



TaqMan-based real-time quantitative fluorescence PCR for detection of Orf virus

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Received: 14 August 2018; Accepted: 11 September 2018

ABSTRACT

Contagious ecthyma, also known as scabby mouth or Orf, is a zoonosis, which is caused by the Orf virus (ORFV). Human contact with infected animals can cause cutaneous lesions. To prevent and control ORFV effectively, rapid detection method is very important and highly needed. Real-time quantitative fluorescence PCR (qPCR) assay is considered as a rapid technology to detect ORFV, and has been used for clinical diagnosis and epidemiological investigation. In present study, we developed a TaqMan-based qPCR assay for detection of ORFV. Beacon Designer 7.9 was used to design specific primers and probes were based on the ORFV020 gene sequence of the virus (GenBank Accession No. KF666563.1). The method had no cross-reactions with other common bacteria and viruses, was highly specific; the sensitivity test result showed that it could detect 10 copies of ORFV genomic DNA, and was more sensitive than conventional PCR. Both intra- and inter-variabilities were less than 2%, indicating the high stability and repeatability of the method. Additionally, 99 clinical samples from sheep and goats with suspected contagious ecthyma were tested using the developed assay and conventional PCR. The results showed that the developed assay was more sensitive and faster than conventional PCR. It can be concluded that the assay was suitable for routine detection of the ORFV and the epidemiological investigation.

Key words: Contagious ecthyma, Orf virus, Real-time PCR, TaqMan probe

Contagious ecthyma, also known as scabby mouth, is a zoonotic contact dermatitis caused by Orf virus (ORFV) that affects the epithelia of sheep and goats (Karabasanavar *et al.* 2018). There were many cases reported that human were infected by ORFV through direct contact (Scagliarini *et al.* 2004, Töndury *et al.* 2010, Hasheminasab *et al.* 2016, Flores *et al.* 2017). Contagious ecthyma is a worldwide disease which had been reported in many countries including New Zealand, Finland, America, Japan, Norway, Italy, India, Iran, Ethiopia, Australia and south Asia (Hosamani *et al.* 2009, Du *et al.* 2013, Tedla *et al.* 2018). In China, contagious ecthyma is one of the main epidemic diseases, especially in farms located in West, South and Northeast (Du *et al.* 2013). Fujian province, which is located in south of China, is one of the most epidemic areas for contagious ecthyma. The disease had resulted in \$70 million loss for farmers in Fujian every year. Therefore, it is very important to establish a rapid assay for detecting ORFV, which can provide a basis for scientific prevention and control of the disease.

It is hard to differentiate ORFV infection from other diseases, therefore laboratory confirmation is necessity. Traditional laboratory diagnostic methods have certain limitation, and are laborious and time-consuming. A number

of rapid nucleic acid detection techniques including PCR, RPA, LAMP, SYBR Green I and TaqMan based real-time quantitative fluorescence PCR (qPCR) have been developed for accurate and rapid diagnosis of ORFV (Gallina *et al.* 2006, Yu *et al.* 2013, Yang *et al.* 2015). Among these assays, qPCR has shown to be a highly sensitive and specific assay for detection of ORFV in clinical samples and can distinguish related viruses. Du *et al.* (2013), Venkatesan *et al.* (2014) and Wang *et al.* (2017) had reported that SYBR Green I or TaqMan based qPCR assay for the detection of ORFV based on B2L, ORFV024 or ORFV025 gene is faster and more sensitive than conventional PCR. ORFV020 (virus interferon resistance, VIR) gene is relatively conserved and has been used to study the molecular epidemiology and genetic variation of the ORFV strains (Haig *et al.* 2002, Wang *et al.* 2015, Kottaridi *et al.* 2006, Peralta *et al.* 2015, Ahanger *et al.* 2018), but no one used it as the target gene to develop a qPCR method for detecting the ORFV.

To further understand the prevalence of this disease in Fujian, south of China and the associated temporal dynamics, we established a TaqMan based qPCR assay for detecting the ORFV in sheep and goats.

MATERIALS AND METHODS

Cells, viruses and bacteria: Madin-Darby bovine kidney (MDBK) cells served as a host for ORFV and were cultured in a minimal essential dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum

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(FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). The ORFVCHA vaccine strain was purchased from Biological Products (Shandong Taifeng, China); Goat pox virus CHA vaccine strain and sheep pox virus CHA vaccine strain were purchased from the Spirit Jinyu Biological Pharmaceutical (Inner Mongolia, China); foot-and-mouth disease virus CHA vaccine strain was purchased from China Animal Husbandry Industry (Lanzhou, China); *Escherichia coli* strain ATCC25922 (Guangdong Huankai Microbial), *Pasteurella multocida* strain CVCC44801 (China Institute of Veterinary Drug Control), and *Staphylococcus aureus* strain 261111 (National Institute for Food and Drug Control) were gifts from Long-fei Cheng (Institute for Animal Husbandry and Veterinary Medicine, Fujian Provincial Academy of Agricultural Sciences). *Mannheimia haemolytica*, *Acholeplasma laidlawii*, *Mycoplasma bovis*, *Mycoplasma ovipneumoniae*, *Mycoplasma mycoides subsp. capri*, and ORFV-FZ strain (Lin *et al.* 2015) were all isolated, identified, and preserved in our laboratory. Viruses DNA were extracted by using the EasyPure Viral DNA/RNA kit (TransGen, China) according to the manufacturer's instructions; bacteria and *Mycoplasma* DNA were extracted by using the Ezup Column Animal Genomic DNA Purification kit (Sangon, China) according to the manufacturer's instructions. Extracted DNA was stored at -70°C for further use.

Design and synthesis of primers and probes: Based on the sequence of the goat Orf virus ORV020 (GenBank, KF666563.1), Beacon Designer 7.9 was used to design specific primers and probes. The upstream primer was named F, the downstream primer was named R, and the probe was named P. The sequence of the upstream primer was F-5'-CATCGACATCATGACTCA-3', and that of the downstream primer was R-5'-CATCGACATCATGACTCA-3'; the probe sequence was P-FAM-AAAGTCTTAACCCGGTCAGCG-Eclipse. The target fragment was 85 bp long. The above primers and probe were analyzed by BLAST searches against GenBank. Previously published primer sequences were used to amplify the whole region of the ORFV020 gene of the ORFV (Yang *et al.* 2015). All the primers and probe were synthesized by Takara Bio.

Preparation of standards: Total DNA from the extracted ORFV was used as the template, and PCR amplification was conducted using the pre-designed primers. The reaction mixture for PCR amplification contained 2 μl DNA template, 10 μl Premix TaqTM Version 2.0 plus dye, 1 μl each of the upstream and downstream primers (10 pmol/ μl), and sterile deionized water to achieve a total volume of 20 μl . The reaction conditions were as follows: 94°C for 2 min; 37 cycles of 94°C for 30 sec, 55°C for 15 sec, and 72°C for 15 sec; 72°C for 10 min. The PCR reaction mixture was then used for 1% agarose gel electrophoresis. The product was recovered from the gel and ligated into the pMD19-T vector, which was then transformed into the genetically engineered bacteria DH5. The recombinant plasmids were extracted and identified by PCR and double

enzyme digestion. Next, the plasmids were sent to Takara Bio for sequencing. The confirmed correct recombinant plasmids were used as the standard, and a NanoDrop 2000 was used to determine the concentration of the recombinant plasmid, which was converted to the copy number according to the formula

$$\text{copies}/\mu\text{l} = (\text{ng}/\mu\text{l} \times 10^{-9}) \times (6.02 \times 10^{23}) / (\text{DNA length} \times 660)$$

Optimization of reaction conditions for the TaqMan qPCR: In this study, 25 μl reactions were set up according to the instructions of the Premix Ex TaqTM (Probe qPCR) kit as follows: 0.5 μl each of the downstream and upstream primer, 1 μl of probe, 2 μl of DNA template, 12.5 μl of Premix Ex TaqTM (Probe qPCR), and 8.5 μl of water. The amplification was performed according to the following parameters: 95°C for 30 sec and 40 cycles of 95°C for 5 sec, 55°C for 10 sec, and 72°C for 20 sec. Primer and probe concentrations were diluted to 5 pmol/ μl , 10 pmol/ μl , 15 pmol/ μl , and 20 pmol/ μl , respectively. The matrix method was used for optimization, and the optimal combination of concentrations was screened.

Specificity analysis of the TaqMan qPCR: The ORFV vaccine extracted DNA was used as the positive control and double distilled water was used as the blank control. Using the same PCR conditions, nucleic acids from goat pox virus, sheep pox virus, foot-and-mouth disease virus; *Mycoplasma ovipneumoniae*, *Mycoplasma mycoides subsp. capri*, *Escherichia coli*, *Pasteurella multocida*, *Staphylococcus aureus*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Acholeplasma laidlawii* were detected by the qPCR to verify the specificity of this method.

Sensitivity analysis of the TaqMan qPCR: Standards of different concentrations were used as templates and TaqMan qPCR and conventional PCR were performed. The sensitivities of the two methods were compared.

Analysis of repeatability and stability of the TaqMan qPCR: Standards diluted to 1×10^7 , 1×10^5 and 1×10^3 copies/ μl were selected, and for each concentration, the tests were repeated three times. The coefficient of variation within each group was calculated according to Ct values. The test was conducted once every three days. The coefficient of variation between groups was calculated using the Ct values and the repeatability and stability of the method were tested.

Generation of the standard curve: Sixteen concentration gradients of 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 copies/ μl were obtained through serial dilution with EASY Dilution and used as positive templates. Reactions (25 μl) were set up according to the instructions of the Premix Ex TaqTM (Probe qPCR) kit as follows: 0.5 μl each of the upstream and downstream primers (10 μM), 1 μl of the probe (5 μM), 2 μl of template, 12.5 μl of Premix Ex TaqTM (Probe qPCR), and 8.5 μl of water. PCR was performed using the Mastercycler ep realplex RF-PCR system with the following parameters: 95°C for 30 sec and 40 cycles of 95°C for 5 sec, 55°C for 10 sec, and 72°C for 20 sec. Upon completion of the reaction, a standard curve was automatically

generated by the software.

Clinical sample testing: Field samples (99) were collected from suspected sheep and goats of contagious ecthyma in Fuzhou, Putian, Sanming, Zhangzhou, Longyan, and Ningde cities in the province of Fujian from September 2014 to June 2018. Clinical samples DNA were extracted by using the EasyPure Viral DNA/RNA kit (TransGen, China) according to the manufacturer's instructions. Then, extracted DNA was used for real-time PCR and conventional PCR detection. Positive samples were used to infect MDBK cells for further observation of CPEs.

RESULTS AND DISCUSSION

Optimal conditions of the TaqMan qPCR: After optimization using the matrix method, the optimal primer conditions for the upstream and downstream primers were 10 μ M and 0.5 μ l, respectively, and those for the probe were 5 μ M and 1 μ l. The optimal reaction conditions were as follows: 40 cycles of 95°C for 30 sec, 95°C for 5 sec, 55°C for 10 sec, and 72°C for 20 sec.

Specificity of the TaqMan qPCR: The established quantitative fluorescence PCR technique was used to detect the ORFV, goat pox virus, sheep pox virus, foot-and-mouth disease virus; *Mycoplasma ovipneumoniae*, *Mycoplasma mycoides* subsp. *capri*, *Escherichia coli*, *Pasteurella multocida*, *Staphylococcus aureus*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Acholeplasma laidlawii*. Double distilled water was used as the blank control. The results showed that, except for the ORFV, which could be amplified to form an S-curve, no samples were amplified, indicating good specificity of the qPCR method described in this study.

Sensitivity of the TaqMan qPCR: The standards were diluted from 1×10^8 to 1×10^0 copies/ μ l, and conventional PCR and qPCR were conducted to compare sensitivities. Results showed that the detection limit of conventional PCR was 1000 copies/ μ l, whereas that of qPCR was 10 copies/ μ l, indicating that the sensitivity of the latter technique was

100-fold greater than that of the former technique (Fig. 1). The results was similar with the previous reports (Bora *et al.* 2011, Du *et al.* 2013, Wang *et al.* 2017). The high sensitivity may be due to high amplification efficiency.

Evaluation of the repeatability and stability of the TaqMan qPCR assay: The standards, diluted to 1×10^7 , 1×10^5 , and 1×10^3 copies/ μ l, were used for repeated intra- and inter-assay tests. The results showed that both intra- and inter-assay coefficients of variation were all less than 2% (Table 1), indicating that the established method has good repeatability and stability.

Standard curve for the TaqMan qPCR: Six standards, obtained by serial dilution using EASY dilution (10^7 , 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 copies/ μ l), were used as templates. Quantitative PCR amplification was conducted using a Mastercycler ep realplex to generate a standard curve. The linear equation was $y = 3.246 \log(x) + 32.49$, with a correlation coefficient (R^2) = 0.999, and an amplification efficiency (E) = 103%. Wang *et al.* (2017) report, suggesting that the DNA of the target gene can be accurately detected with a large concentration range.

Testing of clinical samples: Nine-nine clinical samples suspected of contagious ecthyma collected from different city of Fujian province were tested using both TaqMan qPCR assay established in this study and conventional PCR for comparing the sensitivity of these two assays. The total positive detection rate by TaqMan qPCR assay was 87.9% (87/99), whereas that of the conventional PCR was 85.6% (85/99) (Table 2). This relatively high rate could be related to unimmunized vaccines, as the sheep and goats industry was small in the south of China, which made vaccine sellers reluctant to sell. Therefore, strengthening the epidemiological surveillance of contagious ecthyma and understanding the epidemic trends associated with the disease and the molecular genetic variation of the virus could not only improve the effective prevention and control of the disease but are also critical for public health. In all samples, conventional PCR tested positive samples were

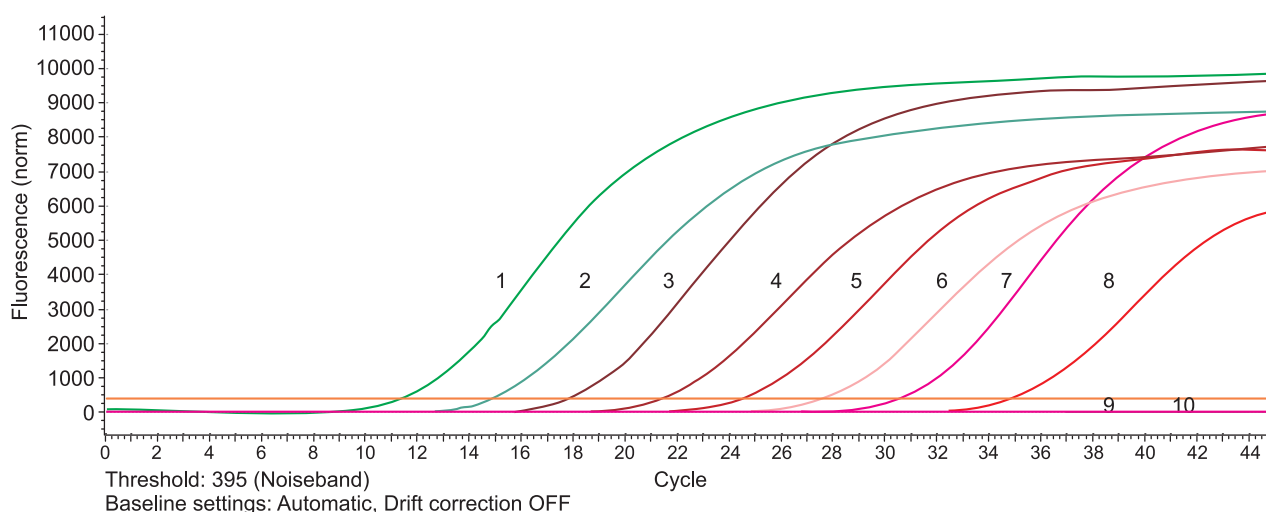


Fig. 1. Sensitivity test for the qPCR assay.

also positive by TaqMan qPCR (data not shown). All the samples that were positive by TaqMan qPCR assay were sent to Shangon Bio for sequencing, and then the sequences were subjected to a BLAST search in the NCBI database. The results showed all the sequences were consistent with the ORFV020 gene sequence of the ORFV, implying that the TaqMan qPCR assay was more sensitive than conventional PCR and can be used for rapid/and accurate detection of ORFV.

Highly conserved sequences in genome are usually used as a detecting target, as previously reported that ORFV B2L gene (Gallina *et al.* 2006), DNA polymerase (DNA pol) gene (Bora *et al.* 2011, Venkatesan *et al.* 2012), ORFV F1L gene (Yao *et al.* 2012), ORFV024 gene (Du *et al.* 2013), a major component of the viral RNA polymerase (Torfason *et al.* 2002) and ORF007 gene (dUTPase) (Töndury *et al.* 2010) are used as detection target to apply to qPCR or conventional PCR for detecting ORFV. As these genes are relative conserved in ORFV, so they can be used as target gene to detect the ORFV by developed qPCR, conventional PCR, LAMP (Inoshima *et al.* 2016) and RPA (Yang *et al.* 2015) assay. The ORFV020 gene is one of the major conserved genes of the ORFV and is usually used for construction of the phylogenetic tree by comparing the relatedness among different strains (Yang *et al.* 2014). There are poxvirus conservative initiators and transcriptional control elements in the upstream of the ORFV020 gene initiation codon, and a poxvirus conservative early gene transcription termination sequence T5NT after the TAA termination codon. When the virus was suppressed in late stage, the expression of the ORFV020 gene was still confirmed to be present in the early stage of infection (Kottaridi *et al.* 2006, Yang *et al.* 2014, Wang *et al.* 2015). Hence ORFV020 could be use as target gene to detect ORFV.

Table 1. Reproducibility of the qPCR for ORFV detection

Concentration of ORFV (copies/μl)	Intra-assay reproducibility (Mean $Ct \pm SD$ CV)	Inter-assay reproducibility (Mean $Ct \pm SD$ CV)		
1×10^7	14.39 $\pm$ 0.17	1.18	14.63 $\pm$ 0.18	1.23
1×10^5	21.72 $\pm$ 0.14	0.64	21.82 $\pm$ 0.24	1.09
1×10^3	27.85 $\pm$ 0.23	0.83	27.93 $\pm$ 0.32	1.15

Table 2. Virus detection in clinical samples using TaqMan qPCR and conventional PCR assays

Regions of Fujian province	Number of clinical samples	TaqMan qPCR positive rate (%)	Conventional PCR positive rate (%)
Nongyan	14	85.71 (12/14)	78.57 (11/14)
Sanming	25	88.00 (22/25)	84.00 (21/25)
Fuzhou	17	88.23 (15/17)	88.23 (15/17)
Zhangzhou	10	80.00 (8/10)	80.00 (8/10)
Putian	18	88.89 (16/18)	88.89 (16/18)
Ningde	15	86.67 (13/15)	86.67 (13/15)
Total positive	99	87.88 (87/99)	85.86 (85/99)

In conclusion, the ORFV TaqMan-based qPCR assay is reliable and sensitive, which can be used for early rapid detection of ORFV genomic DNA with low virus content in clinical samples or cell culture. The TaqMan qPCR assay could be used for the diagnosis and epidemiological investigation of contagious ecthyma, limit the spread of infection in sheep and goats and will also provide a basis for the development of commercial kits.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research Project of China (2016YFD0500906), Fujian Science and Technology Innovation Platform Construction Project (2014N2003-5), Fujian Public Welfare Project (2014R1023-6), Science and Technology Innovation Team PI Project of FAAS (2016PI-7), and the Innovative Research Team for Fujian Academy of Agriculture Science (STIT2017-3-10).

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