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Original Research Article

Antigenic and Genetic Stability of Rabies Virus

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Rabies virus (RABV) is a single stranded RNA genome virus that encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA dependent polymerase (L). However, RABV seem to be remarkably stable antigenic and genomic differences among isolates from different species have been recognized for many years. Analysis of RABV isolates from different natural reservoirs reveals antigenic variants and/or genetic lineages with specific characteristics, suggesting selection and adaptation of viruses to each of the particular species. Such selections and adaptations are so specific that they allow for the identification of the natural reservoir of origin of a determined isolate. This work was conducted to investigate the genomic and antigenic stability of four different genetic lineages of RABV, originated from different host species, following successive passages in mice. Four RABV isolates (IP4005/10, IP964/06, IP3629/11 and IP4871/11) were inoculated intracerebrally into 3-4 weeks-old mice. After each passage, the viruses were examined in their antigenic profile with a panel of monoclonal antibodies to rabies virus antigens. Viral RNA was extracted from the 1st, 5th and 10th passages and submitted to reverse transcription (RT) followed by polymerase chain reaction (RT-PCR), sequencing and phylogenetic analyses. Antigenic profile of the isolates did not reveal any recognizable alteration throughout. No nucleotide substitutions were noticed in the final sequences in any of genes sequenced from the four RABV isolates, with the exception of one nonsynonymous substitution in the putative protein P in position of amino-acid 222 in the isolate of non-hematophagous bat origin. These findings highlight the high antigenic and genetic stability of RABV, as opposed to the alleged high genomic variability of viruses with RNA genomes. On the other hand, it seems that different isolates may present different degrees of genetic stability, what may be related to the degree of adaptations of the isolate to the reservoir.

Keywords: Rabies, Successive passages, Reservoirs.

INTRODUCTION

Rabies virus (RABV) is a neurotropic virus, currently classified in the genus *Lyssavirus* of the family *Rhabdoviridae*. The single stranded RNA genome of RABV encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA dependent polymerase (L). The single stranded RNA genome has frequently been blamed for the putative genomic variability of RNA-containing viruses. In particular, the high error tendency of viral RNA polimerases, whose lack of fidelity would facilitate quasispecies generation and adaptation to new hosts.

Despite that, RABV isolates seem remarkably stable. However, antigenic and genomic differences among RABV isolates from different species have been recognized for many years; isolates from different natural reservoirs reveals antigenic variants and/or genetic lineages with unique characteristics, suggesting selection and adaptation of the virus to a particular species. Such selections and adaptations necessarily alters the composition of nucleotides of the gene and are so specific that allow identification of the reservoir and in the majority of cases the origin of the isolate.

This is remarkable since, in the vast majority of species, RABV leads almost invariably to fatal infections, which do not provide opportunities for co-evolution and, for this reason, instances of co-evolution are identified only after many generations of virus interaction with the population of host species. Within a few species, the virus has somehow co-evolved, which have become “natural hosts” or reservoirs, and where RABV can be maintained and the perpetuated without the need for reintroduction of virus from another host source. To date, the genetic and phenotypic alterations necessary for

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such adaptation of the virus to occur in a host species remain unknown.

This work was conducted to investigate the genomic and antigenic stability of four different genetic lineages RABV, originated from different host species, when subjected to subsequent passages in a non-natural host, using mice as an experimental model.

MATERIALS AND METHODS

Viruses

Four distinct RABV isolates recovered from central nervous system (CNS) tissues of different species and considered representative of different RABV genetic lineages were used in the present study. Isolate IP4005/10 is a virus genetic lineage whose natural host is the hematophagous bat *Desmodus rotundus*. Isolate IP964/06 was recovered from a non hematophagous bat *Epitesicus furinalis*. Isolate IP3629/11 was originated from a domestic dog.

Isolate IP4871/11 was recovered from the wild canid *Cerdocyon thous*. All RABV isolates had only one passage in mice and displayed genetic characteristics distinctive in relation to each other, as determined by the sequencing of the part of nucleoprotein gene. This work complies with Protocol nº 02/2014 issued by the Ethics Committee of the Pasteur Institute of São Paulo.

Mouse inoculation

Each of the four RABV isolates was submitted to ten successive passages in swiss albino adult mice. Animals were inoculated by intracerebral route, following standard protocols (Koprowski, 1996). Briefly, 10% (w/v in PBS) suspensions of infected CNS tissues were prepared and inoculated in groups of six, 3-4 weeks old mice. Animals were kept in cages in controlled environmental conditions and with food and water *at libidum*. The mice were monitored daily and the development of clinical signs until death, when brains were harvested, were recovered and described in days post infection (dpi).

Multiplication of RABV in CNS of mice was confirmed by direct immunofluorescence. A transversal section of the CNS of infected mice was used to prepare a fresh inoculum (as above) and the process repeated up to the ten successive passages.

Antigenic analyses

Samples of each of the ten successive passages were examined by indirect immunofluorescence (IIF) with a panel of 8 monoclonal antibodies (Mabs) raised to RABV nucleoprotein (CDC, Atlanta, GA, USA), as described elsewhere (Diaz *et. al.* 1994).

Genomic analyses

The first, fifth and tenth passages of each of the four RABV isolates were submitted to reverse transcription (RT) followed by polymerase chain reaction (RT-PCR), sequencing and phylogenetic analyses. For RT-PCR, total RNA was extracted with Trizol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. Extracts from mice infected with the "fixed" strain Challenge Virus Standard (CVS-31) were included in all reactions as positive controls; DEPC-treated water was included as negative control throughout.

The RT was carried with primer R.11904 (Table 1). The reaction was performed in 50 volumes with aid of the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA), following instructions of the manufacturers. The reaction was incubated for 3 hours at 42 ºC followed by a final incubation at 72 ºC for 15 minutes. PCR was carried separately for each gene out on cDNA as previously described (Carnieli, *et. al.* 2008), using primers targeting regions of nucleoprotein gene (N), glycoprotein gene (G), phosphoprotein gene (P), matrix gene (M) and polymerase gene (L) (Table 1). For sequencing, amplicons obtained at PCR of each one gene were purified and sequenced with same primers used for PCR.

The RABV sequences obtained were edited with the CHROMAS software (version 2.24 Copyright© 1998–2004 Technelysium Pty Ltd.). After editing, sequences from different parts of genome of RABV, nt 50-1474 corresponding to the whole N protein, nt 1514-2407 corresponding to the whole P protein, nt 2496-3105 corresponding to to whole M protein, nt 3312-4088 corresponding to part of G protein and nt 7086-7506 corresponding to part of L protein were obtained. (Positions along the RABV genome based on the PV strain-GenBank acession number M13215). The output sequences were aligned with CLUSTAL/W using the BioEdit software version 7.1.3.0. The putative amino acid (aa) sequences of each gene in the RABV sequences obtained were deduced with the program BioEdit.

In silico Restriction Endonuclease Analysis (REA)

The final RABV nucleotide sequences obtained were submitted to *in silico* REA with the NEBcutter software (version V2.0).

RESULTS

Mouse inoculation

Data on the course of infection following the passages of RABV isolates in mice are shown in table 2. After each passage, invariably, the animals showed typical clinical signs of rabies, characterized by anorexia, apathy, ataxia, muscular spasms and death. An important reduction in the incubation period was observed with two isolates. IP4005/10 (recovered from haematophagous bat origin) in which it was reduced from 9 to 11 days after the first passage to 5 days after the tenth passage. For isolate IP4871/11 (recovered from wild dog *Cerdocyon thous*) incubation period was 7–17 dpi in first passage of isolate and only 6 dpi after ten passages. An equal incubation period of 5-6 days was observed for all isolates after ten successive passages.

Antigenic analyses

The antigenic profile of the isolates did not reveal any recognizable alteration, as determined by indirect immunofluorescence with the Mab panel (Table 3). The viruses retained its patterns of antigenic reactivity unaltered, even after ten successive passages.

Genomic analyses

The genomic sequences of each of the RABV isolates were compared after the first, fifth and tenth passages in mice. No nucleotide substitutions were noticed in the final sequences in any of genes sequenced from the four RABV isolates.

Table 1. Description of primers (nucleotide sequences and annealing positions) used for RT-PCR and sequencing

Name of primers	Sense	Nucleotide sequence (5'-3')	Position*	Reference
JW12	Forward	ATGTAACACCYCTACAATTG	55-73	Mochizuki *et al*., 2011
304	Reverse	TTGACGAAGATCTTGCTCAT	1514-1523	Orciari *et al*., 2001
Psense1	Forward	CGAATCATGATGAATGGAGG	1292-1311	Mochizuki *et al*., 2011.
Panti1	Reverse	TCATTTTATCAGTGGTGTTG	2479-2499	Mochizuki *et al*., 2011.
RVP860-879	Forward	TGCAAGACGACCTGAACCGT	2273-2292	Mochizuki *et al*., 2011.
RVG62-81	Reverse	TGGTATCGTGTAGACGGGGA	3379-3398	Mochizuki *et al*., 2011
Ga3222-40	Forward	CGCTGCATTTTRTCARAGT	3221-3239	Mochizuki *et al*., 2011
Gb4119-39	Reverse	GGAGGGCACCATTTGGTMTC	4116-4135	Mochizuki *et al*., 2011
PV04	Forward	RAAGGYAGRTTTTTYKCDYTRATG	7068–7088	Bourhy *et al*., 2005
PV03	Reverse	CCADMCBTTTTGYCKYARRCCTTC	7526–7503	Bourhy *et al*., 2005
R.11904	Reverse	ACGCTTAACAAATAAACAACA	11924–11904	Campos *et al*., 2011

*Positions along the RABV genome based on the PV strain (GenBank acession number M13215).

Table 2. Course on infection (from day 0 post infection to death) of each of the ten intracerebral passages of the rabies virus (RABV) isolates, included in the present study. Data in days post infection (dpi).

	Passage number									
RABV isolate number	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**	**7th**	**8th**	**9th**	**10th**
IP4005/10	9–11	7–8	6–7	6 – 7	6	6–7	6–7	6	6	5
IP964/06	7–8	5-8	6–7	6 – 7	6	6–7	6 –7	6 – 7	5 – 6	5–6
IP3629/11	7–9	7–9	5	5	4 - 5	3–4	3–4	5	5	5
IP4871/11	7–17	6-7	6	5 – 6	6	5–6	6–7	6 – 7	6	6

Table 3. Monoclonal antibody reactivity profile of the four rabies virus (RABV) isolates passaged in used in the present study. The profiles remained unaltered after ten successive intracerebral passages in mice.

	Monoclonal antibodies (Mabs)							
RABV isolate number	**C1**	**C4**	**C9**	**C10**	**C12**	**C15**	**C18**	**C19**
IP4005/10	-	+	+	+	+	-	-	+
IP964/06	-	+	-	+	+	-	-	-
IP3629/11	+	+	-	+	+	+	-	+
IP4871/11	+	+	+	-	+	+	-	+

Table 4. Specific nucleotide and amino acid substitutions found in the viral phosphoprotein (P) of rabies virus (RABV) isolate IP964/06.

RABV isolate number	Passage Number	Nucleotide position 2177*	Amino acid position 222*
IP964/06	1	Adenine (A)	Isoleucine (Ile)
IP964/06	5	Guanine (G)	Valine (Val)
IP964/06	10	Guanine (G)	Valine (Val)

*Positions along the RABV genome based on the PV strain (GenBank acession number M13215).

One nucleotide substitution was identified and resulted in a nonsynonymous substitutions in putative protein P in isolate (IP964/06) as shown in table 4.

In silico Restriction Endonuclease Analysis (REA)

No change in the profile of restriction endonuclease was found for all RABV isolates with any restriction endonuclease enzyme. Despite of nonsynonymous substitutions in putative protein P in isolate (IP964/06) no change in restriction profile was also identified for isolate IP964/06.

DISCUSSION

In this study, four rabies virus isolates recovered from different rabies reservoirs were submitted to ten successive passages in mice. The antigenic pattern of reactivity was determined by examining all passages by indirect immunofluorescence (IIF) with a panel of 8 monoclonal antibodies (Mabs) raised to RABV nucleoprotein, in addition, the nucleotide sequences of genes for N, G, P, M and L were determined on the first, fifth and tenth passages. The ten successive passages of the four different genetic lineages of RABV did not lead to any alteration neither in the incubation period nor in clinical signs observed in inoculated mice.

Yamaoka et al, 2013, studied the fixed RABV strain Nishigahara and the Ni-CE strain, which has been established after 100 passages in chicken embryo fibroblast cells. The Nishigahara strain killed all mice, whereas the Ni-CE strain caused nonlethal infections. Here, ten successive passages led to no apparent alteration in the capacity to induce lethality in mice of isolates. However, Yamaoka et al, 2013 evaluated strains with a significantly higher number of passages *in vitro*.

The generation of heterogeneity in RABV as in other viruses is related to many factors as period of infection, route of transmission, viral load, immune system of host and interaction between virus and host. Kissi et al, 1999, made successive passages of RABV in different animal species and found some mutations to identify quasispecies in RABV. The mice in study of Kissi were inoculated by intramasseter route and here mice were inoculated by intracerebral route. In the same study, considering inoculation in different species as dogs and cats three successive passages were not enough to identify variations in RABV.

Carnivora and chiroptera include the main reservoirs of RABV in nature. Here, two representative isolates of viruses adapted to species of each of these orders were chosen for the experiments. For RABV isolates recovered from carnivores (IP3629/11- isolated from domestic dogs and IP4871/11 isolated from canids *Cerdocyon thous*) the results were similar in that no identified any antigenic or genetic alterations after ten successive passages. However, genome sequencing revealed an alteration in isolate IP964/06 recovered from non-hematophagous bat *Eptesicus furinalis.*

This finding represents that RABV could be less adapted to non-hematophagous bat *Eptesicus furinalis* once RABV recovered from this specie, shown to be less stable than strain IP4005/10 isolated from haematophagous bat *Desmodus rotundus* that remains the same genetic characteristics after ten successive passages. A nonsynonymous substitution was found in the putative protein of the P gene of RABV isolate recovered from non-hematophagous bat (IP 964/06). One amino acid substitution was identified fifth passage and was maintained in tenth passage. Despite of many random substitution in viruses the maintenance of substitution, is an important point and could be indicate an evolutionary aspect of this isolate because the mutation was fixed, that is, it was identified in the fifth passage and maintained in the tenth passage. Phosphoprotein is the less conserved gene of RABV and an identification of nonsynonimous substitution in the P gene is hope.

Despite many studies have been conducted attempting to identify different genetic lineages related to various species of non-hematophagous bats, studies on the adaptation of the viruses to a particular species are scarce. The findings reported here highlight the need for deepening studies on RABV adaptation to different hosts or reservoir species.

In conclusion the findings reported here highlight the remarkable antigenic and genetic stability of RABV from different hosts or reservoirs of origin. The few genetic alterations detected may be related to the degree of adaptation of the virus to a particular host or reservoir.

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