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Modification of the fluorescent antibody virus neutralisation test—Elimination of the cytotoxic effect for the detection of rabies virus neutralising antibodies

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A B S T R A C T

The virus neutralisation test is used for the quantitation of specific antibodies in serum samples. However, the success of the test depends on the quality of samples. In the case of poor quality samples, a cytotoxic effect can be observed and the results of the test can be compromised. Additionally, the cytotoxic effect limits the use of different substances, such as muscle extract or liquid from thoracic cavity (thoracic liquid), as a sample for the detection of rabies virus neutralising antibodies in the follow-up of fox oral vaccination campaigns. To eliminate the cytotoxic effect, a modified fluorescent antibody virus neutralisation (mFAVN) test was developed and evaluated. In the mFAVN test, inocula were removed after a 1 h and the cytotoxic effect was prevented. According to the results obtained, the specificity of the mFAVN test compared to the FAVN test was 88.8% and the sensitivity was 94.4%. The diagnostic validity of the test was 0.99 (CI = 0.98–1.00). To evaluate the possibility of using muscle extract and thoracic liquid as samples for the virus neutralisation test, 102 sera, muscle extract and thoracic liquid samples of dog origin were tested with the mFAVN test. The correlation between sera and muscle extracts was 87.9% ($r = 0.88$, $p < 0.001$). The correlation between sera and thoracic liquid was 94.2% ($r = 0.94$, $p < 0.001$). These findings indicated that both muscle extract and thoracic liquid could be used as samples for detection of rabies virus neutralising antibodies in the follow-up of oral vaccination campaigns. To evaluate the level of elimination of the cytotoxic effect, the 102 samples of sera, muscle extracts and thoracic liquid of dog origin were also tested in parallel using the mFAVN and FAVN tests. In the mFAVN test, no instance of cytotoxic effect was observed in the cells. In the FAVN test, two sera (1.9%), 35 muscle extracts (34.3%) and 56 thoracic liquid samples (54.9%) showed cytotoxic effect. The results of this study strongly suggest that cytotoxic effect can be eliminated completely from the rabies virus neutralising antibody detection tests used in the follow-up of oral vaccination campaigns and that very poor quality samples, such as muscle extract and thoracic liquid, can be used.

1. Introduction

The virus neutralisation test is the only method that allows quantitation of specific antibodies. However, in some samples a cytotoxic effect on cells can be observed, and this effect can compromise the reliability of the virus neutralisation test results (Cliquet et al., 2003). This happens particularly when very poor quality sera of foxes are used for the detection of rabies virus neutralising antibodies in the follow-up of fox oral vaccination campaigns. To eliminate the cytotoxic effect on cells, pre-treatment (heat inactivation, centrifugation) and dilution of sera are performed routinely. However, often this is not enough to eliminate completely the cytotoxic effect on cells, especially at lower dilutions. The main reason for the cytotoxic effect on cells is usually the time taken between shooting the animals and sampling, resulting in poor quality samples. Currently, two virus neutralisation tests, rapid fluorescence foci inhibition test (RFFIT) (Smith et al., 1973) and fluorescence virus neutralisation test (FAVN) (Cliquet et al., 1998), are used to evaluate the immunogenicity of human and animal rabies vaccines and both are OIE listed tests (OIE, 2011). As an alternative to the virus neutralisation test, the OIE listed indirect enzyme-linked immunosorbent assay (ELISA) (Platelia Rabies II – Biorad, Marnes-La-Coquette, France) can be used (Cliquet et al., 2003). Recently, a new blocking ELISA (BioPro, Prague, Czech Republic) was developed (Wasniewski and Cliquet, 2012). This new blocking ELISA has yet to be standardised and evaluated by the EU Reference Laboratory for rabies. The monitoring of oral vaccination campaigns is generally conducted by examining the sera of the

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target species for neutralising antibodies (Cliquet et al., 1998). The detection of rabies virus neutralising antibodies in follow-up of fox oral vaccination campaign is generally performed using various modifications of the original RFFIT and ELISA tests (Cliquet et al., 1998). ELISA is particularly suitable for cases of poor quality sera (Cliquet et al., 1998). According to standardisation and validation studies, ELISA has potential to replace the virus neutralisation test (Cliquet et al., 2003) but, agreement between the ELISA and virus neutralisation test was poor (Knoop et al., 2010). In any case, the purpose of both tests is to demonstrate the immunity, which means the presence of rabies virus specific antibodies in animals vaccinated against rabies in either pet travel schemes or follow-up oral vaccination campaigns The collection of non-haemolytic blood is possible if it is obtained immediately after the fox is shot, but this type of sample collection is not possible to organise with volunteer hunters and often the sera cannot be collected until later because of the inappropriate storage of carcasses and external temperature conditions. In these cases, it is possible to use the liquid from thoracic and abdominal cavities or muscle extract as a replacement.

The aim of this study was to modify the virus neutralisation test in order to eliminate the cytotoxic effects on the cells used for the detection of neutralising antibodies in the follow-up studies to fox oral vaccination campaigns. To obtain the most relevant results and because of the ease of sampling, dogs were used as the animal model. Another goal was to evaluate the possibility of using liquid from the thoracic cavity and muscle extracts as samples for detection of specific antibodies against rabies. A modification of the FAVN (mFAVN) test was developed and evaluated in comparison to the FAVN test. In the mFAVN test procedures, an additional step was included. The inocula were removed after 1 h incubation and the cytotoxic effect on cells was eliminated. To prove that the elimination of cytotoxic effect cells was successful, extremely poor quality samples of liquid from the thoracic cavity and samples of muscle extract were tested in parallel with the mFAVN and FAVN tests.

2. Material and methods

2.1. Samples

This study was approved by ethics committee of Veterinary Faculty University of Zagreb; number: 251-61-01/139-11-72. Samples from 102 domestic dogs were collected. The dogs were euthanised because of injury, illness or old age. To obtain sera samples before euthanasia from each dog, blood was taken using vacutainer system. After the euthanasia, the carcasses of each dog were transported to the Croatian Veterinary Institute. In the order to simulate field conditions and sample quality during an oral vaccination campaign, carcasses were placed at ambient temperature for three to five days. Afterwards, the fluid from the thoracic cavity and a piece of *m. femoralis* (approximately 5 cm × 7 cm) was taken from each dog.

The collected blood samples and liquid samples from the thoracic cavity were centrifuged at 220 × *g* for 10 min and the separated sera were stored at −20 °C until testing. The muscles in sterile flasks were frozen for four days and then placed at 4 °C for 3–5 days. From each piece of muscle, a sample approximately 200–300 μl of the muscle extract was collected, centrifuged at 220 × *g* for 10 min and stored at −20 °C prior to analysis. On the day of testing, all samples were heat-treated at 56 °C for 30 min and centrifuged (220 × *g*) one more time.

2.2. Reference serum

Each test (mFAVN and FAVN) included a positive and negative control. The standard used was the OIE positive sera of dog origin with an activity 6 IU (ANSES, Malzeville, France).

The ampoule was reconstituted with 0.5 ml of distillate water and transferred to a sterile tube. The suspension was diluted by 1/12 (titrating 0.5 IU/ml), divided into aliquots of 500 μl and stored at −20 °C. OIE negative serum of dog origin (ANSES, Malzeville, France) was included in each test as a negative control.

To evaluate accuracy of the mFAVN test, known titres of the positive reference serum were titrated in three independent tests. Dilutions of the OIE positive serum (6 IU/ml) were prepared in order to obtain titres of 0.6, 0.3 and 0.15 IU/ml.

2.3. Establishment of the control chart

Before testing, control charts for the reference sera and for the CVS 11 strain of rabies virus (ATCC VR 959) were established. Reference sera and back titration of the working dilution of the virus were titrated in 20 independent trials. The results, expressed as decimal logarithms (D_{50}), were marked and the standard deviation for reference sera was calculated. Allowed values for the virus were 30–300 $TCID_{50}$/50 μl. These values were taken from the procedures described for the FAVN test in the OIE manual for terrestrial animals (OIE, 2011). The mFAVN and the FAVN tests were considered as valid only when the titre of the virus control and D_{50} of the negative and positive sera samples were not significantly different (within one standard deviation) from the mean of the values from the control charts.

2.4. Modified FAVN test (mFAVN)

The modified FAVN test was conducted on 96-well micro tissue culture plates (Nunc, Roskilde, Denmark) using BHK21-13s (ATCC CCl-10) cells and the CVS 11 strain of rabies virus. To prepare the virus stock, suspended cells were infected with 0.5 ml of the virus in 25 ml flasks (Nunc) containing DMEM (PAA, Pasching, Austria) supplemented with 10% heat inactivated foetal calf serum (PAA). The flask was incubated at 37 °C for 48 h and then frozen. After thawing, suspension was centrifuged (300 × *g*). The 75 ml flask (Nunc) with suspended cells was then infected with 8 ml of supernatant from a 25 ml flask and incubated at 37 °C for 72 h. After that, the flask was frozen and thawed and suspension was centrifuged at 300 × *g* for 10 min, dispensed in 1 ml aliquots and stored in liquid nitrogen. The virus was titrated as described below and working dilutions were prepared to obtain 50–300 $TCID_{50}$/50 μl.

For each test, two 96-well plates (Nunc) were used. In each well of the first plate, 100 μl of the cell suspension (diluted in the growth medium to a concentration of 10^6 cells/ml) was added. Plates with cells were incubated for 3 h at 37 °C in a humidified incubator with 5% CO_2. In parallel, on the second plate, serial twofold dilutions (up to 1/256) of the samples (referent sera, sera, liquid from thoracic cavity and muscle extracts) were performed in DMEM (PAA) in 100 μl volumes. For the virus back titrations, 50 μl of the virus was added in four adjacent wells and four-fold serial dilutions (up to 1/65,536) in DMEM (PAA) in 200 μl volumes were made.

In each well with control sera and tested sample, 50 μl of virus at each of the working dilutions was added. Plates with cells and virus/serum mixture were incubated for 1 h at 37° C. After incubation (3 h for cells and 1 h for virus/serum mixture), the medium from the cells was carefully removed with a multichannel electronic pipette. Using a multichannel electronic pipette, 100 μl of virus–serum mixture, virus control and reference sera control from the second plate were transferred to each well in the plate with cells. The second plate was wasted in virucidal disinfectant and discharged. Plates with cells were then incubated for 1 h at 37 °C. After incubation, the supernatant was carefully removed with a multichannel electronic pipette and 100 μl of DMEM (PAA) supplemented with 10% heat inactivated foetal calf serum (PAA) and 1% antibiotic (Zell Shield, Minerva Biolab, Berlin, Germany) was