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Development and evaluation of a RT-qPCR assay for fast and sensitive rabies diagnosis

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ABSTRACT

Rabies virus is endemic to Russia, among other countries. It is therefore critical to develop a high-quality and high-precision diagnostic procedure for the control and prevention of infection.
The main objective of the research presented here was to develop a reliable RT-qPCR assay for rabies diagnostics. For this purpose, a RABV strains from various biological and geographical origins were used. In addition, rabies-positive and rabies-negative samples, as well as nucleic acids from other viruses and DNA extracted from the brain tissues of mice, dogs, cats, bats and humans, were studied using the developed assay.
The analytical sensitivity of the assay, as assessed using armored recombinant positive control dilutions, was 10^3 copies/ml, and the sensitivity measured using characterized strains was between 0.1 LD50/ml and 1.0 LD50/ml. A broad range of RNA from RABV strains circulating in different regions of Russia, as well as RNA from RABV-positive primary brain samples from 81 animals and two humans, was detected using the developed assay. No false-positive or false-negative results were obtained.
Given that high analytical and diagnostic sensitivities and a high specificity were verified for this assay, it has high potential as a screening test that may be suitable for the epizootiological monitoring of animals and for the fast postmortem diagnosis of rabies.

1. Introduction

Classical rabies virus (RABV) is a neurotropic negative-sense single-stranded RNA virus that belongs to the genus Lyssavirus within family *Rhabdoviridae*. RABV causes one of the deadliest zoonotic diseases, namely, rabies, and is estimated to be responsible for up to 70,000 human deaths worldwide each year (WHO Expert Consultation on Rabies, 2013). Apart from RABV, the genus Lyssavirus includes 15 species, some of which are circulating in the territory of the former Soviet Union, including European bat lyssaviruses 1, Khujand lyssavirus, Aravan lyssavirus, Irkut lyssavirus, and West Caucasian bat lyssavirus (https://talk.ictvonline.org/ictv-reports/ictv_online_report/, 2016). All of these viruses are neurotropic infectious agents that cause irreversible brain lesions in humans and other warm-blooded animals, resulting in rabies-like diseases (Delmas et al., 2008). However, the greatest epidemiological and epizootiological significance is attributed to RABV because it is widespread and causes the vast majority of the known disease cases related to Lyssavirus species in both humans and animals. In contrast, other members of the genus Lyssavirus are known to have restricted areas of distribution and a limited number of host species, with bats being the main host (WHO Expert Consultation on Rabies, 2013).

All currently known RABV can be divided into seven major genetic groups. Two of these groups, the Cosmopolitan and the Arctic/Arctic-like groups, circulate in the Russian Federation. Within these two major groups, members of six subgroups have been reported in Russia: A. Arctic rabies (northern parts of Siberia), B. Arctic-like rabies (Khabarovsk Krai, Transbaikal region), C. steppe rabies (Eurasian Steppe), D. Central European Russian rabies, E. Northeast European Rabies, and F. Caucasian rabies (Deviatkin et al., 2017).

Rabies is a zoonotic disease transmitted by direct contact with infected domestic and wild animals through bites or scratches, usually via saliva (http://www.who.int/mediacentre/factsheets/fs099/en/). Globally in up to 99% of human cases, RABV is transmitted by domestic

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dogs. Despite significant advances in research within the fields of epidemiological surveillance and prophylaxis (Dietzschold et al., 2003; Simonova and Khadarcev, 2014), cases of infection are still being registered on all continents except for Antarctica (http://www.who.int/mediacentre/factsheets/fs099/en/).

In Russia alone, 39,895 disease cases were registered within the period of 2000–2016 (39,783 in animals and 114 in humans) (http://www.who-rabies-bulletin.org/Queries/Surveillance.aspx). Approximately 400,000 people come into contact with potentially rabid animals and receive post-exposure prophylaxis each year (Deviatkin et al., 2017). Rabies is enzootic in Russia from the western borders to the Far East. According to surveillance data, this disease is maintained in Russia by wild canids, including the arctic fox, raccoon dog, steppe wolf and wolf. However, the majority of rabies cases are associated with the red fox, which is the main RABV reservoir (Kuzmin et al., 2004). Therefore this is a peculiarity of enzootic process in Russia.

Currently, the most applicable rabies diagnostic method in Russia is the fluorescent antibody test (FAT), which is applied to autopsy material. In cases of negative or ambiguous results, additional diagnostic procedures are applied, including virus isolation from animals (mouse inoculation test (MIT)) or cell cultures and an enzyme-linked immunosorbent assay (ELISA) (Anonymous, 2013; *Methodological guideline for laboratory diagnostics of rabies in animals and Hismatullina,* 1997; WHO Expert Consultation on Rabies, 2013). For now, antemortem diagnosis is not performed, and all of the utilized methodology is based on World Health Organization (WHO) recommendations. Within these guidelines, molecular diagnostic techniques are considered as auxiliary or additional for rabies diagnosis. However, molecular techniques have high potential and could be faster confirmatory methods due to their prevailing procedural simplicity and the ease of interpreting their results. The reported diagnosis of two rabies cases in humans from the Astrakhan region supports this opinion (Dedkov et al., 2016).

The use of molecular methods for RABV diagnosis or for the diagnosis of all members of the Lyssavirus genus is not new. The efficiency of PCR techniques for the routine diagnosis of rabies has been convincingly demonstrated by many researchers (Ermine et al., 1990; Heaton et al., 1997; Kamolvarin et al., 1993; Kulonen et al., 1999; Limaa et al., 2005; McColl et al., 1993; Sacramento et al., 1991). In the last decade, a great number of molecular assays for RABV and pan-lyssavirus diagnostics have been developed and evaluated (Wadhwa et al., 2017). These assays are based mainly on real-time reverse transcription-PCR (RT-qPCR) via intercalating dye or TaqMan probe methods (Coertse et al., 2010; Dupuis et al., 2015; Hayman et al., 2011; Mani et al., 2014; Nadin-Davis et al., 2009; Suin et al., 2014; Wakeley et al., 2006). However, no reliable real-time RT-qPCR assay has been developed for RABV diagnostics in Russia and neighboring countries. Such a method is necessary for the diagnosis of rabies and surveillance of the virus in wildlife.

The main objective of the research presented here was to develop a reliable RT-qPCR assay for postmortem rabies diagnosis in both humans and animals. We deliberately focused on the diagnosis of RABV only because other members of the Lyssavirus genus, although prevalent in Russia, are extremely rare, and the diseases associated with them are unique (Leonova et al., 2010).

2. Materials and methods

2.1. Sample collection

In total, 87 RABV strains collected in various regions of Russia from 2008 to 2014 were used in the present study (Table 1). These strains belong to four of the six RABV subgroups (A, C, D, F) prevalent in Russia and neighboring territories. All strains were isolated using white outbred mice and characterized prior to the study by the staff of Omsk Research Institute of Natural Foci Infections. Frozen secondary brain samples were stored at −70 °C until the beginning of the study. A subset (n = 8) was characterized quantitatively and used for sensitivity measurement, and another subset (n = 79) was used to obtain sequences of the N gene and formed the control panel of RABV strains (n = 27). The strains were specifically isolated from the primary brain tissues of 64 wild, 9 domesticated, and 12 farm animals and two humans from different regions, including the Tver region (n = 6), Tuva (n = 9), Khakassia (n = 6), the Voronezh region (n = 5), the Omsk region (n = 8), the Belgorod region (n = 3), the Lipetsk region (n = 17), Altai krai (n = 6), the Krasnoyarsk region (n = 16), the Astrakhan region (n = 2) and Primorsky krai (n = 1). In addition, 26 rabies-positive and 23 rabies-negative (according to MIT results) primary brain samples were studied (Tables 2, 3), as were nucleic acids from the following viruses and mammals: fam. *Rhabdoviridae* (Aravan and Khujand viruses); fam. *Bunyaviridae* (Tahyna, Batai, Crimean-Congo hemorrhagic fever and Inkoo viruses); fam. *Filoviridae* (Zaire ebolavirus, Marburg virus); fam. *Orthomyxoviridae* (Dhori virus); fam. *Reoviridae* (Kemerovo virus and rotavirus); fam. *Togaviridae* (Chikungunya, Sindbis and Rubella viruses); *Flaviviridae* (Yellow fever, West Nile and Tick-borne encephalitis viruses); fam. *Picornaviridae* (enterovirus ECHO-71); fam. *Retroviridae* (HIV); and DNA extracted from brain tissues taken from mice, dogs, cats and humans. The RABV strains were isolated, characterized, stored and propagated by the Omsk Research Institute of Natural Foci Infections. RNA was extracted from the RABV strains at the Omsk Research Institute of Natural Foci Infections.

Additional negative brain tissue samples and viral non-*Rhabdoviridae* RNA are part of the collection stored at the Central Research Institute for Epidemiology, Moscow, Russia. RNA from the Aravan and Khujand viruses was provided by the Omsk Research Institute of Natural Foci Infections. All negative brain tissue samples and viral RNAs were stored at −70 °C until the beginning of the study.

2.2. RABV propagation, RNA purification and reverse transcription

RABV propagation was performed in vivo (Koprowski, 1996). Briefly, 10% suspensions of the primary brain tissues from rabies-infected animals and humans were homogenized manually in phosphate-buffered saline at pH 7.0 (Dako, Denmark). The suspensions were then centrifuged at 2000 rpm for 10 min to precipitate debris, and 0.03 ml of the supernatant was inoculated intracerebrally into three-week-old white outbred mice. The mice were subsequently observed for specific signs of neurotropic disorders (i.e. anorexia, flaccidity, paresis, and plegia), after which they were euthanized and their brains extracted. The cause of death was validated using the FAT. Finally, 10% brain suspensions were prepared as described above, and RNA extraction was performed. The standard RABV strain Rus(Lipetsk)8054f_2011 (GenBank #KC595282) was used as a positive control. The FAT was performed using Centocor FITC-Anti-Rabies Monoclonal Globulin (Fujirebio Diagnostics, Inc., Malvern, PA, USA). All of the procedures involving mice were performed with the approval of the institutional committees for animal care and use.

Total RNA from all type of samples (RABV-positive and RABV-negative) was extracted using a RIBO-zol-C RNA extraction kit (AmpliSens, Russia). Reverse transcription was performed using a Reverta-L RT kit (AmpliSens, Russia). Both procedures were performed according to the manufacturer's instructions.

2.3. Sequencing of the N gene

Two fragments with 76 bp overlap were used for the N-gene sequencing. Two primer pairs (Table 4) were used in a 25 μL reaction mix containing 10 μL of PCR-mix-2-blue (CRIE, Moscow) with 0.4 μM direct and reverse primers, 2.5 μL of dNTPs (1.76 mM; CRIE, Moscow), 2 μL of the RABV cDNA and H_2O (Milli-Q) up to 25 μL.

Hot-start PCR was performed on a Tercyc Conventional PCR Thermal Cycler (DNA-Technology, Russia) with the following thermal cycling

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Table 1
List of RABV strains used in the study.

N	Name	Gen Bank accession no	Collection date	Region	Isolation source	Subgroup
1	Rus(Altai)7564	KJ958221	2008	Altai krai	Cattle	C
2	Rus(Altai)7565	KJ958222	2008	Altai krai	Red fox	C
3	Rus(Altai)7566	KJ958223	2008	Altai krai	Red fox	C
4	Rus(Altai)7572	KJ958224	2008	Altai krai	Cattle	C
5	Rus(Khakassia)7891	KJ958225	2011	Khakassia rep.	Cattle	C
6	Rus(Khakassia)7892	KJ958226	2011	Khakassia rep.	Cow	C
7	Rus(Khakassia)7893	KJ958227	2011	Khakassia rep.	Cattle	C
8	Rus(Khakassia)7894	KJ958228	2011	Khakassia rep.	Wolf	C
9	Rus(Khakassia)7895	KJ958229	2011	Khakassia rep.	Red fox	C
10	Rus(Khakassia)7896	KJ958230	2011	Khakassia rep.	Red fox	C
11	Rus(Krasnoyarsk)7499	KJ958231	2008	Krasnoyarsk krai	Red fox	C
12	Rus(Krasnoyarsk)7499	KJ958232	2008	Krasnoyarsk krai	Red fox	C
13	Rus(Krasnoyarsk)7500	KJ958233	2008	Krasnoyarsk krai	Red fox	C
14	Rus(Krasnoyarsk)7501	KJ958234	2008	Krasnoyarsk krai	Red fox	C
15	Rus(Krasnoyarsk)7502	KJ958235	2008	Krasnoyarsk krai	Red fox	C
16	Rus(Krasnoyarsk)7503	KJ958236	2008	Krasnoyarsk krai	Red fox	C
17	Rus(Krasnoyarsk)7504	KJ958237	2008	Krasnoyarsk krai	Red fox	C
18	Rus(Krasnoyarsk)7508	KJ958238	2008	Krasnoyarsk krai	Horse	C
19	Rus(Krasnoyarsk)7937	KJ958239	2011	Krasnoyarsk krai	Red fox	C
20	Rus(Krasnoyarsk)7938	KJ958240	2011	Krasnoyarsk krai	Red fox	C
21	Rus(Krasnoyarsk)7941	KJ958241	2011	Krasnoyarsk krai	Red fox	C
22	Rus(Krasnoyarsk)7943	KJ958242	2011	Krasnoyarsk krai	Red fox	C
23	Rus(Krasnoyarsk)7972	KJ958243	2011	Krasnoyarsk krai	Red fox	C
24	Rus(Krasnoyarsk)7973	KJ958244	2011	Krasnoyarsk krai	Red fox	C
25	Rus(Krasnoyarsk)7979	KJ958245	2011	Krasnoyarsk krai	Red fox	C
26	Rus(Krasnoyarsk)7980	KJ958246	2011	Krasnoyarsk krai	Red fox	C
27	Rus(Omsk)7444	KJ958247	2008	Omsk region	Red fox	C
28	Rus(Omsk)7445	KJ958248	2008	Omsk region	Red fox	C
29	Rus(Omsk)7461	KJ958249	2008	Omsk region	Red fox	C
30	Rus(Omsk)7586	KJ958250	2009	Omsk region	Dog	C
31	Rus(Omsk)7604	KJ958251	2009	Omsk region	Cat	C
32	Rus(Omsk)7701	KJ958252	2010	Omsk region	Red fox	C
33	Rus(Omsk)7703	KJ958253	2010	Omsk region	Red fox	C
34	Rus(Omsk)8000	KJ958254	2011	Omsk region	Red fox	C
35	Rus(Omsk)8001	KJ958255	2011	Omsk region	Red fox	C
36	Rus(Tuva)7463	KJ958256	2008	Tuva rep.	Wolf	C
37	Rus(Tuva)7466	KJ958257	2008	Tuva rep.	Cattle	C
38	Rus(Tuva)7467	KJ958258	2008	Tuva rep.	Dog	C
39	Rus(Tuva)7468	KJ958259	2008	Tuva rep.	Red fox	C
40	Rus(Tuva)7471	KJ958260	2008	Tuva rep.	Red fox	C
41	Rus(Tuva)7719	KJ958261	2010	Tuva rep.	Wolf	C
42	Rus(Tuva)8060	KJ958262	2011	Tuva rep.	Cat	C
43	Rus(Tuva)8092	KJ958263	2012	Tuva rep.	Wolf	C
44	Rus(Tuva)8093	KJ958264	2012	Tuva rep.	Camel	C
45	Rus(Tver)7573	KJ958265	2008	Tver region	Dog	D
46	Rus(Tver)7574	KJ958266	2008	Tver region	Red fox	D
47	Rus(Tver)7575	KJ958267	2008	Tver region	Red fox	D
48	Rus(Tver)7576	KJ958268	2008	Tver region	Raccoon dog	D
49	Rus(Tver)7577	KJ958269	2008	Tver region	Red fox	D
50	Rus(Tver)7579	KJ958270	2008	Tver region	Red fox	D
51	Rus(Voronezh)7510	KC794007	2008	Voronezh region	Red fox	C
52	Rus(Voronezh)7511	KC794008	2008	Voronezh region	Red fox	C
53	Rus(Voronezh)7512	KC794009	2008	Voronezh region	Red fox	C
54	Rus(Voronezh)7513	KC794010	2008	Voronezh region	Red fox	C
55	Rus(Voronezh)7514	KC794011	2008	Voronezh region	Red fox	C
56	Rus(Belgorod)7532	KC794012	2008	Belgorod region	Cattle	C
57	Rus(Belgorod)7539	KC794013	2008	Belgorod region	Red fox	C
58	Rus(Belgorod)7548	KC794014	2008	Belgorod region	Red fox	C
59	Rus(Lipetsk)8052	KC538849	2011	Lipetsk region	Red fox	C
60	Rus(Lipetsk)8053	KC538850	2011	Lipetsk region	Cat	C
61	Rus(Lipetsk)8054	KC538851	2011	Lipetsk region	Red fox	C
62	Rus(Lipetsk)8057	KC538852	2011	Lipetsk region	Red fox	C
63	Rus(Lipetsk)8061	KC538853	2012	Lipetsk region	Cattle	C
64	Rus(Lipetsk)8064	KC538854	2012	Lipetsk region	Red fox	C
65	Rus(Lipetsk)8067	KC538855	2012	Lipetsk region	Cattle	C
66	Rus(Lipetsk)8069	KC538856	2012	Lipetsk region	Red fox	C
67	Rus(Lipetsk)8072	KC538857	2012	Lipetsk region	Cattle	C
68	Rus(Lipetsk)8075	KC538858	2012	Lipetsk region	Cat	C
69	Rus(Lipetsk)8080	KC538859	2012	Lipetsk region	Red fox	C
70	Rus(Lipetsk)8083	KC538860	2012	Lipetsk region	Red fox	C
71	Rus(Lipetsk)8089	KC538861	2012	Lipetsk region	Cat	C
72	Rus(Lipetsk)8052	KC595280	2011	Lipetsk region	Red fox	C
73	Rus(Lipetsk)8053	KC595281	2011	Lipetsk region	Cat	C
74	Rus(Lipetsk)8054	KC595282	2011	Lipetsk region	Red fox	C

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Table 1 *(continued)*

N	Name	Gen Bank accession no	Collection date	Region	Isolation source	Subgroup
75	Rus(Lipetsk)8057f_	KC595283	2011	Lipetsk region	Red fox	C
76	Rus(Altai)8041	KY172632	2011	Altai krai	N/A	C
77	Rus(Astrakhan)_8329	KT728348	2003	Astrakhan region	Human	C
78	Rus(Astrakhan)_8330	KT728349	2003	Astrakhan region	Human	C
79	PO-01_2014_Primorye	KP997032	2014	Primorsky krai	Brown bear	C
80	8202	N/A	2013	Omsk	Red fox	C
81	8247	N/A	2013	Omsk	Raccoon dog	C
82	8300	N/A	2013	Omsk	Raccoon dog	C
83	8313	N/A	2013	Omsk	Red fox	C
84	8315	N/A	2013	Novosibirsk	Cattle	C
85	8317	N/A	2013	Novosibirsk	Red fox	C
86	1p8318	N/A	2013	Novosibirsk	Red fox	C

parameters: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 25 s, 72 °C for 30 s, and finally 72 °C for 3 min.

PCR products of the appropriate length were separated by electrophoresis on 1.5% agarose gel supplemented with ethidium bromide. Amplicon purification was performed using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified PCR product was then sequenced using an ABI Prism 3500 (Applied Biosystems, USA).

The N-gene sequences of the 79 RABV strains were assembled using a CLC Main Workbench 5 (Qiagen, Germany).

2.4. Design of the primers and the probe for RT-qPCR

The primers and the probe were designed in accordance with the current guidelines regarding TaqMan primers and probes for real-time PCR techniques (*PCR primer design and Yuryev*, 2007; Tyagi and Kramer, 1996; Van Pelt-Verkuil et al., 2008). The melting temperature for the primers was calculated using the Oligonucleotide Properties Calculator (Kibbe, 2007). Additionally, the Oligonucleotide Properties Calculator and MFOLD were used to assess the thermodynamic characteristics of the probe and the probability of the appearance of secondary structures (http://unafold.rna.albany.edu/?q=mfold/download-mfold).

Specifically, the RABV-specific probe was covalently attached to the fluorescent reporter dye rhodamine 6G (R6G) and to black hole quencher 1 (BHQ1) at the 5′ and 3′ ends, respectively. The primers and the probe were synthesized by the branch related to bioorganic synthesis at the Central Research Institute for Epidemiology, Moscow, Russia.

2.5. Reaction mixture and amplification mode

The total volume of the RT-qPCR reaction mix was 25 μL, including the following: 10 μL of the RNA sample, 0.2 μM of each primer and the probe (Rt_Rab-2f_Neuer, Rt_Rab-2r_Neu, Rt_Rab-z2_ne) and 0.2 μM of each STI-87-rec detection primer and the probe, 2.5 μL of dNTPs (1.76 mM; AmpliSens, Russia), 5 μL of RT-PCR mix2 FEP/FRT (AmpliSens, Russia), 0.25 μL of MMLV reverse transcriptase (AmpliSens, Russia), 0.25 μL of RTG-mix2 (AmpliSens, Russia) and 0.5 μL of TaqF polymerase (AmpliSens, Russia). The thermal cycling parameters were as follows: 50 °C for 15 min; 95 °C for 15 min; and then 45 cycles of 95 °C for 10 s, 57 °C for 25 s, and 72 °C for 10 s. Fluorescence was observed at 57 °C in a Rotor-Gene 6000 (Qiagen, Germany) in the yellow and green channels for the specific signals and the IC signals, respectively. The threshold value of fluorescence was chosen as the middle of the linear increase in the positive-control fluorescence, expressed in the logarithmic units. Amplification results were considered positive if the level of fluorescence crossed the threshold.

Table 2
Rabies-positive primary brain samples.

N	Sample ID	GenBank accession no.	Collection date	Region	Source	C_T value	Subgroup
1	7985	N/A	2011	Resp. Yakutia	Reindeer	22.0	N/A
2	8202	N/A	2013	Omsk	Red fox	23.5	N/A
3	7496	KY002892	2008	Nenets distr.	Reindeer	22.6	A
4	7510	KC794008	2008	Voronezh	Red fox	17.8	C
5	7539	KC794013	2008	Belgorod	Red fox	15.4	C
6	7555	KY002907	2008	Tyumen	Reindeer	18.7	A
7	7557	KY002909	2008	Tyumen	Polar fox	19.1	A
8	7558	KY002886	2008	Resp. Dagestan	Cattle	16.5	C
9	7559	KY002888	2008	Resp. Dagestan	Dog	17.5	F
10	7560	KY002887	2008	Resp. Dagestan	Cat	12.2	C
11	7561	KY002889	2008	Resp. Dagestan	Dog	17.7	F
12	7576	KJ958268	2008	Tver	Raccoon dog	17.8	D
13	7941	KJ958241	2008	Krasnoyarsk	Red fox	15.3	C
14	7990	KY002905	2011	Resp. Yakutia	Polar fox	28.3	A
15	8093	KJ958264	2011	Resp. Tuva	Camel	15.8	C
16	8300	N/A	2013	Omsk	Raccoon dog	26.6	C
17	828 f 816	N/A	1989	Voronezh	Red fox	22.4	N/A
18	8247	N/A	2013	Omsk	Raccoon dog	14.0	N/A
19	8313	N/A	2013	Omsk	Red fox	16.6	N/A
20	8315	N/A	2013	Novosibirsk	Cattle	23.4	N/A
21	8317	N/A	2013	Novosibirsk	Red fox	22.3	N/A
22	8318	N/A	2013	Novosibirsk	Red fox	21.2	N/A
23	1p8202	N/A	2013	Omsk.	Red fox	13.3	N/A
24	8229H	KT728349	2003	Astrakhan	Human	33.9	C
25	8330H	KT728348	2003	Astrakhan	Human	29.4	C
26	PO-01-2014	KP997032	2014	Primorsky Krai	Brown bear	15.9	C

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Table 3
Non-rabies samples.

N	Sample ID	Collection date	Region	Source
1	8215	2013	Omsk	Mink
2	8217	2013	Omsk	Mink
3	8218	2013	Omsk	Mink
4	8220	2013	Omsk	Mink
5	8221	2013	Omsk	Mink
6	8222	2013	Omsk	Mink
7	8224	2013	Omsk	Raccoon dog
8	8225	2013	Omsk	Red fox
9	8226	2013	Omsk	Red fox
10	8229	2013	Omsk	Raccoon
11	8230	2013	Omsk	Raccoon
12	8233	2013	Omsk	Red fox
13	8234	2013	Omsk	Red fox
14	8235	2013	Omsk	Red fox
15	8236	2013	Omsk	Red fox
16	8237	2013	Omsk	Red fox
17	8238	2013	Omsk	Red fox
18	8239	2013	Omsk	Red fox
19	8241	2013	Omsk	Red fox
20	8270	2013	Omsk	Sable
21	8271	2013	Omsk	Marten
22	8310	2013	Novosibirsk	Dog
23	8316	2013	Novosibirsk	Dog

To control all stages of the RT-qPCR reaction, a positive control for PCR (C+) and an armored recombinant positive control for reverse transcription (ARC+) were developed. The commercially available STI-87rec internal control (IC) was used to monitor RNA extraction; for that purpose, STI-87rec-specific primers and the probe were added to the reaction mixture. Additionally, a negative control for extraction (EC-) and a negative control for PCR (C-) were used to exclude false-positive results due to cross-contamination.

2.6. Generation of the positive-control samples

The cDNA region (905 bp) equivalent to the N gene of RABV (strain 8202f_Omsk_2013) that included the primer and the probe target sequences was amplified, extracted using a MinElute Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions and ligated into the pGEM-T plasmid vector (Promega, USA) under the control of the T7 RNA polymerase promoter. The resultant plasmid was transformed into *Escherichia coli* (XL1 blue strain) (Maniatis et al., 1989). Recombinant plasmids from the individual clones were extracted using a Plasmid Miniprep kit (Axygen) according to the manufacturer's instructions, and the orientation and absence of mutations in the cloned PCR fragment were evaluated by Sanger sequencing using an ABI-Prism 3500 XL (Applied Biosystems, USA). Diluted plasmid solutions of known concentrations were used as C+ as the PCR-step efficiency control.

The same cDNA region was also used to prepare ARC+ based on a previously described procedure for MS2-phage-based armored RNA phage particles (Cheng et al., 2006; Pasloske et al., 1998), with certain minor modifications. Briefly, the PCR fragment containing the target region and additional flanking nucleotides (see above) was ligated into a linearized in-house plasmid vector containing the MS2 coat protein gene. After verification by DNA sequencing, the generated recombinant plasmid was transformed into *E. coli* (strain B21), and protein expression was induced with isopropyl-L-thio-D-galactopyranoside (IPTG). After induction, the cells were collected, lysed using a method combining lysozyme and freeze-thawing, and treated with DNase I (Fermentas, USA) and RNase A (Fermentas, USA). The derivate was then purified using CsCl gradient centrifugation, quantified and diluted in RNAlater Stabilization Solution (Life Technologies, USA). The absence of residual DNA in the treated sample was tested using the developed qPCR assay without the reverse transcription step. The C+ and ARC+ concentrations were also measured with a QX100 system (Bio-Rad) using a PCR Supermix for Probes kit (Bio-Rad), a One-step ddPCR Supermix for Probes kit (Bio-Rad), specific primers and a suitable probe according to the manufacturer's instructions (Table 4).

Table 4
Oligonucleotides used for the amplification and sequencing of RABV genome fragments.

Primer	Nucleotide sequence, 5′-3′	Reference sequence, nt JQ944704 (GenBank (NCBI)).	Amplicon length, bp
Rab1f	ACGCTTAACAACAAAATCATAGAAG	1–25	709
Rab1r	AACATGTCGTAGGTTCCGGC	689–708	
Rab2f	ATGACAACTCACAAAATGTGYGC	632–653	905
Rab2r	GGATTGACGAAGATCTTGCTCAT	1514–1536	

2.7. Internal control samples

To access the efficiency of the RNA extraction, an STI-87rec (AmpliSens, Russia) exogenous internal control (IC) was added to the reagent mixture. The STI-87rec is specifically an artificial RNA sequence (150 nt, GC content 50%) surrounded by an MS2-derived protective protein coat.

2.8. Assessment of cross-contamination risks

To assess the absence of cross-contamination due to high concentration of viral RNA in the studied rabies-positive samples, one EC- every 12 extracted samples and one C- every 12 RT-qPCR samples were intercalated. Therefore, there were 6 EC- and 6 C- in each run. All procedures were performed in complete accordance with good molecular diagnostics laboratory practice.

2.9. Analytical sensitivity

Analytical sensitivity was assessed using a series of 10-fold dilutions of ARC+ at a known concentration in the 10% human brain tissue suspension, which was prepared in phosphate-buffered saline at pH 7.0 (Dako, Denmark). Homogenization was performed in a Tissue Lyser LT (Qiagen, Germany) for 5 min. The resulting solutions were tested to detect the minimal threshold value for sample positivity. The limit of detection (LOD) was set as the minimal dilution detected in three replicates. Analytical sensitivity was further assessed using 8 RABV strains for which the concentrations were calculated using the Reed-Muench method (LD50/ml) (Reed and Muench, 1938). The LD50/ml values were converted into RNA copies using the QX100 system (Bio-Rad) with the One-step ddPCR Supermix for Probes kit (Bio-Rad) and specific primers and probe, as described above. The sensitivity threshold was set as the minimal dilution detected in three replicates (Table 5).

Table 5
Features of the RABV strains used for the analytical sensitivity measurement.

Strain	Isolation date	Region	Source	Copies of RNA per 1 LD50/ml	LOD LD50/ml	LOD ARC copies/ml
8202	2013	Omsk	Fox	43,165	0.1	4317
8247	2013	Omsk	Raccoon dog	2760	1.0	2760
8300	2013	Omsk	Raccoon dog	32,150	0.1	3215
8313	2013	Omsk	Red fox	11,515	0.1	1152
8315	2013	Novosibirsk	Cattle	5960	0.1	596
8317	2013	Novosibirsk	Red fox	2970	1.0	2970
8318	2013	Novosibirsk	Red fox	58,945	0.1	5895
1p8202	2013	Omsk	Red fox	41,595	0.1	4160

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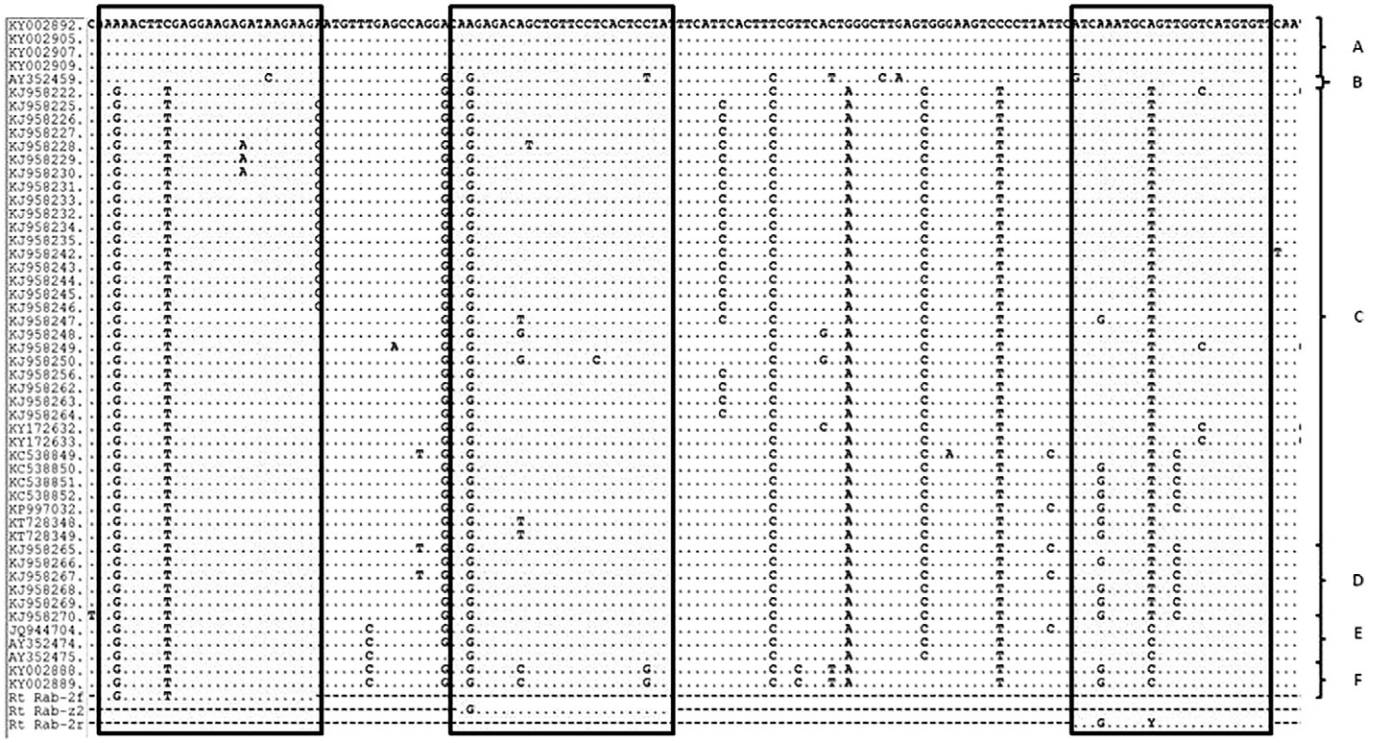

Fig. 1. Alignment of the RABV sequences, primers and probe. RABV sequences with the following GenBank (NCBI) accession numbers were aligned: KY002892, KY002905, KY002907, and KY002909 (subgroup A); AY352459 (subgroup B); KJ958221-KJ958264, KC794007-KC794014, KC538849-KC538861, KC595280-KC595283, KY172632, KT728348, KT728349 and KP997032 (subgroup C, some shown in the figure); KJ958265-KJ958270 (subgroup D); JQ944704, AY352474, and AY352475 (subgroup E); and KY002888 and KY002889 (subgroup F).

2.10. Analytical specificity

The ability of the developed assay to detect a broad range of RABV strains circulating in Russia was evaluated using a control panel of RNA from 79 RABV strains with a wide geographical distribution (see above); this RNA was extracted from mouse brain tissues after inoculation.

Potential cross-reactivity was assessed using high-titer RNA from 19 viral species belonging to 9 viral families and the DNA extracted from the brain tissues of RABV-negative animals and humans.

2.11. Diagnostic sensitivity and specificity

Diagnostic sensitivity and specificity were assessed during the primary sampling of the brain homogenates prepared from the RABV-infected animals and humans (26 samples, Table 5) and from the RABV-negative animals (23 samples, Table 3). All of the samples were tested previously using the FAT, which was performed using Centocor FITC-Anti-Rabies Monoclonal Globulin (Fujirebio Diagnostics, Inc., Malvern, PA, USA) on all of the samples before assessment.

3. Results

Nucleotide sequences of the N-gene region were obtained from 79 RABV strains collected throughout Russia to evaluate the similarity of the target region for RABV-specific PCR. The sequences were submitted to GenBank (NCBI) under the following accession numbers: KJ958221-KJ958270, KC794007-KC794014, KC538849-KC538861, KC595280-KC595283, KY172632, KT728348, KT728349 and KP997032. Multiple alignments (Fig. 1) of the obtained sequences and the sequences of RABV available in GenBank at the beginning of the work allowed the identification of highly conserved regions required for the design of the RABV-specific primers and a respective probe (Table 6).

Based on the sequencing data, oligonucleotide primers and the fluorescent probe were designed and synthesized, and the RABV-specific assay was developed.

The developed assay included all components required for RT-qPCR and allowed control of all stages of the assay, including RNA extraction, reverse transcription and PCR. Using EC- and C-, the risk of false-positive results due to cross-contamination was also minimized.

The analytical sensitivity, as assessed using ARC+ dilutions, was 10^3 copies/ml, and the sensitivity measured using characterized strains was between 0.1 LD50/ml and 1.0 LD50/ml (Table 7). Standard detection was linear in the range from 10^6 copies/ml ($C_t = 29.5$) to 10^3 copies/ml ($C_t = 38.5$) of the RABV ARC+ ($R^2 = 0.996$) (Fig. 2).

A broad range of RNA from 79 RABV strains circulating in different regions of Russia was detected using the developed assay, as was RNA from 26 RABV-positive primary brain samples from 81 animals and two humans. No false-positive or false-negative results were obtained. In addition, the potential for cross-reactivity was assessed using high-titer RNA from 19 viral species, including RNA from Aravan and Khujand lyssaviruses. No species tested positive. Consequently, the evaluated diagnostic sensitivity and specificity were both 100%. Our results demonstrated a low risk of false-positive results due to cross-contamination

Table 6
Features of the primers and the probe used in the assay, including the sequences, positions and length.

Primer or probe	Sequence, 5′-3′	Reference sequence, nt JQ944704 (GenBank (NCBI)).	Amplicon length, bp
Rt_Rab-2f_Neuer	AAGAACTTTGAGGAAGAGATAAGAAG	857–882	140
Rt_Rab-z2_Neu	(R6G)-AGGAGACAGCTGTTCCTCACTCCTAT-(BHQ1)	900–925	
Rt_Rab-2r_Neu	AACACATGACCAACRGCATTCGAT	973–997	

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Table 7
Analytical sensitivity assessment performed using strains with known concentrations.

Strain	10 LD50/ml			1 LD50/ml			0.1 LD50/ml		
8202f_Omsk_2013	+	+	+	+	+	+	+	+	+
8247rd_Omsk_2013	+	+	+	+	+	+	+	–	–
8300rd_Omsk_2013	+	+	+	+	+	+	+	+	+
8313f_Omsk_2013	+	+	+	+	+	+	+	+	+
8315c_Novosibirsk_2013	+	+	+	+	+	+	+	+	+
8317f_Novosibirsk_2013	+	+	+	+	+	+	+	+	–
8318f_Novosibirsk_2013	+	+	+	+	+	+	+	+	+
1p8202f_Omsk_2013	+	+	+	+	+	+	+	+	+

because of commitment to good practice in the molecular diagnostics laboratory, including the use of appropriate negative-control samples in all steps of the study, including extraction, reverse transcription and PCR.

4. Discussion

As mentioned above, FAT remains the most applicable diagnostic technique, but based on our own experience, the sensitivity of this method significantly depends on the quality of the studied sample (Dean et al., 1996; Praveena et al., 2007). While numerous studies have shown concordance between FAT and RT-qPCR assays (Appolinário et al., 2015; Dacheux et al., 2016; Dupuis et al., 2015; Faye et al., 2017; Tricou et al., 2016), some have reported that RABV has been detected by RT-qPCR in FAT-negative samples (Dupuis et al., 2015). In the absence of any evidence to indicate that the PCR methodology is producing false-positive responses, these findings suggest that RT-qPCR may be more sensitive than FAT, particularly if the sample quality is compromised. Ultimately, the status of a sample may need to be determined based on a consensus of multiple tests; such evidence may eventually establish the superiority of molecular tests over the traditional FAT.

On the other hand, 16 European laboratories jointly performed a comparative trial of 14 assays for lyssavirus detection using a sample panel provided by the Friedrich Loeffler Institute (FLI). This comparative trial revealed that none of the 14 assays was effective at detecting all of the genetic variants of RABV included in the panel (Fischer et al., 2013).

In recent years, several diagnostic kits for RABV detection based on RT-qPCR techniques have been developed in Russia. However, those kits do not possess the appropriate certificates for state registration, and their diagnostic value is yet to be determined.

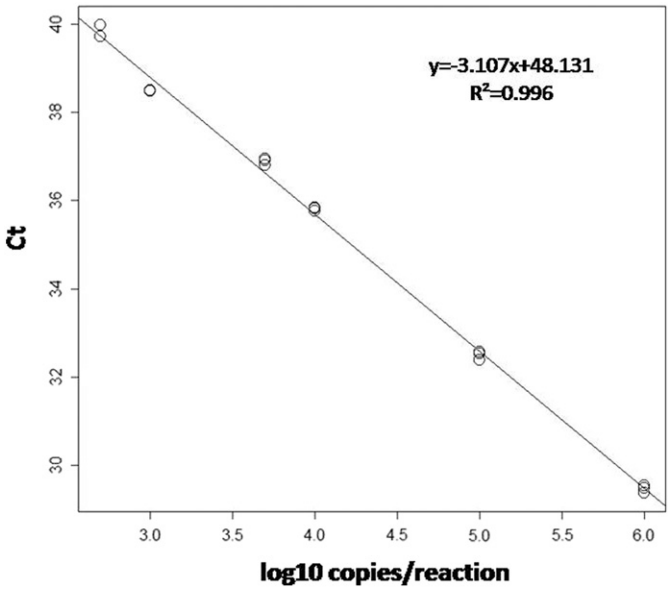

Fig. 2. Assessment of the RABV assay sensitivity.

Here, we report the development of an RT-qPCR-based diagnostic assay specific for RABV and primarily suitable for the detection of the strains most abundant in Russia. We evaluated our assay for the majority of RABV subgroups prevalent in Russia and neighboring territories, excluding subgroups B (Arctic-like rabies) and E (Northeast European Rabies). However, the circulation of strains belonging to subgroup B was documented in Russia for the last time more than 40 years ago (Kuzmin et al., 2008), whereas strains belonging to subgroup E had only one additional mismatch with the reverse primer according to the sequence alignment. Analytical sensitivity assessment for the 8 strains with known concentrations was in the range of $6x10^3$ to $6x10^2$ copies/ml for the different strains. This tenfold difference in sensitivity was considered insignificant because the viral concentration in the sampled primary brain tissues was significantly higher than concentrations corresponding to the Ct values of any of the investigated strains (Table 4).

The developed assay was evaluated only for postmortem diagnosis of rabies because we did not have an appropriate amount of antemortem samples from rabies-infected humans or animals. Nevertheless, given its high analytical sensitivity, the assay could be suitable for the detection of RABV in saliva or skin biopsy, although this possibility requires confirmation in further studies.

The high genetic heterogeneity of RABVs challenges the application of a single RT-qPCR method for the broad detection of all viral subtypes. Therefore, our assay developed for the detection of RABVs of Russian origin may have limited use for the detection of viruses from other parts of the world, but this ability remains to be thoroughly evaluated; such an analysis is outside the scope of the present work.

5. Conclusions

Here, we report the development and evaluation of an RT-qPCR assay for the diagnosis of RABV infection. High analytical and diagnostic sensitivities were demonstrated, supporting the notion that this assay has the potential to become a faster confirmatory method than virus isolation or mouse inoculation in the Russian Federation. Thus, this assay can be used for epizootiological monitoring of animals and for laboratory postmortem diagnosis of RABV infection in humans. Nevertheless, until enough research supports the value of molecular methods in the diagnosis of RABV infection, the FAT or MIT is still required for the final verification of rabies infection.

5.1. Ethical considerations

The study has been evaluated and approved by the Ethics Committee of the Central Research Institute for Epidemiology.

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