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Characteristics of quality control indicators of the virus neutralization test in cell culture for the detection of rabies antibodies (FAVN-test)

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Abstract. *The paper presents information on the results of continuous monitoring of rabies virus titers and standard serum in the FAVN - test, which is a guarantee of the quality and reliability of the results of blood serum testing for the presence of rabies antibodies. The FAVN-test is the main method for assessing the immune response to rabies vaccination, recommended for use by the WHO and WOAH. Testing the strength of individual post-vaccination rabies immunity is a requirement of the European Union and many other countries around the world, including Ukraine, for the non-commercial movement of companion animals. The FAVN-test method was introduced for the first time in Ukraine at the Research Virology Department of the State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise (SSRILDVSE). To ensure the quality of research, forms were developed and control cards were introduced. The log_{50} values of the positive standard serum (0.5 IU/cm^3), the log_{50} values of the negative serum, and the log_{50} values of the infectious titer of the CVS-11 rabies virus obtained in each reaction are entered. During 2019 – 2024, 929 were performed using the FAVN-test, in which all specified indicators were calculated, and corresponding graphs were constructed. Constant monitoring of the specified indicators made it possible to take corrective action when changing the rabies virus series or updating cell culture lines. In 2025, we participated in the Rabies Serology Proficiency Test. Studies of the coded blood serum panel showed a coefficient of determination of «R^2» = 0.9879. Our results indicate a high (≥ 0.95) degree of correspondence with the expected results.*
Keywords: rabies virus, reference strain, blood serum, antibodies, virus neutralization test.

Surveillance of the spread of many infectious diseases, including rabies, is based on the results of laboratory tests. One of the many elements of the modern rabies surveillance system is the evaluation of vaccination effectiveness, i.e., testing blood sera for the presence of antibodies to the rabies virus in a virus neutralization test in cell culture (FAVN-test – Fluorescent Antibody Virus Neutralization test). Today, the FAVN-test is the main method for assessing the immune response to rabies vaccination, recommended for use by the WHO and WOAH (WHO, 2018, 2019; WOAH, 2023).

In addition, testing the strength of individual post-vaccination rabies immunity is a requirement of the European Union (EU Regulation No. 576/2013), as well as many other countries around the world and Ukraine (Order of the Ministry of Agrarian Policy and Food of Ukraine No. 553 of 16.11.2018), during the non-commercial movement of domestic carnivorous animals – companion animals. These regulatory documents stipulate that dogs, cats, and ferrets must be vaccinated against rabies and have a quantitative volumetric analysis of rabies antibodies at a level of at least 0.50 IU/cm^3 (Regulation (EU) No 576/2013).

The FAVN-test has been introduced for the first time in Ukraine at the Research Virology Department SSRILDVSE. Research on rabies immunity using the FAVN-test at the SSRILDVSE is accredited in accordance with the requirements of the international standard

DSTU ISO/IEC 17025:2017 “General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2017, IDT)”, which is an integral part of the test result recognition procedure.

Annual validation of the FAVN-test method was carried out under the auspices of the European Reference Laboratory for Rabies (ANSES, France). During the validation, the specificity and reproducibility of the method were assessed, the range of variation in values for three repetitions of the tests according to the coefficient of variation and the experimental value of the theoretical indicator of 0.5 IU/cm^3 with the determination of the range of expected titers.

Of course, ensuring reliable results requires strict adherence to the reaction parameters. This is especially true for neutralization reactions involving live objects – in our case, the rabies virus CVS-11 and the BHK-21 C13 cell line (ATCC CCL-10) (Polupan, 2021).

Obtaining virus-containing material with a sufficient infectious titer is a certain guarantee of the reliability of determining the virus-neutralizing activity of animal blood sera. However, an important step, along with virus accumulation and FAVN-test development, is determining the titer of the rabies virus and the dose taken in each tests (Cliquet, 1998; Hostnik, 2000).

To ensure the appropriate quality of research and monitoring of the stability of parameters of the FAVN-test, forms have been developed and control cards have been introduced. The cards contain the $\log_{50}$ values of the positive standard serum (0.5 IU/cm^3), the $\log_{50}$ values of the negative serum, and the $\log_{50}$ values of the titer of the rabies virus CVS-11 obtained in each tests.

The aim of study was the stability characteristic of the parameters of the FAVN-test during 2019 – 2024.

Materials and methods. The study was conducted at the Research Virology Department SSRILDVSE. The reaction was performed in BHK-21 C13 (ATCC SCCL-10) cell line grown in 96-well microplates with a constant dose of the rabies virus, strain CVS-11 (ATCC VR 959) (Cliquet, 1998).

In each reaction, the $\log_{50}$ of the positive standard serum (reconstituted and diluted to the prescribed theoretical value of 0.5 IU/cm^3), the $\log_{50}$ of the negative serum, and the $\log_{50}$ value of the titer of the CVS-11 rabies virus were tested and calculated.

After entering all the values of the determined indicators into MS Excel, a corridor of acceptable deviations of the reaction parameters within 15% was automatically set. Based on the results of all three indicators, graphs were generated every six months.

Results. Direct determination of the rabies virus titer of the CVS-11 is based on the selection of the optimal dilution factor and initial virus dilution, since the infectious titer of the experimental virus-containing suspension is the limiting dilution of the virus at which fluorescence foci are observed. The virus titer was determined after obtaining virus-containing raw materials in order to further establish the limiting dilution and calculate the infectious dose for use in the reaction by preparing and studying sequential 10-fold dilutions from 10^{-1} to 10^{-12}. During 2019 – 2022, six series of the CVS-11 virus were obtained and studied in three replicates. The virus was stored in duplicate at temperatures of minus 80±1 °C and minus 196 °C in liquid nitrogen (Polupan, 2011).

For use in the FAVN-test of the CVS-11 strain of rabies virus, a theoretical calculation of the infectious dose of the virus was performed, which was 100 $TCID_{50}$/0.05 cm^3. The infectious dose of the new virus series was calibrated by threefold testing of the infectious titer and the virus-neutralizing activity of the reference serum with an activity of 0.5 IU/cm^3, comparing the values obtained with the previous rabies virus series. Only after obtaining the expected reaction parameters (virus titer/reference serum titer) was the new series of the CVS-11 rabies virus used in tested.

In total, 929 reactions were performed between 2019 and 2024 (145 in 2019, 134 in 2020, 124 in 2021, 134 in 2022, 182 in 2023, 210 in 2024) using the FAVN-test, in which the $\log_{50}$ of positive standard serum (reconstituted and diluted to the prescribed theoretical value of 0.5 IU/cm^3), $\log_{50}$ of negative serum, and $\log_{50}$ of the titer of the CVS-11 rabies virus. Corresponding graphs were constructed based on these values.

In 2019, a new series of viruses was introduced in test No. 21 (Fig. 1), but in tests No. 23-25, the titer of the CVS-11 was lower than the expected 4.63 log_{50} $TCID_{50}$ (lower calculated limit of virus titer 4.74 log_{50} $TCID_{50}$), although the titer of positive serum was within the specified range (Fig. 2).

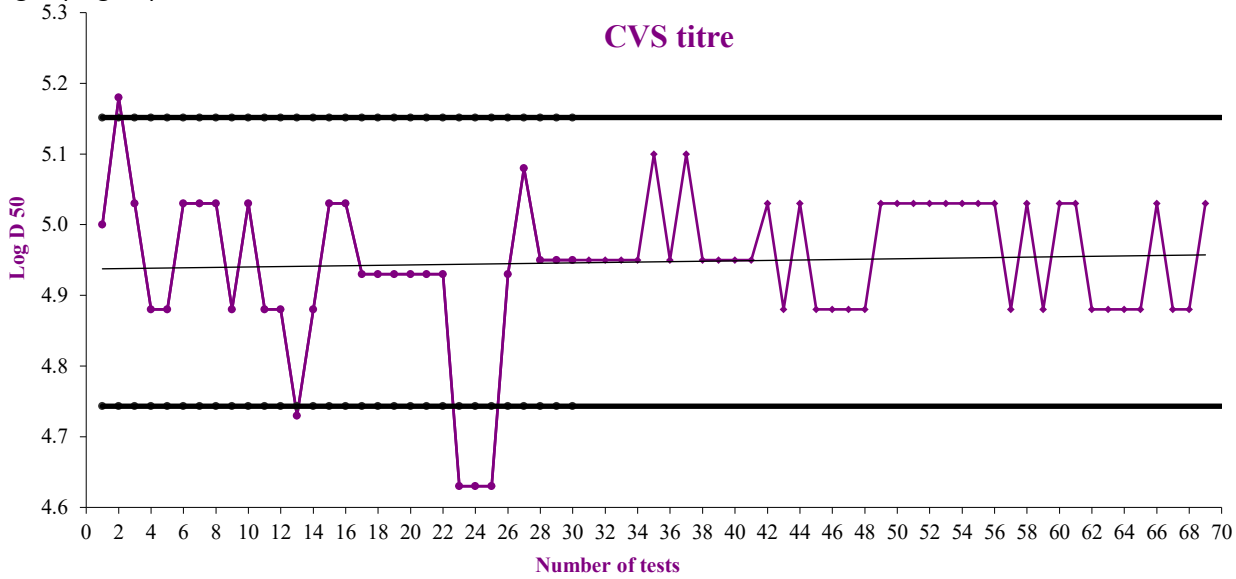

Fig. 1. Titers of the rabies virus, strain CVS-11, in the FAVN-test in 2019 (test 1-69)

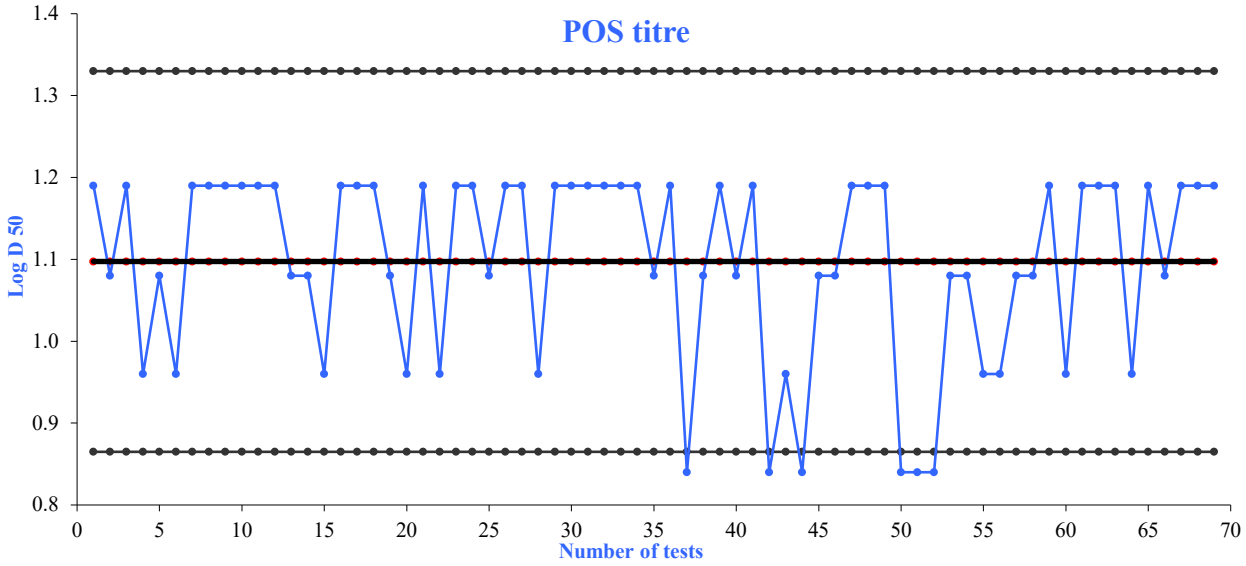

Fig. 2. The significance of positive WHO standard serum titers (reconstituted and diluted to a theoretical value of 0.5 IU/cm^3) when performing the FAVN-test in 2019 (test 1 – 69)

The implementation of corrective measures (gradual increase in the virus dose with monitoring of standard positive serum indicators) stabilized the virus titer in subsequent tests, which varied between 4.73 and 5.08 log_{50} $TCID_{50}$, which is a satisfactory indicator. In six reactions, further analysis revealed lower values of positive WHO standard serum, although the virus titers in these protocols were within the specified range. Starting with reaction 110, the following series of titrated and characterized rabies virus CVS-11 was used. During the next 15 tests, a tendency towards higher titers of infectious activity than expected parameters was noted – 5.0±0.2 log_{50} $TCID_{50}$, with peak values of 5.33 log_{50} $TCID_{50}$ (Fig. 3).

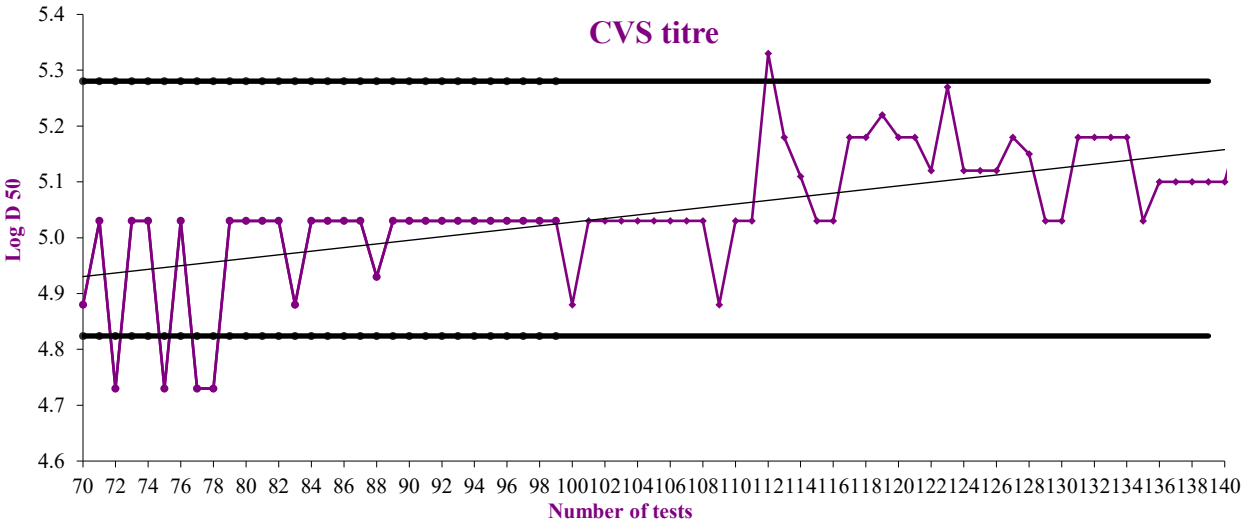

Fig. 3. Titers of the rabies virus, strain CVS-11, in the FAVN-test in 2019 (test 70 – 145)

According to corrective measures (gradual reduction of the virus dose with control of standard positive serum indicators), the virus titer was stabilized within 4.87-5.21 log_{50} $TCID_{50}$ in subsequent tests. During the reduction of the infecting virus dose used in each reaction, the minimum value of positive standard serum was established in six reactions. However, these values (117 – 133 tests) fluctuated within the specified range (Fig. 4).

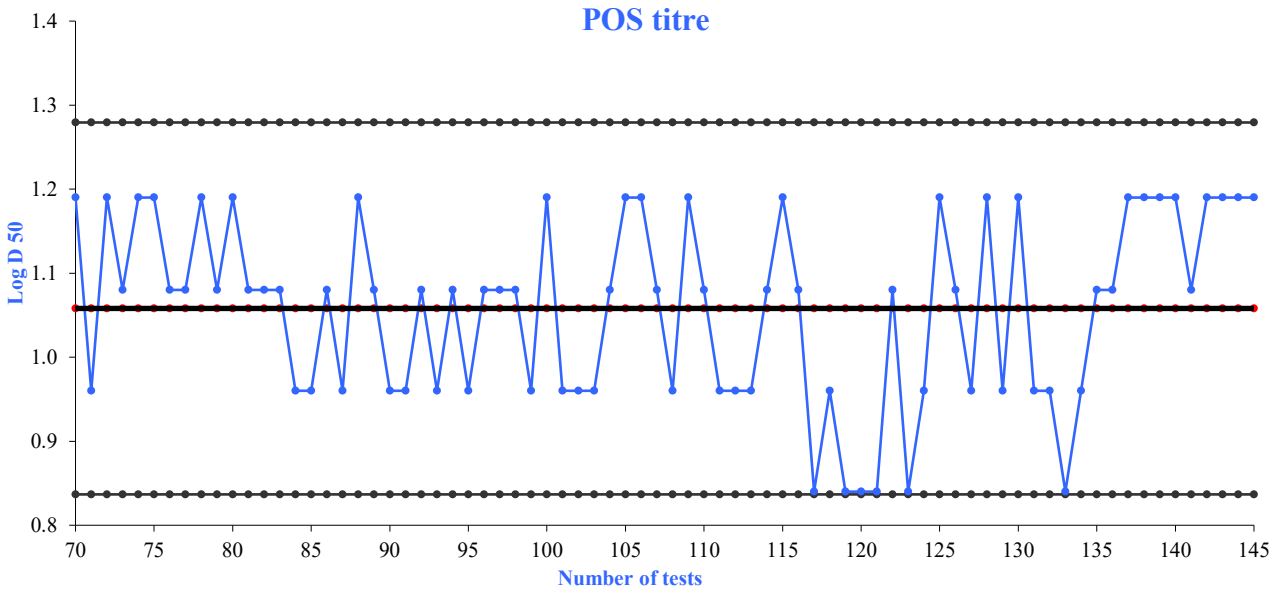

Fig. 4. The significance of positive WHO standard serum titers (reconstituted and diluted to a theoretical value of 0.5 IU/cm³) when performing the FAVN-test in 2019 (protocol 70 – 145)

Four more characterized series of the CVS-11 strain of rabies virus were subsequently used for routine studies. There were no significant fluctuations or deviations in the titer of the virus infectious activity in the reaction. The virus was titrated within the expected range of 4.97-5.27 log_{50} $TCID_{50}$ (except for test No. 172 in 2023 and No. 43 in 2024 (titer – 5.42 log_{50} $TCID_{50}$).

The log_{50} values of the positive standard serum also ranged within the expected range – 1.08-1.19 (except for test No. 58, No. 82 in 2023, where the log_{50} of positive serum was 1.43 and 1.31, respectively, and test No. 53 in 2024, where the log_{50} of positive serum was 0.96).

The minor deviations obtained in both the rabies virus titer and the positive serum were not systemic and were not associated with changes in the virus series or other changes in the

reagent or medium series used to perform the FAVN-test, but were most likely associated with human factors, and therefore did not require any corrective action.

Discussion. The control of intensity of humoral rabies immunity in animals is an unconditional factor that makes the entire system of laboratory diagnosis of rabies complete. Modern effective and reliable serological tests, as well as the principles of their application, are defined in all international diagnostic guidelines, such as those of the WHO or WOAH (WHO, 2018, 2019; WOAH, 2023). The assessment of rabies immunity is of great importance, as these studies have been and remain a key element in evaluating the effectiveness of rabies vaccination (Regulation (EU) No 576/2013; Corey, 1976; Shiraishi, 2014).

The most common methods for such studies are the neutralization reaction in white mice, the FAVN-test, or the RFFIT (Rapid Fluorescent Focus Inhibition test) in cell culture (Haase, 1985; Cliquet, 1998; Kostense, 2012). All these reactions involve the use of standard blood serum with a defined virus-neutralizing activity against the rabies virus (WOAH, 2023). The standard positive rabies serum from dogs recommended by the WOAH, produced by the European Reference Laboratory for Rabies (AFSSA, France), is used for this purpose (Wasniewski, 2017). This standard preparation is intended for the assessment of rabies immunity in domestic animals using in vitro methods. Batches of this product are calibrated to the Second International Standard for Human Immunoglobulin (Wasniewski, 2019).

The stable and continuous laboratory capacity to test blood sera using the FAVN-test in accordance with the requirements of the international standard DSTU ISO/IEC 17025:2017 at the Research Virology Department SSRILDVSE was ensured by the implementation of a complex of continuous monitoring of indicators: $\log_{50}$ of positive standard serum (reconstituted and diluted to the prescribed theoretical value of 0.5 IU/cm^3), $\log_{50}$ of negative serum, and $\log_{50}$ of the infectious titer of the CVS-11 strain of rabies virus. It was the continuous monitoring of these indicators that made it possible to take corrective action when changing the rabies virus series or updating cell culture lines.

Taking into account the requirements of international (European) regulatory documents, one of the main criteria for assessing the technical competence of laboratories, in addition to accreditation in accordance with DSTU ISO/IEC 17025, is the results of their participation in rounds of inter-laboratory comparative tests. Participation in such rounds is a mandatory and integral part of external quality control of research in a testing laboratory (Wasniewski et al., 2019).

In 2025, a panel of blood sera was received from the European Reference Laboratory for Rabies (ANSES, France) to participate in a round of interlaboratory comparative tests. Tested of the coded panel of blood sera conducted at the Research Department of Virology SSRILDVSE showed a coefficient of determination of «R^2» = 0.9879. The results we obtained for the rabies activity of blood sera indicate their high (≥ 0.95) degree of correspondence to the expected results (theoretical $\log_{50}$) (Wasniewski, 2019).

Conclusions. The obtained results indicate the need for constant monitoring of rabies virus titers and standard serum in each reaction, as the stability (especially when changing the rabies virus series or updating cell culture lines) of these indicators indicates the quality and reliability of the results of blood serum testing for the presence of rabies antibodies.

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Характеристика контрольних показників якості реакції віруснейтралізації в культурі клітин для виявлення антитіл до вірусу сказу (FAVN-тест)

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Резюме. *В статті представлена інформація щодо результатів постійного контролю за показниками титрів вірусу сказу та стандартної сироватки в FAVN-test, що є запорукою якості і достовірності результатів дослідження сироваток крові на наявність антитіл до вірусу сказу. FAVN-test є основним методом оцінки імунної відповіді на введення антирабічної вакцини, що рекомендується до використання ВООЗ і ВООЗТ. Дослідження напруженості індивідуального поствакцинального антирабічного імунітету є вимогою країн Європейського Союзу та багатьох інших*

країн світу та України під час некомерційного переміщення тварин-компаньйонів. В Україні вперше впроваджено метод FAVN-test в науково-дослідному вірусологічному відділі ДНДІЛДВСЕ. Для забезпечення якості досліджень розроблено форми і впроваджено картки контролю. Вносяться отримані в кожній постановці реакції значення log_{50} позитивної стандартної сироватки ВООЗТ (0,5 МО/см^3), значення log_{50} негативної сироватки та значення log_{50} титру вірусу сказу штаму CVS-11. Протягом 2019 – 2024 рр. проведено постановку 929 реакцій методом FAVN-тест, в яких досліджено та обраховано усі визначені показники та побудовані відповідні графіки. Постійний контроль визначених показників дав змогу здійснювати коригувальні дії при зміні серії вірусу сказу або оновлені лінії культури клітин. В 2025 році прийнято участь в міжлабораторному раунді (Rabies serology proficiency test). Проведені дослідження отриманої закодованої панелі сироваток крові, показали значення коефіцієнта детермінації «R^2» = 0,9879. Отримані нами результати свідчать про їх високу (≥ 0,95) ступінь відповідності очікуваним результатам.

Ключові слова: вірус сказу, референс-штам, сироватка крові, антитіла, віруснейтралізація

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