Detection of rabies virus RNA and antigen in tissues from naturally infected Nigerian dogs: *In situ* hybridization and immunohistochemical studies

S.S. Baba[1]

■ PATHOLOGIE INFECTIEUSE

Key words

Dog - Rabies virus - Antigen - Nucleic acid - Hybridization - Immunohistochemistry - Nigeria.

Summary

Postmortem formalin-fixed and paraffin-embedded tissues (cerebral cortex, hippocampus, cerebellum, trigeminal ganglion and salivary glands) from 25 dogs were acquired from a veterinary diagnostic laboratory in Nigeria and assayed for rabies virus RNA and antigen. Rabies virus genomic RNA and messenger RNA (mRNA) encoding the glycoprotein were detected in tissues with *in situ* hybridization (ISH) using ^{3}H-labeled single-stranded RNA probes. Rabies virus antigen was demonstrated with the avidin-biotin-complex (ABC) immunoperoxidase staining method. Viral mRNA was detected in tissues using digoxigenin-labeled RNA probes. Histopathological diagnosis by hematoxylin and eosin staining revealed Negri bodies in tissues from 8 dogs (32%), and 5 (20%) had inflammatory changes of viral encephalitis without Negri bodies. Antigen was detected in 11 (44%) of the dogs tested, i.e., in the tissues of all 8 dogs with Negri bodies, in 2 of 5 with inflammatory changes, and in 1 of 12 with negative histopathology. Genomic RNA and mRNA were detected in tissues from all dogs that were antigen positive, and the distribution of staining signals for both methods in the neurons and acinar cells of salivary glands was similar. mRNA was more abundant than genomic RNA, and radioactive grains had a diffuse distribution in the perikarya and dendritic processes of neurons, and in salivary acinar cells and ducts. Abundant mRNA was also found in neurons and acinar cells with digoxigenin-labeled probes, and the methodology was simpler. Signals indicating antigen and mRNA were most abundant, followed by genomic RNA, and Negri bodies/histopathology in order of decreasing quantities. The immunoperoxidase staining method and ISH technique using digoxigenin-labeled probes may be useful for rabies research and diagnosis, especially in developing countries, where the disease continues to be a major public health problem.

■ INTRODUCTION

The rabies virus is highly neurotropic in humans and animals and causes acute infection of the central nervous system (CNS).

1. Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Maiduguri, PMB 1069, Maiduguri, Nigeria

Tel.: 234 76 23 29 72

E-mail: yaysib@infoweb.abs.net

Despite the severity of clinical signs, there are no consistent macroscopic lesions and relatively scant microscopic lesions in animals that die of rabies. The most significant microscopic lesions occur in the CNS, and cranial and spinal ganglia. They consist of perivascular cuffing, focal and diffuse gliosis, neuronal degeneration, intracytoplasmic inclusions, or Negri bodies, in neurons (9). Lesions in the salivary glands consist of degeneration and necrosis of acinar epithelial cells, with infiltration of lymphocytes, neutrophils, and plasma cells.

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In most developing countries, formalin-fixed tissues are often used for making a diagnosis of rabies by examining histological sections prepared with acid fuchsin-methylene blue (16) for the presence of Negri bodies. Negri bodies can also be demonstrated in impression smears from fresh (glycerol-saline preserved) brain tissue, stained by the method of Sellers (14). However, these methods lack sensitivity. The standard diagnosis method is to reveal the rabies virus antigen in impression smears of fresh brain by immunofluorescence (IF) (5). The test takes only one to three hours to perform, and is of comparable sensitivity to mouse inoculation, with a concordance of 95-99% between the two methods when the IF test is performed by experienced investigators (2). Therefore, detection of the rabies virus antigen in CNS tissues, performed with either the IF or immunohistochemical (IHC) methods (3, 11) is very useful as a confirmation of rabies. The avidin-biotin-complex (ABC) immunoperoxidase staining technique (IP) is often used particularly when good morphology is important in addition to requirements for sensitive and specific detection of antigens (1, 11). Besides, the method becomes relatively suitable in laboratories where a fluorescent microscope is not available or where facilities for its operation are lacking. Furthermore, since immunoperoxidase-stained slides can be stored longer than IF slides without losing staining intensity, the method is convenient for staining of samples in the field and examination with a light microscope later on. The IF and IP techniques have been modified for retrospective studies involving detection of rabies virus antigens in postmortem formalin-fixed and paraffin-embedded tissues (4, 7, 15).

Advances in recombinant DNA technology have resulted in the ability to detect viral nucleic acids in tissues using hybridization (ISH) techniques (6). ISH allows the localization of viral nucleic acids in tissues with preservation of the morphology of cells and tissues. The detection of messenger RNA (mRNA), that encodes different rabies virus proteins in the CNS of experimentally infected mice and in brain tissues from fatal cases of rabies, has been reported by ISH (11, 12). The method has potential applications for the diagnosis of rabies in selected cases to complement the results of IF or IHC and in future investigation of rabies pathogenesis.

In this study, postmortem tissues from 25 dogs submitted to a veterinary diagnostic laboratory in Nigeria for rabies diagnosis were examined for presence of rabies virus antigen by the ABC immunoperoxidase method. The presence of rabies genomic RNA and mRNA coding for rabies virus glycoprotein was demonstrated by ISH. Differences were noted between the quantities of genomic RNA, mRNA, and antigen in dog tissues.

■ MATERIALS AND METHODS

Dog tissues

Postmortem formalin-fixed paraffin-embedded blocks of tissues (cerebral cortex, hippocampus, cerebellum, trigeminal ganglion, and salivary glands) from 25 dogs were acquired from a veterinary diagnostic laboratory in Nigeria. The dogs were either sacrificed for rabies diagnosis after biting humans or submitted to the laboratory after death from unknown causes. In most cases fresh or frozen whole heads were submitted to the laboratory. The tissues were fixed in 10%-buffered formalin for 10-14 days before they were processed and embedded in paraffin for histopathologic examination. Tissue sections were initially stained by hematoxylin and eosin (H&E) for histopathologic examinations. Tissue blocks from an experimental puppy used in physiological studies were also examined as controls.

Immunoperoxidase staining

Tissue sections were stained for rabies virus antigen by the avidin-biotin-complex peroxidase method as previously described by Jackson and Wunner (12). Deparaffinized slides were successively reacted with 0.004% pepsin (Boehringer, Mannheim, Germany) in 0.01 N HCl at 37°C for 30 min, with 5% normal goat serum, with rabbit anti-rabies serum diluted 1:2000 (obtained from K.M. Charlton, Animal Diseases Research Institute, Nepean, Ontario, Canada), with biotinylated goat anti-rabbit immunoglobulin G diluted 1:200 (Vector Laboratories, Burlingame, CA, USA), with 1% hydrogen peroxide in methanol, with elite avidin-biotinylated horseradish peroxide complex (Vector Laboratories), 3,3'-diamino-benzidine tetrachloride (Polysciences, Warrington, PA, USA) with 0.01% hydrogen peroxide, and finally with 0.5% cupric sulfate in 0.015 M sodium chloride, and the slides were counterstained with hematoxylin. Normal rabbit serum diluted 1:2000 was used as a primary antibody for tissues as control.

Preparation of RNA probes

^{3}H- and digoxigenin-labeled RNA probes were used for localization of rabies virus RNA in tissues. cDNA clone containing the coding sequences for rabies virus glycoprotein (gp) was used to prepare the radiolabeled as well as digoxigenin-labeled RNA probes. A 1.5 kb fragment of the sequence coding for the rabies virus gp of the ERA strain (obtained from Connaught Research Institute, Willowdale, Ontario, Canada) was excised with EcoRI and BamHI from cDNA clone and subcloned into the dual promoter-containing pGEM-2 vector (Promega, Madison, WI, USA). Positive-sense mRNA and negative-sense probes were synthesized in the presence of 5,6-^{3}H UTP (ICN Radiochemicals, Irvine, CA, USA) using either SP6 or T7 RNA polymerase. Negative-sense genomic RNA was also produced in the presence of digoxigenin-11-UTP (Boehringer, Mannheim) using SP6 polymerase. An irrelevant control template was used to prepare ^{3}H-labeled RNA transcripts as control for the specificity of the hybridization reaction.

In situ *hybridization*

In situ hybridization for rabies virus RNA and mRNA was performed as previously described by Jackson and Wunner (12). Briefly, tissue sections were deparaffinized, hybridized with labeled probes at 45°C for 4 h using hybridization mixture containing 0.2 µ of labeled RNA transcripts per milliliter, dipped in NTB2 nuclear tract emulsion (Eastman Kodak Company, Rochester, NY, USA) and exposed for eight weeks at 4°C and developed with D19 developer (Eastman Kodak Company) for 5 min. After development, the sections were counterstained with hematoxylin. With digoxigenin-labeled probe, hybridization products were detected immunologically by application of polyclonal sheep anti-digoxigenin Fab-fragments conjugated to alkaline phosphatase (Boehringer, Mannheim). For both methods, controls included tissue sections pretreated with RNase A (Boehringer, Mannheim), *in situ* hybridization on uninfected tissues with rabies virus RNA probes, and hybridization of rabies virus-infected tissues with the RNA probe prepared from the Riboprobe Gemini control template.

■ RESULTS

Histopathological examination using the H&E staining technique revealed Negri bodies in brain tissues from 8 (32%) dogs, and 5 (20%) dogs had inflammatory changes of viral encephalitis without Negri bodies (table I). The salivary glands from all 8 dogs

Table I

Distribution of test results performed on tissues from suspect dogs (n = 25)

Dog num.	NB	HTP	IP	ISH-^{3}H	ISH-dig.
1	+	-	+	+	+
2	-	-	-	-	-
3	-	+	+	+	+
4	+	-	+	+	+
5	-	-	-	-	-
6	-	-	-	-	-
7	+	-	+	+	+
8	-	-	-	-	-
9	-	+	+	+	+
10	+	-	+	+	+
11	-	+	-	-	-
12	-	-	-	-	-
13	+	-	+	+	+
14	-	-	-	-	-
15	-	+	-	-	-
16	+	-	+	+	+
17	-	-	-	-	-
18	-	-	-	-	-
19	+	-	+	+	+
20	-	+	-	-	-
21	-	-	-	-	-
22	-	-	-	-	-
23	+	-	+	+	+
24	-	-	-	-	-
25	-	-	+	+	-
Total positive (%)	8 (32)	5 (20)	11 (44)	11 (44)	10 (40)

+: positive;
-: negative
NB: Negri bodies
HTP: histopathology
IP: ABC immunoperoxidase method
ISH-^{3}H: *in situ* hybridization using ^{3}H-labeled probes
ISH-dig.: *in situ* hybridization using digoxigenin-labeled probes

with Negri bodies and 2 of 5 with inflammatory changes (table II) had degeneration of acinar epithelial cells with marked infiltration of lymphocytes and neutrophils (figure 1). Histopathological examinations did not reveal changes suggesting significant postmortem autolysis. The method appeared more sensitive for detection of pathological changes in the salivary glands than in other tissues (table II). Abundant rabies virus antigen was also detected in tissues from all 10 dogs with lesions in their salivary glands, as well as in 1 of 12 with negative histopathology and absence of Negri bodies (table I, figures 2 to 6). The sensitivity of ISH using different probes for detection of viral RNA was comparable with the IP method used for localization of viral antigen (tables I and II). Genomic RNA and mRNA were detected in tissues from all dogs that were antigen positive, and the distribution of staining signals for both methods in the infected neurons (figure not shown) and acinar cells of salivary glands (figure 6 to 8) was similar. However, the antigen was demonstrated with greater staining intensity than viral RNA by ISH in neurons (figure not shown). The staining intensity on salivary glands using both methods was comparable (figures 6 to 8). No definite signal was found in the control using digoxigenin (figure not shown) and ^{3}H-labeled probes (figure 9).

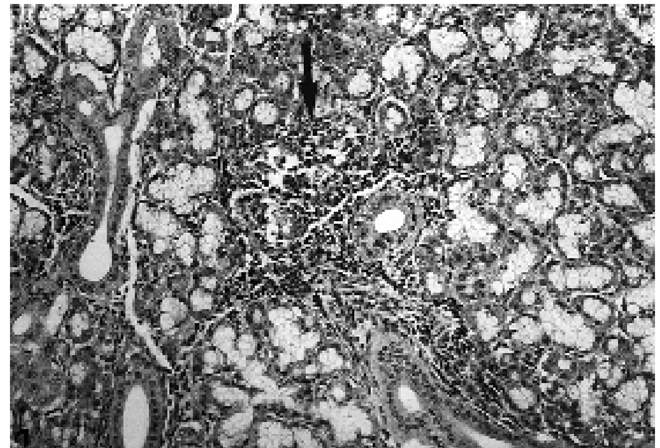

Figure 1: *Dog with rabies; salivary gland. Marked infiltration of lymphocytes in the acinar cells and ducts (arrow). X 155 hematoxylin and eosin.*

Table II

Results of tests performed on different tissues from suspect dogs

Tissues	Total num. tested	Num. positive for NB only (%)	HTP only (%)	IP (%)	ISH-^{3}H (%)	ISH-dig. (%)
Cerebral cortex	25[a]	8 (32)	5 (20)	11 (44)*	11 (44)	10 (40)
Hippocampus	25	8 (32)	5 (20)	11 (44)	11 (44)	11 (44)
Cerebellum	25	8 (32)	5 (20)	11 (44)	11 (44)	11 (44)
Trigeminal ganglion	20[b]	0	5 (25)	2 (10)	2 (10)	2 (10)
Salivary glands	20	NT	10 (50)	10 (50)	10 (50)	10 (50)

NB: Negri bodies; HTP: histopathology
IP: ABC immunoperoxidase method
ISH-^{3}H: *in situ* hybridization using ^{3}H-labeled probes
ISH-dig.: *in situ* hybridization using digoxigenin-labeled probes
[a, b] Number of tissues available from 25 dogs (each tissue from individual dog)
* Consisting of 8 positive for NB, 2 positive for HTP, and 1 negative for NB and HTP
NT: not tested

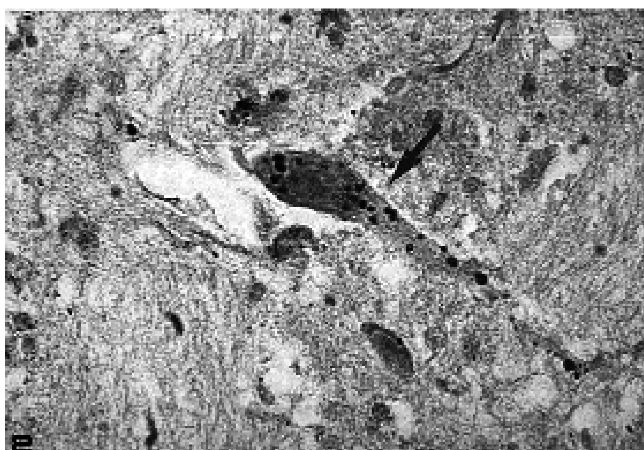

Figure 2: *Dog with rabies; cerebral cortex. Multiple cytoplasmic inclusions (antigen) of various sizes within the perikarya and neuronal process (arrow). X 288 ABC immunoperoxidase and hematoxylin counterstain.*

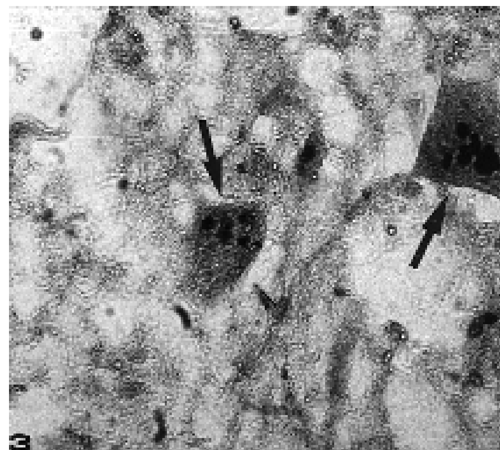

Figure 3: *Dog with rabies; brain stem. Note multiple cytoplasmic inclusions (antigen) of various sizes within the perikarya of the neurons (arrow). X 288 ABC immunoperoxidase and hematoxylin counterstain.*

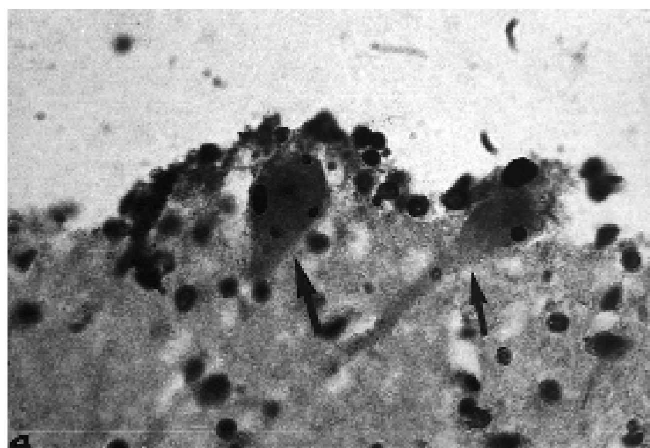

Figure 4: *Dog with rabies; cerebellum. Multiple cytoplasmic inclusions of various sizes within the perikarya of Purkinje cells and processes (arrows). X 288 ABC immunoperoxidase and hematoxylin counterstain.*

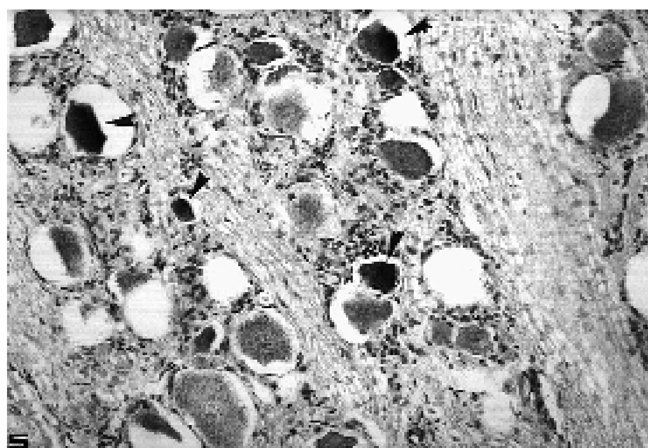

Figure 5: *Dog with rabies; trigeminal ganglion. Massive viral antigen within the neurons (arrowheads). X 288 ABC immunoperoxidase and hematoxylin counterstain.*

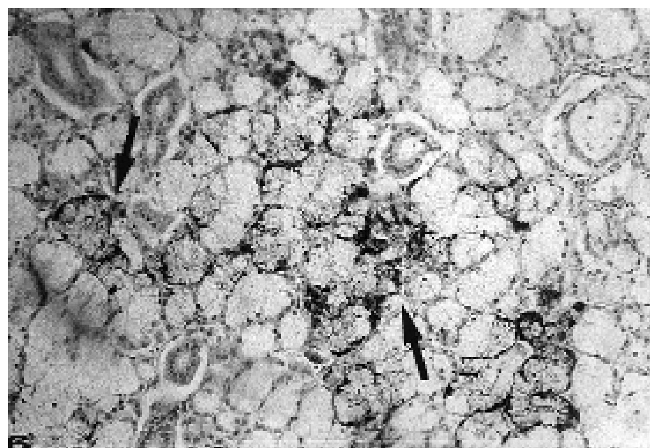

Figure 6: *Dog with rabies; salivary gland. Massive viral antigen within the acinar epithelial cells and ducts (arrows). X 288 ABC immunoperoxidase and hematoxylin counterstain.*

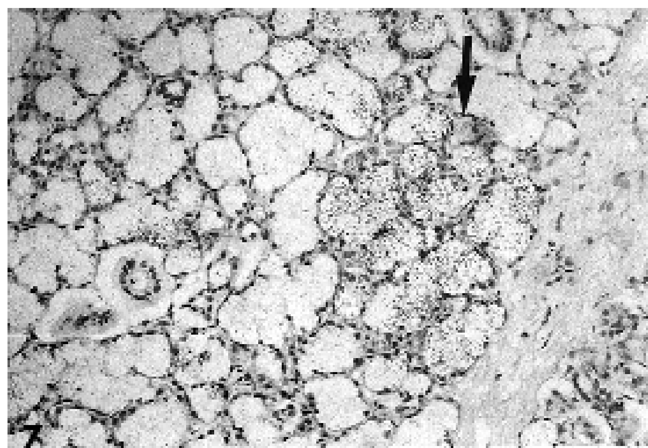

Figure 7: *Dog with rabies; salivary gland. Abundant signal for mRNA in a multifocal distribution in the acinar cells (arrow). X 288* in situ *hybridization (^{3}H-labeled probe) and hematoxylin counterstain.*

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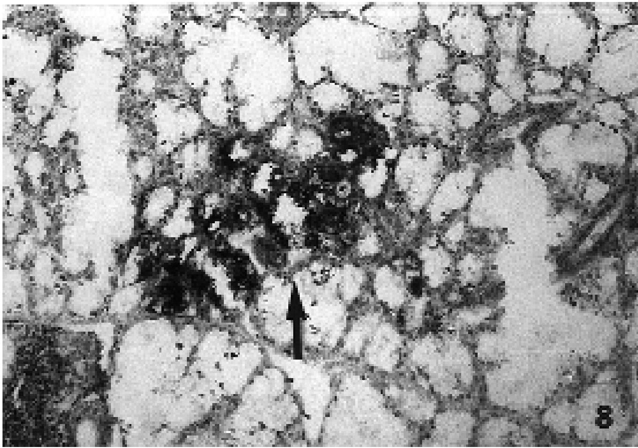

Figure 8: Dog with rabies; salivary gland. Abundant signal for mRNA in a multifocal distribution in the acinar cells (arrow). X 288 in situ *hybridization (digoxigenin-labeled probe); detected by polyclonal sheep anti-digoxigenin Fab-fragment conjugated to alkaline phosphatase.*

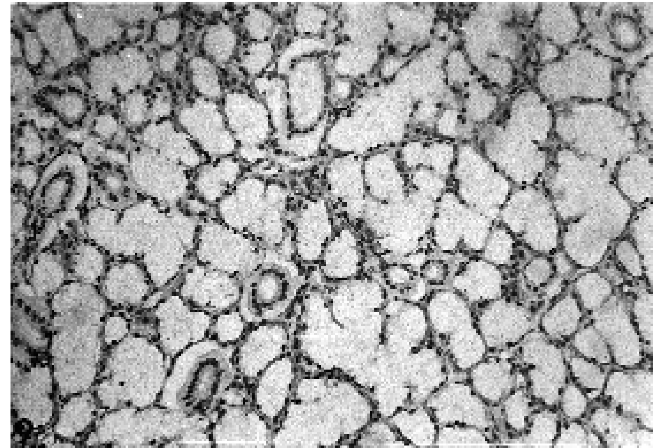

Figure 9: Nonrabid dog; salivary gland (negative control). X 288 in situ *hybridization (^{3}H-labeled probe) and hematoxylin counterstain.*

Expectedly, signals indicating presence of mRNA were more abundant than genomic RNA, and radioactive grains had a diffuse distribution in the perikarya and dendritic processes of neurons, and in salivary acinar cells and ducts. Similar quantities of mRNA (based on distribution of staining signals in tissues) were found in neurons and acinar cells with digoxigenin-labeled probes, and the methodology was simpler than the ISH using ^{3}H-labeled probes. Generally, staining signals indicating antigen and mRNA are more abundant when compared with other methods, followed in decreasing order of quantities by genomic RNA and Negri bodies (figures 2 to 8).

■ DISCUSSION

Viral proteins of rabies virus have been demonstrated in tissues for many years using antigen-detection techniques. Specimens are routinely submitted to diagnostic laboratories for the rapid detection of rabies virus antigens using immunofluorescence (5). Immunoperoxidase staining techniques have also been used to detect rabies virus antigens in tissues, especially when good morphology is important in addition to requirements for sensitive and specific detection of antigens (1, 4, 11).

In this study, the IP method was used to detect rabies virus antigen in formalin-fixed, paraffin-embedded tissues from naturally infected dogs. There was no difference in the sensitivity of the method in the detection of rabies virus antigen either in the brain (cerebral cortex, hippocampus, cerebral cortex) or in the salivary glands of infected dogs. As a result, when the test is used for the detection of virus antigen in paraffinized sections, it is expected to facilitate retrospective studies of the disease and is therefore useful for field studies (7, 11). The disadvantages of IF test, which include the requirement for specialized laboratories and properly immunized personnel to carry out the test on fresh or frozen tissues, and the difficulty in using the technique on formalin-fixed, paraffin-embedded tissues, could be alleviated by the use of the IP method (7). Because of the inadequate transportation infrastructure and power supply as well as the high cost and maintenance of fluorescent microscopes, developing countries may find the use of the IF test prohibitive. Therefore, IP has potential applications for the diagnosis and investigation of rabies especially in developing countries as an alternative to the IF method.

This study has also demonstrated that rabies RNA can be detected in paraffin-embedded tissues in a sensitive and specific manner using *in situ* hybridization. The sensitivity of detection of rabies virus RNA correlated well with the sensitivity of detection of antigen as observed with the IP staining. Since routinely prepared formalin-fixed, paraffin-embedded blocks from naturally infected dogs were used for this study, the *in situ* hybridization technique can also be performed on routine autopsy specimens and may be done retrospectively. Formalin-fixed, paraffin-embedded tissues from routine autopsy materials could also be examined, even after storage for many years (13). A greater abundance of mRNA than genomic RNA was found in infected dog tissues. This finding contrasted with the observation for detection of rabies virus RNA in brains from fatal cases of human rabies, but agreed with that noted in brains of street-virus-infected mice (11, 12). The relative abundance of genomic RNA observed in the human brain has been associated with the possibility of a long-duration postmortem period and the temperature of the body during this interval, which might have influenced the integrity or recovery of mRNA. Changes in the levels of specific RNAs may have occurred in human cases during the agonal state (8, 12). In the case of tissues from infected dogs used in this study, histopathologic examination did not reveal changes suggesting significant postmortem autolysis. Besides, the results of the tests on dog tissues are in concordance with those obtained in other animals including mice (11).

Development of non-isotopic methods, e.g., using biotin-labeled or digoxigenin-labeled probes, have in most cases comparable sensitivity with radiolabeled probes and could be quicker, less expensive, and technically less difficult for wide applications (10). This study revealed that there is little difference in sensitivities between the ^{3}H-labeled and digoxigenin-labeled probes in the detection of rabies virus mRNA in the tissues from infected dogs. However, the methodology using the digoxigenin-labeled probe was simpler and could be adopted for use in developing countries where facilities and expertise for handling radioactive elements are lacking. *In situ* hybridization is not likely to become a routine diagnostic test for rabies in the near future. Nevertheless, the method could be applied in selected cases when antigen detection techniques (IF or IP staining) are equivocal and viral isolation is negative or tissues are unavailable for isolation studies. *In situ* hybridization also has the potential for applications in the study of rabies pathogenesis.

■ CONCLUSION

ABC-elite immunoperoxidase and *in situ* hybridization have been used for the specific detection of rabies virus antigen and RNA, respectively, in postmortem tissues from naturally infected dogs. The methods can be applied to infected and paraffin-embedded tissues to facilitate retrospective studies and ensure good cell morphology in addition to sensitive and specific detection of the viral antigen and RNA. The use of non-isotopic probes for detection of rabies virus RNA in infected tissues has comparable sensitivity with radiolabeled probes and could be quicker, less expensive and technically less difficult than the use of the latter probes. The non-isotopic probe has the potential for applications in developing countries for the diagnosis and investigation of virus diseases.

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Retour au menu

Résumé

Baba S.S. Détection de l'ARN et de l'antigène du virus de la rage dans des tissus de chiens infectés naturellement au Nigeria : hybridation *in situ* et études immunohistochimiques

Des tissus post mortem (du cortex cérébral, de l'hippocampe, du cervelet, des ganglions trigéminaux et des glandes salivaires), fixés dans du formaldéhyde et inclus dans de la paraffine, de 25 chiens ont été obtenus dans un laboratoire de diagnostic vétérinaire du Nigeria et soumis à des tests de recherche de l'ARN et de l'antigène du virus de la rage. L'ARN génomique et l'ARN messager (ARNm) du virus de la rage codant pour la glycoprotéine ont été détectés dans les tissus par la technique d'hybridation *in situ* (HIS) en utilisant des sondes d'ARN à simple brin marquées au ^{3}H. L'antigène du virus de la rage a été mis en évidence par immuno-marquage avec de la peroxydase et le complexe avidine-biotine. L'ARNm viral a été détecté dans les tissus en utilisant des sondes d'ARN marquées à la digoxigénine. Le diagnostic histopathologique par coloration à l'hématoxyline-éosine a révélé des corps de Negri dans les tissus de 8 (32 p. 100) chiens ; 5 (20 p. 100) autres chiens ont présenté des modifications inflammatoires dues à des encéphalites virales sans corps de Negri. L'antigène a été détecté chez 11 (44 p. 100) des chiens examinés, soit dans les tissus des 8 chiens chez lesquels des corps de Negri ont été observés, de 2 des 5 chiens ayant présenté des modifications inflammatoires et d'un chien sur les 12 dont l'histopathologie était négative. L'ARN génomique et l'ARNm ont été détectés dans les tissus de tous les chiens où se trouvait l'antigène, et la distribution des signaux colorant pour les deux méthodes dans les neurones et dans les acini des glandes salivaires a été similaire. L'ARNm était plus abondant que l'ARN génomique et les signaux radioactifs étaient distribués de manière diffuse dans les périkaryons et les processus dendritiques des neurones, et dans les acini et les canaux salivaires. L'ARNm a également été trouvé en abondance dans les neurones et dans les acini avec des sondes marquées à la digoxigénine ; la méthode était plus simple. Par ordre décroissant de quantités, les marqueurs de l'antigène et de l'ARNm étaient les plus nombreux, venaient ensuite ceux de l'ARN génomique et enfin ceux des corps de Negri et d'histopathologie. La méthode de coloration par immunoperoxydase et la technique HIS utilisant des sondes marquées à la digoxigénine peuvent permettre d'effectuer des recherches sur la rage et d'en établir le diagnostic, en particulier dans les pays en développement où cette maladie continue de poser un problème de santé publique de grande importance.

Mots-clés : Chien - Virus de la rage - Antigène - Acide nucléique - Hybridation - Immunohistochimie - Nigeria.

Resumen

Baba S.S. Detección del virus ARN y del antígeno de la rabia en tejidos de perros nigerianos infectados en forma natural: estudios inmunohistoquímicos y de hibridación *in situ*

Tejidos post-mortem fijados en parafina y en formalina (corteza cerebral, hipocampo, cerebelo, ganglio trigémino y glándulas salivales), provenientes de 25 perros, se adquirieron en un laboratorio de diagnóstico veterinario en Nigeria y fueron sometidos a pruebas para la detección del virus ARN y del antígeno de la rabia. El ARN genómico del virus de la rabia y el ARN mensajero (ARNm) que codifican la glicoproteína fueron detectados en tejidos con una hibridación *in situ* (ISH), mediante probadores de ARN uni linear marcados con ^{3}H. El antígeno del virus de la rabia se determinó mediante el método de tinción con inmunoperoxidasa del complejo biotina avidina (ABC). El ARNm viral fue detectado en los tejidos gracias a probadores de ARN marcados con digoxigenina. El diagnóstico histopatológico con tinciones de hematoxilina y eosina reveló corpúsculos de Negri en tejidos provenientes de 8 perros (32%) y 5 (20%) presentaron cambios inflamatorios de encefalitis viral sin corpúsculos de Negri. El antígeno fue detectado en 11 (44%) de los perros examinados, o sea, en los tejidos de todos los perros con corpúsculos de Negri, en 2 de los 5 con cambios inflamatorios y en 1 de los 12 con histopatología negativa. El ARN genómico y el ARNm se detectaron en los tejidos de todos los perros que fueron positivos para el antígeno, con una distribución similar de las señales de tinción mediante ambos métodos, tanto en las neuronas como en las células acinares de las glándulas salivales. El ARNm fue más abundante que el ARN genómico y los gránulos radioactivos presentaron una distribución difusa en los procesos dendríticos y en la pericarya de las neuronas, así como en los ductos y las células acinares. El ARNm también se encontró en forma abundante en las neuronas y en las células acinares con probadores marcados con digoxigenina y con una metodología más simple. Las señales indicadoras del antígeno y del ARNm fueron las más abundantes, seguidas por el ARN genómico y en orden decreciente, la histopatología/corpúsculos de Negri. El método de tinción con inmunoperoxidasa y la técnica ISH, mediante el uso de probadores marcados con digoxigenina, pueden ser útiles para la investigación y el diagnóstico de la rabia, principalmente en los países en desarrollo, en donde la enfermedad representa aún un problema importante para la salud pública.

Palabras clave : Perro - Virus de la rabia - Antígeno - Acido nucleico - Hibridación - Inmunohistoquímica - Nigeria.