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EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND
NEWCASTLE DISEASE**



**SOP VIR 1002
DETECTION AND PATHOTYPING OF AVIAN ORTHOAVULAVIRUS TYPE 1
(AOAV-1) BY ONE-STEP RT-PCR AND SANGER SEQUENCING OF THE
FUSION PROTEIN CLEAVAGE SITE
(Kant et al., 1997)**

Standard operating procedure used by the EURL for AI and ND at the Istituto Zooprofilattico Sperimentale delle Venezie. Released on 07/04/2021.

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1. Purpose and field of application

This protocol describes the procedure to detect avian orthoavulavirus type 1 (AOAV-1) in biological samples by reverse transcription polymerase chain reaction (RT-PCR), and to determine the pathotype based on the sequence analysis of the fusion protein gene cleavage site.

The protocol targets the gene region encompassing the F0 cleavage site of different AOAV-1 genotypes, based on the oligonucleotides developed by Kant et al. (1997).

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

- A. Kant, G. Koch, D.J. Van Roozelaar, F. Balk, A. Ter Huurne. Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 hours by polymerase chain reaction. *Avian Pathol* 26(4):837-49, 1997. doi: 10.1080/03079459708419257;
- 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.

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);
- AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems);
- Sense primer A 5'-TTG ATG GCA GGC CTC TTG C-3';
- Antisense primer B 5'-GGA GGA TGT TGG CAG CAT T-3';
- 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 AOAV-1 and stored at $\leq -70^{\circ}\text{C}$, 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 AOAV-1 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 ($2-8^{\circ}\text{C}$) until the completion of the analysis. For long term conservation, store samples at $\leq -70^{\circ}\text{C}$.

Purified nucleic acids can be refrigerated for a few hours prior RT-PCR, otherwise they must be stored at $\leq -70^{\circ}\text{C}$.

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
Nuclease-free water	-	-	5.5
2X RT-PCR Buffer	2X	1X	12.5
Sense primer A	10 µM	0.2 µM	0.5
Antisense primer B	10 µM	0.2 µM	0.5
25X RT-PCR Enzyme Mix	25X	1X	1
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 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, PTC. In case of NTC, 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	45°C for 45 min	1
Initial PCR activation	94°C for 2 min	1
Denaturation	94°C for 1 min	40
Annealing	55°C for 1 min	
Extension	72°C for 1 min	
Final Extension	72°C for 10 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. AOA-1 identification is confirmed via BLAST (Basic Local Alignment Search Tool; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) by comparing the partial F gene nucleotide sequence with the sequences available in the NCBI (National Center for Biotechnology Information) database. Pathotyping is determined by analyzing the deduced amino acid sequence of the F0 cleavage site, according to the guidelines reported in the OIE Terrestrial Manual, Chapter 3.3.14.

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 362 bp amplification product	Repeat the analysis from the nucleic acids extraction and check the PPC stock
NPC	Negative, i.e. absence of the 362 bp amplification product	Repeat the analysis from the nucleic acids extraction and check nucleic acids isolation reagents
PTC	Positive, i.e. presence of the 362 bp amplification product	Repeat the analysis from the RT-PCR and check the PTC stock
NTC	Negative, i.e. absence of the 362 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 AOA-1 positive controls (PPC and PTC). The expected amplicon size for AOA-1 positive samples is 362 bp.

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

Result	Interpretation and action
Presence of the 362 bp amplification product	Suspect. Proceed with Sanger sequencing and BLAST analysis for AOA-1 confirmation, genotyping and pathotyping (see paragraphs 5.6. and 6.3.)
Absence of the 362 bp amplification product	Negative

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

6.3. AOA-1 confirmation and pathotype interpretation

The quality of chromatograms covering the F0 cleavage site should be sufficient to allow the correct sequence 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.

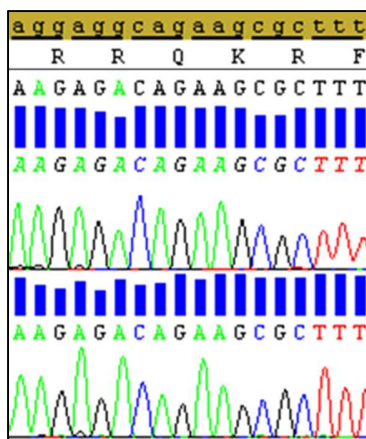


Figure 1. Chromatogram of a velogenic AOA-1 cleavage site

The AOA-1 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 AOA-1 F0 cleavage site, that is therefore eligible for pathotype identification. Most AOA-1 viruses that are pathogenic for chickens have the motif ¹¹²R/K-R-Q/K/R-K/R-R¹¹⁶ at the C-terminus of the F2 protein and F¹¹⁷ at the N-terminus of the F1 protein, whereas the viruses of low virulence have the residues ¹¹²G/E-K/R-Q-G/E-R¹¹⁶

and L¹¹⁷. Some of the pigeon variant viruses (PPMV-1) have the sequence ¹¹²G-R-Q/K-K-R-F¹¹⁷, but give high ICPI values. Thus, at least one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at 113 seem to be required to show virulence for chickens. However, some PPMV-1 may have virulent cleavage sites with variable ICPI values.

7. Characteristics of the method

The IZSve assessed the performances of the method employing AIV samples and clinical specimens available at the IZSve repository, as well as materials, equipment and procedures as described above.

Because of the high variability of AOAV-1, assay inclusivity might be strain-dependent. For this reason, it is advisable to use this method in combination with another RT-PCR with different specificity performances (e.g. SOP VIR 063).

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