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Research Article

Establishment and Application of a Real-time, Duplex PCR Method for Simultaneous Detection of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*

Yuzi WU [1,2] Hassan Z. A. ISHAG [3] Lizhong HUA [1] Lei ZHANG [1] Beibei LIU [1] Zhenzhen ZHANG [1] Haiyan WANG [1] Yanna WEI [1] Zhixin FENG [1] Hafizah Yousuf CHENIA [2] Guoqing SHAO [1] Qiyan XIONG [1]

[1] Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences· Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture· National Center for Engineering Research of Veterinary Bio-Products, Nanjing 210014 CHINA
[2] Discipline: Microbiology, School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, SOUTH AFRICA
[3] College of Veterinary Science, University of Nyala, Nyala, SUDAN

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Abstract

The objective of this study was to develop a TaqMan probe-based, sensitive, specific duplex real-time PCR assay for simultaneous detection of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*. The specific primers and probes, labeled with FAM and Texas Red, respectively, were designed to amplify the *p97* gene of *M. hyopneumoniae* and *p37*gene of *M. hyorhinis*. The duplex real-time PCR reaction mixtures were established and optimized and the sensitivity, specificity and reproducibility of the assay were assessed. The sensitivity of the duplex real-time PCR was found to be 10 copies/μL for both *M. hyopneumoniae* and *M. hyorhinis,* respectively. There was no cross reaction with other common viral and bacterial pathogens. The concentration of standard coefficient of variation of Ct values was less than 5%, indicating a good reproducibility. Clinical samples (n = 937) were tested by the duplex real-time PCR assay, including broncho-alveolar lavage fluids, nasal swabs, tissues and cell culture supernatant. Duplex real-time PCR for simultaneous detection of *M. hyopneumoniae* and *M. hyorhinis* was highly sensitive and can be utilized for diagnosing clinical samples. It is time-efficient and economic, thereby providing a new approach to control both *M. hyopneumoniae* and *M. hyorhinis*.

Keywords: *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, duplex real-time PCR, Swine, Detection*

Mycoplasma hyopneumoniae ve *Mycoplasma hyorhinis*'in Aynı Anda Tespitinde Gerçek Zamanlı, Dubleks PCR Metodunun Uygulanması

Öz

Bu çalışmanın amacı, *Mycoplasma hyopneumoniae* ve *Mycoplasma hyorhinis*'in aynı anda tespitinde TaqMan prob temelli, hassas, spesifik dubleks gerçek zamanlı PCR yönteminin geliştirilmesidir. FAM ve Teksas Kırmızısı ile işaretli spesifik primer ve problar *M. hyopneumoniae p97* geni ile *M. hyorhinis p37* geninin amplifikasyonu amacıyla dizayn edildi. Dubleks gerçek zamanlı PCR reaksiyon karışımları oluşturularak optimize edildi ve yöntemin hassasiyetliği, özgüllüğü ve tekrarlanabilirliği hesaplandı. Dubleks gerçek zamanlı PCR'ın hassasiyetliği hem *M. hyopneumoniae* hem de *M. hyorhinis* için 10 kopya/μL olarak bulundu. Diğer yaygın viral ve bakteriyel patojenler ile çapraz reaksiyon yoktu. Ct değerlerinin varyasyonlarının standart katsayısının konsantrasyonu %5'ten az olup iyi bir tekrarlanabilirliğe işaret etmekteydi. Bronkoalveoler lavaj sıvısı, nazal svablar, dokular ve hücre kültürü süpernatantlarını içeren klinik örnekler (n = 937) dubleks gerçek zamanlı PCR ile test edildi. *Mycoplasma hyopneumoniae* ve *Mycoplasma hyorhinis*'in aynı anda tespitinde dubleks gerçek zamanlı PCR oldukça yüksek hassasiyetliğe sahip olup klinik örneklerde tanı amacıyla kullanılabilir. Yöntem kısa zamanda uygulanabilmesi ve ekonomik olması sebebiyle hem *M. hyopneumoniae* hem de *M. hyorhinis*'in kontrolünde yeni bir yaklaşım olarak kullanılabilir.

Anahtar sözcükler: *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Dubleks gerçek zamanlı PCR, Domuz, Tespit*

İletişim (Correspondence)
+86 025 84390880 Fax: +86 025 84391973
qiyanxiongnj@163.com

INTRODUCTION

Mycoplasma hyopneumoniae and *Mycoplasma hyorhinis* are members of the Mycoplasmatales family that affect swine health and production in worldwide [1]. *M hyopneumoniae* is the etiological agent of enzootic pneumonia in swine, a chronic respiratory disease characterized by highly infectious, high morbidity and low mortality rates [2]. In the acute phase of the disease, catarrhal pneumonia is observed, with exudates in the airways. The bronchial and mediastinal lymph nodes are often enlarged. In the chronic stage of the disease, recovering lesions, consisting of fissures of collapsed alveoli adjoining areas of alveolar emphysema, are observed [3]. *M. hyopneumoniae* is a very contagious bacterium and may be transmitted *via* direct contact between pigs [4] or *via* the environment [5,6].

M. hyorhinis is a common pollutant in cell culture and is associated with the development of certain human tumor diseases *in vitro* [7], with unknown the mechanisms. It may cause arthritis, polyserositis, ear infections, pneumonia, pleurisy, peritonitis, pericarditis, pharyngeal tube inflammation and otitis media [8-10], with high morbidity and low mortality rates. The mixed bacterial infection with porcine enzootic pneumonia and porcine reproductive and respiratory syndrome was thought to facilitate the development of disease. *M. hyorhinis* generally occurs in 3 to 10-week-old pigs and is generally transmitted through nasal secretions by sows to piglets. It has been isolated from the nasal secretions of about 30-40% of weaning pigs or from lung tissue with typical lesions.

The establishment of detection methods for *M. hyopneumoniae* and *M. hyorhinis* is crucial for epidemiological and pathogenesis studies. Many methods are mainly based on clinical diagnosis (slaughterhouse monitoring), bacterial culture, serology and molecular biology diagnostic methods [11-15]. The culture isolation detection method is often regarded as the gold standard method for *M. hyopneumoniae* detection. Molecular detection systems have the potential to provide a higher degree of sensitivity and time-saving compared to culture isolation. PCR methods have been applied to lung tissue [16-18], aerosol samples [19], nasal swabs [20-23], broncho-alveolar lavage fluids and cell culture. Fluorescent, quantitative PCR technology is a method of choice to diagnose diseases because of its high sensitivity/specificity as well as being rapid, quantitative and accurate [24]. This study established a method for simultaneous detection of *M. hyopneumoniae* and *M. hyorhinis*. The double fluorescent quantitative PCR method of *M. hyopneumoniae* is helpful for rapid qualitative and quantitative monitoring of *M. hyopneumoniae* and *M. hyorhinis* infections, providing a useful technology for the prevention and control of animal diseases caused by these organisms. It is simpler, faster, more accurate and has wide application prospect when compared to conventional PCR, nested PCR and singleplex real-time PCR.

MATERIAL and METHODS

The laboratory in which this study was conducted practices strict physical separation of all the various steps involved in PCR, and a unidirectional workflow was employed to reduce risk of contamination.

Bacterial Strains, Virus and Cells

Fourteen bacterial and viral strains were detected. Bacterial strains: *Actinobacillus pleuropneumoniae*, *Escherichia coli*, *Haemophilus parasuis*, *M. hyopneumoniae*, *M. hyorhinis*, *M. flocculare*, *M. gallisepticum*, and *Staphylococcus aureus,* as well as viruses: Porcine circovirus type 2 (PCV2), Porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus infection (PPI), classical swine fever virus (CSFV) and Swine influenza virus were isolated, identified and provided by the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, China. *M. hyosynoviae* (M60, ATCC® 27720™) was obtained from the American Type Culture Collection (Rockville, Md.).

Twelve cell lines, including the parental porcine mono-myeloid cell line (3D4/21; ATCC CRL-2843), St. Jude porcine lung cells (SJPL; ATCC PTA-3256), porcine kidney cell (PK15; ATCC PTA-8244) and swine tracheal epithelial cells (STEC) were provided by the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, China.

DNA and RNA Extraction

Processing of the lung tissue: The dead swine to be tested were euthanatized and fresh lung tissues were taken and rinsed with sterile phosphate buffered saline (PBS) solution. The junctions of normal and diseased tissue were cut, and DNA was extracted from the tissue using Column Animal DNA_{OUT} Kit (Tiandz Inc., Beijing, China) following the manufacturer's instructions [19].

Processing of bronchial alveolar lavage fluids: The trachea was filled with sterile PBS solution and gently kneaded to ensure full immersion of PBS solution into the lung tissues and BALF samples were collected [25]. DNA was extracted using Column Bacterial DNA_{OUT} (Tiandz Inc.).

Processing of aerosol samples: Aerosol samples were collected using an electromagnetic air pump [26] in pig herds, injected into Erlenmeyer flask, and centrifuged at 12000 rpm/min. The precipitate was collected and used to extract DNA using the phenol-chloroform method [27].

Processing of nasal swabs sample: Pigs were tethered and a cotton swab was gently touched to the nasal septum to stimulate swine sneezing 3 times. The swab was pulled and placed into sterile PBS solution at 4°C for 12 h. Following centrifugation at 10000 rpm/min for 5 min, the precipitate was collected and used to extract DNA using Column Swab DNA_{OUT} Kit (Tiandz Inc.) according to the manufacturer's instructions.

Processing of bacterial and viral strains: DNA of *A. pleuropneumoniae*, *E. coli*, *H. parasuis*, *M. flocculare*, *M. gallisepticum*, *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae*, *S. aureus*, and PPI was extracted using the Column Bacterial DNA_{OUT} kit (Tiandz Inc.). RNA of PCV2, PRRSV, CSFV, Swine influenza virus was extracted using the One-Tube Viral DNA-RNA_{OUT} kit (Tiandz Inc.).

Primers and Probes

The real-time PCR method for *M. hyopneumoniae p97* assay has been described previously by Strait et al.[28]. The *M. hyorhinis*-specific real-time PCR assay developed according to our previous studies [29] was modified slightly. The difference was reflected on the labeling of the probe. Optimization included using *M. hyorhinis p37* sequence as the probe instead of the previously described labeling with 5′-6-carboxyfluorescein (FAM) and a 3′ minor grove binder (MGB) non-fluorescent quencher, a Texas Red-labeled probe was used *(Table 1)*. All oligonucleotides were synthesized by TaKaRa (Dalian, China).

Optimization of Duplex Real-Time PCR Assay

The concentrations of the primers and the probe were optimized to establish the optimum duplex real-time PCR reaction system. DNA of *M. hyopneumoniae* and *M. hyorhinis* were used as template, the primers concentration range (3 μM to 10 μM), a probe concentration range (0.5 μM to 5 μM), and an annealing temperature (50°C to 60°C). The duplex real-time PCR reaction system (20 μL) was composed as follows: 10 μL AceQ qPCR probe Master Mix (Vazyme Biotech Co., Ltd), 1 μL template (approximately 0.1 ng/μL), 1 μL ddH_2O, the primers and probes (concentrations described in *Table 1)* were merged as a master mix. Each run included a positive control (the gradient dilution of recombinant plasmid), a negative control (ddH_2O). The reaction conditions were as follows: 40 cycles of 50°C for 2 min, 95°C for 10 min; 95°C for 15 s, 60°C for 60 s). The reaction was carried out in Quant Studio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Templates were tested in triplicate and the Cycle threshold (CT) values were plotted against the copy number in order to verify the reproducibility.

Testing Inter- and Intra-Detection Specific of Assay

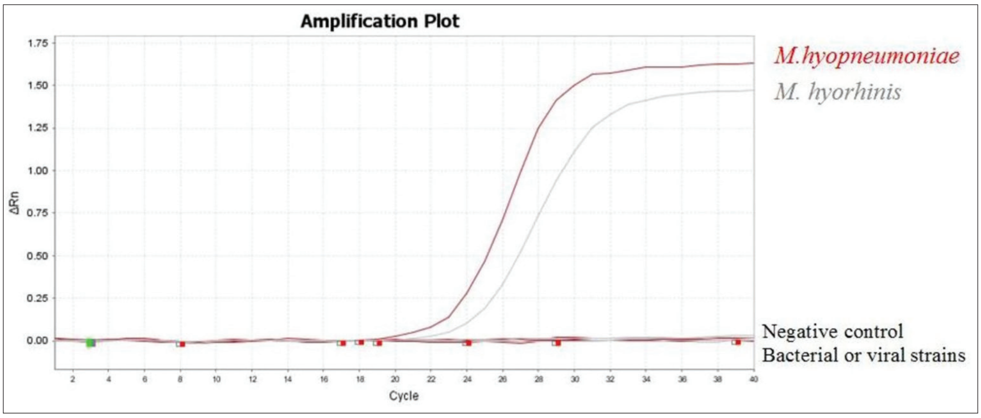
Positive plasmid of *M. hyopneumoniae* and *M. hyorhinis* was prepared as described by Strait et al.[28] and Bai et al.[29]. The plasmids were diluted 10 times as standard template, and optimized reaction mixtures and conditions were utilized to detect the sensitivity of the method. DNA and RNA extracted from 14 bacterial and viral strains were used to confirm the specificity of the assay. These strains included *A. pleuropneumoniae*, *E. coli*, *H. parasuis*, *S. aureus*, *M. flocculare*, *M. gallisepticum*, *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae*, PCV2, PRRSV, PPI, CSFV, and Swine influenza virus *(Fig. 1)*.

Table 1. *The primers and probes selected for singleplex and duplex real-time PCR*

Primers or Probes	Sequence 5′-3′	Genomic Target	Concentration (pmol/reaction)		References
			Singleplex Real-Time PCR	Duplex Real-Time PCR	
*p97*F	CCAGAACCAAATTCCTTCGCTG	*p97*	1	0.5	[28]
*p97*R	ACTGGCTGAACTTCATCTGGGCTA		1	0.5	
*p97*P	FAM[a]-AGCAGATCTTAGTCAAAGTGCCCGTG–TAMRA[b]		0.5	0.5	
*p37*F	AGAAGGTTCTTTTGCTTGAACACA	*p37*	1	0.5	[29]
*p37*R	TGCTTCCATCTTTTCATTTGCTT		1	0.5	
*p37*P	TXR[c]-ATCAGCAACAAAACCTT-BHQ[d]		0.5	1.5	

[a] FAM, 6-carboxyfluorescein, fluorescence reporter dye; [b] TAMRA, Carboxytetramethylrhodamine; [c] TXR, texas-red, fluorescence reporter dye; [d] BHQ, Black Hole Quencher

Fig 1. The amplification curve of specific experiments: *M. hyopneumoniae, M. hyorhinis* and other strains were tested using the duplex real-time PCR. *M. hyopneumoniae* and *M. hyorhinis* tested positive, while the other samples (i.e., *M. flocculare, M. gallisepticum, M. hyosynoviae, A. pleuropneumoniae, E. coli, H. parasuis, S. aureus,* PCV2, PRRSV, PPI, CSFV, and Swine influenza virus) tested negative. The negative control and other common bacterial or viral pathogens did not amplify, were straight lines. There was no cross reaction with other common bacterial or viral pathogens



Varying concentrations of *M. hyopneumoniae* and *M. hyorhinis* plasmid DNA (1×10^6, 1×10^5, 1×10^4 copies/µL respectively), were incorporated into three reaction mixtures. Three batches of intra- and inter-assay testing were performed in order to calculate the Coefficient of Variation (CV) and reproducibility was also measured.

Evaluation of Clinical Samples

The duplex real-time PCR was evaluated for the detection of different clinical samples. Clinical samples tested included broncho-alveolar lavage fluids, nasal swabs and tissues.

One hundred negative samples from known mycoplasma-negative pigs (15 lung tissues, 65 BALF, 20 nasal swabs) were frozen at -70°C by the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, China. Nasal swabs (*n*=583) were obtained from different eleven pig herds in Jiangsu province, China.

Twelve pigs were used in animal experiments to obtain different samples. Seven of them were experimentally infected with *M. hyopneumoniae* [7], while the remaining five pigs were not inoculated with *M. hyopneumoniae*. The different samples including BALF, blood and tissue samples (hilar lymph nodes, lung tissue, muscle, kidney, heart, spleen, liver, stomach, brain, pancreas, duodenum, jejunum, ileum, colon, rectum and cecum) were from these twelve pigs. All experimental procedures were approved by the Ethical and Animal Welfare Committee of the Jiangsu Academy of Agricultural Sciences (No.161028).

RESULTS

Analytical Specificity, Sensitivity and Reproducibility of the Duplex Real-Time PCR

Singleplex assays integrated in the newly developed duplex real-time PCR assay have been assessed previously with respect to sensitivity and specificity. The sequences of all primers and probes included in the duplex real-time PCR *(Table 1)* were aligned with publically available sequence information (NCBI GenBank) with a special focus on porcine viruses. There was no indication of possible cross-reactions.

The specific detection: The duplex real-time PCR approach has been established to exclude non-specific reactions. Nucleic acids extracted from lung tissue, BALF and nasal swabs collected from healthy pigs were tested. All samples scored negative in assays included in the duplex real-time PCR *(Table 2,* sample ID 01-100). *M. hyopneumoniae*, *M. hyorhinis* and other strains were tested using the duplex real-time PCR. *M. hyopneumoniae* and *M. hyorhinis* tested positive, while the other samples (i.e., *M. flocculare*, *M. gallisepticum*, *M. hyosynoviae*, *A. pleuropneumoniae*, *E. coli, H. parasuis*, *S. aureus,* PCV2, PRRSV, PPI, CSFV, and Swine influenza virus) tested negative *(Table 2,* sample ID 101 to 114). There was no cross reaction with other common bacterial or viral pathogens *(Fig. 1)*.

Establishment of the standard curve: The recombinant plasmid of *M. hyopneumoniae* and *M. hyorhinis* was diluted 10 times with 1×10^9 copies/µL to 1×10^4 copies/µL dilution as a template for duplex real-time PCR. The concentration of the amplification results was the abscissa, and the corresponding Ct value was the ordinate, and two standard curves were obtained *(Fig. 2)*. The linear correlation, coefficient R^2 and the amplification efficiency E of *M. hyopneumoniae* and *M. hyorhinis* were -3.207, 1, and 104.68%; -3.215, 1 and 105.04% respectively. The linear relationship of the amplified product was good between the Ct value and the concentration.

The sensitivity test: The analytical sensitivity in the duplex real-time PCR was evaluated using a series of 10-fold dilutions of recombination plasmid of *M. hyopneumoniae* and *M. hyorhinis* in three replicates per run on three different days. The results indicated that the sensitivity was 10 copies/µL for both *M. hyopneumoniae* and *M. hyorhinis (Fig. 3)*.

The reproducibility test: To test the reproducibility of the duplex real-time PCR, standard plasmids of *M. hyopneumoniae* and *M. hyorhinis* at three different concentrations, 1.0×10^7 - 1.0×10^5 copies/µL were used *(Table 3)*. The variations were assessed by three replicates per run on three different days. The results demonstrated that the duplex TaqMan Ct values are easily achieved at the end of the process with a CV of Ct values between the intra-assay test and the inter-assay test being less than 5% *(Table 3)*. The study showed that the reproducibility were good.

Clinical and Experimentally Infected Sample Detection Using Duplex Real-Time PCR

In total, 126 individual samples were tested by the duplex real-time PCR, and in the respective singleplex assays, simultaneously *(Table 2,* sample ID 1 to 126). Overall, a high agreement could be observed between the Ct values obtained in the duplex real-time PCR and each single-target PCR assay for the clinical samples.

Twelve cell lines of STEC, PK15, SJPL and 3D4/21 were examined *(Table 2,* sample ID 115 to 126). Only a single STEC cell line was positive for *M. hyorhinis*. The detection result was accordant to that of the above Single-target real-time PCR. It appeared to be contaminated with *M. hyorhinis (Table 2,* sample ID 115). .

Following collection of nasal swabs from 11 pig farms *(Table 2,* sample ID 127 to 709), *M. hyopneumoniae* and *M. hyorhinis* could be detected, although the Ct values were relatively low. The positive rate of *M. hyorhinis* was higher than *M. hyopneumoniae*, with only a single pig farm where the positive rate of *M. hyorhinis* was lower than *M.*

Table 2. *Assessment of diagnosis of M. hyoneumoniae and M. hyorhinis in clinical samples using real-time PCR*

No.	Sample Material	Duplex Real-Time PCR (Ct*)		Single-Target Real-Time PCR (Ct)	
		M. hyopneumoniae	***M. hyorhinis***	***M. hyopneumoniae***	***M. hyorhinis***
Negative samples					
1-15	lung tissue	no Ct	no Ct	no Ct	no Ct
16-80	BALF	no Ct	no Ct	no Ct	no Ct
81-100	nasal swabs	no Ct	no Ct	no Ct	no Ct
Bacterial or viral strains					
101	*M. hyopneumoniae*	20.26	no Ct	20.94	no Ct
102	*M. hyorhinis*	no Ct	22.58	no Ct	23.01
103	*M. hyosynoviae*	no Ct	no Ct	no Ct	no Ct
104	*M. flocculare*	no Ct	no Ct	no Ct	no Ct
105	*M. gallisepticum*	no Ct	no Ct	no Ct	no Ct
106	*H. parasuis*	no Ct	no Ct	no Ct	no Ct
107	PCV2	no Ct	no Ct	no Ct	no Ct
108	pleuropneumoniae	no Ct	no Ct	no Ct	no Ct
109	PRRSV	no Ct	no Ct	no Ct	no Ct
110	PPI	no Ct	no Ct	no Ct	no Ct
111	*S. aureus*	no Ct	no Ct	no Ct	no Ct
112	CSFV	no Ct	no Ct	no Ct	no Ct
113	Swine influenza virus	no Ct	no Ct	no Ct	no Ct
114	*E. coli*	no Ct	no Ct	no Ct	no Ct
Cell culture supernatant					
115	STEC	no Ct	37.3	no Ct	37.5
116	STEC	no Ct	no Ct	no Ct	no Ct
117	STEC	no Ct	no Ct	no Ct	no Ct
118	STEC	no Ct	no Ct	no Ct	no Ct
119	PK15	no Ct	no Ct	no Ct	no Ct
120	PK15	no Ct	no Ct	no Ct	no Ct
121	SJPL	no Ct	no Ct	no Ct	no Ct
122	SJPL	no Ct	no Ct	no Ct	no Ct
123	SJPL	no Ct	no Ct	no Ct	no Ct
124	3D4/21	no Ct	no Ct	no Ct	no Ct
125	3D4/21	no Ct	no Ct	no Ct	no Ct
126	3D4/21	no Ct	no Ct	no Ct	no Ct
Clinical samples from different pig herds					
127-181	nasal swabs	11/55	39/55	n.t#	n.t
182-211	nasal swabs	13/30	9/30	n.t	n.t
212-311	nasal swabs	18/100	31/100	n.t	n.t
312-359	nasal swabs	19/47	34/47	n.t	n.t
360-417	nasal swabs	18/58	25/58	n.t	n.t
418-452	nasal swabs	17/35	18/35	n.t	n.t
453-494	nasal swabs	18/42	31/42	n.t	n.t
495-552	nasal swabs	30/58	37/58	n.t	n.t
553-591	nasal swabs	2/39	27/39	n.t	n.t
592-649	nasal swabs	20/58	26/58	n.t	n.t
650-709	nasal swabs	21/60	23/60	n.t	n.t

**Ct –cycle threshold; # n.t.-not tested*

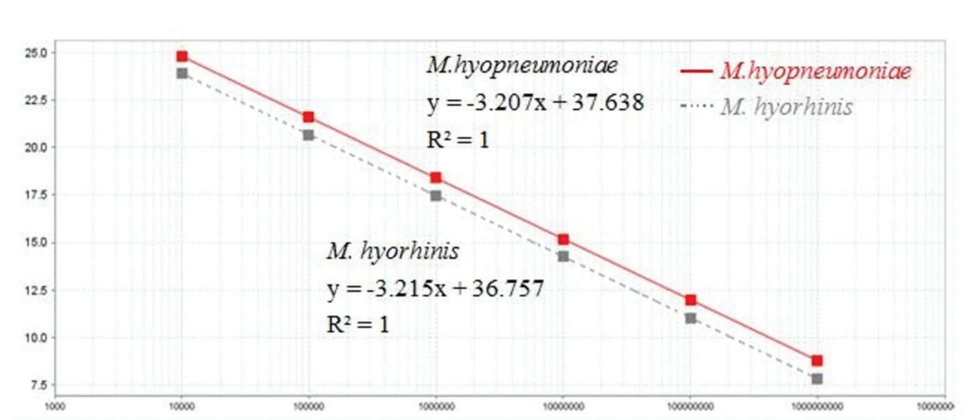
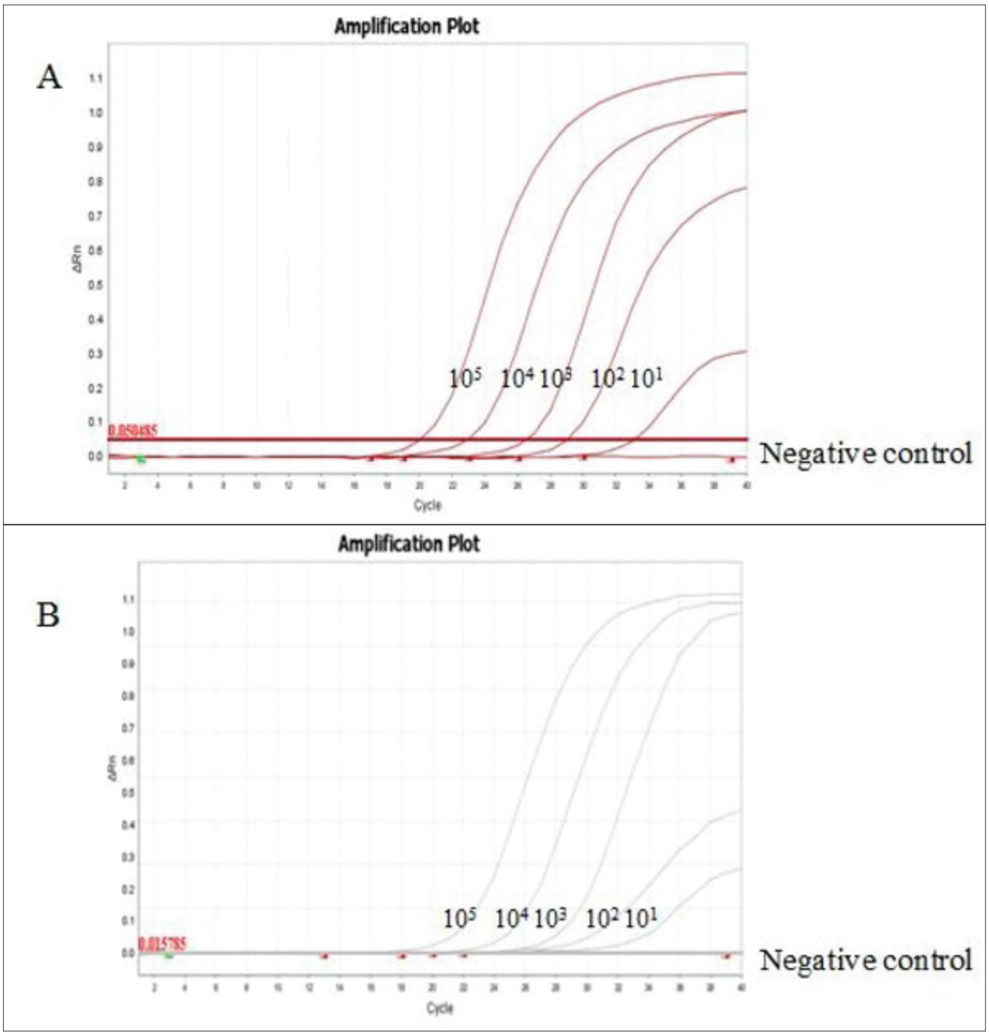


Fig 2. Standard curve of duplex real-time PCR: The linear correlation, coefficient R2 and the amplification efficiency E of *M. hyopneumoniae* were -3.207, 1, and 104.68%, respectively. The linear correlation, coefficient R2 and the amplification efficiency E of *M. hyorhinis* were -3.215, 1 and 105.04%, respectively. The standard curve of *M. hyopneumoniae* and *M. hyorhinis* were y = -3.207x + 37.638, y = -3.215x + 36.757. The linear relationship of the amplified product was good between the Ct value and the concentration

Fig 3. Sensitivity test of the real-time PCR. A: Amplification curve of *M. hyopneumoniae*, B: Amplification curve of *M. hyorhinis*, The sensitivity of both *M. hyopneumoniae* and *M. hyorhinis* was 10 copies/µL, respectively



hyopneumoniae (Table 2, sample ID 182 to 211).

Seven animals were inoculated with *M. hyopneumoniae*, while other five animals were not inoculated. The clinical samples from twelve pigs, including nasal swabs, BALF, blood, lung tissue, hilar lymph nodes, muscle, kidney, heart, spleen, liver, stomach, brain, pancreas, duodenum, jejunum, ileum, colon, rectum and cecum *(Table 4)* were detected by using the established duplex real-time PCR assay. All the samples of hilar lymph nodes, lung tissue from seven pigs inoculated with *M. hyopneumoniae*, BALF were positive for *M. hyopneumoniae (Table 4* Pig No.1, 2, 3, 4, 5, 6, and 7). In a few nasal swabs samples of the experimentally infected animals with *M. hyopneumoniae*, *M. hyorhinis* was detected, although the Ct was relatively low *(Table 4)*. Pig No.4, 6, 9 and 10, these four nasal swabs were positive for *M. hyorhinis*, whether or not to be challenged *M. hyopneumoniae.*

Table 3. *The intra- and inter-detection result of duplex real-time PCR*

Agent	Concentration of Standard (copies/µL)*	Intra-Assay	Inter-Assay
		CV (%)[#]	CV (%)[#]
M. hyopneumoniae	1×10^7	0.03	0.23
	1×10^6	0.11	2.27
	1×10^5	0.15	1.62
M. hyorhinis	1×10^7	0.08	1.32
	1×10^6	0.22	1.77
	1×10^5	0.17	2.33

** **Copies/µL:** the DNA copy numbers per microliter, [#] **CV (%):** Ct coefficients of variations*

Table 4. *Detection of M. hyopneumoniae and M. hyorhinis in experimentally infected tissues using a duplex Real-time PCR assay*

Specimen	Ct[#] Values for Detection of ***M. hyopneumoniae*** (A) and ***M. hyorhinis*** (B)															
	Pig															
	1		2		3		4		5		6		7		8-12	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Nasal Swabs	33.7	no Ct	35.1	no Ct	33.8	no Ct	34.1	35.7	34.1	no Ct	33.8	36.5	31.7	no Ct	no Ct	no Ct 37.2 38.2 no Ct no Ct
Hilar lymph nodes	33.4	no Ct	31.8	no Ct	33.2	no Ct	33.4	no Ct	32.7	no Ct	32.9	no Ct	36.1	no Ct	no Ct	no Ct
Lung tissue	33.0	no Ct	31.5	no Ct	28.5	no Ct	33.5	no Ct	34.6	no Ct	32.1	no Ct	32.8	no Ct	no Ct	no Ct
BALF	31.8	no Ct	28.5	no Ct	31.9	no Ct	33.3	no Ct	33.1	no Ct	29.2	no Ct	32.2	no Ct	no Ct	no Ct
Blood	32.3	no Ct	no Ct	no Ct	no Ct	no Ct	36.4	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Muscle	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Kidney	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Heart	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Spleen	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Liver	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Stomach	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Brain	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Pancreas	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Duodenum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Jejunum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Ileum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Colon	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Rectum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
Cecum	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct

A: *M. hyopneumoniae;* ***B:*** *M. hyorhinis; Ct –cycle threshold*

DISCISSION

Diseases associated with *M. hyopneumoniae* and *M. hyorhinis* are difficult to control because of the long survival of the organism in the environment, shedding by apparently healthy but infected animals and the unreliability of diagnostic tests [26,27]. Therefore, a rapid diagnosis of the causative agent is crucial [30]. A variety of detection methods for swine viruses have been developed during recent years, for instance, multiplex PCR [31,32], real-time PCR [26,33]. Wu et al.[32] established a duplex PCR detection method based on Hps *p2* protein gene and *M.*

hyorhinis p37 protein gene of *Haemophilus parasuis* and *M. hyorhinis*. The results showed that the sensitivity was 100 copies/reaction. This method could determine the pathogenicity of *Haemophilus parasuis,* according to the size of amplification products.

For detection of several pathogens, or for multiple genetic tests of the same pathogen, singleplex PCR is severely limited, because it will lead to waste of time, human resources and detergent. By using multiplex PCR systems, several infectious agents can be detected and differentiated simultaneously in a single reaction, reducing costs and efforts as well as the amount of sample material and time required [34]. Duplex real-time PCR has several advantages, combining a reduced risk of cross-contamination with a high sensitivity and the possibility of quantitative analysis. Oligonucleotide probes labeled with different fluorophores permit multiplexing in a qPCR format, enabling the detection of different target sequences as well as the co-amplification of internal controls. The duplex real-time PCR assay for the simultaneous detection of *M. hyopneumoniae* and *M. hyorhinis* was developed and validated in this study. The probes specific for genome detection of the two notifiable bacteria were labeled with the different fluorophores, i.e., Texas Red and FAM. A rapid (time to completion, <4 h, including DNA extraction), convenient, quantitative and reliable screening system is beneficial for monitoring the clinical course of *M. hyopneumoniae* and *M. hyorhinis* and enhances the clinical utility of molecular testing. Duplex real-time PCR assay labelling with FAM and Texas Red for detection can yield results within 2 h. It does not require post-PCR processing, reduces sample handling, minimizes the risks of contamination [35], and is beneficial for monitoring the clinical course of *M. hyopneumoniae* and *M. hyorhinis*, which will enhance the clinical utility of molecular testing. It is simple, rapid and particularly useful for clinic detection, including BALF, nasal swabs, blood, and tissues. The test revealed a specificity of 100%, has higher sensitivity than Normal PCR [36-38], and equal as Real-time PCR described by Dubosson et al.[39], Marois et al.[18] Fourour et al.[26] increased detection of *M. flocculare*, established a multiplex real-time PCR targets the *p102, p37* and *fruA* genes for *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare*. The detection limits reached 14, 146, and 16 genome equivalents μl^{-1}, respectively, the sensitivity is more than five times lower than this study.

One of the main problems caused by a large number of oligonucleotides in the same reaction tube is a possible interaction of those molecules with each other, resulting in inhibition of the amplification reactions and a subsequent reduced sensitivity [5,34,40]. In our assays, only two pairs of primers and probes were used in the same reaction tube, for each of the large number of clinical samples tested. Similar Ct results were achieved in both the single and duplex approaches, which is consistent with results of Wernike et al.[41].

The newly developed duplex real-time PCR is suitable for use with diverse sample materials, such as BALF, nasal swabs, blood, tissues (lung tissue, hilar lymph nodes, muscle, kidney, heart, spleen, liver, stomach, brain, pancreas, duodenum, jejunum, ileum, colon, rectum cecum) and cell culture supernatant. The application of real-time PCR in diverse clinical samples has been replicated many times [18,29,42,43]. *M. hyopneumoniae* was the persistent organism in the trachea and bronchial lymph nodes, and could be re-isolated from inner organs like liver, spleen and kidneys of experimentally infected pigs. The observed persistence cannot be explained by dissemination of *Mycoplasma* spp. in internal organs, as this phenomenon seems to be transient with no *Mycoplasma* spp. being re-isolated from internal organs at the end of the studies. This suggests that *M. hyopneumoniae* can ephemerally colonize the internal organs of the host, indicating that *M. hyopneumoniae* exists in these tissues without causing disease, and maybe spread through the lymph circulation or blood circulation. Friis [44] isolated *M. hyopneumoniae* from brains of infected pigs. Jin [45] detected *M. hyopneumoniae* in heart, liver, brain, and muscles, indicating that *M. hyopneumoniae* could colonize the internal organs of the host. Wang et al.[46] detected *M. hyorhinis* in blood with the positive rate of 20% (16/80). In this study, it was observed that *M. hyopneumoniae* could be detected in a blood sample of two pigs experimentally infected with *M. hyopneumoniae (Table 4, 1A, 4A)*. Whether *Mycoplasma* spp. spreads through lymphatic circulation or blood circulation remains a problem needed for further research.

The STEC cell line, derived from tracheal epithelial, is more susceptible to contamination from *M. hyorhinis* than other cells, therefore, it is easier to do *M. hyopneumoniae* infestation experiment. The availability of accurate, sensitive and reliable detection duplex real-time PCR and the application of robust and successful elimination methods provides a powerful means for overcoming the problem of mycoplasma contamination in cell cultures. The contamination of cell cultures by *Mycoplasma* spp., especially *M. hyorhinis,* remains a major problem in cell culture. Ideal detection methods for contaminating mycoplasma should be highly sensitive and specific, but also simple, rapid, efficient and cost effective.

In conclusion, the newly developed duplex real-time PCR allows the simultaneous detection of *M. hyopneumoniae* and *M. hyorhinis* combined in a single tube assay with a rapid, convenient, and reliable screening system. The new system could therefore significantly improve the early detection of diseases of swine and could lead to a new approach in syndromic surveillance. Our study indicates that the reported duplex Real-Time PCR could be an accurate diagnostic tool for assessing infection *M. hyopneumoniae* and *M. hyorhinis*. Future detailed studies in diverse geographical locations are warranted to investigate the clinical value of this technique.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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