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Veterinary laboratory testing protocols for priority zoonotic diseases in Africa

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Introduction

BACKGROUND

Veterinary laboratories play a critical role in disease diagnosis and surveillance, fulfilling national reporting obligations for notifiable diseases and implementing national disease control measures. Enhancing the diagnostic capacity of laboratories is an essential step for generating reliable and accurate data, particularly in settings where fundamental gaps and capacity constraints may prevent the early and rapid detection of many animal diseases, including those of economic or zoonotic importance. At the same time, rapidly evolving technology in assay development calls for ongoing guidance on current protocols and techniques, and the use of carefully developed and validated tests is extremely important. Appropriate training is vital in addressing the needs of a given laboratory, and this includes ensuring that laboratories have an understanding of how to use and interpret test results.

New or updated diagnostic techniques should be introduced in accordance with a sustainable quality management system relating to validated tests to ensure accurate and reproducible results. Several benefits can be gained through the use of modern assays: reduced costs, increased ease and safety of use, and improved data for sensitivity and specificity. While many advances have been made in disease detection in the past ten years, particularly in the field of novel, biotechnology-based diagnostic techniques, it is important to be aware of the strengths and weaknesses of each assay to ensure appropriate interpretation of results, and to know when it is necessary to refer samples to more specialized laboratory settings. In this regard, it is important to distinguish between (i) the appropriate and informed use of front-line diagnostic assays that have been robustly validated, and preferably proven in outbreak and surveillance settings, and (ii) investigative techniques which are more suited to specialist laboratories with relevant experience. Additionally, it is essential to sustain classical virology, bacteriology, pathology and parasitology techniques, which allow further *in vitro* and *in vivo* characterization and remain the gold standards for the diagnosis of many diseases.

SELECTION OF THE DISEASES

This compendium of protocols has been compiled by FAO as part of a Global Health Security Agenda (GHSA) project funded by USAID.

The selected diseases included in this document are the more common priority zoonotic diseases (PZDs) identified in Africa through the One Health Zoonotic Disease Prioritization (OHZDP) workshops organized by the Centers for Disease Control and Prevention (CDC) and supported by FAO among other partners.

The OHZDP process brings together representatives from human, animal and environmental health sectors, as well as other relevant partners, to prioritize zoonotic diseases of greatest concern for multisectoral, One Health collaboration in a country, region or other area. It uses a transparent approach and incorporates equal input from all represented One Health sectors working at the human–animal–environment interface.

The goals of the OHZDP process are to use a multisectoral, One Health approach to 1) prioritize zoonotic diseases of greatest concern, and 2) develop next steps and action plans to address the PZDs in collaboration with One Health partners.

The protocols listed in this document focus on the following PZDs:

- Anthrax
- Avian influenza
- Bovine tuberculosis
- Brucellosis
- Crimean–Congo haemorrhagic fever (CCHF)
- Lassa fever (LASV)
- Middle East respiratory syndrome coronavirus (MERS-CoV)
- Rabies (RABV)
- Rift Valley fever (RVF)
- Salmonellosis
- West Nile virus

SCOPE

This compendium of protocols is intended to provide a practical and pragmatic resource for laboratories seeking to update, enhance or expand their diagnostic assays. For each selected disease, diagnostic protocols are explained, focusing on updated assays that have been tested to ensure their fitness for purpose by FAO Reference Centres or World Organisation for Animal Health (WOAH)[1] Reference Laboratories, or have been published in peer-reviewed journals. All of the protocols are WOAH-recommended techniques as set out in the *WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022*.

Final selection of a particular assay should be made with respect to its fitness for purpose and within the context of the disease control strategy employed for each country.

Note that reference to commercially available diagnostic assays cannot be considered as an automatic FAO endorsement in any sense, particularly if no independent validation data (e.g. outlined in a peer-reviewed publication) is readily available. However, some commercial diagnostic assays may have been assessed and approved through national policies in some countries (e.g. by the Friedrich-Loeffler-Institut in Germany and the United States Department of Agriculture), where there is a requirement to examine the performance and suitability of such kits for the purposes of veterinary diagnostic product registration.

Diagnostic technologies continue to evolve, and so it is important that this compendium is a living document which is updated regularly. Ongoing validation of established diagnostic assays is recommended by the WOAH (2022, Chapter 1.1.6), and should remain a responsibility of reference laboratories, who must communicate any new developments or changes to others involved in the same disease programmes.

[1] Founded as the International Office of Epizootics (OIE).

Risk assessment and biosafety instructions

Before beginning any activity, a risk assessment and risk analysis must be carried out, enabling assignment of a biosafety level to the planned work, selection of appropriate personal protective equipment, and development of standard operating procedures incorporating other safety interventions to ensure the safest possible conduct of the work.

Please refer to the FAO Biosafety Primer for details (FAO, 2018).

Disease-specific biosafety advice is provided in the relevant protocol if required.

Molecular protocols for priority zoonotic diseases

GENERAL GUIDELINES ON MOLECULAR TECHNIQUES

Sample preparation

For the detection of priority diseases, several matrices, such as lymph nodes, genital swabs, tissue, organs, fluids and environmental samples, can be tested. Matrices should be handled according to Chapter 1.1.2 of the WOAH Terrestrial Manual, and as specified for each disease in Chapter 3 of the Terrestrial Manual (WOAH, 2022).

RNA extraction

This section gives an overview of the different steps involved in ribonucleic acid (RNA) extraction. Please refer to the protocol provided by the manufacturer of the extraction kit for more details.

Equipment required

- Tubes (1.5–2 ml)
- Centrifuge with a rotor for 2 ml tubes
- RNA extraction kit
- Absolute ethanol
- Rack for 1.5–2 ml tubes
- Gloves
- Virkon (1% solution)
- Sodium hypochlorite (0.6% solution)
- Positive control

Extraction steps

The entire procedure must be performed under a Class II Type A2 Safety Cabinet (BSC-II) to avoid any contamination of staff, working materials and samples (cross-contamination).

Start the work session by marking the tubes with the corresponding sample identification number.

If the extraction kit is new, it is recommended that each stock solution of each of the reagents be aliquoted to avoid contamination.

For each batch of samples, a negative control must be included by adding phosphate buffer saline solution (PBS) or RNase-free water instead of the sample.

The extraction procedure starts with the lysis of the tissue to release the nucleic acid from the cells. This is done by adding the sample to the lysis buffer provided in the kit, in accordance with the proportions recommended by the manufacturer. This buffer contains reagents such as guanidine and Triton X-100 that destroy cells, denature proteins, inactivate RNases and create appropriate binding conditions that favour adsorption of RNA to the silica membrane.

Dispense the required volume of lysis buffer into each tube, remembering that guanidine[2] is harmful if inhaled or swallowed, or if it comes into contact with skin. In some kits, the addition of ethanol is required to adjust RNA binding conditions.

The correct amount of homogenized tissue under examination is then added to the lysis buffer (see kit protocol).

Vortex the solution to mix the reagents and avoid pelleting of any precipitate in the tube.

Follow the manufacturer's instructions for the next steps.

After washing, RNA elution is possible. Prepare sterile, properly identified collection tubes to collect and, ultimately, store the RNA eluate.

Place each column into the collection tube and discard the flow-through from the final wash in the correct waste vessel.

RNA is eluted by adding RNase-free water. The water dissolves the low ionic bindings between the silica membrane and the nucleic acid.

After a final centrifugation, the RNA is eluted and the extraction is completed. The total RNA of the sample is collected in the tube.

To ensure RNA stability, store the RNA at minus 70 °C (+/- 10 °C).

DNA extraction

This section describes nucleic acid extraction from various animal samples including blood and faeces.

Equipment required

- Tubes 1.5–2 ml
- Centrifuge with a rotor for 2 ml tubes
- DNA extraction kit
- Absolute ethanol
- Rack for 1.5–2 ml tubes
- Gloves
- Positive control

Extraction steps

The entire procedure should be performed under a Class II Type A2 Safety Cabinet (BSC-II) in order to avoid any contamination of staff, working materials and samples.

Start the work session by marking the tubes with the corresponding sample identification number.

If the extraction kit is new, it is recommended to aliquot each stock solution of each of the reagents to avoid contamination.

For each batch of samples, a negative control must be included by adding PBS or RNase-free water instead of the sample.

The extraction procedure starts with the lysis of the tissue to release nucleic acid from the cells. This is done by adding the sample to the lysis buffer provided in the kit according to the proportions recommended by the manufacturer. Lysis buffer contains reagents such

[2] Please refer to the material safety data sheet (MSDS) for details.

as proteinase-K to digest proteins. In most DNA extraction protocols, addition of ethanol is required, followed by a spin column procedure with silica. Follow washing steps, including vortex and centrifuge, as recommended in the protocol.

Prior to elution, prepare sterile, properly identified collection tubes to collect and, ultimately, store the DNA elute.

Place each column into the collection tube and discard the flow-through from the final wash in the correct waste vessel.

DNA is eluted by adding RNase-free water. The water dissolves the low ionic bindings between the silica membrane and the nucleic acid.

After a final centrifugation, the DNA is eluted and the extraction is completed. The total DNA of the sample is collected in the tube. To quantify the amount of eluted DNA, and to detect possible contaminates with proteins, use an absorption spectrophotometer to measure the optical density at a wavelength of 260 nm.

To ensure DNA stability, store the DNA at minus 70 °C (+/- 10 °C).

PCR master mix preparation

This section describes a protocol for preparing a master mix for polymerase chain reaction (PCR) applications, including reagents and machinery, and highlights the precautions to be taken before entering the laboratory.

Equipment required

For RT-PCR or PCR

- RNase-free microtubes (200 µl)
- Microtube rack (200 µl)
- Thermocycler
- Centrifuge for microtubes
- Vortex
- Reagents (refer to reagents list in the protocols)
- RNase-free water

For real-time RT-PCR

- Thermocycler for real-time PCR
- Reagents (refer to reagents list in the protocols)
- Microtubes (depending on the type of thermocycler)

PCR master mix preparation

Preparation of the PCR master mix is a crucial part of molecular diagnosis and it should be carried out in a dedicated clean room with its own equipment.

Leave lab coats outside and put on a clean one to work inside the clean room.

All steps must be carried out under a Class I Biological Safety Cabinet (BSC-I).

Use clean, disposable nitrile or latex gloves (powder free) to avoid any risk of contamination.

No samples, DNA or RNA templates should ever enter this room.

There should be a dedicated freezer inside the mix room for the storage of molecular reagents. All stock solutions of reagents are stored at minus 20 °C to maintain shelf life.

All reagents should be thawed on ice, except the enzymes (reverse transcriptase and Taq polymerase), which should be kept at minus 20 °C until use to avoid degradation.

Aliquots of the reagent stock solutions should also be prepared.

Vortex and spin each reagent before use to prevent pellets of material forming in the tube.

Before the reaction mix is prepared, it is necessary to calculate the final amounts of reagents required for all samples under examination, according to the protocol in use.

The addition of a positive PCR control and a negative PCR control will need to be considered in the calculation.

Prepare and label a sufficient number of PCR tubes according to the number of samples to be amplified.

To ensure homogeneous distribution of PCR reagents, it is advisable to prepare the mix in a single-reaction tube and then aliquot the necessary amount into each individual PCR tube.

When preparing the master mix for real-time PCR, a florescent probe is also added to the mix solution. Probes need to be kept in the dark to avoid any reduction in fluorescence.

When all reagents are included in the mix, vortex the tube to mix them, spin it briefly, then distribute the single-reaction mixture into each sterile PCR tube. Keep the tubes on ice. If the mix contains fluorescent probes, remember to keep the tubes in the dark, away from light sources, to prevent probe degradation.

Addition of RNA or DNA to master mix

Addition of RNA or DNA template

Templates should be added to the master mix in a **dedicated room** with its own equipment.

All steps must be carried out under a Class I Biological Safety Cabinet (BSC-I).

Keep the RNA or DNA samples and the PCR tubes containing the master mix on ice to avoid degradation of the RNA/DNA or enzyme. Note that if the master mix contains fluorescent probes, it should be kept away from light sources (e.g. by turning off the BSC light during manipulation then wrapping the Eppendorf tubes in aluminium foil once the RNA or DNA has been added).

Since this procedure carries a high risk of contamination, it is important to add the RNA or DNA sample by **opening one tube at a time** and using a separate tip for each sample.

Add a positive and a negative control to each batch of samples.

Loading samples into the thermocycler

Before loading the samples into the thermocycler, spin the tubes for a few seconds.

Put the tube in a thermocycler, program the correct parameters according to the protocol, and start the reaction.

Note that the amplicon is stable for four to seven days at 4 °C.

Agarose gel electrophoresis

The PCR product can be detected and analysed using gel electrophoresis (for conventional PCR only), which allows for the DNA fragments to be separated based on their molecular sizes through an agarose gel matrix placed in an electric field.

Equipment required

- Analytical balance
- Gel tray
- Gel combs
- Graduated cylinder
- Conical flask to mix reagents
- Electrophoresis chamber and power supply
- Buffer solution (Tris–Acetate–/EDTA (TAE) 0.5 X, or Tris–Borate–EDTA (TBE) 1X and 10X)
- Ultraviolet fluorescent dye (ethidium bromide (10mg/ml) or GelRed)
- Agarose powder
- Molecular weight ladder
- Gel loading buffer

Gel preparation

Assemble the gel casting, tray and comb.

The volume of the gel to be prepared depends on the dimensions of the gel tray and the concentration.

Example:

Width of the gel tray: 10 cm
Length of the gel tray: 7.5 cm
Thickness: 0.5 cm

10 cm x 7.5 × 0.5 cm = **37 ml TAE 0.5 X** and 0.37 **gr of agarose** (for 1 percent gel)
0.74 gr of agarose (for 2 percent gel)

Note: The agarose gel concentration (percentage) determines the range of DNA fragment separation:

- Low percentage agarose gels are best for the separation of large DNA fragments;
- Higher percentage gels are best for smaller DNA fragments.

Agarose concentration (% in m/v)	Length of fragment (in kb)
0.3	5–60
0.5	1–30
0.7	0.8–12
1	0.5–10
1.2	0.4–7
1.5	0.2–3
2	0.05–2

Weigh the correct amount of agarose powder to obtain the expected concentration.

Add the proper volume of electrophoresis buffer solution and carefully bring the solution to the boil, preferably in a microwave oven, until the agarose is completely dissolved.

Cool the solution to prevent the release of vapours when adding the intercalating dye to the gel solution.

The most common intercalating ultraviolet fluorescent dye used to make DNA bands visible for agarose gel electrophoresis is GelRed® in the proportion of 1 µl per 10 ml of agarose gel.

A series of safety tests have confirmed that GelRed® is non-cytotoxic, non-mutagenic and non-hazardous at concentrations well above the working concentrations used in gel staining. As a result, working strength GelRed® can be safely disposed of down the drain or with regular waste, providing convenience and reducing waste disposal costs.[3]

Fill the gel tray with the agarose gel, avoiding the formation of air bubbles, and allow the gel to polymerize at room temperature for about 20 minutes.

A support for sample preparation, such as Parafilm®, sample loading buffer and an appropriate molecular weight ladder are required, along with DNA samples.

Prepare the samples for the electrophoresis run by mixing at least 5 µl of the sample with loading buffer on the Parafilm® (pay attention to the order of the sample; do not mix it).

Note that due to the billions of copies of DNA produced by PCR, this procedure carries a high risk of contamination. It is strongly recommended that you open one PCR tube at a time and use separate tips.

When the gel is solid and the samples are prepared, carefully remove the gel comb from the solidified gel. Note that the holes left by the comb are the wells for sample loading.

Place the gel in its tray in the electrophoresis chamber and fill it with enough electrophoresis buffer solution to cover the gel completely.

Load the samples following the scheme described on the working data sheet.

Now load the molecular weight ladder, which should be chosen based on the expected molecular weight of the samples.

Close the gel chamber and connect it to the power supply, setting up the electrophoresis conditions according to the length of the target fragment and the concentration of the gel.

When the run is finished, turn off the power supply and disassemble the gel chamber.

Place the gel on an ultraviolet ray source such as a transilluminator or in an ultraviolet light box to visualize the DNA bands.

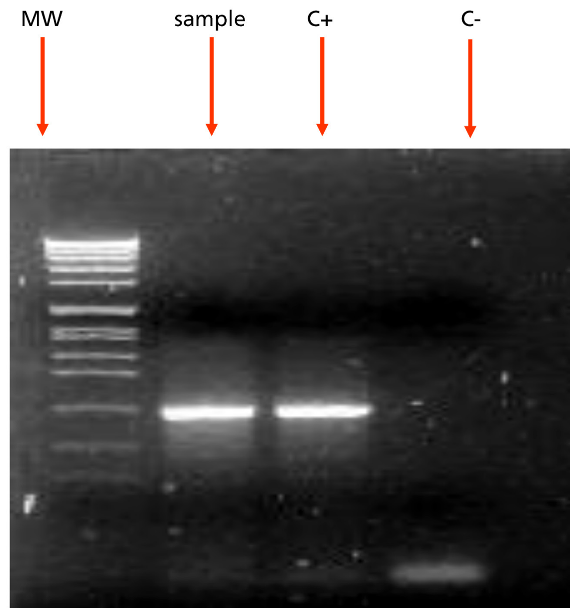
Note: Use protective goggles to limit ultraviolet exposure.

Results from agarose gel electrophoresis represent a qualitative analysis of the sample.

Positive results are characterized by bands of the expected molecular weight compared to the molecular weight ladder (MW). The positive PCR control (C+) helps to identify the correct molecular weight and verifies the efficiency of the run. No bands should be visible in the lane corresponding to the negative PCR control (C-) nor in the extraction negative control, indicating respectively the absence of contamination during the PCR and RNA extraction procedure.

[3] Safety Report for GelRed® and GelGreen®.

FIGURE 1
Agarose gel electrophoresis results



Source: Authors' own elaboration.

MOLECULAR PROTOCOLS

Anthrax

Detection of anthrax (capsule and protective antigen) by real-time RT-PCR

See Ellerbrok *et al.* (2002).

This protocol detects the protective antigen (PA).

Reagent name	Company/Code	Storage condition
Probe Pa PA-Probe: FAM-CTC GAA CTG GAG TGA AGT GTT ACC GCA AAT-BHQ1	-	20 °C (+2–10) °C
Forward primer Pa 5′- CGG ATC AAG TAT ATG GGA ATA TAG CAA-3′	-	-20 °C (+2–10) °C
Reverse primer Pa 5′- CCG GTT TAG TCG TTT CTA ATG GAT-3′	-	-20 °C (+2–10) °C
Quantabio PerfeCTa PCR Master Mix (2x)	Quantabio 95112-012	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
RNase-free water	/	4.5	
Primer Pa (7.5 µM)	300 nM	1	
Primer Pa (7.5 µM)	300 nM	1	
Quantabio PerfeCTa PCR Master Mix (2x)	1X	12.5	
Probe FAM Pa (2.5 µM)	100 nM	1	
VOLUME		20	
RNA		5	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

Starting denaturation	Denaturation	Annealing
95 °C 10 min	95 °C 15 sec	60 °C 1 min
	40 cycles	

This protocol detects the capsule (Ellerbrok *et al.*, 2002).

Reagent name	Company/Code	Storage condition
Probe Ca FAM-CCA CGG AAT TCA AAA ATC TCA AAT GGC AT-BHQ1	-	-20 °C (+2–10) °C
Forward primer Pa 5'- ACG TAT GGT GTT TCA AGA TTC ATG-3'	-	-20 °C (+2–10) °C
Reverse primer Pa 5'- ATT TTC GTC TCA TTC TAC CTC ACC-3'	-	-20 °C (+2–10) °C
Quantabio PerfeCTa PCR Master Mix (2x)	Quantabio 95112-012	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	.... Reactions µL Total*
RNase-free water	/	4.5	
Primer **Pa** (7.5 µM)	300 nM	1	
Primer **Pa** (7.5 µM)	300 nM	1	
Quantabio PerfeCTa PCR Master Mix (2x)	1X	12.5	
Probe FAM **Pa** (2.5 µM)	100 nM	1	
VOLUME		20	
RNA		5	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

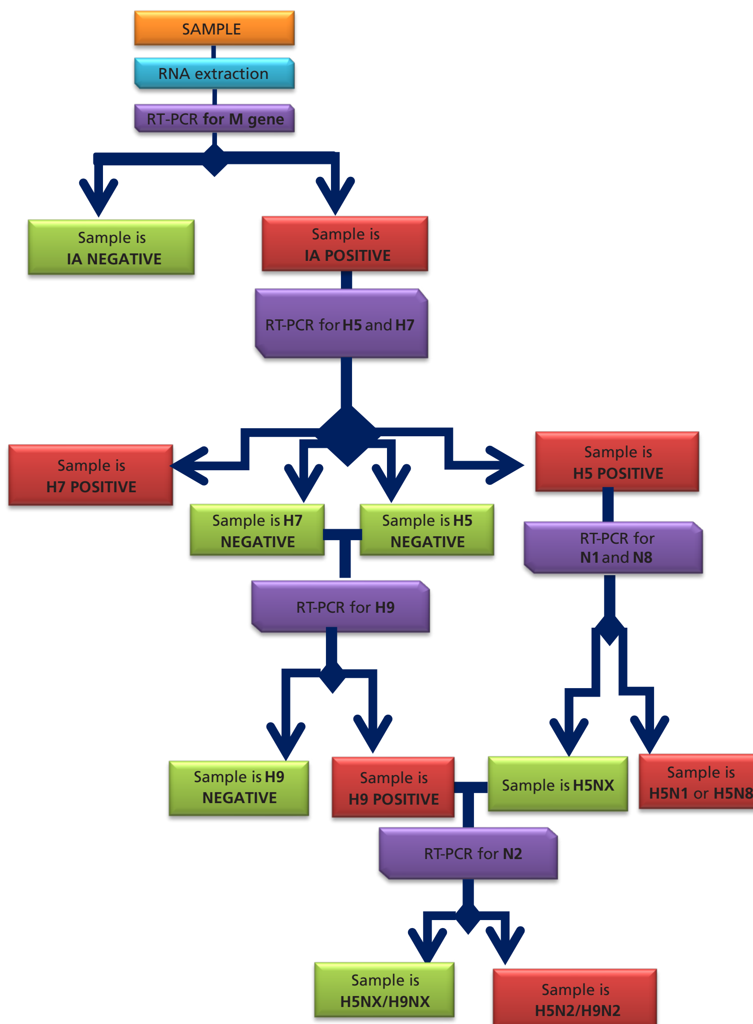
Thermal profile

Starting denaturation	Denaturation	Annealing
95 °C 10 min	95 °C 15 sec	60 °C 1 min
	40 cycles	

Avian influenza

Note: Any modification to the standard operating procedures described herein by third-party laboratories (e.g. employment of lyophilized RT-PCR reagents) should be supported by proper verification/validation of data assessing that the methods are still fit for purpose.

FIGURE 2
Avian influenza molecular diagnostic algorithm
(The workflow refers exclusively to the diagnostic protocols reported in this document; these methods were prioritized based on relevant AIV subtypes and on the epidemiological situation in the African countries in 2022)



Note: In the event of H5, H7 and H9 negative samples or inconclusive results, please refer to a FAO/WOAH Reference Laboratory for guidance and support.
Source: Authors' own elaboration.

Detection of avian influenza (M gene) by one-step real-time RT-PCR

See Heine *et al.* (2015)

Note: Protocol in use at the EU/WOAH/National Reference Laboratory for Avian Influenza and Newcastle Disease and FAO Reference Centre for Animal Influenza and Newcastle Disease hosted by the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, PD, Italy)

Reagent name	Company/Code	Storage condition
Nuclease-free water	**any**	Room temperature (RT)
AgPath-ID One-Step RT-PCR Reagents	Applied Biosystems	-20 °C (+2–10) °C
Sense primer IVA D161M1 5′-AGA TGA GYC TTC TAA CCG AGG TCG-3′	-	-20 °C (+2–10) °C
Antisense primer IVA D162M1 5′-TGC AAA AAC ATC YTC AAG TCT CTG-3′		-20 °C (+2–10) °C
Antisense primer IVA D162M2 5′-TGC AAA CAC ATC YTC AAG TCT CTG-3′	-	-20 °C (+2–10) °C
Antisense primer IVA D162M3 5′-TGC AAA GAC ATC YTC AAG TCT CTG-3′	-	-20 °C (+2–10) °C
Antisense primer IVA D162M4 5′-TGC AAA TAC ATC YTC AAG TCT CTG-3′	-	-20 °C (+2–10) °C
Probe IVA MA 5′-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3′	-	-20 °C (+2–10) °C, away from light

Reaction mix preparation

Reagent/Stock concentration	Initial concentration	Final concentration	µL per reaction	…….. Reactions µL Total*
Nuclease-free water	n/a	n/a	2.51	
2× RT-PCR buffer	2×	1×	12.5	
Sense primer IVA D161M	20 µM	0.9 µM	1.125	
Antisense primer IVA D162M1	10 µM	0.225 µM	0.56	
Antisense primer IVA D162M2	10 µM	0.225 µM	0.56	
Antisense primer IVA D162M3	10 µM	0.225 µM	0.56	
Antisense primer IVA D162M4	10 µM	0.225 µM	0.56	
Probe IVA MA	10 µM	0.250 µM	0.625	
25× RT-PCR enzyme mix	25×	1×	1	
MIX VOLUME			20	
RNA template			5	
FINAL REACTION VOLUME			25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	RT inactivation/ Initial denaturation	Denaturation	Annealing Data collection
45 °C 10 min	95 °C 10 sec	95 °C 15 sec	60 °C 45 sec
		45 cycles	

Detection of type A influenza virus by real-time RT-PCR

Adapted from Nagy *et al.* (2021)

Note: The protocol is included in the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022 *and can detect all type A influenza virus in avian and non-avian species.*

Reagent name	Company/Code	Storage condition
Nuclease-free water	-	RT
Forward SVIP-MP-F 5′ GGC CCC CTC AAA GCC GA 3′	-	-20 °C (+2–10) °C
Reverse primer SVIP-MP-R 5′CGT CTA CGY TGC AGT CC 3′	-	-20 °C (+2–10) °C
Probe SVIP-MP_P2-MGB 5′-FAM-TCA CTK GGC ACG GTG AGC GT-MGB-NFQ(EQ)-3′	-	-20 °C (+2–10) °C, away from light
QuantiTect Probe RT-PCR Kit	Qiagen	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
Nuclease-free water		3.72	
2× QuantiTect Probe RT-PCR Master Mix	1x	12.5	
Sense primer SVIP-MP-F	0.6 µM	1.5	
Antisense primer SVIP-MP-R	0.6 µM	1.5	
Probe SVIP-MP_P2-MGB	0.21 µM	0.53	
QuantiTect RT Mix	-	0.25	
MIX VOLUME		20	
RNA Template		5	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	PCR Initial activation	Denaturation	Annealing Data collection	Elongation
50 °C 30 min	95 °C 15 sec	95 °C 10 sec	64 °C 30 sec	72 °C 10 sec
			45 cycles	

Detection of avian influenza H5 subtype by one-step real-time RT-PCR

See Hassan *et al.* (2022)

Reagent name	Company/Code	Storage condition
Primer for H5-HA1-F 5′- GAT TYT AAA RGA TTG TAG YGT AGC -3′	-	-20 °C (+2–10) °C
Probe H5-FAM3-RC FAM 5′-CGC ACA TTG GRT TYC CRA GGA GCC- 3′ BHQ1	-	-20 °C (+2–10) °C, away from light
Primer H5-HA1-R1 5′- CTC TCY ACC ATG TAR GAC CA - 3′	-	-20 °C (+2–10) °C
Primer H5-HA1-R2 5′- CTC TCY ACT ATG TAR GAC CA -3′	-	-20 °C (+2–10) °C
Primer H5-F2 5′- GTTCCCTAGYAYTGGCAATCAT -3′	-	-20 °C (+2–10) °C
Primer H5-R2 5′- AATTCTARATGCAAATTCTGCAYTG -3′	-	-20 °C (+2–10) °C
Probe H5-FAM2 FAM 5′-CTGGTCTATYYTTRTGGATGTGCTCC- 3′ BHQ1	-	-20 °C (+2–10) °C, away from light
AgPath-ID One-Step RT-PCR Reagents	Applied Biosystems	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	.... Reactions µL Total*
Nuclease-free water	-	2.25	
2× RT-PCR buffer	1×	6.25	
Primers and probes pre-mix [(1)]	-	1	
25X RT-PCR enzyme mix	1×	0.5	
MIX VOLUME		10	
RNA		2.5	
FINAL REACTION VOLUME		12.5	

* to be calculated according to the number of samples and filed.

[(1)] Pre-mix stock preparation (200 µL)

Oligo	µL of 100 pmol/µL stock solutions
H5-HA1-F	20
H5-FAM3-RC	6
H5-HA1-R1	15
H5-HA1-R2	15
H5-F2	20
H5-FAM2	6
H5-R2	15

Finally, add 103 µL 0.1 × TE buffer to reach a final volume of 200 µL, sufficient for approximately 200 reactions.

Thermal profile

RT	RT inactivation/ Initial denaturation	Denaturation	Annealing Data collection	Elongation
45 °C 10 min	95 °C 10 sec	95 °C 15 sec	56.5 °C 20 sec	72 °C 30 sec
			45 cycles	

Detection of avian influenza H7 subtype by one-step real-time RT-PCR

See Slomka *et al.* (2009).

Reagent name	Company/Code	Storage condition
Probe H7 Pro11 FAM 5'- CCG CTG CTT AGT TTG ACT GGG TCA ATC T-3' BHQ-1	-	20 °C (+2–10) °C, away from light
Forward primer LH6 H7 F 5'- GGC CAG TAT TAG AAA CAA CAC CTA TGA- 3'	-	-20 °C (+2–10) °C
Reverse primer RH4 H7-R 5'-GCC CCG AAG CTA AAC CAA AGT AT -3'	-	-20 °C (+2–10) °C
OneStep RT-PCR Kit (PCR buffer 5X, dNTPs mix)	Qiagen cod. 210212	-20 °C (+2–10) °C
$MgCl_2$	-	-20 °C (+2–10) °C
RNase inhibitor 40 U/µl	-	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
Sterile RNase-free water	-	13.625	
Primer LH6 H7 F (50 µM)	400 nM	0.2	
Primer RH4 H7 R (50 µM)	400 nM	0.2	
PCR buffer 5X	1X	5	
dNTPs mix (10 mM)	0.4 mM	1	
$MgCl_2$ (25 mM)	1.25 mM	1.25	
Probe H7 Pro11 (6 µM)	150 nM	0.625	
RNase inhibitor (40 U/µl)	4 U	0.1	
One-step RT-PCR enzyme mix	-	1	
MIX VOLUME		23	
RNA		2	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	Initial denaturation	Denaturation	Annealing Data collection	Elongation
50 °C 30 min	95 °C 15 sec	95 °C 10 sec	54 °C 30 sec	72 °C 10 sec
			40 cycles	

Detection of avian influenza H9 by real-time RT-PCR

See Panzarin *et al*. (2022)

Reagent name	Company/Code	Storage condition
Nuclease-free water	-	RT
Probe Pan-H9 5′-FAM-TTC TGG GCY ATG TCH AAY GG-BHQ1-3′ (*);	-	-20 °C (+2–10) °C, away from light
Sense primer Pan-H9 for 5′-ATR GGG TTT GCT GCC-3	-	-20 °C (+2–10) °C
Antisense primer Pan-H9 rev1 5′-TCA TAT ACA AAT GTT GCA YCT G-3′;	-	-20 °C (+2–10) °C
Antisense primer Pan-H9 rev2 5′-TTA TAT ACA GAT GTT GCA YCT G-3′	-	-20 °C (+2–10) °C
RNase inhibitor 40U/µl.	-	-20 °C (+2–10) °C
AgPath-ID One-Step RT-PCR reagents	Applied Biosystems	-20 °C (+2–10) °C

(*) LNA-modified bases of Probe Pan-H9 are in bold and underlined.

Reaction mix preparation

Reagent/Stock concentration	Final concentration	µL × 1 reaction	.... Reactions µL Total*
Nuclease-free water	-	1.5	
Sense primer Pan-H9 for (10 µM)	0.4 µM	1	
Antisense primer Pan-H9 rev1 (10µM)	0.2 µM	0.5	
Antisense primer Pan-H9 rev2 (10 µM)	0.2 µM	0.5	
Probe Pan-H9 (10 µM)	0.2 µM	0.5	
2× RT-PCR buffer	1X	12.5	
RNase inhibitor (40U/µL)	20U/rxn	0.5	
25× RT-PCR enzyme mix	1X	1	
REACTION MIX VOLUME		20	
RNA TEMPLATE		5	
TOTAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT Reaction	RT inactivation/ initial denaturation	Denaturation	Annealing	Extension
50 °C 10 min	95 °C 10 sec	95 °C 15 sec	54 °C 30 sec	72 °C 15 sec
		45 cycles		

Detection of avian influenza HPAI H5N1 by duplex ONE-STEP real-time RT-PCR

Adapted from Slomka *et al.* (2007a) and developed by the Friedrich-Loeffler-Institut; Federal Research Institute for Animal Health.

Reagent name	Company/Code	Storage condition
Probe AIV-H5-1_HEX 5′ HEX-TCA ACA GTG GCG AGT TCC CTA GCA - BHQ1 3′	-	-20 °C (+2–10) °C, away from light
Forward primer LH1 H5 F 5′ ACA TAT GAC TAC CCA CAR TAT TCA G 3′	-	-20 °C (+2–10) °C
Reverse primer RH1 H5 R 5′ AGA CCA GCT AYC ATG ATT GC 3′	-	-20 °C (+2–10) °C
Probe AIV-N1-3_FAM 5′ FAM-ATY TGG ACY AGT GGG AGC AGC AT - BHQ1 3′	-	-20 °C (+2–10) °C, away from light
Forward primer N1-3-F 5′ AGR CCT TGY TTC TGG GTT GA 3′	-	-20 °C (+2–10) °C
Reverse primer N1-3-R 5′ ACC GTC TGG CCA AGA CCA 3′	-	-20 °C (+2–10) °C
iTaq™ Universal Probes One-Step Kit	Bio-Rad 172-5141	-20 °C (+2–10) °C

Mix H5 (20 reactions)	Volume (µL)
H5LH1 (100 µM)	4
H5RH1 (100 µM)	4
AIV-H5-1_HEX (100 µM)	0.5
0.1 TE (pH 8.0)	31.5
FINAL MIX VOLUME	40

Mix N1 (20 reactions)	Volume (µL)
N1-3-F (100 µM)	5
N1-3-R (100 µM)	5
AIV-N1-3_FAM (100 µM)	0.6
0.1 TE (pH 8.0)	29.4
FINAL MIX VOLUME	40

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
RNase-free water	-	3	
Probe mix H5 (10 µM)	800 nM	2	
Probe mix N1 (10 µM)	800 nM	2	
iTaq Master Mix	1×	12.5	
iTaq Reverse Transcriptase	-	0.5	
REAGENT VOLUME		20	
RNA		5	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	Initial denaturation	Denaturation	Annealing Data collection	Elongation
50 °C 10 min	95 °C 2 min	95 °C 30 sec	56 °C 30 sec	68 °C 30 sec
		42 cycles		

Detection of avian influenza N1 subtype by one-step real-time RT-PCR

See Hassan *et al.* (2022)

Reagent name	Company/Code	Storage condition
Primer for N1-F 5'- GRC CTT GYT TCT GGG TKG A-3'	-	-20 °C (+2–10) °C
Probe N1-FAM FAM 5'- CAA TYT GGA CYA GTG GRA GYA GCA T- 3' BHQ1	-	-20 °C (+2–10) °C, away from light
Primer N1-R 5'- ACC GTC TGG CCA AGA CCA- 3'	-	-20 °C (+2–10) °C
AgPath-ID One-Step RT-PCR reagents	Applied Biosystems	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
Nuclease-free water	-	2.25	
2× RT-PCR buffer	1×	6.25	
Primers and probes pre-mix [(1)]	-	1	
25× RT-PCR enzyme mix	1×	0.5	
MIX VOLUME		10	
RNA		2.5	
FINAL REACTION VOLUME		12.5	

* to be calculated according to the number of samples and filed.

[(1)] Pre-mix stock preparation (200 µL)

Oligo	µL of 100 pmol/µL stock solutions
N1-F	40
N1-FAM	6
N1-R	40

Finally, add 114 µL 0.1 × TE buffer to reach a final volume of 200 µL, sufficient for approximately 200 reactions.

Thermal profile

RT	RT inactivation/ Initial denaturation	Denaturation	Annealing Data collection	Elongation
45 °C 10 min	95 °C 10 sec	95 °C 15 sec	56.5 °C 20 sec	72 °C 30 sec
		45 cycles		

Detection of avian influenza N2 subtype by one-step real-time RT-PCR

See Hassan *et al.* (2022)

Reagent name	Company/Code	Storage condition
Primer for N2-F1 5′- AGTC TGG TGG ACY TCA AAY AG-3′	-	-20 °C (+2–10) °C
Primer for N2-F2 5′- CAG AGT RTG GTG GAC ITC-3′	-	-20 °C (+2–10) °C
Probe N2-FAM FAM 5′- CAT CAG GCC ATG AGC CTG TYC CAT- 3′ BHQ1	-	-20 °C (+2–10) °C, away from light
Primer N2-R 5′- TTG CGA AAG CTT AYA TNG VCA T- 3′	-	-20 °C (+2–10) °C
AgPath-ID One-Step RT-PCR reagents	Applied Biosystems	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
Nuclease-free water	-	2.25	
2× RT-PCR buffer	1×	6.25	
Primers and probes mix (1)	-	1	
25× RT-PCR enzyme mix	1×	0.5	
MIX VOLUME		10	
RNA		2.5	
FINAL REACTION VOLUME		12.5	

* to be calculated according to the number of samples and filed.

(1) Pre-mix stock preparation (200 µL)

Oligo	µL of 100 pmol/µL stock solutions
N2-F1	20
N2-F2	20
N2-FAM	4
N2-R	40

Finally, add 116 µL 0.1 × TE buffer to reach a final volume of 200 µL, sufficient for approximately 200 reactions.

Thermal profile

RT	Denaturation	Denaturation	Annealing Data collection	Elongation
45 °C 10 min	95 °C 10 sec	95 °C 15 sec	56.5 °C 20 sec	72 °C 30 sec
		45 cycles		

Detection of avian influenza N8 subtype by one-step real-time RT-PCR

See Hassan *et al.* (2022)

Reagent name	Company/Code	Storage condition
Primer for N8-F1 5'- TCC ATG YTT TTG GGT TGA RAT GAT-3'	-	-20 °C (+2–10) °C
Primer for N8-F2 5'- CTG ATC TCT CTT ACA GGG TTG-3'	-	-20 °C (+2–10) °C
Primer for N8-F3 5'- TCC ATG YTT TTG GGT IGA AAY GAT-3'	-	-20 °C (+2–10) °C
Probe N8-FAM1 FAM 5'- TCH AGY AGC TCC ATT GTR ATG TGT GGA GT- 3' BHQ1	-	-20 °C (+2–10) °C, away from light
Probe N8-FAM2 FAM 5'- TGC CCA GTG ACA CTC CAA GAG GGG AA- 3' BHQ1	-	?
Primer N8-R1 5'- GCT CCA TCR TGC CAY GAC CA- 3'	-	-20 °C (+2–10) °C, away from light
Primer N8-R2 5'- GTG CAT GAA CCG ACA AAT TGA G- 3'	-	-20 °C (+2–10) °C
AgPath-ID One-Step RT-PCR reagents	Applied Biosystems	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
Nuclease-free water	-	2.25	
2× RT-PCR buffer	1×	6.25	
Primers and probes mix (1)	-	1	
25× RT-PCR enzyme mix	1×	0.5	
MIX VOLUME		10	
RNA		2.5	
FINAL REACTION VOLUME		12.5	

* to be calculated according to the number of samples and filed.

(1) Pre-mix stock preparation (200 µL)

Oligo	µL of 100 pmol/µL stock solutions
N8-F1	15
N8-F2	15
N8-F3	15
N8-FAM1	6
N8-FAM2	6
N8-R1	20
N8-R2	20

Finally, add 103 µL 0.1 × TE buffer to reach a final volume of 200 µL, sufficient for approximately 200 reactions.

Thermal profile

RT	RT inactivation/ Initial denaturation	Denaturation	Annealing Data collection	Elongation
45 °C 10 min	95 °C 10 sec	95 °C 15 sec	56.5 °C 20 sec	72 °C 30 sec
			45 cycles	

Detection of avian influenza H5 subtype by one-step conventional RT-PCR

See Slomka *et al.* (2007b).

Note: This protocol has been adapted from the original paper by the EU/WAHO/National Reference Laboratory for Avian Influenza and Newcastle Disease and FAO Reference Centre for Animal Influenza and Newcastle Disease hosted by the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, PD, Italy).

Reagent name	Company/Code	Storage condition
Nuclease-free water	-	
Qiagen OneStep RT-PCR Kit	Qiagen	-20 °C (+2–10) °C
Sense primer H5-Kha 1 5′ CCTCCAGARTATGCMTAYAAAATTGTC 3′	-	-20 °C (+2–10) °C
Antisense primer H5-Kha 3 5′ TACCAACCGTCTACCATKCCYTG 3′	-	-20 °C (+2–10) °C
RNase inhibitor 40 U/µl	Promega cod. no. N2611	-20 °C (+2–10) °C

Reagents/Stock concentration	Initial concentration	Final concentration	µl per reaction	…….. Reactions µL Total*
RNase-free water	-	-	14.3	
OneStep RT-PCR buffer	5×	1×	5	
dNTPs mix	10 mM	0.4 mM	1	
Sense primer H5-Kha1	50 µM	1 µM	0.5	
Antisense primer H5-Kha3	50 µM	1 µM	0.5	
RNase inhibitor	40 U/µl	8 U	0.2	
OneStep RT-PCR enzyme mix	-	-	1	
REAGENT VOLUME			22.5	
RNA			2.5	
FINAL REACTION VOLUME			25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	Initial PCR activation	Denaturation	Annealing	Extension	Final Extension	Cooling
50 °C 30 min	94 °C 15 min	94 °C 30 sec	58 °C 1 min	68 °C 1 min	68 °C 7 min	4 °C ∞
		40 cycles				

The amplicon is detected on an agarose gel. The size of the expected fragment is 300–320 bp.

Note: In the event of positive results, the PCR products can be subjected to Sanger sequencing to confirm H5 detection and determine the pathotype based on the sequence of the cleavage site.

Detection of avian influenza H7 subtype by one-step conventional RT-PCR

See Slomka *et al.* (2007b).

Note: This protocol has been adapted from the original paper by the EU/WAHO/National Reference Laboratory for Avian Influenza and Newcastle Disease and FAO Reference Centre for Animal Influenza and Newcastle Disease hosted by the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, PD, Italy).

Reagent name	Company/Code	Storage condition
Nuclease-free water	-	?
Qiagen OneStep RT-PCR Kit	Qiagen	-20 °C (+2–10) °C
Sense primer GK7.3 5′-ATG TCC GAG ATA TGT TAA GCA-3′	-	-20 °C (+2–10) °C
Antisense primers GK7.4 5′-TTT GTA ATC TGC AGC AGT TC-3′;	-	-20 °C (+2–10) °C
RNase inhibitor 40 U/µl	Promega cod. no. N2611	-20 °C (+2–10) °C

Reagents/Stock concentration	Initial concentration	Final concentration	µl per reaction	…….. Reactions µL Total*
RNase-free water	-	-	14.3	
OneStep RT-PCR buffer	5×	1×	5	
dNTPs mix	10 mM	0.4 mM	1	
Sense primer GK7.3	50 µM	1 µM	0.5	
Antisense primer GK7.4	50 µM	1 µM	0.5	
RNase Inhibitor	40 U/µl	8 U	0.2	
OneStep RT-PCR enzyme mix	-	-	1	
REAGENT VOLUME			22.5	
RNA			2.5	
FINAL REACTION VOLUME			25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	Initial PCR activation	Denaturation	Annealing	Extension	Final extension	Cooling
50 °C 30 min	94 °C 15 min	94 °C 30 sec	52 °C 45 sec	68 °C 1 min	68 °C 7 min	4 °C ∞
		40 cycles				

The amplicon is detected on an agarose gel. The size of the expected fragment is 200–220 bp.

Note: In the event of positive results, the PCR products can be subjected to Sanger sequencing to confirm H7 detection and determine the pathotype based on the sequence of the cleavage site.

Brucellosis

Protocols provided by the IZSAM, Italy, WOAH Reference Laboratory for Brucellosis

Real-time PCR detection for Brucella *spp.*

See Bounaadja *et al.* (2009).

Below are details of real-time PCR assays using a TaqMan probe targeting the insertion sequence IS711 to detect *Brucella* at genus level.

For each batch of samples, it is necessary to analyse one positive control consisting of DNA from the *Brucella* strain and a no template control (NTC).

Reagent name	Storage condition
Forward primer: IS421 fw 5′- CGCTCGCGCGGTGGAT -3′	-20 °C (+2–10) °C
Reverse primer: IS511 rw 5′- CTTGAAGCTTGCGGACAGTCACC 3′	-20 °C (+2–10) °C
Probe: ISTq 5′- FAM-ACGACCAAGCTGCATGCTGTTGTCGATG-BHQ1- 3′	-20 °C (+2–10) °C
PCR kit containing master mix with Taq DNA Polymerase, dNTPs and reaction buffer	-20 °C (+2–10) °C

Sample preparation

Several matrices, such as lymph nodes, genital swabs, organs and fluids, can be tested.

The matrix should be handled for bacteriological isolation according to Chapter 3.1.4 of the WOAH Manual, *Brucellosis* (WOAH, 2022).

The tissues are macerated and ground in PBS, the swabs are hydrated in PBS solution, and the homogeneous suspensions are used for genomic DNA extraction.

Milk samples are pelleted and the pellet is used for genomic DNA extraction.

The optimal DNA quantity for the assay ranges between 2 and 40 ng.

DNA is extracted from single *Brucella* spp. colonies using commercial kits.

Reagent/Stock concentration	Final concentration	μL × 1 reaction	…….. Reactions μL Total*
Ultrapure sterile water	-	9.7	
TaqMan universal master mix	1x	12.5	
Primer (50 μM)	0.3 μM	0.15	
Primer (50 μM)	0.3 μM	0.15	
Probe (10 μM)	0.2 μM	0.5	
VOLUME		23	
DNA		2	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

Starting denaturation	Denaturation	Annealing/Extension
95 °C 10 sec	95 °C 15 sec	60 °C 1 min
	40 cycles	

Interpretation of results

The cut-off cycle threshold (Ct) value is 35.

Samples showing a Ct value below Ct ≤ 35 are considered POSITIVE.

Samples showing 35 < Ct < 38 are considered DOUBTS.

Samples showing Ct ≥ 38 are considered NEGATIVE.

Conventional PCR detection for Brucella *spp*

Below are details of conventional PCR assays targeting the insertion sequence IS711 to detect *Brucella* at genus level.

For each batch of samples, it is necessary to analyse one positive control consisting of DNA from the *Brucella* strain and an NTC.

Reagent name	Storage condition
Forward primer BB1: 5′- CATATCTTCCGGGGCGAGTTGGTA -3′	-20 °C (+2–10) °C
Reverse primer BB2: 5′- TCTGAGCCGTTGCCTTGAGATYG- 3′	-20 °C (+2–10) °C
PCR kit containing master mix with Taq DNA Polymerase, dNTPs and reaction buffer	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
Nuclease-free water	-	21	
Master mix	2×	25	
Primer (25 µM)	0.5 µM	1	
Primer (25 µM)	0.5 µM	1	
VOLUME		48	
DNA		2	
FINAL REACTION VOLUME		50	

* to be calculated according to the number of samples and filed.

Thermal profile

Initial PCR activation	Denaturation	Annealing	Extension	Final extension	Cooling
94 °C 5 min	94 °C 30 sec	60 °C 30 sec	72 °C 30 sec	72 °C 7 min	4 °C ∞
		33 cycles			

Interpretation of results

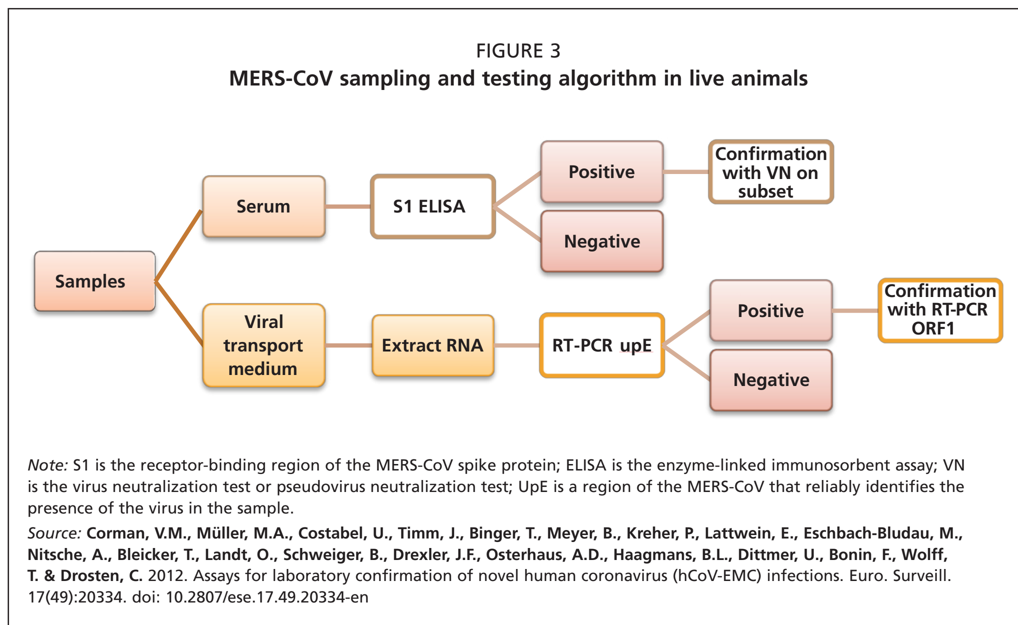
- NTC (NEGATIVE CONTROL) has no band.
- POSITIVE CONTROL shows band size of 600 bp.

Middle East respiratory syndrome coronavirus (MERS-CoV)

MERS-CoV testing algorithm for live animals

For MERS-CoV sampling and testing in live animals (from both camelids and non-camelids), the following algorithm has been developed.

FIGURE 3
MERS-CoV sampling and testing algorithm in live animals



Note: S1 is the receptor-binding region of the MERS-CoV spike protein; ELISA is the enzyme-linked immunosorbent assay; VN is the virus neutralization test or pseudovirus neutralization test; UpE is a region of the MERS-CoV that reliably identifies the presence of the virus in the sample.

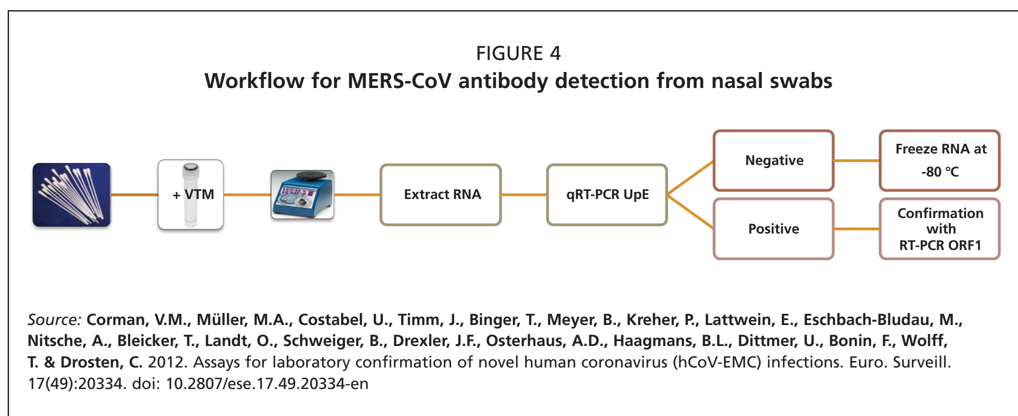
Source: **Corman, V.M., Müller, M.A., Costabel, U., Timm, J., Binger, T., Meyer, B., Kreher, P., Lattwein, E., Eschbach-Bludau, M., Nitsche, A., Bleicker, T., Landt, O., Schweiger, B., Drexler, J.F., Osterhaus, A.D., Haagmans, B.L., Dittmer, U., Bonin, F., Wolff, T. & Drosten, C.** 2012. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro. Surveill. 17(49):20334. doi: 10.2807/ese.17.49.20334-en

Testing algorithm for post-mortem samples

For sampling of camelids at slaughterhouses, the same algorithm can be applied, provided that unclotted blood can be obtained. The workflow for these standard samples will be the same as the workflow described in this document.

In addition, for MERS-CoV surveillance, the following tissue samples should be prioritized: nasal epithelium (especially mid/caudal nasal cavity), proximal trachea and any lung lesions.

FIGURE 4
Workflow for MERS-CoV antibody detection from nasal swabs



Source: **Corman, V.M., Müller, M.A., Costabel, U., Timm, J., Binger, T., Meyer, B., Kreher, P., Lattwein, E., Eschbach-Bludau, M., Nitsche, A., Bleicker, T., Landt, O., Schweiger, B., Drexler, J.F., Osterhaus, A.D., Haagmans, B.L., Dittmer, U., Bonin, F., Wolff, T. & Drosten, C.** 2012. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro. Surveill. 17(49):20334. doi: 10.2807/ese.17.49.20334-en

Detection of MERS-CoV (E gene) by real-time RT-PCR

See Corman *et al.* (2012).

Reagent name	Company/Code	Storage condition
Probe upE-Prb 5′ FAM- CTCTTCACATAATCGCCCCGAGCTCG-6-carboxy-N,N,N,N′-tetramethylrhodamine -TAMRA′	-	-20 °C (+2–10) °C
Forward primer upE-Prb 5′ GCAACGCGCGATTCAGTT 3′	-	-20 °C (+2–10) °C
Reverse primer upE-Prb 5′ GCCTCTACACGGGACCCATA 3′	-	-20 °C (+2–10) °C
SuperScript III One-Step RT-PCR System with Platinum Taq Polymerase	Invitrogen	-20 °C (+2–10) °C
$MgCl_2$	Invitrogen	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
RNase-free water	-	3.6	
Forward primer ORF1a	400 nM	1	
Reverse primer ORF1a	400 nM	1	
PCR buffer 2×	1×	12.5	
$MgCl_2$ (50 mM)	0.8 mM	0.4	
Probe ORF1a	200 nM	0.5	
One-step RT-PCR enzyme mix	-	1	
REAGENT VOLUME		20	
RNA		5	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	Initial denaturation	Denaturation	Annealing Data collection
55 °C 20 min	95 °C 3 min	95 °C 15 sec	58 °C 30 sec
		45 cycles	

If UpE PCR is positive:

For the purposes of diagnosing suspicious cases, if the screening test for MERS-CoV is positive, samples should be confirmed with a second RT-PCR to a different viral gene target using ORF1.

Confirmation of MERS-CoV by real-time RT-PCR

See Corman *et al.* (2012).

Reagent name	Company/Code	Storage condition
Probe ORF1a 5′ FAM- TTGCAAATTGGCTTGCCCCCACT6-carboxy-N,N,N,N′-tetramethylrhodamine -TAMRA′	-	-20 °C (+2–10) °C
Forward primer ORF1a 5′ CCACTACTCCCATTTCGTCAG 3′	-	-20 °C (+2–10) °C
Reverse primer ORF1a 5′ CAGTATGTGTAGTGCGCATATAAGCA 3′	-	-20 °C (+2–10) °C
SuperScript III One-Step RT-PCR System with Platinum Taq Polymerase	Invitrogen	-20 °C (+2–10) °C
Non-acetylated bovine serum albumin	Sigma	4 °C
$MgCl_2$	Invitrogen	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
RNase-free water	n/a	2.6	
Forward primer ORF1a	400 nM	1	
Reverse primer ORF1a	400 nM	1	
PCR buffer 2X	1X	12.5	
$MgCl_2$ (50 mM)	0.8 mM	0.4	
Probe ORF1a	200 nM	0.5	
BSA	-	1 µg	
One-step RT-PCR enzyme mix	?	1	
REAGENT VOLUME		20	
RNA		5	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	Initial denaturation	Denaturation	Annealing Data collection
55 °C 20 min	95 °C 3 min	95 °C 15 sec	58 °C 30 sec
		45 cycles	

Rabies

Sample preparation

It is recommended that the samples for the confirmatory test by means of conventional RT-PCR be prepared in parallel with the immunofluorescence slides, and that the RNA be extracted as soon as possible.

If the RNA is not extracted directly after sample preparation, tubes must be kept at minus 80 °C.

Prepare a 10% suspension by collecting 100 µl (or 1 gram) of central nervous system (CNS) tissues and adding 900 µl of sterile PBS.

Mix the CNS homogenates thoroughly by vortexing or using a disposable stirrer such as a 1 ml pipette tip.

Use a clean, sterile tip for each sample, open only one tube at a time, and change gloves between each sample. The risk of cross-contamination is high during this step.

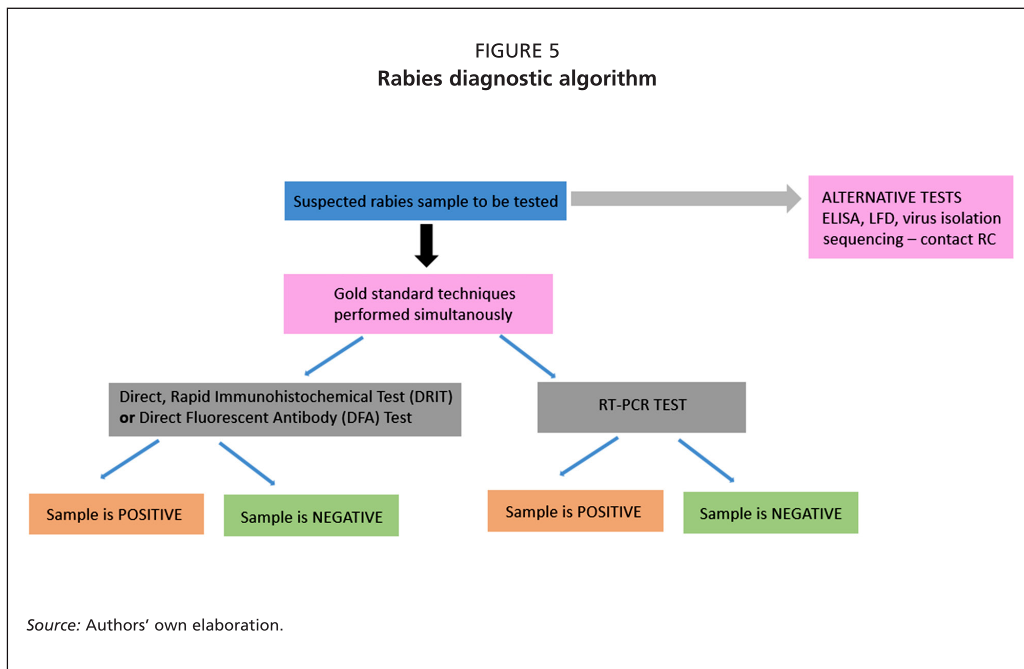
Samples are then centrifuged for 15 minutes at 4 °C at 3 000 g. At the bottom of the tube, a pellet of cerebral material should be visible.

The supernatant is the starting material for the confirmatory test.

Collect the necessary volume of sample in accordance with your RNA extraction protocol and proceed with extraction. Do not forget to include a negative control for the extraction step.

Once the RNA has been extracted, the reaction mix for conventional RT-PCR can be prepared.

FIGURE 5
Rabies diagnostic algorithm



Source: Authors' own elaboration.

Detection of rabies virus (N gene) by one-step RT-PCR

See De Benedictis *et al.* (2011).

Reagent name	Company/Code	Storage condition
RabPyro forward primer 5′ – AACACYYCTACAATGGA – 3′	-	-20 °C (+2–10) °C
RabPyro reverse primers		
5′ - TCCAATTNGCACACATTTTGTG – 3′	-	-20 °C (+2–10) °C
5′ – TCCARTTAGCGCACATYTTATG – 3′	-	-20 °C (+2–10) °C
5′ – TCCAGTTGGCRCACATCTTRTG – 3′	-	-20 °C (+2–10) °C
OneStep RT-PCR kit	Qiagen cod. 210212	-20 °C (+2–10) °C
RNase inhibitor 40 U/µl	-	-20 °C (+2–10) °C

Primer mix preparation

The primers used to detect rabies by conventional RT-PCR are: primer RabForPyro (forward primer), RabRevPyro 1 (reverse primer 1), RabRevPyro 2 (reverse primer 2), RabRevPyro 3 (reverse primer 3).

Resuspend the primers with an adequate volume of TE buffer or RNase-free water to obtain a concentration of 100 µM (follow the manufacturer's instructions).

Dilute the primers 1:10 in TE buffer or RNase-free water to a final working concentration of 10 µM as explained below:

- Prepare the forward primer: pipette 10 µl of the 100 µM dilution RabForPyro and add 90 µl of TE buffer or RNase-free water.
- Prepare the reverse primer mix: pipette 10 µl of the 100 µM dilution RabRevPyro 1, 10 µl of the 100 µM dilution RabRevPyro 2, and 10 µl of the 100 µM dilution RabRevPyro 3, and add 70 µl of TE buffer or RNase-free water.

Reagent/Stock concentration	Final concentration	µL × 1 reaction	.... Reactions µL Total*
RNase-free water	/	13.75	
RabPyro forward primer (10 µM)	400 nM	1	
RabPyro reverse primer mix (10 µM)	400 nM	1	
PCR buffer 5×	1X	5	
dNTPs mix (10 mM)	0.4 mM	1	
RNase inhibitor (40 U/µl)	10 U	0.25	
One-step RT-PCR enzyme mix	/	0.5	
REAGENT VOLUME		22.5	
RNA		2.5	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

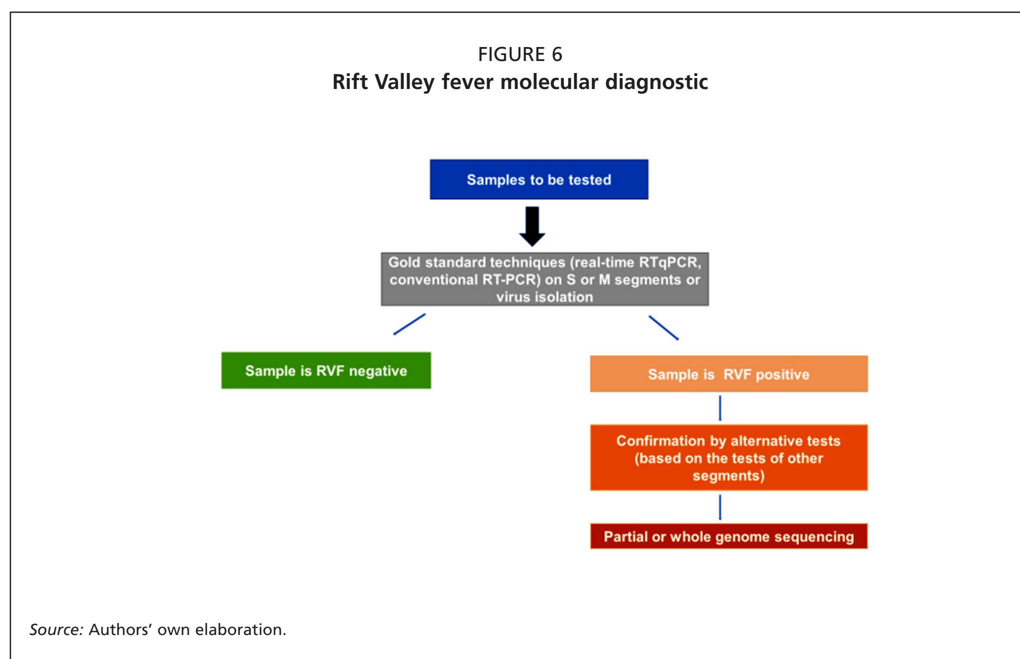
Thermal profile

RT	Activation Taq Polymerase	Denaturation	Annealing	Elongation	Final elongation	On hold
50 °C 30 min	95 °C 15 min	94 °C 30 sec	52 °C 30 sec	72 °C 40 sec	72 °C 5 min	4 °C ∞
		45 cycles				

Product of amplification is 603 bp.

Rift Valley fever

FIGURE 6
Rift Valley fever molecular diagnostic



Source: Authors' own elaboration.

Detection of Rift Valley fever virus (RVFV) by real-time RT-PCR – M segment, G2 gene

See Drosten *et al.* (2002).

Reagent name	Company/Code	Storage condition
Forward primer RVS 5'-AAAGGAACAATGGACTCTGGTCA-3'	-	-20 °C (+2–10) °C
Reverse primer RVAS 5' -CACTTCTTACTACCATGTCCTCCAAT-3	-	-20 °C (+2–10) °C
Probe RVP **5'-FAM**-AAAGCTTTGATATCTCTCAGTGCCCCAA-3' TAMRA	-	-20 °C (+2–10) °C
SuperScript™ One-Step RT-PCR System with Platinum™ Taq DNA Polymerase	Invitrogen™ Cat. 10928042	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total**
RNase-free water	-	3.8	
Primer RVS- (10 µM)	1 µM	2	
Primers RVAS- (10 µM)	1 µM	2	
Probe RVP (20 µM)	0.2 µM	0.2	
PCR buffer 2× RT-PCR*	-	10	
REAGENT VOLUME		18	
RNA		2	
FINAL REACTION VOLUME		20	

* The reaction mix contains additional $MgSO_4$ to a final concentration of 3.7 mM per reaction.

** to be calculated according to the number of samples and filed.

Thermal profile

RT reaction	Starting denaturation	Denaturation	Annealing
45 °C 30 min	95 °C 5 min	95 °C 5 sec	57 °C 35 sec
		45 cycles	

Detection of RVFV based on the L gene using a real-time RT-qPCR method

See Bird *et al.* (2007).

Reagent name	Company/Code	Storage condition
Forward primer RVFL-2912fwd 5'-TGAAAATTCCTGAGACACATGG-3'	-	-20 °C (+2–10) °C
Reverse primer RVFL-2981rev 5'-ACTTCCTTGCATCATCTGATG-3'	-	-20 °C (+2–10) °C
Probe RVFL-probe-2950 5'-FAM-CAATGTAAGGGGCCTGTGTGGACTTGTG-3'-BHQ1		
AgPath-ID One-Step RT-PCR Kit	Ambion-Applied Biosystems AM1005	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total
RNase-free water	/	3.5	
Primer RVFL-2912fwd (10 µM)	0.4 µM	1	
Primers RVFL-2981rev (10 µM)	0.4 µM	1	
Probe RVFL-probe-2950 (5 µM)	0.2 µM	1	
Real-time probe-based PCR buffer 2X RT-PCR (AgPath One-Step RT-PCR or similar)	/	12.5	
Real-time probe-based enzyme 25X RT-PCR (AgPath One-Step RT-PCR or similar)	/	1	
REAGENT VOLUME		20	
RNA		5	
FINAL REACTION VOLUME		25	

Thermal profile

RT reaction	Starting denaturation	Denaturation	Annealing
45 °C 10 min (if using AgPath One-Step RT-PCR)	95 °C 10 min	95 °C 15 sec	60 °C 1 min
		45 cycles	

Detection of RVFV based on the S segment using a conventional RT-PCR method

See Bird *et al.* (2007b).

Reagent name	Company/Code	Storage condition
Forward primer RVFS-AFwd 5'-ACACAAAGCTCCCTAGAGATAC-3'	-	-20 °C (+2–10) °C
Reverse primer RVFS-ARev 5'-ACACAAAGACCCCCTAGTG-3';	-	-20 °C (+2–10) °C
OneStep RT-PCR Kit	Qiagen cod. 210212	-20 °C (+2–10) °C
RNase inhibitor 40 U/µl	-	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total
RNase-free water	/	12	
Primer (10 µM)	0.4 µM	1	
Primers (10 µM)	0.4 µM	1	
dNTPs mix (10 mM)	0.4 mM	1	
PCR buffer 5x	1X	5	
RNase inhibitor (40 U/µl)	40 U	1	
One-step RT-PCR enzyme mix		1	
REAGENT VOLUME		22	
RNA		3	
FINAL REACTION VOLUME		25	

Thermal profile

RT	Starting denaturation	Denaturation	Annealing	Elongation	Final elongation	On hold
50 °C 30 min	95 °C 15 min	95 °C 30 sec	56 °C 30 sec	72 °C 2 min	72 °C 5 min	4 °C ∞
		45 cycles				

Amplicon (PCR product) size: **1690 bp**. Detection of PCR product: 1 percent agarose gel (ultraviolet (UV) illumination).

Detection of RVFV by conventional RT-PCR – M segment

See Bird *et al.* (2007b).

Reagent name	Company/Code	Storage condition
Forward primer RVFM-AFwd 5′-ACACAAAGACGGTGC-3′,	-	-20 °C (+2–10) °C
Reverse primer RVFM-ARev 5′-ACACAAAGACCGGTGC-3′.	-	-20 °C (+2–10) °C
OneStep RT-PCR kit	Qiagen cod. 210212	-20 °C (+2–10) °C
RNase inhibitor 40 U/µl	-	-20 °C (+2–10) °C

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
RNase-free water	/	12	
Primer (10µ M)	0.4 µM	1	
Primers (10 µM)	0.4 µM	1	
dNTPs mix (10 mM)	0.4 mM	1	
PCR buffer 5×	1×	5	
RNase inhibitor (40 U/µl)	40 U	1	
One-step RT-PCR enzyme mix	/	1	
REAGENT VOLUME		22	
RNA		3	
FINAL REACTION VOLUME		25	

* to be calculated according to the number of samples and filed.

Thermal profile

RT	Starting denaturation	Denaturation	Annealing	Elongation	Final elongation	On hold
50 °C 30 min	95 °C 15 min	95 °C 30 sec	56 °C 30 sec	72 °C 2 min	72 °C 5 min	4 °C ∞
		45 cycles				

Amplicon (PCR product) size: **3885 bp**. Detection of PCR product: 1% agarose gel (UV illumination).

West Nile fever

Detection of West Nile virus by real-time RT-PCR (lineage 1 and 2)

See Del Amo *et al.* (2013).

Reagent name	Company/Code	Storage condition
Forward primer WN-LCV-F1 5′-GTG ATC CAT GTA AGC CCT CAG AA-3′	-	-20 °C (+2–10) °C
Reverse primer WN-LCV-R1 5′-GTC TGA CAT TGG GCT TTG AAG TTA-3′		
Forward primer NS5-2-Fwd* 5′-GAA GAG ACC TGC GGC TCA TG-3′	-	-20 °C (+2–10) °C
Reverse primer NS5-2-Rev* 5′-CGG TAG GGA CCC AAT TCA CA-3′	-	-20 °C (+2–10) °C
Probe WN-LCV-S1 (Lineage 1) FAM-5′-AGG ACC CCA CAT GTT-3′-MGB	-	-20 °C (+2–10) °C
Probe WN-LCV-S2 (Lineage 2) VIC-5′-AGG ACC CCA CGT GCT-3′-MGB	-	-20 °C (+2–10) °C
Probe NS5-2-NED* 5′NED-CCA ACG CCA TTT GCT CCG CTG-3′MGB	-	-20 °C (+2–10) °C
QuantiTect Probe RT-PCR Kit	Qiagen	-20 °C (+2–10) °C
Armored RNA West Nile Virus (HNY1999)	Asuragen, Inc., cod. 42040	

* Probe and primers for internal positive control.

Reagent/Stock concentration	Final concentration	µL × 1 reaction	…….. Reactions µL Total*
H_2O	n/a	4.85	
QuantiTect RT-PCR Master Mix	1×	12.50	
Primer WN-LCV-F1 (20 µM)	0.4 µM	0.50	
Primer WN-LCV-R1(20 µM)	0.4 µM	0.50	
Probe WN-LCV-S1 (Lineage 1) (10 µM)	0.2 µM	0.50	
Probe WN-LCV-S2 (Lineage 2) (10 µM)	0.2 µM	0.50	
Probe NS5-2-**NED** (20 µM)	80 nM	0.20	
Primer NS5-2-Fwd (50 µM)	150 nM	0.10	
Primer NS5-2-Rev(50 µM)	150 nM	0.10	
QuantiTect RT Mix	1X	0.25	
Vol. MIX TOTAL		20.0	

* to be calculated according to the number of samples and filed.

Thermal profile

RT reaction	Starting denaturation	Denaturation	Annealing
50 °C 30 min	95 °C 15 min	95 °C 15 sec	60 °C 1 min
		45 cycles	

Detection of West Nile virus by real-time RT-PCR (all lineages)

See Vasquez *et al.* (2016).

Reagent name	Company/Code	Storage condition
Forward primer WNRT-F 5'-CGG AAG TYG RGT AKA CGG TGC TG-3'	-	-20 °C (+2–10) °C
Reverse primer WNRT-Re 5'-CGG TWY TGA GGG CTT ACR TGG-3'	-	-20 °C (+2–10) °C
Probe WNV-P 5'FAM-WCC CCA GGW GGA CTG-3'MGB-NFQ	-	-20 °C (+2–10) °C
Forward primer NS5-2-Fwd* 5'-GAA GAG ACC TGC GGC TCA TG-3'	-	-20 °C (+2–10) °C
Reverse primer NS5-2-Rev* 5'-CGG TAG GGA CCC AAT TCA CA-3'	-	-20 °C (+2–10) °C
NS5-2-Probe* 5'VIC-CCA ACG CCA TTT GCT CCG CTG-3'TAM	-	-20 °C (+2–10) °C
SuperScript III Platinum One-Step Quantitative RT-PCR System	Invitrogen	-20 °C (+2–10) °C
Armored RNA West Nile Virus (HNY1999)	Asuragen, Inc., cod. 42040	-80 °C (+2–10) °C

* Probe and primers for internal positive control.

Reagent/Stock concentration	Initial concentration	Final concentration	µL × 1 Reaction	…….. Reactions µL Total*
H_2O	**n/a**	**n/a**	5.51	
2× RxN mix	2×	1×	12.50	
ROX ref dye	25 µM	500 nM	0.50	
Primer WNRT-F	100 µM	800 nM	0.20	
Primer WNRT-Re	100 µM	800 nM	0.20	
Probe WNV-P	50 µM	400 nM	0.20	
Probe NS5-2	20 µM	80 nM	0.20	
Primer NS5-2-Fwd	50 µM	150 nM	0.10	
Primer NS5-2-Rev	50 µM	150 nM	0.10	
SS III Platinum Taq			0.50	
Vol. MIX TOTAL			20.00	

* to be calculated according to the number of samples and filed.

Thermal profile

RT reaction	Starting denaturation	Denaturation	Annealing
50 °C 15 min	95 °C 10 min	95 °C 15 sec	60 °C 1 min
		40 cycles	

Serological protocols for priority diseases

This section describes protocols for priority diseases including avian influenza, brucellosis and MERS-Cov that require serological assays to be detected.

AVIAN INFLUENZA

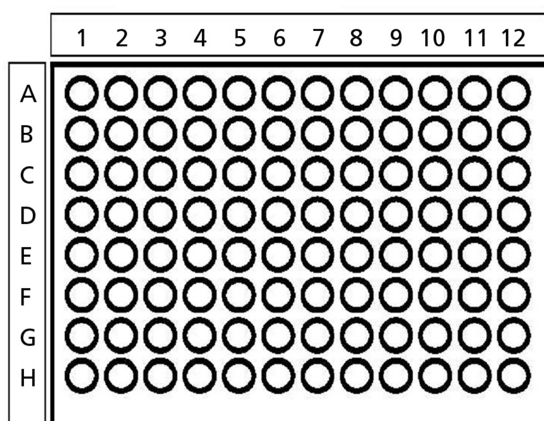
Haemagglutination inhibition (HI) test

This method is based on a simple reaction between the virus and the specific antiserum. When the specific antibodies present in the serum react with the virus, they will prevent the virus from binding to red blood cell (RBC) receptors. Consequently, haemagglutination is inhibited and RBCs will precipitate as a spot in the bottom of the microplate wells. The HI test is used to detect antibodies for specific subtypes of avian influenza viruses. Before performing an HI test, titration of the reference antigen by means of a haemagglutination test (HA test) is necessary to prepare the 4 hemagglutinating unit (HAU) antigen solution.

Materials and reagents

- PBS
- Freeze-dried reference antigen diluted with PBS to obtain 4 HAU per 25 µl
- 1 percent chicken RBC suspension
- Negative control chicken serum
- Positive control chicken serum

FIGURE 7
HI microplate



Source: Authors' own elaboration.

HA test in microtitre plate (micro HA test):

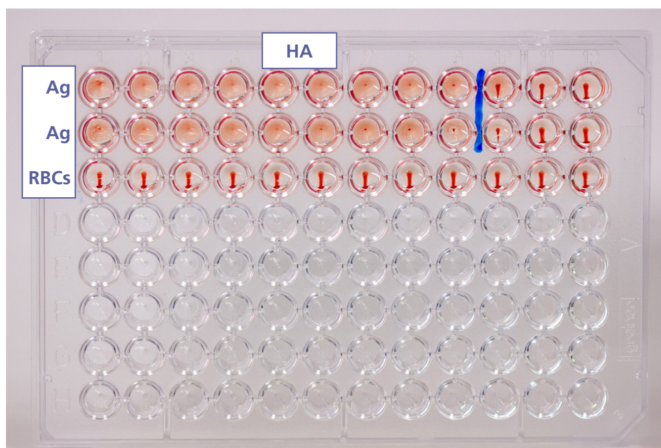
- Dispense 25 µl of PBS into each well of lines A, B and C of a plastic microplate (V-bottomed wells).
- Place 25 µl of the reference antigen in the first well of lines A and B to titre it in double. Use line C for the RBC control.
- Make twofold dilutions (from 1:2 to 1:4096) of virus across the plate from the first to the twelfth well and discard the last 25 µl.
- Dispense 25 µl of PBS into each well of the three rows.
- Gently shake the RBC suspension to mix the solution and prevent pelletting of the cells.
- Add 25 µl of 1 percent RBC suspension to each well of lines A, B and C.
- Mix by tapping the microplate gently and incubate it for 30 minutes at room temperature (+20–24 °C) or 60 minutes at + 4 °C.

Read results when the RBC control wells have settled, forming a distinct pellet in the bottom of the well. Results are read by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. Wells with no haemagglutination should flow at the same rate as the control cells with no virus.

When the tear-shaped streaming in the RBC control wells is complete, mark the highest dilution giving complete hemagglutination of the RBCs in the first row. This dilution contains 1 HAU.

To prepare a 4 HAU antigen solution, it is necessary to calculate the total amount of antigen solution required to test all serum under examination. Calculate the hemagglutinating titre containing 4 HAU by dividing the antigen titre by four.

FIGURE 8
HA test



Source: Authors' own elaboration.

For example:
If the HA titre is 1:512 (1 HAU), the 4 HAU will be obtained by dividing four times that titre (512 divided by 4=128).

The antigen dilution for carrying out the HI test with 4 HAU is 1:128. Therefore, to perform the test, use:

- 1 ml of antigen in 127 ml of PBS; or
- 0.5 ml of antigen in 63.5 ml of PBS

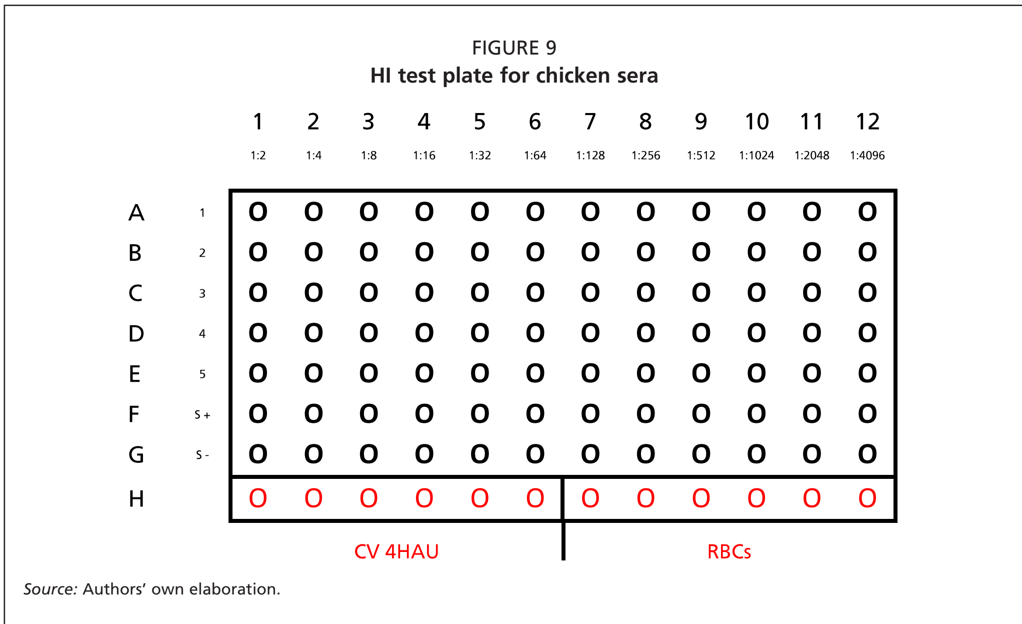
N.B.: This count means that it is possible to prepare only the amount of antigen solution required, considering that 2.5 ml of solution are sufficient for an entire microplate.

HA titre is 1:512; RBCs: control of red blood cells

HI test for chicken sera

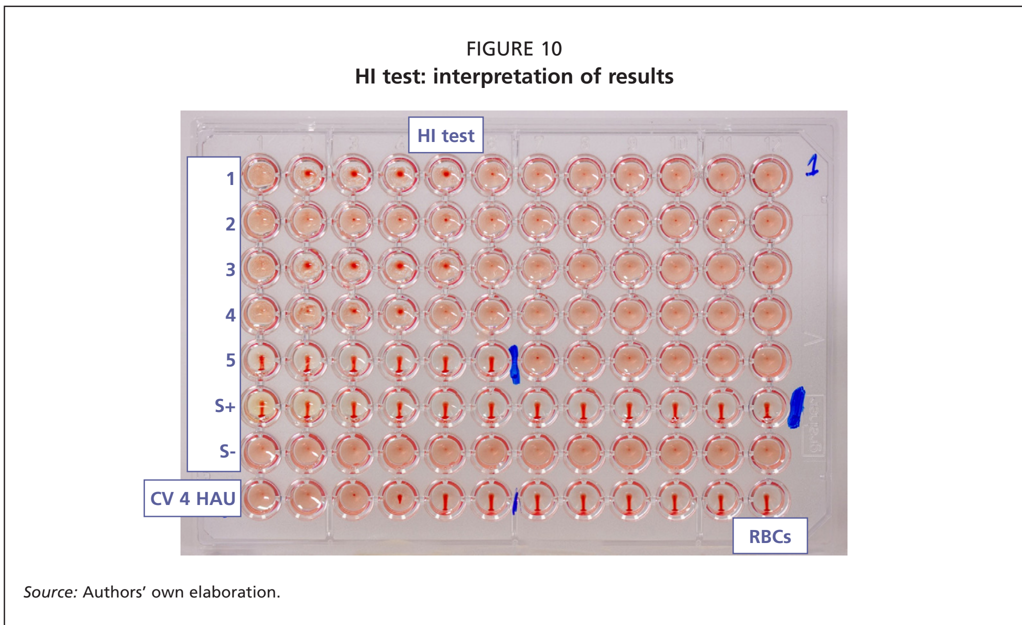
- Identify the samples under examination on the microplate with an indelible marker.
- The last three lines will be used for the positive control serum, the negative control serum, the 4 HAU antigen solution control, and the RBC control, respectively.
- Using the indelible pen, mark the division between the sixth and seventh well of the last row, and use the first six wells for the back titration of the antigen solution control and the last six for the RBC control.
- Dispense 25 µl of PBS into all the wells of a microplate with the exception of the first well of the antigen solution control wells, in H1.
- Dispense 25 µl of serum into the first wells of the first column, using a new tip for each sample (from A1 to E1).
- Add 25 µl of the positive control serum (with known HI titre) in the F1 well and 25 µl of negative control serum in the G1 well.
- Using a multichannel micropipette, make twofold dilutions of the serum (A1–A12) across the plate (from row A to G). Discard the last 25 µl.
- Add 25 µl of 4 HAU antigen suspension across the plate, with the exception of row H.
- Add 25 µl of 4 HAU antigen suspension in the first two wells of row H (4 HAU control from H1 to H6), make twofold dilution from H2 to H6, and discard the last 25 µl in order to obtain 4 HAU, 2 HAU, 1 HAU, 0.5 HAU, 0.25 HAU and 0.125 HAU. This control must be included in each microplate.
- Add 25 µl of PBS in all wells in row H to bring the volume of the wells to 50 µl.
- Gently tap to mix and incubate the plate between +20 °C and +24 °C for 30 minutes or at +4 °C for 60 minutes.
- Add 25 µl of 1 percent RBC suspension into all wells.
- Gently tap to mix and incubate the plate at between +20 °C and +24 °C for 30 minutes or at +4 °C for 60 minutes.
- Results are recorded when the RBCs control has settled. To read results, tilt and observe the presence or absence of tear-shaped streaming of the RBCs at the same rate as the control wells containing RBCs alone. The HI titre is the highest dilution of serum causing the complete inhibition of the haemagglutination.

FIGURE 9
HI test plate for chicken sera



Source: Authors' own elaboration.

FIGURE 10
HI test: interpretation of results



Source: Authors' own elaboration.

From the picture we can see:

- Samples 1, 2, 3 and 4 are negative
- Sample 5 is positive: titre 1:64
- Titre of positive serum: 1: 4096

CV 4 HAU: control of 4 haemagglutinanting viral units (HAU)

RBCs: control of red blood cells

HI test on sera of avian species other than chicken

- To perform a valid HI test, sera from species other than chicken must be pre-treated with a 10 percent RBCs suspension following the procedure below in order to remove factors that may cause non-specific agglutination. For some species such as guinea fowls, quails, ostriches and species bred for hunting purposes, an additional inactivation pre-treatment in a water bath at 56 °C for 30 minutes should be carried out prior to performing the procedure described hereafter.
- Identify the samples under examination on the microplate (V-bottomed wells) with an indelible marker. The last three lines will be used for the positive control serum (S+), the negative control serum (S-), the control of the 4 HAU antigen solution (CV) and the RBCs control respectively.
- Using the indelible pen, mark the division between the sixth and the seventh well of the last row, and use the first six wells for the back titration of the antigen solution (CV) and the last six for the RBCs control.
- Add 50 µl of PBS to the first well of the test serum row (A1).
- Add 25 µl of PBS to all test wells excluding the well of column 2 (A3-A12).
- Add 25 µl of PBS to all wells of rows F (positive control serum (S+)) and G (negative control serum (S-)).
- In row H (control of the 4 HAU antigen solution (CV) and the RBCs control), with the exception of the first well, add 25 µl of PBS (H2-H12).
- Add 25 µl of test serum to the appropriate wells in column 1 (from A1 to E1), using a new tip for each sample.
- Add 25 µl of 10 percent chicken RBCs suspension to the test well of column 1 (A1).
- Gently shake the plate and incubate at room temperature for at least 30 minutes.
- Add 25 µl of the positive control serum (with known HI titre) in the F1 well and 25 µl of negative control serum in the G1 well and make serial twofold dilutions across the plate by transferring 25 µl of positive and negative control sera from the first well to the following ones (from F-G1 to F-G12).
- Discard the remaining 25 µl.
- Dilute the test serum by transferring 25 µl from A1 to A2 (dilution 1:4) and a further 25 µl from A1 to A3 (dilution 1:8).
- Make serial twofold dilutions across the plate by transferring 25 µl of the test serum from A3 to A12 and discard the remaining 25 µl. The test serum is now ready to be treated as chicken serum by following the next steps. Column 1 is not considered in the interpretation of results.
- Add 25 µl of standardized antigen dilution to all wells containing test serum, positive and negative control sera and to H1 and H2 wells. This control must be included in each microplate.
- Make serial twofold dilutions from H2 to H6 to obtain 4, 2, 1, 0.5, 0.25 and 0.125 HAU and discard the remaining 25 µl.
- Add 25 µl of PBS to all wells of row H, with the exception of H1; bring the volume of the wells to 50 µl.
- Gently shake the plate and incubate at room temperature for 30 minutes or at +4 °C for 1 hour.

- Add 25 µl of 1 percent chicken RBCs suspension to all wells.
- Gently shake the plate and incubate at room temperature for at least 30 minutes or at +4 °C for 1 hour.
- Results are recorded when the RBCs control has settled. This is done by tilting and observing the presence or absence of tear-shaped streaming of the RBCs at the same rate as control wells containing RBCs alone. The HI titre is the highest dilution of serum causing the complete inhibition of the haemagglutination.

Interpretation of results

- When the HI titre is ≤1:8, the sample is considered as negative.
- When the HI titre is ≥1:16, the sample is considered as positive.

The 4 HAU control should show:

- a complete haemagglutination in the first three wells (H1-H3, containing respectively 4, 2, 1 HAU), a partial haemagglutination (small spot at the bottom of the well) in the well H4 containing 0.5 HAU, an apparent total inhibition of haemagglutination in wells H5 and H6 (containing 0.250 and 0.125 HAU respectively).

The test is valid if:

- the negative control serum has a lower titre than 1:8 against the 4 HAU antigen solution;
- the positive control (reference) serum has a titre which matches the expected value of the reference serum. Note that a difference of ± 1 dilution is permitted.

BRUCELLOSIS

Detection of brucellosis by Rose Bengal test (RBT)

See Chapter 3.1.4 of the WOAH Manual, *Brucellosis* (WOAH, 2022).

Necessary material

- *Brucella* antigenic suspension (with Rose Bengal)
- Pipettes (10–100 µl) with tips
- White tile, enamel or plastic plate or World Health Organization (WHO) haemagglutination plate.

Procedure

Bring the serum samples and antigen (commercially available) to room temperature (22 °C ± 4 °C); only sufficient antigen for the day's tests should be removed from the refrigerator.

- Place 25–30 µl of each serum sample on a white tile, enamel or plastic plate, or in a WHO haemagglutination plate.
- Shake the antigen bottle well, but gently, and place an equal volume of antigen near each serum spot.
- Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2 cm in diameter.
- The mixture is agitated gently for 4 minutes at room temperature (22 °C ± 4 °C).

FIGURE 11
RBT: positive and negative samples

Source: Authors' own elaboration.

Read for agglutination immediately after the 4-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day's tests are begun, to verify the sensitivity of test conditions.

The RBT is very sensitive. However, like all other serological tests, it may sometimes give a positive result in cattle because of B. abortus S19 vaccination or false positive serological reactions (FPSR). The same phenomenon occurs in small ruminants or pigs, where the test is affected by FPSR, and in small ruminants vaccinated with B. melitensis Rev.1. Positive reactions should therefore be investigated using suitable confirmatory or complementary strategies (including epidemiological investigation). Conversely, false negative reactions occur rarely.

Nevertheless, RBT appears to be adequate as a screening test for detecting infected herds or to guarantee the absence of infection in brucellosis-free herds or flocks.

MERS-CoV

PROTOCOL FOR EUROIMMUN ANTI-MERS-CoV ELISA CAMEL (IgG)

Preparation and stability of the reagents

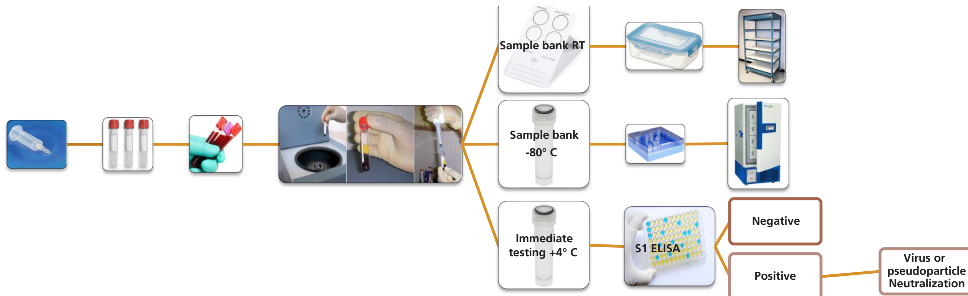
All reagents must be brought to room temperature (18 °C to 25 °C) approximately 30 minutes before use. After first use, the reagents are stable for four months if stored at between 2 °C and 8 °C and protected from contamination, unless stated otherwise. The thermostat-adjusted ELISA incubator must be set at 37 °C ± 1 °C.

Stability: Samples to be investigated can generally be stored at between 2 °C and 8 °C for up to seven days. Diluted samples should be incubated within one working day.

Sample dilution: Samples are diluted 1:100 in sample buffer. For example, dilute a 10 µl sample in 1 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

Please note: The calibrator and controls are pre-diluted and ready for use; do not dilute them.

FIGURE 12
Workflow for MERS-CoV antibody detection from sera



Source: **Corman, V.M., Müller, M.A., Costabel, U., Timm, J., Binger, T., Meyer, B., Kreher, P., Lattwein, E., Eschbach-Bludau, M., Nitsche, A., Bleicker, T., Landt, O., Schweiger, B., Drexler, J.F., Osterhaus, A.D., Haagmans, B.L., Dittmer, U., Bonin, F., Wolff, T. & Drosten, C.** 2012. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro. Surveill. 17(49):20334. doi: 10.2807/ese.17.49.20334-en

Step 1 – sample incubation:

Transfer 100 µl of the calibrator, positive and negative controls or diluted samples into the individual microplate wells according to the pipetting protocol. For manual processing of microplate wells, cover the finished test plate with the protective foil.

Incubate for 30 minutes at 37 °C ± 1 °C.

Washing:

- Remove the protective foil, empty the wells and subsequently wash three times using 300 µl of working strength wash buffer for each wash.
- Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing, thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer. Note: Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g. less than three wash cycles, inadequate wash buffer volumes, or residence times that are too short) can lead to false high extinction values.
- Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Step 2 – conjugate incubation:

- Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-camel IgG) into each of the microplate wells. The wells must be covered with the protective foil when incubating manually. Incubate for 30 minutes at +37 °C ± 1 °C.

Washing:

- Remove the protective foil and empty the wells. Wash as described above.

Step 3 – substrate incubation:

- Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for 15 minutes at room temperature (+18 °C to +25 °C). Protect from direct sunlight.

Stopping the reaction:

- Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:

- Photometric measurement of the colour intensity should be made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm within 30 minutes of adding the stop solution. Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

If S1 ELISA positive:

Positive samples, or a subset thereof as calculated using the previously mentioned algorithm, should be tested using confirmatory tests such as **virus neutralization** or **pseudo-particle mirconeutralization**.

Immunochemical identification for priority zoonotic diseases

RABIES

This section describes immunochemical protocols, including the direct fluorescent antibody test (dFAT) and the direct rapid immunohistochemistry test (dRIT) for the detection of lyssaviruses.

Detection of rabies using the dFAT

Required equipment and reagents

- Sterile PBS (pH 7.2–7.4)
- Distilled/deionized water
- Acetone (min. 99.8%)
- Bio-Rad fluorescein isothiocyanate (FITC) anti-rabies conjugate (reference number: 3572112)
- Glycerol 20 percent mounting medium (pH 9.6)
- Evans blue dye solution
- Virkon 1 percent disinfectant
- 3 percent sodium hypochlorite solution
- Glass microscope slides, frosted one end
- Coverslips (22 × 22 mm)
- Hellendhal/Coplin containers (10 or 16 slides)
- Humid chamber
- Pipets and tips
- Fluorescence microscope (FITC filter)
- Thermostat at 37 °C

The most widely used test for rabies diagnosis is the dFAT, which is recommended by both WHO and WOAH (see Chapter 3.1.17, *Rabies*, of WOAH, 2022). This test is used directly on a brain impression smear. It is also used to confirm the presence of rabies virus antigen in cell culture or in the brain tissue of mice that have been inoculated for diagnosis. The dFAT test is highly sensitive and specific (between 96 percent and 99 percent), and gives reliable results on fresh specimens within 2 hours.

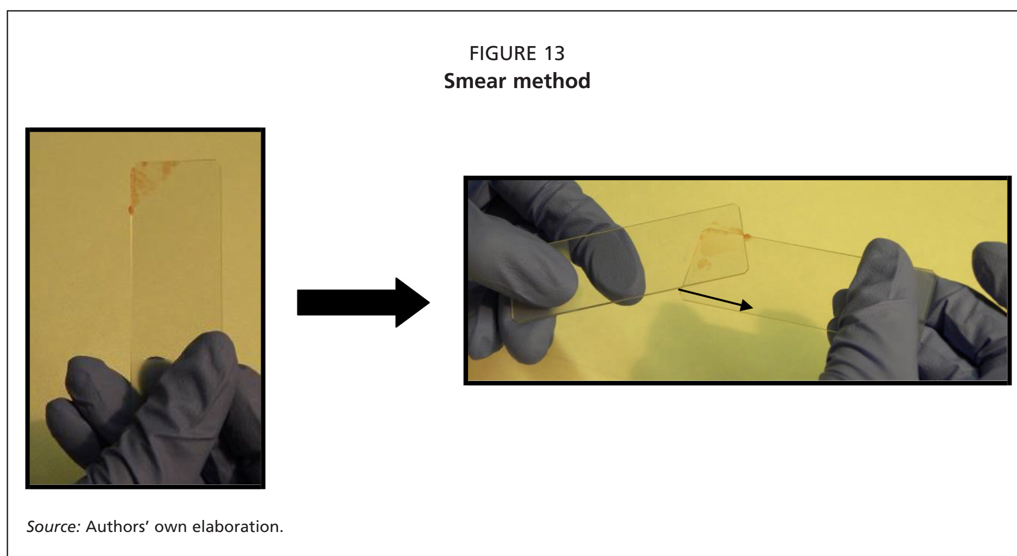
N.B. Each step of the manipulation must be carried out under a **Biosafety Cabinet Class II (BSC-II)**.

If the BSC-II does not have a specific acetone filter, please reduce the working time under the BSC-II when acetone is handled, and take appropriate countermeasures (keep the acetone bottle closed when not in use, keep the tubes closed as much as possible, and so on).

Please note that according to WHO recommendations, all technicians who are manipulating suspected rabies samples should be vaccinated against rabies.[4]

[4] See www.who.int/ith/vaccines/rabies/en

FIGURE 13
Smear method



Source: Authors' own elaboration.

Smear method[5]

- Place a small section of brain tissue on one end of the slide.
- Use another slide to crush the section of tissue against the first slide.
- Spread the sample homogeneously over a small portion of the slide.

Care should be taken not to use a tissue section which is too large, as this results in an excessively thick film, making proper staining and microscopic examination impossible.

Do not forget to prepare positive and negative controls together with the samples.

- Leave the slides to dry for a few minutes under the BSC-II.

Fixation

- Once dried, both the test slides and the positive/negative control slides are immersed in slide racks (glass container), filled with cold pure acetone and fixed at room temperature for at least **1 hour** (under a chemical cabinet), or at 8 °C overnight.

Use a Hellendhal/Coplin staining jar (previously stored in a freezer (minus 20 °C) to prevent it from breaking when the acetone is added) for fixing. The positive control should be fixed in separate containers to avoid transferring tissues from the sample slides to the positive control during fixation.

- Remove the slides from the acetone and air dry the slides at room temperature for 15–20 minutes (under BSC-II).

[5] Protocol provided by FAO Reference Centre for Rabies (IZSVe-Padova, Italy).

Immunofluorescent staining

There are a large number of polyclonal and monoclonal commercial antibodies. The laboratory is free to choose whichever one works best, paying attention to the dilution that should be applied before use.

The use of the anti-rabies nucleocapsid conjugate (polyclonal) corresponds to the protocol in use at the IZSVe laboratory (the FAO Reference Centre for Rabies).

These polyclonal antibodies target the ribonucleoprotein complex of the rabies virus.

- Calculate the total volume of conjugate you need by multiplying the number of slides that will be analysed by the amount that will be distributed on each slide (we recommend using between 20 µl and 50 µl). ***Number of slides × volume (µl) of antibody per slide = total volume (µl) of antibodies required for the work session***.
- To this volume, add the required amount of Evans blue to obtain 1:20 dilution.

For example, ***for a total volume of 100 µl of antibodies, add 5 µl of 1 percent Evans blue.***

- Add 20 µl to 50 µl of conjugate and Evans blue mix to each slide. Incubate the slides for 30 minutes at 37 °C in a humid chamber to prevent the conjugate from drying.

Washing

- After incubation, slides are washed twice with PBS (5 minutes for each wash) and once with distilled water for a few seconds. Drain the slides on filter paper.
- When dry, one drop of mounting medium is added to each slide.
- Mounting medium is a solution of 20 percent glycerol in 50 mM Tris buffer (supplemented with 150 mM of sodium chloride), pH 9.6.
- Carefully place the coverslip, ensuring that no bubbles form.

Reading the slide microscopically

The procedure involves allowing a labelled antibody (FICT) to react with a specific antigen (if present), and observing the reaction under a fluorescence microscope.

Control slides are examined before the test slides to ensure that the equipment is operating satisfactorily and the slides are properly stained.

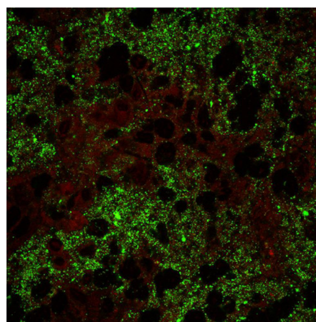
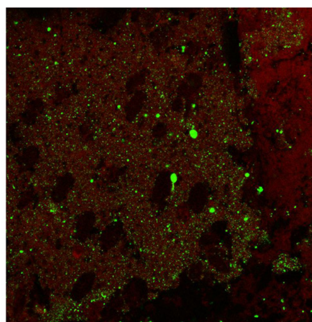
The positive control and test slides containing rabies antigen will contain brilliantly fluorescing green or greenish-yellow structures.

A diagnosis session is considered valid provided that:

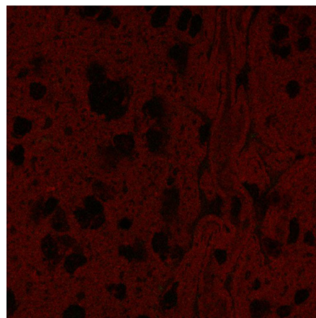
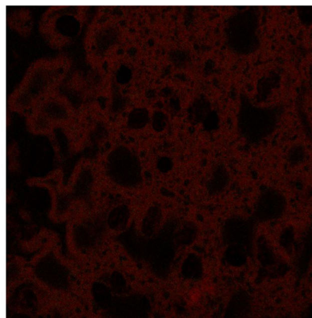
- Fluorescence is detected on the positive control slide; and
- No fluorescence is detected on the negative control slide.

FIGURE 14
Reading slides: positive and negative samples

Positive sample

Negative sample

Source: Authors' own elaboration.

Direct rapid immunohistochemistry test (dRIT)

See Chapter 3.1.17 of the WOAH Manual, *Rabies* (WOAH, 2022).

A novel diagnostic assay for lyssavirus diagnosis, known as the dRIT, was developed by Rupprecht and Niezgoda during the early part of the twenty-first century, while at the CDC. The development of the dRIT assay, as it is currently known, is based on a simplified version of the standard avidin-biotin-complex immunohistochemical (IHC) diagnostic assay. The main differences between the dRIT and standard IHC tests are that the original IHC test takes many hours to conduct, requires a linker antibody conjugated to a biotin moiety, and is performed upon cut sections of fixed tissue, while the dRIT is accomplished in under 1 hour, utilizes either a polyclonal or monoclonal antibody that is directly labelled with a biotin moiety, and is performed upon a simple touch impression of brain tissue, allowing an anatomic-pathologic appreciation of viral inclusions in neurons. Additionally, the need for a linker antibody is negated by the subsequent addition of a streptavidin that has a high affinity for the biotin moiety. The streptavidin is tagged to a reporter enzyme that is used to catalyse the formation of a coloured precipitate once the antibodies are bound

to the RABV antigens in the presence of an appropriate substrate (H_2O_2) and chromogen (amino-ethylcarbazole [AEC]).

The below protocol was provided by the GARC. For further information please contact info@rabiesalliance.org

Apparatus

- Single-edge, frosted microscope slides
- Tissue-Tek slide staining kit or multiple individual glass staining dishes
- Slide rack
- Tissue culture plate lids or commercially supplied humidity chamber
- Pipettor (10–100 µl)
- Pipettor (100–1000 µl)
- Universal pipettor tips (5–100 µl)
- Universal pipettor tips (100–1000 µl)
- Plastic dropper bottle
- Corning cover glass
- Light microscope with 20x and 40x objectives

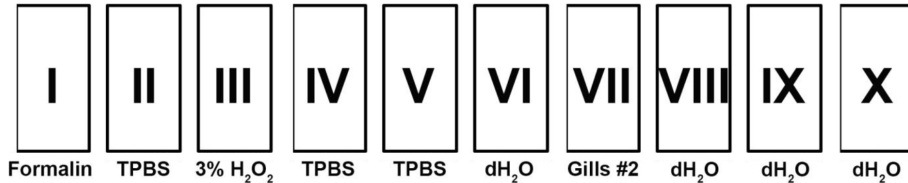
Reagents

- Formalin, 10 percent neutral buffered *(Sigma-Aldrich, Cat # HT501128*)
- 1x PBS (*Fisher Scientific, Cat # SH30256,02*)
- Tween 80 (*Sigma-Aldrich, Cat # P1754*)
- Hydrogen peroxide (3 percent) (*Use any commercial supplier*)
- Biotinylated antibody preparation (*Use any commercial supplier*)
- Streptavidin-peroxidase (*Kirkegaard & Perry Laboratories Inc, Cat # 71-00-38*)
- AEC staining kit (*Sigma-Aldrich, Cat # AEC101*)
- Gills formulation #2 (*Sigma-Aldrich, Cat # GHS232*)
- Gel/mount (*Fisher Scientific, Cat # BM-M01*)
- Distilled water (*Fisher Scientific, Cat # S75232*)

Staining dish set-up for reagents

The staining dish set-up has been designed to simplify the diagnostic process (Niezgoda and Rupprecht, 2006). The staining dishes should be prepared before starting the diagnostic procedure. The contents of each staining dish can be used for different time periods and care should be taken to label the dishes with the date on which they were filled and make sure that reagents are replaced when necessary as described in the next section.

FIGURE 15
Staining dish set-up for dRIT



Note: Image modified from Niezgoda and Rupprecht (2006).
Source: Modified by GARC from the publication Lembo T, Niezgoda M, Velasco-Villa A, Cleaveland S, Ernest E, Rupprecht CE. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. Emerg Infect Dis. 2006 Feb;12(2):310-3. doi: 10.3201/eid1202.050812. PMID: 16494761; PMCID: PMC3294322.

Preparation

Reagent preparation and usage:

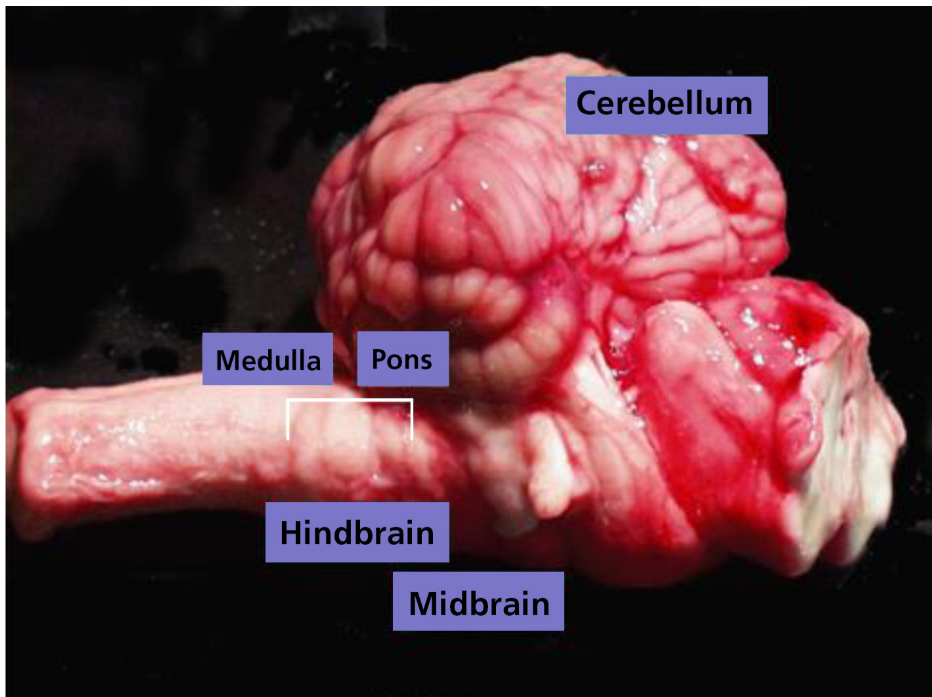
1. Formalin, 10 percent: ready-to-use. [*Change out after two runs or once a week*]
2. TPBS buffer: Add 990 ml 1xPBS and 10 ml Tween 80. Shake well until the Tween 80 is completely dissolved. [*Change out after each run*]
3. 3 percent hydrogen peroxide: ready-to-use. [*Change out after each run*]
4. Biotinylated antibody: apply working dilution as determined by means of a titration series of the stock solution of biotinylated antibody.
5. Streptavidin-peroxidase: ready-to-use.
6. AEC staining kit: The staining kit contains three dropper bottles (vials) filled with the required reagents. Create the working solution immediately before use as follows: *Mix 4 ml distilled water, two drops of vial 1 (acetate buffer, 2.5 M, pH 5.0), one drop of vial 2 (3-amino-9-ethylcarbazole in N,N-dimethylformamide) and one drop of vial 3 (3 percent hydrogen peroxide in distilled water).*
7. Gills formulation #2: The Gills formulation #2 is diluted 1:1 in distilled water [*Change out every week*]
8. Gel/mount: ready-to-use.
9. Distilled water: ready-to-use. [*Change out after each run*]

What sections of the brain should be collected for dRIT diagnosis?

A fresh section of cerebellum or medulla oblongata (or any other available brain tissue) should always be tested.

If available, a cross-section of the brain stem (consisting of either the mid-brain, pons or medulla oblongata) should always be included in the material being tested.

FIGURE 16
Lateral view of whole CNS tissue



Note: Image modified from the Protocol for Post-mortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing (CDC, 2003).

Source: Modified by GARC from the Protocol for Post-mortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing (CDC, 2003). (www.cdc.gov/rabies/pdf/rabiesdfaspv2.pdf). GARC, https://rabiessurveillanceblueprint.org/IMG/pdf/drit_training_manual_4th_edition_final.pdf.

Biological material: deterioration or decomposition

The dRIT diagnostic assay relies on the detection of viral antigens that are present in the CNS tissue. As such, the physical condition of the tissue is very important, with tissue being graded according to the following criteria (Coetzer, Nel and Rupprecht, 2014):

- Good: Tissue is fresh and shows no signs of decomposition.
- Fair: Slight tissue decomposition can be observed. Although slight discoloration might be observed, the tissue still has clearly identifiable anatomical features.
- Poor: The tissue shows signs of discoloration (substantial green colour), liquefaction, desiccation or an unrecognized gross anatomy.

If negative results are obtained from a tissue sample in "poor" condition, the test report should state only that the tissue condition of the sample is such that the test cannot rule out the presence of RABV in the specimen – the sample may thus not be reported as rabies-negative (Coetzer, Nel and Rupprecht, 2014).

Procedure

Performing the diagnostic assay:

1. Create a tissue impression of a fresh section of cerebellum or medulla oblongata (or any other available brain tissue) by lightly pressing a degreased microscope slide on the brain pieces. The slides with tissue impressions are subsequently dried on a piece of absorbent tissue paper and left to dry at room temperature for 5 minutes. *Positive and negative control slides (freshly prepared from stored CNS tissue of known rabies infection status) must be included in every staining session. These control slides should be fixed and stained alongside the samples, using the same reagents.*
2. Immerse the tissue impressions in 10 percent neutral buffered formalin for 10 minutes (Dish I).
3. Remove tissue impressions from the 10 percent neutral buffered formalin and dip rinse slides several times in fresh TPBS buffer (Dish II).
4. Immerse the tissue impressions in 3 percent hydrogen peroxide for 10 minutes (Dish III).
5. Remove excess hydrogen peroxide by dip rinsing the slides in fresh TPBS buffer (Dish IV).
6. Immerse all tissue impressions in fresh TPBS buffer (Dish V). *Work with one slide at a time while leaving the remaining tissue impressions submerged in the TPBS buffer.*
7. Remove one slide from the immersed TPBS buffer and shake off the buffer. After shaking off the buffer, the excess buffer can be blotted from the sides surrounding the tissue impression.
8. Place tissue impression in a humidity chamber (the tissue culture plate lid can be lined with moistened paper towel and used to replace commercially available humidity chambers). Add the biotinylated antibody preparation drop by drop until the entire tissue impression is covered. Repeat steps 7 and 8 for all slides before continuing to the next step.
9. After all of the slides have been prepared, place the lid on the humidity chamber and incubate for 10 minutes at room temperature (the incubation can be done on a bench top in the laboratory).
10. Shake off the biotinylated antibody after incubation and immerse the tissue impressions in TPBS buffer (Dish V; this TPBS buffer can be used until step 16 in the diagnostic process). *Work with one slide at a time while leaving the remaining tissue impressions submerged in the TPBS buffer.*
11. Remove one slide from the immersed TPBS buffer and shake off the buffer. After shaking off the buffer, the excess buffer can be blotted from the sides surrounding the tissue impression.
12. Place tissue impressions in a humidity chamber and add the ready-to-use streptavidin-peroxidase to each tissue impression until it is completely covered. Repeat steps 11 and 12 for all slides before continuing to the next step.
13. After all of the slides have been prepared, place the lid on the humidity chamber and incubate for 10 minutes at room temperature (the incubation can be done on a bench top in the laboratory).

14. Shake off the streptavidin-peroxidase after incubation and immerse the tissue impressions in TPBS buffer (Dish V). *Work with one slide at a time while leaving the remaining tissue impressions submerged in the TPBS buffer.*
15. Remove one slide from the immersed TPBS buffer and shake off the buffer. After shaking off the buffer, the excess buffer can be blotted from the sides surrounding the tissue impression.
16. Place tissue impressions in a humidity chamber and add the working solution of AEC chromogen to each tissue impression until it is completely covered. Repeat steps 15 and 16 for all slides before continuing to the next step.
17. After all of the slides have been prepared, place the lid on the humidity chamber and incubate for 10 minutes at room temperature (the incubation can be done on a bench top in the laboratory).
18. Shake off the excess AEC chromogen after incubation and immerse the tissue impressions in distilled water (Dish VI) for 5 minutes.
19. Immerse tissue impression in counterstain of Gills #2 Hematoxylin (diluted 1:1 with distilled water) for 2 minutes (Dish VII).
20. Immediately remove the excess counterstain from tissue impressions by dip rinsing the slides in distilled water (Dish VIII). Perform a second dip-rinse in fresh distilled water (Dish IX) to ensure that all the excess counterstain is removed from the tissue impression.
21. Immerse the slides in fresh distilled water (Dish X). *Work with one slide at a time.*
22. Shake off the distilled water and blot excess distilled water from the sides surrounding the tissue impression.
23. Mount the slides with a water-soluble mounting medium and cover glass, taking care to ensure that the tissue impressions do not dry out.
24. View the slides by light microscopy. Use a 20x objective to scan the field, and a 40x objective for closer inspection of rose-red inclusion bodies present on the blue neuronal background.
25. Record results.

Reading and recording of results:

Negative results: A sample is considered negative if more than 40 fields are viewed at a magnification of at least 200x and no rose-red inclusion bodies are visible on the blue neuronal background.

Positive results: Both the staining intensity and antigen distribution is based on the presence of the rose-red inclusion bodies than can manifest in the form of round or oval masses of varying sizes.

- *Staining intensity*

  The staining intensity is graded from +1 to +4 and the positive control slides should always contain a tissue impression with a staining intensity of +4. If a slight loss of colour is observed the staining intensity is graded as +3, while noticeably dull stains are graded as +2 or +1.

- *Antigen distribution*
  The antigen distribution is graded by the amount of observed antigen and is scored as follows:
  +4: large numbers of both small and large inclusions of varying shapes are visible in almost every field of the impression.
  +3: numerous inclusions, of varying sizes and shapes, are found in almost every microscopic field.
  +2: between 10 percent and 50 percent of the observed fields contain a few inclusions of varying sizes and shapes.
  +1: very few (usually one or two) inclusions of varying sizes and shapes are visible in less than 10 percent of the observed fields.

Test results are reported if the following observations are made:

Control slides: The positive control slide should contain an accumulation of both large and small rose-red inclusions with a staining intensity of +4 and an antigen distribution of either +3 or +4. No staining should be present on the negative control slide.

Test slides: Samples that are positive should have a staining intensity of +3 or +4 and an antigen distribution of +2 to +4 in slides made from both the brain stem and cerebellum. Samples that are negative should have no specific staining whatsoever.

Other techniques for priority diseases

This section provides protocols for different techniques, including culturing.

ANTHRAX

Identification of the agent by culturing

Demonstration of encapsulated *Bacillus anthracis* in smears of blood or tissues from fresh anthrax-infected carcasses and growth of the organism on blood agar plates is relatively uncomplicated and within the capability of most bacteriology laboratories. Difficulty may be encountered in the case of pigs and carnivores in which the terminal bacteraemia is frequently not marked, or in animals that received antibiotics before death.

Recovery of *Bacillus anthracis* from old, decomposed carcasses, processed specimens (bone meal, hides) or environmental samples (contaminated soil) is often difficult, requiring demanding and labour-intensive procedures. However live spores may be recovered from the turbinate bones of dead livestock and wildlife for an extended period after death.

For any further information about biosafety requirements, please refer to Chapter 3.1.1 of the WOAH Manual, *Anthrax* (WOAH, 2022).

Culture on fresh specimen

Bacillus anthracis grows readily on most types of nutrient agar, however, 5–7 percent horse or sheep blood agar is the diagnostic medium of choice.

Samples: Blood is the primary clinical material to examine.

Swabs of blood, other body fluids, or swabs taken from incisions in tissues or organs can be spread over blood agar plates.

N.B.: To avoid environmental contamination, post-mortem examinations of the carcasses of animals suspected to have died of anthrax are discouraged.

Results: After overnight incubation at 37 °C, *Bacillus anthracis* colonies are grey–white to white, 0.3–0.5 cm in diameter, non-haemolytic, with a ground-glass surface, and very tacky when teased with an inoculating loop. Tailing and prominent wisps of growth trailing back towards the parent colony, all in the same direction, are sometimes seen. This characteristic has been described as a "medusa head" or "curled hair" appearance.

Confirmation: Confirmation of *Bacillus anthracis* should be accomplished by the demonstration of a capsulated, spore-forming, gram-positive rod in blood culture. Absence of motility is an additional test that can be done.

BOVINE TUBERCULOSIS

Bovine tuberculosis is a chronic bacterial disease of animals and humans caused by *Mycobacterium bovis*. In a large number of countries, bovine tuberculosis is a major infectious disease among cattle, other domesticated animals and certain wildlife populations.

Bovine tuberculosis infection in cattle is usually diagnosed in live animals on the basis of **delayed hypersensitivity reactions**. This test is the standard method for detecting bovine tuberculosis. It involves measuring skin thickness, injecting bovine tuberculin intradermally into the measured area, and measuring any subsequent swelling at the site of injection 72 hours later.

After death, infection is diagnosed by necropsy and histopathological and bacteriological techniques. Rapid nucleic acid methodologies, such as the PCR, may also be used, although these are demanding techniques and should only be employed when appropriately validated.

N.B.: The new real-time RT-PCR is now available in Chapter 3.1.13 of the WOAH Manual, *Mammalian tuberculosis* (WOAH, 2022).

Traditional mycobacterial culture remains the gold standard method for routine confirmation of infection, but such techniques require biosafety level 3 conditions.

For any technical assistance, please contact the WOAH Reference Laboratory for Bovine Tuberculosis; María Laura Boschiroli-Cara, Bacterial Zoononses Unit, Animal Health Laboratory, ANSES: Maria-laura.boschiroli@anses.fr.

CRIMEAN–CONGO HAEMORRHAGIC FEVER

Crimean–Congo haemorrhagic fever virus (CCHFV) of the genus *Nairovirus* of the family Bunyaviridae causes a zoonotic disease in many countries of Asia, Africa, the Near East and southeastern Europe. As the distribution of CCHFV coincides with the distribution of its main vector, ticks of the genus *Hyalomma*, the spread of infected ticks into new, unaffected areas facilitates the spread of the virus.

The virus has a single-stranded, negative-sense RNA genome consisting of three segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The virus can be isolated from serum or plasma samples collected during the febrile or viraemic stage of infection, or from the liver of infected animals.

For testing animals for viraemia, as well as in human clinical diagnosis, a rapid diagnosis can be achieved by the detection of viral nucleic acid in serum or plasma using conventional (Burt *et al.*, 1998) or real-time RT-PCR (Drosten *et al.*, 2002; Duh *et al.*, 2006; Wölfel *et al.*, 2007), or by demonstration of viral antigen (Shepherd *et al.*, 1988). Specimens to be submitted for laboratory confirmation of CCHFV include blood and liver samples. Because of the risk of laboratory-acquired infections, work with CCHFV should be conducted in appropriate biosafety facilities.

For more information, please refer to Chapter 3.1.5 of the WOAH Manual, *Crimean–Congo haemorrhagic fever* (WOAH, 2022).

LASSA FEVER VIRUS

The Lassa fever virus (LASV) is a single-strand RNA virus of the Arenaviridae family. It is endemic in West Africa, including Nigeria, Sierra Leone, Guinea, Liberia, Benin, Ghana and Mali.

Reservoir: *Mastomys natalensis* mutimammate rodents are the most common rodents across the African continent, found predominantly in rural areas and human dwellings. The rodents show persistent LASV infection but are largely unaffected by the disease and shed the virus in their excrement.

The LASV belongs to risk group 4, i.e. agents that are likely to cause serious or lethal human disease and for which preventive or therapeutic interventions are not usually available (high individual risk and high community risk).

The collection, storage and handling of LASV specimens require biosafety level precautions similar to those used for the Ebola virus. Biosafety level 4 precautions are recommended when handling specimens which may contain infectious Lassa virus.

Various techniques are available for the diagnosis of Lassa fever in humans, including viral culture, antigen and antibody detection assays, and nucleic acid detection methods. Although viral culture is the gold standard for diagnosis, its use is limited by the non-availability of biosafety level 4 facilities. Antigen and antibody detection assays may provide a rapid diagnosis, but there are high numbers of false positive results once the antigenemia period has resolved in most patients. Thus, nucleic acid-based assays such as the RT-PCR remains the available diagnostic method, and can be carried out using specimens that have been inactivated in a glove box and analysed in biosafety level 2 facilities.

None of the RT-PCR protocols have been validated on animal samples. However, some studies have been done and published. See Mariën *et al.* (2017), Fichet-Calvet *et al.* (2007, 2016), Vieth *et al.* (2007) and Olschläger *et al.* (2010).

Please note that according to WHO, mitigation of human and vector (rats) interaction needs to be prioritized to decrease the recurrence of Lassa fever outbreaks. To achieve this requires effective social mobilization and community engagement strategies targeting vector control and environmental management, especially in the endemic area.

For any further information, please contact the **CDC**, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of High-Consequence Pathogens and Pathology (DHCPP).

SALMONELLOSIS

Salmonellosis is an infectious disease of humans and animals caused by bacteria of the genus *Salmonella*. Salmonellae are aetiological agents of diarrhoeal and systemic infections. They often cause subclinical infections and may be shed in large numbers within the faeces of clinical cases and carrier animals, resulting in contamination of the environment.

The disease can affect all species of domestic animals; young animals and pregnant and lactating animals are the most susceptible. Enteric disease is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis and respiratory disease, may be seen. Many animals, especially pigs and poultry, may be infected but show no clinical illness. Such animals may be important in relation to the spread of infection between flocks and herds, and as sources of food contamination and human infection.

Diagnosis is based on the isolation of the organism either from tissues collected aseptically at necropsy or from faeces, rectal swabs or environmental samples, food products and feedstuffs; prior or current infection of animals by some serovars may also be diagnosed serologically. When infection of the reproductive organs or abortion occurs, it is necessary to culture foetal stomach contents, placenta and vaginal swabs and, in the case of poultry, embryonated eggs.

Several protocols are available, but a preliminary study of the context is fundamental.

For any technical support, please contact the **WOAH Reference Laboratory for Salmonellosis:** Antonia Anna Lettini, IZSVe: alettini@izsvenezie.it.

References

Biotium. n.d. *Safety Report for GelRed® and GelGreen®.* Fremont, United States. https://biotium.com/wp-content/uploads/2013/07/GelRed-and-GelGreen-Safety-Report.pdf

Bird, B.H., Bawiec, D.A., Ksiazek, T.G., Shoemaker, T.R. & Nichol, S.T. 2007a. Highly sensitive and broadly reactive quantitative reverse transcription–PCR assay for high-throughput detection of Rift Valley fever virus. *J. Clin Microbiol.*, 45(11):3506–3513. doi: 10.1128/JCM.00936-07

Bird, B.H., Khristova, M.L., Rollin, P.E., Ksiazek, T.G. & Nichol S.T. 2007b. Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. *J. Virol.*, 81(6):2805–2816. doi: 10.1128/JVI.02095-06

Bounaadja, L., Albert, D., Chénais, B., Hénault, S., Zygmunt, M.S., Poliak, S. & Garin-Bastuji, B. 2009. Real-time PCR for identification of Brucella spp.: a comparative study of IS711, bcsp31 and per target genes. *Vet. Microbiol.*, 137(1–2):156–164. doi: 10.1016/j.vetmic.2008.12.023

CDC. 2003. Protocol for post-mortem diagnosis of rabies in animals by direct fluorescent antibody testing. In: *Centers for Disease Control and Prevention.* Washington, DC. Cited 27 September 2016. www.cdc.gov/rabies/pdf/rabiesdfaspv2.pdf

Coetzer, A., Nel, L.H. & Rupprecht, C.E. 2014. Demonstration of lyssavirus antigens by a direct rapid immunohistochemical test. In: C.E. Rupprecht & J. Nagarajan, eds. *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention*, pp. 27–36. San Diego, USA, Elsevier Ltd. doi:10.1016/B978-0-12-800014-4.00002-0

Corman, V.M., Müller, M.A., Costabel, U., Timm, J., Binger, T., Meyer, B., Kreher, P., Lattwein, E., Eschbach-Bludau, M., Nitsche, A., Bleicker, T., Landt, O., Schweiger, B., Drexler, J.F., Osterhaus, A.D., Haagmans, B.L., Dittmer, U., Bonin, F., Wolff, T. & Drosten, C. 2012. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. *Euro. Surveill*. 17(49):20334. doi: 10.2807/ese.17.49.20334-en

De Benedictis, P., De Battisti, C., Dacheux, L., Marciano, S., Ormelli, S., Salomoni, A., Caenazzo, S.T., Lepelletier, A., Bourhy, H., Capua, I. & Cattoli, G. 2011. Lyssavirus detection and typing using pyrosequencing. *J. Clin. Microbiol.*, 49(5):1932–1938. doi: 10.1128/JCM.02015-10

Del Amo, J., Sotelo, E., Fernández-Pinero, J., Gallardo, C., Llorente, F., Agüero, M. & Jiménez-Clavero, M.A. 2013. A novel quantitative multiplex real-time RT-PCR for the simultaneous detection and differentiation of West Nile virus lineages 1 and 2, and of Usutu virus. *J. Virol Methods.*, 189(2):321–327. doi: 10.1016/j.jviromet.2013.02.019

Drosten C., Göttig S., Schilling S., Asper M., Panning M., Schmitz H. & Gunther S. 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean–Congo haemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. *J. Clin. Microbiol.*, 40(7):2323–2330. doi: 10.1128/JCM.40.7.2323-2330.2002

Duh D., Saksida A., Petrovec M., Dedushaj I. & Avsic-Zupanc T. 2006. Novel one-step real-time RT-PCR assay for rapid and specific diagnosis of Crimean–Congo hemorrhagic fever encountered in the Balkans. *J. Virol. Methods*, 133:175–179. doi: 10.1016/j.jviromet.2005.11.006

Ellerbrok, H., Nattermann, H., Ozel, M., Beutin, L., Appel, B. & Pauli, G. 2002. Rapid and sensitive identification of pathogenic and apathogenic Bacillus anthracis by real-time PCR. *FEMS Microbiol Lett.*, 214(1):51–59. doi: 10.1111/j.1574-6968.2002.tb11324.x.

FAO. 2018. *FAO Biosafety Primer*. Bangkok. www.fao.org/3/i6800en/I6800EN.pdf

Fekadu, M., Greer, P.W., Chandler, F.W. & Sanderlin, D.W. 1988. Use of the avidin-biotin peroxidase system to detect rabies antigen in formalin-fixed paraffin-embedded tissues. *J. Virol Methods.*, 19(2): 91–96. doi: 10.1016/0166-0934(88)90152-8

Fichet-Calvet, E., Lecompte, E., Koivogui, L., Soropogui, B., Doré, A., Kourouma, F., Sylla, O., Daffis, S., Koulémou, K. & ter Meulen, J. 2007. Fluctuation of abundance and Lassa virus prevalence in Mastomys natalensis in Guinea, West Africa. *Vector Borne Zoonotic Dis.*, 7(2):119–128. doi: 10.1089/vbz.2006.0520

Fichet-Calvet, E., Ölschläger, S., Strecker, T., Koivogui, L., Becker-Ziaja, B., Camara, A.B., Soropogui, B., Magassouba, N. & Günther, S. 2016. Spatial and temporal evolution of Lassa virus in the natural host population in Upper Guinea. *Sci Rep.*, 6:21977. doi: 10.1038/srep21977

Hassan, K.E., Ahrens, A.K., Ali, A., El-Kady, M.F., Hafez, H.M., Mettenleiter, T.C., Beer, M. & Harder, T. 2022. Improved subtyping of avian influenza viruses using an RT-qPCR-based low density array: 'Riems Influenza a Typing Array', version 2 (RITA-2). *J. Viruses,* 14(2):415. doi: 10.3390/v14020415

Heine, H.G., Foord, A.J., Wang, J., Valdeter, S., Walker, S., Morrissy, C., Wong, F.Y.K., & Meehan, B. 2015. Detection of highly pathogenic zoonotic influenza virus H5N6 by reverse-transcriptase quantitative polymerase chain reaction. *Virol J. 12:18.* doi: 10.1186/s12985-015-0250-3

Hoffmann, B., Hoffman, D., Henritzi, D., Beer, M. & Harder, T.C. 2016. Riems influenza A typing array (RITA): An RT-qPCR-based low density array for subtyping avian and mammalian influenza A viruses. *Sci. Rep.*, 6, 27211. doi: 10.1038/srep27211

Lembo, T., Niezgoda, M., Velasco-Villa, A., Cleaveland, S., Ernest, E. & Rupprecht, C.E. 2006. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. Emerg Infect Dis. Feb;12(2):310-3.doi: 10.3201/eid1202.050812. PMID: 16494761; PMCID: PMC3294322.

Mariën, J., Borremans, B., Gryseels, S., Soropogui, B., De Bruyn, L., Bongo, G.N., Becker-Ziaja, B., de Bellocq, J.G., Günther, S., Magassouba, N., Leirs, H. & Fichet-Calvet, E. 2017. No measurable adverse effects of Lassa, Morogoro and Gairo arenaviruses on their rodent reservoir host in natural conditions. *Parasit. Vectors.*, 10(210):1–11. doi: 10.1186/s13071-017-2146-0.

Nagy, A., Černíková, L., Kunteová, K., Dirbáková, Z., Thomas, S.S., Slomka, M.J., Dán, A., Varga, T., Máté, M., Jiřincová, H. & Brown, I.H. 2021. A universal RT-qPCR assay for "One Health" detection of influenza A viruses. *PloS One* 16(1):e0244669. doi: 10.1371/journal.pone.0244669

Niezgoda, M. & Rupprecht, C.E. 2006. *Standard operating procedure for the direct, rapid immunohistochemistry test for the detection of rabies virus antigen.* National Laboratory Training Network Course. Atlanta: United States Department of Health and Human Services, Centers for Disease Control and Prevention.

Olschläger, S., Lelke, M., Emmerich, P., Panning, M., Drosten, C., Hass, M., Asogun, D., Ehichioya, D., Omilabu, S. & Günther, S. 2010. Improved detection of Lassa virus by reverse transcription-PCR targeting the 5**'** region of S RNA. *J. Clin. Microbiol.* 48(6):2009–2013. doi: 10.1128/JCM.02351-09

Panzarin, V., Marciano, S., Fortin, A., Brian, I., D'Amico, V., Gobbo, F., Bonfante, F., Palumbo, E., Sakoda, Y., Le, K.T., Chu, D.-H., Shittu, I., Meseko, C., Haido, A.M., Odoom, T., Diouf, M.N., Djegui, F., Steensels, M., Terregino, C. & Monne, I. 2022. Redesign and validation of a real-time RT-PCR to improve surveillance for avian influenza viruses of the H9 subtype. *J. Viruses* 14(6):1263. doi: 10.3390/v14061263.

Shepherd, A.J., Swanepoel, R., Leman, P.A. & Shepherd, S.P. 1986. Comparison of methods for isolation and titration of Crimean-Congo hemorrhagic fever virus. *J. Clin. Microbiol.*, 24(4):654–656. doi: 10.1128/jcm.24.4.654-656.1986

Slomka, M. J., Pavlidis, T., Banks, J., Shell, W., McNally, A., Essen, S. & Brown, I. H. 2007a. Validated H5 Eurasian real-time reverse transcriptase–polymerase chain reaction and its application in H5N1 outbreaks in 2005–2006. *Avian Dis.*, 51:373–377. doi: 10.1637/7664-060906R1.1

Slomka, M.J., Coward, V.J., Banks, J., Löndt, B.Z., Brown, I.H., Voermans, J., Koch, G., Handberg, K.J., Jørgensen, P.H., Cherbonnel-Pansart, M., Jestin, V., Cattoli, G., Capua, I., Ejdersund, A., Thorén, P. & Czifra, G. 2007b. Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union. *Avian Dis.*, 51(1 Suppl):227–234. doi: 10.1637/7674-063006R1.1.

Slomka, M.J., Pavlidis, T., Coward, V.J., Voermans, J., Koch, G., Hanna, A., Banks, J. & Brown, I.H. 2009. Validated real-time reverse transcriptase PCR methods for the diagnosis and pathotyping of Eurasian H7 avian influenza viruses. *Influenza Other Respir. Viruses*, 3(4):151–164. doi: 10.1111/j.1750-2659.2009.00083.x

Vasquez, A., Herrero, L., Negredo, A., Hernández, L., Sánchez-Seco, M.P. & Tenorio, A. 2016. Real time PCR assay for detection of all known lineages of West Nile virus. *J. Virol Methods.*, 236:266–270. doi: 10.1016/j.jviromet.2016.07.026

Vieth, S., Drosten, C., Lenz, O., Vincent, M., Omilabu, S., Hass, M., Becker-Ziaja B., ter Meulen, J., Nichol, S.T., Schmitz, H. & Günther, S. 2007. RT-PCR assay for detection of Lassa virus and related Old World arenaviruses targeting the L gene. *Trans. R. Soc. Trop. Med Hyg.*, 101:1253–1264. doi: 10.1016/j.trstmh.2005.03.018

WOAH. 2022. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.* Paris. www.woah.org/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access

Wölfel, R., Paweska, J.T., Petersen, N., Grobbelaar, A.G., Leman, P.A., Hewson, R., Georges-Courbot, M., Papa, A., Günter, S. & Drosten C. 2007. Virus detection and monitoring of viral load in Crimean-Congo hemorrhagic fever virus patients. *Emerg. Infect. Dis.,* 13(7):1097–1100. doi: 10.3201/eid1307.070068

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Animal health laboratories play a crucial role in veterinary disease diagnosis and surveillance for the prevention and control of transboundary animal diseases, including those of economic impact or zoonotic threat with potential pandemic risk. Enhancing the diagnostic capacity of laboratories is an essential pillar for generating accurate data, particularly in settings where fundamental gaps and capacity constraints may prevent the early, rapid and reliable detection of many animal diseases. Importantly, the constant evolution of pathogens coupled with the rise of technology and assay development calls for ongoing guidance on current protocols and techniques. This is also particularly essential to ensure the use of carefully developed and validated tests.

This compendium of protocols aims to contribute to capacity-building efforts for sustainable and reliable functioning of animal health laboratories in Member States. It provides a practical and pragmatic resource for novel or updated validated diagnostic techniques to be introduced in accordance with a sustainable quality management system to ensure specific, accurate and reproducible results. The selected diseases included in this document are the most common priority zoonotic diseases (PZDs) identified in Africa. For each selected disease, the diagnostic protocols are explained, focusing on assays that have been tested and validated by reference laboratories to ensure their fitness for purpose.

Laboratories around the world may use this compendium of protocols as guidance to update, enhance or expand their diagnostic assays.

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