ECDC TECHNICAL DOCUMENT

Diagnostic preparedness in Europe for detection of avian influenza A(H7N9) viruses

Technical briefing note
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1 Background and objective

In China, a novel avian-origin reassortant influenza A(H7N9) virus has been detected in a number of human cases [1]. In response to the potential cross-border health threat associated with emerging disease caused by the appearance of the novel virus, European laboratories need to be aware of the novel virus and adapt and confirm their diagnostic capability to detect and identify it. This document is a joint CNRL/ECDC/WHO Europe technical briefing note for virology laboratories. It is designed to assist clinical and public health laboratory directors in appraising options for establishing novel diagnostic assays for screening and confirming cases of infection in humans.

This document is intended for use in the area of human influenza surveillance, investigation, risk assessment, and control, encouraging cooperation between expert and reference laboratories in order to 'foster the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents which may threaten public health' [2].

This document contains:

- a list of laboratory preparedness considerations to ensure European-wide diagnostic capability;
- an update on current methods used for molecular detection of human infection with avian influenza A(H7N9) virus by RT-PCR;
- a table of H7 HA assay validation criteria;
- information on positive controls for RT-PCR assays.

Contributing authors

CNRL management team: Maria Zambon, Nichola Goddard, Adam Meijer, John McCauley*, Rod Daniels*
ECDC: Eeva Broberg, Marc Struelens
WHO/Europe: Dimitriy Pereyaslov, Caroline Brown
CNRL: Olav Hungnes, Joanna Ellis, Thedi Ziegler
* WHO CC, London

2 Laboratory preparedness considerations for action

2.1 Epidemiological situation

- Cases of avian influenza A(H7N9) virus infection in humans continue to be reported from China, with distribution over several hundred kilometres without obvious epidemiological links [3].
- Current assessments suggest that cases are most likely to have occurred as a result of sporadic contacts with infected poultry where the avian influenza A(H7N9) virus may be circulating without overt signs of disease [1].

Preparedness action: Member State laboratory partners should follow the situation via reports from WHO [4] and the epidemiological updates of the ECDC [5].

2.2 Surveillance strategy

- The same surveillance strategy applies as for human infections with highly pathogenic avian influenza A(H5N1) virus [6].
- Each WHO-recognised National Influenza Laboratory (NIC) should be able to perform testing for influenza viruses and detect an influenza A virus by RT-PCR assay using primers for a conserved internal gene (e.g. matrix (M) gene and then further perform tests using currently available H1, H1pdm09, H3 and H5 subtyping primers).
- Unsubtypeable influenza A viruses should be sent urgently to a WHO Collaborating Centre for Reference and Research on Influenza (WHO CC) for further analysis.

Preparedness action: NICs should work to develop diagnostic detection capability for novel influenza viruses like A(H7N9) to ensure a 'within country' capability for clinical diagnosis of avian influenza A(H7N9) virus in travellers returning from affected areas or for general response capability if the pandemic risk increases with more widespread distribution.

2.3 Laboratory H7 detection capabilities

- Laboratories testing for H7 will require a molecular detection capability allowing same-day specific detection ability in response to clinical queries or case scenarios.
- A two-step approach for detection and confirmation of avian influenza A(H7N9) virus infection should be followed.
- Laboratories require: (i) a generic influenza A virus testing capability which will assuredly detect an avian influenza A(H7N9) virus within the Eurasian lineage as influenza A and (ii) a specific H7 HA detection capability to confirm the presence of an avian influenza A(H7N9) virus in a sample which is positive for influenza A, but negative for H1, H3 and H5. Further assessment and monitoring of avian influenza A(H7N9) virus detection capability in laboratories is on-going to ensure diagnostic coverage in Europe

Preparedness action: Laboratories should look carefully at the sequence match of their primers and probes for their influenza type A molecular assays against the avian influenza A(H7N9) virus gene sequences published in GISAID. The laboratories would then need to ensure that if any potential mismatch looks unacceptable *in silico* (located at key positions in primers or probes), they update their reagents to improve the probability of detection. In addition, laboratories should await the distribution of inactivated avian influenza A(H7N9) virus material to test for sensitivity/verification with existing protocols. Further information will be provided on how these materials will be distributed, via WHO CC or other agreed channels, once the avian influenza A(H7N9) viruses are available and biosafety requirements confirmed for sharing materials (see section 3.3 on biorisk management considerations). Laboratories will be asked to inform their national public health authorities and European influenza laboratory network coordinators at ECDC and the WHO Regional Office when they are capable of these specific testing assays.

3 Update on current methods used for molecular detection of human infection with avian influenza A(H7N9) virus by RT-PCR

3.1 Generic influenza A virus detection capability

Review of data provided from over 30 influenza reference laboratories in Europe during the *in silico* exercise in 2011/2012 to evaluate capability of European laboratories to detect H3N2v virus indicates that the majority of European influenza reference laboratories are using an M-gene-based target for generic influenza A virus detection [7]. Accurate information about which exact molecular target sequences all laboratories are using for generic influenza A detection is not available, as some laboratories may be using commercial kits for which specific assay information is not available. Data from the WHO Influenza External Quality Assurance Project (EQAP) Panel 11 (2012) which consisted of nine gamma-ray inactivated influenza A(H1N1)pdm09, A(H3N2), A(H5N1), A(H9N2) and B viruses and one negative sample, show that among the 60 participating laboratories that returned results, 40 (67%) laboratories reported correct results for all 10 samples. When considering the influenza A(H9N2) sample only, 50 out of 60 participants (83%) correctly reported this sample as influenza A unsubtypeable. Nine (15%) laboratories from eight countries successfully identified this sample as influenza A(H9) [8]. Based on the data from WHO EQAP Panel 11, the predicted ability of several assays (CDC, PHE/HPA, RIVM) in use by the network, and the conserved nature of the internal genes, most countries are expected to have reasonable capability for detection of H7 Eurasian lineage as influenza A using existing reagents and protocols for detection of M gene targets.

For existing influenza type A virus tests where the molecular target sequence contains differences from the avian influenza A(H7N9) virus that may affect molecular detection, the sensitivity cannot be reliably evaluated until avian influenza A(H7N9) viruses are available in Europe.

Laboratories relying on an influenza type A RT-PCR test for which the molecular target sequence is not available (commercial assays), will need to seek confirmation about test adequacy from the provider.

NICs are also advised to explore/ascertain the adequacy for detecting the avian influenza A(H7N9) virus of the influenza type A tests used in primary diagnostic laboratories within their country where samples may undergo primary testing.

3.2 Specific influenza A(H7) HA detection capability

Development/application of H7 HA detection protocols requires that countries make choices about which protocols they may wish to use at a national level. Factors involved in decision-making include:

- Technical platforms, workflows and algorithms already in use
- Necessity to support regional networks with a variety of platforms
- Technical capabilities within NICs.

The A(H7) HA assay protocols and reagents detailed below (Appendix 1) have been selected based on expert opinion of the authors, after reviewing the best virological evidence available to date on genetic characteristics of the newly recognised reassortant strain of avian influenza A(H7N9) first described in March 2013 [1].

Due to the emerging nature of the disease caused in humans by this novel influenza virus strain of avian origin, the validation of some nucleic acid amplification and hybridisation assays and reagents designed for clinical detection has been limited to *in silico* genomic modelling and *in vitro* analytical validation of strain sensitivity and specificity in the absence of clinical data.

It should be emphasised that clinical validation studies of diagnostic performance (clinical sensitivity and specificity) are still either lacking or only preliminary for some of the described H7-specific assays. Therefore, the clinical diagnostic performance is uncertain for such assays and interpretation for patient management should be made with appropriate caution. Advice for clinical validation of the assays is available in Appendix 2 and Saunders *et al* [9], which set out some of the considerations when bringing into use new molecular assays for human clinical diagnosis for emerging infections. To assist with making an informed decision about clinical result interpretation, a table summarising the currently available data obtained from *in vitro* and clinical validation studies of proposed assays and reagents is included as Appendix 1.

Application of such assays for routine clinical diagnostic purposes is subject to national regulations. In the European Union it is subject to conformity assessment and CE marking as an *in vitro* diagnostic device by competent national notification authorities according to Directive 98/79/EC [10], unless it is used as in-house test qualifying under Article 1(5) of Directive 98/79/EC that provides CE label exemption for devices manufactured and used only within the same health institution.

It is necessary for each country to make decisions about whether and how to develop H7 HA detection capability based on the overall diagnostic detection approach in the country, and the laboratory accreditation system, which governs the operation of the NIC. Decisions about how to disseminate H7 testing capability is a matter for individual countries, but all countries should have an idea about what kinds of generic influenza A tests are being used, to evaluate the potential for missing detection of cases of H7 virus within health systems. Understanding how testing algorithms for generic influenza A detection as opposed to H1, H3 and influenza B are undertaken may also be an important consideration.

Existing protocols which are completely/fully validated may be updated using new reagents or tested against avian influenza A(H7N9) viruses as they arrive in Europe, and these protocols may form the basis of country detection capability. The table in Appendix 1 below sets out some criteria for existing protocols.

3.3 Receipt of avian influenza A(H7N9) virus control material

Handling virus stocks and propagation of live avian influenza A(H7N9) viruses in laboratories undertaking human diagnostics requires BSL3 laboratory containment [11]. The following resources can be used to support individual laboratory biorisk management [12] (CWA 15793) assessments for specimen collection, handling, and other specific laboratory requirements to comply with the relevant EU legislation [13, 14].

Different types of control materials can be provided and will be governed by a number of factors including the provider, and facilities and assays available in recipient laboratories (Table 1).

Table 1. Types, intended uses, and specific requirements for control material distribution

Type of control material	Intended use and specific requirements
Live avian influenza A(H7N9) virus	Appropriate for nucleic acid extraction control as well as amplification control for influenza generic type A RT-PCR and specific H7 targeted RT-PCR. Operational BSL3 facilities required to receive and handle this material Avian influenza A(H7N9) viruses are classified as PIP biological materials[i] and their distribution should comply with the PIP framework[ii]. The transfer should be recorded in the Influenza Virus Traceability Mechanism (IVTM). NIC laboratories and their regional network laboratories (under operational exemption[iii]) may use the viruses for public health testing but not for any commercial applications.
Inactivated avian influenza A(H7N9) virus	Appropriate for nucleic acid extraction control as well as amplification control for influenza generic type A RT-PCR as well as specific H7-targeted RT-PCR.
Extracted avian influenza A(H7N9) virus RNA	Appropriate only as amplification control for influenza generic type A RT-PCR as well as specific H7-targeted RT-PCR.
In vitro generated avian influenza H7 RNA transcripts	Only appropriate as amplification control, H7 HA target-specific. The *in vitro* transcripts need to cover the molecular target sequence of the PCR protocols used.

Laboratories can decide which control strategy to following according to local requirements. Live virus or inactivated virus or extracted virus RNA is available from the WHO CC [15]. *In vitro* transcripts covering the target region of the PHE/H7 HA assay are in progress and others will be developed.

[i] Includes human clinical specimens, virus isolates of wild-type human H5N1 and other influenza viruses with human pandemic potential; and modified viruses prepared from H5N1 and/or other influenza viruses with human pandemic potential developed by WHO GISRS laboratories, these being candidate vaccine viruses generated by reverse genetics and/or high growth re-assortment. Also included in 'PIP biological materials' are RNA extracted from wild-type H5N1 and other human influenza viruses with human pandemic potential and cDNA that encompass the entire coding region of one or more viral genes.

[ii] Pandemic Influenza Preparedness (PIP) Framework http://www.who.int/influenza/pip/en/

[iii] Operational exemption: materials shared within the WHO GISRS or with other laboratories specifically for non-commercial public health uses including surveillance activities, diagnostic applications, and quality assurance, are not handled as PIP Biological Materials. Their onward transfer for purposes other than those specified in the terms of reference of National Influenza Centres, WHO Collaborating Centres, Essential Regulatory Laboratories and H5 Reference Laboratories is not allowed under this operational exemption.

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Appendix 1
Table of H7 HA assay validation criteria

Primers and probes	Known location in HA gene sequence	Specificity tested against human flu (a)	Specificity avian flu (b)	Specificity other resp. viruses (c)	Platform suitability (d)	Preliminary clinical validation (e)	Analytical Sensitivity/ LoD (f)	Control material provision (g)
US CDC, Atlanta	Availability and updates will be given on: https://www.influenzareagentresource.org							
HPA/PHE*	942 – 1048	Yes	Yes	Yes (adenovirus 1,2,3,7, rhinovirus 16, 72, 87, CoV OC43, 229E, NL63, hMPV, RSV A, RSV B	TaqMan 7500	Yes	1pfu/ per reaction	Specific in vitro transcript in preparation, or inactivated virus
China NIC[$] Tested at WHO CC London	489 – 571** (HA1)	Yes A(H1N1)pdm 09; A(H3N2)	Yes* & 4 other Eurasian avian H7 viruses (negative for H11N9)		TaqMan 7500			RNA extracted from egg-grown A/Anhui/1/2013
Friedrich-Loeffler Institute (FLI) Tested at WHO CC London	1544 – 1693** (HA2)	Yes A(H1N1)pdm 09; A(H3N2)	Yes* & 4 other Eurasian avian H7 viruses (negative for H11N9)		TaqMan 7500			RNA extracted from egg-grown A/Anhui/1/2013
VLA Animal Health and Veterinary Laboratories Agency (AHVLA), Weybridge Tested at WHO CC London	1485 –1616** (HA2)	Yes A(H1N1)pdm 09; A(H3N2)	Yes* & 4 other Eurasian avian H7 viruses (negative for H11N9)		TaqMan 7500			RNA extracted from egg-grown A/Anhui/1/2013
VLA HA2 Tested at Animal Health and Veterinary Laboratories Agency (AHVLA), Weybridge (Slomka *et al.***) & NIC Norway	1485 – 1616*** (HA2)	Yes A(H1N1)pdm 09; A(H3N2); B	Yes Negative for H1-6; H8-16 (Slomka et al. 2009)	Yes (PIV1-3, Adenovirus, RSV A/B, rhinovirus, hMPV, *M. pneumoniae*)	MX3000 (Slomka); Rotor-Gene*** (NIC Norway)	Avian yes, human negative yes(n=50); human positive pending	Sensitivity corresponding to CDC M1-gene real-time RT-PCR	Several European avian H7 are suitable

Primers and probes	Known location in HA gene sequence	Specificity tested against human flu (a)	Specificity avian flu (b)	Specificity other resp. viruses (c)	Platform suitability (d)	Preliminary clinical validation (e)	Analytical Sensitivity/ LoD (f)	Control material provision (g)
RIVM	803 – 1076	Yes H1, H1pdm09, H3, BVic, BYam	Yes Negative for H5, H9. Further see Slomka *et al.* 2009	Yes (Rhinovirus, Enterovirus, human Coronavirus, hMPV, PIV 1-4, Adenovirus, RSV, C. pneumonia, *M. pneumonia*)	LightCycler 480	With limited number of specimens from suspect human cases associated with H7 poultry outbreaks in the Netherlands	Sensitivity similar to M real-time RT-PCR	Inactivated virus
Corman *et al.* 2013[#]	See Fig 1. in reference[#]; HA(I) 1501-1562; HA(II) 1549-1630; NA 447-516	Yes H1, H1pdm09, H3N2, B	Yes Negative for H5N1	Yes (Human coronaviruses, rhinovirus, RSV, enterovirus, hMPV, PIV 1-4, adenovirus, and parechovirus)	Not mentioned	With 121 clinical specimens which had tested positive for other respiratory viruses including human influenza A virus among others	7.0 (HA) and 7.8 (NA) copies of RNA per reaction	In vitro transcribed RNA of HA and NA genes of A/Mallard/Sweden/91/2002 (H7N9)
Other/ Commercial								

(a) Specificity tested against human circulating strains H1, H3, B; Yes indicates no detection of seasonal influenza.
(b) Specificity tested against other avian subtypes, e.g. H5 and other non-human subtypes; Yes indicates detection of H7 viruses.
(c) Testing against other respiratory pathogens which might be present in a respiratory clinical sample; Yes indicates no detection of the agents listed.
(d) Platform suitability, e.g. TaqMan, Rotagene, Lightcycler, other; the platform used in the testing laboratory is indicated.
(e) Testing against known positive and negative human clinical respiratory material. This may be preliminary/limited for H7N9 human clinical material.
(f) Sensitivity/limit of detection evaluated by pfu/virus count/copy number.
(g) Strategy for control material (live or inactivated virus, extracted RNA from infected cells, in vitro transcripts).
$ CNIC real-time RT-PCR protocol for the detection of avian influenza A(H7N9) virus – revision 01 (http://www.who.int/influenza/gisrs_laboratory/a_h7n9/en/; http://www.who.int/influenza/gisrs_laboratory/cnic_realtime_rt_pcr_protocol_a_h7n9.pdf)

* Indicates has been tested against RNA from A/Anhui/1/2013 H7N9 virus.
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*** Numbering from the assumed start of the non-coding region (with no polybasic cleavage site in A/Anhui/1/2013).
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Appendix 2
Validation molecular detection: real-time PCR for detecting microbiological targets

Adam Meijer PhD, Marcel Jonges MSc. National Institute for Public Health and the Environment, Ministry of Health, Welfare and Sport, the Netherlands.

This is an English translation of an extract dealing with real-time PCR from a larger document in Dutch on the procedures for validation of diagnostic assays.

Introduction

Before a real-time PCR pathogen detection assay is developed the goals should be set, for example: detection of pathogen X (specificity), should be more sensitive than current assay (sensitivity), suitability for detection pathogen in matrix Y, should have lower costs than current assay, should have shorter hands-on time and should be able to make use of laboratory automation.

At the end of the validation experiments, the data should be checked against the predefined targets before the assay is released by an authorised person.

Types of assays

There are four different types of assays that can be used for the real-time PCR detection of pathogens, which have to be subjected to a different validation process.

1 Commercially available assay that has been CE/IVD certified

Sensitivity and specificity has been validated by the manufacturer. Reports and/or peer-reviewed literature are available from the manufacturer.

Familiar well-defined materials, such as external quality assurance panels and/or at least 10 known positive clinical materials should be used to determine whether the assay or the installed equipment on which it will be used meets the expectations (accuracy and precision) of the assay as described by the manufacturer.

When the recommendations of the manufacturer with regard to equipment and protocol are not followed, it must be demonstrated that the new procedure has an equal or better performance than the recommended procedure.

2 Commercially available assay that has been CE certified

When the manufacturer has studies available which have been used to determine the assay characteristics, the same guidelines as for CE/IVD certified assay should be used for validation.

When the manufacturer does NOT have studies available which have been used to determine the assay characteristics, the validation guidelines for in-house developed assays should be used to validate the assay.

3 Assay developed by another laboratory

When a validation report or a scientific publication describing the validation of the assay is available, only limited validation is needed demonstrating that the assay as performed in the laboratory meets the expected specifications. For guidelines for validation see those for CE/IVD certified assays.

When NO validation report or scientific publication describing the validation of the assay is available, the assay should be validated according to the guidelines described for 'in-house' developed assays.

4 'In-house' developed assays

A real-time PCR assay for the detection of micro-organisms in clinical material that has been developed in-house should be subjected to the entire validation process as described below.

Validation process

The validation process for real-time PCR assays has the following components.

1. Perform an *in silico* analysis of primers and probes with regard to specificity and primer-dimer formation.

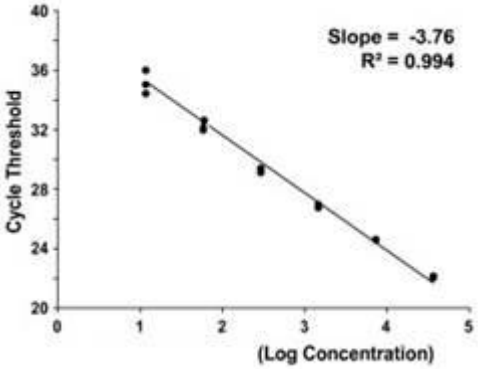
2. Determine (and if necessary, optimise) the efficiency of the PCR assay using a dilution series of positive control material, and (2B) compare the results with the current diagnostics if available.

Plot a calibration graph (Figure 1) using the results of a 10-fold dilution series with at least five dilution steps which has been carried out in at least 3-fold. Determine on the linear portion of the graph the slope and the correlation coefficient R^2.

Next, calculate the amplification efficiency using the following formulas:

E = 10^(-1/slope)
E% = (10^(-1/slope)-1)*100

Figure 1. Calibration curve for determination of the amplification efficiency



3. Determine the analytical detection limit (limit of detection, LOD, 95% is detectable) of the assay using the results of a 2-fold dilution series of positive control material carried out in at least 8-fold, and (3B) compare the results with the current diagnostics if available.

4. Determine the specificity of the primers and probes by analysis of cross-reactivity with the DNA (or RNA) of two types of relevant micro-organisms: (1) evolutionary related micro-organisms and (2) micro-organisms causing similar clinical symptoms.

5. Validate the clinical performance of the assay with a minimum number of both known positive and negative clinical specimens and compare the results with the current diagnostics if available, or, if possible, compare the results with those of another laboratory using the same specimens to determine congruence. The number of known positive and negative clinical specimens depends on whether a known pathogen is being targeted or an emerging 'new' pathogen.

In Table 1 the components required for the different types of validation are summarised.

Table 1. The minimum components in the validation process for real-time PCR for the detection of microbiological targets

Component in validation process	Adjustment of existing assay		Newly developed assay	
	Change in enzyme mixture, PCR cycling program or nucleic acid extraction	Other changes, e.g. nucleotide change in primer or probe	Old assay available	No assay available
1. *in silico* analysis		X	X	X
2. efficiency	X	X	X+2B	X -2B
3. LOD	X	X	X+3B	X -3B
4. cross reactivity		X	X	X
5. clinical specimens	X*	X*	X*	X*

* For exotic/new targets, component 5 cannot be performed completely due to lack of known positive clinical specimens.