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REAL-TIME RT-PCR ASSAY FOR MOLECULAR DETECTION OF PESTE DES PETITS RUMINANTS VIRUS (PPR) IN THE CLINICAL CASES

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SUMMARY

The present study was conducted on clinical cases of suspected Peste des Petits ruminants (PPR) infected sheep during 2013 in Behira Governorate. A total of 60 samples of 6 tissues (2 lungs, 2 spleen and 2 mesenteric lymph nodes), 24 swabs (8 buccal, 8 fecal and 8 ocular), 10 buffy coat and 20 serum samples were collected from sheep suffering from pneumonia, diarrhea with high incidence of mortality for detection of PPRV antigen and its specific antibodies by real time RT-PCR, direct flourecent antibody technique (DFAT) and immuno- capture ELISA (IC-ELISA). Detection of PPRV by real time RT- PCR revealing that, ocular swabs was positive with ct of 20, also tissue samples gave ct of 29, oral swabs gave ct at 32 and finally fecal and buffy coat gave ct at 37. By DFAT 7 samples were positive (1 lung, 1 LN, 2 ocular swabs and 3 buffy coat). Fifteen out of 40 samples were positive (2 lungs, 1 LN, 3 buffy coat, 4 ocular swabs, 3 buccal and 2 fecal) by IC-ELISA. Isolation for PPRV was done by inoculation of positive samples onto VERO and MDBK cell cultures, 5 samples showed cytopathic effect. Detection of PPRV antibodies in serum samples using competitive ELISA (C-ELISA) revealing 7 positive samples. In conclusion real time RT-PCR appears to be a very sensitive, accurate, rapid and specific tool with ELISA for the detection of PPR in clinical samples.

INTRODACTION

Peste des petits ruminants (PPR) is a primarily a disease of sheep and goats. It is highly contagious and economically important viral disease of domestic and wild small ruminants. Several reports of PPRV detection in other captive wild ungulates were recorded **(Abu Elzein et al., 2004 and Kinne et al., 2010).** Clinically, PPR is characterized by the sudden onset of depression, fever, discharges from the eyes and nose, sores in the mouth, disturbed breathing and cough, foul-smelling diarrhoea, with 100% morbidity and 20-90% mortality **(Kul *et al.*, 2007).** PPR was first

described in Côte d'Ivoire in West Africa in 1942. The disease is now widespread in tropical and sub-tropical countries, particularly in sub-Saharan Africa, Middle East and western and southern Asia **(Dhar *et al.*, 2002).In Egypt**, the first outbreak of PPR occurred in January 1987 among goats in a private farm at Giza Governorate **(IKram et al., 1988).**

The causative virus belongs to the genus Morbillivirus of the family Paramyxoviridae. PPRV also known as Ovine rinderpest, it is enveloped single-stranded, negative-sense RNA virus. The nucleoprotein (N) gene is located at the most 3- end of the genome. It is a well-conserved gene and probably one of the best targets for the development of a highly sensitive real-time RT-PCR. The genome of this virus comprises of approximately 16 Kbp arranged in a linear conformation **(Kwiatek et al., 2007 and Grant et al., 2009).** Definitive diagnosis of PPRV infection is based on virus isolation and detection of PPR antigen, and its specific antibodies by conventional different diagnostic tests as FAT, IC-ELISA, agar gel precipitation test and immuno peroxidase **(Singh et al., 2004**).

PCR in combination with nucleotide sequencing is the method of choice for molecular characterization of viruses **(Diallo, 2003).** For genome-based detection, a reverse transcription (RT-PCR) was developed previously with primers targeting the nucleoprotein (N) gene after extraction of the genomic material which is sensitive and specific test for PPR diagnosis **(Brindha *et al*, 2001)** or by direct PCR without nucleic acid extraction **(Michaud et al., 2007).** A one-step real-time RT-PCR assay (RRT-PCR) for PPRV was developed to detect the four lineages of PPRV by targeting N gene of the virus **(Kwiatek et al., 2010).**The present study **aimed** to:

- Apply a reliable and reproducible one-step real-time RT-PCR for detection of PPRV.
- Trials for isolation of PPRV from recent clinical cases by inoculation onto tissue culture.
- Detection of PPRV specific antibodies by competitive ELISA.

MATERIALS AND METHODS

1. Samples

a) Swabs: a total of 24 swabs (8 ocular, 8 buccal and 8 fecal) were collected from sheep suffered from signs of pneumonia and diarrhea for virus isolation and identification (**Table 1**).

b) Tissue samples: A total of 6 tissue samples (2 lungs, 2 spleens and 2 mesenteric LN) were collected from two sheep directly after death. Tissues homogenate were prepared according to **(Rahman et al., 2011).** The suspension was clarified by centrifugation at 3000 rpm for 5 min, The prepared suspension was stored at -70°C until used

c) Blood samples: 30 blood samples were collected from diseased and apparently healthy sheep (10 samples with anticoaggulant for separation of buffycoat for detection and isolation of PPRV and 20 samples without anticoagulant for detection of PPRV antibodies).

Table (1): Different samples collected from Behira Governorate.

Samples	No
Tissues (lung, spleen & mesenteric LN)	6
Occular swabs	8
Buccal swabs	8
Fecal swabs	8
Serum	20
Buffy coat	10
Total	**60**

2. Molecular detection of the virus:

a. RNA extraction

RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocols from the field samples

b. The Primer Design™ genesig Kit: A PPRV specific primer and probe mix is provided by PrimerDesign™ with TaqMan® principle. The PPRV primers have been designed for the specific and exclusive in vitro quantification of all PPRV isolates and do not detect other morbillivirues. The target sequence (Large protein) has a highly

conserved region that is unique to PPRV and is the ideal marker for real time PCR based detection.

c. One step qRT-PCR MasterMix: A one-step real-time RT-PCR assay (RRT-PCR) for peste des petits ruminants virus (PPRV) was developed to detect the four lineages of PPRV by targeting the nucleoprotein (N) gene of the virus **(Kwiatek et al., 2010)** with FAM labeled, BHQ quenched and PPRV positive control template. Amplification using Applied Biosystem one-step apparatus. Reverse transcription at 50ºC for 10 min, followed by 50 cycles of denaturation and extension (**Table 2).** To confirm the absence of contamination, a negative control reaction should be included every time the kit is used.

Table2: Amplification conditions using PrimerDesign One step qRT-PCR_MasterMix.

Step	Time	Temp	Cycle
zyme activation	10 mins	95 oC	
naturation	10s	95 oC	50
ta Collection	60s	60 oC	

3. Detection of PPRV by direct fluorescent antibody technique (DFAT)

PPRV detection was carried out by impression smears of tissue samples and buffy coat smears using anti - PPRV specific fluorescein isothiocyanate conjugate supplied by **Sigma** as described by **Sumption et al., (1998)**.

4. Detection of PPRV antigens by Immunocapture ELISA

ID screen PPR sandwich ELISA kit jointly produced by ID® Vet Innovative diagnostics, France for detection of PPR viral antigen in the tissue samples, buffy coat and swabs. It was carried according to the

instruction described by **Couacy Hymann et al., (2009) and Abubakar et al., (2011).**

5. VERO and MDBK cell culture:

African green monkey kidney (VERO) and Madin Darbey Bovine Kidney (MDBK) cell cultures were obtained from VACCERA and used for isolation of PPRV.

6. Trials for isolation of PPRV:

The prepared tissues, swabs and buffy coat samples were inoculated onto VERO and MDBK cell line for PPRV isolatin according to **Khalafalla et al., (2010)**. The cells were examined daily under inverted microscope to observe the cytopathic effect (CPE).

7. Detection of PPRV antibodies by Competitive ELISA:

All procedure was carried according to the instruction of the manual of PPR Competition ELISA diagnostic kit manufactured by ID Screen Vet Innovative diagnostics, France. The wells are coated with purified recombinant PPR nucleo protein (NP). Anti NP antibodies, if present, form an antibody-antigen complex which masks the NP epitopes. An anti-NP peroxidase conjugate is added to the micro wells. It fixes to the remaining free NP epitopes, forming an antigen- conjugate – peroxidase complex. After adding the substrate solution, the resulting coloration depends on the quantity of specific antibodies present in the samples to be tested **(Libeau et al., 1995).**

RESULTS

Molecular detection of the virus

The primers and probe sequences in PrimerDesign™ genesig Kit have effecint matching with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis. The samples from ocular swabs was positive with ct of 20, also tissue samples gave ct of 29, oral swabs gave ct at 32 and finally fecal and buffy coat gave ct at 37. The control positive gave ct at 17 (Photo1).

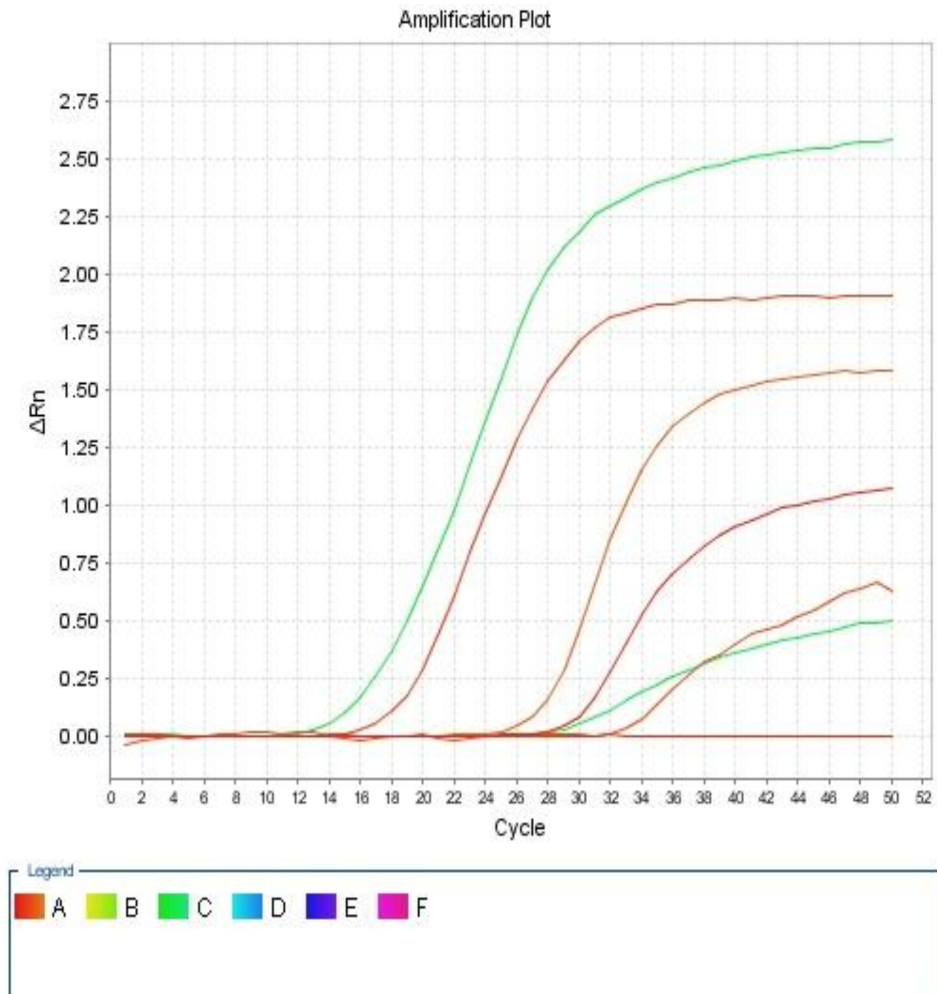

Photo (1): Amplification curves representing samples examined for the detection of PPR virus by Real time RT-PCR

Detection of PPRV by DFAT:

By examination under a fluorescent microscope, 7 samples (1 lung, 1 mesentric LN, 2 ocular smears and 3 buffy coat) revealed specific yellowish green fluorescence (Table 3 and Photo 2).

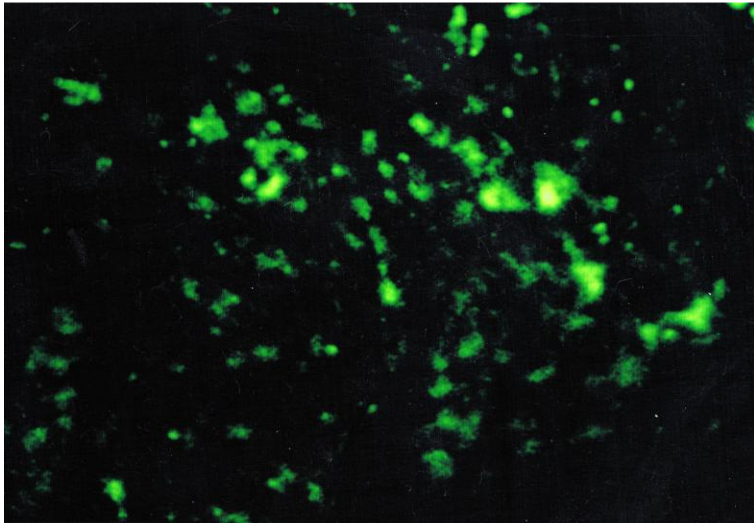

Photo (2): Yellowish green fluorescent granules on buffy coat impression smear (400X)

Table (3): Detection of PPRV by DFAT, Immunocapture ELISA and PCR technique

Type of Samples	No of samples	DFA	ELISA (Ag)	RT-PCR (pooled samples)
Tissue samples (lung and LN)	6	2	3	+ve
Ocular swabs	8	2	4	+ve
Mouth swabs	8	ND	3	+ve
Fecal swabs	8	ND	2	+ve
Buffy coat	10	3	3	+ve
Total	40	7	15	

ND: not done.

Detection of PPR antigen by Sandwich immunocapture ELISA:
Detection of PPRV by immunocapture ELISA, revealed that 2 lung, 1 LN, 4 ocular swabs, 3 buccal, 2 fecal and 3 buffy coat were positive as showed in (Table 3)

Virus isolation on VERO and MDBK cell culture
Fifteen positive samples were inoculated onto VERO and MDBK cells, 5 samples gave cytopathic effect (CPE) in the form of rounding, aggregation of cells, and small syncytia formation 5 days post inoculation after 3 blind passage (Photo 3& 4).

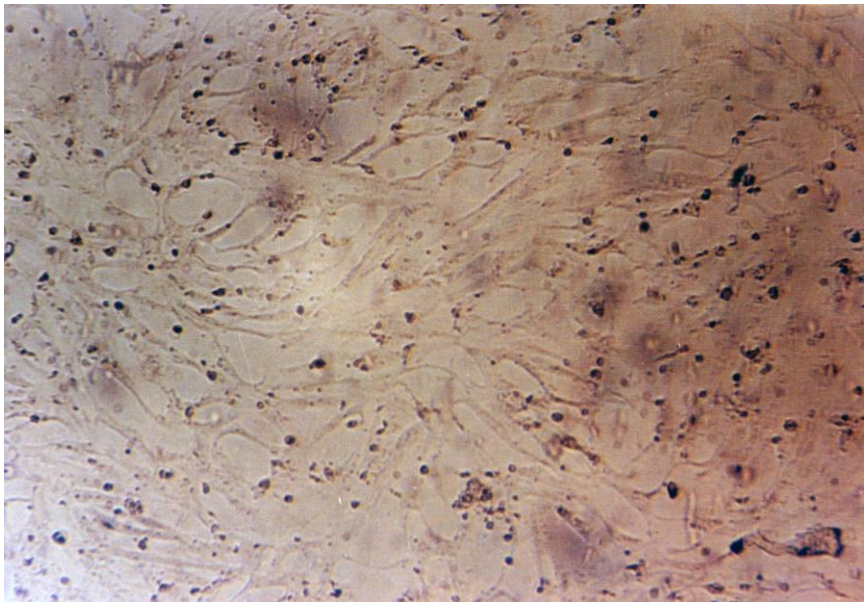

Photo (3): VERO cells infected with PPRV showing aggregation of **c**ells (200X)

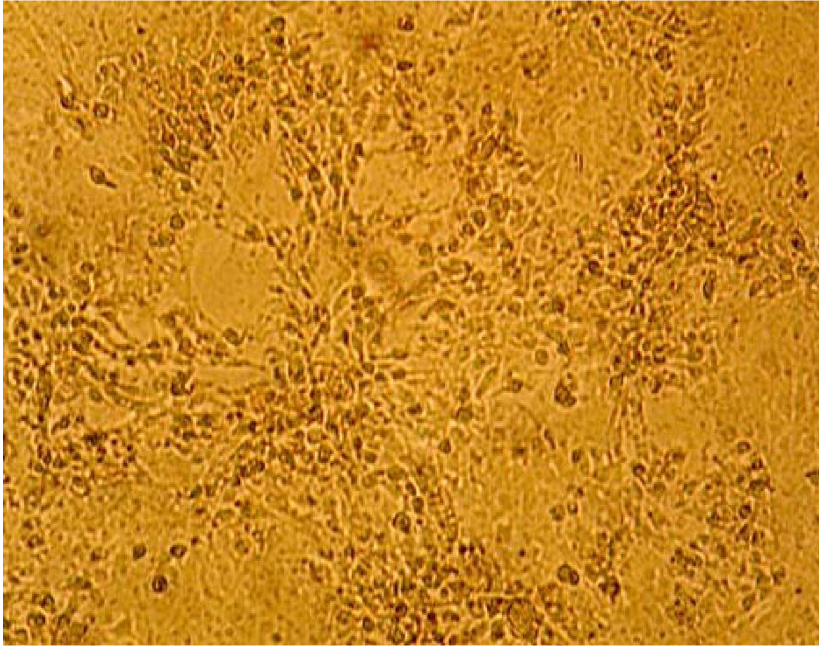

Photo (4): MDBK cells infected with PPRV showing small syncytia formation (400X).

Detection of PPRV antibodies by Competitive ELISA:
By using competitive ELISA for detection of PPRV specific antibodies 7 of 20 serum samples were positive (Table 3).

DISCUSSION

Peste des petits ruminants is an economically important viral disease of goat and sheep. The huge number of small ruminants that are reared in the enzootic areas makes PPR a serious disease that threatens the livelihood of poor farmers **(Diallo et al., 2007).** In Egypt, small ruminants are one of the main sources of meat production. Infection with PPR virus is common causing high morbidity and mortality and leading to severe economic losses **(El-Allawy et al., 1993).**The present study recorded the appearance of PPRV like symptomes during 2013 in Behira Governorate from sheep suffered from fever, anorexia, diarrhea, mucopurelant nasal, ocular discharge, dyspenea and ulcerative stomatitis

were recorded, these agreed with **(Ahmed et al., 2005 and Kul et al., 2008).**

The detection of PPR virus was done by real-time PCR which enables sensitive and specific detection of pathogen nucleic acid in animal samples, allowing for reliable, rapid screening and detection of infected animals (Table 3). This method is also ten times more sensitive and rapid with several advantages over than the conventional RT-PCR, as it is performed in a closed one-tube system to avoids potential cross contamination during sample preparation for post-PCR analysis.Nucleic acid amplification methods for PPR diagnosis have been significantly improved with quantitative real-time RT-PCR **(Bao et al., 2008 and Batten et al., 2011**). Real time RT-PCR was capable of detecting 20% more positive field samples with low viral RNA loads compared to the conventional PCR method **(Kwiatek et al., 2010).**

Similarly, **Albayrak and Alkan (2009)** reported that the PPRV nucleic acid from nasal and conjuctival swap samples from the infected animals were detected, but no viral nucleic acid was detected in the samples of blood samples and oral swap by RT-PCR. They reported that the samples of nasal and conjuctival swaps are more valuable as the diagnostic material from animals with clinical symptoms. We determined that the diagnostic value of necropsy materials such as lymph node, spleen, lung, oro-nasal swap and blood were determined more valuable diagnostic materials in the diagnosis of PPRV infection by RT-PCR (Table 3)..

Fluorescent antibody technique can be used successfully on conjunctival smears and tissues collected at necropsy with fixation of the smears in cold acetone **(OIE, 2013).** In this study DFAT was used for detection of PPR viral antigen revealing that (1 lung, 1 mesentric LN, 2 ocular and 3 buffy coats) samples were positive showing yellowish green fluorescence granules on the impression smears (Photo 2). This agreed with **(IKram et al., 1988 and Sumption et al., 1998)** who stated that FAT has been used successfully on tissues and occular smears. Other study by Abd El-

Rahim et al., (2010) used FAT for detection of PPR antigen in occulonasal smears.

PPR must be confirmed by laboratory methods. Rapid diagnosis of the disease is done by immunocapture enzyme-linked immunosorbent assay (ELISA) and RT-PCR (OIE, 2013) Commercial Immunocapture ELISA (I-c ELISA) can detect and quantities cell free PPRV antigen (**Mohammed et al.; 2008 and OIE, 2013**). I-c ELISA is a very sensitive and rapid test for screening of PPR viral antigen in tissue specimens, swabs and buffy coat and can detect a very low titre of PPR viral antigen; **(Nada et al. 1996**). In this study PPR viral antigen detection using I-C ELISA, revealing that (2 lung, 1 LN, 3 buffycoat, 4 ocular swabs, 3 buccal and 2 fecal swabs) were positive (Table 2). These results agreed with (**Abd El-Rahim et al., 2010**) who detect PPR viral antigen in occulonasal swabs using ELISA. Other studies by **Khalafalla et al., (2010) and Abubakar et al., (2011)** detected PPR viral antigen in lung and lymph nodes using commercial I-c ELISA.

Even when diagnosis has been carried out by rapid techniques, the virus should be isolated from field samples on tissue culture for further studies and provides live virus for biological characterization studies (**Housawil, et al.; 2004 and Zahur et al.; 2009).** In our study positive samples by FA and IC-ELISA were inoculated onto VERO and MDBK cell culture for three blind passages. The cytopathic effect (CPE) appeared at 5th day post inoculation in the form of cell rounding, aggregation and syncytial formation (Photo 3 and 4). This agreed with the study by **(IKram et al., 1988)** which stated that, PPR virus was isolated on Vero cells from lymph nodes and spleen tissues. Other studies by **Khalafalla et al., (2010), Abd El-Rahim et al., (2010)** and **Rahman et al., (2011)** identified the virus through its isolation. PPR virus was isolated and identified through the cytopathic effects and I-c ELISA (**Zahur et al.; 2009**).

Detection of PPR viral antibodies can confirm the diagnosis of PPR (**Mehmood et al., 2009**). In our study detection of PPR viral antibodies by competitive ELISA in sheep serum indicated 7 out of 20 samples were positive, this agreed with **(Khan et al., 2007, Mohammed et al.,**

2008 & Munir and Ali (2009) which concluded that c-ELISA is used as a standard technique since it has the best sensitivity and specificity and can be utilized for samples which are not kept under ideal conditions.

In conclusion: PPRV among sheep in Behira Governorate may be attributed to the presence of circulating virus, real-time RT-PCR and ELISA provides sensitive, specific and rapid tool for diagnosis of PPR antigen and antibodies. The present study recommended that this situation need a lot of studies and regulations to control PPRV infection in Egypt. Restriction of movement of sheep and goat population during appearance of PPRV infection should be applied. Control by vaccination process is very important.

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اختبار تفاعل انزيم البلمرة المتسلسل العكسى ذو الوقت الحقيقى للكشف الجزيئى حقليه عن فيروس طاعون المجترات الصغيرة فى حالات

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الملخص العربى

فى هذه الدراسة تم تسجيل مرض طاعون المجترات الصغيرة فى حالات سريرية لأغ نام فى محافظه البحيرة عام ٢٠١٣، فقد تم تجميع عدد ٦٠ عينـه (٦ أنسجة ،٢٤ مسحات ، ١٠ دم على مانع للتجلط و٢٠ سيرم) من اغنام تعانى من اعراض المرض الحادة وتشمل الحمى والانقطاع عن الطعام واسهال وافرازات من الانف والعين وضيق فى التنفس والتهابات فى الفم مع أعراض تنفسية ومعوية مع نسبة وفيات عالية. تم التعرف السريع والدقيق على الفيروس باستخدام اختبار تفاعل البلمره المتسلسل العكسى حقيقى الوقت لعينات ايجابية للمسحات العينية والفمية والشرجية وكذلك عينات الدم على مانع للتجلط.

باستخدام اختبار الفلورسنت المشع تم التعرف على الفيروس فى عدد ٧عينات (١ رئه ،١ غدد ليمفاويه ، ٢ مسحات من العين و ٣ عينات دم على مانع للتجلط)، كما تم الكشف عن الفيروس باستخدام ساندوتش الاليزا فى ١٥ عينه ايجابيه (٢ رنه ،١ غدد ليمفاويه ،٤مسحات من العين، ٣ من الفم،٢ شرجيه و ٣ دم على مانع للتجلط،، بتمرير العينات الايجابيه على خلايا الزرع النسيجى VERO cell و MDBK فى محاولـه لعزل الفيروس اعطت تغيرات باثولوجيه على الخلايا CPE على شكل تدوير وتجمعات فى الخلايا وتكوين فراغات syncytia formationوذلك فى اليوم الخامس بعد التمريره الثالثة فى خمس عينات وبالفحص السيرولوجى لعينات السيرم باستخدام اختبار الاليزا التنافسى كانت ٧عينات من ٢٠ ايجابيه للاجسام المناعيه للفيروس. وهذا البحث يدل على مدى حساسية اختبارى الاليزا وتفاعل البلمرة المتسلسل حقيقى الوقت فى الكشف على الحالات الاكلينيكية المصابة بطاعون المجترات الصغيرة.