NOTE *Bacteriology*

A Real-Time PCR for Detection and Quantification of *Mycoplasma ovipneumoniae*

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ABSTRACT. A real-time PCR for detection and quantification of *M. ovipneumoniae* was developed using 9 recently sequenced *M. ovipneumoniae* genomes and primers targeting a putative adhesin gene *p113*. The assay proved to be specific and sensitive (with a detection limit of 22 genomic DNA) and could quantify *M. ovipneumoniae* DNA over a wide linear range, from 2.2×10^2 to 2.2×10^7 genomes.
KEY WORDS: *Mycoplasma ovipneumoniae*, quantification, real-time PCR

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Mycoplasma ovipneumoniae has been associated with non-progressive pneumonia in sheep, goats and some wild small ruminants [2, 4, 13]. While *M. ovipneumoniae* can cause pneumoniae in its own right, it can predispose the hosts to invasion by other pathogens, such as *Mannheimia haemolytica* [4], which may enhance the pathological process.

Traditional microbiological culture techniques for isolation and identification of *M. ovipneumoniae* are cumbersome and usually can take up to 2 weeks for completion due to slow growth rate and the fastidious nutritional requirements of *M. ovipneumoniae*. Culture-based diagnosis is thus rarely undertaken routinely, which is often performed at highly specialized laboratories. Although conventional PCR [11] for *M. ovipneumoniae* detection has been developed and widely used in diagnosis and epidemiological investigation of *M. ovipneumoniae* infection [2, 4, 12, 13], tradictional PCR results are merely binary and can not quantify *M. ovipneumoniae* load in samples. Traditional culture techniques for Mycoplasma spp, such as plate-counting method, can also be quantitative; however, mycoplasmas grow slowly in culture medium yielding very small colonies after one week of incubation. Validated diagnostic assays for accurate and rapid quantification of *M. ovipneumoniae* are needed.

Real-time quantitative PCR (qPCR) is a reliable method for quantification of both DNA and RNA molecules in samples and is often more sensitive to conventional PCR assays. Recently, a number of real-time PCR assays have been described for a variety of mycoplasmas [1, 3, 6, 7, 9, 10]. Here, we reported the development and validation of a SYBR Green based qPCR assay specific and sensitive for *M. ovipneumoniae* DNA.

The first step in developing the qPCR was to identify genes common to referent *M. ovipneumoniae* strains and absent in other mycoplasmas. As we previously sequenced the genomes of 9 *M. ovipneumoniae* strains, including the field strain SC01 [16], type strain Y-98 and other 7 isolates (Table 1), we first identified orthologous genes among those 9 fully sequenced genomes using OrthoMCL version 2.0 [8]. Then, we used MUSCLE v3.7 [5] to perform a multiple sequence alignment of conserved genes. BLAST was then use to exclude genes with high homology with genes present in other microorganisms. Subsequently, a manual screening was performed to select genes with highly conserved regions among the 9 strains and further tested them as targets for subsequent primer design using Beacon designer 7.0 (PREMIER Biosoft International, Palo Alto, CA, U.S.A.). BLAST was then used to exclude primers with high homology for other microorganisms, including mycoplasmas. The sequence that was specific for *M. ovipneumoniae in silico* and that had the highest score attributed by the software was chosen for primer development for the real-time PCR. The primers that were deemed appropriate and finally selected for conducting real-time PCR were P113F (5′-TCTCCCAGATGATGCTAACC-3′) and P113R (5′-TGAAAATCAACTGGTCTAA-3′), which amplifies a 295bp fragment of the *p113* gene coding for a putative adhesin P113 [17].

The bacterial strains used as referents to test the performance of the real-time PCR primers and the protocol, are listed in Table 2. Mycoplasmas were propagated in Hayflick broth containing 20% horse serum, whereas other bacterial strains were grown in Trypticase Soy broth. Clinical samples were also collected from animals that exhibited symptoms, such as coughing, nasal discharge and dyspnea, to test the performance of the qPCR. Clinical samples included 98 nasal swabs and 28 lung tissue samples collected from 4 goat herds from the Sichuan province, China. Nasal swabs were swirled in 1 m*l* PBS, which was then centrifuged at 10, 000 g for 30 min to harvest the precipitate for DNA extraction.

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Table 1. *Mycoplasma ovipneumoniae* strains used for primer design and accession numbers

Strain	GenBank ID	Host	Sample type	Country	Source
Y-98	JOTL00000000	sheep	Lung	U.S.A.	CVCC[a)]
SC01	AFHO00000000	goat	Lung	China	SWUN[b)]
TC-1	JOTE00000000	goat	Nasal swab	China	SWUN
TC-2	JOTF00000000	goat	Lung	China	SWUN
TC-3	JOTG00000000	goat	Nasal swab	China	SWUN
TC-4	JOTH00000000	goat	Lung	China	SWUN
TC-5	JOTI00000000	goat	Nasal swab	China	SWUN
TC-7	JOTJ00000000	goat	Lung	China	SWUN
TC-8	JOTK00000000	goat	Nasal swab	China	SWUN

a) China Veterinary Culture Collection Center; b) Southwest University for Nationalities (our lab collection).

Table 2. Mycoplasmas and other bacterial strains used to test the performance of the developed qPCR for *Mycoplasma ovipneumoniae*

Species	Strain	Source	Real-time PCR
M. ovipneumoniae	Y-98	CVCC[a)]	+
	SC01[e)]	Lab[b)]	+
	TC-1[e)]	Lab	+
	TC-2[e)]	Lab	+
	TC-3[e)]	Lab	+
	TC-4[e)]	Lab	+
	TC-5[e)]	Lab	+
	TC-7[e)]	Lab	+
	TC-8[e)]	Lab	+
	JY1001[e)]	Lab	+
	JY1002[e)]	Lab	+
	LZ1001[e)]	Lab	+
	LZ1002[e)]	Lab	+
	CD1001[e)]	Lab	+
	CD1002[e)]	Lab	+
	QH0145[f)]	Lab	+
	QH0146[f)]	Lab	+
	QH0214[f)]	Lab	+
	QH0215[f)]	Lab	+
	GS1001[f)]	Lab	+
	GS1002[f)]	Lab	+
	XJ1001[f)]	Lab	+
M. capricolum subsp. *capripneumoniae*	87001	CVCC	–
	87002	CVCC	–
M. mycoides subsp. *mycoides* Large Colony	Y-goat	CVCC	–
M. putrefaciens	GM1	ATCC	–
M. mycoides subsp. *capri*	PG3	CVCC	–
M. agalactiae	PG2	CVCC	–
M. arginini	G230	CVCC	–
M. bovis	CD-2	Lab	–
	CQ20	SWU[c)]	–
M. conjunctivae	HRC/581T	ATCC	–
	Mc1001	Lab	–
	Mc1002	Lab	–
Mycoplasma capricolum subsp. *capricolum*	California kid	Xie[d)]	–
Staphylococcus aureus	ATCC 6538	CVCC	–
Mannheimia haemolytica	ATCC 31611	ATCC	–
Pasteurella multocida	SC-3	Lab	–
Escherichia coli	013	Lab	–

a) China Veterinary Culture Collection Center; b) Our Laboratory Collection; c) Southwest University, China; d) Genomic DNA, kindly provide by Dr Xiulan Xie at Research Center of Grass and Livestock, Ningxia Academy of Agriculture and Forestry Sciences, China; e) *M. ovipneumoniae* isolates from goats; f) *M. ovipneumoniae* isolates from sheep.

Lung tissues were homogenized in PBS for DNA extraction. Pure DNA was extracted from bacterial cultures, nasal swabs and lung tissues by using a commercial Genomic DNA Isolation Kit (Foregene, Chengdu, China) following the manufacturer's instruction.

All real-time PCRs were performed on an ABI 7300 Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, U.S.A.) using SYBR® Premix Ex Taq™ Kit (TaKaRa, Otsu, Japan). The 20μl reaction mixture consisted of 10 μl of SYBR premix, 2 μl of DNA, 0.5 μl of each primer (10 μM) and 7 μl of deionized water. The cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec and ending with a melting curve analysis at temperatures ranging from 65°C to 95°C.

In order to rule out the false negative results due to DNA losses during DNA extraction and PCR inhibitor in the template, we used duck enteritis virus DNA as an internal control (IC) [15] to evaluate DNA extraction and PCR efficiency as previously described for other mycoplasmas and IC [10]. Ten *M. ovipneumoniae* broth cultures, 10 nasal swabs and 10 lung tissues were used for this experiment. In brief, duck enteritis virus DNA was added to the lysis buffer L1 (20, 000 DNA copies per m*l* of buffer) of the Foregene DNA Isolation Kit prior to DNA extraction. The fixed amount of this internal control had been calculated to yield a mean C_T value of 27.81 with an SD of 0.86 as calculated from 30 independent runs. qPCR for IC detection was carried out using primers Dev-F 5′-CTCTACGCAGCTTTTGACGATTT-3′, Dev-R 5′-AGAAACATACTGTGAGAGTGACGA-3′ and TaqMan probe 5′-6-FAM- CCTCCTCCTCGCTGAGTGGCATCC-TAMRA-3′ as described [15]. The IC was detected in all tested samples with qPCR C_T values below 29.86, indicating minimal and acceptable DNA losses during DNA extraction and the absence of PCR inhibitors with our qPCR amplification protocol.

To construct a standard curve and determine the detection limit of the assay, *M. ovipneumoniae* SC01 genomic DNA was used as a quantification standard. The genomic DNA was quantified by measuring the optical density at 260 nm using a DU 800 spectrophotometer (Beckman Coulter, Brea, CA, U.S.A.). The amount of genomic DNA was then calculated based on the genome size (1,020,601 bp). Subsequently, 10-fold serial dilutions of *M. ovipneumoniae* SC01 genomic DNA were prepared and tested using real-time PCR. Three replicates were tested for each concentration 10-fold dilution. The genome copy numbers in each reaction and their corresponding C_T values were used to plot the standard curve (Fig. 1). The detection limit was found to be 22 genomic DNA copies, and a wide linear dynamic range (2.2×10^2 to 2.2×10^7 genomes) was established. The linear correlation between the C_T values and the logarithm of the DNA copy number was higher than 0.994.

The serial dilutions were simultaneously tested using a gel-based method based on 16S rDNA gene PCR and a conventional PCR protocol as described [11] to determine the level of sensitivity of our qPCR assay with respect to conventional PCR. Results indicated that our *p113* gene qPCR

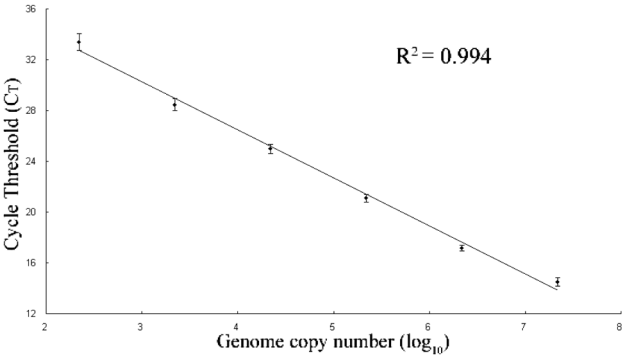

Fig. 1. Correlation between C_T values and genome copy numbers for *M. ovipneumoniae* reference strain SC01. Error bars indicate triplicate testing.

was able to detect *M.ovipneumoniae* three dilutions earlier (1,000 times more sensitive) compared to the gel-based 16S rDNA PCR for which the detection limit was of 2.2×10^4 copies of genomic DNA.

The specificity of the primers P113F/P113R was tested using DNA from 35 mycoplasma strains and 4 bacteria strains listed in Table 2. The qPCR successfully detected *M. ovipneumoniae* and yielded negative results as expected for the other mycoplasmas and bacteria tested. Melting curve analysis confirmed the presence of a single specific melt peak for each amplicon of *M. ovipneumoniae* strains. The melting temperature was calculated to be 79.6°C ± 0.4°C. These results suggested that the qPCR was highly specific for *M. ovipneumoniae* detection and could therefore be used to discriminate *M. ovipneumoniae* from other bacteria, including *Mannheimia haemolytica*, *Pasteurella multocida* and members of the *Mycoplasma mycoides* cluster, which are causative agents of respiratory illnesses in sheep and goats.

To evaluate the use of qPCR in clinical samples, DNA from 98 nasal swabs and 28 lung samples was tested using both the *M. ovipneumoniae* qPCR and the conventional 16S rDNA PCR assay. The qPCR showed positive results for 59.2% (58/98) nasal swabs and 75% (21/28) lung tissues, whereas 16S rDNA-based PCR showed a lower rate of positive results (nasal swabs 38.8%, 38/98; lung tissues 42.9%, 12/28). The qPCR detected *M. ovipneumoniae* in all samples that tested positive by the 16S rDNA PCR assay, indicating good agreement and a higher detection ability with the qPCR for both types of samples (nasal swabs, $P<0.01$; lung tissues, $P<0.05$). This newly developed qPCR assay might have higher clinical sensitivity than previously described gel-based PCR technique.

To confirm the PCR accuracy and exclude the possibility of false-positive amplifications during qPCR, 10 PCR amplicons (5 from nasal swabs and 5 from lungs) were selected, cloned into pMD-T vector (TaKaRa) and sequenced. Sequencing showed that all amplicons were from *M. ovipneumoniae* DNA with a sequence similarity of 95.8–100% compared to reference strain Y-98.

Here, we report the development and validation of a qPCR protocol for the detection and quantification of *M. ovipneumoniae*. The quantitative detection of mycoplasmas via qPCR is valuable, because this approach overcomes the challenges associated with conventional methods. Traditional titration techniques, such as the plate-counting method, which are used for quantitation of bacterial cultures, are impractical for quantitation of mycoplasmas owing to the slow growth and small size of colonies in solid agar surfaces. The qPCR assay presented in this study was shown to be linear over a broad range of 2.2×10^2 to 2.2×10^7 DNA copies with a high correlation coefficient supporting the role of our protocol to accurately quantitate *M. ovipneumoniae* in the samples.

Sensitive detection of mycoplasmas is essential for the diagnosis of mycoplasma infections. The qPCR developed in this study was 1,000 times as sensitive as the existing conventional 16S rDNA-based PCR method. The increased sensitivity of this assay would enable detection of *M. ovipneumoniae* in infected animals with low mycoplasma loads. Although it has been recommended that test sensitivity for conventional PCR can be increased for diagnostic purposes by culture enrichment of clinical specimens [14], such step would not be necessary with a more sensitive qPCR method. Despite the feasibility, enrichment of samples can be cumbersome, because of the fastidious nature and nutritional requirements.

In conclusion, we developed a highly sensitive and rapid SYBR Green-based real-time PCR assay that specifically detects *M. ovipneumoniae.* This assay could be a valuable tool for detection and quantification of *M. ovipneumoniae* and could have applications in clinical diagnostics and epidemiological studies.

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