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Molecular Detection and Epidemiological Insight into Peste des Petits Ruminants Outbreaks in Small Ruminants in Giza, Egypt

Rabie Etman [1], Mahmoud S. Safwat [2], Reham Karam [3*] and Mohamed Khodeir [4]

[1] *Faculty of Veterinary Medicine, Cairo University, Egypt.*
[2] *Infectious Diseases Department, Faculty of Veterinary Medicine, Cairo University, Egypt.*
[3] *Weqaa Canter, MEWA, Kingdom Saudi Arabia.*
[4] *Veterinary Serum and Vaccine Research Institute, Egypt.*

Abstract

Peste des petits Ruminants (PPR) is a highly contagious viral disease affecting mainly goats, sheep and wild ruminants and considered as a major obstacle in small ruminants production. Four migratory flocks of sheep and goats in Giza governorate (Moatamadia village, Zenien, Saft El-Labn and Kafrberak El-Khiam) and 5 sheep admitted to the clinic of faculty of veterinary medicine, Cairo University were suspected to be affected with PPR based on field diagnosis (case history, clinical and Post mortem (P.M.) examination) during the period from October 2014 to November 2014. A total number of 40 samples (21 buffy coats, 18 nasal swabs and one spleen tissue) were obtained from affected animals for confirmation of this suspicion by virus isolation on Vero cells and RT-PCR using primers directed to the highly conserved sequence in nucleoprotein gene of PPRV. The total number of samples which gave positive results were 15 and 37 samples by virus isolation (VI) and RT-PCR, respectively. These results confirmed that the causative agent of these case series was PPRV. Also, it was hypothesized that the disease is endemic in Egypt with regular flourishment in the period around Al-Adha festival and this hypothesis required further analysis.

Keywords: PPR virus, Egypt, SNT, Vero cells

Introduction

Peste des petites ruminants (PPR), is a viral disease affecting small ruminants caused by PPRV. PPRV is an RNA virus under the genus Morbillivirus *caprinae* in the family Paramyxoviridae. It has 4 lineages (I-IV) [1]. PPR has a great socioeconomic importance because of its contagious and transboundary nature [2].

It is manifested as mild in apparent infection and can be fulminant in nature according to the host immune status. It causes clinical affections as oral ulcerations, respiratory manifestations and diarrhea. Zebra tripping is the Laboratory confirmation of virus infection can be done through; virus isolation, serum neutralization test (SNT), Ag detection ELISA and PCR and sequencing either partially or fully [3].

PPR was first reported in Egypt between 1987 and 1990. In 2008 North Africa witnessed several outbreaks of PPR that was reported in Egypt after it was reported in Morocco and spanned most of the North African countries [4]. In 2025, the (Global Framework for the Progressive Control of Transboundary Animal Diseases) GF-TADS meeting in Riyadh, Saudi Arabia, Egypt has presented the current situation of PPR and the future national goals of their eradication. Egypt is in the second stage of the PPR progressive control pathway [5]. Achieving the complete eradiation of PPR in 2030 as proposed by WOAH/FAO has 5 pillars that compose the 4 progressive stages. Accurate laboratory diagnosis is a fundamental pillar to achieve eradication [6].

The current work is a case control study aims at field and laboratory diagnosis of PPR circulated in Egypt Giza governorate as a part of PPR surveillance in 2014.

Material and Methods

A case series study was conducted recruiting sheep and goats showed oculonasal discharges, stomatitis, respiratory and digestive disorders in Giza governorate.

Animals:

Between October and November 2014, (just after Al-Adha festival) suspected cases of Peste des Petits

*Corresponding authors: Reham Karam, E-mail: reham_farag@mans.edu.eg Tel.: +201063384084
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Ruminants (PPR) were identified in Four migratory flocks of sheep and goats located in Moatamadia, Zenien, Saft El-Labn, and Kafr Berak El-Khiam all near from each other. in (Giza Governorate, Egypt), as well as in five individual sheep admitted to the veterinary clinic of the Faculty of Veterinary Medicine, Cairo University were considered as the fifth group of samples. The total number of animals per flock were 51 to 100 in average. The ages of the enrolled animals are presented in Table 1. The suspicion of PPR infection was based on clinical history, physical examination, and postmortem findings.

History

Animals in these flocks depended on migration for feed searching. Feeding mainly on agricultural wastes and garbage (all flocks were migratory). The migration routes of these flocks were identified as either on the borders of- or within the area and there was overlapping migration routes between most of these flocks. Regular introduction of new animals for any reason (outside ewes and does for breeding or purchasing new animals) without quarantine was practiced in all these flocks. One flock contained sheep as the only species and the other flocks were mixed (sheep and goats) but goats were presented in lower number. Most affected animals were sheep and young age (less than 1 year old).

Field diagnosis

History of geographical distribution and management of affected flocks and admitted animals, species and age susceptibility and time of occurrence of the cases were identified and recorded. A total number of 25 diseased animals (24 sheep and 1 goat) were subjected to general clinical examination (rectal temperature, mucous membranes color and presence of lesions, lymph nodes palpation and respiratory rate). One sheep carcass suspected to be dead with PPR was subjected to PM examination (oral mucosa, lung, spleen and rectum lesions using basic pathological techniques according to [1].

Samples collection and preparation

A total of 40 samples were collected for laboratory confirmation, comprising 21 buffy coat samples, 18 nasal swabs, and one spleen tissue sample from a deceased animal. Samples were assorted according to species, age and sex as shown in **table (1)** and according to their location in Giza governorate as shown in **table (2).** Animals were admitted for diagnosis purpose and samples were taken with minimum pain or discomfort possible as a part of PPR routine diagnosis conducted in Giza Governorate, Egypt.

Laboratory confirmation of suspected PPR cases

For laboratory confirmation of this suspicion, a total number of 40 samples (21 whole blood on EDTA, 18 nasal swabs and 1 spleen) were collected from affected animals.

Samples preparation

Blood samples: About 3 mL of blood were collected through jugular vein puncture into EDTA vacutainers. Lymphocyte separation medium (lymphoprep®, Axis shield, Scotland) was used for easy and rapid separation of lymphocytes from whole blood as compared with the conventional technique.

Nasal swabs: were collected in 3mL of virus transport media and spinned for clarification as recommended in [8] A 0.5 mL supernatant was collected and kept in -20 C till further use.

Spleen tissues: one gram of spleen tissue was excised from morbid animal. The tissue was homogenized and clarified according to [8] then kept at -20C till use in PPR laboratory diagnosis.

Isolation of PPR virus on tissue culture

Virus isolation

Three blind successive passages in African green monkey kidney cell cultures (Vero cells) were prepared in cell culture tube as in [9]. When clear Cytopathic effect (CPE) was obtained, infected and cell control slides were fixed and stained with haematoxyline and eosin (H&E) according to the method of [10].

Identification of virus isolates

Serological identification of PPRV isolates was carried out using serum neutralization test (using reference PPR antiserum supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo) according to methods described by [11].

Molecular diagnosis (RT-PCR)

Purification of viral RNA

It was carried out using QIAamp viral RNA minikit (Qiagen, Germany) according to instructions of manufacturer.

Reverse transcriptase polymerase chain reaction

It was carried out for confirmation of PPR in suspected diseased animals by detecting the viral nucleic acid using Qiagen one step RT-PCR kit (Qiagen, Germany) using the primer pair (NP3 and NP4) to 351 base pair as recommended by [12] and shown in table (3, 4).

The PPR reference virus was kindly supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Egypt. It was adapted on Vero cell cultures and had a titer of 10^6 $TCID_{50}$/ml and was used as a positive control in both RT-PCR, cell culture and SNT.

Template RNA and positive and negative controls (nuclease free water) were added to individual PCR tubes containing the prepared QIAGEN OneStep RT-PCR master mix, Germany.

The PCR reaction was performed as: reverse transcription in a single step at 50 °C for 30 minutes (1 cycle). This was followed by the initial PCR activation step, during which the reverse transcriptase was inactivated and the DNA polymerase activated, carried out at 95 °C for 15 minutes (1 cycle).

Subsequently, PCR amplification of the cDNA was performed through 40 cycles consisting of three steps: denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 1 minute.

A final extension step was then carried out at 72 °C for 10 minutes to complete the reaction.

Agar gel electrophoresis

Ten microliters of PCR product were analyzed by agar gel electrophoresis.

Preparation and staining of the agarose gel (1.5%) was done according to [8].

Results

Clinical examination.

All affected animals (24 sheep and one goat) were subjected to general clinical examination which revealed that all animals had fever (rectal temperature 40.1ºC -41.5ºC) except for three animals with normal temperature, congested mucous membrane, normal lymph nodes upon inspection and palpation and some animals showed high resting respiratory rate (40-45/min).

The most clinical signs observed during clinical examination were anorexia, stomatitis which manifested by frothy salivation and erosions in mouth cavity (cheek papillae, dorsal aspect of the tongue, inner side of lips and gums while hard palate was affected in some animals), mucopurulent nasal and ocular discharges, dyspnea and diarrhea of offensive odor as showed in figure (1) plus a history of mortalities in young animals.

PM examination

One dead sheep from affected flock in KafrBerak El-Khiam was subjected to PM examination that revealed consolidation of the lung, enlarged spleen, erosions in mouth cavity and hemorrhages in the PM examination

Fig. (1). sheep suspected to be affected with PPR showing purulent nasal and ocular discharges, a: extensive erosions with caseated material on the inner side of upper lip mucosa, b: erosion on the lower gum mucosa, c: erosions on gum and dental pad, d: erosions and caseation of the tongue, e: frothy salivation

Fig. (2). Erosions in the inner side of lips and gum mucosa (left picture) and zebra marking of rectum (right picture) of a sheep suspected to be dead with PPR

Viral isolation (three blind passages)

Out of 40 samples (21 buffy coats, 18 nasal swabs and one spleen tissue) inoculated into Vero cells as a trial for PPRV isolation, 25 samples (21 buffy coats, 3 nasal swabs and one spleen tissue) were positive with complete cytopathic effect (CPE) in Vero cells in the three blind passages mainly 5-7 days post inoculation. The cytopathic effect was in the form of cell rounding, syncytium formation and detaching of cell sheet as shown in figuress 3 and 4.

Identification of viral isolates by SNT

Out of the 25 viral isolates obtained from the 3rd blind passage, 15 samples (14 buffy coats and one spleen tissue) were serologically identified as PPR by viral neutralization test using known PPR antiserum. The presence of PPR virus in the sample could be neutralized with PPR specific antibody and prevents it from infecting cells. So, no CPE appears which indicate positive results.

Virus titration

All 15 PPRV isolates were titrated in tissue culture plates using Vero cells. The three passages were titrated and PPRV titer was calculated according to [2]. It was noted that virus titers were increased from the first to the third passages.

Reverse transcriptase polymerase chain reaction

Out of 40 samples (21 buffy coats, 18 nasal swabs and one spleen tissue) analyzed by RT-PCR using PPR nucleoprotein gene specific primers, 37 samples (21 buffy coats, 15 nasal swabs and one spleen tissue) were positive as they showed bands of the expected length (351 bp) as compared with the control positive in agar gel electrophoresis as provided in figure (5).

From table (5) that shows the overall positivity of results based on tissue culture isolation, SNT and RT-PCR, it shows that tissue culture is 100% sensitive in detection of PPR from buffy coat samples and rather less sensitive in detection in nasal swab samples 18.3%. SNT is much less senisitve in detection of PPR in buffy coat samples (66.6%) and in nasal swabs (0%). RT-PCR showed the highest sensitivity as (100%) in buffy coat and (83.3%) in nasal swabs.

Discussion

A case series study in Giza governorate as sheep and goats showed oculonasal discharges, stomatitis, respiratory and digestive disorders. Five migratory

sheep and goats flocks in Giza governorate and five sheep cases admitted to the clinic of the faculty of Veterinary Medicine, Cairo University were suspected to be affected with PPR based on field diagnosis.

A total number of 20 diseased animals (19 sheep and 1 goat) and one dead sheep from the affected flocks and the five diseased sheep cases were subjected to field diagnosis of PPR (case history, clinical signs and PM examination).

The case history showed that all affected flocks were depended on migration for feeding, there was an overlapping migration routes for these flocks which facilitated the disease transmission between infected and healthy flocks when come in contact with each other either through direct or indirect manner (communal grazing).The stress put on these animals due to the migration and lacking of appropriate feeding also made them at higher risk for acquiring and developing the disease, these results agree with the results obtained by [14][3][3][3][3][3].

It was noted that the regular introduction of new animals without quarantine was practiced in all of these flocks (opened population). This made these flock at higher risk for introduction of the PPR by animals in incubation period after developing clinical signs (diseased carriers) and recovered animals. Other studies proved that introduction of new animals without quarantine is a risk factors for introduction of PPR in healthy flocks including [15].

Age is an important factor in susceptibility to PPR as most of the samples were positive as they were mostly collected from animals less than 15 month old as agrees with [16].

The disease hit these flocks just after the Adha festival in one flock in Moatamadia village then continued to appear in the other flocks in a short period and generally the disease was regressed from these flocks after a period of 45 days. The relationship between spreading of the disease just before and after Al-Adha festival might be attributed to religious reasons "sacrificing animals for Allah" Islamic Shariah was required increased frequency of visiting animal's markets for selling or purchasing and continuous introduction of new animals, such a relationship was discussed early by [17] who reported that highly contagious viruses burden increase most commonly after the major festive seasons like Pilgrimage. Another reason for spreading of the disease in this period may be due to the lower temperature of the autumn season in Egypt, when compared with the summer season. This climatic factor provided a higher survival rate for PPRV outside the host and subsequently facilitated the disease transmission. This finding was reviewed by [14].

Our obtained results revealed that most animal's species affected in these flocks were sheep. The sheep were more affected than goats (frequency not severity) but this may be due to the lower number of goats contained in such flocks rather than higher susceptibility of sheep to the disease.

Studying the clinical signs showed that fever, anorexia, frothy salivation, stomatitis (erosions on the inner side of lip, gums, upper lingual surface, cheek papillae, hard palate and dental pad), mucopurulent ocular and nasal discharges, dyspnea and diarrhea were the predominant clinical signs observed during clinical examination of affected animals as shown in figures (1). Studying the P.M. picture for the one dead sheep showed erosive stomatitis, consolidation of the lung and hemorrhages on the crests of the longitudinal folds of the rectum as shown in fig. (2). This agree with many studies which addressed the same clinical signs during experimental and natural PPR infection in sheep and goats including [18] and [3].

From the above mentioned case history, clinical examination and postmortem examination it was suspected that these five flocks and the five sheep were affected with PPR and for confirmation of such suspicion a laboratory procedures were carried out.

For laboratory confirmation of suspected cases, total number of 40 samples (21 buffy coats, 18 nasal swabs) and one spleen necropsy) were obtained from diseased animals. The spleen necropsy was taken from the dead sheep (showing necrotic lesions) analyzed for presence of PPRV or its nucleic acid by virus isolation and identification and reverse transcriptase polymerase chain reaction, respectively.

All buffy coat samples gave +ve PPR CPE during isolation in Vero cell line (100% sensitivity). Buffy coats were obtained from diseased animals showed fever (viremia) as the PPRV has a tropism for lymphocytes which explained by the higher affinity of the virus to signaling lymphocytes activation molecules (SLAM) receptor which is a receptor found mainly in lymphocytes and lymphoid organs as concluded by [3].. While nasal swabs give much less sensitivity (16.6 %) supporting thee fact that less virus was present in nasal swabs and T.C isolation requires higher titer of the virus as in [3].. SNT gave much less sensitivtiy as expected because it depends on many variables as the positive serum, the cells and the virus and serum concentration.

The nucleoprotein gene specific primers (NP3 and NP4) used in this study showed higher sensitivity (100%, 83.3%) for buffy coat and nasal swab samples respectively, as these primers were developed based on the assumption that the first two nucleotides in the 3'end of the primers are critical for the success of PCR and any difference in those positions between the primers and the target will make the PCR unsuccessful. Both primers are 24 nucleotides long

and the first three nucleotides in their 3' ends are conserved in the partial NP gene sequence of 10 viruses of different origins. Also the higher sensitivity may be due to the higher rate of transcription of N-gene so that the nucleoprotein is more abundant. These conclusions were given by [19].

This study has lacked sequencing and phylogenetic analysis of the isolated samples. Although phylogenetic analysis is beyond the diagnostic scope of this study as Lineage-IV has circulated in Egypt solely [4] we highlight that future studies will aim to include sequence-based phylogenetic analysis to strengthen epidemiological interpretation.

Conclusion

The PPRV was confirmed to be the causative agent of the disease which hit five migratory flocks in Giza governorate in the period from October to November 2014. It was found that the period just before or after Al-Adha festival is a suitable time for the spreading of many diseases among animals including PPR (descriptive epidemiology) so that such field observations should be tested by other analytical studies for confirmation of this hypothesis and we recommended that the veterinary authorities should control the animals‟ movement and animals markets during this period to reduce the incidence of many diseases.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

Not applied.

TABLE 1. Subdivision of animals according to their species, sex and age recruited in this study

Species	Sex	Age in months	
		≤15	<15 to ≤21
Sheep (n=25)	Male (n=14)	n=14	n=0
	Female (n=11)	n=11	n=0
Goats (n=1)	Male (n=1)	n=0	n=1
	Female (n=0)	n=0	n=0

TABLE 2. Geographical distribution of animals recruited in this study and the samples collected from each group

Locality	Species	Age in months		No of samples collected/ type of sample	
		≤15	<15 to ≤21	Buffy coat	Nasal swab
El- Moatamdia village (n=5)	Sheep (n=3)	n=3	n=0	n= 4	n= 4
	Goats (n=1)	n=0	n=1	n=1	n=1
Kafr Berak Khiam (n=3)	Sheep (n=3)	n=3	n=0	n= 2	n= 1
Saft El-Labn (n=2)	Sheep (n=2)	n=2	n=0	n=1	n=1
Znien (n=11)	Sheep (n=11)	n=11	n=0	n= 9	n= 9
Clinic of Vet. Med. Fac., Cairo Uni., n=5	Sheep (n=5)	n=5	n=0	n= 4	n=3
Total No.	N=25	N=24	N=1	N=21	N=18

TABLE 3. Primers sequence, target gene and expected length of amplicon

Primers	sequence	Target gene	Gene accession number	Expected length
Forward primer (NP3)	5‟-GTC-TCG-GAA-ATC-GCC-TCA-CAG- ACT-3‟	nucleoprotein gene of PPRV	JN647718.1	351 bp
Reverse primer (NP4)	5‟-CCT-CCT-CCT-GGT-CCT-CCA-GAA- TCT-3‟			

TABLE 4. Preparation of master mix and primers for one reaction of RT-PCR

Component	Volume/reaction	Final concentration
RNase-free water	22microliters	-------
5x QIAGEN OneStep RT-PCR Buffer	10 microliters	1x
dNTP Mix (containing 10 mM of each dNTP	2 microliters	400 µM of each dNTP
QIAGEN OneStep RT- PCR Enzyme Mix	2 microliters	-------
Primer NP3	2 microliters	0.6 µM
Primer NP4	2 microliters	0.6 µM
Template RNA added at step 4	10microliters	1 pg – 2 µg/ reaction
Total volume	50 microliters	--------

TABLE 5. Results of the case series of PPR in Giza governorate, Egypt including the species and the numbers of samples collected in detail.

Locality	Animal species	No of samples collected/ type of sample		Positive T.C		Positive SNT		Positive RT-PCR	
		B.C **	N.S***	B.C	N.S	B.C	N.S	B.C	N.S
El- Moatamdia village (n=5)	Sheep (n=4)	n= 4	n= 4	n=4	n=1	n=4	n=0	n=4	n=4
	Goats (n=1)	n=1	n=1	n=1	n=0	n=1	n=0	n=1	n=1
Kafr Berak Khiam (n=3)	Sheep (n=3)	n= 2	n= 1	n=3	n=1	n=2	n=0	n=2	n=1
Saft El-Labn (n=2)	Sheep (n=2)	n=1	n=1	n=2	n=0	n=0	n=0	n=1	n=0
Znien (n=11)	Sheep (n=11)	n= 9	n= 9	n=11	n=1	n=4	n=0	n=9	n=6
Clinic of Vet. Med. Fac., Cairo Uni., n=5	Sheep (n=5)	n= 4	n=3	n=5	n=0	n=3	n=0	n=4	n=3
Total No.*	**N=25**	**N=21**	**N= 18**	**21/21 (100%)**	**3/18 (16.6%)**	**14/21 (66.6%)**	**0/18 (0%)**	**21/21 (100%)**	**15/18 (83.3%)**

*Added one spleen tissue from the vet clinic at Cairo University was positive in T.C, SNT and RT-PCR.
** Buffy coat, *** Nasal swab.

Fig. 1. Sheep suspected to be affected with PPR showing purulent nasal and ocular discharges

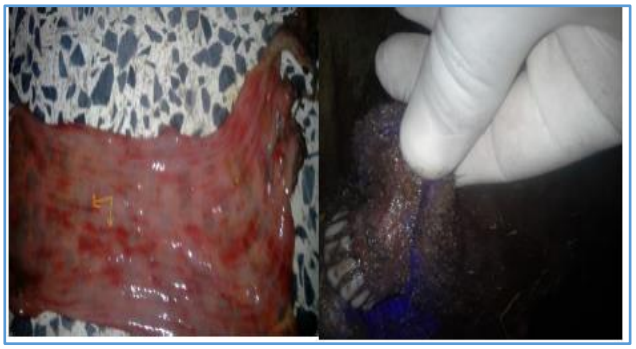

Fig.2. Erosions in the inner side of lips and gum mucosa (left picture) and zebra marking of rectum (right picture) of a sheep suspected to be dead with PPR

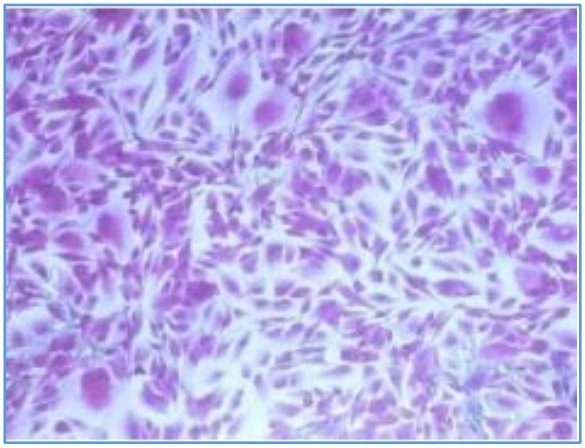

Fig.3. control non infected Vero cells (elliptical in shape)

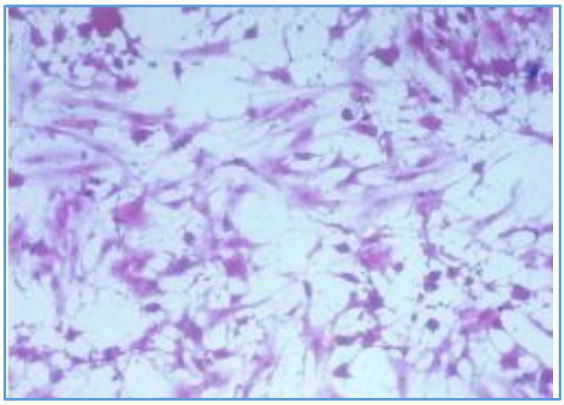

Fig. 4. Infected Vero cells showing CPE (cell rounding and syncytia formation)

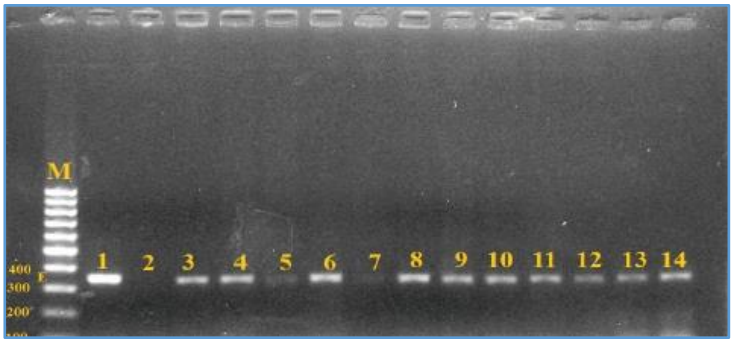

Fig. 5 Agarose gel electrophoresis of RT-PCR products amplified from field samples
M: DNA marker (100 bp), E: expected length of PPRV amplicon (351 bp), 1: control positive, 2: control negative, 3-14: positive samples

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تشخيص حقلي وتأكيد مخبري لاندلاع وباء من مرض طاعون المجترات الصغيرة في محافظة الجيزة، مصر

ربيع عتمان [١] ، محمود صفوت [١] ، ريهام كرم [3] و محمد حسن خضير[4]

[١] قسم الأمراض المعدية، كلية الطب البيطري، جامعة القاهرة، الجيزة، مصر، ١٢٥١٩

[٢] معهد بحوث الأمصال واللقاحات البيطرية، العباسية، القاهرة، مصر، ١١٥١١

[٣] قسم علم الفيروسات، كلية الطب البيطري، جامعة المنصورة، مدينة المنصورة، مصر، ٣٥٥١٦؛

[٤] قسم علم الفيروسات، مختبر وقاء المركزي، مركز وقاء، الرياض، المملكة العربية السعودية.

الملخص

طاعون المجترات الصغيرة (PPR) هو مرض فيروسي شديد العدوى يصيب أساسًا الأغنام والماعز والمجترات البرية، ويمثل عائقًا كبيرًا أمام إنتاج المجترات الصغيرة. خلال الفترة من أكتوبر إلى نوفمبر ٢٠١٤، تم الاشتباه سريريًا في إصابة خمس قطعان راحلة من الأغنام والماعز في محافظة الجيزة (المعتمدية، زنين، صفط اللبن، وكفر برك الخيام)، بالإضافة إلى خمسة رؤوس من الأغنام أُدخلت إلى عيادة كلية الطب البيطري بجامعة القاهرة، وذلك استنادًا إلى التشخيص الحقلي الذي شمل السيرة المرضية، الأعراض السريرية، والفحوصات بعد النفوق. تم جمع ما مجموعه ٤٠ عينة من الحيوانات المصابة، شملت ٢١ عينة طبقة بيضاء (buffy coat)، و١٨ مسحة أنفية، وقطعة نسيجية من الطحال، وذلك لتأكيد الاشتباه السريري من خلال العزل الفيروسي باستخدام خلايا Vero، واختبار RT-PCR لاستهداف منطقة محفوظة من جين النيوكليوبروتين (N) الخاص بالفيروس. أظهرت نتائج العزل الفيروسي إيجابية في ١٥ عينة، بينما كشف اختبار RT-PCR عن وجود الفيروس في ٣٧ عينة. تؤكد هذه النتائج أن فيروس PPR هو العامل المسبب في هذه الحالة. كما تدعم هذه الملاحظات الفرضية بأن المرض متوطن في مصر، وأن زيادة انتشاره قد تكون مرتبطة بحركة الحيوانات وتجمعاتها خلال فترة عيد الأضحى، وهو ارتباط يتطلب مزيدًا من الدراسة الوبائية المتعمقة.

الكلمات الدالة: فيروس طاعون المجترات الصغيرة؛ مصر؛ اختبار التعادل المصلي(SNT) ؛ خلايا فيرو؛ التشخيص الفيروسي.