ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 1007

MOLECULAR DETECTION OF ORTHOAVULAVIRUS JAVAENSE (OAV-J) AND IDENTIFICATION OF GENOTYPE gVII AND AVIRULENT STRAINS BY REAL-TIME RT-PCR

(Wise et al., 2004; Moharam et al., 2019; Fortin et al., 2023)

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 28/07/26.

The content of this document is subject to modification without prior notice.

The commercial products referenced in the SOP are the ones currently in use at the EURL for AI/ND. Their inclusion is intended exclusively to support diagnostic laboratories in the implementation of the protocol and does not constitute any form of commercial promotion. The use of alternative reagents or instruments that prove fit for the intended purpose is not restricted under any circumstances.

Revision history

Revision	Date	Description
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1. Purpose and field of application

This procedure describes the protocol to identify *Orthoavulavirus javaense* (OAV-J), virulent strains of genotype gVII and avirulent strains in biological samples from avian species by real-time reverse transcription polymerase chain reaction (RRT-PCR). The procedure can be applied to RNA purified from OAV-J isolates, organ/tissue homogenates, stool and swabs properly collected and preserved.

The procedure is primarily intended for application during epidemics of Newcastle disease (ND) caused by gVII in order to enable the prompt implementation of control measures, and consists of three independent RRT-PCR reactions:

- the 'OAV-J assay' targeting the matrix (M) gene of both virulent and avirulent OAV-J, based on the protocol developed by Wise et al. (2004);
- the 'gVII assay' targeting the fusion protein (F) gene of OAV-J of genotype gVII, based on the protocol developed by Moharam et al. (2019);
- the 'avirulent OAV-J assay' targeting the F gene of avirulent strains of gI, gII, gX (including vaccine strains), based on the protocol developed by Fortin et al. (2023).

The procedure may be performed in full by carrying out all three reactions in parallel. Alternatively, after proving reactivity with respect to the circulating gVII strains, laboratories may elect to run only the 'gVII assay' in the following circumstances: i) to confirm a clinical suspicion of ND (e.g. increased mortality, egg drop, etc.) in epidemiological scenarios consistent with the circulation of NDV of gVII, ii) to confirm secondary cases epidemiologically linked to primary outbreaks in which the presence of NDV of gVII was confirmed by sequencing, iii) in the event of widespread circulation of NDV of gVII in domestic or wild birds within a defined geographical area. Importantly, when the 'gVII assay' is applied to samples originating from primary outbreaks in any disease-free area, genotype and pathotype confirmation must be performed by sequencing the cleavage site (CS) of the fusion protein gene (e.g. SOP VIR 1002).

The application of the sole 'gVII assay' must be regarded as a targeted investigation for this specific genotype. In the presence of clinical, pathological or epidemiological suspicion of ND, a negative result obtained with this reaction does not rule out infection with virulent OAV-J viruses belonging to genotypes other than gVII. Under such circumstances, all the other reactions included in this procedure must be performed and, where necessary, additional investigations must be carried out.

2. References

- M.G. Wise, D.L. Suarez, B.S. Seal, J.C. Pedersen, D.A. Senne, D.J. King, D.R. Kapczynski, E. Spackman. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J Clin Microbiol 42(1):329-38, 2004. doi: 10.1128/JCM.42.1.329-338.2004;
- I. Moharam, A.A. El Razik, H. Sultan, M. Ghezlan, C. Meseko, K. Franzke, T. Harder, M. Beer, C. Grund. Investigation of suspected Newcastle disease (ND) outbreaks in Egypt uncovers a high virus velogenic ND virus burden in small-scale holdings and the presence of multiple pathogens. Avian Pathol. 48(5), 406–415, 2019. doi: 10.1080/03079457.2019.1612852;

- A. Fortin, A. Laconi, I. Monne, S. Zohari, K. Andersson, C. Grund, M. Cecchinato, M. Crimaudo, V. Valastro, V. D'Amico, A. Bortolami, M. Gastaldelli, M. Varotto, Newcastle Disease Collaborating Diagnostic Group, C. Terregino, V. Panzarin. A novel array of real-time RT-PCR assays for the rapid pathotyping of type I avian paramyxovirus (APMV-1). J Virol Methods 114813, 2023. doi: 10.1016/j.jviromet.2023.114813;
- WOAH - World Organization for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 3.3.10, Newcastle disease (infection with Newcastle disease virus) (Version adopted in May 2021);
- 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 1007.

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 assessment, 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 storage conditions of commercial products, refer to the manufacturer's instructions.

- Nuclease-free water;
- Oligonucleotides resuspension buffer (e.g. TE pH 8.0);
- OneStep RT-PCR kit (QIAgen);
- Sense primer APMV1_M+4100 5'-AGT GAT GTG CTC GGA CCT TC-3';
- Antisense primer APMV1_M-4220 5'-CCT GAG GAG AGG CAT TTG CTA-3';
- Probe APMV1_M+4169 5'-TTC TCT AGC AGT GGG ACA GCC TGC-3';
- Sense primer F_EGY_Fw 5'-CGS ARG ATM CAA GGG TCT-3';
- Antisense primer F_EGY_Rev 5'-CTA CAC TGC CAA TAA CRG C-3';
- Probe F_EGY_FAM FAM 5'-AGG AGA CRA AAA CGY TTT ATA GGT GC-3' BHQ-1;
- Sense primer Class II A for 5'-CTC ACC CCY CTT GGT GA-3';
- Antisense primer Class II A rev 5'- GGA GRA TGT TGG CAG CAT T-3';
- Probe Class II A avir MGB 5'- FAM-CCT ATA AGG CGY CCC TGT YTC-MGB-3';
- RNase Inhibitor 40U/µl;
- $MgCl_2$ 25 mM.

4.2. Equipment

- General molecular biology laboratory equipment and consumables;

- CFX Opus 96 Real-Time PCR System (Bio-Rad) (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 and for all the designated RRT-PCR assays.

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 and stored at ≤ -70°C, that is processed along with the samples starting 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

5.2. Preparation of samples and storage

For samples preparation 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 RRT-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 list of nucleic acids isolation systems validated at the IZSVe on different types of matrices is available in SOP VIR 1000.

5.4. Real-time RT-PCR

The preparation of the master mix for the RRT-PCR reactions has to be carried out in a clean dedicated area. The reagents should be kept refrigerated throughout the entire preparation phase. Probes should be protected from light. For the whole procedure, the use of filter tips and nuclease-free plastics is recommended.

Assays pre-mixes preparation

Oligonucleotides pre-mixes of the three assays can be assembled in advance and stored until use. The volumes reported in Tables 2-4 are sufficient for approximately 200 RRT-PCR reactions.

Component	Initial concentration	µl
TE pH 8.0	-	148
Sense primer APMV1_M+4100	100 µM	20
Antisense primer APMV1_M-4220	100 µM	20
Probe APMV1_M+4169	100 µM	12
Total volume		200

Table 2. Volume of the 'AOV-J assay' pre-mix components (Wise et al., 2004)

Component	Initial concentration	µl
TE pH 8.0	-	108
Sense primer F_EGY_Fw	100 µM	60
Antisense primer F_EGY_Rev	100 µM	20
Probe F_EGY_FAM	100 µM	12
Total volume		200

Table 3. Volume of the 'gVII assay' pre-mix components (Moharam et al., 2019)

Component	Initial concentration	µl
TE pH 8.0	-	85
Sense primer Class II A for	100 µM	50
Sense primer Class II A rev	100 µM	50
Probe Class II A avir MGB	100 µM	15
Total volume		200

Table 4. Volume of the 'avirulent AOV-J assay' pre-mix components (Fortin et al., 2023)

Reaction mix preparation

Prepare a master mix volume sufficient for the number of samples to be tested in each run. The volumes reported in Tables 5-7 are per single reaction.

Component	Initial concentration	Final concentration	µl per reaction
RNase-free water	-	-	10,62
Qiagen OneStep RT-PCR Buffer	5X	1X	5
dNTP Mix	10 mM	0,320 mM	0,8
$MgCl_2$	25 mM	1,25 mM	1,25
Pre-mix 'AOV-J assay'	-	Primers sense and antisense 0,4 µM each; probe 0,24 µM	1

Qiagen OneStep RT-PCR Enzyme Mix	-	-	1
RNAse inhibitor	40U/μl	13U	0,33
Master mix minus template			20
Template			5
Total reaction volume			25

Table 5. Volume and concentration of the reaction mix components for the 'AOV-J assay'

Component	**Initial concentration**	**Final concentration**	**μl per reaction**
RNase-free water	-	-	10,95
Qiagen OneStep RT-PCR Buffer	5X	1X	5
dNTP Mix	10 mM	0,320 mM	0,8
$MgCl_2$	25 mM	1,25 mM	1,25
Pre-mix 'gVII assay'	-	Primers sense 1,2 μM, Primer antisense 0,4 μM; probe 0,24 μM	1
Qiagen OneStep RT-PCR Enzyme Mix			1
Master mix minus template			20
Template			5
Total reaction volume			25

Table 6. Volume and concentration of the reaction mix components for the 'gVII assay'

Component	**Initial concentration**	**Final concentration**	**μl per reaction**
RNase-free water	-	-	10,62
Qiagen OneStep RT-PCR Buffer	5	1X	5
dNTP Mix	0,8	0,320 mM	0,8
$MgCl_2$	1,25	1,25 mM	1,25
Pre-mix 'avirulent AOV-J assay'	5	Primers sense and antisense 1 μM each; probe 0,3 μM	1
Qiagen OneStep RT-PCR Enzyme Mix			1
RNAse inhibitor			0,33
Master mix minus template			20
Template			5
Total reaction volume			25

Table 7. Volume and concentration of the reaction mix components for the 'avirulent AOV-J assay'

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

Template 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, PPC. 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 8, and select the FAM compatible detection channel for fluorescence acquisition.

Step	Temperature and time	Repetitions
Reverse transcription	50°C for 30 min	1
Initial PCR activation	95°C for 15 min	1
Denaturation	95°C for 10 sec	40
Annealing (*)	54°C for 30 sec	
Extension	72°C for 10 sec	

Table 8. Real-time RT-PCR thermal profile. (*) Fluorescence acquisition

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 (c.ca 100 RFU), 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 9. 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	Increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve at the expected Cq value	Repeat the analysis from the nucleic acids extraction
NPC	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	Increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve at the expected Cq value	Repeat the analysis from the RRT-PCR and check the PTC stock
NTC	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 9. Assessment of test reliability. Cq = quantification cycle

6.2. Diagnostic samples

Criteria for data interpretation of diagnostic samples and subsequent actions are reported in Tables 10-12.

Result	**Interpretation and action**
Increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Cq ≤ 36	Positive for AOV-J
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve	Negative for AOV-J
Weak increase in fluorescence from the FAM fluorophore, yielding a sigmoidal (or logarithmic) amplification curve with Cq > 36	Doubtful for AOV-J. It is responsibility of the Head of the Laboratory to identify the actions to be taken

Table 10. Interpretation of 'AOV-J assay' diagnostic results

Result	**Interpretation and action**
Increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Cq ≤ 36	Positive for gVII NDV
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve	Negative for gVII NDV
Weak increase in fluorescence from the FAM fluorophore, yielding a sigmoidal (or logarithmic) amplification curve with Cq > 36	Doubtful for gVII NDV. It is responsibility of the Head of the Laboratory to identify the actions to be taken

Table 11. Interpretation of 'gVII assay' diagnostic results

Result	Interpretation and action
Increase in fluorescence from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Cq ≤ 36	Positive for avirulent AOV-J
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal (or logarithmic) amplification curve	Negative for avirulent AOV-J
Weak increase in fluorescence from the FAM fluorophore, yielding a sigmoidal (or logarithmic) amplification curve with Cq > 36	Doubtful for avirulent AOV-J. It is responsibility of the Head of the Laboratory to identify the actions to be taken

Table 12. Interpretation of 'avirulent AOV-J assay' diagnostic results

In ND vaccinated birds, positive AOV-J samples might be associated with positive or doubtful results for avirulent AOV-J, depending on the vaccination scheme applied. Positive or doubtful results for avirulent AOV-J are also possible in unvaccinated birds following infection with avirulent strains.

Positive and doubtful NDV gVII samples may occur also in the presence of ND vaccination and may be associated with positive results for the 'avirulent AOV-J assay'.

Positive AOV-J samples associated with negative results with both the 'gVII assay' and the 'avirulent AOV-J assay' require additional investigations to rule out the presence of NDV of virulent genotypes other than gVII (e.g. SOP VIR 1002, SOP VIR 1006, NGS).

Doubtful testing results for the 'gVII assay' that cannot be confirmed with other pathotyping methods, might require re-sampling.

7. Characteristics of the method

This method was validated at the IZSVe according to the ISO/IEC 17025, employing AOV-J samples, selected avian viruses and bacteria available at the IZSVe repository, as well as materials, equipment, and procedures as described above.

The 'AOV-J assay' allows sensitive detection of NDV of gVII; however, the assay is known to have imperfect inclusivity with respect to other AOV-J genotypes. In the presence of clinical signs or pathological lesions indicative of ND which are not confirmed by the AOV-J assay', a second screening assay (e.g. SOP VIR 151) might be applied.

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.