MOLECULAR DETECTION AND CHARACTERIZATION OF RABIES VIRUS IN BRAZIL: NEW APPROACHES FOR EPIDEMIOLOGY AND SURVEILLANCE

ABSTRACT

This review describes both Brazilian and worldwide efforts on the use of molecular approaches for rabies virus diagnosis and epidemiology. The main challenges and perspectives of molecular detection, characterization and epidemiology of rabies virus in Brazil for the coming years are also discussed.

INTRODUCTION

The rabies virus is a member of the Rhabdoviridae Family of the Mononegavirales Order, presenting a non-segmented and single-stranded RNA genome with negative polarity (3\'-5\'). The genome codes for five proteins as follows: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L) that are separated by intergenic regions of 2, 2, 5 and 24 nucleotides, respectively, having great importance in genome transcription (Tordo et al. 1986, Finke et al. 2000).

The rabies virus infection causes a fatal encephalopathy in a wide variety of mammalian hosts, determining around 50,000 human deaths/year worldwide (Hemachuda et al. 2002), thus, causing more human deaths than other important infectious diseases such as yellow fever and dengue (Rupprecht et al. 2002). Under-notification must be considered since rabies has already been reported among eight recipients of transplanted corneas in five countries (MMWR 1999), and recently, on June, 2004 the Center for Disease Control and Prevention/USA (CDC) confirmed diagnoses of rabies in three recipients of solid organ transplants (MMWR 2004a, 2004b).

In regions where domestic rabies is controlled, considered to be under control or even not reported, the disease is mainly transmitted to human beings by wild animals such as the fox in Europe, raccoons and skunks in the USA (Acha & Szyfres 1986).
In Brazil, several epidemiological aspects of rabies are unknown, not well understood, or present complex relationships depending on the region. Since the 80's, the southern states of Brazil have been considered free of human rabies in contrast with other regions such as the north and northeastern which still have alarming numbers of human infections, even in major cities such as Salvador, Bahia, where the virus is mainly transmitted to humans by dogs.

In a 11-year period (1980 - 2001), a total of 1,331 human deaths caused by rabies infection were reported in Brazil (FUNASA 2001, OPAS 2001). Moreover, up to USS 15,000,000/year are lost in Brazil due to rabies infection in livestock animals, especially in bovines, causing over 40,000 animal deaths/year (Ministério da Agricultura 1997, Kotait 1998).

Throughout Latin America, vampire bats (especially Desmodus rotundus) are considered virus reservoirs in rural areas and the most important transmitters of the rabies virus to livestock animals (Acha 1967). Several other bat species, especially insectivorous ones, may also play an important role in virus transmission (Favi et al. 2002, Rupprecht et al. 2002). Nowadays, the Pan-American Health Organization (OPAS) considers bats as the second most important rabies transmitters to humans (OPAS 2001).

Considering the presence of naturally infected bats in a wide geographical area and the distinct biology of bat species involved in rabies transmission, it is possible that new human and animal cases of the disease may appear even in areas where rabies is not formerly reported or is considered under control. Several cases of rabies virus transmission from the rural or sylvatic cycle to the domestic cycle in several Brazilian states, as well as in other Latin America countries, have been reported (Carriê et al. 2001, Schaefer et al. 2002, Velasco-Villa et al. 2002).

Rabies virus transmission by vampire bat bites was reported for the first time in 1908 in the State of Santa Catarina, southern Brazil (Carini 1911). Despite the occurrence of numerous cases of bovine rabies in this State, the last cases of human and dog rabies infection were reported in 1981 (FUNASA 2001) and 1987 (Maciel 2000), respectively.

Between January and October 2004, a total of 20 human deaths caused by rabies transmitted by bats have been reported in Brazil. Fifteen of these cases occurred in the Portel locality, Pará State (northeastern Brazil). For the first time in this country, these bat-transmitted cases were superior in number when compared with cases caused by contact with domestic animals. The increasing number of bat-transmitted cases in Brazil could be explained by the human exploitation of wild habitats and the absence of an effective diagnosis network and epidemiological surveillance by the health care system (FIORCruz 2004). Nevertheless, human rabies is certainly under-reported in Brazil, in other Latin American Countries and even in developed countries.

Diagnosis of rabies infection in wild or domestic animals is of utmost importance for health care of exposed patients. Thus, laboratory diagnosis must be fast, sensitive and specific enough to confirm rabies diagnosis in order to allow specific and effective treatment.

Several techniques are currently available and can substantially vary in cost, requirement of hands-on time, human resource training, sensitivity and specificity. Traditionally, rabies diagnosis is carried out by direct Fluorescent Antibody Assay (FAT), and the Mouse Inoculation Test (MIT), but several other techniques such as virus isolation in cell cultures, immunohistochemistry, and reverse transcription coupled with a polymerase chain reaction (RT-PCR) have been proposed (Trimarchi & Smith 2002).

The FAT assay is a fast, low-cost, sensitive and specific method, currently considered the gold-standard technique for rabies diagnosis (Dean et al. 1996). However, due the occurrence of false-negative results in FAT as reported in samples from equines (Peixoto et al. 2000), a confirmatory test is required since misdiagnosis can lead to fatal infections. Considered as a time-consuming method with several ethical issues, the MIT is the most frequently used technique for confirmatory rabies diagnosis, despite the growing ethical issues about experimental animal use (Meslin & Kaplan 1996). Although MIT can be replaced by techniques such as cell culture isolation (King 1996, Wiktor & Clark 1975), it is the preferred diagnostic method in Brazilian laboratories.

The first molecular test conceived for rabies diagnosis was described by Sacramento et al. (1991), who developed a RT-PCR reaction directed to the nucleoprotein gene (N) of rabies virus. The conserved nature and the higher transcription rates

Once in the host cell, the rabies virus polymerase begins to transcribe viral genes in a monocystronic way due to the presence of a polyadenylation signal at the end of each gene (Tordo et al. 1986). Interestingly, the viral polymerase returns to the transcription start point of the same gene and start to transcribe a new copy instead of transcribing the following gene in the genome. This atypical transcription is known as the "cascade model" and considers that transcription efficiency decreases in a rate of -30% at each gene located at the 5' end of the rabies genome (Iverson & Rose 1981).

After the description of RT-PCR for rabies virus diagnosis, several other authors developed PCR-based methodologies aiming to improve the specificity and sensitivity. New oligonucleotides designed to specifically amplify distinct virus variants (Nadin-Davis et al. 1996) or the use of a semi-nested PCR have shown improvements when compared with the original method (Kalmovar et al. 1993, Heaton et al. 1997, Nadin-Davis 1998, Soares et al. 2002). Nevertheless, the use of RT-PCR for rabies virus diagnosis was not endorsed by the World Health Organization despite these encouraging results (Meslin & Kaplan 1999).

Studies for rabies virus detection by RT-PCR were initially carried out in Brazil by Ito et al. (2001a). Using two primer sets to fully or partially amplify the nucleoprotein gene (N), these authors have shown that primers P1/P2 amplified a fragment of 965bp of the N gene in all 49 samples studied, revealing a 100% concordance with FAT and MIT. Moreover, analysis of the RT-PCR results obtained with both primer sets suggested the existence of two different rabies virus populations circulating in Brazil (Ito et al. 2001a).

Soares et al. (2002) evaluated the use of a semi-nested RT-PCR assay for detection of rabies virus in 42 samples from bovines, vampire bats, felines, goats and sheep from the central region of Brazil. The results obtained showed 100% agreement with previous FAT and MIT assays, proving the usefulness of the method for specific and sensitive diagnosis of rabies infection.

Brasil-dos-Anjos (2003) developed a hot start RT-PCR to amplify the whole coding region of the N gene (1,353bp), having tested the assay in a blind study with 72 field samples isolated in Santa Catarina State. In this study, RT-PCR showed 93% agreement with FAT and MIT, corroborating the results of Heaton et al. (1997). In the same study, Brasil-dos-Anjos detected 4 positive samples by RT-PCR that were negative in both FAT and MIT but positive in N2A Cell Culture Isolation (Bordignon et al. 2001).

Phillys et al. (2003) studying the role of vampire bats in rabies transmission in the State of Rio de Janeiro, Brazil, showed that RT-PCR was able to detect all positive samples initially detected by FAT. Moreover, five out of six FAT-negative samples showed evidence of rabies RNA in brain tissues by RT-PCR, corroborating the findings of Brasil-dos-Anjos (2003) that RT-PCR is more sensitive than FAT.

Disagreement between RT-PCR and other assays used for rabies diagnosis has been reported and may have several causes. For instance, the decomposition status of the samples is one of the major concerns, since degradation of rabies proteins and RNA molecules has a strong influence on diagnosis by FAT, MIT or RT-PCR (Heaton et al. 1997, David et al. 2002).

Considering the sensitivity and specificity, as well as the ability to detect the virus in highly decomposed samples, a common situation in tropical countries, the use of RT-PCR directed to the N gene seems to be as efficient as classical techniques for rabies detection. Due to these characteristics, several authors have suggested that RT-PCR could replace MIT as the confirmatory technique for rabies diagnosis, avoiding the use of experimental animals and enhancing the sensitivity of rabies detection (David et al. 2002, Heaton et al. 1997, Kalmovar et al. 1993).

Considering the urgency required for rabies diagnosis, it is well established that RT-PCR is faster than MIT (~6h instead of 21-28 days of MIT), which is of special interest to address medical care to patients possibly exposed to rabies virus and reducing the use of experimentation animals.

Until the mid 1970’s, the rabies virus was considered as antigenically invariable. Studies of cross-protection and determination of antigenic profiles by monoclonal antibodies allowed the distinction of four different serotypes (Schneider et al. 1973, Wiktor et al. 1973, 1980, Wiktor & Kopolowski 1978, Flamand et al. 1980a, 1980b). These assays also
permitted characterization of antigenic variants into distinct serotypes, which are of utmost importance from the public health point of view, allowing the evaluation of the spreading pattern of a virus variant in a particular region as well as its transmission among sylvatic hosts (Diaz et al. 1994, Smith & King 1996, Smith 2002).

One of the most important achievements of molecular diagnosis of rabies infection is the possibility of further molecular typing, revealing important information to public health authorities (Heaton et al. 1997). Nowadays, a total of 8 genotypes of rabies virus are known, as follows: Genotype 1 (Classical rabies) which has a worldwide distribution, Genotype 2 (Lagos Bat), 3 (Mokola) and 4 (Duvenhage) present in distinct areas of the African continent and genotypes 5 and 6 which were isolated from Eptesicus sp. and Myotis sp. bats in Europe, respectively (Kissi et al. 1995). In 1997, Gould et al. proposed the existence of a new genotype (genotype 7), for a rabies virus isolated from Pteropus sp. bats in Australia and involved in human deaths. Recently, the existence of another genotype (genotype 8) was proposed by Arai et al. (2003). The sample was isolated in 1991 from a lesser mouse-eared bat (Myotis blythi) in the Osh region of Kyrgyzstan, central Asia.

Despite this clustering, Badrane et al. (2001) based on the nucleotide sequence of the glycoprotein gene, pathogenicity and immunogenicity characteristics of genotypes 1-7, was able to group all genotypes in two main phylogroups: phylogroup I composed of genotypes 1, 4, 5, 6 and 7, and phylogroup II of genotypes 2 and 3.

In Brazil, two epidemiological cycles of rabies have been identified on the basis of a 203 nucleotide sequence of the N gene: i) a dog-related cycle (DRRV), where virus circulates mainly in dogs and humans and ii) a vampire bat-related cycle (VRRV), mostly present in herbivores, bats and rarely in humans (Ito et al. 2001b). In this study, the authors were pioneers in the molecular characterization of Brazilian rabies virus isolates, providing important information about the molecular epidemiology of rabies in Brazil. Nevertheless, the number of samples and animals examined was quite limited in comparison to the great diversity of infected animals in a wide geographical area of Brazil, revealing the need for more studies.

A third epidemiological cycle for rabies virus in Brazil has been recently proposed on the basis of a sample isolated from marmosets (Callithrix jacchus) in Ceará State, northeastern Brazil. This new cycle was proposed on the basis of immunological and molecular parameters, although no sequence is available for comparison with standard samples from the two well-established cycles (Morais et al. 2000, Favoretto et al. 2001). Despite a single report by Heaton et al. (1997), who identified a genotype 5 rabies virus in a Brazilian bat, all other samples identified in Brazil are grouped as genotype 1 rabies virus despite their epidemiological cycle.

Nucleotide sequencing is the technique most used for molecular characterization of rabies virus (Sacramento et al. 1991, Ito et al. 2001b, Kissi et al. 1995, Brasil-dos-Anjos 2003, Bordinon et al. 2004). Other techniques commonly used are restriction fragment length polymorphism (RFLP) (Ito et al. 2003), RT-PCR labelled with specific probes (Black et al. 2002), RT-PCR assay with specific primers to differentiate between rabies variants (Nadin-Davis et al. 1996), RT-PCR coupled to enzyme-linked immunosorbent assay to distinguish between genotypes (Whitby et al. 1997), and recently a low stringency single and specific primer PCR (LSSP-PCR) based on the G-L intergenic region of rabies virus (Pieri 2003).

Among the above-mentioned techniques, nucleotide sequencing is the most expensive but accurate method for sample characterization requiring, however, specialized personnel to perform the assays and to interpret the results. Thus, the urgent need for a simple and less expensive technique, especially in developing or underdeveloped countries where human and/or animal rabies cases occur.

Another technique is the use of RT-PCR coupled with labelled probes used by Black et al. (2002) for amplification of the N gene, allowing discrimination of the different rabies genotypes through the use of specific probes. This technique is faster than, but as expensive as, sequencing, requiring special equipment and trained technicians. Moreover, probes adapted to the Brazilian rabies isolates, epidemiological cycles and variants must be designed in order to implement the technique in our country.

The use of the RT-PCR assay with specific primers to differentiate between rabies variants as proposed by Nadin-Davis et al. (1996) is very interesting. However, the variability of the virus genome
must be initially revealed in order to confirm the usefulness of the proposed oligonucleotides or the need to design new primers in order to detect regional variants of the virus.

Despite the fact that in Brazil only a few reports of genomic sequences of the rabies virus have been published up to now, Ito et al. (2003) have proposed two sets of primers that were able to discriminate between DRRV and VRRV. However, the existence of new virus variants must be observed and the use of classical or universal primers for the rabies virus followed by sequencing of the PCR products is a powerful tool for characterization and for specific primer design.

The combination of RT-PCR with the enzyme-linked immunosorbent assay (ELISA) reaction as proposed by Whitby et al. (1997) is of limited application due to the requirements of equipment and trained personnel in molecular biology and immunological assays, and showed no increase in sensitivity or specificity in comparison with other techniques such as RT-PCR, RFLP or sequencing.

Recently, Pieri (2003) characterized rabies virus samples isolated in Santa Catarina State by generating fingerprints of the rabies virus G-L intergenic region amplified by RT-PCR (Sacramento et al. 1991) by Low Stringency Single-Specific Primer PCR assay (LSSP-PCR). The G-L intergenic region is a 423bp long fragment and was proposed as a rabies pseudogene by Tordo et al. (1986). The region was characterized as one of the most variable regions of the virus genome, serving as a good target for molecular discrimination of rabies isolates (Sacramento et al. 1991, Sacramento et al. 1992).

Since this region of the rabies genome is little studied among Brazilian isolates, these preliminary results must be expanded in order to define the primers to be used for amplification and fingerprint generation of Brazilian isolates. Nevertheless, the author was able to differentiate standard strains from strains of sylvatic origin. Also, sequencing of the G-L region revealed the absence of some characteristics described by Tordo et al. (1986) when they proposed the pseudogene theory (Bordignon et al. unpublished data). Further studies of the G-L region of the rabies virus genome using a larger number of isolates are being performed at the Federal University of Santa Catarina, Brazil.

Ito et al. (2003) developed a RT-PCR followed by RFLP assay which was able to separate the rabies isolates according to well established epidemiological cycles observed in Brazil, the dog-related (DRRV) and vampire bat-related (VRRV) cycles. Using the BglI and Bsu36 enzymes for digesting the 964bp fragment amplified from the N gene, the authors were able to confirm the origin of virus samples. The technique requires the apparatus for RT-PCR and specific enzymes able to differentiate samples, which is nowadays considered as a relatively simple assay that can easily be adopted as a reference method for diagnosis and typing of rabies virus in Brazil.

The glycoprotein (G), being the only protein of the rabies virus responsible for inducing the production of neutralizing antibodies, is of special importance for rabies vaccine development and efficacy and also another interesting target for studying the molecular epidemiology of the rabies virus (Tordo et al. 1993, Nadin-Davis et al. 1996, Badrane et al. 2001, Sato et al. 2004). Amplification and comparison of both nucleotide and amino acid sequences of the rabies virus glycoprotein from both wild and vaccine strains, could direct vaccine development or improvement, allowing prediction of the vaccine efficacy.

Since the epidemiology of the rabies virus in Brazil is dependent on several biological, geographical and social aspects, which are still not fully comprehended, the studies addressing these questions should face several challenges. Above all, we consider the urgent need to standardize a RT-PCR reaction for rabies detection and characterization, since this technique has proved to be sensitive and specific, being widely spread in Brazilian universities, research centers and governmental institutes. Once having established a national network for rabies diagnosis and molecular epidemiology, the information generated will be of major importance to understand the virus variants and the circulating pattern in each distinct region, to detect the occurrence of cases in a viable, faster and reliable way, to support health authorities in surveillance programs as well as for human resources training.

In conclusion, molecular epidemiological data concerning rabies infection in Brazil is scarce and/or restricted to a specific geographical area (Ito et al. 2001a, 2001b, 2003, Heinemann et al. 2002, Phyllis et al. 2003, Pieri 2003, Bordignon et al. 2005). However, despite revealing promising approaches and some relevant data, the above-mentioned articles are also quite limited in terms of
ACKNOWLEDGMENTS

This work was partially supported by CNPq (Brazilian Government Research Agency).

REFERENCES


Favoretto RS, Mattos CC, Morais BN, Araújo AAF, Mattos CA 2001. Rabies in Marmosets (Callitrix


Maciel RRH 2000. Ocorrência, ciclicidade e evolução de focos de raiva de herbívoros na região da Grande Florianópolis e os morcegos hematofagos Desmodus rotundus (Chiroptera, Phyllostomidae). Monografia do Curso de


Wiktory TJ, Clark HF 1975. Growth of Rabies Virus in Cell Culture. In: GM Baer (ed.). The Natural His-