Journal of Clinical Virology 78 (2016) 74–81

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Retrospective diagnosis of two rabies cases in humans by high throughput sequencing

V.G. Dedkov[a,b,*], A.N. Lukashev[c,d], A.A. Deviatkin[a,b,c], K.V. Kuleshov[a], M.V. Safonova[a,c], E.M. Poleshchuk[e], J.F. Drexler[f,g], G.A. Shipulin[a]

[a] Federal Budget Institute of Science Central Research Institute for Epidemiology, Russian Inspectorate for Protection of Consumer Right and Human Welfare, Moscow, Russia
[b] RAS Institute of Occupational Health, Moscow, Russia
[c] Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia
[d] Martsinovsky Institute of Medical Parasitology and Tropical Medicine, Sechenov First Moscow State Medical University, Moscow, Russia
[e] Omsk Research Institute of Natural Foci Infections, Omsk, Russia
[f] Institute of Virology, University of Bonn Medical Centre, Bonn, Germany
[g] German Centre for Infection Research, partner site Bonn-Cologne, Germany

ARTICLE INFO

Article history:
Received 14 November 2015
Received in revised form 27 February 2016
Accepted 12 March 2016

Keywords:
Broad-range PCR
High throughput sequencing
Rabies virus
Russia

ABSTRACT

Background: Rabies is prevalent in 150 countries and is definitely found in Russian Federation. The average registered incidence of rabies infection among animals in Russia is 3000 cases per year. At least 500,000 cases of animal bites and scratches are registered in the Russia every year, but only 2–4 cases of rabies infection in humans are reported per year. This relatively low incidence of rabies infection among humans is the result of a well-organized program of rabies surveillance and control as well as the readily available vaccination and immunoglobulin therapies. However, physician awareness of rabies infection in patients with acute encephalopathy is low, and some cases of rabies remain undiagnosed.
Objectives: A retrospective study of autopsy materials from patients who died of encephalitis of unknown etiology in the Astrakhan region of Russia in 2003.
Study design: A broad-range polymerase chain reaction (PCR) analysis followed by high throughput sequencing were used for the diagnosis.
Results: Two cases of rabies were detected and subsequently confirmed using a fluorescent antibody test, an enzyme-linked immunosorbent assay (ELISA) and a mouse inoculation test. Two strains of rabies virus were isolated and characterized using virological methods. The entire genome of each strain was sequenced.

1. Background

Rabies virus (RABV) is a neurotropic virus of the order Mononegavirales, family Rhabdoviridae and genus *Lyssavirus*. In addition to RABV, the *Lyssavirus* genus includes 11 virus species: Australian bat lyssavirus (ABLV), Duvenhage virus (DUVV), European bat lyssaviruses 1 and 2 (EBLV1 and 2), Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), Lagos bat virus (LBV), Mokola virus (MOKV), West Caucasian bat virus (WCBV) and Shimoni bat lyssavirus (SHIBV). All lyssaviruses cause acute progressive encephalitis (rabies) in mammals, and a fatal outcome is typically observed. Transmission of lyssaviruses from rabid animals occurs directly by bites, scratches or contamination of mucous membranes with infected saliva [1].

Members of the genus *Lyssavirus* contribute differentially to the incidence of rabies. RABV is widely distributed around the world and is the causative agent of the majority of rabies cases. Other lyssaviruses have confined areas of prevalence and a limited host range, primarily consisting of bats. Nevertheless, these viruses can also cause rabies infection in humans [2].

Rabies is prevalent in 150 countries, including Russia [3]. The average registered incidence of rabies infection among animals in Russia is 3000 cases per year. Of these, 36% are foxes, 21% are dogs, 20% are cattle and 13% are cats [4]. At least 500,000 cases of animal bites and scratches are registered in the Russian Federation every year [5]. However, only 2–4 cases of rabies infection among

* Corresponding author at: 111123, Central Research Institute for Epidemiology, Russian Inspectorate for Protection of Consumer Right and Human Welfare, 3a Novogireevskaya, Moscow, Russia.
E-mail address: vgdedkov@yandex.ru (V.G. Dedkov).

http://dx.doi.org/10.1016/j.jcv.2016.03.012
1386-6532/

humans are reported per year [6]. This relatively low incidence of rabies infection among humans is the result of well-organized surveillance and control for RABV, as well as readily available vaccination and immunoglobulin therapies. However, due to low disease incidence, physician awareness of rabies infection in patients with acute encephalopathy is reduced, and some cases of rabies remain undiagnosed.

2. Objectives

To identify the causative agent, a retrospective study of autopsy materials from patients who died of encephalitis of unknown etiology was conducted using a broad-range polymerase chain reaction (PCR) analysis followed by high throughput sequencing.

3. Study design

3.1. Sample preparation and RNA extraction

During a retrospective study of autopsy material collected from patients who died of encephalitis of unknown etiology, two samples of brain tissue from a boy (seven years of age) and a man (fifty years of age) together with other seven samples were examined to clarify the cause of death. Both samples were collected in the Astrakhan region of the Russian Federation in 2003 and stored in the collection of the Central Research Institute for Epidemiology (Moscow) at −70 °C.

Total RNA was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) according to the recommendations of the manufacturer. The RNA was eluted with 100 μl of RNase free water (Qiagen, Germany) and stored at −70 °C.

3.2. Broad-range reverse transcriptase-polymerase chain reaction (RT-PCR)

Broad-range RT-PCR was performed using a set of broadly reactive degenerate oligonucleotides designed to target each viral species that could be a causative agent of encephalitis within the genera and families as follows: *Cardiovirus*, *Enterovirus*, *Parechovirus*, *Flavivirus*, *Pestivirus*, *Norovirus*, *Astrovirus*, *Hepevirus*, *Alphavirus*, *Filoviridae*, *Arenaviridae*, *Paramyxovirus*, *Nairovirus*, *Lyssavirus*, *Phlebovirus*, *Orthobunyavirus*, *Orthoreovirus*, *Adenoviridae*, and *Herpesviridae* (Table 1).

Reverse transcription and touch-down amplification were performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, USA) in a reaction containing 2 μl of RNA, 0.4 μM sense primer, 0.4 μM antisense primer, 12.5 μl of 2x reaction buffer, 1 μl of enzyme mix, 0.2 μl of 50 mM Mg_2SO_4, 1 μl of bovine serum albumin (BSA; 1 ng/μl), and H_2O, to a total volume of 25 μl.

The following thermal cycling parameters were used: 45 °C for 20 min and 94 °C for 3 min, followed by 10 cycles of 94 °C for 20 s, 55 °C to 45 °C (−1 °C per cycle) for 20 s, and 72 °C for 30 s; and then 40 cycles of 94 °C for 20 s, 45 °C for 20 s, and 72 °C for 30 s; and finally 72 °C for 3 min. All amplification steps were performed using Mastercycler Nexus thermocycler (Eppendorf, Germany). The products of the amplification reactions were analyzed by 1.2% agarose gel electrophoresis.

3.3. Library preparation and high throughput sequencing of PCR products

The products of different PCR assays for each sample were pooled in one tube, purified using QIAamp PCR-purification kit (Qiagen, Germany), eluted in 50 μl of elution buffer and prepared for high throughput sequencing using TruSeq DNA Sample Preparation Kit v.2.0 (Illumina, Inc., USA) in accordance with the manufacturer's recommendations. Separate libraries were prepared for each sample. Sequencing was performed using Illumina MiSeq system to generate 150-bp paired-end reads.

The obtained reads were subjected to analysis via a custom bioinformatic pipeline developed in our lab. At the first stage, the reads were filtered using Trimmomatic v0.35 software to exclude low quality reads [20]. Then, the sequences of degenerate broad-range PCR primers, as well as simple repeats, were masked through a combination of custom Python scripts and software packages. Filtered reads with an unmasked region of greater than 30 bp were collected, clipped and used for further analysis. To assign taxonomic labels to the obtained reads, we compared assembled contigs or individual reads to the NCBI non-redundant nucleotide and protein sequence databases using the blastn, blastx or tblastn algorithms.

3.4. Post mortem verification of diagnosis

To confirm rabies infection, a fluorescent antibody test (FAT), enzyme-linked immunosorbent assay (ELISA), and a mouse inoculation test (MIT) were performed.

FAT was performed using Centocor FITC-Anti-Rabies Monoclonal Globulin (Fujirebio Diagnostics, Inc., Malvern, PA, USA). ELISA was performed using a RABV ELISA test kit manufactured by the Research Institute for Veterinary Medicine, Kazan, Russia. All procedures were performed in accordance with the manufacturers' recommendations. The MIT protocol is described below.

3.5. Whole genome sanger sequencing of RABV strains

The complete genomes of two RABV strains (excluding the 5′ and 3′ ends) were obtained using the 20 primer pairs described in Ref. [21] (Table 2). Reverse transcription was performed using random hexanucleotide primers and Reverta-L kit (AmpliSens, Russia) according to the manufacturer's instructions. cDNA was stored at −70 °C and used as a template for amplification.

Hot-start PCR amplification was performed in a 25-μl total volume containing 2 μl of cDNA, 0.4 μM sense primer, 0.4 μM antisense primer, 2.5 μl of dNTPs (1.76 mM, AmpliSens, Russia), and 10 μl of PCR buffer blue-2 with 7.5 mM $MgCl_2$ (AmpliSens, Russia). The following thermocycling parameters were employed: 94 °C for 1 min; 40 cycles of 94 °C for 10 s, 55–65 °C (depending on the primer pair) for 15 s, and 72 °C for 60 s; and 72 °C for 5 min. The reactions were performed in MaxyGene gradient thermocycler (Axygen, USA). The products were analyzed by 1.2% agarose gel electrophoresis.

All PCR products were purified and sequenced using an ABI Prism 3500 XL (Applied Biosystems, USA).

3.6. In silico analysis

Sanger reads were assembled into complete genomes using CLC Genomics Workbench v5.5.1 software.

To understand phylogenetic relationship of two studied strains among the RABV representatives available in the NCBI GenBank, a phylogenetic analysis of N-gene nucleotide sequences was performed. First, Clustal W 2.0 software was used for multiple sequence alignment [22]. A maximum-likelihood tree (ML) was then reconstructed with MEGA 6.0 software [23] using the Tamura substitution model [24]. The significance of the monophyly of the each clade was evaluated by 1000 bootstrap replicates.