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Evaluation of a rabies ELISA as an alternative method to seroneutralisation tests in the context of international trade of domestic carnivores

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A B S T R A C T

For several years, international movements with pets have greatly increased. Most countries have relaxed their quarantine measures and adopted a scheme combining vaccination of pets against rabies followed by a serological test to check the efficacy of vaccination. This new scheme has been strongly supported by the OIE, WHO and the European Commission to facilitate the free movement of people and pets around the world. Currently, only two reference methods are recognised and prescribed (the FAVN test and the RFFIT) to measure rabies antibody levels in serum samples for international trade. They are reliable and valuable methods of assessing the efficacy of rabies vaccination but they are time-consuming and require well-trained people and specialised laboratory facilities. A few years ago, an ELISA (Platelia™ Rabies II kit *ad usum Veterinarium*) was developed for domestic carnivores and wildlife. To our knowledge, this ELISA is the only one certified and prescribed by the OIE. Following its marketing, one task of the EURL for rabies serology was to evaluate the performance of laboratories using this new kit. The results revealed that 26% of the participants, which were already approved laboratories for rabies serology, failed the inter-laboratory trial. Such unsatisfactory results have never been observed during any of the previous proficiency tests organised annually since 2000 by the EURL for rabies serology using reference methods. More investigations were undertaken through internal and collaborative studies to assess the performance of this newly marketed ELISA kit. The results of the internal study revealed that even with a specificity of 100%, the sensitivity evaluated on 593 samples of domestic carnivores came to 78.2%. An issue regarding the underestimation of serum titres was also revealed during the study. The results of a collaborative study involving 23 international laboratories reinforced the preliminary conclusions regarding lack of sensitivity. Indeed, only 5 laboratories out of the 23 obtained satisfactory results. We therefore suggest adopting a threshold of 0.3 EU/mL instead of 0.5 EU/mL to increase the sensitivity of the test.

1. Introduction

Rabies is a zoonotic disease found in all inhabited countries. According to the World Health Organization (WHO), this disease causes around 55 000 fatalities per year, 99% of which are caused by infected dogs. Preventive vaccination of domestic carnivores is the most successful measure to fight against rabies in countries where it is dog mediated (WHO, 2005). The WHO and OIE consider this vaccination to be valid when the neutralising antibody titre is equal or above 0.5 IU/mL in the serum of vaccinated animals and humans (OIE, 2012; WHO, 2005).

For several years, international travel with pets has greatly increased (AHAW Panel, 2006). Most countries have relaxed their quarantine measures and adopted a scheme combining vaccination of pets followed by serological test (Aubert, 1992; Briggs and Schweitzer, 2001; Fooks et al., 2002; Cliquet et al., 2003a; Mansfield et al., 2004) to maintain their rabies-free status. This new scheme has been strongly supported by the OIE, WHO and the European Commission to facilitate the free movement of people and pets around the world. This increase has led the European Commission to establish a system of EU-level approval to guarantee

Abbreviations: OIE, Office international des Epizooties; WHO, World Health Organization; FAVN test, fluorescent antibody virus neutralisation test; RFFIT, rapid fluorescent focus inhibition test; ELISA, enzyme linked immunosorbent assay; EURL, European Union Reference Laboratory; EFSA, European Food and Safety Authority; O.D., optical densities.

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an effective system for monitoring the laboratories performing these tests (European commission, 2000). This has resulted in a strong network of approved laboratories which participate in a proficiency test organised by the European Union Reference Laboratory for rabies serology each year in order to maintain their "approved laboratory" status (European commission, 2010).

Since 1 January 2012, European Regulation 998/2003 regarding the non-commercial movement of pets has changed (European commission, 2003). Since this date, there has been no need to perform a rabies serological test for a pet entering Ireland, Malta, Sweden or the United Kingdom from another EU Member State (AHAW Panel, 2006). Only valid identification, documents attesting to a valid anti-rabies vaccination and treatments against ticks and tapeworms are mandatory. However, it is necessary to maintain these serological controls for pets coming from non-EU countries, where rabies is endemic and not under control, in order to avoid rabies introduction into rabies-free European countries.

Neutralising antibodies are known to be the most reliable indicator of successful vaccination (Clark and Wilson, 1996; Moore et al., 2005; Johnson et al., 2010) in order to ensure satisfactory protection against rabies (Aubert, 1992; Brown et al., 2011). Currently, to measure the neutralising antibody level in a serum sample for international trade, only two reference methods are recognised and prescribed by WHO and OIE: the RFFIT (Smith et al., 1973) and the FAVN test (Cliquet et al., 1998). Both methods are based on the principle of a seroneutralisation of the live rabies virus in sensitive cells. They are highly reliable and valuable methods to assess the efficacy of rabies vaccination but they are time-consuming and require well-trained technicians and special laboratory facilities.

For this reason, new serological techniques have been developed, especially the enzyme linked immunosorbent assay (ELISA) which has already been used in the serological detection of many infectious diseases. These tests reduce times, facilitate handling and avoid the use of biosecurity level 2 or 3 laboratories. Several tests have been developed to be used as alternative methods to rabies seroneutralisation tests for the veterinary field (Barton and Campbell, 1988; Elmgren and Wandeler, 1996; Hostnik and Grom, 1997; Sugiyama et al., 1997; Shimazaki et al., 2003; Inoue et al., 2003; Ogawa et al., 2008; Zhang et al., 2009; Jeon et al., 2012; Nishizono et al., 2012). Several commercialised ELISA assays have been developed to replace the seroneutralisation tests to check the pet vaccination against rabies (Cliquet et al., 2004; Servat et al., 2007) and have been assessed (Arai et al., 2002; Bahloul et al., 2005; Servat and Cliquet, 2006; Welch et al., 2009; Knoop et al., 2010; De Benedictis et al., 2012; Wasniewski and Cliquet, 2012).

A few years ago, an ELISA (Platelia™ Rabies II kit *ad usum Veterinarium*) for domestic carnivores and wildlife was developed by Bio-Rad (Marnes-La-Coquette, France) and ANSES Nancy Laboratory (Servat et al., 2007) evaluated it before its marketing (Servat et al., 2008). This ELISA was the only one certified by the OIE in 2007 (http://web.oie.int/VCDA/eng/Registre/Abstract%20sheet_OIE%20Register_PlateliaRabiesII_v1.pdf). This ELISA kit was also validated by the Pasteur Institute (France) to check the effectiveness of rabies vaccination in human samples (Feyssaguet et al., 2007) and is currently used in France for this purpose.

Following its marketing in spring 2007, one task of the EURL for rabies serology was to evaluate the performance of laboratories using this new kit in the context of international trade in order to propose a new list of approved laboratories for this test to the European Commission. A proficiency test was undertaken including most of the approved laboratories for rabies serology (Wasniewski and Cliquet, 2007) and the success rate was very low compared with that usually obtained for seroneutralisation methods (Wasniewski et al., 2006). Based on this statement and with the support of the European Commission, the EURL for rabies serology undertook further investigations on this newly marketed kit and coordinated a collaborative study with the international approved laboratories in order to assess the performance of this test.

This paper presents the results obtained for the proficiency test, for the internal studies carried out by the EURL for rabies serology and finally those obtained for the collaborative study.

2. Materials and methods

2.1. Samples

2.1.1. Panel of sera for the proficiency test

Our laboratory provided a panel of 11 coded sera to 46 laboratories which were already approved by the European Commission to undertake the rabies serological controls in the context of international trade. This panel contained 2 naive sera from unvaccinated dogs and 9 sera from vaccinated dogs and cats. Among these 9 sera were one high antibody titre serum (obtained from a pool of sera from dogs and cats brought to the laboratory for routine serological controls prior to international animal movements) and 8 different dilutions of this serum in a buffered saline solution. Dilution factors were odd (therefore unpredictable) and every dilution was made once (using a large volume sufficient to be aliquoted for each participant) directly from the high titre sample in order to avoid the repetition of any possible dilution errors in the series. Each sample of the panel was coded, as were the participating laboratories, so that the assessed laboratories blindly tested the panel of sera and the statistical analyses for each laboratory were performed blindly by the reference laboratory. The codes were randomly drawn. Laboratories were asked to test each serum of the panel in three independent runs using the ELISA (Bio-Rad Platelia™ Rabies II kit *ad usum Veterinarium*).

2.1.2. Panel of sera from vaccinated animals for internal study

Five hundred and ninety-three sera were received by our laboratory in the context of international trade and were tested. One hundred and sixty-seven sera were from vaccinated cats, 413 sera were from vaccinated dogs and 13 sera from vaccinated animals whose species were not identified. These 593 sera were tested using both the FAVN test (Cliquet et al., 1998) and the ELISA commercialised by Bio-Rad (Platelia™ Rabies II kit *ad usum Veterinarium*) (Servat et al., 2007). At reception, the sera were heat inactivated (30 min at 56 ± 2 °C) and stored at −20 °C until they were tested by both methods.

2.1.3. Panel of sera for the collaborative study

The samples to be analysed were serum samples with defined titres selected in each participating approved laboratory from its own collection stored at −20 °C. Opting for a panel representative of each laboratory (a total of 23 laboratories took part in this study) enabled the Platelia™ Rabies II kit to be compared with samples mimicking the conditions met in approved laboratories (samples of species from different countries of origin or destination, vaccinated with different rabies inactivated vaccines, etc.).

2.2. Methods

2.2.1. Standard reference serological method: the FAVN test

Rabies neutralising antibodies were determined by the FAVN test (Cliquet et al., 1998; OIE, 2012). The OIE serum of dog origin adjusted to 0.5 IU/mL was used as a positive control. Briefly, each serum sample as well as the positive and negative controls were each distributed in four consecutive wells and then serially diluted. The challenge rabies virus (CVS-11, ATCC VR 959) containing around 100 TCID50/50 μL (TCID50 = 50% tissue culture infective dose) was then added to each serum dilution well. After 60 min of incubation, a volume of 50 μl of 4×10^5 cells/mL suspension was added to each well and the microplates were incubated