ADVANCES IN DIAGNOSTIC METHODS AND TYPING OF RABIES VIRUS *

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1 INTRODUCTION.

Significant advances in the techniques available for rabies diagnosis have taken place over the past thirty years. The traditional method of histological examination for Negri bodies in the brain sections has been largely replaced by immunofluorescent staining methods for the detection of nucleocapsid antigen in brain smears (fluorescent antibody test: FAT) (Dean and Abelseth, 1973). Nevertheless, new laboratory methods for rabies diagnosis are still developed in order to increase the specificity and the sensitivity, reduce the time required for diagnosis, extend the detection to all the lyssaviruses, extend the sensitivity of the "intra-vitam" diagnosis and lower the cost of the diagnosis.

Today, there is also a need to find methods for typing isolates and for appreciating their variability throughout the world. To meet these requirements, a progressive polymerase chain reaction (PCR) technique for rabies has been developed recently (Sacramento et al., 1991). Already it is a promising alternative tool for diagnosis, a very efficient means of virus typing and a useful tool in molecular epidemiological studies (Tordo et al., 1992; Smith et al., 1992). PCR is a highly sensitive technique in which contamination through sample-handling must be avoided at all costs, high quality sample collection is an absolute prerequisite.

2 ROUTINE PROCEDURES FOR RABIES DIAGNOSIS (FIG. 1)

2.1 Rapid methods of sample collection.

The opening of the skull for the collection of brain specimens for rabies diagnosis is a relatively long and delicate procedure that should be performed by well trained technicians. It also requires special precautions to avoid accidental exposure to the virus through wounds or by aerosol. Internal brain sampling without autopsy by the introduction of a disposable plastic pipette via the occipital foramen (Barrat and Halek, 1986) or via the retro-orbital route (Montano Hirose et al., 1991) appeared to be particularly rapid and safe in field conditions.

2.2 Virus isolation in cell cultures.

In many laboratories the accurate but time-consuming technique of virus isolation by mouse inoculation has been replaced by a cell-culture technique (rabies tissue culture infection test: RTCIT) employing murine neuroblastoma cells (Porthoi et al., 1982) which is equally as accurate but which can be used to detect the products of virus replication in one or two days rather than in weeks (Bourhy et al., 1989).

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2.3 Detection of rabies nucleocapsid by immuno-enzymatic tests

Considering that not all laboratories possess equipment for immunofluorescence or cell culture, an enzyme-linked immunosorbent assay (Rapid Rabies Enzyme Immuno-Diagnosis: RREID), based on the immunocapture of rabies ribonucleoprotein, was developed (Perrin et al., 1986). This was particularly adapted for routine diagnosis and for epidemiological studies of large numbers of specimens. The results obtained with RREID correlate well with those obtained with FAT and RTCIT (Bourhy et al., 1989; Bourhy and Sureau, 1991). The results are not very much impaired if the specimens have not been maintained imperfectly good conditions for transportation. It is strongly recommended as a back up procedure to corroborate the FAT results, as a simple technique for epizootiological surveys, and as a sensitive technique for laboratories which are not equipped for performing FAT.

The RREID can also be modified by using biotinylated antibodies, streptavidin conjugate and a mixture of monospecific polyclonal antibodies against ribonucleoprotein from Pasteur virus (lyssavirus serotype 1), European Bat lyssavirus subtype 1 (EBL1) and Mokola virus (lyssavirus serotype 3) (Perrin et al., 1992). The modified technique (RREID-lyssa) was used for the detection of ribonucleocapsid of different lyssavirus serotypes. Compared to the RREID, the threshold of detection of the modified technique (RREID-lyssa) was 100 times lower (0.2 ng of viral nucleocapsid whatever the serotype). The sensitivity and the specificity were identical to the RREID. Consequently, RREID-lyssa can be a useful tool for diagnostic laboratories that receive specimens infected by rabies-related viruses.

3 CONTRIBUTION OF MOLECULAR BIOLOGY TO THE RABIES DIAGNOSIS AND TYPING

3.1 Diversity of the lyssaviruses.

Four serotypes of rabies and rabies-related viruses were determined on the basis of seroneutralization and monoclonal antibodies studies: classical rabies virus strains (serotype 1) and Lagos bat virus (serotype 2), Mokola virus (serotype 3), Duvenhage virus (serotype 4) (W.H.O., 1990). European bat lyssaviruses (EBL) were not classified. Rabies virus (serotype 1) is present worldwide except in several protected islands. Rabies-related viruses (serotype 2, 3, 4 and EBL) have a large geographic distribution in Africa and in Europe. All the rabies and rabies-related viruses are pathogenic for mammals including man and lead to a rabies-like encephalitis. Monoclonal antibody studies and cross protection experiments have demonstrated that Mokola virus was the rabies-related virus most distantly related to the vaccinal strains of serotype 1 (King and Crick, 1988).

3.2 Genetic study of the Mokola virus

A molecular study of Mokola virus was undertaken in order to appreciate the maximal diversity of the Lyssavirus genus. Viral RNA was extracted from purified virus propagated in BHK-21 cells. Two primers extrapolated from the PV genome sequence generated 5 molecular clones encompassing the complete Mokola virus genome (Bourhy et al., 1989). The cDNA sequence of the 5568 nucleotide of the 3' moiety and the predicted polypeptide sequence of Mokola virus antigenome was determined (Bourhy et al., 1993). The sequence encodes 5 major non overlapping open reading frames (ORF) corresponding from the 3' to the 5' end to the N, M1, M2, G and the beginning of the L polymerase, respectively. The order of protein conservation in lyssaviruses (nucleoprotein > matrix protein > phosphoprotein) is consistent with the vesiculovirus findings and seems more general in the order Mononegavirales. Nevertheless, whatever the protein, N, M1 or G, a weak conservation of the AA sequence of the antigenic sites defined in the rabies virus was noticed in Mokola virus. These mutations observed in these immunogenic regions explain the lack of cross protection between vaccinal strains and Mokola virus (Tordo et al., 1993).
3.3 Detection of rabies virus nucleic acids

Comparison between the sequences of the Mokola virus (serotype 3) (Bourhy et al., 1993) and the PV strain (serotype 1) (Tordo et al., 1988), two lyssaviruses representative of the most divergent serotypes according to antigenic studies, allowed the delineation of conserved regions within the lyssavirus genomes that can be useful targets for primers. The cDNA synthesis and amplification of the viral transcripts by PCR may then be carried out as previously described (Sacramento et al., 1991). Primers allowing the amplification of the whole nucleoprotein (N), glycoprotein or pseudogene were defined (Bourhy et al., 1992; Sacramento et al., 1992; Tordo et al., 1992). Each of these three genomic target regions possess their own purposes: the N gene can be used for diagnosis, taxonomy, typing, epidemiology and immunological studies; the glycoprotein gene is useful for epidemiology and for immunological studies; the pseudo-gene is interesting for typing and epidemiology. For the purpose of diagnosis (Fig. 1), the PCR products were diluted, heat denatured for 10 min, chilled on ice and then filtered on to a nylon membrane using a multi-well vacuum filtration unit. The nylon membranes used in these blotting techniques were air dried before covalent binding of the nucleic acids by UV illumination at 312 nm for three min (Sacramento et al., 1991). For virus typing, 2-10 µl of the PCR products were digested by a panel of selected restriction enzymes and separated by electrophoresis on a convenient agarose gel in buffer containing ethidium bromide. The pattern was analysed on a print of the gel viewed under UV illumination at 312 nm (Bourhy et al., 1992; Sacramento et al., 1991).

3.4 Typing of rabies viruses after genomic amplification

3.4.1 Introduction

The N gene was chosen for molecular epidemiology studies for several reasons. First of all, in a comparative way, since most of the rabies-related viruses were identified according to their reactivity with antinucleocapsid monoclonal antibodies. Secondly, because of the important role of the nucleoprotein in inducing immunity, in particular against infection with heterologous lyssaviruses. Thirdly, the N gene seems to be a good target for comparison among isolates across a relatively long term evolution. Finally, the study of the N gene respects the progressivity of the PCR technique from a simple diagnosis which has already allowed the amplification of the N gene to a very precise typing by determining its nucleotide sequence. For this purpose, a rapid method to sequence selected genomic areas of wild isolates was developed (Sacramento et al., 1991) (Fig. 1). Where possible, we worked with the original infected brain. Otherwise, suckling mouse brains infected with the original virus at the lowest number of passage available were used. The viral RNA extraction, the cDNA synthesis, the PCR amplification, the purification of the amplified fragment on 0.7% NuSieve GTG agarose and its direct sequencing were performed as described by (Sacramento et al., 1991). Alignment of the deduced amino acid sequence of the N proteins was performed by the ClustalV package of multiple alignment programs (Higgins and Sharp, 1989). The phylogenetic trees were also generated by the ClustalV package of multiple alignment programs according to the neighbour joining method (Saitou and Nei, 1987).

3.4.2 Determination of genotypes

The N gene of seventy rabies and rabies-related viruses representative of the diversity of the Lyssavirus genus were analysed and a phylogenetic tree was constructed. Six tight genetic clusters named genotypes were distinguished (Fig. 2 and 3): (1) rabies virus, (2) Lagos bat virus, (3) Mokola virus, (4) Duvenhage virus, (5) European bat lyssavirus biotype 1 (EBL1) and (6) EBL2. Genotypes 1 and 4 corroborated the previous classification in serotype. However, the genetic grouping appeared more powerful than seroneutralization and NC-MAbs studies in establishing that EBL1 and EBL2 must be considered as independent genotypes. It is also more sensitive to appreciate inter-genotype relationships: genotypes 4 and 5 are related and genotypes 2 and 3 are the most phylogenetically distant from the vaccinal and classical rabies virus of genotype 1. The threshold of similarity below which a new genotype should be defined is in the interval of 96-93% AA similarity.
3.4.3 Determination of geographic lineages

An extensive analysis of rabies virus of genotype 1 collected all over the world was also conducted in order to further evaluate the diversity within the genotype 1 of lyssavirus and more precisely to detect wild reservoirs of canine rabies and to appreciate the divergence of the wild isolates with the vaccinal rabies strains. The nucleotide sequences (1531 b) of the whole nucleoprotein gene and the intergenic nucleoprotein - phosphoprotein region of seventy strains originating from forty different countries were determined. The lineages distinguished by the evolutionary analysis correlate with the geographic origin (Fig. 4). This analysis allowed us to distinguish nine groups of phylogenetically distinct viruses of genotype 1: Africa 1, Africa 2, Asia, Arctic rabies, Europa and Middle East, Latin America 1, Latin America 2 and two groups of vaccinal strains. The group Africa 1 can be divided into Africa la circulating in the North and the East of Africa and into Africa 1b, circulating in the South of the African continent. Viruses of the group Africa 2 are distributed in the central part of Africa. Further analysis of other strains would certainly have allowed us to define other groups. Nucleotide sequences evidence also a common ancestor for fox, raccoon dog, dog and wolf isolates in Europe.

3.4.4 Comparison with vaccinal strains

Genetic variability may complicate the efficacy of a vaccine intended to prevent rabies. If this proves to be the case, genetic heterogeneity, rapid evolution and consequently antigenic diversity will offer rabies virus ample opportunity to evade the host immunity induced by rabies vaccines. The % of similarity between vaccinal strains and wild isolates remains relatively high at the AA level (Fig. 5). This is in favour of a good protection given by the vaccinal strains. Nevertheless, particular attention was given to some strains isolated from reported cases of vaccination or treatment failures in animals or in humans. In those cases the sequence at the antigenic sites were also very similar to those of the vaccinal strains and not different from field strains collected in the same area. The cases of vaccination and treatment failures do not seem to be due to a particular genetic variation.

4 Conclusion

New laboratory methods are now available for the diagnosis of rabies. The fluorescent antibody test should remain the reference technique. Virus isolation in cell culture and RREID were shown to be sensitive and specific confirmatory methods. They can be recommended as a back up procedure to corroborate the FAT results. RREID would also be very useful for laboratories which are not equipped for performing FAT. In the last few years, antigenic analysis has played a major role in viral classification by discriminating between variants. Nevertheless the advent of molecular biology brought more rapid and performant tools. Already, PCR applied to rabies provides a progressive technique starting from diagnosis and ending to a precise genetic characterisation by sequencing of viral limited genetic areas, advantageously replacing seroneutralisation and NC-MAbs studies. For this purpose the study of the nucleoprotein gene appears to be an efficient method discriminating between genotypes and defining phylogenetic relatedness between wild isolates. It allows us, for example, to characterise two different phylogenetic groups in Africa. It also provides a way of rapid analysis for mutations in the antigenic sites of strains isolated from reported cases of vaccination or treatment failures in animals or in humans.

References


Figure 1: Principle and time required for the different diagnosis and typing techniques

Figure 2: Radial phylogenetic tree showing the relationships between the different genotypes of lyssavirus.

Alignments were performed by the ClustalV package of multiple alignment programs (Higgins and Sharp, 1989). The tree was generated according to the neighbour joining method (Saitou and Nei, 1987). The dotted line surrounds the branches corresponding to the strains of genotype 1. The lengths of the branches indicates the phylogenetic distance between the different viruses.
RHABDOVIRIDAE FAMILY - LYSSAVIRUS GENUS

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>GEOGRAPHIC DISTRIBUTION</th>
<th>ANIMAL SPECIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rabies</td>
<td>Worldwide, except: Australia, British Islands, Ireland, New Zealand, Japan, Antarctica, Scandinavia, Hawaii</td>
<td>Man, wild and domestic carnivores and herbivores, bats</td>
</tr>
<tr>
<td>2. Lagos-bat</td>
<td>Nigeria, Central African Republic, South Africa, Zimbabwe, Guinea, Senegal, Ethiopia</td>
<td>Frugivorous bats, cats, dog</td>
</tr>
<tr>
<td>4. Duvenhage</td>
<td>South Africa, Zimbabwe</td>
<td>Man, insectivorous bat</td>
</tr>
<tr>
<td>5. EBL 1</td>
<td>European countries</td>
<td>Man, insectivorous bats: genus Eptesicus, Pipistrellus</td>
</tr>
<tr>
<td>6. EBL 2</td>
<td>European countries</td>
<td>Man, insectivorous bats: genus Myotis</td>
</tr>
</tbody>
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Figure 3: Geographic distribution and host species of lyssaviruses (from King and Crick, 1988; Metbatsion et al., 1992; Bourhy et al., 1993)

Figure 4: Radial phylogenetic tree showing the relationships between the different geographic lineages of lyssavirus. Alignments were performed by the Clusta1V package of multiple alignment programs (Higgins and Sharp, 1989). The tree was generated according to the neighbour joining method (Saitou and Nei, 1987). The length of the branches are not indicative of the phylogenetic distance between the different viruses.
Figure 5: Schematic representation of the % of AA similarity between the different groups of viruses identified in Figure 3.