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Multiplex fluorescence quantitative PCR for the detection of eight bacterial pathogens of porcine respiratory disease complex (PRDC)

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Abstract: As rapid and reliable tools for diagnosis of porcine respiratory disease complex (PRDC) bacterial pathogens are getting more important over time, we established a new multiplex fluorescence quantitative polymerase chain reaction (mFQ-PCR) system with a similar symptom. Our results showed that the system could accurately and simultaneously detect 8 pathogens within 1.5 h. The detection limits were 2-5 standard DNA copies. The correlation coefficient of the standard curve was more than 0.998, with a well dynamic range from 10^1 to 10^9, and the intra- and intervariation coefficients (CVs) were less than 2.06%. After setting the positive threshold of Ct value, the coincidence rate between mFQ-PCR and conventional PCR (cPCR) could reach 92%-100%, the specificity and sensitivity of mFQ-PCR exceeded 93.5% and 90.8%, respectively. We also found that the positive rates of 8 bacteria were 12.5%-68.2%, while the rates of co-infection were 90%-100% in the clinical samples. The co-infection among *Streptococcus suis* (Ss), *Haemophilus parasuis* (Hps), and *Mycoplasma hyorhinis* (Mhr) accounted for 78.3%, 91.4%, 100%, respectively of all the positive samples, among *Actinobacillus pleuropneumoniae* (App) and *Mycoplasma pneumoniae* (Mhp) are 45.5% and 35.7%, among *Bordetella bronchis* (Bb), *Pasteurella multocida* (Pm) and toxic *Pasteurella multocida* (T^+Pm) are 52.2%, 41.4%, and 100%, respectively. The results demonstrated that co-infection of 2-4 pathogenic bacteria with similar symptoms was a common situation for PRDC in south of China; our method has the potential to become a reliable detection tool.

Key words: Porcine respiratory disease complex (PRDC), multiplex fluorescence quantitative polymerase chain reaction, bacteria pathogens, detection

1. Introduction

Porcine respiratory disease complex (PRDC) is a common term for mixed respiratory infections of multifactorial etiologies. Postweaning piglets aged 6-10 weeks, and early fattening pigs aged 13-20 weeks are affected typically. The clinical symptoms of PRDC include fever, cough, dyspnea, and anorexia. It decreases feed conversion and growth rate significantly and, in some cases, results in death, which led to major economic losses in the swine industry [1,2]. The most important feature of PRDC is the multiple system damage caused by co-infection of multiple viral and bacterial pathogens. Most of the invading pathogens are initial pathogens; they can destroy the immune barrier of the respiratory tract and invade the organism. The upper respiratory tract of the pigs is typically colonized with bacterial species, most of which belong to the secondary pathogen. The most common bacteria responsible for PRCD are *Actinobacillus pleuropneumoniae* (App), *Mycoplasma pneumoniae* (Mhp), *Bordetella bronchis* (Bb), *Pasteurella multocida* (Pm), *Streptococcus suis* (Ss), *Haemophilus parasuis* (Hps), and *Mycoplasma hyorhinis* (Mhr). The co-infection of bacteria will not only exacerbate symptoms during the disease phase but also increase the risk of developing into a chronic disease and finally lead to the reduction of production efficiency [1,3]. Therefore, rapid diagnostic approach and effective bacterial infection control are the keys to successfully prevent PRDC and reduce economic loss in swine production.

Traditional bacterial isolation and culture was now well established as a standard method for the identification of the above pathogens. However, bacterial isolation and culture was technically time-consuming and demanding. And most elements of the normal bacterial flora colonize the respiratory tract, which could affect the isolation and identification of pathogenic bacteria [4,5]. This led to a decreased efficiency of selecting different antibiotic-resistant mutants [6,7]. Furthermore, it is difficult to detect and differentiate the co-infection of bacteria with

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similar clinical symptoms, lead to misdiagnosis or missed diagnosis [8]. With the wide use of molecular assays, we have previously used the mFQ-PCR assay to detect bacterial pathogens in clinical samples, which gained more acceptance due to its differentiation potential and reliability [9].

In this study, we designed specific probes for conservative genes of different pathogenic bacteria with similar symptoms and labeled them with different fluorescence. We established triple fluorescence quantitative PCR methods for Ss, Hps, and Mhr, duplex quantitative PCR for App and Mhp, triple fluorescence quantitative PCR methods for Pm, T$^+$Pm, and Bb, respectively. By optimizing the amplification conditions, not only multiple pathogens could be detected concurrently, but also pathogens with similar symptoms can be identified. These approaches provide more complete information and technical support for the prevention and control of PRDC.

2. Materials and methods

2.1. Bacterial stains

The bacterial strains were preserved in our institute, including 12 positive control strains and 7 negative control strains. The specific positive control strains are listed in Table 1, including 4 strains of SS (serotype 1, 2, 7, and 9), 2 strains of Hps (serotype 5 and 9), 2 strains of Pm (toxin-producing Pm and non-toxin producing Pm), and 1 strain for each four other pathogens. And the negative control strains included *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (ATCC 49619), *Streptococcus pyogenes* (ATCC 19615), *Enterococcus faecalis* (ATCC 29212), *Streptococcus hemolyticus B* (ATCC 21059), *Streptococcus equi* (CVCC 573).

2.2. Primers and probes

All the sequence information of primers and probes are presented in Table 2. Total 8 sets of primers and TaqMan probes were designed by using MAGA5.1 and Oligo7, including 7 sets of primer combinations for 7 bacteria and 1 set primer combination for toxin associated gene of T$^+$Pm. According to the results of multiple sequence alignments, *gdh*, *omp2*, *p37*, *apx IVA*, *p110, ttt,* and *kmt1* genes were selected as the species-specific target genes of 7 pathogenic bacteria, corresponding to Ss, Hps, Mhr, App, Mhp, Bb, and Pm, with identity range from 77.5% to 99.8%. At the same time, the *toxA* gene was selected as the toxin associated target gene for T$^+$Pm with 97.4% identity. Pathogenic bacteria with similar clinical symptoms in the same reaction system was combined, and the probes of a different bacteria were labeled with different reported fluorophores. Among them, Ss, Hps, and Mhr constituted a triplex-PCR reaction system labeled as No1 reaction; App and Mhp constituted a duplex PCR reaction labeled as No2 reaction; Bb, Pm, and T$^+$Pm constituted a triplex-PCR reaction labeled as No3 reaction.

2.3. Multiple fluorescence quantitative PCR

The multiplex quantitative PCR reactions were performed on an ABI 7500 real- time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) by using Premix Ex Taq (Probe qPCR) (Takara, Kusatsu, Shiga, Japan). Firstly, the annealing temperature of the three multiplex fluorescence quantitative PCR reaction systems was unified as follows: predenaturation at 95°C for 5 min, 40 cycles at 95°C for 15 s, and 58°C for 30 s with fluorescence acquisition. After optimization, the 25 μL reaction system was determined, including 12.5 μL Premix Ex Taq buffer (2 ×), 0.5 μL or 0.75 μL primer (10 μM), 1 μL probe (5 μM), 1 μL DNA sample, 0.25 μL ROX reference dye (50 ×), ddH_2O supplementation

Table 1. Bacterial strains used for positive controls.

Bacterium	Serotype	Isolation time	Source
Streptococcus suis(S.s)	1	2016	brain
	2	2015	joint fluid
	7	2016	tonsil
	9	2018	brain
Haemophilus parasuis(Hps)	5	2016	joint fluid
	9	2016	tonsil
Actinobacillus pleuropneumoniae(App)	3	2017	lung
Mycoplasma hyorhinis(Mhr)	-	2016	lung
Mycoplasma pneumoniae(Mhp)	-	2017	lung
Bordetella bronchis(Bb)	-	2016	nasal cavity
Toxin$^-$Pasteurella multocida(T$^-$Pm)	-	2018	nasal cavity
Toxin$^+$Pasteurella multocida(T$^+$Pm)	-	2018	nasal cavity

Table 2. Primers and probes sequences.

Bacterium	Genes	primers/probes	sequences (5'-3')	GenBank No	Positions	Amplicons
For mFQ-PCR						
Ss	*gdh*	GDH-F	GAGCTCTTCTCTACACTTGAGCC	AY853916	91-113	114 bp
		GDH-R	CCATGGAACACGGAAGCTG		186-204	
		GDH-P	**FAM**-TTGAAGCACACCCAGAATACATCGAAGAA-**TAMRA**		119-147	
Hps	*omp2*	OMP-F	GTTTAGGTGGCTATGGTCATGAA	EU741907	326-348	103 bp
		OMP-R	CCAGTACCAACACCGTATACTTTATC		403-428	
		OMP-P	**CY5**-CCAGCTTGACCAATGCTATCACCGATT-**BHQ2**		372-398	
Mhr	*p37*	P37-F	CTTGAACACATAACAAATCAGCAAC	KC415003	938-962	108 bp
		P37-R	TAAACACTCCAACATCATACGGAA		1022-1045	
		P37-P	**HEX**-AAGCAAATGAAAAGATGGAAGCACT-**BHQ1**		980-1004	
App	*apx IVA*	APXIVA-F	CGGTCGGGTTCGGGATTTGC	GQ332268	2502-2521	82 bp
		APXIVA-R	GGAGGCCTTAGTGTACTGTTGTAATA		2558-2583	
		APXIVA-P	**HEX**-TGAAGCCGCCGCACTTTCTGAGGAGT-**BHQ1**		2523-2548	
Mhp	*p110*	P110-F	GCTTCTGTTTTAGGTAGAGGATTTG	AF279292	4069-4093	90 bp
		P110-R	GGTCATACCCACTCGGTCTTG		4138-4158	
		P110-P	**FAM**-CTATGGACAGATCGGTGATACAACCCC-**BHQ1**		4095-4121	
Bb	*ttt*	TTT-F	TTCTTCGCGCCCAAGGGAA	CP014013	1470626-1470644	115 bp
		TTT-R	TTTCGAGCCCCGACTCCAC		1470722-1470740	
		TTT-P	**CY5**-CCGGCGATCCTGGACAAGCTCA-**BHQ2**		1470650-1470671	
Pm	*kmt1*	KMT1-F	CACTGGGTAAATAGCGGATAGAGC	CP003313	1715148-1715171	95 bp
		KMT1-R	ACAGAAAAGACAGCAATTTCGAGC		1715219-1715242	
		KMT1-P	**FAM**-ACAATGGTGGGGCTTTACGCTGA-**BHQ1**		1715194-1715216	
	toxA	TOXA-F	TACTGCCTGGTTTGTTACGTG	EF441531	2327-2347	73 bp
		TOXA-R	AGGCTATTTTCTATGATACGACACT		2375-2399	
		TOXA-P	**HEX**-ACTTAACATCCCCTTGCTTTGCGAT-**BHQ1**		2349-2373	
For cPCR of standard DNA						
Ss	*gdh*	GDH-SF	GGAATTCCATATGTCAAATGCC	AY853916	1-12	214 bp
		GDH-SR	CCATGGAACACGGAAGCTG		186-204	
Hps	*omp2*	OMP-SF	CGTCGGTTTAGGTGGCTATGGTCATG	EU741907	321-346	581 bp
		OMP-SR	CATAAGAGTAGTTTCCATACACGCCAGAT		873-901	
Mhr	*p37*	P37-SF	CAGGAGTAGTCAAGCAAGAGG	KC415003	80-100	966 bp
		P37-SR	TAAACACTCCAACATCATACGGAA		1022-1045	
App	*apx IVA*	APXIVA-SF	CAAACCAAGATCAGCGTATCGACC	GQ332268	2171-2194	863 bp
		APXIVA-SR	TAAGGCTTCAGTACGAGAATAATC		3010-3033	
Mhp	*p110*	P110-SF	TCTGAAAACTAATCGGGGCAA	AF279292	3635-3655	494 bp
		P110-SR	GGTCATACCCACTCGGTCTTG		4138-4158	
Bb	*ttt*	TTT-SF	GGAACTGTTCAAGGCGCAAAC	CP014013	1470370-1470390	371 bp
		TTT-SR	TTTCGAGCCCCGACTCCAC		1470722-1470740	
Pm	*kmt1*	KMT1-SF	GCTGTAAACGAACTCGCCAC	CP003313	1714710-1714729	533 bp
		KMT1-SR	ACAGAAAAGACAGCAATTTCGAGC		1715219-1715242	
	toxA	TOXA-SF	GGTTCTGGTGCCGCTCGAT	EF441531	2059-2077	341 bp
		TOXA-SR	AGGCTATTTTCTATGATACGACACT		2375-2399	

Note: 1.*gdh*, *omp2*, *p37*, *apx IVA*, *p110, ttt,* and *kmt1* genes were the species-specific target genes of 7 pathogenic bacteria, corresponding to Ss, Hps, Mhr, App, Mhp, Bb, and Pm; t*oxA* gene was the toxin associated target gene for T^+Pm.2.FAM, Cy5, HEX fluorescent groups, TAMRA, BHQ1, BHQ2 were quenching groups in fluorescent quantitative PCR.

Abbreviations: Ss=*Streptococcus suis;* Hps = *Haemophilus parasuis;* Mhr = *Mycoplasma hyorhinis;* App = *Actinobacillus pleuropneumoniae;* Mhp = *Mycoplasma pneumonia;* Bb = *Bordetella bronchis;* Pm = *Pasteurella multocida;* T^+Pm = toxic *Pasteurella multocida;* mFQ-PCR = multiplex fluorescence quantitative PCR; cPCR = conventional PCR.

to 25 µL. The probe concentration of three reaction system was 200 nM, while the primer concentration of reaction No1 and No3 was 200 nM, and that of No2 was 300 nM.

2.4. Construction of recombinant plasmids as standard

The primers labeled SF and SR were used to amplifying DNA as shown in Table 2. The reverse primers for amplifying the standard DNA of Ss, Mhr, Mhp, Bb, Pm, T^+Pm were the same as fluorescence quantitative PCR. Each standard DNA was cloned into the pMD18-T (Takara, Japan). The recombinant plasmids were purified, and the concentration was quantified using NanoDrop ND-2000C (Thermo, Waltham, MA, USA). Then, the standard control plasmid was diluted at concentrations ranging from 10^9-10^1 copies/µL by using the 10-fold serial dilutions. Each dilution was tested in triplicate and used to construct the standard curve. The correlation coefficient (R^2) and amplification efficiency were calculated by using SPSS software (IBM, Armonk, New York, USA).

2.5. Specificity, sensitivity, reproducibility

The specificity, sensitivity, and reproducibility of multiplex fluorescence quantitative PCR were evaluated under the optimal condition. To evaluate the specificity, 12 positive and 7 negative control bacteria were detected respectively by observing the fluorescence amplification signals in the three reaction systems. The detection limit was verified in triplicates per dilution. We repeated the reaction at three different time points using 1×10^3 to 1×10^7 copies/µL plasmid standard as a template. The intra- and inter-coefficient of variation of each dilution were calculated to determine the stability of the method.

2.6. Detection of clinical samples

A total of 88 clinical samples from pigs with PRDC were collected in the south of China in the different farms, including nasal swabs, joint fluid, tonsils, lung tissues, etc. The Genomic DNA Extraction Kit (Takara, Japan) was used to extract the bacterial genome DNA from the samples; parallel detection of DNA was carried out by both multiplex fluorescence quantitative PCR and conventional PCR [10-15]. The conventional PCR was considered as positive for the presence of target fragments, while fluorescence quantitative PCR was considered positive for the presence of typical amplification curve, and the initial copy number of DNA was calculated according to Ct value and the standard curve. The coincidence rate and consistency (Kappa coefficient) of the two methods were analyzed by SPSS software. For samples with different qualitative results, the receiver operating characteristic (ROC) curve of sensitivity and specificity of fluorescence quantitative PCR was calculated compared with conventional PCR as a reference test, and the optimal cut-off value (OCV) of Ct value was derived. After setting the OCV, the qualitative results of fluorescence quantitative PCR were rejudged. The Ct value less than or equal to the OCV was positive, while the Ct value greater than the OCV was negative.

3. Results

3.1. Establishment of the standard curve

The fluorescence signal was generated only if the target sequence for the probe was amplified. The standard curves and their linear formulas of the three multiple reaction systems are shown in Figure 1. The results showed that the amplification efficiency of eight target genes reached 100.1%-105.1%. The slope of the standard curve ranged from -3.320 to -3.205, and the correlation (R^2) ranged from 0.998 to 1. Notably, the R^2 of the standard curve of Bb reached 1, which indicated that the Ct value of the bacteria was completely correlated with the original amplicons under the optimal reaction conditions.

3.2. Specificity, sensitivity, reproducibility of the multiplex fluorescence quantitative PCR

The diagnostic specificity, sensitivity, reproducibility of the multiplex fluorescence quantitative PCR has been evaluated and shown in Table 3. The fluorescence intensity of each strain increased significantly only in the reaction systems containing corresponding target gene probes. There was no amplification of the target occurs in negative control wells in the three reaction systems. The results showed that the method could simultaneously detect 8 pathogenic bacteria including Ss, Hps, Mhr, App, Mhp, Bb, Pm, T^+Pm without cross-reaction with other strains.

We further assessed the sensitivity of the assay, which indicated that the detection limit for Hps, Mhr, Bb, T^+Pm was 2-3 copies/reaction, and for Ss, App, Mhp, and Pm was 5 copies/reaction. Ct values of Mhp and T^+Pm were reproducible, with a standard deviation of 0.11 and 0.03, the variation coefficient of 0.33% and 0.09%. On the other hand, for other pathogens, standard deviation ranged from 0.51 to 0.87, and variation coefficient ranged from 1.43% to 2.46%. These data indicated that the multiplex fluorescence PCR was highly sensitive.

To evaluate the reproducibility of the assay, we repeated the reaction three times and obtained the error bar of the Ct value shown in Figure 2. The results showed that the standard deviations of Ct values were all within the range of 0.05-0.35. The variation coefficients of intra-assay were ranged from 0.08% to 2.06% in three detection systems, of inter-assay were ranged from 0.29% to 1.55% with good reproducibility and low detection limit.

3.3. Comparison of fluorescence quantitative PCR and conventional PCR results for clinical samples

We compared the results of the two PCR assays, and the results are shown in Table 4. The results from both methods were congruent with a coincidence rate of 100% and kappa value 1, while the qualitative results of Ss, Hps, Mhr, App,

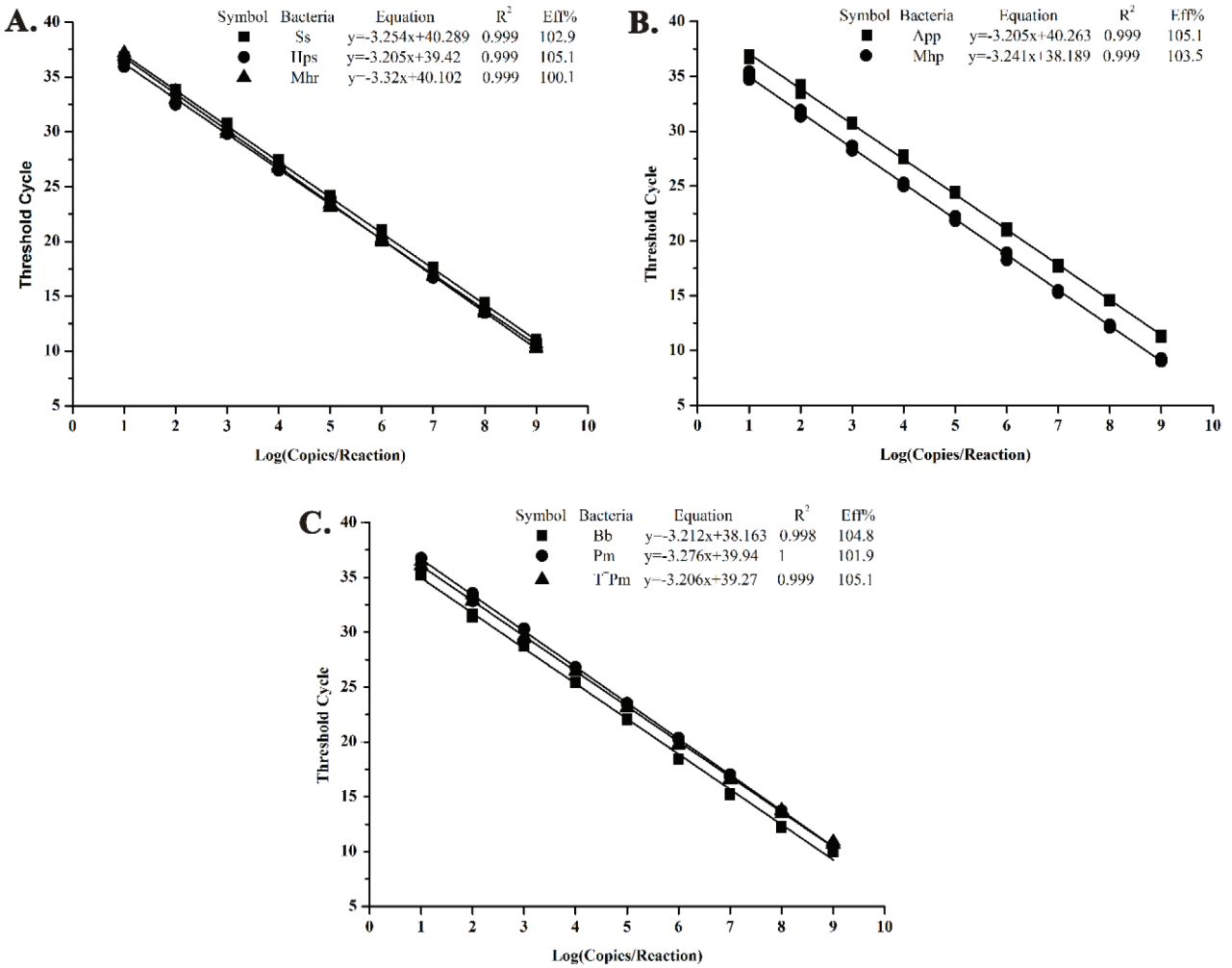

Figure 1. Standard curve of plasmid standard DNA (A. reaction system No1, containing Ss, Hps, Mhr, B. reaction system No2, containing App = Mhp; C. reaction system No3, containing Bb, Pm, T⁺Pm. The copies of standard samples were diluted 10 times by 1×10^9-1×10^1copies/μL with nine gradients, log10 values in longitudinal coordinates, and Eff values in amplification efficiency) Abbreviations: Ss = *Streptococcus suis;* Hps = *Haemophilus parasuis;* Mhr = *Mycoplasma hyorhinis;* App = *Actinobacillus pleuropneumoniae;* Mhp = *Mycoplasma pneumonia;* Bb = *Bordetella bronchis;* Pm = *Pasteurella multocida;* T⁺Pm = toxic *Pasteurella multocida;* R^2 = correlation coefficient; Eff% = amplification efficiency

Mhp, Pm were different with a coincidence rate of 78.4%-95.5% and kappa value of 0.564-0.92. Combined with the quantitative results, we found that these differences mainly occurred in the samples with 1-99 copiers, i.e. the original amplicon was lower than the detection limit of conventional PCR (usually 10^2 copies). Only two HPS samples had more than 100 copies of the template, while conventional PCR was negative with the quantitative results, which are 101 and 104 copies.

And then, we took the qualitative results of conventional PCR as the reference and calculated the ROC curves of fluorescence quantitative PCR, which were shown in Figure 3. We observed that the area under the curve of each bacteria was between 0.997-1 ($p < 0.001$), with OCV of Ss, Hps, Mhr, App, Mhp, and Pm were 35.4, 35.2, 33.8, 35.9, 34.9, and 35.3, respectively. After setting OCV, the coincidence rate of the two PCR assays increased to 92%-100%, and the kappa value was 0.808-1, showing a high consistency. At the same time, the specificity and sensitivity also reached a high level of 93.5%-100% and 90.8%-100%, respectively.

3.4. Analysis of clinical samples

The results of fluorescence quantitative PCR of 88 clinical samples were analyzed, as shown in Figure 4. Under the OCV, the total positive rate was 87.5%. Only 12.5% of all positive samples were infected with single bacteria, while 85.7% of all positive samples were co-infected with 2-7 bacteria (Pm and T⁺Pm double- positive was excluded).

Table 3. Positive fluorescence of positive control bacteria and variation of Ct value in detecting limit template quantity.

Bacteria	Probe fluorescence								Sensitivity (3 replicates)			
	No1			No2		No3			Limit template (copies)	Ct mean value	Standard deviation	Variation coefficient (%)
	gdh	*omp2*	*p37*	*apx IVA*	*p110*	*ttt*	*kmt1*	*toxA*				
Ss	FAM								5	36.77	0.62	1.69
Hps		Cy5							2-3	35.97	0.51	1.43
Mhr			HEX						2-3	37.26	0.67	1.80
App				HEX					5	35.74	0.86	2.42
Mhp					FAM				5	35.21	0.11	0.33
Bb						Cy5			2-3	35.47	0.87	2.46
T^{-}Pm							FAM		5	36.11	0.54	1.51
T^{+}Pm							FAM	HEX	2-3	33.55	0.03	0.09

Note: FAM, Cy5, HEX were fluorescent groups in fluorescent quantitative PCR.
Abbreviations: Ss = *Streptococcus suis;* Hps = *Haemophilus parasuis;* Mhr = *Mycoplasma hyorhinis;* App = *Actinobacillus pleuropneumoniae;* Mhp = *Mycoplasma pneumonia;* Bb = *Bordetella bronchis;* Pm = *Pasteurella multocida;* T^{+}Pm = toxic *Pasteurella multocida;* Ct = cycle threshold.

Specifically, the positive number of Ss, Hps, Mhr, App, Mhp, Bb, Pm, T^{+}Pm were 60, 58, 43, 11, 14, 23, 29, and 11, respectively, with co-infection rates of 68.2%, 65.9%, 48.9%, 12.5%, 15.9%, 26.1%, 33%, and 12.5%, respectively. Furthermore, the results showed that the number of co-infection samples in these positive samples was 54, 54, 43, 11, 14, 23, 28, and 11, respectively, with positive rates (total number of co-infection samples/total number of positive samples) of 90%, 93.1%, 100%, 100%, 100%, 96.6%, 100%, respectively. Among them, the co-infection rates of Ss, Hps, and Mhr were 78.3%, 91.4%, and 100%; co-infection rates of App and Mhp were 45.5% and 35.7%; co-infection rates of Bb, Pm, T^{+}Pm were 52.2%, 41.4%, and 100% (the co-infection samples of Pm and T^{+}Pm were excluded).

4. Discussion

Pathogenic infection is considered as one of the most important causes affecting PRDC. Primary pathogens are usually the leading force, while the secondary pathogens play the role of subsequent destroyers. After infection, primary pathogens mainly destroy the immune system that operates within the respiratory tract and secondary pathogens could leave the colonization site and migrate, which causes systemic infection in different organs. This leads to more serious physical injury than the single infection of the primary pathogen [1,2]. In our study, seven causative pathogens were selected, including three primary pathogens App, Mhp, Bb, and four secondary pathogens Ss, Hps, Mhr, and Pm [4,16]. According to the epidemiological survey, these pathogens were widely distributed all over the world, but the main pathogens involved in PRDC vary significantly from region to region [4,16,17]. In the south of China, the main pathogens associated with PRDC were Ss, Hps, and Mhr, with positive rates of 68.2%, 65.9%, and 48.9%, respectively. And the positive rates of these pathogens in pig farms were 78.3%, 82.6%, and 60.9% (a total of 23 farms were detected, and the data were not shown). At the same time, the positive rates of samples with App, Mhp, Bb, Pm were lower, ranging from 12.5% to 33%, while its positive rates of farms were from 26.1% to 43.5%. Besides, the positive rate of primary pathogens in this research was 43.2%, which was much lower than the secondary pathogens which was 87.5%. It was interesting that the secondary pathogen T^{+}Pm was positive only in a backyard pig farm. And we found that the farm was in the outbreak stage of PRDC, all the pigs in all age groups had symptoms of wasting and dyspnea, and more than half of the pigs had obvious atrophic rhinitis. In their clinical samples, the positive rates of T^{+}Pm and Bb were 100% under the sporadic occurrence characteristics of T^{+}Pm in China [18]. According to our results, the pathogens associated with PRDC in infected pigs were mainly Ss, Hps, and Mhr in the south of China, while Pm and Mhp were the main pathogens in the United States, and App was the major one in Europe [4,16,17]. We hypothesized that the difference may be attributed to the insufficient attention paid to pathogens by Chinese farmers, the lack of vaccination, and the inadequacy of biosafety protection. Furthermore, our research suggests that the threat of T^{+}Pm to these regional farms could not be ignored during the outbreak of atrophic rhinitis disease which may cause serious economic losses.

In our specific experiments, 77/88 (97.5%) of the samples were infected with these pathogens, and

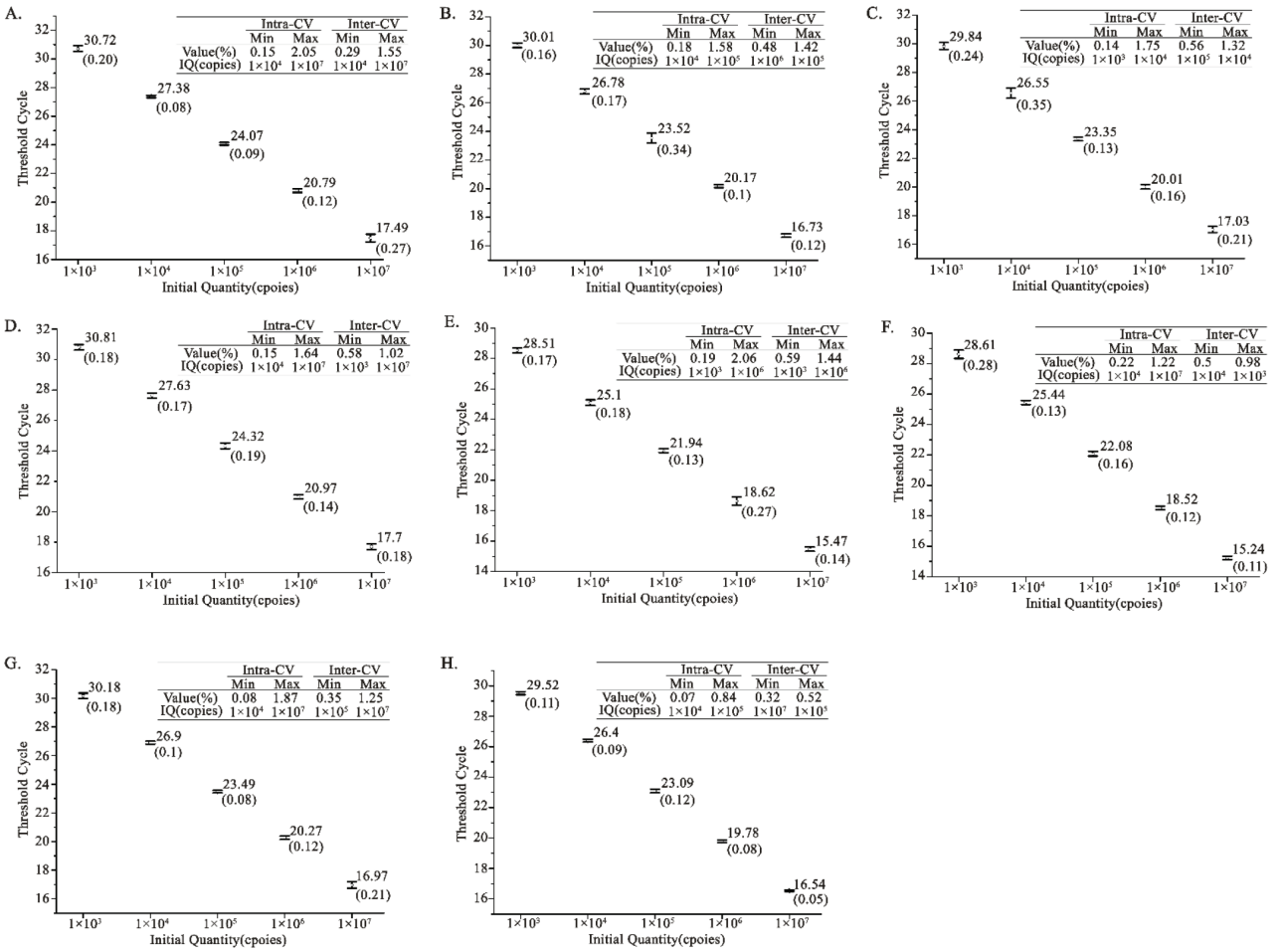

Figure 2. Error limit map for fluorescence quantitative PCR repeated detection of Ct value (The order of A~H is Ss, Hps, Mhr, App, Mhp, Bb, Pm, T⁺Pm. The upper-right table was the maximum and minimum coefficients of variation within or between in the three repeated test of plasmid standard). Abbreviations:Ss = *Streptococcus suis;*Hps = *Haemophilus parasuis;*Mhr = *Mycoplasma hyorhinis;* App = *Actinobacillus pleuropneumoniae;* Mhp = *Mycoplasma pneumonia;*Bb = *Bordetella bronchis;*Pm = *Pasteurella multocida;*T⁺Pm = toxic *Pasteurella multocida;*CV = variation coefficient; Ct = cycle threshold.

54/62 (87.1%) of the tissue samples were infected with these pathogens even after removing the nasal swab samples. Among these positive samples, co-infection samples accounted for 85.7% of the total samples. Our investigation once again confirmed that co-infection of bacteria is a common situation in PRCD cases, especially with 2-4 bacteria, which is similar to the published studies [18-22]. And the co-infection rates of pathogens within similar clinical symptoms were high, where the rates of Ss, Hps, and Mhr were 78.3%-100%, 45.5%, and 35.7% for App and Mhr, 41.4%-100% for Bb, Pm, and T⁺Pm. Therefore, the risk of misdiagnose by relying solely on clinical manifestation is very high. To solve this problem, the multiplex fluorescent PCR assay was developed and 2-8 stains of pathogenic bacteria can be detected simultaneously in one experiment, and the pathogenic bacteria with similar symptoms can be differentiated.

Compared with conventional PCR, firstly, fluorescence quantitative PCR has both primers and probes so the target genes of bacteria could be detected with no cross- reaction, except for the target genes Kmt, which were used to detect Pm and T⁺Pm. Secondly, we could complete the detection of a larger number of samples within 1.5 h. The whole reaction was in a closed-tube, not only reduced the risk of contamination but also greatly decreased processing times and labor costs. Also, the greatest advantage of fluorescence quantitative PCR technology is its high sensitivity, reproducibility, and wide dynamic range. Specifically, the detection limit of our assay is as low as 2-5 copies; the correlation coefficient of the standard curve could reach 0.998-1, and the amplification efficiency was more than 100%. Furthermore, the standard deviation and variation coefficients of repeated multiple fluorescence quantitative PCR were less than 0.35% and 2.06%, respectively, with good sensitivity and stability.

Table 4. Detection results and consistency of 88 clinical samples by two assays.

Copies of fluorescent quantitative (copies/reaction)		Conventional PCR															
		Ss		Hps		Mhr		App		Mhp		Bb		Pm		T^+Pm	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
≥100		49	0	45	2	42	0	7	0	9	0	13	0	23	0	11	0
1-99		16	8	12	9	3	19	2	6	5	2	10	0	6	4	0	0
0		0	15	0	20	0	24	0	73	0	72	0	65	0	55	0	77
Before setting threshold	Consistency (%)	90.9		87.5		78.4		93.2		97.7		100		95.5		100	
	Kappa values	0.735		0.702		0.564		0.713		0.92		1		0.901		1	
After setting threshold	Consistency (%)	92		96.6		97.7		97.7		100		-		100		-	
	Kappa value	0.808		0.924		0.955		0.887		1		-		1		-	

Abbreviations: Ss = *Streptococcus suis;*Hps = *Haemophilus parasuis;* Mhr = *Mycoplasma hyorhinis;* App = *Actinobacillus pleuropneumoniae;* Mhp = *Mycoplasma pneumonia;* Bb = *Bordetella bronchis;* Pm = *Pasteurella multocida;* T^+Pm = toxic *Pasteurella multocida;*

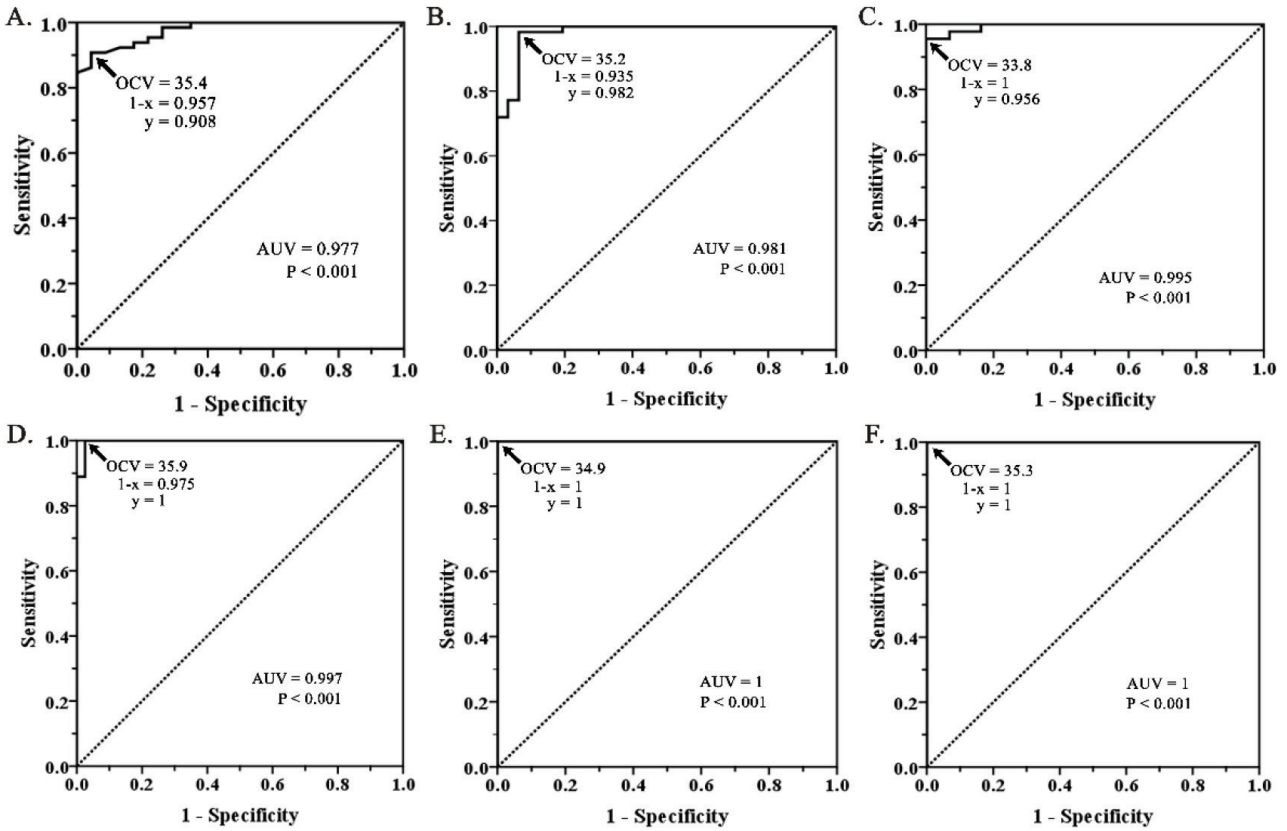

Figure 3. ROC curve of multiplex fluorescence quantitative PCR for clinical samples with the results of common PCR as reference. (A~H was Ss, Hps, Mhr, App, Mhp, Pm. Plotted the ROC curve taking detect Ct as the test variable and common PCR as state variable. AUV indicated the area under the curve, and when it was greater than 9, it showed that the detection method had high diagnostic value, $p < 0.001$ indicated that the diagnosis was valid. OCV was the best threshold, referring to the maximum Youden index of the curve (Youden index = sensitivity + specificity -1), and the point indicated by the arrow on the graph) Abbreviations:Ss = *Streptococcus suis;*Hps = *Haemophilus parasuis;*Mhr = *Mycoplasma hyorhinis;*App = *Actinobacillus pleuropneumoniae;* Mhp = *Mycoplasma pneumonia;*Bb = *Bordetella bronchis;*Pm = *Pasteurella multocida;*T^+Pm = toxic *Pasteurella multocida;*CV = variation coefficient; Ct = cycle threshold;ROC = receiver operating characteristic;OCV = optimal cut-off value; AUV = Area U*nder Curve value.*

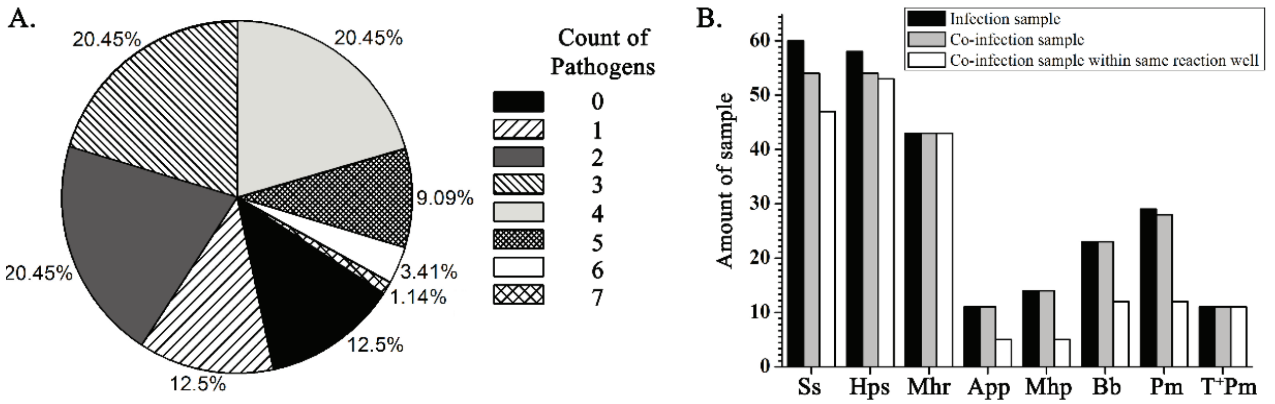

Figure 4. Bacterial infection in clinical samples. (A. pie chart of the number of bacterial species, 0 was negative,1 was single bacterial infection, 2-7 corresponded to 2-7 bacterial co-infection samples in turn. B. bar chart of the number of positive bacterial samples and co-infection samples. "infection samples" referred to the samples with corresponding bacterial infection; "co-infection" referred to the samples with co-infection of aim bacteria and other bacteria; "Co-infection sample within the same reaction system" referred to the co-infection of the bacteria with other bacteria in the same reaction system, respectively ,including No1 between Ss, Hps and Mhr , No2 between App and Mhp , between Bb, Pm and T^+Pm in No3 which Pm and T^+Pm double positive samples were excluded from the number of multiple infection samples. Abbreviations: Ss = *Streptococcus suis;* Hps = *Haemophilus parasuis;* Mhr = *Mycoplasma hyorhinis;* App = *Actinobacillus pleuropneumoniae;* Mhp = *Mycoplasma pneumonia;* Bb = *Bordetella bronchis;* Pm = *Pasteurella multocida;* T^+Pm = toxic *Pasteurella multocida*.

Generally, the detection limit of conventional PCR is more than 100 copies, which is 20-50 times higher than that of fluorescent quantitative PCR [10-15]. Some samples with low amplicons were considered negative in conventional PCR, but typical amplification curves appeared in fluorescent PCR, especially those with original amplicons less than the detection limit of conventional PCR. Only Bb and T^+Pm fluorescent quantitative PCR were completely consistent with the qualitative results of conventional PCR, while the other six pathogenic bacteria had different qualitative results with low amplicons amount. Considering the stability of multiplex fluorescence quantitative PCR for the original amplicons with detection limit decreased, the results could be false positive. To ensure the true positive rate in the detection, we set the OCV of Ct value by calculating the ROC curve of sensitivity and specificity of fluorescence quantitative PCR compared with conventional PCR as a reference test. The detection limit of the conventional Mhr PCR method we referred was only 500-1000 copies [12], the OCV of most bacterial fluorescence quantitative PCR in this study was close to the detection limit, which is about 35, and only the OCV of Mhr was 33.8.

5. Conclusion

A multiplex fluorescence quantitative PCR can simultaneously, quickly, and accurately diagnose 8 pathogenic bacteria of PRCD within 1.5 h. The detection limit of fluorescence quantitative PCR is as low as 2-5 copies. The coincidence rate of fluorescence quantitative PCR and conventional PCR reached 92%-100%, and the specificity and sensitivity of quantitative fluorescence PCR were between 93.5%-100% and 90.8%-100% after setting OCV. Besides, this method is suitable for use as a routine diagnostic test for the detection of a large number of clinical samples, especially those with complicated clinical symptoms. It can also provide potential technical support for the control of PRDC. And the co-infection of 2-4 pathogenic bacteria with similar symptoms is a common situation for PRDC in the south of China.

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