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A new duplex qPCR-based method to quantify *Mycoplasma mycoides* in complex cell culture systems and host tissues

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Abstract

Bacterial pathogen-host interactions are a complex process starting with adherence and colonization followed by a variety of interactions such as invasion or cytotoxicity on one hand and pathogen recognition, secretion of proinflammatory/antibacterial substances and enhancing the barrier function of epithelial layers on the other hand. Therefore, a variety of *in vitro*, *ex vivo* and *in vivo* models have been established to investigate these interactions. Some *in vitro* models are composed of different cell types and extracellular matrices such as tissue explants or precision cut lung slices. These complex *in vitro* models mimic the *in vivo* situation more realistically, however, they often require new and more sophisticated methods for quantification of experimental results. Here we describe a multiplex qPCR-based method to quantify the number of bacteria of *Mycoplasma (M.) mycoides* interacting with their hosts in an absolute manner as well as normalized to the number of host cells. We choose the adenylate kinase (*adk*) gene from the pathogen and the Carcinoembryonic antigen-related cell adhesion molecule 18 (*CEACAM18*) gene from the host to determine cell numbers by a TaqMan-based assay system. Absolute copy numbers of the genes are calculated according to a standard containing a defined number of plasmids containing the sequence which is amplified by the qPCR. The new multiplex qPCR therefore allows the quantification of *M. mycoides* interacting with host cells in suspension, monolayer, 3D cell culture systems as well as in host tissues.

Highlights

- The new TaqMan-based qPCR was developed for absolute quantification of *M. mycoides*.
- The duplex qPCR quantifies *Mmm* and *Mmc* normalized to the interacting host cells.
- Assays are suitable for various *in vitro* models and tissue samples.

1. Introduction

Mycoplasmas are remarkable self-replicating bacteria. They contain small genomes with a low GC content, lack a cell wall and show a strict dependence on the host for nutrients [1, 2]. *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) is the causative agent of Contagious bovine pleuropneumonia (CBPP), a highly contagious disease of cattle with a huge economic impact, notifiable to the World Organization for Animal Health (WOAH) [3]. *Mmm* belongs to the '*Mycoplasma mycoides cluster*', which consists of five closely-related ruminant pathogens. Besides the causative agent of CBPP, the '*M. mycoides* cluster' encompasses four additional pathogens, *M. leachii*, another bovine pathogen, and the small ruminant pathogens *M. capricolum* subsp. *capripneumoniae* (*Mccp*), *M. mycoides* subsp. *capri* (*Mmc*) and *M. capricolum* subsp. *capricolum* (*Mcc*)[4-6]. Clinical signs induced by members of the cluster include pneumonia, septicemia, mastitis, wound infections, arthritis and meningitis [7]. Members of the '*M. mycoides* cluster' have a remarkable degree of host specificity [8]. For example, *Mmm* is strictly adapted to *Bovidae* while its closest relative *Mmc* is pathogenic to goats and to a lesser extent to sheep. Comparative examination of the interaction of these two pathogens with different hosts or host cells will provide new insights into drivers of pathogenicity. The first step of infection is marked by adherence and colonization of host cells [9]. In previous reports adhesion of *Mmm*/*Mmc* was determined by flow cytometry [10], and of *Mmc* by determination of [U-14C] palmitic acid which was used to label *Mmc* [11]. For adhesion assays of other mycoplasmas qPCR assays were established [12-14]. Zou *et al.* measured adhesion of the p19 adhesin to embryonic bovine lung (EBL) cells with confocal laser scanning microscopy [15] and Clampitt *et al.* determined adherent mycoplasmas after isolation by determining the plating efficacy [16]. For high throughput analyses of members of the '*M. mycoides* cluster', new methods are needed. Bischof *et al.* used a *Mmm* specific TaqMan real-Time PCR for the calculation of the copy numbers of the L α-glycerophosphate oxidase gene (*glpO*) to quantify adherent mycoplasmas to a bovine epithelium cell line (ECaNEp) [17]. We describe in this report a duplex qPCR to determine genome numbers of pathogen per host cell, which is based on single copy genes of the mycoplasmas (adenylate kinase (*adk*)) and of the host (CEA related cell adhesion molecule 18 (*CEACAM18*)). Using primers and probes mentioned here *Mmm* and its closest relative *Mmc* could be detected. For detection of *Mccp*, *Mcc* or *M. leachii* the sequence of the probe has to be adapted and the performance of the PCR has to be tested with the primers that fit to all members of the '*M. mycoides* cluster'. Similarly, the probe for detection of *CEACAM18* fits to cattle and goats while the primers differ slightly between both species. The use of a duplex qPCR prevents inter assay variation due to different qPCR efficacies. In addition, we tested the performance of the assay in various applications.

2. Material and methods

2.1. Primer and probe sequences

Primers and probes were purchased from Metabion International AG, Planegg, Germany. The sequences of primers and probes used in the study are listed in Table 1.

Table 1: Primers and probes used in this study

Gene	Oligonucleotide sequence 5`-3`	Amplicon size	Source
Mmm/Mmc_adk	For: AGCTTGTTCTAAAGTTCTTG	184 bp	This study
	Rev: CTGGTGATTTAATGAGAAAG		
	Pro: FAM-CTGACTAACGATTTGATTAACAATTTGAT-BHQ-1		
	For: AGCCAAATCTACATCACCCC	216 bp	

Bta_CEACAM18	Rev: `ACCTCTAATGGACACACTTT`		
Chi_CEACAM18	For: `AGCCAAATCTACATCGCCCC`	216 bp	
	Rev: `ACCTCTAACGGACACACTTT`		
Bta/Chi_CEACAM18	Pro: `HEX-TCATCGGAGTGGAAAGATATTCGAGCT-BHQ-1`		
Mmm_lppQ	For: `ATCAAGATATTTCGAGTTGAAATGTAAG`	81 bp	[18]
	Rev: `TGTATATTTTTTAGATTTCAATCTGAAAGTG`		
	Pro: `FAM-TTTCAGCTCGATAA`**`AACA`**`T`**`AT`**`TT-BBQ1`		
*GAPDH*_bovine	For: `TTCTGCCATGTGGACCCA`	130 bp	This study
	Rev: `CAGGTCAGATCCACAACAGA`		
	Pro: `HEX-AGCTGGGAGGAGCAGTGCAGACTGACC-BHQ1`		

Locked nucleic acids (LNA) containing nucleotides are displayed in bold

2.2. Sequence comparison and phylogenetic analyses

Sequences for the sequence alignments were identified by similarity searches using the NCBI BLAST tool blastn http://blast.ncbi.nlm.nih.gov/Blast.cgi using default parameters. For the search of the *CEACAM18* genes used in Figure 1 we took the sequence of the *CEACAM18* gene from *Bos taurus* as previously described [19]. Sequences with the following accession numbers were identified *Bos indicus*, XM_019978315; *Bos grunniens*, JANCMS010091302.1; *Bubalus bubalis*, XM_044931086; *Ovis aries*, XM_042231059.1; *Capra hircus*, XM_018063197.1. For similarity searches of *adk* we used the sequence of *M. mycoides* subsp. *mycoides* strain Afadé (accession number: JQ673722).

Phylogenetic analyses based on nucleotide sequences were conducted using MEGA X [20]. Alignments were performed with ClustalW. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [21]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Sequences with the following GenBank accession were used for phylogenetic analyses: *M. mycoides* subsp. *mycoides* PG1 (BX293980); *M. mycoides* subsp. *capri* Y-Goat (FQ377874); *M. mycoides* subsp. *capri* PG3 (JFAE01000006); *M. capricolum* subsp. *capripneumoniae* F38 (LN515398); *M. leachii* 99/014/6 (FR668087); *Acholeplasma laidlawii* PG8B (LVCP01000009); *M. agalactiae* PG2 (CU179680); *M. alkalescens* 14918 (AMWK01000004); *M. arginini* 7264 (AORG01000012); *M. bovigenitalium* HAZ596 (AP017902); *M. bovirhinis* HAZ141_2 (AP018135); *M. bovis* PG45 (CP002188); *M. californicum* ST-6 (CP007521); *M. canadense* HAZ360 (AP014631); *M. capricolum* subsp. *capricolum* California Kid/ATCC 27343 (CP000123); *M. dispar* 462/2 (LR214971).

2.3. Generation of standards

The *Mmm* strain Afadé [22] and embryonic bovine lung cells, EBL, (ACC 192, DSMZ, Braunschweig, Germany) were used as template DNA for the preparation of standards (see 2.7. and 2.8.). DNA fragments of *adk*, known to be present in all members of the 'M. mycoides cluster' [5] and *CEACAM18* [19] were synthesized to include terminal BamHI and HindIII restriction sites. These fragments were cloned into a plasmid vector pSC-A, (Agilent, Santa Clara, CA, USA) according to the manufacturer`s instructions. Bacterial strain *Escherichia coli* XL-1 was used as host for creation of cDNA clones. Plasmid extraction was performed using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Presence of specific sequences in clones was verified by restriction enzyme digestion and validated by Sanger sequencing using BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Weiterstadt, Germany).

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For the determination of the nucleic acid concentration, the commercial Quant-iT-dsDNA Assay Kit HS (Thermo Fisher Scientific, Waltham, MA, USA) was used according to vendor's protocol. The conversion of the dsDNA concentration into copies/µl was done with the Copy number Webtool from Thermo Fisher Scientific, assuming that the average weight of a base pair (bp) is 650 Daltons. https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/.

2.4. DNA extraction from bovine tissues

Total DNA from 25 – 50 mg of lung and of lymph node tissues (*Lnn. mediastinales* and *Lnn. pulmonales*) was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). The origin of the tissues used is described below in 2.9. Briefly, Lymph node tissue was homogenized prior to DNA extraction in 500 µl phosphate-buffered saline (PBS) using the Fast Prep 24 tissue homogenizer (MP Biomedicals, Irvine, CA, USA) with Lysing Matrix A followed by DNA isolation according to manufacturer's instructions. DNA extraction from Precision-Cut Lung Slices (PCLS) was performed using the Qiagen DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions, as described earlier [23]. DNA concentration was determined by NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany).

2.5. PCR methods

2.5.1. Semi-quantitative PCR

The primers for amplification of the *adk*, *CEACAM18* and *GAPDH* genes (Table 1) were tested using conventional PCR assays using DreamTaq polymerase (Thermo Fisher Scientific) with the following parameters: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C and elongation at 72 °C for 30 s and final elongation at 72 °C for 10 min. Eight µl of each PCR product were analyzed by electrophoresis on a 1.8 % agarose gel and visualized by GelRed staining.

2.5.2. Standard qPCR (*lppQ*)

The assay was performed as a simplex real-time PCR according to Schnee *et al.* (2011) [18] on a CFX 96 cycler (BioRad, Hercules, CA, USA) with the following parameters: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 20 s, primer annealing at 57 °C and elongation at 68°C for 45 s. The reactions were carried out in duplicates in a total volume of 25 µl per reaction. Serial dilutions of DNA from *Mmm* Afadé were applied as standards and as well as a positive control. The following volumes and concentrations were used: 12.5 µl 2 × QuantiTect® Multiplex PCR Master Mix (Qiagen), 0.4 µM of each primer, 0.5 µM probe, and 1 µl sample template (10 ng/ml).

2.5.3. Single- and duplex qPCR for the detection of *Mmm*

The assays were run on a C1000 thermal cycler (BioRad) using the following cycling parameters: initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 45 s, primer annealing and extension at 55 °C for 45 s. Each 25-µl duplex reaction contained 12.5 µl 2 × QuantiTect® Multiplex PCR Master Mix (Qiagen) with 1 µl of DNA template and a final concentration of 0.5 µM of each primer and 0.2 µM of each probe. All amplified qPCR products were analyzed using the BioRad CFX-Manager, and amplification plots were generated with an adaptive baseline. The threshold cycles were calculated accordingly by the instrument.

2.6. *Mycoplasma* culture and DNA extraction

Frozen or lyophilized *Mycoplasma* stocks were grown in broth cultures of modified Hayflick medium containing 20 % horse serum or commercially available ML - Mycoplasma Liquid Medium, (Mycoplasma Experience, Bletchingley, UK) at 37°C in an atmosphere containing 5 % CO_2 to logarithmic phase for 48 - 96 h. Cells were harvested by centrifugation and chromosomal DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to manufacturer's instructions. The following mycoplasma strains from the mycoplasma strain collection housed at the Friedrich-Loeffler-Institute in Jena were used for qPCR validation: *Mmm* (PG1, Afadé, T1/44, 95014, PO2), *Mmc* (My-325, M-5, M-29, Y-Goat, PG3, Kombolcha, G1313/94, YCpMmyc1.1[11], *Mcc* (California Kid), *Mccp* (F38), *M. leachii* (PG50), *M. bovis* (PG45), *M. bovigenitalium* (PG11), *M. bovirhinis* (PG43), *M. agalactiae* (PG2), *M. californicum* (ST-6), *M. dispar* (462/2), *M. arginini* (G230), *M. alkalescens* (PG51), *M. canadense* (275C), *M. fermentans* (PG18), *M. orale* (CH19299) and *M. hyorhinis* (BTS-7). *Mmm* (Afadé) was used for cell culture, PCLS and animal infection experiments, whereas *Mmc* (Y-Goat) was the inoculum for bovine cell culture and *Mmc* (GM12) for PCLS experiments.

2.7. Culture of bovine cell lines and infection assay

Cells of the bovine macrophage cell line (BoMac) [24] were seeded so that they reached 80 % confluence after 24 h. After renewal of medium, *Mmm* or *Mmc* were added to the cells at a MOI of 30 (according to the number of colony forming units (cfu)) and co-cultured for 24 h. Before harvesting the cells by trypsination, the medium was removed and the adherent cell layer was carefully rinsed with prewarmed PBS. The harvested cells were processed with the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's instructions.

2.8. Precision-Cut Lung Slices (PCLS)

The PCLS were prepared and co-cultured with 10^8 cfu/slice *Mmm*, strain Afadé, and *Mmc*, strain GM12, as previously described [23]. PCLS samples were stored in 20 % glycerol at −20 °C until further use.

2.9. Tissue samples

Tissue samples from Boran cattle (*Bos indicus*) and twenty Holstein-Sbt heifers (*Bos taurus*), which were experimentally infected with the virulent African *Mmm* strain Afadé, were generated in previously conducted animal experiments. Experimental infection of Boran cattle was previously described [25]. Experimental infection of Holstein-Sbt cattle was performed by intranasal administration of 10^8 cfu *Mmm* strain Afadé with an atomizer device on three consecutive days combined with an intratracheal administration of 10^9 cfu/animal on day five by intubation. Tissue samples for this study were taken from lung tissue and from lung lymph nodes (*Lnn. mediastinales* and *Lnn. pulmonales*). The lung tissue samples were taken at the transition from healthy to the tissue with lesions. The experiment was approved by the animal use committee of local authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Rostock, Germany; 7221.3-1-047/17).

3. Results

3.1. Selection of target genes

To establish an assay to quantify the absolute number of either *Mmm* or *Mmc*, normalized to the host cell number, in various *in vitro* culture systems as well as in tissues, we developed a novel duplex TaqMan-based qPCR. We chose the adenylate kinase gene (*adk*) to quantify the number of *Mycoplasma* genomes in the samples since *adk* is a single copy gene present in all members of the 'M. mycoides cluster'. The *adk* gene is relatively conserved in *M. mycoides* (*Mmm* and *Mmc*) enabling the use of the same primers and probes to quantify *Mmm* and *Mmc* (Fig. 1A). Next, we chose the *CEACAM18* gene for quantification of the eukaryotic host cells. We selected a sequence of exon 2 which is identical for *Bos taurus*, *Bos indicus*, and *Bos grunniens*, the most relevant domesticated hosts

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of *Mmm* (Fig. 1B). The same qPCR target region of the gene may be used for other host species such as swine, goats and sheep, since they show minimal sequence variation, however, the sequences for primers and probes have to be slightly modified as shown in this report for sheep and goats. Primers and probes for *Mmm* and *Mmc* and different host species are displayed in Table 1.

A

```
                          Primer 1      Gap 36 bp           Probe             GAP 63 bp    Primer 2
Mmm PG01       GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACGATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mmm T1/44      GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACGATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mmm Gladysdale GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACGATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mmc My-325     GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACGATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mmc M-5        GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACGATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mmc M-29       GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACGATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mmc GM12       GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACAATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mmc G1313/94   GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACAATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mmc Y-Goat     GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAACTGACTAACAATTTGATTAACAATTTGATCTG...TTTCTTTTCTCATTAAATCACCAGTTG
Mcc ATCC 27343 GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAATTGATTAACAATTTGATTAACAATTTGTCTG...TCTCTTTTCTCATTAAATCACCAGTTG
Mccp F38       GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAATTGATTAACAATTTGATTAACAATTGATCTG...TCTCTTTTCTCATTAAATCACCAGTTG
M. leachii PG50 GATTTAGCTTGTTCTAAAGTTCTTGGAT...AAATTGATTAGCAATTTGATTAACATTTGTCTG...TCTCTTTTCTCATTAAATCACCAGTTG
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B

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                    Primer 1                     Probe          GAP 133 bp   Primer 2
Bos taurus      CCTCCAGCCAAATCTACATCACCCCGGATTCACTCATCGGAGTGGAAAGTATTCGAGCTCACTG...GGGAAAGTGTGTCCATTAGAGGTACC
Bos indicus     CTTCCAGCCAAATCTACATCACCCCGGATTCACTCATCGGAGTGGACGCATACTCGAGCTCACTG...GGGAAAGTGTGTCCATTAGAGGTACC
Bos grunniens   CCTCCAGCCAAATCTACATCACCCCGGATTCACTCATCGGAGTGGAAAGTATTCGAGCTCACTG...GGGAAAGTGTGTCCATTAGAGGTACC
Bubalus bubalis CCTCCAGCCAAATCTACATCGCCCCGGATTCACTCATCGGAGTGAAGCTATTCAAGCTCACTG...GGGAAAGTGTGTCCATTAGAGGTACC
Ovis aries      CCTCCAGCCAAATCTACATCGCCCCGGATTCACTCATCGGAGTGAAAGTATTCGAGCTCACTG...GGGAAAGTGTGTCCGTTAGAGGTACC
Capra hircus    CCTCCAGCCAAATCTACATCGCCCCGGATTCACTCATCGGAGGGAAAGATATTCGAGCTCACTG...GGGAAAGTGTGTCCGTTAGAGGTACC
```

Fig. 1. Polymorphisms in the sequences of target genes. A. Sequence alignment of the adenylate kinase (*adk*) gene from various *Mmm* and *Mmc* strains and the type strains of *Mcc*, *Mccp* and *M. leachii*. The sequences were identified by Blast searches using the primer sequences and the NCBI data base. **B.** Sequence alignment from exon 2 of the Carcinoembryonic antigen-related cell adhesion molecule 18 (CEACAM18) gene of various ruminants. Different parts of the sequence were colored. Green, forward primer; red, probe sequence; blue, reverse primer. Differences between the sequences to the selected primer and probe sequences in the colored regions are indicated by black letters.

3.2.   Functionality of primers and probes

After selection of primer sequences, we tested their functionality by amplifying the *CEACAM18* and *GAPDH* fragment from tissues of *Bos taurus* (Bta) and *Bos indicus* (Bin) by conventional PCR. Both PCRs worked equally well with both tissues (Fig. 2A). The primers for amplification of the *adk* gene were tested using different *Mmm* and *Mmc* strains. The *M. bovis* strain PG45 was used as a negative control (Fig. 2B). An *adk*-specific fragment was amplified from both the *Mmm* strains Afadé, PO2 and T1/44 and the *Mmc* strains Y-Goat and GM12::YCpMmyc1.1 [11], but not from the *M. bovis* strain PG45 (Fig. 2B). In addition, the amount of the amplification product strongly correlated with the amount of template (Fig. 2B). Due to the low GC content of the *adk* gene and the *Mycoplasma* genome in general the optimal annealing temperature varied between the *adk* PCR (55 °C) and the *CEACAM18* PCR (60 °C). Thus, we performed a temperature gradient qPCR of *CEACAM18* and *GAPDH* to test which PCR is more suitable in combination with the *adk* PCR in the duplex qPCR. The *CEACAM18* qPCR was very robust concerning the annealing temperature in the range of 55 °C to 60 °C. In contrast the *GAPDH* qPCR required an annealing temperature of 60 °C for optimal performance (Fig. 2C). Finally, we determined the influence of the annealing temperature on the performance of the duplex qPCR. Both qPCRs were robust from an annealing temperature of 55 °C to 58 °C (Fig. 2D).
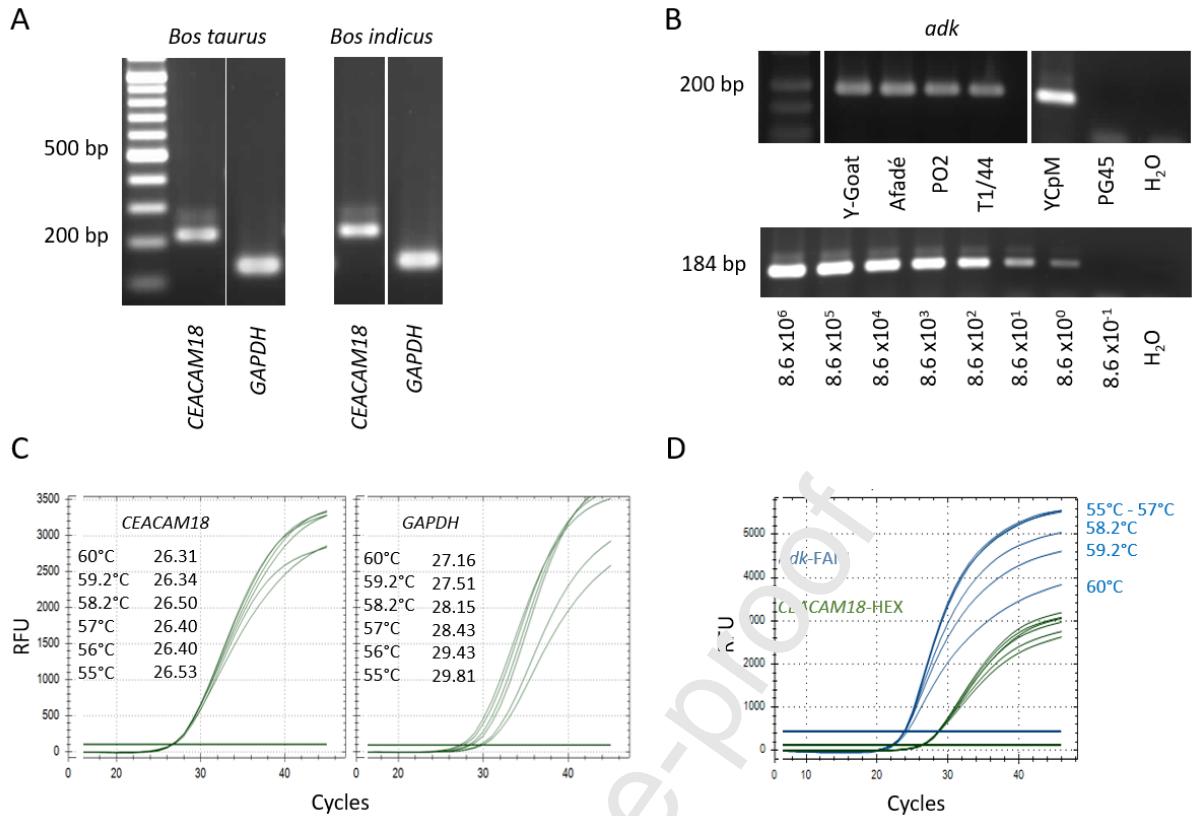
Fig. 2. Functionality of selected primers and probes. A. Semi quantitative PCR of genomic DNA isolated from lung tissues of *Bos taurus* and *Bos indicus* using primers for *CEACAM18* and *GAPDH*. **B.** Conventional PCR of genomic DNA from different *Mmm* (Afadé, PO2, and T1/44 (vaccine strain)) and *Mmc* (Y-Goat, YCpM = GM12::YCpMmyc1.1) strains as well as the PG45 strain (type strain) of *M. bovis* using primers for *adk*. Lower panel: amplification of *adk* using DNA isolated from serial dilution of colony forming units from *Mmm* strain Afadé as template. **C.** qPCR amplification graphs with temperature gradient of bovine specific genes in *Bos indicus*. **D.** Duplex-qPCR of target genes run with temperature gradient in infected lung tissue from *Bos indicus*.

3.3.   Specificity of *adk*-qPCR

Once we had established the *adk*-qPCR for *Mmm* and *Mmc* type strains, we determined the specificity of the assay with a range of *Mmm* and *Mmc* field strains as well as with other members of the '*M. mycoides* cluster' and related mycoplasmas to assess its specificity. Fig. 3A represents the phylogenetic relationship of the *adk* genes from the *M. mycoides* cluster and other ruminant and cell contaminating mycoplasmas based on the complete coding sequence. The *adk*-qPCR amplified all *Mmm* and *Mmc* strains with comparable Cq values and a sigmoidal amplification curve, whereas *Mcc*, *Mccp* and *M. leachii* genomic DNA (gDNA) was amplified with lesser efficiency resulting in higher Cq values and abnormal, flat amplification curves (Fig. 3B and Supplementary Table 1). gDNA of *Mycoplasma* species outside the *M. mycoides* cluster could not be detected which was in concordance with the high number of mismatches with the *adk*-PCR probe (Table 2).

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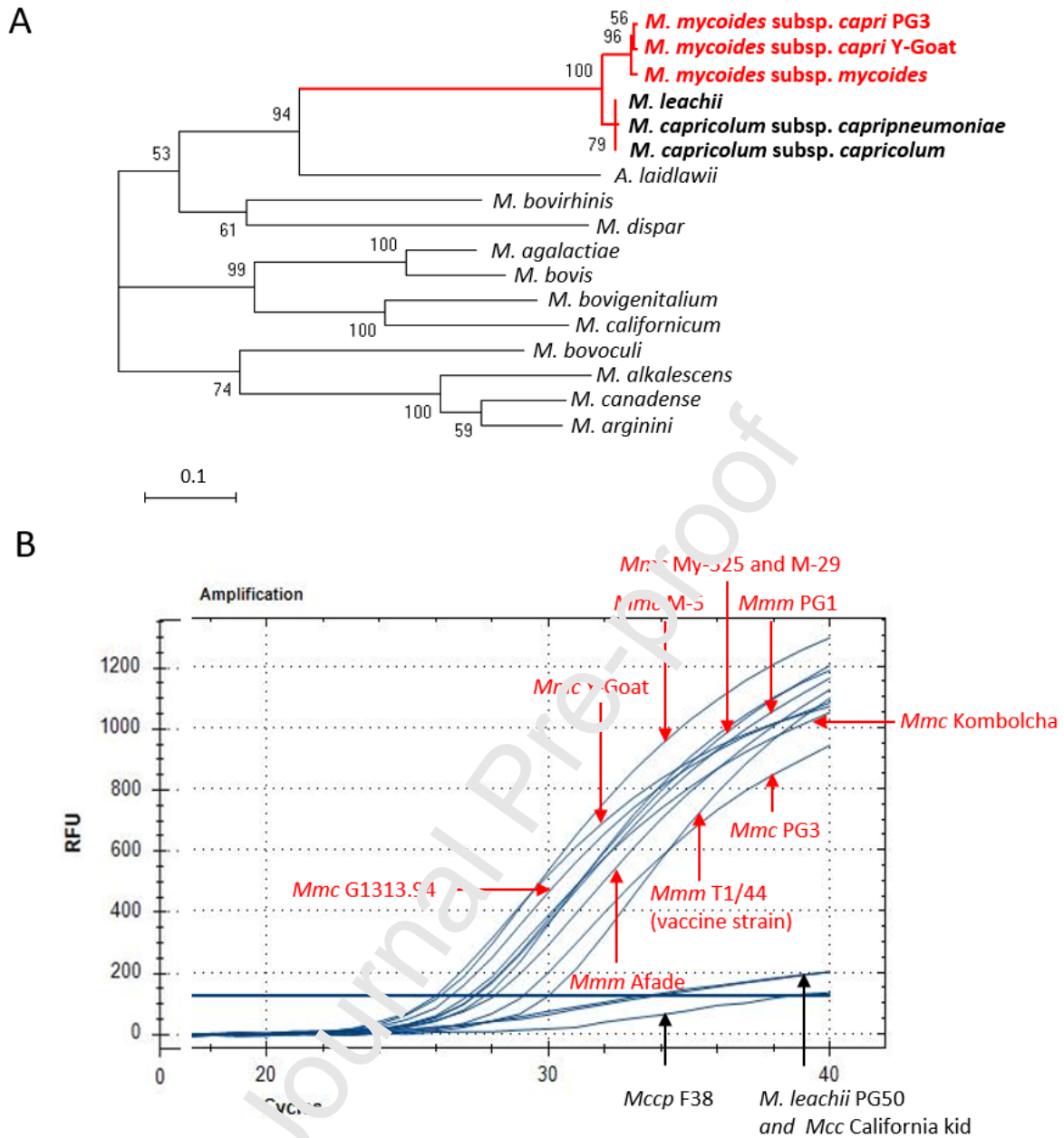
Fig. 3. Specificity of *adk*-qPCR. (A) Phylogenetic relationship of ruminant *Mycoplasma* (M.) subsp. and their cross reactivity in the qPCR assay. Red, strong cross reactivity; bold black, cross reactivity with abnormal curve progression; black, no cross-reactivity. (B) Detection of different strains of *Mmm* and *Mmc* (red labels) and the cross reactivity with *Mccp* and *M. leachii* (black labels). The complete list of tested *Mmm* and *Mmc* strains is shown in Supplementary Table 2.

Table 2. The *adk*-PCR did not show cross reactivity with *Mycoplasma* (*M.*) subsp. outside the *M. mycoides* cluster of ruminant and cell culture origin.

Species	Strain	Number of probe mismatches	Cq adk-PCR*	Amplification curve
Ruminant mycoplasmas				
M. bovigenitalium	PG11	12	no Cq	no
M. bovis	PG45	13	no Cq	no

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M. bovirhinis	PG43	15		no Cq	no
M. agalactiae	PG2	11		no Cq	no
M. californicum	ST-6	13		no Cq	no
M. dispar	462/2	15		no Cq	no
M. alkalescens	PG51	16		no Cq	no
M. canadense	275C	13		no Cq	no
Cell culture contaminants					
A. laidlawii	PG8	11		no Cq	no
M. arginini	G230	14		no Cq	no
M. fermentans	PG18	15		no Cq	no
M. orale	CH19299	12		no Cq	no
M. hyorhinis	BTS-7	15		no Cq	no

*Template 100 pg/µl gDNA

3.4.   Quantification of bacteria and normalization to host cells

In order to perform an absolute quantification, a standard curve was established to convert the Cq values into genome copy numbers (diploid cells). For this purpose, we cloned the cDNA of target genes *CEACAM18* (bovine and caprine) and *adk* (*Mmm* and *Mmc*) into plasmid vectors. The copy number of the vector in a sample was calculated from the DNA concentration and the size of the vectors. Serial dilutions of the standards were used in duplicates to determine the range in which the established qPCR was capable of a linear amplification of the sequence. As shown in Figure 4A and B, the standard curve (using bovine *CEACAM18* and *adk* of *Mmm*) is close to linear in the range of 10^2 - 10^9 copies per µl. We further determined the specificity of the different qPCRs (simplex and duplex) by using either pure gDNA isolated from the cell line (BoMac) for the *adk*-qPCRs or gDNA from Mycoplasmas (*Mmm* and *Mmc*) for the *CEACAM18* qPCRs. In none of the cases was a product amplified. Finally, the efficiency of the simplex qPCRs and the duplex qPCRs was determined. In Fig 4C the results using plasmids encoding caprine CEACAM18 and gDNA from *Mmc* (Y-Goat) are depicted. Similar results were obtained using plasmids encoding bovine CEACAM18 and gDNA from *Mmm* (Afadé) (Supplementary data 1). The efficiency of the duplex qPCRs was in the same range as the efficiency of the simplex qPCRs for both, bovine and caprine samples with the respective pathogens (Supplementary data 1).
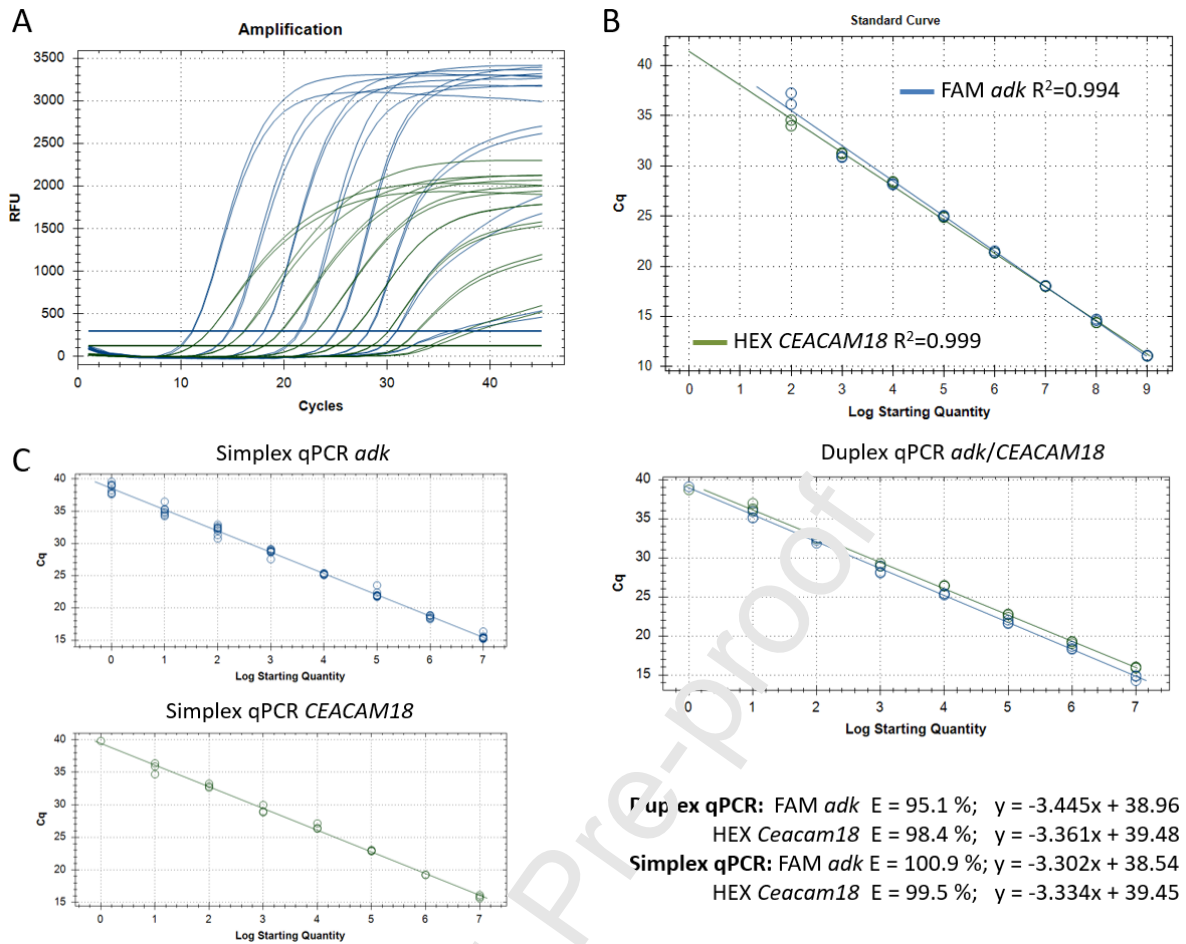
Fig. 4. Amplification of standards and calculation of the standard curve. Dilutions of the DNA standards corresponding to $10^2 - 10^9$ copies were prepared for both bovine *CEACAM18* (HEX- green) and *Mmm adk* (FAM- blue). (A) Using these serial dilutions as template the duplex qPCR was performed and the relative fluorescence units (RFU) were determined. (B) The Quantification cycle (Cq) was correlated with the copy numbers of the template. The correlation coefficient for both curves R^2 is indicated. The equations for the curves are: Cq_(*adk*) = -3.508X + 42,6 and Cq_(*CEACAM18*) = -3.354X + 41,4. (C) Comparison of simplex qPCRs (left panel) for *adk* (*Mmc* Y-Goat) and *CEACAM18* (goat) with the duplex qPCR (right panel) for the same targets. 3-5 replicates in each experiment were performed (see also Supplementary data 1).

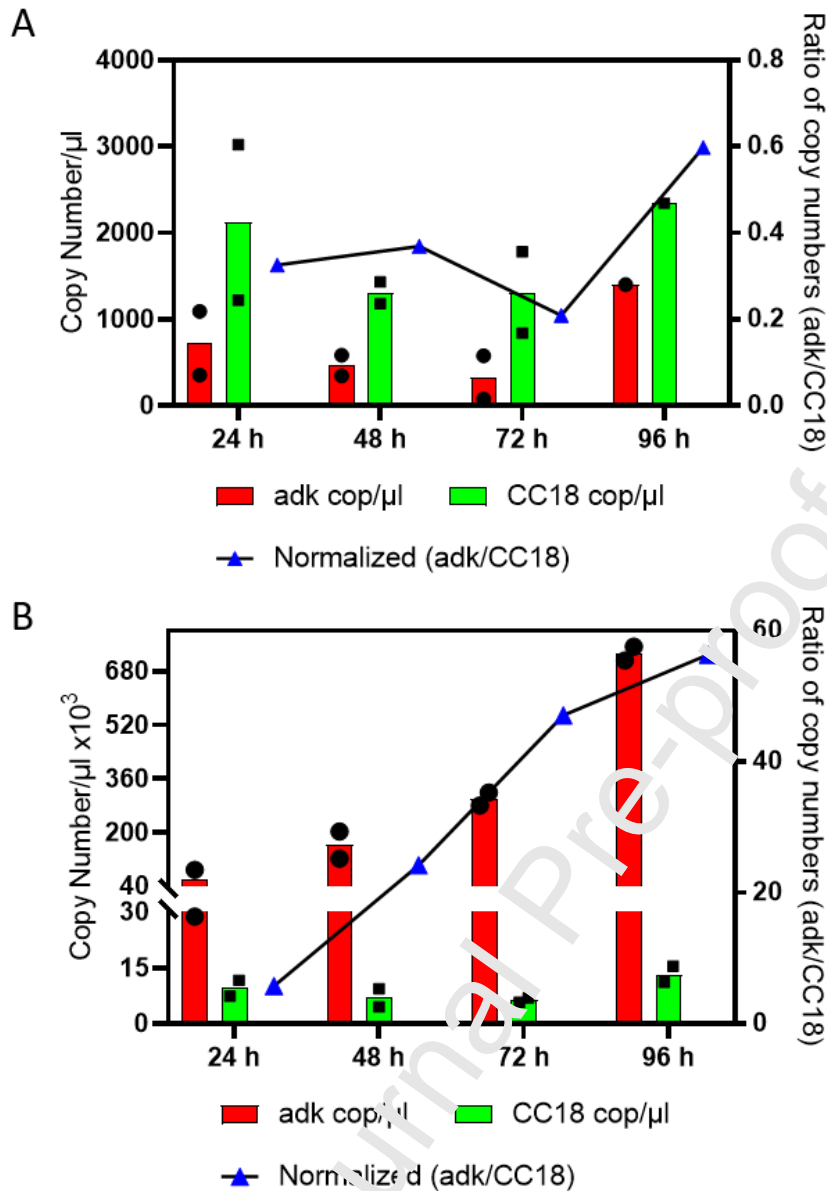### 3.5. Quantification of mycoplasma adherence to bovine cell lines

After establishing the quantification assay for *Mycoplasma* and host genomes we tested the assay in various applications. First, we analyzed the suitability of the assay to determine *Mycoplasma* association to bovine macrophages grown as monolayer. We used the BoMac cell line and incubated them with either *Mmm* or *Mmc* at various MOIs ranging from 30 – 120 for 24 h. After harvesting the surviving BoMac cells, we observed that the cell number significantly decreased with an increasing MOI of *Mmm* but not of *Mmc* (Fig. 5C). The absolute number of *Mmm* in the well reached a maximum at a MOI of 60 (Fig. 5D). This resulted in an MOI-dependent amount of *Mmm* associated with BoMac cells, while the number of *Mmc* associated with vital BoMac was independent from the MOI (Fig. 5E).
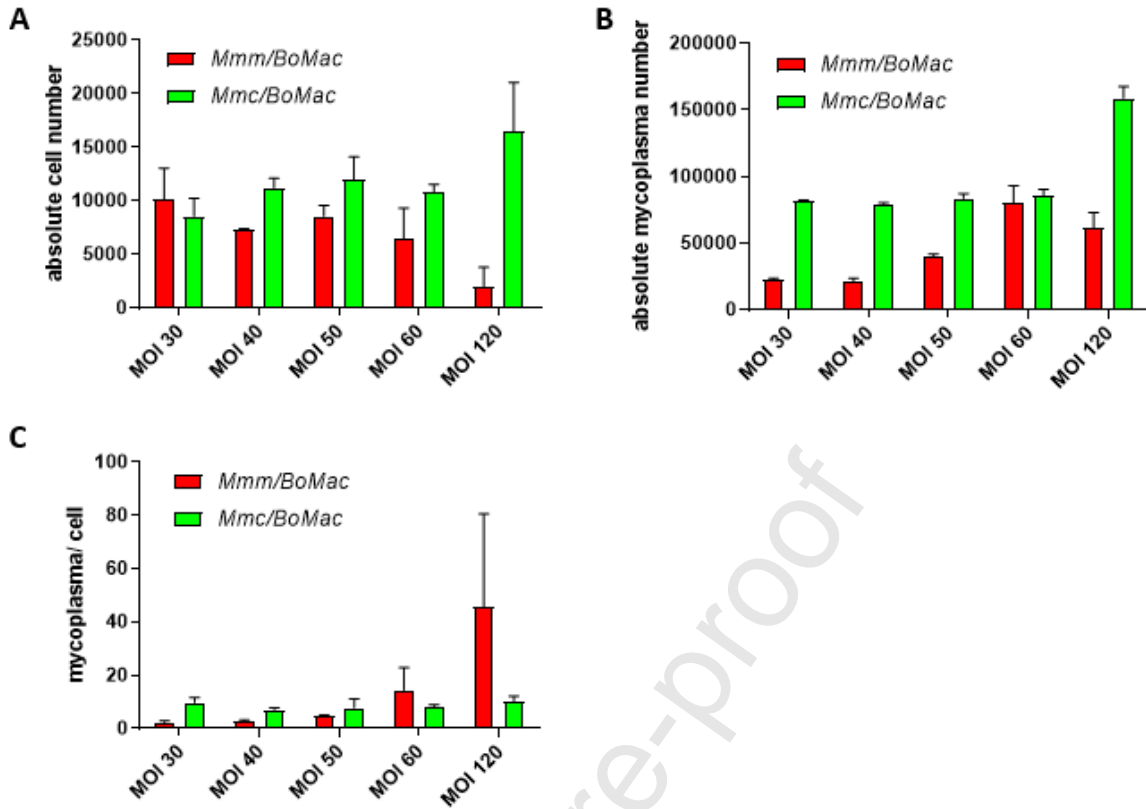
Fig. 5. Association of either *Mmm* or *Mmc* with a bovine macrophage cell line (BoMac) grown as monolayer. A. Number of surviving BoMac cells upon incubation with *Mmm* Afadé (red) and *Mmc* Y-Goat (green) for 24 h at an MOI of 30 to 120. The number of surviving BoMac cells cultured without mycoplasmas was 9377 +- 2070. **B.** Number of harvested *Mmm* Afadé (red) and *Mmc* Y-Goat (green) upon coculture with BoMac cells for 24 h at an MOI of 30 to 120. **C.** Number of *Mycoplasma* genomes per bovine cell genome after 24 h of coculture with BoMac cells at MOI between 30 and 120.

3.6. Adherence and invasion of three-dimensional tissue culture systems.

Next, we wanted to determine the usability of our qPCR assay to quantify the number of mycoplasmas interacting with lung cells in complex three-dimensional tissue culture systems. Therefore, we incubated bovine and caprine precision-cut lung slices (PCLS) with mycoplasmas for increasing times. Thereafter we isolated total DNA and determined the copy numbers of *Mycoplasma*- and host genomes. As shown in Fig. 6 the eukaryotic DNA content of the PCLS varied only marginally between individual PCLS. However, the number of mycoplasmas differed considerably. Via comparing the ratio of prokaryotic and eukaryotic genomes over time we revealed that *Mmm* associated with bovine PCLS did not change significantly during the coculture for 96 h with an average ratio of 0.5 (adk/CC18) genome copies. In contrast, the number of *Mmc* cocultured with caprine PCLS increased continuously reaching a ratio of 56 (adk/CC18) genome copies after 96 h. This experiment revealed that depending on the pathogen-host combination the ratio of them may dramatically differ during the culture period, underlining the importance of pathogen number normalization to the eukaryotic host cell for data interpretation using more advanced *in vitro* models (Fig. 6).

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Fig. 6. Quantification of Mycoplasmas in three dimensional *in vitro* models. Analysis of *Mmm* strain Afadé (Af) adhesion/invasion in bovine (A) and *Mmc* strain GM12 in caprine PCLS (B) at the indicated time points of incubation. The copy numbers of the *adk* gene are shown in red and the copy number of CEACAM18 (CC18) in green, both are depicted at the left y-axis. The right y-axis shows the ratio of the *adk* and the CC18 copy numbers for the curve marked with the blue triangles. PCLS: precision cut lung slices, cop, copies; CC18: CEACAM18 (Carcinoembryonic antigen-related cell adhesion molecule 18).

3.7.   Detection of *Mmm* in bovine tissues

Finally, we wanted to know if the number of mycoplasmas related to the *CEACAM18* copy number provides additional information compared to the relation to tissue weight or total DNA content. Comparable amounts of tissues (25 to 50 mg) from experimentally infected cattle were used for DNA isolation and aliquots of the isolated DNA were used as template in the duplex qPCR. *Mmm*-specific DNA was detected in 15 of 20 samples, 5 samples were negative for *Mmm* (Fig. 7A). *CEACAM18* copy numbers considerably differed between tissue samples, which may reflect the variation of cell content of tissues (Fig. 7A). Next, we investigated the correlation of total DNA content of tissues as measured

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by a NanoDrop photometer and the copy number of *CEACAM18* determined by qPCR (Fig. 7B). The overall correlation was R^2 = 0.71, suggesting that total DNA determination can be used as a first estimate, but using duplex qPCR provides more accurate results.
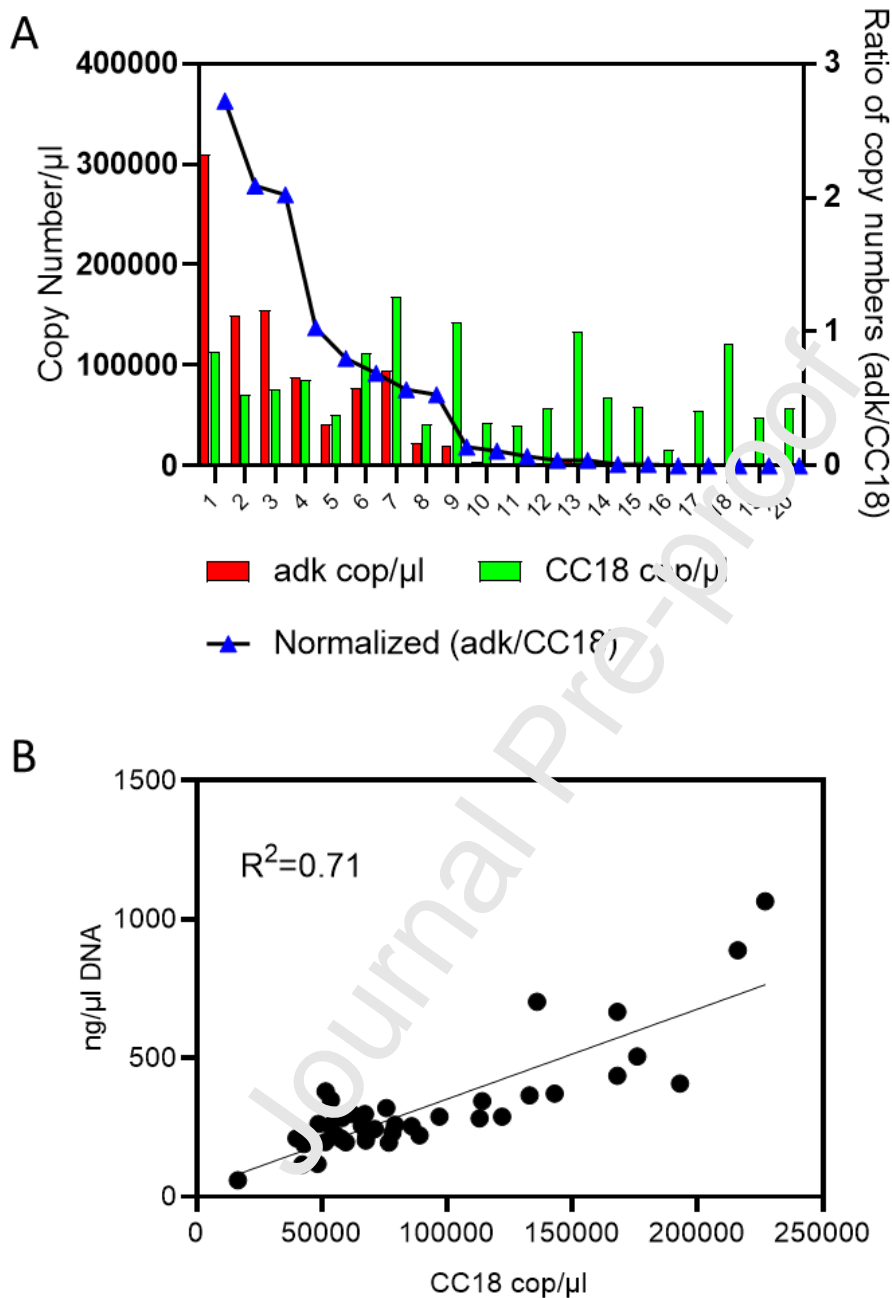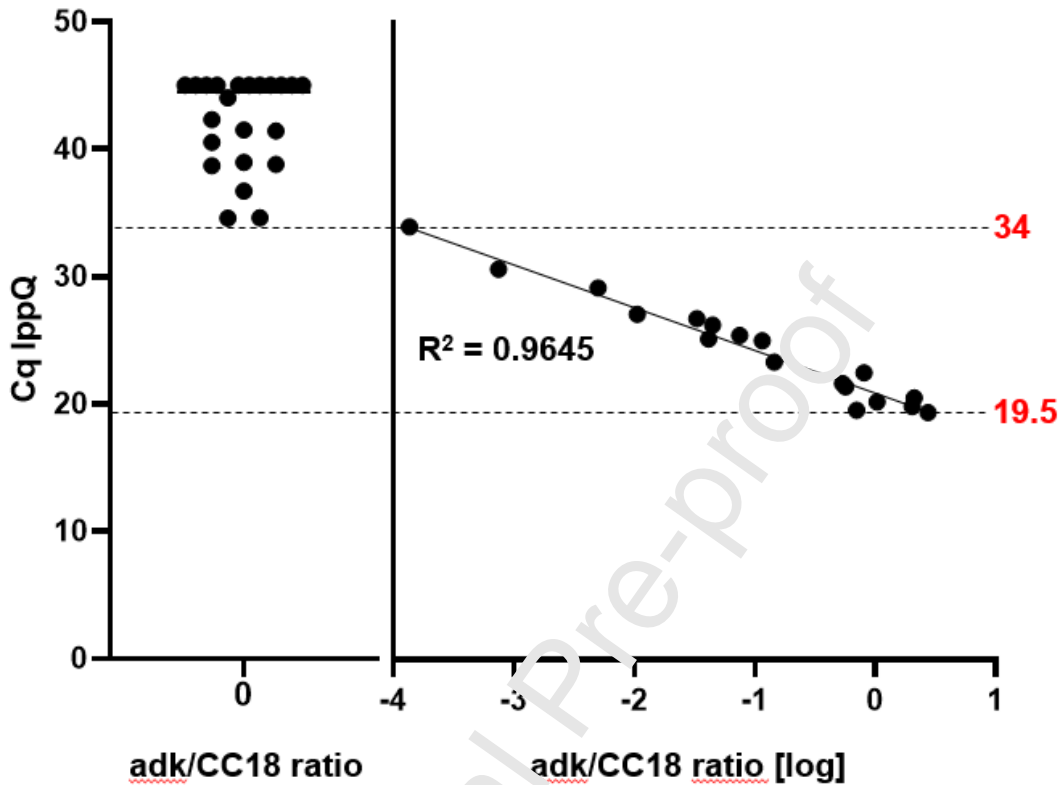

Fig. 7. Detection of *Mmm* tissues after experimental infection of cattle. (A) The number of *adk* gene copies (red) and *CEACAM18* copies (green) in lung tissues as determined by the novel duplex-qPCR compared with the ratio of the copy numbers (blue triangles). (B) Correlation between the concentration of DNA in ng/µl and the copy numbers/µl of *CEACAM18* of the lung tissues shown in A. and one sample per animal of lung draining lymph nodes (n = 40). cop, copies; *adk*, adenylate kinase; CC18, *CEACAM18*.

3.8   Comparison of the sensitivity between the *adk*/CEACAM18 and the *IppQ* qPCR.

We used the tissues described above (Fig. 7B) to compare the sensitivity of the *adk*/CEACAM18 multiplex qPCR with the previously developed *IppQ* qPCR (Fig. 8)[18]. There was a very good

correlation between both assays with a correlation coefficient of $R^2= 0.9645$ for samples with an *adk*/CC18 ratio of more than 10^{-4} and a Cq less than 34. The novel duplex qPCR did not detect *Mmm* in samples that have a Cq > 34 in the *IppQ* qPCR.

Fig. 8. Comparison of the standard *IppQ* qPCR and the TaqMan-based duplex *adk/CC18* qPCR. 40 bovine tissue samples of experimentally infected cattle (Fig. 7B) were used for the detection of *Mmm*. Cq values from standard *IppQ* qPCR (y-axis) were compared with the ratio of *adk/CEACAM18* (CC18) copy numbers (x-axis). *Mmm* in samples with a Cq > 34 in the *IppQ* qPCR were not detected by the duplex qPCR. The correlation coefficient R^2 is indicated.

4. Discussion

Host-pathogen interactions are poorly understood for mycoplasmas of the '*M. mycoides cluster*', as specific factors responsible for the strong host-specificity of these economically important pathogens are mostly unknown [26, 27]. To accelerate research in this field, we have developed a duplex qPCR that allows the determination of the absolute genome number of the closely related *Mmm* and *Mmc* as well as their number per host cell in a robust and reproducible way. Similar methods already exist for other mycoplasmas and have proven to be very useful [12-14]. Josi *et al.* used a real-time qPCR for the high throughput screening of a transposon induced mutant library of *M. bovis* in order to identify adhesion and virulence-related genes [12]. Similarly, qPCRs specific for single members as well as of the complete '*M. mycoides* cluster' were previously established [28-30]. However, from an experimental point of view the simultaneous detection of *Mmm* and its closest relative *Mmc* with the same assay would be desirable. But so far, no qPCR exists that exclusively detect *Mmm* and *Mmc* with comparable sensitivity and specificity. Neither exists a duplex qPCR for the quantification of *Mmm*/*Mmc* normalized to host cells. Normalization to host cells may be of particular interest when dynamic pathogen-host interactions or the interaction with complex tissues or cell culture systems will

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be analyzed. We chose the CEACAM18 gene from the host because the specific detection of this gene can be easily adapted to other host species, due to its favorable degree of conservation between different mammalian species, which may guarantee great experimental flexibility [31]. The PCR described here can be applied for tissues derived from *Bos taurus* as well as for *Bos indicus*. Furthermore, the sequences used to detect the *adk* gene from mycoplasmas were chosen so that the primers match all cluster members (Fig. 1), therefore, species-specificity can be adapted by sequence variations of the probe. Thus, the assay can be adapted to a variety of host-pathogen combinations. In the case of *Mmm* and *Mmc* the assay performed equally well, while cross-reactions with other mycoplasmas did not occur or can be easily recognized by an atypical curve progression (Fig. 3B). The most straight forward application of the established duplex qPCR is the determination and quantification of pathogen adherence to host cells. In these experiments, it is obvious that normalization to host cells is crucial, since the number of host cells will change during the coculture period, due to differences in toxicity of the pathogen. Thus, the number of remaining pathogens in the well may critically depend on the remaining host cells in the incubation chamber, although some adherence of pathogens to the bottom of the well could not be excluded. In the example shown, we could identify a correlation of *Mmm* associated with BoMac cells with the used MOI. In contrast, when the same BoMac cells were infected with *Mmc*, the numbers of *Mmc* were similar in each infection experiment independent of the MOI used. *In vivo*, pathogens interact with three-dimensional tissues – therefore more complex *in vitro* models such as PCLS are frequently used to study pathogen-host interaction in respiratory infectious diseases [32, 33]. However, despite their advances in tissue architecture and composition of different cell types these models have also disadvantages especially concerning methods to control similarity of tissue composition and pathogen-host cell ratio. Indeed, in our experiments we observed a significant variation of the pathogen host ratio over time as well as using different pathogens. This result indicates that the control of pathogen-host cell ratio is important for the interpretation of the results obtain with such PCLS models. We further used the assay to determine the content of *Mmm* in tissue samples obtained from experimentally infected cattle. Tissues may largely vary in respect to tissue density, tissue origin and the ratio of cellular and extracellular components. This is especially the case for healthy and inflamed lung tissue. We compared the relation of the amount of DNA used as template in the qPCR and the copy numbers determined by qPCR. Overall, we found a good correlation, however, there were also some discrepancies. These may be explained by the fact that the efficiency of the PCR is additionally controlled in the duplex qPCR, because potential PCR inhibitors, which may be part of the DNA preparation, will influence the amplification of both the host and pathogen gene. Finally, we compared the new duplex qPCR with the qPCR based on the detection of the *lppQ* gene described by Schnee *et al.* [18]. We found a very good correlation between the absolute gene copy number (new duplex qPCR) and the Cq value of the *lppQ* qPCR between Cq values of 19 to 34. Samples that resulted in a Cq value >34 in the *lppQ* qPCR were not found to be positive in the duplex qPCR, indicating that this assay is slightly less sensitive than the *lppQ* qPCR.

5. Conclusions

In the present report we describe a duplex qPCR for the simultaneous detection and absolute quantification of *Mmm* and *Mmc* in different experimental settings as well as in tissue samples. This qPCR can be applied for different tissue types from *Bos taurus* and *Bos indicus*. We did not intend to verify that the performance of the method in each application is exactly similar to each other. In contrast, validation of the method's performance by users with their own DNA preparations is highly recommended. In addition, the selection of primer and probe sequences makes the duplex qPCR adaptable to other animal species or other members of the '*M. mycoides* cluster'.

Based on this duplex qPCR, future applications may determine simultaneously the number of pathogens in the tissue and differentially expressed transcripts of disease relevant genes.

Abbreviations

adk: adenylate kinase

CEACAM18:   carcinoembryonic antigen-related cell adhesion molecule 18

EBL cells: embryonic bovine lung cells

lppQ: gene of lipoprotein LppQ of *Mmm*

qPCR: quantitative polymerase chain reaction

PCLS: Precision-Cut Lung Slices

Declarations

Ethics approval and consent to participate

All protocols of the study were designed and performed in accordance with the European Legislation for Animal Experimentation and were approved by the animal use committee of local authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Rostock, Germany; 7221.3-1-047/17).

Consent for publication

Not applicable.

Availability of data and materials

All relevant data are presented in this report.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

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Dear Editor

I would like to submit a manuscript entitled: **A new duplex qPCR-based method to quantify members of the 'Mycoplasma mycoides cluster' in complex cell culture systems and host tissues** by Hänske et al. to be considered for publication in the *Journal of Microbiological Methods*.

We declare that we do not have any conflict of interests.

Yours sincerely,

PD Dr. Robert Kammerer

Institut für Immunologie, Friedrich-Loeffler-Institut

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Highlights

- The new TaqMan-based qPCR was developed for absolute quantification of *M. mycoides*.
- The duplex qPCR quantifies *Mmm* and *Mmc* normalized to the interacting host cells.
- Assays are suitable for various *in vitro* models and tissue samples.