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ISOLATION AND STANDARDIZATION OF CAMELPOX VIRUS FROM NATURALLY INFECTED CASES IN CENTRAL REGION OF SAUDI ARABIA 2004

(With 8 Photos and One Table)

By

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عزل فيروس جدري الإبل من حالات إصابة حقلية بالمرض في المنطقة الوسطى بالمملكة العربية السعودية. ٢٠٠٤م

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تم الكشف عن فيروس جدري الإبل في كحتات جلدية مجمعة من إبل مصابة بشدة بأعراض مرض جدري الإبل الإكلينيكية وذلك بواسطة اختبار الفلورسنت المناعي غير المباشر باستخدام المصل المناعي الأرنبي المضاد لفيروس جدري الإبل ، وتم عزل وتنقية المسبب المرضي من خلال خمسة تمريرات متتابعة في مزارع خلايا باستخدام أعلى التخفيفات الإيجابية ، لقد أظهر الفيروس المعزول آثار إمراضية متماثلة في الخلايا المحقونة فيما بين التمريرة الأولى إلى التمريرة الخامسة ، وتم اختبار قياسية الفيروس المعزول كعترة ضارية لفيروس جدري الإبل بواسطة إجراء اختباري الفلورسنت المناعي وتعادل الفيروس إلى جانب اختبار الإمراضية ، وسُميت بعترة العربية السعودية ــ عنيزه ٢٠٠٤.

SUMMARY

Camelpox virus in skin lesion scrapings; collected from camels severely affected with clinical signs of camelpox; suspecion was detected by Indirect Fluorescent Antibody (IFA) test using standard anti Jouf-78 strain camelpox virus rabbit antiserum. The causative agent was isolated and purified throughout five successive passages on vero cell cultures using the highest positive dilutions. The virus isolate showed identical

Cytopathogenic Effects (CPE) in inoculated vero cell cultures between the 1st. and 5th. passages. The isolate was standardized as a virulent camelpox virus by application of IFA, virus neutralization and pathogenicity tests ,and designated as Saudi Arabia, Uniza 2004 strain.

Key words: *Virology, camelpox virus, vaccination*

INTRODUCTION

Camelpox is the most frequent infectious viral disease widely reported in camelids in Asia and Africa (Kriz, 82, Higgin *et al* 1992, and Al-Hendi *et al* 1994). The causative agent is a brick shaped, enveloped, double stranded DNA virus with irregularly arranged superficial protein filaments, measured 260-280 nm x 190-200 nm. The virus was classified as species member of the genus orthopoxvirus belonging family poxviridae, (Munz, 1992 and Renner-Muller *et al* 1995). It causes mild (local) or severe (systematic) disease characterized by fever, development of localized or generalized skin and mucus membrane eruptions of papules, vesicles and pustules with crusts on skin of lips, nostrils, eye lids, legs and thigh region, and mucus of buccal cavity and nares with mortalities in young camels (Munz *et al.* 1986 and Al-Hendi *et al.* 1994). This study was understaken to isolate and standardize a local virulent strain of camelpox virus in order to facilitate the process of quality control potency test of the locally attenuated camelpox virus, Jouf-78 strain vaccine where no challenge virus of camlepox is available.

MATERIALS and METHODS

Animals:

Three clinically health native young camels, one aged 4 months and two aged 6 months purchased from local market. The animals were housed in special isolatd stable in National Agricultural Research & Animal Resources Center (NARARC), Riyadh. Animal were observed and their temperatures were taken daily before inoculation with the virus isolate.

Cells:

Cultures of vero cell line obtained from Veterinary Vaccines Production center, Riyadh, Saudi Arabia were maintained and grown in Eagle's Minimal Essential Medium (EMEM) supplemented with 2-10% fetal bovine serum plus 100 U Penicillin sodium and 100 mg streptomycin sulphate / 1 ml medium.

Antiserum:

Standard anti-camelpox virus serum prepared in rabbits and guinea pigs against Jouf-78 strain of camelpox virus by NARARC (under publication) was used in detection and standardization of the camelpox virus isolate throughout immunofluorescent and neutralization tests.

Reference Virus:

Camelpox virus, (Jouf-78 strain, isolated from clinical cases of the disease in Al-Jouf, north region of Saudi Arabia) attenuated throughout serial passages in primary cell cultures of the kidney of camel by NARARC, Riyadh, Saudi Arabia and reviewed by the Institute of Tropical Veterinary Medicine and Pathology, Faculty of Veterinary Medicine, Munich, Germany was used for preparation of the live attenuated vaccine of camelpox (Hafez *et al.* 1992). Stock vials of the vaccine batch No.1, produced by NARARC in 1995 and kept at –20°C was titrated in vero cell cultures and used in conduction of serum neutralization test.

Clinical History and Sampling:

In January 2004, camelpox was clinically suspected in herd of 160 camels in Unaiza, central region of Saudi Arabia. 70 out of 160 camels had showed clinical signs of fever, appearance of crusted papules and pustules on the thigh region, concurrent with vesicular buccal mucosal lesions. The fatality rate was approximately 13% in youngs. Scrapings were collected from skin lesions in sterile containers without additives and transported in an ice box into the laboratory of veterinary diagnosis, Riyadh for virus isolation.

Preparation of Tissue Samples:

Skin eruption scrapings were prepared for virus detection and isolation as follows:

10% tissue hemoginate in 0.01 M phosphate buffered solution (PBS), pH 7.4 (W/V) was prepared by using electric homogenizer, (Power Gen 125, Fisher Scientific, USA,) then centrifuged at 5000 RPM for 30 minutes at 10°C. The supernatant fluid was kept at -70°C until used for virus isolation. The sediment was washed twice with PBS through resuspension and centrifugation at 3000 RPM for 10 min. Washed cells were dispersed and diluted in appropriate volume of PBS, then dispensed in wells of chamber slide (Lab-tek, Nalgen Nuc) 50 ul / well. After dryness of the cells suspension on slide, 100 ul of 80% cold acetone in PBS was added to each well then kept at +4°C for 1 hour. The

fixed slide was washed twice with PBS and kept at 4°C until examined for camelpox virus detection.

Virus isolation and propagation in vero cell cultures:

Confluent vero monolayers (25 cm^2 tissue culture) flasks were inoculated with unfiltered and undiluted sample preparation as well as several ten-fold dilutions of unfiltered sample in EMEM. After three hours of virus adsorption at 37°C, the inoculums were decanted and washed with appropriate volume of EMEM, 10 ml of EMEM supplemented with 2% fetal calf serum plus 500 U Penicillin sodium and 500 ug streptomycin sulphate / ml medium was added to each flask. Inoculated flaks were incubated at 37° with daily media changes before the appearance of CPE. Inoculated cultures fluids were collected when the maximal CPE completed. The highest dilution of sample showing proper progressive cytopathogenic effect in inoculated cells cultures was selected for repassage in new cultures of vero cells using serial ten-fold dilutions. The 3^{rd}. to 5^{th}. passages were completed with the same way.

Cytopathology:

Infected vero cells monolayers which showed focal and extensive cytological changes in the five passages of the virus isolate, were washed by rinsing in 3 changes of PBS; PH 7.4; then fixed in 10% formalin in PBS. The changes induced by the isolate virus were microscopically examined.

Virus Infectivity Assay:

Virus infectivity was assayed by end point in microtitre plate cultures of vero cells, using serial ten-fold dilutions (four wells per dilution). Titres were calculated by the method of Reed and Munch 1938, and regested as Log_{10} $TCID_{50}$ / ml.

Indirect Fluorescent Antibody (IFA) Test:

IFA test was applied for detection of camelpox virus antigens in infected tissues specimens (skin eruption scrapings) and in infected vero cell cultures (inoculated with the 4^{th}. passage of the virus isolate) in guidience of Woldehiwet and Hussein, 1994 as follows:

Fixed infected and control cells on the slides were incubated with a 1:5 dilution of fetal calf serum in 0.01 M PBS, pH 7.4 for 10 min at 37°C. After through washing with PBS for 10 min at room temperature, standard anti-camelpox rabbit serum diluted 1:20 in PBS was added to the slides (50 ul / Well). The slides were incubated in humid box at 37°C for 1 hour. After washing twice, 50 ul of 1:20 dilution of fluores (Conjugated Swine anti-rabbit IgG Dokocytomation, Lot No. 4918,

Denmark) was added to each well and followed by incubation at 37°C for 45 min. After additional twice washing, the slides were examined.

Virus Neutralization Test (VNT):

VNT was conducted as described by Cottral, 1978 to specify the virus isolate as a camelpox virus using constant dilution (1:10) of standard anti-camelpox-virus rabbit and guinea pig sera against variable ten-fold dilutions (10^{-1} – 10^{-4}) of the virus isolate at the 4th passage. Neutralization period of one hour was used. The virus-serum mixtures and the virus alone were inoculated in a microtitre plate cultures of vero cells. Log neutralization index (NI) was calculated as the Log titre of the virus alone minus Log titre of the virus-serum mixture.

Serum neutralization test (SNT)

SNT was performed basically with the same way as that of Hafez et al 1992 in a microtitre system using monolayer cultures of vero cells, and serial double fold dilutions (1:12 – 1: 256) of serum samples against 10 $TCID_{50}$ of camelpox virus, Jouf-78 strain. The titre was calculated as the reciprocal of the highest serum dilution that completely almost inhibited viral CPE in 50% of inoculated cells.

Pathogenicity Test:

The harvest of 2nd passage of the virus isolate on vero cell cultures was inoculated experimentally in one Camelpox-partially immune camel calf and two camelpox seronegative camel calves. Each animal was injected intramuscularly with 2 ml of the virus which had an infectivity titre of 4.5 Log_{10} TCID 50 / ml. Animals were observed and examined daily for any deviation from normal health and body temperature was recorded until the 10th Day Post Inoculation (DPI). Also, serum samples were collected from each animal at the day of inoculation and the 10th DPI. These serum samples were subjected to SNT for detection of seroconversion rate in the inoculated animals.

RESULTS

Virus Detection:

Emissions of the positive Immunofluorscence (IF) reaction was seen by flourescent microscopy using standard anti-camelpox-virus rabbit-serum and conjugated antispecies in emulsion of skin lesion scrapings, and in the cytoplasm of vero cells inoculated with the 5th. passage of the isolate (Photo1 and 2).

Cytopathogenecity :

The cytopathological changes in vero cells inoculated with a high dilutions of the virus isolate between the 1st and 5th passages were recorded. Foci of rounded cells appeared within the 24th-48th Hour Post Inoculation (HPI), then enlarged progressively with clumping and ballooning of the cells followed by rapid degeneration and detachment between the 96th and 120th HPI (Photo. 3,4, 5 and 6).

Virus Titration and Neutralization:

The infectivity titres of the virus isolate in vero cells were 4.5 Log_{10} and 6.2 Log_{10} $TCID_{50}$ / ml respectively in passages No. 2 and 5. Standard anti-camelpox virus rabbit and guinea pigs sera were reduced the infectivity of the isolate of the 5th passage in vero cells by 2.75 and 1.5 Log_{10} $TCID_{50}$ respectively, (NI 2.75 & 1.5).

Pathogenicity Test:

One of the camel calves; which has a titre of camelpox virus neutralizing antibodies (4) at the time of inoculation with the virus isolate; showed rise of body temperature ranged between 39.5°C and 40° C during the first three days after inoculation. No apparent clinical manifestations were recorded until the 10th DPI (end time of experiment). Camelpox virus neutralizing antibody titre increased to (48) in sera collected by the 10th DPI (Table 1).

One of the seronegative camel calves body temperature was raised 0.5-1.5°C over the normal temperature between 1st and 9th DPI. Appearance of watery ocular discharges on the 2nd DPI and copious mucopurulent oculo-nasal discharges between the 4th and 6th DPI associated with moderate respiratory manifestations (coughing and dyspenia) with severe itching, followed by rapid relief of the respiratory manifestations were recorded (Photo7). No localized or generalized pox lesions were noticed on the affected animal until the 10th DPI. There was an increase in titre of camelpox virus-neutralizing antibodies from (0.0) to (12) in period of ten days after inoculation (Table1).

The other seronegative camel calf had an increase of body temperature between 0.3°C - 1.0°C over the normal temperature. This was noticed between the 3rd and 6th DPI. Appearance of oculo-nasal discharges, anorexisa and severe respiratory distress (coughing and dyspenia) were clearly observed by the 8th DPI followed by rapid prostration, recumbancy and death on the day 10 after inoculation. Focal lobular edema in lung tissue with peterchial hemorrhages and body exudates in the thoracic cavity were the most pronounced post mortum findings (Photo 8).

Photo 1, 2: Positive immunoflurscent reactions in vero cell sheet inoculated with camelpox virus isolate, one day and three days after inoculation (X200)

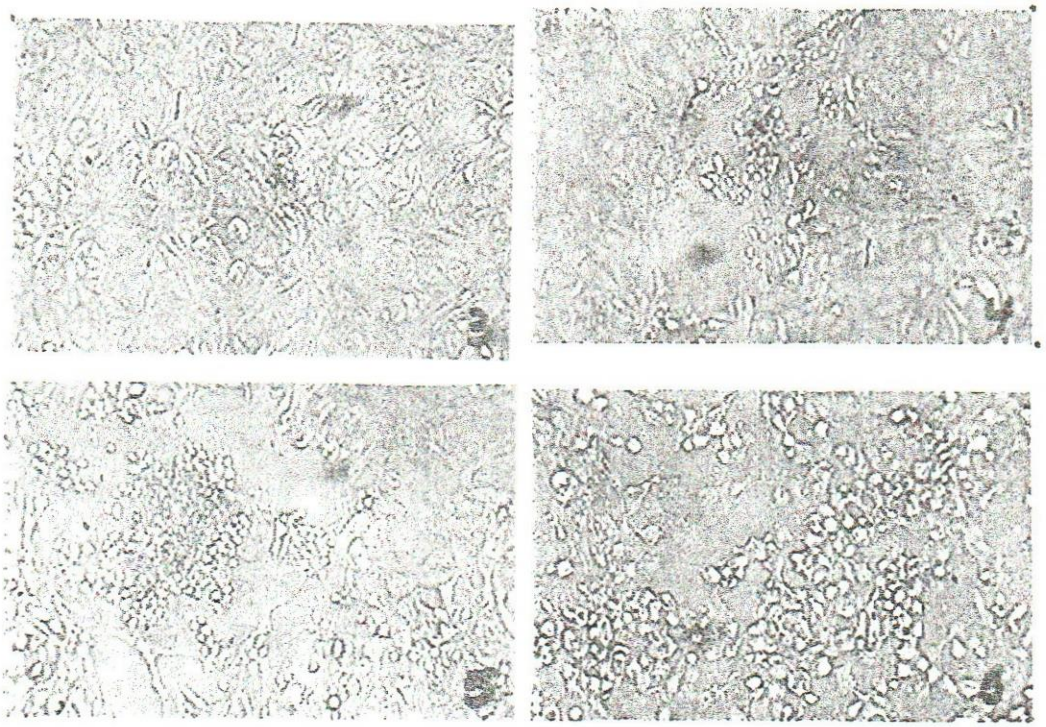

Photo 3, 4, 5 and 6: Cytological changes in vero cells sheet inoculated with camelpox virus isolate, 0, 1, 2 and 3 days after inoculation (x 100)

Photo 7: Oculo - nasal mucopurulent discharges in camel calf inoculated with camelpox virus isolate.

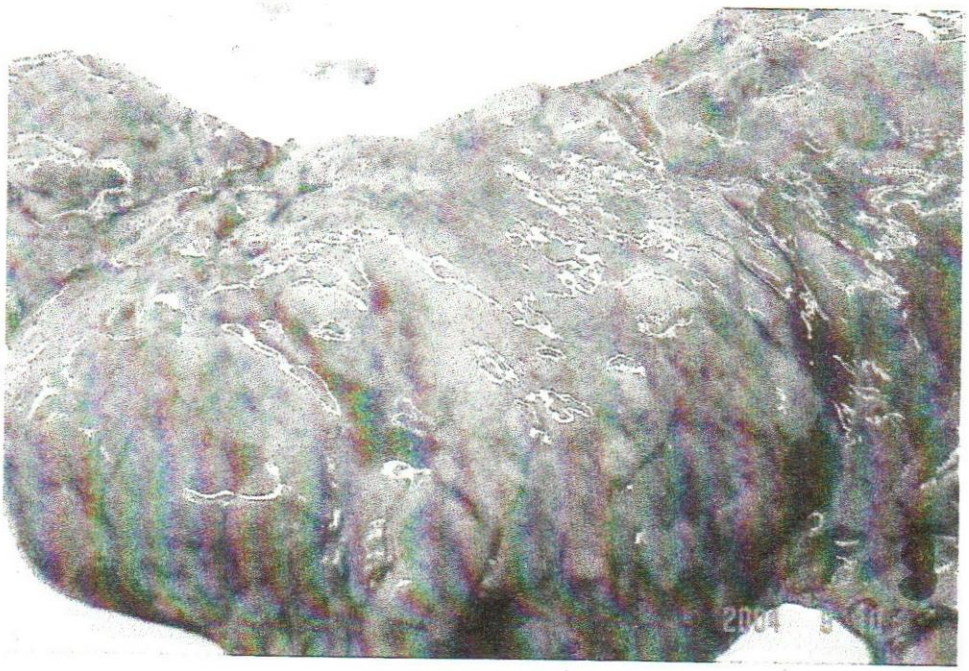

Photo 8: Focal lobular edema in lung of camel calf succumbed 10 days after inoculation with camelpox virus isolate.

Table 1: Temperature and seroconversion in camel calves experimentally inoculated with camelpox virus isolate.

Camel Calf No.	Rectal Temperature, °C DPI											Camelpox virus neutralizing antibodies, DPI	
	0	1	2	3	4	5	6	7	8	9	10	0	10
1	38.8	40	39.9	39.5	39	39.1	39.0	39.2	39	38.9	39.0	4*	48
2	39.0	40	40.3	40.5	40.5	40.4	40.3	40.3	40.0	39.5	39.2	0	12
3	38.5	39	39	40	39.5	39.7	39.3	39.1	38.0	37.9	S	0	4

DPI : Day Post Inoculation
S : Succumbed

DISCUSSION

Routine potency test of live attenuated vaccine of camelpox virus is based on a vaccination challenge test in susceptible adult camels. Vaccinal and challenge strains of camelpox virus are the principal elements in this test. This study aimed for isolation and standardization of a local virulent strain of camelpox virus. It will be used as a challenge virus in need for application of direct potency test of the tissue culture live attenuated vaccine in Saudi Arabia.

The skin lesion scrapings were obtained from a field camelpox suspected case. Once it showed positive fluorescent emissions, serial dilutions of the sample were inoculated on cultures of vero cells to isolate the causative agent. Observations of characteristic cytopathological changes in the inoculated cultures were identical to that described by Ramyar and Hessami, 1972 and Davis *et al,* 1975. The CPE of the reference camelpox virus, Jouf-78 strain encouraged us to repass the virus isolate on vero cell cultures for further four successive passages. Moreover, a strict similarity in the CPE of the virus isolate between the 1st and 5th passages on vero cell cultures proved that the virus isolate required no adaptation to vero cells and the uniformity of the virus isolate. This finding does agree with Kaaden *et al,* 1992 and Renner-Muller *et al,* 1995. Therefore, infectivity titre of 4.5 Log_{10} $TCID_{50}$ / ml of the 2nd passage and 6.2 Log 10 $TCID_{50}$ / ml of the 5th passage of the virus isolate were allowed to conduct the virus neutralization test.

The obtained neutralizing indices of (2.75) against rabbit anti-camelpox, virus serum and (1.5) against guinea pig anti-camelpox virus

serum as well as positive immunofluorescence reaction in vero cells inoculated with the 5th passage of virus isolate against standard rabbit anti-camelpox virus serum were referred significantly to the quite homolog between the virus isolate and Jouf-78 strain of camelpox virus.

Concerning the pathogencity of the virus isolate in three native camel calves aged 4-6 months, one partially immune camel calf that had a residual maternal immunity of camelpox virus neutralizing antibody titre of 4 did not show clinical manifestations other than the rise of body temperature between the 1st and 3rd DPI. Two camelpox seronegative camel calves showed fever, anorexia, muco-purulent nasal discharges and respiratory distress manifestations including coughing and dyspenia that might complicated in one of them leading to death by the 10th DPI. Respiratory manifestations are the predominant clinical symptoms of camelpox particularly in young camlels (Zaitoun *et al* 2000). Focal lobular edema in lung tissue of the dead animal was clearly seen macroscopically but virus isolation from these lesions failed.

Unfortunately, no localized or generalized eruptions of camelpox lesions were noticed in any of the inoculated animals during the period of experiment (10 days after inoculation). Only one out of two Camelpox seronegative suffered from severe itching by the 6th DPI. It was noticed that all of the three Camel calves that were inoculated with the virus isolate showed a significant seroconversion. Camelpox virus-neutralizing antibody titres increased from 4 – 48 in one animal and from 0 to 4 and 12 in the others. It is important to mention here that control seronegative adult camels challenged with camelpox virus did developed a neutralizing antibody titres between 24 and 64 by the 14th DPI (Hafez *et al.* 1992).

In conclusion, Saudi Arabia, Unaiza 2004 (SA, U. 2004) is a suggesting designating name of the present virus isolate and further study on application performance of this isolate, as a challenge virus in camels vaccinated with Saudi vaccine of camelpox virus, Jouf-78 strain is recommended.

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