Bulgarian Journal of Veterinary Medicine, 2017, **20**, Suppl. 1, 162–169
ISSN 1311-1477 (print); ISSN 1313-3543 (online)

EVALUATION OF DIFFERENT ANTI-RABIES CONJUGATES USED FOR RABIES DIAGNOSIS CONFIRMATION

M. A. DASCALU[1], A. SERVAT[2], F. DARABAN[1], E. VELESCU[1], & O.-I. TANASE[1]

[1]Department of Public Health, Faculty of Veterinary Medicine, Ion Ionescu de la Brad University of Agricultural Sciences and Veterinary Medicine, Iași, Romania; [2]Anses, Laboratory for Rabies and Wildlife, WHO Collaborating Centre for Research and Management in Zoonoses Control, OIE Reference Laboratory for Rabies, European Union Reference Laboratory for Rabies, European Union Reference Laboratory for Rabies Serology, Technopôle Agricole et Vétérinaire, Malzéville, France

Summary

Dascalu, M. A., A. Servat, F. Daraban, E. Velescu & O.-I. Tanase, 2017. Evaluation of different anti-rabies conjugates used for rabies diagnosis confirmation. *Bulg. J. Vet. Med.*, **20**, Suppl. 1, 162–169.

Rabies is a lethal zoonotic disease which affects thousands of humans around the world. It is caused by a virus which belongs to the *Rhabdoviridae* family, *Lyssavirus* genus and affects all warm blood mammals. Diagnosis of the disease is performed by using the Fluorescent Antibody Test, recognised as the `gold standard` method by both WHO and OIE. In this study, we tested 3 different conjugates with the purpose to evaluate them and establish whether there was a direct correlation between the quality of the conjugate, to that of the submitted test samples and the consequences for the human victims if an incorrect diagnosis is obtained.

Key words: anti-rabies conjugate, rabies diagnosis

INTRODUCTION

The main diagnostic method for rabies in the past was histopathological exam of brain specimens. Given the disavantages of this method, represented by a low sensibility and a inconstant presence of the corpuscles in the brain, it has been replaced with newer and more sensitive methods (Bingham *et al.*, 2002).

Hence, the rabies diagnostics was revolutionised by the introduction of the fluorescent antibody test (FAT) which is rapid, simple and highly sensitive and specific (99–100%). FAT is the rabies diagnosis reference method recommended by both WHO and OIE.

In case of FAT doubtful results, the Rabies Tissue Culture Infection Test (RTCIT) (Campbell *et al.*, 1988), which has now extensively replaced the Mouse Inoculation Test (MIT), may be used to propagate and amplify the virus on neuroblastoma cells. This method was developed by Coons and Kaplan in 1950, later being modified for rabies diagnosis by

Goldwasser and Kissling in 1958 (Meslin *et al.*, 1999).

The FAT doesn't require sophisticated equipment or expensive materials, it is easy to perform and the results are obtained after a short period of time, generally between 2 to 4 hours depending on the experience of the specialist (Goldwasser *et al.*, 1959; Tekki *et al.*, 2016). It is performed post mortem on brain specimens (Meslin *et al.*, 1999).

Different types of conjugates are currently commercially available for FAT: *Bio-Rad (*Bio-Rad Laboratories, Marnes-la-Coquette, France), *Fujirebio* (FDI FITC Antirabies Monoclonal Globulin, Fujirebio Diagnostics, Inc. Malvern, PA, USA), *Millipore* (Light Diagnostic TM Rabies DFA Reagent, Livingston, United Kingdom), *Bioveta* (Ivanovice na Hane, The Czech Republic), *Sifin* (Berlin, Germany) and *Rabitest* (ROMVAC S.A., Romania).

The aim of this paper is to perform a comparative study using 3 different conjugates, in order to analyse the quality of these products, their ability to detect the rabies virus, the intensity of fluorescence, the distribution of rabies antigens detected and the background coloration.

MATERIAL AND METHODS

Virus isolates

A panel of 21 brain samples isolated in the North-East of Romania was used in this study (Fig. 1). These samples were submitted by the Sanitary Veterinary and Food Safety Direction (SVFSD) from Iași, Piatra Neamț, Vaslui, Bacău, Galați and Vrancea, being tested by FAT and MIT (only when FAT results were negative or doubtful) and were isolated between 2012 and 2015 from 1 *Felix catus*, 3 *Canis canis*, 7 *Bos taurus*, 1 *Canis lupus*, 1 *Capreolus capreolus* and 8 *Vulpes vulpes* (Table 1). The positive samples obtained by SVFSD were submitted for confirmation to the European Union Reference Laboratory for Rabies, Nancy, France.

Fig. 1. Map of Romania representing the geographical origin of the 21 tested samples.

Materials

In addition to the brain samples, different materials were included to perform the FAT. To validate each assay, a positive and a negative controls were required. Thereby, two positive controls (brains from mouse infected with CVS-27 and mouse infected with EBLV-1) and a negative one (brain from uninfected mouse) were included in each test. Another materials like slides (Immuno-Cell Inc.), acetone (Thermo Chemical, UK), phosphate buffered saline (PBS) (Sigma Life Science, UK), distilled water (Sigma Life Science, UK), mounting fluid, low glycerol (Light Diagnostic, USA), coverslips (Knittel glass, 24 × 60 mm), 3 conjugates, UV microscope (Olympus BX41), laminar hood (Holten LaminAir, 2nd class, Denmark) and incubater Incucell (Fisher Bioblock Scientific, MMM Medcenter) were necessary in performing the technique.

The 3 conjugates used in the study were a) Bio-Rad (Bio-Rad Laboratories, Marnes-la-Coquette, France); b) Fujirebio

Table 1. Characteristics of the Romanian samples tested in the comparative study and results of the rabies diagnosis by FAT

No	City	Host species	Sample code	Year of isolation	Source	Rabies diagnosis by FAT Intensity of fluorescence (4+, 3+, 2+, 1+ and -)		
						Bio-Rad	Fujirebio	Rabitest
1	Bârlad	Fox	DR1016	2013	SVFSD VS	–	–	–
2	Bârlad	Cat	DR1017	2014	SVFSD* VS*	4+	3+	1+
3	Vrancea	Fox	DR1018	2015	SVFSD VN*	3+	3+	1+
4	Vrancea	Fox	DR1019	2014	SVFSD VN	3+	3+	2+
5	Vrancea	Fox	DR1020	2013	SVFSD VN	3+	4+	2+
6	Vrancea	Wolf	DR1021	2014	SVFSD VN	3+	4+	2+
7	Vrancea	Cow	DR1022	2013	SVFSD VN	2+	2+	1+
8	Vrancea	Cow	DR1023	2013	SVFSD VN	2+	2+	2+
9	Bacău	Fox	DR1024	2012	SVFSD BC*	4+	4+	2+
10	Bacău	Fox	DR1025	2012	SVFSD BC	4+	4+	2+
11	Bacău	Dog	DR1026	2012	SVFSD BC	3+	3+	3+
12	Bacău	Deer	DR1027	2012	SVFSD BC	4+	4+	2+
13	Iași	Cow	DR1028	2013	SVFSD IS*	3+	2+	1+
14	Iași	Cow	DR1029	2014	SVFSD IS	4+	2+	1+
15	Iași	Cow	DR1030	2013	SVFSD IS	3+	1+	1+
16	Galați	Cow	DR1031	2015	SVFSD GL*	3+	4+	2+
17	Neamţ	Dog	DR1032	2012	SVFSD NT*	2+	1+	1+
18	Neamţ	Fox	DR1033	2013	SVFSD NT	3+	4+	2+
19	Neamţ	Fox	DR1034	2013	SVFSD NT	3+	4+	2+
20	Neamţ	Cow	DR1035	2012	SVFSD NT	2+	2+	2+
21	Neamţ	Dog	DR1036	2013	SVFSD NT	2+	3+	1+

*SVFSD The Sanitary Veterinary and Food Safety Direction; VS: Vaslui city; VN: Vrancea city; BC: Bacau city; IS: Iasi city; GL: Galati city; NT: Neamt city.

(FDI FITC Antirabies Monoclonal Globulin, Fujirebio Diagnostics, Inc. Malvern, PA, USA) and c) Rabitest (ROMVAC S.A., Romania). Their characteristics are described in Table 2.

Reconstitution of conjugates

The conjugates used in the study were delivered in lyophilised form. The protocol regarding the reconstitution was performed according to manufacturer's recommendations. Consecutively, Bio-Rad was reconstituted using 3 mL of distilled water and centrifuged for 5 min at 1500 rpm, then the supernatant was used for slide coloration. The Fujirebio conjugate was re-suspended in 5 mL of distilled water, let to rest for 30 min until complete dissolving, then was used for coloration. Rabitest was presented as 2 different vials, namely: a lyophilised fluorescent active rabies serum (1 mL) and a lyophilised fluorescent negative control rabies serum (1 mL). Both the active and the control vials were re-suspended separately in 1 mL of distilled water, centrifuged for 10 min at 2000 rpm and the resulting supernatant was used for coloration.

Principle of the method

The monoclonal or polyclonal anti-rabies antibodies, when conjugated with fluorescein isothiocyanate (FITC), form antigen-antibody complex in the presence of

Table 2. The fluorescent conjugates used and their characteristics

Conjugate	BIO-RAD	FUJIREBIO	RABITEST
Manufacturer	BIO-RAD LABORATORIES, Marnes-la-Coquette France	Fujirebio Diagnostics, Inc. Malvern, PA, USA	ROMVAC S.A Romania
Composition	Polyclonal antibodies	Monoclonal antibodies	Rabbit hyperimmune serum
Detection	All genotypes of Lyssavirus, irrespective of the animal species tested	Classical rabies virus RABV (fixed and wild strains) and related viruses (Duvenhage, Lagos Bat and Mokola)	It does not specify
Reconstitution	In 3 mL of distilled water, centrifuging for 5 min at 1500 rpm	In 5 mL of distilled water, stand for 30 min to dissolve	In 1 mL of distilled water, centrifuging 10 min 2000 rpm

rabies virus. If the examined tissue doesn't contain viral antigen the specific antigen-antibody complex is lacking.

The technique was performed according to manufacturer's recommendations. For each conjugate studied, two positive and one negative controls were used. Brain smears were performed on teflon slides (4 smears per slide for each sample to test) and the excess of brain tissue was removed with a wooden spatula.

After drying, impressions were then fixed in ice-cold acetone at –20 °C for 30 min. Slides were air dried under the hood and each smear was stained at 37 °C for 30 min in humid chamber with 50 µL of conjugate. Slides were washed once in phosphate buffered saline and once in distilled water, air dried and mounted with a drop of glycerolated medium. After adding coverslips, slides were examined using a fluorescence microscope with a magnification of ×400.

RESULTS

The results obtained were classified in two categories: a) staining intensity and b) antigen distribution.

Regarding the first category of staining intensity, the results provided by FAT are classified into positive, negative or uninterpretable. Out of the 21 brain samples tested, 20 were positive and one negative by FAT, results in concordance with those obtained by the SVFSD.

According to the conjugates manufacturers, the analysis of the intensity of fluorescence was performed respecting the following scores: 4+ (very bright yellow green fluorescence), 3+ (bright yellow green fluorescence), 2+ (dull yellow green fluorescence), 1+ (dim but detectable yellow green fluorescence) and negative (absence of fluorescence).

In the case of the three conjugates used in the study, both Bio-Rad and Fujirebio expressed a dark background, which resulted in a very good highlight of antigen-antibody complexes, compared to Rabitest, which provided a light-coloured background, making it more difficult to identify these complexes and discriminate them from unspecific fluorescence.

The easiest interpretation and best results were obtained with the Bio-Rad conjugate, giving a dark background and a very bright fluorescence of the antigen-

antibody complex for 5 samples (23.80%) (Fig. 2), a bright fluorescence for 10 samples (47.63%), a dull fluorescence for the other 5 (23.80%) and no fluorescence for one sample (4.77%) (Fig. 5).

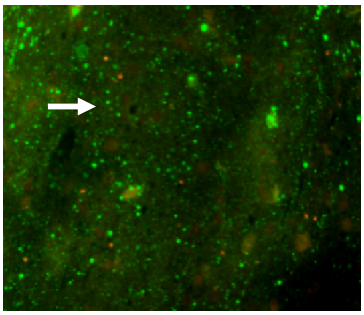

Fig. 2. Positive FAT, stained with Bio-Rad, ×400, DR1027 (arrow).

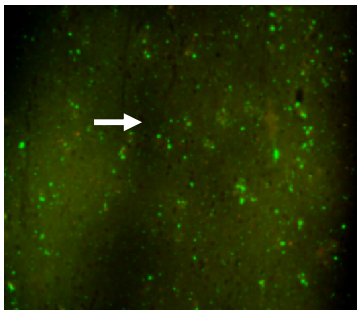

Fig. 3. Positive FAT, stained with Fujirebio, ×400, DR1027 (arrow).

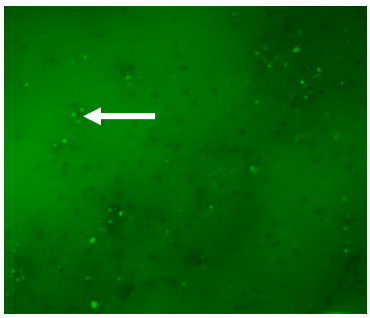

Fig. 4. Positive FAT, stained with Rabitest, ×400, DR1027 (arrow).

Fujirebio ranked second, 8 samples (38.10%) showed a immunosignal scored as 4+ (Fig. 3), 5 samples (23.80%) as 3+, 5 samples (23.80%) as 2+, two samples (9.53%) were scored as 1+ and one sample revealed no fluorescence (4.77%) (Fig. 6).

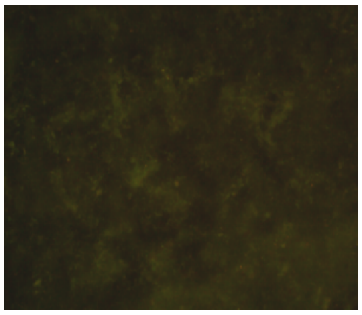

Fig. 5. Negative FAT, stained with Bio-Rad, ×400, DR1016

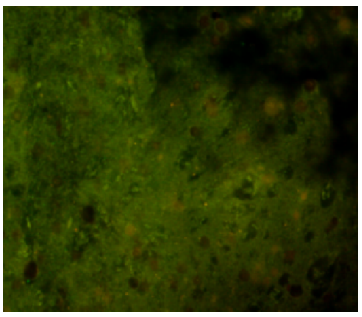

Fig. 6. Negative FAT, stained with Fujirebio, ×400, DR1016.

In the case of Rabitest, the results obtained were inferior to the other two conjugates, no sample was scored as 4+ and only one sample (4.77%) was scored as 3+. Most of the samples were scored in the last two categories: 9 samples (42.85%) were scored as 2+ (Fig. 4), while 10 samples (47.61%) were scored as 1+, with a dull, respectively dim but detectable yellow green fluorescence and one sample with no fluorescence (4.77%) (Fig. 7).

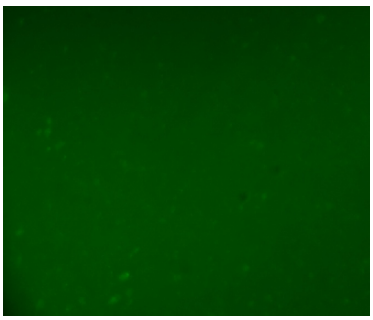

Fig. 7. Negative FAT, stained with Rabitest, ×400, DR1016.

For the second category the antigen distribution was analysed. The amount of the rabies detected antigen was evaluated according to the fluorescence intensity as followed: 4+ (massive infiltration of large and small inclusions in ~100% of the microscopic fields examined per smear), 3+ (inclusions of varying size and shape in ~75% of the microscopic fields examined per smear), 2+ (inclusions of varying size and shape in 10–50% of the microscopic fields examined per smear), 1+ (inclusions of varying size and shape in less than 10% of the microscopic fields examined per smear) and negative (absence of the inclusions in the microscopic field).

Consecutively, the best results were showed by the Bio-Rad conjugate, where 5 samples (23.80%) revealed 100% of the antigen, followed by Fujirebio conjugate, with 3 samples (14.28%) and none for Rabitest. The Fujirebio conjugate revealed 75% of the antigen in 7 samples (33.33%), Bio-Rad conjugate revealed 75% of the antigen in 6 samples (28.57%), while the Rabitest revealed none. With respect to the antigen revealed as 50%, 10 samples were identified by Rabitest (47.61%), 5 samples (23.80%) by Bio-Rad and 3 samples (14.28%) by Fujirebio. Rabitest showed 25% of the antigen in 10 samples (47.61%), the Fujirebio in 7 samples (33.33%), while the Bio-Rad in 4 samples (19.04%), respectively. For the DR1016 sample, no antigen was revealed, being considered negative.

DISCUSSIONS

The fluorescent conjugate is a critical biological factor when performing the FAT. Background for FAT has a remarkable importance for accurate diagnosis in rabies, facilitating the observation of antigen-antibody complexes. It can be useful to differentiate unspecific florescence more easily from antigen-antibody complexes that can lead to an incorrect diagnosis.

Possible reading errors due to unspecific fluorescence may occur when the samples are submitted in bad condition or inadequately preserved, therefore examination of fresh and unaltered tissue is recommended in order to maintain the accuracy of the test (Meslin *et al.*, 1999), because sample degradation will ultimately affect the sensitivity of all diagnostic procedures (Jackson *et al.*, 2002).

In order to avoid false negative or positive results, several criteria must be met, namely: the use of valid controls, the use of a qualitative conjugate with adequate dilution and reconstitution, equipment under optimum operating conditions and very important strict adherence to each step in order to avoid possible cross-contamination between positive and negative controls and the samples (Jackson *et al.*, 2002; Robardet *et al.*, 2013). Moreover, drying of the conjugate in the incubation step (if distilled water is not added in the tray), precipitation of FITC due to inadequate reconstitution, storage or clarifying agents can lead to unspecific fluorescence (Jackson *et al.*, 2002).

The brain areas examined, the duration and type of fixation, the alkalinity of the immersion substance, the amount of glyc-

erol, the use of an appropriate microscope are factors that can contribute to the sensitivity and specificity of the FAT (Rudd *et al.*, 2005; OIE, 2013; Robardet *et al.*, 2013). The immersion substance with a pH of 8.5 is preferred for obtaining a good fluorescence and for maintaining its brightness for a longer period of time (Durham *et al.*, 1986).

To our best knowledge, this is the first study involving the Romanian conjugate on rabies positive samples. To compare his quality, 2 different conjugates were introduced in the study, Bio-Rad and Fujirebio. A paper published in 2013 by Robardet *et al.* compared the last 2 conjugates together with others, used at European level by different national laboratories, obtaining good results. Some results of the conjugates which produced a high background staining and unspecific fluorescence were observed and attributed to the lower concentration of virus in the sample, the working dilution used in the procedure and the lack performance of the laboratories.

Our results showed that the Bio-Rad was the most accurate conjugate able to identify 100% of the antigen, whilst the Rabitest failed in identify such a high concentration of the antigen. According to our study, the most suitable for the analysis of rabies diagnosis by FAT was the Bio-Rad conjugate, giving the highest fluorescence intensity signal and a dark background.

CONCLUSIONS

Our study highlighted the importance of using a qualitative conjugate for the confirmation of rabies diagnosis in animals. In most cases, the quality of the samples subjected for diagnosis next to that of the conjugate are of major importance in obtaining a valid result. False positive or negative results have been reported and may occur if these recommendations are not respected. Although, a false positive result doesn`t have severe consequences, a negative one may have instead tragic consequences when human victims are involved.

REFERENCES

Bingham, J. & M. van der Merwe, 2002. Distribution of rabies antigen in infected brain material: determining the reliability of different regions of the brain for the rabies fluorescent antibody test, *Journal of Virology Methods*, **101**, 85–94.

Campbell, J. B. & K. M. Charlton, 1988. Diagnosis of Rabies Infection In: *Developments in Veterinary Virology – Rabies*, eds W. A. Webster & G. A. Casey, Kluwer Academic Publishers, pp. 201–222.

Durham, T. M., J. S. Smith, F. L. Reid, C. T. Hale-Smith & M. B. Fears, 1986. Stability of immunofluorescence reactions produced by polyclonal and monoclonal antibody conjugates for rabies virus. *Journal of Clinical Microbiol*ogy, **24**, 301–303.

Goldwasser, R. A., R. E. Kissling, T. R. Carski & T.S. Hosty, 1959. Fluorescent antibody staining of rabies virus antigens in the salivary glands of rabid animals. *Bulletin of the World Health Organi*sation, 20, 579–588.

Jackson, A. C. & W. H. Wunner, 2002. Diagnostic evaluation. In: *Rabies*, eds C. Trimarchi & J. S. Smith, Academic Press, USA (Elsevier Science), pp. 307–349.

Meslin, F.-X., M. M. Kaplan & H. Koprowski, 1999. La rage: techniques de laboratoire, quatrieme edition, Organisation mondiale de la sante, Geneve.

OIE Terrestrial Manual, 2013. Rabies (Infection with rabies virus), OIE. Paris.

Palmer, S. R., L. Soulsby& D. I. H. Simpson, 2005. Zoonoze. Editura Științelor Medicale.

Robardet, E., S. Andrieu, T. B. Rasmussen, M. Dobrostana, D. L. Horton, P. Hostnik, I.

Jaceviciene, T. Juhasz, T. Müller, F. Muti¬nelli, A. Servat, M. Smreczak, E. Vanek, S. Vázquez-Morón & F. Cliquet, 2013. Comparative assay of fluorescent antibody test results among twelve European National Reference Laboratories using various anti-rabies conjugates, *Journal of Virology Methods*, **191,** 88–94.

Rudd, R. J., J. S. Smith, P. A. Yager, L. A. Orciari & C. V. Trimarchi, 2005. A need for standardized rabies-virus diagnostic procedures: effect of cover-glass mountant on the reliability of antigen detection by the fluorescent antibody test, *Virus Research*, **111**, 83–88.

Tekki, I. S., Z. N. Ponfa, C. I. Nwosuh, P. R. Kumbish, C. L. Jonah, P. A. Okewole, D. Shamaki & S. M. Ahmed, 2016. Comparative assessment of seller's staining test (SST) and direct fluorescent antibody test for rapid and accurate laboratory diagnosis of rabies, *African Health Science*, **16**, 123–127.