ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 151

DETECTION OF AVIAN ORTHOAVULAVIRUS TYPE 1 (AOAV-1) BY REAL-TIME RT-PCR

(Sutton et al., 2019)

This protocol is a copy of the standard operating procedure used by the EURL for AI and ND at the Istituto Zooprofilattico Sperimentale delle Venezie. Released on 09/04/21.

Contents

1. Purpose and field of application

This protocol describes the procedure to detect avian orthoavulavirus 1 (AOAV-1) in biological samples by real-time reverse transcription polymerase chain reaction (RRT-PCR).

The protocol targets L gene encoding the polymerase of any AOAV-1 genotype, based on the assay developed by Sutton et al. (2019). Modifications to the original method pertain: addition of an exogenous internal control, amplification kit and cycling conditions, lack of ROX passive dye, real-time PCR apparatus and diagnostic cut-off.

The procedure can be applied to RNA purified from AOAV-1 isolates (normally allantoic fluid), tissue homogenates, stool, tracheal and cloacal swabs properly collected and preserved.

2. References

- D.A. Sutton, D.P. Allen, C.M. Fuller, J. Mayers, B.C. Mollett, B.Z. Londt, S.M. Reid, K.L. Mansfield, I.H. Brown. Development of an avian avulavirus 1 (AAvV-1) L-gene real-time RT-PCR assay using minor groove binding probes for application as a routine diagnostic tool. J Virol Methods 265:9-14, 2019. doi: 10.1016/j.jviromet.2018.12.001;
- B. Hoffmann, K. Depner, H. Schirrmeier, M. Beer. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods 136(1-2):200-9, 2006. doi: 10.1016/j.jviromet.2006.05.020;
- OIE - World Organization for Animal Health, Terrestrial Manual, Chapter 3.3.14. Newcastle disease (Version adopted in May 2012);
- SOP VIR 1000 - Sample preparation and nucleic acids isolation for the detection and typing of Avian influenza virus and Avian Orthoavulavirus type 1 by molecular methods;
- IZSVe PDP VIR 151.

3. Safety

Individual laboratories are responsible for ensuring that all the procedures described in this document are conducted under high safety standards, including awareness on chemical and biological risks. According to the risk hazard, either BSL2 or BSL3 facilities must be used. Safety rules at individual laboratories must be agreed with the biosecurity and biosafety officer and acknowledged by all the staff members involved.

4. Materials

4.1. Reagents

For commercial products, refer to the manufacturer's instructions for storage conditions.

- Nuclease-free water;
- Oligonucleotides resuspension buffer (e.g. TE pH 8.0);
- Intype IC-RNA (Indical Bioscience) (required for the nucleic acids isolation phase);
- QuantiTect Multiplex RT-PCR Kit (Qiagen);
- Sense primer NDF 5'-GAG CTA ATG AAC ATT CTT TC-3';

- Antisense primer NDR 5'-AAT AGG CGG ACC ACA TCT G-3';
- Probe LproMGB 5'-FAM-CCA ATC AAC TTC CC-MGB-3';
- Probe LproMGB2 5'-VIC-AAT AGT GTA TGA CAA CAC-MGB-3';
- Sense primer EGFP-11-F 5'-CAG CCA CAA CGT CTA TAT CAT G-3';
- Antisense primer EGFP-2-R 5'-GAA CTC CAG CAG GAC CAT G-3';
- Probe EGFP-Cy5 5'-Cy5-AGC ACC CAG TCC GCC CTG AGC A-BHQ2-3'.

4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- RotorGene Q (Qiagen), RotorGene 6000 (Corbett) (or equivalent real-time PCR platform).

5. Procedure

5.1. Controls

To ensure test reliability, the controls listed in Table 1 must be included in each run.

Control Type	Definition
Internal control (IC)	Non target RNA sequence contained in each sample, and subject to the whole analytical process, starting from the nucleic acids isolation phase
Negative process control (NPC)	Sample containing no target organism that is processed along with the samples starting from the nucleic acids isolation phase
Positive template control (amplification control, PTC)	Sample containing a known amount of the target RNA of AOAV-1 and stored at ≤ -70℃, that is processed along with the samples star ting from the RRT-PCR phase
Negative template control (NTC)	Sample containing all PCR reagents but no target RNA, that is processed along with the samples starting from the RRT-PCR phase

Table 1. Controls to ensure test reliability

The use of the internal control (IC) is usually recommended to prevent false negative results arising from PCR inhibition or failure in nucleic acids extraction. Whenever the internal control cannot be adopted, a positive process control (PPC) (i.e. sample containing the target organism that is processed along with the samples starting from the nucleic acids isolation phase) should be used instead to assess the reliability of the analytical process.

5.2. Preparation of samples and storage

For samples preparation (allantoic fluid, tissues, stool, tracheal and cloacal swabs), see SOP VIR 1000.

Clinical samples and viral isolates must be stored at refrigerated temperature (2-8℃) until the compl etion of the analysis. For long term conservation, store samples at ≤ -70℃.

Purified nucleic acids can be refrigerated for a few hours prior RRT-PCR, otherwise they must be stored at ≤ -70℃.

5.3. Isolation of nucleic acids

RNA can be isolated either by manual or automatic methods.

The IZSVe validated the following nucleic acids isolation systems:

- NucleoSpin RNA kit (Macherey-Nagel);
- QIAamp Viral RNA Mini Kit (Qiagen);
- QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen) on the QIAsymphony SP instrument (Qiagen);
- MagMAX Pathogen RNA/DNA Kit (Applied Biosystems) on the KingFisher Flex Magnetic Particle Processor (Thermofisher Scientific).

The intype IC-RNA must be added to each sample (including the NPC) during the lysis phase. The volume of intype IC-RNA to be spiked into each sample should be 1:10 of the elution volume, as recommended by the manufacturer. For additional details on the nucleic acids isolation procedures, see SOP VIR 1000.

5.4. Real-time RT-PCR

The preparation of the master mix for the RRT-PCR reaction has to be carried out in a clean dedicated area, according to the specifications reported in Tables 2 and 3. The reagents should be kept refrigerated throughout the entire preparation phase. For the whole procedure, the use of filter tips and nuclease-free plastics is recommended.

IC-RNA assay pre-mix preparation

The IC-RNA oligonucleotides pre-mix can be prepared in advance and stored until use. The volumes reported in Table 2 are sufficient for approximately 100 RRT-PCR reactions.

Component	Initial concentration	Final concentration	µl per reaction
TE pH 8.0	-	-	186.25
Sense primer EGFP-11-F	100 µM	2.5 µM	5
Antisense primer EGFP-2-R	100 µM	2.5 µM	5
Probe EGFP-Cy5	100 µM	1.8 µM	3.75
Total reaction volume			200

Table 2. Volume and concentration of the IC-RNA pre-mix components

Reaction mix preparation

Prepare a master mix volume sufficient for the number of samples to be tested in each run. The volumes reported in Table 3 are per single reaction.

Component	Initial concentration	Final concentration	µl per reaction
Nuclease-free water	-	-	1.25
2X QuantiTect Multiplex RT-PCR Buffer	2X	1X	12.5
Sense primer NDF	12.5 µM	0.5 µM	1

Antisense primer NDR	12.5 µM	0.5 µM	1
Probe LproMGB	5 µM	0.2 µM	1
Probe LproMGB2	5 µM	0.2 µM	1
IC-RNA assay pre-mix	-	-	2
QuantiTect Multiplex RT Mix	-	-	0.25
Master mix minus template			20
Template			5
Total reaction volume			25

Table 3. Volume and concentration of the reaction mix components. If the internal control is not used, replace the IC-RNA assay premix volume with 2 µl of nuclease-free water

The master mix should be mixed thoroughly and 20 µl solution per sample must be pipetted in real-time PCR tubes.

Template (5 µl) must be added in a separate room. To minimize the risk of cross-contamination, it is recommended to add controls and samples in the following order: NTC, NPC, nucleic acids isolated from diagnostic samples, PTC. In case of NTC, 5 µl of nuclease-free water should be added.

Cycling conditions

Place the tubes in the real-time PCR apparatus. Set up the loading scheme as well as the thermal profile as per Table 4, and select Cy5, FAM and VIC compatible detection channels for fluorescence acquisition.

Step	Temperature and time	Repetitions
Reverse transcription	50℃ for 20 min	1
PCR initial activation step	95℃ for 15 min	1
Denaturation	94℃ for 45 sec	40
Annealing/extension (*)	50℃ for 45 sec	

Table 4. Real-time RT-PCR thermal profile. (*) Fluorescence acquisition has to be done during the annealing/extension phase

5.5. Data analysis

Upon completion of the amplification reaction, amplification plots must be critically assessed. The baseline and the threshold can be set either automatically or manually. The threshold should be placed above the background fluorescence noise, across the exponential phase of all the amplification curves (corresponding to the early linear phase of the logarithmic view). The use of a PTC with a known target concentration can act as a calibrator to enable standardization of data analysis and an approximate estimation of the viral load.

6. Interpretation of results

6.1. Reliability of controls

Test reliability is assured if the controls yield the expected results, as reported in Table 5. In case of invalid results, the causative reason must be investigated and proper actions have to be taken.

Control	Expected Result	Action in case of invalid control
IC	Positive, i.e. increase in fluorescence from the Cy5 fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Ct ≤ 30 in all samples, including NPC	If an invalid IC result (negative or with Ct > 30) is associated with a negative/dubious AOAV-1 sample, to rule out inhibition issues dilute the purified nucleic acids 1:10 with nuclease-free water and repeat the analysis from the RRT-PCR; in case of recurrent invalid IC, repeat the analysis from the nucleic acids extraction, further diluting the original biological sample (SOP VIR 1000). Notably, an invalid IC can occur in positive AOAV-1 samples, typically characterized by a high viral load, as a result of competition for reagents during the duplex amplification reaction. In this case, the sample can be considered conforming and no action has to be taken
PPC (if used)	Positive, i.e. increase in fluorescence from either of the probe fluorophores (FAM and/or VIC) yielding a sigmoidal (or logarithmic) amplification curve at the expected Ct value	Repeat the analysis from the nucleic acids extraction and check the PPC stock
NPC	Negative, i.e. absence of fluorescence increase from either of the probe fluorophores (FAM and VIC), with no sigmoidal (or logarithmic) amplification curve	Repeat the analysis from the nucleic acids extraction and check nucleic acids isolation reagents
PTC	Positive, i.e. increase in fluorescence from either of the probe fluorophores (FAM and/or VIC) yielding a sigmoidal (or logarithmic) amplification curve at the expected Ct value	Repeat the analysis from the RRT-PCR and check the PTC stock
NTC	Negative, i.e. absence of fluorescence increase from the Cy5, FAM and VIC fluorophores, with no sigmoidal (or logarithmic) amplification curve	Repeat the analysis from the RRT-PCR and check RRT-PCR reagents

Table 5. Assessment of test reliability. Ct = threshold cycle

6.2. Diagnostic samples

Criteria for data interpretation of diagnostic samples and subsequent actions are reported in Table 6.

Result	Interpretation and action
Increase in fluorescence from either of the probe fluorophores (FAM and/or VIC) yielding a sigmoidal (or logarithmic) amplification curve with Ct ≤ 35, associated with any Ct value for the IC	Positive. The fluorophore yielding the lower Ct value has to be used for results reporting. Proceed with downstream molecular and/or virological analyses for AOAV-1
Negative, i.e. absence of fluorescence increase from either of the probe fluorophores (FAM and VIC), with no sigmoidal (or logarithmic) amplification curve, associated with a Ct value ≤ 30 for the IC	Negative. No action has to be taken
Weak increase in fluorescence from either of the probe fluorophores (FAM and VIC) yielding a sigmoidal (or logarithmic) amplification curve with Ct between 35 and 40, associated with a Ct value ≤ 30 for the IC	Dubious. Repeat the analysis from the nucleic acids extraction starting from a new aliquot of the sample together with the original extract. Alternatively, another screening method can be applied to the nucleic acids extracts for confirmatory diagnosis. In case of recurrent dubious results, it is responsibility of the Head of the Laboratory to identify the actions to be taken

Table 6. Interpretation of real-time RT-PCR diagnostic results

7. Characteristics of the method

This method was validated and accredited at the IZSVe according to the ISO/IEC 17025, employing AOAV-1 samples, selected avian viruses and bacteria available at the IZSVe repository, as well as materials, equipment and procedures as described above. The validation dossier is accessible upon request by contacting eurl.ai.nd.secretariat@izsvenezie.it

Any modification to this SOP by third party laboratories should be supported by proper validation data assessing that the method is still fit-for-purpose.