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Short communication

Molecular characterization of rabies virus isolated from dogs in Tunisia: Evidence of two phylogenetic variants

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ABSTRACT

In an attempt to explain temporal and geographical rabies incidence fluctuations in Tunisia, a molecular epidemiological study of rabies virus (RV) was carried out. A panel of RV isolates from dogs, collected between 1992 and 2003, from different regions in Tunisia have been analysed by direct sequencing of PCR-amplified products coding for the nucleoprotein gene. New sequences have been compared to prototype sequences of *Lyssavirus* species and nine lineages of species 1. All Tunisian isolates belonged to species rabies virus and segregated into two rabies lineages geographically distinct: NCS lineage characterizing Northeast, Central and Northern areas of the country and NW lineage more restricted to the North-Western regions. Phylogenetic analyses showed that Tunisian RV clustered most closely to Africa 1a lineage: NCS lineage showed nucleic affiliation with isolates from Algeria and Morocco, whereas, NW lineage shared a strong relationship with Ethiopian and Sudanese strains.

Rabies is a zoonotic viral disease of domestic and wild animals characterized by an infection of the central nervous system, causing encephalopathy and ultimately death.

Rabies virus (RV) belongs to the *Lyssavirus* genus of the *Rhabdoviridae* family and has a, negative-sense, single-stranded, RNA genome of approximately 12 kb, which codes for five proteins designated N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein) and L (polymerase) (Wunner et al., 1988). Gene sequencing and phylogenetic analyses of the N and G genes delineated eleven *Lyssavirus* species: Rabies virus (species 1), Lagos bat virus (species 2) (Boulger and Porterfield, 1958), Mokola virus (species 3) (Shope et al., 1970), Duvenhage virus (species 4) (Meredith et al., 1971), European bat lyssavirus 1 (species 5) (Schneider and Cox, 1994), European bat lyssavirus 2 (species 6) (King et al., 1994), Australian bat lyssavirus (species 7) (Fraser et al., 1996), Aravan virus (species 8) (Arai et al., 2003; Kuzmin et al., 2003), Khudjand virus (species 9) (Botvinkin et al., 2003; Kuzmin et al., 2003), Irkut virus (species 10) and West Caucasian bat virus (species 11) (Botvinkin et al., 2003; Kuzmin et al., 2003, 2005). Rabies virus is prevalent throughout the world and can be carried by domestic or wild animals; however the other species are more specific to hosts and geographic areas. Molecular analysis based on the genetic characterization of the RV isolates is an important tool for the understanding of epidemiological relationships and the investigation of origination and transmission patterns of endemic viruses. Data collected from such analyses lead to a more effective strategy of control and prevention of rabies viral infection.

In Tunisia, rabies is known to be enzootic and endemic, and the dog is the principal reservoir and transmitter of the disease (Sureau et al., 1982). The national rabies control program introduced in 1982, including mass vaccination campaigns of dogs, has reduced significantly the incidence of human and animal rabies cases (Kharmachi and Hammami, 1992). Nevertheless, temporal and geographical fluctuations of the rabies incidence were registered, with clear succession of recrudescence and fall of incidence periods. In 1992, 1996 and 2000, was registered higher rabies incidences in animal and human with 581/25, 187/7 and 266/2 cases, respectively (Kharmachi et al., 2007). Furthermore, until 1994, the rabies was restricted to the North and the Center of Tunisia; after this date, the first cases of rabies were reported in the southern regions of the country (Kharmachi et al., 2007). The genetic characterization of RV endemic isolates may contribute to better understanding and explanation of the rabies epidemiological evolution during last decades; such data are not available in Tunisia.

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Table 1
Tunisian rabies dog isolates with their respective regions, years of isolation and accession numbers.

Samples	State	Year	Isolate	Accession number	
				3′N sequence	5′N sequence
1	Kairouan	1992	DogTN/Kr92	/	EU643557
2	Ariana	1992	DogTN/Ar92	/	EU643556
3	Sidi bouzid	1995	DogTN/Sz95	/	EU643565
4	Medenine	1995	DogTN/Md95	EU643545	/
5	Tozeur	1996	DogTN/Tz96	EU643551	EU643572
6	kesserine	1996	DogTN/Ks96	EU643542	EU643586
7	kef	1996	DogTN/Kf 96	EU643540	EU643583
8	Gabes	1996	DogTN/Gb96	EU643532	EU643564
9	Jendouba	1996	DogTN/Jd96	EU643535	EU643562
10	Jendouba	1998	DogTN/Jd98	EU643536	EU643560
11	Ariana	1998	DogTN/Ar98	EU643555	EU643569
12	Gabes	1998	DogTN/Gb98	EU643533	EU643566
13	Bizerte	1998	DogTN/Bz98	EU643527	EU643589
14	Kesserine	1998	DogTN/Ks98	EU643543	EU643587
15	Kairouan	1998	DogTN/Kr98	EU643539	EU643581
16	Gabes	1999	DogTN/Gb99	EU643530	EU643563
17	Bizerte	1999	DogTN/Bz99	EU643528	EU643561
18	Jenbouba	1999	DogTN/Jd99	EU643537	EU643588
19	Medenine	1999	DogTN/Md99	EU643546	EU643573
20	Ariana	2000	DogTN/Ar00	EU643524	EU643579
21	Zaghouan	2000	DogTN/Zg00	EU643552	EU643568
22	Bizerte	2000	DogTN/Bz00	EU643529	EU643580
23	Nabeul	2000	DogTN/Nb00	EU643548	EU643575
24	Kesserine	2000	DogTN/Ks00	EU643541	EU643585
25	Nabeul	2001	DogTN/Nb01	EU643541	EU643576
26	Jendouba	2001	DogTN/Jd01	EU643534	EU643559
27	Beja	2001	DogTN/Bj01	EU643525	EU643558
28	Bizerte	2002	DogTN/Bz02	EU643525	EU643582
29	kairouan	2002	DogTN/Kr02	EU643538	EU643570
30	Tataouin	2002	DogTN/Ta02	EU643550	EU643567
31	Tunis	2003	DogTN/Tn03	EU643554	EU643578
32	Kairouan	2003	DogTN/Kr03	EU643553	EU643571
33	Sidi bouzid	2003	DogTN/Sz03	EU643549	EU643577
34	Gabes	2003	DogTN/Gb03	EU643531	EU643584
35	Medenine	2003	DogTN/Md03	EU643544	EU643574

This report attempt to respond to this epidemiological shortage by analyzing RV isolates collected between 1992 and 2003 and obtained from different regions of the country. Sequencing and phylogenetic analyses in two different regions of the nucleoprotein gene were conducted.

Among the dog brains specimens available in the laboratory and conserved since 1992, specimens used in this study were selected according to temporal and geographic criteria (Table 1): periods of recrudescence of the rabies incidence (1992, 1996 and 2000) and periods of fall of incidence (1995, 1998, 2001, 2002 and 2003) were included. Samples were also representative of both endemic regions (North and Center of Tunisia) and recently infected areas (South of the country). Viral RNA was extracted from 0.2 g of brain specimen by homogenization in a mono-phasic solution of phenol and guanidine (TRIzol, Gibco BRL) as specified by the manufacturer. Precipitated RNA was resuspended in sterilised water and quantified by spectrophotometry. RT-PCR protocol was performed essentially as described by Nadins Davis (1998). For cDNA synthesis, 2 μg of total RNA was reverse transcribed using 50 pmol of genomic sense primer RabN1 and MMLV reverse transcriptase (Invitrogen) in a total volume of 20 μl. The cDNA product was added to 80 μl of PCR reaction mixture containing 50 pmol antisense primer RabN5 and 0.5 U of *Taq* DNA polymerase (Invitrogen). After an initial heating at 80 °C for 5 min, the N gene amplification reached 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and followed by a final extension at 72 °C for 10 min. For the samples detected as PCR positive, the expected bands of 1472 bp were excised from the agarose gel and purified with the commercial QIAQuik gel extraction kit (QIAGEN) and bi-directionally sequenced by the automate ABI PRISM 377 DNA Sequencer (PE Applied Biosystems) using the PCR primers. The sequencing arrays were performed according to the instructions of ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. Phylogenetic analyses were carried out in order to, firstly, identify the *Lyssavirus* species, and secondly, characterize the Tunisian viral variants (if any) and evaluate their relationship with other rabies strains isolated throughout the world. For the species identification approach, sequences obtained in this work were compared to the nucleoprotein gene of *Lyssavirus* species reference isolates (Table 2). For the phylogenetic affiliation approach, the Tunisian strains were aligned with 17 representative N gene sequences obtained from GenBank (Table 2). These sequences correspond to 9 phylogenetic lineages of the species rabies virus (Kissi et al., 1995), and were chosen as they share a maximum of nucleotide similarity with the Tunisian viral sequences. Sequence alignments were performed using the Clustal X program (Thomson et al., 1997). Phylogenetic and nucleic distance analyses were conducted with Mega 2.1 program (Kumar et al., 2001) using neighbor joining method (Saitou and Nei, 1987) and kimura 2 parameter (Kimura, 1980). Phylogenetic trees were visualised with Mega 2.1 and the Treeview program. Robustness of the tree was accessed with branch supporting-values from bootstrap statistic analyses (1000 replicates). The sequences reported in this study were deposited in the EMBL sequence database under the accession numbers EU643524 to EU643589.

Thirty-two sequences of 388 bp were obtained in the amino terminus region (3′N) and 34 sequences of 343 bp in the carboxy terminus region (5′N). When aligned with prototype sequences of *Lyssavirus* species, all the Tunisian isolates clustered with the Pasteur Virus (PV) strain representative of rabies virus (species 1), with