Isolation and phylogenetic analysis of caprine Orf virus in Malaysia

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Abstract Orf virus is a DNA virus that causes contiguous ecthyma in goat and sheep. Infection of animals with this virus cause high mortality in young animals resulting in huge economic losses. In this study, we investigated an outbreak of Orf in a goat farm in Malaysia. Samples were collected from infected animals and viral isolation was done using both LT and MDCK cell lines. Molecular detection was done by conventional PCR for specific primers; B2L and F1L genes and phylogenetic analysis was done on the sequence data obtained. Cytopathic effects (CPE) were observed in both cell lines after 3 days of inoculation and were 50 % by the sixth day. PCR showed positive bands for both B2L and F1L genes and phylogenetic analysis showed that the Malaysian strain had close homology to the Chinese and Indian Orf virus isolates. This study gives more insight into the existing Orf viral strains in Malaysia and their relationship with other strains globally.

Keywords Orf virus · Contagious ecthyma · Parapoxvirus · Goat · Sore-mouth infection

Introduction

Orf virus (ORFV) the type species of the genus *Parapoxvirus*, subfamily *Chordopoxvirinae* and family *Poxviridae* and is the causative agent of contagious ecthyma (contagious pustular dermatitis; Orf) [16, 23]. ORFV has a linear double-stranded (ds) DNA with approximately 139,962 bp and a high G+C (63 %). The genome encodes for 130 proteins classified as early, intermediate early and late proteins [7, 20].

The virus has a worldwide distribution [22] with an incidence of up to 90 % [6]. Both young and adult animals are susceptible to the virus infection. However, high mortality was reported in young animals [11] which leads to economic problems related with loss of young animals and weight loss in lambs [10]. Interestingly, the virus was reported in humans as a zoonotic disease [8, 21].

Infected animals develop skin disease known as contagious ecthyma “sore-mouth, orf or scabby mouth” that associated with a proliferative dermatitis in the lips, nostrils, gums, tongues and teats results in the development of pustules and scabs around the muzzle, buccal cavity, udder and between the toes [28]. The clinical signs of CE is the first diagnostic tool in the field. Techniques such as cell culture [19, 26, 28] and PCR have been used for virus isolation and detection [9, 14, 15, 19, 26, 28]. PCR was evaluated as a fast, cheap and valid technique for virus detection. The most common genes used are B2L gene; a highly immunogenic envelope gene [19, 26, 28] and Orf F1L gene; an immunodominant gene [1, 9, 12, 14, 15, 18, 19, 26, 28].

In Malaysia, only two studies have so far reported Orf virus infection in animals [2, 3], and molecular or the genomic properties of the virus is not available in GeneBank. This is the first study that attempted to sequence

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partial genome data of Orf virus isolated in Malaysia using the B2L and F1L genes, and to establish a relationship with other existing strains in the database.

Materials and methods

Collection of tissue samples

Samples were collected from infected goats with clinical signs of Orf in the Ladang Angkat program, Universiti Putra Malaysia (Fig. 1a) in 2014. Approximately, 10 % of goats in the farm were infected and two goats (age 6–10 months) died. A total of three (3) samples were collected from the farm. The samples were stored in -20°C prior to virus isolation and molecular detection.

Fig. 1 **a** Showing pox lesions in goats infected with the Orf virus. **b** Gel electrophoresis of Orf virus DNA amplified by conventional PCR from infected samples. Lane M 100 bp molecular weight marker. Lanes 1, 3, 5 samples amplified by Orf virus B2L primer. Lanes 2, 4, 6 samples amplified by Orf virus F1L gene primer

Sample processing

One hundred milligram of infected skin crust was homogenized in Phosphate Buffered Saline solution (PBS) and centrifuged at 1000 rpm for 10 min to collect the supernatant, which was then filtered through a $0.45\ \mu\text{m}$ syringe filter and stored at -70°C .

Isolation of Orf virus in cell line

The virus was isolated in both LT and MDCK cell lines. Confluent monolayer of the cell lines were washed with PBS and 0.1 mL of processed sample was added onto the monolayer together with DMEM media (0 % fetal bovine serum) and incubated at 37°C for 30 min. The monolayer was then washed with PBS and fresh DMEM media containing 1 % FBS was added and incubated at 37°C with 5 % CO_2 . The cells were checked daily for development of cytopathic effects (CPE).

Molecular detection of Orf

DNA extraction was done by using conventional extraction method as described previously [17]. Detection of Orf virus using PCR and semi nested PCR was confirmed using four sets of primers for VIR (interferon resistance gene), B2L gene (ppp1, ppp3 and ppp4 and ORV011) and ORV059 (F1L gene) [3, 14, 19]. The primer sequences are: VIR forward primer (5-ACA ATG GCC TGC GAG TG-3), VIR reverse primer (5-TTA GAA GCT GAT GCC GCA G-3), ORV011 forward primer (5-ATG TGG CCG TTC TCC TCT ATC-3), ORV011 reverse primer (5-TTA ATT TAT TGG CTT GCA G-3), ORV059 forward primer (5-ATG GAT CCA CCC GAA ATC AC-3), ORV059 reverse primer (5-TCA CAC GAT GGC CGT GAC CAG-3), ppp1 forward primer (5-GTC GTC CAC GAT GAG CAG CT-3), ppp3 forward primer (5-GCG AGT CCG AGA AGA ATA CG-3) and ppp4 reverse primer (5-TAC GTG GGA AGC GCC TCG CT-3). PCR was done using Novagen kit (Toyobo, Japan) following the manufacturer's instruction. The thermal cyclor was programmed with the following cycling conditions; 95°C for 2 min as an initial activation step, followed by 35 cycles (94°C , 20 s; T_a $^{\circ}\text{C}$, 30 s, 70°C , 20 s) and one cycle of 72°C for 2 min. The annealing temperature (T_a) were 43, 50, 50, 52°C for VIR, ORV011, ORV 059 and semi nested PCR primers respectively. Gel electrophoresis was run for PCR product on a 1.25 % agarose gel at 100 V for 50 min. The gel was stained with Red safe and DNA bands were viewed using Gel Doc.

Positive samples were amplified using primers Orf virus B2L and F1L primers [28]. The primer sequence are: Orf virus B2L forward primer (5-TATAGGATCCGCCAT

GTGGCCGTTCTCCTCCATC-3), Orf virus B2L reverse primer (5-CCGCTCGAGTTAATTTATTGGCTTGCA G-3), Orf virus F1L gene forward primer (5-CAAGCT TGCCACCATGGATCCACCCGAAATC-3) and Orf virus F1L reverse primer (5-CGAATTCTCACACGATGGCCG TGACC-3). PCR was done as described above and positive PCR samples were sent for DNA sequencing using Sanger sequencing on the Applied Bio-systems 3730XL Genetic Analyzer. Sequence data obtained was analysed using the Applied Bio-systems DNA Sequence Analysis Software v5.2 with KB Base caller.

Phylogenetic analysis

Sequence data obtained was blasted on the NCBI website to check for similarity with other existing archived sequences. Phylogenetic analysis was done using both Orf virus B2L and Orf virus F1L gene sequences. The phylogenetic tree was constructed using MEGA 6 software program.

Result

Virus isolation

CPE were first observed after 3 days post inoculation, approximately 50 % CPE was observed in monolayer culture of LT and MDCK cell lines. Typical CPE observed include cellular ballooning, rounding and vacuolation of cells.

Molecular detection of Orf

All 3 samples were positive for Orf virus with all four sets of primers. The three samples also produced a single positive band with sizes corresponding to each gene; B2L and F1L, respectively (Fig. 1b).

Phylogenetic tree

NCBI nucleotide blast using DNA sequence of all samples was done to search for high similarity with existing DNA sequences. Based on B2L gene sequencing, the Orf virus isolates from the samples had a high percentage (99 and 98 %) of homology with Orf virus isolated from Goats and Sheep in China and India; FJ-SJ2-ORFV011 and ORFV 82/04 isolate. On the other hand, samples for F1L gene sequencing had similar homology with Orf virus isolate FJ-SJ2 ORFV059 gene from Goats in China (100 %) and isolates FJ-SJ1 and FJ-SL (99 %). The three isolates have GeneBank accession numbers KR024023, KR024024,

KR024025 for B2L gene, and KR339014, KR339015, KR339016 for F1L gene respectively (Fig. 2).

Discussion

Recently, numerous outbreaks of Orf virus have been reported among animals [3, 5, 19, 25, 28]. The virus mostly causes an acute infection but chronic infection although rare have been reported. The mortality rate were reported up to 45 % in infected animals [25]. In some cases the high mortality rate was associated with secondary infection with bacteria and other viruses [13, 28]. In this study, only 2 animals died out of 30 animals in the farm sampled. Clinical signs of orf infection was used to identify animals with CE. Typical Orf virus CPE was observed in LT and MDCK cells as reported previously [4, 19, 26]. These include ballooning, rounding and degeneration of cells as early as 3 days post inoculation. The cells were seen to aggregate in a grape-like pattern.

Cell culture method is a time consuming method used for viral isolation [4, 27]. However, PCR technique has been used as a faster and more effective method of Orf virus detection [14]. In this study, 4 sets of primers were used in detection [3, 14, 19], and all samples showed a positive amplification with these primers. Amplification and sequencing using OPFV 011 and ORFV 059 was done due to the importance of these genes in virus infection and pathogenicity [28].

The virus isolates were found to be closely related to Orf strains isolated from china and India (AC: KC568397.1 and DQ263303.1) respectively [5, 12]. On the other hand, alignment of sequence results for F1L genes showed close homology of the three isolates to Orf virus strain (AC: KC568408.1) that distributes in South China [5].

Heretotogency was also reported in China, where some strains share 98.2–99.3 % of nucleotide identity even when they were collected from the same scab in the same farm [5]. Another high variation was reported in China among the B2L and F1L genes, where 96–97 and 77–99 % nucleotide similarity and 97–98 and 95–99 % amino acid identities were observed between these two genes [19]. Similarly, the Jilin Orf virus isolate from china also showed 96.5–98.9 and 95.3–97.4 % nucleotide similarities in their B2L and F1L genes, respectively [28]. In India, analysis of B2L gene showed approximately 98.1–99.8 and 98.4–99.7 % similarity in nucleotide and amino acid sequences [25]. However, the differences between strains did not seem to be associated with the clinical signs of the disease as animals with different strains or similarities between strains showed the same clinical signs and pathogenicity [24].

Fig. 2 Phylogenetic analysis based on nucleotide sequences of ORFV 011 (B21 gene) and ORFV059 respectively. The phylogenetic relationship was constructed by neighbour joining algorithm using MEGA 6 software; one thousand bootstrap replicates were subjected

to nucleotide sequences distance (cut-off value 50 % from 1000 bootstrap replicates. All bootstrap values are displayed above branches

Analysis of worldwide distributing Orf virus strains showed that the maximum nucleotides and amino acids variations were ($n = 33$) 8.1 and 9.6 %, respectively [5].

Orf virus infection mostly affects young animals and can lead to serious economic losses. Both B2L and F1L genes are considered among the most important genes in evaluation of strain variations. Availability of sequence data from regions and countries will be highly beneficial in designing suitable polyvalent vaccines that have wider coverage in the livestock production industry.

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