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Evaluation of new antibodies for the detection of rabies virus in formalin fixed brain tissue samples

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Abstract

Rabies is an important zoonosis with impact on livestock production. The diagnosis is usually based on the rabies virus detection in fresh or refrigerated brain samples by direct immunofluorescence (IF) performed on fixed brain smears. The aim of this study was to evaluate the performance of 6 monoclonal and 1 rabbit-polyclonal new antibodies for rabies diagnostic by using immunohistochemistry (IHC), which detects the agent on formalin fixed paraffin embedded tissues samples. Tested with 2 positive and 2 negative cows for rabies at dilutions 1/200 and 1/1000, obtained immunostaining was strong for one monoclonal and weak for the polyclonal at both dilutions. For 3 monoclonals, the immunostaining was weaker at 1/1000 and was negative at both dilutions for 2 monoclonals. Unwanted background was absent and negative samples remained clear for all antibodies. When the first monoclonal was applied on sections of brain of 19 cows with Rabies and 41 control cows at 1/1000 dilution, immunohistochemistry recognized all positive samples and was negative for all control cows. The number of cases analyzed did not allow estimating sensitivity and specificity of the tested assay, but the correlation observed between IF and IHC in both positive and negative samples suggested that accuracy of the test might be good. The results indicated that the tested antibody can detect the rabies virus on formalin fixed tissue samples, and that immunohistochemistry can complement other confirmatory tests when those cannot be performed.

Key words: Rabies, cattle, diagnostic, immunohistochemistry.

Introduction

Rabies is a neurologic infectious disease of numerous species caused by a rhabdovirus of the genus *Lyssavirus*. Known for centuries, it still causes thousands of deaths in human beings and animals, both domestic and wild species (3). The rabies virus (RV) is transmitted by bites of infected animals. Its infection produces generally fatal encephalitis after a variable incubation period. Cattle and other farm animals may have different clinical signs. Usually, they suffer the paralytic form of the disease showing ataxia, dysphagia, and death (4, 6). In South America, the hematophagous bat, *Desmodus rotundus*, disseminates the disease witch is distributed from the north of Argentina, and from Chile to Mexico (6, 19). Besides the risk for public health, rabies causes economic losses due to the death of infected animals. In spite of the information about the economics of rabies is scant, it should not be dismissed. For example, the impact of the disease in the livestock industry of Mexico was estimated in US$23,000,000 (2).

Diagnostic of rabies is based on the detection of RV in brain samples. A Direct Immunofluorescence test (IF) is currently applied for routine diagnostic. However, it requires specific equipment that cannot be available in many laboratories. On the other hand, antigen detection can be affected by autolysis, and for this, samples should be quickly shipped refrigerated (3). In many regions, these

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conditions are not easy to achieve and the diagnostic efficiency of IF and other virologic assays may be affected. Moreover, due to the RV remains viable in chilled samples, people involved in diagnostic and shipping could be exposed to the risk of infection (3, 10, 13). Finally, under certain conditions fresh or chilled samples cannot be collected or conserved and another diagnostic strategy should be implemented. Since many fixative agents inactivate the virus, histopathology could help for diagnostic of rabies. The presence of compatible changes (lymphomonocytic encephalitis) and intracytoplasmatic inclusion bodies highly specific (Negri bodies) suggest a strong diagnosis of the disease (4, 10). Unfortunately, intensity of inflammatory response may vary and Negri bodies cannot be detected in all infected animals. For that microscopic examination is not a recommendable test for diagnostic of the disease (3).

Immunohistochemistry (IHC) has been successfully used for detection of RV in several species (1, 4, 10, 11, 13, 14). Since it allows detecting specifically viral antigens in formalin fixed tissue samples, it can be useful for diagnosis of rabies when shipping is difficult, because fixation inactivates the virus. For the IHC execution, the primary antibody results essential since the specific identification of the virus depends on it. However, this reagent is not easily available in many regions, which limit the diagnostic performance for veterinary services. The aim of this study was to evaluate the ability of 7 new antibodies locally produced for the detection of RV in formalin fixed- paraffin embedded tissues by IHC and to test the performance of the IHC procedure used with the routine IF test.

Material and Methods

Antibodies evaluated

The antibodies used were previously produced and evaluated for virus detection by other techniques, such as ELISA, fluorescence microscopy and Western Blot (7, 8, 9). Six of them were monoclonal antibodies and the other, a rabbit polyclonal. The antigen used for the production of the antibodies was a commercial human vaccine (VERORAB, Sanofi Pasteur, France). All monoclonal antibodies recognize glycoprotein anchored in the membrane of infected and/or transduced cells while the polyclonal antibody recognize the whole antigen used (7, 8).

Analyzed animals

Paraffin blocks stored in the pathology archive containing samples of central nervous system of 19 infected and 40 control cows were evaluated. Positive animals were confirmed for rabies by IF. Negative control samples belonged to healthy cows, raised and slaughtered in areas where *D. rotundus* was absent, and did not showed histopathological changes.

Evaluation of the IHC test

The IHC test had 2 different phases. First, the procedure and the ability for RV detection of the 7 antibodies was adjusted by testing each one at dilutions 1/200 and 1/1000 on samples of brain from 4 cows, 2 of them confirmed for rabies and 2 negative controls. At this stage, the antibody that allowed better immunostaining was selected for the next step, which consisted in testing that antibody in all samples (positive and negative).

IHC procedure

For IHC assay, 3 µm in thick sections were obtained from each block, containing the brain stem and cerebellum. Those were mounted on positively charged slides and deparaffinized. A routine IHC protocol previously used was followed (6). Briefly, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (20 min) and antigenic retrieval was performed by humid heat treatment (121°C, 15 min) in citrate buffer (monohydrate citrate, 10 mM, pH 6). After cooling, slides were immersed in TBS buffer (50 mM Tris–HCl, 300 mM NaCl pH 7.6) for 20 min. A blocking step was performed (50 µl BSA 2% (Promega) in TBS, 5 min), after which 40 µl of the primary antibody at dilutions 1/200 or 1/1000 in TBS was added and incubated at 4°C overnight. The reaction was revealed by LSAB2 system (Dako Citomation System) and aminoethylcarbazole (AEC) (DAKO Corp.). Slides were counterstained with Mayer Hematoxylin and coverslipped with permanent aqueous medium (Dako).

Slides were considered positive when immunostaining was present at the same spot in the different slides tested. For comparing both dilutions of primary antibodies at the first stage, IHC results were classified as negative (-), weak (+), moderate (++), and intense (+++), according to the number of stained cells at 400X. When abundant staining was clearly detected at 100X it was classified as intense (5). For the second stage, IHC results were evaluated as positive or negative depending on the presence or absence of immunostaining.

Results

Histopathology

Samples of positive animals in the IF test showed different degree of non-suppurative encephalitis, with lymphoplasmacytic perivascular cuffs. Four cases had additionally Negri inclusion bodies in few neurons (Fig. 1A). Additionally, 2 cases showed multifocal gliosis and focal malacia.

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Immunohistochemistry

Obtained results are summarized in Tables 1 and 2. Positive immunostaining consisted in intracytoplasmic small to medium red spots, which involved the neuronal soma and the axon in many cases (Fig. 1B). IHC was negative for all antibodies when the negative cows were tested.

Table 1. Antibodies tested and immunostaining obtained at dilutions 1/200 and 1/1000. IHC results: -: negative; +: weak; ++: moderate; +++: intense.

Antibody	Type/ Source	IHC Result at 1/200	IHC Result at 1/1000
1	Monoclonal/ Mouse	-	-
2	Monoclonal/ Mouse	++	-
3	Monoclonal/ Mouse	-	-
4	Monoclonal/ Mouse	+++	+++
5	Monoclonal/ Mouse	+	-
6	Monoclonal/ Mouse	++	+
7	Policlonal/Rabbit	+	+

Table 2. IHC results for rabies infected and negative control cows, analyzed with antibody 4.

Paraffin blocks	IHC Results		Total
	Positive	Negative	
Rabies Positive Cows	19	0	19
Control Cows	0	41	41
Total	19	41	60

At dilution 1/200, immunostaining was negative for antibodies 1 and 3, weak for antibodies 5 and 7, moderate for antibodies 2 and 6 (Fig. 1C) and intense for antibody 4 (Fig. 1E). At 1/1000 dilution, it remained negative for antibodies 1 and 3 and turned negative for antibody 2 and 5. The obtained with antibodies 6 and 7 was weak (Fig. 1D), and the corresponding to antibody 4 kept intense (Fig. 1F). Based on these results, antibody 4 was selected for testing IHC in all samples.

When applied in 19 infected cows, IHC with antibody 4 was positive in all cases. Staining was present in the cytoplasm of neurons and their axon. Intensity was variable, from weak to strong, but always easily detected. All 41 healthy animals analysed were negative (Table 2).

Discussion

Known for centuries, rabies is still an important zoonosis that causes important economic losses in animal production. Diagnostic of suspicious animals is crucial for the adoption of control measures. For this, critical reagents should be available for diagnostic laboratories. Four of the 7 new antibodies tested allowed detecting the RV in formalin fixed brain samples from naturally infected cows. Among these, the one selected was capable to detect all positive animals while negative cows were correctly identified without background immunostaining.

Although IHQ is considered useful for diagnostic of rabies, IF is the gold standard test and it should be the first choice for its diagnostic (3). However, requirement of fresh or chilled samples cannot be always accomplished. Since brain sampling for histopathology requires conditions easily accessible for storing, diagnostic based on formalin-fixed paraffin-embedded samples can be useful. In previous reports IF was performed on paraffin-embedded tissues samples, both fixed with 10% formalin and Carnoy's solution (12, 18). Performing IF on paraffin blocks could solve the lack of chilled samples but results can be no reliable due to the inherent fluorescence described for such waxed specimens (17). In addition, IF should be performed in laboratories that have a fluorescent light microscope. Since IHC can be easily performed on paraffin embedded tissues and it has not specific requirements for equipment, it can be considered as a useful tool when samples must be shipped for long distances or when they cannot be shipped immediately.

Virologic test as IF (and the immunoperoxidase assay developed for solving the requirement of a fluorescent microscope), detect the RV with proved high sensitivity and specificity (3, 13, 15). However, these assays do not provide additional information useful for achieving a differential diagnostic if animals are negative for rabies. On the other hand, histopathology allows inferring the etiology of the disease but it is not appropriated for rabies since this must be confirmed for executing control measures or treatments (3). IHC allows the detection of RV antigens and the identification of other agents or detection of pathologic changes in animals with neurologic disorder negative to rabies. Based on this, IHC can be particularly useful in areas where other neurologic disorders are highly prevalent or when other disease could be suspected in areas where rabies is endemic.

In spite of IHC is described by the World Organisation for Animal Health in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals as useful for diagnostic of rabies, it is not routinely used in many laboratories. This could be explained because IF can be executed quickly. However, unavailability of the primary antibody could difficult the IF performing especially in countries where there were not providers for diagnostic supplies. The products tested in the present report detect the RV, particularly the antibody number 4 with which the viral antigen was easily detected in all positive samples.

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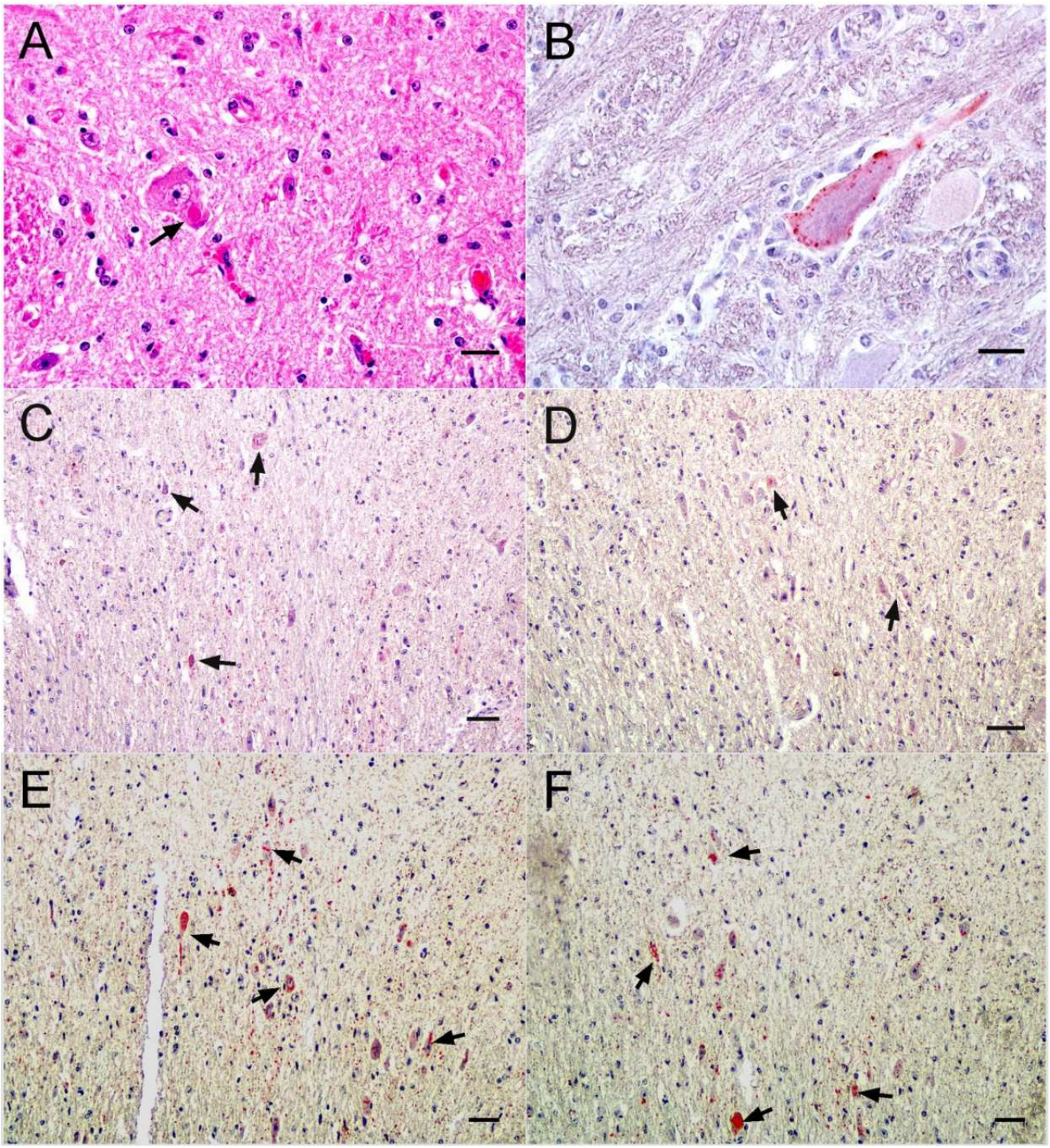


Figure 1. Sections of the brain stem from a cow infected with Rabies Virus. **A.** An intracytoplasmatic Negri inclusion body (arrow) within a neuronal soma from the brain stem. H&E. Scale bar: 50 µm. **B.** Presence of immunostaining in the cytoplasm and the axonal cone of a neuron, obtained with the antibody 4 at 1/1000 dilution. IHC, AEC/Hematoxilin. Scale bar: 50 µm. **C.** Moderate immunostaining easily detected within the cytoplasm of the neurons (arrows), obtained with the antibody 6 at dilution 1/200. IHC, AEC/Hematoxilin. Scale bar: 100 µm. **D.** Weak immunostaining hardly detected within the cytoplasm of the neurons (arrows), obtained with the antibody 6 at dilution 1/1000. IHC, AEC/Hematoxilin. Scale bar: 100 µm. **E.** and **F.** Intense immunostaining within the cytoplasm of the neurons easily detected (arrows), obtained with the antibody 4 at dilutions 1/200 (E) and 1/1000 (F). IHC, AEC/Hematoxilin. Scale bar: 100 µm.

Because the number of positive animals analysed was scanty, sensitivity, specificity and predictive values could not be determined. However, the high correlation between IHC and the IF previously carried out, suggested that the accuracy of the tested technique would be adequate for detection of RV in formalin fixed brain samples. Moreover, all control brain samples (consisted in healthy cows raised and slaughtered in areas free of *D. rotundus*, without microscopic changes) were negative for IHC without background even when dilution of antibodies was low. This indicated that reagents tested did not recognized structures normally present in the brain, which suggested an acceptable specificity for the test. However, further studies should be performed in order to determine the diagnostic ability for this technique.

The antibodies used were produced against G protein but specific epitope of each monoclonal was not determined. In spite of all were successfully tested previously for ELISA and Western blot, 2 of them (antibodies 1 and 3) were negative for IHC even when tested at dilution 1/200. This fact could be related with the masking of epitopes described for many fixative solutions. In spite of an unmasking procedure was performed, it could be possible that the humid head treatment applied was not enough to recover all epitopes (16). Supplementary steps with proteases, acid or alkali should be tested in further studies.

The obtained results with the antibody 4 indicated that it seemed to be able to detect RV in a routine diagnostic. The fact that this is a monoclonal antibody is important, since it can be continuously produced. The technical procedure allows its production in small or big scale by using serum free media that do not require animals or other biologic products. Thus, this antibody can be easily produced, and applied to validated diagnostic procedures, which can be transferred to the animal health systems.

Rabies is still one of the most important zoonosis. IHC can be useful for retrospective studies, when chilled samples of brain cannot be collected or shipped, or in animals that required a differential diagnosis. The obtained results indicated that five of the developed antibodies detected the RV, and one of them was able to identify successfully infected and control animals at high dilution. In spite of further studies are necessary to achieve sensitivity and specificity, the IHC procedure with this monoclonal antibody can be used for diagnostic of rabies as a complement for other virologic tests and collaborate in the control of rabies.

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