UNIVERSITI PUTRA MALAYSIA

DETECTION OF ORF VIRUS USING 4 DIFFERENT SET OF PRIMER FROM CLINICAL CASES OF ORF IN GOAT'S FROM PROGRAM LADANG ANGKAT UPM UNIVERSITI PUTRA MALAYSIA

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It is hereby certified that we have read this project paper entitled "Detection of Orf Virus Using 4 Different Set of Primer from Clinical Cases of Orf in Goat's From Program Ladang Angkat Upm", by Muhammad Farid bin Ismail and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD4999 Project.

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DEDICATION

I dedicate this paper to both my parents, for their love and everlasting support that made me who I am today. To my siblings; my sister and brothers that inspire me to continue my study in tertiary education. To all my teachers and friends that help me all the way to here. Thank you very much.

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Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 –
Projek Tahun Akhir

PENGGUNAAN 4 JENIS PRIMER DALAM REAKSI RANTAI POLIMERASE (PCR) UNTUK MENGESAN VIRUS ORF PADA KAMBING DARIPADA LADANG DI BAWAH PROGRAM LADANG ANGKAT UPM

By

Muhammad Farid Bin Ismail

2015

Penyelia: Prof. Dato' Dr.Mohd Azmi Mohd Lila

Penyelia bersama: Prof. Dr Zeenathul, Prof. Rasedee, Dr Jesse

Penyakit ektima menular merupakan penyakit virus yang akut, berjangkit, melumpuhkan dan mempunyai kepentingan ekonomi kepada bebiri, kambing, dan juga kepada ruminan yang lain. Ejen kepada penyakit tersebut adalah virus Orf di bawah genus virus Parapox dan keluarga Poxviridae. Dua buah ladang angkat UVH, UPM telah dijangkiti penyakit ektima menular dan 3 sampel kuping telah diambil dari ladang tersebut. Sampel kuping itu diekstrak secara manual dan juga menggunakan kit pengekstrakan DNA. DNA yang diperlukan untuk mengesan virus Orf digandakan menggunakan 4 jenis primer, primer tersebut menyasarkan 2 gen iaitu gen B2L (PPP1-PPP4, semi nested PCR PPP3-PPP4, ORFVO11F-ORFVO11R) dan gen VIR (VIRF-VIRR, , ORFVO59F-ORFV059R) dalam tindak balas reaksi rantai polimerase (PCR). Dalam kajian ini, kepekaan primer untuk mengesan virus tersebut tidak dapat ditentukan.Pokok filogenetik telah dihasilkan menggunakan data daripada gen B2L dan VIR tersebut. Berdasarkan gen B2L, sampel 2 dan 3 adalah berkait rapat secara filogenetik berbanding sampel 1. Berdasarkan gen 059 filogenetik untuk semua sampel tersebut adalah kurang berkaitan tetapi mempunyai persamaan dengan strain dari negara China.

Kata kunci : kambing, virus Orf, diagnosis, Reaksi Rantai Polimerase (PCR)

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, UPM in partial requirement of the course VPD 4999- Final Year Project

DETECTION OF ORF VIRUS USING 4 DIFFERENT SET OF PRIMER FROM CLINICAL CASES OF ORF IN GOAT'S FROM PROGRAM LADANG ANGKAT UPM

By

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2015

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Contagious ecthyma is an acute, contagious, debilitating and economically important viral skin disease of sheep, goat and some other domesticated and wild ruminants. The etiological agent for this disease is Orf virus where the virus is under the genus Parapoxvirus and family Poxviridae. Two farms from programme Ladang Angkat had outbreak with contagious ecthyma. Three sample were obtained from the outbreak. The scab samples were collected from animals were homogenized and the DNA was extracted manually. The targeted DNA were then amplified by using

Polymerase Chain Reaction (PCR). 4 set of primers were used in this study in both interferon resistant (VIR) gene of the virus (VIRF-VIRR; ORFVO59F-ORFV059R) and the major envelope proteins (PPP1-PPP4, semi nested PPP3-PPP4, ORFVO11F-ORFVO11R). Phylogenetic trees were built by comparison of the complete coding area for both 011 and 059 genes. The result of this study showed that there is no significant difference between all the primers used in this study. Both samples 2 and 3 were so closed phylogenetically to each other compared to sample 1 according B2L whole coding gene. The Orf virus based on B2L gene isolated from this cases are different from the virus strain from east south Asia strains. For Orf virus based on 059 gene from this case are different to each other but closely related to some Chinese Orf virus strain.

Keywords: Goat (*Capra hircus*), Orf virus, diagnosis, Polymerase Chain Reaction (PCR)

1. INTRODUCTION

Contagious ecthyma is an acute, contagious, debilitating and economically important zoonotic viral skin disease that affects sheep, goat and some other domesticated and wild ruminants. The disease is also known as sore mouth, contagious pustular dermatitis or scabby mouth (Nandi, De, & Chowdhury, 2011). The morbidity of the disease may reach up to 100% and mortality due to secondary bacterial infections may reach to 15%. The disease is not normally fatal but is a debilitating disease that can be fatal if lambs and kids are prevented from suckling or succumb to secondary bacterial or fungal infections. It is also a zoonotic disease affecting humans that called 'milker's node' or 'pseudocowpox' due to the frequent infection of milkers when milking of cows by hand was common (M.Buttner & Rziha, 2002). Etiological agent for contagious ecthyma is Orf virus which is under the genus Parapoxvirus and family Poxviridae (Buchen Osmond, 2003). The disease can affect sheep, goat and some other domesticated and wild ruminants like alpacas, camels, reindeer, big horn sheep, Sichuan takin, domestic Shetland sheep, deer, prong horn antelope ,wapiti and seal squirrels. In Malaysia, Orf virus infection has been reported since 1935 and subsequently, outbreaks have been reported (Zamri-Saad, Al-Ajeeli, & Ibrahim, 1992). Orf virus can be diagnosed on the basis of characteristic lesions on the anatomic areas of predilection which is the lips and muzzle, and also by laboratory test. Commonly used lab test to confirm Orf infection comprise of electron microscopy (EM), serological tests such as agar gel precipitation test (AGPT), agglutination test, complement fixation test (CFT), enzyme linked immunosorbent assays (ELISAs), serum neutralization test (SNT), histopathology of affected tissues, nucleic acid based assays including polymerase chain reaction (PCR) and restricted fragment length polymorphism (RFLP) analysis but among all the diagnostic test, development of PCR methods for the molecular detection of parapox DNA has met the demands for specific and sensitive laboratory diagnosis of orf disease (de la Concha-Bermejillo, et al., 2003). Different set of primers can be used to detect Orf virus, commonly used primer are primer that targeting interferon resistant (VIR) gene of the virus (VIRF-VIRR; ORFVO59F-ORFV059R) and primer that target the major envelope proteins (PPP1-PPP4, semi nested PPP3-PPP4, ORFVO11F-ORFVO11R). Phylogenetic study will help to distinguish the virus strains, where the most common gene used to classify the strain is 011 and 059 genes. The objectives of this study are:

1. To determine the sensitivity of primers for detection Orf virus from clinical cases.
2. To determine the similarity between current and previous isolated strains.

2.0 LITERATURE REVIEW

2.1 Parapoxvirus

2.1.1 Introduction

Orf virus (ORFV) is the prototype member of the genus Parapoxvirus of the subfamily Chordopoxvirinae and family Poxviridae. It is the causative agent of contagious ecthyma (contagious pustular dermatitis; Orf). The genus Parapoxvirus also includes bovine papular stomatitis virus (BPSV) and pseudocowpox virus (PCPV), which infect cattle; squirrel parapoxvirus; and parapoxvirus of red deer in New Zealand (Nandi *et al*.,2011). Contagious ecthyma caused by ORFV is distributed worldwide and has been reported in many countries (Nandi *et al*., 2011). The first cases of parapoxvirus was mentioned by Robinson and Lyttle, 1992 even the Contagious pustular dermatitis was reported since 1787(Steeb, 1787).

2.1.2 Classification

According to International committee on taxonomy of virus, 2014(ICTV, 2014), the virus classified under *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Parapoxvirus*, and species *Orf virus*. There are other species under genus *Parapox* virus including Bovine papular stomatitis virus, Parapox virus of red deer in New Zealand and pseudocowpox virus (Nandi *et al*., 2011)

2.1.3 Virus morphology

The virion are ovoid in shape, it have a long axis of 260 nm and short axis of 160 nm when viewed under electron microscope. Under negative stained, 2 form will appear, capsular form is shown by a finely crenelate membrane appear to surround an inner amorphous core, another form is a regular array of tubule-like structures arranged in a criss-cross manner along the length of the particle. In cell culture, the virion appear surrounded by a membranous structure 9-8 nm thick. (Stephen B. Fleming and Andrew A. Mercer, 2007)

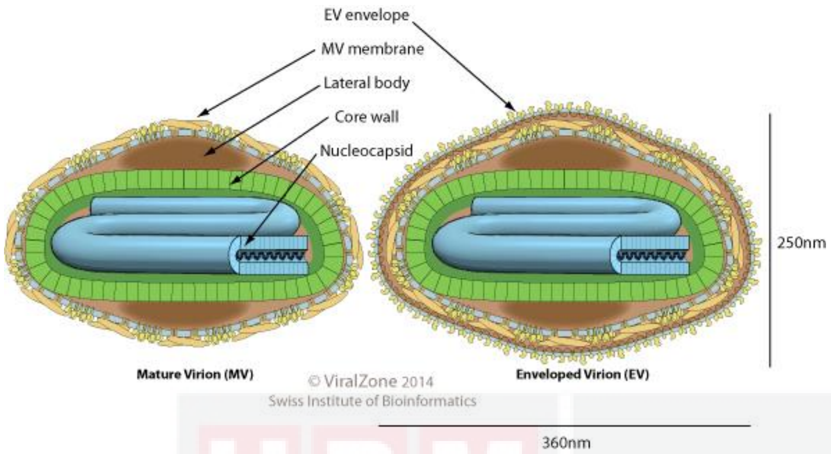


Figure 2.1. Orf virus morphology (ViralZone 2014)

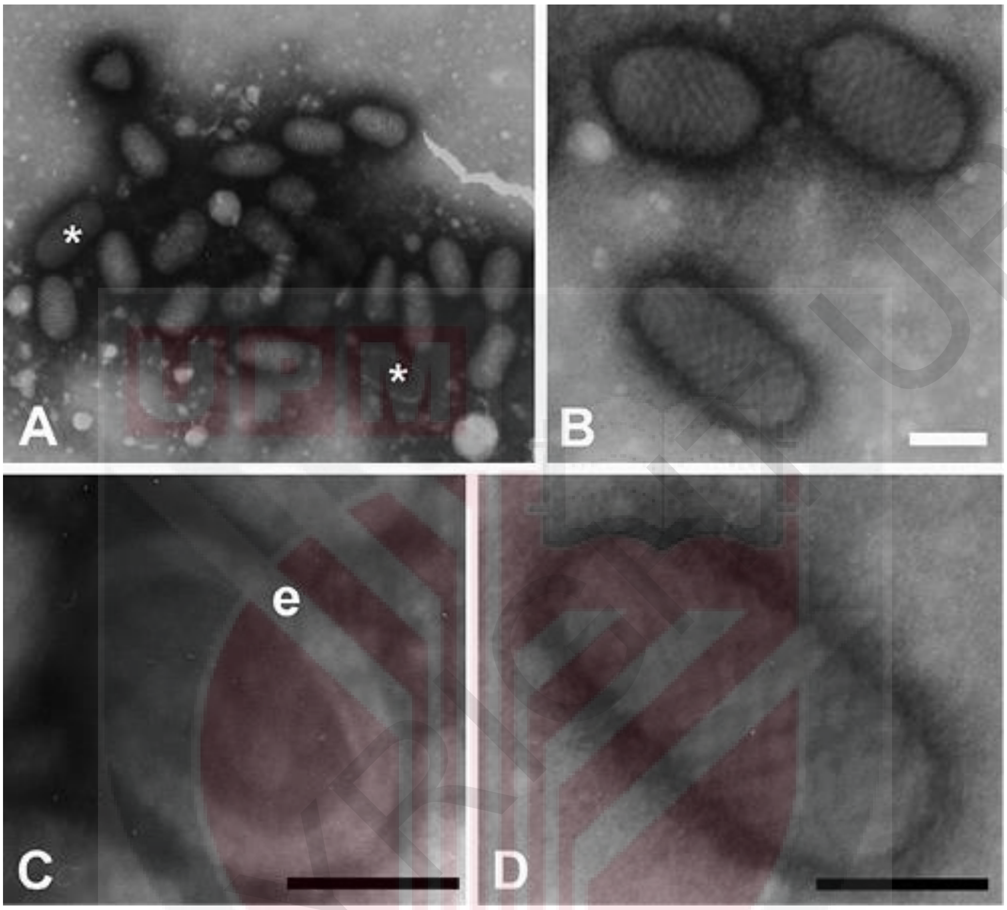


Figure 2.2. . Electron micrographs of Parapoxvirus particles in scrapings from skin lesion. Electron microscopy (negative stain). (A) Virus particles were observed in large groups. (B–D) Higher magnifications of parapox viruses. They occur mainly in the M (mulberry) form (without envelope), but also in the C (capsule) form (* in A, and panel C). The thick viral envelope (e, in panel C) can be clearly recognized. Note also the ovoid shape and the typical spiraled surface pattern. Scale bars indicate 100 nm, i.e. the approximate width of the virus (Kitchen et al., 2013).

2.1.4 Genome structure

Poxviruses are a DNA viruses that perform their replication in the cytoplasm. The genome structure of the family members mostly the same. It is a linear ds DNA. The central region is conserved among members and encode for proteins that necessary for in RNA and DNA synthesis, protein processing, and virion assembly and structural proteins whereas the terminal region encodes for the proteins affecting host range, virulence or interaction with the host immune system (Moss, 2001). ORFV like other poxviruses share the same genome characters linear double-stranded (ds) DNA of approximately 135 kbp with cross-linked ends. The nucleotide composition are high in G+C which approximately 63%. Orf virus contains 132 genes encodes at least three classes of genes, early, intermediate early and late (Stephen B. Fleming and Andrew A. Mercer, 2007)

Comparisons of predicted protein sequences of the three fully sequenced ORFV isolates revealed a degree of inter-isolate sequence variation that is uncommon among chordopoxviruses. For instance, amongst the 20 most variant genes, the average predicted amino acid sequence identity was only 80% (Mercer et al., 2006).

Figure 2.1. ORFV NZ2 genetic map. The assigned open reading frames of ORFV are shown as boxes on a line representing the genome. Boxes above the line represent open reading frames transcribed rightward and those below the line are transcribed leftward. Each line except the last corresponds to 20 kb as indicated by the numbers in italics at the left of each line. The boxes are shaded to indicate the approximate BLASTP bit score with the orthologous gene in VACV (Copenhagen). The four shades of grey from darkest to lightest correspond to bit scores of greater than 300,

100–300, 55–99 and 34–54, respectively. The speckled boxes represent scores of less than 34. White boxes are those ORFV genes for which no significant BLASTP match with a VACV protein was detected. White boxes with cross-hatching represent genes encoding ankyrin repeat proteins (Mercer et al., 2006).

2.1.5 Replication cycle

Replication of the Poxvirus requires the viral transcription complex within the core particle that, after a virion enters the host cell and undergoes a preliminary uncoating, transcribes a large set of early genes into mRNAs (Moss, 2001). Translation of the early mRNAs produces proteins that are involved in blocking host anti-viral responses, DNA replication, and transcription, including transcription factors (TF) for intermediate genes. Intermediate gene transcription occurs only after DNA replication provides a suitable DNA template (Baldick and Moss, 1993). Intermediate gene products continue the gene expression cascade by producing late TFs that stimulate the final wave of late transcription to produce virion proteins, including envelope proteins and transcription machinery that must be packaged into nascent virus particles (Broyles, 2003).

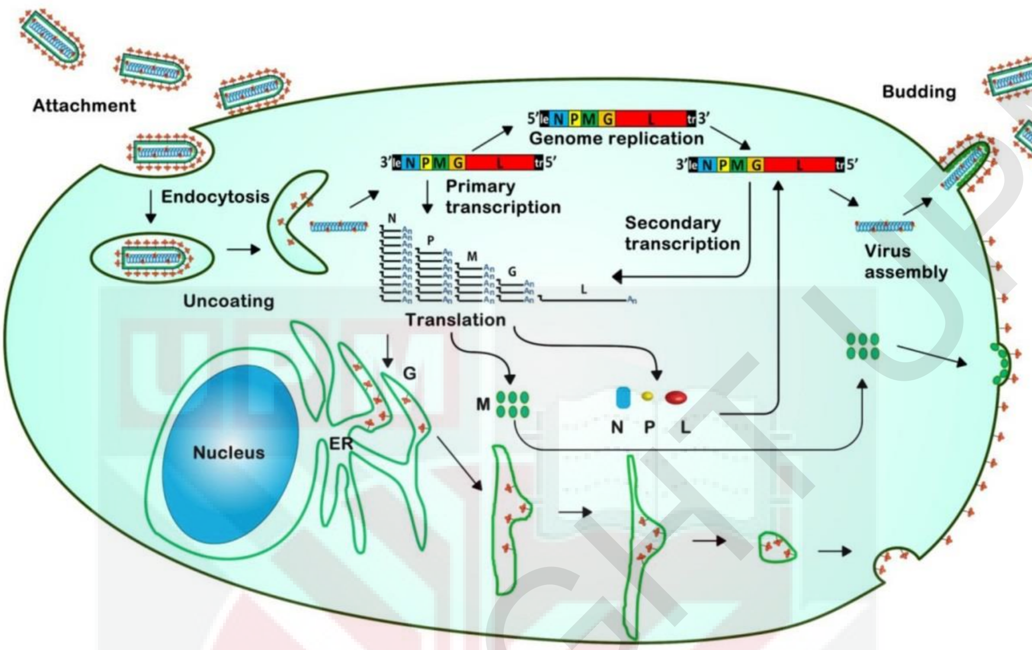


Figure 2.2. Overview diagram of VSV life cycle (as example of Parapoxvirus genus). Steps of virus life cycle: attachment, endocytosis, uncoating, genome replication, mRNA transcription, viral protein translation, viral assembly, and budding are shown (adapted from Li and Zhang, 2012).

2.2. Contagious Ecthyma

2.2.1. Introduction

Contagious Ecthyma is an acute, contagious, debilitating and economically important viral skin disease that affect ruminant .The disease is also known as sore mouth, contagious pustular dermatitis or scabby mouth. Orf virus infection are ubiquitous in sheep-producing and cattle producing countries worldwide. The resistant nature of the virion in the environment and the short lived immunity to reinfection make the disease are can be maintained and spread easily in the environment. (Nandi *et al*., 2011)

2.2.2. Clinical sign

The disease is manifested by proliferative lesions on the mouth and muzzle that usually resolved in 1–2 months. Primary Orf lesions are the most severe with a clinical progression of erythematous macule, papule, vesicle, pustule and scab formation in 4–6 weeks and young animals are at high risk. Reinfection lesions progress through the same clinical stages but are generally smaller, not proliferative and resolve more rapidly usually within 2–3 weeks (Nandi *et al*., 2011).

Image 2.1. Clinical sign of Orf virus. a .bloody lesion in sheep in severe outbreak of contagious ecthyma b. Orf virus manifestation at the udder of lactating ewe c. typical concentric inflammatory bands in a case of bovine papular stomatitis d. milker's node as a consequences of Orf Virus transmission by direct contact with OV infected sheep. (Duhrsen, year 2002).

2.2.3. Virus specificity

The diseases primarily infects animals less than 1 year old, affecting lambs and kids shortly after birth and at 3–4 months. The incidence in a flock may reach 90%, but mortality is usually low. Spread in a flock is rapid and occurs by contact with affected animals or shed scabs (Nandi *et al*., 2011).

2.2.4. Pathogenicity

2.2.4.1 Animal

Contagious Ecthyma is of economic importance due to mortality and weight loss in lambs that are reluctant to eat because of oral and perioral lesion. The infection is confined to the epithelium and oral mucosa. The virus usually infects through abrasions and breaks to the skin, and the clinical pathology observed at sites of infection is typically the formation of pustules and scabs (Nandi *et al*., 2011). Infection begin as reddening and swelling around the sites of inoculation and small vesicles develop within 24 h, the lesion take on a pustular appearance as they develop due to a large infiltration of polymorphonucleocytes. Adjacent lesion that coalesce together will lead to formation of a scab (Stephen B. Fleming and Andrew A. Mercer, 2007).

The histopathological features of natural and experimental infections of ORFV (Robinson and Lyttle 1992; McKeever et al., 1988; Jenkinson et al., 1990 and Jenkinson *et al*., 1991), was described. The infection is proliferative. Mostly infected epidermis showed vacuolation and swelling of keratinocytes in the stratum spinosum, reticular degeneration, marked epidermal proliferation, intra-epidermal microabscesses and accumulation of scale-crust. Intracytoplasmic eosinophilic inclusion bodies may be visible in ballooning keratinocytes 72 h after infection. Epidermal proliferation leads to markedly elongated rete pegs. Neutrophils migrate into areas of reticular degeneration and form microabscesses that subsequently rupture on the surface. A thick layer of scale crust is built up, composed of hyperkeratosis, proteinaceous fluid, degenerating neutrophils, cellular debris and bacteria. Dermal lesions include oedema, marked capillary dilation and infiltration of inflammatory cells. Papillomatous growths that consist of pseudoepitheliomatous hyperplasia and granuloma formation often develop in natural ORFV infections and may become extensive (Stephen B. Fleming and Andrew A. Mercer, 2007)

2.2.4.2. Human

ORFV infection in human have been noted for centuries. A lots of cases were reported (Bassioukas et al., 1993; Georgiades et al., 2005; Huerter et al., 1991; Sanchez et al., 1985; Schimmer et al., 2004) especially in those who in closed contact with the infected animal are usually got infected with the virus. Infection in human are localized as foci of infection and having a several stages as the disease progressed which include maculopapular stage (days 1–7), target stage (days 7–14), acute stage (days 14–21), regenerative stage (days 21–28), papilloma stage (days 28–35) and lastly regressive phase (after 35 days) (*Stephen B. Fleming and Andrew A. Mercer)*

2.3 Diagnosis

The contagious ecthyma can be diagnosed on the basis of characteristic lesions on the anatomic areas of predilection and by performing a laboratory test that able to detect the virus or antibody against the virus (Nandi *et al*., 2011).

2.3.1. Clinical sign

The incubation period of the disease varies from 4 to 8 days with an initial rise in temperature, development of papules and pustules often at oral commissures, skin of lips and nose followed by thick, tenacious scabs covering a raised area of ulceration, granulation and inflammation. Similar skin lesions of few millimeters to several centimeters are also seen in the lips of the kids particularly below two months of age and disseminated to the skin of face, ears, feet, flanks, and scrotum (Nandi *et al*., 2011). In human, the virus infection can confirm through the skin lesion, histology study, clinical course and patients history (Huerter et al., 1991; Rieger et al., 2003; Schimmer et al., 2004; Nourani and Maleki, 2006). It is a self-limiting diseases and no especial treatment need, only some disinfected solution to minimize the secondary bacterial infection. In some cases (immunocompromised patients) treatment can provide such as cryotherapy, surgical excision and 40% topical idoxutidine (Degraeve et al., 1999).

2.3.2. Laboratory test

2.3.2.1. Elisa

ELISA used as screening method of serum sample to detect antibodies against Orf virus (McKeever et al., 1987; Yirrell et al., 1994; Azwai et al., 1995). It is also used to screen serum samples of different species of animals employing purified antigens and peroxidase conjugated protein A or G or chimeric A/G (Inoshima et al., 1999).

2.3.2.2. Electron microscopy (EM)

EM is the most rapid method of diagnosis and differentiation of poxvirus infection in human and animals. Parapoxvirus virion appear as crisscross pattern against an electro dense background core and approximately 200nm × 160nm (Guo et al., 2004). According to Vikoren et al., 2008, Parapox virus appear as negatively stained oval-shaped virus particles with the arrangement of the outer protein filaments and the size of the virus particles is approximately 260nm × 130nm((Vikoren et al., 2008).

2.3.2.3. Virus isolation in cell culture

Many cell lines were used for parapox isolation. The most common cell line Lamb testes (LT) cell line, Canine kidney (MDCK) and (Vero) cell line. Isolation of virus in cell line was failed to confirm the parapox virus (*Kottaridi et al., 2006).*

2.3.2.3. Polymerase chain reaction

Different procedures were used in parapox virus detection. Some of them are time-consuming, laborious and sometimes show lack of specificity and sensitivity with cross-reactions observed (Wittek et al., 1980; Rosenbusch and Reed, 1983 ;Lard et al., 1991). Polymerase Chain reaction (PCR) is a molecular technique that a rapid, sensitive and specific tool in identifying several infectious diseases of veterinary importance (Mangana-Vougiouka et al., 1999, 2000; Inoshima et al., 2001, 2002; Billinis et al., 2001). Several PCR protocols have been described for parapox DNA detection (Mazur et al., 2000; Inoshima et al., 2000, 2001, 2002; Torfason and Gunadottir, 2002; de la Concha-Bermejilo et al., 2003; Guo et al., 2003, 2004; Tryland et al., 2005) emphasizing on the prevention of serological cross reactivity and on diagnosing ecthyma without using cell culture systems or electron microscopy.

2.3.2.3. 1. Standard PCR

PCR base on B2L or VIR gene has been used to diagnose the Parapoxvirus infection. Development of PCR methods for the molecular detection of Parapox DNA has met the demands for specific and sensitive laboratory diagnosis of Orf disease (Inoshima *et al*., 1999). PCR-assay is a powerful tool for the diagnosis of Orf infections which shows increased sensitivity over existing PCR protocols, is more rapid when compared with virus isolation by cell culture and can differentiate successfully poxvirus infections (Kottaridi et al., 2006).

2.3.2.3.2. Semi nested

Semi nested PCR is a PCR technique that run 2 PCR process consecutively. For the second PCR process, 1 of the primer in the 1st PCR are used again in the 2nd PCR process targeting the same sequence. A semi-nested PCR based on the major enveloped protein B2L gene has been reported to detect low copy number of virus particles from clinical samples (Inoshima *et al.*, 1999); using more than one pair of primers can help to increase the sensitivity of the PCR (Mangana-Vougiouka et al., 1999; Guo et al., 2003; Kottaridi et al., 2004 and Kottaridi et al., 2006).

2.3.3. Phylogenetic study

A phylogenetic tree, also known as a phylogeny, is a diagram that depicts the lines of evolutionary descent of different species, organisms, or genes from a common ancestor. Phylogenies are useful for organizing knowledge of biological diversity, for structuring classifications, and for providing insight into events that occurred during evolution. Furthermore, because these trees show descent from a common ancestor, and because much of the strongest evidence for evolution comes in the form of common ancestry, one must understand phylogenies in order to fully appreciate the overwhelming evidence supporting the theory of evolution (**Baum, 2008).** Whole genome information or some genes can be used for study the relationship between different species or close species or strains. Study the variation of orf viruses strains were done by using different genes (Andrew et al., 2005, Kottaridi et al., 2006). The most common genes that were reported are B2L gene (Andrew et al., 2005) and VIR (Kottaridi et al., 2006). B2L of ORFV encodes a highly immunogenic envelope protein that induces a strong antibody response (Chan *et al*., 2009). A polymerase chain reaction (PCR) method based on the B2L gene is typically used to detect ORFV (Abrahao *et al*., 2009). Complete or partial B2L sequences have often been used in phylogenetic analysis in India (Hosamani *et al*., 2006), Korea (Oem *et al*., 2009), China (Chan *et al*., 2009), Brazil (Abrahao *et al*., 2009), and Turkey. The B2L gene has been reported to encode a highly immunogenic envelope protein and play an important role in ORFV immunity (Sullivan et al., 1994). This gene has been widely used for molecular characterization and phylogenetic analysis of strains of the virus (Inoshima et al., 2000; Abrahão et al., 2009; Oem et al., 2009; Zhang et al., 2010).

Interferon resistance gene (VIR) is located in the left terminus of the Orf viral genome (McInnes et al., 1998) and it encode the information for a dsRNA-binding protein that inhibits the antiviral activity of interferon. Recently, the B2L and VIR genes have both been used in studies of genetic variation and molecular epidemiology (Oem et al., 2009). This gene, interferon-resistance (VIR) gene showed some variation since it is encounter in the immune response and can undergo some mutation (de la Concha-Bermejilo et al., 2003; Guo et al., 2004) suggested that this region could serve as a genetic marker for parapox virus.

3.0 MATERIALS AND METHOD

3.1 Tissue sample

Scab samples were used in this study are from clinical cases of Orf in goat's from Program Ladang Angkat UPM in 2014. Total of three (3) scab samples from different outbreak were used in this study. The sample were stored in -20^{o}c refrigerator before proceed to nucleic acid extraction procedure.

3.2 Sample processing

100mg of the scab sample were homogenized in Phosphate Buffer Solution (PBS). The samples were then centrifuged at 3000 r.p.m for 10 minute and supernatant were filtered through 0.45µl syringe filter and stored at -70^{o}C for further use.

3.3. Isolation of Parapox virus in cell line

3.3.1. Cell subculture

Lamb testis (LT) cell line and Madin-Darby canine kidney (MDCK) were used in this study. A confluent monolayer of both cell lines were washed twice with PBS and trypsin zed with ATV. The cells were sub cultured into new cell culture flask and supplied with 10% DMEM. The flasks were incubated at 37^{o}C with presence of 5% CO_2 .

3.3.2. Isolation of Parapox (*Orf*) virus in cell line

A confluent monolayers were used in this study. The cells were washed with DMEM media. 0.1ml of the processed samples were inoculated into the flask. The negative control was inoculated with 0.1 ml DMEM media. The flasks were incubated at 37^{0}C for 30min and was examined daily for development of cytopathic effect.

3.4. Detection of Orf virus by polymerase chain reaction

3.4.1. DNA extraction

Manual extraction of DNA was performed according to the standard protocol. 500µl extract buffer and 30 µl Sarcosyl were added to the virus suspension. The suspension was incubated at 60^{0}C for 2 minute. 15 µl proteinase K was added and the suspension were continued for incubation at 37^{0}c for 2 hour. An equal volume of Phenol equilibrated with Tris CL was added to the suspension and were mixed gently before centrifuged at 4000rpm for 5min at 4^{0}C. Aqueous phase of the suspension was transferred to a new tube, a P: C: I (25:24:1) solution was added and centrifuged at 4000rpm, 5 min, 4 ^{0}C. The process was repeated again with C: I (24:1) solution. The solution salt was adjusted using $MgCl_2$ and NaCl according to total volume of the suspension. Cold alcohol was added 2.5x from the total suspension volume for DNA precipitation. The suspension was kept overnight at -20^{0}C, then proceed with centrifuge at 12000 rpm for 15min. The supernatant was discarded and the DNA was washed with 70% ethanol. The tubes were centrifuged at 12 000 rpm for 15min. The supernatant was discarded and DNA was dissolved in 30 µl RNAse free water.

3.4.2. Primers

The primers for detection of Orf virus that were used in this study are primers targeting both major large envelop protein (B2L) (Inoshima et al 2000; Li et al 2012) and interferon resistant gene (VIR) (Bandy et al 2014; Li et al 2012). Detail about the primers are shown in Table 1.

3.4.3. Polymerase Chain Reaction (PCR)

PCR master mix(Novagen kit) was carried out in 25µl total volume as following; 3 µl of extracted DNA , 2.5 µl Deoxynucleotide (dNTP), 2.5 µl PCR buffer solution, 1.5 µl Magnesium Sulphate (MgSO4), 0.5 µl of polymerase , 0.75 µl forward primer, 0.75 reverse primer and 13.5 µl Rnase-free water. The thermal cycler was programmed for 95 ^{0}C for 2 min following by 35 cycle of denaturation (94^{0}C, 20s), annealing temperature (were set differently according to primer used as shown in table 1) and elongation were carried out at (70^{0}C, 20s). The PCR product was kept at -20^{0}C.

3.4.4. Gel Electrophoresis

1.25% agarose gel was prepared by mixing 1.25g agarose and 100ml 1X TAE buffer, boiled and allowed to solidify under room temperature. 2 µl loading dye and 10 µl PCR product were loaded into well of the agar. The comparison was done by using 100bp DNA ladder. The gel electrophoresis was run at 100v for 50 minute. Then the gel was stained using Red safe for 30 minute and the DNA bands were viewed using Gel Doc.

3.5. DNA sequence

The positive samples with Parapoxvirus was used for DNA sequencing. The samples were amplified by PCR using the whole gene B2L and 059 coding gene primers (table 2). Presence of the virus were confirmed using gel electrophoresis and the positive samples were sent for sequencing at 1st base company, the software used for the sequencing is Applied Biosystems DNA Sequencing Analysis Software v5.2 with KB Basecaller.

3.6. Phylogenetic study

The samples results were blasted at National Center for Biotechnology Information (NCBI) website to verify it is Parapoxvirus. Sequences data of all clinical sample by using both forward and reverse primers were compared with another Parapoxvirus (Orf virus) that available in Gene bank database under NCBI. The reference strains that were used for this study was shown in table 3 and 4 for both gene B2L and 059 respectively. The phylogenetic three was constructed by using MEGA 4 program. Reference strain that used for tree construction were shown in table 3 and 4

3.7. Determined the sensitivity of primers

3.7.1. TCID 50%

The LT cell line was seeded in 96 well plate. Serial 10 fold dilution were prepared on DMEM without serum. The confluent monolayer of the LT cell line in column 1-10 was inoculated with the virus dilutions. The last two columns were kept as –ve control. The cell was examined daily for any cytopathic effect (CPE). The virus titer was calculated by using Spearman & Karber algorithm as described in Hierholzer & Killing (1996), virology method manual, p 374.

3.7.2. DNA extraction and PCR

The DNA extraction were done for all 10 fold serial dilution by using 100µl and 500µl of the virus dilution as describe previously 3.4.1. PCR using 4 sets of primers was done as describe previously 3.4.3.

4.0 Results

4.1 Virus cultivation

Approximately 50% CPE were observed 3 days post inoculation and after 6 days post inoculation in monolayer culture of lamb testis (LT) and Madin-Darby canine kidney (MDCK) cell line. The CPE that was observed are ballooning, rounding and degeneration of cells.

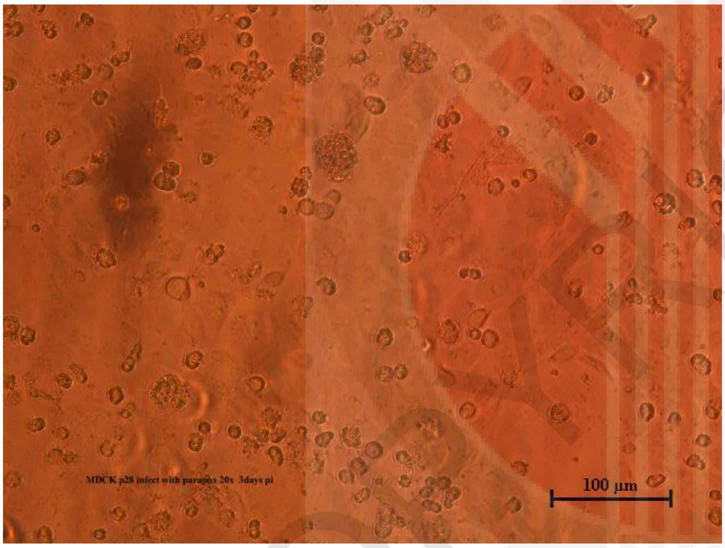


Figure 1 MDCK cell line 3 days pi 20X

Presence of ballooning, rounding and degeneration of cells.

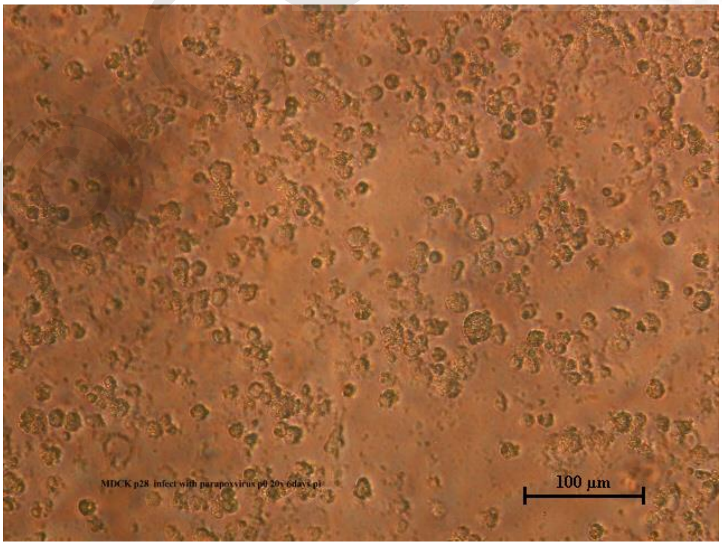


Figure 2 MDCK cell line 6 days pi 20X

Presence of ballooning, rounding and degeneration of cells.

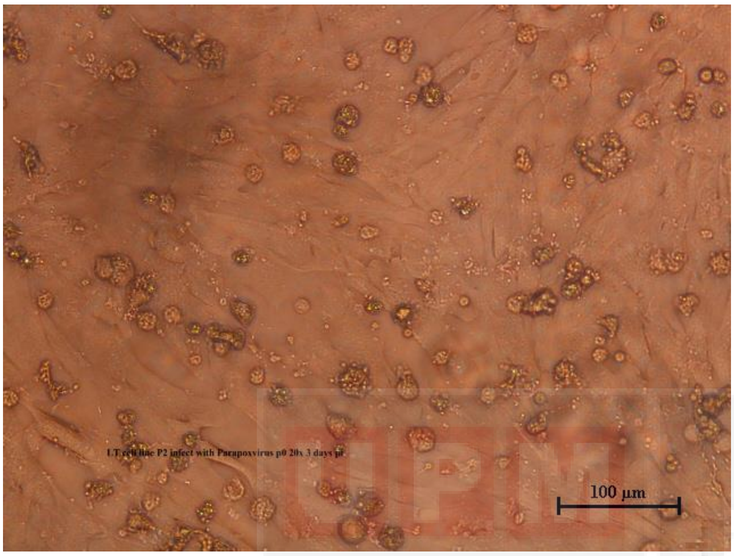


Figure 3 LT cell line 3 days pi 20X

Presence of ballooning, rounding and degeneration of cells.

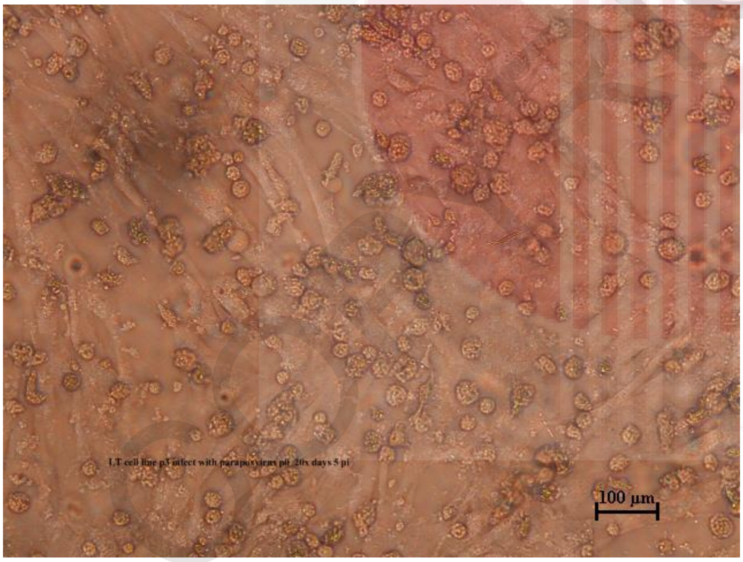


Figure 4 LT cell line 5 days pi 20X

Presence of ballooning, rounding and degeneration of cells.

4.2 Polymerase chain reaction (PCR)

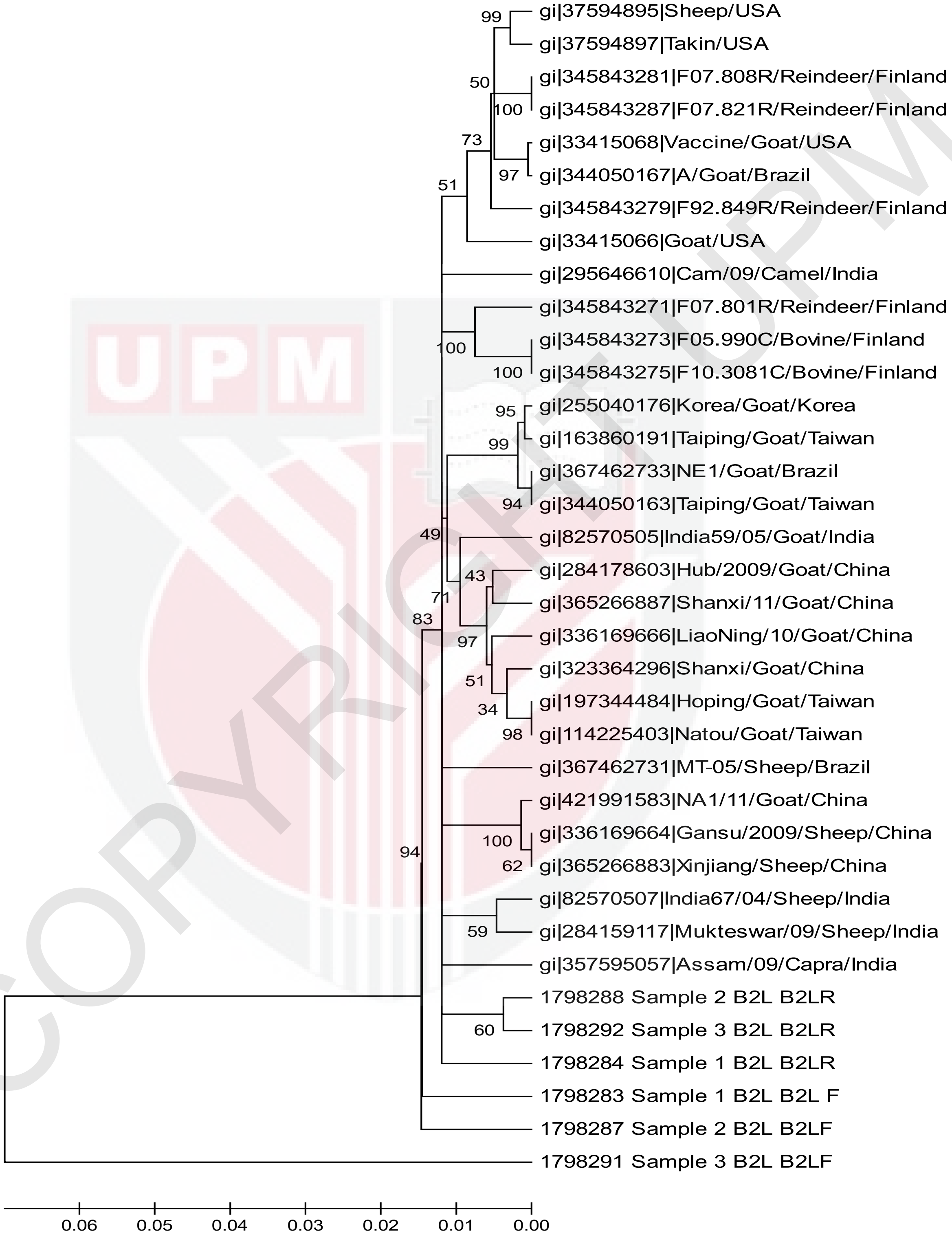
The partial gene primer VIR and PPP1-PPP4, semi nested PCR PPP3-PPP4 and whole gene primer ORFV011 and ORF059 showed positive result in all 3 samples where the amplification was approximately 500+ bp for VIR and PPP1-PPP4 primer, 200+ bp for PPP3-PPP4 primer and 1000+ bp for ORFV011 and ORF059 primer.

Figure 5 Detection of Orf virus from scab sample. Lane M: 100 bp molecular weight marker. Lane 1: VIR primer, Lane 2: PPP1-PPP4 primer, Lane 3: semi nested PPP3-PPP4, Lane 4: 0RFV011 primer, Lane 5: ORF059 primer.

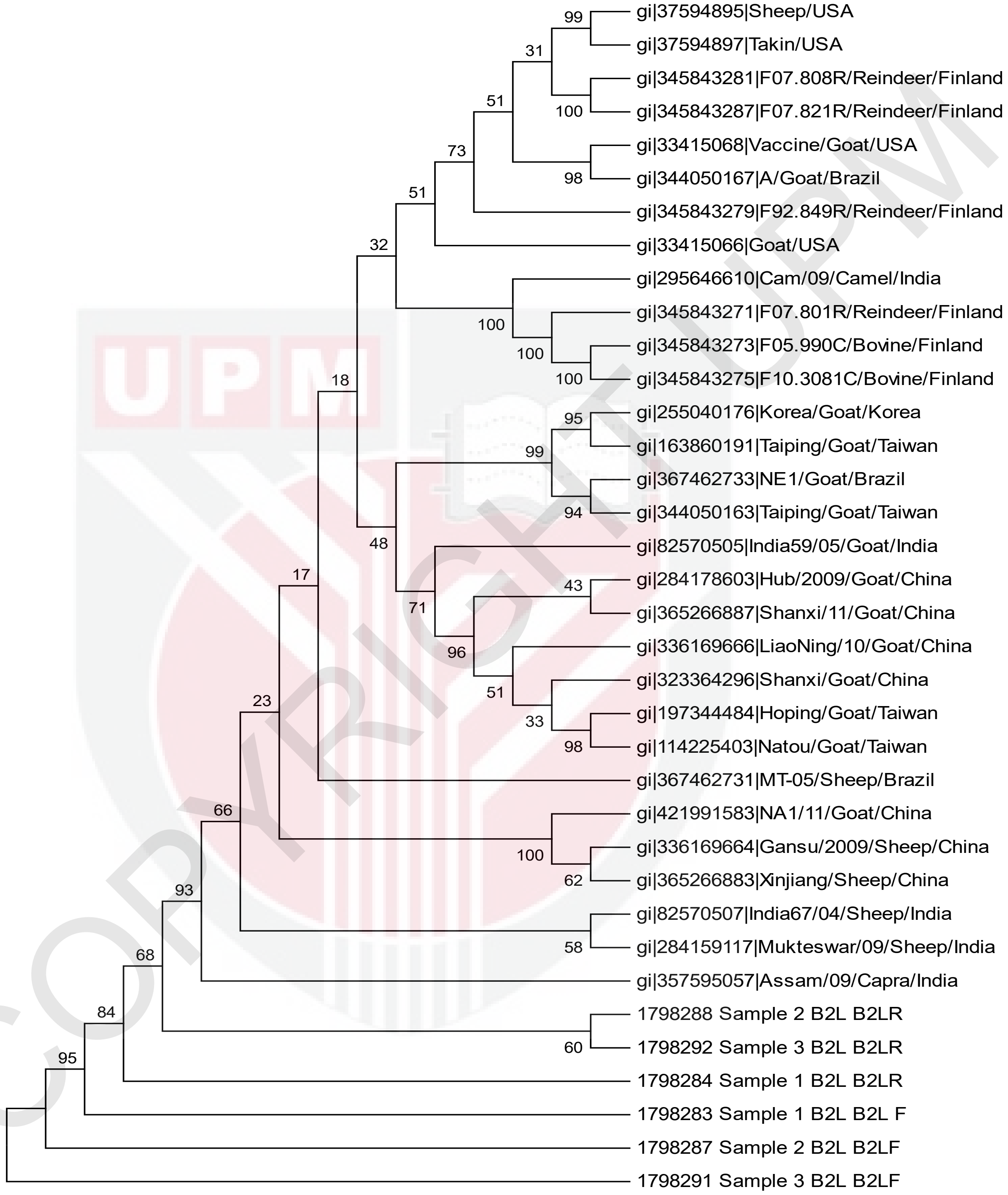
Figure 6 Sequencing of Orf virus from scab sample. Lane M: 100 bp molecular weight marker. Lane 1, 3, 5: primer ORFVB2L. Lane 2, 4, 6: primer ORFV059

4.3 Phylogenetic tree

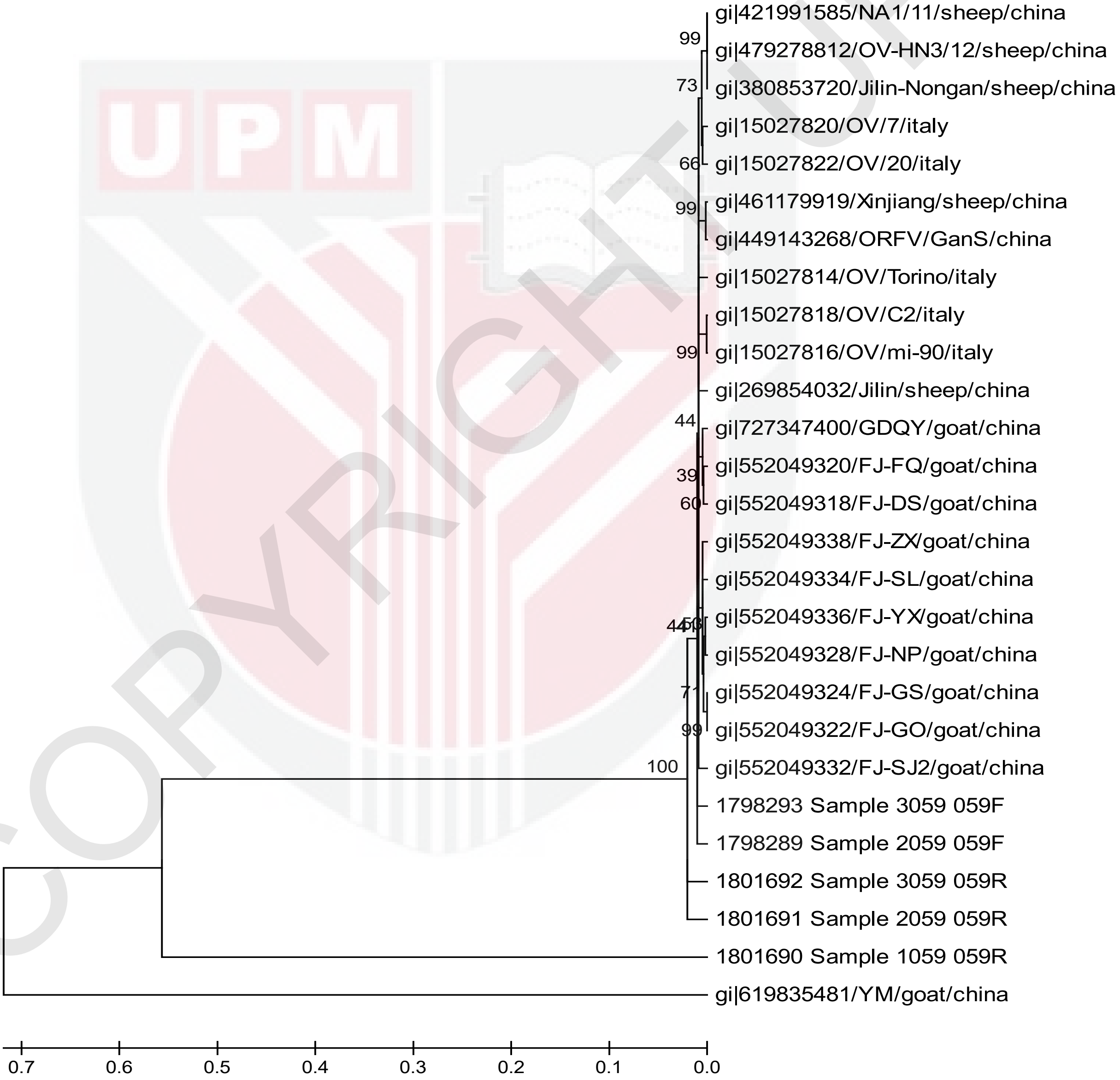
NCBI nucleotide blast on DNA sequence of all sample were done to search for highly similar DNA sequences. The result revealed a high percentage homology with an Orf virus strain. Based on B2L gene, the Orf virus isolate from sample 2 have a high grade of homology to the sample 3, compared to sample 1 and also show high grades of homology with Orf virus isolates obtained from sheep from India and China.



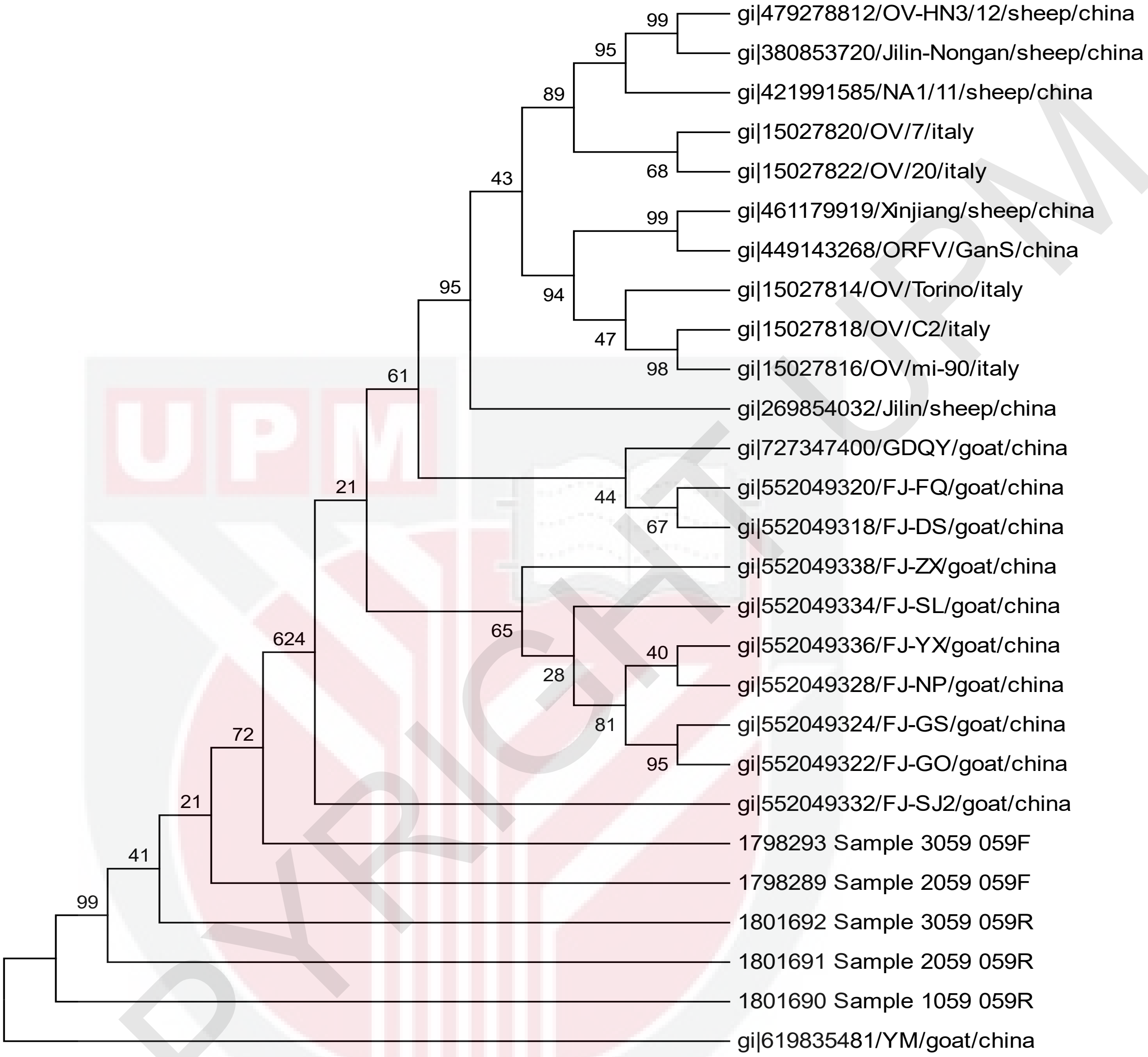
Phylogenetic analysis based on nucleotide sequences of ORFV011 (B2l gene): The phylogenetic relationship was constructed by neighbor joining algorithm using MEGA 4.0 software; one thousand bootstrap replicates were subjected to nucleotide sequence distance (cut-off value 50% from 1000 bootstrap replicates. All bootstrap values are displayed above branches.



Phylogenetic analysis based on nucleotide sequences of ORFV011 (B2l gene): Topology only



Phylogenetic analysis based on nucleotide sequences of ORFV059: The phylogenetic relationship was constructed by neighbor joining algorithm using MEGA 4.0 software; one thousand bootstrap replicates were subjected to nucleotide sequence distance (cut-off value 50% from 1000 bootstrap replicates. All bootstrap values are displayed above branches.



Phylogenetic analysis based on nucleotide sequences of ORFV059: Topology only

4.4. Primer sensitivity

4.4.1. TCID50%

The **virus titer was calculated by** Spearman & Karber algorithm (Hierholzer and Killing 1996) by using Microsoft excel. The titer was found 2.37E+04 TCID50/ml≈ 1.64E+04.

4.5. Primer sensitivity

All primer sets showed a signal band at 10^{-1}.

Figure 7 Result for primer sensitivity Lane M: 100 bp molecular weight marker. Lane 1: 10^{-1} virus dilution, Lane 2: 10^{-2} virus dilution, Lane 3: 10^{-3} virus dilution, Lane 4: 10^{-4} virus dilution, Lane 5: 10^{-5} virus dilution, Lane 6: 10^{-6} virus dilution, Lane 7: 10^{-7} virus dilution, Lane 8: 10^{-8} virus dilution, Lane 9: 10^{-9} virus dilution. Lane 10: 10^{-10} virus dilution

5. DISCUSSION

Virus inoculated in both cell line LT and MDCK both cell line showed the same CPE that occur as ballooning, rounding and degeneration of the cells. The cells aggregated in a grape-like pattern. The same CPE was reported by Kruse and Weber, 2001; Vikoren et al., 2008. The CPE was started to develop from the third day of inoculation as mentioned by Hessami et al., 1979. A plaque-like focal areas of large, highly refractile cells was also reported by another researchers in the BFS monolayers but it was not observed in this study. Moreover, the virus can develop inclusion bodies as it was reported by Yorifumi et al., 1999. Study by Li et al., 2012 showed that all the homogenates from tissues collected from the outbreak that inoculated into cultures of OFTu cells resulting in a cytopathic effect (CPE), indicated by cell rounding, pyknosis, and cell detachment in the cell cultures. The results in our study are in agreement with the researchers.

Different PCR protocol even standard or semi nested were develop for ORF virus detection (Inoshima et al., 1999). In this study, 4 sets of primers; 3 standard PCR and 1 set as semi nested. The methods adapted in this work are from Bandy *et al*., 2014 and Zhao *et al*., 2010. The PCR products were 1199 bp and 1062 bp respectively. The results on this study are in agreement with the researchers. .

Semi nested PCR with PPP1 and PPP4 (594 bp) and following by amplification of PCR product with PPP-3 and PPP-4 primers will lead to (235 bp). All primers used in this study were able to detect the ORF virus. Virus titer were necessary to determine the sensitivity of primers, thus the virus titer were calculated using TCID 50 and in this study the virus titer was very low. A low titer mainly because the virus was in first passage in the cell. The virus need to be passaged several times to let the virus grow and achieve a higher titer. Due to time limitation, the virus not been passaged for long time and it may affect the result of the sensitivity test.

Positive samples were amplified by using the complete gene, ORF V011 and ORF V059 gene primers established by Li et al., 2012. The product size are 1137 and 1017 respectively. Samples 2 and 3 were very close to each other's comparing with the sample 1 and they are so India strain whereas the alignment of sequence result by using 059 genes showed that that the tree samples showed variation from each other and they are more close to china strains. The variation between Orf virus strains were reported by different researches. The global variation between isolates in both nucleotides and amino acid sequences worldwide was (n= 33) 8.1% and 9.6%, respectively. The analysis revealed that the maximum nucleotide and amino acids variation amongst Orf virus strains isolates worldwide was (n= 33) 8.1% and 9.6%, respectively (Fleming *et al.*, 2005)

Heterogenicity also was reported in China, some strains share 98.2% -99.3% of nucleotide identity even they are collected from the same scab coming from the same farm (Chi et al., 2013). Another high variation that showed in China, comparison according to B2L gene 96-97% similarity but through 059 gene showed 95-99% similarity Li et al., 2012. The Jilin Orf isolate from china also showed 96.5-98.9% and 95.3-97.4% in their B2L and 059 genes respectively comparing with another global isolates (Zhao et al., 2010). Looking to Orf virus in India, analysis of B2L gene showed that approximately 98.1-99.8% and 98.4-99.7% in nucleotide and amino acid sequences (Venkatesan et al., 2011).

CONCLUSION

From this studies, it can be concluded that all primer used are able to detect Orf virus and all primer have similar sensitivity. Phylogenetic studies reveal that there are strain variation of the Orf virus in ladang angkat UPM

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TABLE 1 Primer used for Orf virus detection

Primer	Forward primer	Primer	Reverse primer	References	Temperature (^{0}C)
VIRF	5-ACA ATG GCC TGC GAG TG-3	VIRR	5-TTA GAA GCT GAT GCC GCA G-3	Faroque et al 2014	43
PPP1	5-GTC GTC CAC GAT GAG CAG CT-3	Ppp4	5-TAC GTG GGA AGC GCC TCG CT-3	Inoshima et al 2000	52
PPP3	5-GCG AGT CCG AGA AGA ATA CG-3			Inoshima et al 2000	50
ORFV011F	5- ATG TGG CCG TTC TCC TCT ATC-3	ORFV011R	5- TTA ATT TAT TGG CTT GCA G-3	Li et al 2012	50
ORFV059F	5- ATG GAT CCA CCC GAA ATC AC-3	ORFV059R	5- TCA CAC GAT GGC CGT GAC CAG-3	Li et al 2012	50

TABLE 2 Primer used for Orf virus phylogenetic study

Primer	Forward primer	Primer	Reverse primer	References	Temperature (^{0}C)
ORFV B2LW GF	5- TAT AGG ATC CGC CAT GTG GCC GTT CTC CTC CAT C-3	ORFV B2LW GR	5- CCG CTC GAG TTA ATT TAT TGG CTT GCA G-3	Zhao et al 2010	60
ORFV 059 WGF	5- CAA GCT TGC CAC CAT GGA TCC ACC CGA AAT C-3	ORFV 059 WGR	5-CGA ATT CTC ACA CGA TGG CCG TGA CC-3	Zhao et al 2010	60

TABLE 3 B2L gene reference strain

STRAIN	ACESSION NUMBER	HOST	COUNTRY
	37594895	Sheep	USA
	37594897	Takin	USA
F07.808R	345843281	Reindeer	Finland
F07.821R	345843287	Reindeer	Finland
Vaccine	33415068	Goat	USA
A	344050167	Goat	Brazil
345843279	F92.849R	Reindeer	Finland
	33415066	Goat	USA
Cam/09	295646610	Camel	India

F07.801R	345843271	Reindeer	Finland
F05.990C	34584327	Bovine	Finland
F10.3081C	345843275	Bovine	Finland
Korea	255040176	Goat	Korea
Taiping	163860191	Goat	Taiwan
|India59	8257050	Goat	India
Hub/2009	284178603	Goat	China
Shanxi/11	365266887	Goat	China
Hoping	197344484	Goat	Taiwan
Natou	114225403	Goat	Taiwan
MT-05	367462731	Sheep	Brazil
NA1/11	421991583	Goat	China
Gansu/2009	336169664	Sheep	China
Xinjiang	365266883	Sheep	China
India67/04	82570507	Sheep	India
Mukteswar/09	284159117	Sheep	India
Assam/09/	357595057	sheep	India

TABLE 4: 059 gene reference strain

STRAIN	ACESSION NUMBER	HOST	COUNTRY
OV-HN3	479278812	sheep	china
Jilin-Nongan	380853720	sheep	china
NA1/11	421991585	sheep	china
OV/7	15027820		Italy
OV/20	15027822		Italy
Xinjiang	461179919	sheep	china
ORFV/GanS	449143268		china
OV/Torino	15027814		Italy
OV/C2/	15027818		Italy
OV/mi-90	15027816		Italy