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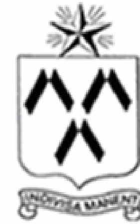

Palestine Polytechnic University

Deanship of Graduate Studies and Scientific Research

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Faculty of Science

Characterization of *Orf virus* Loads in Ecthymous Scabs for Autologous Vaccine Development in Palestinian Sheep

By

Dua'a Deib Abu Shkhaidem

In Partial Fulfillment of the Requirements for the Degree

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

Characterization of *Orf virus* Loads In Ecthymous Scabs For Autologous Vaccine Development In Palestinian Sheep

By

Dua'a Deib Abu Shkhaidem

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Graduate Advisory Committee:

Committee Member (Student's Supervisor) — Date: 4/7/2016

Dr. Robin Abu Ghazaleh, Palestine Polytechnic University

Committee Member (Internal Examine) — Date: 11/7/2016

Dr. Omar Mousa Darissa, Bethlehem University

Committee Member (External Examine) — Date: 13/7/2016

Dr. Suheir Ereqat, Al-Quds University

Approved for the Faculties

Dean of Graduate Studies and Scientific Research

Palestine Polytechnic University

Date 20/7/2016

Dean of Faculty of Science

Bethlehem University

Date 20/7/2016

Characterization of *Orf virus* Loads in Ecthymous Scabs for Autologous Vaccine Development in Palestinian Sheep

by (Dua'a Deib Abu Shkhaidem)

ABSTRACT

In Palestine, there is no certified vaccine for use against *Orf virus*, which is the causative agent of contagious ecthyma. This disease affects sheep and goats, and is characterized by lesion formation around the mouth, nostrils, teats and udder. It is widespread in Palestine, where it causes considerable losses due to fatigue and starvation of infected animals. A recent field survey of sixteen farms in the West Bank, led by Palestine Polytechnic University (PPU) in partnership with the National Agricultural Research Center – Qabatiya, showed that mortality reached 50% amongst new born lambs in the surveyed farms. Lesions and scabs from these animals were collected for confirmation by polymerase chain reaction, and samples that contained *Orf virus* DNA were used in this study, to prepare and validate infected scabs by viral culture in the laboratory for use as an autologous live vaccine suitable for delivery to Palestinian sheep by scarification.

Dried scabs for viral culture were homogenized, suspended in PBS to 10% (w/v) and filtered. The filtrates were inoculated onto the chorio-allantoic membrane (CAM) of embryonated chicken eggs, and also onto confluent Vero cell cultures; each of which were examined for the appearance of pocks on the CAM and cytopathic effects (CPE) in Vero cells, respectively. Cytopathic effects could not be observed on Vero cell cultures, but characteristic, specific pock lesions were observed on the CAMs of embryonated chicken eggs five days post inoculation for thirteen of sixteen different scab filtrates tested through a dilution range of 10^{0} – 10^{-8}.

The sensitivity of two different routes of CAM inoculation was compared, and inoculation through an artificial air-sac was shown to be the more sensitive route. This is the first reported comparison of inoculation routes for detection of *Orf virus* on the CAM of embryonated chicken eggs. Titration for infectious *Orf virus* from one of the scab suspensions was performed by making tenfold serial dilutions ranging from 10^0 - 10^{-9} of a 10% (w/v) scab filtrate where 0.2 ml of each dilution was inoculated (n=4) onto each CAM and a 50% egg infectious dose (EID_{50}) of $5x10^8$ / ml was determined. Counting individual pocks on each of the CAMs at each dilution, followed by linear regression analysis, enabled a supporting value of mean egg infectious dose to be estimated from the same experiment, in addition to the EID_{50}.

After this confirmation of infectivity, scab filtrates of five sheep from different farms were pooled, and mixed with glycerol/PBS as an excipient, for autologous vaccine testing by Dr. Robin Abu Ghazaleh and Dr. Jihad Al Ebrahim on farms with a previous history of *Orf virus* infection in cooperation with the Food and Agriculture Organization of the United Nations (FAO) and with the generous support of the European Union. Initial results from a trial in five sheep in Tammoun district resulted in distinctive signs of viral replication restricted to the site of scarification of the skin. A scab from one of these animals, taken from the site of scarification eight days after vaccination, was tested in this study by PCR upon the *Orf virus* A32l gene (821bp), which suggests replication of the virus at the site of vaccination.

These results demonstrate that *Orf virus* from locally derived scabs retains high infectivity through laboratory processing and storage, and that these scabs have the potential to serve as a reservoir of material for autologous vaccination of Palestinian sheep in the absence of a commercial licensed vaccine.

ملخص

في فلسطين، لا يوجد لقاح معتمد للاستخدام ضد فيروس Orf والذي يعتبر العامل المسبب لمرض الإكثيمة المعدية. هذا المرض يصيب الأغنام والماعز، ويتميز بتكون جروح حول الفم، و الأنف، والحلمات والضرع. ينتشر مرض الإكثيمة في فلسطين ويسبب خسائر كبيرة بسبب الإعياء والجوع الشديدين الذي يصيب الحيوانات. وقد تم إجراء دراسة ميدانية حديثة لستة عشر مزرعة في الضفة الغربية من قبل جامعة بوليتكنك فلسطين بالشراكة مع مركز البحوث الزراعية الوطنية - قباطية والتي أظهرت أن معدل الوفيات بلغ 50٪ بين الحملان المولودة حديثا في المزارع التي شملتها الدراسة. تم جمع القشور(الجلبة) من الحيوانات التي شملتها الدراسة لتأكيد الاصابة باستخدام تفاعل البلمرة المتسلسل، واستخدمت العينات التي أظهرت نتائج إيجابية لفيروس Orf في هذه الدراسة لتحضير وتقييم القشور المصابة عن طريق زراعة الفيروسات في المختبر لاستخدامها كلقاح ذاتي حي مناسب للتسليم والاستخدام للأغنام الفلسطينية عن طريق إحداث خدش في الجلد

تم طحن القشور وخلطها بمحلول PBS ومن ثم تصفية هذا المحلول وترشيحه ليصبح جاهزا للحقن في كل من خلايا Vero و أغشية الكوريون لأجنة الدجاج. ليتم بعد ذلك اختبار ظهور اثار الاعتلال الخلوي على كل من خلايا Vero وأغشية الكوريون لأجنة الدجاج بعد الحقن . لم يتم ملاحظة ظهور اعتلال خلوي على خلايا Vero المحقونة بالفيروس أما بالنسبة لأغشية الكوريون فقد ظهرت بقع وبثور مميزة على ثلاثة عشر عينة مختلفة من القشور من أصل ستة عشر عينة من القشور المصابة وذلك بعد خمسة أيام من حقنها بالفيروس ، وقد تم كذلك اختبار ظهور الاعتلال الخلوي على أغشية الكوريون لدى أجنة الدجاج خلال إجراء سلسلة من التخفيفات للفيروس من 10^0 الى 10^{-8}

تم إجراء مقارنة بين طريقتين مختلفتين للحقن داخل غشاء الكوريون، وتبين أن الحقن عبر كيس الهواء الاصطناعي هو الطريق الأكثر حساسية . وهذه أول مقارنة لطرق حقن فيروس Orf داخل غشاء الكوريون لبيض الدجاج للكشف عنه . ثم بعد ذلك تم إجراء معايرة لفيروس Orf لتحديد الجرعة المعدية وذلك خلال ا لتخفيفات المتسلسلة للفيروس والتي تتراوح ما بين $10^0 - 10^{-9}$. حيث تم حقن 0.2 مل من كل تخفيف أربع مرات منفصلة (ن = 4) وتم تحديد 50٪ من الجرعة المعدية في البيض (EID_{50}) وهي $5x10^8$. وقد ظهرت علاقة انحدار خطية بين عدد البثور التي ظهرت على أغشية البيض المحقون و نسبة تخفيف الفيروس حيث أن عدد البثور المتكونة على غشاء الكوريون تزاد بزيادة تركيز الفيروس والعكس.

بعد التأكد من وجود عدوى فيروس Orf كما تبين سابقا ، تم تجميع خمسة من رواشح القشور لخمسة من الأغنام من مزارع مختلفة وخلطها مع الجلسرين و PBS كسواغ لاختبار لقاح ذاتي، وقد قام باختبار اللقاح الذاتي على هده الأغنام كل من الدكتور روبن أبو غزالة والدكتور جهاد إبراهيم في المزارع التي لديها تاريخ سابق للإصابة بفيروس Orf بالتعاون مع منظمة الأغذية والزراعة للأمم المتحدة (الفاو) وبدعم سخي من الاتحاد الأوروبي.
وقد أسفرت النتائج الأولية من تجربة اللقاح الذاتي على الأغنام الخمسة في منطقة طمون عن ظهور علامات مميزة لتكاثر الفيروس ول هذه العلامات اقتصر ظهورها في موقع إحداث الخدش من الجلد أي مكان التطعيم.
تم أخد عينة من القشور (الجلبة) التي تشكلت في مكان حدوث الخدش في الجلد بعد أسبوع واحد من التطعيم.لدى أحد الحيوانات المطعمة وتم إعادة هذه العينة من القشور بعد التطعيم لجامعة بوليتكنك فلسطين حيث تم استخراج الحمض النووي لفيروس Orf وتأكيده عن طريق تفاعل البلمرة المتسلسل باستخدام الجين الخاص بفيروس Orf وهو جين A32L(821bp)مما يشير الى تكاثر الفيروس في موقع التطعيم.

هذه النتائج تثبت أن فيروس Orf ، المستمد من القشور(الجلبة) المصابة محليا يحتفظ بقدرته على إحداث عدوى عالية و ذلك من خلال تجهيزات ملائمة داخل المختبر بالإضافة الى توفر ظروف التخزين للفيروس، وبالتالي هذه الجلبة لديها القدرة على أن تكون بمثابة مخزن ومصدر لمواد التلقيح الذاتي للأغنام الفلسطيني في ظل عدم وجود لقاح تجاري مرخص.

DECLARATION

I declare that the Master Thesis entitled "Characterization of *Orf virus* Loads in Ecthymous Scabs for Autologous Vaccine Development in Palestinian Sheep" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name: Dua'a Abu Shkhaidem

Signature: ___________________

Date: 28/6/2016

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Dedication

To my mother who has always loved me unconditionally and whose good example has taught me to work hard for the things that I aspire to achieve.

To my father, who has been a constant source of support and encouragement during the challenges of graduate school and life.

To my brothers and sisters who have never left my side and whose words of encouragement and advocacy for tenacity ring in my ears.

To all my professors who taught me in all grades.

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Abbreviations:

CAM	**Chorio-allantoic membrane.**
CPE	**Cytopathic effects.**
DMEM	**Dulbecco's Modified Eagle's Medium.**
dNTPs	**Deoxynucleotides.**
FCS	**Fetal Calf Serum.**
RCF	**Relative centrifugal force.**
PBS	**Phosphate-buffered saline.**
PCR	**Polymerase Chain Reaction.**
SPF	**Specific pathogen free.**
(strep/pen)	**Streptomycin and penicillin.**
TBE buffer	**Tris/Borate/EDTA buffer.**
TE buffer	**Tris/EDTA buffer.**
UV	**Ultraviolet.**
(w/v)	**Weight to volume.**
EID_{50}	**50% Embryo Infectious Dose.**
$TCID_{50}$	**50% Tissue culture Infective Dose.**

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CHAPTER 1

1. Introduction

1.1. Contagious Ecthyma of Sheep and Goats

Contagious ecthyma (CE) is caused by an epitheliotropic virus that affects primarily sheep, goats and wild ruminants, with a worldwide distribution wherever sheep are raised. It has been reported specifically in India (Sharma et al., 2016, Dar et al., 2015, Kumar et al., 2014, Maan et al., 2014, Kumar et al., 2013, Bora et al., 2012, Yogisharadhya et al., 2012), Egypt (Zeedan et al., 2015, El-Tholoth et al., 2015, Zeedan et al., 2014, El-Mahdy et al., 2014, Said et al., 2013, Ali et al., 2013, Mahmoud et al., 2010), China (Zhang et al., 2015, Wang et al., 2014, Li et al., 2012, Chi et al., 2013, Chan et al., 2009), Korea (Oem et al., 2013, Oem et al., 2009), United States (Guo et al., 2003), Brazil (Schmidt et al., 2013, Abrahão et al., 2012), Argentina (Peralta et al., 2015), Canada (Tomaselli et al., 2016), Bahrain (Abubakr et al., 2012), Bangladesh (Alam et al., 2016), Israel (Bouznach et al., 2013), Iran (Mombeni et al., 2012), Iraq (Abdalamer and Alrodhan, 2015), Saudi Arabia (Housawi et al., 2012), Syria and Turkey (Akkutay-Yoldar et al., 2016), Ethiopia (Gelaye et al., 2016), Gabon (Maganga et al., 2016), Sudan (Khalafalla et al., 2015, Sharma et al., 2016). The disease is characterized by the appearance of lesions around the epithelium of the mouth, lips, nostrils, eyelids, teats, udder, and oral mucosa **(Figure 1.1)**. These lesions develop through the stages of local erythema, followed by formation of papules, vesicles, pustules and scabs. Lesions around the mouth can be severe enough to prevent animals from feeding, and suckling lambs are especially susceptible to starvation due to either loss of appetite from the painful lesions on their mouths or to rejection by their dams because lesions on the udder may cause the dam pain during suckling (Haig and Mclnnes, 2002). Furthermore, secondary infections with bacteria and fungi are common and these may increase the seriousness of the disease. Mortality rates range from 10% to 93% depending on the age and physiological status of animals (Li et al., 2012, Lin et al., 2010, Chan et al., 2009, Lojkic et al., 2010) and morbidity commonly reaches 100% (Nandi et al., 2011).

Contagious ecthyma causes lost production and massive negative effects on animal welfare (Bora et al., 2012, Lojkic et al., 2010), and has a significant economic impact on developing countries that depend mainly on livestock farming for their livelihood and income (Oktay and GÖKÇE, 2005, Haig and Mclnnes, 2002). The disease may also have a direct health impact upon farmers as it is a zoonotic disease, although animal to human transmission is infrequent, and requires close and direct contact between infected animals and humans who have a break in their skin. The most common groups of people affected are farmers, and veterinarians, who are at greatest risk during lambing, shearing, or slaughtering of affected animals (Veraldi et al., 2014).

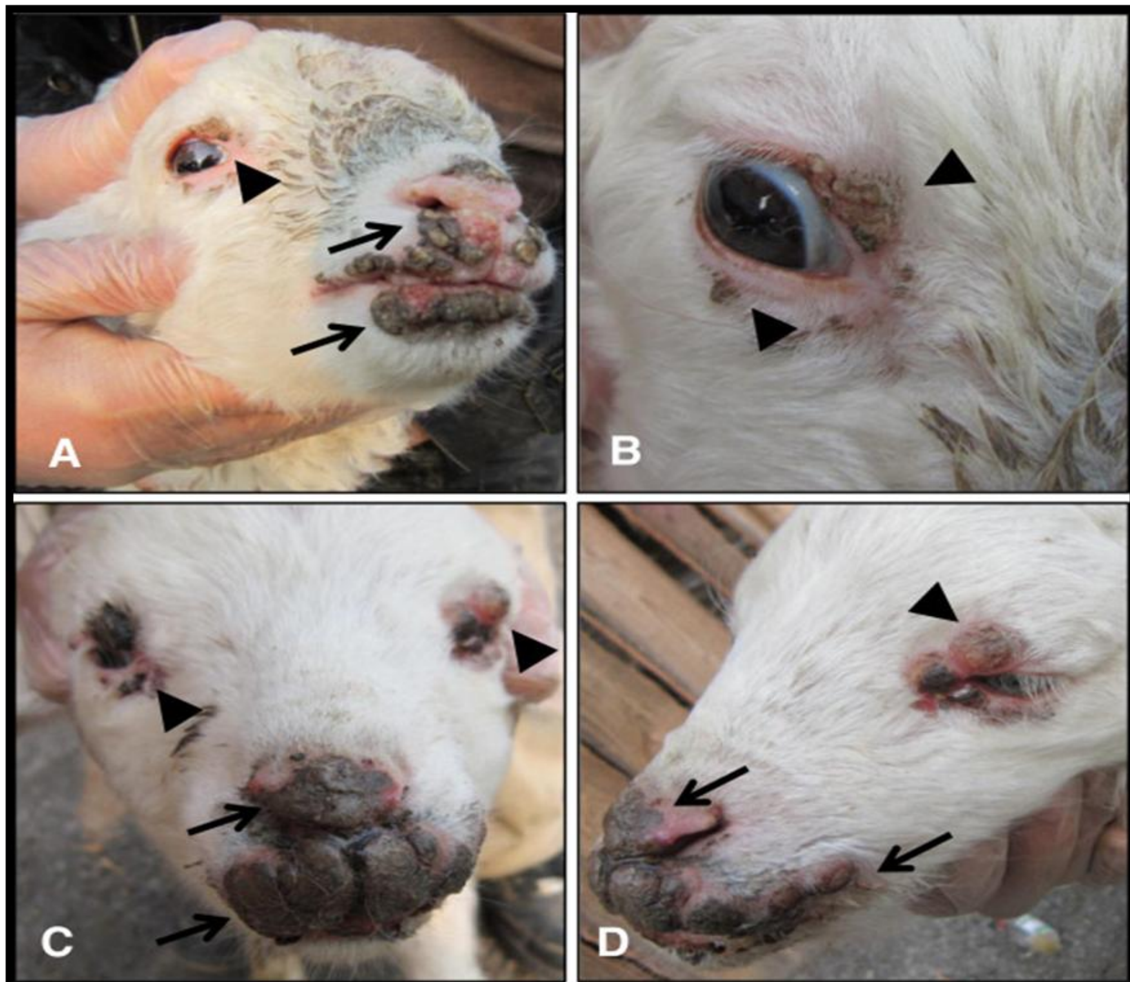

Figure 1.1: Sheep infected with *Orf virus* are characterized by proliferative lesions. The lips and nostrils (black arrows), and the eyelids (black arrowheads) are the most common sites of infection (Li et al., 2012).

1.2. *Orf virus* Classification and Structure

The causative agent of contagious ecthyma in sheep and goats is *Orf virus* (ORFV), which is a member of the genus *Parapoxvirus* (PPV) of the *Chordopoxvirinae* subfamily and family *Poxviridae*. The other members of the genus *Parapoxvirus* include: *Bovine papular stomatitis virus* (BPSV), *Pseudocowpox virus* (PCPV), and *Parapoxvirus* of red deer in New Zealand (PVNZ) (Kottaridi et al., 2006b, Robinson et al., 1982).

The linear double stranded DNA genome of *Orf virus* is the smallest in the family *Poxviridae* with a length of 134-140kbp (Wang et al., 2014, Yogisharadhya et al., 2012, Lin et al., 2010, Chan et al., 2009, Kottaridi et al., 2006b) which contains 132 genes (Li et al., 2012). Conserved genes (ORFs 009–111) (Bora et al., 2012) are found in the central region of the genome and are essential for viral DNA replication and virus particle production in the cytoplasm of infected cells, while variable genes (ORFs 001–008 and 112–134) (Bora et al., 2012) are found at the terminal ends of the genome and encode products that are involved in the interaction between virus and host cells that determine viral pathogenicity and virulence (Yogisharadhya et al., 2012, Mercer et al., 2007, Delhon et al., 2004). Like all the *Poxviridae, Orf virus* replication occurs in the cytoplasm of the host cells.

1.3. *Orf virus* Interactions with the Host Immune Response

When *Orf virus* infection occurs, the host immune response initiates with migration of neutrophil cells to the infection site, followed by accumulation of dendritic cells, $CD4^{+}$TC and $CD8^{+}$TC on the infected dermis cells. Dendritic cells are important at the early stage of viral infection for maintenance of the immune response, and $CD4^{+}$TC and $CD8^{+}$TC also play a crucial role in the host antiviral immunity by killing virus infected cells through the MHC class I pathway (Weber et al., 2013, Haig et al., 1999, Haig and Fleming, 1999). *Orf virus* reduces the primary immune response of T-cells by enhancing apoptosis of antigen presenting cells by a CD 95 pathway (Weber et al., 2013). It encodes apoptotic proteins, which induce CD 95 resulting in apoptosis of monocytes and macrophages via CD95/CD95L (Kruse and Weber, 2001). Moreover, *Orf virus* has its own mechanism to kill epithelial cells and lymphocytes to avoid the active immune response of the infection site, which may

explain why *Orf virus* infection induces a remarkably short-lived immunity post-infection and has the ability to re-infect the host soon after healing (Alcamí and Smith, 1996).

Orf virus has many virulence genes which mediate anti-inflammatory effects and interference with host immune defense mechanisms (Haig and McInnes, 2002, Alcamí and Smith, 1996). These virulence genes include: vascular endothelial growth factor (VEGF) that seems to have been acquired from the mammalian genome by horizontal gene transfer and serves the virus by enhancing the growth of blood vessels that support the proliferation of epithelial cells at the lesion site induced by other *Orf virus* genes, which gives the virus a more receptive target cell population for viral replication (Lyttle et al., 1994, Hughes and Friedman, 2005). Cytokine IL-10 is another viral gene homologue of its host and serves as a major virulence gene involved in viral immune evasion (Fleming et al., 1997) by increasing the apoptosis of antigen presenting cells leading to a reduced T-cell mediated immune response (Kruse and Weber, 2001). A gene encoding ORFV interferon resistance (OVIFNR) acts to resist the host mammalian interferon response to viral infection (Haig and McInnes, 2002, Deane et al., 2000, Haig and Fleming, 1999).

1.4. Diagnosis of *Orf virus*

Contagious ecthyma can be diagnosed by its clinical signs, which are characterized by proliferative diffuse or local lesions, and nodular lesions around the skin of the lips, nose, face, ears, feet and udder of infected animals (Guo et al., 2003). However, laboratory confirmation may be necessary to avoid confusion with the clinical signs of other viral diseases such as Foot-and-mouth disease and Lumpy skin disease.The common laboratory tests employed for *Orf virus* detection include: enzyme linked immunosorbent assays (ELISAs), histopathology of affected tissues, serum neutralization test (SNT), nucleic acid based assays including polymerase chain reaction (PCR), restricted fragment length polymorphism (RFLP) analysis and, historically, electron microscopy (Vikøren et al., 2008, Guo et al., 2004).

Polymerase chain reaction is the most commonly performed diagnostic method for detection of *Orf virus* (Zheng et al., 2007, Gallina et al., 2006, Kottaridi et al., 2006a, Nitsche et al., 2006, Inoshima et al., 2000). Various PCR assays have been developed that target the conserved genes in the central region of the genome, such as the B2L or VIR gene (Kottaridi et al., 2006a, Inoshima et al., 2000). Duplex PCR has been reported as a diagnostic method to detect and differentiate *Orf virus* from *Capripoxvirus* (CPV) using the A29 gene and H3L gene (Zheng et al., 2007). Real time PCR has been developed based on the B2L gene for *Orf virus* detection and differentiation from *Pseudocowpox virus* (PCPV), *Bovine papular stomatitis virus* (BPSV), and *Sealpox virus* (SPV) (Gallina et al., 2006, Kottaridi et al., 2006a, Nitsche et al., 2006).

1.5. Propagation of *Orf virus* in Embryonated Chicken Eggs

Embryonated chicken eggs were first described for the isolation and identification of many viruses in the 1930s (Burnet, 1936, Burnet and Galloway, 1934), and their use preceded the advent of cell culture as a method for virus cultivation (Fenner et al., 2013). Embryonated chicken eggs, which contain the embryo and its membranes, serve as an excellent system for the growth of many viruses because they are readily available, inexpensive, easy to maintain, and provide a sterile environment for virus growth. There are four different routes of inoculation into embryonated chicken eggs available for virus cultivation: chorio-allantoic membrane (CAM), allantoic cavity, amniotic cavity, and yolk sac **(Figure 1.2)**. Some examples of viruses that are propagated by each of these four routes, along with the signs of viral growth, are given in **(Table 1.1)** (Fenner et al., 2013).

Chorio-allanotoic Membrane inoculation of chicken eggs is the preferred route for Poxviruses, and was first used in 1931 to culture Fowl pox virus (Burnet, 1936) and then in 1936 to culture Vaccinia virus (Westwood et al., 1957). Inoculation of the CAM can be done directly through the natural air-sac of the egg **(Figure 1.2),** but a more complex variant of CAM inoculation can give better results and involves producing an artificial air-sac **(Figure 1.3)** for inoculation by drawing air out of the natural air-sac with the teat of a Pasteur pipette through a small hole drilled into the top of the egg to create a negative pressure, which pulls

air in through a second hole drilled into the side of the egg, at the position of the needle shown uppermost in **(Figure 1.2)**. As a result of this, the CAM at this position is pulled down (dropped) away from the inner egg shell, and is more receptive to viral spread and growth than the CAM beneath the natural air-sac. This modified method has been used effectively for *Fowl pox virus* and *Vaccinia virus* (Overman and Tamm, 1956, Frisch, 1950, Burnet and Ferry, 1934, Burnet, 1936).

Table 1.1: **The different Inoculation routes in embryonated chicken eggs for various viruses** (Fenner et al., 2013).

Inoculation route	**Virus**	**Signs of virus growth**
Yolk sac	Herpes Simplex	Death
Chrioallantoic membrane (CAM)	Herpes simplex Poxviruses Rous sarcoma	Pocks
Allantoic cavity	Influenza Mumps and Newcastle disease Avain adenovirus	Hemagglutination Death Death
Amniotic cavity	Influenza Mumps	Hemagglutination Death

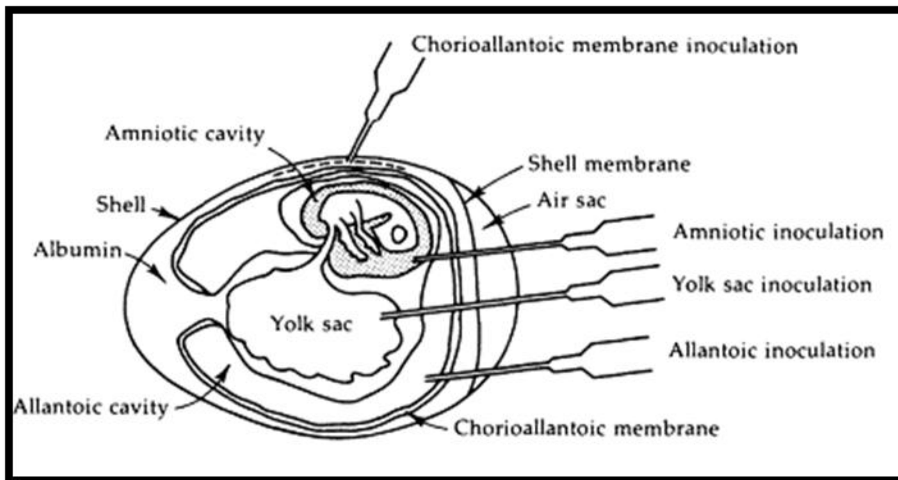

Figure 1.2: The different routes of virus inoculation into embryonated chicken eggs (Fenner et al., 2013).

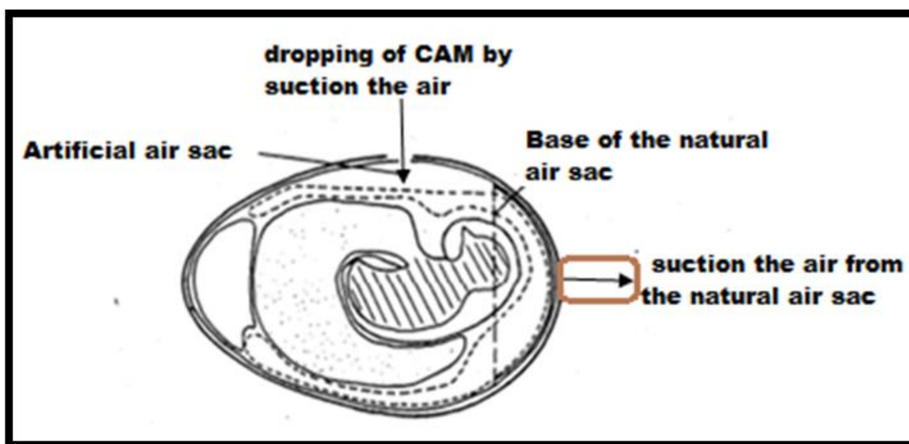

Figure 1.3: **Method of producing an artificial air-sac**. The CAM is dropped through suction of the air from the natural air-sac hole leads to creation a negative pressure which pulls air in through the second hole.

Viral replication on CAM produces visible pocks, white materials, grayish yellow plaques, and thickening areas on the CAM **(Figure 1.4)** (Burnet, 1936).

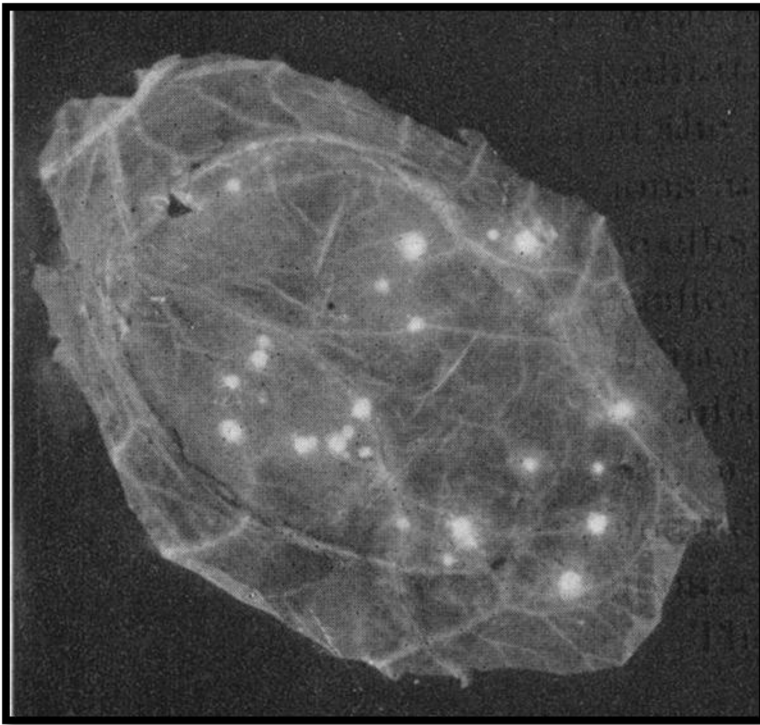

Figure 1.4: Formation of pocks on infected CAM with *Vaccinia virus* (Burnet and Faris, 1942).

1.6. Propagation of *Orf virus* in Animal Cell Culture

Many primary cell cultures and continuous cell lines are used for isolation of *Orf virus*. Primary cell cultures are generally more sensitive for viral replication, but may only be passaged a few times, while cell lines have the great advantage of providing a consistent and long-lasting stock allowing better standardisation and reproducibility of results amongst laboratories world-wide (Pye, 1989).

Primary lamb testis and primary lamb kidney cells are the most commonly used primary cell cultures for *Orf virus* (Tan et al., 2009, Savory et al., 2000, Pye, 1990, Traĭkova, 1981). However, bovine testis cells (Deane et al., 2000, Savory et al., 2000, Balassu and Robinson, 1987), fetal bovine muscle cells (Gumbrell and McGregor, 1997, Robinson et al., 1982), bovine fetal lung cells (Friederichs et al., 2014, Inoshima et al., 2002), ovine fetal turbinate cells (Chi et al., 2013, Li et al., 2012, Delhon et al., 2004), fetal lamb muscle cells (McInnes et al., 2001, Deane et al., 2000), bovine kidney (Fiebig et al., 2011) and bovine lung cells

(Friederichs et al., 2014) have also been used for *Orf virus* culture. *Orf virus* can produce cytopathic effects on the infected primary cell culture after one to three days of incubation (Li et al., 2012, Guo et al., 2003) and sometimes cytopathic effects have been reported to take five to seven days of incubation (Friederichs et al., 2014, Ali et al., 2013). The most common features of cytopathic effects for *Orf virus* are rounding-up of cells, multinucleation of cells, and detachment of cells from the surface **(Figure 1.5)** (Friederichs et al., 2014, Ali et al., 2013, Kottaridi et al., 2006b).

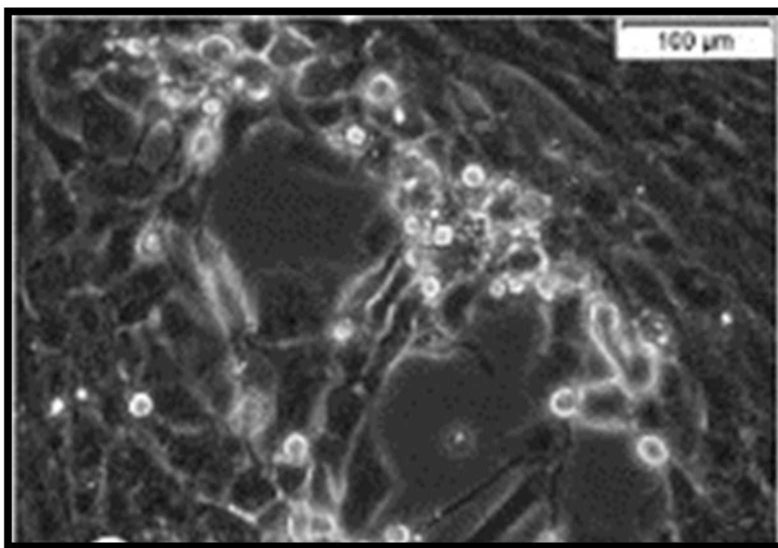

Figure 1.5: Cytopathic effects (CPE) produced by *Orf virus* in primary ovine fetal turbinate (OFTu) cells after thirty six hours of infection (Li et al., 2012).

Among continuous cell lines, Madin-Darby bovine kidney (MDBK) and Madin-Darby ovine kidney (MDOK) cells are the most commonly used for isolation and propagation of *Orf virus* (Ali et al., 2013, Guo et al., 2004, Guo et al., 2003), while RK-13 (Rabbit kidney) (Ali et al., 2013), HEK (Human embryonic Kidney), Hela (derived from cervical cancer), THP-1(Human monocytic cell line), BHK (Baby hamster kidney) (Pye, 1989), CSL (from fetal lamb lung) (Pye, 1989), and OT (Ovine testis) (Oem et al., 2013) have also been used effectively.

Vero cells are a very common continuous cell line, and these were used for the research presented in this study, but results with Vero cells have had mixed success in the past. Live

Orf virus vaccines have been grown in Vero cell cultures (Housawi et al., 2012). In India, primary goat testis and Vero cells were compared for the culture of *Orf virus*, which resulted in cytopathic effects characterized by cell rounding, ballooning, and degeneration, within forty eight hours post-infection for primary goat testis cells, but not for Vero cells, even after five blind passages in Vero cells were performed (Kumar et al., 2014). Another research group from India used the supernatant of a viral suspension prepared from infected scabs for DNA isolation for polymerase chain reaction and for virus isolation using Vero cell cultures, and cytopathic effects were noted in Vero cells in the form of cell rounding and enlargement, karyopyknosis, granularity of the cytoplasm and cell detachment starting from the third or fourth day after infection, which resulted finally in complete destruction of cells (Maan et al., 2014). Also in China, *Orf virus* was isolated in Vero cells, but cytopathic effects were not visible till the fifth passage (Yang et al., 2014).

1.7. Disease Control

1.7.1. Farm Management and Hygiene

Disinfection practices and safety measures must be followed on farms, especially where there is a history of contagious ecthyma, to avoid spread of *Orf virus* infection amongst the herd. These measures are applied to infected animals, uninfected animals, houses and materials used by infected animals and humans. Infected animals should be separated from uninfected animals and fed after feeding the rest of the flock. Newly introduced animals or flocks should be segregated and examined to avoid the possibility of introducing *Orf virus* to an existing flock. Lambs should be prevented from rough grazing on farms with a history of contagious ecthyma to avoid wounding the mouth, which would be an entry point for virus from the environment (Lojkic et al., 2010). Animal houses and materials of infected animals should be disinfected thoroughly after the last infected scabs have been shed, as these scabs may re-introduce the virus in the following lambing season.

Many cases of contagious ecthyma have been reported in humans from various parts of the world (Veraldi et al., 2014, Nougairede et al., 2013, Coskun et al., 2008, Van Lingen et al., 2006, Uzel et al., 2005, Degraeve et al., 1999, Buchan, 1996), and hence those in contact with

infected animals, like farmers, veterinarians, abattoir workers and butchers, should wear protective clothes such as gloves and a gown during animal handling. Infection occurs through direct contact and so handlers should avoid the wounded skin, scabs, and wool of the infected animals. The hands where a cut or graze on the skin or needle puncture has occurred are the most common site of *Orf virus* infection with other sites such as the face only occasionally being involved (Uzel et al., 2005, Gill et al., 1990). Finally, washing hands with soap and water or alcohol 70% is recommended after handling potentially infected animals or materials.

1.7.2. Vaccination against *Orf virus*

A vaccine against contagious ecthyma was developed in the 1930s by Schmidt and Hardy in Texas Agricultural Experiment Station to control the disease and prevent *Orf virus* infection from transmission to the non-infected herd (Boughton and Hardy, 1935). Various types of vaccines have since been prepared against contagious ecthyma virus.

Commercial *Orf virus* vaccines containing live virus prepared in tissue culture have been developed (Zhao et al., 2011, Pye, 1990) and are applied with a small double pointed fork, which holds a small volume of vaccine suspension and simultaneously scratches the skin and dispenses the fluid (Musser et al., 2008).

However, all existing commercial vaccines are live vaccines against contagious ecthyma and there include: Scabivax Forte® (Schering Plough Animal Health Corp., The Netherlands) used in New Zealand is based on live ORFV (strain NZ2), ovine contagious ecthyma vaccine is produced by the Colorado Serum Company (CO, USA), the vaccine Ecthybel®, which is a product of Merial-France, and Vaxall Orf Vaccine®(Willows Francis Veterinary) (Tryland et al., 2013, Bhanuprakash et al., 2012, Gallina et al., 2004).

Autologous vaccines prepared by grinding a piece of removed scab from an infected animal with saline by mortar and pestle and adding antibiotics (Penicillin /Streptomycin) to avoid bacterial contamination have been used informally by veterinary practitioners. These can be maintained for months in a refrigerator (Bath et al., 2005).

DNA vaccines may serve as more convenient and safe vaccines against *Orf virus* than the currently used live attenuated vaccines, because they can induce both neutralizing antibodies and a cellular immune response without viral replication and tis associated contamination risks (Zhao et al., 2011).

Despite the existence of these commercial vaccines, their use is prevented in Palestine because the Israeli occupation authority has not registered a vaccine for use against *Orf virus*. The development and adoption of a local Palestinian alternative means of vaccination would enable farmers to protect their flocks in the face of considerable losses due to this disease.

CHAPTER 2

2. Objectives

Overall objective/aim

To provide a ‘proof of principle’ for autologous vaccination in Palestine using infectious scabs from sheep that have been diagnosed with contagious ecthyma.

Sub objectives

1. To confirm the presence of live *Orf virus* in scabs, from sheep that have been diagnosed with contagious ecthyma.
2. To optimize a method of *Orf virus* propagation in embryonated chicken eggs or Vero cell culture.
3. To quantify the amount of live *Orf virus* in a scab by titration to either an EID_{50} or a $TCID_{50}$ value.
4. To prepare a live *Orf virus* vaccine formulation for an autologous vaccination trial in lambs on a Palestinian farm.

CHAPTER 3

3. Materials and Methods

3.1. Materials:

Table 3.1: Equipment and consumables.

Name	Company	Catalog number
Equipment		
Water jacketed CO_2 incubator	Thermo Scientific; USA	3110
Inverted light microscope	Micros; Austria	MCXI600
Tissue homogenizer	Heidolph; Germany	91126
Gel documentation system	Bio-Rad; America	721
Consumables		
Agarose	SIGMA; Jerusalem	A9539
DMEM media	Biological Industries	01-050-1A
Ethanol 99%	Bio-lab; Jerusalem	05252301
Ethanol 70%	GADOT	64-17-5
Fetal Calf Serum (FCS)	Biological Industries	04-122-1A
Marker (DNA 100bp)	Genedirex	DM011-R500
phosphate-buffered Saline (PBS)	Biological Industries	02-020-1A

QIAamp DNA Mini Kit	QIAGEN, Germany	51304
QIAamp DNA blood Kit	QIAGEN, Germany	51304
Strep/Pen	Biological Industries	1445472
Sterile Glycerol	AMRESCO; Jerusalem	0854
Tris –Borate –EDTA buffer for gel electrophoresis	SIGMA; Jerusalem	T7527-4L
Tris-EDTA buffer	Hy labs; Jerusalem	T7527
25cm^2 Cell culture flasks	SPL Life Sciences	70025
27-gauge needle	KDL Medical Product Company	130410
1ml syringe	KDL Medical Product Company	130410
Israeli chicken eggs- Ross strain	Al Tamimi for trading and agriculture	223
Spanish chicken eggs	Al Tamimi for trading and agriculture	414
Ballade chicken eggs	Ghassan Natsheh farm; Hebron	
Fowl pox live vaccine	Biovac	110
Vero Cells	Kind donation from Konkuk University, Seoul, South Korea.	
Trypsin-EDTA	BIOLOGICAL INDUSTRIES	1430198

3.2. Methods

3.2.1. Viral Isolation from Scabs

Scabs from sheep and goats with clinical signs of contagious ecthyma were collected by Dr. Jehad El-Ibrahim from sixteen farms in the West Bank between 2013 and 2015. Ethical

approval was through the Ministry Of Agriculture, National Agricultural Research Center, and farmers full consent was obtained before sampling. The scab samples were ground by mortar and pestle and suspended to 10% (w/v) sterile phosphate-buffered saline (PBS) contained antibiotics (strep/pen) (El-Mahdy et al., 2014, Ali et al., 2013). The suspensions were frozen at -80°C and thawed three successive times, and then centrifuged at rcf of 2000 g for thirty minutes at 4°C (Kottaridi et al., 2006b). The clarified supernatant was passed through a 0.45μm pore size filter to remove any residual bacterial or fungal contamination (Friederichs et al., 2014) and stored at -80°C until required.

3.2.2. Viral Propagation in Embryonated Chicken Eggs

3.2.2.1. Egg Candling

Embryo development was monitored by candling during the incubation time using a light transmitted through the egg shell against the opaque egg surface. Candling was achieved by shining a strong light source directly above the egg in a dark room. After four days in an egg incubator **(Figure 3.1)**, fertilized eggs were distinguished from unfertilized eggs by the presence of a network of thin clear blood vessels **(Figure 3.2C),** which were absent in unfertilized eggs that appeared as empty except for a visible yellow yolk and the air-sac **(Figure 3.2A)**. As for early dead embryos, these displayed a broken pattern of blood vessels and an immobile embryo inside the egg **(Figure 3.2B),** which were distinguished after checking at seven days of incubation, embryo viability was characterized by mobility of the embryo inside the egg. The dead embryos and unfertilized eggs were discarded.

Figure 3.1: Egg incubator that moves the eggs by tilting automatically after the third day of incubation, with a temperature of 37°C and humidity 55% - 60%.

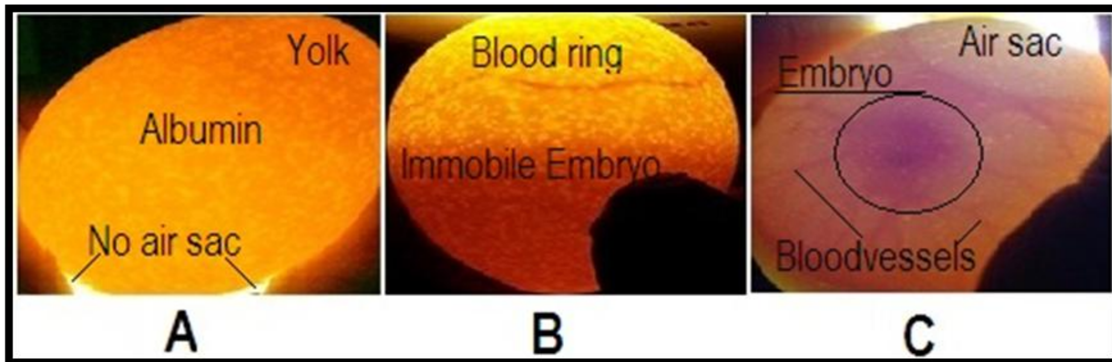

Figure 3.2: checking eggs for viability of embryos by candling. A: unfertilized egg, **B:** fertilized egg with an early dead embryo, **C**: healthy embryo on the sixth day of incubation.

3.2.2.2. Inoculation Routes

Two routes of inoculation were evaluated for propagation of *Orf virus* from scab suspensions on the chorio-allantoic membrane (CAM) of embryonated chicken eggs. One route is through the natural air-sac, and the second route is through an artificial air-sac. *Sheep pox* and *Fowl pox* live vaccines were used as positive controls, and eggs injected with PBS were used as negative controls.

A. CAM Top Route (Natural air-sac)

The shell of the embryonated chicken egg (11 days old) was first disinfected by wiping with 70% alcohol. Then a hole was punched through the shell in the top of the air-sac, with attention that the hole should be far from the large blood vessels, followed by injection through this hole onto the surface of the CAM below the natural air-sac **(Figure 3.3).**

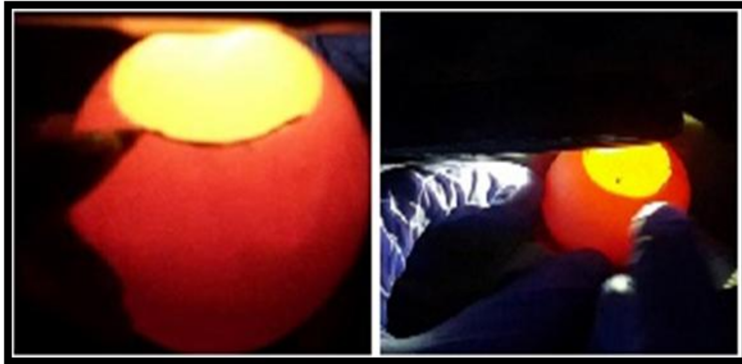

Figure 3.3: marking natural air-sac and inoculation site for inoculation through CAM top route.

B. Dropped CAM Route (Artificial air-sac)

The shell of the embryonated chicken egg (11 days old) was first disinfected by wiping with 70% ethyl alcohol. The natural air-sac was outlined with a ring at the top of the egg and a site for penetrating the shell was marked, keeping away from any blood vessels, with a small cross. After that, the egg was placed on its side and a hole was made, also avoiding any blood vessels, where the artificial air-sac would be generated **(Figure 3.4A).** A second hole was then made at the previously marked site in the natural-air sac, and the air was extracted through this hole by suction using a rubber teat **(Figure 3.4B).**

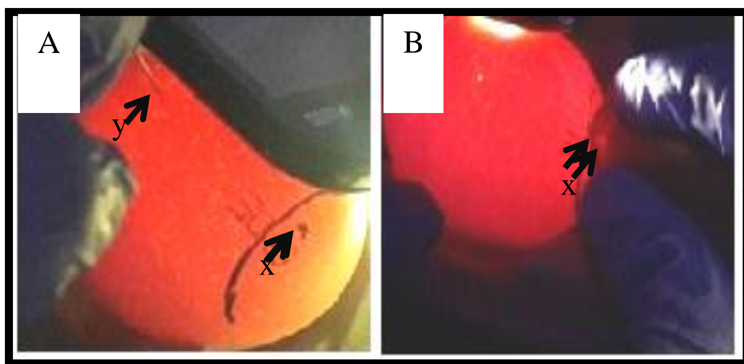

Figure 3.4: Steps in the generation of an artificial air-sac for inoculation by the dropped CAM route. A: punching a whole through the shell at the position marked 'y' where the artificial air-sac will be made is followed by removal of air from the natural air-sac **B:** by

suction through a hole made at position ‘x’, which drops the CAM by displacement forming an artificial air-sac beneath the hole at position ‘y’ at 90^0 to the natural air-sac.

3.2.2.3. Inoculation of CAMs with Scab Suspensions

The filtered supernatants of scab suspensions were removed from storage at $^-80$°C and were thawed on ice completely. A 25-27-gauge needle was inserted into the artificial air-sac or into the natural air-sac through a point above the periphery of the air-sac, and 0.1 ml of inoculum was dropped carefully onto the CAM **(Figure 3.5)**, followed by sealing the inoculation site with a drop of melted wax. After inoculation, the eggs were laid horizontally, and the inoculation site was kept uppermost for twenty-four hours in the incubator at 37°C and 55% - 60% humidity (Brauer and Chen, 2015) without turning, then the eggs were returned to the natural upright position with regularl turning each two hours.

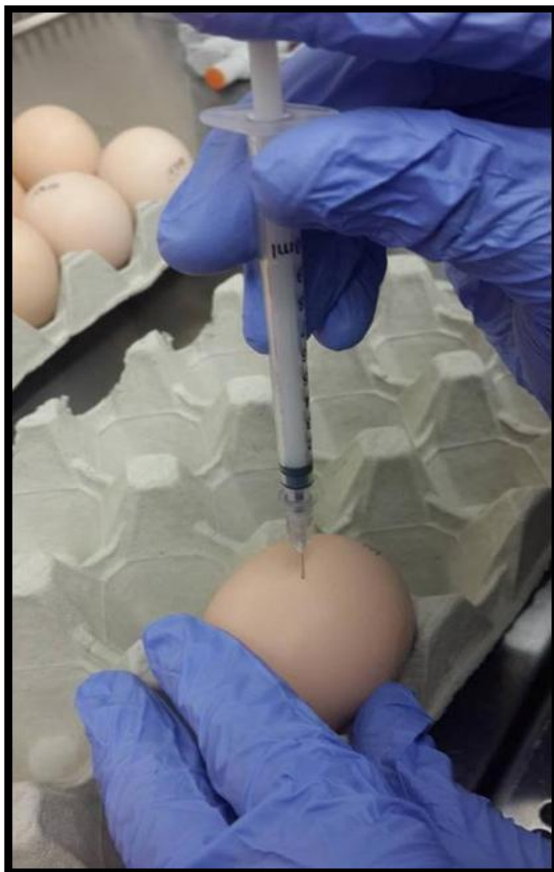

Figure 3.5: Viral inoculation with a needle through the natural air-sac.

3.2.2.4. Harvesting of CAMs

The inoculated eggs were candled at least once daily for five successive days post inoculation to check that the embryos were still alive, and any that died within the first twenty-four hours of inoculation were excluded. At the end of five days post inoculation, the eggs were cut in half **(Figure.3.6b)**, and the CAMs of each half were peeled away from the shell and washed with PBS to examine for the presence of white pock lesions, thickening areas and whitening materials. The infected CAMs were pooled and stored at -80°C to use for further passages on CAM or viral DNA extraction as needed.

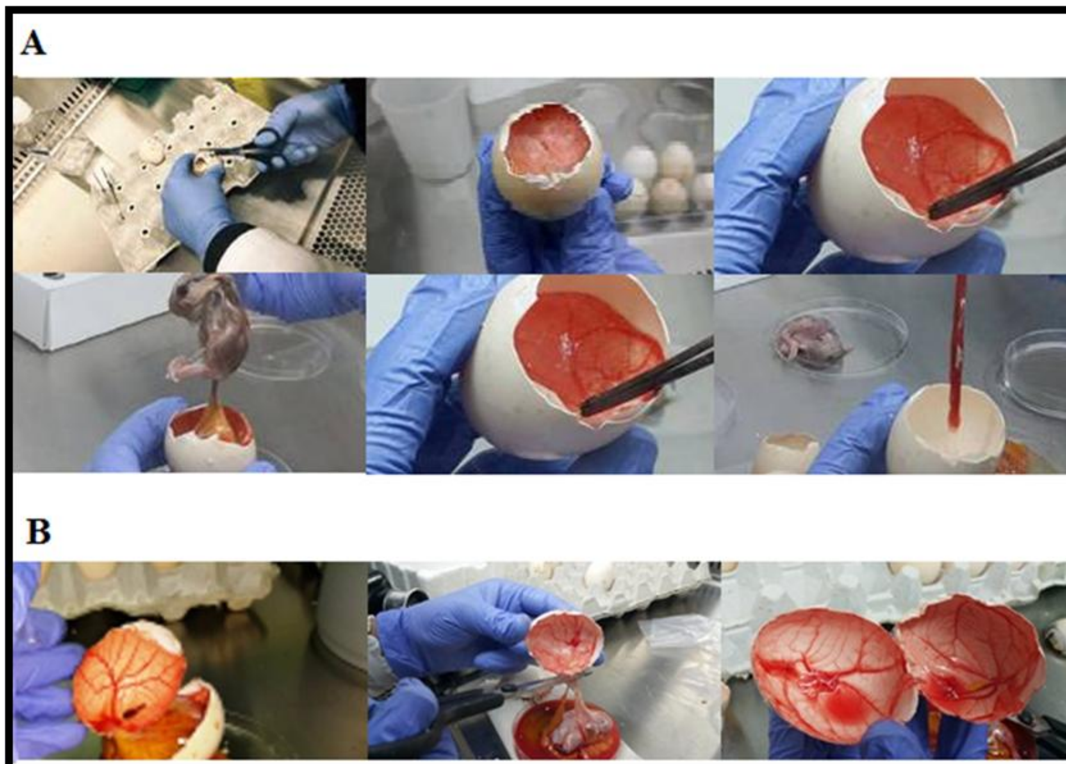

Figure 3.6: Harvesting infected CAM. **A**: from the top of air-sac, this method was used after inoculation through the natural air-sac. **B**: by cutting in half laterally, this method was used after inoculation through the artificial air-sac.

3.2.3. Viral Propagation in Vero Cell Culture

3.2.3.1. Vero Cell Line Propagation

Vero cells preserved in cell culture vials at $^{-}80°C$ were thawed rapidly in a water bath at 37°C and transferred after complete thawing immediately from the vial into a falcon tube, whereupon cells were suspended with DMEM growth media that was supplemented with 10% of fetal calf serum (FCS) and antibiotics (strep/pen). The suspension of cells was centrifuged at rcf of 200g for five minutes to pellet the cells, which were then suspended in DMEM media. The cells were propagated in $25cm^2$ culture flasks and incubated at 37°C in a humidified CO_2 incubator (5%). On the following day, media was changed to remove any non-adherent cells, and culture media was changed thereafter every two to three days until confluent cell monolayers were achieved, as judged by regular observation using an inverted light microscope **(Figure 3.7).**

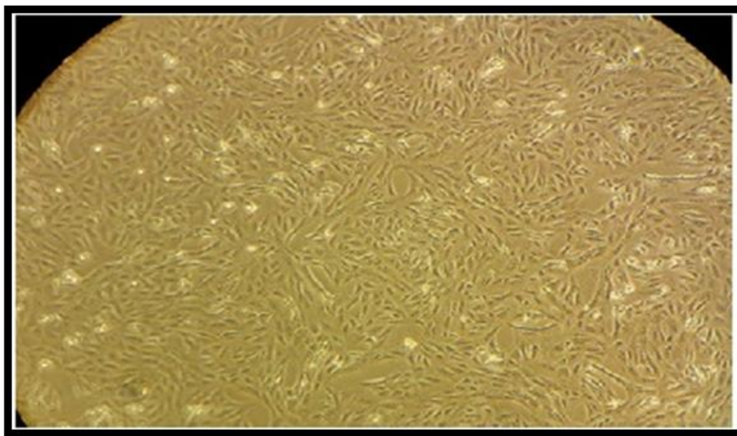

Figure 3.7: A confluent monolayer of Vero cells on a T-$25cm^2$ culture flask.

3.2.3.2. Trypsinization of Vero Cells

Confluent Vero cells were passaged by trypsinization. The old media was removed and the cells were washed with PBS. Then, one ml of trypsin-EDTA was added to detach the cells from the old flask. When detachment had occurred, five ml of DMEM media with 10% FCS were added to neutralize the trypsin, and the suspension of detached cells was centrifuged at rcf of 200 g for five minutes, and the cell pellet was re-suspended in DMEM with 10% FCS and split between as many flasks as required.

3.2.3.3. Inoculation of Vero Cells

Seven of the same scab suspensions used in embryonated chicken eggs were used to inoculate Vero cell culture. Three attempts at virus propagation from scab suspensions were performed in Vero cells, which were subsequently observed for evidence of cytopathic effects, and one of these attempts was repeated for a second round on a fresh flask of cells in the hope that a second 'blind passage' would allow observation of CPE. Different dilutions of virus were prepared in PBS and inoculated onto confluent Vero cells in 25cm^2 culture flasks, where each flask received 200 µl of virus dilution or culture media alone for the negative control. Adequate time for virus adsorption to the host cells was allowed by incubation at 37°C for two hours in a humidified 5% CO_2 incubator. After allowing for viral adsorption, the excess inoculum was removed from the tissue culture flasks, and 4 ml of DMEM media supplemented with antibiotics (strep/pen) and 5% FCS were added to each infected flask and also to the negative control flask (Kottaridi et al., 2006, Mangana-Vougiouka et al., 1999). The culture medium was changed approximately every five days, and microscopic examination for cytopathic effect was continued for three to five weeks.

3.2.3.4. Harvesting Media from Vero Cells

The culture media of infected cells and controls were harvested each time media was changed, and stored at $^-$80°C until the time of viral DNA extraction (Li et al., 2012, Guo et al., 2003).

3.2.4. Viral Titration of Scabs and CAMs in Embryonated Chicken Eggs

Titration of *Orf virus* samples was performed in 11 day-old embryonated chicken eggs. Tenfold Serial dilutions from suspensions of ground filtered scabs and ground infected CAM were prepared to a maximum dilution of 10^{-9} in phosphate buffered saline, and 0.2 ml of each dilution was inoculated per egg. Eggs injected with 0.2 ml of PBS and uninfected eggs were used as negative controls.

3.2.5. Vaccine Preparation

Orf virus live vaccine was prepared from the viral suspension of scabs and also from infected CAMs. In both cases, several isolates from each of scabs or CAMs were pooled to be more representative than a single scab or CAM.

3.2.5.1. Infected Scabs

The frozen viral suspensions of scabs were thawed on ice and vortexed for twenty seconds, and 2000 µl of glycerol 50% was added to 1000 µl of total viral suspensions. The glycerol was added as a protective agent to prevent vaccine from freezing and freeze damage by occurrence of sedimentations and agglomeration as described by (Braun et al., 2009). Then, the mixture was vortexed for twenty seconds and two aliquots were prepared in 1.5ml eppendorf tubes and stored at $^{-}$80°C ready for a future vaccination trial in sheep.

3.2.5.2. Infected CAMs

The frozen infected CAMs were ground by mortar and pestle and suspended to 10% (w/v) sterile phosphate buffered containing antibiotics (strep/pen). The suspensions were centrifuged at rcf of 600 g for twenty minutes at 4°C and 300 µl of each sample was taken and mixed to each other, 2000 µl of glycerol 50% was added to 1500 µl of total viral suspensions and mixed by vortexing for twenty seconds, and two aliquots were prepared in 1.5ml eppendorf tubes. The prepared vaccines were stored at $^{-}$80°C ready for vaccination trials in sheep.

3.2.6. Viral DNA Extraction

Viral DNA was extracted from the infected chorio-allantoic membranes with pock lesions as described in (Mahmoud et al., 2010). The method involved homogenization of infected chorio-allantoic membranes, by grinding with a mortar and pestle, from which 20 mg of ground tissue was weighed into a 2 ml microfuge tube. Lysis buffer and proteinase K were then added and incubated at 56°C in a water bath until complete lysis of the tissue occurred

(one-three hours). DNA was then extracted from the lysed tissue using a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions.

Viral DNA was extracted from the scabs of vaccinated animals according to (Pignatti et al., 1979). The scab sample was frozen at $^-$80°C for twenty minutes, and then lysis solution was added to the scab, which was then incubated at room temperature for ten minutes. After that, 0.5M NaCl was added to the suspension and centrifuged at a rcf of 1000g for ten minutes at 4°C. After that, DNA was extracted from the supernatant using an RNA virus nucleospin kit (Macherey-Nagel), which is effective for both DNA and RNA viruses, in accordance with the manufacturer's instructions.

Viral DNA was extracted from 200 µl of cell culture medium as described in (Li et al., 2012) using the QIAamp DNA blood kit (QIAGEN) and following the manufacturer's instructions.

3.2.7. Polymerase Chain Reaction (PCR)

PCR was performed on DNA extracted from infected CAMs, infected cell culture medium, and scabs of vaccinated animals using A32L gene primers **(Table 3.2)** as described by (Chan et al., 2009). Primers delivered from Hy laboratories Ltd. Rehovot, were dissolved in 1x TE buffer to get a concentration of 100 pmol/µl from which working dilutions were made to a final concentration of 10 pmol/µl and these were stored at $^-$20°C until needed.

Polymerase chain reactions were carried out in a total volume of 25µl reaction mixture, which contained 2.5 µl of 10x reaction buffer, 0.5 µl of 10 µM dNTPs, 0.5 µl of each the forward and reverse primer working stock,1 µl of template DNA, 2.5 µl of 20 mM $MgSO_4$, and 0.6 units of Taq DNA polymerase. PCR was performed in a thermo cycler (MJ Mini; BioRad) under the following program: Initial denaturation at 94°C for four minutes followed by thirty-five cycles of 94°C for forty seconds, 62°C for forty seconds, 72°C for forty seconds, and a final extension of 72°C for ten minutes.

Table 3.2: Description of primers for PCR that target the A32L gene of *Orf virus*. The sequences, primer lengths, Tm, and the amplicon size are shown (Chan et al., 2009).

Primer	Sequence (5'-3')	length	Tm	Size of amplicon
Forward	GAGGGCGCGAGCACCATTTA	20 mer	63°C	821bp
Reverse	CGGAGCCGGTAATTTAGTGACAGT	24 mer	65°C	

3.2.8. Agarose Gel Electrophoresis

PCR products were analyzed using 1% agarose gel electrophoresis in 1X TBE buffer, stained with ethidium bromide dye. The voltage power ranged between 100 and 130 volts, and amplicons were visualized as bands under ultra violet light (UV) and photographed using a gel documentation system. A 100 base pair DNA ladder was used as the DNA molecular weight marker.

CHAPTER 4

4. Results

4.1. Optimum Route of *Orf virus* Inoculation onto the Chorio-Allantoic Membrane

The optimum route for *Orf virus* inoculation onto the chorio-allantoic membrane was determined based on the clarity of the pock lesions that appeared on the infected CAM. CAMs inoculated through the natural air-sac and artificial air-sac route were compared. CAM inoculations through the artificial air-sac showed more satisfactory results than inoculation through the natural air-sac, because the pocks resulting from inoculation through the natural air-sac were much harder to see. Distinct pocks and white materials appeared more clearly on CAM inoculated through the artificial air-sac **(Figure 4.1),** while CAM inoculated through the natural air-sac displayed few signs of the presence of *Orf virus*

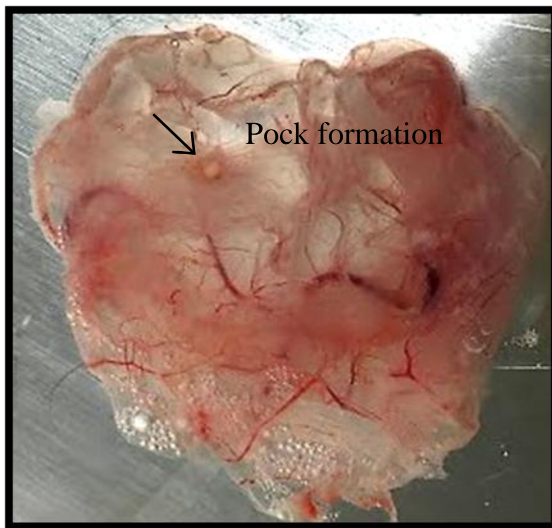

Figure 4.1: Infected CAM after *Orf virus* inoculation through an artificial air-sac, showing a distinct pock and thickened area.

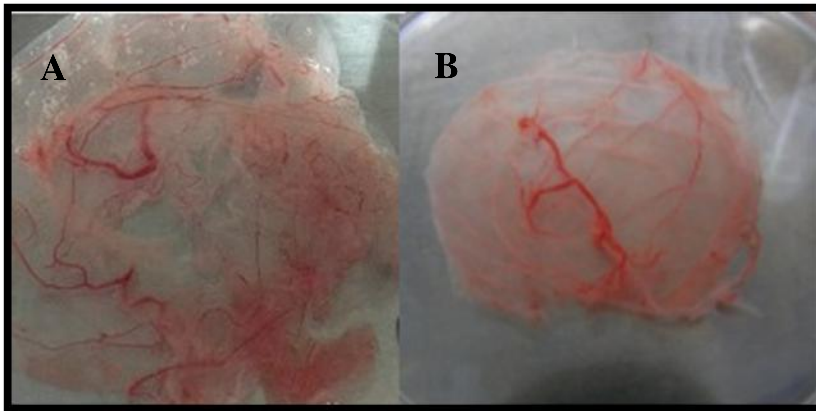

Figure 4.2: Typical result of CAM infected with *Orf virus* through the natural air-sac route. **A**: did not show the viral infection signs, and could not be distinguished from **B**: the negative control.

4.2. *Orf virus* Isolation and Propagation

Viral suspensions prepared from scabs of animals were propagated by inoculation onto chorio-allantoic membrane of embryonated chicken eggs and onto Vero cells.

4.2.1. Virus Propagation in the CAM of the Embryonated Chicken Eggs

Sixteen samples of scabs, taken from different sheep on nine farms, that were previously determined to be positive for *Orf virus* were inoculated onto the CAM of embryonated chicken eggs **(Table 4.1).** The proportion of these that produced signs of viral infection on the CAMs was 13/16 (81%). The nature of these pathological signs on CAM were pock lesions **(Figure 4.3),** whitening materials and thickening areas **(Figure 4.4),** in addition to the peeling of CAM, hemorrhage and small black or gray foci **(Figure 4.5),** compared with the negative controls where CAMs were normal without pocks or any of the other signs of virus infection **(Figure 4.6).** Positive controls were needed in setting up the technique for the first time in the laboratory and were obtained from commercial vaccines containing live *Sheep pox* or *Fowl pox* virus (note that commercial *Orf virus* vaccines are not available in Palestine); however, *Sheep pox* or *Fowl pox* virus are both well characterized for CAM inoculation, and these gave distinct signs of viral infection with very well-defined primary pocks **(Figure 4.7).** *Orf virus* infection was confirmed further by screening the positive

samples of CAMs with PCR using OVA32LF1 and OVA32LR1 primers that are specific for the A32L gene of *Orf virus*. An amplicon of the appropriate size, 821bp, was successfully amplified from infected CAMs **(Figure 4.8).**

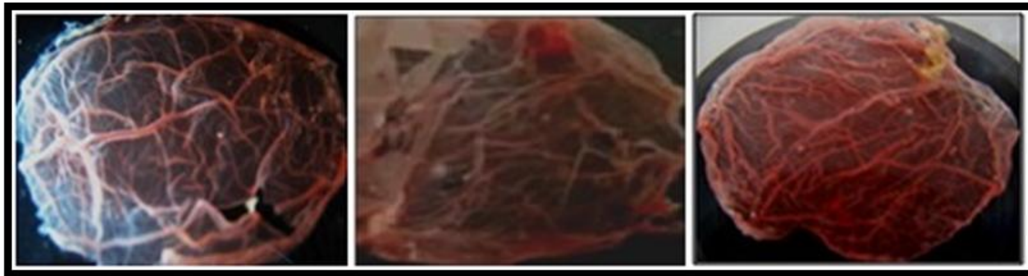

Figure 4.3: Positive Infected CAMs with *Orf virus* showing pock formation on CAM.

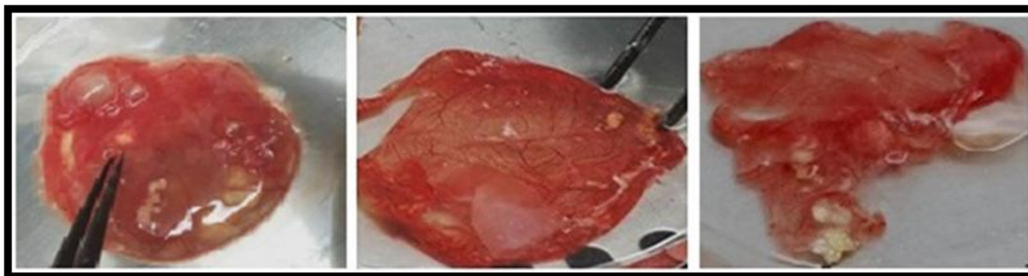

Figure 4.4: Positive infected CAM with *Orf virus* showing white materials and thickening areas on CAM.

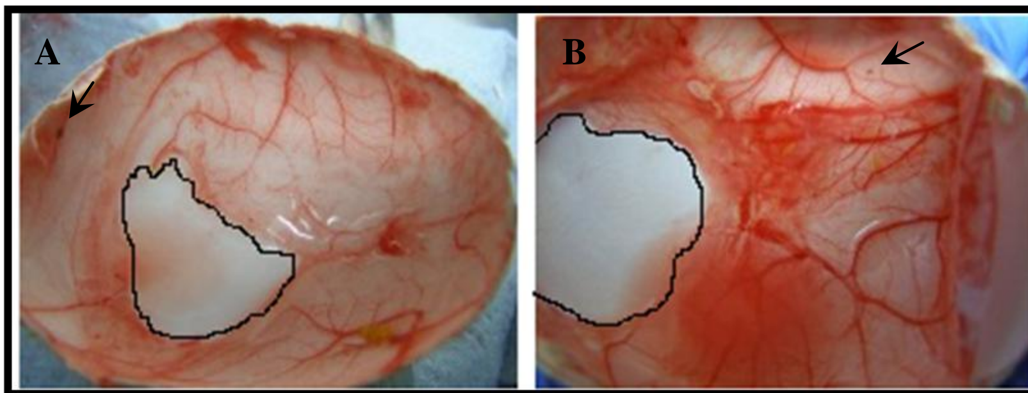

Figure 4.5: Positive infected CAM with *Orf virus*. showing peeling on CAM (outlined), and small black and gray foci (arrows).

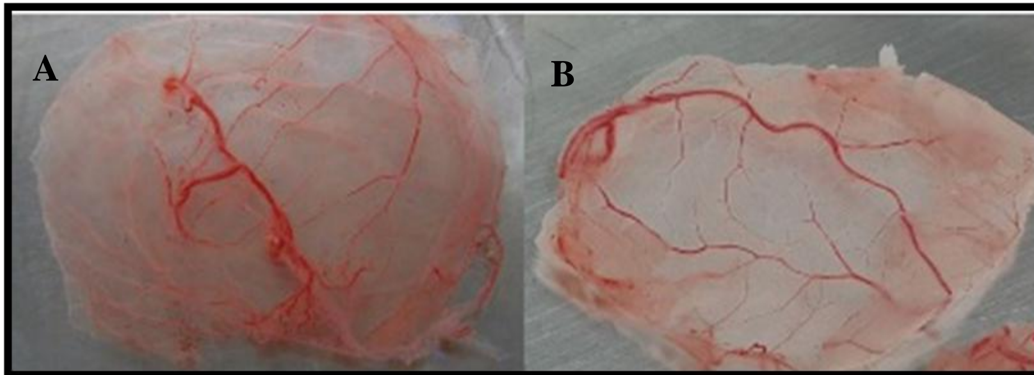

Figure 4.6: Negative control CAMs. A: uninfected CAM, B: injected CAM with PBS.

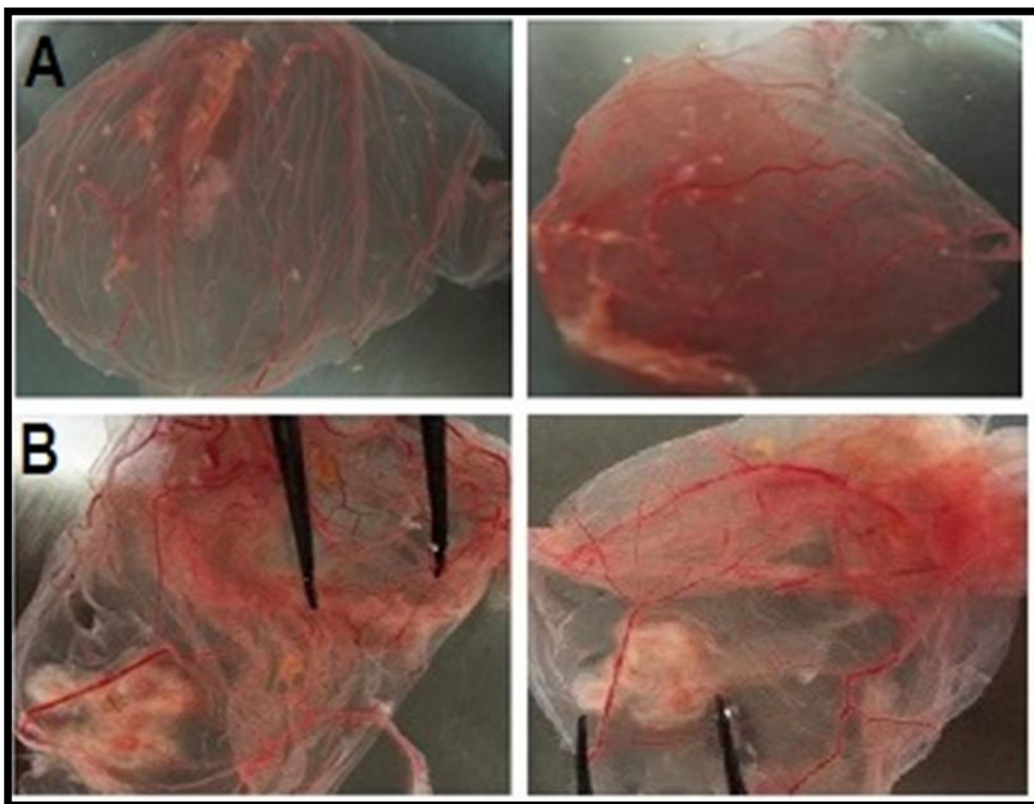

Figure 4.7: Positive control CAMs. A: *Sheep pox* live vaccine showing distinct pock marks, **B**: *Fowl pox* live vaccine showing large lesions.

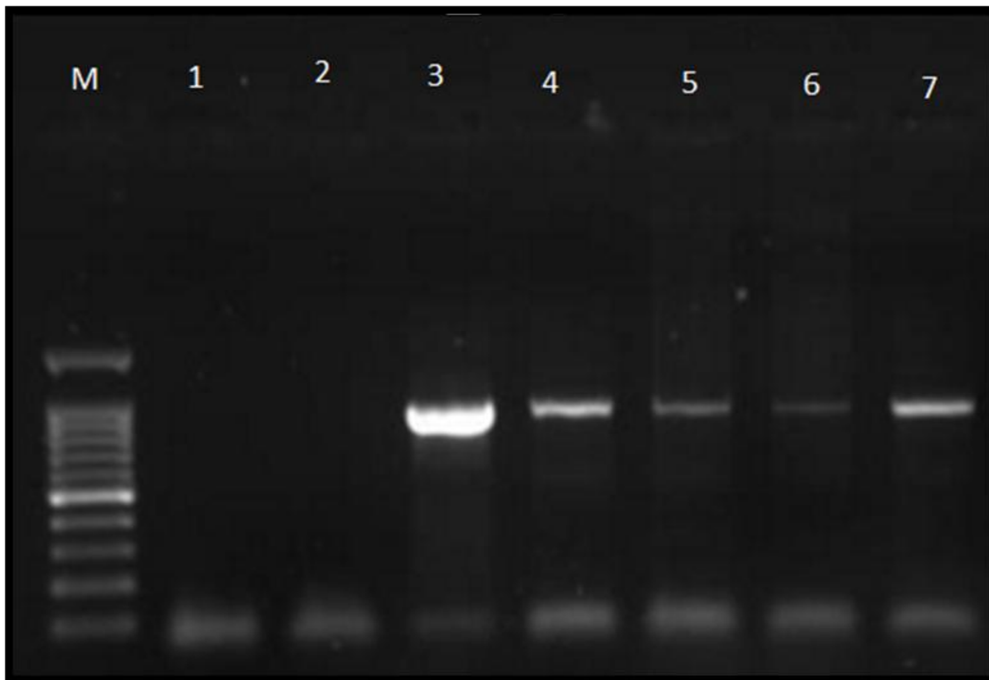

Figure 4.8: Agarose gel electrophoresis of 821bp DNA fragment of A32L gene of *Orf virus* amplified by PCR. M: 100 bp molecular weight marker. **Lane 1**: Negative template control (NTC). **Lane 2**: DNA extracted from non-infected CAM as an additional negative control. **Lane 3**: Positive control. **Lanes 4,5,6,7** are positive samples of infected CAM where the same sized 821bp amplification products are visible as for the positive control. The isolate designations for these lanes are: **Lane 4**: Orf 5.6. **Lane 5**: Orf 8.11. **Lane 6**: Orf 8.4. **Lane 7**: Orf 5.5.

4.2.2. Virus Propagation Attempts in Vero Cell Culture

Orf virus samples used to infect Vero cells with different dilutions failed to result in detectable signs of cytopathic effect. Cells were observed daily under an inverted light microscope from the first twenty-four hours of inoculation even up to one month without evidence of cytopathic effects, and the infected cells at different passages post infection were no less healthy than the controls **(Figure 4.9).** In order to rule out the possibility that viral replication was occurring too slowly to produce obvious cytopathic effects, samples of culture media were collected for testing by polymerase chain reaction using the A32L gene. Viral DNA was not successfully amplified from any tissue culture supernatants, thus supporting the conclusion that Vero cells were not supporting viral replication from our scab isolates **(Figure 4.10).**

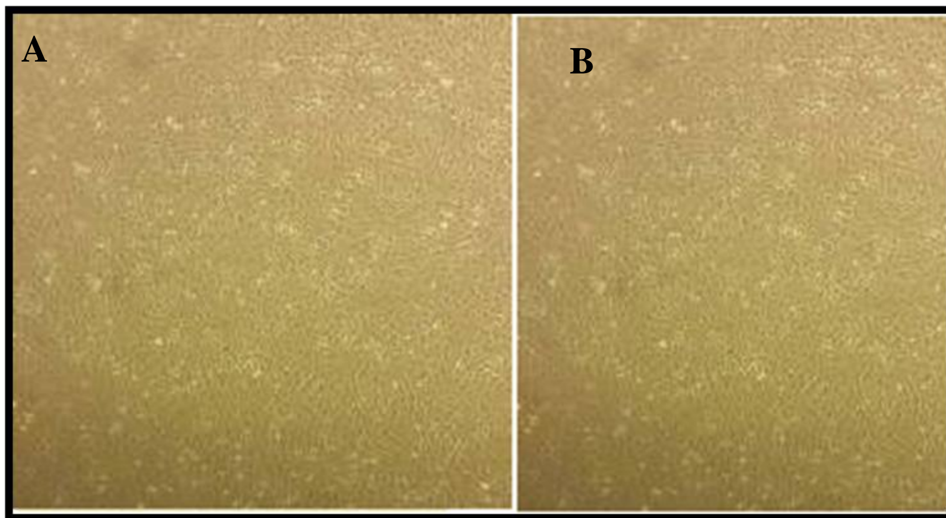

Figure 4.9: Vero cell monolayer after seven days in culture on T-25cm^2 flasks. **A**: inoculated with *Orf virus,* and **B**: inoculated with PBS as a negative control.

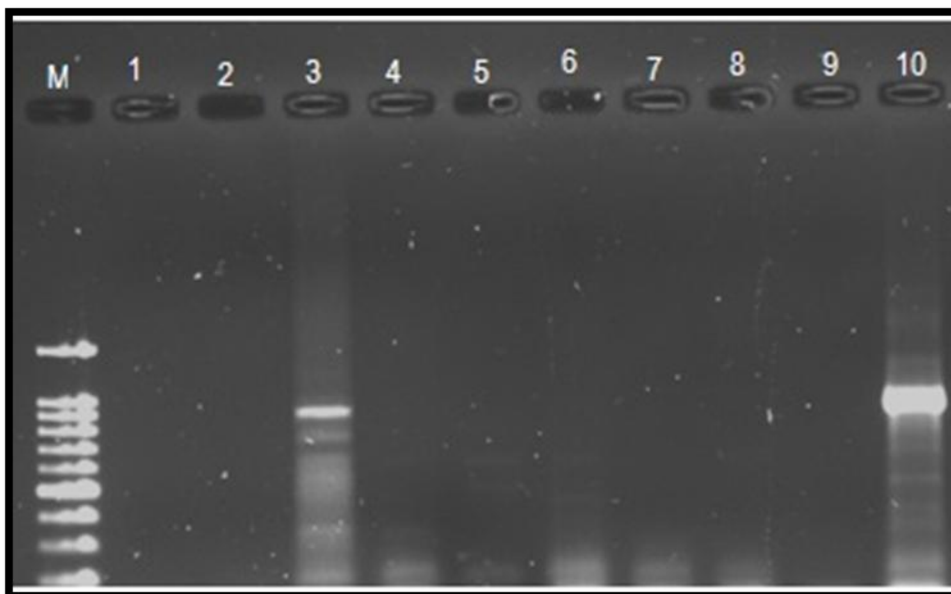

Figure 4.10: Confirmation of absence of *Orf virus* DNA in Vero cells that were inoculated with filtered scab suspensions from *Orf virus* infected sheep by PCR of A32L gene. M: 100 bp molecular weight marker**. Lane 1**: NTC. **Lane 2**: Negative control from uninfected cells. **Lanes 3 and 10:** Positive controls. **Lanes 4,5,6,7,8,9** are negatives from the infected cells at different passages, as following: **Lane 4:** Orf 8.4 on the 7th day post infection. **Lane 5:** Orf 8.11 on the 7th day post infection. **Lane 6:** Orf 5.6 on the 7th day post infection. **Lane 7:** Orf 8.4 on the 26th day post infection. **Lane 8:** Orf 8.11 on the 26th day post infection. **Lane 9:** Orf 5.6 on the 26th day post infection.

Table 4.1: Recovery of infectious virus from scabs from nine farms previously surveyed that were positive for *Orf virus* by PCR.

Region -Farm	*Orf virus* samples	System for *Orf virus* detection	
		CAM of embryonated chicken eggs	Vero cell culture
Jenin-Tamoun-Cooperative farm	Orf 1.9	Positive	Not tested
Jenin-Jalbon-Mohamad Husein Abu Rob	Orf 5.5 Orf 5.6 Orf 5.7	Positive Positive Negative	Negative Negative Not tested
Jenin-Jaba'	Orf 8.7 Orf 8.9 Orf 8.11	Positive Positive Positive	Negative Not tested Negative
Jreicho – Jiftlik	Orf 3.1 Orf 3.2	Positive Positive	Not tested Negative
Jericho – Nasiriyah	Orf 8.4	Positive	Negative
Jericho-Ahmad Nawaja	Orf 5.1 Orf 5.2	Negative Positive	Not tested Not tested
Jericho - Nashat Dweikat	Orf 9.1	Positive	Not tested
Nablus - Beit Hassan-Mazin Dawoud	Orf 6.9 Orf 6.10	Positive Negative	Not tested Not tested
Tobass - Montaser Ishtayieh	Orf 2.3	Positive	Negative

4.3. Virus Titration

One of the thirteen scab samples that tested positive was studied further to assess three things: the relationship of the various indicators of *Orf virus* replication in the CAM with viral inoculum dose, the ability of virus extracted from CAMs to be passaged a second and third time onto the CAMs of fresh embryonated chicken eggs and thirdly, the *Orf virus* titre from a scab. After carrying out a series of virus dilutions, the signs of *Orf virus* infection on

CAM at different dilutions separately were observed and recorded in **(Table 4.2)**, they were classified as primary signs which include: pocks, whitening and a localized thickening of the CAM as well as secondary signs which include: hemorrhage, and necrosis or peeling on CAM.

Table 4.2: The signs of *Orf virus* infection on CAM at different dilutions from suspensions of scabs from sheep sample Orf 8.4 (left half of the table) followed by subsequent passage of virus extracted from one of these CAMs in fresh CAMs for a second and third passage.

Virus dilution	From scab on CAM (Orf 8.4)				From CAM on CAM (Orf 8.4)		
	First passage				Second passage	Third passage	
	N=1	N=2	N=3	N=4	N=1	N=1	N=2
10^{0}	n.d.	P, W, T	P, W, T	P, W, T	P, W, T	P, W, T	P, W, T
10^{-1}	P, W, T	P	n.d.	n.d.	n.d.	D	P, W, T
10^{-2}	P, W, T	P	d	P	P, W, T	P, W, T	P, W, T
10^{-3}	P, W, T	P	n.d.	n.d.	n.d.	P, W, T	P, W, T
10^{-4}	P, W, T	P, W	P	P, W	n.d.	P, W, T	P, W, T
10^{-5}	P, W, T	HN	p	HN	P, N	P, W, T	P
10^{-6}	P, W, T	HN	P	HN	n.d.	P	p
10^{-7}	P	HN	d	HN	n.d.	P	d
10^{-8}	P	d	HN	d	n.d.	P	d
10^{-9}	d	n.d.	n.d.	n.d.	n.d.	No signs	p

Primary signs:
P: Pock, W: Whitening, T: Thickening area
Secondary signs:
H: Hemorrhage, N: Necrosis
n.d. = not determined

The pocks that were observed on CAMs were further sub classified into two types of pocks: primary pocks and secondary pocks. The primary pocks were generally few in number and had a larger diameter between 0.5 cm and 2.0 cm with a thick white mass as shown in (**Figure 4.11**), while the secondary pocks had a diameter less than 0.5 cm and were sometimes clustered together and otherwise dispersed over the CAM (**Figure 4.12),** and these were most commonly associated with peeling and hemorrhage on the CAM.

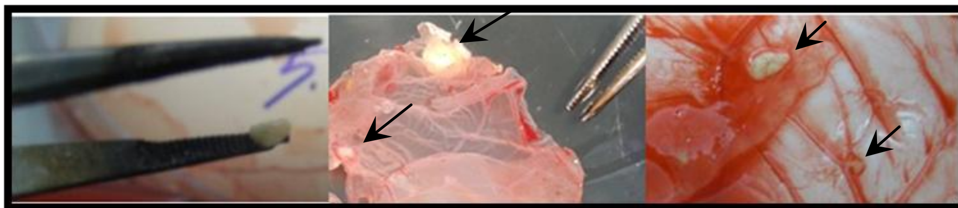

Figure 4.11: Primary pocks on different CAMs that show the large diameter of the pocks (0.5cm-2.0cm) and white thickened appearance.

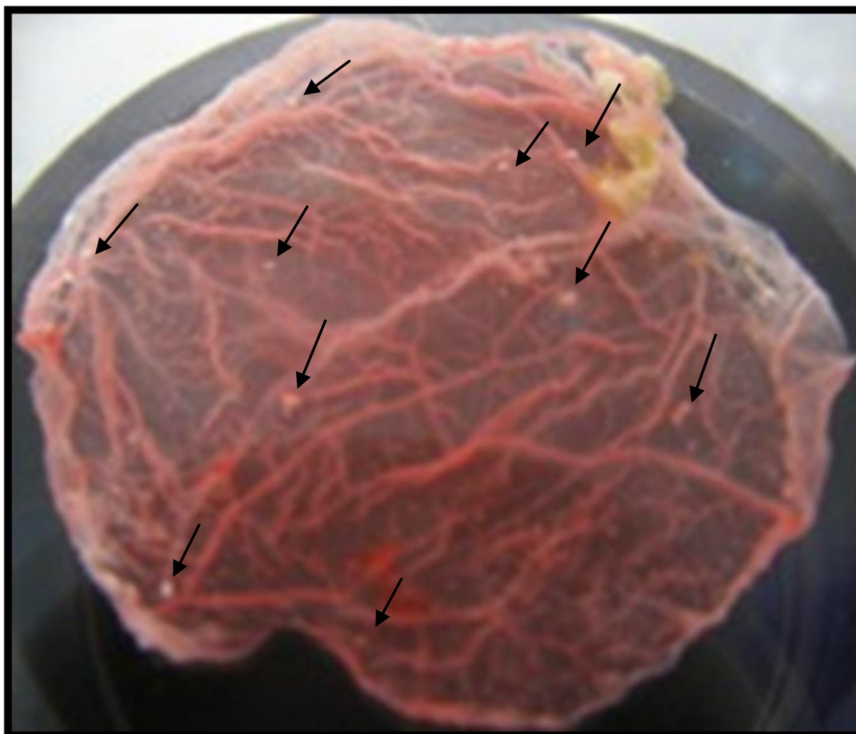

Figure 4.12: Secondary pocks on different CAMs where the diameter was less than 0.5cm. Black arrows show the distribution of small pocks formed on the CAM.

Both primary and secondary pocks were counted at all different dilutions of virus and recorded in **(Table 4.3)** and **(Figure 4.13).** The secondary signs of hemorrhage and necrosis appeared only at dilutions of 10^{-5} or greater, and this pattern was consistent through the two subsequent passages on fresh CAMs **(Table 4.2).**

Quantification of virus from the scab was performed by counting the pocks that appeared **(Table 4.3).** The first observation to note is that primary pocks gave way to secondary pocks as the scab suspension was diluted such that only secondary pocks were found from the dilutions of 10^{-4} through to 10^{-9}. The mean number of pocks, and their standard deviations, for each dilution over the range from 10^{-1} to 10^{-8} was graphed in **(Figure 4.13)** where linear regression was performed that gave a high R^2 value of 0.7543, which is indicative of a correlation between dilution and pock number. It appears that there is a negative linear relationship between the mean number of pocks and the dilution of scab filtrate (virus concentration) such that an egg infectious dose EID to the order of 10^8 is observed. Lack of availability of eggs was the reason for some specific values being not determined for certain dilutions of the four repeat experiments shown, and some embryos died within the first twenty-four hours resulting in further loss of data. Accordingly, the reason that the 10^0 and 10^{-9} dilutions were not used for the graph **(Figure 4.13)** was that only a single data point was available at those dilutions. From the data shown in **(Table 4.3)** it can also be seen that the EID_{50} for the same set of experiments was greater than 10^8, which is reasonably consistent with the EID value inferred from the linear regression shown in **(Figure 4.13).**

Prior to performing this titration, two other scabs (Orf 5.5 and Orf 5.6) from a different farm were titred twice each by the same method down to a dilution of 10^{-5}, and all three of the surviving embryos (a fourth died within 24 hours and was excluded) had pock marks at this dilution. These preliminary results from Orf 5.5 and Orf 5.6 along with the more definitive titration of Orf 8.4 down to a dilution of 10^{-9} indicate that high viral titres in *Orf virus* scabs are common

Table 4.3: Counting *Orf virus* pocks (described qualitatively in the first half of Table (4.2) on CAM from scab material from sample (Orf 8.4).

Virus dilution	Number of pocks on CAM			
	Sample (Orf 8.4)			
	1	2	3	4
10^{0}	n.d.	n.d.	10(P)	d
10^{-1}	10(P)	7(S)	n.d.	n.d.
10^{-2}	3(S)	5(S)	d	3(P)
10^{-3}	3(S)	5(P)	n.d.	n.d.
10^{-4}	d	5(S)	4(S)	4(S)
10^{-5}	3(S)	d	2(S)	2(S)
10^{-6}	3(S)	2(S)	1(S)	1(S)
10^{-7}	1(S)	1(S)	d	1(S)
10^{-8}	2(S)	1(S)	1(S)	D
10^{-9}	d	1(S)	n.d.	n.d.

P= primary pock which is a well-developed pock that has a large diameter between 0.5 cm and 2.0 cm.
S= secondary pocks have small diameter less than 0.5 cm
d= dead embryos at the first 24 hours post infection.
n.d. = not determined

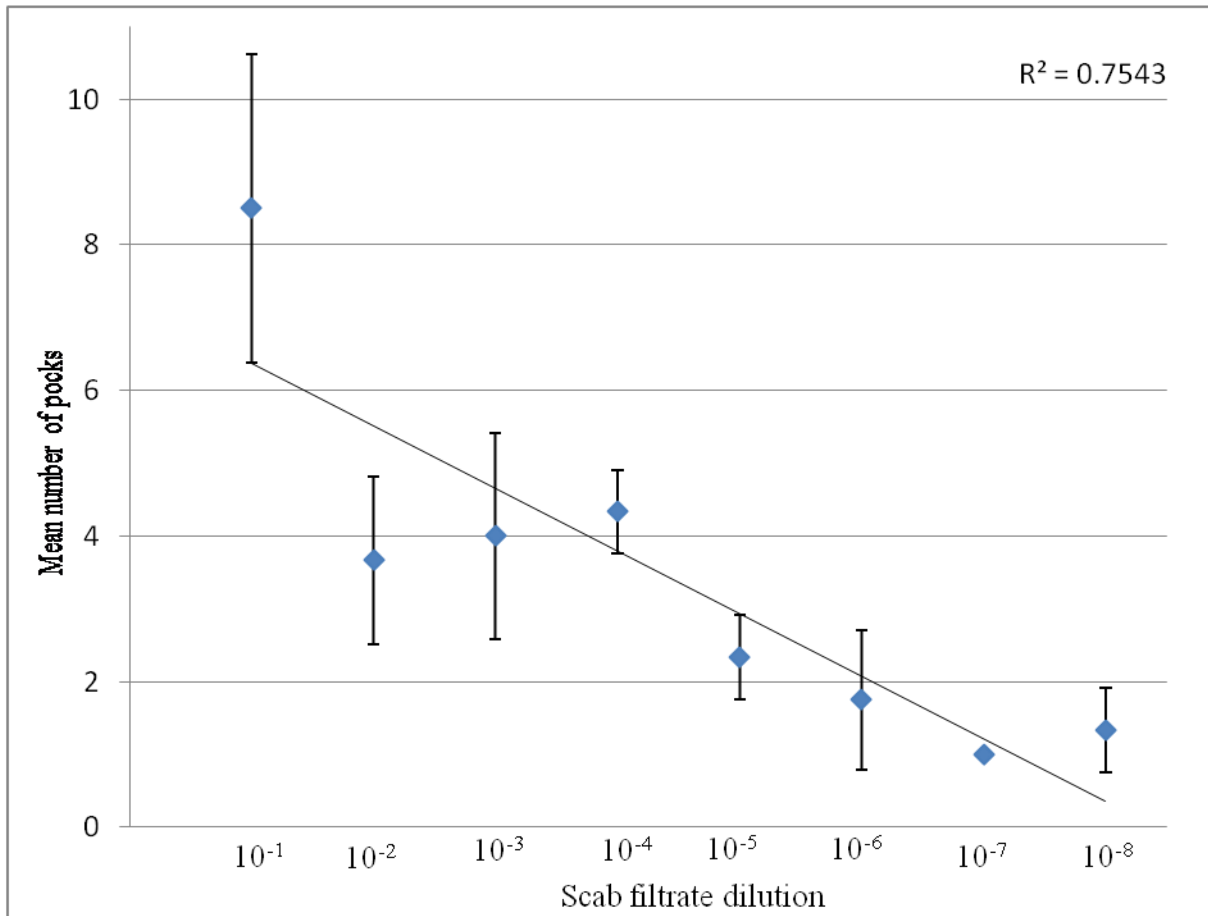

Figure 4.13: Relation between scab filtrate dilution and mean number of *Orf virus* pock counts on the CAM at different dilutions. Means were counted for all dilutions where data was available for two or more separate experiments. The error bars represent the standard deviations about each mean, and a negative linear regression with an R^2 value of 0.7543 was obtained.

4.4. Vaccine Preparation and Vaccination

If the high dose of infectious *Orf virus* found in the titred scab suspension from sample (Orf 8.4) **(Figure 4.13)** is representative of *Orf virus* scabs in general, then these scabs could be suitable for use as a stock for autologous vaccination in Palestine. Therefore, a suspension of scabs (described in 3.2.5) was prepared for a preliminary field trial conducted in a separate investigation by Dr. Robin Abu Ghazaleh and Dr. Jihad Al Ebrahim in five Palestinian sheep from a farm in Tammoun district. Classical signs of mild local infection were observed post-vaccination by Dr. Robin Abu Ghazaleh and Dr. Jihad Al Ebrahim, and scabs were collected

after eight days post-vaccination and delivered to the laboratory to be tested by PCR **(Figure 4.14)** for confirmation of *Orf virus* DNA. The very strongly positive result provides proof of principle that collection, preparation and long term storage of scab suspensions retain infectivity and can be delivered to the field in a stable glycerol formulation to provide an effective material for autologous vaccination in Palestine.

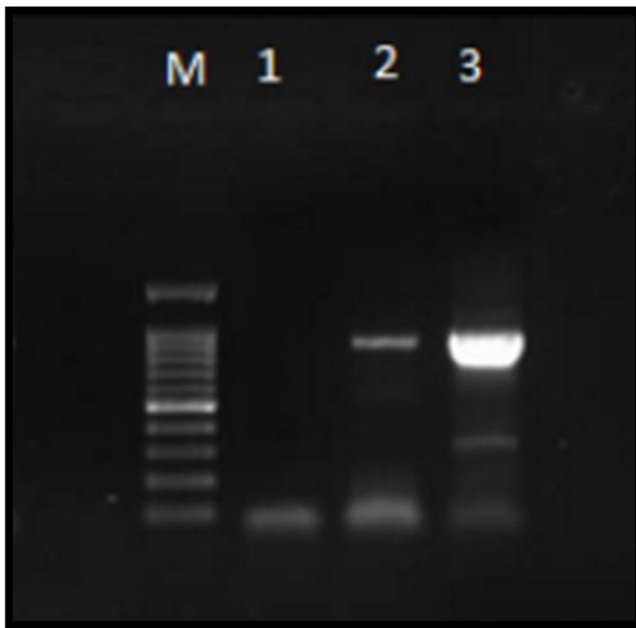

Figure 4.14: PCR amplification of A32L gene of *Orf virus* from scabs that formed on sheep as a result of a vaccination trial. **M**:100bp molecular weight marker. **Lane 1**: Negative template control. **Lane 2**: Positive control. **Lane 3**: demonstrates 821bp amplification product of genomic DNA extracted from scab of vaccinated sheep after eight days post vaccination.

CHAPTER 5

5. Discussion

Contagious ecthyma is a common disease of sheep and goats on Palestinian farms, where the deaths amongst new born sheep can reach 50%. Despite this urgent problem, no certified vaccine is present in the market, and Palestinian farmers are in need of an effective local solution to the problem of contagious ecthyma. Some veterinarians use scabs from infected animals as an autologous vaccine for protecting healthy animals on the same farm, but the results not always satisfactory. The main problems are that the viral dose in these scabs has not been quantified, and secondary bacterial and fungal infections may be carried over to the vaccinated sheep in these crude extracts.

This work aimed to overcome the existing limitations and to evaluate the use of scabs collected from sheep with contagious ecthyma for the purpose of autologous vaccination against *Orf virus* amongst sheep in Palestine. In order to do this, a method of propagating live virus was developed to determine the amount of infectious virus that could be recovered from a scab. Then, a vaccine formulation was made for testing in the field, and confirmation of efficacy was demonstrated by recovery of viral DNA from vaccine-induced scabs eight days post vaccination. Over the course of this research, two main additions have been made to scientific knowledge generally in the *Orf virus* domain.

This work provides the first report of a comparison between two different methods of *Orf virus* inoculation onto the chorio-allantoic membrane (CAM) of embryonated chicken eggs. *Orf virus* inoculation onto the CAM was performed by the top route (natural air-sac) and was compared with the dropped CAM route (artificial air-sac). Inoculation onto the CAM with *Orf virus* through an artificial air-sac was shown to give more satisfactory results in this study than the natural air-sac route. *Orf virus* inoculation onto CAM by the artificial air-sac produced distinctive signs of viral propagation and specific pocks characterized by having a

circular form, a more opaque central area and a surrounding haze as described previously for other viruses, where those pocks displaying any two of the listed features were considered as specific pocks (Burnet and Faris, 1942). Previous research that described specifically *Orf virus* inoculation in embryonated chicken eggs on CAM did not report whether the route of inoculation was by the natural air-sac or an artificial air-sac, although it may be assumed to have been inoculation through the natural air-sac as this is the "original method" of inoculation onto the CAM of embryonated chicken eggs (Ali et al., 2013, Mahmoud et al., 2010). Nevertheless, the dropped CAM route (artificial air-sac) has been used in previous studies for propagation of other poxviruses such as Fowlpox virus and Vaccinia virus (Overman and Tamm, 1956, Frisch, 1950, Burnet and Ferry, 1934) and hence it was considered preferable to compare both routes of *Orf virus* propagation on CAMs before attempting to measure the viral load in scabs from infected sheep.

This work provides the first report of live *Orf virus* load in a scab suspension, which is important because this study aimed at assessing the suitability of scabs as a source of live virus for autologous vaccination in Palestine. Vaccine companies report the titres of live virus vaccines in terms of either a $TCID_{50}$ or EID_{50} value, and *Orf virus* tires up to $10^{7.2}$ $TCID_{50}$ / ml have been reported for cell culture-adapted virus (Pye, 1990) (Mercer et al., 1997). However, $TCID_{50}$ values were not considered appropriate for our study with non-cell culture-adapted viruses from field isolates because these viruses do not induce signs of CPE without prior cell culture adaptation over multiple rounds of blind-passage. Therefore, an EID_{50} value was calculated for this study after optimizing the route of infection onto the CAM of embryonated chicken eggs, and this is the first report of an EID_{50} value for *Orf virus*. Using 0.2ml of serial dilutions from a 10% scab suspension, an EID_{50} of 5 x10^{8} / ml was deduced in this study, which is at least 10 fold higher than the $TCID_{50}$ values reported previously for tissue culture-adapted *Orf virus*.

After measuring the *Orf virus* titre of an infected scab, a vaccine preparation was made and tested successfully, which represents the first controlled vaccination of sheep in Palestine against *Orf virus* using material that has been stored and titred in the laboratory. In the absence of any national capacity to procure an Israeli-licensed commercial vaccine, some

local veterinarians have been using autologous vaccination on the farm with mixed success, and without the benefit of knowing the titres of virus being delivered in their autologous vaccination procedures. In the future it is hoped to secure funding to better test the parameters of scab suspension use as a vaccine source

by setting up trials to determine the minimum EID_{50} values that gives a measurable local immune response in sheep. In addition, it is difficult to know how well an EID_{50} value for scab isolates could be compared with a commercial $TCID_{50}$ value for cell culture-adapted strains, and further research would enable the most efficient procedure of autologous vaccine preparation to be adopted.

Generation of a Vero cell culture-adapted *Orf virus* strain would have been a useful addition to vaccine development in Palestine, and many cell lines such as Vero, Madin-Darby bovine kidney (MDBK) and Madin-Darby ovine kidney (MDOK) cells have been used successfully for *Orf virus* isolation in the past (Ali et al., 2013, Li et al., 2012, Guo et al., 2004, Guo et al., 2003, Gumbrell and McGregor, 1997), but adaptation could not be achieved in this study, and after two blind passages in Vero cells CPE was not observed. Previous reports of *Orf virus* propagation in Vero cells had mixed results. Some studies showed negative results of *Orf virus* isolation in Vero cells, with no cytopathic effects observed even after five blind passages (Kumar et al., 2014, Yang et al., 2014). However, others were successful in reaching cytopathic effects after blind-passaging (Maan et al., 2014, Housawi et al., 2012). Future attempts in this direction might benefit from extending the number of blind passages performed and from performing these passages in a highly parallel fashion by inoculating separate aliquots of each scab suspension onto each of the wells of a 96-well plate of cells rather than a single T-25cm^2 flask as used in this study. It is recommended to continue this work as success in tissue culture adaptation could generate a seed-stock vaccine that would allow commercial vaccine production in the region using a Palestinian strain of *Orf virus* for which Palestinian researchers hold the intellectual property rights. Confidential discussions with a regional vaccine producer in Jordan and patent preparations are ongoing.

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