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ISOLATION AND MOLECULAR ANALYSIS OF RABIES VIRUS IN BATS IN EGYPT

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ABSTRACT

Rabies vaccination campaigns target foxes, dogs, cats, and other carnivores as potential disease carriers. Otherwise, without immunization, animals such as cattle, donkeys, and bats coexisted with those carnivores and other domestic animals could be infected. A freshly dead cat and 250 deceased Egyptian tomb bats (*Taphozous perforatus*) were examined in this study. N-gene-specific primers were used in RT-PCR to detect the rabies virus. Rabies virus was confirmed by immunofluorescence, histopathology, and baby hamster kidney cells (BHK-21) propagation. The incomplete sequence of the rabies genome's (N) gene was also sequenced using 720 bp PCR fragments. It may be easier for the virus to spread from bat tissue to carnivores by hunting or even by feeding on recently dead bats, as evidenced by the isolation of rabies from bats that may be fed on the beetles, ticks, and other arthropods contaminated with the saliva of rabid animals. Indeed, this study reports the isolation and characterization of the rabies virus from Egyptian tomb bats (*T. perforatus)* in Egypt. It is worth noting that, to our knowledge, this is the first report of rabies detection in bats in Egypt.

Keywords*:* Bat, Cat, Rabies virus.

INTRODUCTION

Bats are known to maintain the transmission of the rabies virus (RABV) and influence the prevalence of RABV infection in other species (Markotter *et al.*, 2020). The vampire bat is recognized as the primary reservoir of RABV in South America. It is believed that other bat species are also involved in the viral life cycle. Without a doubt, among the insectivorous bats of Brazil, seven genus-specific rabies lineages have been found. A considerable number of bats in French Guiana were found to carry the virus, including 27 insectivorous bats (Duarte *et al.*, 2020). Bats found to be seropositive for the RABV virus included those living in monospecific colonies (Favi *et al.*, 2002).

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T. perforatus is a member of the *Emballonuridae* family of bats. weighing about 30 g (1.1 oz.) (Bendjeddou *et al.,* 2020), considered as an airborne insectivore that feeds in open areas, living in northern Sudan and southern Egypt (Kom Ombo) and its primary food source is moths, but it also consumes ticks, termites, beetles, and other insects (Mahmood-ul-Hassan *et al.,* 2012).

Australian Bat Lyssavirus (ABLV) and rabies virus spread to fruit bats through direct contact with saliva or tissues of an infected animal, usually via bites or scratches. Rabies virus (RABV) is widely distributed within bat colonies and transmitted when the saliva of one vampire bat species comes into direct contact with the broken skin and mucous membranes of another (Shipley *et al.,* 2019; Coertse *et al.,* 2020). According to recent research, afforestation habitats supply enough food for bat survival and can prevent the spread of RABV through infected bat feces. On the other hand, the vampires might have to use different resources. (Castilho *et al.,* 2017; Fooks *et al.,* 2021).

Due to their scratching habits, dogs and cats are more likely than humans to be exposed to bat-acquired rabies (Mayen, 2003; Begeman *et al.,* 2018), as within six months after the appearance of rabies symptoms, cat scratching can infect a human through a prior cut or injury (Fogelman *et al.,* 1993).
Since the rabies virus type detected in cats was the same as that linked to vampire bats (*Desmodus rotundus*), they were responsible for spreading the disease in Brazil (Castilho *et al.,* 2017). Although cat rabies prevalence has remained constant over time, it has increased in comparison to canine rabies, making cats the primary human rabies exposure source in various nations (Eng and Fishbein, 1990). When an Egyptian tomb bat consumes rabies-contaminated beetles, ticks, and other arthropods in contact with the contaminated saliva of rabid cattle or donkeys, the virus in their gut can be spread to carnivorous animals that feed on freshly dead contaminated bats; also, some cats hunt live bats that fall on the ground, which facilitates acquiring the infection. The direct fluorescent antibody (dFA) technique and the reverse transcription polymerase chain reaction (RT-PCR) are dependable methods for rabies laboratory diagnosis. Histopathological investigation can also be utilized (Salheen *et al.,* 2021; Ahmed *et al.,* 2022).

MATERIALS AND METHODS

1. Ethical approval

The use of animals and protocols were approved by Agricultural Research Center - The Institutional Animal Care and Use committee (ARC-IACUC), Egypt (approval numbers ARC-AHRI-39-24).

2.Collection of samples

A total of 250 dead *T. perforatus* bats hat had fallen on the ground were collected, and only one rabid cat was obtained from Aswan governorate (Kom Ombo), Egypt, in 2021–2022.

Under complete hygienic conditions and precautions in a necropsy chamber to avoid contamination, samples from the cat brain, including the central vermis, left and right hemispheres of the cerebellum, and brainstem, were obtained by cutting off the caudal half of the cerebellum. Intestinal and gut tissues from bats were homogenized and used for viral isolation, dFA, and RT-PCR (Ashwini *et al.,* 2024).

3. Virus detection

3.1. Direct fluorescent antibody technique (dFA)

Tissue examination was done using a fluorescent microscope according to (Dean, 1996). Formalin-fixed, paraffin-embedded tissues were cut at 5 µm. Slides were heated at 55°C to melt the paraffin, deparaffinized in xylol, hydrated through graded ethanol, and finally rinsed in phosphate-buffered saline (PBS); then the section was left to dry in air for 30 minutes, fixed with acetone for 10 minutes, and then washed with PBS (pH 7.6). A few drops of 1:100 dilutions of reference

FITC-conjugated anti-RABV antibodies (Chemi-Con, Temecula, CA, USA) and the slides were kept in a humidified chamber (1 h/37°C). The slides were thoroughly washed with PBS for 15 minutes three times. They were then mounted with buffered glycerin, covered with a cover slip, and examined under a fluorescent microscope.

3.2. Reverse transcriptase PCR (RT-PCR)

Primers (TIB MOLBIOL Syntheselabor GmbH), were designed by (Langoni *et al*., 2005), and used in the RT-PCR reaction to amplify a specific segment of 720 bp from N protein gene of the RABV genome, The partial N-gene forward primer,5´ATG GAT GCCGACAAGATTGT-3´and reverse primer 5´CCCACTCTGATTGCCGAA TA-3, and the PCR reaction was performed according to (Nadin-Davis *et al*., 2009). RNA extraction was done using QIAamp Viral RNA Mini Kit (Cat. No. 52906) according to the manufacturer's instructions. Normal non-infected fruit bat gut tissue homogenates were included as a negative control.

3.3. Histopathological investigation

The stained histopathological sections were examined under light microscopy according to Bancroft and Gamble (2008), Sections from the samples revealed that RABV by RT-PCR was carried out by fixing tissue specimens in 10% neutral buffered formalin solution and staining with hematoxylin and eosin.

3.4. Viral isolation

Virus isolation (VI) using Baby Hamster Kidney-21 was done in the three positive RABV samples by RT-PCR and examined under a fluorescent microscope for RABV detection in the inoculated cells according to Rudd *et al*. (1980). Although BHK-21 cells that grow faster and are much easier to maintain represent an adequate alternative for the mouse inoculation test (MIT) as a confirmatory test for rabies diagnosis in bat specimens, yielding reliable results in a short time.

3.5. Phylogenetic analysis

The phylogenetic tree was constructed with the sequences found in GenBank, and evaluation was carried out according to Parrish *et al.* (2016), The obtained sequence data were analyzed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) , and then the alignment *.aln output file was used for performing the neighbor-joining (N-J) phylogenetic analysis with 1000 repeats of bootstrap tests analysis, and calculation of divergence and identity percent was performed using Meg-Align (DNASTAR, Lasergene®, Madison, WI, USA).

RESULTS

1. DFA technique

The presence of RABV in the cat brain tissue was detected using the dFA technique (Fig. 1).

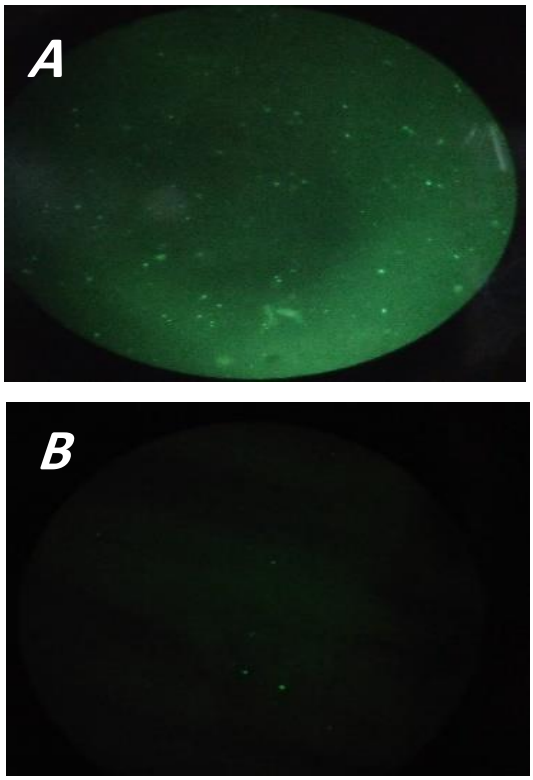

Fig. 1: (A) Results of the dFA technique from cat brain tissue show green color (stars in cloud appearance) viral nucleoprotein **(B)**. Normal brain tissue sample shows negative results with ×400 magnification.

2. RT-PCR

Analysis of the PCR products revealed the positive amplification of N-gene (720 bp) (Fig. 2).

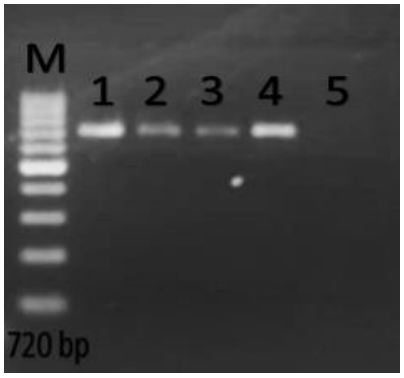

Fig. 2: Gel electrophoresis of 720bp RT-PCR products of partial N-gene of rabies virus genome. M - DNA marker, Lane 1 - positive control, Lane 2, 3 & 4 - amplified products prepared from gut tissue samples of bats, Lane 5 - negative control.

3. Histopathological investigation

The characteristic cytoplasmic inclusion bodies in the examined sections, besides perivascular cuffing (infiltration) with lymphocytes, plasma cells, and a few macrophages, were reported. Also, necrotic or degenerative tissue was found. (Fig. 3)

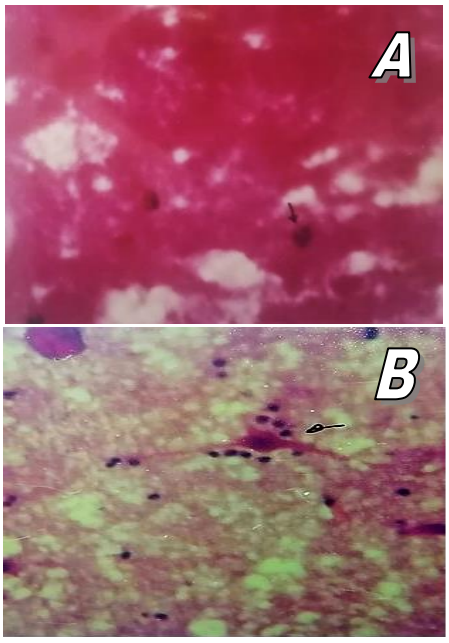

Fig. 3: (A) Brain tissue specimen of rabid Cat revealed signs of encephalitis varying from (satellitosis) perivascular cuffing / neuronophagia/pericellular edema /demyelination/severe congestion and hemorrhage. (B) Intracytoplasmic eosinophilic inclusions (Negri bodies) in neurons (arrows). By sellers' stain (A) H&E stain with ×400 magnification.

4. Viral isolation

Using the strong positive RABV samples by RT-PCR (Fig. 4), isolation of RABV in BHK-21 was monitored with fluorescent dye using reference FITC-conjugated anti-RABV antibodies (Fig. 5).

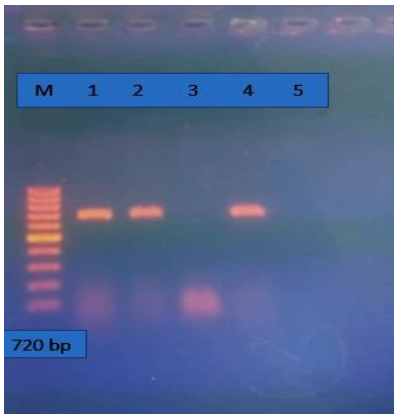

Fig. 4: Samples after three passages on BHK-21 revealed 720 bp of amplified partial N-gene of the RABV genome. M - DNA marker, Lane 1 - positive control, Lane 2, 3 & 4 - the amplified products prepared from gut tissue samples of bats, Lane 5 - negative control.

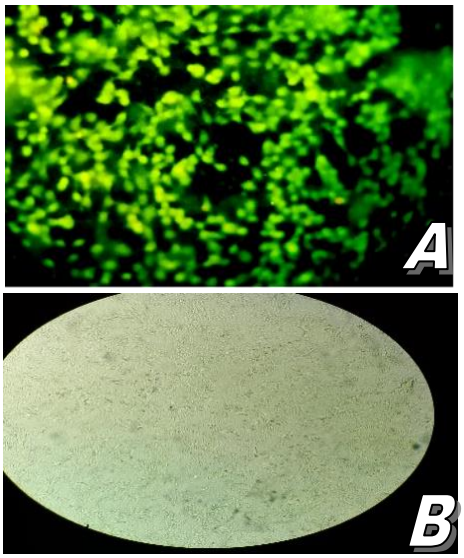

Fig. 5: (A) Positive fluorescence RABV in BHK-21 cell line after 3 successful passage 100x, (B) positive BHK-21 for the RABV by light microscopy 50X.

5. Phylogenetic analysis

The 720 bp PCR fragments representing N-gene nucleotide sequences were compared with available RABVs sequences in GenBank. Sequences were submitted to GenBank; the obtained accession numbers were MZ889656, MZ889654, and MZ889655 for Egyptian tomb bats and MZ889657 for the cat isolate. The Phylogenetic tree was constructed and the multiple alignments exposed a similarity of ~99 % at the nucleotide level between three Egyptian tomb bat isolates and Egyptian cattle isolates carrying accession number MT03606 (Fig. 6), and ~95 % similarity of the RABVs isolate from Egyptian cat, but about ~50 % far from bat origin RABVs (Fig. 7).

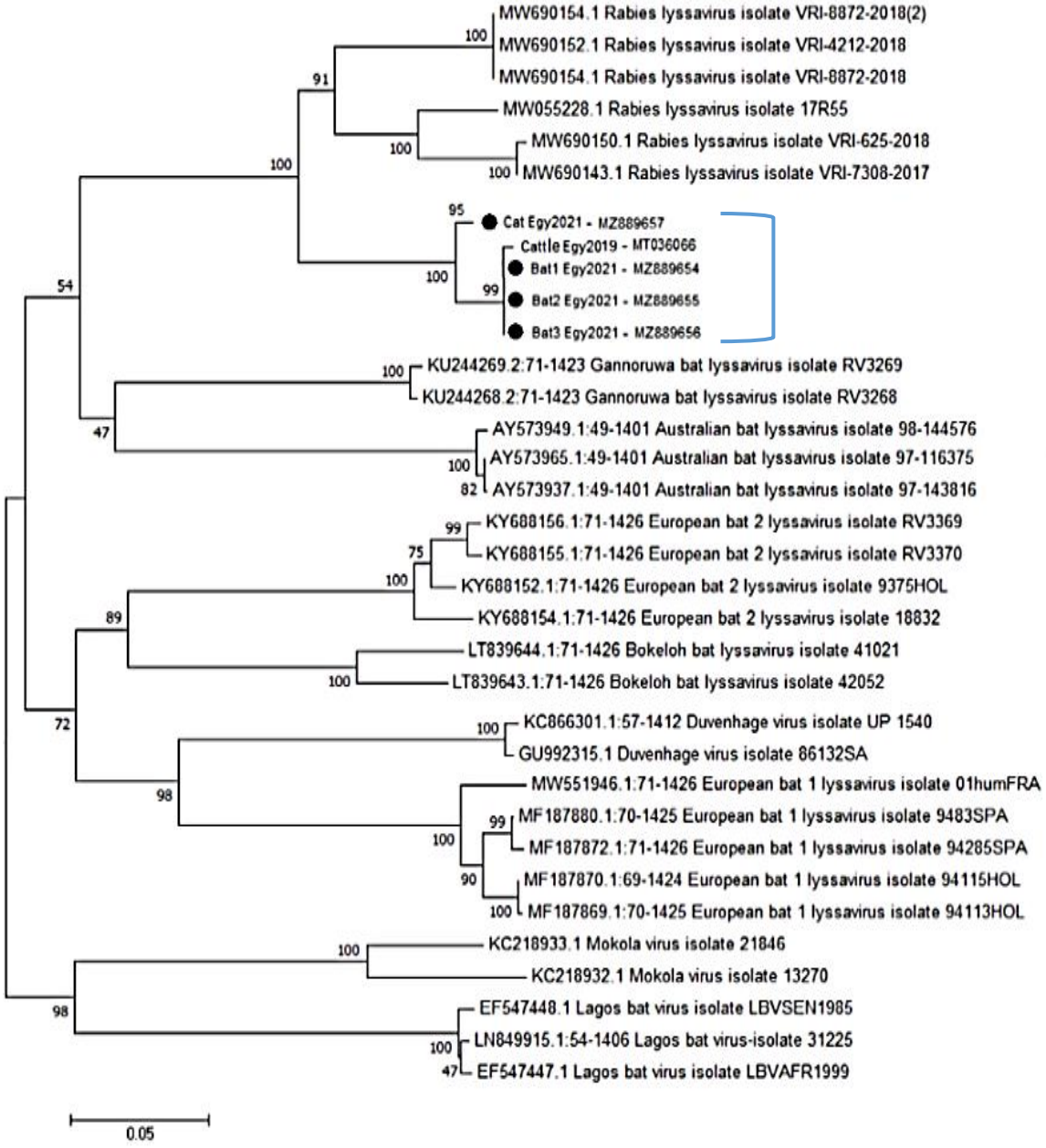

Fig. 6: Tree based on N-gene nucleotide sequences of RABV from Egyptian tomb bats with the rabid cat and other rabies viruses whose N genes were retrieved from the GenBank database sequences. Numbers at the internal nodes signify the bootstrap likelihoods (1000 replicates).

Seq->	Rabies virus	Lyssavirus	Eu.bat lyssa1	Eu.bat lyssa2	Aust.bat lyssa	Lagos bat	Mokola virus	Bat isolate 1	Bat isolate 2	Bat isolate 3	Cat isolate
Rabies virus		0.919	0.709	0.714	0.721	0.614	0.616	0.872	0.872	0.872	0.872
Lyssavirus	0.919		0.716	0.707	0.721	0.63	0.611	0.94	0.94	0.94	0.948
Eu.bat lyssa1	0.709	0.716		0.723	0.704	0.638	0.666	0.68	0.68	0.68	0.683
Eu.bat lyssa2	0.714	0.707	0.723		0.719	0.638	0.607	0.68	0.68	0.68	0.674
Aust.bat lyssa	0.721	0.721	0.704	0.719		0.638	0.635	0.687	0.687	0.687	0.683
Lagos bat	0.614	0.63	0.638	0.638	0.638		0.752	0.591	0.591	0.591	0.594
Mokola virus	0.616	0.611	0.666	0.607	0.635	0.752		0.588	0.588	0.588	0.582
Bat isolate 1	0.872	0.94	0.68	0.68	0.687	0.591	0.588		1	1	0.95
Bat isolate 2	0.872	0.94	0.68	0.68	0.687	0.591	0.588	1		1	0.95
Bat isolate 3	0.872	0.94	0.68	0.68	0.687	0.591	0.588	1	1		0.95
Cat isolate	0.872	0.948	0.683	0.674	0.683	0.594	0.582	0.95	0.95	0.95	

Fig. 7: Three bat isolates were similar to the cat RABV isolate identified in Aswan. The RABV was shown to have ~95% identity at the nucleotide level.

DISCUSSION

The results of this study support the hypothesis that Egyptian tomb bats could serve as transient hosts for rabies and should be taken into consideration in Egypt's rabies control plan. They might be a major factor in the short-term preservation of the rabies virus in Egypt's ecosystem and could spread to animals that eat them like carnivores (Castilho *et al.,* 2017). RABV is the cause of one of the most common, deadly, and horrifying zoonotic infections. Such a neurotropic virus replicates and causes neurological symptoms in both humans and animals after traveling from the infection site through peripheral nerves to the central nervous system (Regnault *et al.,* 2021). A prompt test for diagnosis is an appropriate action required to be taken at once.

In this study, gut tissue samples from Egyptian tomb bats (*T. perforatus*) were tested for detection of RABV by RT-PCR and dFA methods. Diagnosis for RABV was made based on a histological study of the distinctive intracytoplasmic inclusion bodies of the virus in the cat brain. This study reports a close genetic relationship between Egyptian RABVs isolated from Egyptian tomb bats (*T. perforatus*) in South Egypt - Aswan and other RABV isolates from cattle in south of Egypt - Western Desert (~99 %), and from the recently deceased stray cat (~95%). It does not match with bat-originated RABV reported from different species, which indicates that RABVs in cattle, cats, and bats have the same Lyssavirus origin and not bat-originated virus. Analysis showed that it is classical type and not bat type. This suggested that these viruses may have shared an ancestor. Spillover from cattle to bats and from bats to cats, may be infected by contacting bats with RABV-contaminated saliva or infected insects dropped off cattle, or by hunting bats or feeding on freshly dead bats (Cheetham and Markotter, 2021), particularly carnivorous creatures that coexist with bats.

The spread of RABVs in Egypt may have been caused by dogs and cats roaming freely, feeding on recently deceased, recently infected bats. Nucleotide sequence comparisons between the isolated RABV from bats and those from cats and cattle revealed a tight genetic link, suggesting a possibility of RABV transmission from and/or to stray animals. Hence, it could be concluded that RABV may circulate among Egyptian tomb bats, with sporadic transmission to stray carnivores. Additionally, the RABV from bats in the Aswan Governorate showed nucleotide sequence similarities to RABVs from cats, suggesting a potential transmission and may play a role in the zoonotic action.

To prevent samples from being autolyzed, which would result in falsely negative rabies virus tests, only freshly dead or alive bats that had fallen to the ground were collected. The underscoring significance of rabies virus surveillance in bats may be due to viral variation in this species and difficulty in sampling and isolating the virus in such

species. On the other side, results of the previous study revealed that bats had been attacked by carnivorous animals, raising the possibility of bats that carry the virus transmitting rabies on a national scale, emphasizing the importance of carnivorous rabies virus transmission for people and the necessity of public health (Duarte *et al.,* 2020 ; Coertse *et al.,* 2021).

Detection of RABV in the bat gut tissue indicates the virus shedding in bat fecal matter, which has a hazardous importance. As a rapid control measure in case of wound contamination (Benavides *et al.,* 2019). Since (*T. perforatus*) typically preys on moths, ticks, termites, beetles, and other insects, Dogs and cats could be more likely to contract the disease through unintentional contact with bats by hunting or feeding on freshly dead bats (Antunes *et al.,* 2018; Regnault *et al.,* 2021).

To prevent stray animals in Egypt from playing a zoonotic or reverse zoonotic role in the spread of the rabies virus, mass vaccination campaigns should be implemented (Cheetham and Markotter, 2021). In line with this, it is important to further characterize the rabies virus lineages from (*T. perforatus*) bats to study more about the dynamics of rabies transmission between bats and domestic animals, as well as to gather more data for the implementation of control strategies.

The increasing activities of rabies surveillance in bats are significant since insectivorous bats were a focus of the control programs. Data from the present study showed that the percentage of RABV-positive bat samples during analysis was low (3 out of 250 sample size), indicating the need for additional research and surveillance among insectivore bat populations throughout the Egyptian governorate, particularly those that reside close to human populations. Also, in stray cats, surveillance is important due to the silence of rabies signs among cats and the difficulty of catching them or control their movement as this is the main reason of cat samples shortage.

CONCLUSION

The results of the present study concluded that RABV is circulating among bats and carnivorous animals in Egypt. This study reports that we detected 3 RABV bats with RABV-infected gut tissue, which indicates they had fed on RABV-contaminated arthropods that may be infected or soaked in infected saliva of rabid cattle, which revealed similarity at the nucleotide level, indicating circulation of the same virus from animal origin and not from bat origin.

Continued studies of epidemiology and transmission of RABV are needed to update the control strategy of the disease in Egypt. Optimization of diagnostic protocols and surveillance strategy to study the evolution of circulated strains of RABV should be taken into consideration. Mandatory epidemiological studies of arthropod vectors (arthropod-borne virus diseases), Determine preventive and control strategies for the disease.

SUBMISSION DECLARATION

The work submitted has not been published previously and is not under consideration for publication elsewhere. This publication is approved by all authors.

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عزل وتوصيف فيروس داء الكلب في الخفافيش والقطط في مصر

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تستهدف حملات التطعيم ضد داء الكلب الثعالب والكلاب والقطط وغيرها من الحيوانات آكلة اللحوم باعتبارها حاملات محتملة للأمراض. وبخلاف ذلك، فمن دون التحصين، يمكن أن تصاب الحيوانات مثل الماشية والحمير والخفافيش التي تتعايش مع تلك الحيوانات آكلة اللحوم وغيرها من الحيوانات الأليفة. في هذه الدراسة، تم فحص ٢٥٠ خفاش من خفافيش القبور المصرية الميتة وقطة ميتة واحدة، تم اكتشاف فيروس داء الكلب باستخدام البادئات الخاصة بالجين (N gene) , و أكده التشريح المرضى واختبار البلمرة المتسلسل العكسي . قد يوفر عزل داء الكلب من الخفافيش أيضًا دليلاً على أنها تتغذى على الخنافس والقراد والمفصليات الأخرى الملوثة بلعاب الحيوانات المصابة بداء الكلب، و هذا قد يسهل انتقال الفيروس من أنسجة الخفافيش إلى الحيوانات آكلة اللحوم عن طريق اصطياده من قبل القطط او الكلاب او الحيوانات اكله اللحوم أو عن طريق التغذيه على الخفافيش الميتة حديثا قبل التحلل . تشير الدراسة إلى عزل وتوصيف فيروس داء الكلب من خفافيش القبور المصرية في مصر. وعلى حد علمنا، يعد هذا أول تقرير عن اكتشاف داء الكلب في الخفافيش في مصر.