Molecular characteristics isolates of rabies virus isolated from humans in Ukraine

I M Polupan¹, V V Nedosekov², T V Stepanova³, O V Rudoi¹, A V Parshikova³ and E I Drozdova³

¹ Research Virology Department, State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise, Donetska str. 30, Kyiv, Ukraine
² National University of Life and Environmental Sciences of Ukraine, Heroyiv Oborony st., Kyiv, 03041, Ukraine
³ Federal State Budget Scientific Institution “Federal Scientific Centre VIEV”, Ryazanskiy prospekt, 24, 1, Moscow, 109428, Russia

E-mail: i.n.polupan@gmail.com

Abstract. The article presents the results of molecular-genetic investigation of isolates of rabies virus was isolated from 2 rabies patients in Ukraine. It has been confirmed that the virus that caused the deaths belongs to animal lyssavirus, namely the rabies virus. It has been proved that street isolates from humans by their genetic characteristics belong to the first genotyp (RABV), the first phylogroup of animal lyssavirus. High homology between experimental samples and materials from cats, dogs, and foxes from the territory of Ukraine and with isolates in fox populations in South-Eastern Europe was revealed.

RT-PCR confirmed that 2 people died of rabies, the isolates belong to the first phylogroup of lissavirus, the first genotype (RABV). It is proved that 2 street isolates from people from Ukraine are similar in degree of homology to the isolates isolated in fox populations in South-Eastern Europe.

1. Introduction

Rabies is a neglected viral disease that damage to the central nervous system (polyencephalomyelitis) and is caused by a neurotropic virus, that belongs to the family Rhabdoviridae, genus Lyssavirus [1, 2, 3, 4, 5]. Yearly in the world, about 59,000 infected people die from rabies (main deaths occurred in Asia - 59.6% and Africa - 36.4%) and more than 20 million persons receive post-exposure rabies treatment (immunoglobulin + vaccines) after bites [6, 7, 8, 9, 10].

In Ukraine (UA), rabies is constantly registered among animals (domestic and wild). More than 2,000 rabies cases per year were recorded in 2003, 2005, 2006, 2007, and 2008. In addition, 37 % of all rabies cases were reported in foxes. Epidemiological investigation shown, that cause rabies in the all species animals was plural factors. The most significant factors appear to be the foxes ecology, level densities (more than 5–6 foxes/100 km²), the presence and density of stray dogs, the level of foxes contact with domestic dogs and cats and their frequency and level of human contact [11, 12, 13]. Another reason for the rabies epizootic (epidemic) is low levels of specific rabies prevention in domestic animals. This contributed to a high rabies incidence in dogs (more than 19 % of total rabies cases) and in cats (more than 25 % of total cases of rabies) in Ukraine [14].
According to the SSUFSCP (State Service of Ukraine on Food Safety and Consumer Protection), each year, 90,000-110,000 cases of animal bites are registered in Ukraine, and 15-20% from this cases, receive post-exposure rabies treatment. Notwithstanding, almost every year, despite significant level of post-exposure rabies treatment in Ukraine, people die from this zoonosis. In the last 20 years (1999-2018) were registered 58 human deaths with a diagnosis of rabies (hydrophobia) [15].

Perhaps, that one of the cause of infected and human death was the gap or low level of post-exposure rabies treatment.

On the other hand, the increased incidence of rabies in humans may be due to synanthropization of animals [16]. An Epidemiological investigation of the rabies outbreak and epidemic situation in total in Ukraine has shown that as the number of cases of rabies in domestic animals increases, so does the incidence of rabies in humans. Possible causes for such situation are insufficient level of specific prophylaxis of rabies in animals [17, 18, 19].

Furthermore, in distribution of rabies significant role play the circulation of different genotypes of rabies virus that need to understanding of molecular – genetic biology of rabies virus [20, 21, 22].

To rabies control in Ukraine, also is important to understand that different species of animals may be reservoirs and/or vectors of plural genotypes of rabies virus (include classified and unclassified), and this way, why molecular-genetic investigation should be a part of the accurate and reliable laboratory diagnosis system of rabies [5]. For this needed isolation of RNA of rabies virus, phylogenetic analysis, and comparison with known virus genotypes that are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank).

However, there is currently scantily information on the molecular epidemiology of rabies virus in Ukraine and especially about human rabies virus isolates.

For molecular investigation, the N gene of rabies virus is the optimal target due to its high conservatism and can be used to indicate genetic differences between rabies isolates [23].

These research presents on the molecular characterization of the street rabies virus isolates from humans in Ukraine namely, the comparison investigation of rabies virus isolates sequensis from Ukraine and the sequensis of different molecular-genetic version of the rabies virus from GenBank.

2. Methods

Pathological material. For investigation was used 2 brain human samples from Lviv and Kyiv regions (UA), who became ill with rabies (hydrophobia) in 2009 and 2011, respectively.

Fluorescent antibody test (FAT). The brain of laboratory mouse infected with rabies virus reference strain (Rabies virus ATCC VR-959) was used as a positive control, and the brain of an uninfected mouse was used as a negative control. FITC Anti-Rabies Monoclonal Globulin (Fujirebio) was used to stain the smears [16]. Luminescent microscopy was performed using a microscope ZEISS Axio Lab.A1.

Mouse inoculation test (MIT). Isolation of the virus (in vivo) was performed on white mice (6-8 g) by the method of Koprowski H. (1996) [24]. The specificity of the bioassay was confirmed by FAT.

Reverse transcription chain polymerase reaction (RT-PCR). Primers complementary to the N-gene of rabies virus JW6DPL (CAATTCGCACATATTGTG) position 660-641 and JW12 (ATGTAACACC(C/T) CTACAATTG) position 55-73 were used for RT-PCR.

RNA extraction was performed with the QIAampViral RNA MiniKit. Pre-prepared samples, with the addition of the reaction mix, MixSuperscript III / PlatiniumTaq enzymes, 2 oligonucleotide primers JW6DPL and JW12 and water for PCR were used to perform RT-PCR. The reaction consisted of 35 cycles. Each cycle of 6 stages, 1 and 2 denaturation, 3-4 annealing of primers, 5-6 polymerization (completion of complementary primers). Amplification of samples was performed in a PCR amplifier SystemProFlex. Next, the obtained samples were placed in a 1.5% agarose gel, which was prepared on TAE buffer with the addition of 0.004% ethidium bromide, through which a current of 110 V was passed for 35 minutes.

The amplification products were purified using a kit QIAquick PCR Purification Kit, QIAGEN.
Purified amplicons were sequenced in both directions using a pair of oligonucleotide primers used in RT-PCR, automated sequencer HELICON "Applied Biosystems ABIPRISM" and a BigDye Sequencing Kit (Applied Biosystems) with GeneScan analysis software.

**Phylogenetic analysis.** The nucleotide sequences of the strands after sequencing were canceled using the Reverse Complement program.

The nucleotide sequences were aligned using Clustal W multiple alignment and visualized using BioEdit v. 7.0.5.3.

Test samples were compared with selected from GenBank sequences of virus isolates of rabies: from humans to compare them with each other; from foxes, as they are the main vectors of the rabies virus in Europe; from domestic dogs and cats, because this carnivores are the main cause and source of rabies virus for humans; samples from bats and material from Ukraine (isolated in the Kharkiv region in 2010), because these animals confirmed the circulation of different genotypes of the virus in Europe, as well as belonging to different phylogroups and/or genotypes; and vaccine strains of rabies virus (table 1, 2).

**Table 1** Isolates of rabies virus that are included in the phylogenetic investigation.

| Number in GenBank | Genotype | Approved Species (ICTV) | Isolates | Year | Species |
|-------------------|----------|-------------------------|----------|------|---------|
| EU293121          | 1        | Rabies virus            | 8743THA  | 1983 | human   |
| EU293111          | 1        | Rabies virus            | 8764THA  | 1983 | human   |
| EU293115          | 1        | Rabies virus            | 9147FRA  | 1991 | fox     |
| EU293116          | 1        | Rabies virus            | 9704ARG  | 1997 | dog     |
| AY705373          | 1        | Rabies virus            | SHBRV-18 | 1983 | bat     |
| EF437215          | 1        | Rabies virus            | NNV-RAB-H | 2006 | human   |
| AY956319          | 1        | Rabies virus            | RABV     | 2004 | human   |
| M31046            | 1        | Rabies virus            | SADB19   | vaccine strain |
| NC_001542         | 1        | Rabies virus            | PV       | vaccine strain |
| EU293110          | 2        | Lagos bat virus         | 8619NGA  | 1956 | bat     |
| EU293108          | 2        | Lagos bat virus         | 0405SEN  | 1985 | bat     |
| NC_006429         | 3        | Mokola virus            | MOKV     | 1981 | cat     |
| EU293118          | 3        | Mokola virus            | 86101RCA | 1981 | shrewmouse |
| EU293120          | 4        | Duvenhage virus         | 94286SA  | 1981 | bat     |
| EU293119          | 4        | Duvenhage virus         | 86132SA  | 1971 | human   |
| EU293112          | 5        | Europen bat lyssavirus1 | 8918FRA  | 1989 | bat     |
| EU293109          | 5        | Europen bat lyssavirus1 | 03002FRA | 2003 | bat     |
| EF157976          | 5        | Europen bat lyssavirus1 | RV9      | 1968 | bat     |
| EU293114          | 6        | Europen bat lyssavirus2 | 9018HOL  | 1986 | bat     |
| EF157977          | 6        | Europen bat lyssavirus2 | RV1333   | 2002 | human   |
| AF418014          | 7        | Australian bat lyssavirus | ABLh    | 1986 | human   |
| NC_003243         | 7        | Australian bat lyssavirus | ABLb    | 1996 | bat     |

**Table 2** Isolates of rabies virus from Ukraine that are included in the phylogenetic investigation.

| Number in GenBank | Isolates   | Year | Species |
|-------------------|------------|------|---------|
| JN656511          | Rvu-10-33  | 2010 | Dog     |
| JN656510          | Rvu-09-06  | 2009 | Cat     |
Analysis of sequencing results was carried out using the software package MEGA 6.06. The phylogenetic tree of nucleotide sequences of test rabies samples, vaccine strains and street isolates of virus was constructed due to method of NJ (nearest neighbors), Kimura's model with 1000 bootstrap replications.

3. Results and discussion

A case of human rabies (hydrophobia) was registered in Ukraine in 2009. A 32-year-old resident of Lviv Oblast went to the doctor with complaints of general weakness, numbness in his right arm, and a fever of 37.5°C over the past few days. These symptoms are a typical sign for the first period of classical rabies, a period of predictors, which is typical of increased reflex excitability and mental disorders.

In serious condition on the 8th day of the disease he was hospitalized, and the next day was transferred to the intensive care unit. The patient's period of paralysis lasted three days, the condition was extremely severe: did not respond to treatment, body temperature up to 37.4 °C. Resuscitation measures did not yield results and the patient died of cardiac arrest.

Epidemiological data: a man was bitten on the thumb of his right hand by a fox, but it was not possible to establish how much time passed (according to him, it happened 3 years ago). The man did not seek for rabies treatment. The diagnosis was confirmed by FAT. At the MIT, all experimental mice died within 23-28 days. The disease in mice proceeded with an erased clinical picture for 7-9 days, which is 4 times longer than usual when infected with the street rabies virus. In our opinion, the possible cause of the unusually long illness could be the properties of the rabies virus isolate, which infected the deceased.

Another case occurred in Kyiv in 2011. A man (was born in 1984) died with a pathognomic clinical manifestation of hydrophobia. When collecting the anamnesis, the victim's contact with the dog was established about 2-3 months before death. The diagnosis was also confirmed by FAT. The virus was isolated in a bioassay (MIT) by intracerebral administration to white mice 20% brain suspension. The disease in mice proceeded with the classic "paralytic" form for 24-48 hours, the incubation period was 7-11 days.

After confirmation of the diagnosis by traditional methods, the samples were examined in RT-PCR. RT-PCR also confirmed the presence of rabies virus genetic material in both samples of pathological material. Then was carry out sequencing of the isolated RNA samples of rabies virus.

The next step was to compare the obtained sequences with known and classified genotypes and samples from GenBank. For phylogenetic analysis of experimental samples, a dendrogram was constructed using the program MEGA 6.06 (figure 1).

| JN656509 | Rvu-10-29 | 2010 | Dog |
| JN656508 | Rvu-10-01 | 2010 | Cat |
| JN656507 | Rvu-10-08 | 2010 | Marten |
| JN656506 | Rvu-10-14 | 2010 | Dog |
| JN656505 | Rvu-10-09 | 2010 | Fox |
| JN656504 | Rvu-08-22 | 2008 | Cat |
| JN656503 | Rvu-10-04 | 2010 | Dog |
| JN656502 | Rvu-02-16 | 2002 | Fox |
| JN656494 | Rvu-02-13 | 2002 | Fox |
| JN656501 | Rvu-10-20 | 2010 | Ferret |
| JN656499 | Rvu-10-18 | 2010 | Cat |
| JN656498 | Rvu-10-31 | 2010 | Fox |
| U89465 | 9443UKR | 1985 | Human |
| «YULI» |
| - | BAT 22.02.10 | 2010 | Bat |
Figure 1. Phylogenetic trees comparing the two Ukrainian isolates with the isolates of rabies virus based on N gene nucleotide sequences. The phylogenetic analysis was carried out by the NJ method. Bootstrap values were obtained for 1000 replicates. HUMANUKRKIEV 01.10.11, HUMANUKRKLIV 07.10.09, BAT 22.07.10 - isolates from Ukraine.

It is established that the samples HUMANUKRKIEV 01.10.11 and HUMANUKRKLIV 07.10.09 belong to the first phylogroup. In terms of their genetic characteristics, the samples are close to the field isolate isolated from the fox in France in 1991 (9147FRA, EU293115 in GenBank) and is a representative of the 1st genotype of lissaviruses (RABV).

Analysis of part of the N gene sequence showed high genetic affinity (96–99%) in terms of amino acid composition of street isolates isolated from humans from the territory of Ukraine and samples presented in GenBank.

Also, the genetic sequences of the studied samples are characteristic of isolates isolated in South-Eastern Europe, Western Siberia, Kazakhstan and the Russian Federation.

The research revealed a high homology level between prototypes and materials from cats, dogs and foxes from Ukraine (figure 2). However, our results shown high affinity between different isolates of rabies virus from Ukraine and indicates that the main reservoir of rabies virus in Ukraine is the red fox (Vulpes vulpes).
Figure 2. Phylogenetic trees comparing the two Ukrainian isolates with the others Ukrainian isolates of rabies virus based on N gene nucleotide sequences. The phylogenetic analysis was conducted by the NJ method. Bootstrap values were obtained for 1000 replicates. HUMANUKRKIEV 01.10.11, HUMANUKRLVIV 07.10.09, BAT 22.07.10 - isolates from Ukraine.

In addition to the genetic sequences of rabies virus isolates from Ukraine, sequences of 4 vaccine strains were used in the construction of the phylogenetic tree. Considering the high genetic relatedness between isolates and vaccine strains, it can be assumed that in the case of timely and efficacy post-exposure treatment, the consequences could be avoided.

Thus, after epidemiological and molecular research on rabies virus isolates isolated from humans and analysis of results, it can be concluded that the virus that caused human death belongs to lissaviruses (RABV). And the fact that a man from Lviv region showed clinical symptoms of the disease after a long period of time (according to the epidemiological history) can be explained by the fact that the amount of virus that got into the wound during a fox bite was small. In addition, a significant factor is the peculiarity of the development and functioning of the patient's neuromuscular system, and the number of nerve endings at the site of the bite. However, the detected long incubation period and the uncharacteristic clinical picture of rabies in experimental mice during the bioassay may be evidence of genetic changes in this isolate of rabies virus, which requires further research.

4. Conclusion

Thus, RT-PCR confirmed that two men died of rabies, the isolates belong to the first phylogroup of lissavirus, the first genotype (RABV). It is proved that two street isolates from people from Ukraine are similar in degree of homology to the isolates isolated in fox populations in South-Eastern Europe.

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