Received: 4 November 2019 | Revised: 7 March 2020 | Accepted: 11 April 2020

DOI: 10.1111/tbed.13585

Transboundary and Emerging Diseases WILEY

ORIGINAL ARTICLE

Simultaneous identification of 6 pathogens causing porcine reproductive failure by using multiplex ligation-dependent probe amplification

Yingshan Zhou[1] | Lin Chen[1] | Lifei Zhang[1] | Chunyan Shao[1] | Jing Sun[1] | Sheng Jiang[1] | Quanjiang Song[1] | Bin Zhou[1] | Yongchun Yang[1] | Wanyu Dong[1] | Yang Yang[1] | Fangfang Wei[1] | Weihuan Fang[1,2] | Xiaodu Wang[1] | Houhui Song[1]

[1]Key Laboratory of Applied Technology on Green-Eco-Healthy Animal Husbandry of Zhejiang Province, Zhejiang Provincial Engineering Laboratory for Animal Health Inspection and Internet Technology, College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang A&F University, Lin'an, Zhejiang, China

[2]Zhejiang Provincial Key Lab of Preventive Veterinary Medicine, Institute of Preventive Veterinary Medicine, College of Animal Sciences, Zhejiang University, Hangzhou, Zhejiang, China

Correspondence
Houhui Song and Xiaodu Wang, College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang A&F University, Key Laboratory of Applied Technology on Green-Eco-Healthy Animal Husbandry of Zhejiang Province, Zhejiang Provincial Engineering Laboratory for Animal Health Inspection and Internet Technology, Lin'an, Zhejiang, China.
Emails: songhh@zafu.edu.cn (H. S.) and xdwang@zafu.edu.cn (X. W.)

Funding information
National Key Research and Development Program of China, Grant/Award Number: 2017YFC1200500; Key Research and Development Program of Zhejiang Province, Grant/Award Number: 2019C02043, 2019C02052 and 2018C02028; National Natural Science Foundation of China, Grant/Award Number: 31902249, 31972656, 31502127, 31602062, 31802258, 31602119 and 31502034; Zhejiang Provincial Natural Science Foundation, Grant/Award Number: LQ19C180003, LQ15C180002, LGN18C180001 and LQ19C180002; Zhejiang A&F University, Grant/Award Number: 2013FK023, 2013FR076, 2015FR042,

Abstract

We developed a multiplex ligation-dependent probe amplification (MLPA) assay for the simultaneous detection of 6 clinically relevant viral pathogens causing porcine reproductive failure, that is porcine reproductive and respiratory syndrome virus (PRRSV), Japanese encephalitis virus (JEV), classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), pseudorabies virus (PRV) and porcine parvovirus (PPV). The limits of detection for the assay varied among the 6 target organisms from 1 to 8 copies per MLPA assay. The MLPA assay was evaluated with 346 heparinized porcine umbilical cord blood specimens, and the results of the assay were compared to those of real-time PCR. The MLPA assay showed specificities and sensitivities of 99.2% and 100%, respectively, for PRRSV; 100% and 100%, respectively, for CSFV, PCV2, PRV and PPV. No sample was found to be positive for JEV by either the MLPA assay or the real-time PCR. In conclusion, the MLPA assay has comparable clinical sensitivity to that of real-time PCR assay and provides a useful tool for fast screening porcine reproductive failure-associated viruses.

KEYWORDS
Multiplex Ligation-dependent Probe Amplification (MLPA), porcine reproductive failure, viral disease

Yingshan Zhou and Lin Chen are contributed equally.

2018FR009 and 2018FR015; Department of Education of Zhejiang Province, Grant/Award Number: Y201533341; National Students' platform for innovation and entrepreneurship training program, Grant/Award Number: 201810341022

1 | INTRODUCTION

Agents that cause reproductive failure in sows lead to a broad spectrum of sequelae, including abortions, weak-born piglets, stillbirth, mummification, embryonic death and infertility. The major viral infectious causes of reproductive failure in pigs include porcine reproductive and respiratory syndrome virus, Japanese encephalitis virus, classical swine fever virus, porcine circovirus type 2, pseudorabies virus and porcine parvovirus (Lefebvre, 2015).

Porcine reproductive and respiratory syndrome (PRRS), caused by an arterivirus, is of great importance in China and throughout most of the world. This disease has caused tremendous economic losses to pig industry in recent years (Nan et al., 2017). Japanese encephalitis is a vector-borne disease that causes reproductive failure in pigs and encephalitis in humans in the Asia-Pacific region (Heffelfinger et al., 2017; Ricklin et al., 2016). Classical swine fever (CSF) is a highly contagious viral disease of swine. CSF is currently under control yet far from being eradicated in China (Luo, Li, Sun, & Qiu, 2014). Porcine circovirus type 2 (PCV2) is found ubiquitous in pigs worldwide and is associated with several conditions, including sporadic outbreaks of late-term abortions and term litters with increased numbers of dead piglets (Karuppannan & Opriessnig, 2017). Pseudorabies (PRV), caused by suid herpesvirus 1, is a major viral disease manifested in swine characterized by central nervous system disorders, respiratory symptoms or reproductive failure (Sun et al., 2016). Porcine parvovirus (PPV) is one of the most common and important infectious agents of infertility in most major swine-producing countries (Meszaros, Olasz, Csagola, Tijssen, & Zadori, 2017).

Multiplex Ligation-dependent Probe Amplification (MLPA) is a variation of the multiplex polymerase chain reaction that allows simultaneous amplification of up to 40 different targets with only a single primer pair (Schouten et al., 2002). Each MLPA probe consists of two parts: the left probe oligonucleotide (LPO) and the right probe oligonucleotide (RPO), which recognize directly adjacent target sites on the DNA. Only when LPO and RPO are hybridized to their respective targets, they can be ligated to form a complete probe. The ligated probes are simultaneously amplified exponentially by PCR with a universal primer pair. Each complete probe has a unique length, so the amplicons can be separated and distinguished by capillary electrophoresis.

The MLPA assay is a technique in detection of DNA copy number changes (Stuppia, Antonucci, Palka, & Gatta, 2012) and has mainly been used to study hereditary disorders (Sulek et al., 2013), tumours (Johann et al., 2017) and methylation status of DNA sequences (Nygren et al., 2005). It has also been applied in detecting human respiratory viruses (Reijans et al., 2008), sexually transmitted disease pathogens (Muvunyi et al., 2011), agents causing central nervous system infections (Wolffs et al., 2009) and foodborne pathogens (Kim et al., 2016). However, it has not yet been widely used in the field of veterinary virology, except for the bee virus screening (De Smet et al., 2012).

Our report here shows the application of MLPA for simultaneously detecting 6 porcine reproductive failure-associated viruses. Because of its high sensitivity and specificity, the MLPA assay is also a useful tool for differential diagnosis and epidemiological studies of viruses in pig populations.

2 | MATERIALS AND METHODS

2.1 | Viruses and real-time PCR

The virus isolates used in this study were PRRSV (2015-jx-01), JEV (SA-14-14-2), CSFV (shimen strain), PCV2 (GU325754), PRV (Hangzhou strain) and PPV (purchased from China Veterinary Culture Collection Center, CVCC AV30). The results of the real-time PCR obtained with nucleic acid preparations from fivefold serial dilutions of 6 viruses were kindly provided by Zhejiang Provincial Engineering Laboratory for Animal Health Inspection.

2.2 | Samples

A total of 346 heparinized porcine umbilical cord blood specimens were obtained from farms in Zhejiang Province between December 2017 and September 2018. Sampling was authorized by the piggery owner or manager. We selected 12 farms with reproductive issues (such as irregular oestrus returns, low farrowing rate, low number of piglets born alive, abortion, mummification, stillbirth), 12 farms with clinical concerns in weaners, growers and finishers, 3 farms with issues in both sows and progeny. The detailed information of the farms is shown in Table S1. To collect umbilical cord blood, expelled placenta was inverted and 3–4 umbilical cords (attached to the placenta and not visibly contaminated) from the same litter were milked into a heparinized test tube. The samples were split into 2 portions and stored at 4°C until shipment to the laboratory for the MLPA assay evaluation and testing by other assays as described below. Clinical specimens with known viral infection status proven by PCR from previous studies were selected to test the diagnostic sensitivity and specificity of the MLPA assay. These clinical specimens were positive for PRRSV, JEV, CSFV, PCV2, PRV, PPV, Porcine epidemic diarrhoea virus (PEDV) and transmissible gastroenteritis virus (TGEV).

2.3 | Stuffer-free probe and primer design

MLPA probes and pre-amplification primers were designed for 6 virus targets. For each virus, a pair of probes was designed following the rules described in the manual ''Design synthetic MLPA probes, Version 15'' (MRC Holland, Amsterdam, Netherlands). The probes and primers were designed using the Geneious software (Biomatters Ltd, Auckland, New Zealand) against the most conserved regions within each virus as determined by aligning all available gene sequences in the GenBank using MUSCLE alignment programme. The target region of the MLPA probes was flanked by the pre-amplification primers. The specificity of the probes was confirmed by running the LPO hybridizing sequence (LHS) and RPO hybridizing sequence (RHS) together by BLAST in the NCBI database. The LPO probe contained a universal forward primer binding sequence (5'-GGGTTCCCTAAGGGTTGGA-3') and LHS. The RPO probe contained RHS and a universal reverse primer binding sequence (5'-TCTAGATTGGATCTTGCTGGCAC-3'). The RPO were 5' phosphorylated. Oligos longer than 60 nt were synthesized with 'ultramers' quality. The pre-amplification primers and MLPA probes used in the experiments were ordered from GENEWIZ (Suzhou, China) and are listed in Tables 1 and 2.

TABLE 1 Sequences of the pre-amplification primers

Pathogen	Primer	Sequence (5'–3')
PRRSV	Forward	GGTACATGACATTCGCGCAC
	Reverse	CGGATGAAAGCCCGCGGCACT
JEV	Forward	AGAAGCGAGCTGATAGTAGCT
	Reverse	GCCGCCTGGGACGCCCCAACTTG
CSFV	Forward	TGGACATGTGTGAAAGGCGA
	Reverse	CATCTGATGCATGCACCTTGAC
PCV2	Forward	ATCTCATCATGTCCACCGCC
	Reverse	GACGTATCCAAGGAGGCGTT
PRV	Forward	TGATGACCGTGACGTACTCG
	Reverse	CGTGCAGCTTCACCTCG
PPV	Forward	CTGTGAATGTTAGTGTTCCTTTCC
	Reverse	GAACTTGATACAGATCTAAAACCTAGACTACA

2.4 | Nucleic acid extraction

Viral RNA or DNA was extracted by using the automated TIANLONG® Nucleic Acid Extractor Workstation (Tianlong, China). The Viral DNA/RNA Kit (Tiangen, China) and the total nucleic acid lysis extraction protocol were applied. Extraction was performed according to the manufacturer's instructions. Briefly, 200 μl of starting material was used, and the purified nucleic acid was eluted in a final volume of 100 μl.

2.5 | Pre-amplification

A one-step RT-PCR protocol was performed with a One-Step RT-PCR kit (Qiagen, Hilden, Germany). Reverse transcription and PCR were carried out sequentially in the same tube. A total volume of 25 μl reaction mixture contains 10 μl of OneStep Ahead RT-PCR Master Mix, 1 μl OneStep Ahead RT-Mix, all pre-amplification primers (0.5 μM of each primer) and 10 μl of the RNA or DNA sample. Amplification was performed on Bio-Rad T100 thermocycler with an initial reverse transcription at 50°C for 10 min, followed by PCR activation at 95°C for 5 min, 40 cycles of 95°C for 15 s, 57°C for 20 s and 72°C for 20 s with a final elongation at 72°C for 2 min.

TABLE 2 Target genes and sequences of the left probe oligonucleotide (LPO) and right probe oligonucleotide (RPO)

Virus	Target gene	Probe	Sequence (5'–3')	Amplicon Size(bp)
PRRSV	M gene	LPO	gggttccctaagggttggaGAAACCTGGAAATTCATCACCTCCAG	94
		RPO	ATGCCGTTTGTGCTTGCTAGGCCGCAtctagattggatcttgctggcac	
JEV	E glycoprotein gene	LPO	gggttccctaagggttggaGTGGACTTTTCGGGAAGGGAAGCATTGACA	100
		RPO	CATGTGCAAAATTCTCCTGCACCAGTAAtctagattggatcttgctggcac	
CSFV	E2 gene	LPO	gggttccctaagggttggaGGTAAGTGCATTTTGGCAAATGAGACAG	106
		RPO	GTTACAGAATAGTAGATTCAACGGACTGTAACAGAGtctagattggatcttgctggcac	
PCV2	ORF2	LPO	gggttccctaagggttggaGGTGCGGGAGAGGCGGGTGTTGAAGATGCCATTTTTCC	116
		RPO	TTCTCCAGCGGTAACGGTGGCGGGGGTGGACGAGCCtctagattggatcttgctggcac	
PRV	gE	LPO	gggttccctaagggttggaTCGGGGACCGGTGGCTGACCGCCTGCCCCTTCGACGCCTT	122
		RPO	CGGCGAGGAGGTGCACACGAACGCCACCGCGGACGAGTCGtctagattggatcttgctggcac	
PPV	VP2	LPO	gggttccctaagggttggaGCACCAAACCTAACAGATGATTTCAATGCTGACTCTCCT	127
		RPO	CAACAACCTAGAATAATAACTTATTCAAACTTTTGGTGGAAAGGAAtctagattggatcttgctggcac	

Note: Universal primers binding sequences are shown in lower-case letters, the target-specific sequences are shown in upper-case letters. Each RPO probe is 5'-phosphorylated.

2.6 | Construction of a MLPA ladder

Synthetic viral target genes including both pre-amplification PCR primer regions and the MLPA probe binding sequence were constructed by Generay. Constructs were mixed in equal amounts and used as template in a MLPA reaction to amplify the expected products. The amplified product was loaded as a positive control to facilitate the interpretation of the results.

2.7 | MLPA reaction

MLPA reaction was performed using a commercial SALSA MLPA EK1 reagent kit (MRC Holland) per the manufacturer's instructions with a minor adjustment. All MLPA reaction steps were performed with a Bio-Rad T100 thermocycler. Five microlitres pre-amplification mixture was denatured at 98°C for 5 min and cooled at 25°C. A mixture of 1.5 μl of probe mix containing all half-probe oligos (1.33 nM of each oligo) and 1.5 μl of MLPA buffer (10 mM Tris-HCl, pH 8.5, KCl, EDTA and PEG-6000) was added to the pre-amplification mixture. The mixture was then denatured at 95°C for 1 min and hybridized at 60°C for 4 hr. After hybridization, the ligation reaction was performed at 54°C for 15 min with the ligation mixture (32 μl), which contained 3 μl ligase buffer A (coenzyme NAD, pH3.5), 3 μl ligase buffer B (Tris-HCl, $MgCl_2$, non-ionic detergents, pH 8.5), 1 μl ligase-65 enzyme (glycerol, EDTA, KCl, Tris-HCl, pH 7.5) and 25 μl ultrapure water. Subsequently, ligase inactivation was performed at 98°C for 5 min. A volume of 2 μl ligated mixture was used in a 20-μl reaction volume containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1 U *Taq* polymerase and 0.2 μM of the two PCR primers (forward primer, 5-GGGTTCCCTAAGGGTTGGA-3; reverse primer, 5-GTGCCAGCAAGATCCAATCTAGA-3). PCR amplification was performed on Bio-Rad T100 thermocycler with an initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min with a final extension step at 72°C for 20 min. The amplified MLPA products were analysed using a high-resolution gel cartridge on Qsep100 DNA Analyzer (BIOptic, Taiwan, China).

2.8 | In vitro transcripts of viral targets

Cloned viral targets mentioned above were linearized with a restriction enzyme and transcribed in vitro from the T7 promoter by using the MEGAscript™ T7 Transcription Kit (Life Technologies). Template DNA was removed by TURBO DNase treatment. The concentration of the T7 RNA transcripts was determined by measuring the A_{260} of 1:300 diluted aliquot of the reaction (for single-stranded RNA, 1 A_{260} unit corresponds to 40 μg/ml).

2.9 | Confirmation of the MLPA results

Species-specific real-time PCR of each pathogen was conducted on all the samples to confirm the specificity of the MLPA targets. For PRRSV, the result of the MLPA assay was compared with the method recommended by National standard in China and the Office international des epizooties (Kleiboeker et al., 2005). For JEV, a real-time RT-PCR was used as comparative assay (Huang et al., 2004). For CSFV, the MLPA results were first compared to the method of Zhao (Zhao et al., 2008). A *virotype*® CSFV RT-PCR Kit (Qiagen) was used as a confirmatory test to test samples with discrepant results. The commercial assay was performed according to the manufacturers' instructions. For PCV2, PRV and PPV, the MLPA results were

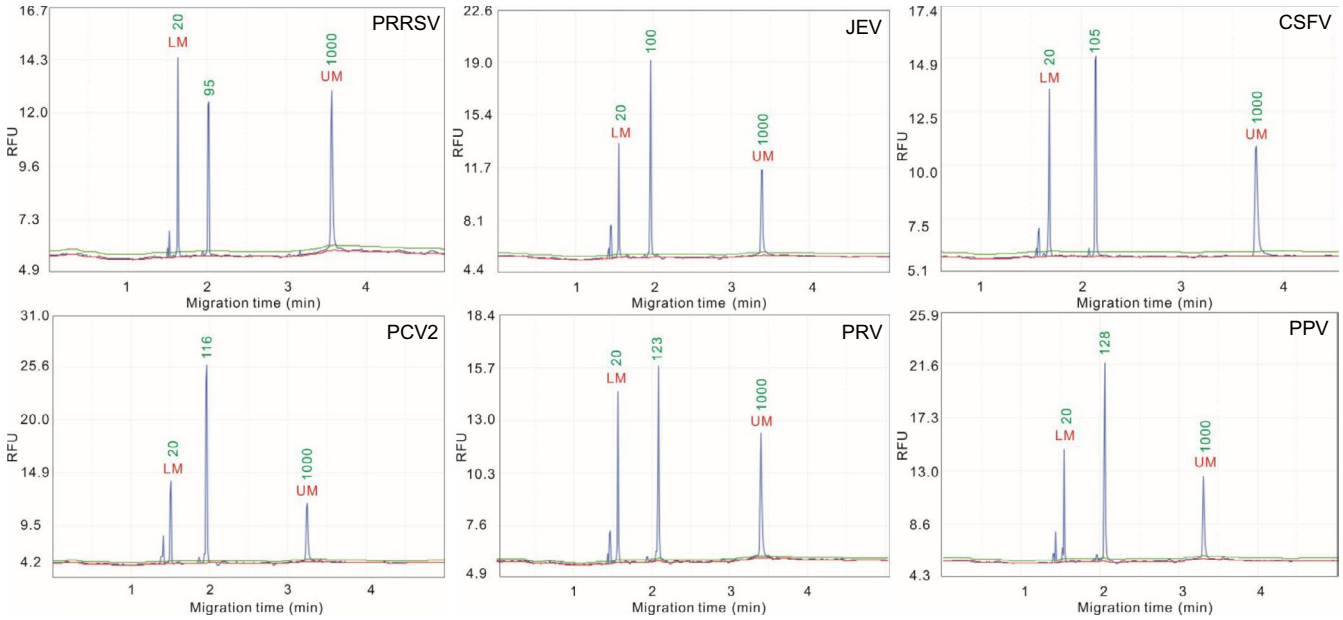

FIGURE 1 Electropherograms obtained from the capillary electrophoresis analysis of the MLPA products for each pathogen. LM: lower marker, UM: upper marker

compared to the methods described previously (Ma et al., 2008; Song, Zhu, Zhang, & Cui, 2010; Zhu et al., 2012). Determination of true-positive cases for the pathogens studied was based on a positive result by at least 2 PCR assays.

3 | RESULTS

3.1 | Specificity

The specificity of the pre-amplification primers and the MLPA probes was tested in a monoplex or multiplex MLPA reaction with different viruses. There was no cross-reactivity between any of the targets when tested with cell culture samples of each virus. The electropherogram of each viral pathogen that was analysed using MLPA is shown in Figure 1. All 6 target viruses were identified from a single peak and were also analysed simultaneously from a single reaction. The peaks of the 6 viruses were separated from one another (Figure 2). The diagnostic ability of the MLPA assay was tested on previously PCR-proven virus-positive samples (Figure 3). No other pathogens showed cross-reactivity with the MLPA probes (see Figure S1).

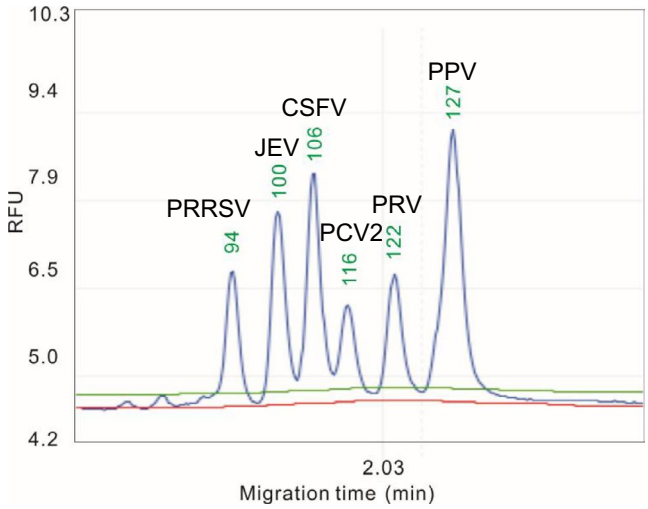

FIGURE 2 Electropherogram obtained from the capillary electrophoresis analysis of the multiplex MLPA products for 6 viral pathogens

3.2 | Detection limit

The analytical detection limit of the MLPA assay was evaluated using ten-fold serial dilutions of viral RNA transcripts of all RNA viruses and plasmids with target virus sequences of DNA viruses and was found to be 1 copy/MLPA assay for PRRSV, 8 copies/MLPA assay for JEV, 1 copy/MLPA assay for CSFV, 1 copy/MLPA assay for PCV2, 7 copies/MLPA assay for PRV and 8 copies/MLPA assay for PPV.

The sensitivity of the MLPA assay was compared to that of the real-time PCR. Nucleic acid preparations from fivefold serial dilutions of culture supernatants of 6 viruses were tested. The results are shown in Table 3. The results indicate that the sensitivity of the MLPA assay was comparable to that of the real-time PCR.

3.3 | Evaluation with clinical specimens

The MLPA assay was evaluated on 346 porcine umbilical cord blood specimens collected from pig farms in Zhejiang province. Altogether,

TABLE 3 Sensitivity of the MLPA assay compared to that of real-time PCR[a]

Virus	Highest viral dilution detected by:	
	MLPA assay	Real-time PCR
PRRSV	10	7
JEV	6	7
CSFV	8	5
PCV2	12	11
PRV	6	7
PPV	8	8

[a]Fivefold serial dilutions of all indicated viruses were analysed.

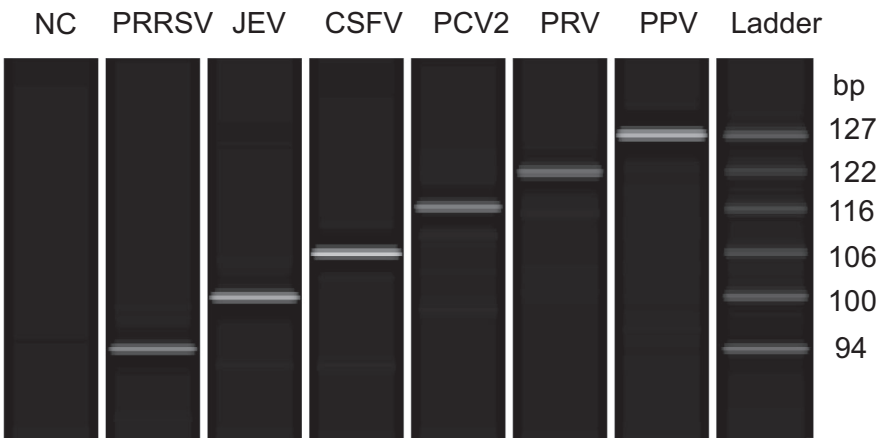

FIGURE 3 Results of the MLPA assay with clinical samples which were analysed by capillary electrophoresis. MLPA reactions were performed on PCR-proven virus-positive samples which are indicated on the top of each lane. The MLPA amplicons were analysed via capillary electrophoresis using a high-resolution gel cartridge on Qsep100 DNA Analyzer. MLPA ladder showing the different sized bands representing the respective viruses are indicated at the right site of panel

28.0% (97/346) of samples were positive for PRRSV, 9.5% (33/346) for CSFV, 24.9% (86/346) for PCV2, 5.8% (20/346) for PRV, two samples were positive for PPV, no sample was found positive for JEV (Table 4).

The specimens were allocated and examined with virus-specific real-time PCR, and the results were compared to those of the MLPA assay (Table 4). No sample was tested positive for JEV by either the real-time PCR or the MLPA assay. For 86 PCV2-positive samples, 20 PRV-positive samples and 2 PPV-positive samples, concordant results were obtained by both the MLPA assay and the real-time PCR.

The MLPA assay and the PCR results were the same for PRRSV in 340 of 346 samples. The MLPA assay detected 6 more positive samples for PRRSV. All aberrant results were detected by the real-time RT-PCR recommended by the Office international des epizooties (Kleiboeker et al., 2005), which confirmed 4 of the positive samples. The remaining 2 discrepant samples were further sequenced and found to be false positive. In agreement with the results of real-time RT-PCR, the MLPA assay identified 31 samples positive for CSFV, in addition, the MLPA assay detected 2 more positive samples. The commercial kit confirmed all positive results by the MLPA assay.

The sensitivity and specificity of the MLPA assay were calculated after resolving the inconsistent results in PRRSV and CSFV detection (Table 5). For PRRSV, the sensitivity and specificity were 100% and 99.2%, respectively. The MLPA assay was both 100% sensitive and specific for the detection of CSFV, PCV2, PRV and PPV. The sensitivity for JEV could not be calculated due to the absence of JEV-positive specimens.

TABLE 4 Comparison of the results obtained by the MLPA assay with the real-time PCR in 346 clinical specimens

	No. of positive/negative by comparator assays:		
Virus	**Positive**	**Negative**	**Total**
PRRSV			
Positive	91	6	97
Negative	0	249	249
JEV			
Positive	0	0	0
Negative	0	346	346
CSFV			
Positive	31	2	33
Negative	0	313	313
PCV2			
Positive	86	0	86
Negative	0	260	260
PRV			
Positive	20	0	20
Negative	0	326	326
PPV			
Positive	2	0	2
Negative	0	344	344

4 | DISCUSSION

Viral culture is the diagnostic gold standard in clinical virology, however, isolation of viruses in cell culture is time-consuming and difficult to perform. The evolvement of molecular methods has greatly improved the timeliness, accuracy and sensitivity of viral diagnosis. Molecular testing has replaced traditional viral culture approach in many laboratories (Hodinka & Kaiser, 2013). The MLPA assay takes the advantage of multiplex PCR in which a primer set permits amplification of multiple targets, which is suitable for detection of a large scale of pathogens simultaneously.

Pig viral diseases are a complex problem owing to potential co-infection with several viruses, therefore, our study aims to develop a MLPA approach that is able to detect 6 porcine reproductive failure-associated pathogens simultaneously including porcine reproductive and respiratory syndrome virus, classical swine fever virus, Japanese B encephalitis virus, porcine circovirus type 2, pseudorabies virus and porcine parvovirus.

A standard MLPA reaction requires at least 6,000 copies of target DNA (Schouten et al., 2002). In order to achieve the required sensitivity for the effective detection of infectious pathogens, we added a pre-amplification step. This step combines a reverse transcription step and a 40-cycle target amplification step, which is then present at levels above the detection level of MLPA. Due to the introduction of the pre-amplification step, the hybridization time in the MLPA reaction could be reduced from 16 hr set by the original

TABLE 5 Sensitivities and specificities of the MLPA assay compared with those of the real-time PCR

	No. of samples with the following results					
Virus	**True positive**	**False positive**	**True negative**	**False negative**	**Sensitivity (%)**	**Specificity (%)**
PRRSV	95	2	249	0	100	99.2
CSFV	33	0	313	0	100	100
PCV2	86	0	260	0	100	100
PRV	20	0	326	0	100	100
PPV	2	0	344	0	100	100

protocol to 4 hr, therefore, the whole assay could be accomplished within a day.

MLPA probes can discriminate two closely-related sequences or one that specifically detects single-nucleotide polymorphism or a point mutation (Schouten et al., 2002). A major consideration in the design of MLPA probes for viruses, especially RNA viruses, is the genetic variance. Viral sequences retrieved from GenBank database were aligned and used to design virus-specific probes that would compatible with as many sequences as possible. However, the specificity of the probes should not be compromised. Sequence variations within two nucleotides of the ligation site can result in a reduced probe signal due to the less effective ligation of the two probe oligonucleotides. Besides, mismatches in the middle of the hybridizing sequence may have a strong effect on the final probe signal by negatively affecting the stability of the probe binding, due to the reduction of the remaining melting temperature. Therefore, the probes we designed were positioned in highly-conserved regions. The effect of mismatches was minimized by situating the mutations at least 8 nucleotides from the ligation site. No cross-reactivity was detected with any of the probes.

The sensitivity and the specificity of the MLPA assay were assessed by comparison of the results of the MLPA assay to those of real-time PCR obtained with 346 clinical samples. The MLPA assay detected 6 more virus-positive samples which were missed by the PCR. For PRRSV detection, in resolving the MLPA positive/real-time PCR negative specimens, a second real-time PCR confirmed 4 specimens (4/6) as truly positive. The remaining 2 discrepant samples were further sequenced and found to be false positive, resulting in a final specificity of 99.2%.

In order to facilitate the analysis of the MLPA results, a control was run each time by adding equimolar amounts of recombinant plasmid as template each corresponding to a specific virus. Due to the small length differences between the target sequences, agarose gel electrophoresis was unable to distinguish all product amplicons. The results were analysed by a high-resolution gel cartridge on Qsep100 DNA Analyzer. The amplified MLPA products can be also analysed on other platforms, such as by Beckman GenomeLab GeXP and ABI-Prism 310 Genetic Analyzer.

In summary, we have developed a MLPA approach to detect 6 porcine reproductive failure-associated viruses simultaneously. The analytical sensitivity of the MLPA assay is comparable to the sensitivity of the real-time RT-PCR. The evaluation with clinical samples shows that the MLPA assay gives a satisfying overall performance. The assay only needs a PCR machine and a capillary electrophoresis system. Compared to other techniques, MLPA assay is easy-to-perform, cost-effective and allows fast detection for a broad spectrum of viruses. The MLPA technology allows the analysis of up to 45 sequences, which gives room for future expansion of the number of probes in the assay.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

ACKNOWLEDGEMENTS

This study was supported by the National Key Research and Development Program of China (2017YFC1200500), the Key Research and Development Program of Zhejiang Province (2019C02043, 2019C02052, 2018C02028), National Natural Science Foundation of China (No. 31902249, 31972656, 31502127, 31602062, 31802258, 31602119, 31502034), Zhejiang Provincial Natural Science Foundation (No. LQ19C180003, LQ19C180002, LGN18C180001, LQ15C180002), the grant from Zhejiang A&F University (2013FK023, 2013FR076, 2015FR042, 2018FR009, 2018FR015), the Department of Education of Zhejiang Province (No. Y201533341). National Students' platform for innovation and entrepreneurship training programme (201810341022). The funding bodies had no role in the design of the study and collection, analysis and interpretation of data and in writing the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Yingshan Zhou https://orcid.org/0000-0002-8011-431X
Xiaodu Wang https://orcid.org/0000-0002-0512-9212
Houhui Song https://orcid.org/0000-0001-6530-5794

REFERENCES

De Smet, L., Ravoet, J., de Miranda, J. R., Wenseleers, T., Mueller, M. Y., Moritz, R. F., & de Graaf, D. C. (2012). BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. *PLoS ONE*, *7*(10), e47953. https://doi.org/10.1371/journal.pone.0047953

Heffelfinger, J. D., Li, X. I., Batmunkh, N., Grabovac, V., Diorditsa, S., Liyanage, J. B., … Marfin, A. A. (2017). Japanese encephalitis surveillance and immunization—Asia and Western Pacific Regions, 2016. *Morbidity and Mortality Weekly Report*, *66*(22), 579. https://doi.org/10.15585/mmwr.mm6622a3

Hodinka, R. L., & Kaiser, L. (2013). Is the era of viral culture over in the clinical microbiology laboratory? *Journal of Clinical Microbiology*, *51*(1), 2–8. https://doi.org/10.1128/JCM.02593-12

Huang, J.-L., Lin, H.-T., Wang, Y.-M., Weng, M.-H., Ji, D.-D., Kuo, M.-D., … Lin, C.-S. (2004). Sensitive and specific detection of strains of Japanese encephalitis virus using a one-step TaqMan RT-PCR technique. *Journal of Medical Virology*, *74*(4), 589–596. https://doi.org/10.1002/jmv.20218

Johann, P. D., Hovestadt, V., Thomas, C., Jeibmann, A., Heß, K., Bens, S., … Hasselblatt, M. (2017). Cribriform neuroepithelial tumor: Molecular characterization of a Smarcb1-deficient non-rhabdoid tumor with favorable long-term outcome. *Brain Pathology*, *27*(4), 411–418. https://doi.org/10.1111/bpa.12413

Karuppannan, A. K., & Opriessnig, T. (2017). Porcine circovirus type 2 (PCV2) vaccines in the context of current molecular epidemiology. *Viruses*, *9*(5), 99. https://doi.org/10.3390/v9050099

Kim, S.-Y., Chung, B., Chang, J.-H., Jung, G. Y., Kim, H. W., Park, B.-Y., … Oh, M.-H. (2016). Simultaneous identification of 13 foodborne pathogens by using capillary electrophoresis-single strand conformation polymorphism coupled with multiplex ligation-dependent probe

amplification and its application in foods. *Foodborne Pathogens and Disease*, *13*(10), 566–574. https://doi.org/10.1089/fpd.2016.2143

Kleiboeker, S. B., Schommer, S. K., Lee, S.-M., Watkins, S., Chittick, W., & Polson, D. (2005). Simultaneous detection of North American and European porcine reproductive and respiratory syndrome virus using real-time quantitative reverse transcriptase–PCR. *Journal of Veterinary Diagnostic Investigation*, *17*(2), 165–170. https://doi.org/10.1177/104063870501700211

Lefebvre, R. C. (2015). Fetal mummification in the major domestic species: Current perspectives on causes and management. *Veterinary Medicine: Research and Reports*, *6*, 233–244. https://doi.org/10.2147/VMRR.S59520

Luo, Y., Li, S., Sun, Y., & Qiu, H. J. (2014). Classical swine fever in China: A minireview. *Veterinary Microbiology*, *172*(1–2), 1–6. https://doi.org/10.1016/j.vetmic.2014.04.004

Ma, W., Lager, K. M., Richt, J. A., Stoffregen, W. C., Zhou, F., & Yoon, K. J. (2008). Development of real-time polymerase chain reaction assays for rapid detection and differentiation of wild-type pseudorabies and gene-deleted vaccine viruses. *Journal of Veterinary Diagnostic Investigation*, *20*(4), 440–447. https://doi.org/10.1177/104063870802000405

Meszaros, I., Olasz, F., Csagola, A., Tijssen, P., & Zadori, Z. (2017). Biology of porcine parvovirus (Ungulate parvovirus 1). *Viruses*, *9*(12), 393. https://doi.org/10.3390/v9120393

Muvunyi, C. M., Dhont, N., Verhelst, R., Crucitti, T., Reijans, M., Mulders, B., … Padalko, E. (2011). Evaluation of a new multiplex polymerase chain reaction assay STDFinder for the simultaneous detection of 7 sexually transmitted disease pathogens. *Diagnostic Microbiology and Infectious Disease*, *71*(1), 29–37. https://doi.org/10.1016/j.diagmicrobio.2011.06.005

Nan, Y., Wu, C., Gu, G., Sun, W., Zhang, Y. J., & Zhou, E. M. (2017). Improved vaccine against PRRSV: Current progress and future perspective. *Frontiers in Microbiology*, *8*, 1635. https://doi.org/10.3389/fmicb.2017.01635

Nygren, A. O., Ameziane, N., Duarte, H. M., Vijzelaar, R. N., Waisfisz, Q., Hess, C. J., … Errami, A. (2005). Methylation-specific MLPA (MS-MLPA): Simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Research*, *33*(14), e128–e128. https://doi.org/10.1093/nar/gni127

Reijans, M., Dingemans, G., Klaassen, C. H., Meis, J. F., Keijdener, J., Mulders, B., … Simons, G. (2008). RespiFinder: A new multiparameter test to differentially identify fifteen respiratory viruses. *Journal of Clinical Microbiology*, *46*(4), 1232–1240. https://doi.org/10.1128/JCM.02294-07

Ricklin, M. E., García-Nicolás, O., Brechbühl, D., Python, S., Zumkehr, B., Nougairede, A., … Summerfield, A. (2016). Vector-free transmission and persistence of Japanese encephalitis virus in pigs. *Nature Communication*, *7*, 10832. https://doi.org/10.1038/ncomms10832

Schouten, J. P., McElgunn, C. J., Waaijer, R., Zwijnenburg, D., Diepvens, F., & Pals, G. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Research*, *30*(12), e57–e57. https://doi.org/10.1093/nar/gnf056

Song, C., Zhu, C., Zhang, C., & Cui, S. (2010). Detection of porcine parvovirus using a taqman-based real-time pcr with primers and probe designed for the NS1 gene. *Virology Journal*, *7*(1), 353. https://doi.org/10.1186/1743-422X-7-353

Stuppia, L., Antonucci, I., Palka, G., & Gatta, V. (2012). Use of the MLPA assay in the molecular diagnosis of gene copy number alterations in human genetic diseases. *International Journal of Molecular Sciences*, *13*(3), 3245–3276. https://doi.org/10.3390/ijms13033245

Sulek, A., Elert, E., Rajkiewicz, M., Zdzienicka, E., Stepniak, I., Krysa, W., & Zaremba, J. (2013). Screening for the hereditary spastic paraplaegias SPG4 and SPG3A with the multiplex ligation-dependent probe amplification technique in a large population of affected individuals. *Neurological Sciences*, *34*(2), 239–242. https://doi.org/10.1007/s10072-011-0899-3

Sun, Y., Luo, Y., Wang, C. H., Yuan, J., Li, N., Song, K., & Qiu, H. J. (2016). Control of swine pseudorabies in China: Opportunities and limitations. *Veterinary Microbiology*, *183*, 119–124. https://doi.org/10.1016/j.vetmic.2015.12.008

Wolffs, P. F. G., Vink, C., Keijdener, J., Habek, B., Reijans, M., Simons, G., … van den Brule, A. J. C. (2009). Evaluation of MeningoFinder, a novel multiplex ligation-dependent probe amplification assay for simultaneous detection of six virus species causing central nervous system infections. *Journal of Clinical Microbiology*, *47*(8), 2620–2622. https://doi.org/10.1128/JCM.02436-08

Zhao, J.-J., Cheng, D., Li, N. A., Sun, Y., Shi, Z., Zhu, Q.-H., … Qiu, H.-J. (2008). Evaluation of a multiplex real-time RT-PCR for quantitative and differential detection of wild-type viruses and C-strain vaccine of Classical swine fever virus. *Veterinary Microbiology*, *126*(1), 1–10. https://doi.org/10.1016/j.vetmic.2007.04.046

Zhu, B., Xu, F., Li, J., Shuai, J., Li, X., & Fang, W. (2012). Porcine circovirus type 2 explores the autophagic machinery for replication in PK-15 cells. *Virus Research*, *163*(2), 476–485. https://doi.org/10.1016/j.virusres.2011.11.012

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhou Y, Chen L, Zhang L, et al. Simultaneous identification of 6 pathogens causing porcine reproductive failure by using multiplex ligation-dependent probe amplification. *Transbound Emerg Dis*. 2020;00:1–8. https://doi.org/10.1111/tbed.13585