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POLYMERASE CHAIN REACTION FOR DIFFERENTIATION BETWEEN SOME CAPRIPOX VIRUS ISOLATES

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ABSTRACT

A total number of 38 clinical specimens of skin lesions from diseased animals (17 cattle and 21 sheep) of both sex and of different ages were investigated for lumpy skin disease virus (LSDV) and sheep pox virus (ShPV). Bovine samples obtained from (Shubra Shehab station, Qalubia Governorate) with 60% morbidity and 8% mortality especially in young animals. The clinical examination revealed the presence of nodules found most numerous on head and neck of the animal and may cover whole body with some eruption of skin nodules. Ovine samples obtained from sheep housed in (Sakha station, Kafr El-sheikh Governorate) with 70% morbidity and 20% mortality especially in kids 3-4 month old. The affected sheep was suffering from papules with hard swelling may covered by fluid filled vesicles in axilla and perineum region with enlargement of superficial lymph nodes. The two viruses were successfully isolated on ECE and tissue culture and identified using agar gel precipitation test (AGPT), fluorescent antibody technique and virus neutralization test (VNT) with sensitivity of 42.2%, 71.1% and 65.8% respectively. The two viruses were differentiated using multiplex polymerase chain reaction (MPCR) with species-specific primers for LSDV and ShPV of different amplicon sizes about 192 bp and 289 bp respectively with high specificity and sensitivity with speed and do not require nucleotide sequencing or restriction analysis of PCR products. Therefore MPCR shown to be the method of choice for differentiation between some Capripox virus isolates and diagnosis directly from clinical specimens.

INTRODUCTION

Capripoxviruses (CaPVs) represent one of eight genera within the Chrodopox virinae (ChPV) subfamily of the Poxviridae family. The capripoxvirus genus is currently composed of three closely related viruses: Lumpy skin disease virus (LSDV), sheep poxvirus (ShPV), and goat poxvirus (GPV) ***(Tulman et al., 2001, Adama and Gerrit 2007 and Balinsky et al., 2007). OIE*** includes these diseases in list A of the most dangerous animal diseases, which can cause epizootics entailing great economic loss. ***(Orlova et al., 2006).***

The virion is brick shaped particle 170 to 260 by 300 to 450-nm-diameter capsid contain a linear, non segmented, double-stranded DNA genome of approximately 150 kilo bases that is surrounded by lipid that is not true envelope ***(Moss, 2001).*** CaPVs tend to be host specific, however incidences where ShPV and GPV have crossed species into goats and sheep respectively have documented ***(Munz and Dumbell, 1994).***

ShPV and GPV often transmitted by respiratory route during close contact, abraded skin, or transmitted mechanically by insect, but LSDV transmitted primary by biting insects ***(OIE, 2008)***.

LSD is mainly confined to Sub-Saharan Africa suggesting that they are caused by distinct viruses ***(Mondal et al., 2004).*** ShPV and GPV are endemic to Asia, and Africa south of the equator, particulary to the north and west of the Sahara ***(Orlova et al., 2006)***. Lastly, theses viruses could be isolated from recent outbreaks in Yemen and Vietnam ***(Babiuk et al., 2009).*** The only confirmed LSD case in cattle occurred in Israel in years 1989, 2006 and 2007 ***(Starm et al., 2008).***

ShPV and GPV genomes are very similar to that of LSDV, sharing 97% nucleotide identity. However LSDV contain its unique genes not found in the ShPV and GPV. The absence of these genes in ShPV and GPV suggests a significant role for them in bovine host range **(*Tulman et al., 2002).***

LSD (psudourticaria, Neethling virus disease) is a pox viral disease of cattle with significant morbidity. Losses occur from decreased milk production, abortion, infertility, loss of condition and damaged hides. The development of nodules may penetrate the full thickness of skin and sometimes the underlying muscle. The necrotic material separated from adjacent skin (sit fast). Deep holes or scars are often left in skin ***(OIE, 2008)***.

LSD disease was recognized in Egypt in Suez Governorate in May 1988 and Ismailia by ***Ali et al., (1990), House et al., (1990) and (Saber, 1992).*** Another outbreak was reported among cattle in El- Menia Governorates, Upper Egypt in July 1998 **(*Abd El- Rahim et al., 2002).*** A new rash of LSD cases appeared in July 2006 and has proven more difficult to control ***Daily Star Egypt Staff (2006).*** About 15 outbreaks in 5 Governments Ismailia, BeniSweif, Al Beheira, New Valley and Monoufiya.

Sheep pox is contagious viral disease of sheep. This disease may be mild in endemic areas, but are often fatal in newly introduced animals. Economic losses result from decreased milk production, damage to the quality of hides and wool. The disease can limit trade and prevent the development of intensive livestock production ***OIE (2008).*** The disease was reported in Egypt by many authors ***Sabban (1957), Soad et al., (1996),Agag et al.,(1997),Tawfik et al.,(2001) and Nawal et al.,(2006).***

In Egypt, although the vaccination programs of both viruses were applied annually, epidemics occurred in Egypt from time to time ***(Agag et al., 1997 and Mohammed, 2000).***

Unfortunately serological distinction between CaPVs is not possible, previous classification was based only on animal host origin but today differentiation is possible using genomic DNA. Therefore our article was planed to isolate these viruses and the application of modern, recent and accurate technique as multiplex PCR as a sensitive reliable test for detection and differentiation of CaPVs.

MATERIAL & METHODS

Animals: A total number of 38 diseased animals (17 cattle and 21 sheep) of both sex and of different ages were subjected for this work. Bovine samples obtained from Shubra Shihab station, El Kanater El Khairea, El Qalubia Governorate with 60% morbidity and 8% mortality especially in young animals. All the animals were vaccinated by sheep pox vaccine. Ovine samples obtained from Sakha station and Kafr EL Sheikh Governorate with 70% morbidity and 20% mortality especially in kids 3-4 month old.

Sample collection and preparation: The samples were sent to the laboratory as soon as possible and treated according to ***OIE (2008).*** Skin biopsies comprising epidermis, dermis and subcutis of the nodules were minced and ground in a sterile pestle and mortar with sterile sand then 10% suspension was prepared in sterile PBS containing sodium penicillin (1000 IU/ml), streptomycin sulphate (1mg/ml) and mycostatin (100 IU/ml). The suspension is freeze-thawed three cycles and then partially clarified by centrifugation at 3000 rpm/10 minutes, the supernatant fluid was stored at -20̊c until used for virus isolation on ECE and Tissue culture.

Virus isolation:

1) **On embryonated chicken egg (ECE):** The isolation was applied according to ***House et al., 1990.*** Specific pathogen free (SPF)-ECE were obtained from National Laboratory for Quality Control on poultry production (NLQP), Dokki, used for virus isolation by inoculation via CAM rout (11-13days old embryos) and incubated at 35̊c and 70% humidity for 5 days post inoculation.

2) **On tissue culture:** Vero cell culture as well as Maddien Derby bovine kidney(MDBK) were used for virus isolation. The cultures were obtained from Virology Dept, Animal Health Res. Institute, Cairo. Samples were inoculated for three passages on tissue culture (Vero cell for ovine sample and MDBK cell for the bovine sample) according to ***Carn and Kitching (1995).*** The infectivity titer was $10^{3.2}$ $TCID_{50}$ /ml for LSDV, while it was $10^{3.4}$ for ShPV.

Virus identification:

1) **Agar Gel precipitation test (AGPT):** The technique was applied according to ***OIE (1989)*** for identification of the isolated viruses in concentrated infected tissue cultures and in 10% suspensions of inoculated CAM by using hyperimmune serum kindly obtained from pox Dept, Serum and Vaccine Research Institute, Abbasia,

2) **Direct fluorescent antibody technique (FAT):** It was carried out according to ***Kitching and Hammond (1992).*** Briefly, the infected cultures fixed in acetone at -20̊C for 10 minutes and stored at 4̊C till staining with antibovine fluorescein isothiocyanate conjugates was supplied by Virology Dept Animal Health Res. Institute, Dokki,

3) **Virus neutralization test:** The test performed according to ***Pandy and Singh (1970).*** Neutralization procedure using hyperimmune serum supplied from pox department, serum and vaccine research Institute, Abbasia, Cairo.

4) **Multiplex polymerase chain reaction (MPCR):** The technique was applied according to ***Orlova et al., (2006),*** using species-specific different primers with different amplicons in a singl tube.

DNA Extraction: The DNAs extracted from samples as well as from isolated viruses as described by ***Sambrook et al., (1989)*** phenol chloroform method. The infected cell lysate 10% scab suspension were clarified by centrifugation at 1500 g for 10 min followed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1). The DNA was precipitated with absolute ethanol and finally dissolved in 30ul of nuclease free water after air drying the pellet.

DNA amplification: The extracted DNAs was amplified using oligonucleotid primers for LSDV designed according to the sequence of viral attachment protein encoding gene published by ***Ireland and Binepal (1998).*** The size of the amplicon is 192 bp the primers have the following gene sequences:

- Forward pimer 5'- TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'

- Reverse primer 5'-AAA-TTA-TAT-ACG-TAA-ATA-AC-3'

The oligonucleotide primers for sheep pox were prepared according to the published data of the inverted terminal repeats (ITRs) gene by ***Black et al.,(1986)*** and ***Gershon and Black (1989).*** The size of amplicon is 289 bp the primer have the following gene sequence:

- Forward primer 5'-AGA-AAC-GAG-GTC-TCG-AAG-CA-3'

- Reverse primer 5'- GGA-GGT-TGC-TGG-AAA-TGT-GT-3'

The primers were synthesized by MWG-Biotech(Eberberg, Germany). The amplification was carried out according to ***OIE (2008).*** A final volume of 50ul containing: 5ul of 10X PCR buffer, 1.5ul of $MgCL_2$ (50mM), 1ul of dNTP (10mM), 1ul of each primer, 1ul of DNA template, 0.5ul of Taq DNA polymerase then added nuclease-free water to the final volume. The sample were incubated in the Thermal cycler (MJ incorporation, USA) programmed to perform initial denaturation step 2 minutes at 95°c followed by 35 cycles consisting of (45 second at 95°c for denaturation, 50 second at 50°c for primer annealing, 1minute at 72°c for extension) then 2 minutes at 72°c for final extension. Lastly the products holded in the thermal cycler at 4°c until analysis.

Analysis of PCR products using agar gel electrophoresis: The analysis was carried out according to ***Sambrook et al., (1989).*** Briefly 10ul of each PCR product was loaded on 1.5% agarose gel, containing 1ul/ml ethidium bromide in Tris-acetate buffer and visualized under ultra violet transilluminator. Positive control viruses (supplied from pox Dept. Serum and Vaccine Res. Institute, Abbasia, Cairo) as well as negative control and 100 bp DNA-Marker Ladder were also included in the test.

RESULTS

In this study, the clinical observation of affected animals revealed presence of fever as first clinical signs, skin lesions, in addition to swelling of lymph nodes, congestion of oral, nasal and ocular mucous membranes. The lesions range from few skin nodules in mild cases to large number of nodules covering the whole body in severely affected cases. By inoculation on CAM of SPF-ECE characteristic inflammatory edematous spots (pock lesions) were seen five days post inoculation (after 1st passage and become clear after 3rd passage) while embryonic death does not occur **(photo 1).**

The inoculations on tissue cultures have successfully produced characteristic CPE consisting of retraction of cell membrane from surrounding cells and eventually rounding of cells 8 days post inoculation. Three passages were done for viruses isolation. The isolated viruses were identified using AGPT, the results are presented in **table (1).** A clear precipitation lines were appeared (as a positive results) against reference LSDV and ShPV antisera. Regard with fluorescent antibody technique positive samples showed small fluorescent granules in the cytoplasm **(photo 2),** while the stained non infected control cells showed no fluorescence**.** Results of the test are recorded in **table (2).** The isolated viruses were identified also using virus neutralization test the results are documented in **table (3).** The viral attachment protein primers as well as the inverted terminal repeats gene primers used in PCR amplification produced the expected amplicon sizes of 192 bp and 289 bp for LSDV and ShPV respectively, while no PCR product was observed from negative control **(photo 3)**.

Table (1): Identification of the isolated virus by Agar gel precipitation test (AGPT) using reference anti LSDV and ShPV antisera.

Animal	No. of sample	Results		Suspected virus	%
		+Ve	-Ve		
Cattle	17	7	10	LSDV	41.2
Sheep	21	9	12	ShPV	42.9
Total No.	**38**	**16**	**22**		**42.1**

LSDV: Lumpy skin disease virus. ShPV: Sheep pox virus.

Table (2): Identification of the isolated viruses by fluorescent antibody technique.

Animal	No. of sample	Results		Suspected virus	%
		+Ve	-Ve		
Cattle	17	12	5	LSDV	70.6
Sheep	21	15	6	ShPV	71.4
Total No.	**38**	**27**	**11**		**71.1**

Table (3): Identification of the isolated viruses using virus neutralization test (VNT) with antiserum (1:20).

Animal	No. of sample	Results		Titer No. of isolates					Suspected virus	%
		+Ve	-Ve	1/8	1/16	1/32	1/64	1/128		
Cattle	17	11	6	-	1	3	5	2	LSDV	64.7
Sheep	21	14	7	2	4	1	4	3	ShPV	66.7
Total No.	**38**	**25**	**13**							**65.8**

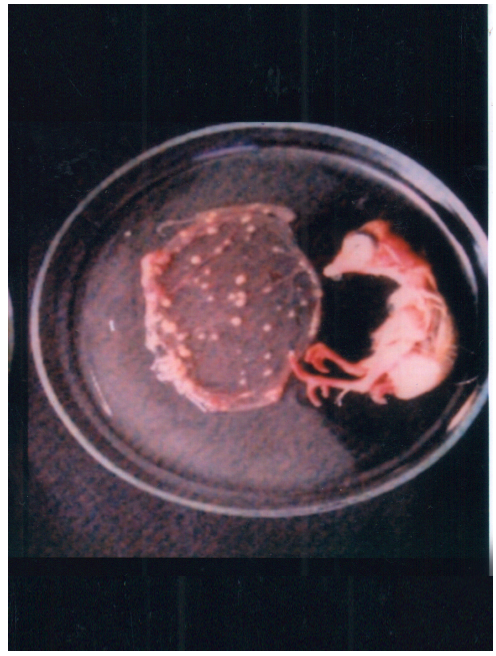

Photo (1): Characteristic inflammatory odematous spots (pock lesions) on specific pathogen free (SPF) - embryonated chicken egg (ECE).

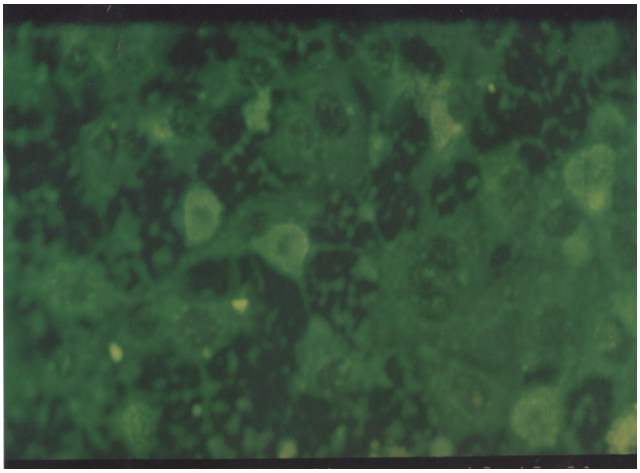

Photo (2): Fluorescent antibody technique in the cytoplasm of infected MDBK cells.

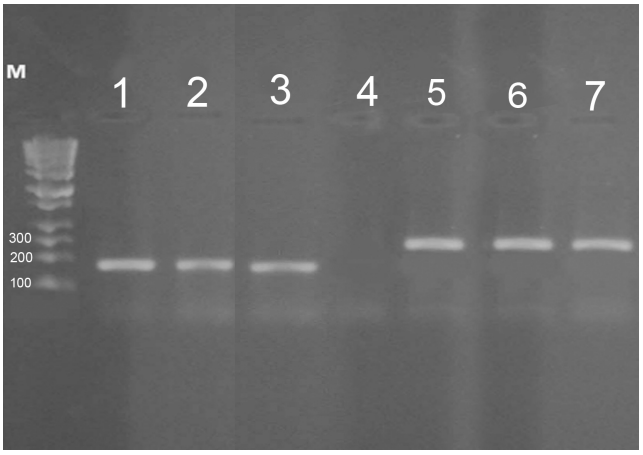

Photo (3): PCR amplification of isolated LSDV and ShPV. PCR products were electrophoresed on 1.5% agarose gel, with ethidium bromide and visualized under an ultraviolet transilluminator. **M:** Molecular weight marker, 100 bp DNA Marker Ladder. **Lan (1):** PCR amplification product of LSDV from tissue culture. **Lan (2):** LSDV from skin biopsy. **Lan (3):** Positive control for LSDV. **Lan (4):** Negative control. **Lan (5):** ShPV from tissue culture. **Lan (6):** ShPV from skin biopsy. **Lan (7):** Positive control for ShPV.

DISCUSSION

Capripoxvirus infections including ShPV, GPV and LSDV, which are listed by the OIE, are malignant, severe and highly contagious diseases in sheep, goats and cattle respectively ***(Yazici et al., 2008).*** Sheep pox occupies an important place in the livestocks industries and contributes significantly to the world economy as the disease threatens animal population, inflicts substantial losses, reduces productivity and lower quality of wool ***(Kitching and Carn, 1996).*** In addition, sheep pox virus are extremely host specific ***Rao and Bandyo-padhyay (2000).***

Comparative genomic data by ***Tulman et al., (2002)*** indicate the close genetic relationship among Capripoxviruses, and they suggest that ShPV and GPV are distinct and likely derived from an LSDV – like ancestor. Laboratory diagnosis is based on clinical signs and serological tests such as virus neutralization, immunofluorescence, agar gel immunodiffusion techniques, ELISA, virus isolation or electron microscopy ***(Oguzoglu et al., 2006).*** These technique are time-consuming, difficult to apply routinely with difficulty in term of cross-reaction with the ORF virus ***(Managana-Vougioka et al., 2000)***.

Although recent molecular studies suggest that capripox virus genus including sheep pox, goat pox and LSDV are very similar in term of antigenic characteristics; these viruses are phylogenetically distinct and can be differentiated by accurate molecular techniques **(*Bhanuprakash et al., 2006).***

Capripoxvirus P32 antigen is a structural protein present in all strains of CaPV. Western blot analysis for LSDV, P32 reacts with hyper immune serum after analyzed by sodium dodecyl sulfate (SDS - PAGE)

in plyacrylamide gel electrophoresis ***(Hanan and Aggour, 2002).*** Restriction endonuclease analysis can be used to distinguish LSDV from ShPV after digestion of their DNA with Hind III and electrophoresis of the DNA fragment in agarose gel ***(Hanan, 2003).***

In this study, the clinical observation of the animals revealed difference in severity of the disease reflecting individual variation between animals and the susceptibility of small animals the same observation was previously reported by ***House, (1992) Barnard et al., (1994) and Joshi et al.,(1999).***

The skin biopsies used in this work for the detection of the viruses as biopsy material is easy to obtain ***(Ireland and Binepal, 1998).*** Regard with AGPT table (1), FAT table (2), photo (2) and VNT table (3) used in this work for the identification of the viruses both tests gave sensitivity of 42.2% 71.1% and 65.8% respectively these results agree with previous study of **(*Mangana-Vougiouka et al., 2000).***

Virus neutralization test is the most specific serological tests, but because the immunity to LSDV and sheep pox virus infection is predominantly cell mediated so the test is not sufficiently sensitive to identify animals that have had contact with LSDV or sheep pox virus and developed only low level of neutralizing antibody**.** AGPT table (1) and FAT table (2) are less specific due to cross reaction with antibody to other poxvirus ***OIE (2008)***.

As animals are most contagious before neutralizing antibodies develop, that occurs approximately a week after the onset of the clinical signs. These neutralizing antibodies can interfere with virus isolation and some antigen-detection tests**.** As we know that, all viruses of capripox virus

genus share a common major antigen for neutralizing antibodies and it is not possible to distinguish strains of Capripox virus from cattle, sheep and goats using serological techniques ***(OIE, 2008).*** Therefore our article planed to fulfill towards the application of PCR technique in detection and differentiation of some Capripox virus.

The PCR technique could be done on the biopsy materials and does not require low temperature storage, results can be obtained in a few hours, and can be used later in the course of the disease when virus-specific antibodies are present ***(Iman et al., 2007).*** The test is also suitable for use in those countries in which the disease is not endemic and live virus is not available **(*Heine et al., 1999).***

In this work, a fast and simple method for capripox virus species identification has been applied. The method is based on multiplex polymerase chain reaction (MPCR) with species-specific primers **photo (3)** and does not requires nucleotide sequencing or restriction analysis of PCR products as mentioned by ***Orlova et al., (2006)***. Previous studies reported that multiplex PCR technique is an essential cost-saving technique for large-scale genotyping with significant scientific, clinical and commercial applications ***Shi, (2001).*** A duplex PCR was developed and optimized for simultaneous detection and differentiation of capripox virus and orf virus ***(Zheng et al., 2007).***

Regard with the results of multiplex PCR technique **photo (3),** the viral attachment protein gene primers as well as the inverted terminal repeats gene primers used in PCR amplification produced the expected amplicon sizes of 192bp and 289bp for LSDV and ShPV respectively. Even samples that were negative were proved positive with PCR technique

using these specific primers. This observation indicates that PCR technique is more sensitive than other diagnostic tests. This result agreed with those obtained by ***(Managana-Vougiouka et al., 2000 and Tuppurainen et al., 2005)***. Polymerase chain reaction (PCR) assays targeting viral attachment gene sequences of capripox viruses have also been reported for detection of capripox viruses in skin biopsies and infected culture fluid ***(Ireland and Binepal, 1998, Heine et al., 1999, Managana-Vougiouka et al., 1999, Parthiban et al., 2005 and Ahmed and Kawther 2008).*** The same primers of inverted terminal repeats gene sequence were successfully used by ***(Black et al., 1986 and Gershon and Black 1989).***

CONCLUSION

Outbreaks can be controlled by quarantines, movement controls, followed by stringent cleaning and disinfection of farms and equipment. Proper disposal of infected carcasses. Vaccination may also be considered. Insecticides and repellent may also be helpful to control spread of LSDV. Antibiotics are used to control secondary infection. We can conclude that PCR technique described combines high specificity and sensitivity with speed. Also MPCR assay can be successfully used for simultaneous detection and differentiation of capripox virus.

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تفاعل البلمرة المتسلسل للتفرقة بين بعض معزولات مجموعة فيروسات الكابرى

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في هذه الدراسة تم فحص عدد 38 عينة جلدية من حيوانات مصابة إكلينيكياً (عدد 17 عينة من الأبقار وعدد 21 عينة من الأغنام) لكل من الجنسين وأعمار مختلفة ضد فيروسي مرض الجلد العقدي في الأبقار وجدري الأغنام.

تم أخذ عينات الأبقار من محطة شبرا شهاب بمحافظة القليوبية حيث كانت نسبة الإصابة 60% ونسبة الوفيات 8% وخصوصا فى الحيوانات الصغيرة, وقد أثبت الفحص الإكلينيكي وجود عقد جلدية وكانت عديدة فى الرأس والرقبة للحيوان وقد تغطى الجسم كله مع فقد الجزء المصاب من الجلد وفقدان في الشهية ونقص في الوزن مع قلة إدرار اللبن.

كما تم أخذ عينات الأغنام من محطة سخا بمحافظة كفر الشيخ حيث كانت نسبة الإصابة 70%ونسبة الوفيات 20% وظهرت حويصلات على الأغنام المصابة مع تضخم في الغدد الليمفاوية.

تم بنجاح عزل الفيروس على خلايا البيض المخصب وخلايا الزرع النسيجي ،كما تم التعرف على الفيروس باستخدام اختبار الأجار الترسيبي والفلورسنت واختبار التعادل مع حساسية 42.2 % ، 71.1% ، 65.8 % على التوالي.

وكانت التفرقة بين فيروس الجلد العقدي وفيروس جدري الأغنام عن طريق تفاعل البلمرة المتسلسل المتعدد مع استخدام بادئ للتفاعل خاص بكل فيروس وكانت النتيجة 192 وحدة مزدوجة لمرض الجلد العقدي، 289 لفيروس جدري الأغنام.

وخلص البحث أن تفاعل البلمرة المتسلسل لديه الكفاءة والسرعة للتفرقة بين بعض فيروسات مجموعة الكابرى والتشخيص مباشرة من الحالات الإكلينيكية.