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Establishment of a Multiplex Real-Time TaqMan-MGB Polymerase Chain Reaction (PCR) Method for the Simultaneous Detection of Three Animal Chlamydia Species

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Chlamydiae are spread globally and cause infectious diseases in both humans and animals. The existing detection methods for this disease have numerous shortcomings, including low sensitivity, time consuming procedures, and high contamination vulnerability.

Material/Methods: To overcome shortcomings for detecting animal chlamydiosis, a multiplex quantitative polymerase chain reaction (PCR) assay was established for simultaneously detecting and differentiating 3 *Chlamydia* species *(C. pecorum, C. abortus, and C. psittaci*) by real time PCR based on TaqMan-MGB technology.

Results: The limit of detection was 20.2 copies/μL for *Chlamydophila (Cp.) abortus*, 30.8 copies/μL for *Cp. pecorum*, and 16 copies/μL for *Cp. psittaci*. This method has good repeatability and stability as coefficients of variation range from 0.04% to 1.38%. Furthermore, compared with OIE (World Organization for Animal Health) recommended PCR assay and previously reported animal chlamydia shell PCR, this multiplex PCR assay demonstrated 99% concordance in detecting clinical samples of porcine nasal swabs and vaginal swabs.

Conclusions: The novel established method in this study was able to detect 3 types of *Chlamydia* species simultaneously, and had high sensitivity, strong specificity, and good stability. It provided a rapid, reliable, and convenient method for epidemiological and clinical diagnosis of *chlamydiosis* in animals.

MeSH Keywords: Chlamydophila • Diagnosis • Real-Time Polymerase Chain Reaction • Sensitivity and Specificity • Zoonoses

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Background

Chlamydiae are spread globally and cause diseases in humans and animals. They may provoke ocular, pulmonary, genital, articular, and intestinal illness [1]. Outbreaks of chlamydiosis cause considerable economic damage for health care system and agriculture [2]. According to current classification system, the *Chlamydia* family is divided into the genera *Chlamydia* (*C.*) and *Chlamydophila (Cp.*). *Chlamydia* includes *C. trachomatis*, *C. suis*, *C. muridarum*, and *C. caviae*. *Chlamydophila* includes *Cp. abortus*, *Cp. felis*, *Cp. pecorum*, *Cp. pneumoniae*, and *Cp. psittaci* [3–5]. All *Chlamydia* species are potential zoonotic pathogens, and especially *Cp. pecorum*, *Cp. Abortus,* and *Cp. psittaci* pose the greatest threat to livestock breeding [1]. Besides, *Cp. psittaci* and *Cp. abortus* are classified as class B diseases according to a previously published study [3], therefore, quarantine inspection for Chlamydia is *inter alia* recommended in the course of international trade.

Cp. pecorum infected animals were found to suffer from intermittent encephalomyelitis, multiple arthritis, pneumonia, enteritis, vaginitis, and endometritis [4–6]. Pigs infected with *Cp. pecorum* suffer pneumonia, polyarthritis, pleurisy, pericarditis and abortion [7–9]. *C. trachomatis* and *Cp. pecorum* infections may be the main reasons for porcine chlamydial abortion [10]. *Cp. abortus*, known as ovine enzootic abortion (OEA), is mainly confined to the epithelial cells of reproductive tract. *Cp. abortus* infection usually results in damage to the reproductive tract, leading to miscarriage dams, stillbirth, low tire, sire orchitis, urethritis, and inflammation of the glans and the foreskin as characterized by chronic contagious disease [11–13]. In the United Kingdom, Longbottom et al. [12] showed that *Cp. abortus* infection causes up to 50% of all ovine abortions. *Cp. psittaci* is a type of intracellular parasitic zoonosis pathogen, which has a strong tendency to infect birds, poultry, and livestock. Through contact or inhalation of infectious secretions and excretions of poultry, humans have become infected, causing atypical pneumonia, sepsis, conjunctivitis, myocarditis, meningitis, etc. [1,14,15]. It thus was designated as a World Organization for Animal Health (OIE)-listed notifiable disease in 2018 [16]. Therefore, there is an urgent need to develop a rapid, reliable method for sensitive and specific detection of Chlamydia in animals.

Currently, the diagnostic methods for detection of Chlamydia including enzyme linked immunosorbent assay (ELISA), indirect hemagglutination test (IHA), complement fixation test (CFT), and polymerase chain reaction (PCR) [17,18]. Isolation of the pathogen is still considered to be the gold standard for diagnosis of chlamydiosis, however, the sensitivity is relatively low. Moreover, chlamydia-mycoplasma contamination is a common problem in cell culture [19,20]. Mukherjee et al. [21], using PCR and enzyme immune assay (EIA), compared the level of Chlamydia by direct detection of PCR and found it had a high positive rate and good sensitivity. Khan et al. [22] used RT-PCR detection of Chlamydia in children with bronchitis to show that this method was superior to conventional PCR. Opota et al. [23] improved the molecular diagnosis for the *C. abortus* and *C. psittaci* infection using the species-specific duplex RT-PCR assay. However, the use of conventional PCR imposes greater limitations, such as ease of contamination, time-consuming, and low sensitivity makes diagnostic testing of chlamydial zoonosis pathogens unsatisfactory. Thus, it is necessary to improve diagnostic methods of Chlamydia detection.

Material and Methods

Chlamydia strains

C. pneumoniae (ATCC 53592), *C. psittaci* (ATCC), *Cp. abortus* (ATCC 656), *Cp. pecorum* (ATCC 1575), *C. muridarum* (ATCC VR123), *C. suis* (ATCC VR1474), and *C. trachomatis* (ATCC VR878) were purchased from American Type Culture Collection (ATCC). *Cp. abortus*, *Cp. pecorum*, and *C. psittaci* were used as positive controls. Other related strains of *Chlamydia* were used for optimizing multiple quantitative PCR conditions.

Sample collection and DNA extraction

The nasopharyngeal swabs (n=246) and vaginal swabs (n=960) were collected from animals in farm with an abortion history. The samples were stored at –80°C until usage. DNA was extracted from clinical samples or cell culture supernatants using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden) according to manufacturer's instructions. DNA was eluted in 50 µL of elution buffer and kept at –80°C until further analysis. The quality and concentrations of DNAs were determined by spectrophotometer (BioMATE3, Thermo Scientific, Wilmington, DE, USA).

Primers and probes

The primers and probe for this experiment were designed based on the sequences of major outer membrane protein of chlamydial (including *Cp. pneumoniae*, *Cp. abortus*, *Cp. pecorum*, *C. muridarum*, *C. suis*, *C. trachomatis*, *Cp. psittaci*, *Cp. Felis*, *C. caviae*, and Cp. *abortus*), The sequences of Chlamydia major outer membrane protein were downloaded from the GenBank and aligned by MEGA 5.05 software. The conservative areas were used to design a pair of ordinary PCR primers (used in the construction of a positive quality) by using Primer Express 3.0 software. In addition, 3 pairs of primers and specific probes of individual Chlamydia were designed for multiplex PCR. The primer and probe sequences were listed in Table1.

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Table 1. Sequence of primers and probes.

Species	Primers and probes	Nucleotide sequence (5´-3´)	Product length (bp)
Chlamydiaceae	OMP Fw	CTCCTTRCAAGCYYTGCCTGT	1100
	OMP Rev	GTGAGCWGCTCTTTCRTYRATTAARCG	
Cp. abortus	Abortus Fw	GCATGGGTGCAGTTCCTACA	63
	Abortus Rev	TGGGTCTATCCGTAGGAGTTTTG	
	Abortus probe	5'NED-ACCGCAGCAGCTAA-MGB3'	
Cp. pecorum	Pecorum Fw	GCAGAGCCAAGTTTATTAATTG	125
	Pecorum Rev	TAGCGCAAGGATCACATG	
	Pecorum probe	5'FAM-ATCTCCTGACATACCTTC-MGB3'	
Cp. psittaci	Psittaci Fw	GCAACTCCTACGCAGGCTACA	67
	Psittaci Rev	TCGGTCTGCCATTTGCTTCT	
	Psittaci probe	5'VIC-AACGCAAGTAATACTAATCA-MGB3'	

Plasmids

Three specific targets of each Chlamydia were amplified with 3 pairs of primers (Table 1). Meanwhile, a most abundant chlamydial protein, major outer membrane protein (MOMP), was also detected (Table 1). The produced DNAs were extracted with agarose gels using Gel Extraction Kit (Cat. No. E.Z.N.A.TM, Omega Bio-Tek, Doraville, GA, USA), according to instructions of manufacturer. The amplified DNA fragments were subcloned into pMD19-T plasmid (Cat. NO. D102A, Takara, Dalian, China) according to manufacturer's protocol. Then, the recombined plasmids were transfected into the *Escherichia coli*. The MiniBEST Plasmid Extraction Kit (Cat. NO. 9760, Takara, Dalian, China) was employed to isolate the plasmids containing targeting genes. Subsequently, the targeting DNAs in subcloned plasmids were sequenced and analyzed with DNASTAR software 4.0 (DNASTAR, Madison, WI, USA). Eventually, the gene sequences were compared with the associated sequencing data recorded in the GenBank, to identity the cloned DNA fragments.

Measuring the concentration of each plasmid and calculating its copy number, the positive plasmid copy number (copies/μL)=plasmid concentration×10^{-9}×diluted multiples×6.02×10^{23})/(660 Dalton/bases x bases) to make the copy number positive. The copy numbers of *Cp. abortus*, *Cp. psittaci*, and *Cp. pecorum* were 2.02×10^{9} copies/μL, 1.6×10^{9} copies/μL and 3.08×10^{9} copies/μL respectively. The resultant recombinant plasmids contain the fragment of each *Chlamydia* strains were stored at –80°C and used as positive controls plasmids for subsequent PCR optimization.

Multiplex quantitative PCR

The constructed plasmids carrying the targeting DNA fragments were employed to optimize the multiple real-time PCR, as the PCR templates. The real-time PCR assay conditions were optimized by varying various single parameters and locking the other parameters. Based on findings of orthogonal experiments or tests selecting optimal primers proportion, we also optimized the appropriate effects of annealing-temperature and the other conditions on the PCR assay.

The optimized real-time PCR reaction (20 μL) was comprised of 1×Premix Ex Taq (Probe qPCR) (TaKaRa), 0.4 μmol/L *Cp. abortus* primers, 0.2 μmol/L *Cp. abortus* probe, 0.2 μmol/L *Cp. pecorum* primers, 0.05 μmol/L *Cp. pecorum* probe, 0.6 μmol/L *Cp. psittaci* primers, 0.1 μmol/L *Cp. psittaci* probe, and 2 μmol/L of DNA extract. The following thermal-cycling conditions were applied: 95°C for 30 seconds, following with 40 cycles in 2 steps: a) 95°C for 5 seconds and b) 58°C for 34 seconds. The fluorescence levels were measured at the end of each cycle.

Three strains of *Chlamydia* were mixed according to their individual optimized reaction systems: sterile water was added to bulk the volume to 20 μL. PCR optimization was carried out by modulated the concentration of primers, probe and cycling conditions, The multiplex PCR results were generated by following the PCR protocols described as follows: 1×premix Ex TagTm, 0.4 μmol/L *Cp. abortus primers*, 0.2 μmol/L *Cp. abortus* probe, 0.2 μmol/L *Cp. pecorum* primers, 0.05 μmol/L *Cp. pecorum* probe, 0.6 μmol/L *Cp. psittaci* primers, 0.1 μmol/L *Cp. psittaci* probe, and 1 μL of extracted plasmid DNA. Cycling conditions were as follows: 95°C for 30 seconds following with 40 cycles composing of 95°C for 5 seconds, and then 58°C for 34 seconds.

Diagnostic specificity and sensitivity

For specificity testing, plasmid contains of *Cp. pecorum*, *C. muridarum*, *C. trachomati*, *Cp. psittaci*, *Cp. abortus*, *Cp. pneumoniae*, and *C. suis* were used as PCR templates separately.

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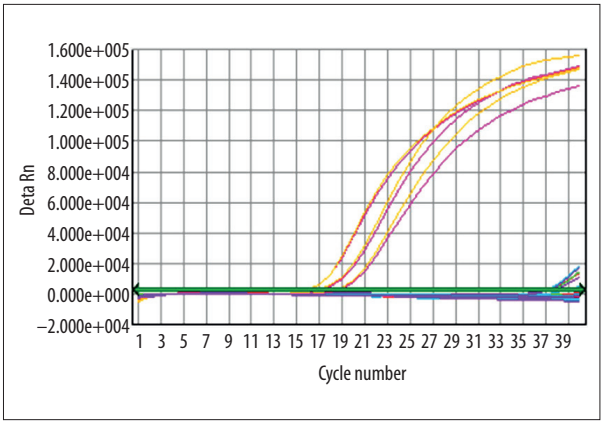

Figure 1. Multiplex polymerase chain reaction assay specific identification results.

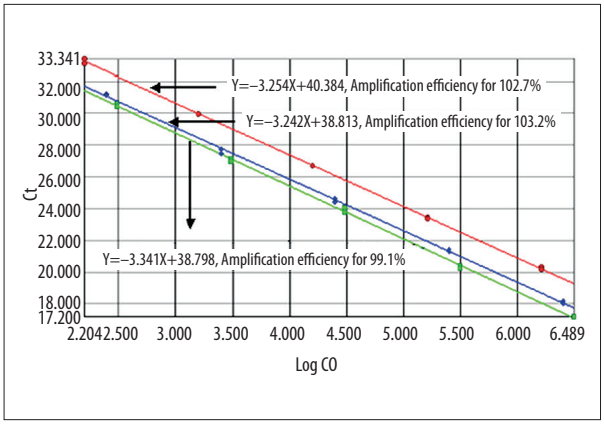

Figure 2. Multiplex real-time quantitative polymerase chain reaction standard curve.

The detection limit was determined using decimal serial dilutions of purified recombinant plasmid which contains chlamydial segments as template in multiplex PCR reaction (*Cp. abortus* 2.02×10^9 copies/μL to 2.02 copies/μL, *Cp. psittaci* 1.6×10^9 copies/μL to 1.6 copies/μL, *Cp. pecorum* 3.08×10^9 copies/μL to 3.08 copies/μL).

Standard curve, repeatability and stability assessment

To calculate the standard curve of each gene, serially diluted concentrations of *Cp. abortus*, *Cp. psittaci* or *Cp. pecorum* recombinant plasmids were used as template in multiplex real-time PCR. The repeatability and stability of multiplex real-time PCR was evaluated by several cycle of amplification in serial dilutions (10^6, 10^5, 10^4 copies/μL) for all of the aforementioned plasmids. All the reactions were run in triplicates.

Detection of clinical samples

Clinical specimens were employed to assess efficacy of the established multiplex real-time PCR assay. The specimens were 660 porcine nasopharyngeal swabs and 960 vaginal swabs collected from Chongqing at the same time. Here, multiplex real-time PCR assay established was referred as Test 1, while OIE recommended fluorescence quantitative PCR [16] was referred as Test 2 and reported animal chlamydia shell PCR was referred as Test 3 [23]. The detection methods established in this study detected 17 positive samples from 1620 (660 porcine nasopharyngeal swabs and 960 vaginal swabs) clinical samples and could distinguish the type of infection. Moreover, sequencing of the 17 chlamydial positive samples (Takara Biotechnology (Dalian) Co., Ltd., China) were used to confirm the PCR results.

Results

Analysis of specificity of multiplex quantitative PCR

To assess the specificity co-amplification of chlamydial strains using the established multiplex real-time PCR assay, a series of the different concentrations of *Cp. pecorum*, *C. muridarum*, *C. trachomati*, *Cp. psittaci*, *Cp. abortus*, *Cp. pneumoniae*, and *C. suis* DNA were co-amplified. *Cp. psittaci*, *Cp. abortus*, and *Cp. pecorum* were amplified in presence of other chlamydial strains. The results showed high specificity and no cross-reactivity with other viruses. The crossing threshold (Ct) values were locked for specific target DNA when the other strains were co-amplified (Figure 1).

Analysis of sensitivity of multiplex quantitative PCR

To assess the sensitivity of established multiplex real-time PCR assay, limit of the detection (LOD) was evaluated for *Cp. psittaci*, *Cp. abortus*, and *Cp. pecorum* using a 10-fold dilution of the plasmid standards. The LOD for *Cp. abortus was* 2.02×10 copies/μL, the LOD for *Cp. pecorum was* 3.08×10 copies/μL and the LOD of *Cp. psittaci was* 1.6×10 copies/μL.

Linear range, intra-and inter-run variation

Validation experiments were performed to determine the amplification efficiencies of 3 chlamydial strains by developed multiplex quantitative PCRs. Briefly, serially diluted plasmid was used as template DNA in the multiplex amplification. A good linear regression plot was found by plotting the Cq-values against the values of $\log_{10}$ DNA for the 3 curves and the corresponding standard curve showed that each of the 3 chlamydial strains had a regression factor R^2 of 0.999 and efficiency ranging from 99.1% to 103.2% (Figure 2).

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Table 2. Intra-assay and inter-assay reproducibility test of multiple fluorescence quantitative PCR.

Names	Standard dilution	Inter-coefficient of variation			Intra-coefficient of variation		
		Mean	SD	CV (%)	Mean	SD	CV (%)
Cab	1000	18.1094	0.0625	0.35	18.3380	0.0173	0.09
Cab	10000	21.3336	0.1034	0.48	21.4749	0.0057	0.03
Cab	100000	24.5173	0.1429	0.58	24.6650	0.1520	0.62
Cps	1000	19.7522	0.1349	0.68	19.7710	0.1621	0.82
Cps	10000	23.0197	0.0279	0.12	23.1184	0.0087	0.04
Cps	100000	26.0750	0.3001	1.15	26.0434	0.3595	1.38
Cpe	1000	17.0897	0.0194	0.11	17.1113	0.0157	0.09
Cpe	10000	20.1744	0.0890	0.44	20.1733	0.0865	0.43
Cpe	100000	23.7497	0.2211	0.93	24.0050	0.0105	0.04

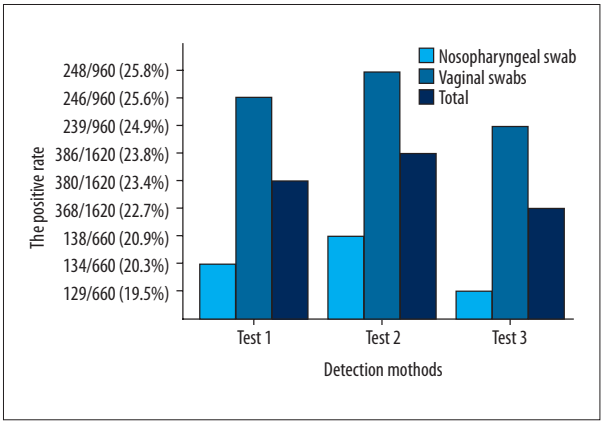

Figure 3. Comparison of positive testing rates of the 3 detection methods.

Reproducibility test of multiple fluorescence quantitative PCR

In order to assess repeatability, a serial 10-fold dilution (from 10^6 copies/μL to 10^4 copies/μL) of 3 chlamydial segments containing plasmids were used as PCR templates. Experiments were carried out in triplicate on 3 independent PCR reactions. The inter- and intra-batch coefficients of variation were range from 0.04% to 1.38% (Table 2). The results indicated the validity of the developed multiplex quantitative PCR.

Diagnostic application of the established multiplex real time PCR on clinical specimens

To assess reliability of established multiplex real-time PCR assay for diagnosing the chlamydial pathogens, performance of our developed assay was compared to those of the OIE recommended [16] and a previously reported reference method [23]. The 3 assays mentioned were used to test 660 porcine nasopharyngeal swabs and 960 vaginal swab samples. By using the OIE recommended PCR method, chlamydial infection was diagnosed with a positive rate of 20.9% in nasopharyngeal swabs and 25.8% in vaginal swabs. The positive rate was 19.5% and 24.9% respectively with previously reported nested PCR assay (Figure 3, Table 3). And with the multiplex PCR assay we developed in this study, the chlamydial pathogens of interest were detected in 20.3% of nasopharyngeal swabs and 25.6% of vaginal swabs (Figure 3, Table 4). The detection positive rates of multiplex quantitative PCR developed in this study displayed 99% identification agreement with the OIE recommendations and slightly higher than the nested PCR methods.

Table 3. Comparison of the results from developed multiplex realtime PCR and reference PCRs for detection of *Cp. abortus*, *Cp. pecorum*, and *C. psittaci* in clinical specimens.

Type	Detection methods		
	Test 1	Test 2 [16]	Test 3 [23]
Nasopharyngeal swabs	134/660 (20.3%)	138/660 (20.9%)	129/660 (19.5%)
Vaginal swabs	246/960 (25.6%)	248/960 (25.8%)	239/960 (24.9%)
Total	380/1620 (23.4%)	386/1620 (23.8%)	368/1620 (22.7%)

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Table 4. Multiple types of infection: quantitative PCR positive samples.

Type of infection	Positive (a)	The positive rate (%)
Cpe/Cps/Cab	1	5.88
Cpe/Cab	1	5.88
Cpe/Cps	2	11.76
Cps/Cab	1	5.88
Cab	4	23.53
Cps	3	17.64
Cpe	5	29.41
Total	17	100.00

Therefore, the application of multiplex quantitative PCR assay was comparable with the reference PCRs and has high levels of analytical and diagnostic accuracy of detection with suspected clinical specimens.

Discussion

The traditional detection methods of Chlamydia infection mainly include serological experiments, trace immunofluorescence methods and ELSA. However, the cross-reaction of immune-detecting method thus engendering a lack of specificity and sensitivity.

PCR is a sensitive, specific, and stable nucleic acid amplification technique. Currently, there are enormous methods of PCR for testing for Chlamydia infection. At present, the conventional PCR detection methods are mainly focused on the major outer membrane protein gene 16S/23S rRNA, and some of them performed well as detection methods for Chlamydia [19,24,25]. Condon designed a PCR method according to the chlamydial 16S rRNA and compared with a serological method and a pathogen separation method and found that PCR method have high sensitivity and strong specificity [26]. However, in this study, we detected the abundantly expressed chlamydial protein, MOMP, but not the chlamydial 16S/32S rRNA, for detecting Chlamydia infection. Meanwhile, the MOMP gene is usually attenuated in the chronic, persistent chlamydial infections, which has not been considered in this study and represented. In the following study, we would exclude the clinical specimens with the chronic, persistent chlamydial infections. Kaltenbock and colleagues developed a nested PCR assay, and tested 407 specimens from several host animals with various clinical conditions to evaluate their genus-specific amplification [27].

Real-time PCR developed in earlier studies mainly designed for a certain chlamydial strain [28,29]. Note that animal chlamydiosis may be caused by mixed infection. Several multiplex real-time PCR assays had been developed during the past few years. Pantchev and colleagues developed real-time PCR tests for species-specific detection of *C. psittaci* and *C. abortus* [30]. Rodolakis et al. [31] reported that their multiplex PCR assay could simultaneous differential detection of *Cp. abortus*, *Cp. pecorum*, and *Coxiella burnetii* from aborted ruminant's clinical samples. In this study, for the first time we developed and optimized a method of TaqMan-MGB based fluorescence PCR that can simultaneously detect the most important chlamydial species *Cp. pecorum, Cp. abortus*, and *Cp. psittaci* in a single reaction.

The probe was an MGB probe and its quenching was a kind of fluorescence quenching of the group. It will not produce fluorescence, and thus the quenching efficiency can reach a high level. At the same time, it can reduce the fluorescence background signal intensity of the PCR reaction. On the MGB probe there are special chemical groups known as small groove binders. The combination of these can makes the Tm value increased 10-fold and it greatly increased the specificity of the amplification reaction.

Compared to conventional PCR methods, multiple fluorescent quantitative PCR developed by this study was more sensitive and accurate [27]. The LOD of the multiplex PCR was 2.02×10, 3.08×10, and 1.6×10 for *Cp. Abortus*, *Cp. pecorum*, and *Cp. psittaci,* respectively. Although a previous study [23] also developed a TaqMan PCR method to detect the *Cp. abortus* and *Cp. psittaci,* with a higher sensitivity and specificity compared with the present results which did not detect *Cp. pecorum* with a higher efficacy. Although, the multiple fluorescent quantitative PCR established in this study had a high sensitivity. In addition, the CV value of inter- and intra-batch ranged from 0.04% to 1.38% which indicated good stability and repeatability of the multiplex PCR assay. However, this result did not evaluate the sensitivity and specificity of our established multiplex PCR method using the receiver operating characteristic (ROC) curve against the standard, which was a limitation of our study. Meanwhile, previously published studies [32,33] also demonstrated higher sensitivity, even higher than 97%, and higher specificity even higher than 90%. However, in our study, we assigned the LOD to represent the sensitivity of our multiplex real-time PCR, illustrating sensitivity of *Cp. abortus, Cp. pecorum,* and *Cp. psittaci with* 2.02×10 copies/μL, 3.08×10 copies/μL, and 1.6×10 copies/μL, respectively. Actually, the LOD represented sensitivities even achieve results up to 100%, therefore, the sensitivity of our established PCR assay was higher compared to previous studies [32,33]. For the specificity of the established PCR assay, although we have not quantified the value of specificity, and there was no cross-reactivity with other viruses, therefore, our results showed high specificity for diagnosing the *Cp. abortus, Cp. pecorum*, and *Cp. psittaci.*

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Finally, we tested porcine swab samples and compared the results with that of OIE recommendations and published animal Chlamydia nested PCR methods to assess application of multiplex quantitative PCR assay. The performances were in accordance with recommendations of the OIE and better than that of conventional PCR assay. We found that OIE ranks PCR detection methods as higher than multiple fluorescence quantitative PCR with regard detection rates because the OIE recommends PCR detection method as able to detect all chlamydial strains in animals while multiple fluorescence quantitative PCR can detect only 3 strains. Compared with the method of multiple fluorescent quantitative PCR for animal chlamydia shell PCR, we found that the detection accuracy of multiple fluorescence quantitative PCR was higher. The method of multiple fluorescent quantitative PCR established in this study had a high sensitivity and specificity. In addition, the most important advantage of this method was its ability to distinguish 3 different strains of Chlamydia. Moreover, the PCR method reported by Everett, could only detect *Chlamydiaceae* family not species-specific test, subsequent sequencing of PCR product is needed in order to identify the species [34]. While the multiplex method developed in this study could detect and differentiate the 3 most harmful *Chlamydia* species and capable of detecting mixed infections.

Actually, one PCR method might bring different diagnosing results of chlamydiosis for different species, such as human and other animal species [35]. A few established PCR assays are suitable for the chlamydiosis of humans, while some established PCR assays are suitable for the chlamydiosis of animals. However, in this study, we only identified the diagnosis efficacy of established multiplex real-time PCR for chlamydiosis of animals, which was a limitation of this study.

Conclusions

The multiplex real-time PCR method was found to be time saving, highly sensitive and specific and should be suitable for routine diagnostic testing. The fast diagnosis of animal *chlamydiosis* could thus provide a meaningful contribution to prevention and clinical treatment of animal Chlamydia infection in the future.

Conflict of interest

None.

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