بسم الله الرحمن الرحيم

GROWTH CHARACTERISTICS OF VACCINIA AND CAMELPOX VIRUSES IN EMBRYONATED EGGS AND CELL CULTURE AND THEIR DETECTION BY POLYMERASE CHAIN REACTION

BY

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A THESIS

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DEDICATION

To my great family, for their great efforts

With ever lasting love

.

To all those whom I love

I dedicate this thesis

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Abbreviations

ATI:	Acidophilic-type inclusion body.
BHK:	Baby hamster kidney.
CAM:	Chorioallantoic membrane.
CPE:	Cytopathic effect.
CPV:	Camelpox virus.
DNA:	Deoxyribonucleic acid.
Dubca:	Dubai camelpox vaccine.
DDW:	Deionized distilled water.
ELISA:	Enzyme-linked immunosorbent assay.
GMEM:	Glasgow Modified Eagles Medium.
H&E:	Haematoxillin and Eosin.
HIS:	Hyperimmune serum..
OPV:	Orthopoxvirus.
PBS:	Phosphate buffer saline.
PCR:	Polymerase chain reaction.
PDB:	Phosphate diluent buffer.
TCID/50:	Tissue culture infective dose 50%.
TPB:	Tryptose phosphate broth.

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Abstract

Camelpox virus isolates, VD45 (African camelpox strain) and Vaccinia virus were used for propagation in the chorioallantoic membrane of embryonated eggs (CAM). Lesions were seen as pocks ranging in size from 1 to 1.5mm in diameter, and they increase in size with serial passage and taking opaque- white and opaque-yellow colours. When propagated in Vero cells, these viruses gave clear CPE, characterized by rounding of cells, plaque formation, syncytia and detachment of cells from glass.

Neutralization test was used as the method of diagnosis of camelpox virus in cell cultures, and two methods of DNA extraction were employed to conduct PCR to compare it with neutralization test. The first method of DNA extraction was accomplished by using viral DNA in CAMs and tissue culture supernatant, which was released by initial heating for 15 min at 99c˚ followed by ordinary PCR. In the second method DNA was extracted by using DNA Isolation Kit from tissue culture supernatant as a template.

Rapid identification and differentiation of camelpoxvirus and Vaccinia virus were achieved by PCR and this assay proved to be fast and feasible, and can be an alternative to orthodox serological methods.

ملخص الأطروحة

سبعة عزلات من فيروس جدري الإبل وفيروس جدرىالابل الأفريقي وفيروس الفاكسينيا استعملت فى هذه الدراسة وتم إكثارها في الغشاء المشيمي السقائى لجنين الدواجن فأعطت آفـات واضـحة يتراوح حجمها ما بين 1-1.5ملم وتأخذ اللون الأبيض المعتم والأصفر المعتم. وهذه الفيروسات تم إكثارها أيضا في خلايا كلى القرد الأخضر الأفريقي و أعطت أثرا مرضيا خلويا. وعلى هذه الآفات يمكن التفريق بين فيروس جدري الإبل و الفاكسينيا.

استخدم في هذه الدراسة اختبار التعادل المصلى كإحدى الطرق لتشخيص مرض جـدري الإبـل و مقارنته باختبار تفاعل البلمرة التسلسلي و تم استخدام طريقتين لاستخلاص الحمض النـووي مـن خلايا الزرع الخلوي والغشاء المشيمي السقائى المصابة بالفيروس ، الطريقة الأولى تم فيها استخلاص الحمض النووي بواسطة التسخين الابتدائي في درجة حرارة 99°م لمدة 15 دقيقة وفى الطريقة الثانية تم استخلاص الحمض النووي بواسطة استخدام طقم لعزل الحمض النووي.

أوضحت هذه الدراسة انه يمكن استخدام اختبار تفاعل البلمرة التسلسلي للتمييز بين فيروسي جدري الإبل و الفاكسينيا واستخدامه كطريقة سريعة لتشخيص المرض بديلا للاختبارات المصلية المـستعملة حاليا.

Introduction

> Camels are important livestock resource adapted to hot and arid enviroment. They have been utilized by man for meat, milk, wool, hides and traction power. Sudan is the second most densely camel populated country in the world after Somalia (Schwartz & Dioli, 1992)

Camelpox (CP) is a highly contagious viral disease affecting mostly young animals and characterized by papular pustular eruptions on the skin and mucous membranes. The mortality in infected herds range from 25% to 100% for young animals, and from 5% to 25% for older animals (Ramyar and Hessami, 1972; Kriz, 1982). The presence of CP in Sudan was first reported in 1953(Anon., 1954). However, identification of its causative agent has not been made then (Shommein &Osman, 1987).

The CP virus which is a typical representative of the genus Orthopoxvirus, Family Poxviridae was isolated in many camel raising countries (Ramyar and Hessami, 1972; Davies, Mungai and Shaw, 1975; Alfalluji, Tantawi and Shony, 1979; Hafez, AL-Sukayran, Dela Cruz, Mazloum, AL-Bokmy, AL-Mukayel, Amjad, 1992; Chauhan and Kaushik, 1987; Nguyen, Richard and Gillet, 1989; Higgins, Silvey,

Abdelghafir and Kitching, 1992 and Khalafalla, Mohamed and Ali, 1998).

Camelpox genome consists of a single molecule of linear double stranded DNA, the genome has cross links that join the two DNA strands at both ends, the end, of each DNA strand has long inverted tandemly repeated nucleotide sequences that form single- stranded loops (Murphy, Gibbs, Marian, Horzinek and Studdert 1999). The central region of the genome contains genes that are highly conserved in all sequenced orthopoxvirus.

Objectives of the study:

1- Differentiate between orthopoxviruse species (Camelpox virus and Vaccinia virus) by biological methods including propagation in Vero cell (African green monkey kidney cells) and on chorioallantoic membrane of embryonated eggs.

2- Introduce gene base techniques for diagnosis of camelpox virus which is expected to help implement measures to control this important disease resulting in improving health and productivity of camels and help to understand the epidemiology of the disease and support efforts to control it.

3- Introduce direct PCR assay as method of a rapid and accurate technique for diagnosis more than serological tests.

Chapter I

Literature Review

Camel pox

1.1. Definition

Camel pox (CP) is a systemic disease. A typical pox exanthema appears over the entire body and on the head, in particular.It is caused by poxvirus with a relatively restricted host spectrum (Mayr& Czerny, 1990). Camel pox causes a severe generalized disease in camel, with extensive skin lesion (Murphy, Gibbs, Marian, Horzinek and Studdert, 1999). Munz, Moallin, Mahnel, Reimann, (1990) described CP as a species –specific highly contagious disease of camel characterized by pox lesions that cause severe outbreaks mainly in young animals with mortality rates up to 10 %.

1.2 Etiology of CP

Camel pox is caused by a virus which has been classified under the orthopoxvirus genus of family Poxviridae (Mahnel, 1974; Meyer,.Osterrieder and Pfeffer, 1993 and Wernery & Kaaden, 2002).

The virus has the characteristics and properties of a true poxvirus and is closely related to the vaccinia –variola group (Mahnel & Bartenbach, 1973). Andrews, Pereira and Wildey (1978) considered

the virus to be a member of the orthopoxvirus group with resemblance to the smallpox virus.

1.3 Historical perspective

Pox in camels was recognized in the 19th,when it was first described by Masson in 1840 in India, where it was known by the local population under the name photohitur (Buchnev, Tulepbaev and Sansyzbaev, 1987). The second report of the disease was made by Vedernikov in 1893 in the former USSR (Wernery & Kaaden, 1995).

Later on, the disease was reported in India by Leese (1909) and continued to be mentioned in the literature thereafter (Wernery & Kaaden, 1995).

1.4 Geographical distribution of the disease

Camel pox is widespread in Asia and Africa, especially Algeria, the Sahara, Egypt, Sudan, Kenya and Somalia; the Asiatic part of the Soviet Union and India (Baxby, 1972).In addition, the disease was reported from Iran (Baxby, 1972; Ramyar & Hessami, 1972) ,Saudi Arabia (Hafez Eissa, Amjad, AL-sharif, AL-Sukayran, Dela Cruz, 1986), United Arab Emirates (Kaaden, Walz, Cerny and Wernery, 1992), lrag (Al- Falluji,.Tantawi and Shony, 1979), Egypt (Tantawi,.Saban, Reda and EL Dahaby, 1974), Kenya (Davies, Mungai and Shaw, 1975), Niger

(Nguyen, Richard, and Gillet, 1989), Somalia (Kriz, 1982), Ethiopia (Oden Hal, 1983) and Morocco (Fassi-Fehri, 1987; ELHarrak,.Loutif, and Bertin, 1991).

The presence of CP in Sudan was first reported in 1953. According to Shommein and Osman (1987) the disease was also reported in the late seventies in Kassala province of Eastern Sudan. Khalafalla and Mohamed (1994) isolated CP virus (CPV) from Butana area of Eastern Sudan.

1.5 Epidemiology of CP

Camelids may become infected with the poxvirus through small abrasions of the skin, by aerosol infection of the respiratory tract or by mechanical transmission through biting arthropods. Several scientists have reported an increase in CP outbreaks during wet seasons (Munz, 1992; Wernery, Meyer and Pfeffer, 1997 and Wernery, Kaaden and Ali, 1997) when the disease becomes more severe. During the dry season, it usually follows a milder course (Pfahler and Munz, 1989).

Since the CPV has been isolated from the camel tick *Hyalomma dromedarii*, it is generally belived that a larger arthropod population builds up during rainy seasons, forcing a greater virus pressure and virus doses onto the camel populations (Wernery and Kaaden, 2002). Camels' aging 2-4 years often develop the localized form, with lesions on skin and mucous membranes of lips and nose. Young camels up to one year old and female camels in the final month of pregnancy are affected mainly by the generalized form (Khalafalla and Mohamed, 1998). In two principal camel-rearing areas of Kenya, the disease was

found in Turkana where outbreaks were detected in two herds of young animals, while in Samburu, outbreaks were found in two herds of adult animals, as well as in two herds of young camels. In all cases, there was 100% morbidity in the affected herds.When the young camels were involved, the main lesions were confined to the mouth, nose and muzzle as distinct pustular lesions. In adult animals, there was also extensive odema of the head and neck (Gitao, 1997).

According to Munz (1992) the high prevalence of antibodies against CPV in camel sera and the occurrence of clinical outbreak in Kenya, Somalia and Sudan indicate that the disease may be enzootic, or sometimes epizootic in these countries. In four areas of the Sudan Khalafalla,Mohamed and Agab (1998) found that the prevalence of seropositive animals was higher in adults more than 4 years old (87%) than in calves less than 1 year old (40%) and than in young animals of 1- 4 years old (75%) and the prevalence rates were higher in female camels (76.5%) than in males (66.7%).

1.6 Clinical Symptoms

The incubation period ranges from 9 -13 days, pustules develop on the nostrils and eyelids as well as on the oral and nasal mucosa in mild cases. In more severe cases, presenting with generalized clinical signs such as fever, lassitude, diarrhea and anorexia; the eruptions are distributed over the entire body (Wernery and Kaaden, 2002,). Abortion of female camels has been reported (Borisovich & Orekhov, 1966, Buchnev and Sadykou, 1967; Munz, 1992). Mortality can reach 28% in generalized forms of the disease (Jezek, Kriz and Rothbauer, 1983). Secondary bacterial and mycotic infections can complicate the course of the disease (Wernery and.Kaaden, 2002).

Pox-lesions were also observed in the trachea and lungs of young dromedaries (Wernery and, Kaadan, 1995; Kinne, Cooper and Wernery, 1998). Classical lesions in the skin start as erythematous macules, which develop into papules and vesicles. Vesicles develop into pustules with depressed centers and raised erythematous borders the so-called pock. After the pustules have

ruptured, they become covered by crusts. Healing of pustules might take 4-6 weeks with or without scars (Wernery &Kaaden, 2002). Associated lymph nodes are often swollen (Munz, 1992). Mammary glands,genitalia and anal areas are also frequently affected (Kriz, 1982;.Murphy *et al*, 1990). Lesions also develop on the mucous membranes of the oral cavity resulting in difficulty in eating and consequent loss of condition (Munz, 1992).

1.7 Morbidity and Mortality

The more severe forms of the disease usually occur in young animals, and during epidemics, the case- fatality rate may be as high as 25% (Murphy *et al*., 1999). The mortality rate in infected herds ranges from 25 -100% for young animals, and 5 -25% for older ones. Mortality rate is higher in males than in females, it was reported by Ramyar& Hessami (1972) and Kriz (1982) to be 8.8% in males and 4.4% in females.

1. 8 Diagnosis of CP

Preliminary diagnosis of CP is based on clinical epizootiological and pathological findings (Buchnev *et al*, 1987). Confirmatory diagnosis may be accomplished by electronmicroscopic detection of orthopoxvirus particles in pox lesions, cultivation of the virus in tissue culture cells or on the chorioallantoic membrane (CAM) of embryonated chicken eggs as well as by serological tests (Munz, 1992).

The systematization and laboratory differentiation is of great importance in demarcating the orthopoxvirus from the parapoxvirus, as both viruses can be found in the same

camel (Wernery and Kaaden, 1995). Newer diagnostic methods include the ELISA technique with monoclonal antibodies, DNA restriction enzyme analysis (Munz *et al*., 1992) and a dotblot assay digoxigenin-labeled DNA probes (Meyer *et al,* 1993). Czerny,.Meryer and Mahnel, 1989;Johann and Czerny, 1993 and Pfeffer, Wernery,Kaaden and Meyer, 1998) have described various laboratory methods for the diagnosis of CP. They include electron microscopy, ELISA, immuno-histochemistry and polymerase chain reaction (PCR). CPV-antigen detection by immuno–histochemistry is a new method for the diagnosis of CP, which can easily be performed in laboratories not possessing an electron microscope.In addition to the diagnosis, immuno-histochemistry is of particular interest for histopathologists because it facilitates visualization of the morphological changes induced by the poxvirus (Wernery and Kaaden, 2002). Khalafalla and Mohamed (1998) identified CPV using virus neutralization, agar gel diffusion, and immuofluorecent tests and histopathological pictures of the skin lesions.

Ropp, Jin, Knight, Massung and Esposito, (1995) developed a PCR strategy to differentiate between orthopoxvirus species including CPV. They have successfully used this strategy to identify virus DNA in clinical materials, infected cell culture and CAMs. Meyer, Pfeffer and Rziha, (1994) established PCR and restriction enzyme protocols for detection and differentiation of species of the genus orthopoxvirus.

1.9 Control of CP

In order to minimize secondary infections, it is advisable to treat severe cases by local application or parental administration of broad-spectrum antibacterial antibiotics and vitamins.

Although CP has a great economic significance, only few scientists have concerned themselves with the production of specific vaccine. Camel owners recognized the importance of CP long ago, and even today, they protect their camel calves by dissolving scabs from affected animals in milk and rubbing the mixture on the scarified lips (Leese, 1909; Higgin, 1986). An inactived vaccine was developed in Morocco and has been used in prophylactic campaigns since 1991.Attenuated virus strains were employed in Saudi Arabia and in UAE. The UAE group established a permanent fetal dromedary skin cell line (Dubca) for isolation of the CPV

(Kaaden *et al,* 1992; Klopries, 1993; Kaaden *et al,* 1995). The UAE attenuated CP vaccine called Ducapox (Dubai Camelpox vaccine) has been in use since 1994 with appreciable success (Wernery, 1994).

1.10 Physico-chemical properties of CPV

The CPV, as a typical representative member of the genus orthopoxvirus is, brick-shaped, measuring 260-300x190-200 nm with superficial protein filaments arranged irregularly (Mahnel & Bartenbach, 1973; Nguyen *et al*., 1989; Munz, 1992). There are conflicting reports as to the sensitivity of CPV to ether and chloroform. Some authors (Alfalluji *et al*., 1979) stated that the virus (Iraqi isolate) was resistant to both ether and chloroform. Ramyar and Hessami, (1972) found the virus (Iranian isolate) to be sensitive to ether and chloroform. Others (Davies *et al*., 1975 and Nguyen *et al*., 1989) found that the CPV isolates from (Kenya & Niger) were resistant to ether but sensitive to chloroform. (Khalafalla & Mohamed, 1998) stated that the CPV (Sudanese isolates) was sensitive to both ether and chloroform.

Effect of temperature on virus infectivity also varied between CPV isolates in different countries. Alfalluji *et al*., (1979) reported on the heat stability of an Iraqi isolate of CPV .The virus was found to be resistant to heat at 56c^{o} for 1hr. Reduction of 50% of virus infectivity and complete inactivation occurred at 60c^{o} after 1 and 2hrs, respectively.

The virus infectivity was greatly reduced at70c^{o} after 10 minutes and completely disappeared after 30 minutes. On the other hand, Nguyen *et al*., (1989) found that a CPV isolated in Niger was thermolabile at 56c^{o} for 10 minutes. The CP isolate lost infectivity of cell culture when heated at 56c^{o} for

10 minutes and above (Khalafalla & Mohamed, 1998). The virus is readily inactivated by heat, direct sun light, weak acids and alkali, iodine and potassium permanganate (McGrane & Higgins, 1985).

1.11 Biological Properties of CPV

1.11.1 Host Spectrum

Camel pox virus has a narrow host range. Apart from camels, the virus induced typical pox lesions in monkeys from which the virus can be isolated (Alfalluji *et al*, 1979). However, in cattle, buffaloes, sheep, goats, horses, donkeys, guineapigs, monkeys and rabbits it does not produce pox lesions (Ramyar&Hessami, 1972; Tantawi 1974; Davies *et al*, 1975; Alfalluji *et al*., 1979; Khalafalla and Mohamed, 1998). In chickens, the virus produces vesicles, about 2.5mm in diameter after 4-5 days postinoculation (Khalafalla & Mohamed, 1998). Camel pox is most probably not a zoonosis, although various articles have reported the possibility of transmission of CPV to man (Wernery & Kaaden, 1995).

1.11.2 Growth of CPV in Embryonated Chicken Eggs

Most CPV isolates from different countries grow readily on primary inoculation of embryonated chicken eggs (Ramyar & Hessami, 1972; Marrennikova *et al*., 1974; Alfalluji *et al*, 1979; ElHarrak *et al*, 1991). Camel pox virus produces several distinct pocks, which were opaque-white, round (approximately 0.5-1.5mm in diameter) and without haemorrhagic or necrotic centers.(Khalafalla & Mohamed, 1998)

1.11.3 Growth of CPV in Cell Culture

The virus replicates and causes complete CPE in a wide range of cell cultures (Mayr& Czerny, 1990). Clear CPE was observed in baby hamster kidney (BHK) cell and lamb lung, calf and lamb testis and chick embryo kidney cell cultures (Khalafalla & Mohamed, 1998).

Primary inoculation of material from CP lesion produced CPE in Vero and BHK cells, lamb testis, lamb and calf kidney cells,

characterized by round cell formation and detachment of cells from the surface (Davies *et al*., 1975). According to Nguyen *et al* (1989), the CPE produced by the VD45 isolate of CPV in Vero and porcine kidney cells has been observed under different forms. First, infected cells may be seen as round refractive, second formation of syncytium by progressive fusion of a greater number of affected cells which terminates by detachment of these cells to form plaques; the third form was a mixed type consisting of a synctium of round cells and plaques.

1.12 Antigenic Properties of CPV

Camelpox virus is closely related immunologically to other representatives of the orthopox genus of the family poxviridae such as vaccinia and variola virus (Wernery &Kaaden, 1995). The differences demonstrated between the behavior of the viruses (Camelpox and smallpox) is discussed in the light of their otherwise close relationship and the limited information available about CP infections in man, (Baxby *et al*., 1972).

The biological properties of the isolates from Iraq indicated that it was probably identical with strains of CPV isolated from Iran, Egypt, Kenya and the USSR (AL-Falluji *et al*, 1979).

A virus isolated from pock –like vesicular eruptions of camel in Northern Kenya, was shown to be poxvirus with many characteristics of members of the orthopox group .It appears to be identical with CP strains from Iran and has similar properties to certain East African variola virus (Davies *et al*., 1975).

Five orthopoxvirus isolates of camels from different geographic regions of Africa and Asia were analyzed with respect to their biological and genomic attributes, these findings confirm that the orthopox viruses of camels constitute a separate species within the genus orthopoxvirus (Renner *et al*, 1995).

1.13 Viral Replication

The unique terminal structure of the poxvirus genome, is an evidence that initiation occurs near the ends of the molecule, and the presence of concatemer junctions in replicating DNA suggests a model

similar to that proposed for replication of single stranded parvovirus DNA. A hypothetical nick occurring at one or both ends of the genome provides a free 3` end for priming. The replicated DNA strand then folds back on itself and copies the remainder of genome. Concatemer junctions form by replication through the hairpin; very large branched concatemers can arise by initiating new rounds of replication before resolution occurs. Recombinational strand invasion may further contribute to the formation of complex multibranched molecules. After the onset of late –stage transcription, unit –length genomes are resolved, and the incompletely base-paired terminal loops, with inverted and complementary sequences, are regenerated (Moss, 2001).

Chapter II

Materials and Methods

2.1 Virus strains

Seven camelpox viruses (CPVs) isolated from outbreaks in the Sudan (Khalafalla and Mohamed, 1998) and VD45 strain of CPV isolated in Niger (Nguyen *et al*., 1989) supplied by CIRAD-IEMVT, France, and a prototype vaccinia virus (Elstree strain) was obtained from the virus stock of the Federal Research Centre for Virus Diseases of Animals, Tübingen and used as a reference strain for OPV, were all employed in this study.

2.2 Hyperimmune serum

This was prepared in rabbits using VD45 strain of CPV (Kalafalla *et al*., 1998).

2.3 Preparation and Sterilization of Glassware

Flasks, beakers, bijou, universal and volumetric bottles, measuring cylinders, tissue culture bottles, tubes and other glassware were rinsed in running tapwater, brushed with soap and then rinsed several times in tap and distilled water (DW). The clean dry glassware were sterilized in the hot-air oven at 160c^{o} for 1 hr. Volumetric glass pipettes were soaked

overnight in potasium dicromate, then, they were washed several times in tap and DW and left to dry. The clean dry pipettes were cotton plugged, placed in canisters and sterilized in hot air oven at 160c^{o} for 2 hrs.

2.4 Preparation of Solution and Cell Culture Media

2.4.1 Phosphate buffer saline (PBS) and phosphate diluent(PD)

A. Ingredients:

Solution (a)

NaCl	16.0g
KCl	0.4g
Na_2HPO_4 (anhydrous)	2.3g
KH_2PO_4	0.4g
DDW	1500.0 ml

Solution (b)

$MgCl_2.6H_2O$	0.426g
DDW	**200.0 ml**

Solution (c)

$CaCl_2.2H_2O$	0.264g
DDW	***200.0 ml***

B. Preparation:

Solutions a, b and c were autoclaved separately at 121c^{o} for 10 minutes and left to cool. To prepare working solution of PBS (1x) solutions a and b were mixed. Solution c was then added and the final volume was brought to 2 litres with sterile DDW. To prepare PD (1x) solution a was made up to 2 litres with sterile DDW.

2.4.2 Glasgow Minimum Essential Medium (GMEM)

A- Stock Solution (5x)

The entire content (125.7g) of one bottle of powdered media (Sigma) was dissolved in 2 litre of DDW.The solution was immediately filtered through a Millipore filter (0.22 u) under positive pressure, tested for sterility using thioglycolate media and store at –20c^{o}.

B- Outgrowth and Maintenance Media

Outgrowth and maintenance media were prepared according to Ali (1971) as is shown below:

	Medium (liter)	
Stock solutions		**Outgrowth maintenance**
GMEM	200 ml	200ml
Lactalbumin hydroysate	25 ml	25 ml
Yeast extract (1%)	25ml	25 ml
Sodium bicarbonate (7.5%)	7.5 ml	10 ml
Penicillin-streptomycin solution	1 ml	1 ml
Fungizon(5000µg/ ml)	1 ml	-
DDW	to 1L	to1L

Tryotose phosphate broth (TPB)	50 ml	50 ml
Bovine serum	100 ml	20 ml

2.4.3 Tryptose Phosphate Broth (TPB)

Three grams of TPB powder were dissolved in 100 ml DDW, sterilized by autoclaving at 121c^{o} for 10 min and stored at 4c^{o}.

2.4.4 Lactalbumin hydrolysate

Five grams of lactalbumin powder were dissolved in 100 ml of DDW, sterilized by autovlaving at 121c^{o} for 10 min and stored at 4c^{o}.

2.4.5 1% Yeast extract solution

One gram of yeast extract powder was dissolved in 100 ml of DDW, autoclaved at 121c^{o} for 10min. and stored at 4c^{o}

2.4.6 7.5% Sodium bicarbonate solution ($NaHCO_3$)

Seven and half grams of $NaHCO_3$ were dissolved in 100ml DDW and autoclaved at 121c^{o} for 10 min and stored at 4c^{o}.

2.4 7 Thioglycolate medium

Twenty-nine and half grams of thioglyconate medium were dissolved in 100 ml of DDW, dispensed in bijou bottles and autoclaved at 121c^{o} for 10 min and stored at 4c^{o}.

2.4.8 Trypsin –Versene solution

Trypsin (2.5 % solution) was sterlized by filteration (Millipore filter 0.22ul), versene (5 % solution) sterilized by autoclaving, then 6 ml of trypsin were added to 4 ml of versene and the mixed solution completed to 100ml with sterile Phosphate diluent (1x).

2.4.9 Penicillin- Streptomycin Solution

One gram of streptomycin powder and 2 million iu penicillin were dissolved in 10 ml of sterile DDW so that 1mlof the prepared solution contained 100 mg streptomycin and 20.000iu pencillin .The solution was kept at 4c^{o}.

2.4.10 Fungizon solution

The content of one vial of fungizon (Amphotericin B 50mg) was dissolved in 10ml of sterile DDW and kept at 4c^{o}.

2.5 Embryonated Chicken Eggs

Embryonated chicken eggs were obtained from the poultry unit of Virology Research Laboratory (VRL) Dept. Of Vet Microbiology, Shambat. The eggs were cleaned, disinfected and incubated at 37c^{o} in a humidity range of 60-65%. Embryonated chicken eggs were used for production of pocks on the chorioallantoic membrane (CAM) at the age of 10-12 days. Embryonated eggs were candled in dark room to check for embryo viability.

2.5.1 Chorioallantoic Membrane Inoculation

A cross was made in an area over the air-cell and another one on the egg side using a pencil. Eggs were then swabbed with 70% alcohol and a pore was made at the crosses drilling just deep enough to penetrate the shell membrane to ensure that each chorioallantoic membrane (CAM) drops to form a false air sac. To drop the membrane, a rubber bulb was used .The bulb was placed over the hole in the air cell end of the egg and slowly aspirated from the cell by releasing pressure on the deflated bulb. Holes were drilled to the proper depth, to create a false air sac to form in the area of the second hole. Eggs were inoculated by chorioallantoic membrane (CAM) route with sterile disposable 1ml syringes.Using just

the tip of the needle, 0.1-0.2ml of inoculum was injected from the syringe. The pores were sealed with melted paraffin wax. Eggs were incubated at 37c^{o} for 5 days with daily candling to check for embryo death.

Embryos that died during 24 hours of inoculation were considered as nonspecific and discarded and those that survived, thereafter were killed by chilling at 4c^{o}.

2.5.2 Harvest of Chorioallantoic Membrane

Eggs were removed from the refrigerator, disinfected with 70% alcohol and the shell over the air sac was removed using sterile forceps.The embryo and yolk were extracted with forceps. Care was taken not to disturb the CAM, and the area of inoculation was examined for lesion before removal from the shell; the CAM was then detached from the shell with sterile forceps, stripped of excess fluid with another forceps and placed in a sterile Petri dish, then examination of the pock lesions was performed. CAM samples showing pock lesion were collected and homogenized using sterile mortars and pestels with aid of sterile sand. Samples were centrifuged at 1000 rpm for 10 min.

Supernatant fluids were collected into sterile bottles and treated with antibiotics and then stored in sterile bijou bottles at +4cº.

2.6 Preparation of Cell Culture

A flask containing confluent monolayer culture of Vero cell was obtained from the Central Veterinary Research Laboratory (Soba), the growth medium was removed and the cells were briefly washed with PD. 0.5ml of warm trypsin –versene solution was added and the flask incubated at 37cº until cells flew freely when the flask was tilted. Few drops of bovine serum were added to stop the action of trypsin and versene. The cells were diluted in GMEM growth medium and subcultured in appropriate plastic tissue culture flasks and incubated at 37cº.

2.7 Virus Propagation

A 24 wells plate containing semi-confluent monolayers was inoculated with 50 ul volume of inocula (every 3 wells inoculated with one sample). The inoculated plate was kept at 37cº for 60 minutes adsorption time. Incula were then removed and monolayers washed twice

with PD and refed with maintenance medium. The plate and a set of control wells were examined daily with an inverted microscope and, when cytopathic effect (CPE) involved 70 % or more of the sheet cover, the whole cultures were harvested by three repeated cycle of freezing and thawing. The harvested cell lysate was used as inculum to infect new plastic tissue culture flasks.

2.8. Virus Titration

Determination of tissue culture infective dose 50/ml (TCID 50/ml) for CPV was performed following the procedure described by Vilegas and Purchase (1983). Micro plates containing 96 wells were used. Ten-fold dilution of virus (10^{-1}_10^{-8}) was prepared in GMEM. 100ul of each dilution was added to five wells then 100ul of 2X Vero cell suspension was added to each well. A set of 5 wells was left as noninoculated control. The micro plate was incubated at 37c^{o} and examined for the presence of CPE every other day for 8 days postinoculation. Titers were expressed as TCID 50/ml calculated according to the method of Reed and Muench (1938).

2.9 Virus Neutralization test

The alpha neutralization procedure (constant-serum, diluted virus) as described by Beard (1983) was followed. In this method, micro-plate containing 96 wells were used, 10 fold serial dilutions (10^{-1}_ 10^{-8}) of virus under test were prepared in GMEM and each of the 5 wells received 100ul of a virus dilution. Each virus dilution was mixed with an equal volume of 1/5 dilution of rabbit hyper immune serum (HIS) against CPV (100ul) and another set of virus dilutios were mixed with normal rabbit serum. The mixtures were shaken vigorously and incubated at 37ºc for ½ hour. After that 100ul of Vero cells suspension were added to each well and the plate was incubated at 37cºThe end point titer of each serum –virus mixture was determined. Determination of the neutralization index (NI) was made by calculating the difference between the Log titer of the virus control (incubated with normal rabbit serum) and the Log of HIS – virus mixture.

2.10 PCR Procedure

2.10.1 DNA Extraction

When 100% CPE appeared, tissue cultures in plastic flasks were harvested by freezing and thawing three times, then pelleted by ultracentrifugation (Suprafuge 22,fixed angle, rotors HFA 22.50,8 X 50 ml, Heracus Sepatech) for 1 hour at 20000 rpm to pellet the virus. The supernatant was discarded, the pellet drained, covered with 0.5ml of PBS and kept at 4cº

over night. The pellet was then dissolved and transferred to eppendorf tube (1.5ml). The DNA was extracted by using a DNA Kit (Genomic DNA Isolation Kit,Puregene,Gentra System USA), in these steps:-

-300ul Cell Lysis Solution was added to the cells by pipetting up and down.

-1.5ul RNase A solution was added to cell lysate.

-Then 100ul of Protein Precipitation Solution was added, and the mixrure centriguged at 10.000xg for 3min.

-300ul 100% Isopropanol (2-propanol) was added to supernatant, which contain the DNA and the mixture centrifuged at 16000xg to precipitate the DNA.

-300ul 70% Ethanol was added to DNA pellet to wash it.

-10ul DNA Hydration Solution was added to rehydrate DNA.

2.10 2 Primers and PCR Condition

The nucleotide primers were derived from the Acidophilic-Type Inclusion body protein gene (ATI up 1 and ATI low 1) described by Meyer et al (1994). The ATI up 1 sequence is (5˜to3˜) AAT ACA AGG AGG ATCT with molecular weight of 5193.2 and the sequence of ATI low 1 is (5˜ to3˜) CTT AAC TTT TTC TTT CTC with a molecular

weight of 5645.6. The PCR condition was described by Meyer et al (1994), amplification was achieved by 35 cycles each including a denaturation step at 94c^{o} for 1 min, an annealing step at 40c^{o} for 1min and an extension step at 72c^{o} for 2.5 min .The final step was prolonged to 10 min to ensure complete extension of amplification products .The PCR reaction was carried out in a Biometra T3 Thermocycler (BIOMETRA, Germany).

2.10. 3 PCR Product Analysis

The PCR products were separated electrophoretically in 1% agrose gels (SIGMA) (1gm of agrose was dissolved in 100ml TAE buffer) containing ethdium bromide 1µl/40 ml agrose (PROMEGA, Madison, USA, 10mg /ml). 10 µl of 100 bp DNA Ladder (INVITRRGEN) which prepared by adding 20ml of ladder to 80ml of blue dye was loaded in the first slot of the gel. The 10µl of the PCR products were mixed with 5µl of

dye (bromophenol blue 11% +glycerol 40µl +DDW 50µl) and loaded on the rest wells. Electrophoresis was performed in a Minigel electrophoresis (BIOMETRA) using 75 volt for 45 min after the gel was covered with TAE buffer (40 ml of 40m M Tris-Hcl PH 8.0 +20 ml of 20m M Na-acetate) using standard Power Pak P 25(BIOMETRA). DNA bands were visualized using the BIODOC ANALAYZ gel documentation system (BIOMETRA).

Chapter III
Results

3.1 Growth of CPV and Vaccinia virus on the Chorioallantoic Membrane

CPV isolates CP/Mg/92/1, CP/Nw/92/2, CP/Dbg/92/3, CP/Nh/92/4, CP/Ab/93/5, CP/Tm/93/6 grew readily on the CAM of chicken embryonated eggs and produced pock lesions on the 5th day after inoculation. They were small, opaque- white, round and approximately 0.5- 1.5 mm in diameter and without hemorrhagic or necrotic centers. These pocks were usually accompanied with thickening of membranes and oedema and the virus was not lethal to the embryos. Also VD45 produced small pock lesions 0.5 -0.8 in diameter, white in colour and round with thickening of membranes.

Vaccinia virus, on the other hand, produced small pock lesions, round, opaque-yellow in colour and approximately 0.1-0.2mm in diameter and irregular in shape and induce hemorrhage of membranes with death of the embryos 3 days post inoculation. fig (1.b).

3.2 Growth of CPV and Vaccinia in Cell Culture

Clear CPE was observed when the 6 CPV isolates were inoculated in Vero cells starting on the 5th day after inoculation. The CPE consisted of rounding of cells, plaque formation, syncytia, cytoplasmic elongation and detachment from glass and give 80% CPE in 8 days post inoculation. VD 45 strain of CPV produced rounding of cells, few syncytial formation, detachment and plaque formation,detachment and ploque formation.fig (2B)

Vaccinia virus replicated in Vero cells and produced CPE characterized by cell rounding, plaque formation and destruction of the cell sheet, the time taken for

appearance of complete CPE (100%) was three days post inoculation.

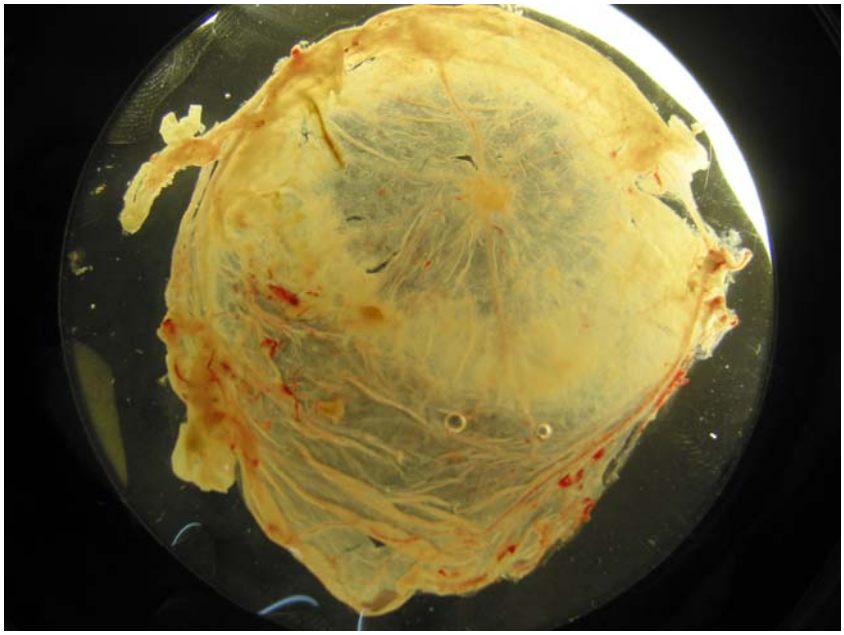

Fig 1.a: Pock lesions produced by CPV on CAM of embryonated eggs

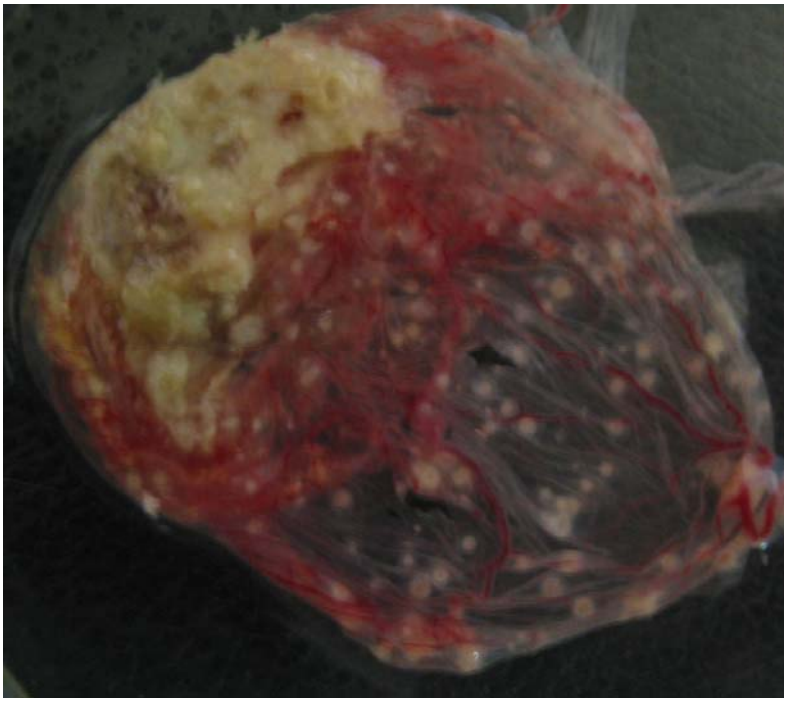

Fig 1.b Pock lesions produced by Vaccinia virus on CAM.

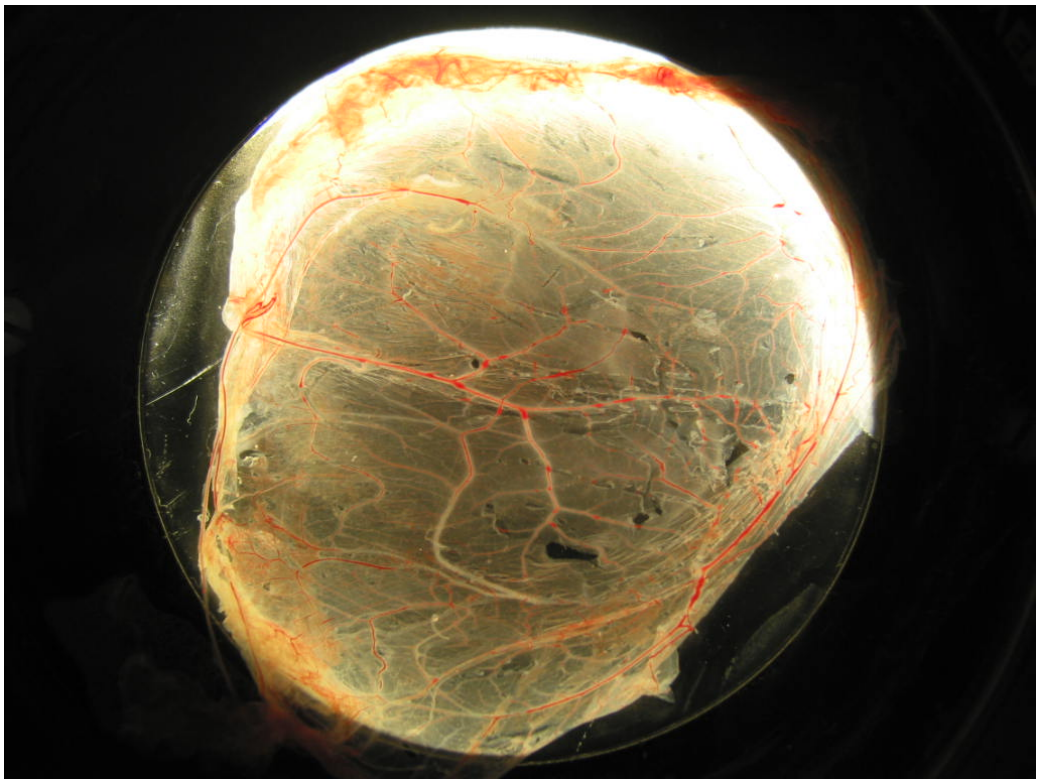

Fig 1 c: Uninfected CAM used as control.

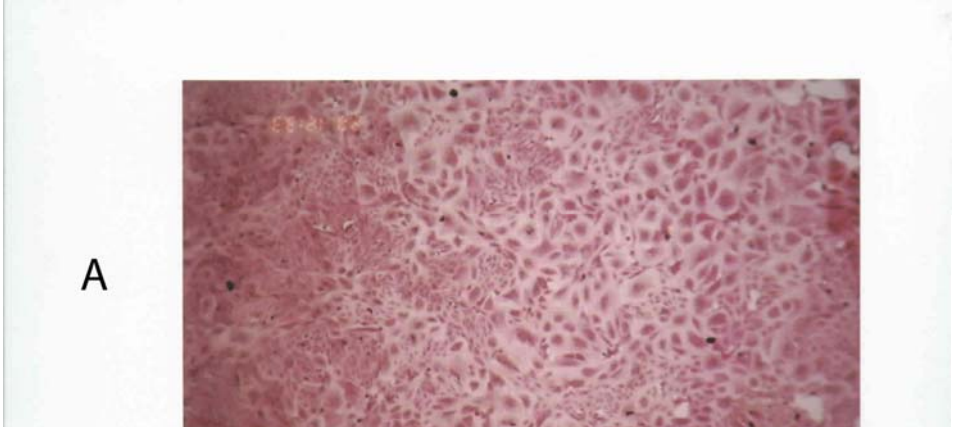

Fig 2. A: Normal Vero cell line , stained with H&E (X 40)

Fig 2. B: Vero cell line infected with CPV showing syncytia stained with H & E (X40).

3.3 CPV titration

Isolate CP/Dbg/92/3 was selected for virus titration in Vero cells.The tissue culture infective dose 50% (TCID50/ml) was found to be $10^{5.2}$/ml.

3.4 Neutralization of CPV

Isolate CP/Dbg/92/3 was selected to perform neutralization test as confirmatory test for diagnosis of CP. The titer Virus- HIS mixture was $10^{3.8}$/ml. Accordingly the neutralization index was calculated to be 1.4.

3.5 PCR amplification

3.5.1 PCR amplification from CAMs homogenates and tissue culture supernatant (Direct PCR)

No amplification products were detected when CAMs infected with CPV, Vaccinia and VD45 viruses were used. However, positive results were obtained with cell culture supernatant of CPV and Vaccinia viruses. The expected product size of CPV was 881bp fig (3). On theother hand, Vaccinia virus DNA gave product size of 300bp(data not shown).

3.5.2 PCR amplification from extracted DNA

Amplification products were detected when DNA was extracted by Puregene DNA Isolation Kit from cell culture supernatant infected with Vaccinia viruse, VD45 and CPV fig (4).

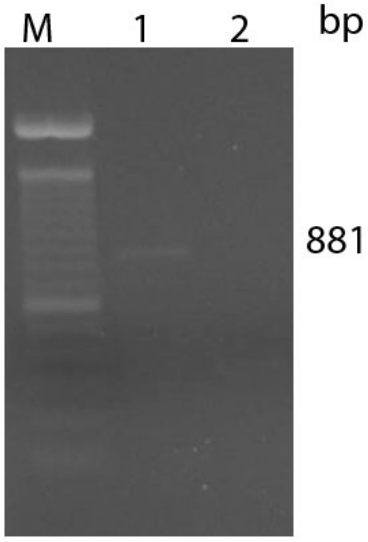

Fig 3: Visualization of CPV products by agrose gelelectrophoresis, direct PCR.

Lane1:tissue culture supernanant. Lane 2: CAM homogenate.

Lane M: 100bp marker.

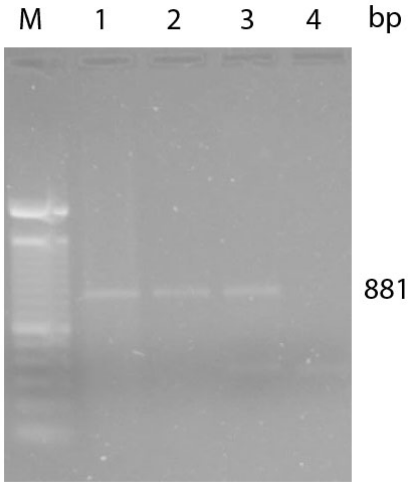

Fig 4: Visualization of CPV products by agrose gelelectrophoresis. Lane1-3: CPV DNA extracted by Kit. Lane M: 100 bp marker.

Chapter IV

Discussion

Biological methods like growth on Chorioallantoic Membrane of embryonated eggs (CAM) and cell cultures were used to distinguish between CPV, VD45 and Vaccinia viruses. Some distinctive features and differences in the character of lesions produced by these viruses were noticed.

In this study, CPV was able to grow on CAM and produced distinct pock lesions, which were opaque- white small in size about 0.5-1.5mm and increased in serial passage without hemorrhages or necrotic centers. VD45 produced small pock lesions, which were opaque- white, its size 0.3-0.5 mm. On the other hand, Vaccinia virus gave large pock lesions, which were round, opaque-yellow in colour, diameter was about 1.5-2mm. The results in this study confirm the observations of Marennikova *et al*, (1974) about CPV who reported that when CPV was propagated on CAM at 37c˚, monomorphic punctated, rather dense white pock 0.2-0.3mm in size developed, similar to those produced by Vaccinia but smaller in size; however, in this study, Vaccinia virus produced larger pocks.

The present study also yielded results which agree with those of Khalafalla *et al* (1998) who stated that CPV grew on CAM and produced distinct pock lesions which were opaque –white in colour, round and 0.5-1.5 in diameter. Renner-Muller *et al* (1994) observed that CPV produced small opaque- white pocks at ceiling temperature of 39c˚ and clearly separates CPV from Vaccinia, which induced large pocks.

When CPV, VD45 and Vaccinia viruses were grown in Vero cells they produced clear CPE consisting of rounding of cells, plaque formation, refrectile cells, destruction of cell sheet, syncytia and cytoplasmic elongation in about 8 days. Vaccinia virus produced cell rounding, plaque formation and detachment from glass in just 3 days after inoculation and no syncytia were observed; thus one can easily differentiate between CPV and Vaccinia viruses when they replicate in Vero cells. The latter observation is contradictory to the data of Baxby (1974) who observed that

the two viruses could easily be distinguished in Hela (Human cervical carcinoma, WISH (Transformed human amnion) and in some like Vero, HEL (Diploid human embryo lung) no differences were seen. The present data confirm the reported evidence on the growth of CPV in all cell cultures types by Khalafalla *et al* (1998) who observed that the CPE produced by CPV consisted of rounding of cells, plaque formation, cytoplasmic elongation and multinucleated giant cells and also agree with Renner-Muller *et al* (1994); Ramyar and Hessami (19972) and Nguyen *et al* (1989). Wernery *et al* (1997) reported that, the virus in scab samples collected from camels grow well in Vero and Dubca cells and developed syncytia within 2 to 3 days post inoculation. The syncytia consisted of giant cells, further inoculation led to lysis and detachment of infected cells resulting in a plaque type CPE. The initial appearance of CPE in rabbit kidney cells infected with CPV was characterized by rounding and increased granularity of cells 36- 48 hr post inoculation. Within 4 days, small syncytia appeared, detachment of the cells from the glass occurred on the 6 th day post inoculation (Tantawi and Sokar, 1975) and these confirm our results.

The neutralization test was used in this study as confirmatory test for diagnosis of CP and for the purpose of comparing the test with PCR. Vero cells infected with CP were used, and results were taken 8 days post inoculation. The results confirmed the identity of CPV isolates used in the present study since the test gave a neutralization index of 1.4. Virus isolation in cell culture followed by neutralization test, though is the best and accurate method of diagnosis of CPV (Davies *et al*., 1975 and Alfalluji., *et al* 1979) however, it was regarded as a time-consuming technique. To complete this procedure at least 2-3 weeks were required.

To overcome difficulties in virus propagation and substitute the time consuming serological techniques, rapid PCR screening methods are being developed for orthopoxvirus (Meyer *et al*., 1997). A PCR protocol was established that not only allows the detection of, but the differentiation of species of the genus orthopoxvirus. This assay was accomplished by the detection of oligonucleotides located within the gene that encodes the A- type inclusion protein of cowpox virus. The primer pair flanked a region exhibiting distinct and specific DNA deletion in the corresponding sequence of Vaccinia and Camelpox. For this reason PCR resulted in DNA fragments of different size (Meyer *et al*., 1994). A PCR

strategy to differentiate orthopoxviruses species based on the hemaggltinin gene has been described (Ropp *et al*., 1995).

In the present study, PCR using two methods of DNA extraction were employed for detection of CP and Vaccinia. PCR was performed according to the method described by Meyer *et al* (1994) using a single primer- pair based on sequences coding for the major protein component of the cowpox virus acidophilic-type inclusion body (ATI). The PCR product sizes differ depending on the species of orthopoxvirus. The first method of DNA extraction was accomplished by using viral DNA in CAMs, which was released by initial heating for 15min at 99c˚ in PCR tubes followed by ordinary PCR. This method gave poor results but when tissue culture supernatant was used good results were obtained and amplification products were detected. Product size when CPV DNA was amplified was 881bp, which is different from Vaccinia virus DNA which is 300bp. In the second method DNA was extracted by using DNA Isolation Kit from tissue culture supernatant and used as a template to conduct PCR. The size of CPV product was 881 bp corresponding exactly to that drived from purified DNA of the CPV reference strain and the size of Vaccinia virus was 300bp. These findings agree with those of Meyer *et al (*1997) who reported that, PCR experiments with primer pair ATI-up-, ATI-low-1 and template DNA extracted from cowpox, variola, Vaccinia, ectromelia and camelpox virus produced individual virus amplicons of variable sizes. The size differences corresponded to differences in base sequences of the ATI coding region in the GenBank database. The same result was obtained with Khalafalla *et al* (2003) for using PCR for rapid diagnosis and differentiation of parapoxvirus and orthopoxvirus infection in camels who used three procedures of DNA extraction to prepare DNA from scab specimens collected from camels infected by camel contagious ecthyma and camelpox and vaccinia virus as reference strain for orthopoxvirus. He found that the size of orthopoxvirus amplification products differed in size from that obtained with the reference orthopoxvirus.

Conclusion

From this study, it can be concluded that the camelpoxvirus and Vaccinia virus can be grown in chorioallantoic membrane of embryonated eggs and Vero cell line and give lesions by which differentiation between the two viruses can be achieved.

PCR assay provided an additional method to identify and differentiate camelpoxvirus and Vaccinia virus isolates. Also PCR assay was found to be superior to the current methods in use for identification of viruses grow in cell cultures; it is rapid, reliable, and can be applied to replace the conventional method of neutralization test.

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