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Comparative assay of fluorescent antibody test results among twelve European National Reference Laboratories using various anti-rabies conjugates

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Twelve National Reference Laboratories (NRLs) for rabies have undertaken a comparative assay to assess the comparison of fluorescent antibody test (FAT) results using five coded commercial anti-rabies conjugates (Biorad, Bioveta, Fujirebio, Millipore, and SIFIN conjugates). Homogenized positive brain tissues infected with various lyssavirus species as well as negative samples were analyzed blindly using a standardized FAT procedure. Conjugates B, C, D, and E were found to be significantly more effective than conjugate A for GS7 (French RABV) diluted samples (1/8 and 1/100) while the frequency of concordant results of conjugates C and D differ significantly from conjugates A, B and E for CVS 27. For detection of EBLV-1 strains, conjugates C and D also presented a significantly lower frequency of discordant results compared to conjugates A, B and E. Conjugates B, C and D were found to be significantly more effective than conjugates E and A for EBLV-2 and ABLV samples. In view of these results, conjugates C and D set themselves apart from the others and appeared as the most effective of this 5-panel conjugates. This study clearly demonstrates that the variability of conjugates used by National Reference Laboratories can potentially lead to discordant results and influence assay sensitivity. In case of false negative results this could have a dramatic impact if the animal under investigation is responsible for human exposure. To avoid such situations, confirmatory tests should be implemented.

1. Introduction

Considering the efforts that are being undertaken to eliminate canine and wildlife rabies in the world, rabies surveillance is of the utmost importance. A condition precedent to the systematic ongoing collection, analysis and interpretation of rabies data and the dissemination of information is a reliable rabies diagnosis (Cliquet et al., 2010). The latter is also an indispensable condition in human medicine as regards the administration of adequate and timely post-exposure prophylaxis (WHO, 2005). Various methods are used for the detection of lyssavirus of the family rhabdoviridae. The fluorescent antibody test (FAT) remains the "gold standard" and consequently the most commonly used rabies diagnostic technique (Meslin et al., 1996; OIE, 2012). Rabies virus antigens are detected in the brain tissue of infected animals using Fluorescein isothiocyanate (FITC) labelled anti-rabies antibodies (Dean et al., 1996). Because of its high sensitivity and specificity this simple technique provides reliable results on fresh specimens within a few hours in 98–100% of cases (OIE, 2012). However, small variations in the procedure, including the area of the brain examined (Bingham and

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van der Merwe, 2002), the duration and type of fixation (Upcott and Markson, 1971), the alkalinity of the mounting medium (Pital and Janowitz, 1963; Durham et al., 1986), the proportion of glycerol (Rudd et al., 2005) and the use of appropriate microscopy filters (Lewis et al., 1973), have also been highlighted as affecting, even critically, the sensitivity and specificity of the FAT (OIE, 2012). This high sensitivity may also be reduced in the case of autolysed and putrefied samples (Lewis and Thacker, 1974; Cliquet et al., 2010). The nature of the FITC labelled anti-rabies antibody conjugate, whether mono or polyclonal, is also one of these critical variables (Rudd et al., 2005). While in-house products are used occasionally by laboratories (Trimarchi and Debbie, 1974; Tzianabos et al., 1976; Ribas Antunez et al., 2005; Caporale et al., 2009) most of the conjugates used are commercial reagents (Robardet et al., 2011). An assessment on a broader international level has never been conducted. Considering that the quality and use of different conjugates could influence the results obtained during routine rabies diagnosis, the comparison of FAT results using different FITC-labelled conjugates was assessed through an international collaborative study in which National Reference Laboratories (NRLs) of Member States of the European Union (EU) participated.

2. Materials and methods

A collaborative study on FITC-labelled conjugates comparison was organized in 2011 by the EURL for Rabies, ANSES Nancy, France. Twelve NRLs from EU Member States participated in the study on a voluntary basis. Each laboratory tested an identical panel of thirty coded lyssavirus positive brain tissue samples, infected potentially with RABV, EBLV-1, EBLV-2 or ABLV, with five different blindly-coded commercially available FITC-labelled anti-rabies conjugates.

2.1. Test sample preparation

Every lyssavirus strain represented in the panel of positive brain tissue samples was produced by intra-cerebral inoculation of either mice or red fox to reproduce standard conditions as closely as possible during the routine rabies diagnosis test (Table 1). Animal experiments to produce positive brain tissues were approved according to animal experimentation directives issued by the French Ethics Committee. Red foxes were killed humanely at the onset of the paralytic stage of the disease while virus production in mice was continued until the death of the animal to collect the maximum amount of virus. For each lyssavirus included in the test panel, e.g. RABV ($N = 3$), EBLV-1 ($N = 2$), EBLV-2 ($N = 1$) or ABLV ($N = 1$), the entire brain tissue of inoculated animals was removed after the death of the animal then homogenized and diluted when needed. To mimic varying amounts of RABV antigen in brain tissue after onset of clinical disease in infected animals, a red fox RABV strain from France was propagated in foxes and the resulting viral stock brain tissue was diluted in a negative red fox brain homogenate to obtain 3 different dilutions (1/1 = undiluted; 1/8, 1/100). Subsequently, all samples were aliquoted into 1 ml tubes and freeze–dried prior to shipment and further testing. A total of 8 different lyssavirus positive brain tissue preparations and a negative control were used for this study (Table 1). Each sample was provided in triplicate in the test panel to improve the statistical power of the study. Consequently the test panel contained 27 randomly coded samples.

2.2. Conjugate preparation

Five different commercially FITC-labelled anti-rabies conjugates used currently within Member States were tested such as:

- Bio-Rad (Marnes-La-Coquette, France); Lyophilized, adsorbed anti-rabies nulceocapsid conjugate 357-2112; batch number 1E0052.
- Bioveta (Ivanovice na Hane, Czech Republic); Anti-rabic Conjugate; batch number 09C03609.
- Fujirebio (Malvern, United States); FITC Anti-Rabies Monoclonal Globulin; batch number 9k02410.
- Millipore (Livingston, United Kingdom); Light Diagnostic TM Rabies DFA Reagent; batch number JH1824149.
- SIFIN (Berlin, Germany); Monoclonal Anti-Rabies, FITC; batch number 59 07 10.

Conjugates were coded randomly (A, B, C, D, E) in order to perform the study blindly. Several vials from the same batch of each conjugate were received. Each of vial conjugate was reconstituted by following strictly the manufacturer's instructions the week before shipping. After reconstitution, the different vials of each conjugate were pooled and aliquoted in opaque tubes in 500 μl (A, B, C, D) or 1.5 ml (E) volumes and then frozen at −20 °C until shipment to participating laboratories. For products for which the impact of freezing was not specified in the manufacturer's instructions (E), laboratory tests were conducted to exclude any negative impact of freezing on conjugate efficacy (data not shown). According to manufacturer instructions, one conjugate (D) had to be diluted extemporaneously; hence, diluents were consequently sent along with the dissolved conjugates to be diluted by the participating laboratories prior to each use. These diluents contained physiological phosphate buffered saline (PBS) (composition: 1.15 Na^2HPO_4, 0.20 g KCL, 0.20 g KH^2PO_4, 8 g NaCl) "Diluent I" or a 20% suspension of Negative Mouse Brain in PBS (Diluent II). Diluent II was prepared by collecting normal mouse brains (NMB). Brains were weighed and pooled to obtain a 20% suspension (weight/volume) in PBS solution. The preparation was homogenized and allowed to stand for 30 min then centrifuged at 330 × *g* for 20 min to remove cell debris.

The working dilutions of conjugates A and D used in the study were those recommended by the manufacturer instructions. When no specific instructions were available like for conjugate B and C, the working dilution was tested previously in the EURL prior to shipment. Final selected optimal working dilutions were 1:8 for conjugate A, 1:50 for conjugate B, 1:70 for conjugate C and 1:20 for conjugate D. Reconstituted conjugate E did not require a

Table 1
Rabies virus strains used in the study.

Batch name	Passaged on	Strain origin	Country	Year of isolation	Infected animal
GS7 1/1	Red fox	GS7	France	1986	*Vulpes vulpes*
GS7 1/8	Red fox	GS7	France	1986	*Vulpes vulpes*
GS7 1/100	Red fox	GS7	France	1986	*Vulpes vulpes*
CVS27 05-10	Mouse	CVS27	Fixed strain	Fixed strain	*Mus musculus*
EBLV-1a 07-10	Mouse	EBLV-1a	France	2000	*Eptesicus serotinus*
EBLV-1b 17-09	Mouse	EBLV-1b	France	2002	*Eptesicus serotinus*
EBLV-2 01-11	Mouse	EBLV-2	United Kingdom	2002	*Myotis daubentonii*
ABLV 08-10	Mouse	ABLV	Australia	1997	*Pteropus alecto*
Negative 19-10	Red fox	Negative	France	2009	*Vulpes vulpes*