THE NUCLEOPROTEIN MOLECULAR DIVERSITY OF FOX AND RACCOON DOG RABIES VIRUSES IN LITHUANIA

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ABSTRACT

The present study was done to determine the molecular epidemiology of rabies virus (RV) in Lithuania wildlife. The 28 red foxes and raccoon dogs RV isolates had been collected during the 2005-2006 period and came from different Lithuanian districts. In order to investigate the roles of the host species and geographical distribution correlation with molecular epidemiology of RV viruses, a 400 bp region of the nucleoprotein gene was sequenced and compared with 75 European virus isolates. Phylogenetic analysis revealed that the foxes and raccoon dogs RV isolates from different part of Lithuania were closely associated, belonged to RV genotype 1 and show significant bootstrap support inside the North East Europe (NEE) group of rabies virus. Phylogenetically the foxes RV isolates from Central and Western Lithuania were clustered together with the same isolates from Poland. The raccoon dogs RV isolates from North and Eastern part of Lithuania exhibited 97.7-99.0% nt identity to previously published sequences from Estonia, Latvia and Russia. The data obtained here show the critical role of geographical isolation and limitation for the genetic clustering and evolution of the rabies virus and also help in predicting its distribution from rabies-affected to rabies free areas in the multi-host scenario.
KEY WORDS: Lithuania, rabies, molecular epidemiology**.**

INTRODUCTION

Advances in molecular epidemiological methods over the past 15 years have resulted in an increase in genetic data on rabies viruses (RV) circulating in a range of host species in a worldwide. The main focus of molecular epidemiology studies of lyssaviruses is the nucleoprotein (N) gene, which was chosen for several reasons including the effectiveness of their region for genotyping and evolutionary analysis (9). More recently, the large numbers of partial N gene sequences available in various databases have facilitated detailed virus, host, geographical and temporal comparisons (7; 12). Johnson e al, (6) provide further insights into epidemiology of rabies in Southeast Europe with evidence for the role of both domestic and sylvatic canine species for the spread and maintenance of the disease across borders. An increasing quantity of rabies sequence data is now available for regions of Eastern Europe, primary as a direct result of successful collaborative programmes. However, rabies molecular epidemiology remains uninvestigated in large areas of Europe including countries as Belarus, Lithuania and the Ukraine, making it difficult to understand the dynamics of viral dispersion and host adaptation fully (13). Only one dog rabies virus isolate sequence (1992) from Lithuania was used in rabies virus phylogenetic analysis (3). In contrast, rabies virus isolates from Latvia, Estonia, Poland and European part of Russia (18; 12; 15; 10) were used in phylogenetic analyses of rabies virus genotypes correlation with geographical distribution and specific host's adaptation. Consequently, the molecular typing methods are playing an increasingly important role in the understanding of rabies host and geographical distribution in Lithuania.

The main objective of this work was to characterize the genetic properties of the field rabies viruses isolated in the Lithuania during the 2005-2006 period, by using reverse transcriptase-polymerase chain reaction (RT-PCR) and partial N gene sequences analysis.

MATERIALS AND METHODS

28 Lithuanian RABV isolates (red foxes (n=10), raccoon dogs (n=18), obtained from brain samples received from the National Veterinary Laboratory were diagnosed as rabies-positive by the FAT and the MIT (2) were used in this study. The isolates had been collected during the 2005-2006 period and came from different Lithuanian districts. For molecular epidemiological study isolates were analysed together with reference to GenBank sequences of the rabies virus isolates from various regions of Eastern, Central, and Northern Europe including the nearest regional Lithuanian neighbours. Total RNA was extracted from infected brain samples using the TRIzol method (Invitrogen, Life Technologies, MD, USA) following the manufacturer's recommendations. RT and PCR were performed according to Amengual et al. (1) and, with primer set N12 (5'-GTAACACCTCTACAATGG-3', nucleotides 57-74) and N8 (5'-AGTTTCTTCAGCCATCTC-3', nucleotides 1585-1568), according to the PV strains sequence. PCR was carried out in 50 µl volumes containing 5 µl of extracted RNA and the following reagents: 5 µl 10x PCR buffer (Fermentas), 5 µl $MgCl_2$ (25 mM, Fermentas), 2 µl dNTPs (10 mM, Fermentas), 5 pmol of each outer primers N12 and N8, 0.5 µl (2.5 U) Taq DNA polymerase (Fermentas), 0.25 µl (10 U) RNasin (Promega, Madison, WI, USA), and 0.5 µl (100 U) MMLV reverse transcriptase (Life Technologies). The tubes were then subjected to the following cycle parameters: 42°C for 30 min, 95°C for 5 min, and then 35 cycles at 94°C for 40 s, 56°C for 40 s, and 72°C for 1 min.

Nested PCR was carried out with primers N53 (5'-GGATGCCGACAAGATTGTAT-3', corresponding to bases 73-92 of the PV sequence) and N55 (5'-CTAAAGACGCATGTTCAGAG-3', corresponding to bases 491-472 of the PV sequence). For the nested PCR as a template was used 2 µl of PCR I product and following reaction mixture: 5 µl 10x PCR buffer (Fermentas), 5 µl $MgCl_2$ (25 mM, Fermentas), 2 µl dNTPs (10 mM, Fermentas), 20 pmol of each nested primers N53 and N55, 0.5 µl (2.5 U) Taq DNA polymerase (Fermentas). The tubes were then subjected to the following cycle parameters: 95°C for 3 min followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 40 s. A single extension step of 72°C for 10 min completed the amplification process. The nested PCR resulted a final amplicon of 400 bp.

Prior to sequencing, the PCR products were elctrophoresed on 1.5 % agarose gel. The DNA was recovered using Nucleospin Extract II kit (Macherey-Nagel GmbH, Germany) following the manufacturer's recommendations. Gel purified PCR products were cycle sequenced using the BigDye™ Terminator Cycle Sequencing kit (v2.0, Applied Biosystems, Foster City, CA, USA) and the ABI310 genetic analyzer (Applied Biosystems). Sequences of both strands of the nucleoprotein N gene products were determined using the same primers as used for the nested PCR amplification. The obtained sequences were assembled by using SeqMan program (Laserge, program package, DNASTAR, Inc., Madison, USA). Clustal W programme from MegAlign Lasergene program package was used for sequence alignment and phylogenetic analyses. For boot strap analyses was used neighbour-joining (NJ) algorithm from CLC Free Workbench 3.2.3 program package (CLC bios A/S, Denmark).

RESULTS AND DISCUSSION

Based on the simplest form of analyses, examination of percentage nucleotide identity, a phylogenetic analysis of obtained sequences from different parts of Lithuania revealed that all 28 rabies virus isolates were closely related and had high identity among themselves (97.7-100.0% nt). All of the RV isolates from red foxes clustered together with the 99.5-100.0% nt identity, whereas identity among the raccoon dogs sequences was 98.8-100.0% nt, irrespective of geographical distribution of RV isolates. Previously published Lithuanian rabies sequence 9345LT (dog, 1992) also showed very close phylogenetic relationship to newly obtained isolates (97.7-99.0 % nt identity). The phyligenetic relationships between Lithuanian RV isolates and those from

neighbouring countries for the 400 bp region of N-gene are show, that red foxes RV isolates from Central and Western Lithuania were related (98.5% nt identity) with foxes RV isolates from Poland (96152POL, 1995 and 9634POL, 1987). The raccoon dogs RV isolates from North and Eastern part of Lithuania exhibited 97.7-99.0% nt identity to previously published sequences from Estonia (9339EST, 1991), Latvia (9904LV, 2003) and Russia (RV309RUS, RV245RUS, 2004). The phylogenetic analysis of Lithuanian isolates in the present study showed that they could not be subdivided according to geographical location or host distribution. All new Lithuanian sequences were well supported from the other group of rabies viruses, which joined isolates from different parts of Europe, with bootstrap support of 96%nt. All sequences from different parts of Lithuania belonged to RV genotype 1 and show significant bootstrap support inside the North East Europe group of rabies virus (NEE) group described earlier by Bourhy or McElhinney (3; 12; 13).

The North East Europe (NEE) group was represented by RV isolates from Western Russia, Finland, Estonia, Latvia, Lithuania, Poland and Slovakia. Polish RV strains belong to four different phylogenetic groups and two are dominant. Their geographical spread is strictly dependent on the geographical barrier of the Vistula River. The NEE group is limited to the Eastern side of the river and the Central European (CE) RV cluster has been isolated mainly in the West and South of Poland – on the west side of the Vistula River (14). In North the NEE distribution is limited in Northwest Russia, Estonia and Finland where RV isolates is closely related to non-Arctic European RV lineages (4). The majority of isolates within NEE group originated from both raccoon dogs and red foxes. This indicates that this variant is equally well supported by both reservoir species or that it represents an emergent biotype for raccoon dogs (12).

The epidemiological analyses, laboratory studies, and modelling suggested that the recent rabies epizootic in Western Europe was propagated and maintained by a single species, the red fox (*Vulpes vulpes*), but his is not necessarily true for Eastern and Northern Europe, where arctic foxes (*Alopex lagopus*) and introduced raccoon dogs (*Nyctereutes procyonides*) are implicated in sustaining the chain of infection (19). The rabies cases in red foxes decreased since 1994, but at the same time, raccoon dog rabies has increased in North-Eastern Europe and more than doubled in the Baltic States: in 2005 the largest number (599) of raccoon dog rabies cases was found in Lithuania, more than the 533 cases found in foxes; in 2006 – 987 rabies cases in raccoon dog, which was 300 cases more than the number found in foxes (11). The involvement of two competent rabies vectors can substantially alter the epidemiology of the disease. Foxes and raccoon dogs provide a larger reservoir for the virus. Even if the population density of a single species is below the threshold for a sustained outbreak, the density of the combined vectors community might be sufficient (5). Moreover, behavioural differences of the vectors might alter transmission spatially or temporally, and might affect transmission within species (e.g. due to different social behaviour) or between species, although cross-species transmission is poorly understood. With respect to the hibernation of raccoon dogs, an important aspect has to be considered: the pathogenesis of the disease might be changed. Raccoon dog hibernation was assumed to affect both incubation and transmission. Effectively, in the model, hibernation impedes all transmission with raccoon dog's involvement during winter. At the same time, the animals cannot fall ill. Thus the incubation period is prolonged until spring (16). However, the hibernation of raccoon dogs depends on climatic condition. Warmer winter could reduce or stop raccoon dogs hibernation, as recent observed in Lithuania and even Southern Finland. Arise in temperature can directly correlate with length of growing season and higher population density (8). The model results showed that in rabies-free regions, contingency control has to take into account the spread of rabies in a community of vectors – in this cases foxes and raccoon dogs. Vaccinating foxes give some protection, but is effective only if the density of racoon dogs in the area is low (17). For this reason, it is vital to take raccoon dogs into account for future planning of rabies control in Europe. Not only because raccoon dogs have become more important in existing rabies zoonotics, but

because they pose an additional potential complication following the reintroduction of this disease to rabies-free areas (16).

CONCLUSION

Our present study suggest that the Lithuanian RV and others NEE group RV isolates might have been evolved from the same progenitor and also indicate that the infection cycle of RV in this area tends to be maintained endemically. Molecular epidemiological data for countries such as Lithuania is essential in gaining a greater understanding of the viral variants responsible for the rabies epizootics which continue to pose a threat to public health. The community of vectors – red foxes and raccoon dogs – strongly enhances the risk of rabies in comparison to the disease in a single vector. The raccoon dog may represent reservoir for the NEE RV and play an important role in its Western spread circulation.

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