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Review

Biotechnology advances: A perspective on the diagnosis and research of *Rabies Virus*

S.R. Silva [a,1], I.S.S. Katz [a,1], E. Mori [a], P. Carnieli Jr. [a], L.F.P. Vieira [b], H.B.C.R. Batista [a], L.B. Chaves [a], K.C. Scheffer [a,*]

[a] *Pasteur Institute, São Paulo, Brazil*
[b] *Espírito Santo Institute of Agricultural and Forestry Defense, Espírito Santo, Brazil*

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ABSTRACT

Rabies is a widespread zoonotic disease responsible for approximately 55,000 human deaths/year. The direct fluorescent antibody test (DFAT) and the mouse inoculation test (MIT) used for rabies diagnosis, have high sensitivity and specificity, but are expensive and time-consuming. These disadvantages and the identification of new strains of the virus encourage the use of new techniques that are rapid, sensitive, specific and economical for the detection and research of the *Rabies Virus* (RABV). Real-time RT-PCR, phylogeographic analysis, proteomic assays and DNA recombinant technology have been used in research laboratories. Together, these techniques are effective on samples with low virus titers in the study of molecular epidemiology or in the identification of new disease markers, thus improving the performance of biological assays. In this context, modern advances in molecular technology are now beginning to complement more traditional approaches and promise to revolutionize the diagnosis of rabies. This brief review presents some of the recent molecular tools used for RABV analysis, with emphasis on rabies diagnosis and research.

1. Introduction

Rabies disease, caused by neurotropic viruses of the genus *Lyssavirus*, family Rhabdoviridae, is a widespread zoonotic disease that is most commonly transmitted by the bite of a rabid animal [1]. The virus infects the central nervous system, causing encephalopathy and, ultimately, death. This terrifying illness is responsible for approximately 55,000 human deaths/year, principally in Asia and Africa [2].

Lyssavirus genus is presently divided into 12 species based on nucleotide and amino acid sequences [3,4]. *Rabies Virus* (RABV) represents the prototype virus of the genus, and the other eleven species are known as "rabies-related" lyssaviruses. The RABV genome is single-stranded, non-segmented RNA, which has a negative-sense (minus-strand) polarity, and five structural proteins are coded by the genome: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase or large protein (L). The five genes are separated by four non-coding intergenic sequences [5].

Accurate laboratory results influence the course of treatment and epizootic investigation, besides ensuring the quality of the biological products used for treatment and prevention in humans and animals [6]. Thus, the development of techniques used in the diagnosis and research of RABV has allowed advances in both prophylaxis and control of the disease.

The direct fluorescent antibody test (DFAT) and the mouse inoculation test (MIT) are the gold standard methods for rabies diagnosis [2]. Both DFAT and MIT have high sensitivity and specificity; however, the first method requires an expensive fluorescence microscope, and MIT requires live animals and is a time-consuming test. In addition, DFAT and MIT cannot detect the antigenic and genetic variability of RABV. The initial studies that indicated the variability of RABV were performed with monoclonal antibodies (mAbs) by Wiktor and Koprowski [7,8]. In 1986, the first sequencing of RABV was performed by Noel Tordo [9]. Since then, many studies

* Corresponding author. Pasteur Institute, 393, Paulista Avenue, 01311-000 São Paulo, SP, Brazil. Tel.: +55 11 3145 3183.
E-mail addresses: sandrianabr@gmail.com (S.R. Silva), ianasuly@gmail.com (I.S.S. Katz), eniomori@gmail.com (E. Mori), pedrocarnielijunior@ymail.com (P. Carnieli), luizuenf@yahoo.com.br (L.F.P. Vieira), hruthner@yahoo.com.br (H.B.C.R. Batista), lbchaves@pasteur.saude.sp.gov.br (L.B. Chaves), ksferreira@pasteur.saude.sp.gov.br (K.C. Scheffer).
[1] These authors contributed equally to this work.

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involving the antigenic and molecular characterization of RABV have been conducted [10–18].

The disadvantages of the traditional methods employed for rabies diagnosis and the identification of distinct strains of the virus has encouraged the use of genomic methods for the detection and characterization of RABV. A reverse transcription polymerase chain reaction (RT-PCR) technique has been developed for the detection of rabies viral RNA, and this method has been used in the antemortem diagnosis of human cases [19–21]. Similarly, real-time RT-PCR has been developed for the quantification of viral RNA. The discrimination of the distinct strains of RABV can be performed by sequencing and real-time RT-PCR [16–18]. Genomic methods promise to be transformative for clinical practice, and the rapidly falling cost and turnaround time suggest that this will become a viable technology in diagnostic and reference laboratories in the near future.

In addition, the temporal dynamics of RABV has been determined with phylogeographic studies based on nucleoprotein and glycoprotein sequences of the virus. These analyses reveal the biogeography of the virus by comparing estimates of the phylogenies of populations or species with their geographic distributions over time and space [22–24].

Proteomic studies with different proteins of RABV can be used to elucidate pathogenic and immunogenic properties of the virus, as well as for characterizing new biomarkers, and may be useful in antemortem diagnosis of human rabies [25].

The monoclonal antibody technology initially employed for detection of antigenic variants of the RABV has recently been used as an alternative possibility for treatment in humans and in the development of different tests for the detection of virus or antibodies [26,27]. The recent development of methods for the preparation of recombinant DNA libraries make it possible to create antibodies using gene engineering approaches, improving the characteristics of existing antibodies; this significantly increases the applicability of these molecules [28].

The application of these new techniques for research and diagnosis and a comparison between new and classical methods should improve our understanding of the maintenance of the virus in nature. In this brief review, we discuss some of the recent methodologies used to detect and characterize RABV, with emphasis on diagnosis and research.

2. Current methods for rabies diagnosis and evaluation

2.1. Application of real-time RT-PCR

2.1.1. Rabies diagnosis

In 1985, the polymerase chain reaction (PCR) technique was developed, enabling the identification of specific sequences of DNA, increasing the chances of success in molecular investigations. Over time, the PCR technique has been refined, and today, there are derivations of the methodology used for the detection and identification of RABV. Specific techniques include random amplification of polymorphic DNA (RAPD-PCR), reverse transcription polymerase chain reaction (RT-PCR), nested or hemi-nested PCR (hn-PCR), PCR-enzyme-linked immunosorbent assay (ELISA), multiplex PCR (mPCR) and real-time PCR [19–22,29,30].

A major advance in the diagnosis and characterization of lyssaviruses occurred in 1991, when Sacramento et al. [31] developed the first RT-PCR protocol for RABV. Due to its sensitivity and specificity, this RT-PCR technique did not require viral isolation. Furthermore, it was more than a diagnostic tool because it enabled the genetic characterization of the virus, which has become important for epidemiological studies of human and/or animal rabies [31].

Another technique of interest for the diagnosis of rabies is hemi-nested RT-PCR (hnRT-PCR). In this technique, several cycles of amplification using a set of primers are performed, and the product of this amplification is then reamplified using another set of primers targeted to a sequence that lies within the sequence selected by the first amplification [19]. The first report of an hnRT-PCR protocol described for RABV was by Heaton et al. [32], who was able to detect six species of RABV and rabies-related lyssaviruses. Studies have shown that the ability to detect RABV could be increased by using a second amplification stage [32–34].

Reverse transcription polymerase chain reaction, nested or hemi-nested RT-PCR and other amplification techniques based on genetic material are increasingly used but are not recommended by the World Health Organization (WHO) for routine diagnosis of rabies [2]. However, laboratories with strict quality control procedures and proven experience and knowledge have successfully used molecular techniques to confirm diagnoses and to conduct epidemiological surveys [35].

The utilization of conventional RT-PCR and its derivations in rabies diagnosis provides a rapid and sensitive tool for the diagnostic laboratory; however, it has some disadvantages, such as the generation of spurious bands in some specimens, a high risk of cross-contamination due to the manipulation of amplified product, and exposure to potential carcinogens (ethidium bromide and ultraviolet light) [36–38]. Nucleotide sequencing following RT-PCR of different genes is an additional step that is essential to confirm the specificity of the amplification, allowing the identification of new strains of RABV.

More recently, the traditional molecular technique for the diagnosis of infectious agents has been replaced with real-time RT-PCR, which has many advantages over conventional RT-PCR and overcomes the above-mentioned problems. The real-time RT-PCR assay involves amplification and detection occurring at the same time in a closed tube system, thereby eliminating post-PCR manipulations such as second-round amplification and gel electrophoresis, thereby reducing potential cross-contamination. This technique is rapid and requires less handling, with results available within 4 h (including reverse transcription and melting curve analysis) as opposed to 12 or 20 h using nested or hemi-nested RT-PCR [38]. Continuous real-time RT-PCR monitoring enables a high throughput of large numbers of specimens in a highly standardized format.

Real-time RT-PCR is widely implemented as a detection and quantification method for rabies and other lyssaviruses that are isolated from a variety of sample types and has been found to be sensitive and specific. Compared to the nested/hemi-nested RT-PCR method, real-time RT-PCR achieves at least a similar level of sensitivity for the detection of RABV isolates [20,39]. In addition, using real-time RT-PCR, it is possible to notify the responsible authorities more rapidly, allowing appropriate decisions to be made concerning patient management or disease control.

One of the most common real-time PCR assays utilizes the 5′-nuclease activity of Taq DNA polymerase on a probe called TaqMan™. In this format, amplicon production results in enzymatic cleavage of the probe and the physical separation of the fluorescent reporter from the quencher, producing a fluorescent signal that increases in proportion to the accumulation of a specific amplification product. Generally, the use of fluorogenic probes as detection systems for real time RT-PCR has improved the analytical sensitivity of the assay due to very short amplification products (~100 bp) [40].

An SYBR Green real-time PCR assay is able to detect all known lyssavirus species using a single universal primer pair, making this method an attractive option for laboratory use as a surveillance