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ORIGINAL ARTICLE

Identification and genetic characterization of rabies virus from Egyptian water buffaloes (*Bubalus bubalis*) bitten by a fox

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Abstract Rabies is caused by negative strand RNA-virus classified in the genus *Lyssavirus*, family *Rhabdoviridae* of the order *Mononegavirales*. The aim of the present study was to identify and analyze nucleotides sequence of nucleoprotein (N) gene of rabies virus (RABV) from two cases of water buffaloes (*Bubalus bubalis*) bitten by a fox in Egypt, 2013. The diseased buffaloes showed nervous manifestations with fever. Specimens from brains of the buffaloes with suspected rabies were collected. RABV in collected samples was identified using direct fluorescent antibody (dFA) technique, histopathological examination and reverse transcription-polymerase chain reaction (RT-PCR). Also, nucleotides sequence of partially amplified nucleoprotein (N) gene was compared with the other street strains of RABV available on GenBank. The results revealed that RABV antigen was identified in the brains of diseased buffaloes by dFA technique and the characteristic intracytoplasmic inclusions (Negri bodies) and RABV nucleic acid were detected by histopathology and RT-PCR, respectively. The identified virus showed close genetic relationship with street strains identified previously from dogs in different Governorates in Egypt and with strains identified in Israel and Jordan indicating transmission of the virus between Egyptian Governorates with a potential transmission from and/or to our neighboring countries.

Keywords Direct fluorescent antibody (dFA) technique · Egypt · Rabies virus (RABV) · Reverse transcription-polymerase chain reaction (RT-PCR) · Sequence analysis · Histopathology · Water buffaloes

Introduction

Rabies is a widespread neurological zoonotic disease which affects any warm blooded animals (including humans). Rabies virus (RABV) belongs to the genus *Lyssavirus* of the family *Rhabdoviridae* within the order *Mononegavirales*. The RABV genome is almost 12 kb in size consisting of five monocistronic RNAs, which encodes the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (large protein). Between the monocistronics there are four intergenic regions (IGRS) with different lengths. Besides, at the end of the genome there are two untranslated regions (UTRS), which play an important role in virus replication. The order of relative conservation of these five genes from high to low could be N > L>M > P>G [16, 24].

There are seven recognized genotypes of lyssavirus defined on the basis of their genetic similarity: rabies virus (genotype 1), Lagos bat virus (genotype 2), Mokola (genotype 3), Duvenhage virus (genotype 4), European bat lyssavirus type 1 (genotype5), European bat lyssavirus type 2 (genotype 6) and Australia bat lyssavirus (genotype 7). Additionally four novel genotypes: Aravan virus, Khujand

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virus, Irkut virus and West Caucasian [3, 8] were recently recovered from bats in Eurasia. Genotype 1 is responsible for classical rabies in terrestrial mammals throughout the world.

The nucleoprotein (N) gene has been extensively used for genetic typing and evolutionary studies because of its relatively lower variation among reservoir-associated variants and geographic lineages [4, 7, 14, 23].

The original method for typing RABV was a panel of monoclonal antibodies (mAbs) to the viral nucleoprotein (N) [13, 22]. However that method is quite antiquated and was replaced by phylogenetic analysis [11, 14].

Rabies transmission usually begins with the bite of an infected host, although transmission has been documented via mucous membrane contamination, aerosol transmission, and corneal and organ transplants [2, 15, 18].

Reliable laboratory diagnosis of rabies could be done by the direct fluorescent antibody (dFA) technique and the reverse transcription polymerase chain reaction (RT-PCR) as both techniques can detect the virus in 98–100 % of the disease cases. Histopathogical examination could also be used on the diagnosis but with much less sensitivity [12]. The aim of the present study was to diagnose two cases of water buffaloes *(Bubalus bubalis)* rabies in Egypt as well as comparing the partial nucleotides sequence of the nucleoprotein (N) gene with the other street strains of rabies virus (RABV) available on GenBank.

Materials and methods

Sample collection

Brain specimens were collected from two water buffaloes *(Bubalus bubalis)* bitten by a fox in Dakahlia Governorate, Egypt in 2013 with suspected rabies clinical signs in form of different nervous manifestations such as paralysis of throat, excessive salivation, extension of neck and grinding of teeth in association with fever. Part of these samples was taken to −20 °C for virus identification by reverse transcription-polymerase chain reaction and nucleotide sequencing. Another part was put in bottles containing 10 % neutral buffered formalin for dFA technique and histopathological testing. Brain sample from normal buffalo were included as a negative control.

Identification of the causative agent

Direct fluorescent antibody (dFA) technique

It was carried out as described previously [6, 19] as follows:

Formalin-fixed, paraffin embedded brain tissues were cut at 5 µm. Slides were heated at 55 °C to melt the paraffin, deparaffinized in xylol, hydrated through graded ethanols and finally rinsed in phosphate buffered saline (PBS) and then the section were left to dry in air for 30 min, fixed with acetone for 10 min and then washed with PBS, pH 7.6. A few drops of 1:100 dilutions of FITC-conjugated anti-RABV antibodies (Chemicon, Temecula, CA, USA) and the slides were kept in a humidified chamber for 1 h at 37 °C. The slides were thoroughly washed with PBS for 15 min three times. They were then mounted with buffered glycerin, covered with a cover slip and examined under a fluorescent microscope (Carl Zeiss, Germany).

Histopathological examination

Histopathological sections were carried out by fixing of brain specimens in 10 % neutral buffered formalin solution. 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 hematoxyline and eosin [1]. The stained sections were examined under light microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR)

Oligonucleotide primers used in the RT-PCR reactions were designed by Langoni et al. [9] and synthesized by TIB MOLBIOL Syntheselabor GmbH. The primers were received in lyophilized form and resuspended in Tris/EDTA (TE) buffer to reach a final concentration of 100 pmol/µl and were designed to amplify a specific segment of 295 bp from N protein gene of RABV genome. The primers sequences for RT-PCR amplification were as follows: forward primer, 5́-ATAGAGCAGATTTTCGAGACAGC-3́ and reverse primer, 5́-CCTCAAAGTTCTTGTGGAAGA-3. RNA extraction was done using QIAamp Viral RNA Mini Kit (Cat. No. 52906) according to the manufacturer's instructions. Normal non-infected brain samples were included as a negative control sample.

RT-PCR amplification was carried out as described before [9] using QIAGEN OneStep RT-PCR Kit (Cat. No. 210212). Briefly, 10 µl 5× QIAGEN OneStep RT-PCR Buffer, 2 µl dNTP Mix (containing 10 mM of each dNTP), 2 µl Forward primer, 2 µ reverse primer, 2 µl ofQIAGEN OneStep RT-PCR Enzyme Mix, 30 µl RNase-free water and 5 µl template RNA. The RT- PCR had reverse transcription step of 50 °C for 30 min then an initial cycle of 94 °C for 10 min, followed by 34 cycles of 94 °C for 1 min, 56 °C for 30 s, 72 °C for 1 min and a final elongation step of 72 °C for 5 min.

Amplified product analysis was carried out as described previously [21]. Briefly 10 µl of the PCR product was mixed with 1 µl 10× gel loading buffer and loaded to the

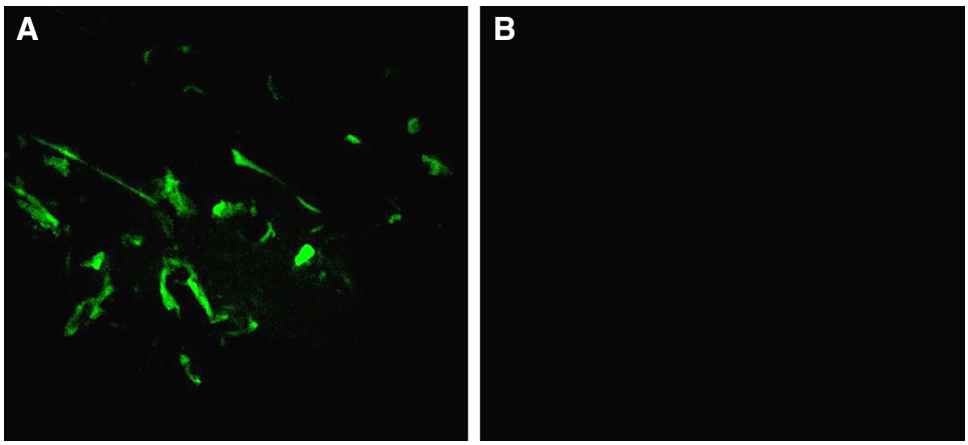

Fig. 1 Results of direct flourescent antibody (dFA) technique of specimen from brain of diseased buffalo shows bright apple green viral nucleoprotein (N) antigen targeted by FITC-conjugated anti-RABV antibodies (**a**) and normal buffalo brain sample shows no viral antigen (**b**). With ×400 magnification

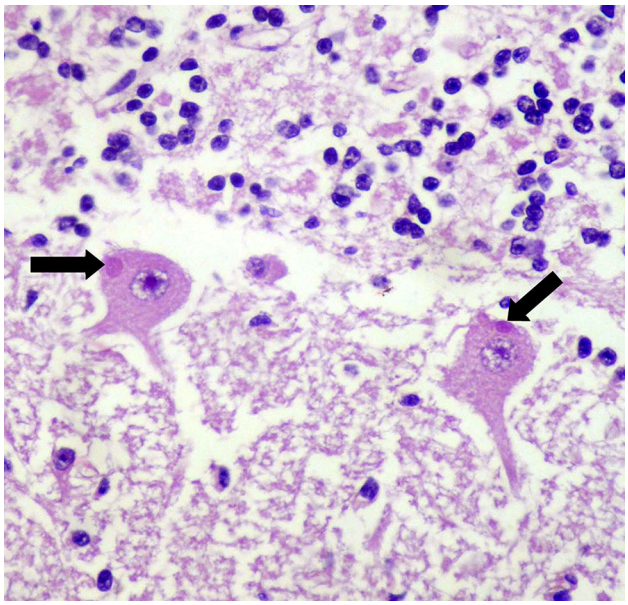

Fig. 2 Brain section of diseased buffalo reveals intracytoplasmic eosinophilic inclusions (Negri bodies) in neurons (*arrows*). HE stain. With ×400 magnification

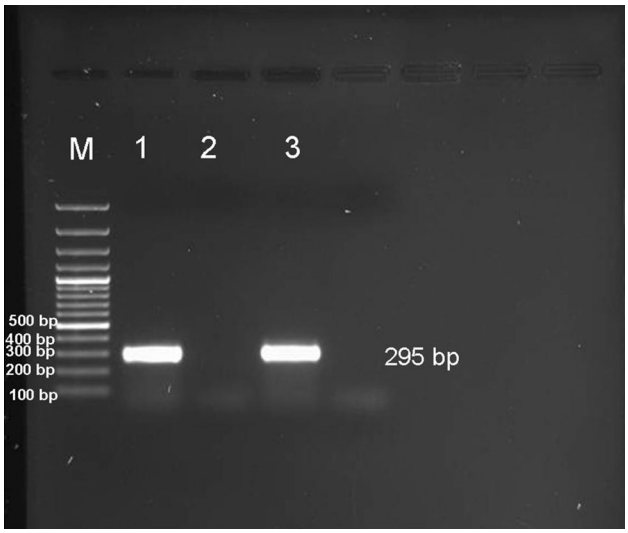

Fig. 3 Gel electrophoresis of PCR products of 295 bp of nucleoprotein (N) protein gene of rabies virus genome. *M* DNA marker, *Lane 1 & 3* The amplified products prepared from brain samples of diseased buffaloes, *Lane 2* negative control buffalo brain sample

individual wells of a 1 % agarose gel. In addition, 2 µl of a 100 bp DNA molecular weight marker was loaded with 2 µl loading buffer in a single outside well to be used as DNA ladder. The amplified DNA products were detected in comparison with DNA ladder using the U.V. transilluminator. The gel was photographed.

Nucleotide sequencing and analysis of sequencing data

Sequencing of the PCR amplicons were performed by SeqLab Gottingen GmbH (Gottingen, Germany). The obtained sequence data were analysed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and then, the alignment *.aln output file was used for performing the neighbour-joining (N-J) phylogenetic analysis with 1000 repeats of bootstrap tests analysis, and calculation of divergence and identity per cents were performed using MegAlign (DNASTAR, Lasergene®, Madison, WI, USA).

Results

Identification of the causative virus

Direct fluorescent antibody (dFA) technique revealed presence of RABV in the brain specimens from the diseased buffaloes while the control sample (normal brain) showed a negative reaction (Fig. 1). On histopathological examination, the characteristic cytoplasmic inclusions (Negri bodies) was detected in examined section (Fig. 2) beside perivascular infiltration (cuffing) with lymphocytes, few plasma cells, and macrophages. Also, some neurons were also found to be degenerative or necrotic.

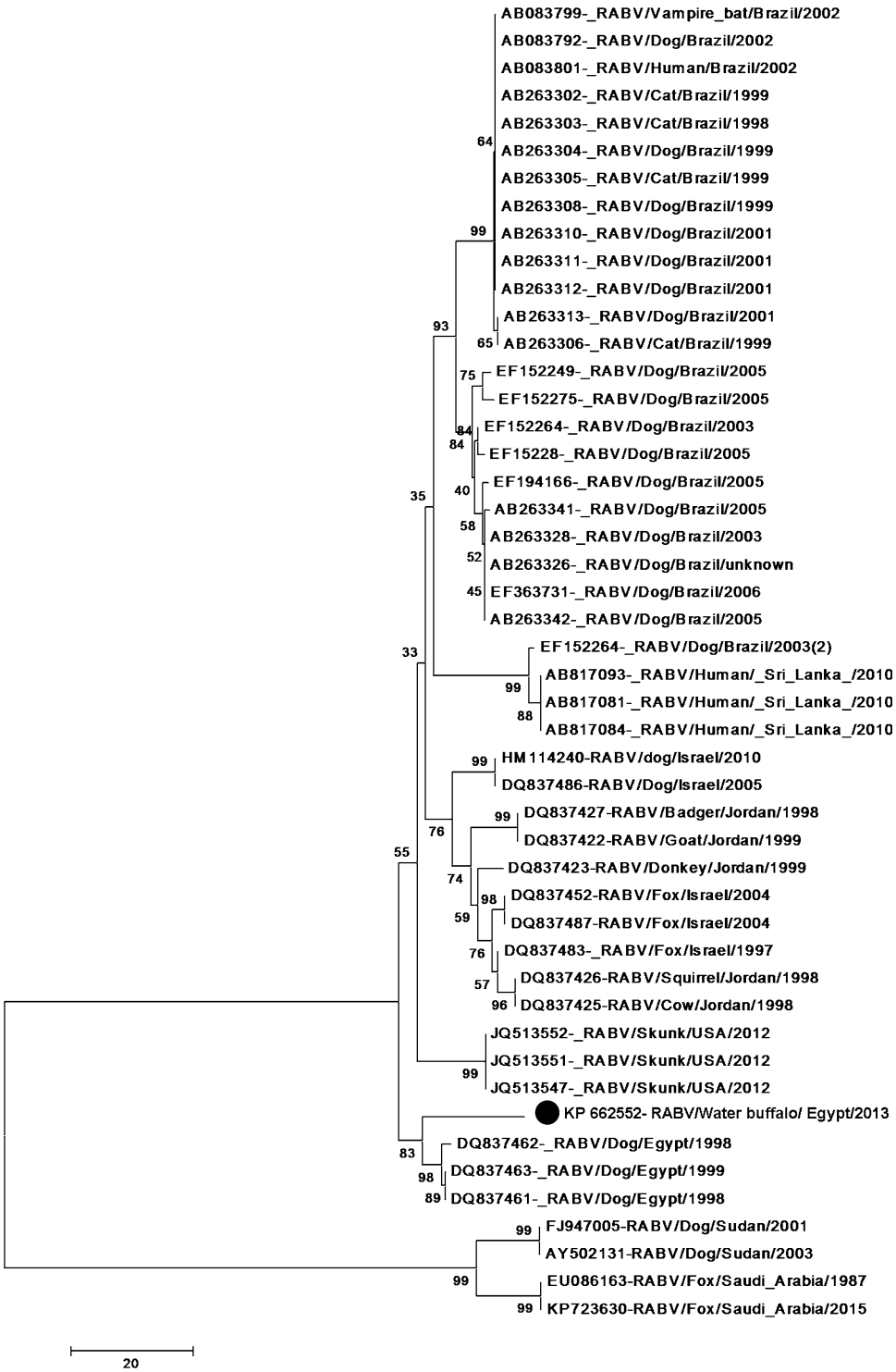

Fig. 4 Phylogenetic tree based on nucleoprotein (N) protein gene nucleotide sequences of our rabies virus from water buffalo with others rabies viruses whose N genes were retrieved from the GenBank database sequences. *Numbers at the internal nodes* represent the bootstrap probabilities (1000 replicates)

Analysis of the PCR products obtained from the amplification reaction of extracted RNA by agarose gel electrophoresis revealed the positive amplification of the nucleoprotein (N) gene with correct size (295 bp) (Fig. 3). The negative control sample did not show any amplified product.

Nucleotide sequencing and analysis of sequencing data

The obtained nucleotide sequence of 295 bp PCR fragments representing the partial sequence of the nucleoprotein (N) gene were analyzed and compared with the

published sequences of RABVs available in GenBank (Supplementary Table-1). Amplicons from the two samples were of the same nucleotides sequence and only one sequence was submitted to GenBank (accession no. KP662552).

The multiple alignments revealed that our RABV is found to be different from Egyptian dog RABV identified in cairo (DQ837463 & DQ837461) and from Faium (DQ837462), at about 17 positions. The RABV was showed to have ~93 % identity at the nucleotide level with dog RABVs from cairo and faium (Egypt) with accession no. DQ837463, DQ837461 & DQ837462. Also, Comparing nucleotides sequences of the identified RABV with those from neighboring countries revealed that the virus shared an average identity of 87.3 % with RABVs of Israel (accession no. HM114240, DQ837452, DQ837487, DQ837486 & DQ837483)while shared identity of 86 % with Jordan RABVs (DQ837427 & DQ837422) and 86.7 % with Jordan viruses with accession no. DQ837425& DQ837423.

The Phylogenetic tree pattern for the alignment is shown in (Fig. 4).

Discussion

Rabies is caused by a neurotrophic virus in the genus Lyssavirus. RABV is the causative agent of one of the most widespread, terrifying and deadly zoonotic disease. This virus is highly neurotropic, moving along peripheral nerves from the site of infection to the central nervous system, where it replicates and causes a progressive and invariably fatal neurologic disease [10]. The disease needs a rapid laboratory diagnosis after being suspected for rapid performing of appropriate measures such as slaughter or vaccination.

In this study, dFA and RT-PCR techniques used for detection of RABV in brain samples from two cases of water buffaloes (*Bubalus bubalis*) bitten by a fox in Egypt serve as a rapid, effective and economic method for laboratory confirmation of the disease [5, 20, 25]. The use of these techniques for direct detection of virus reduces the dependence on tissue culture and the time required to isolate RABV which may delay implementation of appropriate measures for control. Also, histopathological examination was used for diagnosis depending on the characteristic intracytoplasmic inclusion bodies (Negri bodies) but it is time consuming and less sensitive than FAT [12].

Sequence analysis of partial nucleotides sequence of the nucleoprotein (N) gene of our RABV showed close genetic relationship with Egyptian RABVs from dogs in 1998 and 1999 in Cairo and Faium. This indicated that these viruses may have originated from common ancestor and spread between different Governorates. These findings are in agreement with that previously reported [4, 7, 17]. This transgovernmental spread of RABVs occurred in Egypt may be through dog and foxes moving between Governorates without any restrictions. Comparing nucleotides sequences of the identified RABV with those from neighboring countries showed close genetic relationship with those from Jordan & Israel that indicate a potential transmission of RABV from and/or to our neighboring countries.

From this study we could concluded that, dFA and RT-PCR revealed the presence of RABV antigen and nucleic acid, respectively in the brain specimens from the buffalo bitten by fox and could help in rapid diagnosis of the disease. RABV circulate in Egypt among foxes, with occasional transmission to water buffaloes and the RABV from fox in Dakahlia Governorate showed close relationship at nucleotide sequences to RABVs from dogs in different Egyptian Governorates and with strains identified in Israel and Jordan indicating transmission of the virus between Egyptian Governorates with possible transmission from and/or to our neighboring countries through uncontrolled movement of foxes and dogs through the boundaries.

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