ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 014

DETECTION OF H9 AVIAN INFLUENZA VIRUS BY REAL-TIME RT-PCR (Monne et al., 2008)

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 07/04/2021.

Contents

1. Purpose and field of application

This protocol describes the procedure to detect H9 avian influenza virus (AIV) in biological samples by real-time reverse transcription polymerase chain reaction (RRT-PCR).

The protocol targets the segment 4 region encoding the HA2 subunit of the H9 subtype, based on the assay developed by Monne et al. (2008). Modifications to the original method pertain: de-multiplexing of the RRT-PCR to detect the H9 subtype only.

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

2. References

- I. Monne, S. Ormelli, A. Salviato, C. De Battisti, F. Bettini, A. Salomoni, A. Drago, B. Zecchin, I. Capua, G. Cattoli. Development and validation of a one-step real-time PCR assay for simultaneous detection of subtype H5, H7, and H9 avian influenza viruses. J Clin Microbiol 46(5):1769-73, 2008. doi: 10.1128/JCM.02204-07;
- Commission Decision 2006/437/EC approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC;
- OIE - World Organization for Animal Health, Terrestrial Manual, Chapter 3.3.4. Avian influenza (Version adopted in May 2015);
- 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 014.

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);
- QuantiTect Multiplex RT-PCR Kit (Qiagen);
- Sense primer H9-For 5'-ATG GGG TTT GCT GCC-3';
- Antisense primer H9-Rev 5'-TTA TAT ACA AAT GTT GCA YCT G-3';
- Probe H9 5'-FAM-TTC TGG GCC ATG TCC AAT GG-TAMRA-3'.

4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- CFX96 Touch Deep Well Real-time PCR Detection System (Biorad), 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
Positive process control (PPC)	Sample containing the target organism that is processed along with the samples 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 the AIV H9 subtype and stored at ≤ -70℃, that is processed along with the samples st arting 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

This procedure is applied as a downstream method to samples which have tested positive for AI by a different molecular diagnostic assay. In case this procedure is applied as a frontline method from a new sample aliquot, 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) can be used 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).

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 Table 2. 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.

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 2 are per single reaction.

Component	Initial concentration	Final concentration	µl per reaction
Nuclease-free water	-	-	0.55
2X QuantiTect Multiplex RT-PCR Buffer	2X	1X	12.5
Sense primer H9-For	5 µM	0.3 µM	1.5
Antisense primer H9-Rev	5 µM	0.3 µM	1.5
Probe H9	1 µM	0.15 µM	3.75
QuantiTect Multiplex RT Mix	-	-	0.2
Master mix minus template			20
Template			5
Total reaction volume			25

Table 2. Volume and concentration of the reaction mix components

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

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, PPC, PTC. In case of NTC, 5 µl of nuclease-free water should be added.

Cycling conditions

Place the tube/strip/plate in the real-time PCR apparatus. Set up the loading scheme as well as the thermal profile as per Table 3, and select a FAM compatible detection channel 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 (*)	54℃ for 45 sec	

Table 3. 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 4. 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
PPC (if used)	Positive, i.e. increase in fluorescence from the FAM fluorophore 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 the FAM fluorophore, 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 the FAM fluorophore 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 FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve	Repeat the analysis from the RRT-PCR and check RRT-PCR reagents

Table 4. 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 5.

Result	Interpretation and action
Increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Ct ≤ 35	Positive. Proceed with downstream molecular and/or virological analyses for H9 characterization
Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve	Negative. No action has to be taken
Weak increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Ct between 35 and 40	Dubious. Repeat the analysis from the nucleic acids extraction starting from a new aliquot of the sample together with the original extract. Alternatively, another confirmatory method can be applied to the nucleic acids extracts. If PCR inhibition is suspected, dilute the purified nucleic acids 1:10 with nuclease-free water and repeat the analysis from the RRT-PCR and/or repeat the analysis from the nucleic acids extraction, further diluting the original biological sample (SOP VIR 1000). In case of recurrent dubious results, it is responsibility of the Head of the Laboratory to identify the actions to be taken

Table 5. Interpretation of real-time RT-PCR diagnostic results

7. Characteristics of the method

This method was validated at the IZSVe according to the ISO/IEC 17025, employing AIV 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

The method usually yields positive results for samples with Ct values < 35 by M-gene real time RT-PCR. However, sensitivity can be strain-dependent and might be affected by poor RNA quality, low viral load and the presence of PCR inhibitors. A sub-optimal amplification was observed for recent strains from Africa and the Middle East. The use of a degenerate probe (i.e. 5'-FAM-TTC TGG GCY ATG TCC AAT GG-TAMRA-3') might increase assay performances and allow an improved recognition of these strains. However, the degenerate probe lacks full validation.

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.