Avian Influenza Community Reference Laboratory

Avian Influenza virus (AIV) typing by reverse transcription PCR using a universal primer set

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Purpose and field of application

The protocol describes the operating procedures for the RNA amplification and sequencing of the hemagglutinin (HA) gene of the Avian Influenza virus type A (AIV) by reverse transcription PCR using a universal primer set. The following specimens from avian species have been used:

- Allantoic fluid
- Homogenate of organs/tissues
- Faeces
- Cloacal/Tracheal swabs

Target of the analysis is segment 4 of the AIV genome encoding the HA gene. This procedure should only be used to test AIV isolates and diagnostic samples with a high viral concentration (see Recommendations for further details).

Reference documents

Gall A, Hoffmann B, Harder T, Grund C, Beer M. Universal primer set for amplification and sequencing of HA0 cleavage sites of all influenza A viruses. J Clin Microbiol. 2008 Aug; 46(8): 2561-7. *doi: 10.1128/JCM.00466-08*.

Definitions and acronyms

- **AIV**: avian influenza virus
- **HA**: hemagglutinin gene
- **RT-PCR**: reverse transcription-polymerase chain reaction
- **LoD**: limit of detection
- **PTC**: positive template control
- **NTC**: Negative template control
- **NPC**: Negative process control
- **EID_{50}**: embryo infectious dose 50
- **Ct**: cycle threshold

Reagents/solutions/diagnostic kits

Component	Supplier/Specification	Storage temperature
SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* High Fidelity DNA Polymerase	Thermo Fisher Scientific (Cat. No. 12574-030)	≤ -18°C
Sense Primer HA-1057.1	5' – GGRGAATGCCCCAAATAYGT – 3'	≤ -18°C
Sense Primer HA-1057.2	5' – GGRARATGCCCCAGRTATGT – 3'	≤ -18°C
Sense Primer HA-1057.3	5' – GGRGAATGCCCCAARTAYAT – 3'	≤ -18°C
Antisense Primer HA-1232.1(555)	5' – *CTGAGTCCGAACATTGAG*TTGCTATGVTGRTAWCCATACCA – 3'*	≤ -18°C
Antisense Primer HA-1232.2(555)	5' – *CTGAGTCCGAACATTGAG*TTYTGATGYCTGAADCCRTACCA – 3'*	≤ -18°C
RNA extracted from avian influenza (AIV) antigen	Positive template control (PTC)	≤ -70°C

Table 1. Reagents and kit used in the RT-PCR assay. *Non-viral sequences are shown in Italic

RNA extraction

Validation data of this method were obtained by preparing the samples according to the specifications of the QIAamp Viral RNA Mini Kit (Qiagen) and the QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen). The laboratory performing the analysis will proceed with the sample preparation in compliance with the manufacturer's recommendations of the kits in use.

Preparation of controls/reference material

The following controls must be prepared and used in the RT-PCR amplification phase:

- Positive template control (amplification control, PTC): RNA extracted from certified avian influenza A type antigen stored at ≤ -70 °C
- Negative template control (NTC): aliquot containing all the reagents necessary for the amplification reaction except for the nucleic acid, which is substituted by nuclease-free water
- Negative process control (NPC): a definite negative sample extracted along with the samples under analysis

RT-PCR amplification

The preparation of the master mix for the RT-PCR reaction is carried out according to the specifications reported in Tables 2 and 3.

Procedure:

- Prepare the RT-PCR primer mix as reported in Table 2

Component	Initial Concentration	Final Concentration	Volume (µl)
Sense Primer HA-1057.1	100pmol/µl	5pmol/µl	10
Sense Primer HA-1057.2			
Sense Primer HA-1057.3			
Antisense Primer HA-1232.1(555)			
Antisense Primer HA-1232.2(555)			
TE pH 8.0			150

Table 2. Reagent concentrations and volumes of the RT-PCR primer mix

- Prepare the RT-PCR reaction master mix in a sterile 1.5 ml test tube by adding the reagents in the order indicated in Table 3, with the exception of the RNA. The volumes, calculated on the quantity expected per single reaction, must be multiplied by the total number of samples to be analyzed (including controls)
- Thoroughly mix the reagents using a vortex and centrifuge for a few seconds
- Aliquot 20 µl of the mixture per sample into 0.2 ml sterile test tubes

Component	Initial Concentration	Final Concentration	Volume per reaction (µl)
Nuclease-free water			4.5
RT-PCR Enzyme Mix			1
Reaction Mix	2X	1X	12.5
Primer mix (see Table 2)			2
TOTAL VOLUME			20
Template RNA			5
FINAL VOLUME			***25***

Table 3. Reagent concentrations and volumes of the RT-PCR assay mix

- Add 5 µl of both sample and standard RNA to each corresponding test tube. For the NTC control add 5 µl of nuclease-free water

- Place the reaction in the preheated thermal cycler following the temperature setting as reported in Table 4

Step	Temperature (°C)	Time	Cycle Number
Reverse transcription	50	30 min	1
Initial denaturation	94	2 min	1
Amplification	95	45 sec	45
	50	45 sec	
	68	45 sec	
Final extention	68	5 min	1
Hold	4	∞	

Table 4. RT-PCR thermal profile

Expression of the results

Compare the size of the obtained amplification products with those of the positive AIV standard by using gel or capillary electrophoresis. The amplification test is considered conforming if the pre-arranged controls give the following results:

- PTC: presence of a 164-176 bp band (the size depends on the hemagglutinin subtype)
- NPC: absence of the band indicated above
- NTC: absence of the band indicated above

The amplification test is considered non-conforming if the pre-arranged controls do not provide the expected results. In this case, the test must be repeated starting from:

- Amplification reaction if the PTC turns out as non-conforming
- Amplification reaction if the NTC turns out as non-conforming. In this case, use fresh reagents
- Extraction if the NPC turns out as non-conforming

The sample is considered as RT-PCR positive if amplification product of the expected size as the PCR positive control is detected after electrophoresis. On the contrary, the sample is classified as RT-PCR negative if no amplification product of the expected size as the PCR positive control is detected after electrophoresis.

Analysis of the sequencing results and expression of the results

Sequencing and sequence analysis should be carried out according to the methods and the software in use in the laboratory performing the analysis. Subtyping is performed via BLAST (Basic Local Alignment Search Tool) by comparing the nucleic acid sequence of the partial hemagglutinin segment with the sequences available in the NCBI (National Center for Biotechnology Information) database. In case of H5/H7 subtyping, pathotyping is performed by comparing the deduced amino acid sequences of the hemagglutinin cleavage site with the sequences of low and high pathogenic avian influenza viruses (LPAI and HPAI) listed on OFFLU website (http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf), as recommended in the OIE Terrestrial Manual 2015 (Chapter 3.3.4., Avian influenza).

Characteristic of the method

a. *Analytical sensitivity*

Strain	**Subtype**	**Titre (EID_{50}/100 µl)**	**LoD (EID_{50}/100 µl)**	
			PBS	**Liquid stool matrix**
A/duck/Bavaria/77	H1N1	$10^{6.62}$	$10^{3.62}$	-
A/CK/IT/3582-51/10	H3N8	$10^{7.48}$	$10^{2.48}$	-
A/chicken/Japan/AQ-HE144/2015	H5N6	$10^{8.50}$	$10^{3.50}$	$10^{5.50}$
A/TK/IT/4776-48/2015	H6N8	$10^{7.83}$	$10^{4.83}$	-
A/CK/IT/1670/2015	H7N2	$10^{7.50}$	$10^{3.50}$	-
A/TK/IT/13VIR1864-45/2013	H9N2	$10^{7.77}$	$10^{4.77}$	-
A/mallard/IT/4518/07	H10N1	$10^{7.63}$	$10^{4.63}$	-
A/chicken/Italy/1279/99	H7N1	$10^{8.50}$	-	$10^{5.50}$

Table 5. Analytical sensitivity of the RT-PCR method

b. *Inclusivity*

Strain	Subtype	NCBI or GISAID* Accession Number
A/duck/Italy/1447/2005	H1N1	ACN98097
A/Verona-Italy/2810/2009 - pmd	H1N1	EPI211620*
A/duck/Germany/1215/73	H2N3	AY586422
A/pass/Italy/6000/V00	H3N8	-
A/cockatoo/England/1972	H4N8	GQ247847
A/turkey/Italy/1980	H5N2	GQ247849
A/green-winged teal/Wisconsin/103/1976	H5N2	CY180540
A/turkey/Canada/1965	H6N2	GQ247851
A/turkey/Italy/1067/99	H7N1	AF364134
A/turkey/Ontario/6118/1968	H8N4	CY087776
A/turkey/Scotland/70	H9N7	AF218086
A/ostrich/SouthAfrica/2001	H10N1	GQ247860
A/duck/England/1956	H11N6	CY130062
A/duck/Alberta/60/1976	H12N5	GU052216
A/gull/Maryland/704/1977	H13N6	CY130086
A/mallard/Gurjev/263/1982	H14N5	GQ247868
A/wedge-tailed shearwater/Western Australia/2576/1979	H15N9	CY006010
A/gull/Denmark/68110/2002	H16N3	GQ247872

Table 6. Amplified and sequenced subtypes which have been used to assess the inclusivity of the RT-PCR method

Recommendations

The use of this protocol is recommended to type clinical samples showing Ct values of approximately 22 or lower by performing the qRT-PCR for M gene.