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WOAH Reference Laboratory for Rabies

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Founded as OIE

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Production of in-house (internal) positive control serum for rabies antibody testing

Background

Serological tests for rabies are used worldwide in national and private laboratories involved in rabies activities. To harmonise serological testing and to promote the mutual recognition of results for international trade, WOAH and the Biological Standards Commission organise programmes to validate WOAH-approved International Standard Reagents (https://www.woah.org/en/what-we-offer/veterinary-products/reference-reagents/). Rabies serological tests are also widely used in research related to rabies pathogenesis, immunity, vaccine development and routine monitoring of rabies-vaccinated animals (dogs vaccinated during mass parenteral vaccination campaigns are often tested for rabies antibodies) and humans.

For rabies, WOAH added the second batch of WOAH anti-rabies positive standard serum from dog origin to the list of International Standard Reagents in 2014 [1]. This reagent has been produced in Anses-Nancy laboratory since 1991 and is currently recommended by WOAH for rabies serology [2]. The procedure given in the WOAH *Terrestrial Manual* [2] mentions that "this control serum may be used to calibrate an additional internal control that is used for regular FAVN[1] testing". This document describes different ways of production of an in-house positive control serum for serology, which has to be properly calibrated with the WOAH international anti-rabies positive serum prior to its use.

Laboratories producing internal controls are encouraged to have well-documented and detailed protocols and procedures. They should also use the same methodologies [3] when replacing these

1 FAVN: fluorescent antibody virus neutralisation

controls, in order to ensure a similar biological quality between the different internal controls produced over time.

This document can be used for the production of a rabies control serum in different animal species. As an example, the methods presented in parts A and B of the document use the dog as an animal matrix for production of control serum.

Production methods

Different methods can be used for creating an in-house positive control serum for rabies serology carried out on animal samples. The in-house positive control corresponds to a polyclonal antibody preparation. Different production methods are possible:

- Immunisation of experimental animals with rabies virus (RABV) strains previously inactivated.
- Immunisation of experimental animals with commercial inactivated and adjuvanted anti-rabies vaccines.
- Pooling of serum samples from field animals previously vaccinated against rabies.

As the aim is to obtain a control serum with a broad spectrum of antigenic variation, it is important that different RABV strains or different vaccines are used for serum production in experiment animals.

Irrespective of the production method used some important aspects will need to be considered:

- The in-house serum should be prepared using the same animal species, e.g. all experimental animals are foxes or all pooled serum samples are from vaccinated dogs.
- Large quantities should be prepared, aliquoted and stored in appropriate conditions to ensure the biological integrity of the in-house positive control over time [3].
- The laboratory in charge of serum production in caged experimental animals should have all the necessary national authorisations and equipment. The protocols should be approved by an ethics committee, and the experimental facilities should be registered and regularly inspected.
- It is recommended to store the produced serum undiluted and to periodically prepare working aliquots [3]. The target for the control serum should be a final neutralising antibody titre of approximately 10 IU[2]/ml.
- Laboratory methods should be those recommended by WOAH [2].
- Determining the neutralising antibody titres during the different steps of the in-house reference serum production must be done using the WOAH anti-rabies positive standard serum as a positive control. The latter shall be used to determine the IU content of each tested sample.

A Serum production in experimental animals

The serum produced should be representative of the variability of RABV and of the specific use intended by the laboratory.

For example, if the serum is produced as a positive control for titration of field serum samples from one particular infected country, or from one particular infected region, it is recommended to immunise the dogs with different isolates of the RABV circulating in these areas. A maximum of three RABV variants, previously inactivated, representative of the antigenic variability of the virus are used for the

2 IU: international units

immunisation step. In such case, several groups of from two to four dogs are constituted, each group receiving a specific inactivated RABV isolate.

If the control serum is produced to check the immunity of dogs vaccinated parenterally for the purpose of international trade, then it is recommended to use three commercial monovalent rabies vaccines to immunise the dogs. For this purpose, the best way to mimic the variability of the antigenic determinants is to use vaccines with different virus strains (for example Pasteur virus [PV], Pitman–Moore [PM] and Flury low egg passage [Flury LEP] strains, which are the most frequently used vaccine strains) to obtain a broad spectrum polyclonal response. In such case, several groups of from two to four dogs are constituted, each group receiving a specific anti-rabies vaccine.

1 Animals

Pathogen-free dogs, naïve for rabies are housed in suitable experimental facilities, in individual cages or all together and monitored regularly, at least once a day during feeding [1]. They are identified and dewormed. Animals are randomly divided in different subgroups. Any unusual event is recorded.

2 Antigen/vaccine selection and preparation

The isolates selected for immunisation correspond to brain or salivary glands of naturally infected animals. It is necessary to passage them on mice or preferably in cells (neuroblastoma cells [ATCC CCL131] and BHK21 C13 cells [ATCC CCL10]). The methods classically contain one or several passages on cells, concentration of the harvests by centrifugation, inactivation and quality controls (for example, controls of purity, concentration of proteins, etc.). The protocols may differ according to the laboratory equipment and facilities. They have been previously described in detail [see 4].

The inactivated adjuvanted anti-rabies veterinary vaccines selected for immunisation should be tested for potency with the NIH test [5] prior to being used and must contain at least one antigenic unit per dose, according to international recommendations. The different aliquots of a particular vaccine should have the same batch number.

3 Immunisation of animals

A blood sample shall be collected from all dogs prior to the immunisation to check the serological naïve status for rabies. Each dog in a particular group is immunised with the same suspension of antigen or with the same batch of vaccine.

Immunisations with antigens require the use of complete or incomplete Freund's adjuvant mixed (volume/volume) with the antigen and injected by the subcutaneous route (2 ml) in several points on the flanks of each dog. The immunisations with the vaccines are conducted preferably by the intramuscular route (1 ml) and require three injections (for example day 0, week 3 and week 5) [1]. The kinetics of the neutralising antibody response are followed on blood samples collected from all dogs a few days after each injection.

4 Serum collection

Dogs are anesthetised and a catheter is surgically installed in the carotid artery to collect blood in sterile dry tubes [1]. After clotting, the tubes are centrifuged, the different harvests of clear serum from each dog are pooled and filtered, and then quality controls are performed [1]. Before freezing (–20°C) the pools, several aliquots are kept for rabies antibody titration [1].

Once the neutralising titre of each harvest (from each antigen or each vaccine immunisation) has been established, the in-house serum is prepared so that the final control serum contains the same number of IUs from each antigen or from each vaccine, in order to reach a balance for the different antigens/vaccines used in the final preparation [1]. The in-house control serum is then heat-inactivated and frozen (–20°C) after sampling an aliquot for rabies antibody testing. The final

titre of the serum is determined by using the WOAH standard serum as a positive control and the frozen serum is then either freeze-dried [1] in small aliquots or stored frozen in small aliquots. After freeze–drying or freezing, some aliquots are randomly tested for homogeneity and for rabies antibody content. Each aliquot should contain around 10 IU/ml corresponding to the final titre targeted. Several quality controls must be conducted (sterility tests, detection of mycoplasma and stability) [1].

B Serum production by pooling serum from vaccinated dogs

In laboratories that store under appropriate conditions, large quantities of individual serum samples from vaccinated dogs (e.g. samples received for rabies serological testing in the context of international trade), WOAH accepts the principle of producing a control derived from a single animal or from a pool of samples from different animals [3]. Pooling sera from several animals, rather than a single animal, is preferable to mimic as much as possible the response of field samples and serum quality at the population level (diversity of epitopes used during the vaccination step as the animals have been vaccinated by different vaccines, leads to diversity of the paratopes in the pool).

Numerous serum samples having a known rabies serological titre above 5 IU/ml should be selected to constitute a pool with a large volume. Once the pool is obtained, it shall be filtered, heat-inactivated and frozen (–20°C) after sampling aliquots for further rabies antibody titrations. The pool should be titrated several times by using the WOAH standard serum as a positive control to assess if the IU/ml titre is in the expected range. Then, the in-house serum could be either freeze-dried in small aliquots or stored frozen also in small aliquots. After freeze-drying or freezing, some aliquots are randomly tested for homogeneity and for rabies antibody content. Several quality controls have to be conducted on the pool (sterility tests, detection of mycoplasma and stability) [1].

Methods for determining the final titre of the in-house positive control serum

The neutralising antibody titre of the new serum is determined using one of the two WOAH recommended tests (FAVN test or RFFIT[3]) [2] and using the WOAH anti-rabies positive reference serum. This latter reference serum is serially diluted to prepare a calibration range. A correlation curve is obtained between neutralising antibodies determined by the dilutions, and the experimental data found (logarithm of the dilution showing a 50% inhibition [logD_{50}] of the positive wells or fields) [1, 6]. Around 10 serological tests are carried out independently, and for each test, the serial dilutions of the WOAH standard are tested as well as several titrations (at least 5) of the in-house serum. For each test, the mean of the 5 logD_{50} values is calculated and its titre in IU/ml is determined using the standard calibration range of the WOAH serum. Then the mean in IU/ml is calculated from the results of the 10 serological tests.

Another way to determine the final titre of the in-house control serum is to titrate it several times (between 30 and 50 times) under conditions of repeatability and intermediate precision. For each test, the titre of the in-house serum (in IU/ml) is calculated using the WOAH standard serum as a positive control. The geometric mean of the IU/ml titres is calculated to determine the final titre of the in-house serum.

Irrespective of the method used, once the final titre is set, the theoretical dilution to reach the theoretical titre of 0.5 IU/ml has to be defined. It is strongly recommended to assess this theoretical dilution, set to reach 0.5 IU/ml, prior to routine use.

3 RFFIT: Rapid fluorescent focus inhibition test

Bibliography

1. Wasniewski M., Barrat J., Fooks A.R., Franka R., Müller T., Sabeta C. & Cliquet F. (2017). Production and calibration of the second batch of OIE anti-rabies positive reference serum. *Rev. Sci. Tech.,* **36**, 779–788. doi: 10.20506/rst.36.3.2713. PMID: 30160702.

2. World Organisation for Animal Health (WOAH) (2018). Chapter 3.1.18. Rabies (infection with rabies virus and other Lyssaviruses). *In:* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. WOAH, Paris, France. Available at: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.01.18_RABIES.pdf

3. World Organisation for Animal Health (WOAH) (2018). Chapter 2.2.6. Selection and use of reference samples and panels. *In:* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. WOAH, Paris, France. Available at: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.02.06_REFERENCE_SAMPLES.pdf

4. World Health Organization (1996). Laboratory Techniques in Rabies, Fourth Edition, Meslin F.-X., Kaplan M.M. & Koprowski H., eds. WHO, Geneva, Switzerland.

5. World Health Organization (2018). Laboratory Techniques in Rabies, Volume 2 Abela-Ridder B., Rupprecht C.E. & Fooks A.R. World Health Organization, Geneva, Switzerland.

6. Cliquet F., Aubert M. & Sagné L. (1998). Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *J. Immunol. Methods*, **212**, 79–87. doi: 10.1016/s0022-1759(97)00212-3. PMID: 9671155.

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