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DIRECCIÓN GENERAL DE SANIDAD DE LA PRODUCCIÓN AGRARIA

DIVISIÓN DE LABORATORIOS DE SANIDAD DE LA PRODUCCIÓN AGRARIA

LABORATORIO CENTRAL DE VETERINARIA

EU Reference Laboratory for
African horse sickness and Bluetongue

OIE Reference Laboratory for
African horse sickness

EU DIAGNOSTIC MANUAL

FOR

AFRICAN HORSE SICKNESS

AND

BLUETONGUE

Elaborated by: Central Veterinary Laboratory, Algete, Madrid, Spain

EU Reference Laboratory for African Horse Sickness and Bluetongue

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Date: 22/06/2021	Document code: GL-LCV-14	*Rev. 01*

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1. PURPOSE

It is necessary to lay down at European Union (EU) level validated diagnostic laboratory tests for the confirmation of relevant animal diseases as well as procedures and criteria for the collection of samples and evaluation of the results.

This matter is of very technical nature and is subject to frequent modifications due to the evolution of scientific standards. Therefore, to ensure that they are up to date, the rules for the laboratory diagnosis should indicate in a flexible manner which methods should be used and how they should be interpreted.

Delegated Regulation adopted within the framework of Animal health law (Regulation EU 2016/429) establishes that specific legislation and the relevant details and guidance made available on the website of the European Union Reference Laboratories (EURL) or of the Commission are at the top of the hierarchical order into which they should be considered in this matter.

In this framework, the main purpose of this document elaborated by the EURL for African horse sickness and Bluetongue, is to make available to official animal health laboratories in the EU, especially National reference laboratories (EU-NRLs), guidelines for the best implementation of the validated diagnostic procedures and criteria for the collection and storage of samples, as well as provide keys for the interpretation of the results of laboratory tests for the surveillance and confirmation of African horse sickness and Bluetongue.

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2. BRIEF DESCRIPTION OF THE DISEASES: AHS & BT

African horse sickness and Bluetongue are two very different OIE notifiable diseases: AHS affects *equidae* and it is considered one of the most lethal diseases of horses, killing between 50 to 95% of infected animals, while BT virus infects ruminants causing ranges from subclinical infection to mild disease, and just in some cases to acute or even fatal disease.

In the other hand, both diseases have many things in common, mainly they are non-contagious viral diseases transmitted to animals primarily by Culicoides midges vectors and are produced by members of the same genus (Orbivirus). These similarities mean that similar diagnostic tools and control strategies are used in both diseases.

The genus Orbivirus, which is 1 of 15 genera within the family *Reoviridae*, contains 22 distinct virus species recognized by the International Committee for the Taxonomy of Viruses (ICTV). Although AHSV and BTV are the most economically important members of this genus, and therefore the best studied orbiviruses, there are any other pathogens in this genus causing relevant animal diseases such as Epizootic hemorrhagic disease or Equine Encephalosis.

Orbiviruses are non-enveloped virus particles consisting of ten-segmented double stranded (ds) RNA genome (Seg-1–10) encapsulated in the VP3 subcore, surrounded by serogroup specific protein VP7 (core), and the outer shell of the particle is formed by serotype specific proteins VP2 and VP5. . When two segmented viruses of the same species co-infect the same cell, their progeny may incorporate segments picked up from any “parental” virus. This process is called “reassortment” and represents an important way for segmented viruses to evolve.

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Nine (9) serotypes of AHSV and, until 2008, twenty-four (24) serotypes of BTV were documented. The further discovery of novel BTV serotypes added complexity to this specie; however, only the 24 classical serotypes (1-24) are considered for disease notification. The serotype is defined by the interaction of neutralizing antibodies with the VP2 outer capsid protein.

With VP2 exhibiting high levels of amino acid sequence variability between serotypes, it is not surprising that low levels of cross-protection exist between serotypes, which complicates vaccination strategies.

African horse sickness

AHS is one out of the five diseases already identified in the EU Regulation 2016/425 (art. 5) with the potential to pose serious risks to animal health and to result in a high impact on the economy. Therefore, EU regulation applies the higher protection against AHS and this disease is categorized as A, requiring stringent measures to immediately eradicate them as soon as they occur.

The clinical disease caused by AHSV is usually classified into four forms according to characteristic pathology and varies according to host species as well as prior exposure to the specific serotype of AHSV. The horse is the soliped most susceptible to the disease and species of zebra and the African donkey are primary maintenance reservoirs of AHSV.

AHS is endemic in several countries of Sub-Saharan Africa. Outside Africa, AHS outbreaks were reported as follows:

- The first non African cases due to serotype 9 of the AHS virus (AHSV-9) were reported in 1959 -1961 (Saudi Arabia, Lebanon, Syria, Jordan, Iraq, Turkey, Cyprus, Iran, Afghanistan, Pakistan and India).
- In 1965 new AHS-9 outbreaks were recorded in Morocco, Algeria and Tunisia. In October 1966 the disease appeared in Spain.
- In September 1987 an outbreak of AHS caused by serotype 4 was reported in Spain and the next years in Morocco and Portugal.

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- Early 2020, an outbreak of AHS caused by serotype 1 occurred in Thailand, affecting also Malaysia.

Vaccination of animals remains the most successful method of prevention and control. The protection provided by antibodies against the AHS virus is serotype-specific, with known cross protection for some of the serotypes. The rapid diagnosis of AHS and the identification of the serotype are important, in order to allow proper planning of vaccination. At present, only attenuated (monovalent and polyvalent) live vaccines are commercially available for use in horses, mules and donkeys.

Bluetongue

Recent animal health EU regulation lists infection with bluetongue virus (serotypes 1 to 24) as category C disease for optional eradication programme. This implies a change in the policy against this disease since Council Directive 2000/75/EC, which was previously applied to this Regulation, provided for its immediate eradication.

Among ruminants, severe clinical disease is mostly seen in sheep and white-tailed deer, including fever, facial oedema, haemorrhages into, and ulceration on, the oral mucosa and coronitis; whereas, cattle, goats and camelids usually show asymptomatic or sub-clinical disease. However, outbreaks of BTV-8 in Europe during 2006 caused clinical disease in both goats and cattle.

BTV is present around the world and, traditionally, the virus was present in a geographic band between the latitudes 40°N and 35°S, where its vectors, certain species of biting midges, were living. Regarding Europe, during the 20th century, only short outbreaks of BT were occasionally recorded in Southern European countries (Spain, Portugal, Greece and Cyprus). However, since 1998 BTV has been present in Southern European and Mediterranean countries from which it has gradually spread to areas previously free of the virus.

In August 2006, BTV8 epidemics broke out suddenly and unexpectedly in Northern Europe. This strain was highly virulent not only for sheep, but also cattle and, in addition, it could cross the placenta, which was not

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typical of the field strains of BTV in the past. After it, BT incursions of a variety of serotypes occurred and on several occasions became widespread across many parts of Europe.

The disease control strategy in EU is primarily based on vaccination of the relevant targeted animal population using inactivated serotype-specific vaccines, supported by other control measures, such as movement restrictions. In the EFSA opinion to succeed in the eradication, the vaccination coverage should be at least of 95% of the susceptible bovine and ovine animals for a minimum period of five years.

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3. COLLECTION, TRANSPORT AND STORAGE OF SAMPLES

Samples must be obtained with adequate biosafety measures in the farm. Stress, injury and unnecessary physical risk to animals, as well as to people handling them, must be avoided. The outcome of a diagnostic test is strongly influenced by the sample quality, the storage conditions and the way in which the sample is collected.

Samples must be labelled with an indelible marker to trace identity of the animals. Relevant epidemiological information on the animals, such as vaccination status, age or presence of clinical symptoms, should be provided to the laboratory, so that the laboratory staff can assign the most appropriate tests or test sets, as well as interpret the results properly.

Choose the tissues or fluids most likely to contain the infectious agent or analyte to be detected, to the best suitability of samples:

For direct diagnosis (*agent detection*):

- In live animals, whole blood collected in tubes with anticoagulant (EDTA for PCR) is the recommended sample.
- In dead animals, in addition to whole blood collected in tubes with anticoagulant (EDTA for PCR) if possible, organ samples should be collected, especially spleen, lungs and lymph nodes.

For indirect diagnosis (*antibodies detection*), blood must be collected in a tube without anticoagulants to obtain serum sample. Allow the blood to settle at ambient temperature, protected from extreme heat until clear. Better separation can be achieved by placing tubes at a 45° angle. That serum is suitable to be used directly in the test.

If assay will be delayed serum sample will be stored after removing the clot: decant in a sterile vial and discard the blood clot. Do not delay in decanting to avoid haemolysis.

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Otherwise, the whole blood samples can be subjected to a low speed centrifugation (780g for 10 min) for serum portion separation.

For the proper **preservation**, all samples collected must be maintained at a **temperature of +4°C** for up to one week and analysed in the laboratory as soon as possible. **Samples for virus diagnosis must never be stored at freezing temperatures (between 0ºC and -40ºC).**

If storage is required for > 1 week:

- Serum at – 20° C
- EDTA Blood at +4° C (blood sample freezing -80ºC causes the virus release associated to the erythrocyte membrane, resulting in its neutralisation by the antibodies present in the plasma. It could interfere with virus isolation)
- Organs at –80° C.

If storage is required for long periods (as biobank of samples):

- Serum at – 20° C is enough
- EDTA Blood at -80ºC
- Organs at –80° C.

Regarding **orbiviruses isolates**, deep freezing (<-60ºC) is recommended for storage. **Never must be stored at freezing temperatures (between 0ºC and -40ºC).**

Related to the **transport** of biological material, IATA and ADR categorize both clinical samples from animal suspicious of a disease produced by an Orbivirus as well as Orbivirus isolates as Category B (UN 3373).

Therefore, triple packaging system according to Packing Instructions P650, as well as labelling and documentation requirements for this category, must be taken into account.
Information about transport is available at: www.who.int/ihr/publications/WHO-WHE-CPI-2019.20/en/

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4. PROCEDURES

Orbivirus diagnosis could be structured in three steps:

I. Serogroup detection techniques (direct and indirect diagnosis)
II. Serotype identification techniques (direct and indirect diagnosis)
III. Further characterization methods

For the purposes of this document, concepts of technique, method (or assay) and kit have been defined separately. RT-PCR, ELISA, Virus isolation and Seroneutralization test are considered as techniques, while per example "rRT-PCR based on Hofmann *et al* 2008 for detection of BTV genome" or "Blocking ELISA for detection of antibodies against AHSV" are considered as methods. Finally, commercial kits based on the methods sometimes are available.

Standard Operating Procedures (SOPs) provided by the EURL on its website are referred to the methods. Some of them apply to Orbivirus, and others are just to detect a specific serogroup (AHSV or BTV) / serotype.

Validated fit for purpose serogroup diagnostic methods based on RT-PCR and ELISA techniques for AHS and BT diagnosis must be implemented in all EU National reference laboratories.

EURL provides methods based on these techniques, available in the website, and their validation report to facilitate their implementation in the EU-NRLs. Anyway, similar methods based on rRT-PCR and ELISA techniques could be used by the EU-NRLs if these methods comply with the minimum criteria described in the validation chapter.

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In relation to BTV, accordingly to the validation report, for surveillance purpose competitive/blocking ELISA is the recommended method, and double recognition ELISA is recommended to be used as a complementary method.

In addition, EURL provides SOPs for the serotyping and characterization of BTV and AHSV as well as for orbivirus isolation.

There must be considered that to carry out the provided diagnostic procedures, each laboratory must have in force any others procedures aimed at preparing reagents or samples, such as trypsinization of cell lines, growth and full titration of viruses or RNA extraction in clinical samples. Although these additional procedures are not available in the EURL website, the EURL could provide them under request.

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5. INTERPRETATION OF THE RESULTS

According to the OIE Manual, direct and/or indirect (serological) diagnosis could be recommended for different purposes:

- Population freedom from infection
- Individual animal freedom from infection prior to movement
- Contribute to eradication policies
- Prevalence of infection – surveillance
- Confirmation of clinical cases
- Immune status in individual animals or populations post-vaccination

Clinical sign and histological lesions are not pathognomonic. Although differential characteristics of diseases presentation can guide a field diagnosis, only laboratory diagnosis can provide a definitive diagnosis. Therefore, it is essential to perform a differential diagnosis to other infectious diseases, such as, Equine Encephalosis, Equine Infectious Anaemia, Morbillivirus Pneumonia, Equine Viral Arteritis, Babesiosis or Purpura Haemorrhagica in case of AHS, and Foot-and-mouth, Peste des petits ruminants, Malignant catarrhal fever or Capripoxvirus-induced disease in case of BT.

Anyway, laboratory diagnosis should be interpreted considering field diagnosis based on clinical signs, and lesions in organs from dead animals , as well as epidemiological link to a suspected or confirmed case. Moreover, it is recommended to use at least two different diagnostic methods to confirm an AHS or BT infection.

There must be considered that both, AHS and BT disease control strategy, is primarily based on vaccination of the relevant targeted animal population. At the moment, a DIVA (differentiating infected from vaccinated animals) vaccination strategy is not ready-for-use neither AHS nor BT.

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Therefore, both the reason of sampling for laboratory diagnosis and vaccination status of the animal/population must be considered to interpret the laboratory diagnosis results.

FIRST STEP OF DIAGNOSIS: SEROGROUP DETECTION

The first step in approaching the diagnosis is the serogroup detection. At the present, serogroup specific real time RT-PCR in EDTA-blood samples is the main diagnostic tool used in surveillance and control of AHS and BT, because of their analytical and diagnostic sensitivity and specificity, as well as easier automation and processing of large numbers of samples. In addition, long viremia period detected by RT-PCR in animals infected with orbiviruses invites you to choose this methods as technique of choice.

ELISA methods are able to detect antibodies against AHSV or BTV VP7 protein since as early as 7 to 14 days up to several years, being a suitable methods to detect infection on serum samples from unvaccinated animals. In vaccinated animals antibodies are detected as well (at different dpi depending on the vaccine) but it is not possible to differentiate vaccinated from infected animals using the commercial VP7 ELISA kits available. Otherwise, it is not possible to correlate presence of antibodies against VP7 with protection.

ELISA for antibodies detection is simple to use and it has high sensitivity and specificity. In addition, it provides a high accuracy and ability to proportion quick results, in around 3 - 4 hours. Anyway, serological diagnosis is an indirect measure that needs to be interpreted cautiously.

SECOND STEP OF DIAGNOSIS: SEROTYPE IDENTIFICATION

Second step in the diagnosis, serotyping, must be done as soon as possible in order to determinate an adequate vaccination strategy, because vaccines currently available for AHS and BT control are serotype specific.

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In case of epidemiological situation involving different field or attenuated vaccine serotypes, it is recommended to serotype all serogroup positive animals detected.

Neutralization techniques, seroneutralization and virusneutralization test, are serotype specific diagnostic tools which have been historically used for serotyping, neutralizing antibodies in serum sample and isolated virus, respectively. In fact the different serotypes of AHSV and BTV were stablished based on this techniques.

Nowadays virusneutralization test has some disadvantages in comparison to serotype specific RT-PCRs:

- It requires to get the virus isolated and cultured in cells
- Cross-reactions have been described among serotypes. In case AHSV, cross-reaction between serotypes 1 and 2, serotype 6 and 9, and between serotypes 5 and 8, are well known. And in case of BTV, there is significant immunological cross-reactivity among members of the BTV serogroup.
- It takes longer to complete technique (5 – 7 days to read the results)
- A large number of samples cannot be analyzed by VNT

Serotype specific RT-PCRs are nowadays the faster tool for this purpose. Considering the high number of serotypes, molecular strategies, some of them based on RT-PCR, to detect serotypes in multiplex has been developed.

SNT detects serotype specific neutralizing antibodies (anti-VP2-VP5) and requires to handle cultured infectious virus. Interpretation of the results should be done carefully, due to the presence of cross-reactions, as it has been mentioned. Moreover, in case of BTV, infection with a second serotype can broaden the neutralising antibody response to include antibodies to serotypes to which the animal has not been exposed.

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SNT can be used to serological studies, complementing the ELISA technique (anti-VP7). In general, neutralising antibodies can be considered protective against infection, although a clear and specific threshold of a protective titre of specific neutralising antibody cannot be identified neither AHS nor BT. Even more, when some of the AHS and BT vaccinated animals not showing detectable neutralising antibodies have been protected to infection.

THIRD STEP OF DIAGNOSIS: FURTHER CHARACTERIZATION

The third step to complete the diagnosis of AHS and BT includes a further characterization of the pathogens detected and serotyped. It includes molecular and phylogenetic studies, such as detection of novel strains emerged by mutation or reassortment, a characteristic event in viruses with a segmented genome.

Although genome sequencing methods have developed enormously in recent years, especially since the advent of massive sequencing technology, commonly known as Next Generation Sequencing (NGS), which allows for rapid, massive and inexpensive sequencing of genome regions or entire genomes, not always it is possible to get genome sequences from clinical samples. Therefore, virus isolation techniques are of great relevance allowing to have the pathogen cultured and in high concentrations.

Segment 2 (VP2) is the most variable region of the orbiviruses genome and varies in a manner that correlates with virus serotype. Therefore, sequencing this segment allows to confirm the serotype previously assigned by serotype-specific RT-PCR or virusneutralization test. In addition, high variability exists among different strains within each serotype in segment 2 sequence, in both AHSV and BTV, which has demonstrated to be useful in molecular epidemiology to establish the genetic relationship between isolates, to assign isolates within a serotype to particular topotypes and to assist in tracing an outbreak to a source.

Full-genome sequencing studies have increasingly revealed evidence of segment reassortment between multiple strains of BTV or AHSV in the field.

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BTV ATYPICAL STRAINS

For decades, only 24 serotypes of BTV were recognized. As for the development of new molecular technologies, in the last years novel BTV serotypes such as BTV-25 (TOV strain) from Switzerland, BTV-26 from Kuwait, BTV-27 (variants 01, 02 and 03) from Corsica (France), BTV-XJ1407 from China or a BTV strain isolated from a sheep pox vaccine (SP vaccine-derived BTV), have been described by researchers.

Since these novel BTV atypical strains have not caused economic losses, restriction measures have not been implemented in the affected regions so far. In fact, EU Regulation does not consider these new atypical strains as BTV for notification and control purposes.

Anyway, questions regarding the origin of these viruses and their role in relation to BTV classical strains remain open, also considering that, at least for some of them, new biological characteristics with respect to classical BTV serotypes, including goat as the main target species, low levels of RNAemia, a reduced or undetectable humoral immune response, horizontal transmission capabilities and the inability to grow in cell cultures, have been demonstrated.

From the laboratory diagnostic point of view, BTV serogroup RT-PCR targeted to the viral RNA segment 10 (such as Hofmann et al 2008) has been able to detect all news atypical strains emerged at the moment. In contrast, BTV serogroup RT-PCRs targeted to the viral segment 5 or 1 have not detected them or with much reduced sensitivity. Therefore, to have fit for purpose BTV serogroup RT-PCRs targeting to different segments of the BTV genome (p.e segments 10 and 5) is recommended.

All field and laboratory indications mentioned in previous paragraphs, together with impossibility of typing by specific RT-PCRs against the 24 classical serotypes and a seroconversion detected by ELISA, may indicate that we are facing an atypical strain. A definitive diagnostic confirmation is only possible by means molecular methods targeted to segment 2. For it, to try virus isolation using all available methods is highly recommended to get cultured virus in high concentration to carry out a definitive characterization.

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6. GUIDELINES FOR THE BEST IMPLEMENTATION

The purpose of the guidelines included in this chapter is to provide general guidance to the best implementation and quality control of the diagnostic techniques, described as specific methods in the Procedures chapter:

- **ELISA** for the detection of antibodies against different *Orbivirus* (African horse sickness -AHS- virus and Bluetongue -BT- virus) using commercial validated diagnostic kits.
- **RT-PCR methods** to rapidly detect the specific sequences of serogroup / serotype Orbivirus RNA by real time reverse transcription polymerase chain reaction assay.
- **Virus isolation of Orbivirus** (specifically AHSV, BTV and EHDV) *in vitro* system
- **Seroneutralization test** to measure neutralizing antibodies (serotype specific) against *Orbivirus* in animal sera

These guidelines 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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GUIDELINE 1: ELISA FOR THE ANTIBODIES DETECTION

1. TEST PRINCIPLE

The exploitation of the ELISA has been increased through continued development of specifically produced reagents, for example, monoclonal and polyclonal antibodies and peptide antigens coupled with the improvement and expansion of commercial products such as enzyme-linked conjugates, substrates and chromogens, plastics technology and design of microwell plates, instrumentation advances and robotics. However, the principle of the ELISA remains the same.

The main reason for this success is test flexibility, whereby reagents can be used in different combinations. A key feature of the flexibility of ELISA is that more than one system can be used to measure the same thing.

The principle of the ELISA test is the use of a conjugate and a substrate to detect the immuno-complex antigen (viral protein) and specific antibodies. Subsequently, a microplate reader (spectrophotometer) detects colour development.

For antibodies against Orbivirus there are several ELISA systems described, mainly competitive/blocking (c/b), double recognition (dr) and indirect (i). All of them detect antibodies against the structural viral protein VP7 and are not able to discriminate between antibodies from vaccinated or infected animals.

The competitive/blocking (c/b) as well as the double recognition (dr) ELISA assays are designed to detect virus specific antibodies in serum samples from animals of any specie, preventing the problem of species specificity experienced occasionally using indirect ELISA.

Reagents are supplied by the manufacturers in kit format that includes specific reagents and test control for the correct performance.

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ELISA assay is a qualitative test. Optical Density (ODs) and other parameters such as Blocking or S/M percentages are not reported to the customer but could be taken into account at internal level in the quality control.

2. GENERAL REQUIREMENTS FOR TEST'S PERFORMANCE

Protocol

The manufacturer's kit instructions shall be followed as the basis for test implementation.

The laboratory will keep records of the current instruction edition in force and, with this purpose, will revise systematically the booklet inserted in each purchased kit to ensure that always the information of latest edition is used.

Obsolete instruction editions shall be archived for the time period determined by the corresponding Accreditation Body.

The laboratory shall made available a Standard Operating Procedure (SOP) that, taking as the basis manufacturer's kit instructions, 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, actions to take in case of doubtful results, etc).

According to ISO 17025 requirements, reagents and equipment must be used under control (i.e. reagents after the expiring date stablished is strongly not recommended).

All reagents and samples must be allowed to come to room temperature before use.

Mix reagents by gentle inverting or swirling.

Use a separate pipette tip for each sample and control.

Do not expose the substrate solution to strong light or any oxidizing agent.

Do use clean and clear out bucket to dispense the reagents and do not pour unused reagents back into containers in order to prevent contamination of kit components.

Use distilled or deionized water for preparation of reagents that require it (as wash solution).

Mix the content of the plate after the addition of the samples using a microplate shaker.

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Samples

Sample's biological properties should be guaranteed by applying correct sampling, preservation and transport procedures until arrival at the laboratory. Any relevant issue regarding the conservation of the samples during the transport must be registered in the laboratory. Relevant information about collection, transport of storage of clinical samples is included in Chapter 3 of this document.

The laboratory should implement a protocol to reject samples in poor conditions to be analyzed (e.g. strongly hemolyzed, putrefaction).

The laboratory should implement protocols to prepare samples (p. e. centrifugate at 700g during 10 min.to separate the serum fraction, filtrate using 0,45 µm filters and/or centrifugate at 5000g during 15 min. to eliminate bacterial contamination).

Traceability

The traceability system in the laboratory shall preserve the correct identity of samples during laboratory operations.

Precautions

Please, refer to the manufacturer's kit instructions. In particular:

Reagents and samples must be kept at room temperature for 30 minutes before testing. Frozen samples might need extra time to reach the required temperature.

Plates and other kit components must be stored at 5 ºC (3º-8ºC). Use clean and sterile material (e.g. pipettes, tips and vessels) in contact to kit reagents. Such practices avoid contamination and preserve kits reagents until expiring date.

Do not mix reagents from different kit batches.

Use equipment (pipettes, incubators, spectrophotometer) correctly maintained, and periodically verified and calibrated.

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Quality control

The kit includes positive and negative controls that shall be assayed in duplicates for the best reliability. Although the manufacturer's kit instructions usually don´t include repeatability criteria, taken into account that the average of OD obtained for duplicates in controls can be used for the calculation of quantitative result, it is highly recommended to control the variability between replicates. A variation less than 10% between duplicates will be considered as the best performance, although sporadic greater variation (≤ 20%) might be accepted.

If available, a weak positive serum assayed in duplicate should be included in each test run as sensitivity control. In general, qualitative assessment should be enough; nevertheless, a quantitative assessment could be done too.

In addition to test repeatability, it is advisable to test a number of samples in duplicate (e.g. at least 2 samples per assay). In general, qualitative assessment should be enough, both replicates positive or both negative. Nevertheless, a quantitative assessment could be done too. In case of samples close to grey zone (inconclusive), a variation of blocking percentage less than 10% between duplicates will be considered as the best performance, although sporadic greater variation (≤ 20%) might be accepted.

Proficiency test

The laboratory shall participate regularly in *external proficiency testing schemes*. Participation in such scheme is a requirement for accredited laboratories.

According to EU rules, National Reference Laboratories for Bluetongue and African horse sickness in each EU Member State, have to participate in the Annual Proficiency Tests organized by the European Union Reference Laboratory (EURL).

Proficiency testing material has been well characterized and any spare material, once the proficiency testing has been completed, can be useful to demonstrate staff competence, or to regularly check test performance.

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3. ASSAY VALIDATION

It is advisable to develop a safety system for calculations that avoid mistakes, such as a blocked excel sheet or using the Software included in the own reader. A kit specific excel template is available at the EURL under request. Original readings values should be backed up.

Plate validation

Although an assay could include two or more plates, each assayed plate must be validated individually according to manufacturer's kit instructions.

When a weak positive serum is used as sensitivity control, it is recommended to include it in each plate and it must give the expected result.

In case of not acceptance plate validation, it is important to investigate the causes and according to the reasons of the deviation, to take corrective action and then to repeat the assay.

Interpretation of sample results

Once the plate has been validated, the formula provided by the manufacturer must be applied in order to know the status of the sample (Positive, Negative or Doubtful)

Doubtful results should be further investigated by retesting the sample, analyzing using other method if available or by sampling again the animal.

A protocol to apply when the replicates generate a different qualitative result (NEG or POS) must be available.

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GUIDELINE 2: rRT-PCR FOR THE GENOME DETECTION

1. TEST PRINCIPLE

The polymerase chain reaction (PCR) is a powerful molecular technique which allows the specific detection of DNA by the enzymatic exponential amplification of a short DNA fragment (target sequence) delimited by a specific primer set

A first step of DNA or RNA extraction from the original material to be analysed is required previous to the amplification, which will be the template for the PCR.

In the case of Orbiviruses (segmented RNAds virus), detection is performed through reverse transcription followed by PCR (RT-PCR). It is necessary in order to convert RNA template into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as template for exponential amplification by PCR, which consists of repetitive series of cycles involving template denaturation, primer annealing, and extension of the annealed primers by DNA polymerase, resulting in exponential accumulation of the specific fragment whose termini are defined by the 5´ends of the primers (amplicon).

The process is specific due to the use of primers, short oligonucleotide sequences complementary to the ends of the nucleic acid fragment to be amplified, that serves as a starting point for DNA synthesis by the DNA polymerase.

The real-time PCR or RT-PCR (rPCR or rRT-PCR) assay allows the automated detection of the amplicon within each cycle of the PCR by the incorporation in the reaction mix of a Taqman probe (a short oligonucleotide complementary to one strand of the amplicon) labelled with a fluorescent dye. Detection of the amplicon is done by using an instrument that combines thermal cycling with fluorescent dye scanning capability. This approach reduces the risk of carry-over contamination increasing the specificity and, in most cases, the sensitivity of the assay.

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By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

During the first cycles, the increment of fluorescence is low and is not detected by the thermocycler. If the sample is negative, there is not a noticeable increase of the fluorescence. The fluorescence signal at the beginning of the amplification process constitute the baseline. Slightly above of the baseline the threshold is determined. For each positive samples, the threshold cycle (Ct) is define as the amplification cycle in which the fluorescence signal is detected above the threshold. The Ct values is inversely proportional to the amount of target nucleic acid present in the sample.

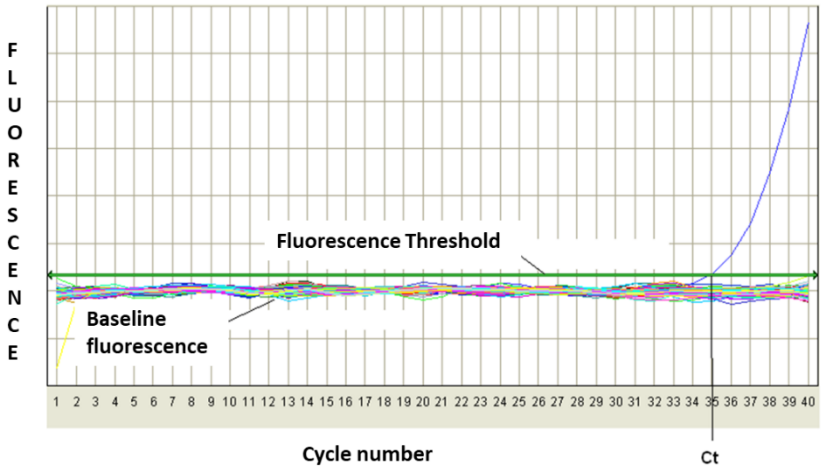

Figure1: Fluorescence vs. Cycle number. Amplification plots are created when the fluorescence signal from a sample is plotted against cycle number; therefore, amplification plot represents the accumulation of product over the duration of the real-time PCR experiment.

Due to the high level of sensitivity reached with the PCR, special care, and indeed special precautionary steps have to be taken to prevent contamination to subsequent PCR sample analyses. This is more likely to be a problem where reaction tubes are opened in the laboratory at the end of PCR for further processing (for example, to run gels or to perform nested PCR assays). To avoid such contamination, strict laboratory

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protocols should be employed involving separate rooms or cabinets for particular stages of the assays, changes of laboratory gowns and gloves, and stringent cleaning programs.

Although rPCR and rRT-PCR are mostly employed as qualitative assays, a threshold cycle (Ct) value is determined and reported together with the qualitative result (POS/NEG/Inconclusive).

2. GENERAL REQUIREMENTS FOR TEST PERFORMANCE

Samples

Relevant information about collection, transport of storage of clinical samples is included in Chapter 3 of this document.

Traceability

The traceability system in the laboratory shall preserve the correct identification of samples during laboratory operations.

Controls

The inclusion of positive and negative controls during the whole process helps to identify the presence/absence of false negative and false positive PCR results, respectively.

- Extraction controls are used to validate the success of the extraction step (extraction positive control) as well as to test the correct manipulation as to avoid cross contamination (extraction negative control), reducing the chance of obtaining a false negative or a false positive result in the sample.

  It is recommended to include extraction controls, both positive and negative, per set of samples to be extracted.

  - *Extraction Positive Control* (EPC) should contain the target virus at a low concentration (weak positive sample). The source of an EPC can be viral suspension (inactivated or not) or, in case of availability, a well characterized positive clinical specimens.
  - *Extraction Negative Control* (ENC): Water or buffer is normally used.

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- Amplification controls: every PCR assay should be validated by incorporating at least a positive control and one negative control into the PCR batch prior to amplification.
  - *Amplification Positive Control (APC) helps to exclude the possibility of "false negative" PCR amplification results, which may be brought about by either the poor quality of one or more of the reaction reagents (e.g. reaction reagents that are "out of date" or damaged), or by erroneous omission of one of the reagents of the PCR mix.*

    Positive amplification controls comprise a PCR mix prepared with the same volume and concentration of reagents as the PCR mix being used to test specimens, but with the addition of target nucleic acid at a low concentration (weak positive).
  - *Amplification Negative Control (ANC) helps to verify that any specific PCR product amplified within the PCRs assay is not the result of contamination, which may be introduced into the PCR batch via contamination of the laboratory environment or contamination of "stock" PCR mix reagents. Negative amplification controls comprise a PCR mix prepared with the same volume and concentration of reagents as the PCR mix being used to test specimens, but with the addition of a volume of sterile nuclease free water or sterile nuclease free buffer instead of extracted template nucleic acid.*

After completion of thermocycling, all negative controls should contain no amplification products and the finding of PCR amplification products within any negative control means that the whole assay should be repeated.

Additionally, to include an internal control in the PCR is recommended to detect poor condition or inhibitors on the sample that would produce false negative results. If the internal control yields a negative result, this infers that the sample is not in good conditions or contains inhibitory substances and that a negative test result for the test sample cannot be interpreted as "negative" because the assay did not perform correctly.

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Certain kind of samples often contain inhibitors whereas this may be less of a problem when testing blood samples or suspension of cultured organisms. Data collected during the validation process concerning assay performance using the sample matrices being analyzed will allow for a risk-based decision as to whether an inhibition control should be included for each sample or whether the test system is unlikely to be affected by inhibition.

There is considerable debate about the most suitable and effective internal control. Examples include the following:

- An artificial target, such as a length of DNA contained in a plasmid, which is added to the extracted sample and amplified with the same primers as the test target, but contains a different internal sequence so that it can be differentially detected with a specific probe.
- An alternative strategy for an internal control is to co-amplify a housekeeping or structural gene such as β-actin which always is present in the sample. If the sample is in poor conditions or contains inhibitors of the PCR, housekeeping gene will not be detected and the inference is that amplification of the gene targeted by the test may also be inhibited. This option is the chosen in the EU-RL.

Cut-off selection

The selection of a cut-off value for rRT-PCR assay is highly recommended.

The linear operating range of the method must be determined, including the estimation of the limit of detection defined as the minimum concentration of analyte detected with a known certainty (dilution of the analyte at which at least 95% of the assays are positive). An analytical cut-off, therefore, could be justified by selecting the Ct value that corresponds to the defined limit of detection of the test. Any Ct value above this defined limit would, thereafter, be considered not reliable (inconclusive).

Precautions

Read and follow carefully the complete procedure.

Always wear disposable nitrile or latex gloves.

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Frozen (-70ºC) extracted RNA should be thawed and kept in ice throughout the reverse transcription preparation and frozen (-70ºC) immediately after using.

Repeated freeze/thaw of RNA could cause significant degradation in RNA integrity.

Keep reagents to the appropriate temperature before and after use.

Fluorescent labelled probes are highly sensitive to light, so they should be manipulated for the minimum time and must be kept always protected from light.

Do not pool reagents from different batches.

Avoid any contamination of reagents. It is highly recommended to manipulate reagents in a dedicated clean area.

Do not use reagents after expiry date.

Critical points

As with conventional PCR, real time PCR reactions can be affected by nucleic acid contamination, leading to false positive results. Some of the possible sources of contamination are:

- Cross-contamination between samples.
- Contamination from laboratory equipment.
- Carryover contamination of amplification products from previous PCRs, being this point the most common source of false positive PCR results.

Therefore, it is compulsory that personnel working on PCR follow and carry out some strict rules in order to minimize the contamination risk associated to PCR technique:

- All steps of sample analysis PCR should be performed in separate areas, using dedicated equipment and material for pre-PCR (sample preparation and extraction), PCR, and post-PCR.
- Personnel must always wear clean disposable gloves while working in the PCR laboratory, that will be replaced frequently and when personnel goes into a different PCR area.
- The work surface should be decontaminated with the appropriate product before starting.
- PCR reagents shall be stored in a separate freezer and handled in a separated area using material and equipment dedicated that are not in contact with nucleic acid or samples.

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- Use a new sterile RNase filter pipette tip each time that pipetting from a tube containing any sample or RNA/cDNA.
- If necessary, tubes containing amplified products should be opened and manipulated only in the post-PCR area, where will be discarded.

Proficiency Test

The laboratory shall participate regularly in external proficiency testing schemes. Participation in such scheme is a requirement for accredited laboratories.

According to EU rules, European Union National Reference Laboratories have to participate in the Annual Proficiency Test organized by the European Union Reference Laboratory (EU-RL).

Proficiency testing material has been well characterized and any spare material, once the proficiency testing has been completed, can be useful to demonstrate staff competence, or to regularly check test performance.

3. ASSAY VALIDATION

The PCR assay will be valid if after completion of thermocycling program:

- All positive controls (EPC, APC) should produce amplification curves. Positive control Ct values should be in the range of the previously assigned Ct value ±2. If not, the assay should be repeated.
- All negative controls (ENC, ANC) should not produce any amplification curve and the finding of PCR amplification curves in any negative control means that the assay should be repeated. If ANC showed amplification, the new PCR test could be performed using the same extracted nucleic acid, otherwise the sample should be retested from the nucleic acid extraction.
- Internal control should produce amplification curves. Positive control Ct values should be in the range of the previously assigned Ct value ±2. For each single well, if IC result is negative but the target sample result is positive, the sample result will be accepted. In case of the IC result is negative and target sample result is also negative, the sample should be re tested

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4. TROUBLESHOOTING

Nucleic acid extraction

Critical Point	Prevention actions	Verification actions
Sample contamination (false positive)	Extraction area separated from areas where extracted nucleic acid or amplified products are being manipulated Good laboratory practices when managing samples or positives controls.	Use of ENC
Low extraction efficiency (false negative)	Preventive equipment maintenance Periodical equipment verification Proper extraction reagents storage	Use of EPC with Ct~32 (tolerance threshold ±2Ct)
Poor condition of sample or inhibition effect (false negatives)	Proper sample preservation Method validation in each matrix	Use of internal control

PCR assay

Critical Point	Prevention actions	Verification actions
Sample contamination (false positive)	PCR performing area separated from areas where amplified products are being manipulated Good laboratory practices when managing extracted nucleic acids	Use of ANC
Low PCR efficiency (false negative)	Proper PCR reagents preservation: Do not use out of expired date reagents, limited freezing/thawing cycles Primers (proper storage depending on its concentration, limited freezing/thawing cycles) Probe (proper storage protected from light depending on its concentration, limited freezing/thawing cycles)	Use of APC with Ct~32 (tolerance threshold ±2 Ct; storage at -20ºC avoiding more than 8 freezing/thawing cycles)

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GUIDELINE 3: ORBIVIRUS ISOLATION

1. TEST PRINCIPLES

Published works have shown that orbiviruses can be isolated by several *in vitro* systems and *in vivo* inoculation and the choice of method(s) may then depend on local circumstances. As a result of adaptation of orbiviruses to grow in cell cultures, it has been possible to develop a range of diagnostic test and vaccines.

This guideline applies when the objective is isolation of virus from clinical samples. Although, the performance of inoculation is almost the same when the purpose is to propagate a virus isolate to produce a new virus batch.

Although BTV, AHSV and EHDV share the same *in vitro* virus isolation protocols, there must be considered that the most efficient conditions of virus isolation method (mammalian cell line, insect cell line or embryonated chicken eggs) could depend on the specific serotype or even strain (field or live vaccine strains).

Virus isolation of orbiviruses, especially ECE method, is a laborious procedure that requires well-trained personnel having a good understanding of the overall isolation procedure.

2. GENERAL REQUERIMENTS FOR TEST´S PERFORMANCE

Definitions

Cell culture medium: diluent with a % v/v of inactivated bovine foetal serum according to each cell line. It is used in the proliferation phase.

Cell suspension: cells in cell culture medium after trypsinized or scrapped

Diluent: enriched liquid medium containing nutrients suitable for replacing the natural environment of the cells. The specific composition depends on the cell line. It is used to dilute the inoculum before adsorption phase and to prepare the cell culture medium.

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General considerations

Traditionally orbiviruses have been isolated through the inoculation of embryonated chicken eggs. However, diagnostic OIE Manual based on publications, includes insect cell cultures, especially KC cells, as an effective method to isolate most strains. Although the *Directive 2010/63/EU on the protection of animals used for scientific purposes* states for the research use of live embryonated avian eggs that the protection of animal policy is only applicable after hatching, nowadays there are several international institutions recommending that ECE should no longer be used for research, diagnostic and therapeutic applications, especially if alternative methods have demonstrated similar efficacy. Therefore, the EURL recommend using ECE only where isolation attempts in cell culture have repeatedly failed.

Although virus isolation methods for Orbivirus have been used in the past to demonstrate presence of the virus for the purpose of disease reporting, nowadays RT-PCR is the election method for specific detection of orbiviruses (serogroup or even serotype). Because of the high sensitivity of RT-PCR methods, it is highly recommended to select only RT-PCR positive samples to perform virus isolation assays.

The presence of adequate concentrations of virus in the original specimen (evaluated by quantitative RT-PCR), as well as intensity of immune response and interferon activity in the animal when the specimen was obtained, are highly related to the chances of virus isolation success.

The close association of orbiviruses with circulating erythrocytes by hemagglutination activity, is likely responsible for prolonged viremia in presence of circulating antibodies. To avoid erythrocytes lysis by means of conservation at refrigeration temperature, as well as to stablish a method for blood sample preparation removing plasma containing antibodies, is highly recommended for a successful isolation.

EDTA blood and tissues (especially spleen) are the samples of choice. Quality of starting samples (blood or tissues) is a relevant point that must be noted. Cell blood lysis, tissues collected too much time *post morten* or bacterial contaminated samples, decrease the chances of success.

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Most of BTV, AHSV and EHDV strains usually produce CPE in mammal cell lines (BHK and Vero are frequently used). However, sometimes several passages must be done before to observe CPE.
CPE is unspecific and virus replication must be confirmed by a specific identification method (virus neutralization test or quantitative RT-PCR mainly). Orbivirus usually do not produce CPE in KC cell line.

Traditionally, three blind passages are compulsory to confirm that virus replication is not happening.

There must be selected one specific virus isolation strategy according to the source of samples and background using this techniques in the laboratory.

Table 1 is an example of EURL flow-chart of virus isolation selection system.

	AHSV	BTV	EHDV
1st passage	Insect cells: KC	Insect cells: KC	Insect cells: KC
2nd passage	Mammal cells: Vero	Mammal cells: BHK	Mammal cells: BHK
3rd passage	Mammal cells: Vero	Mammal cells: BHK	Mammal cells: BHK

If vaccine strain presence is suspected, first passage could be done using mammal cells (Vero or BHK) in parallel to insect cells.

ECE could be used in first passage when virus isolation in cell culture has not been successful and it is highly relevant to get the virus isolation (i.e. first outbreak in a region)

All virus isolation available systems in laboratory could be used in the first passage (included any other cell line such as AA C6/36 or BSR) when a BTV atypical strain is suspected.

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Protocol

The laboratory shall made available Standard Operating Procedures (SOPs) that, taking as the basis the OIE Manual ,adapt 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).

The performance of virus isolation in cell culture or ECE are highly different and requires differentiated SOPs, but sample preparation protocol is shared.

In the framework of the personnel qualification/training, virus isolation SOPs could be divided into two different activities (a/performance and b/microscopically reading and interpretation).

Several different configurations of tubes/flasks/plates are available. Depending on the number of samples and/or the volume of each sample, the most appropriate format could be choose. In the EU-RL, 24-wells plate is the format usually used for virus isolation in cell culture.

Mock inoculum (negative control) allows to check the viability of cell culture along the days.

Sensitivity of cell line to the serogroup virus to isolate has to be under control. Moreover, to determine the maximum number of passages recommended for each cell line , a positive control containing a known low concentration of a reference virus strain of the same serogroup could be inoculated each assay or periodically to check the sensitivity of cell culture.

Adequate forms to record relevant information regarding performance and results, including sample identification, reagent batches or microscopy reading results should be established.

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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.

Although lysed EDTA blood sample (by ultra frozen and defrost) could be directly used, it is highly recommended to stablish a sample preparation protocol to wash erythrocytes and remove plasma containing antibodies before lysis. Sonication and osmotic shock are two methods to get lysed erythrocytes.

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 employed in the assay and with all applicable laboratory SOPs.

Successful inoculation of orbiviruses in ECE by the intravenous route requires a degree of technical expertise.

Preparation of Equipment/Instrumentation

As a general consideration, 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.

Nevertheless, volumes used within virus isolation procedures are not critical and therefore equipment to measure volumes that has not been calibrated could be used.

Preparation of reagents

Cell culture: plates containing semiconfluent monolayer cells must be prepared the day before inoculation using suspension cells adjusted to get 80%-100% confluent monolayer the inoculation day.

ECE: specific pathogen free 9 – 11 day old embryonated chicken eggs could be provided by external or internal producer.

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Preparation of control

Negative and positive control samples must be added as a test sample.

Mock inoculum (negative control) could be the same medium used as diluent.

To avoid and detect (if produced) a cross-contamination with positive control, it is recommended to use a serotype different to the one suspected in samples and add it after test samples and using a different plate (when multi-wells plate is used).

Performance of the assay

The virus isolation assays require the management of cells or ECE in sterile conditions as well as samples containing live virus. Therefore, all the process should be performed within a Class II biological safety cabinet using the appropriate personal protective equipment (nitrile gloves and sleeves).

- Cell culture

If 24-wells plate is used, take into account to distribute samples in different plates considering the previous information (serotype, farm..) in order to avoid cross-contamination among samples from different sources.

Considering the volume of sample available and the chance of a cytotoxic effect, it would be recommended to prepare one or more dilutions of inoculum. In EURL a 1/5 dilution is employed in washed blood, 1/10 dilution in homogenated tissue and 1/100 in case of whole blood would be inoculated.

During adsorption phase it is critical to be sure that cell monolayer does not dry out, so the volume of inoculum should be adequate to avoid drying and humidity should be controlled in incubator. It is highly recommended to remove the inoculum after adsorption before the addition of fresh cell culture medium.

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For microscopic reading use an inverted microscope to visualize CPE (4x and eventually 10x). Evaluate daily the appearance of controls initially and subsequently the test samples.

Inoculated cell lines of mammal must be observed for the presence/absence of CPE. The CPE of Orbivirus in mammalian cells usually occurs from 2 to 7 days post-infection. It 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 are common. The presence of a CPE could indicate virus isolation positive.

Infected KC cells appear normal under a microscope (without CPE). In case of virus replication, the amount of virus inside the cells is similar to the amount of virus obtained in the supernatant.

- ECE

Isolation of Orbivirus using embryonated chicken eggs inoculated by the intravascular (IV) route is 100–1000-fold more sensitive than the yolk sac route (at least on BTV).

10-day-old ECE are examined for viability with a strong light source and only viable eggs are selected and marked with a lead pencil indicating an appropriate blood vessel to be used for inoculation.

IV inoculation requires experience: the egg is illuminated from above in a dark area employing a cold light source. The injection is made by inserting the needle at an acute angle while the egg is held in the other hand beneath the light source.

Due to the difficulty to perform the IV inoculation, it is recommended to inoculate at least five eggs per sample.

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Embryos dying within 24h are discarded (virus is not considered as cause of death). Tissues (brain, liver, spleen, heart and lungs) from embryos dead before day 7 and those sacrificed at day 7 are then removed and processed as any other tissue sample. Embryo deaths are not always an indication of positive virus isolation. Confirmation is required.

The removal of the embryo must be carried out carefully so as not to break the yolk sac which may affect the cell culture.

Any other part of egg (such as allantoid and amniotic fluid) must be avoided to be included with tissues to process.

Quality Controls

When 24-wells plates are used, negative control wells could be distributed in the same plates, that allows to control cross-contamination among samples.

The only purpose of positive control (if used) is to demonstrate that cell line batch (passage) is as sensitivity for a specific strain as it had been verified in the validation assays.

It is recommended to inoculate at least two replicates from each sample.

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.

If it is not possible to get a PT available, internal control must be strengthen by means blind testing of well characterized material or exchange of samples between laboratories.

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3. ASSAY VALIDATION

Quality Control validation

Positive and negative controls are only indicative that cell culture is suitable. But efficiency of virus isolation depends on condition of each sample (virus concentration, presence of neutralizing antibodies and quality condition).

Moreover, virus isolation is a qualitative assay, therefore samples and control results are not measurable.

Interpretation of the results

An identification assay is compulsory to confirm virus isolation. It should be carried out on all wells/ flasks/tubes/embryos regardless of the presence of CPE or embryo death.

Although immunodetection and neutralization techniques have been historically available, nowadays quantitative RT-PCR is the faster method to confirm the virus isolation result.

A positive result of RT-PCR is not confirmatory of isolation, due to the remaining inoculated virus could produce a positive result. Band intensity or Ct value of the RT-PCR of cultured virus and the inoculum should be compared to confirm virus replication (when the Ct value decrease significantly after inoculation). If there are any doubt about it, it is recommended to do a new passage.

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GUIDELINE 4: SERONEUTRALIZATION TEST FOR THE ANTIBODIES DETECTION

1. TEST PRINCIPLE

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 titre is reported together with the qualitative result (POS/NEG).

The SNT involves an initial incubation, on a well of the selected plate, of diluted serum sample or control sera with a known amount of a serotype of an Orbivirus, during 1 -2 hours. 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). After the addition of susceptible cells, plates are incubated for 5 to 7 days.

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.

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.

2. GENERAL REQUERIMENTS FOR TEST´S PERFORMANCE

Definitions

Antibody titre: 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

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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.

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 and serum dilution to the well. 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.

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.

<u>General considerations</u>

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 described, but some cross-reaction has been observed between 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.

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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.

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.

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 trypsinization, 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, 96-well plates containing 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.

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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.

Test sera that are severely haemolysed or visibly contaminated should not be used. Sera that are partially haemolysed 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.

Personnel qualification/training

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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.

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.

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 titration of the virus in the same conditions of the SNT assay 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.

Preparation of control sera

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

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, a proper dilution to add have to be prepared according its titre so that the appropriate endpoint antibody titre is reached.

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Performance of the assay

The virus neutralization assay requires the management of mammalian cells and live virus in sterile 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).

Labelling 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.

Addition of the cells

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:

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 sample cytotoxicity 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).

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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.

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.

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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:

- Sample cytotoxicity 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 this control at the same dilution that the most concentrated final dilution of the serum when testing

- 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 must be added in several dilutions (i.e. 1/5, 1/10, 1/20 and 1/40) in order to determinate the titre. 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 (each dilution) and negative serum control is included.

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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.

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.

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).

3. ASSAY VALIDATION

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).

If the virus titre 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.

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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 with virus within the valid range, as they can be false positive or at least the titer is potentially artificially high.

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

-**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 *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.

<u>Interpretation of the results</u>

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 titre for the tested samples.

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In case of samples tested in replicate, apply a criteria to determinate the qualitative result (or re-tested), in case of qualitative differences. Regarding quantitative result (titre), average or estimated titre 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 titre, positive less than or equal to a titre).

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

-**Estimation method** (i.e. Spearman-Karber 50% endpoint antibody titre): 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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7. VALIDATION

Methods used in the official control must be validated in accordance with internationally accepted scientific protocols accordingly to the Regulation EU 2017/625. Validation is a process that determines the fitness of an assay, which has been properly developed, optimised and standardised, for an intended purpose.

Methods provided by EURL have demonstrated their usefulness on a national, regional or international basis. In fact most of them have been recognised internationally by the OIE when they are designated as prescribed or alternative tests for trade purposes.

EURL makes available to the EU-NRLs documents that declare the validation of the methods provided. Therefore this methods could be used after limited verification to determine whether the assay complies with validation criteria, in the context of the intended application. This may require a limited verification of both analytical Specificity and Sensitivity using available reference materials. Once the laboratory is confident that the assay is performing as described from an analytical perspective, then proceeding to a limited validation should be considered in the context of the intended application and target population before the assay is put into routine diagnostic use.

In case of using a method / kit not provided by the EURL, validation data must be available in the laboratory, according to the OIE validation chapter.

Validated fit for purpose serogroup diagnostic methods based on RT-PCR and ELISA techniques for AHS and BT diagnosis must be implemented in all EU National reference laboratories. Although EURL provides methods based on these techniques and their validation report, EU-NRLs could use other methods based on the rRT-PCR and ELISA techniques if these method comply to the minimum criteria required in the validation process:

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A/ Serogroup rRT-PCR

Analytical specificity: At least the most relevant related Orbivirus strain/s (EHDV and EEV) should be analysed in order to check cross-reactions.

To verify inclusivity, at least all the notifiable serotypes (24 BTV serotypes or 9 AHSV serotypes) should be detected. Additionally, in silico testing should be done with Orbivirus (BTV or AHSV) sequences available through data base, in particular with the strains currently circulating and/or detected in recent years.

In case of BTV, it is highly recommended to use a rRT-PCR method able to detect atypical strains.

Analytical sensitivity: reference material available in EURL (List of reference material) should be detected (100%) at least until recommended dilution:

BTV (strain BTV5 reference collection, batch 4P BHK 09.10.2019 inactivated 23.10.2019), should be detected at least to 10^{-6}

AHSV (strain AHSV4 reference collection, batch 5P Vero 16.03.2015 inactivated 06.08.2019), should be detected at least to 10^{-5}

Diagnostic specificity: >99% (sample size accordingly OIE Manual Chapter 1.1.6)

Diagnostic sensitivity: >95% (sample size accordingly OIE Manual Chapter 1.1.6)

Repeatability intra-laboratory: >97% (qualitative) and <3,3 Ct values (quantitative)

Repeatability inter-laboratory: to complete the validation of the test (Stage 3 according OIE Manual), reproducibility of the assay should be evaluated. At least three laboratories should test the same panel of samples (blinded), with identical aliquots going to each laboratory.

B/ Serogroup VP7 ELISA

Analytical specificity: to test positive serum sample/s against related Orbivirus (EHDV or EEV) if available. To verify inclusivity, antibodies against at least all the notifiable serotypes (24 BTV serotypes or 9 AHSV serotypes) should be detected.

Analytical sensitivity: reference material available in EURL (List of reference material) should be detected, including weak positive samples.

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Diagnostic specificity: >99% (sample size reduced)

Diagnostic sensitivity: >95% (sample size reduced)

Repeatability intra-laboratory: >97% (qualitative)

Repeatability inter-laboratory: to complete the validation of the test (Stage 3 according OIE Manual), reproducibility of the assay should be evaluated. At least three laboratories should test the same panel of samples (blinded), with identical aliquots going to each laboratory.

In case of commercial kit, validation report or a summary of the validation report (validated method certificate) should be available by the manufacturer including these items. In this case, just a verification (reduced validation) should be performed by the laboratory.

To retain the status of a validated assay a quality assurance programme characterised by carefully monitoring the assay's daily performance, primarily through internal controls have to be performed. Such monitoring provides critical evidence that the assay retains its "validated" designation. Reproducibility is assessed through external quality control programmes such as proficiency testing.

A validated assay must be continuously assessed to assure it maintains its fitness for purpose. In this framework, it is suitable to analyse all new strains emerged in order to demonstrate that validated methods are fit to detect them and/or specific antibodies against it. To perform this relevant task, EURL ask for to all EU-NRLs to share the biological material from outbreaks in their countries to be tested in EURL, included in the EURL collection and sent in the Proficiency test to be analysed by all NRLs.

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8. BIOSAFETY AND BIOSECURITY

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

The assays described in this document shall be carried out following Biosafety, Biocontainment and Bioprotection guidance and rules established according to international and national regulations (see references).

Considering the social and economic impact of these pathogens, the EURL, according to the recommendation of the main Biosafety guidances included in the reference, has classified BTV and AHSV in the **group risk 3 under the animal health point of view**. Therefore, in the EURL, viral suspensions of these viruses as well as clinical samples (blood and tissues) from suspicious animals are handled in level 3 containment facilities. Serum samples could come from infected animal during the viremia phase. Orbivirus particles have haemagglutination activity that allows them to adhere to circulating erythrocytes. Thus, the presence of infectious virus in the serum fraction is generally residual. Only when the viremia is very intense, there can be infective Orbivirus particles also in the serum sample. Therefore, the risk assessment of the serum samples must be carried out and safe procedure have to be stablished.

Other pathogens, apart from Orbivirus presence, must be considered in the risk assessment of clinical samples, especially with samples from countries that do not have “free status” for other relevant animal diseases.

The RT-PCR and ELISA reagents have not a specific biological risk. The remaining kit reagents and containers, once finished, must be separated between biological and chemical reagents and dispose according to environmental rules for waste management.

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9. DEFINITIONS

Cross-reaction: occurs when an antibody directed against one specific antigen is successful in binding with another, different antigen as a consequence of sharing some epitopes either by its linear aminoacid sequence or by similar structural conformation

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.

Live attenuated vaccine: vaccine consisting of a live attenuated virus strain that retains the ability to replicate (grow) and produce immunity, but usually does not cause illness.

Monovalent vaccine: designed to immunize against a single strain or serotype of a microorganism.

Polyvalent vaccine: designed to immunize against two or more strains or serotypes of the same microorganism, or against two or more microorganisms.

Primer forward and reverse: short synthetic oligonucleotides which sequence is complementary to the ends of the DNA fragment to be amplified in a PCR. Each primer anneals to one of the strands determining the start and end sites of the amplicon.

Probe: short synthetic oligonucleotide, which sequence is complementary to one of the strands of the amplicon, usually labelled with a fluorescent reporter, which permits detection of PCR products in real-time PCR after hybridization with its complementary sequence.

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Reassortment: process which occurs when two or more segmented viruses of the same species infect the same cell, and their progeny incorporates segments picked up from different "parental" virus. It represents an important way for segmented viruses to evolve.

RNAemia: the presence of RNA of a virus in the bloodstream.

Reverse transcription: process performed by an enzyme (reverse transcriptase) that uses a template strand of RNA to synthesize a complementary strand of DNA. The DNA polymerase component of reverse transcriptase requires an existing 3' end to begin synthesis.

Serogroup: is a group of infectious microorganism, antigenically related, belonging to a 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 or serogroup by means of serological tests. Therefore, the immune responses to a serotype of a microorganism may not protect against other serotype of the same species. Serotypes enable to differentiate organisms at the subspecies level, which is epidemiologically important.

Topotypes: A biological specimen that is of the same species or subspecies (serotype in this case) as a type specimen that has been collected in a particular location.

Viremia: the presence of infectious virus in the bloodstream.

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10. LIST OF ACRONYMS

AA C6/36: cell line derived from *Aedes albopictus* clon C6/36

ADR: European Agreement concerning the International Carriage of Dangerous Goods by Road

AHS: African Horse Sickness

AHSV: African Horse Sickness virus

ANC: Amplification Negative Control

APC: Amplification Positive Control

bELISA: blocking Enzyme-Linked Immunosorbent Assay

BHK: Baby Hamster Kidney cells

BT: Bluetongue

BTV: Bluetongue virus

BSR: cells derived from baby hamster kidney (BHK) cells

cELISA: competitive Enzyme-Linked Immunosorbent Assay

CPE: cytopathic effect

Ct: Threshold cycle

cDNA: Complementary deoxyribonucleic acid

DNA: Deoxyribonucleic acid

DIVA: differentiation of infected from vaccinated animals

drELISA: double recognition Enzyme-Linked Immunosorbent Assay

dsRNA: double stranded ribonucleic acid

ECE: embryonated chicken eggs

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Authority

EHDV: Epizootic Haemorrhagic Disease virus

EHD: Epizootic Haemorrhagic Disease

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

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ENC: Extraction Negative Control

EPC: Extraction Positive Control

EU-NRL: European Union National Reference Laboratory

EURL: European Union Reference Laboratory

IATA: International Air Transport Association

IC: Internal control

iELISA: indirect Enzyme-Linked Immunosorbent Assay

IV: intravascular

KC: cell line derived from *Culicoides variipennis*

MAb: monoclonal antibody

NGS: New Generation Sequencing

NS: non-structural viral protein

OIE: World Organization for Animal Health

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

RT-PCR: Reverse transcriptase polymerase chain reaction

rRT-PCR: Real time reverse transcriptase polymerase chain reaction

SNT: Seroneutralization test

SOP: Standard Operating Procedure

$TCID_{50}$: Fifty-percent tissue culture infective dose

TOV: Toggenburg orbivirus

Vero: epithelial cell line derived from an African green (verda reno) monkey

VI: virus isolation

VNT: Virusneutralization test

VP: structural viral protein

WD: Working dilution

WHO: World Health Organization

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11. REFERENCES

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Chapter African horse sickness (Infection with African horse sickness virus)

Chapter: Bluetongue (Infection with Bluetongue virus)

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