Development and validation of the isothermal recombinase polymerase amplification assays for rapid detection of *Mycoplasma ovipneumoniae*

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Methodology article

Keywords: *Mycoplasma ovipneumoniae*; 16SrRNA gene; real-time RPA; lateral flow strip; isothermal amplification

Posted Date: March 18th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-17865/v1>

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Version of Record: A version of this preprint was published at BMC Veterinary Research on June 1st, 2020. See the published version at <https://doi.org/10.1186/s12917-020-02387-3>.

Abstract

Background Mycoplasmal pneumonia is an important infectious disease that threatens sheep and goat production worldwide, and *Mycoplasma ovipneumoniae* is one of major etiological agent causing mycoplasmal pneumonia. It is an urgent need to develop a rapid and accurate method to detect *M. ovipneumoniae*. Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification technique, and RPA-based diagnostic assays have been described for the detection of different types of pathogens. Results The RPA assays using real-time fluorescence detection (real-time RPA) and lateral flow strip detection (LFS RPA) were developed to detect *M. ovipneumoniae* targeting a conserved region of the 16S rRNA gene. Real-time RPA was performed in a portable fluorescence scanner at 39 °C for 20 min. LFS RPA was performed in a portable metal bath incubator at 39 °C for 15 min, and the amplicons were visualized with the naked eyes within 5 min on the lateral flow strip. Both assays were highly specific for *M. ovipneumoniae*, as there were no cross-reactions with other pathogens tested, especially the *M. capricolum* subsp. *capripneumoniae*. The limit of detection of LFS RPA assay was 1.0×10^1 copies per reaction using a recombinant plasmid containing target gene as template, which is 10 times higher than the limit of detection of the real-time RPA and real-time PCR assays. The RPA assays were further validated on 46 clinical sheep nasal swab and fresh lung samples, and *M. ovipneumoniae* DNA was detected in 17 samples in the RPA assays and 19 samples in the real-time PCR assay. The real-time RPA and LFS RPA showed diagnostic specificity of 100%, diagnostic sensitivity of 89.47%, and a kappa coefficient of 0.909. Conclusions The developed real-time RPA and LFS RPA assays provide the attractive and promising tools for rapid, convenient and reliable detection of *M. ovipneumoniae*, especially in resource-limited settings.

Background

Mycoplasma ovipneumoniae is one of the major pathogens that cause mycoplasma pneumonia in sheep, goats, and wild ruminants [1–5]. *M. ovipneumoniae*-associated respiratory disease is characterized by cough, gasp, runny noses, progressive weight loss, pulmonary interstitial hyperplasia inflammation, and variable morbidity and mortality rates between flocks [6, 7]. Moreover, upon *M. ovipneumoniae* infection, sheep and goats become susceptible to other common pathogens causing respiratory disease, such as *Mannheimia haemolytica*, *Pasteurella multocida* and *Parainfluenza-3* virus [8, 9]. Since first confirmed in Australia in 1972, infections by *M. ovipneumoniae* have been an endemic problem worldwide and have caused severe economic losses to the sheep and goat industry [10–12]. It is an urgent need to develop a rapid and accurate method to detect *M. ovipneumoniae*.

Bacteriological culture of *M. ovipneumoniae* is currently the gold standard for diagnosis, however, the culture is cumbersome and time-consuming due to the fastidious nature of the bacterium as well as that the follows required species identification by biochemical, serological or molecular tests, which make the assay burdensome for the routine applications [13–15]. In addition, the bacterial isolation may be hampered by sample contamination and prior antibiotic treatments received by the diseased animals. Serological tests, such as ELISA, Indirect hemagglutination assay, are the most common, economic, and

in general a convenient method for *M. ovipneumoniae* herd surveillance [12, 16]. However, seroconversion to *M. ovipneumoniae* is often delayed after natural infection, which makes the serology less effective in detecting early-stages of infection in herds, and unsuitable for detecting acute mycoplasmal pneumonia in the field [9, 14]. Different nucleic acid amplification-based methods have been described to be sensitive and specific for *M. ovipneumoniae*, i.e. PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP) [9, 14, 15]. PCR assays require a well-equipped laboratory, expensive equipment, and trained personnel, which limits their application in the under-equipped laboratories and the point-of-need (PON) diagnosis [9, 15]. Compared to the PCR assays, the isothermal amplification methods have advantages regarding convenience, minimal equipment requirement, and rapid results. A LAMP assay for the detection of *M. ovipneumoniae* has been described for low requirement of experimental conditions, however, the assay requires 60 min to complete the reaction [14].

Recombinase polymerase amplification (RPA), an isothermal DNA amplification technique, is rapid, reliable and considered to be an alternative approach for PON diagnosis [17, 18]. RPA-based diagnostic assays have been described for the detection of different types of pathogens from different clinical samples [19, 20]. In this study, a real-time RPA assay using the *exo* probe and a LFS RPA assay using the *nfo* probe combined with lateral flow strip were developed for rapid, specific and sensitive detection of *M. ovipneumoniae*. The performance of the assays was further assessed by collecting and detecting the clinical sheep nasal swab and lung samples.

Results

Analytical specificity and sensitivity of the RPA assays

Specific amplification was only observed with *M. ovipneumoniae*, and there was no cross-reactions of other pathogens tested in both real-time RPA and LFS RPA assays (Fig.1 A, B). Five independent reactions were repeated and similar results were observed, demonstrating the good repeatability of the assays.

The limit of detection of LFS RPA assay was 1.0×10^1 copies *M. ovipneumoniae* standard DNA per reaction (Fig.2A), and the LOD of real-time RPA was 1.0×10^2 copies per reaction (Fig.2B), which was same as that of the real-time PCR (data not shown). The real-time RPA assay was further performed eight times on the molecular standard, in which 1.0×10^7 - 1.0×10^2 copies DNA molecules were detected in 8/8 runs, 1.0×10^1 - 1.0×10^0 , 0/8, which demonstrated the good reproducibility (Fig. 3).

3.2 Validation of the RPA assays on clinical samples

Of the 46 clinical samples, 17 (36.95%) and 19 (41.30%) samples were positive for *M. ovipneumoniae* by the RPA and real-time PCR, respectively (Table 2). Compared to real-time PCR, the real-time RPA and LFS RPA assays showed diagnostic specificity (DSp) of 100%, diagnostic sensitivity (DSe) of 89.47%, positive predictive value (PPV) of 100%, negative predictive value (NPV) of 93.10%, and kappa value of 0.909 (Table 3). The real-time RPA and the LFS RPA demonstrated the same performance in detecting the 46

clinical samples. It took less than 20 min in the RPA assays to obtain the positive results, and only the portable while it took 32 - 46 min in the real-time RT-PCR with the Ct values ranging from 20.77 to 36.49.

Discussion

The two developed real-time RPA and LFS RPA assays for detection of *M. ovipneumoniae* demonstrated to be rapid, specific, sensitive, and easy to perform. Both RPA assays performed well at 39 °C within 20 min. The real-time RPA assay and LFS RPA assay were performed on the tube scanner Genie III and a metal bath incubator, respectively. These two pieces of equipment are portable and can be charged by battery for working a whole day. Moreover, RPA reagents are cold chain independent and RPA is tolerant to most of the PCR inhibitors [19, 21]. The above characteristics make the developed RPA assays ideal for PON detection of *M. ovipneumoniae*, which is especially important for farms located in rural areas.

Previous studies had demonstrated the efficacy of the PCR and LAMP to detect 16S rRNA and other conserved regions of genomic DNA of *M. ovipneumoniae* in different clinical specimens, including the nasal swabs and lung samples [4, 9, 14, 15]. In this study, the RPA primers and probes were also designed based on the 16S rRNA gene. To ensure that the target sequences were unique to *M. ovipneumoniae*, we screened the selected primers and probes in silico using the pattern searching tool function from the EMBOSS package against the genomes of the common mycoplasmas causing infections in ruminants [22]. We were unable to find complementary regions when allowing 1 or 5 sequence mismatches for the primer sequences. Furthermore, there was no mismatch in the reverse primers and probes in the *M. ovipneumoniae* strains available in Genbank, only one mismatch in the forward primers in the strains 2013-12928-46 (MN028079) and NCTC10151 (LR215028.1). According to the above in silico analysis, the designed primers and probes fulfilled the specificity requirements [23]. In the specificity analysis, both the real-time RPA and LFS RPA only amplified the genomic DNA of *M. ovipneumoniae*, and no other mycoplasmas, bacteria and PPRV. Most importantly, *M. capricolum* subsp. *capripneumoniae*, the etiological agent of contagious caprine pleuropneumonia, was not amplified by the new developed RPA assays. Although the in silico sequence analysis support that all the *M. ovipneumoniae* strains are detectable, it should be further confirmed by testing more genomic DNA of different strains of *M. ovipneumoniae*.

The diagnostic performances of the developed RPA assays were evaluated and compared to a real-time PCR assay in this study. The real-time RPA and LFS RPA demonstrated the same diagnostic performance with clinical samples. Seventeen and 19 samples were positive for *M. ovipneumoniae* in the RPA and real-time PCR, respectively. Only two nasal samples showed discrepant results, which were negative in RPA assays and positive in real-time PCR with Ct values of 36.49 and 35.50. Compared to real-time PCR, the real-time RPA and LFS RPA assays showed DSp of 100%, DSe of 89.47%, PPV of 100%, NPV of 93.10%, and kappa value of 0.909. Although the above results are inspiring, the RPA assays need be further validated by testing of more *M. ovipneumoniae* DNA positive clinical samples.

Conclusions

In this study, we describe the development of the real-time RPA and LFS RPA assays for the simple, rapid and reliable detection of *M. ovipneumoniae* directly from the sheep nasal and lung samples. The developed RPA assays could be performed in field conditions without the need of any expensive equipment, and could also become a routine test for rapid and direct detection of *M. ovipneumoniae* in the farm.

Methods

Bacteria, virus strains, clinical samples and DNA extraction

Genomic DNA of *M. Ovipneumoniae* (Y98) and genomic DNA or cDNA of a panel of other close related pathogens considered dangerous to sheep and goats were maintained in our laboratory and used in the study, which were the following 4 mycoplasmas, 2 non-mycoplasma bacteria and 1 virus: *M. capricolum subsp. capripneumoniae* (F38), *M. mycoides subsp. capricolum* (PG3), *M. arginini* (G230), *M. agalactiae* (PG2), *Klebsiella pneumoniae* (strain F21W3), *Pasteurella multocida* (strain F91G3) and *Peste des petits ruminants virus* (Nigeria 75/1 vaccine strain).

A total of 46 sheep clinical samples (30 nasal swabs and 16 fresh lungs) were collected in Baoding City, Hebei Province from October to November 2019. The nasal swabs were collected from the sheep with coughing symptom in Fangzhuang farm in Dingzhou County, Baoding City, and the sheep fresh lungs were obtained from Zhuanluzhen slaughter house in Tang County, Baoding City. The sheep nasal swabs and lung samples were treated and the total DNA was extracted as described previously [24]. All DNA were quantified using a ND-2000c spectrophotometer (NanoDrop, Wilmington, USA) and stored at -80 °C until use.

Generation of standard DNA

To generate a *M. Ovipneumoniae* standard DNA for the RPA assays, a PCR product containing 361 bp covering the region of interest of 16SrRNA gene was amplified from the *M. Ovipneumoniae* DNA using LMF1 and LMR1 as primers (Table 1) and cloned into the pMD19-T (Takara, Dalian, China) for standards. The resulting plasmid, pMO-16SrRNA, was transformed into *Escherichia coli* DH5α cells, purified with the SanPrep Plasmid MiniPrep Kit (Sangon Biotech, Shanghai, China) and quantified using a ND-2000c spectrophotometer. The copy number of DNA molecules was calculated by the following formula: amount (copies/μL) = [DNA concentration (g/μL)/ (plasmid length in base pairs×660)] ×6.02×10²³. Ten-fold dilutions of the pMO-16SrRNA, ranging from 1.0×10⁷ to 1.0×10⁰copies/μL, were prepared in nuclease-free water and aliquots of each dilution were stored at -80 °C.

RPA primers and probe

Nucleotide sequence data for different *M. ovipneumoniae* strains available in GenBank were aligned to identify the conserved regions in the 16SrRNA gene, which was determined as the molecular target for RPA. According to the reference sequences of *M. ovipneumoniae* (Accession numbers: NR_025989.1,

LR215028.1, MN028361, MN028184, MN028079, MH133233), the primers, exo and nfo probes were designed following RPA manufacturer guidelines (TwistDx. Cambridge, UK). Primers and probe are listed in Table 1 and were synthesized by Sangon Biotech Co., Shanghai, China.

Real-time RPA and LFS RPA assays

The *M. ovipneumoniae* real-time RPA assay was performed as described previously [24]. The total reaction volume was 50 µL including 40.9 µL of Buffer A (rehydration buffer), 2.0 µL of each RPA primers (MO-exo-F and MO-exo-R, 10 µmol/L), 0.6 µL of exo probe (MO-exo-P, 10 µmol/L) and 2.5 µL of Buffer B (magnesium acetate, 280 mmol/L). Furthermore, 1 µL of genomic DNA or recombinant plasmid was used for the specificity and sensitivity analysis, or 2 µL of sample DNA was used for the clinical sample diagnosis.

The *M. ovipneumoniae* LFS RPA assay were performed as described previously [24]. The total reaction volume was 50 µL including 29.5µL of rehydration buffer, 2.1 µL of each RPA primers (MO-nfo-F and MO-nfo-R, 10 µmol/L), 0.6 µL of exo probe (MO-nfo-P, 10 µmol/L) and 2.5 µL of magnesium acetate (280 mmol/L). In addition, 1 µL of bacterial genomic DNA or recombinant plasmid was used for the specific and sensitive analysis, or 2 µL of sample DNA was used for the clinical sample diagnosis. The assay was performed in a metal bath incubator at 39 °C for 15 min. Furthermore, the lateral flow strips (Milenia Biotec GmbH, Germany) were used to detect the RPA amplicons dual-labeled with FAM and biotin.

Analytical specificity and sensitivity analysis

Both RPA assays were performed to amplify the nucleic acids of a panel of pathogens including *M. Ovipneumoniae*, *M. capricolum subsp. capripneumoniae*, *M. mycoides subsp. capricolum*, *M. arginini*, *M. agalactiae*, *P. multocida*, *K. pneumoniae*, PPRV, which are considered to be dangerous to the sheep and goat respiratory system. The analytical specificity analysis was repeated five times.

The standard DNA of *M. Ovipneumoniae*, ranging from 1.0×10^7 to 1.0×10^0 copies/µL, was prepared in nuclease-free water and used for the RPA analytical sensitivity analysis. One microliter of each dilution was amplified by both RPA assays to determine the limit of detection (LOD). The analytical sensitivity analysis was repeated five times. Furthermore, the real-time RPA was tested using the standard DNA in 8 replicates, the threshold time was plotted against the molecules detected and a semi-log regression was calculated using Prism software 5.0 (Graphpad Software Inc., SanDiego, California).

Validation with clinical samples

The real-time RPA assay was assessed with 30 sheep nasal swabs and 16 sheep fresh lungs. All samples tested with the two RPA assays were also tested by a real-time PCR in parallel. The real-time PCR for *M. oviopneumoniae* was performed on a ABI 7500 instrument (Applied Biosystems, Foster City, California) described previously [4].

Declarations

List of abbreviations

CT: Cycle threshold; DSp: diagnostic specificity; DSe: diagnostic sensitivity; ELISA: enzyme linked immunosorbent assay; K. pneumonia: Klebsiella pneumoniae; LAMP: loop-mediated isothermal amplification; LFS: lateral flow strip; M. agalactiae: Mycoplasma agalactiae; M. arginini: Mycoplasma arginini; M. capricolum subsp. capripneumoniae: Mycoplasma capricolum subsp. capripneumoniae; M. mycoides subsp. capricolum: Mycoplasma mycoides subsp. capricolum; M. ovipneumoniae: Mycoplasma ovipneumoniae; NPV: negative predictive value; PCR: polymerase chain reaction; P. multocida: Pasteurella multocida; PON: Point-of-need; PPRV: Peste des petits ruminants virus; PPV: positive predictive value; RPA: Recombinase polymerase amplification; TT: Threshold time.

Ethics approval and consent to participate

The sheep nasal swabs and sheep fresh lungs used in this study were collected in a sheep husbandry farm and a slaughter house, respectively. The written consents for the use of the samples before participation in the study were obtained from the farmer and the slaughter house's owner. This study was approved by the Institutional Animal Care and Ethics Committee of Hebei Agricultural University (approval no. IACECHEBAU20110509).

Consent for publication

Not applicable.

Availability of data and materials

The dataset analyzed during the current study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Project for Key Common Technologies for High Quality Agricultural Development of Hebei Province (19226636D), and the Earmarked Fund for Hebei Sheep and Goat Innovation Team of Modern Agro-industry Technology Research System (HBCT2018140204). The funding agencies had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Authors' contributions

JCW and WZY conceived and designed the study. JFW and RWL developed the real-time RPA and LFS RPA assays and analyzed the data. XXS, LBL and XPH performed the clinical samples testing, helped in the data analysis and manuscript revision. JCW and WZY wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank the laboratory staff in the animal hospital of Agricultural University of Hebei and the staff in the Hebei Animal Disease Control Center for samples collection.

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Tables

Due to technical limitations, Tables 1 - 3 are only available for download from the Supplementary Files section.

Figures

Figure 1

Analytical Specificity of *M. Ovipneumoniae* real-time RPA (A) and LFS RPA (B) assays. Only the *M. ovipneumoniae* was amplified, but not other pathogens tested (n=5). lane 1, *M. ovipneumoniae*; lane 2, *M. capricolum* subsp. *capripneumoniae*; lane 3, *M. mycoides* subsp. *capricolum*; lane 4, *M. arginini*; lane 5, *M. agalactiae*; lane 6, *P. multocida*; lane 7, *K. pneumoniae*; lane 8, PPRV.

Figure 2

Analytical Sensitivity of *M. Ovipneumoniae* real-time RPA (A) and LFS RPA (B) assays. The LOD of the real-time RPA was the same as that of the LFS RPA, 1.0×10^2 copies per reaction of *M. ovipneumoniae* standard DNA. lane 1, 1.0×10^7 copies; lane 2, 1.0×10^6 copies; lane 3, 1.0×10^5 copies; lane 4, 1.0×10^4 copies; lane 5, 1.0×10^3 copies; lane 6, 1.0×10^2 copies; lane 7, 1.0×10^1 copies; lane 8, 1.0×10^0 copies.

Figure 3

Reproducibility of *M. Ovipneumoniae* real-time RPA assay. The analytical sensitivity was determined on DNA molecular standard (8 runs) for real-time RPA. Semi-logarithmic regression of the data collected from real-time RPA test runs on the DNA molecular standards using Prism Software. The run time of the real-time RPA was between 4 min-13 min for 1.0×10^7 - 1.0×10^2 copies *M. ovipneumoniae* standard DNA.

Supplementary Files

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