Production and calibration of the second batch of OIE anti-rabies positive reference serum

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M. Wasniewski [(1)*], J. Barrat [(1)], A.R. Fooks [(2)], R. Franka [(3)], T. Müller [(4)], C. Sabeta [(5)] & F. Cliquet [(1)]

(1) Agency for Food, Environmental and Occupational Health Safety (ANSES) – Nancy Laboratory for Rabies and Wildlife, Technopôle Agricole et Vétérinaire, CS 40009, 54220 Malzéville, France (World Health Organization [WHO] Collaborating Centre for Research and Management in Zoonoses Control, World Organisation for Animal Health [OIE] Reference Laboratory for Rabies, European Union Reference Laboratory for Rabies, European Union Reference Laboratory for Rabies Serology)

(2) Animal and Plant Health Agency (APHA), Wildlife Zoonoses and Vector-borne Diseases Research Group, Weybridge–London, United Kingdom (World Health Organization [WHO] Collaborating Centre for the characterisation of rabies and rabies-related viruses/World Organisation for Animal Health [OIE] Reference Laboratory for Rabies)

(3) Poxvirus and Rabies Branch, Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, United States of America

(4) Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald – Insel Riems, Germany

(5) Agricultural Research Council–Onderstepoort Veterinary Institute (ARC-OVI), 100 Old Soutpan Road, Onderstepoort 0110, Pretoria, South Africa

* Corresponding author: marine.wasniewski@anses.fr

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Summary

The Biological Standards Commission of the World Organisation for Animal Health (OIE) oversees the preparation and validation of OIE-approved International Reference Standards for use in serological assays for detecting infectious diseases of animals or the adequacy of their immune response following vaccination against those diseases. The principal use of OIE-approved International Reference Standards is to harmonise serological testing and to promote the mutual recognition of test results for international trade. In the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, the OIE recommends the use of the OIE anti-rabies positive reference serum of dog origin to titrate serum samples in International Units (IU)/ml for use in rabies serological tests. The first batch of OIE reference serum of dog origin was produced in 1991 and was used internationally until the beginning of 2010. The preparation of the new batch began in 2012 and, in contrast to the previous batch, three commercial inactivated rabies vaccines based on the most frequently used vaccine strains (Pasteur Virus and Flury Low Egg Passage) were selected for the immunisation of dogs in accordance with OIE guidelines. In 2013, calibration was completed through an inter-laboratory test involving five OIE Reference Laboratories for Rabies with the 2nd World Health Organization (WHO) International Standard for Anti-Rabies Immunoglobulin being used as a reference standard in this calibration. After statistical analysis of the results, the consensus titre was established as 5.59 IU/ml. The technical and statistical data were submitted to the OIE for assessment. In February 2014, the OIE

Biological Standards Commission adopted this serum as an OIE-approved standard reagent for rabies serology.

Keywords

Dog – Fluorescent Antibody Virus Neutralisation test – Inter-laboratory test – International reference standard – Rabies – Rabies neutralising antibodies – Rapid Fluorescent Focus Inhibition Test.

Introduction

In order to harmonise serological testing and to promote the mutual recognition of test results for international trade, the World Organisation for Animal Health (OIE) Biological Standards Commission manages a programme for the preparation and validation of OIE-approved International Reference Standards for antibody assays for infectious diseases of animals. Usually, such standard preparations are generated by an OIE Reference Laboratory in accordance with OIE guidelines and are subsequently designated by the OIE as primary reference standards for use in conjunction with tests described in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (mammals, birds and bees) (OIE *Terrestrial Manual*) (1).

Comprehensive rabies control programmes in both dogs and wildlife have helped to eliminate canine and wildlife mediated rabies in a number of countries around the world. However, there is a constant threat of reintroduction through the importation of animals incubating the disease, especially from rabies endemic countries, considering the increasing trend in the non-commercial movements of animals around the world (2). Therefore, to avoid the spread of rabies and reintroduction of the disease into rabies free areas via companion animals (dogs, cats and ferrets), regulations and schemes for the movement of such animals have been in place for many years. Depending on the requirements, these schemes allow companion animals to enter countries via a process involving pet passports, microchipping for identification, vaccination against rabies and serological testing for rabies-specific antibodies. With regard to the

latter, virus neutralisation assays in cell cultures are prescribed tests for checking vaccination response prior to international non-commercial movement or trade of animals, including pets. The OIE reference methods for serology include the Fluorescent Antibody Virus Neutralisation (FAVN) test (3) and the Rapid Fluorescent Focus Inhibition Test (RFFIT) (4), with resulting virus neutralising antibody (VNA) titres expressed in International Units (IU) relative to an international standard antiserum. Therefore, both tests require an international positive reference control (PC) to validate test runs and to obtain harmonised titres between laboratories worldwide. In contrast to rabies serology in human medicine, where a concentrated, purified anti-rabies immunoglobulin (2nd World Health Organization [WHO] International Standard for Anti-Rabies Immunoglobulin, National Institute for Biological Standards and Control, Potters Bar, United Kingdom [UK]) is used as the PC, the OIE *Terrestrial Manual* (1) recommends the use of an OIE reference serum of dog origin as a PC, to mimic:

– adequate vaccination with demonstrable seroconversion and

– the biological characteristics of serum in serological assays.

Since 1991, the production and the determination of the VNA titre of the OIE anti-rabies positive reference serum of dog origin have been undertaken by the OIE/ANSES Reference Laboratory for Rabies (Malzéville, France). The first batch of OIE reference serum of dog origin was produced in 1991, on the request of the OIE, with the objective of using inactivated ‘representative’ street rabies virus (RABV) strains to provide a dog anti-rabies whole serum that could be used worldwide for assessing the immunity of vaccinated dogs and validating newly developed serological tests. For this first batch, three ‘street’ RABV strains representative of the antigenic variability of the virus were selected, adapted and produced on cells, then concentrated and inactivated: one dog strain from Thailand, one dog strain from Morocco and one red fox strain from France. Three groups of five dogs were immunised with these inactivated virus strains to induce rabies virus neutralising antibodies. After sampling and freeze-drying,

the titre of this serum (6.7 IU/ml) was established through an inter-laboratory test involving OIE Reference Laboratories. The 2nd WHO International Standard for Anti-Rabies Immunoglobulin (5) was used as the reference standard for its titre determination. This batch consisted of 1,200 vials containing 0.5 ml of freeze-dried serum and was used until the beginning of 2010, when stocks were depleted. In 2009, a new batch of standard serum of dog origin for rabies serology was prepared as a replacement. The protocol was identical to that used for the production of the first batch. Unfortunately, the titre of the freeze-dried serum was found to be decreasing over time, even when stored at –20°C. This decrease was confirmed at the beginning of 2011 during the inter-laboratory calibration test. For this reason this batch was discontinued.

Therefore, there was an urgent need to produce a new batch of the OIE anti-rabies positive reference serum of dog origin to meet international demand. Indeed, the use of the first batch has shown that more than 99% of vials have been used by more than 53 international laboratories as positive controls in seroneutralisation tests to assess the level of rabies antibodies in vaccinated animals. The second reference serum batch was produced by immunising dogs with commercial vaccines using three inactivated monovalent anti-rabies veterinary vaccines based on Pasteur Virus (PV) and Flury Low Egg Passage (Flury LEP), the most frequently used vaccine strains. Details of the production and calibration of the second batch of OIE reference canine serum for rabies are reported here.

Materials and methods

Ethical aspects

The experiments were conducted in 2012. The dogs were housed in the facilities of the OIE Reference Laboratory, Nancy Laboratory for Rabies and Wildlife (Malzéville, France), approved with # C-54-431-1, and the CRBM (BioMedical Research Center) of the National Veterinary School of Maisons-Alfort (France), approved with # F 94-046-2. The experiment was conducted according to the European Community Directive 2010/63/EU on animal experimentation (6).

Each vaccine was administered to a single group of five dogs that were housed together to guarantee social contacts between animals. The animals were given food and water *ad libitum*. Animal welfare was ensured by providing all animals with regular human contact and games and by allowing them to rest on dedicated platforms. Animals were monitored regularly (at least twice a day) and any unusual event or incident potentially affecting the health of animals was immediately recorded, reported and addressed by a veterinarian. In order to comply with the 'reduction' objective of the 3Rs, 10 of the 15 dogs were 're-used' animals. The protocol for dog immunisation was approved by subject matter experts from the OIE Reference Laboratories for Rabies as well as the OIE Biological Standards Commission.

Selection of vaccines

Three inactivated monovalent cell-culture anti-rabies vaccines were selected. These vaccines represent the principal vaccine strains used in anti-rabies inactivated veterinary vaccines: Nobivac Rage (Intervet, PV strain), Rabisin (Mérial, derived PV strain) and Enduracell rage mono (Pfizer, Flury LEP strain). These vaccines contain at least one antigenic unit per dose, according to the recommendations from WHO.

Dogs

Fifteen adult beagle dogs, of both sexes, were immunised. Five adult dogs, 1 to 2 years old, were re-used after a mild or moderate severity procedure and five others, 5.5 to 10 years old, could not be used any more for reproduction by the authorised breeder. The five others were bought from the same authorised breeder for animal experimentation; they were 6 to 8 months old when immunisation began. The dogs were dewormed and vaccinated against canine diseases, including rabies, before arrival in the facility.

Immunisation protocol

The fifteen adult dogs were divided into three groups of five animals, each corresponding to a unique vaccine. Each dog in a particular

group was immunised with the same batch of vaccine. For all vaccinated animals, the unique immunisation protocol consisted of three intramuscular injections of one dose of 1 ml of vaccine at day zero, week three and week five. The kinetics of the VNA response was followed in blood samples collected at week three, week four and week six.

Serum collection

Two weeks after the second booster (i.e. seven weeks after the first immunisation), the dogs were anaesthetised using induction with ketamine–medetomidine followed by inhalational anaesthesia (isoflurane), using a vaporiser to accurately control delivery of the correct concentration of anaesthetic. A respirator was included in the circuit to maintain a longer oxygen supply, providing a larger harvest of blood, which addresses both the reduction and refinement aspects of the '3Rs' (7). Each dog was placed on a heated surgery table and a catheter was surgically installed in the carotid artery (*arteria carotis*) to collect blood in 50 ml sterile dry tubes. While under general anaesthesia, the dogs were killed via terminal blood collection, complying with the European Directive 2010/63/EU on the 'protection of animals used for scientific purposes' (6). The death of each dog was confirmed by assessment of the loss of cardiac function. The blood tubes were stored at room temperature to allow clotting and clot retraction before centrifugation on the following day.

Samples

After the blood had clotted, the tubes were centrifuged at 2,000 ×*g* for 30 min at 4°C. Serum was harvested in a biosafety cabinet in two steps. The different harvests corresponding to a single dog were pooled and filtered through 0.45 µm and 0.22 µm Durapore membranes. Several sterility controls were performed on the filtered products in the OIE/ANSES Reference Laboratory for Rabies to verify the absence of bacteria, fungi and mycoplasmas (see below). Once the rabies VNA titre of each harvest had been established by using five replicates, the selected harvests were mixed so that the final reference serum contained the same number of IU from each vaccine

(i.e. from each of the three selected vaccinal strains). The serum batch was then heat-inactivated (56°C, 30 min) and frozen at –20°C. Before freezing, an aliquot was also prepared for further serological titrations and sterility tests. Several titrations were performed by the FAVN test to ensure that no cytotoxicity occurred on cells and to check whether the VNA titre was still satisfactory, i.e. high enough (around 10 IU/vial). The frozen batch was then sent to a specialised service provider (Lyophal, France) for freeze-drying in 650 µl aliquots in 2 ml vials.

Seroneutralisation test

Rabies-neutralising antibodies were determined with the FAVN test as described previously (1, 8).

Sterility controls

Several sterility controls were performed on the final product at the OIE/ANSES Reference Laboratory for Rabies to ensure the absence of bacteria, fungi and mycoplasmas. Columbia agar (CA) plates containing 5% sheep blood were used to check for the presence of bacteria. After inoculation by streaking, the agar plates were incubated for between 48 and 72 h at 37 ± 2°C. Sabouraud Gentamicin Chloramphenicol (SGC) agar plates were used to check for the presence of fungi. After inoculation by streaking, the agar plates were incubated for between 48 and 72 h at 32.5 ± 2.5°C. After incubation, both the CA and SGC plates were checked for the presence of colonies.

For detection of *Mycoplasma* spp., a commercial kit (MycoAlert™ Mycoplasma Detection Kit, Lonza) was used. Briefly, test samples were mixed with a lysis buffer. If *Mycoplasma* spp. were present, released enzymes would react with the supplied specific substrate and catalyse the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). The latter reaction was then converted into a light signal via the luciferase enzyme. By measuring the ATP levels in a sample both before and after the addition of the luciferase enzyme, a ratio indicating the presence or absence of mycoplasmas was obtained.

Pre-determination of the titre of the proposed reference serum

The 2nd WHO International Standard for Anti-Rabies Immunoglobulin of human origin, with a titre of 30 IU/ml (5), provided by the National Institute for Biological Standards and Control (Hertfordshire, UK; code: RAI), was used as a defined reference standard to calibrate the VNA titre of the new batch of OIE reference serum of dog origin, as previously done for the first batch. The use of the same reference standard (WHO), as far as possible, for the calibration of the different batches of OIE reference canine rabies antiserum allows the avoidance of bias in the titre calibration of the newly produced batches.

In order to prepare for the inter-laboratory comparison test among OIE Reference Laboratories, repeated FAVN test runs performed by multiple operators were conducted at the OIE/ANSES Reference Laboratory for Rabies to determine the approximate VNA titre of the reconstituted freeze-dried serum. After 45 iterations, the mean VNA titre of reconstituted serum samples was equal to 18.58 IU/ml, which was considered too high. Therefore, to guarantee a more accurate calibration against the 2nd WHO International Standard, reconstitution of the proposed OIE reference serum was conducted with double the volume of distilled water (1,300 µl). By decreasing the VNA titre, large dilutions were not required to be made by the laboratories to achieve the target titre of 0.5 IU/ml in individual test runs.

Inter-laboratory study

The neutralising antibody titre of this new batch was determined through an inter-laboratory comparison test involving different OIE Reference Laboratories.

Participants

The following five OIE Reference Laboratories took part in this study:

– ANSES Nancy Laboratory for Rabies and Wildlife, France

– Friedrich-Loeffler-Institut, Germany

– Animal and Plant Health Agency, UK

– Centers for Disease Control and Prevention, United States of America

– Onderstepoort Veterinary Institute, South Africa.

Each laboratory was randomly assigned a number independent of the order listed above (L1 to L5).

Instructions to participants

The participating laboratories were invited to test a panel of coded sera in three independent runs using one of the two seroneutralisation methods prescribed by the OIE, i.e. the FAVN test (3, 8) and the original RFFIT (4).

Design of the panel

The panel consisted of 12 coded samples, including serum from a naïve dog as the negative control (NC). The specificity was assessed by checking that the laboratories titrated this sample as negative, i.e. with titres close to 0 IU/ml.

Three samples were replicates of the new batch of the proposed OIE anti-rabies positive reference serum of dog origin reconstituted in 1,300 µL of distilled water, for determination of the VNA titre. The remaining eight samples corresponded to different dilutions of the 2nd WHO International Standard for Anti-Rabies Immunoglobulin, containing 30 IU/ml, to obtain an appropriate and reliable calibration range. The latter allowed establishment of a correlation curve between VNA titres and the $\log D_{50}$ (logarithm of the dilution showing 50% inhibition of the positive wells [for FAVN], or positive fields [for RFFIT]) for VNA titres ranging between 0.5 and 10 IU/ml.

For each laboratory, a log–log linear regression analysis was undertaken to obtain the WHO standard curve corresponding to each run. The mean of the three $\log D_{50}$ values obtained for the new batch of

positive reference serum was calculated for each run. From the linear equation ($\log y = b\log x + a$) of the curve obtained with the WHO standard, the IU/ml value of the positive reference serum under test was calculated according to its mean $\log D_{50}$ value for each run.

Stability study

After determination of the consensus VNA titre, a stability study was conducted for a period of 26 weeks (between January and July 2014) at the OIE/ANSES Reference Laboratory for Rabies. Three conditions were tested: storage of the reconstituted serum at +4°C, storage of the reconstituted serum diluted to 0.5 IU/ml at +4°C and storage of the reconstituted serum diluted to 0.5 IU/ml at –20°C.

To test the first condition, several vials were reconstituted as described above, pooled and then 500 µl aliquots were distributed in sterile vials and subsequently stored at +4°C. To test the stability of the OIE reference serum at a pre-dilution of 0.5 IU/ml, several freeze-dried vials were reconstituted, pre-diluted using sterile phosphate-buffered saline (PBS) and subsequently pooled to ensure homogeneity. Aliquots of 500 µl were distributed in sterile vials and stored either at +4°C or at –20°C. Once a week, the three different samples were titrated in a single test, using the FAVN test.

Statistical methods

The results obtained by individual laboratories were analysed using statistical tools and a consensus VNA titre of the new batch of the OIE positive reference serum of dog origin was defined.

Two statistical tests previously described (9) were utilized. The first (‘overall test for coincidental regressions’) was used to assess whether k population regressions were coincident in general. The second (‘comparing more than two slopes’) is complementary to the first statistical test. Applied to test for differences among three regression functions, this test allows the differences between slopes and y intercepts to be distinguished independently. These two statistical tests use the Fisher distribution ($\alpha = 0.05$). The Kruskal Wallis test was

used to compare the different values of the positive reference serum ($\alpha = 0.05$).

For the linear regression analysis obtained for the WHO standard curve, the statistical analysis consisted of a Student's test performed with a 95% confidence limit. Two points were considered to validate each run independently: the regression with the coefficient of determination R^2 and the slope (b). The acceptance criteria were the following for each run:

– the coefficient of determination (R^2) should be equal to or above 0.8; if not, the run was excluded from the calculation of the titre

– the slope should not be statistically different from 1.0; if not, the run was excluded from the calculation of the titre.

Results

Inter-laboratory study

Specificity

For the NC serum, a mean VNA titre of 0.07 IU/ml ($n = 9$, standard deviation [SD] = 0.03) was determined from the individual VNA titres. The titres obtained by participating laboratories ranged between 0 and 0.10 IU/ml. The specificity was therefore considered satisfactory for all laboratories.

Determination of the titre of the proposed anti-rabies positive reference serum of dog origin per run for each laboratory

The mean VNA titres of the three replicates of the proposed OIE anti-rabies positive reference serum obtained by each participating laboratory in three independent runs are shown in Fig. 1. For laboratories L2 and L3 the coefficient of determination obtained for run 1 was too low ($R^2 = 0.77$) and the VNA titres of the three replicates obtained in the first run were outside the WHO standard curve. Therefore, these runs were not included in the determination of

the final VNA titre of the proposed OIE anti-rabies positive reference serum.

Insert Fig. 1

Determination of the titre of the proposed anti-rabies positive reference serum of dog origin per laboratory

If no statistical differences were observed between the WHO standard curves obtained by each participating laboratory, the mean of the (IU/ml) values obtained for the positive serum under test was calculated per laboratory. While for laboratories L1, L4 and L5 there was no statistical difference between the three WHO standard curves, laboratories L2 and L3 deviated from one of their own WHO standard curves. Therefore, for these two laboratories, the mean values (IU/ml) were calculated from the two validated runs.

Determination of the titre of the proposed anti-rabies positive reference serum of dog origin

The VNA titres (in IU/ml) of the proposed OIE anti-rabies positive reference serum obtained per run, and the resulting mean VNA titre for each participating laboratory are shown in Table I. A Kruskal Wallis test on the ranks, performed on the 13 values (Table I), considered these values to be not significantly different. Therefore, the consensus VNA titre of the second batch of the proposed OIE anti-rabies positive reference serum was established to be 5.59 IU/ml.

Insert Table I

Stability study

The control card obtained in the stability study of the reconstituted OIE anti-rabies positive reference serum stored at +4°C, the diluted 0.5 IU/ml serum stored at +4°C and the diluted 0.5 IU/ml serum stored at –20°C for a 26-week period is presented in Fig. 2. After 26 weeks at +4°C, the reconstituted OIE reference serum remained fairly stable with a mean VNA titre ($logD_{50}$) of 2.47 (n = 26; SD = 0.22) and a

$logD_{50}$ median value of 2.51. All values remained inside the limits of the control card, i.e. the mean ± 2SD.

Insert Fig. 2

The stability of the OIE reference serum diluted to 0.5 IU/ml and stored at +4°C for 26 weeks was also assessed. The mean VNA titre (in $logD_{50}$) and the $logD_{50}$ median value were 1.35 (n = 26; SD = 0.16) and 1.31, respectively. All values obtained, except one at week 24, were included between the upper and lower limits of the control card. At week 24, the $logD_{50}$ value was 0.96.

The mean VNA titre (in $logD_{50}$) of the OIE reference serum diluted to 0.5 IU/ml and stored at –20°C for 26 weeks and the $logD_{50}$ median value were 1.36 (n = 26; SD = 0.17) and 1.31, respectively. All values remained inside the limits of the control card.

Sterility control

No bacteria, fungi or mycoplasmas were detected in the different samples tested.

Discussion

International reference antibody standards are necessary to ensure that a given serological assay measures the antibody activity with a specified level of sensitivity. According to the OIE, positive reference standards should be prepared from animals that exhibit a typical humoral (i.e. antibody) response to the antigen (10). As mentioned in the OIE Guide 3 for 'International Reference Antibody Standards for Antibody Assays', the immune response may be obtained by immunisation with vaccines. In this way, three commonly used cell-culture, inactivated monovalent anti-rabies veterinary vaccines were used to immunise the dogs in this study. The decision to use three commercial inactivated vaccines instead of field inactivated strains appeared logical following the analysis of the use of the first batch of OIE reference serum: the new batch should be mainly used as a reference in serological testing of parenterally vaccinated domestic carnivores. It is also accepted by the OIE that the standard may be

derived from a single animal or from a pool of samples from different animals. Three groups of five adult dogs were immunised, one group per vaccine, to collect a sufficient volume of serum of adequately high titre.

The OIE/ANSES Reference Laboratory for Rabies used the same reference standard (WHO, calibrated at 30 IU per ampoule) for establishing the titre of the different batches of OIE reference serum, avoiding the creation of a bias in determining the titre of the new batch.

The calibration of this new batch was undertaken through an inter-laboratory test involving five OIE Reference Laboratories for rabies. Four laboratories (L1, L3, L4 and L5) used the FAVN test to titrate the panel of coded samples, and one (L2) used the RFFIT on Labtek chamber slides. Both techniques are prescribed by the OIE (1) and recommended by WHO (11) to titrate rabies antibodies in serum samples and they gave similar results, as previously demonstrated (3, 12). The inclusion of a serum samples from a naïve dog allowed analysis of the specificity obtained by each participating laboratory and validation of their results for the statistical analyses. All laboratories obtained satisfactory results for this criterion as they confirmed this sample to be negative, with a titre close to 0 IU/ml. The titre of the positive reference serum of dog origin per run for each laboratory was also determined. Among the 15 runs performed, only two runs were not taken into account when calculating the mean of three $\log D_{50}$ values obtained per laboratory. The value obtained by laboratory L2 for the first run was deleted because the coefficient of determination of the WHO standard curve was considered too low to be reliable (below 0.80) when compared with the others. The value obtained by laboratory L3 for the first run was also not considered for the calculation because the mean value of the future OIE anti-rabies positive reference serum was too high and therefore outside the limit of the WHO standard curve. Finally, the titre of the anti-rabies positive reference serum of dog origin was calculated from the 13 reliable values obtained by the five OIE Reference Laboratories, which gave a consensus titre of 5.59 IU/ml.

All the technical and statistical data on the evaluation of the new batch were submitted to the OIE so that the OIE Standards Commission was able to review the information dossier before giving its consent. The stability study was performed after storage of the new batch of OIE anti-rabies positive reference serum for a period of 26 weeks and the data obtained demonstrated the good stability of the product after reconstitution and dilution to 0.5 IU/ml, even when stored at +4°C. Moreover, the lyophilised product was shown to be free from bacteria, fungi and mycoplasmas.

Conclusion

In 2014, the OIE Biological Standards Commission adopted the serum as an OIE-approved standard with a consensus titre of 5.59 IU/ml. It was added to the list of International Reference Standards available. This list is supplied to all OIE Member Countries on request, and may also be accessed on the OIE Website (www.oie.int/en/our-scientific-expertise/veterinary-products/reference-reagents/).

Conflict of interest

The authors declare that they do not have any competing interests.

Dr Fooks was elected to the OIE Standards Commission in May 2015, which occurred after the adoption of the anti-rabies positive reference serum as an OIE-approved standard in February 2014.

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Table I
Titres (in IU/ml) of the evaluated positive dog reference serum obtained per run for each participating laboratory

	L1	L2	L3	L4	L5
Run 1	3.82	/	/	6.47	6.19
Run 2	4.42	4.22	6.26	7.48	4.44
Run 3	4.41	5.41	6.36	6.36	6.83
Mean titre in IU/ml	4.22	4.82	6.31	6.77	5.82

Fig. 1
Mean IU/ml values of the evaluated positive dog reference serum for each run and for each laboratory

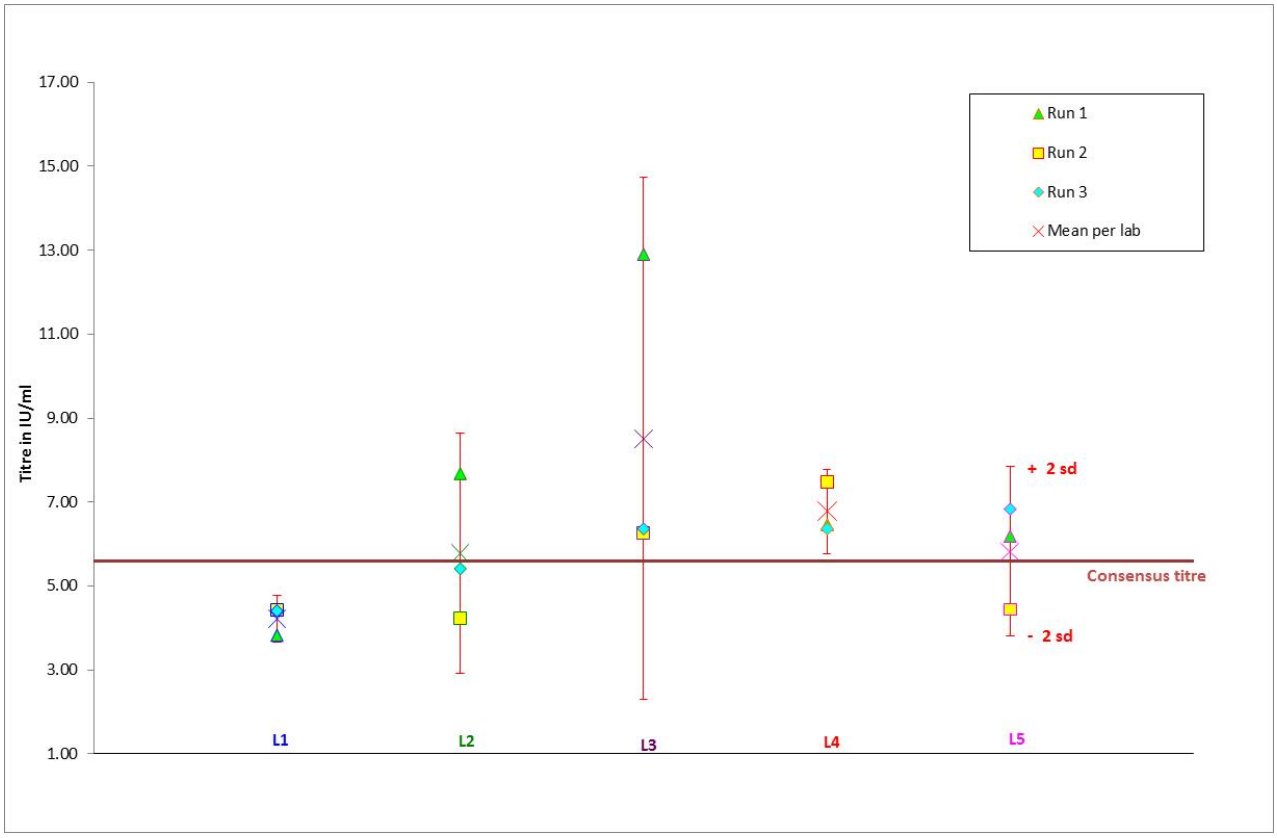

Fig. 2

Control card for the stability study of the reconstituted serum stored at +4°C, the diluted 0.5 IU/ml serum stored at +4°C and the diluted 0.5 IU/ml serum stored at –20°C for 26 weeks