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

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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 ectyma. 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.
(FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). The ORFV/CHA 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 Huan Kai Microbial), Pasteurella multocida strain CVCCC44801 (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 Euzp 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 synthesized by Takara Bio. 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:

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

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 Taq™ (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, and 10^3 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 Taq™ (Probe qPCR) kit as follows: 0.5 µl each of the upstream and downstream primers (10 pmol/µl), 1 µl of the probe (5 µM), 2 µl of template, 12.5 µl of Premix Ex Taq™ (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 µM and 0.5 µl, respectively, and those for the probe were 5 µM and 1 µl. The optimal reaction conditions were as follows: 40 cycles of 95ºC for 30 sec, 95ºC for 5 sec, 55ºC for 10 sec, and 72ºC 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 × 10^3 copies/µl, and conventional PCR and qPCR were conducted to compare sensitivities. Results showed that the detection limit of conventional PCR was 1000 copies/µl, whereas that of qPCR was 10 copies/µl, 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/µl, 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/µl), 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
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 ±SD CV) | Inter-assay reproducibility (Mean ±SD CV) |
|----------------------------------|-------------------------------------------|------------------------------------------|
| 1×10⁷                            | 14.39±0.17                                | 14.63±0.18                               |
| 1×10⁸                            | 21.72±0.14                                | 21.82±0.24                               |
| 1×10⁹                            | 27.85±0.23                                | 27.93±0.32                               |

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.

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REFERENCES

Ahanger S A, Parveen R, Nazki S, Dar Z, Dar T, Dar K H, Dar A, Rai N and Dar P. 2018. Detection and phylogenetic analysis of Orf virus in Kashmir Himalayas. Virus Disease 29(3): 405–10.
Bora D P, Venkatesan G, Bhanuprakash V, Balamurugan V, Prabh M, Siva Sanker M and Yogisharadhyari R. 2011. TaqMan real-time PCR assay based on DNA polymerase gene for rapid detection of Orf infection. Journal of Virological Methods 178(1–2): 249–52.
Du H, Li W, Hao W, Liao X, Li M and Luo S. 2013. Taqman real-time PCR assay based on ORFV024 gene for rapid detection of orf infection. Toxicology Mechanisms and Methods 23(5): 308–14.
Flores C, González E, Verna A, Peralta A, Madariaga C, Odeón A and Cantón G. 2017. Orf virus in human, confirmation in case report from Chile. RevistaChilena de Infectología 34(6): 607–09.
Gallina L, Dal Pozzo F, McInnes C J, Cardeti G, Guercio A, Battilani M, Ciulli S and Scagliarini A. 2006. A real time PCR assay for the detection and quantification of orf virus. Journal of Virological Methods 134(1–2): 140–45.
Haig DM and McInnes C J. 2002. Immunity and counter-immunity during infection with the parapoxvirus orf virus. Virus Research 88(1–2): 3–16.
Hasheminasab S S, Mahmoodi A, Mahmoodi P and Maghsood H. 2016. Orf virus infection in human ecthyma contagiosum: a report of two cases in the West of Iran. Virus Disease 28(2): 209–10.
Hosamani M, Yadav S, Kallesh D J, Mondal B, Bhanuprakash V and Singh R K. 2007. Isolation and characterization of an Indian orf virus from goats. Zoonoses and Public Health 54(5): 204–08.
Hosamani M, Scagliarini A, Bhanuprakash V, McInnes C J and Singh R K. 2009. Orf: an update on current research and future perspectives. Expert Review of Anti-infective Therapy 7(7): 879–93.
Inoshima Y, Takasu M and Ishiguro N. 2017. Establishment of an on-site diagnostic procedure for detection of orf virus from oral lesions of Japanese serows (Capricornis crispus) by loop-mediated isothermal amplification. Journal of Veterinary Medical Science 78(12): 1841–45.
S S and Veeranna K C. 2018. Phylogenetic analysis of Orf virus associated with contagious ecthyma (orf) outbreak in Tellicherry goats (Capra hircus). Indian Journal of Animal Sciences 88(2): 144–49.

Kottaridi C, Nomikou K, Teodori L, Savini G, Lelli R, Markoulatos P and Mangana O. 2006. Phylogenetic correlation of Greek and Italian orf virus isolates based on VIR gene. Veterinary Microbiology 116(4): 310–16.

Lin S, Lin Y, Jiang B, Jiang J, Chen M and Hu Q. 2015. Isolation and identification of a Fuzhou strain of the orf virus. Fujian Journal of Agricultural Sciences 30(1): 6–8.

Peralta A, Robles C, Martínez A, Alvarez L, Valera A, Calamante G and König G A. 2015. Identification and molecular characterization of Orf virus in Argentina. Virus Genes 50(3): 381–88.

Scaqliarini A, Gallina L, Dal Pozzo F, Battilani M, Ciulli S, Prosperi S and Pampiqlione S. 2004. Diagnosis of orf virus infection in humans by the polymerase chain reaction. New Microbiologica 27(4): 403–05.

Töndury B, Kühne A, Kutzner H, Palmedo G, Lautenschlager S and Borelli S. 2010. Molecular diagnostics of parapox virus infections. Journal der Deutschen Dermatologischen Gesellschaft 8(9): 681–84.

Tedla M, Berhan N, Molla W, Temesgen W and Alemu S. 2018. Molecular identification and investigation of contagious ecthyma (Orf virus) in small ruminants, North west Ethiopia. BMC Veterinary Research 14(1):13.

Torfason E G and Gundadhottir S. 2002. Polymerase chain reaction for laboratory diagnosis of orf virus infections. Journal of Clinical Virology 24(1–2): 79–84.

Venkatesan G, Bhanuprakash V, Balanmurugan V, Bora D P, Prabhu M, Yogisharadthy R and Pandey A B. 2012. Rapid detection and quantification of Orf virus from infected materials of sheep and goats. Acta Virologica 56(1): 81–83.

Wang L, Liu Y, Zheng H, Zhang K and Liu X. 2015. Progress on virus interferon resistance gene encoded by Orf virus. Chinese Journal of Zoonoses 31(12): 1181–84.

Wang G, Shang Y, Wang Y, Tian H and Liu X. 2013. Comparison of a loop-mediated isothermal amplification for orf virus with quantitative real-time PCR. Virology Journal 10: 138–47.

Yang Y, Qin X, Wang G, Zhang Y, Shang Y and Zhang Z. 2015. Development of a fluorescent probe-based recombinase polymerase amplification assay for rapid detection of Orf virus. Virology Journal 12: 206.