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RESEARCH ARTICLE

Molecular characterisation of rabies virus detected in livestock animals in the southern part of Egypt during 2018 and 2019

SERAGELDEEN SULTAN[1*], SOHEIR ABDOU HUSSEIN AHMED[2], MOHAMED WAEL ABDELAZEEM[3] and SABRY HASSAN[4]

[1] Department of Microbiology, Virology Division, Faculty of Veterinary Medicine, South Valley University, 83523, Qena, Egypt

[2] Veterinary Services Section, Aswan, Egypt

[3] Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt

[4] Department of Biology, College of Science, Taif University, Taif, Saudi Arabia

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ABSTRACT

Brain samples were collected from 33 animals of different species, including buffalo, cattle, dog, donkey, fox and wolf, that had been suspected to be infected by rabies virus (RABV) in different geographical regions of Aswan and Luxor governorates in Egypt. The samples were submitted for histopathological examination and the presence of the nucleic acid and antigens of RABV was tested by RT-PCR and indirect fluorescent antibody technique (IFAT), respectively. Sixteen samples were found positive by all the three examinations. Three samples were selected for further study from animals in which the highest virus loads were detected. The partial sequence of the RABV N gene was determined and analysed from the samples of a buffalo, a cow and a donkey. The viruses in the samples were found to share 95–98% and 95–97% nucleotide and amino acid sequence identities, respectively. In comparison to reference sequences, a few amino acid substitutions occurred in the N protein antigenic sites I and IV in the immunodominant epitopes of the viruses detected in the cow and the donkey but not in the one from the buffalo. The phylogenetic analysis revealed that the RABVs sequenced from the samples belonged to genotype 1, Africa-4 clade, and formed two distinct sub-clades within the Egyptian clade. These findings indicate the circulation of RABV among livestock animals in the southern part of Egypt and raise public health concerns. The amino acid changes detected in this work may contribute to the antigenic diversification of RABVs.

KEYWORDS

rabies virus, nucleoprotein, phylogenetic analysis, Africa-4 clade, Egypt

INTRODUCTION

Rabies is one of the most severe and widespread zoonotic diseases in the world, which is endemic in most Asian and African countries (Bitek et al., 2019; Naji et al., 2020). Most developing countries have limited facilities in their laboratories for rabies diagnosis which makes the risk of rabies underestimated in many regions (Naji et al., 2020). Rabies virus (RABV) infects a wide range of mammals with almost 100% fatality. RABV has a non-segmented linear negative-stranded RNA genome, and belongs to the genus *Lyssavirus,* family *Rhabdoviridae*, order *Mononegavirales*. Its genome encodes 5 proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and an RNA polymerase enzyme (L) (Dietzgen et al., 2017). The N protein is essential for viral replication, during which it forms an active cytoplasmic ribonucleoprotein (RNP) complex (Vidy et al., 2007). It

*Corresponding author.
Tel./fax: +20 96 321 1223.
E-mail: sultanserageldeen@gmail.com; s.sultan@vet.svu.edu.eg

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is a highly conserved gene, so it is frequently used for genotyping lyssaviruses (Kuzmin et al., 2005). The conserved antigenic sites of the N protein have been considered a useful tool for the detection and identification of lyssaviruses in brain tissues (Goto et al., 2000; Johnson et al., 2002; Khawplod et al., 2006; Nagarajan et al., 2009; Yang et al., 2011; Orłowska and Zmudziński, 2014; de Souza et al., 2017; Manjunatha Reddy et al., 2018). The G protein is responsible for the viral attachment to the host cell surface and for the membrane fusion (Dietzgen et al., 2017). In addition, it also influences viral pathogenicity and affinity to brain cells and enhances the production of neutralisation antibodies to protect hosts against RABV (Ito et al., 2001; Li et al., 2019).

Seventeen distinct species have been distinguished within the genus *Lyssavirus*, *Rabies lyssavirus* (RABV), *Lagos bat lyssavirus*, *Mokola lyssavirus*, *Duvenhage lyssavirus*, *European bat-1 lyssavirus* and *European bat-2 lyssavirus*, *Australian bat lyssavirus*, *Aravan lyssavirus*, *Khujand lyssavirus*, *Irkut lyssavirus*, *West Caucasian bat lyssavirus*, *Shimoni bat lyssavirus*, *Taiwan bat lyssavirus*, *Lleida bat lyssavirus*, *Ikoma lyssavirus*, *Bokeloh lyssavirus* and *Gannoruwa lyssavirus* (Walker et al., 2018). RABV is the most prevalent species worldwide, and causes the majority of rabies cases reported in animals and humans. Although other lyssavirus species isolated from bats have a restricted host range and geographical distribution, they cause a clinical disease indistinguishable from RABV (Badrane et al., 2001; Colombi et al., 2019).

Many diagnostic techniques have been developed for the identification of rabies, including the fluorescent antibody test (FAT) for the detection of rabies virus antigen, RT-PCR for the detection of RABV RNA and histopathological examination for the demonstration of the characteristic intracytoplasmic inclusion bodies (Negri bodies) in the brain tissues of animals (Johnson et al., 2002; Bordignon et al., 2005; Fooks et al., 2009; Zhang et al., 2011; Wisser et al., 2020).

Egypt has been categorised as a high-risk country for RABV infection. Many reports are available on the molecular epidemiology of RABV worldwide (Páez et al., 2005; Khawplod et al., 2006; Barbosa et al., 2008; Zhang et al., 2011; Orłowska and Zmudziński, 2014; Manjunatha Reddy et al., 2018), yet the information concerning Egypt is limited to individual rabies cases reported from Al Wadi Al Jadid and Al Buhayrah governorates (OIE, 2018). Therefore, the current epizootiological study was performed to determine the occurrence of RABV among different animal species including cattle, buffalo, donkey, dog, fox and wolf in the southern part of Egypt during 2018–2019 using molecular, immunological, and histopathological techniques. A phylogenetic analysis was made on the partial N gene sequences obtained from PCR products to determine the RABV genotypes occurring in Egypt.

MATERIALS AND METHODS

Collection of brain tissues from suspected animals

A total of 33 brain samples were collected from different animal species, including 10 from cattle, 9 from dogs, 5 each from donkeys and wolves, and 2 each from foxes and buffaloes, freshly succumbed during 2018–2019. Some cases showed clinical signs suspicious for rabies and others were hunted nearby clinical rabies cases. The complete data concerning animal species, clinical signs, year of collection and location are recorded in Table 1. One part of the brain sample was stored at −80 °C for virus identification by immunological and molecular tools and another part was kept in 10% neutral buffered formalin for histopathological examination (OIE Terrestrial Manual, 2018).

Table 1. Data of the collected brain specimens from suspected rabies-infected animal species in the southern part of Egypt during 2018 and 2019

No.	Sample ID	Species	Location	Year
1	1	Cattle	Aswan	2018
2	5	Cattle	Aswan	2018
3	6	Cattle	Aswan	2018
4	23	Cattle	Aswan	2019
5	24	Cattle	Aswan	2019
6	27	Cattle	Aswan	2019
7	29	Cattle	Aswan	2019
8	30	Cattle	Aswan	2019
9	31	Cattle	Aswan	2019
10	33	Cattle	Aswan	2019
11	28	Buffalo	Aswan	2019
12	32	Buffalo	Aswan	2019
13	7	Donkey	Luxor	2018
14	14	Donkey	Aswan	2019
15	15	Donkey	Aswan	2019
16	25	Donkey	Aswan	2019
17	26	Donkey	Aswan	2019
18	2	Dog	Aswan	2018
19	4	Dog	Aswan	2018
20	16	Dog	Aswan	2019
21	17	Dog	Aswan	2019
22	18	Dog	Aswan	2019
23	19	Dog	Aswan	2019
24	20	Dog	Aswan	2019
25	21	Dog	Aswan	2019
26	22	Dog	Aswan	2019
27	8	Wolf	Aswan	2018
28	10	Wolf	Aswan	2018
29	11	Wolf	Aswan	2018
30	12	Wolf	Aswan	2018
31	13	Wolf	Aswan	2018
32	3	Fox	Aswan	2018
33	9	Fox	Aswan	2018

Indirect fluorescent antibody technique (IFAT)

The IFAT was conducted on impression smears prepared from suspected frozen brain samples according to Soliman et al. (1989). Anti-rabies immune serum and anti-dog conjugate with fluorescent isothiocyanate (FITC) were supplied kindly by the Department of Pet Animals, Vaccine Research Institute, Abbasia, Cairo, Egypt. Briefly, brain smears were prepared on glass slides from the collected samples and allowed to air-dry at room temperature for 5 min. After that,

the slides were kept in acetone (96–100%) at −20 °C for 45 min, then left to air-dry. On the acetone-fixed air-dried slide, 25 µL anti-rabies immune serum was added over the marked smear area and incubated at 37 °C for 1 h. The anti-dog conjugate with fluorescent isothiocyanate (FITC), in a same volume as the anti-rabies immune serum (25 µL), was added on the same marked area and incubated at 37 °C for 30 min prior to examination under fluorescent microscope. The samples were scored as very strongly (++++), strongly (+++) or mildly (++) positive according to antigen intensities in the fixed brain specimens.

Histopathological examination

Histopathological sections were prepared from brain specimens fixed in 10% neutral buffered formalin solution. In brief, the fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, cleaned in xylene, embedded in paraffin, then sectioned (5 µm) and stained with haematoxylin and eosin (HE). The stained sections were examined by light microscopy.

RNA extraction and OneStep reverse transcription polymerase chain reaction (RT-PCR)

The viral RNA was extracted directly from frozen brain tissues by using RNeasy Mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was subjected to the OneStep RT-PCR using OneStep RT-PCR ready mix (Thermo Fisher Scientific, USA) and a primer pair of RABV-NF (5′-GGA AGA GAT AAG AAT GTT TG-3′) and RABV-NR (5′-TTG GAG CTG ACT GAA GAC ATA-3′) which targeted the N gene nucleotide sequence with a PCR product size of 491 bp (Barbosa et al., 2008). The RT-PCR reaction mixture was conducted as a final volume of 25 µL: 12.5 µL 2 × OneStep RT-PCR ready mix, 1.0 µL RABV NF, 1.0 µL RABV NR, 2.75 µL RNase-free water, 1.25 µL RT enhancer, 0.5 µL verso enzyme and 5 µL template RNA. The RT-PCR reaction was conducted in a thermo cycler (A200 Gradient, Long Gene, Japan). The RT-PCR conditions were as follows: one cycle of RT at 50 °C/30 min, one cycle of initial denaturation at 94 °C/1 min, followed by 30 cycles of denaturation at 94 °C/1 min, annealing at 55 °C/1 min, extension at 72 °C/1 min, and then final extension at 72 °C/10 min. The PCR products were separated by gel electrophoresis using a 1.5% w/v agarose gel stained with ethidium bromide (0.5 µg/mL), visualised under ultraviolet light and photographed with a Canon digital camera.

Sequencing analysis of the 491 bases of the N gene of RABV

Sequencing of the PCR amplicon was performed for 3 representative samples (1, 7 and 28) obtained from a bovine animal, a donkey and a buffalo, respectively, by using the Qiaquick purification kit (QIAGEN, Valencia, CA) and sequenced using Big-Dye™ terminator V3.1 cycle sequencing kit (Thermo Fisher scientific, USA) and ABI prism 310 genetic analyzer (Applied Biosystems, USA). The obtained sequence data were analysed using Bio-edit package, Ver.7.2 software, and the phylogenetic tree was constructed using nucleotide sequences deposited in GenBank, which represented the various RABV genotypes worldwide, by maximum likelihood method at bootstraps 1,000 repeats in the Mega 6.0 software (Tamura et al., 2013).

Accession numbers

The nucleotide sequences of EG/RABV/buffalo/28/19, EG/RABV/cattle/1/18, and EG/RABV/donkey/7/18 were deposited to the GenBank under accession numbers MT684779, MT684780, and MT684781, respectively.

Table 2. Results of RT-PCR for partial (N) gene, indirect fluorescent antibody technique (IFAT) and histopathological examination

No.	ID	Species	N gene	IFAT	Histopathology	NS[a]	Year	Location
1	1	Cattle	+	++++	+	Yes	2018	Aswan
2	5	Cattle	+	++	+	Yes	2018	Aswan
3	6	Cattle	+	++	+	Yes	2018	Aswan
4	23	Cattle	+	+++	+	Yes	2019	Aswan
5	24	Cattle	+	++	+	Yes	2019	Aswan
6	27	Cattle	+	++	+	Yes	2019	Aswan
7	29	Cattle	+	++	+	Yes	2019	Aswan
8	30	Cattle	+	+++	+	Yes	2019	Aswan
9	31	Cattle	+	++++	+	Yes	2019	Aswan
10	33	Cattle	+	++++	+	Yes	2019	Aswan
15	28	Buffalo	+	++++	+	Yes	2019	Aswan
16	32	Buffalo	+	+++	+	Yes	2019	Aswan
11	7	Donkey	+	+++	+	Yes	2018	Luxor
12	15	Donkey	+	+++	+	Yes	2019	Aswan
13	25	Donkey	+	++	+	Yes	2019	Aswan
14	26	Donkey	+	++++	+	Yes	2019	Aswan

[a] NS (neurological signs) such as irritation, difficulty swallowing, impaired corneal reflex, muscle tremors, self-biting, recumbency and paralysis.

RESULTS

Immunological detection of the RABV N protein antigen in brain specimens by IFAT

Examination of the brain smears by IFAT indicated the presence of RABV antigen in 16 specimens including 10 from cattle, 2 from buffaloes and 4 from donkeys. All animals had shown neurological signs (Table 2). The samples obtained from dogs, wolves, and foxes as well as one of the five donkey samples were found to be negative.

Identification of Negri bodies by histopathological examination

The characteristic intracytoplasmic, sharply outlined eosinophilic inclusions, the so-called Negri bodies were detected in all 16 samples (Table 2). The Negri bodies were observed in intact or degenerated neurons as a single or many oval inclusions (Fig. 1a–g) with mild congestion, oedema, and mild diffuse gliosis (Fig. 1e) in the brain tissues. In some sections, degenerated neurons associated with satellitosis were seen (Fig. 1c and g). The brain sections from the other 17 animals were negative with normal neurons (Fig. 1h).

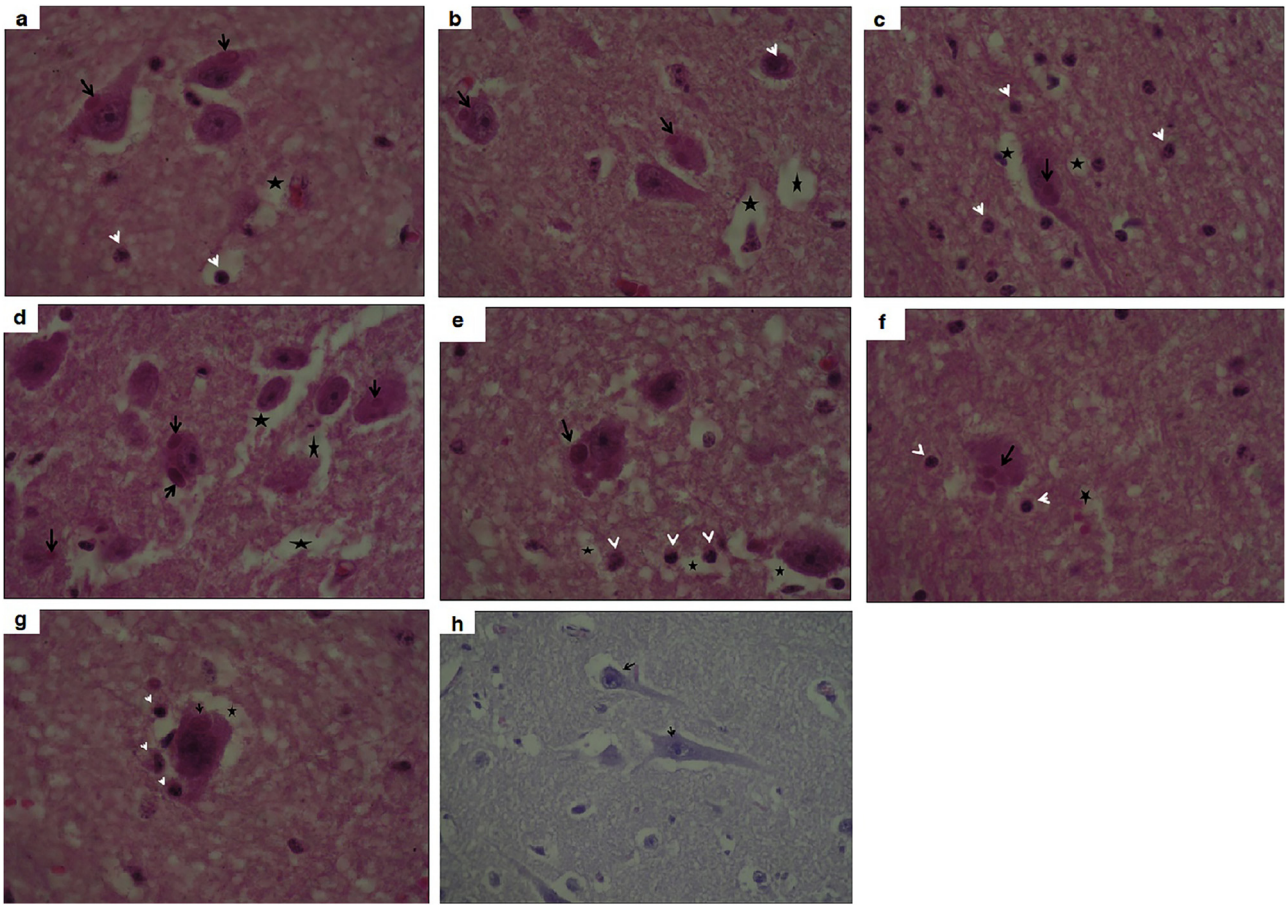

Fig. 1. Histopathological sections of brain from different animals stained with haematoxylin and eosin (HE) and magnified ×1,000 show: (a) Dense eosinophilic and sharply outlined Negri bodies (arrow) of various sizes in the cytoplasm of intact neurons in the brainstem section from cattle. The brain tissue shows mild diffuse gliosis (white arrowhead) and oedema (black star). (b) Dense eosinophilic and sharply outlined Negri bodies (arrow) of various sizes in the cytoplasm of intact, degenerated neurons in the brainstem section from buffalo. The brain tissue shows mild diffuse gliosis (white arrowhead) and oedema (black star). (c) Brain section from cattle shows degenerated neurons associated with satellitosis. A Negri body is also visible in the degenerated neuron (arrow). The brain tissue shows marked severe diffuse gliosis (white arrowhead) and oedema (black star). (d) Brain section of buffalo showing degenerated neurons, and more than one Negri body is visible in the degenerated neurons (arrow). The brain tissue shows mild oedema (black star). (e) Dense eosinophilic and sharply outlined Negri bodies (arrow) in the cytoplasm of an intact neuron in the brainstem section of a donkey. The brain tissue showing mild diffuse gliosis (white arrowhead) and oedema (black star). (f) Dense eosinophilic and sharply outlined Negri bodies (arrow) of various sizes in the cytoplasm of degenerated neurons in the brainstem section of a donkey. The brain tissue show mild diffuse gliosis (white arrowhead) and oedema (black star). (g) Brain section from cattle showing degenerated neurons associated with neurophagia (satellitosis) as indicated by the white arrowhead. A Negri body is also visible in the degenerated neuron (arrowhead) and oedema (black star) can be seen. (h) The brain section shows normal neurons and glial cells from negative samples

Genetic and phylogenetic characterisation of RABV from livestock animals

The RT-PCR resulted in a 491-bp product from the 16 samples that had been found positive by IFAT and by histopathological examination. No amplicons were detected from the RNA of the remaining samples (Table 2). Three samples representing the RABV-positive animal species (cattle, buffalo, and donkey) were sequenced. The nucleotide and deduced amino acid sequences alignment of the samples, EG/RABV/cattle/1/18, EG/RABV/donkey/7/18, and EG/RABV/buffalo/28/19, revealed 95–98% and 95–97% identities to each other, respectively. Amino acid substitutions were detected in EG/RABV/Cattle/1/18 at positions 374, 415, 420 and 421, and in EG/RABV/donkey/7/18 at position 417 but no amino acid substitution occurred in EG/RABV/buffaloes/28/19 in comparison to other Egyptian and Pasteur vaccine strains as shown in Table 3. These mutated amino acids are located at antigenic sites 374–383 and 404–418 of the N protein.

Phylogenetic analysis of N genes from representative strains of different lyssavirus genotypes retrieved from the GenBank and those from the current study revealed that EG/RABV/cattle/1/18, EG/RABV/donkey/7/18, and EG/RABV/buffalo/28/19 belong to the classical rabies virus genotype 1 sub-lineage Africa-4, and forms two distinct subclades in the Egyptian clade of G1-Africa 4 (Fig. 2).

Table 3. Amino acid substitutions among RABV isolates from livestock animals at N protein antigenic sites

Position[a]	Pasteur (PV)	Cattle/ 1/18	Donkey/ 7/18	Buffalo/ 28/19	Egyptian isolates[b]
374	L	R	–[c]	–	–
379	V	L	L	L	L
410	I	M	M	M	M
415	R	G	–	–	–
417	L	-	M	–	–
420	H	Q	–	–	–
421	I	V	–	–	–

[a] Numbering based on the full N gene protein sequence of Pasteur (PV) strain (GU992320).
[b] KX148101 (human/1979), U22627 (human/1995), DQ837463 (dog/1999), MG458317 (dog/1999), DQ837462 (dog/1999), MG458316 (dog/1998).
[c] Same as the Pasteur (PV) strain.

DISCUSSION

Human fatality due to RABV infection has been estimated as 59,000 deaths annually in more than 150 countries, 95% of them occurring in Africa and Asia because of the inaccurate and inadequate risk assessment system. Egypt has been categorised among the human rabies endemic countries (WHO, 2018). The current study was performed to provide data about the presence of RABV among different animal species to facilitate the national and international collaborative efforts for virus eradication by 2030. The infected animals exhibited neurological manifestations and were followed up until death. The presence of the virus was confirmed by RT-PCR, IFAT and histopathological investigation. The brain specimen of one donkey with biting history that had been euthanised three days later before the appearance of any clinical signs was found negative. The intensity of the IFAT detection of RABV antigen and the number of Negri bodies in the brain tissue were affected by the duration of the clinical course of rabies and spontaneous animal death as it has been described previously (Wisser et al., 2020).

Based on the reservoir host (dogs or bats), geographical regions, and the genetic diversity estimated on the basis of partial nucleoprotein sequences, lyssaviruses have been classified into 7 genotypes. Among these, genotype 1 has been further divided into the sub-genotypes Asia, North America, South America, Europe, Middle East, Africa-1a, Africa-1b, Africa-1c, Africa-2, Africa-3 and Africa-4 (David et al., 2007; Troupin et al., 2016; Olarinmoye et al., 2019). Comparison of the N gene nucleotide sequences of various RABV genotypes and the previous Egyptian isolates deposited to the GenBank with those obtained in the current study revealed a common clustering within the genotype 1 of Africa-4 clade distant from the Middle East clade. Also, they separated into two sub-clades represented by EG/RABV/cattle/1/18 and EG/donkey/7/18, and EG/RABV/buffalo/19 within the Egyptian RABV clade. This segregation was attributed to the genetic variation of the virus (Reddy et al., 2011, 2014). Interestingly, the number of amino acid substitutions was higher in the virus from cattle than from donkey and buffalo. This latter one did not differ from the previously reported RABV sequences originating from humans or dogs in Egypt. The case history data indicated that the bovine animal had been bitten by a fox and not by a dog as it had occurred for the donkey and the buffalo. This point could not be clarified further since we could not obtain positive results from the hunted fox and dog samples. Although the N protein is highly conserved including its phosphorylation site (serine at 289) and the antigenic site III (313–337), mutations have been observed at positions 374, 410, 415 and 421 for RABV/cattle/1/18 and at position 417 for RABV/donkey/7/18. These mutated amino acids corresponding to the N protein antigenic sites I and IV (374–383) and the immunodominant peptide (404–418) contribute to specific antigen and antigenic variation (Goto et al., 2000; de Souza et al., 2017).

In Egypt, dogs are the main reservoir of rabies but wild animals should also be regarded as a possible source of RABV transmission. In addition to this, the absence of physical barriers and unrestricted movement between domestic and wild animals might contribute to the rising number of rabies cases among domesticated animals and humans.

In conclusion, the prevalence of RABV infection among livestock animals contributes to the public health risk of human infection and economic losses due to fatality in animals. The possible occurrence of antigenic variation due to

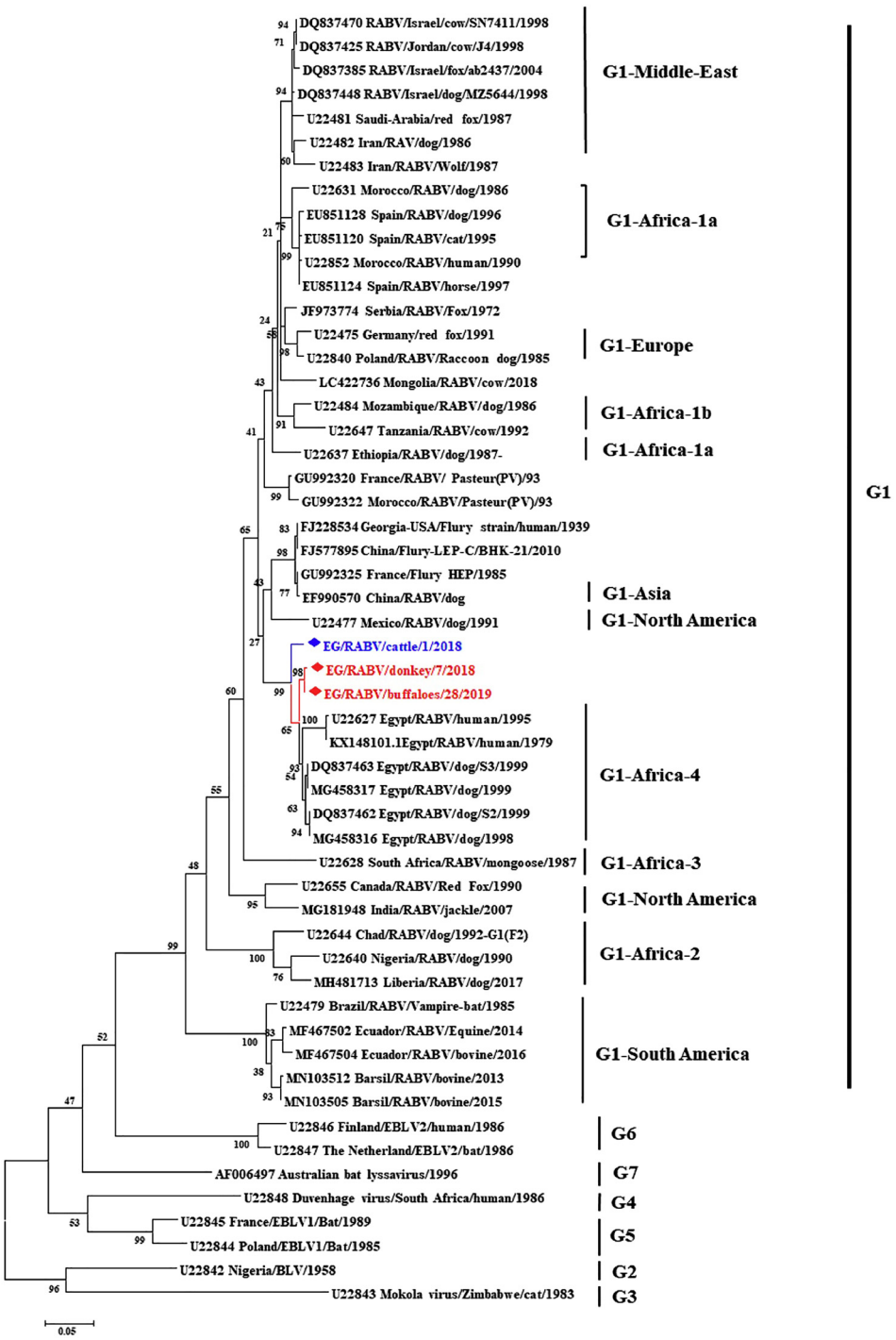

Fig. 2. Phylogenetic analysis of the partial N gene nucleotide sequences (465 nt) using Maximum Composite Likelihood method. The evolutionary distances were computed by General Time Reversible model and bootstrap 1,000 with complete deletion of the gap and missing data. The evolutionary analysis was conducted in MEGA6 software. The two sub-clades were indicated by diamond; cattle/1/18 (blue diamond), donkey/7/18 and buffalo/28/19 (red diamond). The various genotypes of RABV are indicated based on the GenBank database

mutated amino acids at the antigenic sites I and IV as well as immunodominant epitopes may affect the diagnostic procedures and the evolution of the virus. Continuous surveillance of RABV is essential for reaching the international goal of zero cases of RABV infection among humans by 2030.

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