

Molecular Studies on Rabies Isolates from Horses in Cairo

Yanni, M. I¹; Hanaa A. Ahmed²; Lamyaa, A. Ahmed¹; Hanan, A. Fahmy³ and Ibrahim, E.M.⁴ Aggour, M. G.³

Virology¹, Genome Research Unit², Biotechnology³ and Pathology Research Departments⁴ Animal Health Research Institute-Dokki-Giza

ABSTRACT

Rabies causes about 26,000 to 55,000 deaths worldwide per year. More than 95% of these deaths occur in Asia and Africa. In our study, three Arabian horses from Cairo, Egypt, were died after nervous manifestation expecting Rabies. Brain tissues were sent for direct fluorescent antibody technique and isolation on tissue culture using BHK cells. Direct confirmation was performed by Real time RT- PCR and gene sequencing was performed directly from brain samples. Phylogenetic analysis including sequences from all previous Egyptian isolates and neighboring countries isolates was conducted. The % of nucleotide identities of the Egyptian recent isolate (Egy/cairo /2014) with three previous Egyptian isolates were 98.4-98 and 98.4% respectively. Egy/cairo/14 share nucleotide identity with the vaccinal strain flury LEP and kelev with a percentage of (93.1% and 98% respectively). All Egyptian isolates have a common origin. In conclusion ,further epizootological study on a molecular basis is needed.

INTRODUCTION

Rabies virus (RV) is a notorious multi-host pathogen that is capable of infecting all mammals, but paradoxically is maintained in distinct host species-associated transmission cycles, typically within the Carnivora and Chiroptera Rupprecht et al., (2002). The name is derived from the Latin rabies, madness. Simpson D.P., (1979). This, in turn, may be related to the Sanskrit *rabhas*, "to do violence". The Greeks derived the word *lyssa*, from *lud* or "violent"; this root is used in the name of the genus of rabies *Lyssavirus* Rotivel Y., (2009).

Rabies causes about 26,000 to 55,000 deaths worldwide per year (WHO, 2013) and lozano, (2012), more than 95% of which occur in Asia and Africa distributed in 150 countries with more than 3 billion people in risk (WHO, 2013). In most of Europe and Australia rabies is only present in bats (WHO, 2007) and only, many small islands are entirely rabies free (CDC, 2014).

The *Lyssavirus* genus includes seven genotypes: rabies virus (RABV, genotype 1),

Lagos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssavirus 1 (EBLV-1, genotype 5), European bat lyssavirus 2 (EBLV-2, genotype 6), and Australian bat lyssavirus (ABLV, genotype 7) Arai et al.,(2003). In addition, all genotypes except type 2 have been associated with cases of human rabies Jackson, (2002). Within each genotype, sublineages correspond to variants circulating in specific geographical regions and/or animal hosts. The genotypes further segregate in two phylogroups including genotypes 1, 4, 5, 6 and 7 (phylogroup I); and 2 and 3 (phylogroup II). Viruses of each phylogroup differ in their biological properties (pathogenicity, induction of apoptosis, cell receptor recognition, etc.). This classification will evolve, particularly as surveillance for bat lyssaviruses is reinforced. Four recent isolates of bat lyssavirus in Central Asia (Aravan virus (ARAV), Khujand virus (KHUV), East Siberia (Irkut virus (IRKV) and the Caucasian region (West Caucasian bat virus (WCBV) need to be characterized as

new genotypes (WHO, 2002). Genotypes 2 - 4 have a wide geographical distribution in Africa, while genotypes 5 and 6 have a western and Eastern Europe distribution, respectively Heaton *et al.*, (1999).

The rabies virus has a single non-segmented negative strand RNA genome. The genetic information is present in the form of a helical ribonucleoprotein complex (RNP), in which the linear RNA is tightly associated with the viral nucleoprotein. The genome of RV and VSV comprises only five genes encoding viral proteins, namely nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA polymerase (L). The 3' to the 5' end the genomic RNA encodes a leader RNA of about 50 nucleotides, followed by the genes N,P, M, G, and L Finke and Conzelmann, (2005).

In Egypt, five hundred and thirty-two rodents (10 species) and 112 other wild mammals (six species) were examined for rabies infections. Rabies virus was isolated from the brains of three (1-2%) of 249 Gerbils (*Gerbillus gerbillus*) and one (1-8%) of 56 foxes (*Vulpes* species) and by neutralization tests, these isolates were found to be identical to classical rabies virus Botros *et al.*, (1977). Rabies was isolated from different localities in Egypt during the period from 1997 to 2000 Yanni, (2000) and more molecular studies were detailed later by David *et al.* (2007). A recent rabies virus was isolated and sequenced from rabid cattle at Elwadi Elgedid Governorate in 2013 Rawheya *et al.*, (2013). In this study, three suspected rabid horse cases from one farm in Cairo, were tested for Rabies. The aim of our study, is to confirm that these cases were Rabies by different techniques and to relate these isolated cases to those isolated previously together with some neighbourhood isolate to find a relation to them and the vaccinal strains based on the partial *N gene* (nucleoprotein gene) obtained.

MATERIALS AND METHODS

Samples in the form of brains, obtained from 3 Arabian horses that had been reported to examine the cause of death and saliva from one live horse that had shown profound salivation together with some nervous manifestation and died after 2 days.

Direct detection of rabies virus using Fluorescent Antibody technique:

FAT was carried out according to Meslin *et al.* (1996). Impression smear preparations of the hippocampus were placed in a coplin jar containing acetone and fixed at -40 °C for 30 minutes. The slides were air-dried and stained with fluorescein-labelled monoclonal anti-rabies immunoglobulin (BBL Microbiology Systems, Cockeysville, Maryland, USA). These were then incubated at 37°C for 30 minutes in a humid chamber and further washed with Phosphate Buffered Saline (PBS) in 3 successive washes for 5-10 minutes. The slides were rinsed with distilled water; air-dried and mounting buffered glycerol applied, then visualized under an immunofluorescent microscope (Zeiss, Germany) at X400 magnification. Bright/dull/dim apple-green or yellow-green, round to oval intracellular accumulations were observed. Positive and negative controls were run together with the test specimens. The same procedure was carried out on BHK cell scrapping (on glass slides) used for rabies isolation.

Virus isolation on BHK was carried out according to **Rudd *et al.* (1980) and Yanni (2000):**

The BHK-21 cell line {from The Holding Company for Biological Products & Vaccines (VACSERA)} was maintained at the virology department of the animal health research institute-Giza. Eagle basal medium with 3% fetal bovine serum was used as the cell maintenance medium, and a combination of 10% fetal bovine serum and 10% tryptose phosphate broth was used as the cell growth

medium. All media contained 200 IU of penicillin and 0.4 mg of streptomycin per ml. Cell pools for seeding test slides were prepared by trypsinization of confluent cell sheets in tissue culture flasks. The trypsinized cells were suspended in growth medium (25°C) and stored for up to 1 week at 4°C in siliconized glassware. When employed, 50 µg of diethylaminoethyl (DEAE) dextran was added to the suspended cells before seeding the test slides.

All test samples were prepared as 10% suspensions with a mortar and pestle and centrifuged under refrigeration for 30 min at 3,000 x g. The supernatant fluid was used as the undiluted inoculum. The diluent for all suspensions was Eagle growth medium. Sample suspensions were inoculated on tissue culture suspension and incubated at 37°C for 24-48h. Viral growth was confirmed by FAT as before..

Detection of Rabies virus by PCR:

RNA Extraction was done using Total RNA Purification Kit from tissue (Jena bioscience –Germany) according to the manufacturer's instructions (Cat. No. PP-210S).

Real Time PCR was done using PrimerDesign™ genesig Kit (detecting the 3' leader and nucleoprotein region) for Rabies virus Genomes, which was designed for the in vitro quantification of Rabies genomes (UK), and AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystem, USA).

Partial nucleoprotein (N) gene sequencing of RV was done using primers N53 (5'-GGA TGC CGA CAA GAT TGT AT-3', corresponding to bases 73-92 of the RV sequence) and N55 (5'-CTA AAG ACG CAT GTT CAG AG-3', corresponding to bases 491-472 of the RV sequence) according to Amengual et al. (1997) that amplify 400 bp. PCR Product purification done by

QIAquick PCR Purification Kit (Qiagen) and Quantitation of purified product was estimated by Qubit® 2.0 Fluorometer and Qubit® dsDNA HS Assay Kit (Cat. No. Q32851).

Sequencing of the product was done using Big Dye Terminator V3.1 sequencing kit and post sequencing purification was done with Centri-Sep Spin Columns (Invitrogen) and electrophoresis is done in Genetic analyzer 3500 (Applied Bioscience).

Software used for Analysis was BioEdit, multiple and pairwise alignment using Clustal W2 and phylogenetic tree was created by Mega 5 using neighborhood joining tree using some in comparison to other isolates from National Center for Biotechnology Information (NCBI) in table (1).

RESULTS

1-Results of FAT on brain impressions and tissue culture scraping:

The three examined brain tissues were found positive by direct fluorescent antibody technique performed on acetone fixed (brain impressions and tissue culture scraping isolates) (Figure 1, 2 respectively).

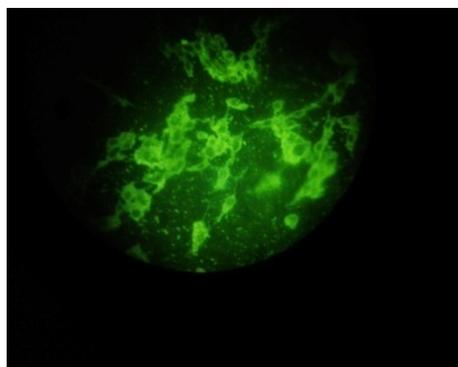


Fig. 1 Positive fluorescence on stained brain impression 40X

Table (1) The Description of isolates from NCBI used for comparison in this study:

No	Accession No	Name	Host	Isolation year	country	Variant	% of similarity with Egy/cairo/2014
1	DQ837414	HB1064	donkey	2002	Israel	III	92.3
2	DQ837423	J2	donkey	1999	Jordan	VI	92.7
3	DQ837452	NS3056	fox	2004	Israel	III	92.3
4	DQ837469	SN4414	cow	2005	Israel	VII	93.8
5	DQ837470	SN7411	cow	1998	Israel	I	93.1
6	DQ837472	SO0047	dog	1996	Israel	III	91.9
7	DQ837473	SP1662	wolf	1997	Israel	IV	92.7
8	DQ837474	T1	cow	2000	Turkey	VII	
9	DQ837481	TH6967	fox	1997	Israel	III	92.3
10	DQ837482	TL8852	jackal	2004	Israel	III	92.3
11	DQ837483	TM3205	fox	1997	Israel	II	93.8
12	DQ837484	VS3370	fox	1995	Israel	II	93.8
13	HG764581	wadi2013	cattle	2013	Egypt		97.3
14	DQ837382	145	human	1997	Israel	II	93.4
15	DQ837383	329	human	1996	Israel	I	93.1
16	DQ837422	J1	goat	1999	Jordan	V	92.7
17	DQ837423	J2	donkey	1999	Jordan	VI	92.7
18	DQ837424	J3	cow	1998	Jordan	VI	92.7
19	DQ837425	J4	cow	1998	Jordan	I	93.1
20	DQ837431	Kelev	dog	1950	Israel	Africa 4	98
21	DQ837460	RZ4879	fox	1998	Israel	III	92.3
22	DQ837461	S1	dog	1998	Egypt	Africa 4	98.4
23	DQ837462	S2	dog	1999	Egypt	Africa 4	98
24	DQ837463	S3	dog	1999	Egypt	Africa 4	98.4
25	DQ837464	SI2236	dog	2005	Israel	VII	93.8
26	DQ837465	SL9655	Dog	1997	Lebanon	I	93.4
27	DQ837466	SM0034	fox	2002	Israel	I	93.1
28	DQ837467	SM0313	cow	2005	Israel	VII	93.8
29	DQ837468	SM3563	dog	2006	Israel	VII	93.8
30	DQ837470	SN7411	cow	1998	Israel	I	93.1
31	DQ837480	T7	dog	2000	Turkey	VII	
32	RVU22840	8618POL		1995	France	I	
33	RVU22482	8681IRA		1995	France	I	
34	HM114241	6628B	dog	2010	Israel	VII	93.8
35	EU282381	CHVC06	mouse	2007	China		
36	EU853569	9016MAR	dog	1990	Morocco		
37	EU853571	9108MAR	human	1991	Morocco		
38	FJ577895	Flury-LEP	vaccine		China		
39	FJ228520	33CAUSCY	coyote	1990	USA	Genotype 1	
40	FJ228534	fluryGA	human	1939	USA	Genotype 1	
41	FJ228535	DgNYKprwsky	Dog	1950	USA	Genotype 1	
42	GU992324	Flury LEP	Vaccine	1985	France	Genotype 1	93.1
43	GU798110	9108MAR		1990	France		
44	RVU22627	8692EGY	human	1979	Egypt	genotype 1	95.4

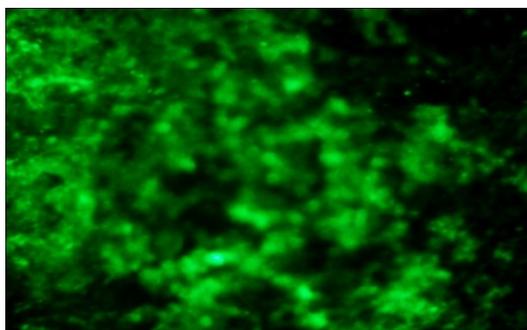


Fig. 2: Positive fluorescence in stained BHK cells 40X

2-Real Time RT-PCR:

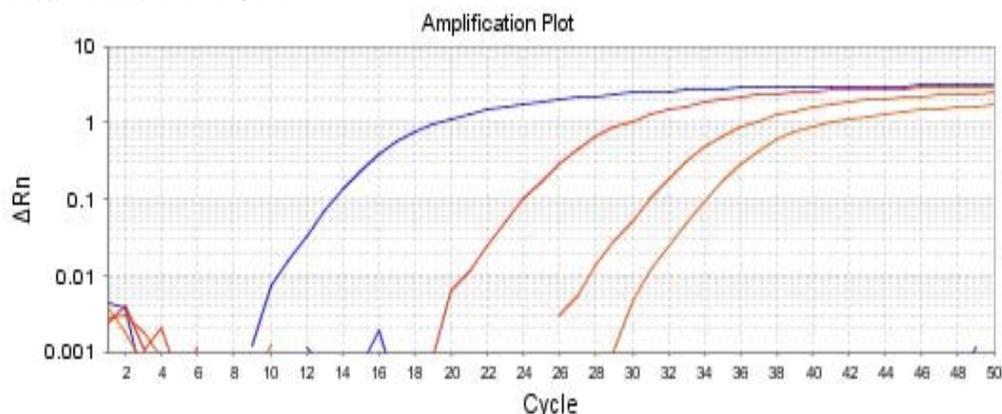


Fig. (3) showing the log curve of amplification plot indicating the exponential increase in fluorescence to the examined samples from the three died animals (Red) CT 25, 31, 34 respectively compared to the control positive (blue) CT 14.

3-Results of PCR for nucleoprotein gene sequencing:

Two of the RT-PCR products were sequenced and the obtained nucleotide was blasted in Gene Bank (NCBI- blastn). The nucleotides was submitted to gene bank (Bankit) with these accession numbers KP119662 and KP119663 with 261 and 230 bp respectively that were 100% identical.

The partial NP from 92to492 protein sequence of the obtained isolates Egypt/cairo/2014was:
 MDADKIVFRVNNQVVSLKPEIADQYE
 YKYP AIKDLKKPCITLGKAPDLNKAYK
 SVLSGMNAAKLPDDVCSYLAAMQL
 FEGTCPE

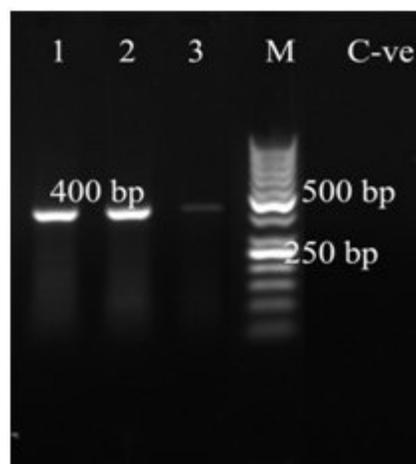


Fig. 4: The electrophoretic pattern of PCR products (400 bp) to the brain samples taken from the 3 died animals.(Brain sample number 3 was -ve) 1-3 brain samples. M 50bp marker (Fermentas). C-ve (water).

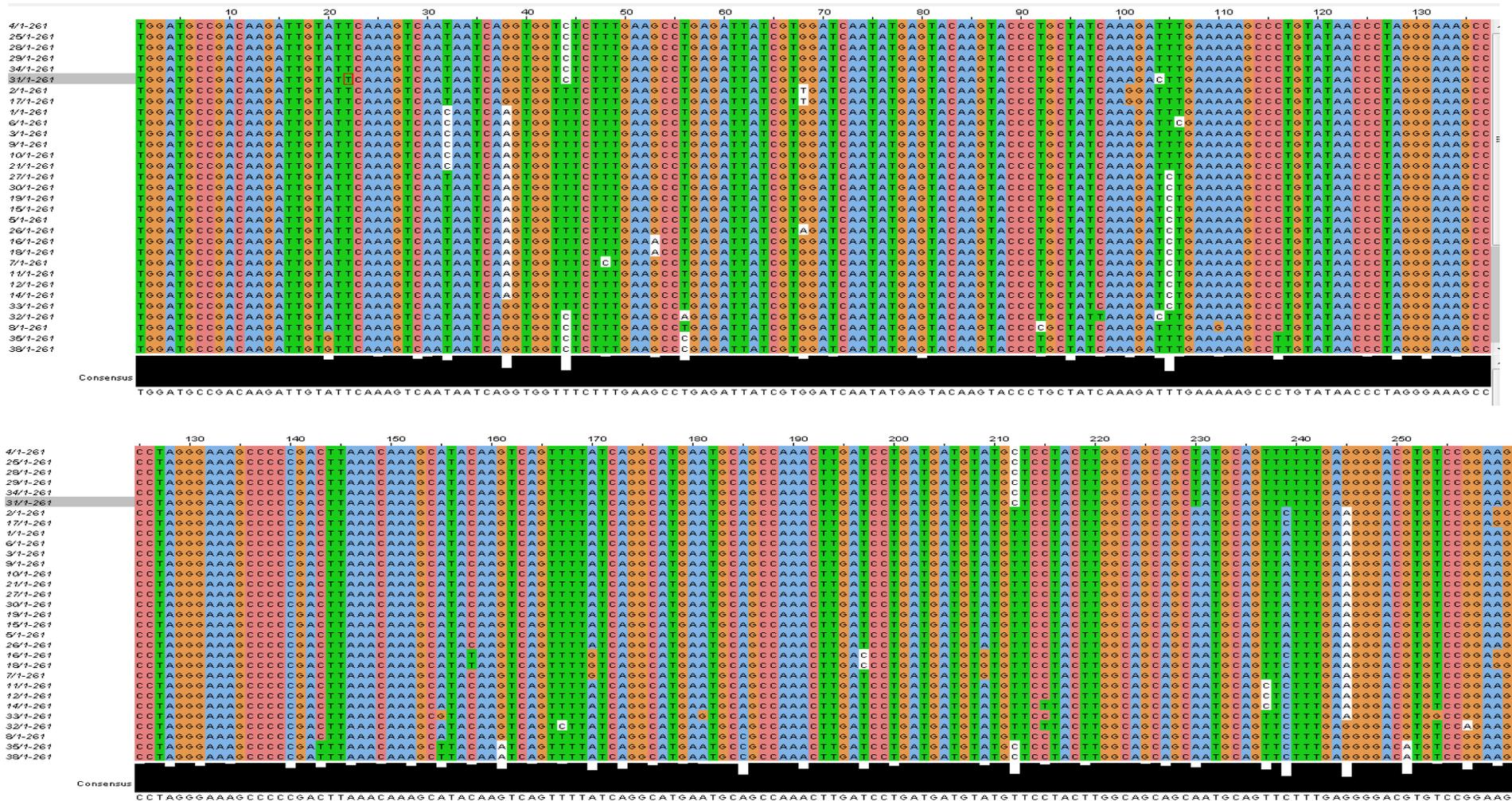


Figure 5: illustrating the nucleotide sequence analysis of the new Egyptian isolates in comparison with other rabies isolates.

Seq	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	35	36	37	38	39	40	41	42	43	44	34 EGY/cai	33	32		
1	ID	0.977	1	0.973	0.992	0.996	0.98	0.969	1	1	0.984	0.984	0.904	0.98	0.992	0.965	0.977	0.965	0.992	0.934	1	0.938	0.934	0.938	0.973	0.988	0.992	0.973	0.973	0.992	0.969	0.946	0.927	0.927	0.946	0.95	0.946	0.946	0.946	0.927	0.911	0.973	0.923	0.973	0.954	
2	0.977	ID	0.977	0.969	0.977	0.973	0.965	0.969	0.977	0.977	0.977	0.977	0.904	0.973	0.977	0.965	1	0.965	0.977	0.934	0.977	0.938	0.934	0.938	0.969	0.977	0.977	0.969	0.969	0.977	0.965	0.946	0.927	0.927	0.946	0.95	0.946	0.946	0.946	0.927	0.908	0.969	0.927	0.973	0.954	
3	1	0.977	ID	0.973	0.992	0.996	0.98	0.969	1	1	0.984	0.984	0.904	0.98	0.992	0.965	0.977	0.965	0.992	0.934	1	0.938	0.934	0.938	0.973	0.988	0.992	0.973	0.973	0.992	0.969	0.946	0.927	0.927	0.946	0.95	0.946	0.946	0.946	0.927	0.911	0.973	0.923	0.973	0.954	
4	0.973	0.969	0.973	ID	0.973	0.969	0.961	0.977	0.973	0.973	0.969	0.969	0.919	0.965	0.973	0.95	0.969	0.95	0.973	0.95	0.973	0.938	0.934	0.938	1	0.969	0.973	1	1	0.973	0.996	0.961	0.934	0.934	0.961	0.965	0.961	0.961	0.961	0.934	0.931	1	0.938	0.965	0.961	
5	0.992	0.977	0.992	0.973	ID	0.988	0.988	0.969	0.992	0.992	0.992	0.992	0.911	0.988	1	0.973	0.977	0.973	1	0.942	0.992	0.946	0.942	0.946	0.973	0.996	1	0.973	0.973	1	0.969	0.946	0.927	0.934	0.946	0.957	0.946	0.946	0.946	0.934	0.911	0.973	0.931	0.98	0.954	
6	0.996	0.973	0.996	0.969	0.988	ID	0.977	0.965	0.996	0.996	0.98	0.98	0.9	0.977	0.988	0.961	0.973	0.961	0.988	0.931	0.996	0.934	0.931	0.934	0.969	0.984	0.988	0.969	0.969	0.988	0.965	0.942	0.923	0.923	0.942	0.946	0.942	0.942	0.942	0.923	0.908	0.969	0.919	0.969	0.95	
7	0.98	0.965	0.98	0.961	0.988	0.977	ID	0.957	0.98	0.98	0.98	0.98	0.908	0.977	0.988	0.977	0.965	0.977	0.988	0.938	0.98	0.942	0.938	0.942	0.961	0.984	0.988	0.961	0.961	0.988	0.957	0.934	0.923	0.923	0.934	0.946	0.934	0.934	0.923	0.908	0.961	0.927	0.969	0.942		
8	0.969	0.969	0.969	0.977	0.965	0.957	ID	0.969	0.969	0.969	0.969	0.969	0.919	0.965	0.969	0.95	0.969	0.95	0.969	0.95	0.969	0.946	0.942	0.946	0.977	0.965	0.969	0.977	0.969	0.973	0.961	0.942	0.942	0.942	0.942	0.946	0.961	0.961	0.961	0.942	0.923	0.977	0.938	0.965	0.961	
9	1	0.977	1	0.973	0.992	0.996	0.98	0.969	ID	1	0.984	0.984	0.904	0.98	0.992	0.965	0.977	0.965	0.992	0.934	1	0.938	0.934	0.938	0.973	0.988	0.992	0.973	0.973	0.992	0.969	0.946	0.927	0.927	0.946	0.95	0.946	0.946	0.946	0.927	0.911	0.973	0.923	0.973	0.954	
10	1	0.977	1	0.973	0.992	0.996	0.98	0.969	1	ID	0.984	0.984	0.904	0.98	0.992	0.965	0.977	0.965	0.992	0.934	1	0.938	0.934	0.938	0.973	0.988	0.992	0.973	0.973	0.992	0.969	0.946	0.927	0.927	0.946	0.95	0.946	0.946	0.946	0.927	0.911	0.973	0.923	0.973	0.954	
11	0.984	0.977	0.984	0.969	0.992	0.98	0.98	0.969	0.984	0.984	ID	1	0.919	0.996	0.992	0.973	0.977	0.973	0.992	0.95	0.984	0.954	0.95	0.954	0.969	0.988	0.992	0.969	0.969	0.992	0.965	0.946	0.927	0.934	0.946	0.957	0.946	0.946	0.946	0.934	0.915	0.969	0.938	0.98	0.954	
12	0.984	0.977	0.984	0.969	0.992	0.98	0.98	0.969	0.984	0.984	1	ID	0.919	0.996	0.992	0.973	0.977	0.973	0.992	0.95	0.984	0.954	0.95	0.954	0.969	0.988	0.992	0.969	0.969	0.992	0.965	0.946	0.927	0.934	0.946	0.957	0.946	0.946	0.946	0.934	0.915	0.969	0.938	0.98	0.954	
13	0.904	0.904	0.904	0.919	0.911	0.9	0.908	0.919	0.904	0.904	0.919	0.919	ID	0.915	0.911	0.908	0.904	0.908	0.911	0.961	0.904	0.965	0.961	0.965	0.919	0.908	0.911	0.919	0.911	0.923	0.919	0.923	0.923	0.919	0.927	0.919	0.919	0.919	0.923	0.934	0.919	0.973	0.908	0.911		
14	0.98	0.973	0.98	0.965	0.988	0.977	0.977	0.965	0.98	0.98	0.996	0.996	0.915	ID	0.988	0.969	0.973	0.969	0.988	0.946	0.98	0.95	0.946	0.95	0.965	0.984	0.988	0.965	0.965	0.988	0.961	0.942	0.923	0.931	0.942	0.954	0.942	0.942	0.942	0.931	0.911	0.965	0.934	0.977	0.957	
15	0.992	0.977	0.992	0.973	1	0.988	0.988	0.969	0.992	0.992	0.992	0.992	0.911	0.988	1	0.973	0.977	0.973	1	0.942	0.992	0.946	0.942	0.946	0.973	0.996	1	0.973	0.973	1	0.969	0.946	0.927	0.934	0.946	0.957	0.946	0.946	0.946	0.934	0.911	0.973	0.931	0.98	0.954	
16	0.965	0.965	0.965	0.95	0.973	0.961	0.977	0.95	0.965	0.965	0.965	0.973	0.973	0.908	0.969	0.973	ID	0.965	1	0.973	0.938	0.965	0.942	0.938	0.942	0.95	0.969	0.973	0.95	0.95	0.973	0.946	0.927	0.915	0.915	0.927	0.938	0.927	0.927	0.927	0.915	0.904	0.95	0.927	0.961	0.934
17	0.977	1	0.977	0.969	0.977	0.973	0.965	0.969	0.977	0.977	0.977	0.977	0.904	0.973	0.977	0.965	ID	0.965	0.977	0.934	0.977	0.938	0.934	0.938	0.969	0.977	0.977	0.969	0.969	0.977	0.965	0.946	0.927	0.927	0.946	0.95	0.946	0.946	0.946	0.927	0.908	0.969	0.927	0.973	0.954	
18	0.965	0.965	0.965	0.95	0.973	0.961	0.977	0.95	0.965	0.965	0.973	0.973	0.908	0.969	0.973	1	0.965	ID	0.973	0.938	0.965	0.942	0.938	0.942	0.95	0.969	0.973	0.95	0.95	0.973	0.946	0.927	0.915	0.915	0.927	0.938	0.927	0.927	0.927	0.915	0.904	0.95	0.927	0.961	0.934	
19	0.992	0.977	0.992	0.973	1	0.988	0.988	0.969	0.992	0.992	0.992	0.992	0.911	0.988	1	0.973	0.977	0.973	ID	0.942	0.992	0.946	0.942	0.946	0.973	0.996	1	0.973	0.973	1	0.969	0.946	0.927	0.934	0.946	0.957	0.946	0.946	0.946	0.934	0.911	0.973	0.931	0.98	0.954	
20	0.934	0.934	0.934	0.95	0.942	0.931	0.938	0.95	0.934	0.934	0.95	0.95	0.961	0.946	0.942	0.938	0.934	0.938	0.942	ID	0.934	0.973	0.969	0.973	0.95	0.938	0.942	0.95	0.95	0.942	0.946	0.942	0.931	0.931	0.942	0.95	0.942	0.942	0.942	0.931	0.957	0.95	0.98	0.938	0.927	
21	1	0.977	1	0.973	0.992	0.996	0.98	0.969	1	1	0.984	0.984	0.904	0.98	0.992	0.965	0.977	0.965	0.992	0.934	ID	0.938	0.934	0.938	0.973	0.988	0.992	0.973	0.973	0.992	0.969	0.946	0.927	0.927	0.946	0.95	0.946	0.946	0.946	0.927	0.911	0.973	0.923	0.973	0.954	
22	0.938	0.938	0.938	0.938	0.946	0.934	0.942	0.946	0.938	0.938	0.954	0.954	0.965	0.95	0.946	0.942	0.938	0.942	0.946	0.973	0.938	ID	0.996	1	0.938	0.942	0.946	0.938	0.946	0.934	0.931	0.934	0.934	0.934	0.931	0.946	0.931	0.931	0.931	0.942	0.946	0.938	0.984	0.942	0.931	
23	0.934	0.934	0.934	0.934	0.942	0.931	0.938	0.942	0.934	0.934	0.95	0.95	0.961	0.946	0.942	0.938	0.934	0.938	0.942	0.969	0.934	0.996	ID	0.996	0.934	0.938	0.942	0.934	0.934	0.942	0.931	0.927	0.931	0.931	0.927	0.942	0.927	0.927	0.927	0.931	0.942	0.934	0.98	0.938	0.927	
24	0.938	0.938	0.938	0.938	0.946	0.934	0.942	0.946	0.938	0.938	0.954	0.954	0.965	0.95	0.946	0.942	0.938	0.942	0.946	0.973	0.938	1	0.996	ID	0.938	0.942	0.946	0.938	0.938	0.946	0.934	0.931	0.934	0.934	0.931	0.946	0.931	0.931	0.931	0.934	0.946	0.938	0.984	0.942	0.931	
25	0.973	0.969	0.973	1	0.973	0.969																																								

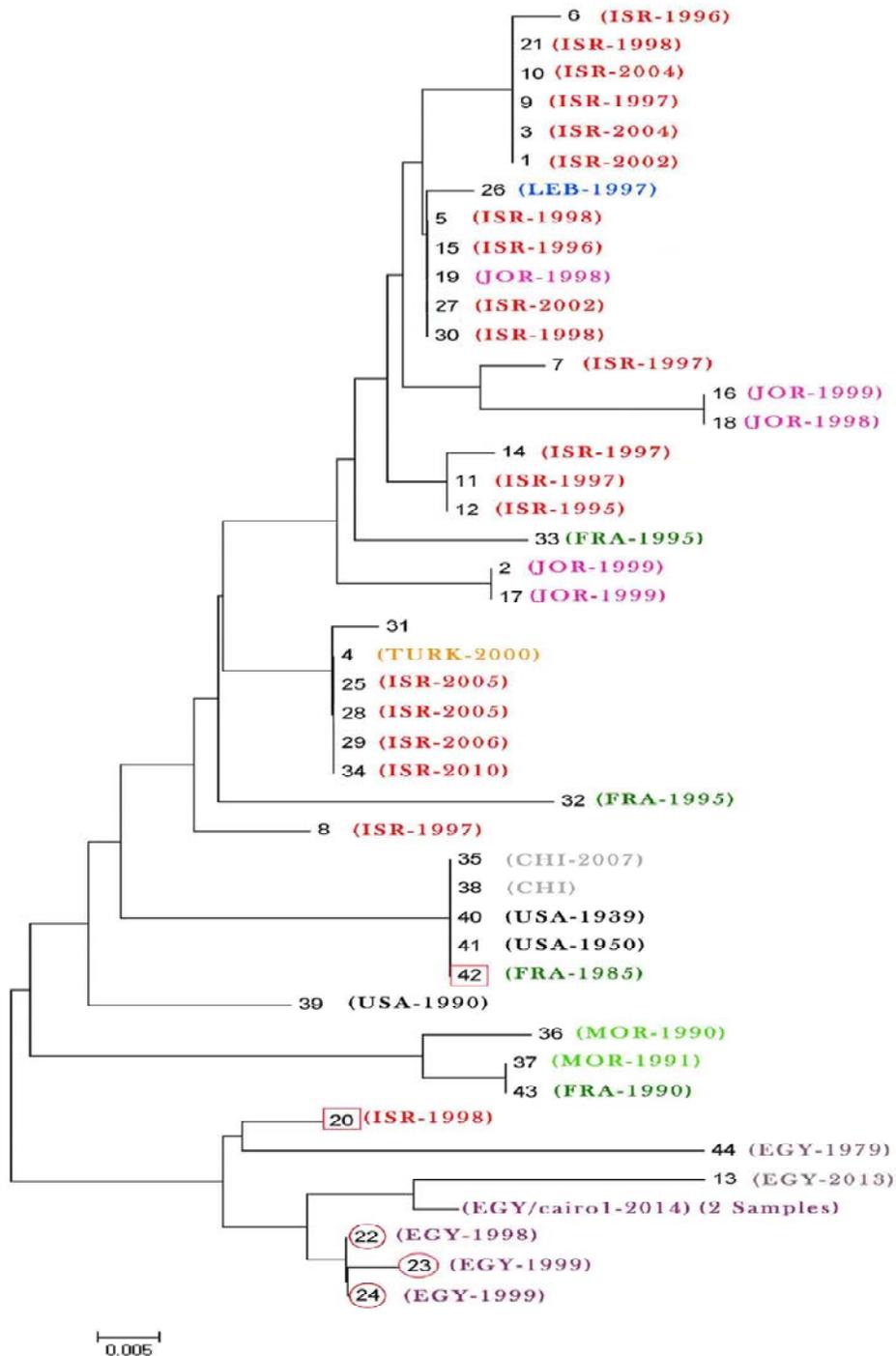


Figure 7: Phylogenetic tree illustrating the relationship of some Egyptian rabies isolates with other isolates.

(Square: vaccinal strains; circle: strains having 98% or more of nucleotide identities with egy/cairo/2014)

After aligning the census sequence with the isolates used in table (1), Fig (5) showed the aligned sequences using ClustalW (EBI European Bioinformatics Institute).

Fig 6 shows the identity matrix of the used isolates from some neighboring countries and some isolates from Egypt and the recently isolated one. It is clear that Egy/Cairo/2014 is 97.3% similar to that isolated from Wadi in 2013 (sample no 13) and equal to and more than 98 % similar to three Egyptian isolates in 1998 and 1999. Fig 7 showed the phylogenetic tree and Cairo isolates were much nearer to Wadi 2013 than to the Egyptian isolates in 1998 and 1999, the vaccinal Kelev strain and the human sample of 1979. Egy/cairo/14 share nucleotide identity with the vaccinal strain flury LEP (38) and kelev (20) with a percentage of (93.1% and 98% respectively). A summary of the identities between isolates was illustrated in table (1).

DISCUSSION

The three horse brain samples were tested positive by FAT and RT-PCR.

The most widely used diagnostic test for rabies is the fluorescent antibody test (FAT), which is recommended by both WHO and OIE . This 'gold standard' test may be used directly on a brain tissue, impression smear and can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnostic purposes. The FAT gives reliable results on fresh specimens within a few hours in more than 95–99 % of the cases (OIE, 2011).

Reverse transcription-polymerase chain reaction (RT-PCR) assays has been reported to be a sensitive and specific tool for routine diagnostic purposes (Tordo et al., 1995 and 1996).

The RABV isolates, originating from three dogs in Egypt (22, 23, 24)(1998and 1999) were typed by mAbs as members of

the V1 antigenic variant(the one most frequently found in Israel, identified in the northern and southern parts of Israel) The three Egyptian dog RABV isolates(22,23and 24), together with the israili Kelev vaccine strain(20)belong to africa 4 clade (David, 2007). The Africa 4 clade is part of the cosmopolitan canine RABV lineage that was believed to have originated in Europe and widely disseminated as a consequence of colonial activity during the 16th to 19th centuries . The Egyptian human isolate(44) wasnt classified within any African lineages (Kissi *et al.*, 1995). The Kelev RABV vaccine strain (20) and the Egyptian human RABV isolate 8692 EGY (44) share 97.8% nucleotide identity, and show 97.3–97.6% identity with the Egyptian isolates 22, 23 and 24. These samples also shared 98.2–99.3% amino acid identity (David, 2007). Egy/cairo/2014 , all Egyptian isolates cited at this study(old and recent isolates) and the israili vaccinal kelev strain have a common origin. Egy/cairo/14 share nucleotide identity with the Egyptian isolate22, 23 and 24 % of nucleotide identities of 98.4%, 98% and 98.4% respectively .

Egy/cairo/14 share nucleotide identity with the vaccinal strains kelev and flury strain with a % of nucleotide identities of 98%, 93.1% respectively. Because of the limited studies on the egyptian rabies isolates, we cannot determine the circulation of other RABV lineages, which are phylogenetically related to the other African(other than africa 4 clade) or Eurasian groups, within Egypt.Rabies african lineage clades in neighbouring countries to Egypt are 7 clades in Israel,3 clades in jordan(1,5 and6) (David, 2007) and unstudied in Sudan and Libya.

In conclusion, this work describes detection ,isolation, partial nucleoprotein sequencing of rabid cases of horses in Egypt . Further epizootological study on a molecular basis at egyptian fauna is needed.

N.B: Biosafety and biosecurity measures were taken during all steps of this assay.

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