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Molecular Genetic Analysis of the Rabies Virus Genome Isolated in Azerbaijan

Shalala Zeynalova
Azerbaijan, Ministry of Agriculture, 3-rd Biosafety Level Central Reference Laboratory
shzeynalova@mail.ru

Introduction

Rabies virus is a high pathogenic disease for humans and animals. In July 1885, Louis Pasteur obtained his first success against rabies by vaccinating Joseph Meister, a 9-year-old boy presenting with multiple deep bite wounds. After more than 700 successful inoculations, Pasteur launched an international subscription and opened the world's first research institute dedicated to the prevention of rabies and other infectious diseases (Lontai, 2004; European Commission, 2011; Picard-Meyer *et al.*, 2007; Heaton, *et al.*, 1997). However, the rabies is still neglected for developing countries. Rabies virus is particularly useful for the study of neuronal circuits because of its ability to spread transsynaptically in the retrograde direction. RABV is distributed worldwide among specific mammalian reservoir hosts comprising various carnivore and bat species (William, 2013; Conrad, *et al.*, 2011; Johnson *et al.*, 2016).

The virus belongs to the genus of *Lyssavirus* (from the Greeklyssa, meaning "rage") in the family *Rhabdoviridae.* Twelve of the fourteen lyssaviruses, which differentiate genetically, segregate into two phylogroups based on phylogenetic analyses. Phylogroup I includes the classic (prototype) RABV, Duvenhage virus (DUVV), European bat lyssavirus, type 1 (EBLV-1), and type 2 (EBLV-2), and Australian bat lyssavirus (ABLV), the putative species Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), and Bokeloh bat lyssavirus (BBLV), the newest species (McElhinney, et al., 2011) to be characterized and considered as an independent species within phylogroup. Phylogroup II includes LBV, Mokola virus (MOKV), and Shimoni bat virus (SHIBV), the newest species to be characterized and considered an independent species within phylogroup II. West Caucasian bat virus (WCBV) and Ikoma Lyssavirus (IKOV), isolated from an African civet (Marston *et al.*, 2012), which do not cross-react serologically with other two members of phylogroups, could tentatively belong to a third phylogroup (Picard-

Meyer *et al.* 2004; Dietzgen *et al.*, 2011; Kuzmin *et al.*, 2010). Phylogenetic analyses show that all lyssaviruses are originated from a precursor bat virus.

Azerbaijan is a European country with 10 million of population and the 86,600 km^2 covering area. Azerbaijan's history of rabies began with the occurrence of rabies in dogs –especially stray dogs – followed by a second epidemic phase with the development of rabies in wildlife via the jackals, foxes and wolfs (Zeynalova *et al*., 2015).

National measures on rabies control include the mandatory vaccination of dogs and cats. Since 2001 average of 170 rabies cases per year were detected until 2018, through passive surveillance, which was conducted by the veterinary offices in regions.

The aim of the present study was to determine the antigenic and genetic profiles of rabies virus circulation in the region of the Azerbaijan Republic, using antigenic and genetic characterization of samples from stray dogs, ruminants and wild animals.

Materials and Methods

Sample collection

In 2017, 10 samples were sent to the OIE/WHO/EU National Rabies Laboratory in France. All samples were taken from Republican Veterinary Laboratory (Baku) as investigation material of the study. The 10 rabies field samples were stabilized on the FTA cards for typing. Each FTA sample was impregnated in the RVL in Azerbaijan with a final volume of 25µl of a 10% suspension of brain tissue according to the procedure of the OIE/WHO/EU rabies laboratory.

Table 1. Characteristics of the field virus samples tested

Sample	Species	City	Year of isolation
Azer 1	Cat	Baku (Nizami r.)	2016
Azer 2	Cattle	Salyan	2016
Azer 3	Cattle	Qusar	2016
Azer 4	Dog	Masalli	2016
Azer 5	Cat	Balakan	2016
Azer 6	Donkey	Cebrayil	2016

Azer 7	Cat	Beylaqan	2016
Azer 8	Cat	Qabala	2016
Azer 9	Jackal	Qabala	2016
Azer 10	Jackal	Goranboy	2016

Molecular biology methods used

RNA extraction

Viral RNA was extracted from the half of the impregnated FTA card. Briefly, half of the specific area ,stained with rabies virus, was cut into small pieces and placed into a 1-5 ml Eppendorf tube. A volume of 300 µL of PBS1x was added to the tube containing the paper disk and incubated two hours at room temperature After incubation, the disk was removed and viral RNA was extracted from 200 µL of eluate using Iprep™ PureLinkTMVirus kit (Invitrogen, France) according to the manufacturer's instructions. Extracted RNA was stored at -80 'C until use. The remaining FTA card was stored at -20°C.

Real-time TaqMan qRT-PCR

RT-PCR was performed in a final volume of 25 µLwith 2 µL of viral RNA and 23 µL of master mix (QuantiTect probe RT-PCR Mater mix, Qiagen, France) containing 800 nmol each of pan-Lyssavirus primers N165-N146 and JW12, 100 nmol of the TaqMan RABV probe3 (Lys-Gtl). The universal Pan Lyssavirus primers (JW12 5' ATGTAACACCYCTACAATG and N165-146: GCAGGGTAYTTRTACTCATA) and the TaqMan RABV probe (Lys-Gtl 5ACAAGATTGTATTCAAAGTCAATAATCAG) are described in Table 2. Amplification was performed on Rotor Gene Q real-time cycler (Qiagen, France) to the following heating and cooling program: 50'C for 30 minutes, one cycle of 95'C for 15 minutes followed by 45 cycles of 95*C for 30 sec, 55*C for 30 sec and 72*C for 30 sec A critical threshold cycle number (Ct)of 0.03 was used as reference. The critical threshold cycle number corresponds to the PCR cycle number at which the fluorescence of the reaction exceeded a value determined to be statistically higher than the background by the rotor gene Q's software. Negative and positive controls were included to validate the assays. All PCR reactions were tested in duplicate. The PCR efficiency and coefficient of determination (R^2) values were calculated by the Rotor Gene Q Series software.

Table 2. Description of primers used in the study

Primers	Sequence (5'-3'	Localisation	Characteristics
Primers used in real-time RT-PCR:			
JW12	ATG TAA CAC CYC TAC AAT	55-73 RT-PCR/forward	Amplification of all known Lyssavirus species
N165-146	GCA GGG TAY TTR TAC TCA TA	165-146 PCR/reverse	Specific amplification of
LysGT1	ACAAGATTGTATTCAAAGTCAATAATCAG	81-109 Probe	RABV
Primers used in conventional RT-PCR:			
Amplification of partial N gene:			
JW12	ATG TAA CAC CYC TAC AAT G	55-73RT-PCR/ forward	
JW6	CAR TTC GCA CAC ATT TTR TG	660-641 PCR1/ reverse	
JW10.1	GTC ATC AAA GTG TGR TGC TC	636-617 PCR2/ reverse	
JW10.2	GTC ATC AAT GTG TGR TGT TC		
JW10.3	GTC ATT AGA GTA TGG TGT TC		
Amplification of the complete N gene:			
JW12	ATG TAA CAC CYC TAC AAT G	55-73 RT-PCR/ forward	
PVN8	AGT TTC TTC AGC CAT CTC	1585-1568 PCR1/ reverse	
M13-JW12	GTAAAACGACGGCCAGATGTAACACCYCTACAATG	PCR2/Forward	
M13-PVN8bis	CAGGAAACAGCTATGACCTTGCTCATAYTTGGG	PCR2/ Reverse	
Sequencing primers:			
JW662-684-F	CAC GGT TGT TAC TGC TTA TG	Sequencing primers	
JW938-959-R	CAA AGT GAA TGA GAT TGA ACA C		
JW938-959-F	GTGTTCAATCTCATTCACTTTG		
M13-rev (-29)	CAG GAA ACA GCT ATG ACC		
M13-F	GTAAAACGACGGCCAG		

Amplification of the partial Nucleoprotein gene by conventional RT-PCR

The viral RNA was subjected to the partial Nucleoprotein gene amplification by hnRT-PCR, as described previously[4]. First (one-step RT-PCR) and round polymerase chain reactions (nested PCR) were performed with pan- Lyssavirus primers described by Heaton et al.[5], giving a PCR product of 606-bp.

Amplification of the full nucleoprotein gene

The amplification of the full Nucleoprotein gene was performed by a one-step RT-PCR with specific rabies primers designed to specifically amplify the rabies virus, followed by a second round of PCR with nested primers for typing.

RT-PCR; the entire N gene amplification (1353-bp) was performed as follows; 5µL of extracted RNA was mixed with 0.6µl of OneStep RT-PCR Enzyme Mix (Qiagen, France), 0.6µl of dNTP 10mM and 10 pmol each of specific rabies primers JW12 and PVN8. The size of the amplified product was of 1520-bp. The PCR conditions consisted of one cycle of reverse- transcription for 30 min at 50°C for 30 min, one cycle of denaturing for 15 min at 95°C, followed by a touch down protocol consisting of 16 cycles for 30 s at 94°C, 30 secs at 57°C minus 1°C per cycle and for 2 min at 72°C, then 29 cycles for 30 s at 94°C, 30 s at 55°C, 2 min at 72°C and a final extension for 10 min at 72°C (Bourhy *et al.* 1999; Picard-Meyer et al., 2004; Marston *et al.,* 2006).

Second round of PCR; 2µL of PCR products (1520-bp) was re-amplified in a final volume of 20 µl using 1.25U of Platinum Taq DNA polymerase (Invitrogen, France) and 2.5 pmol each of sequencing primers M13-JW12 and M13- PVN8bis (Table 2). The PCR conditions were initial denaturation for 2 minat94°C, followed by 45 cycles with denaturation for 30 s at 94°C, annealing for 30s at 48°C and extension for 2 min at 72°C, and a final extension for 7 min at 72°C.

Electrophoresis

The amplification products were resolved by electrophoresis on a 2% agarose gel stained with SYBR Safe at a final concentration of 1/10000 then photographed under UV trans-illumination. All amplified PCR products were stored at -20º C.

Sequencing

Following amplification, PCR products were sequenced in both directions by Eurofins Genomics (Ebersberg, Germany) with the same M13 primers used in PCRs (M13 Forward and M13 Reverse (-29) and three internal forward and reverse primers

(JW662-684F, JW938-959R, JW938-959F). The nucleotide primers used in the sequencing of PCR products are summarized in Table 2.

Nucleotide analysis

The sequences were assembled using the Contig Express program of the Vector NTI software, version 11 (Invitrogen, France). Multiple sequence overlapping was achieved using the five sequencing primers detailed in Table 2. Editing of the alignments was performed in MEGA6. BIOEDIT program was used to translate the gene sequence. Percentage identities and similarity scores were determined in BIOEDIT.

Phylogenetic analyses

First analysis undertaken with the entire n/N gene; A phylogenetic tree was determined by comparing the entire N gene sequence (1353-bp) of the seven sequences from Azerbaijan (LN879480), 61 referenced field rabies sequences representing the Indian subcontinent(n=2), Asian(n=4) and Continental sequences representing the following sub- clades; Middle- east, ME1(n=17) and ME2(n=6), Central-Asia, CA1(n=9), CA2(n=7) and CA3(n=2), and Europe WE(n=2), EE(n=2), and NEE(n=2).

The phylogenetic analysis was performed using the PhyML method (GTR calcul,1000 replicatec) with the software SeaView. The graphic PhyML tree of n gene was visualized with Evolviwew (http; \\www.evolgenius.info\evolview). The bootstrap probabilities of each node were calculated using 1000 replicates. Bootstraps values higher than 70%were regarded as evidence for phylogenetic grouping.

Secondary analysis undertaken with the partial N gene including referenced sequences from Azerbaijan; An additional phylogenetic analysis was performed by including in the previous dataset of sequences (n=69), 10 referenced partial N gene sequences (400-bp0, issued by the study of Zeylonava et al., 2015). All sequences came from Azerbaijan and were isolated between 2012 and 2013 on domestic animals; dog(n=4), cow (n=4), horse(n=2). Referenced sequences extracted from GenBank used in the sequence analysis of partial N gene sequences(400-bp) are detailed in Table 3B.

The phylogenetic analysis was performed on a dataset of 79 sequences(400-bp) using the Phy Mlmethod (GTR calcul,1000 replicates) with SeaView. The graphic PhyML tree of N gene was visualized with Evolviw. The bootstrap probabilities of each node

were calculated using 1000 replicates. Bootstraps values higher than 70% were regarded as evidence for phylogenetic grouping [15].

Estimation of the evolutionary rate and an application of a molecular clock

The substitution rate of the N gene (substitutions per site per year) and divergence times for individual clades of sequences were obtained by analyzing the dataset of 69 sequences (-1353bp) using the BEAST program v1.7.4. In additionally information on sampling time(years)was added for each sequence. The MCC tree was performed using the HKY model (SRDO6)- the constant population size model as a coalescent, prior and the strict, relaxed molecular clock for the estimation of nucleotide substitutions and divergence times, respectively. Five independent MCMC analyses were run for 100 million steps and sampled every 10,00 states.

Table 3. Characteristics of rabies virus isolates used in the phylogenetic analysis of the entire N gene (1353-bp)

					Phylogenetic	
Country reference	Virus name	City	Species	Year	Clade	Sub-clade
Azerbaijan	AZER2	Ghakh	Cattle	2016	Cosmopolitan	CA4
Azerbaijan	AZER3	Gusar	Cattle	2016	Cosmopolitan	CA4
Azerbaijan	AZER4	Masali	Dog	2016	Cosmopolitan	CA4
Azerbaijan	AZER5	Balakan	Cat	2016	Cosmopolitan	CA4
Azerbaijan	AZER6	Jabrayil	Donkey	2016	Cosmopolitan	CA4
Azerbaijan	AZER7	Beylaghan	Cat	2016	Cosmopolitan	CA4
Azerbaijan	AZER9	Gabala	Jackal	2016	Cosmopolitan	CA4
Azerbaijan	5989/lib2		Dog	2002	Cosmopolitan	CA4

The log and tree files of each MCMC chains were combined using the software Log combiner v1.8.2, with a burn-in of 10% The convergence of each parameter in this combined file was checked using TRACER v1.6 and indicated by an effective sample size200. The MCC tree was obtained using Tree Annotator v1.8.2 and visualized using FigTree v1.4.2. Posterior probability values show the degree of support for each node on the tree.

Results

Real Time TAGMAN QRT-PCR and conventional HNRT PCR

7 out of 10 samples fixed on FTA were shown by both conventional and Real time RT-PCR. The species found for the 7 positive RT-PCR samples was classical rabies virus.

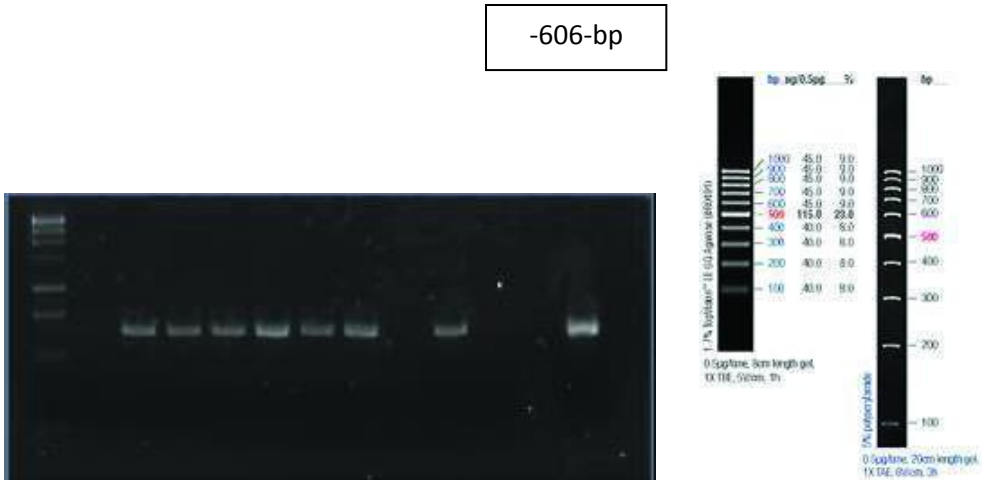

Figure 1. Rabies RNA detection by conventional PCR in a 7 stabilized FTA samples

Amplification of the complete N gene (= 1353-BP) by RT-PCR

Table 2. Results of conventional RT-PCR for the amplification of the entire N gene in the 10 tested FTA samples

Tested samples	**Amplification of the entire N gene1**	
Samples	**Species**	**Detection**
AZER 1	Cat	Neg
AZER2	Cattle	Pos
AZER 3	Cattle	Pos
AZER 4	Dog	Pos
AZER 5	Cat	Pos
AZER 6	Donkey	Pos
AZER 7	cat	Pos
AZER 8	cat	Neg
AZER 9	Jackal	Pos
AZER 10	Jackal	Neg
Neg: negative; Pos; Positive		

Of 10 samples tested, 7 were shown positive for the amplification of the entire N gene. Amplification was performed by one-step RT-PCR with rabies primers JW12 and PVN8 followed by a second round of PCR with M13-JW12 and M13-PVN8bis (Table 2).

Conventional RT-PCR allows the specific amplification of RABV with the amplification of a fragment of N gene (1520-bp).

Phylogenetic Analysis of the Seven Stabilized FTA Samples with Referenced Rabies Sequences

Two phylogenetic analyses of two datasets of sequences (400-bp and 1353-bp) using PhyML produced trees with an identical topology. The PhyML trees represented in Figures 3A and B showed, with a high significant bootstrap, probability that the seven tested FTA samples were characterized within the cosmopolitan clade.

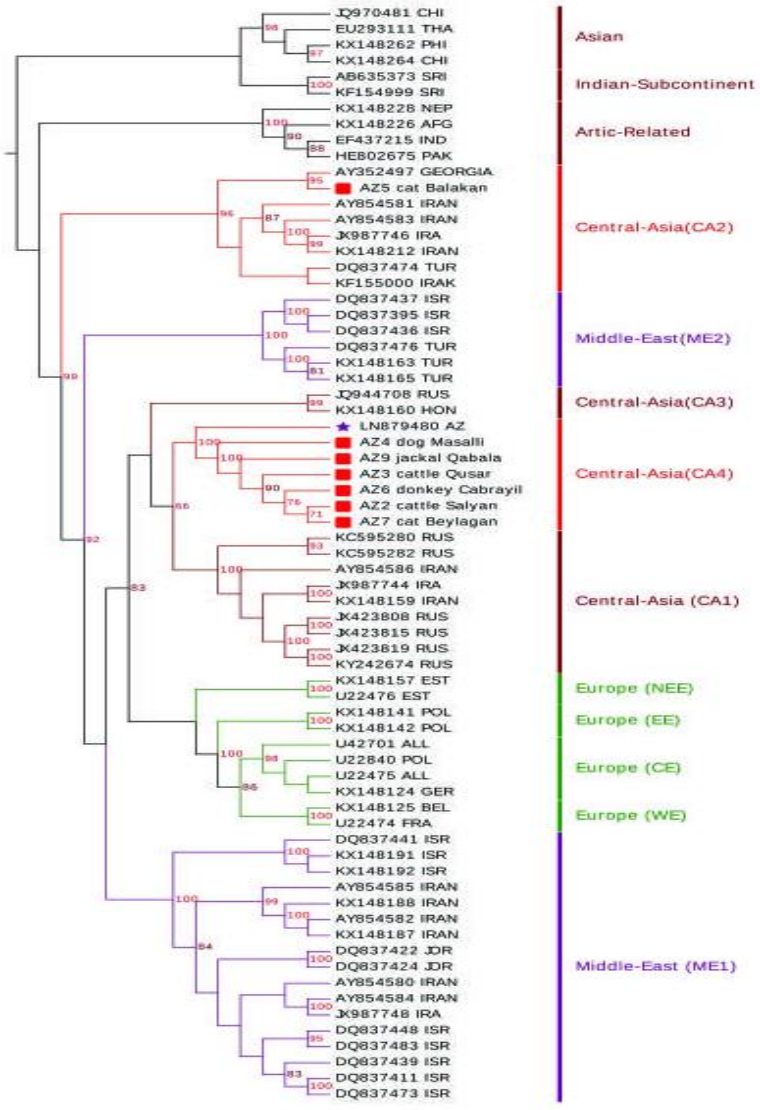

Figure 3. The phylogenetic analysis between the entire N gene of the seven Azeri sequences and 62 referenced sequences.

The seven samples tested in the study fell into two distinct phylogenetic subclades circulating in Central Asia (CA), denoted CA4 and CA2, of which CA2 has been previously described (Troupin et al.). The central Asia clade has been shown to be divided in three sub-clades by Troupin et al. Three subclades encompass viruses originating in China, Iran and Russia for CA1, from Iraq, Iran and Russia for CA2 and a cluster of viruses (named CA3); although, its precise geographical distribution at the border is uncertain.

Phylogenetic Analysis Between the Entire N Gene of the Seven Sequences from Azerbaijan and 62 Representative Sequences

The analysis showed that the majority of tested samples from this study (with 6 of 7 sequences) belong to the group of rabies virus formed with/in the Central Asia subclade CA4 constituted with one representative canine rabies virus from Azerbaijan (LN879480), while the seventh isolate (cat numbered AZER5) from the city Balkan belong to the group of sequences formed with the Central Asia subclade CA2 constituted with isolates from Iraq, Turkey, Iran and Georgia.

The subclade CA4 is formed with the six sequences of this study and one Azeri sequence extracted from Genbank (LN879480). This referenced isolate was isolated from a dog in 2002 in Azerbaijan.

Discussion

Out of ten FTA stabilized samples from Azerbaijan, seven samples were shown positive by real-time RT-PCR and by conventional RT-PCR for the amplification of the partial and full N gene.

The phylogenetic analysis based on the comparison of the full N gene sequences between the seven Azeri sequences and 62 published Lyssavirus sequences showed that seven samples belong to the Cosmopolitan clade. The study showed that out of seven samples analyzed, six samples belong to the subclades CA4 and one goes to CA2 of Central Asia, respectively.

The phylogenetic analysis is based on the comparison of the partial N gene sequences between seven Azeri sequences of the study. 11 referenced to Azeri sequences and 61 referenced Lyssavirus sequences which showed that viruses from dogs predominate in the lineage CA4. 6 dogs, 1 jackal and 6 domestic animals were constituted the lineage CA4. By contrast, the subclade CA2 that is constituted by 9 strains isolated from Iran, Turkey, Iraq and Azerbaijan with two Azeri strains-KJ645928 and the sequence AZER5 isolated from a cat in Balakan is formed with 2

jackals and 6 domestic animals (4 cow, 1 cat and 1 sheep). The phylogenetic study among seven isolated from Azerbaijan and referenced Azeri sequences showed the existence of at least two lineages occurring in the country, with a predominance of dogs in the subclade CA4 (Zeynalova *et al.*, 2015).

Evolutionary analysis of selected rabies isolated from Azerbaijan suggests that there are multiple strains occurring in the country concurrently. In general, the studies of rabies in Eurasia similarly demonstrated more than one strain occurring in Georgia (Kuzmin *et al*., 2004; Kuzmin *et al*., 2006). Dogs and other wild canids and their infection with different rabies virus variants were included in the present study. It is impossible to conclude on a specific vector species occurring in the country and/ or to a possible translocation of an infected animal from bordering countries, for explaining the presence of the subclade ME or CA2 identified in Azerbaijan. The analysis of further strains including wild and domestic canids- including past and recent isolates- would be needed to draw conclusions regarding the interaction between wildlife and dog rabies cycles in the country.

Abbreviations:

Bp - bases pairs
CA- Central – Asia
CE- Central Europe
EE- Eastern Europe,
FTA- paper-based system designed to fix and store nucleic acids directly from fresh tissues pressed into the pre-treated paper, allowing the collection and the archiving of nucleic acids
MCC-Maximum clade credibility
ME- Middle- East
N- Nucleoprotein
NEE- North- East Europe
NJ-Neighbor – Joining
RT-PCR- Reverse transcription polymerase chain reaction
TMRCA- Time to the Most Recent Common Ancestor
WE-Western Europe

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