GUIDELINE: DETECTION OF ANTIBODIES AGAINST ORBIVIRUS BY SERONEUTRALIZATION TEST

Date: 27/02/2020	Document code: GL-LCV-03	*Rev. 01*

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1. PURPOSE/SCOPE

The purpose is to provide general guidance to the best implementation and quality control of the seroneutralization test (SNT) to measure neutralizing antibodies against *Orbivirus* in animal sera. Theoretically, the seroneutralization assay is able to detect antibodies regardless of the animal species. However, it is recommended to verify this item.

Although BTV, AHSV and EHDV share the same protocol, epidemiological and diagnostic interpretation of the results obtained must be done considering the different epidemiological situations of each pathogen agent.

The assay measures the ability of a serum to neutralize a fixed dose of virus and prevent the appearance of a readily observable cytopathic effect (CPE) when both are co-inoculated in susceptible cells culture.

SNT is considered a semi-quantitative assay and an antibody titer is reported together with the qualitative result (POS/NEG).

This guideline take into account applying principles and rules under the accreditation scope of ISO/IEC 17025, *General Requirements for the competence of testing and calibration laboratories* of the International Organization for Standardization.

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2. BACKGROUND INFORMATION

2.1. Abreviation

AHSV: African horse sickness virus // AHS: African horse sickness

BTV: Bluetongue virus // BT: Bluetongue

CPE: Cytopathic effect

EHDV: Epizootic hemorrhagic disease virus // EHD: Epizootic hemorrhagic disease

EMEM : Eagle's Minimum Essential Medium with Earle's balanced salt solution.

IBFS: Inactivated bovine fetal serum

OIE: World Organization for Animal Health

SNT: Seroneutralization test

WD: Working dilution

2.2. Definitions

Antibody titer: the highest dilution of a serum sample containing antibodies sufficient to neutralize the action of a predetermined concentration of the virus on the cell culture, thus the last dilution of the serum sample where the CPE is not visualized. It can be expressed as a dilution or log of the inverse of the highest dilution that neutralizes the virus.

Back titration: it is the virus control of the assay. A ten-fold titration of the working dilution (WD) of virus that is done to confirm the number of $TCID_{50}$/well present in the assay.

Cell culture medium: diluent with 10% v/v of inactivated bovine foetal serum.

Cell suspension: cells in cell culture medium containing the established concentration of viable cells/ml.

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Cytopathic effect (CPE): morphological and cell viability changes, visible to light microscopy, caused during the cycle of viral replication. The cellular alterations that occurs sequentially or simultaneously in the infected cell concludes with cell lysis and new viral particles released to the medium.

Diluent: enriched liquid medium suitable for replacing the natural environment of the cells. In the SNT protocol is made of EMEM + 1% Antibiotic/Antimycotic (100x) + 1% L-Glutamine + 1% Non - essential Aminoacids.

Full titration: A titration of the stock virus for determine the number of $TCID_{50}$/ml using ten-fold dilutions.

Initial / Final serum dilutions: initial serum dilution is the serum dilution in the diluent. The final serum dilution takes into consideration that a further 1/2 dilution of the initial serum dilution is made following the addition of an equal volume of virus to each serum dilution. When reporting results, this issue must be explained.

Reed and Muench 50% endpoint viral titer: a statistical calculation to determine the dilution of the virus that causes a CPE in 50% of the replicate wells in a microtiter plate.

Serogroup: is an infectious microorganism species that differs from other species of the same genus through serological tests.

Serotype: is a subpopulation of an infectious microorganism that differs from other subpopulations of the same species by means of serological tests. Therefore, the immune

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responses to a serotype of a microorganism may not protect against another serotype of the same species. Serotypes enable to differentiate organisms at the subspecies level, which is epidemiologically important.

Stock virus: the batch of undiluted virus that is used to prepare the working dilution (WD) of virus.

Working dilution of virus (WD): the dilution of virus that ideally contains 100 $TCID_{50}$ / well, as determined from previous full titration. It is the dilution of virus that is added to the test plates in the assay.

2.3. Background

Some species of the genus *Orbivirus* (Fam. *Reoviridae*) produce infectious, non-contagious and insect-transmitted diseases in animals. Among these species of Orbivirus there are 3 serogroups that produce diseases in animals of obligatory declaration: African horse sickness, Bluetongue and Epizootic Hemorrhagic disease. All three are transmitted by insects of the genus Culicoides.

The structural conformation of the Orbivirus presents an internal and an external capsid. The major capsid protein, VP2, is also the main responsible for the different serotypes within each serogroup and, together with the VP5 protein, is the target for the virus neutralization. This is why seroneutralization is used as a serotyping technique in the case of Orbivirus.

Each serogroup have antigenically distinct serotypes identified by virus neutralization test: 9 serotypes of AHSV, at least 27 serotypes of BTV and 8 serotypes of EHDV. No cross-reactions among serogroups have been observed, but some cross-reaction has been observed between

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serotypes: in case of AHSV, 1 and 2, 3 and 7, 5 and 8, and 6 and 9. BTV multiple infections or polyvalent vaccination can induce serotype cross-reactive neutralising antibody responses.

It is important to note that antibodies detected by SN (anti-VP2 and anti-VP5) are different from those detected by ELISA (anti-VP7) techniques, which are majority.

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4. SAFETY PRECAUTIONS

The assay shall be carried out following Biosafety, Biocontainment and Bioprotection guidance and rules established according to international and national regulations (see references) considering the target pathogen.

According to OIE Manual, there is no evidence that humans become infected with any field strain of Orbivirus, either through contact with naturally or experimentally infected animals or by virus manipulation in laboratories.

According to OIE recommendations, AHSV, BTV and EHDV are classified as group risk 3 pathogens in the EURL. Therefore, viral suspensions of these viruses as well as clinical samples (blood and tissues) from suspicious animals are handled in level 3 containment facilities.

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Regarding serum samples, Orbivirus particles have hemagglutination activity that allows them to adhere to circulating erythrocytes. Thus, the presence of infectious virus in the serum fraction is generally residual. Only when viremia is very intense, Orbivirus could be isolated from serum samples.

Therefore, biological risk assessment must be done considering two main different sources of biological risk: serum samples and standard Orbivirus strains used to neutralize the antibodies.

Apart from Orbivirus presence, risk assessment of clinical samples must be considered especially when samples from countries that do not have “free status” for other relevant animal diseases.

5. GENERAL REQUERIMENTS FOR TEST´S PERFORMANCE

5.1. Description

The SNT involves an initial incubation of diluted sample and control sera with a known serotype of an Orbivirus. For each serotype, the quantity of virus is standardized to ideally contain 100 $TCID_{50}$/well and is referred to as the working dilution of virus (WD). A second incubation of 5 to 7 days occurs following the addition of susceptible cells.

The assay is evaluated for the presence or absence of degenerative changes in the cells known as cytopathic effect (CPE). Serum containing specific antibodies towards a particular serotype of Orbivirus will neutralize the virus and the cells will remain uninfected. Upon microscopic examination, the cells will be normal in appearance. If the serum does not contain specific antibodies the virus will infect the cells and produce a CPE.

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5.2. Protocol

The laboratory shall made available a Standard Operating Procedure (SOP) that, taking as the basis the OIE Manual ,adapts operations to the laboratory quality system established according to ISO 17025, as well as to every aspect that might be relevant for a correct assay performance (e.g. quality control of assay, interpretation of results).

For an adequate performance, SNT needs other SOPs, such as cell culture maintenance and tripsinization, production of virus stock, full titration of virus stock, estimation of 50% endpoint virus and antibody titer, action in case of discordant results in replicates, biosafety procedures or/and use and calibration of equipment.

In the framework of the personnel qualification/training, the SNT SOP should be divided into 2 different activities (performance and microscopically reading).

In the EU-RL SNT SOP, 25µl of serum, 25µl of virus and 50µl of cells (Final volume per well 100 µl) are used. Slight differences in the final volume per well could be among SOPs established in different laboratories. This fact is not relevant if the final volume is enough to avoid well-drying.

5.3. Traceability and preparation of samples

A sample identification system must be set up in the laboratory in order to guarantee the traceability of the samples. When samples are transferred among different departments into the laboratory, proper records must be planned.

Serum is the sample of choice for performing the SNT assay. If serum sample is received without clot, it must be stored at 5 ± 3ºC until assay is performed within a maximum of seven days. In

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case of whole blood samples, if the serum portion is clearly splitted from the clot, that serum is suitable to be used directly in the test and it must be stored as previously described until the assay is performed. Otherwise, the whole blood samples can be centrifuged at 780g for 10 min for serum portion separation.

Test sera that are severely hemolyzed or visibly contaminated should not be used. Sera that are partially hemolyzed may be tested. However, it may not be possible to report results if the serum is toxic to the cells (as determined from the serum control results). Sera that appear slightly contaminated or sera that were found to be contaminated after performing the SNT assay may be filtered using a 0.45 or 0.2 micron filter and/or centrifuged at 5000g for 15 min. to eliminate bacterial contamination, and the SNT assay performed. However, it may not be possible to report a result if the contamination remains even after filtering. Another serum (re-sampling the animal) is requested and tested, if possible, when unsuitable samples are received.

All sample manipulations such as filtration or centrifugation must be properly recorded.

Serum samples can be stored refrigerated (4ºC) up to seven days until assayed. For longer periods, they must be stored at freezing temperature (-20ºC). Serum samples must be at room temperature before using in the assay.

Volume of the test samples needed should be taken and prepared according to the number of serotypes that are included in the assay.

Test samples must be heat inactivated at a 56 ± 2°C for 30 ± 5 minutes in order to inactivate complement, a group of proteins present in the serum that are part of the immune response and may have an unwanted influence.

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5.4. Preparation of the assay

5.4.1 Personnel qualification/training.

Complete training according to SOP in force in the laboratory/department.

Be familiar with the preparation and proper handling of assay reagents and samples along with proper precautionary measures, with the calibration, maintenance, and use of equipment used in the SNT assay and with all applicable laboratory SOPs.

5.4.2 Preparation of Equipment/Instrumentation

The appropriate equipment, such as pipettes or incubators, must be calibrated and certified according to the current version of their respective SOP in force in the laboratory.

5.4.3 Preparation of reagents

Cell suspension: Although the EURL uses VERO cells, other susceptible cell lines such as BHK-21 or BSR could be used instead of.

Working dilution of virus (WD): In addition to the full titration assay, it is recommended to carry out a back titration assay before testing samples with each new batch of stock virus.

Moreover, in order to check the virus stock conservation, a full titration should be performed from time to time.

5.4.4 Preparation of control sera

Forms to record the relevant information regarding control sera (species, source …) and the information of the batches (dilution of positive serum, liophilization, heat inactivation…) should be established.

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Negative control serum can be the same for all serotypes. while positive control serum must be prepared for each serotype.

Negative control serum must be added as a test sample (heat inactivated and diluted in diluent). Regarding positive control serum to prepare a proper dilution to add so that the appropriate endpoint antibody titer is reached.

5.5. Performance of the assay

The virus neutralization assay requires the management of mammalian cells and live virus in sterility conditions. Therefore, all the process is performed within a Class II biological safety cabinet using the appropriate personal protective equipment (nitrile gloves and sleeves).

The number of serotypes that are tested will depend upon the epidemiological circumstances/need. The plate layout (number of replicates and serum dilution for testing) varies depending of the purpose of the testing (p.e. screening or titration protocol).

Labeling of the microtiter plates with the appropriate information is recommended to guarantee traceability (i.e. accession number, serotype, test date, the plate number if there is more than 1 plate per serotype..).

It is recommended distributing the whole diluent before handling sera samples and virus.

<u>Addition of the cells</u>

Add cell suspension at the appropriate cell concentration to the wells after the incubation of the plates in a manner in which there is no possibility of transferring virus to plates or wells in which virus was not originally added. i.e. the following procedure:

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Add cells to the plates in the following order using the same sterile reservoir and tips per serotype:

1. Plates/wells containing neither virus nor serum: the cell control wells
2. Plates/wells containing no virus: the serum controls wells
3. Lastly, the plates/wells containing virus in the following concentration order: the control virus wells containing ideally 0,1 $TCID_{50}$/well; control virus wells containing 1 $TCID_{50}$/well; control virus wells containing 10 $TCID_{50}$/well; wells containing 100 $TCID_{50}$/well (control virus and test sera).

Reading of plates

Evaluate the controls initially and later the test plates for the presence/absence of a CPE. This can be done either microscopically or macroscopically. The presence of a CPE indicates the absence of neutralizing antibodies in the serum sample to a particular serotype. Conversely, the absence of a CPE indicates the presence of neutralizing antibodies in the serum sample to a particular serotype. Record the results of the controls and test sera.

For microscopic reading use an inverted microscope to visualize CPE (4x and eventually 10x). The CPE of Orbivirus is characterized initially by the formation of plaques of rounded refractive cells and eventually by the destruction of the entire cell monolayer. There must be taken into account that slight differences regarding the extension/characterization of CPE in the well among serogroups and even serotypes is common.

There must be clearly established in the procedure what must be considered CPE or not (inclusion of photographs is recommended). Personnel training protocols have to include this issue to avoid different readings between technicians.

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Stain all the plates using a crystal violet staining solution allows reading the plates macroscopically for the presence or absence of stained cells. The crystal violet staining solution stains uninfected cells purple. The presence of stained cells indicates that the virus was neutralized by antibody present in the serum sample and therefore the virus did not infect the cells. The absence of stained cells indicates that the virus was not neutralized by antibody and therefore the virus was able to infect the cells.

If there is partial CPE within a well, it may be necessary to read the plates microscopically following staining to determine if what was observed was due to the presence of a CPE. Considering that partial CPE is very usual in Orbivirus, it would be the main disadvantage of macroscopic method. Macroscopic reading is not included in the EURL SOP.

5.6. Quality Controls

SNT is a biological assay. This fact can produce greater variability making it necessary to strengthen internal controls, as well as to provide a system to monitor the variability between assays (trends).

Internal control can be distributed in the same plates that test serum plates or in separate plate/s.

Internal controls included in each assay are:

-Serum sample controls: consisting of serum samples and cells (no virus). It is included to determine if each sample serum would have a toxic effect on the cells and to confirm that the serum is not contaminated.

To include this control only in one serotype´s plates is enough.

Serum has to be included in the Serum control at the same dilution that the most concentrated final dilution of the serum when testing

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-Cell control: consisting only of cells. It is included to determine if there is a proper cell growth without contamination of the cells while processing. It is recommended to include several replicates of cell control.

-Virus control (back titration): a ten-fold titration of the working dilution (WD) of virus that is done to confirm the number of $TCID_{50}$/well present in the assay.

Usually 4 ten-fold dilutions (100, 10, 1 and 0,1 $TCID_{50}$ / well) are included in the assay. It is recommended to use at least four wells for each virus dilution.

-Positive and negative serum control: for each serotype. They are included to ensure that the assay is sensitive and specific. Positive serum control titer must be valued as a SNT is a semi-quantitative assay.

Positive serum control must be added in several dilutions (i.e. 1/5, 1/10, 1/20 and 1/40) in order to determinate the titer. Negative serum could be included using only the most concentrated dilution (i.e. 1/5).

It is recommended that at least two replicates of positive and negative serum control is included.

In addition, considering the purpose of the assay (screening or titration) it is advisable to test repeatability by assaying a number of samples in replicate (duplicate or more). Qualitative and semi-quantitative (titer) assessment should be considered.

The internal control results must be regularly reviewed for detection of trends.

5.7. Proficiency Test (PT)

According to the ISO 17025, the laboratory shall participate regularly in external proficiency testing schemes. Participation in such scheme is a requirement for accredited laboratories.

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Serogroup specific methods, such as ELISA VP7, are the method of choice for Orbivirus antibodies detection. SNT in Orbivirus is able to identify and quantify the serotype specific antibodies (serotyping). In this context, Orbivirus SNT is not the method of choice to perform the first serological diagnostic and it is not easy to find available PTs for it.

In case that there is not a PT which includes SNT, internal controls must be strengthened (i.e. by means of testing blind samples).

6. ASSAY VALIDATION

6.1. Quality Control validation

Determine the validity of the assay for each serotype using the following criteria:

-Control virus for each serotype: the control virus (back titration) should be between 30 and 316 $TCID_{50}$/well of virus (Reed and Muench 50% endpoint method).

Note: If the virus titer is low (less than 30 $TCID_{50}$/well) and all other test validity criteria are valid, the test is considered as a valid assay only for those sera that result negative.

Under these conditions, the test is highly specific; a negative sample will continue to be negative if the test were repeated with virus within the valid range. Test sera that are positive under these conditions have to be repeated as they can be false positive or at least the titer is potentially artificially high.

-Positive control serum: for each serotype, the antibody titer (Spearman-Karber 50% endpoint method) of the positive control serum shall be within two-fold of the expected titer established from previous titrations.

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-Negative control serum: for each serotype, the negative serum shall not neutralize the virus (there is a CPE within all replicate wells).

-Serum sample control: there should be no toxicity (abnormal appearance of the cell monolayer) in the serum sample control wells for the test sera.

If toxicity or contamination is present in serum sample control, a determination of the presence/absence of antibody could not be possible depending on the concentration of the toxicity or contaminating factor. If so, criteria to report or not should be included in your SOP.

Test serum that was contaminated, could be appropriately filtered (such as it is explained at the above point 5.3 Traceability and preparation of samples) and, consequently, the SNT assay could be repeated.

Repeatability of samples tested in replicate must be according to the qualitative and quantitative variability criteria established in the SOP.

If the assay fails to meet all the acceptance criteria listed then review the assay for any technical errors and repeat the assay for whatever serotype(s) that failed.

6.2. Interpretation of the results

If the assay is valid, read and record the wells with CPE for the test plates for each serotype. Determine the presence/absence of antibody or antibody titer for the tested samples.

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In case of samples tested in replicate, apply a method to determinate the qualitative result (or re-tested) in case of qualitative differences. Average or estimated titer of positive samples should be calculated (i.e. Spearman-Karber 50% endpoint method).

Report the results of the assay for whatever dilutions in which toxicity or contamination did not occur, taking into consideration that the result reported may need to include modifiers (i.e. negative less than or equal to a titer, positive less than or equal to a titer).

In case of using at least two replicates, the antibody titer can be determinated in two different ways:

-Estimation method (i.e. Spearman Karber 50% endpoint antibody titer): a statistical calculation to determine the dilution of the serum sample that prevent a CPE in 50% of the replicate wells in a microtiter plate.

-100% endpoint method: a determination of the dilution of serum that prevents a CPE in 100% of the replicate wells for that dilution and all preceding (more concentrated) dilutions while not preventing a CPE in either one or both of the replicate wells for further (less concentrated) dilutions in a microtiter plate.

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