UNIVERSITI PUTRA MALAYSIA

STUDY ON PATHOGENICITY OF ORF VIRUS STRAIN UPM 1/14 AND 2/14 IN MICE AT DIFFERENT SITES OF INOCULATION WITH AND WITHOUT DEXAMETHASONE TREATMENT

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TAY KIMMY

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Study on Pathogenicity of Orf Virus Strain UPM 1/14 and 2/14 in Mice at Different Sites of Inoculation With and Without Dexamethasone Treatment", by Tay Kimmy and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project

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DEDICATION

This project paper is dedicated

To my family,

Father

Mother

Brother

& Sister

And to all my teachers who have committed themselves towards the noble cause of education.

ACKNOWLEDMENTS

It is with deepest appreciation and gratitude that I thank all those who have made this project paper a reality.

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LIST OF ABBREVIATIONS

%	Percent
℃	Degree Celsius
kg	Kilogram
ug	Microgram
mg	Milligram
ml	Milliliter
nm	Nanometer
x g	Times gravity
bp	Base pair
CE	Contagious ecthyma
DNA	Deoxyribonucleic acid
G+C	Guanine+Cytosine
kbp	Kilo-base pair
MDBK	Mardin-Darby bovine kidney
MDOK	Mardin-Darby ovine kidney
ORFV	Orf virus
p.i.	Post-inoculation
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
TAE	Tris-acetate-EDTA
UPM	Universiti Putra Malaysia

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 –Projek Tahun Akhir

PENYELIDIKAN KEPATOGENAN ORFV UPM 1/14 DAN 2/14 PADA MENCIT DI TEMPAT INOKULASI YANG BERLAINAN DENGAN DAN TANPA RAWATAN DEXAMETHASONE

Oleh

Tay Kimmy

2016

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

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Dr. Faez Firdaus Jesse Abdullah

Sejak kebelakangan ini, dua jenis ORFV (UPM 1/14 Malaysia; UPM 2/14 Malaysia) telah diasingkan tetapi tiada penyelidikan telah dijalankan pada mencit dengan menggunakan dua jenis ORFV ini. Penyelidikan ini bertujuan untuk memerihalkan kesan dua jenis ORFV ini, tempat inokulasi serta kesan dexamethasone pada kepatogenan jangkitan ORFV pada mencit. Suntikan intradermis 0.2ml 1% UPM 1/14 Malaysia (Kumpulan 1) dan UPM 2/14 Malaysia (Group 2) telah dilakukan dalam

kumpulan berlainan yang terdiri daripada 5 mencit dalam Kumpulan 1 dan Kumpulan 2 di dorsum (Group 1A; Kumpulan 2A), cuping telinga (Group 1B; Kumpulan 2B) dan sudut bibir (Group 1C; Kumpulan 2C). Suntikan intradermis 0.2ml 1% UPM 1/14 Malaysia telah dilakukan dalam kumpulan dexamethasone (n=5) dan kumpulan bukan dexamethasone (n=5). Mencit dalam kumpulan dexamethasone dirawat dengan dexamethasone, 5mg/kg/hari, intraperitoneum tiga hari sebelum cabaran ORFV dan diteruskan sehingga hari kelima selepas cabaran ORFV. Secara umum, hiperemia diperhatikan dalam semua kumpulan rawatan. Hasil statistik menunjukkan tiada perbezaan yang signifikan dalam min lesi skor antara kumpulan tempat inokulasi ($p>0.05$) dan antara kumpulan dexamethasone dan kumpulan bukan dexamethasone ($p>0.05$). Kumpulan 1 dan Kumpulan 2 juga menunjukkan tiada perbezaan yang signifikan dalam min lesi skor ($p>0.05$). Sungguhpun begitu, Kumpulan 2B dan Kumpulan 2C mempunyai min stratum ketebalan yang lebih tinggi ($p<0.05$). Secara keseluruhan, pemeriksaan histopatologi menunjukkan keratosis, akantosis dan penggelembungan degenerasi. ORFV telah dikesan dalam tisu kulit mencit yang menunjukkan lesi kulit melalui tindak balas reaksi rantai polimerase (PCR). Kesimpulannya, inokulasi intradermis dengan menggunakan kedua-dua ORFV tempatan ini mampu menghasilkan lesi kulit dan perubahan histopatologi pada mencit. Selain itu, terdapat tiada kesan yang berbeza pada kepatogenan jangkitan ORFV dengan menggunakan tempat inokulasi yang berlainan pada mencit. Dalam penyelidikan ini, dexamethasone tidak mempunyai kesan yang signifikan pada lesi kulit ORFV. Oleh itu,

drug alternatif seperti cyclosporin boleh dicadangkan untuk mengganti dexamethasone dalam kajian dari segi aspek ini.

Kata kunci: Virus Orf, mencit, tempat inoculasi, dexamethasone, reaksi rantai polimerase (PCR)

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Project.

STUDY ON PATHOGENICITY OF ORF VIRUS STRAIN UPM 1/14 AND 2/14 IN MICE AT DIFFERENT SITES OF INOCULATION WITH AND WITHOUT DEXAMETHASONE TREATMENT

by

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2016

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Recently, two local ORFV strains (UPM 1/14 Malaysia; UPM 2/14 Malaysia) have been isolated. However, there is no study done in mice using these strains. This study aims to describe the effect of different ORFV strains and inoculation sites as well as dexamethasone effect on pathogenicity of ORFV in mice. Intradermal injection of 0.2ml 1% UPM 1/14 Malaysia (Group 1) and UPM 2/14 Malaysia (Group 2) were done in each group of 5 mice in Group 1 and Group 2 at dorsum (Group 1A; Group 2A), ear

pinna (Group 1B; Group 2B) and labial commissure (Group 1C; Group 2C). Intradermal injection of 0.2ml 1% UPM 1/14 Malaysia was performed in dexamethasone group (n=5) and non-dexamethasone group (n=5). Mice in dexamethasone group were treated with dexamethasone, 5mg/kg/day, intraperitoneally three days prior to challenge and continued until day five post-challenge. In general, mild hyperaemia was observed in all treatment groups. There were no significant difference in mean lesion score among the groups of inoculation site ($p>0.05$) and between dexamethasone-treated group and non-dexamethasone group ($p>0.05$). Mice in Group 1 and Group 2 showed no significant difference in mean lesion score as well ($p>0.05$). However, mice in Group 2B and Group 2C had significantly higher mean stratum thickness ($p<0.05$). Overall histopathological examination revealed keratosis, acanthosis and ballooning degeneration. ORFV was detected by means of PCR on skin tissues of mice with skin lesions. In conclusion, intradermal inoculation of both local strains is able to produce mild skin lesion and histopathological changes in mice. Besides, there is no significant effect of variation in inoculation sites on pathogenicity of ORFV in mice model. In this study, dexamethasone has no statistical effect on pathogenicity of ORFV. Therefore, alternative drug such as cyclosporine can be used for further studies on this aspect.

Key words: Orf virus strains, mice, inoculation site, dexamethasone, Polymerase Chain Reaction (PCR)

1.0 INTRODUCTION

Orf virus (ORFV) is the prototype species of the genus *Parapoxvirus* (PPV) of the *Poxviridae* family that includes *Pseudocowpox* (PCPV), *Bovine papular stomatitis virus* (BPSV) and the *Parapoxviruses of red deer in New Zealand* (PVNZ) (Fleming *et al*., 2007). It is the etiological agent of contagious ecthyma (CE), a severe exanthematic dermatitis that affects domestic and wild small ruminants (Peralta *et al*., 2015). It is commonly known as contagious pustular dermatitis, scabby mouth, sore mouth or orf (Fleming *et al*., 2007). It has also been reported in camels and camelids, members of the Cervidae family and various other ruminants (chamois, serows, tahr, steenboks); dogs, cats and squirrels can also been affected (Spyrou *et al*., 2015). The disease also has a zoonotic potential particularly to people working with animals such as veterinarians, farmers and animal attendants (Kumar *et al*., 2013).

Orf virus usually gains access to host's tissue through breaks and abrasions of skin and replicating in regenerating epidermal keratinocytes (Markey *et al*., 2013). This viral replication will result in oedematous and granulomatous inflammation of dermal cells (Spyrou *et al*., 2015). It is clinically recognized by the appearance of papules, vesicles, pustules and rapidly growing scabs confined to the lips and muzzle of the affected animals (Cargnelutti *et al*., 2011). CE is not usually lethal, and lesions typically disappear within 2 to 4 weeks, but death may result if secondary complications, such as bacterial infections or myiasis, develop (Wilson *et al*., 2012). The most frequent

invaders includes Staphylocci, alpha haemolytic Streptococci and Corynebacteria. *Dermatophilus congolensis* and *Fusobacterium necrophorum* can also be found (Nandi *et al*., 2010). Morbidity rates can be up to 70% in flocks where the disease occurs for the first time (Zhao *et al*., 2010). Besides disruption of the national and international trade of animal and animal products, the lesions produced can also affect the optimum productivity and reduce the market value of the meat, leather and wool (Nandi *et al*., 2010). In immunocompromised animals, extensive and recurrent lesions can occur (Guo *et al*., 2003). This will undoubtedly produce certain economic loss to small stock farming. Although gross clinical signs can be used as a good reference to diagnose this disease, the gold standard is to carry out virus isolation (Chan *et al*., 2003).

Many researches had been done to study the unique genes of ORFV and to develop functional vaccines. These advances would not be possible without the use of laboratory animals such as mice. According to Cargnelutti *et al*. (2010), clinical lesions were successfully reproduced accompanied by virus isolation in mice inoculated with ORFV despite consistent failure by other researchers. This gives rise to the questions where choice of viral strains and sites of inoculation can be the determining factors for successfulness of ORFV research in mice model. Isolation of caprine ORFV was carried out recently to give more insight into the Orf viral strains in Malaysia and ORFV strain UPM 1/14 Malaysia, ORFV strain UPM 2/14 Malaysia and ORFV strain UPM 3/14 Malaysia had been isolated (Abdullah *et al*., 2015). To date, there is still lack of work in determining the effect of these viral strains differences on pathogenicity in mice

model. Besides, Dexamethasone has the ability to suppress immune function thereby increases susceptibility to infections and their severity. Thus, this study is to:

1. Determine the severity of ORFV (UPM 1/14 and UPM 2/14) in mice.

2. Determine differences in lesion produced following different inoculation sites in mice.

3. Study the effects of Dexamethasone (simulating stress/non-stress situations) on the severity of Orf.

For this research, the following hypotheses were proposed:

1. ORFV infection in mice causes relevant skin lesions similar to that of the natural host.

2. Different inoculation sites resulted in different disease severity.

3. Use of Dexamethasone resulted in more severe ORFV lesion in mice.

2.0 LITERATURE REVIEW

2.1 Virus Structure

Orf virus, the causative agent of orf, is the prototype member of the genus Parapoxvirus which belongs to the subfamily Chordopoxviridae of the Poxviridae (Li *et al*., 2012). In contrast to other poxviruses, it is relatively small size with presence of crisscross pattern on the particle surface with high G+C content of the genome (Moss, 2001). Virions are cocoon in shape with about 260nm in length and 160 nm in width and covered with long thread like surface tubules resembling a ball of yarn (Nandi *et al*., 2011). The Orf virus genome with a size of 138kbp, is the smallest within the *Chordopoxvirus* subfamily (Mercer *et al*., 2006). It consists of a linear double stranded DNA molecule encoding around 133 gene products (Delhon *et al*., 2004). Conserved genes are found in the central region while variable genes are at the terminal ends (Mercer *et al*., 2002). Conserved genes are involved in viral replication and morphogenesis of mature and extracellular virions whereas factors associated with virulence, pathogenesis, immune invasion/modulation and host range are encoded in the variable genes (Buttner *et al*., 2002).

2.2 Contagious Ecthyma

CE which is also known as contagious pustular dermatitis, sore mouth, scabby mouth and Orf is a disease with worldwide distribution and significant financial importance (Spyrou *et al*., 2015). It is an epitheliotrophic disease that primarily affects sheeps, goats, wild ruminants and humans (McElroy *et al*., 2007).

2.2.1 Clinical Signs

The lesions are characterized by maculopapular, vesicular pustules that mainly affect the skin around the lips, mouth muzzle, nostrils, teats and oral mucosa and rarely extend into the esophagus, stomach, intestines or the respiratory tract (Zhao *et al*., 2010). In ewes, lesions are primarily observed on the teat or the udder skin and less often in the inguinal area and the thigh (Nandi *et al*., 2011). It is not usually lethal, and lesions typically disappear within 2 to 4 weeks, but death may result if secondary complications, such as bacterial infections or myiasis, develop (Wilson *et al*., 2012).

2.2.2 Pathogenesis

Dried stemmy and spiny feed may abrade the tissues of lips, nostrils, mouth as well as fore stomach at the time of grazing (Nandi *et al*., 2011). This allows virus to penetrate and replicate in regenerating epidermal keratinocytes (Markey *et al*., 2013). As a result, there will be oedematous and granulomatous inflammation of dermal cells

(Spyrou *et al*., 2015). Lesions typically evolve through stages of erythema, macules, vesicles, pustules and proliferative scab (Fleming *et al*., 2007).

2.2.3 Diagnosis

Although gross clinical signs can be used as a good reference to diagnose this disease, the gold standard is to carry out virus isolation (Chan *et al*., 2003). Primary lamb testis, lamb kidney, fetal lamb dermis cells, fetal lamb muscle cells, ovine fetal turbinate cells, fetal bovine muscle cells, fetal bovine lung cells as well as cell line MDBK, MDOK, Vero cells are generally used to isolate Orf virus (Delhon *et al*., 2004). Cytopathic effect is characterized by cell rounding, ballooning and degeneration (Kumar *et al*., 2014). Another most widely used diagnostic tool is PCR. B2L gene; a highly immunogenic envelope gene and Orf F1L gene; an immunodominant gene are the two most common genes used in PCR for virus detection (Li *et al*., 2012). Since immunity to Orf virus is mainly cell-mediated, serum neutralization test is not the primary choice for diagnosis.

2.3 Isolation of Orf Virus in Malaysia

To date, there are two studies on isolation of Orf virus that had been carried out in Malaysia. One was done in year 1995 while another in year 2014. In the recent study, three virus strains were isolated, namely, UPM 1/14 Malaysia, UPM 2/14 Malaysia and

UPM 3/14 Malaysia (Abdullah *et al*., 2015). They were found to be closely related to Orf strains isolated from China and India.

2.4 Study of Orf Virus in Mice

According to Cargnelutti *et al*., (2010), their study is the first that had successfully reproduced Orf virus infection and lesions in mice. The lesion progressed from focal erythema, macules, papules, few vesicles and lastly to formation of small scab which can be observed between day 5 and day 12 post-inoculation (p.i.). Among three sites of inoculation (ear, labial commissure and dorsum), only ear produced significant lesions. The lesions produced are consistent with Huda *et al*., (2014). However, in Huda *et al*., (2014), the lesions were produced one day earlier which is day 4 p.i. and lasted for a longer period of time; 6-10 days. In both studies, the histological examination of the lesions revealed focal proliferative dermatitis with ballooning degeneration. In addition, there was presence of eosinophillic intracytoplasmic inclusion bodies in keratinocytes from histological examination in Cargnelutti *et al*., (2010).

2.5 Immunosuppressive effect of Dexamethasone

Dexamethasone has the ability to suppress both cell-mediated and humoral immunity (Rathee *et al*., 2012). It had been used in some researches and results in increased in disease severity. Studies have shown that in immunocompromised animals,

ORFV lesion will be more extensive. In immunocompromised individual, a medical condition known as Giant Orf will occur (Geerinck *et al*., 2001). There was a research done on sheep where it was treated with Cyclosporin, a cell-mediated immunosuppressive drug and resulted in development of more severe ORFV lesions (Haig *et al*., 1996). However, study on effects of Dexamethasone on ORFV lesion is still underexplored.

3.0 MATERIALS AND METHODS

3.1 Tissue sample

Scab samples used in this study are from clinical cases of Orf in goats from Program Ladang Angkat UPM in 2014 which are kept under -20℃. Virus isolation and phylogenetic analysis had previously been done on the scab samples in a study carried out by Abdullah et al., (2015). As a result, three virus strains were isolated, namely UPM 1/14 Malaysia, UPM 2/14 Malaysia and UPM 3/14 Malaysia. In this study, only two virus strains were used which are UPM 1/14 Malaysia and UPM 2/14 Malaysia.

3.2 Preparation of virus suspension

Scab samples which contain the virus were made up as a 10% virus suspension in Phosphate Buffered Saline (PBS). Following centrifugation at 1000rpm for 10 minutes, the supernatant was collected and antibiotics were added at a concentration of 10000 units/ml Penicillin and 10000 µg/ml Streptomycin (HyClone®). The supernatant was further diluted to make 1% virus suspension and kept at -20℃ for further use.

3.3 Experimental design

A total of 35 mice were used. 5 of the mice were used as control group and inoculated intradermal with 0.2ml of sterile Phosphate Buffered Saline (PBS) at the dorsum, ear pinna and labial commissure. The other 30 mice were divided into two equal group of 15 mice each; Group 1 (UPM 1/14 Malaysia) and Group 2 (UPM 2/14 Malaysia). Intradermal injection of 0.2ml 1% UPM 1/14 Malaysia and UPM 2/14 Malaysia were done in each group of 5 mice in Group 1 and Group 2 at dorsum (Group 1A; Group 2A), ear pinna (Group 1B; Group 2B) and labial commissure (Group 1C; Group 2C) (FIGURE I). Following inoculation, the mice were observed for 14 days and sacrified via cervical dislocation.

Another 15 mice were used to study effect of Dexamethasone on Orf virus infection. 5 of the mice acted as Control group and inoculated with 0.2ml of sterile PBS intradermal at the dorsum, ear pinna and labial commissure. The other 10 mice were divided into two equal group of 5 mice each; Dexamethasone group and Non-Dexamethasone group. Mice in both groups were inoculated with 0.2ml of 1% Orf Virus Strain UPM 1/14 Malaysia intradermal at dorsum, ear pinna and labial commissure. Mice in Dexamethasone group were injected with Dexamethasone, 5mg/kg/day, intraperitoneal, 3 days prior to inoculation of Orf virus and continued for another 5 days after challenge (FIGURE II). Following inoculation, the mice were observed for 6 days and sacrified via cervical dislocation.

FIGURE I: Experimental design 1

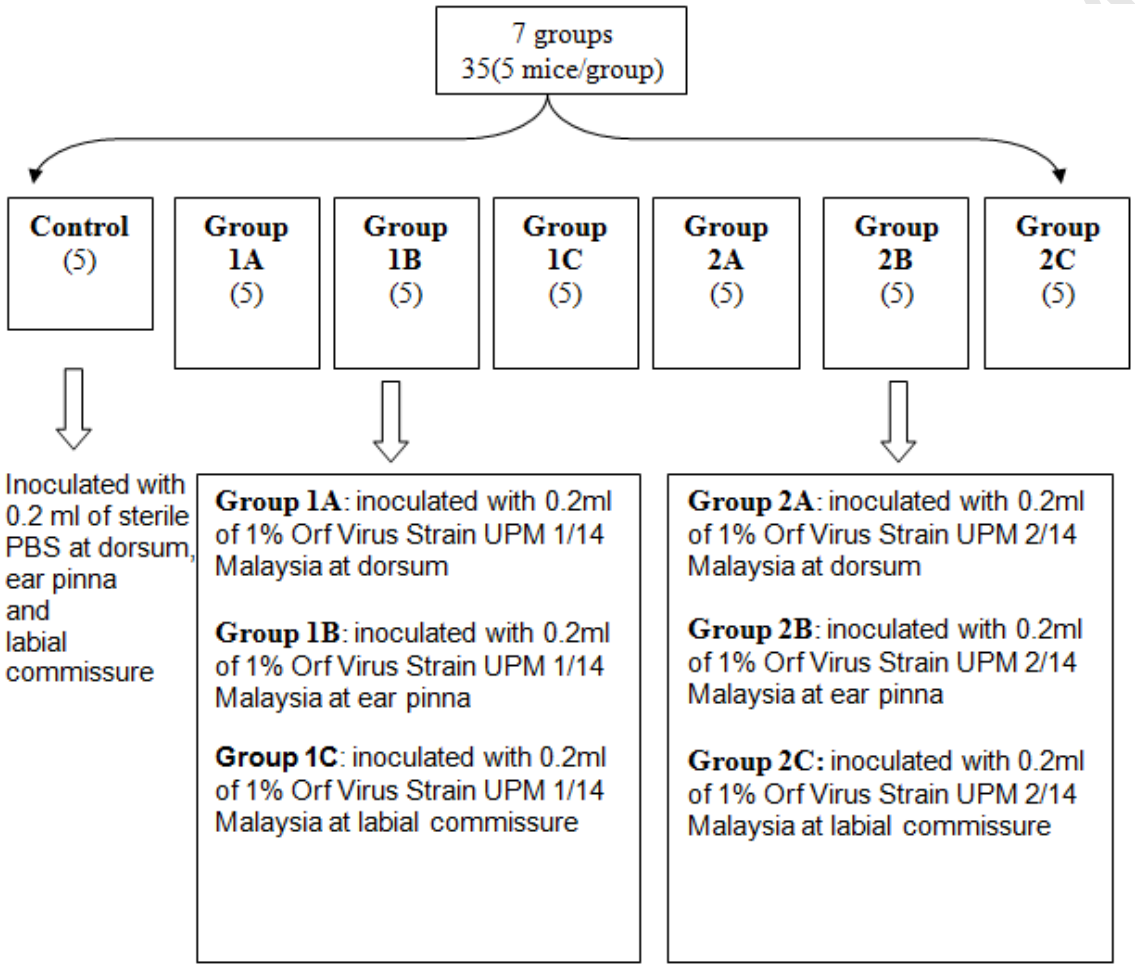
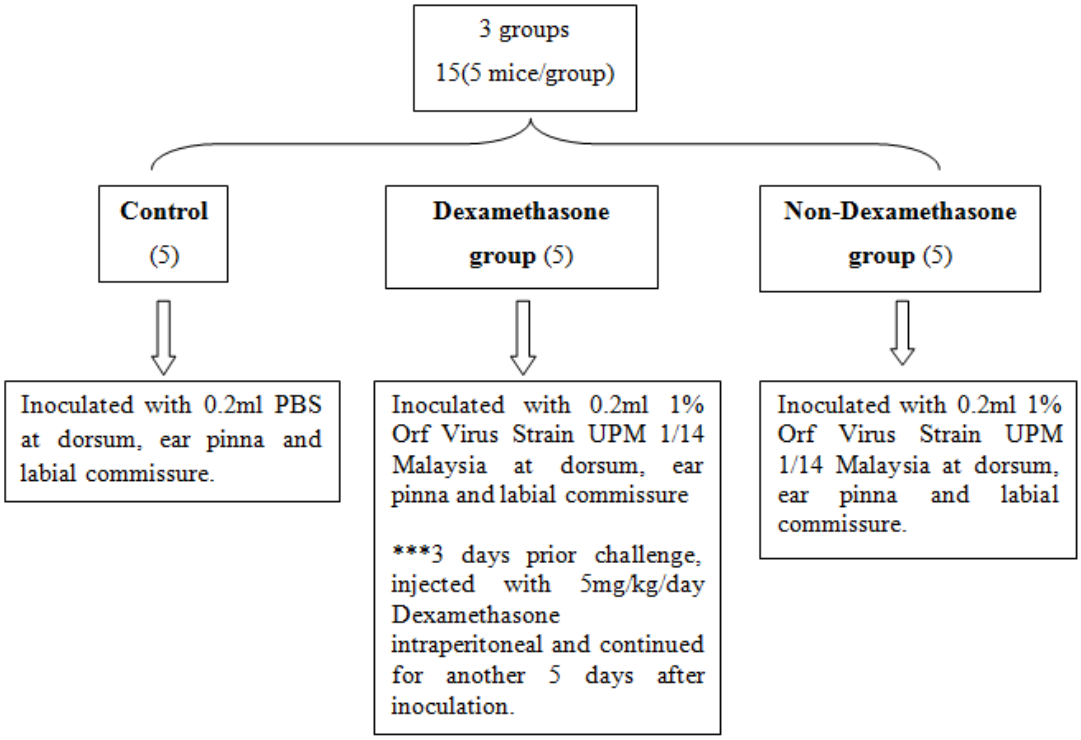


FIGURE II: Experimental design 2



3.4 Clinical scoring

Lesions (hyperaemia, vesicles, pustules, scabs) and clinical signs such as ruffled hair coat, responsiveness and ocular discharges were assessed. For lesion assessment, each indicator was scored from 0 (absence), 1 (mild), 2 (moderate) to 3 (severe). Below is the detailed on the clinical scoring for clinical signs:

TABLE I: Clinical scoring for clinical signs

Clinical signs	Scoring	Interpretation
Ruffled fur	0	Normal fur
	2	Ruffled fur at head
	4	Ruffled fur at head and thorax
	6	Ruffled fur at head, thorax and abdomen
Ocular discharge	0	Normal eyes
	2	Discharge at upper eyelid
	4	Discharge at upper and lower eyelid
	6	Discharge at upper, lower eyelid and medial canthus
	8	Discharge at upper, lower eyelid, medial and lateral canthus
Level of alertness	0	Alert
	1-5	(Number of mice reduced in alertness)

3.5 DNA extraction

Skin tissues from affected animals were collected for DNA extraction by using Vivantis GF-1 Nucleic Acid Extraction Kits. Virus suspensions were prepared from each sample. 200µl of sample was placed in a Eppendorf tube. 20µl of Proteinase K, 2µl of Lysis Enhancer and 200µl of Buffer TB were added into the sample and mix thoroughly by pulsed-vortexing. It was then incubated at 65℃ for 10 minutes. 200µl of absolute ethanol was added and mix immediately and thoroughly by pulsed-vortexing to obtain a homogenous solution. 600µl of sample was then transferred into a column assembled in a clean collection tube and centrifuged at 5000 x g for 1 minute. After that, flow-through was discarded and column washing was done by adding 750µl of Wash Buffer and centrifuged at 5000 x g for 1 minute. Flow-through was discarded and column washing was repeated. Next, column drying was done by centrifuging the column at 10000 x g for 1 minute to remove all traces of ethanol. Lastly, the column was placed into a clean microcentrifuge tube and added with 200µl of preheated Elution Buffer and stand at room temperature for 2 minutes. DNA was eluted by centrifuging at 5000 x g for 1 minute and DNA was stored at -20℃.

3.6 Primers

The primers used to detect Orf virus in this study were B2L and F1L genes. The primer sequences are; B2L forward primer (5-ATG TGG CCG TTC TCC TCT ATC-3),

B2L reverse primer (5-TTA ATT TAT TGG CTT GCA G-3), F1L forward primer (5-ATG GAT CCA CCC GAA ATC AC-3) and F1L reverse primer (5-TCA CAC GAT GGC CGT GAC CAG-3).

3.7 Polymerase chain reaction (PCR)

PCR master mix (iNtRON kit) was carried out in 24µl total volume as following; 1µl of extracted DNA, 2.5µl of Deoxynucleotide (dNTP), 2.5µl of PCR 10x buffer solution, 1.5µl of Magnesium Chloride solution ($MgCl_2$), 0.5µl of DNA polymerase, 0.75µl of forward primer, 0.75µl of reverse primer and 15.5µl of RNase-free water. The PCR was performed on SensoQuest Thermocycler. The thermal cycler was programmed with the following cycling conditions ; 95℃ for 2 minutes which was then followed by 35 cycles; denaturation, 95℃ for 30 seconds; annealing, 60℃ for 30 seconds and final elongation was carried out at 72℃ for 30 seconds. The PCR products were stored at -20℃.

3.8 Agarose gel preparation

1.25% agarose gel was prepared by mixing 0.38g of agarose powder and 30ml of 1xTAE buffer. The mixture was heated in microwave oven for about 2-3 minutes until all the precipitate was gone. Before it was poured into gel cassette, 1µl of FloroSafe DNA Stain (1st BASE) was added into the solution. The gel was then left to solidify in the cassette for 30 minutes before it was ready for PCR loading and electrophoresis.

3.9 Electrophoresis

Agarose gel was placed in a gel holder tank and submerged with 1xTAE buffer. 5µl of 100bp dyed marker (GeneDireX) was used as ladder and 10µl of PCR product together with 2µl of loading dye was loaded into the well carefully. The electrophoresis was run for about 30 minutes at 100V. Lastly, the gel was viewed using Gel Doc and results were captured.

4.0 Histopathological examination

Skin tissues from affected animals were collected for histopathological examination. The samples were processed using Leica TP1020 tissue processor and embedded in paraffin wax using Leica EG1150 tissue embedder. They were then microtomed at 6µm thick using Microtome Jung Multicut RM2045 and lastly stained with Haematoxylin and Eosin. The slides were viewed and images were captured using Moticam Pro 285A.

4.1 Statistical Analysis

Data collected such as lesion score, clinical signs score and stratum thickness were subjected to statistical analysis using IBM SPSS Statistics 20. Significance between data was evaluated by one-way ANOVA/Kruskal-Wallis H test at level $p<0.05$.

4.0 RESULTS

4.1 Clinical signs

Some mice in all experimental groups (Group 1, Group 2, Dexamethasone Group and Non-Dexamethasone Group) showed hyperaemia at their respective inoculation sites within 24 to 72 hours post-inoculation (FIGURE III). It resolved between day three and day nine post-inoculation (p.i.). Small scabs were formed by day five p.i. in small number of mice particularly at dorsum and labial commissure. The overall duration of signs observed at the ear pinna was longer than dorsum and labial commissure. The progression of lesion for mice in Dexamethasone Group could not be assessed as acute death occurred in mice in Dexamethasone Group. One of the mice in Dexamethasone Group died on day one p.i., another two mice died on day three p.i. which was then followed by death of last two mice on day four p.i.

None of the mice exhibit clinical signs such as reduce in responsiveness, ruffled hair coat and presence of ocular discharges. However, exception was given to Dexamethasone Group where ruffled hair coat was observed and most of the mice had reduced in responsiveness (FIGURE IV). Besides, the clinical signs observed were statistically significant different from Non-Dexamethasone Group and Control Group ($p<0.05$). TABLE II shows mean scores of clinical signs observed in three of the groups.

FIGURE III: Mild hyperaemia observed 24 hours p.i. at A: Dorsum; B: Ear pinna; C: Labial commissure

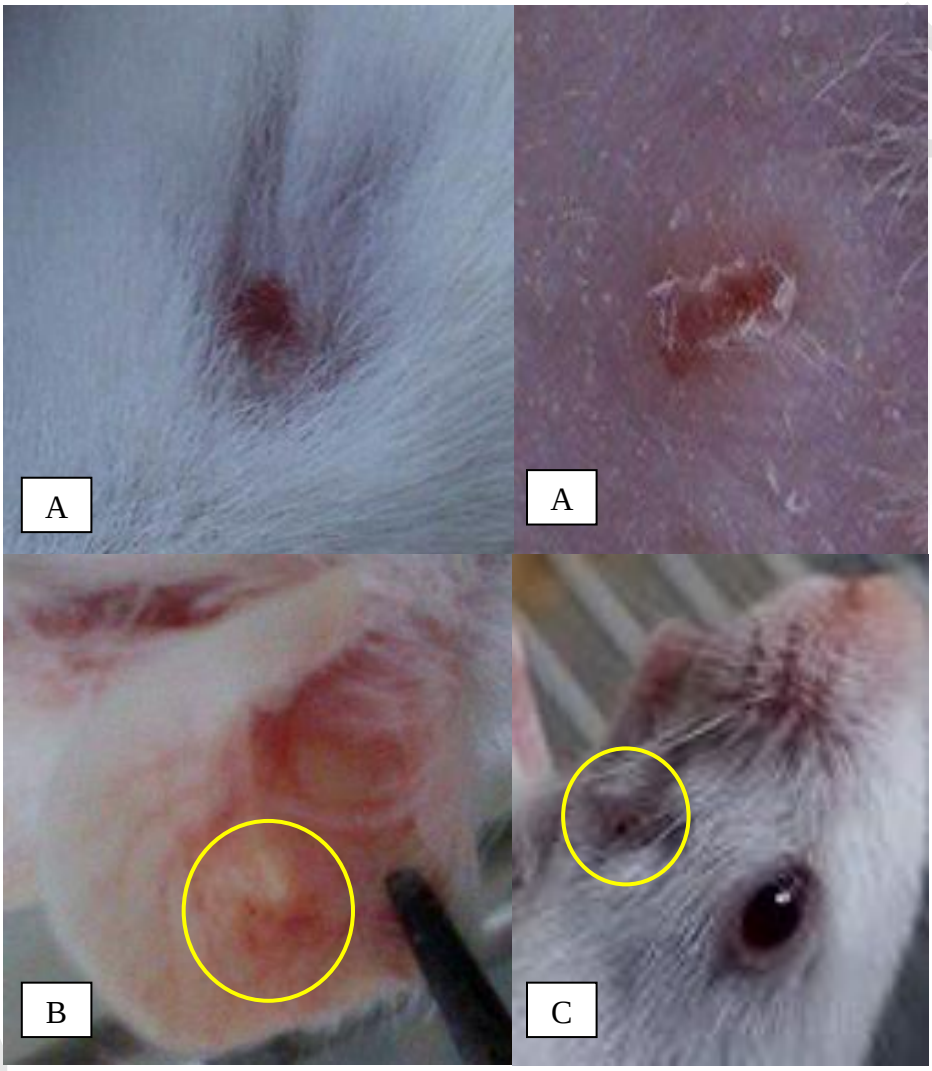


FIGURE IV: Ruffled hair coat at head shown by one of the mice in Dexamethasone Group.

TABLE II: Means scores of clinical signs observed in Dexamethasone Group, Non-Dexamethasone Group and Control Group after 6 days of p.i.

Clinical Signs	Dexamethasone Group	Non-Dexamethasone Group	Control Group
Ruffled hair coat	0.311±0.108[a]	0.000±0.000[b]	0.000±0.000[b]
Alertness	0.292±0.136[a]	0.000±0.000[b]	0.000±0.000[b]

Values are expressed as mean±SE

Mean in the same row with different superscript are significantly different at $p<0.05$

4.2 Mean lesion score

For Group 1, the mean lesion score for dorsum, ear pinna and labial commissure were 0.048, 0.067 and 0.034 respectively. On the other hand, for Group 2, the mean lesion score for dorsum, ear pinna and labial commissure were 0.052, 0.043 and 0.019 respectively. TABLE III shows mean lesion scores for Group 1, Group 2 and Control Group at each inoculation site. There were no significant difference between the groups of inoculation site for both groups ($p>0.05$). However, they were significantly different from Control group ($p<0.05$). Besides, Group 1 and Group 2 showed no significant difference in total mean score ($p>0.05$) but significantly different from Control Group (TABLE IV).

TABLE III Mean lesion score for Group 1, Group 2 and Control Group at three inoculation sites following 14 days of p.i.

Groups	Dorsum	Ear pinna	Labial commissure
Group 1 UPM 1/14	0.048±0.015[a]	0.067±0.020[a]	0.034±0.009[a]
Group 2 UPM 2/14	0.052±0.017[a]	0.043±0.015[a]	0.019±0.008[a]
Control Group	0.000±0.000[b]	0.000±0.000[b]	0.000±0.000[b]

Values are expressed as mean±SE

Mean in the same column with different superscript are significantly different at $p<0.05$

TABLE IV Total mean lesion score for Group 1, Group 2 and Control Group following 14 days of p.i.

Groups	Total mean lesion score
Group 1 UPM 1/14	0.049±0.008[a]
Group 2 UPM 2/14	0.038±0.008[a]
Control Group	0.000±0.000[b]

Values are expressed as mean ±SE

Mean in the same column with different superscript are significantly different at $p<0.05$

For Dexamethasone Group and Non-Dexamethasone Group, there was no significant difference in their total mean lesion score ($p>0.05$) but significantly different from Control Group ($p<0.05$) (TABLE V).

TABLE V: Total mean lesion score for Dexamethasone Group, Non-Dexamethasone Group and Control Group following 6 days of p.i.

Groups	Total mean lesion score
Dexamethasone Group	0.093 ± 0.031^{a}
Non-Dexamethasone Group	0.126 ± 0.013^{a}
Control Group	0.000 ± 0.000^{b}

Values are expressed as mean ±SE

Mean in the same column with different superscript are significantly different at $p<0.05$

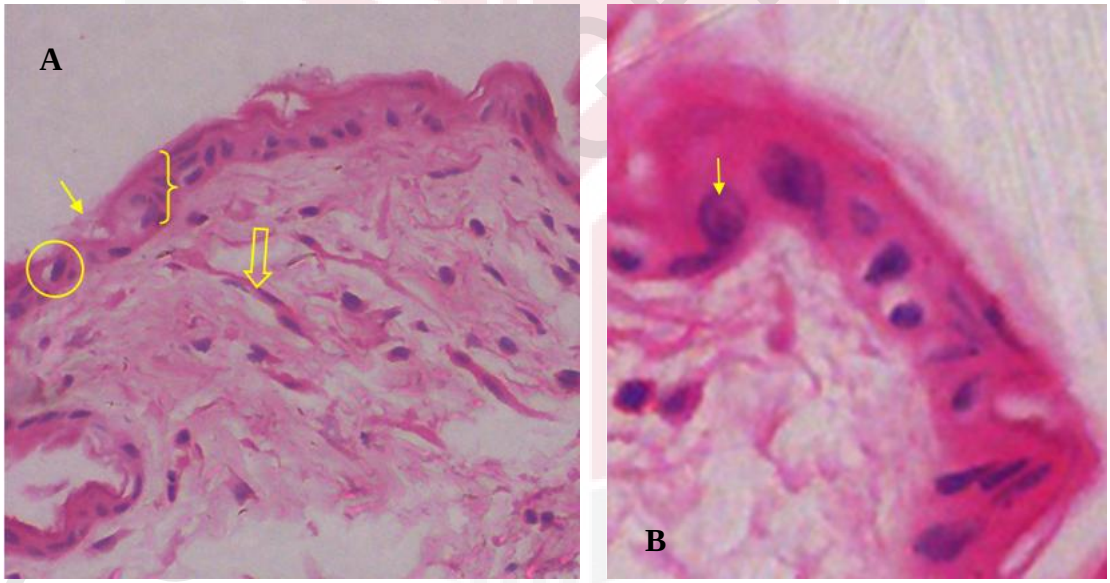
4.3 Histopathological lesion

In general, keratosis, acanthosis, ballooning degeneration were observed in all experimental groups. Congestion and vasculitis can be seen at ear pinna and labial commissure respectively. Inflammatory cells and Langerhans cells were seen in small number of samples. There was presence of intracytoplasmic eosinophilic inclusion body too. FIGURE V shows some pictures of histopathological lesion observed.

FIGURE V: Pictures of histopathological lesions observed.

A: UPM 1/14 Dorsum (H&E stain; 400x magnification) → : Keratosis } : Acanthosis ○ : Ballooning degeneration ⇨ : Langerhans cells

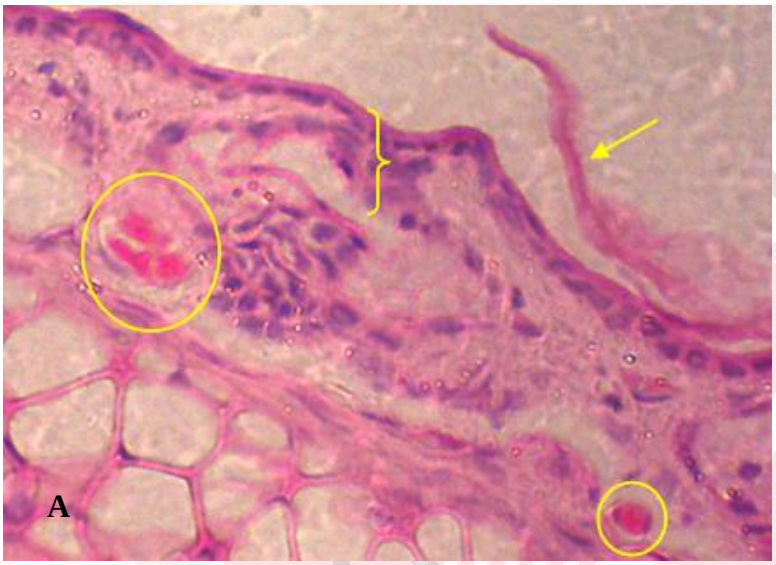
B: UPM 1/14 Dorsum (H&E stain; 400x magnification) → : Eosinophilic intracytoplasmic inclusion body

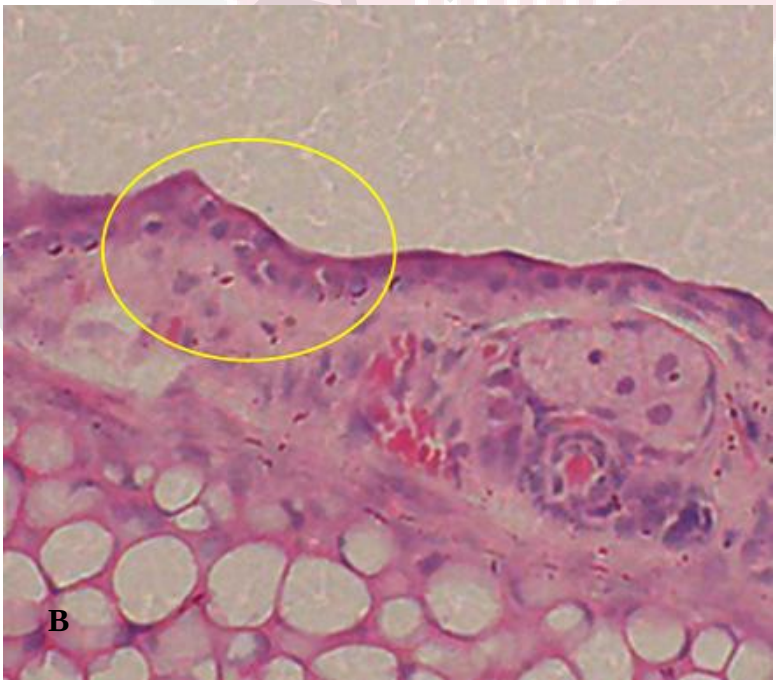


A: UPM 1/14 Ear pinna (H&E stain; 200x magnification) → : Keratosis } : Acanthosis

◯ : Congestion

B: UPM 1/14 Ear pinna (H&E stain; 200x magnification) ◯ : Ballooning degeneration

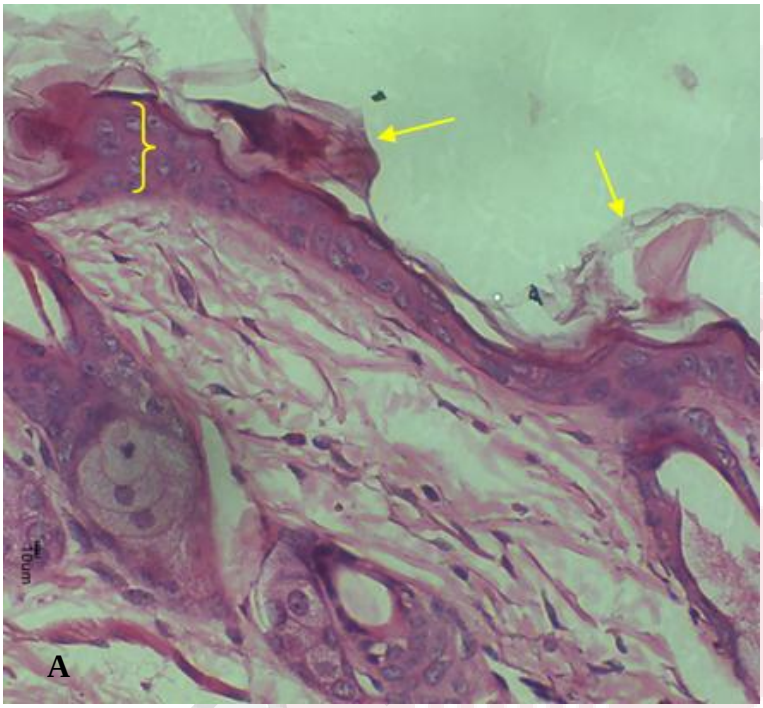


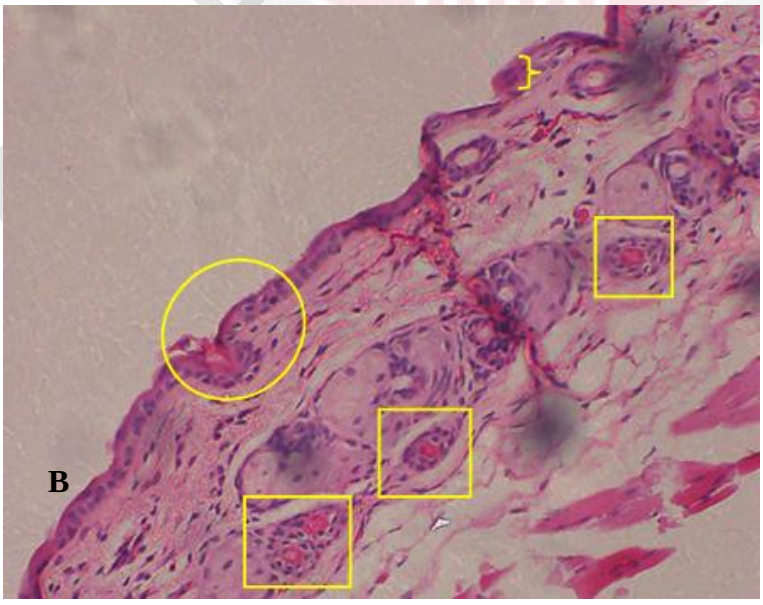


A: UPM 1/14 Labial commissure (H&E stain; 200x magnification) }: Acanthosis

○: Ballooning degeneration □: Vasculitis

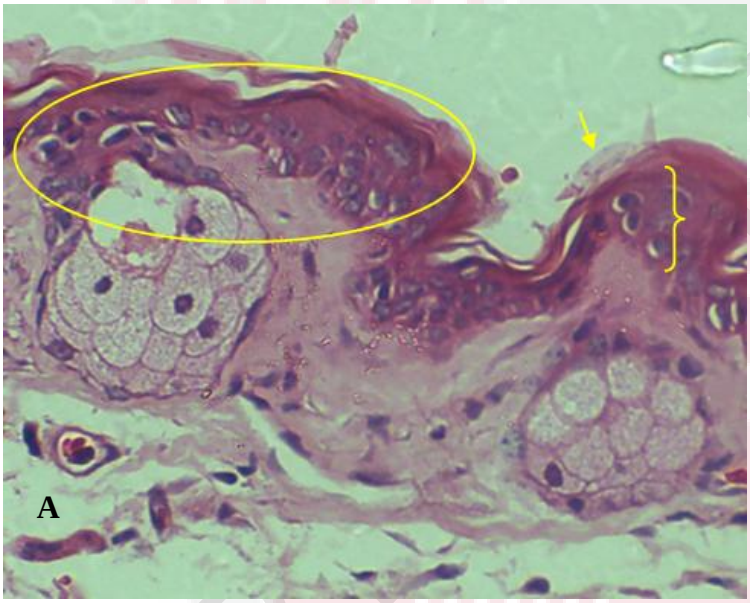
B: UPM 2/14 Dorsum (H&E stain; 400x magnification) →: Keratosis }: Acanthosis

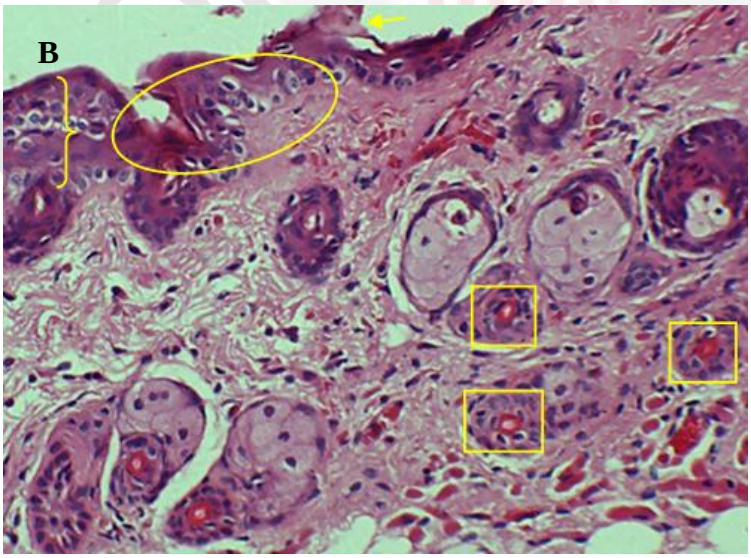




A: UPM 2/14 Ear pinna (H&E stain; 400x magnification) → : Keratosis } : Acanthosis ○ : Ballooning degeneration

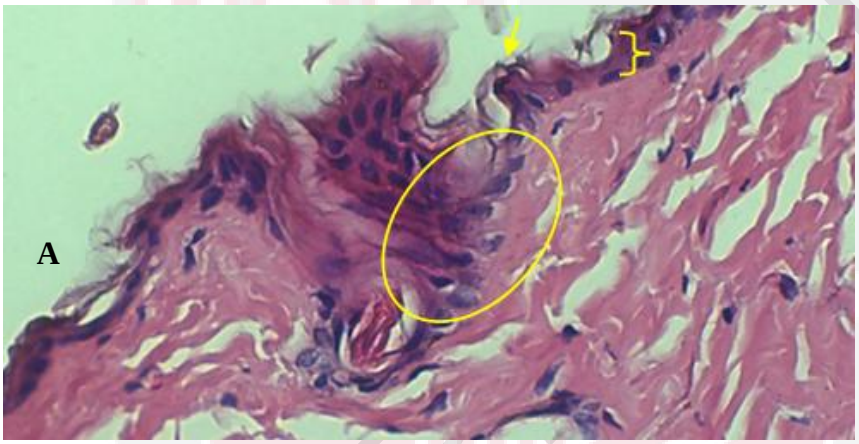
B: UPM 2/14 Labial commissure (H&E stain; 200x magnification) → : Keratosis } : Acanthosis ○ : Ballooning degeneration □ : Vasculitis

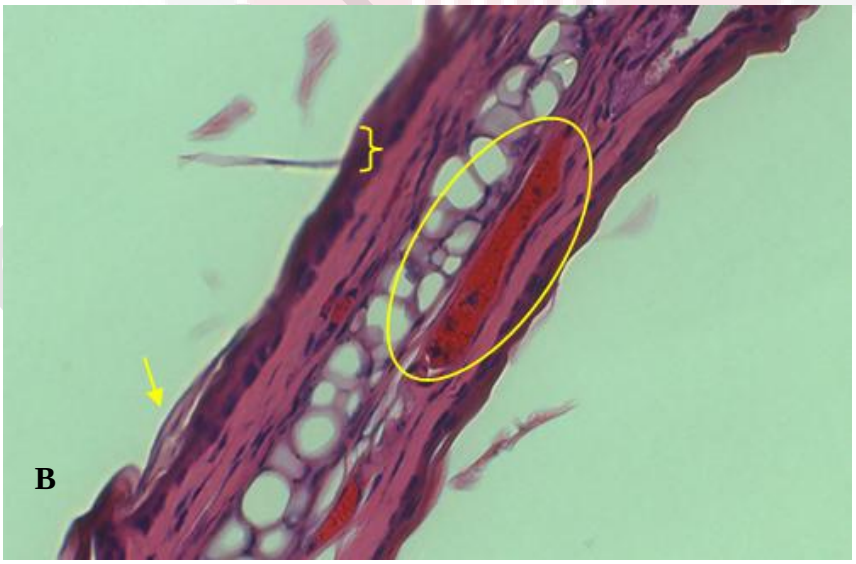




A: Dexamethasone Group Dorsum (H&E stain; 400x magnification) → : Keratosis } : Acanthosis ○ : Ballooning degeneration

B: Dexamethasone Group Ear pinna (H&E stain; 400x magnification) → : Keratosis } : Acanthosis ○ : Congestion



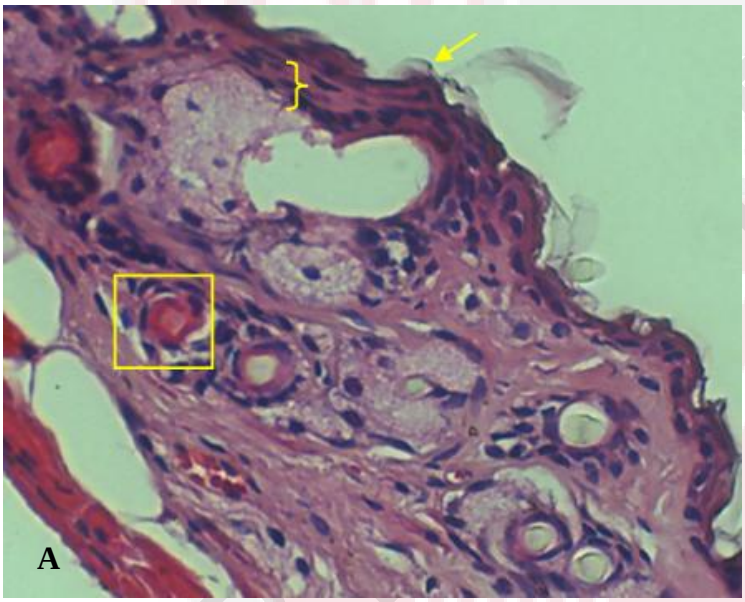


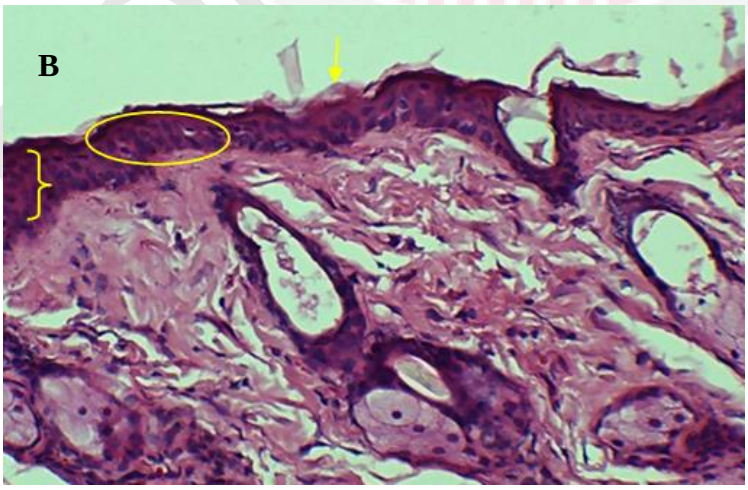
A: Dexamethasone Group Labial commissure (H&E stain; 400x magnification)

→ : Keratosis } : Acanthosis □ : Vasculitis

B: Non-Dexamethasone Group Dorsum (H&E stain; 200x magnification)

→ : Keratosis } : Acanthosis ○ : Ballooning degeneration



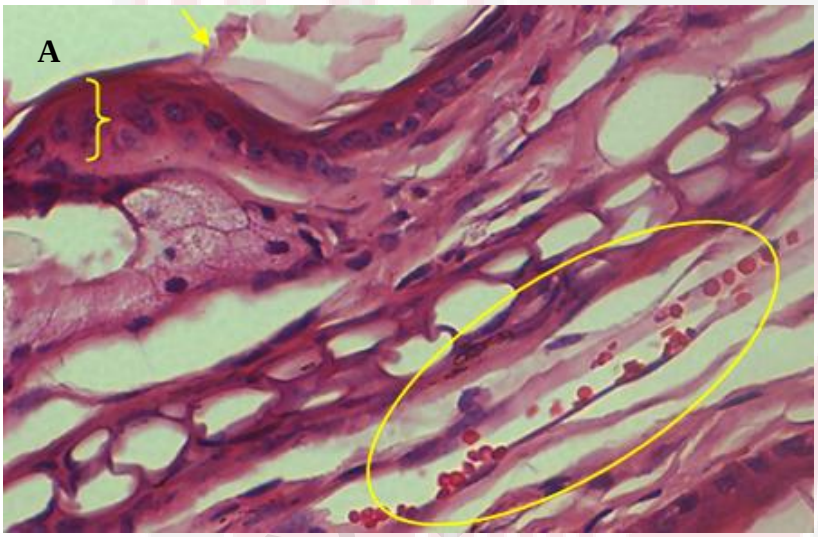


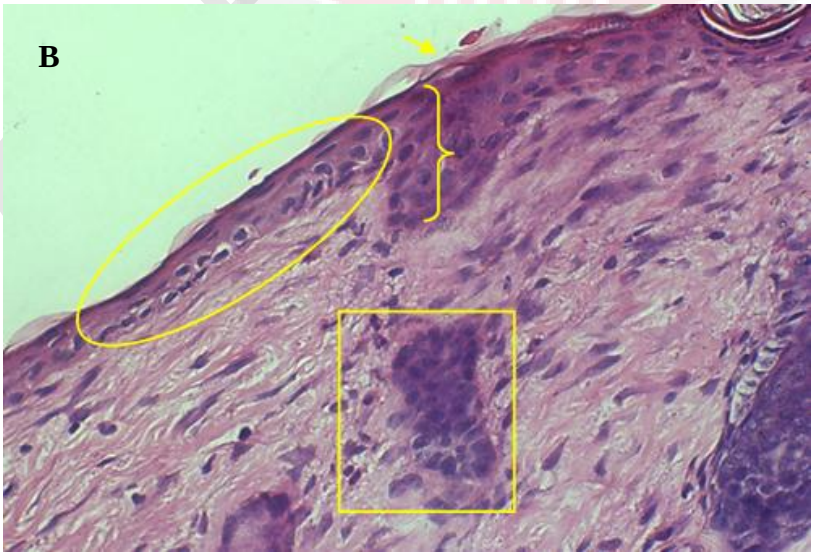
A: Non-Dexamethasone Group Ear pinna (H&E stain; 400x magnification)

→: Keratosis }: Acanthosis ◯ : Congestion

B: Non-Dexamethasone Group Labial commissure (H&E stain; 400x magnification)

→: Keratosis } : Acanthosis ◯ : Ballooning degeneration □ : Inflammation





4.4 Mean histopathological lesion score

The mean stratum thickness for Group 2B (Ear) and Group 2C (Labial commissure) were significantly different when compared to Group 1B and Group 1C ($p<0.05$). TABLE VI shows mean stratum thickness for Group 1, Group 2 and Control Group at each inoculation site.

TABLE VI: Mean stratum thickness for Group 1, Group 2 and Control Group at each inoculation site

Groups	Group 1	Group 2	Control
Dorsum	24.40 ± 0.35^{a}	27.20 ± 2.85^{a}	14.63 ± 0.09^{c}
Ear pinna	13.50 ± 0.17^{a}	25.50 ± 1.53^{b}	6.60 ± 0.31^{c}
Labial commissure	24.77 ± 1.92^{a}	34.30 ± 1.44^{b}	11.00 ± 0.83^{c}

Values are expressed as mean±SE

Mean in the same row with different superscript are significantly different at $p<0.05$

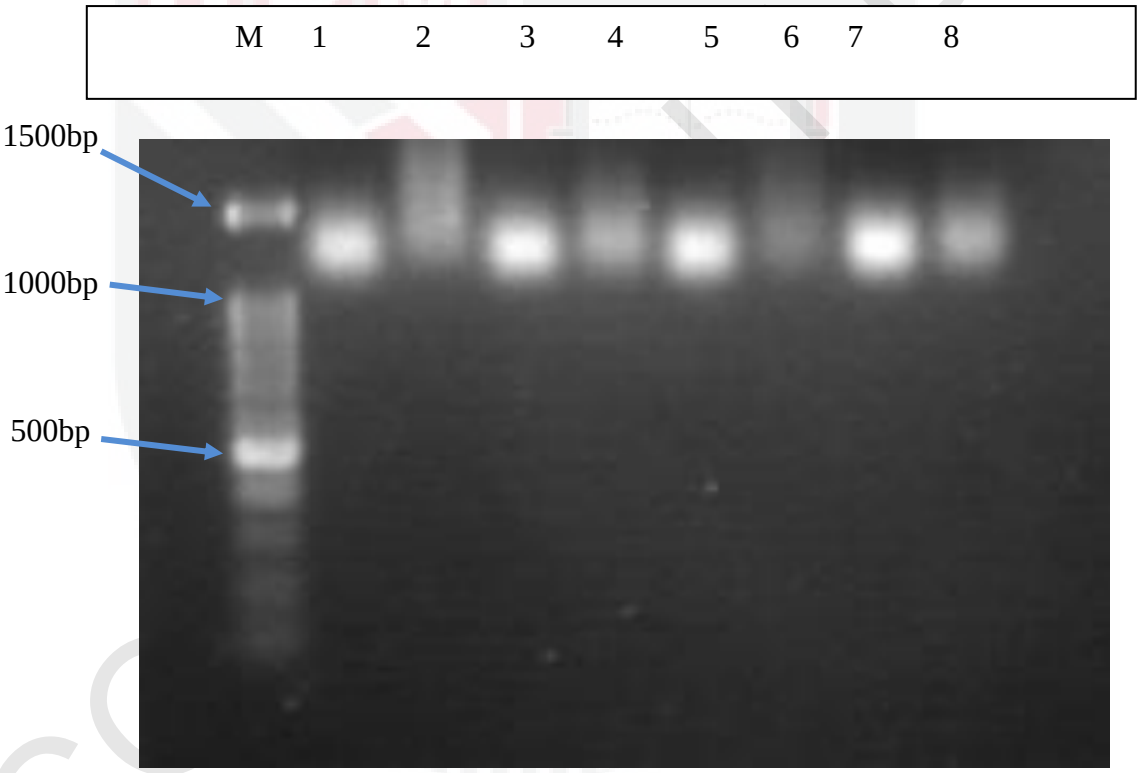
4.5 Polymerase Chain Reaction (PCR)

Skin tissues of affected animals from each experimental group were subjected to PCR and yielded positive result. An example of gel electrophoresis was shown for Non-Dexamethasone Group with ORFV positive bands of 1062bp (F1L gene) shown in well 1, 3, 5 and 7; and positive bands of 1199bp (B2L gene) shown in well 2, 4, 6 and 8 (FIGURE VI). Electrophoresis result for other groups was shown in the appendices.

FIGURE VI: Electrophoresis result for Non-Dexamethasone Group

Lane M: 100bp DNA ladder RTU (GeneDireX) DNA marker; **Lane 1, 2**: Positive control; **Lane 3, 4**: Sample from dorsum; **Lane 5, 6**: Sample from ear pinna; **Lane 7, 8**: Sample from labial commissure

(Lane 1, 3, 5, 7: F1L primer; Lane 2, 4, 6, 8: B2L primer)



5.0 DISCUSSION

Inoculation of both strains, UPM 1/14 Malaysia and UPM 2/14 Malaysia resulted in mild hyperaemia within 24 hours p.i. at respective inoculation sites which could be due to local injection site reaction. This is because in natural host, lesion often develops four to seven days after ORFV exposure (Mashayekhi *et al*., 2013). Nevertheless, ORFV was detected by means of PCR on skin tissues of mice with skin lesions. Small scab formation was seen in small number of animals at dorsum and labial commissure by day five p.i. In this study, the lesions observed in mice are not similar to natural host (goats and sheep) where the lesion progressed from erythema to macule, papules, vesicles, pustules and scab (Abbas & Mughal, 2014). However, the lesion observed was in line with Cargnelutti *et al*., (2011) where the lesion progressed from erythema, macule and finally to scab but papules and few vesicles were observed in one of the experimental mice. Besides, the lesion was not reproducible at dorsum and labial commissure which differed from this study. The rate of appearance for the lesion was also slower; the lesion only started to appear four days p.i.

Huda *et al*., (2014) observed that blisters and crust fully developed by day 4 p.i. and the skin lesion lasted for a longer period of time than the current study. These variations could be due to difference in types of virus strain inoculated and immune variation of the laboratory mice. Furthermore, low virus titre could be one of the contributing factors for production of mild skin lesions.

There was no significant difference in mean lesion scores among three of the inoculation sites. Grossly, the skin lesions produced were almost similar across the inoculation sites and there was no one particular inoculation site that can produce a more prominent and severe lesion. In terms of ease of handling, dorsum can be the primary choice of inoculation site. This is because the skin lesion can be assessed and monitored easily without much handling of animals, thereby reduce unnecessary stress to them.

In goats and sheeps, Orf lesions are often observed at labial commissure which could be due to their feeding behavior. At the time of grazing, spiny forages might abrade the tissues of labial commissure and muzzle, thus allowing virus to penetrate and replicate (Nandi *et al*., 2011). Moreover, in ewes, Orf lesions are primarily observed on the teat due to suckling by the ORFV-infected lambs.

Although Group 1 and Group 2 showed no significant difference in total mean lesion score, from the aspect of histopathological lesion score, Group 2B (Ear pinna) and Group 2C (Labial commissure) had a significant higher mean score in stratum thickness when compared to Group 1B (Ear pinna) and Group 1C (Labial commissure). Since, ORFV is an epitheliotrophic virus which replicates in newly proliferative keratinocytes population, this will lead to epidermal proliferation and result in increase in stratum thickness (Maxie, 2015). Thickness of the stratum will reflect the extent to which the virus replicates and the severity of the tissues involved. It was more reliable as mean lesion score was quite subjective. In this study, based on the mean score in

stratum thickness, ORFV strain UPM 2/14 Malaysia was thought to be more pathogenic. However, further studies need to be carried out on the virulence factors of both virus strains.

Histopathological lesions such as keratosis, acanthosis and ballooning degeneration observed in this study were consistent with a study carried out by Kinley et *al*., (2013) in goats. Acanthosis which is diffuse epidermal hyperplasia was observed in almost all of the samples as ORFV encodes Viral Vascular Endothelial Growth Factor which induces endothelial cell proliferation, vascular permeability and angiogenesis in skin, thus enhances epithelial proliferation (Wise *et al*., 2003). Intracytoplasmic eosinophilic inclusion body was found only in one of the samples. According to Barraviera (2005), eosinophilic inclusion bodies are demonstrable in the cytoplasm of the infected cells but may not be a consistent feature.

On the other hand, immunosuppression under the influence of dexamethasone did not affect the pathogenesis of Orf infection. However, theoretically, dexamethasone might works in enhancing ORFV infection. This is because dexamethasone is able to inhibit T-cell proliferation as well as cytokine production of activated CD4+ T cells (Spies *et al*., 2010) and CD4+ T cells were shown to have a critical role in ORFV clearance; a depletion of CD4+ T cells will result in increased lesion size and resolution time (Lloyd *et al*., 2000). Besides, dexamethasone had been used in some researches and results in increased in disease severity. For example, a study had been carried out to

determine effect of immunosuppression on pathogenesis of peste des petits ruminants virus infection in goats by using dexamethasone. As a result, immunosuppressed goats had more extensive, severe disease advancement and antigen distribution was more extensive and diffused than non-immunosuppressed goats (Jagtap *et al*., 2012). The use of Dexamethasone to simulate stress situation is not only confined to study of viruses, it was also used in parasitic diseases such as toxocariasis and resulted in higher larval burden (de Avila *et al*., 2012).

Since the immunity against ORFV infection is mainly cell-mediated immunity, cyclosporine which is an immunosuppressant that focuses on cell-mediated immune response can be used as an alternative drug. This is supported by a study carried out by Haig *et al*., (1996), where a sheep treated with cyclosporine and resulted in more severe ORFV lesion. Other immunosuppressive drugs such as Cyclophosphomide is less suitable as it suppresses mainly B cells; humoral immunity (Cupps et al., 1982). Furthermore, in ORFV infection, neutralizing antibodies are usually produced at small concentrations (Haig & Mercer, 1998).

ORFV was detected in skin tissues of mice showing skin lesions via PCR using two different primers which were F1L and B2L genes. In this study, it is suggested that F1L gene is more specific in detecting ORFV in mice as the results produced were sharper. The PCR results produced were poor as optimization of PCR was not carried out.

6.0 CONCLUSION

In conclusion, intradermal inoculation of both local strains is able to produce skin lesions and histopathological changes in mice. However, variation in sites of inoculation has no significant effect on pathogenicity of ORFV in mice. Lastly, in this study, dexamethasone did not give significant effects on the disease development and ORFV-associated lesion in mice.

7.0 RECOMMENDATIONS

Although intradermal inoculation of ORFV in mice is able to produce skin lesion and histopathological changes, the development of Orf is not that obvious when compared to natural host. Therefore, it is suggested to use other animal models such as rabbit and rat. Isolated virus from cell culture can be used instead of virus suspension which is made from scab. This is because virus suspension might contain other pathogens such as bacteria and fungi despite addition of antibiotics. Moreover, the virus titre from virus suspension was not known. Cyclosporin, a cell-mediated immunosuppressant which suppresses T cells, can be used to replace dexamethasone in future studies on the same aspect.

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8.0 APPENDICES

8.1 Gel Electrophoresis Results of PCR Assay

FIGURE VII: Electrophoresis result for Group 1 and Group 2

Lane M: 100bp DNA ladder RTU (GeneDireX) DNA marker; **Lane 1, 2**: Positive control; **Lane 3, 4**: Sample from dorsum (Group 1); **Lane 5, 6**: Sample from dorsum (Group 2); **Lane 7, 8**: Positive control; **Lane 9, 10**: Sample from ear pinna (Group 1); **Lane 11, 12**: Sample from ear pinna (Group 2); **Lane 13, 14**: Sample from labial commissure (Group 1); **Lane 15, 16**: Sample from labial commissure (Group 2)

(Lane 1, 3, 5, 7, 9, 11, 13, 15: F1L primer; Lane 2, 4, 6, 8, 10, 12, 14, 16: B2L primer)

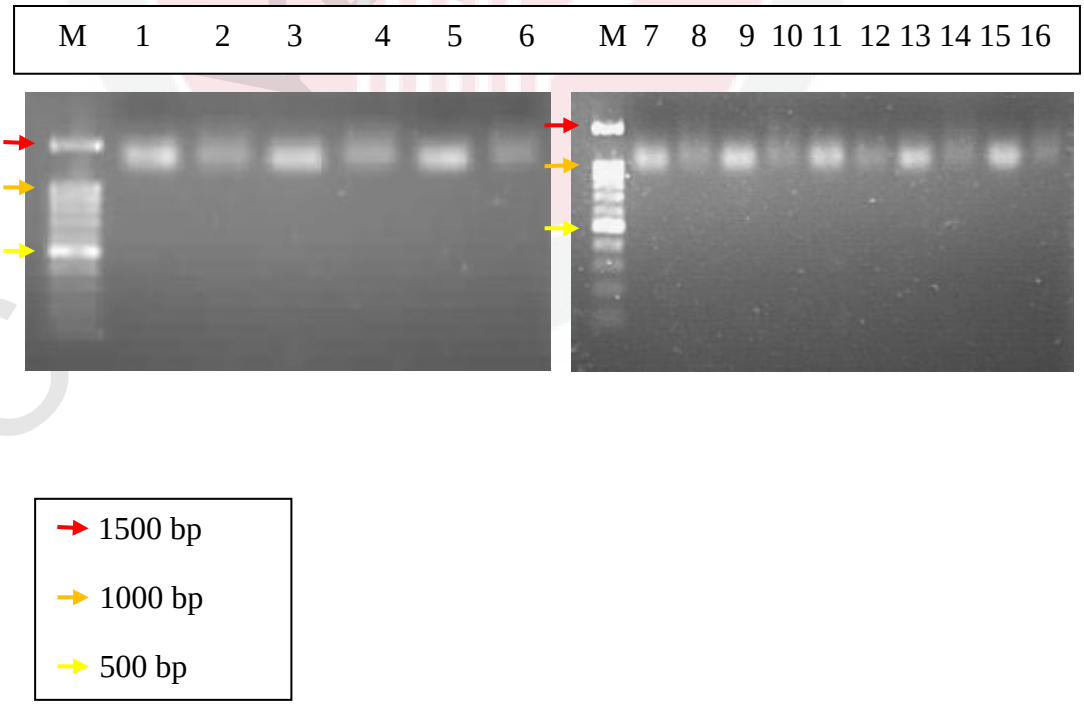

FIGURE VIII: Electrophoresis result for Dexamethasone Group

Lane M: 100bp DNA ladder RTU (GeneDireX) DNA marker; **Lane 1, 2**: Positive control; **Lane 3, 4**: Sample from dorsum; **Lane 5, 6**: Sample from ear pinna; **Lane 7, 8**: Sample from labial commissure

(Lane 1, 3, 5, 7: F1L primer; Lane 2, 4, 6, 8: B2L primer)