. bovirhinis single and RespoCheck real-time PCR showed a linear detection range from from 1 ng to 100 fg DNA per assay, with a \(R^2\) value of 0.999 (Table 3; Fig. 1). In BALF

| A | PCR          | Agent      | Real time PCR | R²     | Slope  | Efficiency (%) | Linearity (ng) | Detection limit (ng/assay) |
|---|--------------|------------|---------------|--------|--------|----------------|----------------|----------------------------|
|   | Singleplex   | M. dispar  | 0.9995        | 3.3836 | 97.49  | 10 ng-10 fg    | 10 fg          |                            |
|   |              | M. bovis   | 0.9966        | 3.1137 | 109.49 | 10 ng-10 fg    | 10 fg          |                            |
|   |              | M. bovirhinis | 0.9955      | 3.5033 | 92.95  | 10 ng-10 fg    | 10 fg          |                            |
|   | Triplex      | M. dispar  | 0.9989        | 3.1175 | 109.3  | 10 ng-10 fg    | 10 fg          |                            |
|   |              | M. bovis   | 0.9955        | 3.6240 | 88.8   | 10 ng-100 fg   | 100 fg         |                            |
|   |              | M. bovirhinis | 0.9939      | 3.3735 | 97.9   | 10 ng-100 fg   | 100 fg         |                            |
| B | PCR          | Agent      | Real time PCR | R²     | Slope  | Efficiency (%) | Linearity (CFU/ml; log 10) | Detection limit (CFU/assay) |
|---|--------------|------------|---------------|--------|--------|----------------|--------------------------------|------------------------------|
|   | Singleplex   | M. dispar  | 0.995         | 3.248 ± 0.1276 | 103.2  | 6.5–2.5      | 1–2                          |                              |
|   |              | M. bovis   | 0.995         | 3.453 ± 0.1178 | 94.8   | 6.5–1.5     | 0.5                          |                              |
|   |              | M. bovirhinis | 0.981      | 3.395 ± 0.2698 | 97.0   | 5.5–1.5     | 0.5                          |                              |
|   | Triplex      | M. dispar  | 1.000         | 3.534 ± 0.04608 | 91.9   | 6.5–2.5     | 1–2                          |                              |
|   |              | M. bovis   | 0.993         | 3.462 ± 0.1440 | 94.5   | 6.5–1.5     | 0.5                          |                              |
|   |              | M. bovirhinis | 0.965      | 2.750 ± 0.3014 | 131.0  | 5.5–1.5     | 0.5                          |                              |
spiked samples, the detection limit of the RespoCheck triplex real-time PCR was 300 cfu/mL for *M. dispar*, and 30 cfu/mL for *M. bovis* or *M. bovirhinis* (Table 3; Fig. 2). In the RespoCheck *Mycoplasma* real-time PCR, 5 μL was tested and the analytical sensitivity was therefore 1–15 cfu/assay. A good linear correlation (R² > 0.96) was found between the values of BALF spiked samples and the Ct-values in the RespoCheck *Mycoplasma* triplex or singleplex real-time PCR for the three Mycoplasmas (Table 3).

**Analytical specificity of the RespoCheck triplex PCR**

RespoCheck Mycoplasma triplex PCR in silico BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi for the specificity of the *M. dispar* amplicon revealed a 100% identity (E-value 1E⁻⁴⁶) for 2 hits for *M. dispar* complete genome sequence. The in silico BLAST search for amplicon of *M. bovis* we found a 99–100% identity (E-values 4E⁻⁴³ - 6E⁻⁴⁵) to 31 complete genome or 16S ribosomal partial sequence, and for the amplicon of *M. bovirhinis* we found a 99–100% identity (E-values 5E⁻⁵⁰ - 1E⁻⁵¹).

For the *M. bovirhinis* amplicon a 97% identity (3E⁻⁴⁰) was found for 11 hits for *Mycoplasma canis* (Taxid:29,555).

Seventeen *Mycoplasma* strains (Table 1) and 107 bacterial strains (Table 2) were used to calculate the analytical specificity of the RespoCheck *Mycoplasma* triplex PCR. The RespoCheck *Mycoplasma* triplex real-time assay for detecting *M. bovis*, *M. dispar* and *M. bovirhinis* possessed an analytical specificity of 100% (0 FP), 98.2% (2 FP) and 99.1% (1 FP), respectively. No cross-reactivity in the RespoCheck *Mycoplasma* triplex real-time assay was observed with any of the 107 bacterial strains. The *M. dispar* PCR did however cross-react with *Acholeplasma axanthum*
S743 and *M. alkalescens* PG 31/D 12. In silico sequence data analyses from the 16S V3 genomic DNA region showed no similarity of *M. dispar* specific sequences with the *A. axanthum* S743 and *M. alkalescens* PG 31/D 12 isolates. The *M. bovirhinis* RespoCheck triplex PCR cross-reacted with *M. canis* with a Ct-value of 20.4. In the *M. bovis* RespoCheck triplex PCR we found a cross-reaction with *M. agalactiae*. Based on the almost 100% similarity of *M. canis* PG14 16S rRNA gene and the *M. bovirhinis* 16S rRNA, it is not possible to prevent for this cross-reaction.

**Diagnostic sensitivity and specificity of the RespoCheck triplex compared with DGGE**

To study the influence of centrifugation of the BALF samples on the PCR results we compared the PCR results from the BALF samples before and after centrifugation (10 min at 4600×g). A significant lower Ct-value (*P* < 0.05; non-parametric Wilcoxon statistics) in the RespoCheck *Mycoplasma* triplex real-time PCR was found for *M. bovis* and *M. dispar* in the pellet of the centrifuged BALF samples. Several *M. bovis*, *M. bovirhinis* and *M. dispar* mix-infections could be detected in one BALF sample with a difference of 10 Ct-values between the three species and were in accordance with the PCR/DGGE analysis (Fig 3). Therefore we used the pellet of the centrifuged BALF samples (50× concentrated) to determine the presence of the three *Mycoplasma* species in 44 BALF samples by real-time PCR.

The calculated diagnostic sensitivity and specificity the RespoCheck triplex PCR is reported in Table 4. As the diagnostic specificity is very low (0.1944, 0.739, 0.3889 for *M. dispar*, *M. bovis* and *M. bovirhinis* respectively) we analysed the sequence of the produced amplicon of five DGGE negative/PCR positive and five DGGE positive PCR positive samples. The sequence of both products was confirmed as *M. bovis*, *M. dispar* or *M. bovirhinis*, as all sequences had a high E-value (3e-44) and 100% Query cover (100%) against the homologue sequence using the BLAST of the NCBI-NIH. Comparison of the Ct-values of PCR positive/ DGGE negative and the PCR positive/ DGGE positive samples with a non-parametric Mann Whitney test, showed that the Ct values of *M. dispar* and *M. bovis* were significantly lower, *p* = 0.0026 and 0.0282, respectively. In the *M. bovis* and *M. dispar* PCR, the difference in Ct value between PCR positive/ DGGE positive and PCR positive/ DGGE negative samples is at least 3.2, which indicates a factor of 10 difference in concentration of *M. bovis* and *M. dispar* DNA between these two groups (Fig. 4). As a consequence the diagnostic
specificity of the RespoCheck triplex PCR is undervalued by this method. We compared the results of the \textit{M. bovis}, \textit{M. dispar} and \textit{M. bovirhinis} RespoCheck triplex PCR with the results of the PCR/DGGE analysis. The detection limit of the \textit{M. bovis}, \textit{M. dispar} and \textit{M. bovirhinis} PCR/DGGE analysis was, as determined by APHA, $0.7 \times 10^3$ cfu/mL, $16 \times 10^3$ cfu/mL and $0.5 \times 10^3$ cfu/mL, respectively (Fig. 5).

**Discussion**

PCR assays for the detection of \textit{Mycoplasmas} generally target sequences on the 16S rRNA gene [29, 30]. In this study we used the highly conserved 16S rRNA sequence to set up the RespoCheck \textit{Mycoplasma} triplex real-time PCR assay for the specific detection of \textit{M. bovis}, \textit{M. dispar} and \textit{M. bovirhinis} in BALF samples of calves.

The lowest concentration of \textit{M. dispar} which could be detected with the RespoCheck triplex PCR assay is around 300 cfu/mL. With a copy number of 16S rRNA of one or two (https://rrndb.umms.med.umich.edu/) and with a test volume of 5 μl the lowest concentration which could be detected is around 1–2 cfu/assay. The lowest concentration of \textit{M. bovis} and \textit{M. bovirhinis} which could be detected with the RespoCheck triplex for \textit{M. bovis}, and \textit{M. bovirhinis} is around 0.5 cfu/assay. From the calculated analytical sensitivity of the \textit{M. bovis}, \textit{M. dispar} and \textit{M. bovirhinis} RespoCheck triplex PCR (0.5–2 cfu/assay) we conclude that the RespoCheck triplex PCR has a good analytical sensitivity. It was shown that the use of a pellet from 25 mL BALF after centrifugation instead of not-centrifuged BALF samples increased the analytical sensitivity of the RespoCheck triplex PCR assay. In order to determine the analytical specificity of the RespoCheck triplex PCR we analysed the DNAs from panels of \textit{Mycoplasma} and bacterial strains. In the \textit{M. bovis} RespoCheck \textit{Mycoplasma} triplex real-time PCR we found a cross-reaction with \textit{M. agalactiae}. Phylogenetic analyses on 16S rRNA sequences and comparing the 16S rRNA sequences of \textit{M. bovis} and \textit{M. agalactiae} [25] at NCBI (www.ncbi.nlm.nih.gov), we found a close relationship between \textit{M. agalactiae} and \textit{M. bovis}, with a 99% nucleotide identity between their 16S rRNA sequences. However, \textit{M. bovis} causes calf pneumonia, mastitis, and arthritis in cattle [16, 31], \textit{M. agalactiae} is the causal agent of contagious agalactia in goats and sheep [32]. Although unusual, \textit{M. agalactiae} has been detected from cattle samples [33, 34]. Therefore the cross reactivity for \textit{M. agalactiae} might be a problem for the intended BALF samples in the \textit{M. bovis}

| Table 4 | Diagnostic sensitivity and specificity of the RespoCheck \textit{Mycoplasma} triplex real-time PCR compared with the PCR/DGGE method |
|---------|---------------------------------------------------------------|
| M. dispar |                      |                      |                      |                      |
| DGGE + | 8 | 0 | 8 | Sensitivity = 1 |
| DGGE - | 29 | 7 | 36 | Specificity = 0.1944 (95% CI: 0.0819–0.3602) |
| Total | 37 | 7 | 44 |                      |
| M. bovis |                      |                      |                      |                      |
| DGGE + | 20 | 1 | 21 | Sensitivity = 0.9524 (95% CI: 0.7618–0.9988) |
| DGGE - | 6 | 17 | 23 | Specificity = 0.7391 (95% CI: 0.5159–0.8977) |
| Total | 26 | 18 | 44 |                      |
| M. bovirhinis |                      |                      |                      |                      |
| DGGE + | 7 | 1 | 8 | Sensitivity = 0.8750 (95% CI: 0.4735–0.9968) |
| DGGE - | 22 | 14 | 36 | Specificity = 0.3889 (95% CI: 0.2314–0.5654) |
| Total | 29 | 15 | 44 |                      |

**Fig. 4** The Ct-level of DNA derived from BALF samples from \textit{M. bovis}, \textit{M. dispar} and \textit{M. bovirhinis} infected calves of PCR/DGGE analyses of DGGE APHA negative and positive samples. Significant $P$ values are indicated by *.
PCR. In the *M. bovirhinis* RespoCheck triplex real-time PCR one false positive reaction was obtained on DNA from *M. canis*. *M. canis* can be isolated from the reproductive tract of dogs, but has not been proved to cause disease in dogs. However, it has been shown to cause clinical signs of pneumonia in experimentally challenged calves [35] and *M. canis* has been isolated from ruminants in Britain [36, 37]. Depending on the incidence of *M. canis* in ruminants, this may give false-positive results in the *M. bovirhinis* RespoCheck triplex real-time PCR. DNA samples from *M. alkalescens* and *A. axanthun* showed high Ct-values (>35) for *M. dispar* in the RespoCheck triplex real-time PCR, and were therefore classified as false-positive (Ct of 40 cycles or less were considered to be positive). *M. alkalescens* and *M. bovigenitalium* are important *Mycoplasmas* that can infect cattle and cause mastitis, arthritis and respiratory disease [17]. However, in the sequence analyses of the PCR-positive and DGGE-negative *M. dispar* BALF samples, we did not find any indication for the presence of *M. alkalescens*, underlining the high specificity for *M. dispar* in the RespoCheck triplex real-time PCR.

Monitoring for *Mycoplasma* species in BALF samples through collection and testing of BALF samples by culture is hampered by the fastidious nutritional requirements, lengthy culture of mycoplasmas, and their susceptibility to growth inhibitors. As a consequence, *Mycoplasma* culture is time-consuming, costly, and requires specific expertise. Moreover, *Mycoplasma* species may easily be overgrown by bacterial contaminants or by more rapidly growing *Mollicutes*, notably *Acholeplasmas*. The PCR/DGGE method of the APHA can differentiate 13 bovine *Mycoplasma* species [18] including the target *Mycoplasmas* of the RespoCheck *Mycoplasma* triplex real-time PCR and in contrary to the RespoCheck can differentiate between *M. bovis* and *M. canis*. Additional the PCR/DGGE is capable of detecting mixed cultures, which would have been difficult to detect by culture methods [18]. Therefore we used this method as a reference for determining the diagnostic sensitivity and specificity of the RespoCheck *Mycoplasma* triplex real-time PCR.

Possibly due to the lower sensitivity of the DGGE analysis compared to the RespoCheck triplex PCR (almost factor 10) and its use as reference method to validate the RespoCheck triplex PCR, the latter test method scores 29, 6 and 22 for *M. dispar*, *M. bovis* and *M. bovirhinis* respectively out of 44 more samples as false-positive and therefore the diagnostic specificity of the RespoCheck triplex PCR is underestimated. The transport and storage conditions or differences in DNA preparation of particularly the more diluted BALF samples for the PCR/DGGE method could have induced a lower sensitivity of
the PCR/DGGE analysis. The Ct values of the *M. bovis*, and *M. dispar* PCR positive and DGGE positive samples are significant (*P* < 0.05 Mann Whitney test) lower than the *M. bovis*, and *M. dispar* PCR positive DGGE negative samples, which confirms the difference in the analytical sensitivity between the RespoCheck triplex PCR and DGGE analysis. In the *M. bovis* and *M. dispers* PCR, we found a 10 fold difference in the Ct values between the DGGE positive/PCR positive and DGGE negative/PCR positive samples, which indicates a higher diagnostic sensitivity of *M. bovis* and *M. dispers* PCR than the DGGE analyses. Results by DGGE from BALF samples with mixed infections could be reproduced by the triplex PCR, suggesting that there is no significant PCR bias when the triplex PCR is used for Mycoplasma detection in field samples. The PCR has thus a higher analytical sensitivity than the DGGE.

**Conclusion**

In conclusion, the RespoCheck *Mycoplasma* triplex PCR test appears to be a sensitive and specific test for the detection of *M. bovis*, *M. dispers* and *M. bovirhinis* in BALF samples of calves.

**Abbreviations**

BALF: Bronchoalveolar lavage fluid; BRDC: Bovine respiratory disease complex; PCR/DGGE: PCR with denaturing gradient gel electrophoresis fingerprinting

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**Availability of data and material**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

JC authored the manuscript, designed the study, performed the qPCR and analysed the data. AB and FB build an Insignia-based database from which pathogen-specific sequence regions were extracted, designed the oligonucleotide primers and probes and assisted in drafting and editing the manuscript. HW and FW assisted in study design, interpretation of data and editing the manuscript. MK was involved in the microbiological analyses of the samples and participated in the drafting of the manuscript. AA designed and coordinated the field study for the collection of BALF samples and also helped to draft this manuscript. BS conducted and coordinated the field sample collection, performed microbiological analysis of samples and managing the database with results. BK participated in the design of the study and helped in the interpretation of the triplex PCR data. All authors read and critically revised and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Sampling of the calves was granted an exemption from requiring ethics approval by the institutional Animal Experiment Commission “Dier Experimenten Commissie (DEC) Lelystad” because sampling was performed for diagnostic purposes. Ethics approval is not applicable. Animal handling, including BALF sample collection, was performed or supervised by approved veterinarians. Consent was obtained from the farmers for the samples collected at their farm.

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