ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 126

DETECTION OF EURASIAN H7 AVIAN INFLUENZA VIRUS BY ONE-STEP RT-PCR AND SANGER SEQUENCING OF THE HEMAGGLUTININ CLEAVAGE SITE

(Slomka et al., 2007)

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 Eurasian H7 avian influenza virus (AIV) in biological samples by reverse transcription polymerase chain reaction (RT-PCR), and to determine the AIV pathotype based on the sequence analysis of the hemagglutinin (HA) gene cleavage site.

The protocol targets the segment 4 region encoding the HA2 subunit of the H7 subtype, based on the assay developed by Slomka et al. (2007). Modifications to the original method pertain: final reaction volume, amplification kit and cycling conditions.

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

- M.J. Slomka, V.J. Coward, J. Banks, B.Z. Löndt, I.H. Brown, J. Voermans, G. Koch, K.J. Handberg, P.H. Jørgensen, M. Cherbonnel-Pansart, V. Jestin, G. Cattoli, I. Capua, A. Ejdersund, P. Thorén, G. Czifra. Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union. Avian Dis 51:227-234, 2007. doi: 10.1637/7674-063006R1.1;
- 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);
- OFFLU - Network of expertise on animal influenza, Influenza A Cleavage Site update. Accessible at https://www.offlu.org/wp-content/uploads/2021/01/Influenza_A_Cleavage_Sites-1.pdf;
- 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 126.

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);
- Qiagen OneStep RT-PCR Kit (Qiagen);

- Sense primer GK7.3 5'-ATG TCC GAG ATA TGT TAA GCA-3';
- Antisense primer GK7.4 5'-TTT GTA ATC TGC AGC AGT TC-3';
- RNase Inhibitor 40 U/µl;
- DNA molecular marker.

4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- PCR thermal cycler;
- QIAxcel Advanced system (Qiagen) (or equivalent electrophoresis system and imaging system for stained gels);
- 3130xl Genetic Analyzer (LifeTechnologies) (or equivalent sequencing system/service).

5. Procedure

5.1. Controls

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

Control Type	Definition
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 the target RNA of the AIV H7 subtype and stored at ≤ -70°C, that is processed along with the samples starting from the RT-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 RT-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°C) until the completion of the analysis. For long term conservation, store samples at ≤ -70°C.
Purified nucleic acids can be refrigerated for a few hours prior RT-PCR, otherwise they must be stored at ≤ -70°C.

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).

For additional details on the nucleic acids isolation procedures, see SOP VIR 1000.

5.4. One-step RT-PCR

The preparation of the master mix for the RT-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

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
RNase-free water	-	-	14.3
Qiagen OneStep RT-PCR Buffer	5X	1X	5
dNTP Mix	10 mM	0.4 mM	1
Sense Primer GK 7.3	50 µM	1 µM	0.5
Antisense Primer GK 7.4	50 µM	1 µM	0.5
RNase Inhibitor	40 U/µl	8 U	0.2
Qiagen OneStep RT-PCR Enzyme Mix	-	-	1
Master mix minus template			22.5
Template			2.5
Total reaction volume			25

Table 2. Volume and concentration of the reaction mix components

The master mix should be mixed thoroughly and 22.5 µl solution per sample must be pipetted in PCR tube/strip/plate.

Template (2.5 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, 2.5 µl of nuclease-free water should be added.

Cycling conditions

Place the tube/strip/plate in the thermal cycler. Set up the thermal profile as per Table 3.

Step	Temperature and time	Repetitions
Reverse transcription	50°C for 30 min	1
Initial PCR activation	94°C for 15 min	1
Denaturation	94°C for 30 sec	40
Annealing	52°C for 45 sec	
Extension	68°C for 1 min	
Final Extension	68°C for 7 min	1
Cooling	4°C	∞

Table 3. One-step RT-PCR thermal profile

5.5. PCR product detection

The presence of PCR products and their size analysis can be performed either by automatic (e.g. QIAxcel Advanced system, Qiagen) or manual (7% acrylamide gel or 2% agarose gel) electrophoresis systems, using appropriate DNA molecular markers.

5.6. Sanger sequencing

All the positive samples showing PCR products of the expected size are subject to Sanger sequencing to obtain the sequence of the cleavage site. Sequencing and sequence analysis should be carried out according to the methods and software in use at each laboratory. AIV subtyping is confirmed via BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi) by comparing the partial HA nucleotide sequence with the sequences available in the NCBI (National Center for Biotechnology Information) database. Pathotyping is determined by comparing the deduced amino acid sequence of the hemagglutinin cleavage site with that of low and high pathogenic avian influenza viruses (LPAI and HPAI) of the H7 subtype listed in the OFFLU website https://www.offlu.org/wp-content/uploads/2021/01/Influenza_A_Cleavage_Sites-1.pdf.

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. presence of the 200-220 bp amplification product	Repeat the analysis from the nucleic acids extraction and check the PPC stock
NPC	Negative, i.e. absence of the 200-220 bp amplification product	Repeat the analysis from the nucleic acids extraction and check nucleic acids isolation reagents

PTC	Positive, i.e. presence of the 200-220 bp amplification product	Repeat the analysis from the RT-PCR and check the PTC stock
NTC	Negative, i.e. absence of the 200-220 bp amplification product	Repeat the analysis from the RT-PCR and check RT-PCR reagents

Table 4. Assessment of test reliability

6.2. Diagnostic samples

The size of the amplification product has to be compared by electrophoresis with that of the AIV positive controls (PPC and PTC). The expected amplicon size for H7 AIV positive samples is 200-220 bp, depending on the length of the sequence encoding the cleavage site.

Criteria for data interpretation of diagnostic samples and subsequent actions are reported in Table 5.

Result	**Interpretation and action**
Presence of the 200-220 bp amplification product	Suspect. Proceed with Sanger sequencing and BLAST analysis for H7 subtype confirmation and pathotyping (see paragraphs 5.6. and 6.3.). If samples originate from confirmed outbreaks or are epidemiologically related to previously confirmed cases, they can be considered as positive
Absence of the 200-220 bp amplification product	Negative. If needed, proceed with RT-PCR for other HA subtypes

Table 5. Interpretation of One-step RT-PCR diagnostic results

6.3. H7 identification and pathotype interpretation

The quality of chromatograms covering the region encoding the HA cleavage site should be sufficient to allow the correct identification of the H7 subtype and its pathotype interpretation. The example in Figure 1 shows the chromatogram of two complementary sequences in opposite orientations, of acceptable quality (i.e. clear and high signal, no background noise, distinct peaks, unambiguous base calling). Contrarily, chromatograms showing high background, superimposed peaks and numerous ambiguous nucleotides are considered of poor quality and therefore unfit, and should require further RT-PCR and/or sequencing attempts.

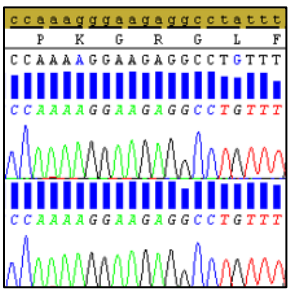

Figure 1. Chromatogram of a H7 LPAI AIV cleavage site

The AIV-H7 subtype identification using BLAST, is confirmed by the similarity with the reference sequences available in GenBank showing the highest “max score” value.

Nucleotide sequence data of acceptable quality result in a reliable translation into deduced amino acid sequence of an AIV-H7 cleavage site, that is therefore eligible for pathotype identification by comparison with HPAI and LPAI reference sequences. An avian influenza virus is considered HPAI if one of the following changes occurs in the proteolytic cleavage site of the hemagglutinin: 1) substitutions of non-basic with basic amino acids (arginine or lysine); 2) insertions of multiple basic amino acids deriving from duplication of HA cleavage site codons; 3) insertions of basic and non-basic residues from unknown source; 4) insertions from other gene segments that lengthen the proteolytic cleavage site via recombination; 5) loss of the shielding glycosylation site at residue 13 in combination with the presence of multiple basic amino acids at the HA cleavage site. As a rule, a cleavage site sequence of an H7 influenza subtype harbouring more than two basic amino acid residues should be able to identify HPAI viruses or viruses that can become highly pathogenic to poultry after simple mutations. The cleavage site is identified by a proline P at the beginning, and by the motif GLF at the end. A list of the common cleavage site nucleotide sequences and amino acid motifs, as well as their pathotype interpretation, is available at the OFFLU website https://www.offlu.org/wp-content/uploads/2021/01/Influenza_A_Cleavage_Sites-1.pdf.

7. Characteristics of the method

This method was validated and accredited 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. False positive results can rarely occur, as assessed during the validation process (i.e. $H_{14}N_5$), thus it is always recommended to confirm the H7 subtype with Sanger sequencing followed by BLAST analysis and pathotype interpretation.

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.