UNIVERSITI PUTRA MALAYSIA

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

CHOOK CHIAN LIN

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A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan.

MARCH 2016

It is hereby certified that we have read this project paper entitled "Study on Pathogenicity of Orf Virus Strain Upm 1/14 Malaysia and Upm 2/14 Malaysia in Rat via Different Inoculation Sites with and without Dexamethasone Treatment", by Chook Chian Lin 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- Final Year Project.

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DEDICATION

I would like to dedicate my humble effort to my beloved family for their support and love. A special feeling of gratitude I send to both of parents who inspire me and siblings who provide joy in my life.

I also like to dedicate this dissertation to all my friends who are always beside me whenever I need them. They are always great cheerleaders. Besides, I also dedicate this work and give special thanks to my supervisor and co-supervisor for their guidance and knowledge.

ACKNOWLEDGEMENT

Firstly, I would like to thank to my family and friends who have supported me throughout this final year project. Their invaluable contributions are highly appreciated.

Besides, I wish to extend my appreciation to my supervisor, Prof. Dato' Dr. Mohd. Azmi Mohd Lila and my co-supervisor, Dr. Faez Firdaus Jesse Abdullah who were generous for their expertise and precious time. I would also like to thank them for their guidance. I wish to extend my deepest appreciation to Prof Noordin too for his guidance on histopathology. Moreover, special thanks to Mr. Jefri and Dr Aimi for their kind assistance throughout the project.

I would also like to acknowledge to all staffs of virology lab, especially Mr. Kamaruddin and Mr. Rosdam for allowing me to conduct my research successfully for their guidance, research and equipment needed. Apart from staffs in virology lab, I wish to thank to Mrs. Latifah of the histopathology lab.

I wish to express my special thanks to my classmates for their assistance and sharing of their invaluable knowledge. Lastly, I would like to acknowledge Mayday Ah Shin for all his motivation and inspiration throughout my life.

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KAJIAN KEPATOGENAN TERIKAN ORF VIRUS UPM 1/14 MALAYSIA DAN UPM 2/14 MALAYSIA KEPADA TIKUS MELALUI PERBEZAAN LOKASI INOKULASI DENGAN DAN TANPA RAWATAN DEXAMETHASONE

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2016

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Orf virus (ORFV) menyebabkan penyakit ektima menular yang mengakibatkan kerugian dalam sektor ekonomi. Kajian ORFV amat penting tetapi kajian menggunakan tikus kurang diterokai. Melalui kajian ini, kepatogenan ORFV kepada tikus dinilai berdasarkan kesan daripada dua terikan virus tempatan iaitu ORFV UPM 1/14 Malaysia dan ORFV UPM 2/14 Malaysia, lokasi inokulasi, serta penindasan immunisasi. Inokulasi secara intradermal dengan 0.5 ml 1% ORFV UPM 1/14 Malaysia (Kumpulan 1) dan ORFV UPM 2/14 Malaysia (Kumpulan 2) telah dilaksanakan dalam kumpulan 1 dan kumpulan 2 yang terdiri daripada 5 tikus

masing-masing pada kulit dorsum (Kumpulan 1A; Kumpulan 2A), daun telinga (Kumpulan 1B;Kumpulan 2B) serta sudut bibir (Kumpulan 1C; Kumpulan 2C). Selain itu, inokulasi secara intradermal dengan 0.5ml 1% ORFV UPM 1/14 Malaysia telah dilaksanakan dalam kumpulan dexamethasone (n=5) dan kumpulan non-dexamethasone (n=5). Tanda-tanda klinikal dan perubahan histopatologi telah dinilai selama 14 hari bagi kumpulan 1 dan kumpulan 2 manakala 7 hari bagi kumpulan dexamethasone dan kumpulan non-dexamethasone. Hyperemia sederhana telah didapati pada kulit dorsum, daun telinga dan sudut bibir daripada 27 tikus dalam kumpulan rawatan. Kumpulan 1 mempunyai rata skor kelukaan kulit yang lebih signifikan tinggi ($p<0.05$) daripada Kumpulan 2. Kumpulan 1A mempunyai rata skor kelukaan kulit yang lebih signifkan tinggi ($p<0.05$) daripada Kumpulan 1B dan Kumpulan 1C. Kumpulan dexamethasone juga mempunyai rata skor kelukaan kulit yang lebih signifkan tinggi ($p<0.05$) daripada kumpulan non-dexamethasone. Keratosis, acanthosis dan degenerasi jenis belon telah diperolehi daripada tikus yang menunjukkan kelukaan kulit dalam kumpulan rawatan. Kumpulan dexamethasone mempunyai rata ketebalan lapisan sel spinosum dan lapisan sel basal sudut bibir yang lebih signifikan tinggi ($p<0.05$) berbanding dengan kumpulan non-dexamethasone. Rata ketebalan lapisan sel spinosum dan lapisan sel basal kulit dorsum, daun telinga dan sudut bibir tidak mempunyai perbezaaan signifikan antara kumpulan 1 dan kumpulan 2. ORFV telah dikesan daripada kulit tikus yang mempunyai kelukaan kulit dalam kumpulan rawatan dengan teknik reaksi rantai polimerase (PCR). Kesimpulannya, kepatogenan ORFV mampu dihasilkan dalam tikus dan ia berbeza

disebabkan oleh terikan virus, lokasi inokulasi dan rawatan dexamethasone. Tikus boleh digunakan sebagai model pengajian ORFV.

Kata kunci: Orf virus, ORFV UPM 1/14 Malaysia, ORFV UPM 2/14 Malaysia, tikus, kepatogenan, lokasi inokulasi, penindasan immunisasi

ABSTRACT

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

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

By

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2016

In this study, pathogenicity of ORFV in rat was evaluated by using of two virus strains, ORFV UPM 1/14 Malaysia and ORFV UPM 2/14 Malaysia with variation in inoculation sites and the effects of induced-immunosuppression. Intradermal inoculation of 0.5 ml 1% ORFV UPM 1/14 Malaysia (Group 1) and ORFV UPM 2/14 Malaysia virus suspension (Group 2) were performed in each group of 5 rats 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) respectively. Intradermal inoculation of 0.5 ml 1% ORFV UPM 1/14 Malaysia virus suspension was performed in dexamethasone-induced immunosuppressed group (n=5) and non-dexamethasone group (n=5). Clinical signs and histopathological changes were

evaluated for 14 days post virus inoculation for rats in Group 1 and Group 2 and 7 days for dexamethasone-induced immunosuppressed group and non-dexamethasone group. Mild hyperemia was observed in dorsum, ear pinna and labial commissure of 27 rats in the treatment group. Rats of Group 1 had significantly higher ($p<0.05$) mean skin lesion scores than Group 2. Rats of Group 1A had significantly higher ($p<0.05$) mean skin lesion scores than Group 1B and Group 1C. Dexamethasone-treated group had significantly higher ($p<0.05$) mean skin lesion scores than non-dexamethasone group. Keratosis, acanthosis and ballooning degeneration were observed in rats showed skin lesions in the treatment group. Dexamethasone-treated group had significantly higher ($p<0.05$) mean thickness of stratum spinosum and stratum basale of labial commissure than non-dexamethasone group. There was no significant difference ($p>0.05$) of mean thickness of stratum spinosum and stratum basale of dorsum, ear pinna and labial commissure between Group 1 and Group 2. ORFV was detected by means of PCR on skin tissues of rats with skin lesions in rats. In conclusion, ORFV is pathogenic in rats, and it varies due to strains, inoculation sites and dexamethasone treatment. Disease and lesions produced in rats are similar to that of the normal hosts. Thus, rat is a suitable laboratory animal model to study OFRV infection.

Keywords: Orf virus, ORFV UPM 1/14 Malaysia, ORFV UPM 2/14 Malaysia, rat, pathogenicity, inoculation sites, immunosuppression

1.0 INTRODUCTION

Contagious ecthyma is caused by Orf virus (ORFV) infection. ORFV is species of the genus *Parapoxvirus* which belong to the family Poxviridae and subfamily Chordopoxvirinae. There are several alternative names of contagious ecthyma, which are orf, soremouth, scabby mouth and contagious pustular dermatitis (Smith & Sherman, 2009). The viral infection cause erythematous spots at the beginning, and then formation of papules, vesicles, pustules and scabs which finally become dry and shed. (Spyrou & Valaikos, 2015) Although it is self-limiting, it is an important disease due to its contagious characteristic, zoonotic potential, world-wide distribution and economic importance.

Regarding on its contagious characteristic and zoonotic potential, the virus is transmitted through direct contact via damaged skin, and then replication occurs in epidermal cells. Transmission usually occurs during grazing and through abrasions developed on lips, nostrils and mouth (Spyrou & Valiakos, 2015). Although the disease affects primarily sheep and goat, it has been reported in other animals too, such as camels and camelids, chamois, serows, tahr, steenboks, deer, reindeer, bighorn sheep, dall sheep, musk oxen, mountain goats, dogs, cats and squirrels (Spyrou & Valiakos, 2015). Besides, it is zoonotic. It causes occupational hazard to people working with the animals. According to Spyrou & Valiakos (2015), it is affecting people who are in direct or indirect contact with infected livestock such as farmers, veterinarians, animal caretakers.

As mentioned in the earlier paragraph, ORFV is a pathogen with world-wide distribution which affects livestock economics. According to Essbauer *et. al.* (2010), its world-wide distribution is described with incidence up to 90%. Economic impact of ORFV infection is undoubtedly significant. Haig and Mercer (1998) stated that in severely affected and young animals, the disease reduces food intake, leading to transient growth impairment, and consequently resulting in economic losses. In addition, morbidity is high which is up to 70 % in flocks which the disease is occuring for the first time (Spyrou & Valiakos, 2015). Although mortality is low which is less than 1 % (Spyrou & Valiakos, 2015), but complications such as myiasis (Housawi & Abu Elzein, 2000), co-infections with papilloma virus and sheep pox virus (Spyrou and Valiakos, 2015) and secondary bacterial infections (Zhao et al., 2010) increase the severity, leading to more treatment costs and labour costs.

ORFV infection undoubtedly impairs the development of small ruminant industry in Malaysia with its contagious ability, zoonotic potential and significant economic impact. According to AADGN country report 2013/14, there are only 8195 heads of dairy goats in Peninsular Malaysia, and the ex-farm price of goat milk is RM 20/liter as compared to cow milk of RM 2.20/ liter. Expansion and development of small ruminant industry is impaired by orf disease which is one of the common diseases in small ruminant. Therefore, study of ORFV is extremely important.

Malaysia strains of ORFV should be studied as they are the specific etiological agents involved in outbreak of contagious ecthyma which affects the small ruminant industry in Malaysia. ORFV UPM 1/14 MALAYSIA and ORFV UPM 2/14 MALAYSIA are suggested in this study. These two ORFV strains have been isolated in the study entitled Isolation and phylogenetic analysis of caprine orf virus in Malaysia done by Ashwaq *et al.* (2015) which claimed to be the first study that sequenced partial genome data of ORFV isolated in Malaysia with B2L genes and F1L genes. Their relationships with the existing strains in the database were determined, and the findings were close homology to the Chinese and Indian strains in term of DNA sequence.

ORFV can be studied with laboratory animal model which provide benefits. Studies of biology of ORFV is impaired by the difficulty to find seronegative normal hosts, which are goat and sheep (Cargnelutti, *et al.,* 2010). Experiment in goat or sheep is more expensive. There are also difficulties in obtaining a non-endemic farm. Moreover, studies on suitable animal models would also benefit vaccine and antiviral drug development and testing (Dal Pozzo *et al*., 2007).

ORFV is underexplored in rat model. This study focuses on the study of pathogenicity of ORFV in rat model to determine more suitable animal models. More variations in suitable animal model undoubtedly will help in more future ORFV study. Virus strain and inoculation sites that are able to provide significant effect in rat are

unexplored. Therefore, by determining the strains and sites of inoculation that produce positive result in rat, more and more studies can be done.

The objectives of this study are:-

1. to evaluate suitability of rat as animal model for ORFV infection.
2. to determine the effect of inoculation sites on disease development
3. to determine the effect of dexamethasone treatment simulating a stress and non-stress situations on the severity of orf disease.

The hypotheses of this study are:-

1. Different inoculation sites resulted in different disease severity.
2. Dexamethasone treatment resulted in severe Orf disease.
3. Rat is a good experimental animal model to study ORFV.

2.0 LITERATURE REVIEW

2.1 Orf virus (ORFV)

2.1.1 Classification

ORFV is a prototype species of the genus Parapoxvirus which belongs to the family Poxviridae and sub-family Chordopoxvirinae. Parapoxvirus virions are characterized by regular surface structures, which distinguish it from other genus. The distinguishing features of ORFV from the other viruses in the genus Parapoxvirus include their ovoid shape which is 220-300 x 140-170 nm in size with a surface filament appearing as regular cross-hatched spiral coil involving a continuous thread (International Committee on Taxonomy of Viruses, 2011). This unique spiral, criss-cross tubule like pattern is the most characteristic feature of their coat (Spyrou & Valiakos, 2015). Besides, the G+C content of ORFV is about 64% as compared to the low G+C content (30%-40%) of other viruses in the subfamily (International Committee on Taxonomy of Viruses, 2011).

2.1.2 Structure

ORFV consists of linear double stranded DNA. The DNA is 130-150 kbp in size (International Committee on Taxonomy of Viruses, 2011). It has homologues of almost all of the vaccinia virus (VACV) structural genes (Tan *et al*., 2008). Besides, according to Tan *et al.* (2008), ORFV has both mature virion and wrapped virion formed, and extracellular virion as VACV. The mature virion is formed in which the outer membrane is derived from the endoplasmic reticulum whereas wrapped virion is

produced when two additional membranes are derived from the trans-golgi network wrapping the mature virion. Extracelluar virion is then produced from wrapped virion when the outermost membrane is lost during virus egress.

2.1.3 Genome

ORFV has 132 genes, and the genome is about 138 kbp long in length. The genetic organization consists of a central region with essential genes needed in position, orientation and spacing. On the other hand, the terminal parts are related to factors influencing virulence, pathogenesis or host range (Buttner, M. & Rziha, H.J. , 2002). Besides, according to Spyrou and Valiakos (2015), inverted terminal repeats (3 kbps in length) which are identical but oppositely oriented are found at the two ends of the ORFV genome. The double strands of DNA are covalently ended with hairpin loops (100 bp in size). Moreover, immune-modulatory genes are encoded to interfere with host immune and inflammatory effector mechanism, such as orf virus interferon resistant gene, viral IL-10 gene and GM-CSF inhibitory factor gene (Haig, D.M., 2002).

2.2 ORFV infection (Contagious ecthyma)

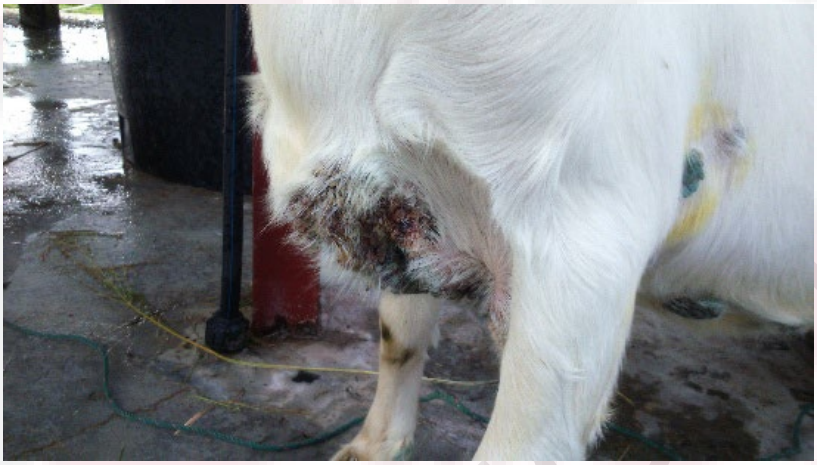
ORFV is the causative agent of contagious ecthyma with alternative names of orf, soremouth, scabby mouth and contagious pustular dermatitis, which causes prominent skin lesions.

2.2.1 Clinical presentations in sheep or goat

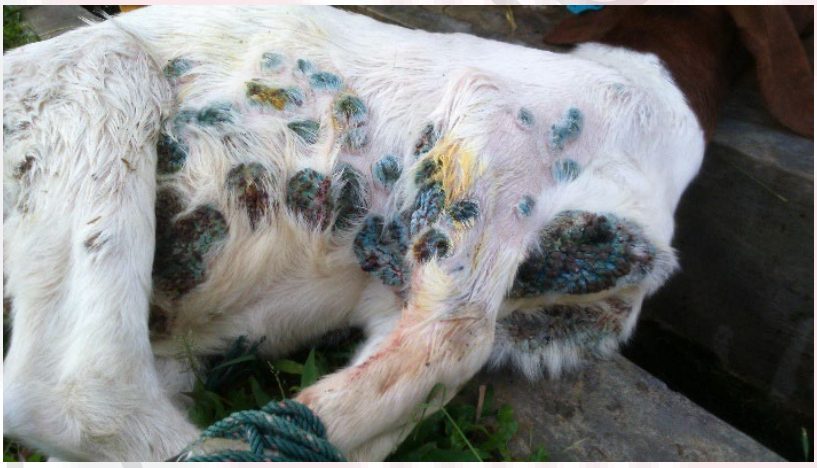
The classical orf lesions are characterized by a progressive course of erythema, macules, vesicles, pustules and proliferative scabs. The incubation period is three to eight days (Smith & Sherman, 2009). The location of lesions varies. According to Smith and Sherman (2009), proliferative lesions are typically formed on the lips, but also face, ears, coronary band, scrotum, teats or vulva (FIGURE I). Moreover, lesions vary among young and adult in terms of severity and location of lesions. Lesions are more severe in young animals less than two months old (Syprou & Valiakos, 2015). Lambs and kids are suffered due to difficulty in suckling and grazing (Chan *et al*., 2007) whereas most adults with lesions on the lips continue to eat and milk well (Smith & Sherman, 2009). In addition, lesions can also involve esophagus, abomasum, intestines or respiratory tract in lambs and kids (Chan *et al*., 2007). On the other hand, lesions on teats and genital organs can be observed in adults, and bacterial mastitis may developed (Mavrogianni *et al*., 2006).

FIGURE I: PROLIFERATIVE LESIONS ON GOATS FROM LADANG ANGKAT, UNIVERSITY PUTRA MALAYSIA

A: lesions observed at the cranioventral of body **B:** lesions observed at right lateral of body



A



B

2.2.2 Diagnosis

Diagnosis of ORFV is based on the clinical signs and history. The definitive diagnosis is confirmed based on virus isolation and molecular detection. Clinical signs and lesions are characteristic of the disease, and they distinguish the disease from others that cause pustular lesions too (Spyrou & Valiakos, 2015). Viral isolation can be done using cell cultures. Cell cultures that can be used are primary chicken fibroblast (Huda & Hussien, 2014), lamb testis, Madin-Darby canine kidney cell line (Ashwaq *et al*., 2015) and Madin-Darby bovine kidney (Klein, J. & Tryland, M., 2005). On the other side, methods of molecular detection that can be carried out to detect ORFV are PCR and semi-nested PCR with primers such as B2L, VIR and F1L gene (Ashwaq *et al*., 2015; Klein, J. & Tryland, M., 2005 ; Bande *et. al.*, 2014). Besides, loop mediated isothermal amplication assay (Wang *et al.,* 2013) and phylogenetic analysis (Ashwaq *et al*., 2015; Klein, J. & Tryland, M., 2005) are important molecular detection methods too. Besides, histopathology using skin biopsies can be used in diagnosing ORFV. Histopathology helps to distinguish ORFV from peste des petis ruminants (Smith & Sherman, 2009).

2.2.3 Immunity

The host immune response to ORFV infection involves cell-mediated and humoral immunity. According to Haig, D.M. & McInnes, C.J. (2002), immune reaction occur after infection involve neutrophils, dermal dendritic cells, T cells, B cells

and antibody. CD4+ T cells, IFN-γ and CD8+ T cells are also involved in partial protection against ORFV infection (Haig, D.M. & McInnes, C.J., 2002). Besides, according to Haig, D.M. (1997), anti-viral response occur, and it involves local inflammatory response in skin and cytokine response in lymph. Repeated infection of ORFV may result in reduced lesion size and time of resolution. This is due to the interference of immune response caused by immune-modulatory protein encoded. (Haig, D.M. & McInnes, C.J., 2002; Haig *et al*., 2002). In addition, regarding on antibodies, neutralizing antibodies have been successfully detected with enzyme linked immunosorbent assay (ELISA), immunofluorescent antibody test (IFAT) and viral neutralization test (VNT) in several orf virus studies, confirming the existence of involvement of neutralizing antibodies in ORFV infection. (Housawi *et al.*, 2008; Zeedan *et al.*, 2015).

2.3 Reviews on experimental infection of ORFV

In the past 60 years, a lot of experimental studies of ORFV on various different animal models have been attempted. Interestingly, positive and negative findings have been generated. Asakawa *et. al.* (1952) produced exanthema which then with the lapse of time formed papules, vesicle and pustule in sheep and goats, but failed to produce significant results in rabbits, mice, guinea pigs and hens. Wheeler *et al.* (1956) successfully produced ORFV lesions in experimental infected sheep and slightly more erythematous lesion than control at the scarified site of skin in rabbits. However, there was no evidence of lesions after inoculation at corneal of rabbits, skin and cornea of

guinea pigs and skin, peritoneum and brain of mice. Besides, Greig, A.S. (1958) reported outstanding susceptibility of lamb and kids to infection of ORFV and very slight reaction which was small reddened areas around sites of inoculation in rabbits. However, mice and guinea pigs did not respond to infection.

Recently, ORFV experimental infections in many countries using particular strains in their countries and various kinds of animals were continued. Positive results were successfully generated. Martins *et al.* (2014) produced classical ORFV lesions and studied pathogenesis of ORFV infection in lambs after inoculation in the labial commissure using three Brazilian ORFV isolates. In addition, Carneglutti *et al.* (2010) described clinical course characterized by erythema, macules, papules or pustules which eventually formed scabs in all intradermal inoculated rabbits and severe lesions in inoculated lambs which were the controls; however, milder clinical course similar as in rabbit was observed in intradermal inoculated mice. Huda and Hussien (2014) successfully reported local signs of erythema, macules, papules, pustules or vesicles and scabs which started around day 4 post inoculation in mice using ORFV isolated from Iraqi sheep. Moreover, Tryland *et al.* (2012) also produced typical ORFV lesions in semi-domesticated reindeer inoculated in scarified oral mucosa.

3.0 MATERIALS AND METHODS

3.1 Experiment design

Group of rats (n=5) were served as control whereas treatment group of rats (n=30) were inoculated intradermal at different sites with two different ORFV Malaysia strains. Treatment group was further divided into Group 1 (n=15) and Group 2 (n=15). Group 1A (n=5), Group 1B (n=5)and Group 1C (n=5) were inoculated with ORFV UPM 1/14 MALAYSIA virus suspension at dorsal skin, ear pinna and labial commissure respectively. Group 2A (n=5), Group 2B (n=5)and Group 2C (n=5) were inoculated with ORFV/ UPM 2/14 MALAYSIA suspension dorsal skin, ear pinna and labial commissure respectively. For study on immunosuppressive effect, experiment was designed with involvement of control group (n=5), non-dexamethasone group (n=5) and dexamethasone group (n=5) The non-dexamethasone group and dexamethasone group were inoculated with ORFV UPM 1/14 MALAYSIA virus suspension at dorsal skin, ear pinna and labial commissure. Infection was monitored by daily clinical examination with clinical scoring system, viral detection via Polymerase Chain Reaction (PCR) method and histological examination.

3.2 Viral suspension preparation

ORFV UPM 1/14 and ORFV UPM 2/14 virus suspension were prepared from scabs samples collected from infected goats in Ladang Angkat, University Putra Malaysia in the year 2014. The virus was isolated and detected by PCR using B2L gene and F1L gene as target templates (Ashwaq *et. al.*, 2015). Firstly, 10% viral suspension

was prepared by grinding 1 gram of scabs with 1 gram of sterile sand, mixing with1 ml of phosphate buffer saline (PBS) and then titrating to 10% by adding 9ml of PBS. After centrifuged at 1000 rpm for 10 mins, supernatant with added penicillin and streptomycin was further diluted to 1 % viral suspension by adding 9ml of PBS to 1 ml of supernatant and stored at -20 ^{o}C.

3.3 Animals

Forty five 7 to 8 weeks old Sprague-Dawley rats were used in this study. Animals were acclimatized for 3 days prior to experiment.

3.4 Immunosuppression

The dexamethasone-treated group were injected with dexamethasone 5mg/kg twice daily 2 days before virus inoculation and continued for 5 days post virus inoculation to induce immunosuppression. Dexakel 05 with concentration of 5mg/ml was used.

3.5 Viral inoculation

The control group was inoculated with 0.5 ml of PBS via intradermal route at three sites, which are dorsal skin, ear pinna and labial commissure. Group 1A, Group 1B and Group 1C were inoculated via intradermal route with 0.5 ml of 1 % ORFV UPM 1/14 virus suspension at dorsal skin, ear pinna and labial commissure respectively. On the other side, Group 2A, Group 2B and Group 2C were inoculated via intradermal

route with 0.5 ml of 1 % ORFV UPM 2/14 virus suspension at dorsal skin, ear pinna and labial commissure respectively.

The non-dexamethasone group and dexamethasone-treated group were inoculated with 0.5ml 1% ORFV UPM 1/14 virus suspension at dorsal skin, ear pinna and labial commissure. The control was inoculated with 0.5 ml PBS at dorsal skin, ear pinna and labial commissure.

Hair at the injection sites were clipped before inoculation. All the intradermal inoculation were performed using 27 G hypodermic needle and 1 ml syringe by positioning needle parallel to the skin and inserting needle just until the bevel of needle is within the dermal layer. Small bleb at injection site was indicated as injected intradermal correctly.

Each group of rats was then kept in separate cages with food and water which was changed twice daily. Bedding was changed once every 3 days. The experiment was approved by Institutional Animal Care and Use Committee (IACUC).

3.6 Clinical monitoring

Following inoculation, the control group, Group 1 and Group 2 were monitored daily for 2 weeks whereas the non-dexamethasone group and dexamethasone induced immunosuppressed group were monitored daily for a week. General clinical

examination and lesions examination were performed daily. Clinical scoring was recorded daily, and meaningful event was recorded in the form of digital picture. Five clinical signs were evaluated and scored which were level of alertness, ruffled hair coat, ocular discharge and skin lesions (TABLE I).

TABLE I: CRITERIA OF EVALUATION OF CLINICAL SIGNS AND THEIR RELEVANT SCORES

Clinical signs	**Score and Interpretation**
Ruffled hair coat	0 : Normal fur Scale 1-6: number of ruffled hair determined at left and right side of cranial, middle and caudal part of body.
Ocular discharges	0 : Normal eyes Scale 1-8: number of eye discharges present at lateral and medial canthus of left and right eyes.
Level of alertness	0: Alert Score scale 1-5: number of rats reduced in alertness examined upon poke
Skin lesions	4 clinical indicators: hyperemia; vesicles and/or pustules; scabs; exudation and/or bleeding 0: absence 1:mild or smaller number 2: moderate or medium number 3: severe or high number

3.7 Histological examination

Rats were euthanized, and skin specimens of rats with skin lesions from each group were collected for histological examination. Skin specimens were fixed in 10% formalin, processed, embedded in paraffin, sectioned with 5-10 μm, stained using Harris' hematoxylin and eosin (H&E) staining and examined microscopically.

Mean thickness of stratum spinosum and stratum basale of treatment group and control were evaluated by measuring the thickness of stratum layer examined under microscope under 200 X magnification.

3.8 Molecular detection

Scabs and skin tissue specimen from rats with skin lesions were collected. Polymerase chain reaction (PCR) was then carried out to confirm the presence of ORFV. Skin tissue specimens were grinded with sterile sand and mixed with PBS to prepare viral suspension. DNA extraction was done according to Vivantis GF-1 tissue DNA extraction protocol involving steps from tissue lysis, homogenization, addition of ethanol, loading to column, column washing, column drying to DNA elution, using GF-1 tissue DNA extraction kit.

Detection of ORFV by PCR was then carried out using B2L gene and F1L gene. The primers sequence used were 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 CAG-3) and F1L reverse primer (5-TCA CAC GAT GGC CGT GAC CAG-3). PCR was done using Intron PCR master

mix kit by preparing PCR products stored at -20ºC with 2.5 µl of PCR buffer solution, 2.5 µl deoxynucleotide, 1.5 µl magnesium chloride ($MgCl_2$), 0.5 µl of DNA polymerase, 15.5 µl Ribonuclease (RNase) free water, 0.75 µl forward primer, 0.75 µl reverse primer and 1 µl extracted DNA. Thermal cycler was programmed with initial activation of 95ºC for 2 mins following by 35 cycles of denaturation (94 ºC, 20 seconds), annealing (60 ºC for 30 seconds) and extension (72 ºC for 30 seconds), and lastly hold at 16 ºC.

After 2µl loading dye and 10 µl PCR product were loaded on 1.25% agarose gel, and gel electrophoresis was run at 100V for 30 minutes, positive DNA bands (B2L:1199 bp; F1L:1062 bp) were viewed with gel documentation system and compared with 100 bp DNA ladder.

3.9 Statistical analysis

Comparison between each inoculation sites group and between immunosuppressed and non-immunosuppressed group were done using analysis of variance (ANOVA) and Tukey's post hoc test if data was tested normally distributed. Mann-Whitney U test and Kruskal-Wallis H test were carried out if data was tested non-normally distributed. All the statistical analysis were analyzed using IBM SPSS version 20.

4.0 RESULTS

4.1 Clinical signs

Clinical signs such as ruffled hair coat (FIGURE II), reduced in alertness and skin lesions were observed in the treatment group. Ocular discharge was absent in all treatment group.

Regarding on skin lesions, mild hyperemia was observed at dorsum, ear pinna and labial commissure in rats of both group 1 and group 2 inoculated with ORFV UPM 1/14 and ORFV UPM 2/14 respectively. Without a clinical course of infection, scabs formed in 2 rats in group 1 and group 2. The dexamethasone group showed mild to moderate hyperemia at dorsum, ear pinna and labial commissure while the non-dexamethasone group showed only mild hyperemia. Macules, raised rash skin resembling papule and crusts were observed too in dexamethasone-treated group and non-dexamethasone-treated group. Control group showed transient skin redness after inoculation, and the redness disappeared after day 1 post inoculation.

Hyperemia started to appear and resolved at different days for different rats. Most of the signs started around day 1 to day 2 post virus inoculation. These skin lesions are described in FIGURE III, FIGURE IV, FIGURE V and TABLE II.

FIGURE II: RUFFLED HAIR COAT AT CRANIAL AND MIDDLE PART OF THE BODY

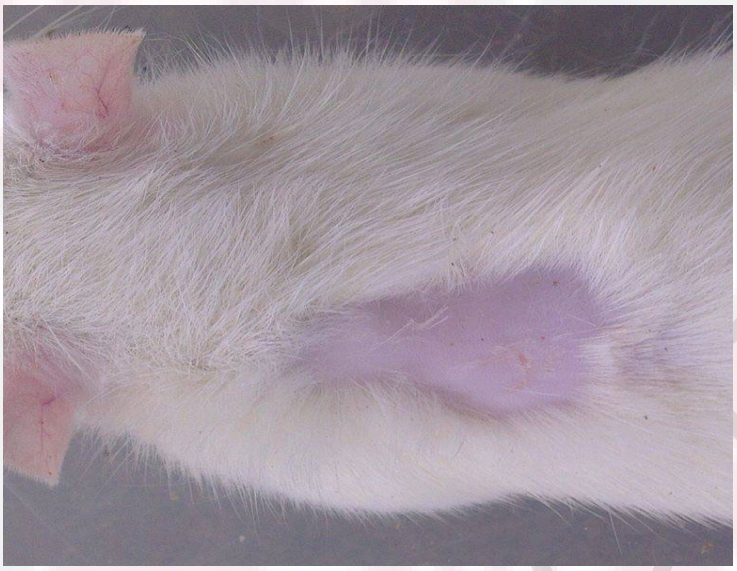


FIGURE III: SKIN LESIONS AT DORSUM OF THE RATS IN TREATMENT GROUP

A: moderate hyperemia of dorsum of rat in dexamethasone group; **B**: scabs and hyperemia of dorsum of rat inoculated with ORFV UPM 1/14

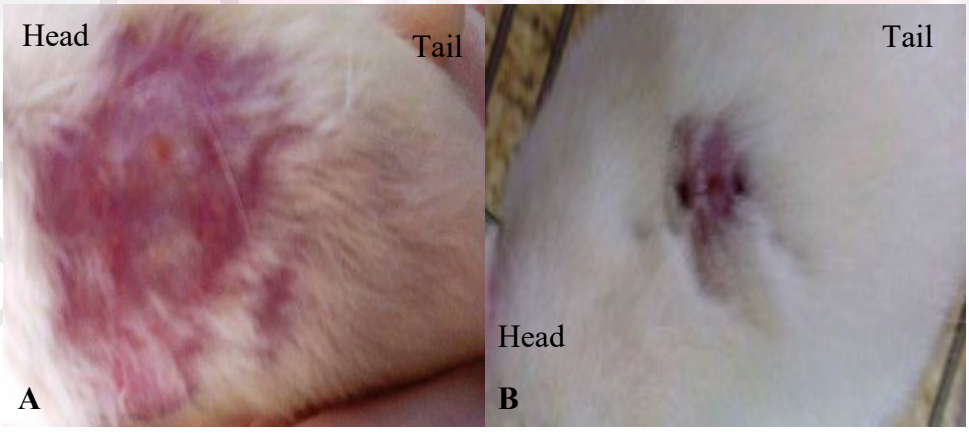


FIGURE IV: SKIN LESIONS AT EAR PINNA OF RATS IN TREATMENT GROUP

A: moderate hyperemia (black arrow) of ear pinna of rat inoculated with ORFV UPM 1/14; B: mild hyperemia (black arrow) of ear pinna of rat inoculated with ORFV UPM 2/14

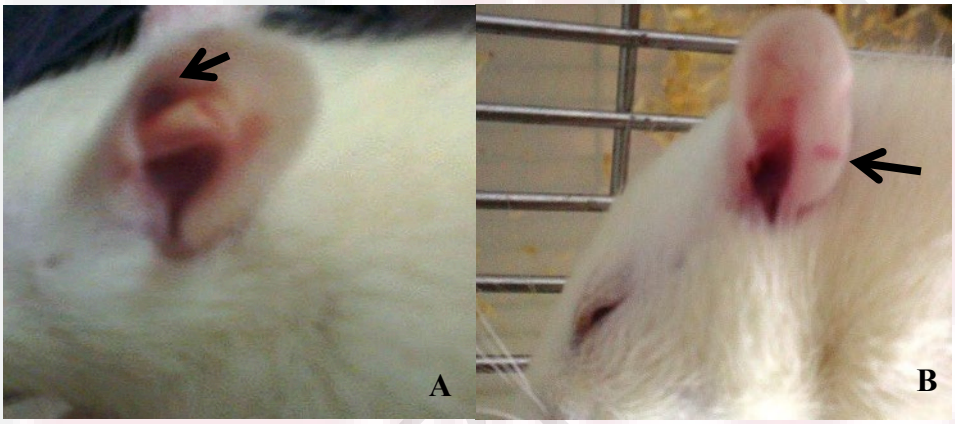


FIGURE V: Skin lesion at labial commissure of rat in treatment group

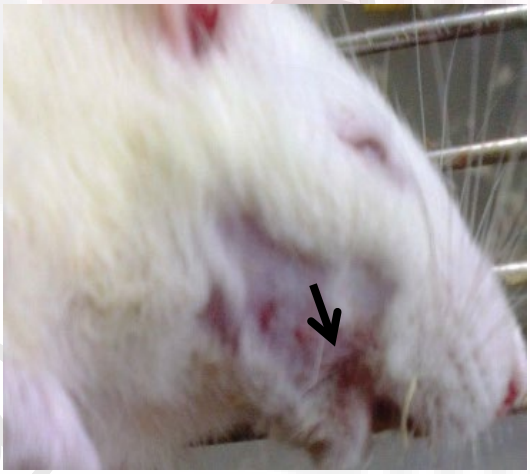


TABLE II: SKIN LESIONS OF EACH RATS IN GROUP 1 (UPM 1/14), GROUP 2 (UPM 2/14), DEXAMETHASONE GROUP AND NON-DEXAMETHASONE GROUP.

Group 1: ORFV UPM 1/14 Malaysia			
Animal	Skin lesion (Days post inoculation)		
	Dorsum	Ear pinna	Labial commissure
Rat 1	H,S (1-7)	H (1- 14)	H (3- 7)
Rat 2	H (1-14)	H (1- 14)	H (6- 14)
Rat 3	H (3- 6)	H (1- 7)	H (11- 14)
Rat 4	H (3- 14)	-	-
Rat 5	-	-	-
Group 2: ORFV UPM 2/14 Malaysia			
Animal	Skin lesion (Days post inoculation)		
	Dorsum	Ear pinna	Labial commissure
Rat 1	H, S (4- 9)	H (1-3)	H (7-14)
Rat 2	H (4- 14)	H (1-3)	H (9-14)
Rat 3	-	H (1- 5)	-
Rat 4	-	H (1- 7)	-
Rat 5	-	-	-
Dexamethasone-treated group			
Animal	Skin lesion (Days post inoculation)		
	Dorsum	Ear pinna	Labial commissure
Rat 1	-	H (1)	H (1)
Rat 2	H (1-2)	-	H (2)
Rat 3	H (1-3)	H (2-3)	H (2-3)
Rat 4	H(1-3)	H, M (2-4)	H (2-4)
Rat 5	-	-	-
Non-dexamethasone-treated group			
Animal	Skin lesion (Days post inoculation)		
	Dorsum	Ear pinna	Labial commissure
Rat 1	-	H (2- 7)	H (2- 7)
Rat 2	-	H, M (2-7)	H (2)
Rat 3	H (2- 7)	H (2-3)	-
Rat 4	-	H (2-7)	-
Rat 5	H (2-5)	-	-

Abbrevation: H-hyperemia; M-macule, V-vesicle, P-pustule, S-scabs, E-exudative/bleeding

4.2 Mean clinical score

The average score for each clinical signs were evaluated and expressed as mean ± standard error of mean (SEM) in tables below.

4.2.1 Ruffled hair coat

TABLE III: MEAN RUFFLED HAIR COAT* OF ORFV UPM 1/14 (GROUP 1), ORFV UPM 2/14 (GROUP 2) AND CONTROL.

Group	**Mean scores of ruffled hair coat**		
Group 1	Dorsum (Group 1A)	Ear pinna (Group 1B)	Labial commissure (Group 1C)
	0.007±0.004	0.000±0.000	0.000±0.000
Group 2	Dorsum (Group 2A)	Ear pinna (Group 2B)	Labial commissure (Group 2C)
	0.000±0.000	0.000±0.000	0.000±0.000
Control	0.000±0.000	0.000±0.000	0.000±0.000

The value is expressed as mean±SEM.*observed for 14 days post inoculation

Statistical analysis revealed that there was significant difference of mean score of ruffled hair coat between Group 1A, Group 1B and Group 1C and control (Kruskal-Wallis H test; $\chi^2(2)=9.340;p=0.025$) with mean rank of 33 for Group 1A and 27 for Group 1B, Group IC and control. However, multiple comparisons using Mann-Whitney U tests showed no significant differences of mean ruffled hair coat score between Group 1A and Group 1B (U=77, p=0.072), between Group 1A and Group 1C (U=77, p=0.072) and between Group 1B and 1C (U=98, p=1.000). On the other side, there was no ruffled hair coat observed in all rats in Group 2.

TABLE IV: MEAN SCORES OF CLINICAL SIGNS* OF ORFV UPM 1/14 (GROUP 1), ORFV UPM 2/14 (GROUP 2) AND CONTROL.

Clinical signs	Group 1	Group 2	Control
Ruffled hair coat (score)	0.002±0.001	0.000±0.000	0.000±0.000
Ocular discharges (score)	0.000±0.000	0.000±0.000	0.000±0.000
Level of alertness (score)	0.048±0.018[a]	0.007±0.007	0.000±0.000[a]
Skin lesions (score)	0.165±0.014[b,c]	0.077±0.012[b,d]	0.000±0.000[c,d]

The value is expressed as Mean±SEM. *observed for 14 days post virus inoculation. Groups sharing common superscripts are statistically significant different at $p<0.05$.

Moreover, there was significant difference of mean ruffled hair coat score between Group 1, Group 2 and control ($X^2(2)=6.026$, $p=0.049$) as shown in Kruskal-Wallis H test. Mean ranks of 65.96, 61.50, 61.50 were determined for Group 1, Group 2 and control respectively. However, Mann-Whitney U test showed no significant difference ($U=799.5$, $p=0.083$) between Group 1 and Group 2.

TABLE V: MEAN SCORES OF CLINICAL SIGNS* IN DEXAMETHASONE-TREATED GROUP AND NON-DEXAMETHASONE-TREATED GROUP

Clinical signs	Dexamethasone-treated group	Non-dexamethasone group	Control
Ruffled hair coat (score)	0.390±0.134[a]	0.229±0.065	0.000±0.000[a]
Ocular discharges (score)	0.000±0.000	0.000±0.000	0.000±0.000
Level of alertness (score)	0.740±0.166[b,c]	0.200±0.0345[b,d]	0.000±0.000[c,d]
Skin lesions (score)	0.316±0.078[e,f]	0.156±0.029[e,g]	0.000±0.000[f,g]

The value is expressed as mean±SEM. * observed for 7 days post inoculation. Groups sharing common superscripts are statistically significant difference at $p<0.05$.

As the data was normally distributed, ANOVA was run for dexamethasone-treated group and non-dexamethasone-treated group. ANOVA revealed significant difference of mean ruffled hair coat score between dexamethasone-treated group, non-dexamethasone-treated group and control ($F(2,16)=6.911$, $p=0.007$). However, post hoc tests revealed no significant difference of mean ruffled hair coat score between dexamethasone-treated group and non-dexamethasone group ($p=0.314$). Besides, mean ruffled hair coat of dexamethasone-treated group (0.390±0.134, $p=0.006$) was significantly higher than the control (0.000±0.000) whereas non-dexamethasone-treated group had no significant difference as compared to control.

4.2.2 Ocular discharge

No ocular discharges were observed in all treatment groups; therefore, there were no comparisons between groups.

4.2.3 Level of alertness

TABLE VI: MEAN SCORES OF ALERTNESS* ORFV UPM 1/14 (GROUP 1), ORFV UPM 2/14 (GROUP 2) AND CONTROL.

Group	**Mean scores of alertness**		
Group 1[a]	Dorsum (Group 1A)	Ear pinna (Group 1B)	Labial commissure (Group 1C)
	0.086±0.046	0.029±0.0194	0.029±0.019
Group 2	Dorsum (Group 2A)	Ear pinna (Group 2B)	Labial commissure (Group 2C)
	0.014±0.014	0.000±0.000	0.000±0.000
Control[a]	0.000±0.000	0.000±0.000	0.000±0.000

The value is expressed as mean±SEM. *observed for 14 days post inoculation. Group sharing common superscripts are statistically significant difference at $p<0.05$.

TABLE IV: MEAN SCORES OF CLINICAL SIGNS* OF ORFV UPM 1/14 (GROUP 1), ORFV UPM 2/14 (GROUP 2) AND CONTROL.

Clinical signs	Group 1	Group 2	Control
Ruffled hair coat (score)	0.002±0.001	0.000±0.000	0.000±0.000
Ocular discharges (score)	0.000±0.000	0.000±0.000	0.000±0.000
Level of alertness (score)	0.048±0.018[a]	0.007±0.007	0.000±0.000[a]
Skin lesions (score)	0.165±0.014[b,c]	0.077±0.012[b,d]	0.000±0.000[c,d]

The value is expressed as Mean±SEM. *observed for 14 days post virus inoculation. Groups sharing common superscripts are statistically significant different at $p<0.05$.

Mean level of alertness had not statistically significant difference between Group 1A, Group 1B, Group 1C and control ($X^2(2)=3.351$, $p=0.341$) and between Group 2A, Group 2B, Group 2C and control ($X^2(2)=3$, $p=0.392$) as shown in Kruskal-Wallis H test. Besides, there was significant difference of mean alertness score between the two local strains (Group 1; Group 2) and control (Kruskal-Wallis H test; $X^2(2)=9.521$, $p=0.009$). However, multiple comparisons done using Mann-Whitney U test revealed no significant difference between Group 1 and Group 2 ($U=509.5$, $p=0.088$) and between Group 2 and control ($U=567$, $p=0.221$). Group 1 was statistically significant higher than control ($U=735$, $p=0.006$) with mean rank of 46 for Group 1 and 39 for control.

TABLE V: MEAN SCORES OF CLINICAL SIGNS* IN DEXAMETHASONE-TREATED GROUP AND NON-DEXAMETHASONE-TREATED GROUP

Clinical signs	**Dexamethasone-treated group**	**Non-dexamethasone group**	**Control**
Ruffled hair coat (score)	0.390 ± 0.134^{a}	0.229 ± 0.065	0.000 ± 0.000^{a}
Ocular discharges (score)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Level of alertness (score)	$0.740\pm0.166^{b,c}$	$0.200\pm0.0345^{b,d}$	$0.000\pm0.000^{c,d}$
Skin lesions (score)	$0.316\pm0.078^{e,f}$	$0.156\pm0.029^{e,g}$	$0.000\pm0.000^{f,g}$

The value is expressed as mean±SEM. * observed for 7 days post inoculation. Groups sharing common superscripts are statistically significant difference at $p<0.05$.

Kruskal Wallis H test showed significant difference of alertness between dexamethasone-treated group and non-dexamethasone-treated group ($X^2(2)=13.945, p=0.01$). Mann Whitney U tests showed that dexamethasone-treated group had higher mean level of alertness than non-dexamethasone-treated group ($U=5, p=0.036$) with mean rank of 9 for dexamethasone-treated group and 4.71 for non-dexamethasone group. Dexamethasone-treated group had higher mean level of alertness ($U=0, p=0.001$) than control with mean rank of 10 for dexamethasone-treated group and 4 for control while non-dexamethasone-treated group had higher mean level of alertness ($U=3.5, p=0.003$) with mean rank of 10.5 for non-dexamethasone-treated group and 4.5 for control.

4.2.4 Skin lesions

TABLE VII: MEAN SCORES OF SKIN LESIONS* ORFV UPM 1/14 (GROUP 1), ORFV UPM 2/14 (GROUP 2) AND CONTROL.

Group	**Mean scores of skin lesions**		
Group 1[h,j]	Dorsum (Group 1A)	Ear pinna (Group 1B)	Labial commisure (Group 1C)
	0.224±0.0184[a,b,c]	0.160±0.026[a,d]	0.110±0.021[b,e]
Group 2[i,j]	Dorsum (Group 2A)	Ear pinna (Group 2B)	Labial commisure (Group 2C)
	0.0947±0.0194[f]	0.0754±0.0258[g]	0.0613±0.0162
Control[h,i]	0.000±0.000[c,f]	0.000±0.000[d,g]	0.000±0.000[e]

The value is expressed as mean±SEM. *observed for 14 days post inoculation Group sharing common superscripts are statistically significant at $p<0.05$

ANOVA revealed that there was significant difference of mean skin lesion score between Group 1A, Group 1B, Group 1C and control ($F(3,52)=26.839$, $p=0.000$). Post hoc test showed that mean skin lesion score of Group 1A (0.224±0.0184) was significantly higher than Group 1B (0.160±0.026, $p=0.037$) and Group 1C (0.110±0.021,$p=0.000$). There was no significant difference between Group 1B and Group 1C ($p=0.340$). Group 1A (0.224±0.0184, $p=0.000$), Group 1B (0.160±0.026, $p=0.000$), Group 1C (0.110±0.021,$p=0.000$) were significantly higher than control (0.000±0.000). Besides, there was significant difference of mean skin lesion score between Group 2A, Group 2B, Group 2C and control as determined by ANOVA ($F(3,52)=5.189$, $p=0.003$). However, post hoc revealed no significant difference between Group 2A and Group 2B ($p=0.841$), between Group 2A and Group 2C ($p=0.525$) and between Group 2B and Group 2C ($p=0.949$). Group 2A (0.0947±0.0194, $p=0.002$) and Group 2B (0.0754±0.0258, $p=0.026$) had significantly

higher mean skin lesion scores than control (0.000). However, Group 2C had no significant difference as compared to the control (p=0.093).

TABLE IV: MEAN SCORES OF CLINICAL SIGNS* OF ORFV UPM 1/14 (GROUP 1), ORFV UPM 2/14 (GROUP 2) AND CONTROL.

Clinical signs	Group 1	Group 2	Control
Ruffled hair coat (score)	0.002±0.001	0.000±0.000	0.000±0.000
Ocular discharges (score)	0.000±0.000	0.000±0.000	0.000±0.000
Level of alertness (score)	0.048±0.018[a]	0.007±0.007	0.000±0.000[a]
Skin lesions (score)	0.165±0.014[b,c]	0.077±0.012[b,d]	0.000±0.000[c,d]

The value is expressed as Mean±SEM. *observed for 14 days post virus inoculation. Groups sharing common superscripts are statistically significant different at $p<0.05$.

Kruskal-Wallis H test revealed significant difference between Group 1, Group 2 and control ($X^2(2)=69.494$, p=0.000) with mean rank of 42 for Group 1, 41 for Group 2 and 41 for control. Mean skin lesions score of Group 1 was significantly higher than mean skin lesions score of Group 2 as determined by Mann-Whitney U test (U=395, p=0.000) with mean rank of 53.10 for Group 1 and 30.63 for Group 2. Besides, Group 1 was significantly higher than control (Mann-Whitney U test;U=84,p=0.000) with mean rank of 61.50 for Group 1 and 23.50 for control. On the other sides, Group 2 was significantly higher than the control too (Mann-Whitney U test;U=336, p=0.000) with mean rank of 54.80 for Group 2 and 29.50 for control.

TABLE V: MEAN SCORES OF CLINICAL SIGNS* IN DEXAMETHASONE-TREATED GROUP AND NON-DEXAMETHASONE-TREATED GROUP

Clinical signs	Dexamethasone-treated group	Non-dexamethasone group	Control
Ruffled hair coat (score)	0.390±0.134[a]	0.229±0.065	0.000±0.000[a]
Ocular discharges (score)	0.000±0.000	0.000±0.000	0.000±0.000
Level of alertness (score)	0.740±0.166[b,c]	0.200±0.0345[b,d]	0.000±0.000[c,d]
Skin lesions (score)	0.316±0.078[e,f]	0.156±0.029[e,g]	0.000±0.000[f,g]

The value is expressed as mean±SEM. * observed for 7 days post inoculation. Groups sharing common superscripts are statistically significant difference at $p<0.05$.

Mean skin lesions score of dexamethasone-treated group, non-dexamethasone group and control showed significant difference according to ANOVA ($F(2,16)=14.824, p=0.000$). Post hoc tests showed that the mean skin lesion score of dexamethasone-treated group (0.316±0.078, $p=0.037$) was significantly higher than the non-dexamethasone group (0.156±0.029). Moreover, both dexamethasone-treated group (0.316±0.078, $p=0.000$) and non-dexamethasone group (0.156±0.029, $p=0.025$) had significantly higher mean skin lesion sore than control (0.000±0.000).

4.3 Histopathological changes

Keratosis, acanthosis and ballooning degeneration were observed in all treatment group. Occasionally, intracytoplasmic inclusion bodies were found. Other findings were vasculitis and congestion which found in some skin tissues specimen. Besides, infiltration of inflammatory cells was observed too in dexamethasone-treated group.

FIGURE VI: HISTOLOGY OF DORSUM OF CONTROL

A: dorsum of control (100 X magnification) **B:** one cell thick of epidermis (black arrow) showed in dorsum of control (400 X magnification)

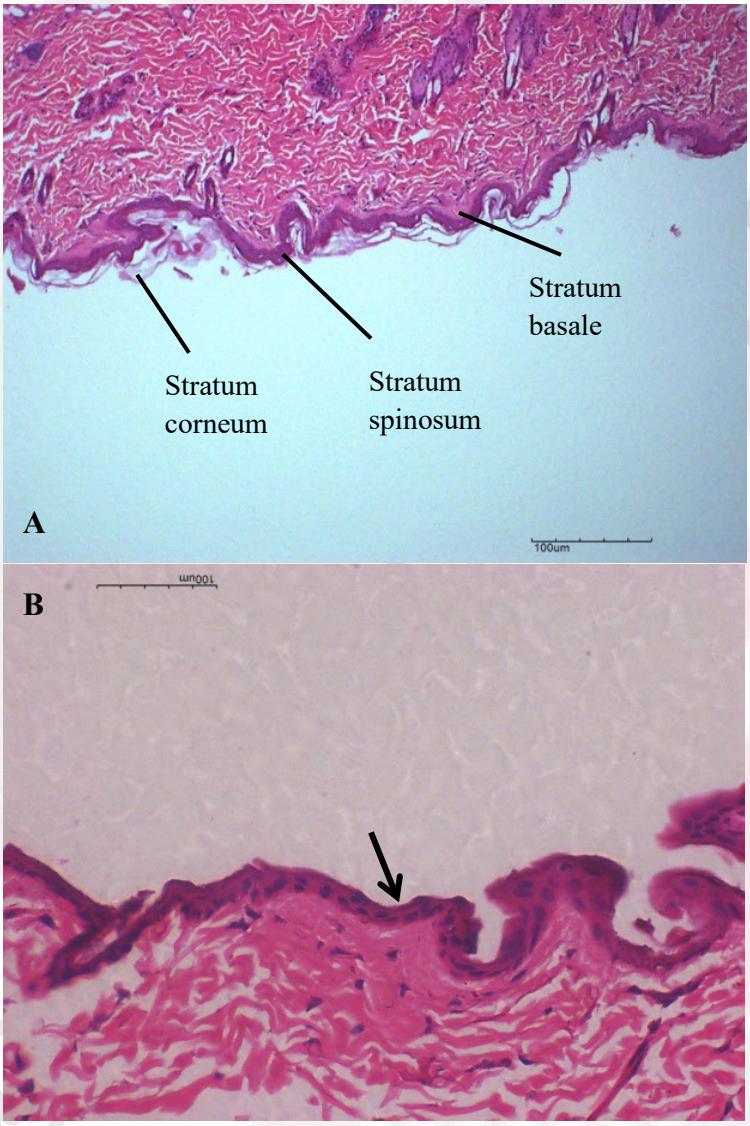


FIGURE VII: HISTOLOGY OF EAR PINNA OF CONTROL

Upper ear pinna of control (100 X magnification)

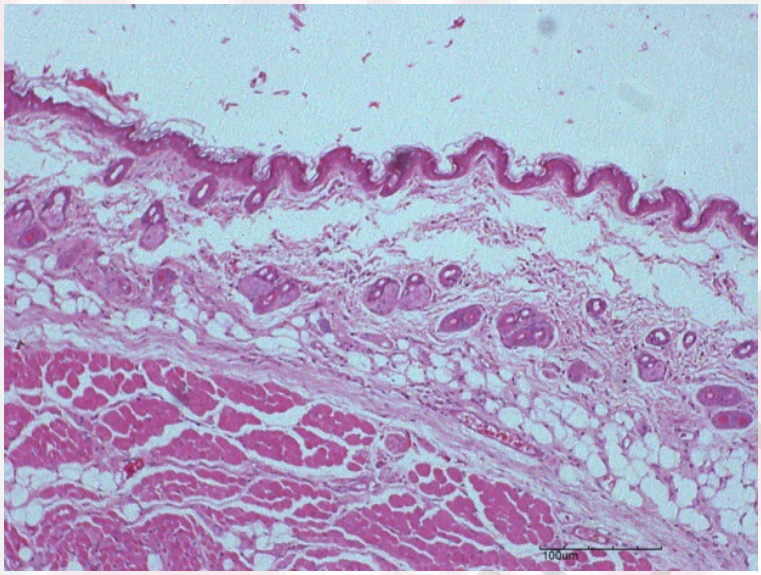


FIGURE VIII: HISTOLOGY OF LABIAL COMMISSURE OF CONTROL

Labial commissure of control (100 x magnification)

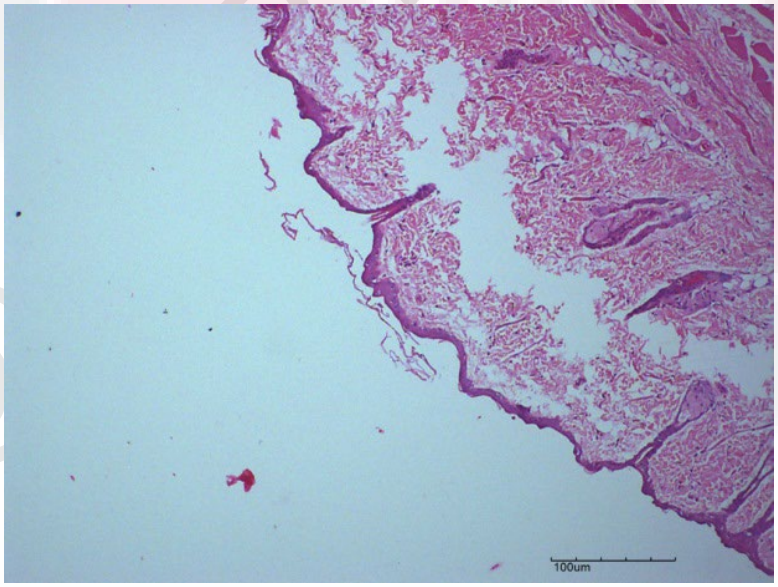


FIGURE IX: HISTOLOGY OF GROUP 1

A: Group 1A (200 X magnification) acanthosis (black arrow) and keratosis (red arrow) **B:** Group 1B (200 X magnification); acanthosis (black arrow) and keratosis (red arrow) **C:** Group 1C (200X magnification); acanthosis at stratum spinosum (black arrow) and karyorrhexis (red arrow) **D:** Group 1C (400X magnification) ballooning degeneration of cells at stratum spinosum (black arrow)

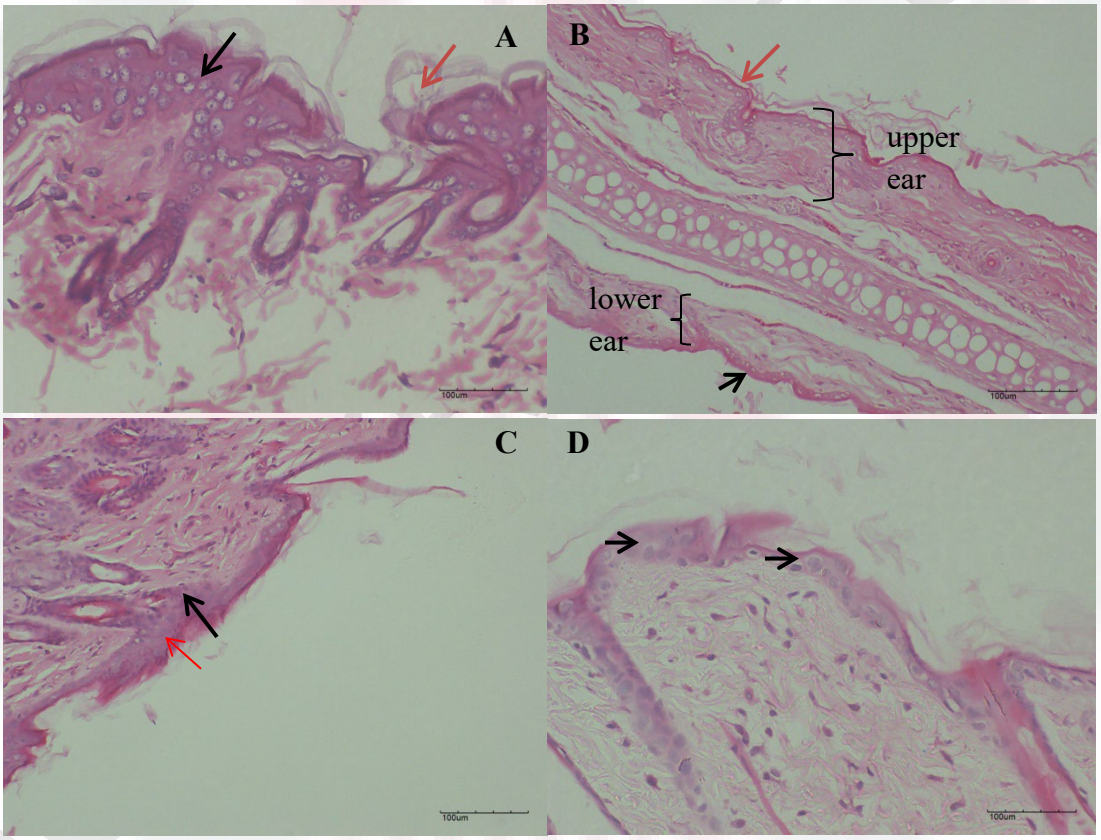


FIGURE X: HISTOLOGY OF GROUP 2

A: Group 2A (400 X magnification); keratosis (black arrow) and ballooning degeneration (red arrow) of cell at stratum spinosum **B:** Group 2B (400 X magnification); acanthosis (black arrow) at stratum spinosum of upper ear pinna **C:** Group 2C (400 X magnification);ballooning degeneration (black arrow) and karyorrhexis (red arrow) **D:** Group 2C (400 X magnification) ballooning degeneration (black arrow) and acanthosis (red arrow)

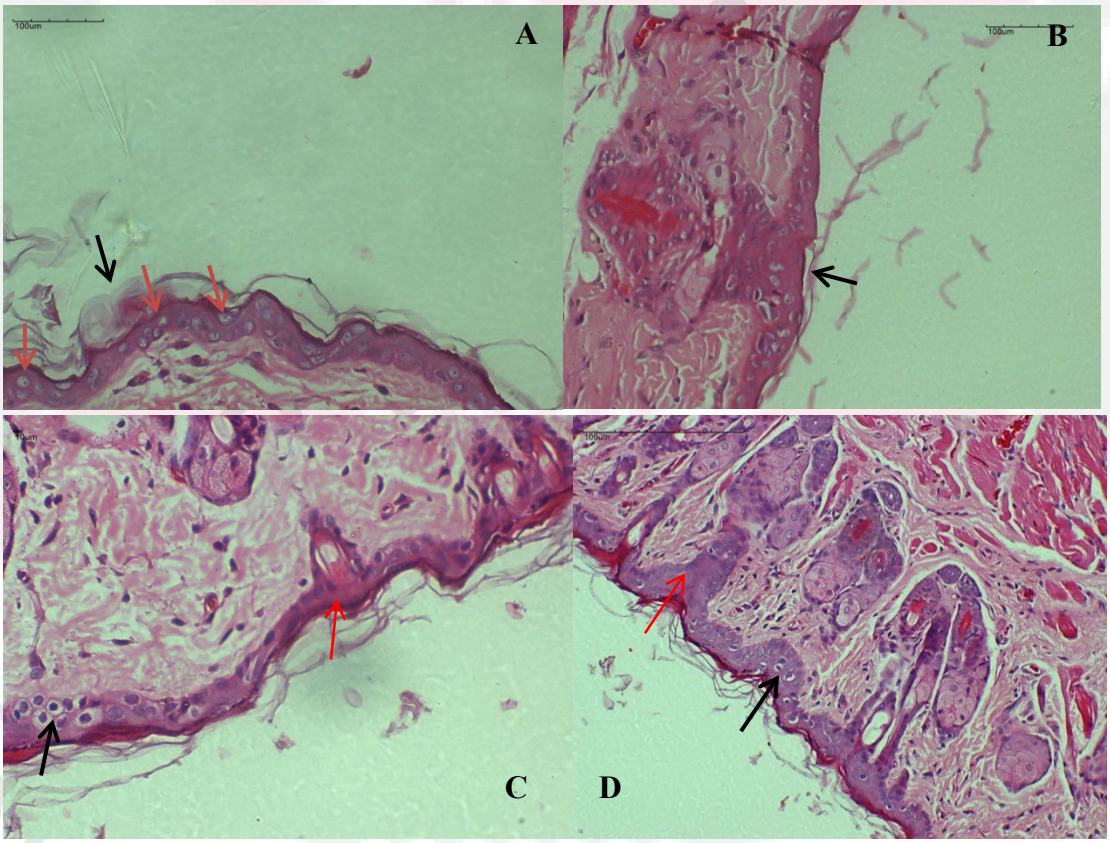


FIGURE XI: INTRACYTOPLASMIC EOSINOPHILIC INCLUSION BODIES (black arrow) observed in dorsum of dexamethasone group (400 x magnification)

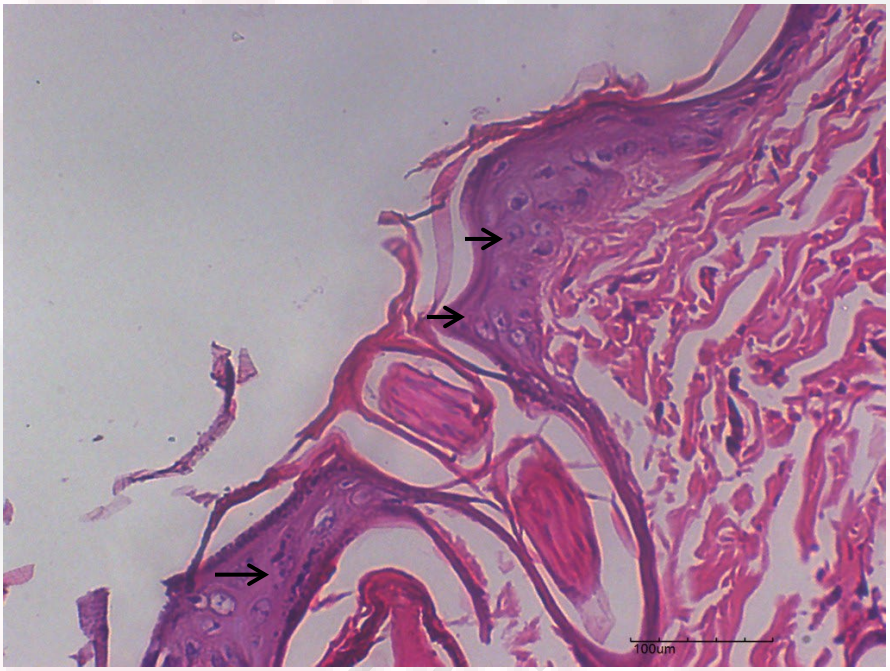


FIGURE XII: HISTOLOGY OF DEXAMETHASONE-TREATED GROUP.

A: bulging of epidermis with infiltration of inflammatory cells of labial commissure (black arrow) and congestion at dermis (red arrow) (100 X magnification) **B:** presence of cysts at epidermis of dorsum (200 X magnification) and keratosis (black arrow)

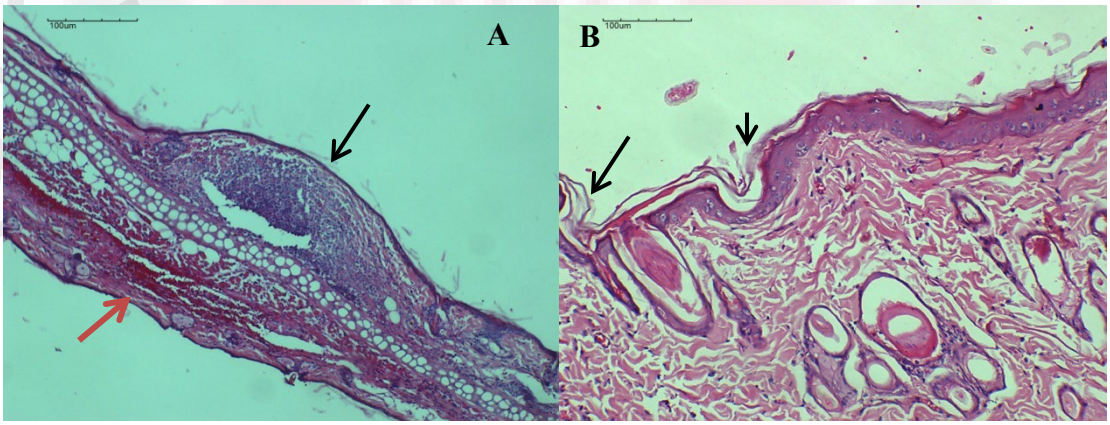


FIGURE XIII: HISTOLOGY OF NON-DEXAMETHASONE GROUP.

A: acanthosis (black arrow) at stratum spinosum of upper ear pinna (200 X magnification) **B:** keratosis (red arrow), acanthosis (black arrow) and ballooning degeneration (yellow asterisk) at dorsum (200 X magnification)

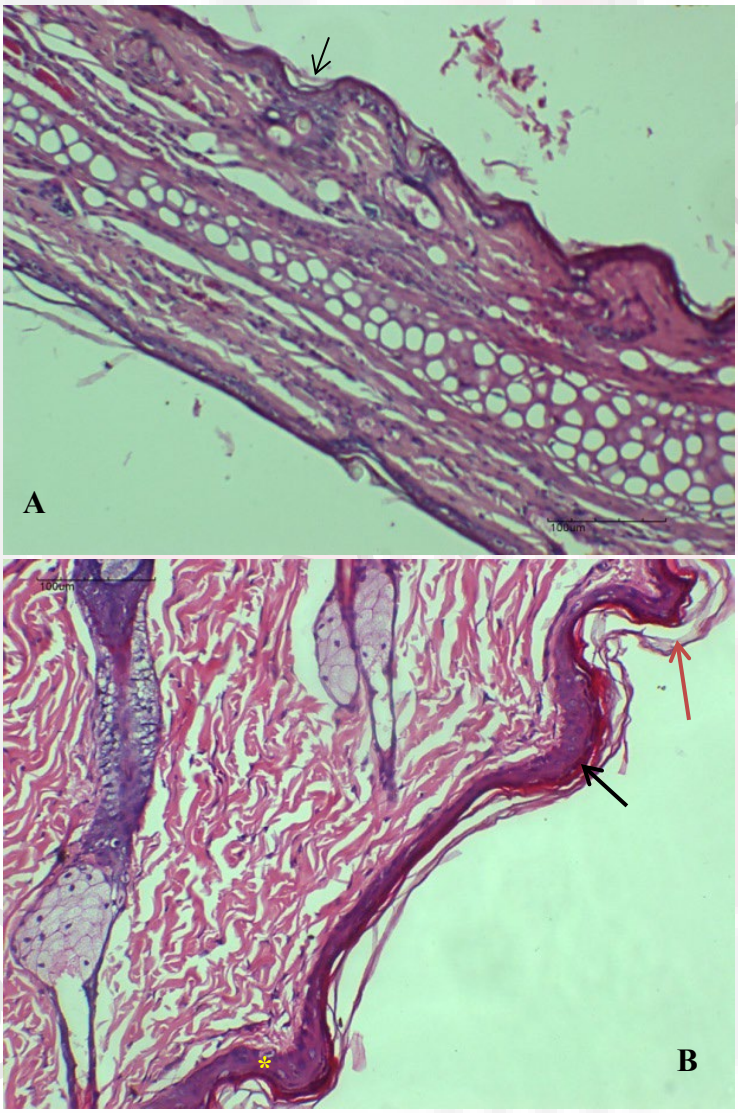


TABLE VIII: HISTOPATHOLOGICAL FINDINGS OF RATS WITH SKIN LESIONS FROM GROUP 1,GROUP 2, DEXAMETHASONE-TREATED GROUP AND NON-DEXAMETHASONE GROUP.

Group	**Histopathological findings**	
Group 1	Group 1A	Keratosis; acanthosis and ballooning degeneration of cells at stratum spinosum and stratum basale
	Group 1B	Keratosis;acanthosis and ballooning degeneration of cells at stratum spinosum and stratum basale
	Group 1C	Keratosis; karyorrhexis, acanthosis and ballooning degeneration of cells at stratum spinosum and stratum basale
Group 2	Group 2A	Keratosis; acanthosis and ballooning degeneration of cells at stratum spinosum and stratum basale
	Group 2B	Acanthosis and keratosis
	Group 2C	Keratosis; acanthosis, karyorrhexis, keratosis and ballooning degeneration of cells at stratum spinosum and stratum basale
Group	**Histopathological findings**	
Dexamethasone-treated group	Keratosis; acanthosis and ballooning degeneration of cells at stratum spinosum and stratum basale; infiltration of inflammatory cells at epidermis and dermis; congestion at dermis; intracytoplasmic inclusion bodies;presence of cyst at epidermis	
Non-dexamethasone group	Keratosis;acanthosis and ballooning degeneration of cells at stratum spinosum and stratum basale.	

4.4 Mean thickness of stratum spinosum and basale

Acanthosis were observed, and the thickness of stratum spinosum and stratum basale were measured and compared between groups. The results were shown in tables below.

TABLE IX: MEAN THICKNESS OF STRATUM SPINOSUM AND STRATUM BASALE OF DORSUM, EAR PINNA, LABIAL COMMISSURE OF GROUP 1 (ORFV UPM 1/14), GROUP 2 (ORFV UPM 1/14) AND CONTROL

	Mean thickness	
	Stratum spinosum	Stratum basale
Group 1A	65.560±6.001[a,h]	10.340±0.945[b,d,I]
Group 2A	71.050±10.221[c,j]	8.638±0.763[k]
Dorsum of control	29.900±0.006[a,c,h,j]	4.700±0.006[b,d,I,k]
Group 1B	24.788±6.694	5.100±0.568
Group 2B	31.325±8.459	8.700±1.707
Ear pinna of control	18.000±0.006	4.700±0.006
Group 1C	49.775±11.951	5.450±0.222[c,f]
Group 2C	44.000±6.618[e,]	5.025±0.549[d,g]
Labial commissure of control	14.950±0.006[e,]	3.100±0.006[c,d,f,g]

The value is expressed as mean±SEM. Groups sharing common superscripts are statistically significant different at $p<0.05$.

Statistical analysis revealed that Group 1A (Independent Sample T-test; 65.560±6.001,t(6)=4.457,p=0.04) had significant higher mean thickness of stratum spinosum than control (29.900±0.006). Besides, Group 1A had also significant higher mean thickness of stratum basale (Independent Sample T-test; 10.340±0.945, t(6)=4.478,p=0.004) than control (4.700±0.006). Group 1B had no significant difference with control for both mean thickness of stratum spinosum (Independent Sample T-test; t(5)=0.857, p=0.431) and mean thickness of stratum basale than control (Independent Sample T-test; t(5)=0.595,p=0.578). On the other side, Group 1C had no significant difference with control for mean thickness of stratum spinosum (Independent Sample T-test; t(5)=2.392, p=0.062) but Group 1C (Independent Sample T-test; 5.025±0.549, t(5)=8.957, p=0.000) had significant higher than control (3.100±0.006).

On the other side, in Group 2, Independent Sample T-test showed that Group 2A had significantky higher mean thickness of stratum spinosum (71.050±10.221, t(5)=3.403,p=0.019) and mean thickness of stratum basale (8.638±0.763, t(5)=4.361,p=0.007) than control (stratum spinosum: 29.900±0.006; stratum basale: 4.700±0.006). Moreover, Group 2C had significantly higher mean thickness of stratum spinosum (44.000±6.618,t(5)=3.710,p=0.014) and mean thickness of stratum basale (5.025±0.549, t(5)=2.965,p=0.031) than control (stratum spinosum: 14.950±0.006; stratum basale: 3.100±0.006) as shown by Independent Sample T-test. Group 2B had no significant difference for both mean thickness of stratum spinosum (t(5)=1.331,p=0.241) and mean thickness of stratum basale (t(5)=1.980,p=0.105) with control according to Independent Sample T-test.

Comparison between Group 1 and Group 2 were analyzed with ANOVA and post hoc tests. The results include significant difference between Group 1, Group 2 and control in mean thickness of stratum spinosum and mean thickness of stratum basale of dorsum (ANOVA; stratum spinosum: F(2,9)=7.599, p=0.012; stratum basale: F(2,9)=10.890,p=0.004) and between Group 1, Group 2 and control in mean thickness of stratum basale of labial commissure (ANOVA, F(2,8)=9.832, p=0.007). There were no significant differences between Group 1, Group 2 and control in mean thickness of stratum spinosum of ear pinna (F(2,8)=0.877,p=0.453) and labial commissure (F(2,8)=0.065,p=0.065) and mean thickness of stratum basale of ear pinna (F(2,8)=3.756,p=0.071) as shown in ANOVA.

However, post hoc test showed no significant difference of mean thickness of stratum spinosum and stratum basale of dorsum (stratum spinosum:p=0.848;stratum basale:p=0.324), ear pinna (stratum spinosum:p=0.770, stratum basale:p=0.112) and labial commissure (stratum spinosum:0.915; stratum basale:p=0.697) between Group 1 and Group 2. Besides, post hoc showed that Group 1 (5.450±0.222,p=0.007) and Group 2 (5.025±0.549,p=0.020) had significantly higher mean thickness of stratum basale of labial commisure than control (3.100±0.006).

Group 1 (65.560±6.001,p=0.023) and Group 2 (71.050±10.221,p=0.014) had significant higher mean thickness of stratum spinosum of dorsum than control (29.900±0.006). Besides, Group 1 (10.340±0.945,p=0.003) and Group 2 (8.638±0.763,p=0.031) had significant higher mean thickness of stratum basale of dorsum than control (4.700±0.006). There were no significant difference of mean thickness of stratum basale of ear pinna between Group 1 and control (p=0.969) and between Group 2 and control (p=0.101). Besides, mean thickness of stratum spinosum of ear pinna showed no significant difference between Group 1 and control (p=0.785) and between Group 2 and control (p=0.424). Mean thickness of stratum spinosum of labial commissure showed no significant difference between Group 1 and control (p=0.068) and between Group 2 and control (p=0.118).

TABLE X: MEAN THICKNESS OF STRATUM SPINOSUM AND STRATUM BASALE OF DORSUM, EAR PINNA, LABIAL COMMISSURE OF DEXAMETHASONE GROUP, NON-DEXAMETHASONE GROUP AND CONTROL

		Mean thickness	
		Stratum spinosum	Stratum basale
Dorsum	Dexamethasone-treated group	56.938±10.156	8.975±1.132[a]
	Non-dexamethasone group	29.267±1.988	5.833±0.371
	Control	29.900±0.006	4.700±0.006[a]
Ear pinna	Dexamethasone-treated group	41.833±6.578	5.800±0.643
	Non-dexamethasone group	28.825±5.465[b]	5.500±0.850
	Control	18.000±0.006[b]	4.700±0.006
Labial commisure	Dexamethasone-treated group	33.133±0.033[c,d]	5.900±0.006[f,g]
	Non-dexamethasone group	19.700±0.608[c,e]	5.100±0.306[f,h]
	Control	14.950±0.006[d,e]	3.100±0.006[g,h]

The value is expressed as mean±SEM. Groups sharing common superscripts are statistically significant different at $p<0.05$.

There were significant differences of mean thickness of stratum spinosum and stratum basale of dorsum and labial commisure between dexamethasone-treated group, non-dexamethasone group and control as shown in ANOVA (stratum spinosum of dorsum: $F(2,7)=4,980$, $p=0.045$; stratum basale of dorsum: $F(2,7)=7.547$, $p=0.018$; stratum spinosum of labial commissure: $F(2,6)=107.828$, $p=0.000$; stratum basale of labial commissure: $F(2,6)=66.857$, $p=0.000$). Regarding on ear pinna, mean thickness of stratum spinosum was significantly difference between dexamethasone-treated group, non-dexamethasone-treated group and control (ANOVA

F(2,7)=4.847,p=0.048); however, mean thickness of stratum basale was significantly difference between dexamethasone group, non-dexamethasone group and control (ANOVA, F(2,7)=0.617,p=0.566).

However, post hoc tests showed no significant difference of mean thickness of stratum spinosum of dorsum between dexamethasone-treated group and non-dexamethasone group (p=0.070); between dexamethasone-treated group and control (p=0.076) and between non-dexamethasone group and control (0.998). There were no significant difference between mean thickness of stratum basale of dorsum between dexamethasone-treated group and non-dexamethasone group (p=0.070) and between non-dexamethasone group and control (p=0.650). However, dexamethasone-treated group (56.938±10.156, p=0.019) had higher thickness of stratum basale of dorsum than control (29.900±0.006).

Regarding on ear pinna, mean thickness of stratum spinosum of non-dexamethasone (28.825±5.465,p=0.040) was significantly higher than control (18.000±0.006). Other differences of mean thickness of ear pinna between groups were not significant (p>0.05). On the side of labial commissure, dexamethasone-treated group (stratum spinosum: 33.133±0.033; stratum basale: 5.900±0.006) had significantly higher mean thickness of stratum spinosum and stratum basale than non-dexamethasone-treated group (stratum spinosum: 19.700±0.608, p=0.000; stratum

basale: 5.900±0.006, p=0.042) and control (stratum spinosum, . 14.950±0.006, p=0.016; stratum basale: 3.100±0.006, p=0.000).

4.5 Molecular detection of virus by Polymerase Chain Reaction (PCR)

ORFV was detected in the 1% virus suspension used to inoculate in the rats. ORFV was able to be detected using F1L and B2L gene by PCR from the skin tissues of rats with skin lesions in treatment group collected at day 14 for Group 1 and Group 2 and day 7 for dexamethasone-treated group and non-dexamethasone group. ORFV was detected in the 1% virus suspension used to inoculate in the rats. Positive bands were produced. PCR products were amplified at 1000+ bp. However, PCR bands obtained were thick and not bright.

FIGURE XIV: GEL ELECTROPHORESIS OF ORFV DNA AMPLIFIED BY PCR FROM VIRUS SUSPENSION

M: 100 bp marker **1:**+ve control;primer F1L, **2:**+ve control;primer B2L,**3:** UPM 1/14; primer F1L**4:** UPM 1/14;primer B2L **5:** UPM 2.14 primer F1L **6:** UPM 2/14;primer B2L

FIGURE XV: GEL ELECTROPHORESIS OF ORFV DNA AMPLIFIED BY PCR

M: 100 bp marker **1:**+ve control;primer F1L, **2:**+ve control;primer B2L,**3:** Group 1A; primer F1L**4:** Group 1A;primer B2L **5:**Group 1B, primer F1L **6:** Group 1B;primer B2L **7:** Group 2A;primer F1L **8:** Group 2A;primer B2L **9:** Group 2B;primer F1L **10:** Group 2B;primer B2L

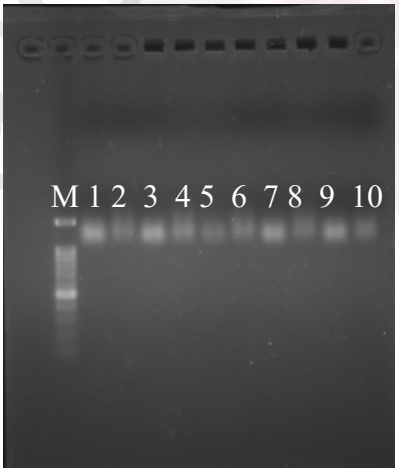


FIGURE XVI: GEL ELECTROPHORESIS OF ORFV DNA AMPLIFIED BY PCR

M: 100 bp marker **1:**+ve control;primer F1L, **2**:+ve control;primer B2L,**3:** Dorsum of DEX Group; primer F1L**4:** Dorsum of DEX group;primer B2L **5:** Ear pinna of DEX group, primer F1L **6:** Ear pinna of DEX group;primer B2L **7:** Labial commissure of DEX group;primer F1L **8:** Labial commissure of DEX group;primer B2L **9:** Group 1C;primer F1L **10:** Group 1C;primer B2L

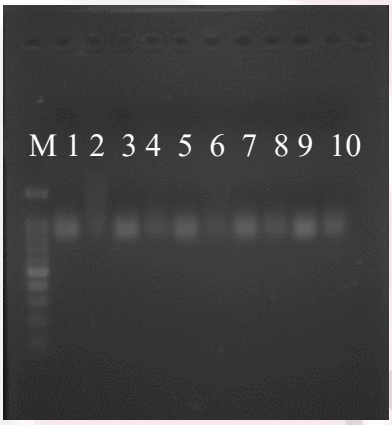


FIGURE XVII: GEL ELECTROPHORESIS OF ORFV DNA AMPLIFIED BY PCR

M: 100 bp marker **1:**+ve control;primer F1L, **2:**+ve control;primer B2L,**3:** Dorsum of non- DEX Group; primer F1L**4:** Dorsum ofnon- DEX group;primer B2L **5:** Ear pinna of non-DEX group, primer F1L **6:** Ear pinna of non-DEX group;primer B2L **7:** Labial commissure of non-DEX group;primer F1L **8:** Labial commissure of non-DEX group;primer B2L **9:** Group 2C;primer F1L **10:** Group 2C;primer B2L

5.0 DISCUSSION

5.1 Pathogenicity of ORFV infection in rat model

Intradermal inoculation of both local strains, which are ORFV UPM 1/14 Malaysia and ORFV UPM 2/14 Malaysia at dorsum, ear pinna and labial commissure are able to produce clinical signs and histopathological changes in rat model. Mild hyperemia was reproduced in rats, and macule in two rats. These results were in line with result of ORFV inoculation in mice by Cargnelutti *et al.* (2010). Besides, these results were also in line with Huda and Hussien (2014) which showed main skin manifestation of thickening and redness at injection sites in mice. However, these result were different from Cargnelutti *et al.* (2010) where clinical course of classical orf lesion involving maculopapular, vesicular and pustular lesion observed in rabbits; Gallina *et al.* (2008) showed typical ORFV lesion in goats and Martins, M (2014) showed typical ORFV lesion in lambs. Therefore, the pathogenicity of ORFV infection varies among species. Immune variation among animal hosts maybe the possible factor.

Histopathological changes which include acanthosis, ballooning degeneration, keratosis, intracytoplasmic eosinophilic inclusion bodies and infiltration inclusion bodies were able to be produced in rat. All these histopathological changes are in line with histopathological changes in normal hosts for ORFV, which are goat and sheep described by Gallina *et al.* (2008) and Pompei et al. (2010). These histopathological changes are in line with intradermal inoculation of ORFV in rabbit described by Cargnelutti, J.F et al. (2010) and Huda and Hussien (2014) in mice.

Moreover, ORFV was detected in skin samples from rats showed skin lesions by PCR. This showed that the skin lesions and histopathological changes produced in the study were due to ORFV infection.

This study elaborated the clinical signs and histopathological changes for the first time in rats. The success of producing mild skin lesions and histopathological changes showed that rat is susceptible to obtain ORFV infection. Based on the results, rat can be used as a suitable animal model for ORFV model. Although the pathogenicity developed was mild, disease produced in rat was similar to that of the natural hosts which are goats and sheeps.

The mild pathogenicity shown in this study may be due to virus titer in the scab suspension. Failure of producing orf disease via inoculation with scab suspension from outbreak of contagious ecthyma was reported by Asakawa *et al.* (1952) in guinea pig and mice; Greig (1956) in guinea pigs and mice and Wheeler *et al*. (1956) in rabbits, guinea pig and mices. Meanwhile, Cargnelutti *et al.* (2010) and Huda and Hussien (2014) reported orf disease in rabbits and mice respectively using isolated virus from cell culture.

Other reason of mild pathogenicity could be slow growing characteristic of ORFV. The monitoring period may be insufficient. The incubation period in natural

cases is 2 to 3 weeks (Zamri-Saad *et al.*, 1992). Moreover, the natural cases of human infection happened within 3 to 6 days post exposure and developed a course of 3 to 4 weeks (Kumar *et al.*, 2015).

5.2 Effect of strains on pathogenicity in rat

The two local strains in 1% virus suspensions produced various pathogenicity in rats. This was supported by other studies of ORFV using different strains. For example, Martins *et al.* (2014) demonstrated ORFV isolates in Brazil display differential virulence in lambs and the differences might be due to genetic changes in virulence genes.

In this study, there are more rats produced the disease following inoculation of ORFV UPM 1/14 than ORFV 2/14. Besides, although no significant difference for other clinical signs and mean thickness of stratum spinosum and stratum basale, ORFV UPM 1/14 showed significantly higher mean skin lesions score than ORFV UPM 2/14. The higher mean skin lesions score is due to more number of rats produce the disease following virus inoculation with more long-lasting lesions. Therefore, ORFV UPM 1/14 Malaysia is more pathogenic than ORFV UPM 2/14 Malaysia based on the mean skin lesions. However, more evidence from other parameters are necessary to access the pathogenicity of the two local strains

5.3 Choice of strain in rat model

The two local strains were able to produce mild pathogenicity in rat model. Therefore, both strains can be used to study ORFV in rat model. Besides, based on the significant higher mean skin lesions score of ORFV UPM 1/14, ORFV UPM 1/14 may be a better choice since it produces more skin lesions.

5.4 Effect of inoculation sites on pathogenicity of ORFV infection in rat

Dorsum, ear pinna and labial commissure displayed different pathogenicity of ORFV infection in rat. Inoculation sites is a critical factor for orf disease development (Cargnelutti *et al.* (2010), Gallina, L. (2008), Wheeler *et al.* (1956) and Asakawa *et al*. (1952).

Intradermal inoculation of ORFV UPM 1/14 Malaysia at dorsum produced significant higher mean skin lesion scores than ear pinna and labial commissure. However, no significant difference between ear pinna and labial commissure and between inoculation sites among intradermal inoculation of ORFV UPM 2/14 Malaysia determined in statistical analysis. Therefore, the rank between these three inoculation sites in term of pathogenicity cannot be determined.

5.5 Choice of inoculation sites of ORFV study in rat model

Based on the results, in inoculation of ORFV 1/14 Malaysia, dorsum was statistically proved that better than ear pinna and labial commissure; however, no significant results generated in inoculation of ORFV 2/14 Malaysia. Therefore, no

conclusion can be made regarding on best inoculation sites that can be used in rat model. The three inoculation sites were able to produce skin lesions and histopathological changes in rat model in both two local strains; thus, the three inoculation sites can be used in rat model.

5.6 Effect of dexamethasone treatment on pathogenicity of ORFV in rats

Dexamethasone treatment and without dexamethasone treatment resulted in varies pathogenicity of ORFV in rats. This study first demonstrated that rats treated with dexamethasone produced more disease than non-dexamethasone group based on mean level of alertness, mean skin lesions score, mean thickness of stratum spinosum of labial commissure and mean thickness of stratum basale of labial commissure. However, the difference in pathogenicity should be identified with evidence of other parameters.

Haig, D.M. and McInnes, C.J. (2002) described vigorous immune reaction in sheep involving neutrophils, dermal dendritic cells, T cells, B cells and antibody occur after ORFV virus infection. There are also involvement of CD4+ T cells, IFN-γ and CD8+ T cells in partial protection against ORFV infection (Haig, D.M. and McInnes, C.J., 2002). Prolonged immunosuppressive dose of dexamethasone suppress all these immune response, resulting in more pathogenicity of ORFV infection.

5.7 Molecular detection of ORFV

ORFV was able to be detected from skin tissues of rats showing skin lesions via PCR. This was in line with Cargnelutti *et al.* (2010) and Huda and Hussien (2014) which demonstrated recovery of virus from lesions. This result also supports that rat able to harbor ORFV replication. There are many factors that cause variation in PCR results, which include degraded primer, contamination carried over from previous PCR, excessive quantity of DNA polymerase, high magnesium ion concentration, incorrect amount of PCR products loaded in the well of gel, incorrect number of cycles of PCR assays and incorrect gel preparation (Elizabeth *et al*., 2008)..

6.0 CONCLUSION

Based on the results, ORFV UPM 1/14 Malaysia and ORFV UPM 2/14 Malaysia are pathogenic in rats. Besides, the pathogenicity of ORFV varies due to strains, inoculation sites and dexamethasone treatment. Based on result of higher mean skin lesions, ORFV UPM 1/14 Malaysia produces more severe disease than ORFV UPM 2/14 Malaysia. Both local strains can be used to study ORFV in rat. Moreover, different inoculation sites result in different disease severity. For ORFV UPM 1/14 Malaysia, inoculation at dorsum produces more severe lesions. All three inoculation sites, dorsum, ear pinna and labial commissure can be used to study ORFV in rat. Dexamethasone treatment which induces immunosuppressive effect results in severe orf disease. Overall, rat can be used as a good model for ORFV study.

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