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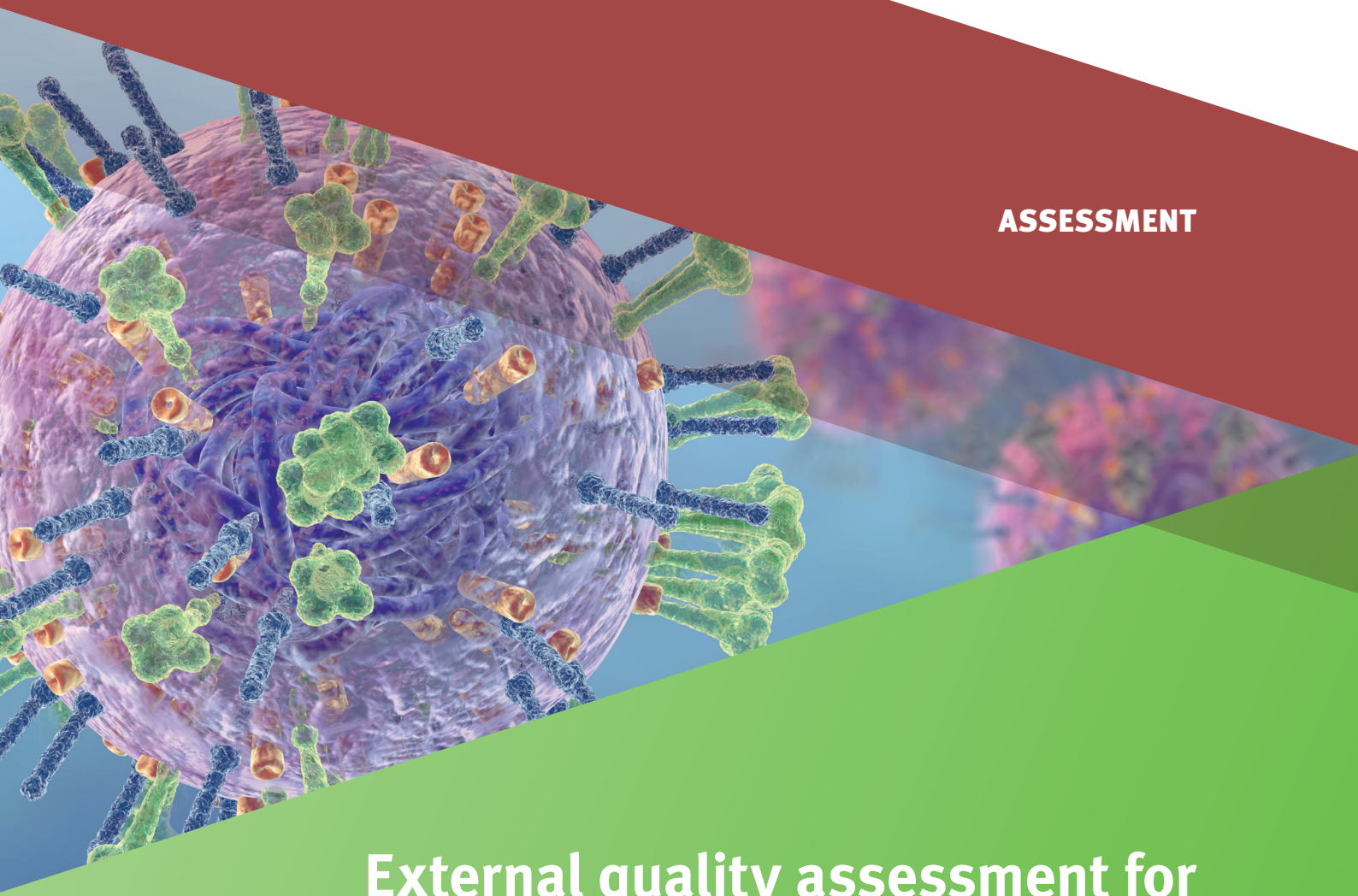

External quality assessment for molecular detection, subtyping and characterisation of potentially zoonotic type A influenza viruses, 2024

An ECDC-funded AURORAE laboratory support activity for national influenza centres/reference laboratories in the European Union/European Economic Area (EU/EEA), Western Balkan countries and Türkiye

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Authors
Adam Meijer, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.

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Abbreviations

a	avian
AURORAE	Laboratory Support for SARS-CoV-2, influenza and RSV for Europe
A(H1N1)	H1N1 subtype type A influenza virus of pre-pandemic 2009 human or swine specificity
A(H1N1)pdm09	H1N1 subtype human seasonal type A influenza virus of 2009 pandemic lineage
A(H1N2)	H1N2 subtype type A influenza virus of human or swine specificity
A(H3N2)	H3N2 subtype type A influenza virus of seasonal human or swine specificity
A(H5N1)	H5N1 subtype avian type A influenza virus
A(H5N8)	H5N8 subtype avian type A influenza virus
A(H7N2)	H7N2 subtype avian type A influenza virus
A(H7N3)	H7N3 subtype avian type A influenza virus
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
cGLP	current Good Laboratory Practice
Ct	Cycle threshold
dPCR	digital Polymerase Chain Reaction
d1	dilution 1
d2	dilution 2
d3	dilution 3
d4	dilution 4
d5	dilution 5
ECDC	European Centre for Disease Prevention and Control
EEIQAP	European External Influenza Quality Assessment Programme
EFSA	European Food Safety Authority
EISN	European Influenza Surveillance Network
EQA	External Quality Assessment
EQAP	External Quality Assessment Project
Erasmus MC	Erasmus Medical Centre
ERLI-NET	European Reference Laboratory Network for Human Influenza
EU	European Union
EURL	European Reference Laboratory
EU/EEA	European Union/European Economic Area
F	Forward primer
g	generic
gt	H5N1 genotype
GC	Genetic Characterisation
GISAID	Global Initiative on Sharing All Influenza Data
h	human
H	Hemagglutinin subtype
HA	Hemagglutinin protein
HEp-2	Human Epithelial 2
HPAI	highly pathogenic avian influenza
Hx	N-subtype in which x indicates any number from 1-18
H1g	H1 subtype NAAT with generic specificity
H1p	H1 subtype NAAT with N1pdm09 specificity
H1pp	H1 subtype NAAT with 2009 pre-pandemic specificity
H1s	H1 subtype NAAT with swine specificity
H1N1	H1N1 subtype type A influenza virus of pre-pandemic 2009 human or swine specificity
H1N1p	H1N1 subtype human seasonal type A influenza virus of 2009 pandemic lineage
H1N1pdm09	H1N1 subtype human seasonal type A influenza virus of 2009 pandemic lineage
H1N1s	H1N1 subtype swine type A influenza virus
H1N1v	H1N1 subtype variant type A influenza virus of swine origin that has been isolated from a human
H1N2	H1N2 subtype type A influenza virus of human or swine specificity
H1N2s	H1N2 subtype swine type A influenza virus
H1N2v	H1N2 subtype variant type A influenza virus of swine origin that has been isolated from a human
H3g	H3 subtype NAAT with generic specificity
H3h	H3 subtype NAAT with human seasonal specificity
H3s	H3 subtype NAAT with swine specificity
H3N2	H3N2 subtype type A influenza virus of seasonal human or swine specificity
H3N2h	H3N2 subtype human seasonal type A influenza virus
H3N2human	H3N2 subtype human seasonal type A influenza virus
H3N2s	H3N2 subtype swine type A influenza virus
H3N2swine	H3N2 subtype swine type A influenza virus

H5a	H5 subtype NAAT with avian specificity
H5N1	H5N1 subtype avian type A influenza virus
H5N1a	H5N1 subtype avian type A influenza virus
H5N1avian	H5N1 subtype avian type A influenza virus
H5N1bhg	A/Black-headed Gull/Netherlands/5/2023 H5N1 virus
H5N1ew	A/Eurasian Wigeon/Netherlands/1/2020 H5N1 virus
H5N1st	A/Sandwich Tern/Netherlands/7/2022 H5N1 virus
H5N8	H5N8 subtype avian type A influenza virus
H6a	H6 subtype NAAT with avian specificity
H7a	H7 subtype NAAT with avian specificity
H7N2	H7N2 subtype avian type A influenza virus
H9a	H9 subtype NAAT with avian specificity
H10a	H10 subtype NAAT with avian specificity
ID	Identifier
INF	Influenza
IRMA	Iterative Refinement Meta-Assembler
ITEMS	Information Technology EQA Management System
IZSVe	Istituto Zooprofilattico Sperimentale delle Venezie
LoD	Limit of Detection
LoD95%	95% Limit of Detection
MD	Molecular Diagnostics
MEM	Minimum Essential Medium
MP	Matrix protein
M2	Matrix protein 2
N	Neuramindase subtype
NA	Neuraminidase protein
NAAT	Nucleic Acid Amplification Test
NGS	Next Generation Sequencing
NIC	National Influenza Centre
NIRL	National Influenza Reference Laboratory
NP	nucleoprotein
NS	non-structural protein
Nx	N-subtype in which x indicates any number from 1-11
N1a	N1 subtype NAAT with avian specificity
N1g	N1 subtype NAAT with generic specificity
N1p	N1 subtype NAAT with N1pdm09 specificity
N1pdm09	N1 subtype of human seasonal type A influenza virus of 2009 pandemic lineage
N1pp	N1 subtype NAAT with 2009 pre-pandemic specificity
N1s	N1 subtype NAAT with swine specificity
N2a	N2 subtype NAAT with avian specificity
N2g	N2 subtype NAAT with generic specificity
N2h	N2 subtype NAAT with human seasonal specificity
N2s	N2 subtype NAAT with swine specificity
N3a	N3 subtype NAAT with avian specificity
N4a	N4 subtype NAAT with avian specificity
N5a	N5 subtype NAAT with avian specificity
N6a	N6 subtype NAAT with avian specificity
N7a	N7 subtype NAAT with avian specificity
N8a	N8 subtype NAAT with avian specificity
N9a	N9 subtype NAAT with avian specificity
n.a.	not applicable
p	pdm09
PA	polymerase acidic protein
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PCR	Polymerase Chain Reaction
pp	pre-pandemic
Pr	Probe
QC	Quality Control
QCMD	Quality Control for Molecular Diagnostics
R	Reverse primer
RIVM	National Institute for Public Health and the Environment
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Reverse Transcription semi-Quantitative Polymerase Chain Reaction
s	swine
sc	subclade
TE	Tris-EDTA

TESSy	The European Surveillance System
UNI	Universal
US	United States
WHO	World Health Organization
WHO CC	World Health Organization Collaborating Centre for Reference and Research on Influenza
%cov	percentage coverage of nucleotides in result sequence compared to reference sequence
%id	percent identical nucleotide in result sequence compared to reference sequence

Executive summary

Since 2022, the continuous spread of highly pathogenic type A subtype H5N1 avian influenza virus has caused outbreaks in poultry worldwide. This has led to infections in various wildlife and domestic mammalian species, including recent outbreaks among cows in the United States (US), as well as sporadic human infections.

Although no ongoing human-to-human transmission has been identified in the European Union/European Economic Area (EU/EEA) to date, the European Centre for Disease Prevention and Control (ECDC) considered that it would be important to assess the pandemic preparedness of influenza reference laboratories in the EU/EEA and Western Balkan countries and Türkiye for the detection, identification and characterisation of potentially zoonotic influenza viruses. As a result, an external quality assessment (EQA) was developed addressing these aspects of type A subtype H5N1 avian influenza viruses as well as other potential zoonotic avian type A influenza virus subtypes and swine type A influenza viruses that caused or are able to cause human infection.

This European External Influenza Quality Assessment Programme (EEIQAP) was performed in 2024. Included in the EQA panel were 14 heat-inactivated type A influenza viruses of different hemagglutinin subtype (H) and neuraminidase subtype (N); five of avian subtype (three different genotypes of genetic clade 2.3.4.4b H5N1 type A influenza viruses of which one in different concentrations to measure sensitivity of tests; one H5N8 and one H7N2), three of swine subtype (H1N1, H1N2 and H3N2) and two of human seasonal subtype (H1N1pdm09 and H3N2) as a reference; and one specimen without a virus as negative control.

Thirty laboratories in 27 countries participated. Regarding the diagnostic capability, all laboratories detected all type A influenza virus subtypes in the panel with their generic type A influenza virus detection Nucleic Acid Amplification Test (NAAT), meaning that type A influenza virus will be detected but further tests (H-subtyping by NAAT and/or sequencing) would then be needed to identify the human seasonal, avian or swine 'likely host species' and specific subtype of the detected type A influenza virus. This is critical for identifying and confirming an infection with a zoonotic type A influenza virus, and this capacity of national influenza reference laboratories has therefore also been assessed in the EEIQAP 2024.

Some difference in sensitivity between laboratories was observed, with 20/30 (67%) laboratories successfully detecting type A influenza virus in the lowest concentration specimen (8.11 genome copies/µl specimen) of the dilution series of an H5N1 strain expected to be found positive. This suggests there is room for improvement in the sensitivity of the laboratories' NAAT for generic detection of type A influenza viruses for those laboratories that failed to detect the lowest concentration specimen that was expected to become positive. This is especially important in infections with animal influenza viruses in humans where upper respiratory specimens may have a lower viral load, especially later after the infection occurred. This also highlights that routine diagnostic laboratories using mostly commercial NAAT should be capable of at least detecting a type A influenza virus in specimens from patients infected with an animal type A influenza virus (if their assay is sensitive enough and the specimens are well taken), to be able to submit influenza virus positive specimens from such patients to the national influenza reference laboratory for further characterisation. In an EQA study in the Netherlands among routine clinical diagnostic laboratories using a similar panel of viruses to the current EEIQAP 2024, it was confirmed that commercial NAAT can indeed detect type A influenza virus in specimens containing potential zoonotic viruses of avian and swine origin.

Most of the national influenza reference laboratories (26/30; 87%) determined the H-subtype of included human seasonal and avian influenza viruses using NAAT as first line diagnostic testing; all laboratories determined the seasonal human influenza virus H1 and H3 and avian influenza virus H5 subtype using NAAT, and 25/29 (86%) the H7 subtype. However, using NAAT, only 6/30 (20%) laboratories identified one or both H1 swine influenza viruses as H1, but 20/30 (67%) laboratories identified the H3 swine influenza virus as H3. However, it should be noted that detecting and identifying swine type A influenza viruses as H1 or H3 by NAAT often cannot in itself distinguish between the respective human seasonal and swine viruses. This means that a zoonotic infection with swine influenza virus can go unnoticed, especially if a treating physician does not include questions on possible animal exposure in reviewing the disease history of the patient.

Almost half of the laboratories (14/30; 47%) did not use N-subtyping NAAT to determine the N-subtype of the panel viruses, similar to as has been observed in all previous EEIQAP studies. However, as 27/30 (90%) laboratories reported in the questionnaire that accompanied the reporting of EQA results having one or more N-subtyping NAAT readily available in the laboratory capable of determining the N-subtype of one or more of the panel strains, in theory more laboratories could have performed and reported on N-subtyping. Nevertheless, those using N-subtyping NAAT used assays for only a limited number of N-subtypes; most often using NAAT only, the human N1pdm09 and/or N2 (by 18/30; 60% laboratories) subtypes and avian N1 subtype (10/30; 33% laboratories) were identified among the panel virus strains. N-subtyping NAAT for most relevant N-subtypes is desirable for national influenza reference laboratories to have in the repository to allow for rapid identification and confirmation of potential zoonotic influenza viruses and for discovering H/N reassortant viruses. The level of mismatched nucleotides with target site in the panel viruses did not predict whether a virus was detected or subtyped or not using NAAT, even with low viral load specimens at or just above the 'Limit of Detection' (LoD) of the used NAAT. This highlights the need for thorough validation/evaluation when a specific NAAT is implemented.

Regarding sequencing capability, using sequencing of the viral genome by 21/30 (70%) laboratories, around half (12/21; 57%) reported the correct H- and N-subtype and 'likely host species' (human, avian or swine) for all human seasonal, avian and swine type A influenza viruses in the panel that were sequenced. However, of the remaining nine laboratories, eight obtained and successfully uploaded correct hemagglutinin protein (HA) and neuraminidase protein (NA) coding genome segment sequences and/or H-subtyping and/or N-subtyping NAAT results, suggesting that errors were made in the reports of the final conclusion on the H- and N-subtype and 'likely host species' of virus strains in the panel rather than failures in NAAT or sequencing. Consequently, full correct subtyping and 'likely host species' identification could have been delivered by the vast majority of laboratories performing sequencing.

Following bioinformatics analysis by laboratories that performed this analysis, genetic clades of the panel viruses were assigned correctly for human H1pdm09 virus by 17/18 (94%) laboratories, for human H3 virus by 16/19 (84%) laboratories, for avian H5 viruses depending on the virus strain by 16–21/17–21 (94%-100%) laboratories, for H7 virus by 9/9 (100%) laboratories, for swine H1 virus depending on the virus strain by 15-16/15-16 (100%) laboratories and for swine H3 virus by 14/17 (82%) laboratories. Subclades for human influenza viruses were assigned correctly by 16/17 (94%) laboratories that performed this analysis. Five laboratories performed genotyping of H5N1 virus strains (for which sequences of all eight genome segments are needed) and all provided the correct genotype for the H5N1 virus strains in the panel they detected.

Similar to the subtyping following sequencing, more laboratories could have performed this analysis because the required sequences were obtained and successfully uploaded. Depending on the specific virus strain included in the panel, 1–9 laboratories more could have provided a clade assignment of one or more viruses, 2–3 laboratories more for subclade assignment and 13–15 laboratories more for H5N1 genotyping. Results for identification of markers for antiviral reduced susceptibility and for mammalian adaption were highly diverse, indicating lack of knowledge on how to interpret genetic data for this purpose, not having tools available or not correctly using the provided guidance (in the instructions that came with the panel and provided by the World Health Organization (WHO) and ECDC) on which amino acid substitutions are important, as all sequences obtained and successfully uploaded by the laboratories contained the expected markers.

In conclusion, this EEIQAP 2024 activity fulfilled its goal of providing a comprehensive assessment of the pandemic preparedness of national influenza reference laboratories in the whole of the EU/EEA and Western Balkan countries and Türkiye for the detection, identification and characterisation of potentially zoonotic influenza viruses. All 30 laboratories detected all virus strains in the panel. Depending on the virus strain, using NAAT, 17%–100% of 30 laboratories identified the H-subtype correctly and 6%–100% of 16 laboratories performing N-subtyping identified the N-subtype correctly. Depending on the virus strain, using NAAT combined with sequencing, 76–100% of 21 laboratories that reported sequencing results identified the H- and N-subtype of the virus strains in the panel correctly. Eight laboratories that did not perform sequencing were unable to provide an N-subtype due to not conducting N-subtyping NAAT. Overall, 9/30 (30%) laboratories did not perform sequencing and therefore could not provide a definitive result or confirmation on the H- and N-subtype and 'likely host species' of all virus strains in the panel, especially for the included swine influenza viruses. Depending on virus strain, the clade, subclade and H5N1 genotype were assigned correctly by 82%–100%, 94% and 100% of the laboratories that reported results on clade (n=9–21), subclade (n=17) and genotype (n=4–5), respectively.

However, the assessment of the laboratory pandemic preparedness is limited because national influenza reference laboratories in only 27/37 (73%) countries in the EU/EEA (n=23), Western Balkan countries (n=3) and Türkiye participated. Nevertheless, the overall results of the laboratories that participated highlights that there is a need for implementing highly specific NAAT for H- and N-subtyping, and/or universal type A influenza virus sequencing at more national influenza reference laboratories to increase the local capacity to recognize zoonotic influenza viruses for the purpose of rapid national response and international alert. Laboratories are encouraged to participate in ECDC EQA activities and WHO External Quality Assessment Project (EQAP) for molecular diagnosis and sequencing of influenza viruses (including potentially zoonotic influenza viruses) and to participate in the training activities (on using laboratory tests and on data processing and analysis using bioinformatics) organised by ECDC.

Patients suspected of infection with a zoonotic type A influenza virus, identified through generic detection in local or regional clinical diagnostic laboratories, must be confirmed by the national influenza reference laboratory. Therefore, these reference laboratories should be capable of performing these tests and determining the H- and N-subtypes of the virus. If the national influenza reference laboratory lacks capability to do so, ECDC offers trainings and technical laboratory support to help with implementing such tests. All specimens positive for zoonotic type A influenza virus should be sent to the WHO Collaborating Centre for Reference and Research on Influenza (WHO CC) in London for further characterisation.

1 Introduction

Since the initiation of this external quality assessment (EQA) in early 2024, when the global outbreak of highly pathogenic avian influenza (HPAI) H5N1 (clade 2.3.4.4b) type A influenza virus affected over a 100 million wild birds and poultry in 2022 - 2024, an expanding range of mammals have been found infected including most recently cows in the United States (US) [1-3].

Since increased activity of clade 2.3.4.4b viruses from mid-2021, and as of 15 May 2025, about 80 confirmed human cases with this clade of different genotypes have been reported to the World Health Organization (WHO), most after direct contact with infected poultry or cows [4,5]. This amplified the concern about the enzootic circulation of H5N1 type A influenza virus and spill-over to humans and the needed preparations to avoid widespread spill-over to humans and the potential of a pandemic.

These preparations include the availability of appropriate diagnostic and molecular characterisation capacities in national reference laboratories for human influenza [6], especially, in the context of this report, of the ECDC European Reference Laboratory Network for Human Influenza (ERLI-Net). There are also concerns about direct transmission of swine influenza viruses to humans and possible reassortment of swine influenza viruses with avian and/or human seasonal influenza viruses, e.g. at mixed poultry and swine farms, increasing the risk that viruses emerge with enhanced potential for transmission to and between humans [7-9].

Current EQAs organised by the WHO [10] and not-for-profit EQA providers provide challenges for detection of potentially zoonotic avian influenza viruses, but not for potentially zoonotic swine influenza viruses. Hemagglutinin (H) and neuraminidase (N) subtyping is often optional, if included in the challenge, especially due to the fact that for routine diagnostic clinical laboratories, subtyping is not an obligation for treatment of an influenza patient, if a patient with clinical suspected influenza is tested at all. However, H-subtyping is required in the WHO External Quality Assessment Project (EQAP) studies carried out since 2007 as a result of the continued threat of avian influenza virus H5N1 and the necessity for WHO National Influenza Centres (NIC) to have this capacity [11]. To our knowledge, only Quality Control for Molecular Diagnostics (QCMD) offers a specific H-subtyping EQA panel, which has included avian influenza viruses since 2006, including in response to the threat represented by avian influenza virus H5N1 [12].

Sequencing for genetic characterisation (clade assignment) and the evaluation of markers for enhanced mammalian transmission and virulence [3] and antiviral reduced susceptibility are not included in these programmes, or only for clade assignment and antiviral reduced susceptibility of human seasonal influenza viruses in WHO's EQAP and ECDC's EEIQAP [13,14]. To fill this gap, to prepare for potential human cases of avian or swine influenza virus infection, and to ensure the reliability and comparability of results reported to The European Surveillance System (TESSy), the National Institute for Public Health and the Environment (RIVM) developed and carried out the EEIQAP 2024 for potentially zoonotic type A influenza viruses at the request of ECDC, in the context of the AURORAE framework contract ECDC/2022/001, LOT 1: 'Centralised laboratory support'.

This ECDC EEIQAP aims to assess the capabilities of the WHO NICs and National Influenza Reference Laboratories (NIRL) in the EU/EEA and Western Balkan countries and Türkiye with respect to detection, subtyping and molecular characterisation of potentially avian and swine zoonotic type A influenza viruses. It comprised of two programmes using the same panel specimens, European Influenza Surveillance Network (EISN) Influenza (INF) Molecular Diagnostics (MD) – Detection and Subtyping 2024 (EISNINF_MD24) and EISNINF Genetic Characterisation (GC) 2024 (EISNINF_GC24).

The overall goal of ECDC-supported EQAs is to appraise the proficiency of public health microbiology laboratories in using microbiological test methods that underpin capabilities in the following areas [15]:

1. Diagnostic confirmation of disease for reporting to TESSy, in accordance with EU case definitions for 52 notifiable diseases and antimicrobial resistance for EU/EEA countries;
2. Outbreak detection, investigation and response;
3. Control of communicable diseases;
4. Preparedness.

Operational public health objectives of ECDC-supported EQAs are [15]:

1. Assessment of the quality and comparability of surveillance data;
2. Support of threat detection capabilities for emerging diseases, epidemic diseases, and drug resistance.

These objectives are consistent with the laboratory and public health objectives of EQAs outlined in the WHO laboratory quality management system handbook [16].

Translated into operational procedures in the laboratory, the main purposes of EQA, as also intended for the EEIQAP 2024, include:

1. Comparison of performance and results among different test sites;
2. Provision of early warning for systematic problems associated with kits or operations;
3. Provision of objective evidence of testing quality;
4. Identification of areas that need improvement;
5. Identification of training needs.

The major objective of the EEIQAP 2024 potentially zoonotic influenza EQA was to assess the capabilities and performance of individual national influenza reference laboratories in the following areas:

1. Rapid detection and hemagglutinin (H)- and neuraminidase (N)- subtyping of influenza A viruses using Nucleic Acid Amplification Test (NAAT), among which Reverse Transcription Polymerase Chain Reaction (RT-PCR), readily available in the laboratory, within a defined reporting timeframe of seven working days;
2. Generate sequences for influenza A viruses (preferably whole genome all eight segments) and determine within a defined reporting timeframe of 36 working days:
    a. Full subtype information, H-subtype and N-subtype;
    b. Genetic clade, subclade and H5N1 genotype as appropriate for the determined subtype;
    c. Markers for reduced susceptibility to matrix protein 2 (M2)-blockers, neuraminidase inhibitors and the cap-dependent endonuclease inhibitor Baloxavir Marboxil, and for human adaptation;
3. For both 1. and 2. determine the most likely host species (human, avian, swine) from which the identified virus originated based on available generated results and provide an overall conclusion.

This report presents the results of the EEIQAP 2024 for molecular detection, subtyping and characterisation of potentially zoonotic type A influenza viruses carried out by national influenza reference laboratories in the EU/EEA and Western Balkan countries and Türkiye, funded by ECDC and designed and prepared by the contractor RIVM in collaboration with ECDC.

2 Study design and methods

2.1 Organisation

The EEIQAP panel was designed by staff from the contractor, RIVM, and the final composition was agreed with ECDC. Human seasonal type A influenza viruses and zoonotic A(H1N1)v and A(H1N2)v swine influenza viruses were taken from the repository at RIVM. The avian influenza viruses were kindly provided by AURORAE consortium member Ron Fouchier and Oanh Vuong, Erasmus Medical Centre, Department Viroscience, Rotterdam, the Netherlands, and the A(H3N2) swine influenza virus was kindly provided by AURORAE consortium member Étienne Simon-Lorière, Institut Pasteur, Paris, France, both for the purpose of this EEIQAP panel only.

The panel was prepared and quality controlled at the Department Emerging and Endemic Viruses, Center for Infectious Disease Research, Diagnostics and laboratory Surveillance (IDS), RIVM, Bilthoven, the Netherlands. All viruses were heat inactivated. Pretesting was performed by AURORAE consortium members National Reference Centre for Respiratory Virus Infections, Virology Laboratory, Institute of Infectious Agents, Lyon, France and Institut Pasteur, Paris, France. The at -80ºC frozen panels were distributed on dry ice to participants by specialist courier, organised by subcontractor QCMD, Glasgow, Scotland, United Kingdom between May through July 2024. Participants received a detailed instructions document for testing of the panel, guidance on which tools can be used for sequence analysis and how to report results and upload sequences (Annex 14).

Participants submitted results to the web-based database Information Technology EQA Management System (ITEMS), which is hosted by QCMD and was adapted to the needs of EEIQAP 2024 potentially zoonotic type A influenza viruses (Annex 15). Briefly, participants were asked to report for each individual NAAT a qualitative judgement positive or negative or the identified H- or N-subtype and the obtained Ct value, if applicable for the used NAAT. Based on all the individual NAAT results the participants were asked to provide a conclusion on the H- and N-subtype and 'likely host species' of the panel viruses.

Laboratories participating in the sequencing part were asked to report the identified H- and N-subtype and 'likely host species' of the panel viruses and an overall conclusion on the H- and N-subtype and 'likely host species' based on the combined NAAT and sequencing results. Laboratories were asked to upload the primers and probes sequences (if known) of the NAAT used for testing the panel specimens and to upload the sequences obtained from the viruses in the panel specimens. Additional questions were asked about readily available NAAT in the laboratory, used equipment and reagents for nucleic acid extraction, NAAT and sequencing and on guidance, bioinformatics software and tools used for raw sequence analysis, clade, subclade and H5N1 genotype assignment and antiviral susceptibility and mammalian adaptation marker analysis.

2.2 Panel composition, preparation and validation

The EEIQAP panel consisted of 15 simulated clinical specimens (Table 1). Five contained different concentrations of the same A(H5N1) virus to get a rough idea about the sensitivity of generic influenza A virus detection protocols and H5 and avian N1 subtyping assays. In total five avian type A influenza viruses were included, of which three A(H5N1) viruses of different genotype (one of these in the five different concentrations) one A(H5N8) and one A(H7N2). Three type A influenza viruses of swine origin were included, one A(H1N1)v and one A(H1N2)v isolated from humans with a swine influenza virus infection and one A(H3N2) isolated from a pig. In addition, for reference and subtype specificity determination by NAAT, one human seasonal A(H1N1)pdm09 and one human seasonal A(H3N2) influenza virus were included. A specimen without virus was included for false positive assessment purposes.

The viruses were not specifically selected for the presence of antiviral reduced susceptibility markers or mammalian adaptation markers or virulence markers. Nevertheless, several of the viruses contained such markers and therefore we have requested to report these to assess the capability of analysing obtained sequences for such markers. The markers that could be identified are listed in Table 2.

Panel specimens have been prepared in Minimum Essential Medium (MEM) with Hank's salts and 10,000 Human Epithelial 2 (HEp-2) cells/ml to simulate a clinical specimen. Before preparing the specimens, the viruses have been inactivated using heat for 2.5 hours at 65ºC [17], and confirmed being inactivated by virus isolation. The Cycle threshold (Ct) values shown in Table 1 were determined by in-house reverse transcription semi-quantitative polymerase chain reaction (RT-qPCR) testing on 5 µl of extract of the panel specimens with Applied Biosystems™ TaqMan™ Fast Virus 1-Step Master Mix on Roche LightCycler® 480 Instrument II, after extracting 200 µl of specimen with Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit on Roche MagNA Pure 96 and elution in 50 µl Tris-EDTA (TE) buffer, which are currently used at RIVM for generic type A influenza virus detection and the indicated subtyping.

As primers and probes of the various RIVM RT-qPCR assays have a perfect or less than perfect match with the targeted genome segments of each of the panel viruses, the Ct values displayed in Table 1 cannot be used directly as a proxy to compare the concentration of viruses in the specimens. In addition, as was stated in the Panel Instructions manual in the 'Note of awareness' (Annex 14), the heat inactivation of the viruses might have an effect on the performance of sequencing protocols, especially those which use whole segment amplification using

universal primers. Therefore, the usual correlation between the Ct value of clinical specimen and the sequencability of the virus in the clinical specimens does not apply to the viruses in the panel specimens. Nevertheless, the panel specimens were prepared in a way that for all individual virus strains included in the panel at least one specimen contained enough intact virus genome segments for whole genome sequencing using universal primers. Therefore, the Ct values for these specimens are relatively low (high viral RNA content with enough intact genome segments to allow for whole genome segment amplicon sequencing using universal primers and long reads, e.g. using Nanopore sequencing). Due to the heat-inactivation restriction for sequencability, only the d1 dilution of the A(H5N1) dilution series prepared for NAAT sensitivity purposes was expected to be sequenced and analysed correctly.

A blinded panel with specimens in a different order than in the final panel was shipped on dry ice to the pretesting laboratories. The same panel was tested at RIVM for internal Quality Control (QC) purposes. Detection and subtyping amplification assays worked as expected at the pretesting laboratories and in the internal QC at RIVM. Sequencing protocols used at RIVM and the two pretesting laboratories, Nanopore or Illumina or a combination of both, on whole genome segment amplicons with UNI universal primers, were able to sequence the whole genomes of all specimens positive in the generic type A influenza virus RT-qPCR; as explained above, only the highest concentration of the A(H5N1) dilution series was evaluated. The sequences were appropriate to determine the 'likely host species' from which the viruses originated (Expected host in Table 1), the full subtype (H and N) and the genetic clade based on the hemagglutinin (HA) genome segment for all viruses, the subclade based on the HA genome segment for human seasonal viruses, and the genotype based on the whole genomes (all eight genome segments needed to determine the genotype) for A(H5N1) viruses for which this is applicable (Table 1).

2.3 Data analysis

For analysis of the reported data and creation of tables and figures, Microsoft Excel 365 version 2402 and GraphPad Prism version 10.2.2 have been used. Where appropriate, summarising percentage datasets or laboratories with correct results were calculated. Apart from the percentage correct nucleotides in uploaded sequences compared to the reference sequences (as explained below), no scoring system for level of performance was used.

For the evaluation of the reported results against the expected results, the overall conclusions based on NAAT results for all laboratories and the overall conclusions based on NAAT results combined with sequencing results for the laboratories that performed sequencing were used. When the reported result differed from the expected result, the underlying individual NAAT and obtained and reported sequences were evaluated and a note added to the reported conclusion (which are displayed as footnotes in the Annexes with participants' individual results).

Similarly, clade, subclade and H5N1 genotype assignments and antiviral susceptibility and mammalian adaptation marker identifications were evaluated against the expected results and deviations checked against the participants' successfully obtained and uploaded sequences of the panel specimens. Where it was considered useful to provide further insight into the overall conclusions reported by the participants, additional analyses were performed using individual test results. Among these additional analyses were the analysis of the possible effect of primers and probes mismatches with target sites in the applicable genome segments of panel viruses on the individual NAAT performance and the analysis of the cross-reactivity of primers and probes in NAAT with viruses of the same H- or N-subtype but different host species (e.g. swine H3 and human seasonal H3 viruses).

Reported primers and probes sequences used in detection and subtyping NAAT (n=516 by 18/30 laboratories; the other laboratories did not upload NAAT primers and probes sequences for evaluation, frequently because for commercial assays this type of information is not released by the manufacturer) were aligned against the appropriate genome segments of the reference genomes of the panel viruses; matrix protein (MP) segments (generic detection) of all panel viruses together, hemagglutinin (HA) genome segments of panel viruses with specific hemagglutinin (H)-subtype (H1, H3, H5 or H7) with for panel viruses with the same H-subtype (H, H3 or H5) together and neuraminidase (NA) genome segments for the panel viruses with specific neuraminidase (N)-subtype (N1, N2, N8) and for panel viruses with the same N-subtype (N1 or N2) together.

All alignments were reviewed and refined manually, where needed, using Bioedit version 7.7.1 software. For each alignment a bitmap figure showing the position of primers and probes sets relative to the virus sequences in the alignment was created using functionality in Bioedit version 7.7.1 software. The number of mismatched nucleotides of primers and probes with each virus in the alignments were manually counted using Bioedit version 7.7.1 software, which takes into account the correct match of ambiguous nucleotides in primers and probes with the appropriate specific nucleotides covered by the ambiguity code (e.g. Y in primer or probe for match with C and T in the genome segment or I in primer or probe for match with A, C and T in the genome segment, etc.). When multiple primers or probes were used in one NAAT rather than one forward primer, probe and reverse primer with ambiguous nucleotides, the best matching primer or probe was counted. Counted mismatches in the order forward primer, probe, reverse primer were converted in a code of three numbers for each primers/probes combination of one NAAT with each panel virus, e.g. mismatched number of forward primer with virus: 0, of probe: 3, of reverse primer: 2, gives the code 032. These codes are used as a proxy for the level of mismatch in the analysis of the potential impact of mismatches on the performance of NAAT for the detection and subtyping of the virus-containing panel specimens.

Reported genome segment sequences (n=1582 by the 21 laboratories that performed sequencing and reported results based on obtained sequences) were aligned for each specific virus in the panel and for each segment separately, using Bioedit version 7.7.1 software to allow evaluation of sequencing performance and the quality of the sequences. For several laboratories, first the uploaded sequences needed further assembly into one consensus sequence because several appeared to consist of concatenated parts of the same segment that also showed overlap. Other uploaded sequences required refined alignment because of reporting of a fragmented genome segment as one sequence without gaps or nnn's (no nucleotide identified) inserted to mimic the not covered parts. After alignment of all reported genome segments of all different panel viruses with those from the expected reference sequence, a detailed assessment of consensus sequence quality of the protein coding reading frames was made. First the percentage identical nucleotides compared to the panel reference sequences was determined, in which gaps (- or n) in the reported sequences were also counted. Second, the percentage coverage of reported nucleotides compared to panel reference sequences was determined, in which gaps were not counted. Third, the percentage identical nucleotides relative to the covered region of a segment using the data from the first two analyses was determined (outcome first analysis divided by outcome second analysis). For these analyses, functionality in Bioedit version 7.7.1 software and Microsoft Excel 365 version 2402 were used. The percentage identical nucleotide data by virus for all segments was turned into a score with 0 = all segments 99.8-100% identical with the reference, 1 = at least one segment less than 99.8% identical but all more than 99.0% identical, 2 = at least one segment less than 99% identical and 3 = at least one segment no sequence available.

This scoring schedule was made based on the observation that the error rate of current sequencing protocols when comparing generated consensus sequences to reference sequences approach 0-0.2% [18,19]. The boundary of 99% was chosen as still acceptable allowing 8 nucleotides difference for the smallest non-structural protein (NS) genome segment up to 23 nucleotides for the largest polymerase basic protein 2 (PB2) genome segment, considering the potential difficulty of sequencing of the panel specimens with high depth because the panel specimens had to be heat inactivated which might have affected the integrity of the segmented genome of influenza viruses. A cumulative score by laboratory was calculated by summing the obtained scores for each virus, with a minimum score of 0 and a maximum of 30 (all 10 viruses a score of 3) for the whole genome and for the HA and NA segments only. Aligned sequences were subsequently used to determine whether with those sequences the appropriate H- and N-subtype, clade, subclade, genotype of a virus correctly could have been determined. Aligned sequences were also used to identify whether expected markers (Table 2) could have been identified.

Table 1. EEIQAP 2024 potentially zoonotic type A influenza virus panel composition and expected results for molecular detection and subtyping and clade and genogroup or genotype assignment following sequencing

Panel coding EEIQAP.1_	Virus	Expected Host	Expected A subtype and abbreviation used in the report	Expected Clade/ genogroup or genotype	Inf A MP dPCR genome copies per µl specimen	Ct value by host specificity, type/subtype and target gene							
						Generic	Human				Avian		
						Inf A MP	H3	N2	H1 pdm09	N1 pdm09	H5 setB	H7 set HA2	N1
AI24-01	A/Sandwich Tern/Netherlands/7/2022[1]	Avian[7]	H5N1 d5[6,7] H5N1st	2.3.4.4b/ AB[7]	0.08	-	-	-	-	-	-	-	-
AI24-02	A/Netherlands/11748/2022[2,3]	Swine	H1N2swine H1N2s	1C.2.2/ n.a.	2.8E+06	16.2	-	-	-	-	-	-	-
AI24-03	A/Chicken/Netherlands/EMC-3/2014[1]	Avian	H5N8	2.3.4.4c/ n.a.	8.6E+05	17.3	-	-	-	-	22.2	-	-
AI23-04	A/Netherlands/11772/2022 MP C124A+G141A[2,4]	Human	H1N1pdm09 H1N1p	6B.1A.5a.2a.1/ C.1.1	1.3E+06	22.7	-	-	24.2	24.1	-	-	34.9[8]
AI24-05	A/Sandwich Tern/Netherlands/7/2022[1]	Avian	H5N1 d3[6] H5N1st	2.3.4.4b/ AB	8.11	33.8	-	-	-	-	30.8	-	35.2
AI24-06	A/Eurasian Wigeon/Netherlands/1/2020[1]	Avian	H5N1 H5N1ew	2.3.4.4b/ C	6.2E+05	19.0	-	-	-	-	18.6	-	18.4
AI24-07	A/Sandwich Tern/Netherlands/7/2022[1]	Avian	H5N1 d1[6]	2.3.4.4b/ AB	8.1E+05	18.2	-	-	-	-	18.4	-	18.0
AI24-08	A/Mallard Duck/Netherlands/2/2019[1]	Avian	H7N2	1.1/ n.a.	6.6E+03	23.9	-	-		-	-	23.5	-
AI24-09	A/Sandwich Tern/Netherlands/7/2022[1]	Avian	H5N1 d2[6] H5N1st	2.3.4.4b/ AB	81.08	30.6	-	-	-	-	29.3	-	31.2
AI24-10	A/Netherlands/10534/2023[2,3]	Swine	H1N1swine H1N1s	1C.2.2/ n.a.	7.6E+04	20.6	-	-	-	-	-	-	26.2[8]
AI24-11	No virus	n.a.	n.a.	n.a.	n.a.	-	-	-	-	-	-	-	-
AI24-12	A/Netherlands/11715/2022 A12T+C136T[2,4]	Human	H3N2human H3N2h	3C.2a1b.2a.2a.1/ G.1.1	7.4E+05	22.1	22.0	24.2	-	-	-	-	-
AI24-13	A/Swine/France/59-150357/2015[5]	Swine	H3N2swine H3N2s	1970.1/ n.a.	1.2E+06	20.6	-	-	-	-	-	-	-
AI24-14	A/Black-headed Gull/Netherlands/5/2023[1]	Avian	H5N1 H5N1bhg	2.3.4.4b/ BB	5.0E+06	15.3	-	-	-	-	15.3	-	15.1
AI24-15	A/Sandwich Tern/Netherlands/7/2022[1]	Avian[7]	H5N1 d4[6,7] H5N1st	2.3.4.4b/ AB[7]	0.81	-	-	-	-	-	-	-	-

n.a. = not applicable; '-' in a cell means tested with a negative result; Inf A = Type A influenza virus; Hx = the hemagglutinin (H)-subtype with x a number; Nx = the neuraminidase (N)-subtype with x a number; MP = the matrix protein genome segment; HA = the hemagglutinin genome segment ; dPCR = digital polymerase chain reaction.

1. *From the Erasmus MC collection, Rotterdam, the Netherlands, courtesy of Ron Fouchier and Oanh Vuong.*
2. *From the RIVM collection, Bilthoven, the Netherlands, courtesy of Adam Meijer and Gabriel Goderski.*
3. *Originating from recent human infections with swine influenza virus in the Netherlands and therefore these viruses are officially labelled A(H1N2)v and A(H1N1)v respectively. However, for the purpose of the panel they should be recognized as originating from swine host and are expected to be reported as A(H1N2)swine and A(H1N1)swine respectively.*

4. *Indicated are recently emerging nucleotide mutations in the MP genome segment of these viruses that have led to underreporting in various assays [20,21 and personal communication UKHSA, WHO National Influenza Centre, London, United Kingdom. The positions of the mutations are relative to the first ATG of the M1/M2 genes in the MP genome segment.*
5. *From the collection of Institut Pasteur, Paris, France, courtesy of Étienne Simon-Lorière.*
6. *H5N1 d1-d5 indicates a dilution series of the same virus A/Sandwich Tern/Netherlands/7/2022 starting with highest viral load in d1, d2 is a 1:10,000-fold dilution of d1, d3-d5 are serial 10-fold dilutions starting from d2. The shown Ct values for all dilutions are average values derived from triplicate testing; for all specimens with a Ct value three out of three replicates were found PCR positive.*
7. *H5N1 dilutions d4 and d5 are below the 95% Limit of Detection (LoD95%) and are expected to become negative in detection and subtyping polymerase chain reaction (PCR)/nucleic acid amplification test (NAAT), unless the used assays have a higher sensitivity than the reference assays used at RIVM that returned for three out of three replicate detection and subtyping assays no presence of virus. Therefore the indicated expected host, subtype and clade/genogroup or genotype for specimens EEIQAP-AI24-01 and EEIQAP-AI24-15 are theoretical. Specimen EEIQAP-AI24-07 with highest viral load is expected to be successfully sequenced and analysed.*
8. *The primers and probe of the used avian N1 subtyping reverse transcription semi-quantitative PCR (RT-qPCR) are known to cross-react with high Cycle Threshold (Ct) values and low fluorescence with the genome segments for the neuraminidase of the human A(H1N1)pdm09 and swine A(H1N1) viruses in the panel.*

Table 2. EEIQAP 2024 potentially zoonotic type A influenza virus panel composition with expected to be identified markers in the viruses present in the panel

Panel coding EEIQAP.1_	Virus	A subtype	Markers present								
			Antiviral reduced susceptibility			Mammalian adaptation/virulence					
			MP	NA	PA	PA	PB1	PB2	HA	NP	NS
AI24-01/05/07/09/15[1]	A/Sandwich Tern/Netherlands/7/2022	H5N1	none	none	none	none	none	none	139P shift[3]	none	none
AI24-02	A/Netherlands/11748/2022	H1N2v	26I;27A;31N	none	none	409N shift[2]	none	292T shift; 607V virulence; 661T shift; 701N shift, virulence[2]	173N shift[2]	none	none
AI24-03	A/Chicken/Netherlands/EMC-3/2014	H5N8	31N	none	none	none	none	none	none	none	none
AI23-04	A/Netherlands/11772/2022	H1N1pdm09	31N	none	none	none	none	none	none	none	none
AI24-06	A/Eurasian Wigeon/Netherlands/1/2020	H5N1	none	none	none	none	none	none	139P shift[3]	none	none
AI24-08	A/Mallard Duck/Netherlands/2/2019	H7N2	none	none	none	none	none	none	none	none	none
AI24-10	A/Netherlands/10534/2023	H1N1v	31N	none	none	409N shift[2]	none	701N shift, virulence[2]	173N shift[2]	none	NS1-215T shift[2]
AI24-12	A/Netherlands/11715/2022	H3N2	31N	none	none	none	none	none	none	none	none
AI24-13	A/Swine/France/59-150357/2015	H3N2	26I;31N	none	none	409N shift[2]	none	701N shift, virulence[2]	175N shift[2]	none	none
AI24-14	A/Black-headed Gull/Netherlands/5/2023	H5N1	none	none	none	none	none	none	none	33I shift[3]	none

Influenza virus genome segments are abbreviated: MP = matrix protein; NA = neuraminidase; PA = polymerase acidic protein; PB1 = polymerase basic protein 1; PB2 = polymerase basic protein 2; HA = hemagglutinin; NP = nucleoprotein; NS = non-structural protein. HA numbering includes the signal peptide.

1. *Although all indicated specimens of the dilution series contain the same virus and specimens EEIQAP-AI24-05, -07 and -09 are expected to be found PCR/NAAT positive, sequencing of the whole genome is expected to be successful for at least specimen EEIQAP-AI24-07 as this specimen has highest viral load in the dilution series.*
2. *Substitutions compared to likely avian predecessor viruses and naturally present in swine influenza viruses, that have been associated previously with a shift from avian to human efficient replication and/or enhanced virulence in humans.*
3. *Substitutions that have been associated previously with shift from avian to human efficient replication.*

3 Results

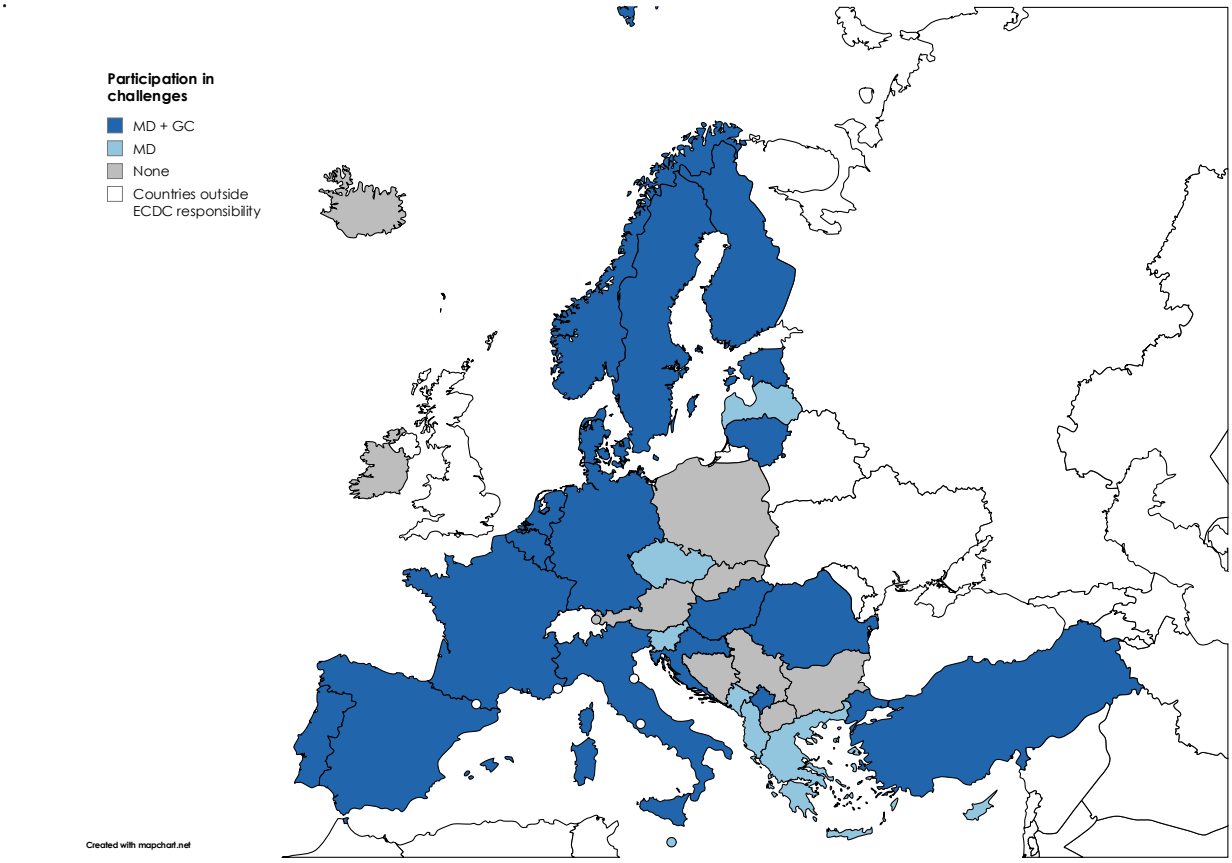
3.1 Participation

Invitations for participation were sent from ECDC to:

- Operational Contact Points for Influenza (Microbiology), National Coordinators and National Focal Points for Influenza on 1 March 2024;
- Operational Contact Points for Influenza (Microbiology) for EU/EEA countries and Western Balkan and Türkiye Contact Points for Operations (Influenza) and National Focal Points for Viral Respiratory Diseases and National Coordinators for EU/EEA countries and Western Balkan and Türkiye Observer National Focal Points for Viral Respiratory Diseases and National Correspondents on 12 March 2024;
- (reminder) Operational Contact Points for Influenza (Microbiology) for EU/EEA countries and Western Balkan and Türkiye Contact Points for Operations (Influenza), National Focal Points for Viral Respiratory Diseases and National Coordinators for EU/EEA countries and Western Balkan and Türkiye Observer National Focal Points for Viral Respiratory Diseases and National Correspondents on 15 March 2024.

Panel distribution from QCMD started 20 May 2024. Return of results was closed second week of August 2024, to allow return of results by the last laboratories that received the panel delayed due to customs challenges. In total 27 of 37 (73%) invited countries participated with 30 WHO National Influenza Centres and/or National Influenza Reference Laboratories (Figure 1; detailed list in Annex 1). Thirty laboratories participated in the Molecular Diagnostics (MD) part with 32 datasets (1 laboratory sent 3 datasets) and 21 laboratories participated in the MD and Genetic Characterisation (GC) part with 21 datasets (Figure 1; detailed list in Annex 2). One laboratory did not receive specimen EEIQAP.1_AI24-06 H5N1ew and another laboratory did not receive specimen EEIQAP.1_AI24-08 H7N2 due to errors in panel box preparation.

Figure 1. Countries that participated in Molecular Diagnostics (MD) and Genetic Characterisation (GC) of the EEIQAP 2024 potentially zoonotic type A influenza virus panel (light blue MD, dark blue both MD and GC) and non-participating countries (grey)



From France, Greece and the Netherlands, two national influenza reference laboratories participated in each. No colour indicates countries outside the EU/EEA and Western Balkan countries and Türkiye, for which this EEIQAP 2024 was not intended.

3.2 Molecular Diagnostics

3.2.1 Type A influenza virus detection

The vast majority (29/32) of the 32 datasets on generic detections used the M genome segment as a target, of which three additionally the HA and one additionally the HA and NA genome segments, while one used only HA, one only NP and one HA and NA as target genome segment. The large variety of nucleic acid extraction and amplification equipment and extraction and amplification reagents used by the participants is listed in Annex 3.

All virus containing specimens in the 32 datasets that reported for specimens containing specific virus strains and for the highest concentration of the dilution series of A/Sandwich Tern/Netherlands/7/2022 H5N1 (specimen EEIQAP.1_AI24-07; H5N1st_d1) were tested positive in generic detection of type A influenza virus (details by laboratory in Annex 4). All 32 datasets reported the no-virus containing specimen as negative (not shown). The dilution series of A/Sandwich Tern/Netherlands/7/2022 H5N1 shows a higher Limit of Detection (LoD) for 12/32 (38%) datasets when compared with the expected result that specimen EEIQAP.1_AI24-05 H5N1st_d3 with 8.11 genome copies/µl specimen should have been found positive (Table 1, footnotes 6 and 7; Figure 2). One of these datasets reported also the 81.08 genome copies/µl specimen negative, suggesting a quite high LoD for this generic type A influenza virus detection NAAT (Annex 4). One other dataset with negative result for specimen EEIQAP.1_AI24-05 H5N1st_d3 in the generic type A influenza virus detection NAAT reported the specimen positive in the H5-subtyping NAAT, suggesting generic detection of type A influenza virus and H-subtyping were performed at the same time and not sequentially performing subtyping on type A influenza virus positive specimens only. Three datasets reported specimen EEIQAP.1_AI24-15 H5N1st_d4 (0.81 genome copies/µl specimen) positive and one of these datasets also reported specimen EEIQAP.1_AI24-01 H5N1st_d5 (0.08 genome copies/µl specimen) positive. Sample volume equivalent of extracted nucleic acid put in NAAT seems not to have an effect on detection of type A influenza virus in specimen EEIQAP.1_AI24-05 H5N1st_d3 (Figure 3).

A variety of NAAT with specific primers and probes have been used. Thirteen laboratories reported the sequences of the primers and probes that were used to target the MP genome segment in 14 different NAAT (Annex 5). The primers and probes are located in two regions at the start of the MP genome segment, with some variation in the exact position, primer or probe length and included ambiguity positions to cover variation in as many type A influenza viruses as possible (Annex 5), the latter either by combining primers or probes with different nucleotides at certain positions in one NAAT or by inclusion of ambiguous nucleotides.

The overall primer and probe match with all virus strains included in the panel is rather variable, from perfect match up to two mismatches for each of the forward primer and/or reverse primer and up to one for the probe (Figure 4). The detected mismatches appeared to have no impact on the detection of the virus strains in the panel at the concentrations included in the panel (Annex 4), apart from A/Sandwich Tern/Netherlands/7/2022 H5N1st for which multiple concentrations were included in the panel. However, in addition no correlation of MP primer and probe combinations with the performance in the dilution series of A/Sandwich Tern/Netherlands/7/2022 H5N1st in the panel was found, despite different levels of nucleotide mismatch with the virus target region of the MP genome segment (Figure 5). Taken together, this illustrates that in principle the variety of primers and probes for generic detection of type A influenza viruses being used in the applied NAAT perform largely equally on the variety of viruses in the panel, but that some differences in the LoD exist, likely due to local setup of NAAT, including used equipment and reagents (Annex 3) and amplification temperature cycling protocol (not asked for to report).

Figure 2. Correlation of MP genome segment copies in each panel specimen of the dilution series of A/Sandwich Tern/Netherlands/7/2022 H5N1st with positive or negative result in NAAT and the associated reported Ct value

Negative results are depicted as Ct 50. Specimens with 8.1E+05 through 8.11 copies per µl specimen were expected to be detected.

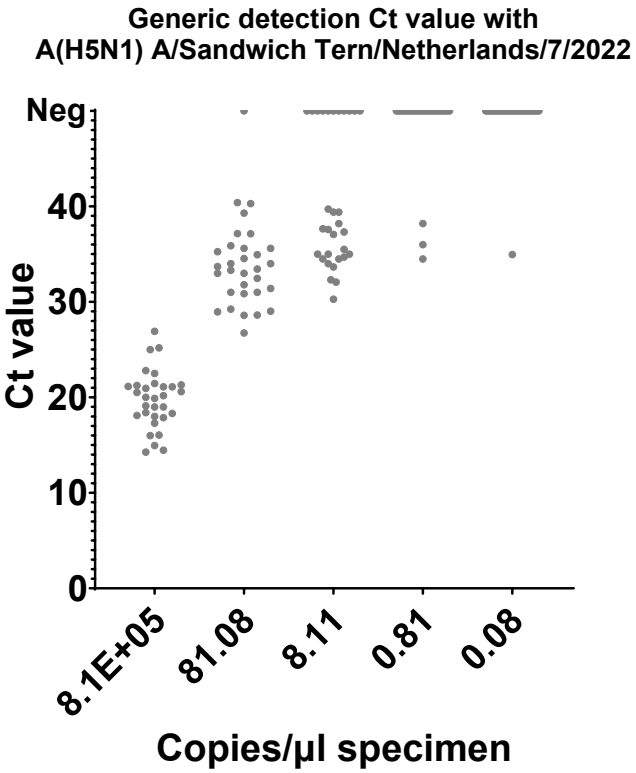


Figure 3. Correlation of specimen volume equivalent input in NAAT of specimen EEIQAP.1_AI24-05 containing 8.11 MP genome segment copies of A/Sandwich Tern/Netherlands/7/2022 H5N1st per µl specimen with positive or negative result in NAAT

The black horizontal lines indicate the median specimen volume input in NAAT.

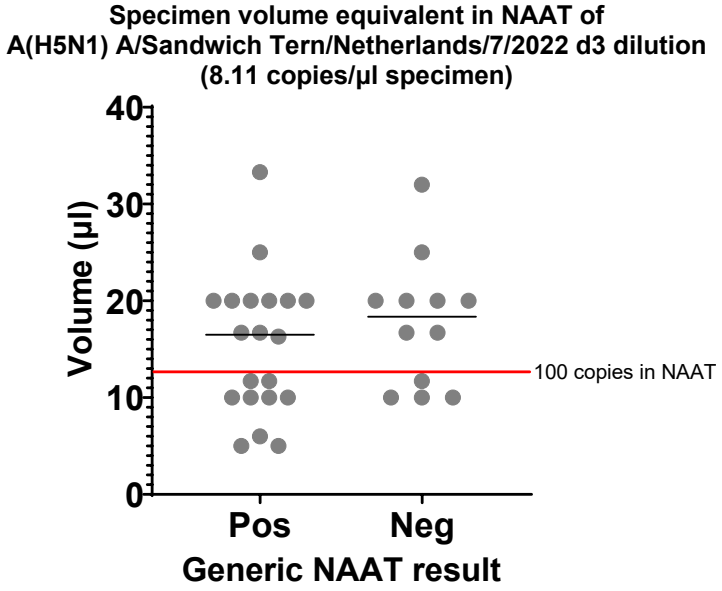


Figure 4. Overview of the overall mismatch profile codes of primers and probe combinations targeting the MP genome segment of 14 NAAT with all 10 virus strains in the panel

The code is composed of nucleotide difference ranges with the MP genome segments of all 10 virus strains in the panel for forward primer (F), probe (Pr) and reverse primer (R) separated by semicolon.

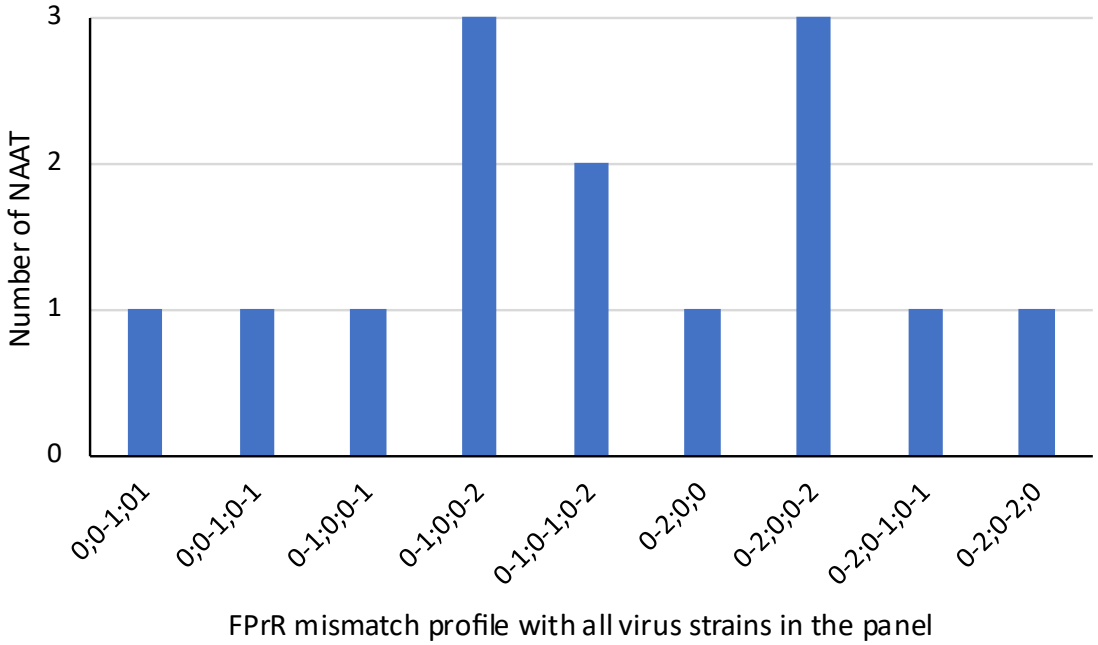
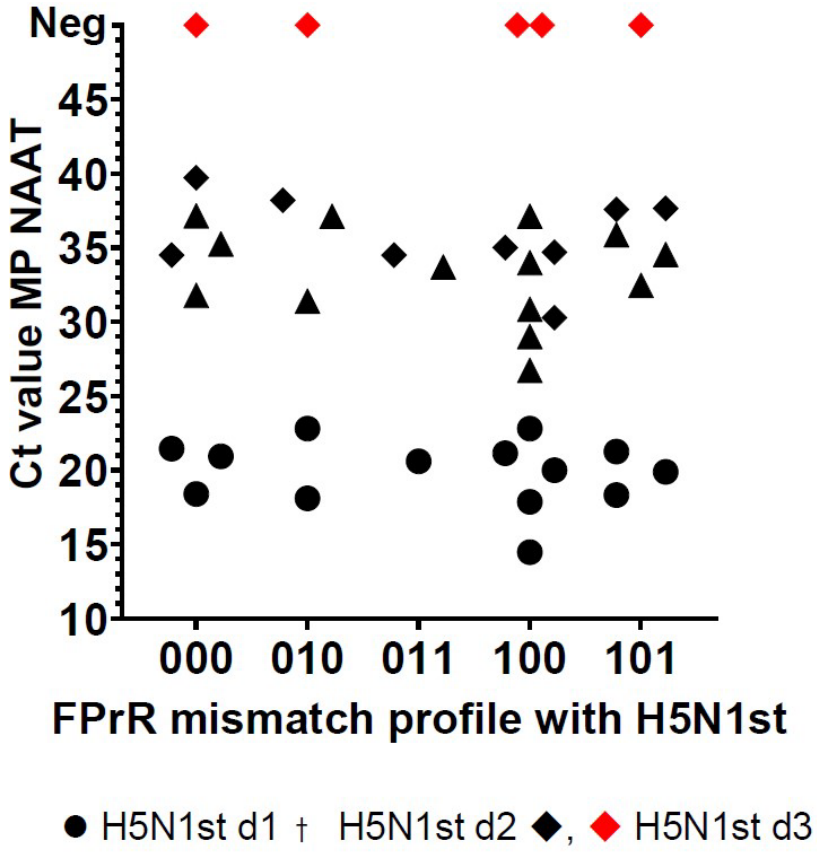


Figure 5. Correlation of MP primer probe match with detection of panel virus A/Sandwich Tern/Netherlands/7/2022 H5N1st by Ct value

Only 8.1E+05 (d1), 81.08 (d2) and 8.11 (d3) copies per µl specimens of the dilution series are included. Negative results are depicted as Ct 50. The mismatch profile code is composed of nucleotide difference of forward primer (F), probe (Pr) and reverse primer (R) with the A/Sandwich Tern/Netherlands/7/2022 H5N1st virus.



3.2.2 Influenza A virus subtyping by NAAT

All 30 participants reported in the answers to the additional questionary questions that they had NAAT capable of subtyping influenza A viruses readily available for 4-12 (median 5) H-subtypes (Figure 6) and 1-14 (median 2) N-subtypes (Figure 7, details by laboratory in Annex 6). The variety of NAAT that participants actually used for the reported results, limited to those used for identification of the H- and N-subtypes of the viruses included in the panel, is summarised in Figure 8 and in detail shown in Annex 7 by individual laboratory and dataset. All 30 laboratories have available and used one or more H-subtyping NAAT (median 4; range 4-6) suitable to determine the H-subtype of one or more of the virus strains in the panel. Although 27/30 (90%) laboratories reported to have one or more N-subtyping NAAT readily available to be able to N-subtype one or more of the virus strains in the panel (median 2; range 1-10 N-subtyping NAAT per laboratory), only 16/30 (53%) laboratories actually used one or more of these N-subtyping NAAT (median 3; range 1-7) to test the panel specimens.

Subtyping results are summarised in Figure 9 and in detail shown in Annex 7. All laboratories determined the H-subtype for H1N1pdm09 (H1N1p), H3N2human (H3N2h) and H5Nx viruses correctly by NAAT. For the N-subtype when such a NAAT was used from the repository of the laboratory (with N1generic, N1pdm09, N1avian, N2generic, N2human and/or N8avian specificity) in the majority (14/16; 88% of laboratories) also the correct N-subtype was reported for these viruses. The H7-subtype of the avian influenza virus H7N2 panel virus was correctly identified using NAAT by 86% (25/29) of the laboratories. Of the laboratories using an N2 subtyping NAAT (with N2human or N2generic specificity) (n=14) on this virus, only one identified the N2 subtype using an NAAT with N2generic specificity. Much less were able to identify the H-subtype of the swine influenza viruses H1N1 (H1N1s) (5/30; 17%) and H1N2 (H1N2s) (6/30; 20%) by NAAT. Of the laboratories using an N1 subtyping NAAT (with N1generic, N1pdm09, N1avian or N1swine specificity), or N2 subtyping NAAT (with N2generic, N2human, or N2swine specificity) on these viruses, the N-subtype of these viruses was identified by 10/14 (71%) and 2/15 (13%) laboratories, respectively. However, three of the laboratories that detected the N1-subtype of the swine H1N1 virus reported 'A no subtype', highly likely because no H-subtype could be determined by applied H-subtyping NAAT and the N1-subtyping NAAT had N1pdm09 or N1avian specificity.

For the H3N2 swine influenza virus (H3N2s), 57% (17/30) of laboratories were able to provide the H3-subtype, highly likely due to higher cross-reactivity of used H3-specific primers and probes with swine H3N2 influenza virus than used H1-specific primers and probes with swine H1Nx viruses in the panel (see below). Of the 15 laboratories using an N2 subtyping NAAT (with N2generic, N2human or N2avian specificity) on this virus, two (13%) identified the N2 subtype of which one however reported the subtype of this virus by NAAT as 'Unknown', highly likely because no H-subtype was obtained.

Several laboratories used multiple NAAT with the same originating host specificity (Figure 8). Nine laboratories used two H5-specific assays; eight had both assays positive with all four H5 virus strains in the panel, whereas one lab had one assay positive with all four H5 virus strains and the other assay only positive with the avian influenza virus H5N8. One lab with four H5-specific assays had all four assays positive with all four H5 viruses. One lab with two H7-specific assays had one positive and one negative with the H7N2 virus in the panel. One lab with three H7-specific assays had all positive with the H7N2 virus in the panel. Of two laboratories with two H1pdm09-specific assays, one had both assays positive with the H1N1pdm09 virus in the panel and the other lab had one assay positive and one assay negative with the H1N1p virus in the panel. One laboratory with two H3human-specific assays had both positive with the H3N2h virus in the panel. For these multiple NAAT with the same originating host specificity per laboratory, primers and probes were different as could be assessed for 17/30 laboratories that uploaded primers and probes (Annex 8). This primers and probes analysis showed that the location of primers and probes of the used H- and N-subtyping NAAT on the target genome segment sequences and the length of the amplicons was variable, although some were roughly identical between several laboratories taken from the same source (e.g. CDC Atlanta) but with some laboratory specific adaptations, likely because of observed mismatches that emerged since the development of such primers and probes.

The specificity of primers and probes sets reported by the laboratories were analysed for cross-reactivity with included panel virus strains for those virus strains that had the same H- and/or N-subtype (Figure 10). Especially, NAAT subtyping with H3human (H3h) and H3generic (H3g) (as expected) specificity showed cross-reactivity with human and swine H3N2 viruses in the panel. Similarly, this was also especially seen for N1-subtyping NAAT for host-specific as well as N1generic NAAT (as expected) and cross-reactivity with viruses with human N1pmd09 (N1p), swine N1 (N1s) and avian N1 (N1a) subtypes in the panel. For H1 and N2 subtyping NAAT only one and two assays showed cross-reactivity. Most of the cross-reactivity of H3human-specific and H3generic NAAT with the human seasonal and swine H3N2 viruses in the panel resulted in a higher Ct value and larger Ct difference (delta Ct) between the subtyping NAAT and the generic type A influenza virus detection NAAT for the swine H3N2 virus compared to the human seasonal H3N2 virus in the panel (Figures 10-12). This higher Ct value and difference in delta Ct is roughly but not absolutely related to the difference in the level of mismatch of primers and probes with the human and swine H3N2 viruses in the panel (Figures 11 and 12). This phenomenon can be used when evaluating testing results, triggering additional testing (e.g. sequencing) when a larger Ct difference is observed between subtyping NAAT and generic influenza A virus NAAT than expected.

Similar to the MP primer and probe analysis for the detection of H5N1st virus in the dilution series (Figure 5), the analysis of primers and probes targeting the H5 subtype hemagglutinin showed that there is no clear relationship between number of mismatches in primers and/or probes and the ability to subtype the H5N1st virus as H5 in the specimens that contained an amount of virus expected to be detected and subtyped (Figure 13). For other

subtyping targets a similar absence of clear association of higher number of mismatches of primers and/or probes with the target gene with a lower ability to H- and N-subtype a virus was seen (not shown). Even, there was no clear trend towards higher Ct values with increasing number of mismatches, as shown for H5-subtyping in Figure 13 for one virus strain. However, as shown for the H3-subtyping analysis with two different H3N2 viruses in Figures 11 and 12, a rough association between level of primers and probes mismatches and Ct value can be seen. The different background sequence of both H3N2 viruses likely plays a role in that [22,23].

Figure 6. H-subtyping NAAT reported as readily available (or used) in the 30 participant laboratories by H-subtype and specificity (a = avian; g = generic; h = human; p = pdm09; pp = pre-2009 pandemic; s = swine)

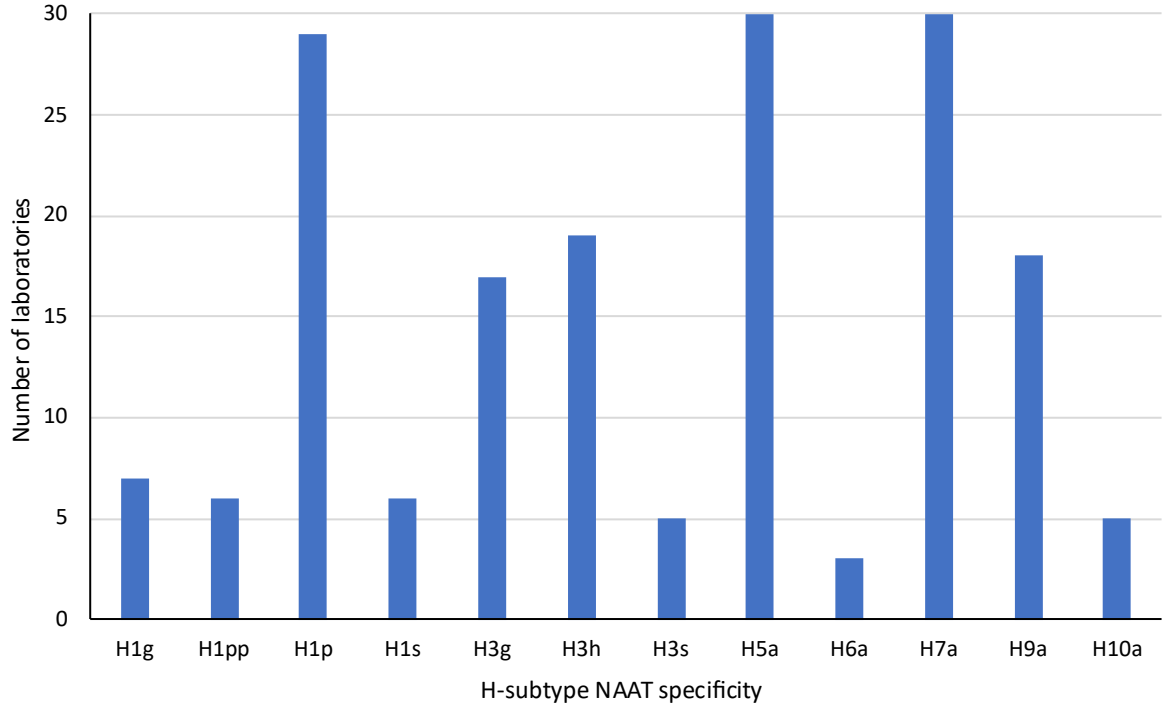


Figure 7. N-subtyping NAAT reported as readily available (or used) in the 30 participant laboratories by N-subtype and specificity (a = avian; g = generic; h = human; p = pdm09; pp = pre-2009 pandemic; s = swine)

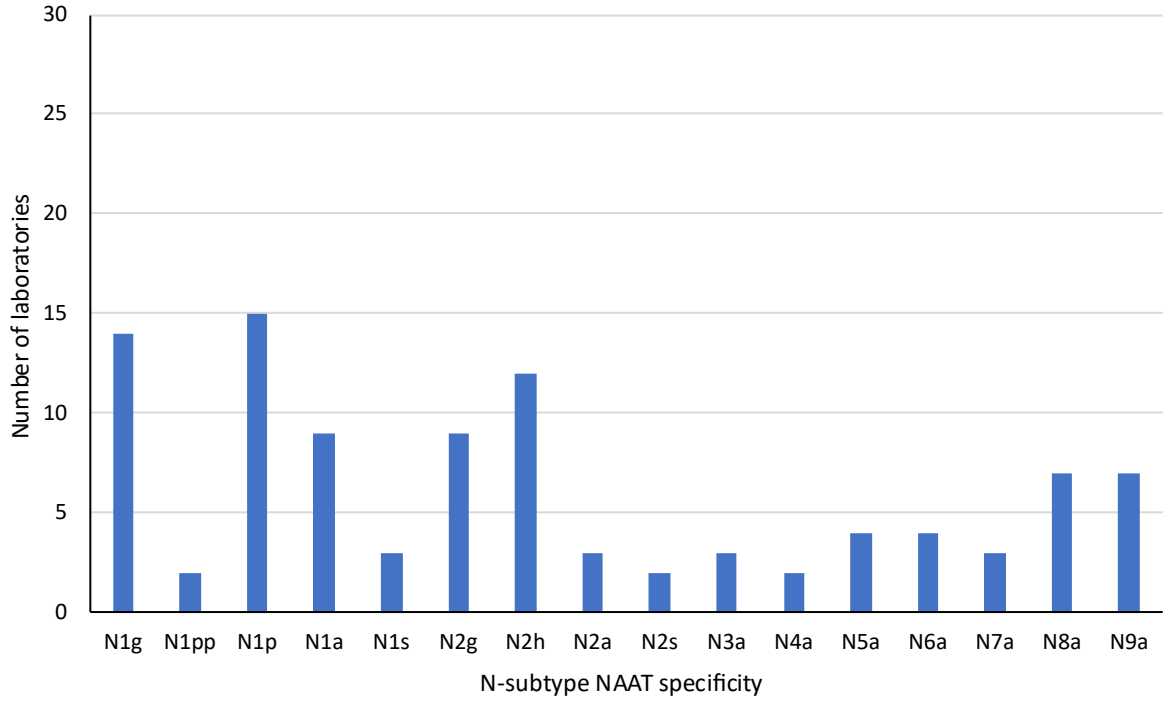
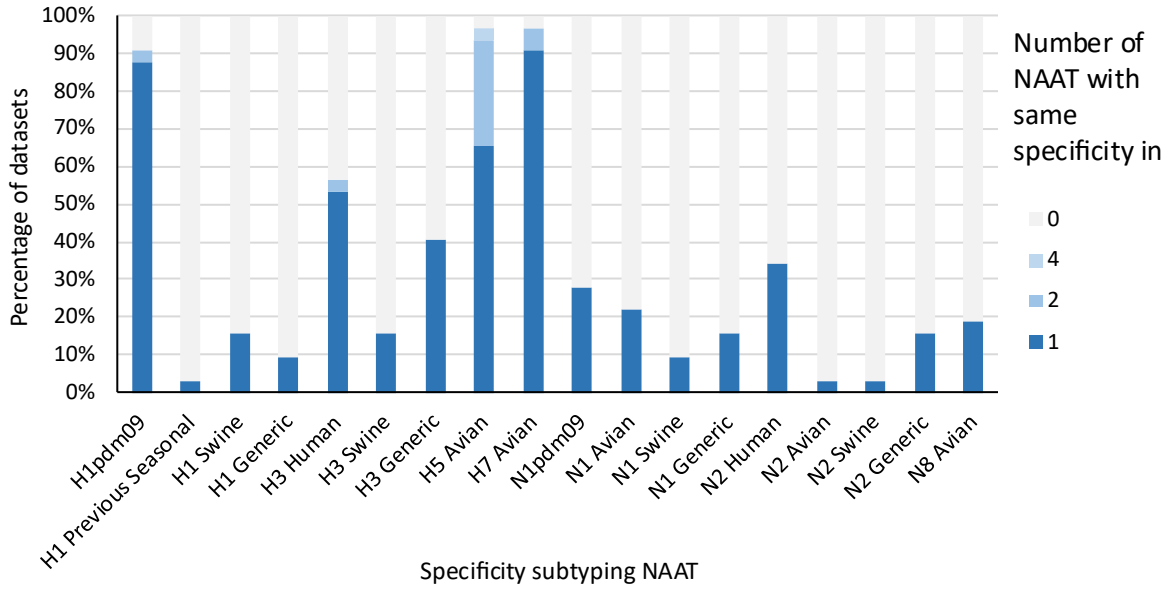
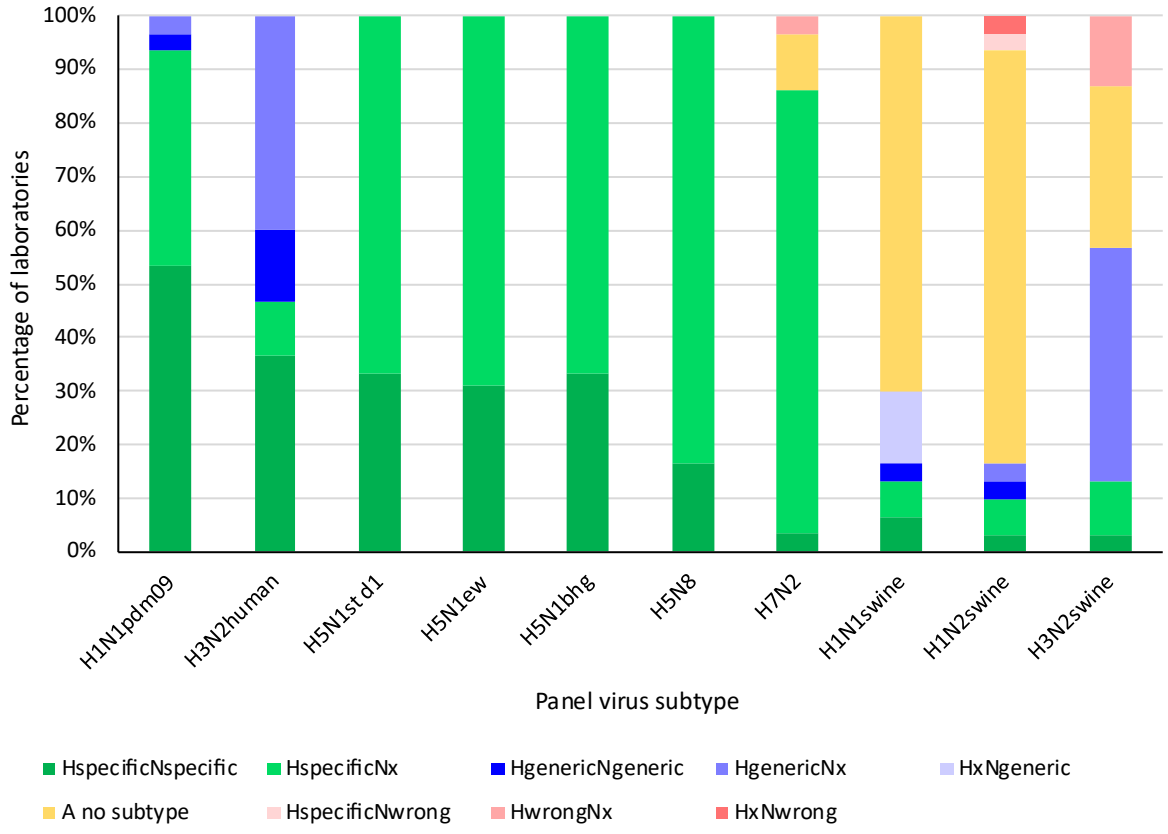


Figure 8. Percentage H- and N-subtyping NAAT used in the 32 datasets for identification of the H- and N-subtypes of the viruses included in the panel



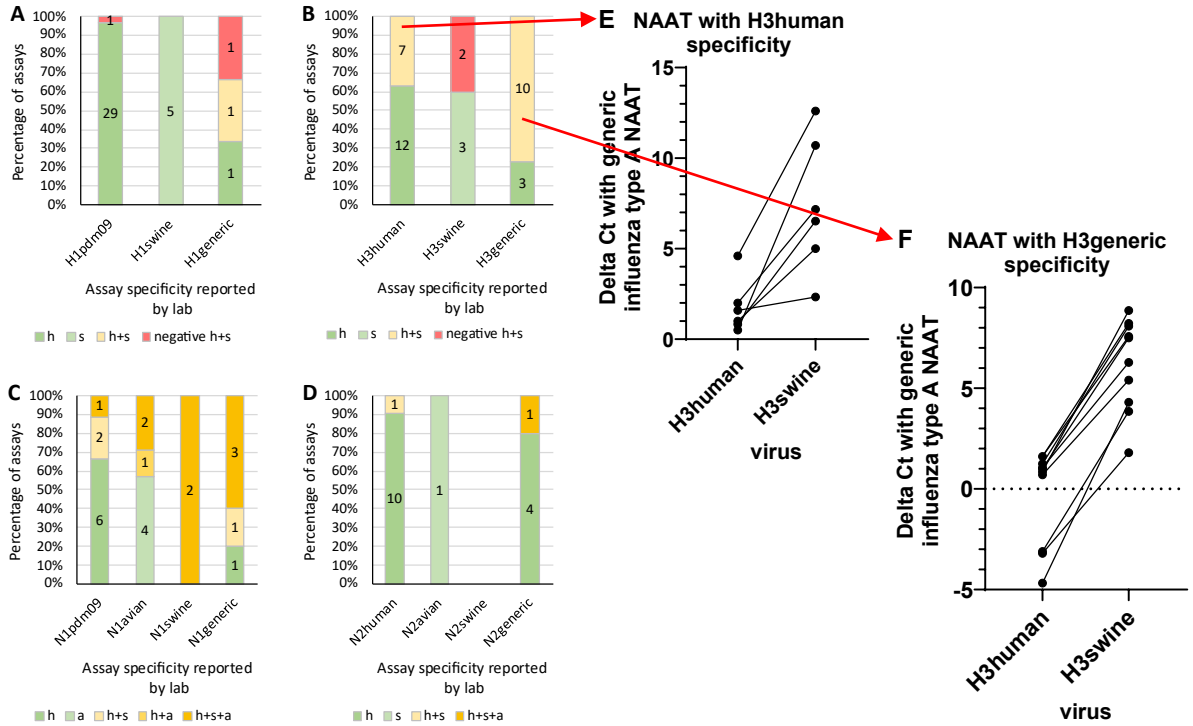
Indicated are the H- and N-subtype followed by the specificity as reported by the participating laboratories.

Figure 9. Subtyping by NAAT for 30 laboratories except for H5N1ew and H7N2 29 laboratories



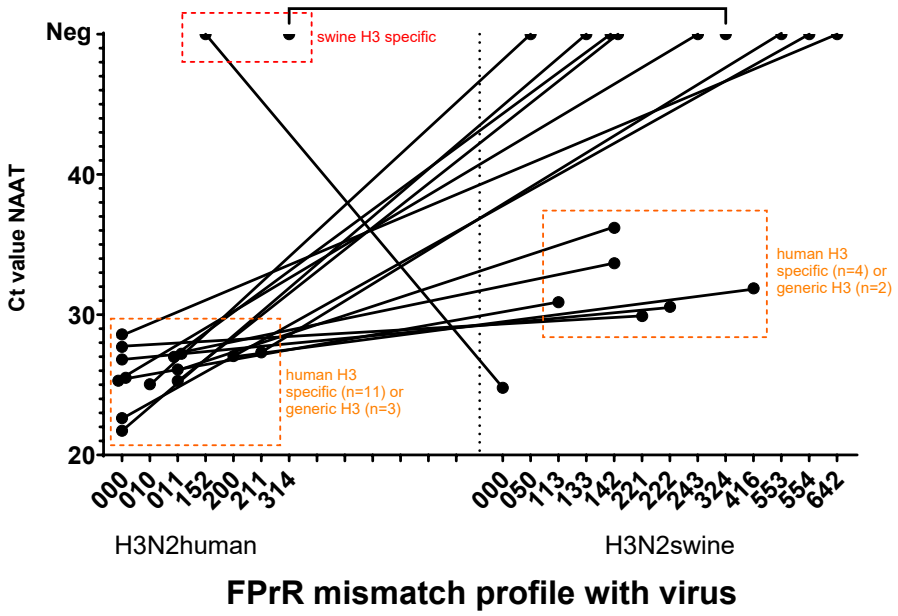
For one laboratory with three datasets reported a single aggregated result is shown. Specific = fully correct subtype for H or N including species, e.g. H1pdm09 or N1swine. Generic = correct generic subtype for H or N, e.g. H1 for virus with H1pdm09 or H1swine. Wrong = incorrect subtype for H or N, e.g. H3human for virus with H3swine or H5 for virus with H3swine. Hx or Nx = H-subtype or N-subtype not known. A no subtype = specimen only found positive in NAAT for generic detection type A influenza virus and negative for all subtyping NAAT used by a laboratory (Annex 7).

Figure 10. Reactivity of H- and N-subtype specific assays with panel viruses with the same H- or N-subtype but different originating species (A, B, C, D), and delta Ct between generic type A influenza virus detection NAAT and H-subtype NAAT for H3human (E) and H3generic (F) reported specificity with H3human and H3swine influenza viruses in the panel



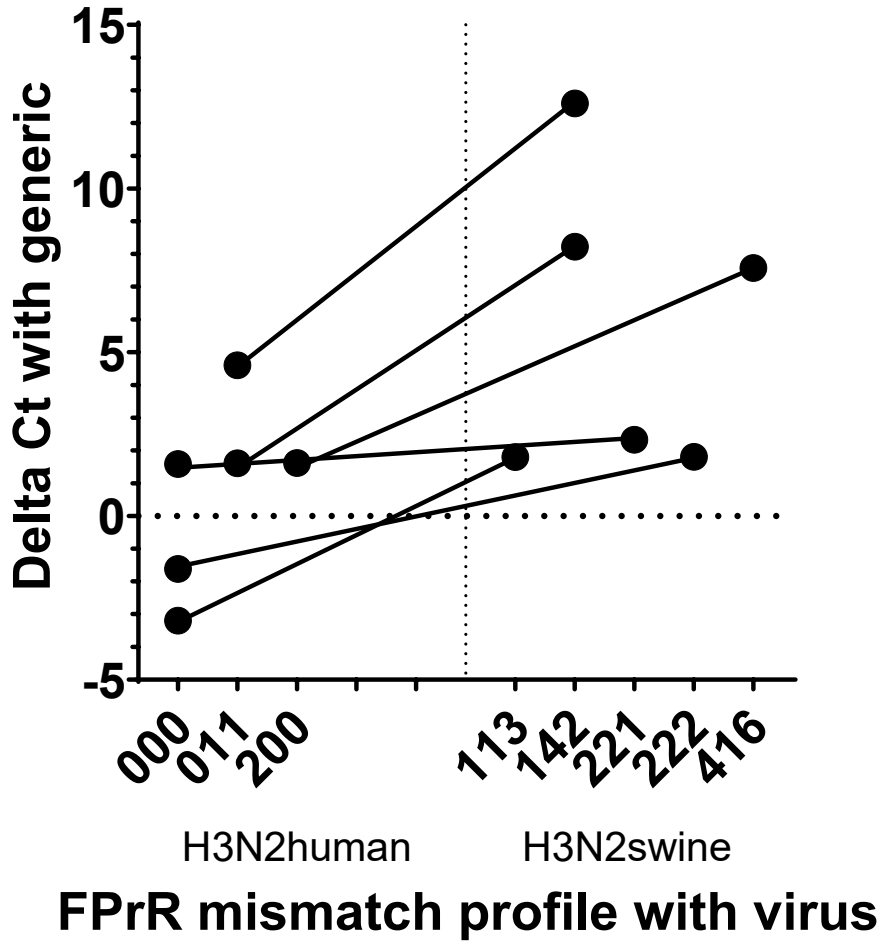
In the figures A-D, the category X-axis shows the by laboratories reported specificity of the NAAT by H- or N-subtype and originating species, human (h), swine (s) or avian (a). The bars show the NAAT positivity with species specific virus only in shades of green, human virus (h), swine virus (s) or avian virus (a); with viruses originating from different species in shades of yellow, h+s, h+a and h+s+a viruses, indicating cross-reactivity of the NAAT on the x-axis; or negative for h+s viruses in red. In the figures E an F, connected dots represent the results with human and swine virus of NAAT with H3human or H3generic specificity.

Figure 11. Correlation of H3 primer probe match with subtyping of human seasonal H3N2 (H3N2human) and swine H3N2 (H3N2swine) panel viruses by Ct value of the subtyping NAAT



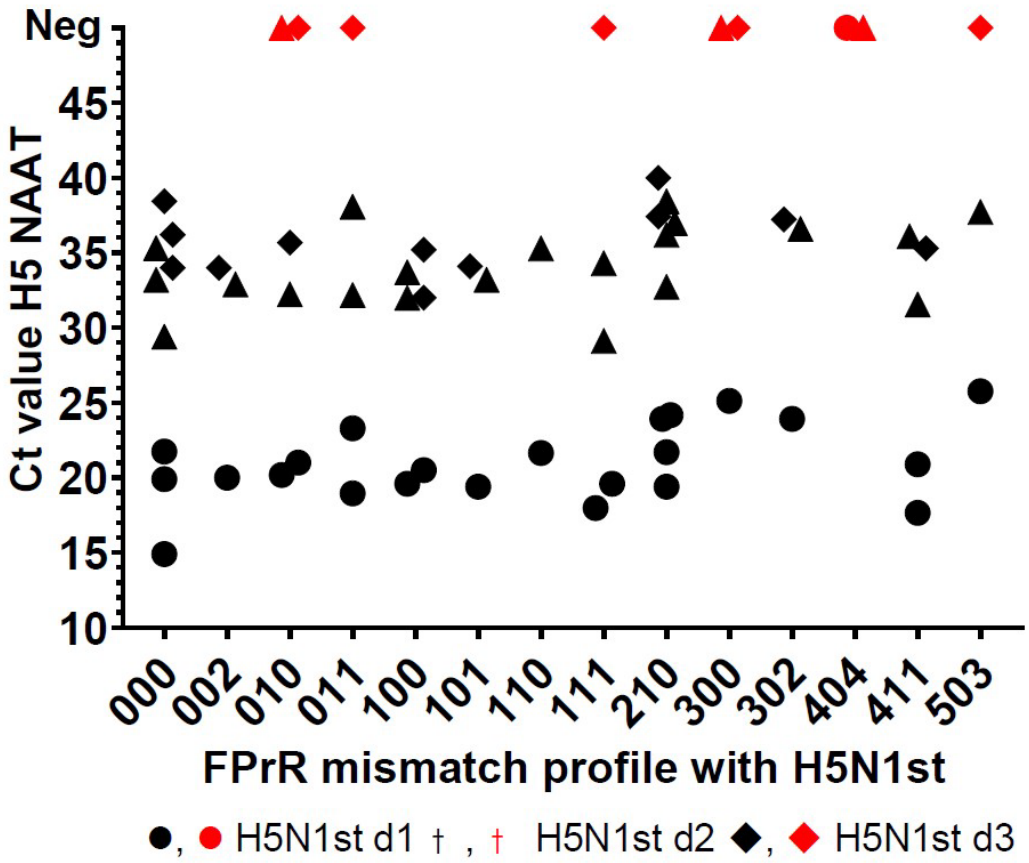
The mismatch profile code per NAAT is composed of nucleotide difference of forward primer (F), probe (Pr) and reverse primer (R) with the specified virus. Each pair of connected dots represents one H3-subtyping NAAT that is used to subtype the H3N2human and H3N2swine viruses in the panel.

Figure 12. Correlation of H3 primer probe match with subtyping of human seasonal H3N2 (H3N2human) and swine H3N2 (H3N2swine) panel viruses by Ct value difference of the subtyping NAAT with the generic influenza A virus detection NAAT



The mismatch profile code is composed of nucleotide difference of forward primer (F), probe (Pr) and reverse primer (R) with the specified virus. Each pair of connected dots represents one H3-subtyping NAAT that is used to subtype the H3N2human and H3N2swine viruses in the panel.

Figure 13. Correlation of H5 primer probe match with H-subtyping of panel virus A/Sandwich Tern/Netherlands/7/2022 H5N1st by Ct value



Only 8.1E+05 (d1), 81.08 (d2) and 8.11 (d3) copies per µl specimens of the dilution series are included. The mismatch profile code is composed of nucleotide difference of forward primer (F), probe (Pr) and reverse primer (R) with the A/Sandwich Tern/Netherlands/7/2022 H5N1st virus. The number of markers in specific mismatch profiles is not identical for each dilution because a number of laboratories did not subject a specimen found negative in generic detection of type A influenza virus to subtyping.

3.3 Sequencing and sequence analysis

3.3.1 Methodologies used

All 21 laboratories that performed sequencing reported using Zhou et al., 2009 [24] UNI12 and UNI13 primers or a variation of them. Platforms used were: Next Generation Sequencing (NGS)/Illumina (n=10); NGS/Nanopore (n=8); NGS/Illumina and Nanopore (n=1); NGS/Ion Torrent (n=1); Sanger and NGS/Ion Torrent (n=1). Pipelines used to turn raw sequencing data into consensus sequences were: In-house developed (n=10), Iterative Refinement Meta-Assembler (IRMA) [25] (n=10), QIAGEN CLC genomics workbench (n=2), and others. For HA-clade, HA-subclade, H5N1-genotype assignment tools a variety of tools were used, several of which were mentioned as an option in the Instructions document sent with the panel (see Annex 14): Nextclade bioinformatics sequence analysis tool [26] (N=18), Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (n=16), Flusurver bioinformatics sequence analysis tool (n=6), bioinformatics tools inside Global Initiative on Sharing All Influenza Data (GISAID) (n=2), Others (e.g. INSaFLU bioinformatics sequence analysis tool [27]) and In-house developed tools. For amino acid substitution analysis related to mammalian adaptation and antiviral susceptibility the following tools were used: Flusurver bioinformatics sequence analysis tool (n=16), Nextclade (n=3) and In-house developed methods based on reference lists (see Annex 14).

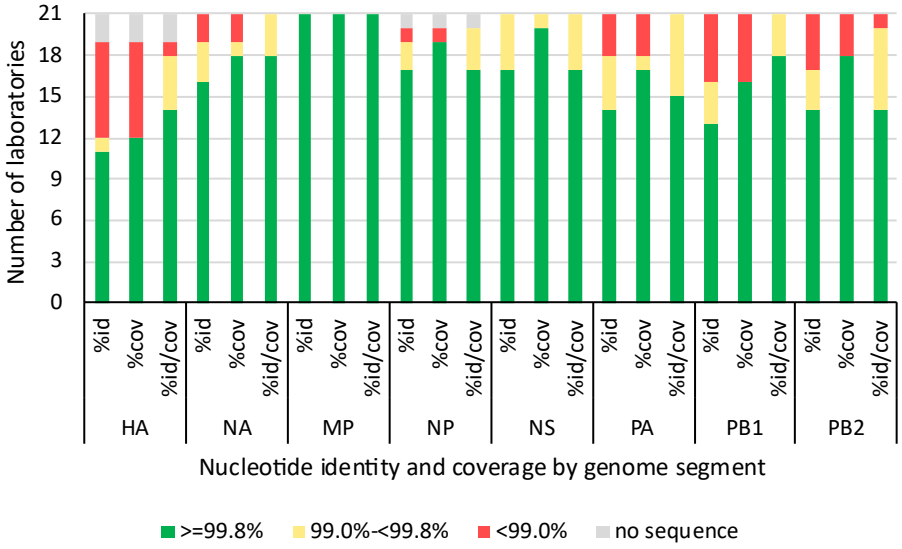
3.3.2 Validation of reported sequences

To be able to interpret and evaluate the clade, subclade, genotype and amino acid substitutions reported by participants, laboratories were asked to report the consensus sequences they obtained. A detailed assessment of consensus sequence quality of the protein coding reading frames is summarised in Figure 14 with all details by laboratory in Annex 9 (for details on methodology see chapter 2.3). The percentage identical nucleotides relative to the covered region of a segment by virus strain, showed that the quality of the sequences obtained, although frequently not for the complete amino acid coding sequence of a segment, fell mostly in the '≥99.8% identical' or '99.0%-<99.8% identical' categories, with the larger segments PA, PB1, PB2 more often in the '<99% identical' category compared to the smaller segments (Figure 14). A wide range of cumulative scores for level of identity of

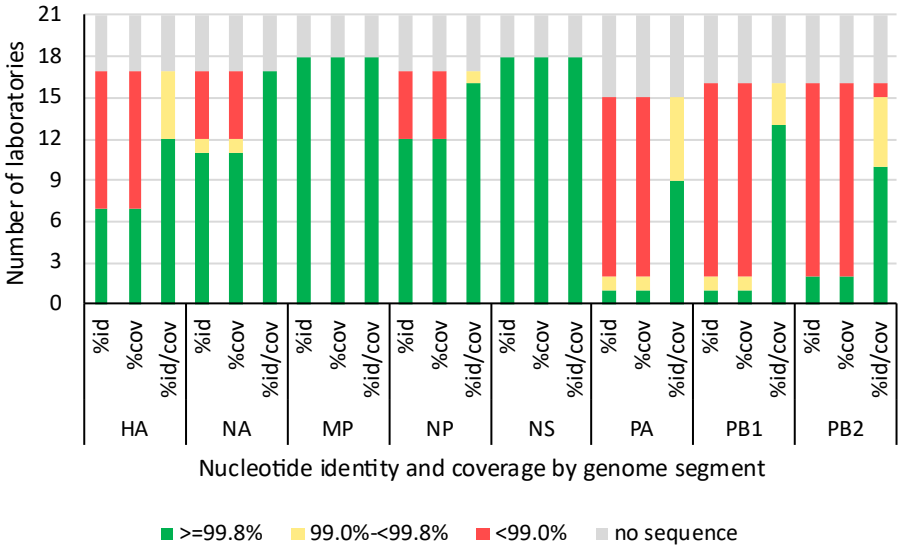
obtained sequences with reference sequences for the whole genome and the HA plus NA segments separately as critical segments for subtyping was observed (Figure 15, Annex 10 by individual laboratory).

Figure 14. Performance of laboratories in sequencing per specific virus in the panel (for H5N1st only the highest concentration specimen d1) by genome segment (A-J)

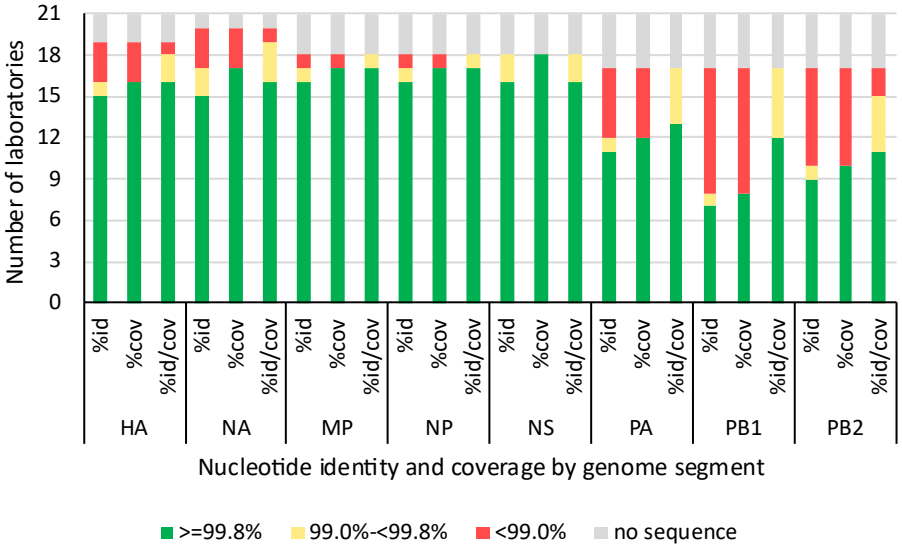
A. H1N2swine



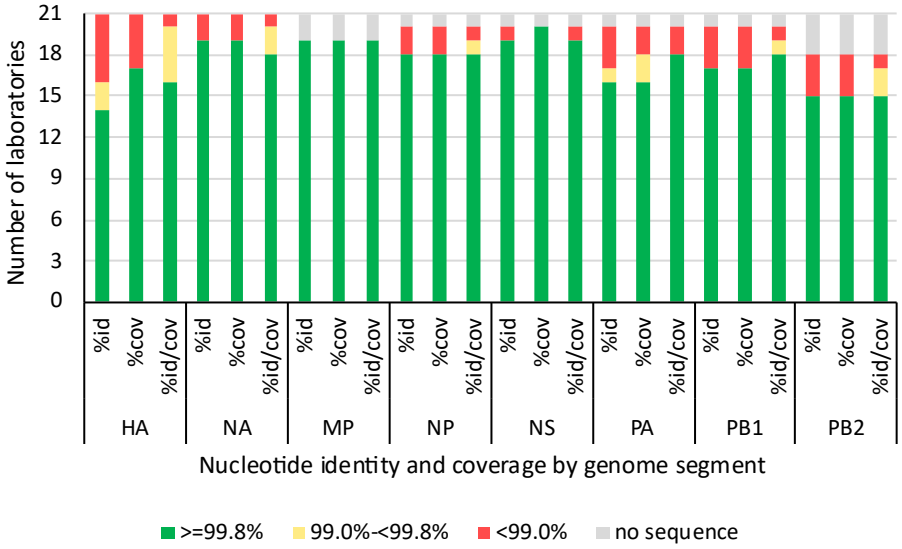
B. H5N8



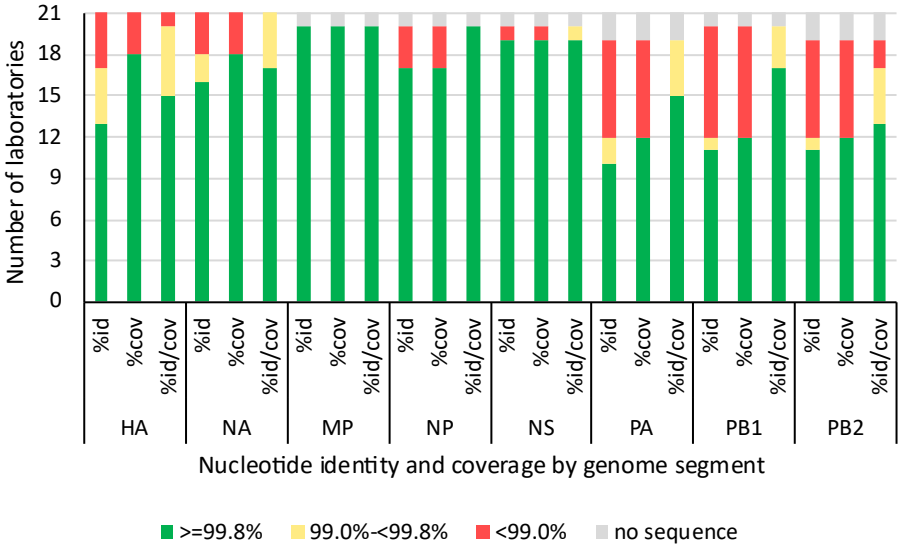
C. H1N1pdm09



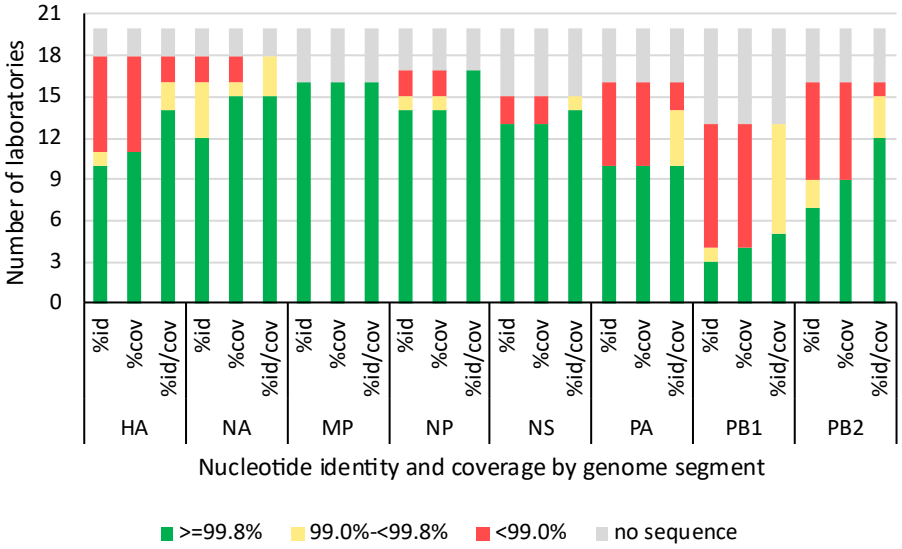
D. H5N1ew



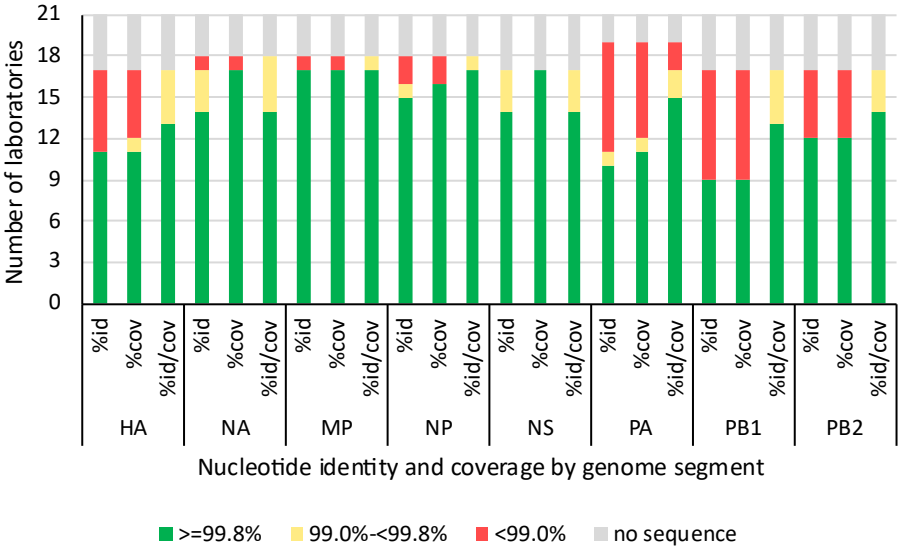
E. H5N1st d1



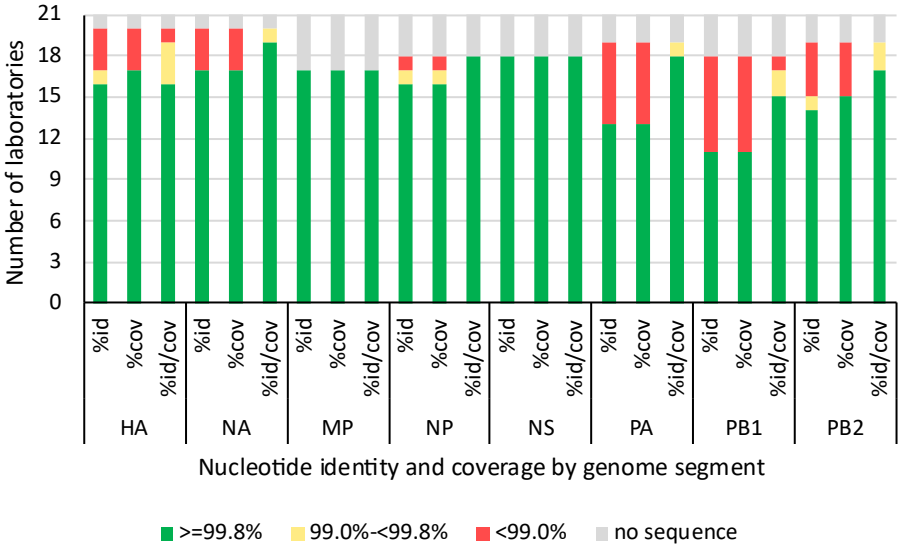
F. H7N2



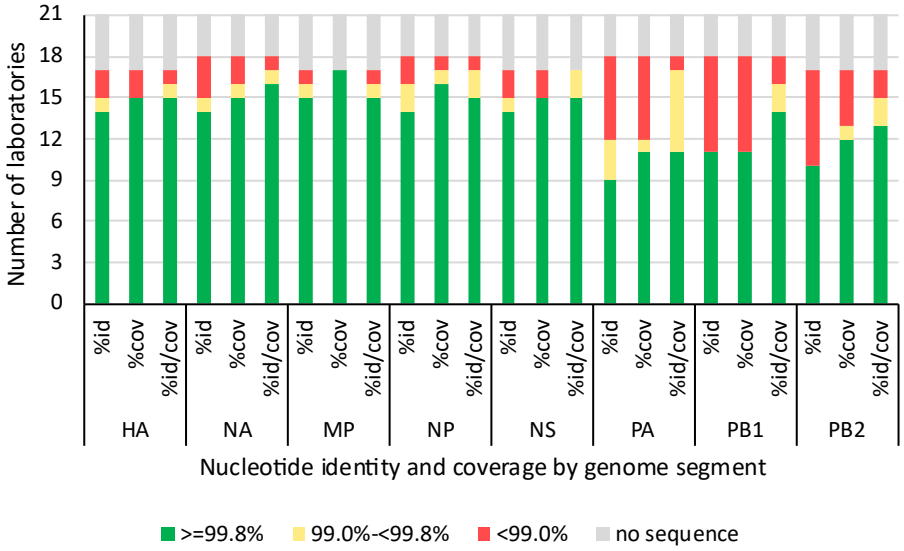
G. H1N1swine



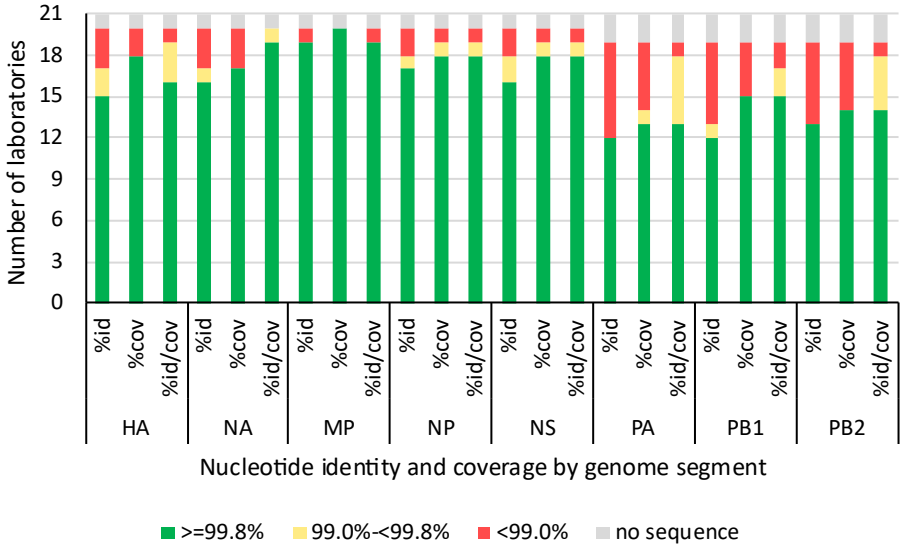
H. H3N2human



I. H3N2swine



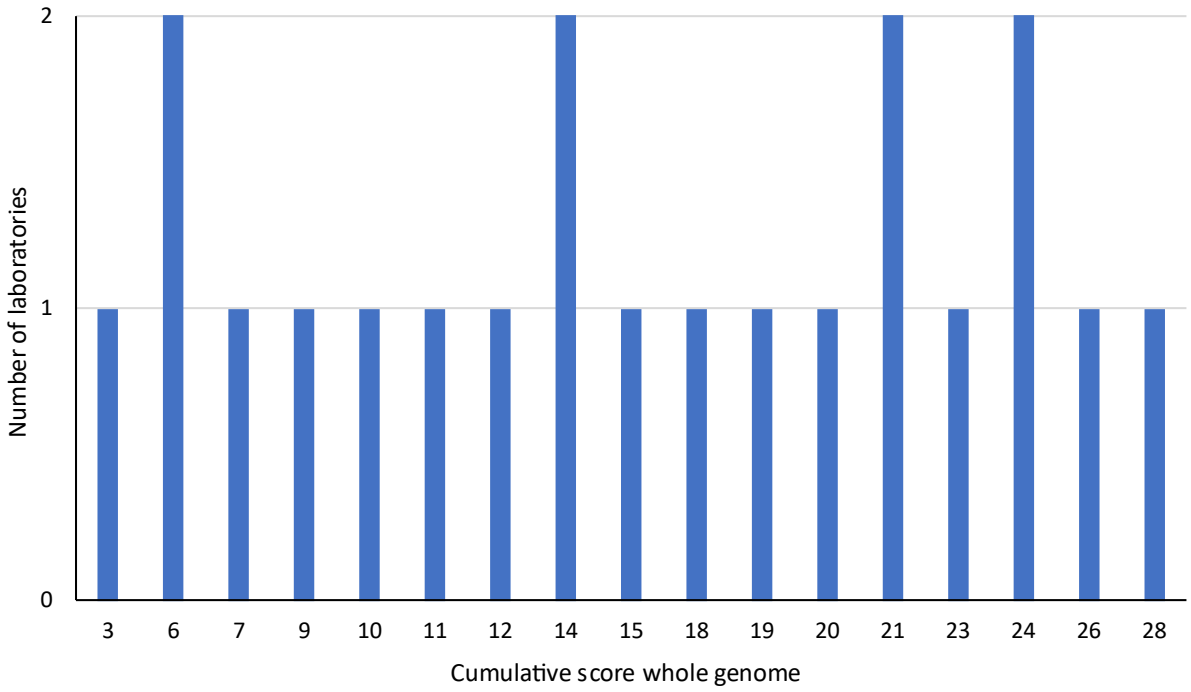
J. H5N1bhg



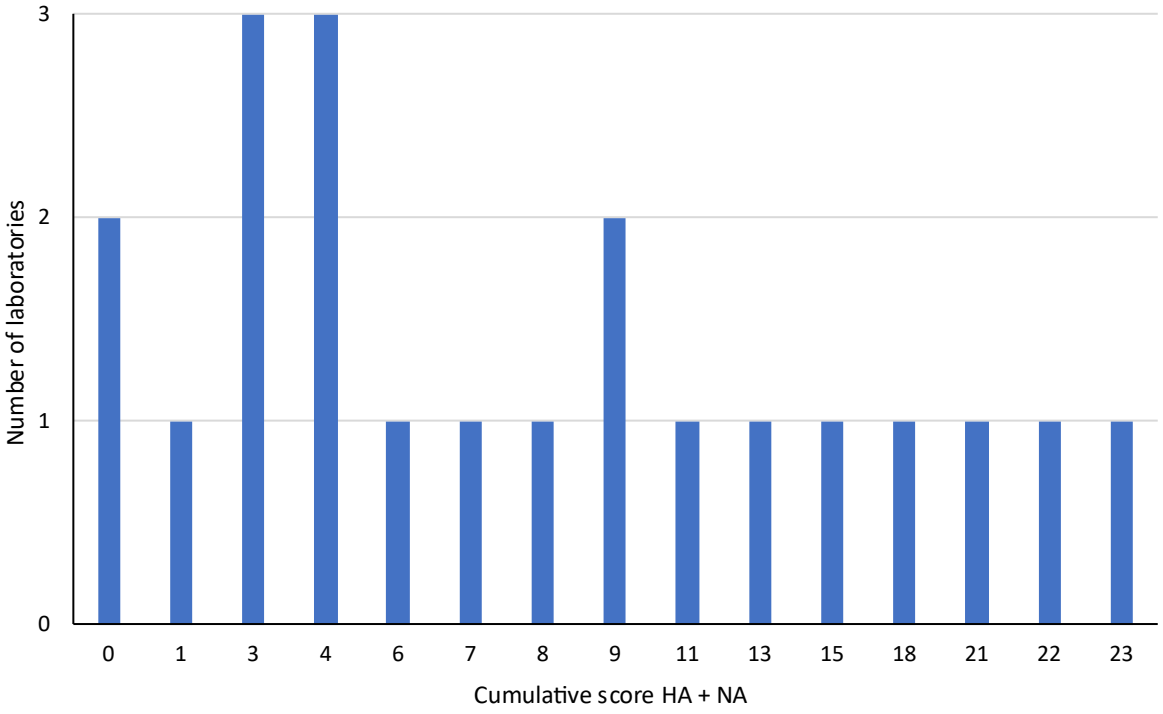
Indicated are percentage identical nucleotide compared to the panel reference sequences in which gaps in the reported sequences are counted (%id), percentage coverage of reported nucleotides compared to panel reference sequences (%cov) and percentage identical nucleotide relative to the covered region of a segment (%id/cov). Percentages have been categorised as >=99.8% (at or above error rate for sequencing), 99.0 to <99.8 (mildly below error rate for sequencing) and <99% (well below error rate for sequencing).

Figure 15. Distribution of laboratories with cumulative score for sequence identity of all viruses in the panel for the whole genome (A) and for the HA plus NA segments only (B)

A. Whole genome



B. HA plus NA segments only



For each laboratory, each virus has been scored 0 = all segments 99.8-100% identical, 1 = at least one segment less than 99.8% identical but all more than 99.0% identical, 2 = at least one segment less than 99% identical and 3 = at least one segment no sequence available (Annex 10). All obtained scores have been summed by laboratory to arrive at the score presented in the figure here (Annex 10 by individual laboratories). The best score would be 0 and the worst score would be 30.

3.3.3 Subtyping following NAAT and sequencing

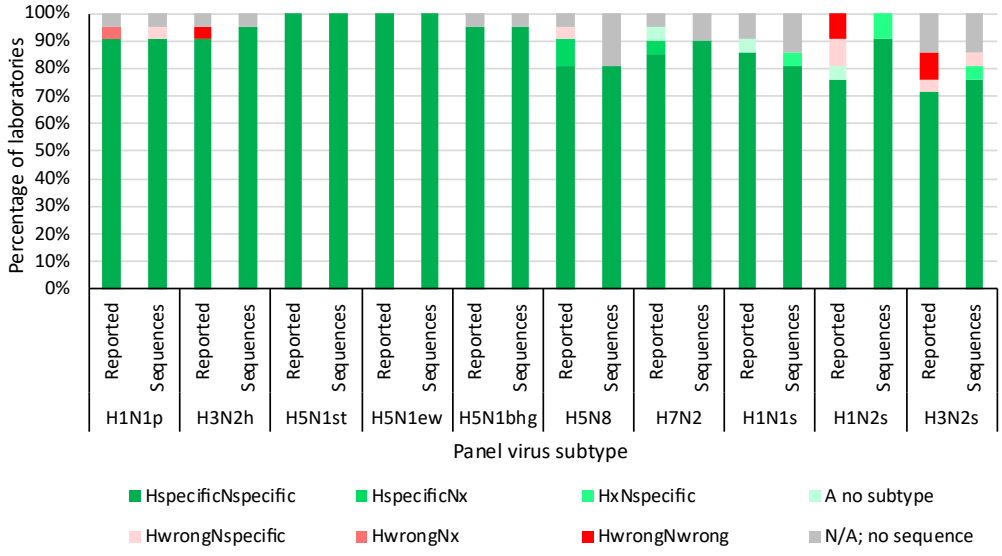
Participants were asked to report the overall conclusion for the H- and N-subtype of viruses and the indication human, avian, swine as the most likely 'likely host species' of the virus based on both the subtyping NAAT and the sequencing results (Figure 16, Annex 11 by individual laboratory). Overall, median 95% (range 76% - 100%) of laboratories reporting the correct H- and N-subtype and host indication by virus strain included in the panel (specimens that were not sequenced were excluded). However, only 12/21 (57%) laboratories reported correct results for all virus strains included in the panel (specimens that were not sequenced were excluded). Swine influenza viruses seemed most difficult to assign correctly (76% - 95% of 18 to 21 laboratories; specimens that were not sequenced excluded).

Several viruses incorrectly assigned to subtype or 'likely host species' technically could have been assigned correctly because when the obtained and successfully uploaded sequences by the participants were analysed, for the vast majority a correct subtype and 'likely host species' could be assigned. The median percentage laboratories with correct subtype and 'likely host species' by virus strain would then increase from median 95% (range 76% - 100%) to median 100% (range 89% - 100%) (specimens that were not sequenced excluded). This analysis of successfully obtained and uploaded sequences by participants showed also that despite the non-optimal quality of many of the HA and NA sequences (shorter than the full coding region of the HA and NA genome segments or with several incorrect nucleotides) (Figure 14 and 15) this did not impact providing the correct subtype and 'likely host species' of the virus for the majority of virus strains included in the panel by the participants (Figure 16).

Only one laboratory submitted two HA sequences of an incorrect subtype, H3human instead of H1pdm09 or not matching the virus in the panel specimen H3human instead of H3swine, which were highly similar to the sequence the laboratory generated from the human seasonal H3N2 virus included in the panel. Causes can be several, from contamination to data processing pipeline error or reporting error. Despite the high number of reported sequences that were suitable for correct subtype and 'likely host species' determination, 8/21 (38%) laboratories reported no subtype or an incorrect subtype and/or 'likely host species' for one or more of the virus strains in the panel (Figure 16, Annex 11 by individual laboratory). Apart from the laboratory that reported incorrect HA sequences, all other errors in reporting the correct subtype and/or 'likely host species' were most likely caused by assessment or reporting errors.

Although all 21 laboratories that reported sequencing results performed H-subtyping using NAAT (median 4; range 4-6 H-subtyping NAAT per laboratory), for 16 of these laboratories sequencing was the only means to report on the H-subtype of swine H1N1 and H1N2 viruses in the panel, for five laboratories on the H-subtype of swine H3N2 virus and for one laboratory on the H-subtype of avian H7N2 virus. Of the 21 laboratories that reported sequencing results, 13 reported N-subtyping by NAAT using median 3 (range 1-7) N-subtyping NAAT per laboratory. Therefore, for eight laboratories sequencing was the only means to be able to report on the N-subtype of panel viruses. Of the other 13, however, for 12 the used N-subtyping NAAT was not capable to identify the N-subtype of many avian and swine influenza viruses in the panel because of the used N-subtyping NAAT missing the right specificity. This shows that sequencing is also for the vast majority of these laboratories crucial for N-subtype determination of all virus strains in the panel.

Figure 16. Conclusion on the H- and N-subtype of panel viruses based on combined results of NAAT and sequencing for 21 laboratories and datasets



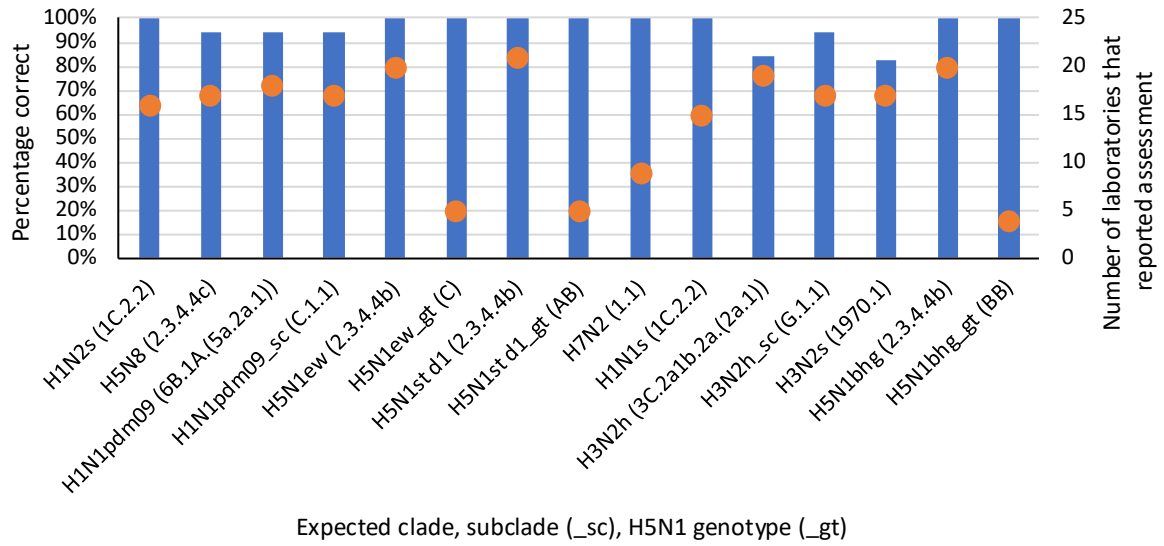
For H7N2 virus 20 laboratories because one laboratory did not receive this panel specimen. For H5N1st only the results for d1 are shown. Reported = the result as reported by the laboratory compared with the expected result. Sequences = the result assessed using the obtained and successfully uploaded HA and NA sequences by the participants. Specific = fully correct subtype for HA or NA including reported 'likely host species', e.g. H1pdm09 or N1swine. Wrong = incorrect subtype for HA or NA or correct subtype but incorrect 'likely host species', e.g. H5 for virus with H3swine or H3human for virus with H3swine respectively. A no subtype = specimen only found positive in NAAT for generic detection of type A influenza virus and unsuccessful subtyping by NAAT and sequencing.

3.3.4 Clade, subclade and H5N1 genotype determination

Of the 21 laboratories that reported a clade (median 18; range 9-21 laboratories), subclade (n=17 laboratories) or H5N1 genotype (median 5; range 4-5 laboratories), by virus strain in the panel, a high number reported the clade (median 16; range 9-21 laboratories), subclade (for human seasonal viruses only); n=16 laboratories) and the H5N1 genotype (median 5; range 4-5 laboratories) correctly for the ten virus strains in the panel (Figure 17 and 18; details by individual laboratory in Annex 12). Based on obtained and successfully uploaded HA genome segment sequences (Annex 9) by participants, technically a higher number of laboratories could have made the clade and subclade assessment of all panel virus strains (in total median 19; range 17-21 laboratories) and could have done this correctly (Figure 18). Also a higher number of laboratories obtained sequences of all segments and could have done the H5N1 genotype assessment; 13 additional laboratories for H5N1ew, 14 additional for H5N1st and 15 additional for H5N1bhg. Nevertheless, a high overall percentage of the laboratories that reported a clade and subclade, reported a correct assessment by virus strain in the panel (median 100%; range 82-100%) (Figure 17). Most difficult seemed the human seasonal H3N2 and swine H3N2 viruses with 84% and 82% of the laboratories that reported a clade, reporting the correct clade.

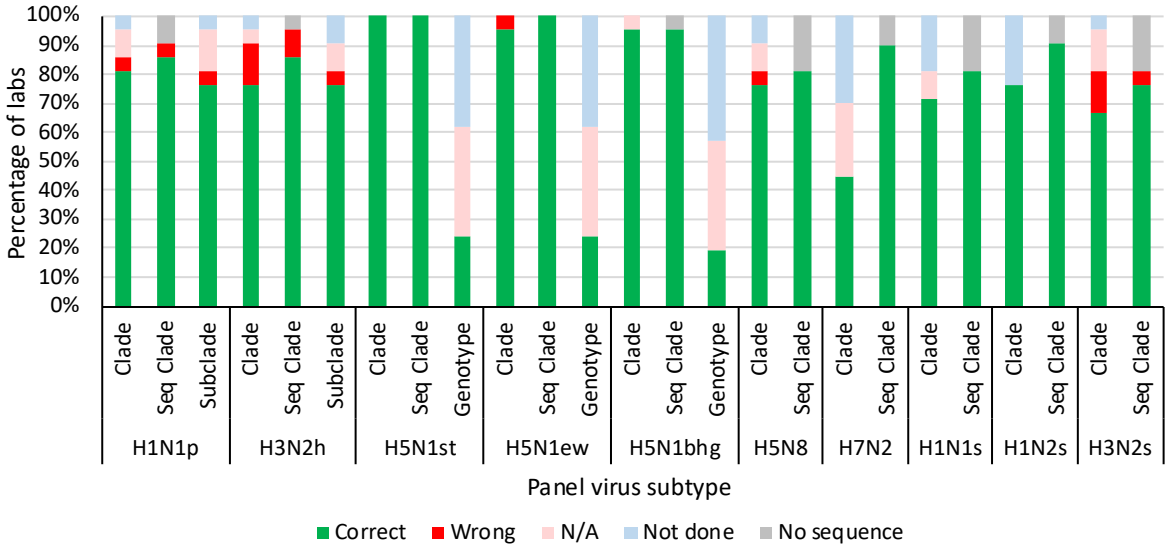
The laboratories that could have reported a clade, subclade or H5N1 genotype, frequently reported 'Not applicable' or ' Not done', for which the reason is not known (Figure 18; Annex 12). Others reporting 'Not applicable' or 'Not done', correctly indicated this, because no HA sequence for clade and subclade assessment was obtained or not all genome segments were obtained that are required for genotype assessment of H5N1 viruses. When laboratories had HA sequences available and uploaded these, in a small number of cases (n=4) still the correct clade could not be obtained due to uploading an HA genome segment sequence of incorrect subtype (n=2) or too high incompleteness (n=2) (Figure 18).

Figure 17. Overview of the percentage of laboratories reporting a clade, subclade and/or H5N1 genotype (orange dot) that reported the expected result (bars)



After the strain subtype between brackets the expected clade, subclade (_sc) or H5N1 genotype (_gt) is displayed.

Figure 18. Results on clade, subclade and H5N1 genotype determination of laboratories that participated in the genetic characterisation part of the EQA



For H5N1st only the results for d1 are shown. N/A and Not done: as in the report by the laboratory either because of not subjected to sequencing, no sequence obtained or sequence obtained but analysis not done. Indicated at 'Seq Clade' are results obtained after analysis by the EQA provider of the HA sequences successfully obtained and uploaded by the participant laboratories. No sequence: the laboratory did not upload a sequence.

3.3.5 Amino acid substitutions

In six panel virus strains, several amino acid changes in the MP genome segment are present that are associated with M2-blocker antiviral reduced susceptibility (Table 2). The percentage of laboratories that correctly identified the mutant amino acid change in the MP among the laboratories that reported amino acid changes by virus strain and amino acid position ranged from 33% to 88% (Figure 19, bars and orange line marker respectively; details by laboratory in Annex 13). Depending on the virus strain 0-5 laboratories did not provide a report on amino acid changes in the MP or reported 'Not assessed' while they had uploaded MP genome segment sequences covering the expected amino acid positions. Based on the obtained and successfully uploaded sequences (Figure 19, grey dot markers), the percentage correct could have been 100% for all virus strains and amino acid positions for those laboratories that obtained and uploaded the MP genome segment sequences (Annex 13).

No virus strains with markers associated with neuraminidase reduced or highly reduced inhibition were included in the panel. However, 13/17 (76%) laboratories reported the NA-Y155H amino acid change in the H1N1v swine influenza virus as a marker associated with Oseltamivir and Zanamivir highly reduced inhibition. Phenotypic testing at RIVM showed that this H1N1v swine influenza virus is normal inhibited by Oseltamivir and Zanamivir, similar to A(H1N1)pdm09 viruses carrying this amino acid change. So far, only for the previous human seasonal A(H1N1) influenza virus it has phenotypically been confirmed that the NA-Y155H amino acid change in the N1 subtype neuraminidase causes highly reduced inhibition by Oseltamivir and Zanamivir.

A detailed overview by laboratory of the detection of the expected amino acid changes related to mammalian adaptation is shown in Annex 13, by polymerase complex proteins (three virus strains for PA and PB2) and by other proteins (five virus strains for HA, one in addition for NS1 and one for NP only). A summary is provided in Figures 20 and 21, respectively. The PB2-701N amino acid was most frequently identified; 64%-82% of laboratories identified PB2-701N in the swine influenza viruses H1N1s, H1N2s and H3N2s. Similar to the observation for MP amino acid changes, a higher number of laboratories could have identified the expected amino acid changes as most sequences uploaded by the laboratories covered the amino acid positions and contained the expected changes; median 95%, range 74%-100%, for uploaded sequences coding for the polymerase proteins PA and PB2 and 90%, range 82%-100%, for uploaded sequences coding for the other proteins HA, NS1 and NP.

Figure 19. Identification of amino acid changes in the matrix protein (MP) previously associated with M2-blocker antiviral reduced susceptibility

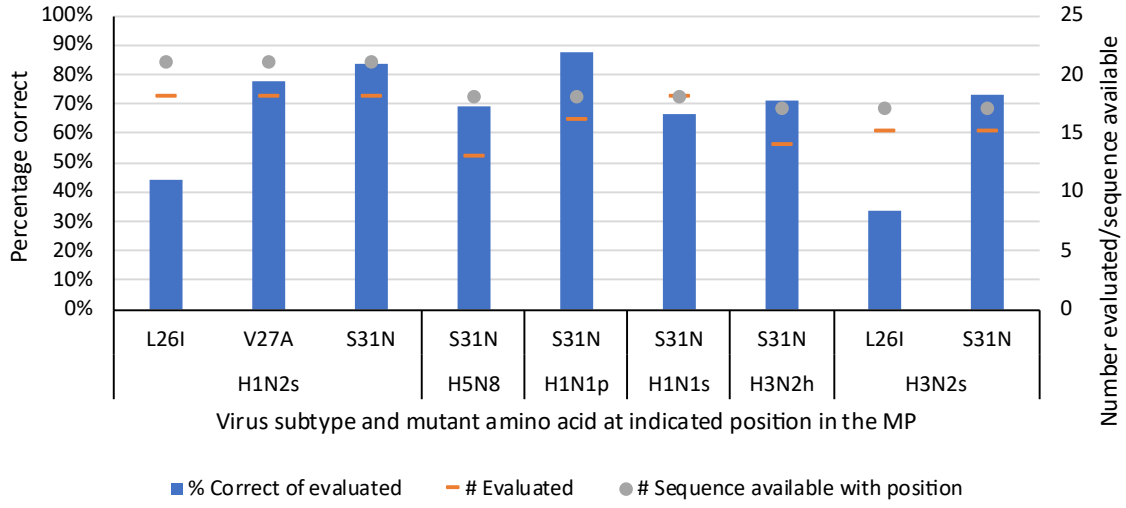


Figure 20. Identification of amino acid changes in the polymerase complex acidic polymerase protein (PA) and basic protein 2 (PB2) previously associated with mammalian adaptation

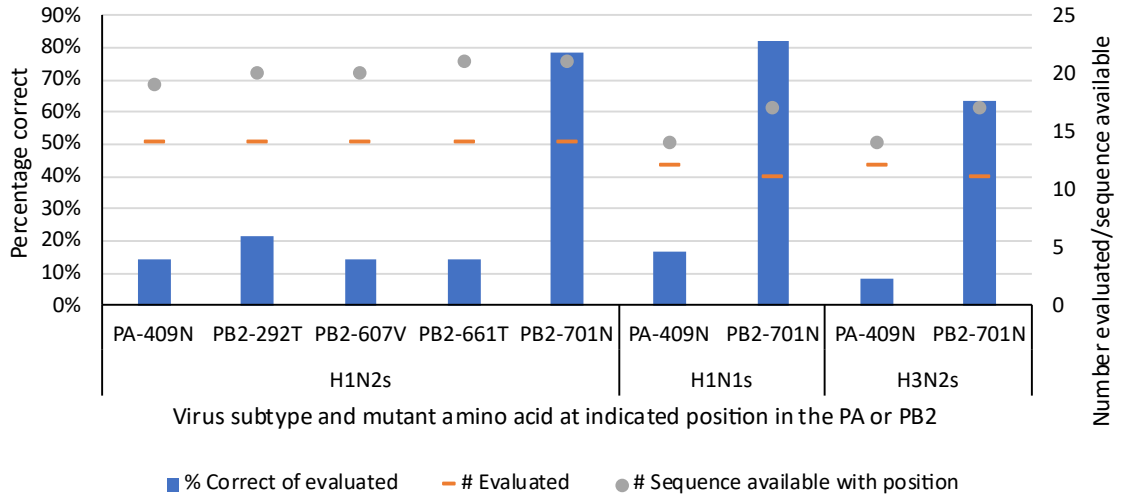
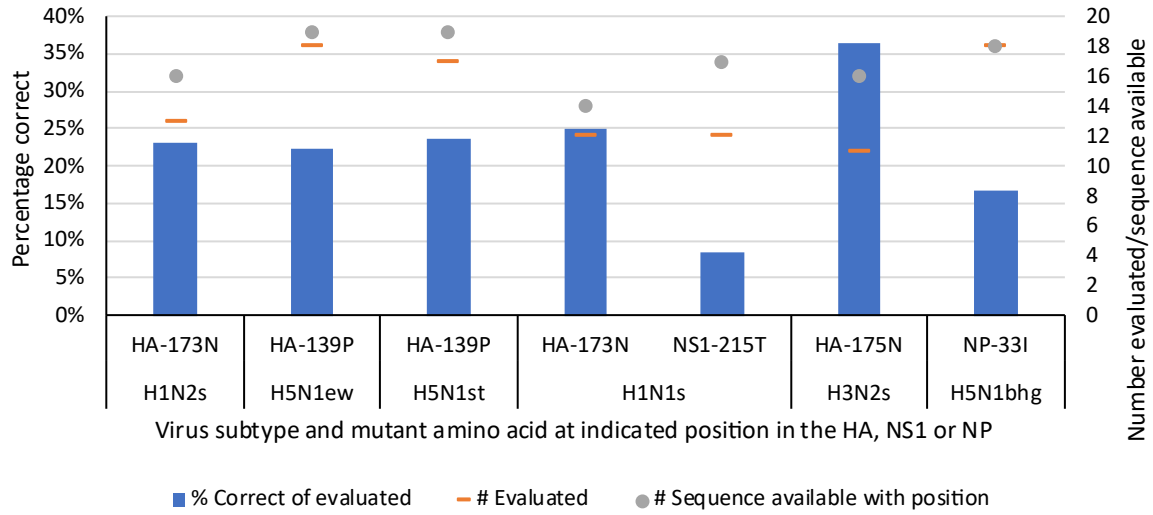


Figure 21. Identification of amino acid changes in the hemagglutinin (HA), non-structural protein 1 (NS1) and nucleoprotein (NP) previously associated with mammalian adaptation



For the evaluation of reported HA amino acid position results, the numbering with an without signal peptide sequence was taken into account.

4 Discussion

This EEIQAP 2024 study on the detection, subtyping and genetic characterisation of potentially zoonotic influenza viruses, including those from avian and swine origin, was the first of its kind in the European Union area. The direct reason for this EEIQAP study was the increased threat of A(H5N1) infections in humans due to the endemic circulation in wild birds since 2022, causing outbreaks among poultry worldwide, and recently also among cows in the United States, and the increased number of mammalian species becoming infected. For a rapid national response and international early warning, it is desirable that national influenza reference laboratories, being often WHO National Influenza Centres, have the capacity to detect and identify animal influenza viruses that infect humans and characterise them genetically [28].

In previous relevant EQA activities, the 2006 EQA on zoonotic influenza virus, organised by QCMD in collaboration with the European Society for Clinical Virology and the European Influenza Surveillance Scheme (EISS), included A(H5N1) and A(H7N3) avian influenza viruses [29]. This assessment identified challenges in the sensitivity of generic detection of type A influenza viruses and the ability to identify the H7 subtype. From the initiation of the WHO External Quality Assessment Project (EQAP) in 2007, most national influenza reference laboratories in the EU/EEA have taken part in this EQAP [11,13]. This annual WHO EQAP always contains human seasonal influenza viruses and H5Nx avian influenza viruses and varying by panel nowadays also A(H7N9) and/or A(H9N2) avian type A influenza virus subtypes. Laboratories from the wider WHO European Region show moderate results in these EQAP studies with about 70-80% of participating laboratories reporting correct detection and H-subtyping results for all specimens in an EQAP panel. For the WHO EQAP as a whole, errors are mainly in reporting the incorrect subtype and false positive and false negative results, giving suggestions for corrective actions by individual laboratories.

A comprehensive EQA panel (EEIQAP 2024) was developed under the auspices of ECDC within the AURORAE laboratory support contract (https://www.rivm.nl/en/international-projects/aurorae), to assess the influenza reference laboratory emergency and pandemic preparedness for the EU/EEA and Western Balkan countries and Türkiye even more completely for detection, identification and characterisation of potentially zoonotic influenza viruses. At the same time addressing the challenges identified with the QCMD/ESCV/EISS and WHO EQAP EQA panels. The aim of this EEIQAP 2024 panel was to address detection (including sensitivity), subtyping and genetic characterisation of avian and swine type A influenza viruses which have the potential to cause or having caused a zoonotic infection and including some human type A influenza viruses as reference. After repeat invitations by ECDC to the network, 27 (73%) of the 37 invited countries participated with 30 laboratories (from three countries, France, Greece and the Netherlands, two national influenza reference laboratories participated per country).

Generic detection of type A influenza viruses using NAAT

Generic detection of type A influenza viruses using NAAT, irrespective of the H- and N-subtype, was excellent for all strains of human seasonal and avian and swine influenza viruses in this EEIQAP 2024 panel as all 30 laboratories in 32 reported datasets detected all strains, similar to earlier EEIQAP studies under the umbrella of ECDC targeting human seasonal viruses only [14,30,31]. This was not surprising, as 29/32 (91%) of reported datasets used at least a NAAT targeting the MP genome segment that has regions highly conserved across all known type A influenza viruses from humans and animals [32].

However, in previous EEIQAP panels, and for all strains in the EEIQAP 2024 panel as well, the concentration of genomic material was always quite high, making sure the other challenges like virus isolation and/or direct sequencing from the panel specimen could be performed using the same panel specimens [14,30,31]. This limits the capacity of these panels to identify subtle differences between NAAT with regard to sensitivity; also because Ct values generated by each laboratory cannot be used to compare sensitivity differences between NAATs. Therefore, this time a dilution series of one of the strains in the panel, a current clade 2.3.4.4b A(H5N1) influenza virus, was included to be able to get a rough estimate of the limit of detection (LoD) of the variety of NAAT used for generic detection of type A influenza viruses by the participating laboratories. Laboratories were expected to detect type A influenza virus in the 8.11 copies/µl specimens A/Sandwich Tern/Netherlands/7/2022 A(H5N1) containing specimen.

Of the 32 reported datasets, 20 (63%) by 19/30 (63%) laboratories (1 laboratory reported two datasets with the 8.11 genome copies/µl specimen found positive) achieved that result, while for 11/32 (34%) datasets by 11/30 (37%) laboratories the rough estimate of the LoD was 81.1 and for 1/32 (3%) datasets by 1/30 (3%) laboratories below 81.1 genome copies/µl specimen. No clear correlation could be found with used specific primers and probes, mismatched nucleotides of the primers and probes with target sequences, specimen equivalent volume put into NAAT, nucleic acid extraction and amplification reagents and equipment. It is well known that many factors apart from match of primers and probes with the target sequence determine the analytical sensitivity of a NAAT and that each implementation should be well evaluated/validated for its analytical sensitivity [33-35].

This is especially true for laboratory-developed tests (LDTs) using a wide variety of extraction and amplification equipment, nucleic acid extraction reagents, reverse transcription and amplification enzymes, primers and probes and specimen equivalent volume nucleic acid put in NAAT, as shown in the current study. However, also using

ready-to-use NAAT kits including primers and probes, nucleotides and enzymes should be well evaluated for analytical sensitivity if not the exact protocol and equipment is used for which it has been validated by the manufacturer of the NAAT kit. Nevertheless, 11/32 (34%) laboratories seem to have room for improvement of the analytical sensitivity of their used NAAT for generic detection of type A influenza viruses, recognising the limitation of the current LoD estimate using only one specific virus and 1/10 dilution steps for a rough estimate of the LoD.

There is a benefit of not standardising used reagents and equipment across laboratories. Implementing a multitude of NAAT essentially reduces the risk of collapse of the complete testing landscape when components fail, but requires well-designed local validation protocols with regular participation in EQA and inter-laboratory comparisons to guarantee equal performance and quality of data reported locally and to ECDC/WHO [35]. Correct detection of type A influenza viruses is of course the first step towards identification of potential zoonotic type A influenza viruses.

Further H-subtyping by NAAT and/or sequencing would be needed to identify the human seasonal, avian or swine 'likely host species' and specific subtype of the detected type A influenza virus. This is critical for identifying and confirming an infection with a zoonotic type A influenza virus, even if there is strong evidence of exposure to animals infected with type A influenza virus.

H- and N-subtyping of influenza type A viruses using NAAT

With regard to H- and N-subtyping using NAAT, also the same observation as in earlier EEIQAP studies for H- and N-subtyping of human seasonal influenza viruses was made that all laboratories use H-subtyping NAAT, but a limited number use N-subtyping NAAT, despite having N-subtyping NAAT available. In the EEIQAP 2024 all 30 participating laboratories used NAAT for H-subtyping of the human seasonal H1pdm09 and H3 influenza viruses as well as for the H-subtyping of H5 and H7 avian influenza viruses included in the panel.

N-subtyping to identify the N-subtype of the panel viruses by NAAT was performed by only about half (16/30; 53%) of the laboratories that participated in the study; most used N1 and N2 NAAT, although with varying 'likely host species' specificity, and less N8. Only 5/30 (17%) laboratories used H-subtyping NAAT specific for swine H1 and swine H3 type A influenza viruses.

One might argue that 'likely host species'-specific N-subtyping is not necessary for a first screening of the subtype of influenza viruses. However, it offers a rapid 'second opinion' when H-subtyping does not give a clear result, as is seen especially for the H1N1, H1N2 and H3N2 swine influenza viruses in the panel. All laboratories in all datasets identified the human seasonal A(H1N1)pdm09 and H3N2 viruses as H1pdm09 (two only H1) and H3 respectively, while only 17/32 (53%) datasets (two despite not having an N1 subtyping NAAT in the report) and 15/32 (47%) datasets reported also the N1 and N2 subtype, respectively.

For all H5 subtype strains in the panel, a 100% correct score was achieved by all 30 laboratories. For the H7 strain 27/31 (87%) datasets by 25 laboratories reported the H7 subtype correctly. Seven of seven (100%) laboratories using avian N1 NAAT and 3/5 (60%) laboratories using generic N1 NAAT reported the N1 subtype for all H5N1 strains. Obviously, it is more difficult to develop a generic N1 subtyping NAAT than an avian N1 specific NAAT, as conserved regions in the N1 genome segment across all influenza HxN1 viruses are limited. The N8 of the H5N8 strain in the panel was correctly identified by the five laboratories using an N8-subtype specific NAAT. The N2 subtype of the H7N2 strain in the panel was only identified by one laboratory using a generic N2 subtyping NAAT while four other laboratories using generic N2 NAAT could not identify the N2 subtype of the H7N2 virus by NAAT. Unfortunately, the laboratory with the positive N2 result did not upload primers and probes sequences nor provided detailed information on the used NAAT and could therefore not be evaluated further. Nevertheless, the four other laboratories using generic N2 NAAT were capable to identify N2 in human seasonal H3N2 virus, but not swine H1N2 and swine H3N2 viruses, suggesting the specificity of these generic N2 NAAT is human seasonal N2, at large.

Considering the H-subtyping using NAAT of the swine H1N1, H1N2 and H3N2 viruses, laboratories performed best on the H3N2 swine virus with 22/32 (69%) datasets by 20/30 (67%) laboratories reporting the H3 subtype of which however only four reported having used a swine H3 specific NAAT (all four have also human H3 specific NAAT), 10 generic H3 and 11 human H3 (of which four have also swine H3 specific NAAT); one laboratory reported H3 based on the subtyping results in another of the three reported datasets while the actual dataset did not use an H3-subtyping NAAT. For the H1N1 and H1N2 swine influenza viruses H-subtyping was worse with 5/32 (16%) and 6/32 (19%) datasets by six laboratories reporting the H1 subtype for these viruses respectively; five of which used swine H1 specific NAAT and one generic H1 NAAT. The N-subtypes of the swine influenza viruses H1N1, H1N2 and H3N2 in the panel were only identified using NAAT by 10 (three with N1dpm09, four with generic N1, two with swine N1 and two with avian N1 NAAT), two (one with human N2 and one with generic N2 NAAT) and two (one with avian N2 and one with generic N2 NAAT) laboratories, respectively. So, H- and N-subtyping of the swine influenza viruses using NAAT relied for a minor part (especially for H3) on intentional specificity (swine or generic specificity) but mainly on incidental cross-reactivity of used primers and probes that have intentionally human or avian specificity.

Cross-reactivity of H- and N-subtyping NAAT independent of intended 'likely host species'-specificity can be considered a benefit. However, it may happen that animal influenza viruses are incorrectly identified as human, which is more likely for H1 and H3 swine influenza viruses as the same H-subtype currently causes human

seasonal influenza virus infections. Therefore, an in-depth analysis of cross-reactivity of used NAAT including primers and probes mismatches with target genome segment sequences was performed.

Somewhat surprisingly there was not a clear correlation of mismatch with capacity to subtype H3 of swine H3N2 influenza virus with NAAT that well identified the H3 of human H3 influenza virus. However, it was clearthat the vast majority of H3 subtyping NAATs had a rather higher Ct difference to that of the generic influenza A virus detection NAAT for swine H3 virus compared to that for human seasonal H3 virus in the panel.

This finding can be used to highlight that there is a need for further investigation of that test virus as this finding can be caused by drift of the HA genome sequence of human seasonal H3 virus resulting in a detrimental mismatch of primers or probes with the target, or because the virus is of animal origin with a natural mismatch with primers and probes that are well suited for human seasonal H3 subtyping. Since the concentration of target RNA was rather high in the panel specimens, this phenomenon was visible. However, with lower target RNA concentrations of swine H3N2 virus these H3 NAAT very likely would have been negative with swine H3N2 virus. Therefore, this observation is limited to specimens that are at least positive in the generic type A influenza virus detection NAAT and H3 subtyping NAAT. Similar observations, but with far less data, were made for H1-, N1- and N2-subtyping NAAT with human seasonal H1N1pdm09, swine H1N1, swine H3N2, avian H5N1 and avian H7N2 viruses.

The bottom line is that one should know the characteristics of used H- and N-subtyping NAAT which target H- and N-subtypes that are shared among host species (H1, H3, N1, N2) well. This can be achieved through thorough validation or evaluation and EQAs that include such H- and N-subtypes of all possible influenza viruses that have infected or are expected to infect humans; this can be considered better than demanding the development of highly 'likely host species'-specific H- and N-subtyping NAAT for such subtypes, which may cause a reduction in sensitivity. However, when intentionally using generic H-subtyping, e.g. for H3, it becomes critical that for treating clinicians to ask questions in the patient disease history review about potential exposure to animals that can infected or are known to be infected with potential zoonotic influenza viruses. It is advisable to do this for at least all critically ill patients who are found type A influenza virus-positive [36,37].

This points to another national challenge, as national influenza reference laboratories do not usually perform primary diagnostics for primary and secondary care apart from being involved in national respiratory illness surveillance programmes where primary testing is performed by national influenza reference laboratories. Routine clinical diagnostic laboratories using mostly commercial NAAT should be able to at least detect a type A influenza virus in a zoonotic influenza virus infection.

In the Netherlands, an EQA study conducted among routine clinical diagnostic laboratories using a similar panel of viruses as in the EEIQAP 2024, confirmed that a wide range of commercial influenza virus detection NAAT detect avian and swine type A influenza viruses equally well compared to human influenza viruses [38]. However, only very few clinical diagnostic laboratories subtyped the detected viruses, because very few commercial tests include some level of subtyping and very few laboratories used laboratory-developed subtyping tests [38]. The latter situation may be likely because of the implementation of EU in vitro diagnostics regulations. When a zoonotic infection is suspected from patient disease history review and initial laboratory results , the national influenza reference laboratory should be consulted immediately to have the virus subtyped and further characterised. It should be noted, however, that even then the detection and identification of animal influenza virus infection of humans, especially with swine influenza virus, can be a coincidence [39].

For avian influenza virus, especially in connection with poultry outbreaks with H5N1 influenza virus, an increasing number of countries have currently implemented active and passive monitoring programmes as outlined in the guidance from ECDC, for which national capacity for sensitive detection and identification of avian influenza viruses is critical [40-42].

H- and N-subtyping following sequencing

Overall, the accuracy of sequencing fell mostly in the '≥99.8% identical' to '99.0%-<99.8% identical' categories considering the successfully obtained parts of the genome segments coding for proteins for which sequences were obtained. However, despite thorough attention paid to creating high quality panel specimen that could be used for any sequencing protocol, whether it sequences fragmented RNA without universal amplification of whole genome segments first (so called 'shotgun' approach) or long read sequencing of whole genome segments after universal amplification of these whole genome segments, none of 21 laboratories that participated in the genetic characterisation part of the panel were able to sequence whole genomes (with full coding region of all eight genome segments) for all panel specimens that were expected to be able to be sequenced.

Mild heat inactivation is a preferred method compared to methods using Triton X-100, beta-propiolactone, gamma irradiation or mild paraformaldehyde inactivation [17]. However, a slightly higher temperature (65 versus 60 degrees Celsius) and prolonged heat inactivation (2.5 hours versus 1 hour) were needed to fully inactivate the avian influenza viruses as compared to human seasonal influenza viruses with the protocol that we used previously [31]. This might have reduced the amount of intact genome segments to a level that is too low for sequencing the full coding region of all eight genome segments of all virus strains present in the panel specimens using whichever sequencing protocol. Nevertheless, as judged from sequences uploaded by the participating laboratories for

evaluation by the EQA provider, the majority of HA and NA sequences were complete enough for determination of the H- and N-subtype and identification of the 'likely host species' of the panel viruses.

Taking NAAT results and sequencing results together for the 21 laboratories, for the majority of virus strains included in the panel, when the reported H- and N-subtype and 'likely host species' were incorrect, these were mainly errors in reporting, as in most cases correct NAAT results and/or sequences were uploaded. Excluding those errors, there was only one laboratory that had two real failures, as wrong sequences were obtained and uploaded suggesting a contamination or bioinformatics pipeline issue. Therefore, 20/21 (95%) laboratories that participated in the genetic characterisation part of the EQA would have had excellent analytical performance in identifying the correct H- and N-subtype, and 'likely host species' if no reporting errors were made. Nevertheless, these findings indicate that reporting is an area of focus for about half of the laboratories that participated.

The genetic characterisation findings illustrate clearly the added value of sequencing to identify the correct H-subtype, and the 'likely host species', because of the use of H-subtyping NAAT with limited range of specificities, especially for the swine influenza viruses. In addition, because of the relatively low number of laboratories using N-subtyping NAAT, or when used it is often for a (very) limited number of N subtypes and regularly with generic specificity, the sequencing results show also clearly the added value for correct N-subtyping of all strains in the panel. Therefore, sequencing of at least the HA and NA genome segments of type A influenza viruses can be considered a critical capacity for national influenza reference laboratories to identify sporadic and emerging infections of humans with animal type A influenza viruses, even if for some of the likely zoonotic type A influenza viruses a highly specific H-subtyping NAAT is available in the test repository of the laboratory [39]. Although the WHO CC in London offers rapid analysis of suspect viruses, from a national perspective having this sequencing capacity in each national influenza reference laboratory would be sensible for a rapid local response.

Genetic characterisation

Of the laboratories that submitted an assessment of genetic clade of all virus strains, of subclade of the human seasonal viruses and of the genotype of H5N1 viruses in the panel, a high proportion submitted correct results, indicating that the variety of methods used by the laboratories worked effectively. However, a higher proportion of laboratories could have made these assessments and could have done this correctly as the vast majority of laboratories that did not report on clade, subclade or H5N1 genotype assessment successfully obtained HA sequences for the strains in the panel and obtained full genomes for H5N1 viruses (with enough coverage for each segment to allow genotype determination).

This highlights the possible need for training in the application of software tools for clade and subclade assignment for a wide variety of type A influenza virus subtypes and for H5N1 genotype assignment. Assigning to clade, subclade and genotype provides important information about the evolution of viruses and especially about the emergence of new clades that might carry specific properties with potential impact to human health, like amino acid substitutions that are markers for antiviral reduced susceptibility and mammalian adaptation. Clade, subclade and genotype assignment should therefore be accompanied with such marker analysis in the HA and also other relevant proteins.

The panel contained viruses not specifically selected for specific amino acid substitutions. Therefore, the best indicator for assessing capacity and capability of laboratories to identify markers was the presence of the well-known M2-S31N substitution that is associated with M2-blocker antiviral resistance [43] in the translation of the MP genome segment into the M2 amino acid sequence in 6/10 strains included in the panel. Indeed, of the laboratories that evaluated the obtained M2 sequence by virus strain, 67%–88% identified the M2-S13N marker. However, all these laboratories, and the laboratories that did not perform an assessment, provided MP genome segments that after translation contained the M2-S31N amino acid change marker.

The less common but well-known M2-V27A marker [43] in one strain was recognised equally well by 78% of laboratories that evaluated the M2 protein. However, the lesser known M2-L26I marker in two strains was only recognised by 33%-44% of laboratories that evaluated the M2 protein. This marker is frequently seen and associated with M2-S31N in H5N1 viruses, especially isolated from humans infected with the H5N1 virus [44]. However, in swine influenza viruses, like those included in the panel, M2-L26I associated with M2-S31N (often together with M2-V27I) is also frequently seen [45,46]. Laboratories performed less well in the identification of markers for mammalian adaption, although the well-known residue PB2-701N marker was identified well by around 80% of the laboratories that performed this assessment. The other expected markers in PB2, PA, HA, NP an NS1 were identified far less, probably because their effect is less well characterised. When sequences uploaded by the laboratories were evaluated, in all sequences the expected markers could be identified. This highlights that if tools were available that were undoubtedly capable of identifying such antiviral reduced susceptibility and mammalian adaptation markers, all laboratories that reported sequences could have correctly identified the markers. Extensive lists are available from Puglia et al. 2024 [47], which were narrowed down to a shortlist in Alvarez et al. 2025 [3]. In the latter paper, only PB2-701N is listed, which might explain why participants mainly focused on this marker. In addition, these lists are for avian influenza virus and human infection only. Less is known about further adaptations in swine influenza viruses that would be needed for efficient ongoing transmission between humans following a zoonotic infection [48,49].

5 Conclusions

From the EEIQAP 2024 activity, the following conclusions are drawn:

- All 30 laboratories from 27 countries detected all virus subtypes of the 10 different strains of various subtype of avian, swine or human origin that were included in the panel, with the generic type A influenza virus detection NAAT, meaning that type A influenza virus will be detected, but further tests (H-subtyping by NAAT and/or sequencing) are needed to identify the specific human seasonal, avian or swine influenza virus H-subtype;
- Some difference in sensitivity between laboratories was observed, with 20/30 laboratories successfully detecting the lower concentration A(H5N1) influenza virus in a dilution series;
- All laboratories determined the seasonal human influenza virus H1 and H3 and avian influenza virus H5 subtype using NAAT, and 26/30 the H7 subtype;
- Using NAAT, only 6/30 laboratories identified one or both H1 swine influenza viruses as H1, and 20/30 laboratories identified the H3 swine influenza virus as H3;
- Detecting and identifying swine type A influenza viruses as H1 or H3 by NAAT often cannot in itself distinguish between the respective human seasonal and swine viruses, highlighting the need for sequencing;
- Many laboratories (14/30) did not use N-subtyping NAAT to determine the N-subtype of the panel viruses;
- Those using N-subtyping NAAT applied them for only a limited number of N-subtypes in the panel viruses; most often only human N1pdm09 and/or N2 (18/30) subtypes and the avian N1 subtype (10/30);
- The level of mismatched nucleotides of primers and probes with target site in the genome segments of panel viruses did not predict whether a virus was detected or subtyped or not using NAAT, even with low viral load specimens at or just above the LoD of used NAAT. This highlights the need for thorough validation/evaluation when a specific NAAT is implemented;
- About half of the 21 laboratories that reported sequencing results (11/21) provided the correct H- and N-subtype and 'likely host species' for all human seasonal, avian and swine influenza viruses in the panel;
- However, of the remaining 10 laboratories, nine provided correct HA and NA genome segment sequences and/or H-subtyping and/or N-subtyping NAAT results, suggesting errors were made in the reports to the EQA scheme rather than failures in NAAT or sequencing. Hence, full correct subtyping and 'likely host species' identification could have been reported;
- Following bioinformatics analysis, clades were assigned correctly for human H1pdm09 virus by 17/21 laboratories, human H3 virus 18/21 laboratories, avian H5 viruses 16–21/21 laboratories depending on which of the four H5 viruses included in the panel, H7 virus 9/20 laboratories (1/21 laboratories did not receive this specimen), swine H1 15–16/21 laboratories depending on which of the two H1 viruses included in the panel and swine H3 virus 14/21 laboratories;
- Subclades for human influenza viruses were assigned correctly by 16/17 laboratories that performed this analysis;
- Five laboratories performed genotyping of H5N1 strains (for which sequences of all eight segments are needed) and all provided the correct genotype for the viruses they detected;
- Antiviral reduced susceptibility and mammalian adaptation marker identification results were highly diverse, indicating lack of knowledge, not having tools available or not correctly using the available guidance on what is important, as all sequences provided by the laboratories that reported sequences contained the expected markers.

In conclusion, this EEIQAP activity fulfilled its goals. All 30 laboratories detected all virus strains in the panel. Depending on virus strain, using NAAT, 17%–100% of 30 laboratories identified the H-subtype correctly. Depending on virus strain, using NAAT, 6%–100% of 16 laboratories performing N-subtyping identified the N-subtype correctly. Depending on the virus strain, using NAAT combined with sequencing, 76–100% of 21 laboratories that reported sequencing results identified the H- and N-subtype of the virus strains in the panel correctly. However, the proportion of laboratories (21/30; 70%) that provided sequencing results shows that 30% (9/30) could not provide definitive result or confirmation on the H- and N-subtype and 'likely host species' of all virus strains in the panel, especially for the included swine influenza viruses. Depending on virus strains in the panel, the clade, subclade and H5N1 genotype were assigned correctly by 82%-100%, 94% and 100% of the laboratories that reported results on clade (n=9–21), subclade (n=17) and genotype (n=4–5), respectively.

Finally, the EQA results provide a comprehensive assessment of preparedness of national influenza reference laboratories and countries in the whole of the EU/EEA, Western Balkan countries and Türkiye for the detection, identification and characterisation of potentially zoonotic influenza viruses, which is substantiating the recent laboratory survey results [28]. However, this assessment is limited because national influenza reference laboratories in only 27 (73%) of the 37 countries in the EU/EEA, WB and Türkiye participated. The overall results highlight the need for implementing highly specific H- and N-subtyping NAAT, as well as universal type A influenza virus sequencing, at more national influenza reference laboratories in order to increase the national capacity to recognise zoonotic influenza viruses for the purpose of rapid national response and international alert.

6 Recommendations

From the overall results of this EEIQAP 2024 study the following recommendations can be concluded to be carried out by national influenza reference laboratories, where needed assisted by ECDC (in collaboration with WHO), when performance is less than that of peers in this EEIQAP 2024, or capacity is lacking:

1. Reviewing and adjusting the analytical sensitivity of used NAAT for generic detection of type A influenza virus;
2. Increasing the capacity for specific H-subtyping using NAAT of the most important H-subtypes of type A influenza viruses that can cause zoonotic infection, especially of swine type A influenza viruses;
3. Increasing the capacity for N-subtyping using NAAT of the most important N-subtypes of type A influenza viruses that can cause zoonotic infection;
4. Increasing the capacity for influenza virus whole genome sequencing;
5. Improving the use of bioinformatics tools (existing or to be developed/improved) for generating correct consensus sequences and further evaluation of these with respect to clade, subclade and H5N1 genotype determination and identification of important amino acid markers for antiviral reduced susceptibility in all type A influenza viruses and of mammalian adaptation markers in zoonotic type A influenza viruses specifically.
6. Continuing to take part and increasing participation in international EQAs for influenza viruses that assess also the subtyping and sequencing as well as phenotypic characterisation.

The above-mentioned recommendations have the ultimate goal to increase the possibility that a zoonotic influenza virus infection would be timely detected for rapid national response and international alert through existing mechanisms of ECDC and WHO.

The above-mentioned recommendations are advised to be facilitated by targeted training through courses and twinning provided by ECDC (AURORAE) or future European Union Reference Laboratory (EURL) for respiratory viruses), were there is a need by a national reference laboratory or laboratories. It is advised to follow up trainings by implementation evaluation by ECDC to identify the effectiveness of training in improving capacity on the identified needs mentioned above.

Furthermore, national influenza reference laboratories (or the appropriate national bodies with which the national reference laboratories are associated) are recommended to do the following:

1. Conducting EQA-type studies among routine clinical diagnostic laboratories in their own country to identify readiness for at least detecting potential zoonotic type A influenza viruses using their routinely used tests in influenza diagnostics. A study performed in the Netherlands can be used as a guidance [38].
2. Increasing awareness among treating physicians in their own country to include exposure to animals known or potentially infected with type A influenza viruses in the patient's review of disease history, especially of severe hospitalised influenza A cases, according to ECDC's surveillance guidance documents [36,37].

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Annex 1. List of participating laboratories

Country	City	Institute
Albania	Tirana	Institute of Public Health 'Hulo Hajderi'
Belgium	Ukkel	Sciensano
Croatia	Zagreb	Croatian Institute of Public Health
Cyprus	Nicosia	Nicosia General Hospital/SHSO
Czechia	Prague	National Institute of Public Health
Denmark	Copenhagen	Statens Serum Institut/NIC
Estonia	Tallinn	Health Board, Laboratory of Communicable Diseases
Finland	Helsinki	Finnish Institute for Health and Welfare (THL)
France	Lyon	Centre National de Référence du Virus Influenza Région Sud
France	Paris	CNR de la Grippe - Institut Pasteur
Germany	Berlin	Robert Koch Institute
Greece	Athens	National Influenza Center for South Greece; Hellenic Pasteur Institute
Greece	Thessaloniki	National Influenza Center for North Greece
Hungary	Budapest	National Center for Public Health and Pharmacy
Italy	Rome	Istituto Superiore di Sanità
Kososvo	Prishtina	National Institute of Public Health of Kosovo
Latvia	Riga	Riga East University Hospital, Laboratory Service, Laboratory of Latvian Centre of Infectious Diseases, National Microbiology Reference Laboratory
Lithuania	Vilnius	National Public Health Surveillance Laboratory
Luxembourg	Dudelange	Laboratoire National de Santé
Malta	Msida	Mater Dei Hospital
The Netherlands	Bilthoven	National Institute for Public Health and the Environment
The Netherlands	Rotterdam	Erasmus MC
Norway	Oslo	Norwegian Institute of Public Health
Portugal	Lisbon	National Institute of Health Dr. Ricardo Jorge
Republic of Montenegro	Podgorica	Institute of Public Health Montenegro
Romania	Bucharest	Cantacuzino National Milirary Medical Institute for Research and Development
Slovenia	Ljubljana	National laboratory for health, environment and food, Laboratory for public health virology
Spain	Madrid	Instituto de Salud Carlos III
Sweden	Solna	Public Health Agency of Sweden
Türkiye	Ankara	General Directorate of Public Health

Participating laboratories are listed here in alphabetical order of the country and then city if more than one. The participant ID assigned for EEIQAP 2024 is a number assigned by QCMD remaining the same across EQA studies, not linked to alphabetical order of country, city or institute name.

Annex 2. Overview of challenge types participated in by each laboratory

Participant ID	Molecular Diagnostics	Genetic Characterisation
95	Yes	Yes
200	Yes	Yes
1299	Yes	Yes
1643	Yes	Yes
1991	Yes	No
2125	Yes	No
2253	Yes	No
2258	Yes	Yes
2270	Yes	No
2271	Yes	No
2272	Yes	No
2274	Yes	No
2278	Yes	No
2812	Yes	No
2819	Yes	Yes
3442	Yes	Yes
4121	Yes	Yes
4209	Yes	Yes
10007	Yes	Yes
10023	Yes	Yes
10080	Yes	Yes
10104	Yes	Yes
10115	Yes	Yes
10144	Yes	Yes
10462	Yes	Yes
10464	Yes	Yes
10466	Yes	Yes
10498	Yes	Yes
11111	Yes	Yes
11119	Yes	Yes

Annex 3. Nucleic acid extraction and amplification equipment and extraction and amplification reagents used by the participants

Participant ID	Extraction equipment	Extraction kit	NAAT equipment	NAAT in house	NAAT kit
95	Roche MagNA Pure 96	Roche MagNA Pure 96 DNA and Viral NA Large Volume kit	Roche LightCycler 480-II	Fast Virus 1-step Master Mix Custom made	
200	Roche MagNA Pure 96	Roche DNA and Viral NA Small Volume kit	Biorad CFX96 and Opus96	Many different	
1299	Macherey Nagel NucleoSpin Core virus	Macherey Nagel NucleoSpin Core virus	Roche Light Cycler 480	Thermo Superscript III RT/Platinum Taq	
1643	Qiagen QIACube	QIAamp minElute Virus Spin	Bio-Rad	SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase	
1991	KingFisher, ThermoFisher Scientific	CDC	ABI 7500 real-time PCR system		CDC Influenza Virus Real-time RT-PCR Kit
2125-1	EXM 3000, Zybio	NA extraction kit Zybio	Roche LightCycler 480 System (dataset 1)		IRR CDC, A (M generic), H1pdm, H3, H5a, H5b, H7 - CDC IRR primers probes positive controls, H9 16.VLA recommended method based on: Alessandra Isabella Monne et al: Development and Validation of a One-Step Real-Time PCR Assay for Simultaneous Detection of Subtype H5, H7, and H9 Avian Influenza VirusesJ. Clin. Microbiol. 2008, 46(5):1769
2125-2	EXM 3000, Zybio	NA extraction kit Zybio	Aus diagnostic system (dataset 2)		Respiratory virus Multiplex 24 panel, Aus diagnostic
2125-3	KingFisher Flex, ThermoFisher Scientific	MaxMax cored DNA/RNA isolation kit	BioRad CFX-96 (dataset 3)	Light CyclerMultiple RNA Virus Master, Roche	
2253	Biomerieux. NucliSens easyMAG	Biomerieux NucliSens easyMAG reagents	Rotor gene Q MDX 5plex, QUAGEN; Rotor-gene 6000, Corbett Research	Invitrogen SuperScript™III Platinum® One-Step Quantitative Kit	

Participant ID	Extraction equipment	Extraction kit	NAAT equipment	NAAT in house	NAAT kit
2258	KingFisher Flex, ThermoFisher Scientific	Applied Biosystems MagMAX Viral/Pathogen II Nucleic Acid Isolation CE-IVD kit	BioRad CFX-96		SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen) + CDC primers and probes (CDC Influenza Virus Real-Time RT-PCR Influenza A/B Typing Panel (VER 2) (RUO) (Catalog No. FluRUO-14); CDC Influenza Virus Real-Time RT-PCR Influenza A (H3/H1pdm09) Subtyping Panel (VER 3) (RUO) (Catalog No. FluRUO-15); CDC Real-Time RT-PCR Influenza Virus A/H5 (Asian Lineage) Subtyping Panel (VER 4) (RUO) (Catalog No. FluRUO-13); CDC Real-Time RT-PCR Influenza Virus A/H7 (Eurasian Lineage) Assay (RUO) (Catalog No. FluRUO-07))
2270	Qiagen EZ1 Advanced XL	EZ1 DSP Virus kit	Qiagen Rotor-Gene Q, Cepheid (Infinity)		XpertXpress CoV-2 plus, Viasure FluA/FluB, Viasure Flu/SARS-CoV-2/RSV
2271	manual extraction	Qiagen Viral RNA mini kit	BioRad CFX-96	PCR BIOSYSTEMS qPCRBIO Probe 1-step Go No-ROX	
2272	EXM 300, Zybio	Nucleic Acid Extraction kit	Qiagen Rotorgene Q	AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems)	
2274	Thermo Scientific KingFisher Flex	MagMAX Viral/Pathogen Kit	ABI 7500 and Rotorgene Q		Fast Track Diagnostics Respiratory Pathogens 33, Sacace Biotechnologies Influenza A H1N1 H3N2 Real-TM, Sacace Biotechnologies Influenza A H5 H7 H9 Typing FRT
2278	QIAGEN, QIA Cube Connect	QIAamp MinElute Virus Spin Kit	QIAGEN, Rotor-Gene Q	Invitrogen, SuperScript III Platinum One-Step qRT-PCR Kit	
2812	Qiasymphonie Qiagen	Qiagen Repiratory protocol	ABI Quantstudio 5		CDC IRR kit
2819	Generotex96, Tianlong, China	RNA/DNA viral extraction kit	QS5 Applied Biosystem, CFX Biorad	TaqMan™ Fast Virus 1-Step Master Mix for qPCR, Applied Biosystems	
3442	StarLet Seegene	StarMag Universal Cartridge Kit	BioRad CFX-96		SACACE - Influenza A H5 H7 H9 Typing FRT; Allplex Respiratory Panel 1A (RP1A)
4121	Manual extraction	QIAamp Viral RNA Mini Kits from QIAGEN	BioRad CFX-96		CDC Influenza Virus Real-Time RT-PCR Influenza A
4209	B-200, Zybio	Zybio nucleic acid extraction kit	BioRad CFX-96	AgPath-ID One-step RT-PCR Kit	
10007	Manual extraction	Qiagen RNeasy mini kit	CFX Opus	QIAGEN QuantiTect Multiplex PCR NoRox Kit	

Participant ID	Extraction equipment	Extraction kit	NAAT equipment	NAAT in house	NAAT kit
10023	Precision System Science	PSS magLEAD 12gC	QuantStudio 6 Flex Applied Biosystems	TaqMan Fast Virus 1-step MM Applied Biosystems	
10080	Roche MagNA Pure 96	Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit	Roche; Lightcycler 480 II	Applied Biosystems; TaqMan™ Fast Virus 1-Step Master Mix for qPCR	
10104	NucliSENS EMAG (bioMérieux)	NucliSens easyMAG	Aria MX (Agilent)	SuperScript™III Platinum® One-Step Quantitative Kit	
10115	Emag (bioMérieux)	Nuclisens lysis buffer (EasyMag or Emag bioMérieux)	QuantStudio Applied	PLATINUM ONE STEP QRT-PCR 11732088 (SUPERSCRIPT III)	
10144	MagNA Pure 96, Roche Diagnostics or QIAcube, QIAGEN	MagNA Pure 96 DNA and Viral NA Small Volume Kit, Roche or QIAamp Viral RNA Mini kit, QIAGEN	RotorGene Q, QIAGEN	AgPath-ID One-Step RT-PCR, Applied Biosystems (subtyping) and UltraPlex 1-Step ToughMix (4X), Quantabio (typing)	
10462	Seegene Starlet	Starmag 96x4 Universal Cartridge	BioRad CFX-96		Seegene Allplex Essential RV used for screening, then Seegene Allplex Respiratory Virus 1A used for H1, H1pdm09, H3 subtyping/In house kit for subtyping H5, H7 and H9
10464	No, we don't use an extractor machine	Qiagen, QIAamp Viral RNA Mini Kit	RT-PCR machine: QuantStudio-5 (Thermo Fisher Scientific); NAAT machine: TProfessional basic gradient (Biometra)		CDC(FLURUO-15); CDC (FLURUO-13); CNIC-H7N9; CNIC-H10; FTD-Respiratory Pathogens 21 (Elitech group)
10466	Roche MagNA Pure 96	Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit	Roche LightCycler 480 II	New England Biolabs Luna® Probe One-Step RT-qPCR Kit (No ROX) (Human and swine subtypes); Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX) (avian subtypes)	
10498	QIAGEN EZ1 Advanced XL	EZ1 Virus Mini Kit V2.0	BioRad CFX-96		CDC Influenza Virus Real-time RT-PCR Panel (RUO), Influenza A(H3/H1 pdm09) Subtyping Panel (VER3) FluRUO-15, Influenza A/H5 (Asian Lineage) Subtyping Panel (VER 3) FluRUO-08, Influenza A/H7 (Eurasian Lineage) Assay FluRUO-07
11111	Roche MagNA Pure 96	Roche MagNA Pure 96 DNA and Viral NA	Roche LightCycler 480 II		CDC Influenza SC2 Multiplex Assay
11119	MagNa Pure, Roche	Roche MagNA Pure DNA and Viral NA Small Volume Kit	QuantStudio 7Pro, Thermo Fisher Scientific	GoTaq Probe 1-Step RT-qPCR System, Promega	

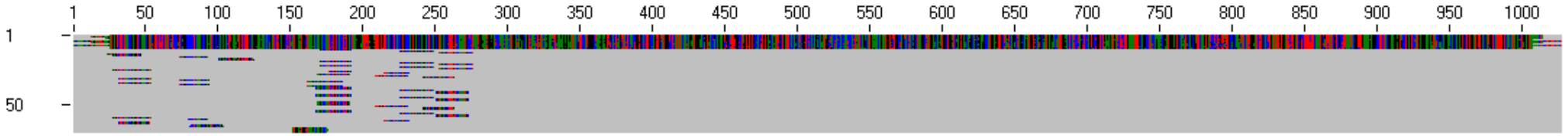
Annex 4. Results for generic detection of type A influenza virus, by laboratory and panel specimen

Participant ID [1]	Generic type A influenza virus NAAT detection result by panel strain Ct value [1]													
	H5N1st d1	H5N1st d2	H5N1st d3	H5N1st d4	H5N1st d5	H5N1ew	H5N1bhg	H5N8	H7N2	H1N1s	H1N2s	H3N2s	H1N1pdm09	H3N2h
95	25.0	39.3	**Neg**	Neg	Neg	25.1	20.9	24.0	29.7	26.6	23.3	29.1	28.6	28.7
200	21.3	34.0	37.3	Neg	Neg	21.0	19.5	21.0	28.2	23.9	19.4	25.8	25.1	24.9
1299	21.5	35.3	39.7	Neg	Neg	20.8	19.8	22.0	27.5	23.8	20.4	25.4	29.2	24.5
1643	18.3	32.5	37.7	Neg	Neg	19.5	16.2	19.1	25.1	21.5	17.5	22.7	24.7	24.4
1991	17.3	31.0	35.5	38.2	Neg	17.9	16.1	17.0	24.9	20.8	16.7	21.2	22.3	22.4
2125-1	21.1	35.6	39.4	Neg	Neg	22.0	21.0	20.5	32.9	24.7	19.9	25.9	27.9	27.9
2125-2	Pos [2]	Pos [2]	**Neg**	Neg	Neg	Pos [2]	Pos [2]	Pos [2]	Pos [2]	Pos [2]	Pos [2]	Pos [2]	Pos [2]	Pos [2]
2125-3	21.1	35.6	39.4	Neg	Neg	22.0	21.0	20.5	32.9	27.7	19.9	25.9	27.9	27.9
2253	14.3	28.6	33.7	34.5	35.0	15.5	11.9	15.5	21.3	18.0	13.8	20.1	18.7	19.1
2258	21.3	34.5	37.6	Neg	Neg	22.2	16.3	22.7	27.7	25.4	22.4	25.5	25.2	25.6
2270	19.1	33.4	**Neg**	Neg	Neg	20.0	18.4	17.8	28.4	21.9	17.9	21.4	22.0	22.9
2271	19.0	33.0	35.0	36.0	Neg		20.0	19.0	26.0	24.0	18.0	24.0	24.0	25.0
2272	16.1	28.6	**Neg**	Neg	Neg	19.0	14.5	20.0	23.4	18.9	19.3	20.2	23.1	21.5
2274	19.0	33.0	34.0	Neg	Neg	21.0	17.0	19.0	27.0	23.0	18.0	23.0	24.0	24.0
2278	15.0	29.2	32.1	Neg	Neg	15.8	14.6	14.8	22.6	19.2	13.8	19.6	20.2	20.2
2812	26.9	**Neg**	**Neg**	Neg	Neg	Pos [3]	21.9	30.4	30.7	30.0	30.9	24.6	31.4	28.9
2819	18.0	31.0	35.0	Neg	Neg	18.0	16.0	17.0	24.0	21.0	16.0	21.0	22.0	23.0
3442	20.9	37.2	**Neg**	Neg	Neg	20.7	18.2	21.4	29.1	24.4	19.4	27.6	24.8	26.1
4121	25.2	40.3	**Neg**	Neg	Neg	25.5	25.1	25.3		27.9	25.1	28.2	31.1	31.0
4209	20.5	33.3	**Neg**	Neg	Neg	22.8	22.4	22.0	27.1	24.4	21.4	26.7	29.7	28.3
10007	22.8	37.1	**Neg**	Neg	Neg	22.0	21.2	22.8	29.9	26.7	22.3	28.6	28.1	29.4
10023	18.4	31.8	34.5	Neg	Neg	18.1	14.8	18.2	24.7	21.3	17.9	22.6	23.0	22.4
10080	17.9	30.9	34.7	Neg	Neg	19.2	15.5	17.6	24.3	20.6	16.6	20.6	22.9	22.1
10104	19.9	35.9	**Neg**	Neg	Neg	21.7	18.0	21.8	27.2	25.0	18.9	25.9	24.7	25.0
10115	18.1	31.4	38.2	Neg	Neg	17.4	15.4	17.2	24.2	21.0	17.3	23.6	23.3	21.5
10144	14.5	26.7	30.3	Neg	Neg	14.8	11.8	13.7	20.7	17.4	13.5	17.5	18.7	18.6
10462	20.2	34.9	37.1	Neg	Neg	22.1	19.2	23.5	28.6	25.1	20.3	28.0	26.2	26.8
10464	20.0	34.0	35.0	Neg	Neg	19.0	18.0	19.0	33.0	23.0	18.0	25.0	24.0	25.0
10466	20.6	33.7	34.5	Neg	Neg	20.4	19.2	21.0	26.8	24.6	20.6	23.7	26.1	26.8
10498	16.0	29.0	32.3	Neg	Neg	16.8	26.5	16.9	22.8	19.0	15.1	19.2	20.6	20.6
11111	21.1	29.0	37.2 [4]	Neg	Neg	22.6	18.6	21.1	27.3	24.2	20.0	24.3	25.8	25.4
11119	22.5	40.4	**Neg**	Neg	Neg	24.7	20.5	22.7	29.7	26.3	23.2	25.6	28.1	28.0

1. NAAT = Nucleic Acid Amplification Test; Ct = Cycle Threshold; d1-d5: dilutions of the H5N1st panel strain; Neg = negative result in NAAT; Pos = positive result in NAAT; orange shading of cells: laboratory that uploaded primers and probes sequences for evaluation; red shading of cells: expected result is positive; grey shading of cells: laboratory did not receive this panel specimen. 2. No Ct value provided by laboratory, as NAAT has touchdown temperature cycling program. 3. No Ct value provided by laboratory for unknown reason. 4. Ct value of H5 subtyping NAAT, as detection in the generic type A influenza virus NAAT was negative.

Annex 5. Location of primers and probes on the MP genome segment and match with panel viruses

For the sequence alignment a bitmap image is displayed. The full detailed alignment is available on request to ECDC. Primers and probes are per NAAT shown in the order from top to bottom: forward primer(s), probe(s), reverse primer(s). MP genome segment sequences of all virus strains in the panel have been included as reference.



Annex 6. H-subtype and N-subtype specificities of readily available and used NAAT

The shown subtype and host species specificity data for H-subtyping NAAT (A) and N-subtyping NAAT (B) has been extracted from the extra survey question on the NAAT readily available in the laboratory (Annex 15), supplemented with specificity data on NAAT that have been used to generate panel testing results if not listed in the survey results.

A. H-subtyping NAAT

Participant ID	NAAT specifity with regard to H-subtype and host species targeted [1]											
	H1g	H1pp	H1p	H1s	H3g	H3h	H3s	H5a	H6a	H7a	H9a	H10a
95		y	y		y [2]	y		y		y		
200	y	y	y	y	y	y	y	y		y	y	y
1299		y	y			y		y		y	y	y
1643	y	y	y	y	y	y	y	y	y	y	y	y
1991	y				y			y		y		
2125 [3]			y		y [2]	y		y	y	y	y	y
2253			y			y		y		y		
2258			y		y			y		y		
2270	y		y		y			y		y	y	
2271			y			y		y		y	y	
2272			y		y	y [2]		y		y		
2274	y		y		y			y		y	y	
2278		y	y		y			y		y	y	
2812			y			y		y		y		
2819			y [2]			y		y [2]		y [2]		
3442			y			y		y		y	y	
4121			y		y			y		y		
4209			y		y			y		y		
10007		y	y			y		y		y	y	
10023			y			y		y		y		
10080			y			y		y		y	y	
10104	y		y [2]	y [2]		y [2]	y [2]	y [2]		y [2]	y [2]	
10115			y	y		y		y		y		
10144			y		y			y		y	y	
10462	y		y		y			y		y	y	
10464			y	y		y	y	y	y	y	y	y
10466			y	y		y	y	y		y	y	
10498			y		y			y		y		

Participant ID	NAAT specifity with regard to H-subtype and host species targeted [1]											
	H1g	H1pp	H1p	H1s	H3g	H3h	H3s	H5a	H6a	H7a	H9a	H10a
11111			y		y			y		y	y	
11119			y		y	y [2]		y		y	y	

1. y = yes, NAAT available; host species: a = avian, g = generic, h = human, p = pdm09, pp = pre-2009 pandemic human, s = swine.
2. Indicated by participant that NAAT with this specificity was used to generate reported testing results; not listed in the participant answer to the survey question on readily available NAAT.
3. Combination of three different assay setups.

B. N-subtyping NAAT

Participant ID	NAAT specificity with regard to H-subtype and host species targeted [1]															
	N1g	N1pp	N1p	N1a	N1s	N2g	N2h	N2a	N2s	N3a	N4a	N5a	N6a	N7a	N8a	N9a
95			y													
200	y	y	y	y	y	y	y	y	y	y	y	y	y		y	
1299		y	y	y		y									y	y
1643	y					y										
1991												y				
2125 [2]	y			y				y		y	y	y	y	y	y	y
2253							y									
2258	y															
2270	y		y			y										
2271			y	y			y									
2272			y	y		y										
2274	y		y			y										
2278																y
2812	y															
2819						y	y [3]									
3442	y						y									
4121	y															
4209	y															
10007			y				y							y		
10023			y													
10080			y	y			y									
10104	y		y [3]				y [3]									y [3]
10115			y	y	y [3]		y								y	y
10144	y															
10462																y

Participant ID	NAAT specificity with regard to H-subtype and host species targeted [1]															
	N1g	N1pp	N1p	N1a	N1s	N2g	N2h	N2a	N2s	N3a	N4a	N5a	N6a	N7a	N8a	N9a
10464			y	y	y		y	y	y	y		y	y	y	y	y
10466			y	y			y						y		y	
10498			y													
11111	y					y										
11119	y					y	y [3]								y	

1. y = yes, NAAT available; host species: a = avian, g = generic, h = human, p = pdm09, pp = pre-2009 pandemic human, s = swine.
2. Combination of three different assay setups.
3. Indicated by participant that NAAT with this specificity was used to generate reported testing results; not listed in the participant answer to the survey question on readily available NAAT.

Annex 7. Result for subtyping by NAAT, by laboratory and panel specimen

The table has been split in two parts, A. all panel viruses with H5 subtype and B. all panel viruses with other H-subtype than H5. Footnotes are listed below part B and are numbered left to right and top to bottom as if both tables were horizontally connected.

A. Viruses in the panel with H5 H-subtype

Participant ID	H subtyping in dataset [1]	N subtyping in dataset [1]	Reported subtype after NAAT [1]							
			H5N1st d1	H5N1st d2	H5N1st d3	H5N1st d4	H5N1st d5	H5N1ew	H5N1bhg	H5N8
95	H1p, H1pp, H3g, H5a, H7a	N1p	A(H5Nx)	A(H5Nx)	Negative	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
200	H1p, H1s, H3h, H3s, H5a, H7a	N1p, N1a, N1s, N2h, N2g, N8a	A(H5N1)	A(H5N1)	A no subtype [2]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
1299	H1p, H3h, H5a, H7a	N1p, N1a, N2g, N8a	A(H5N1)	A(H5N1)	A(H5Nx) [4]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
1643	H1p, H1s, H3h, H3s, H5a, H7a		A(H5Nx)	A(H5Nx)	A(H5Nx)	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
1991	H1g, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	A(H5Nx)	A(H5Nx)	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
2125-1	H1p, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	Other [7]	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
2125-2	H1p, H3h		A(H5Nx) [8]	A(H5Nx) [8]	Other [9]	Negative	Negative	A(H5Nx) [8]	A(H5Nx) [8]	A(H5Nx) [8]
2125-3	H5a, H7a		A(H5Nx)	A(H5Nx) [11]	Negative [12]	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
2253	H1p, H3h, H5a, H7a		A(H5Nx)	A(H5Nx)	A(H5Nx)	A(H5Nx)	Other [14]	A(H5Nx)	A(H5Nx)	A(H5Nx)
2258	H1p, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	A(H5Nx)	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
2270	H1p, H3g, H5a, H7a	N1g, N2g	A(H5N1) [16]	A(H5N1) [16]	Negative	Negative	Negative	A(H5N1) [16]	A(H5N1) [16]	A(H5Nx)
2271	H1p, H3h, H5a, H7a	N1p, N1a, N2h	A(H5N1)	A(H5N1)	A(H5N1)	A(H5N1)	Negative		A(H5N1)	A(H5Nx)
2272	H1p, H3h, H5a, H7a		A(H5Nx)	A(H5Nx)	Negative	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
2274	H1g, H3g, H5a, H7a	N1g, N2g	A(H5Nx)	A(H5Nx)	A no subtype [2]	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
2278	H1p, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	A no subtype [2]	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
2812	H1p, H3h, H5a, H7a		A(H5Nx)	Negative	Negative	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
2819	H1p, H3h, H5a, H7a	N2h	A(H5Nx)	A(H5Nx)	A no subtype [2]	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
3442	H1p, H3h, H5a, H7a	N1g, N2h	A(H5N1) [16]	A(H5N1) [16]	Negative	Negative	Negative	A(H5N1) [16]	A(H5N1) [16]	A(H5Nx)
4121	H1p, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	Negative	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
4209	H1p, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	Negative	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
10007	H1p, H3h, H5a, H7a	N1p, N2h	A(H5Nx)	A(H5Nx)	Negative	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
10023	H1p, H3h, H5a, H7a		A(H5Nx)	A(H5Nx)	A(H5Nx)	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
10080	H1p, H3h, H5a, H7a	N1p, N1a, N2h	A(H5N1)	A(H5N1)	A(H5N1)	Negative	Negative	A(H5N1)	A(H5N1)	A(H5Nx)
10104	H1p, H1s, H3h, H3s, H5a, H7a	N1p, N2h	A(H5Nx)	A(H5Nx)	Negative	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
10115	H1p, H3h, H5a, H7a	N1a, N1s, N2h, N8a	A(H5N1)	A(H5N1)	A(H5N1)	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10144	H1p, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	A(H5Nx)	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
10462	H1p, H1g, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	A(H5Nx)	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
10464	H1p, H1s, H3h, H3s, H5a, H7a	N1p, N1a, N1s, N2h, N2a, N2s, N8a	A(H5N1)	A no subtype [2]	A no subtype [2]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10466	H1p, H1s, H3h, H3s, H5a, H7a	N1p, N1a, N2h. N8a	A(H5N1)	A(H5N1)	A(H5N1)	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10498	H1p, H3g, H5a, H7a		A(H5Nx)	A(H5Nx)	A(H5Nx)	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)
11111	H1p, H3g, H5a, H7a	N1g, N2g	A(H5Nx)	A(H5Nx)	A(H5Nx) [36]	Negative	Negative	A(H5Nx)	A(H5Nx)	A(H5Nx)

11119	H1p, H3h, H5a, H7a	N1g, N2h, N8a	A(H5N1) [16]	A(H5N1) [16]	Negative	Negative	Negative	A(H5N1) [16]	A(H5N1) [16]	A(H5Nx) [37]

B. Viruses in the panel with other H-subtype than H5

Participant ID	H subtyping in dataset [1]	N subtyping in dataset [1]	Reported subtype after NAAT [1]					
			H7N2	**H1N1s**	**H1N2s**	**H3N2s**	**H1N1pdm09**	**H3N2h**
95	H1p, H1pp, H3g, H5a, H7a	N1p	A(H7Nx)	A no subtype	A no subtype	A(H3Nx)	A(H1N1)pdm09	A(H3Nx)
200	H1p, H1s, H3h, H3s, H5a, H7a	N1p, N1a, N1s, N2h, N2g, N8a	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine [3]	A(H1N1)pdm09	A(H3N2)human
1299	H1p, H3h, H5a, H7a	N1p, N1a, N2g, N8a	A(H7Nx)	A no subtype [5]	A no subtype	A no subtype	A(H1N1)pdm09	A(H3N2)human
1643	H1p, H1s, H3h, H3s, H5a, H7a		A(H7Nx)	A(H1swineNx)	A(H1swineNx)	A(H3swineNx)	A(H1pdm09Nx)	A(H3Nx)
1991	H1g, H3g, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A(H3Nx)	A(H1Nx) [6]	A(H3Nx)
2125-1	H1p, H3g, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A(H3Nx)	A(H1pdm09Nx)	A(H3Nx)
2125-2	H1p, H3h		A(H7Nx) [8]	A no subtype	A no subtype	A(H3Nx) [10]	A(H1pdm09Nx)	A(H3Nx)
2125-3	H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A(H3Nx) [13]	A(H1pdm09Nx)	A(H3Nx) [13]
2253	H1p, H3h, H5a, H7a		A(H7Nx)	Other	Other	A(H3humanNx) [15]	A(H1pdm09Nx)	A(H3humanNx)
2258	H1p, H3g, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A(H3Nx)	A(H1pdm09Nx)	A(H3Nx)
2270	H1p, H3g, H5a, H7a	N1g, N2g	A no subtype [17,18]	A(HxN1) [19]	A no subtype [18]	A no subtype [18]	A(H1N1)pdm09 [4]	A(H3N2) [18]
2271	H1p, H3h, H5a, H7a	N1p, N1a, N2h	A(H7Nx)	A no subtype [5]	A no subtype	A no subtype	A(H1N1)pdm09	A(H3N2)
2272	H1p, H3h, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A(H3humanNx)	A(H1pdm09Nx)	A(H3Nx)
2274	H1g, H3g, H5a, H7a	N1g, N2g	A(H7Nx) [20]	A(H1N1) [21,22]	A(H1Nx) [20,21]	A no subtype [20,23]	A(H1N1)pdm09 [21,22]	A(H3N2) [20,23]
2278	H1p, H3g, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A(H3Nx)	A(H1pdm09Nx)	A(H3Nx)
2812	H1p, H3h, H5a, H7a		A no subtype [17]	A no subtype	A no subtype	A no subtype	A(H1pdm09Nx)	A(H3humanNx)
2819	H1p, H3h, H5a, H7a	N2h	A(H7Nx)	A no subtype	A no subtype	A(H3humanNx)	A(H1pdm09Nx)	A(H3N2)human
3442	H1p, H3h, H5a, H7a	N1g, N2h	A(H7Nx)	A(HxN1) [19]	A no subtype	A(H3Nx) [10]	A(H1N1)pdm09 [16]	A(H3N2)human
4121	H1p, H3g, H5a, H7a			A no subtype	A no subtype	A(H3Nx)	A(H1pdm09Nx)	A(H3Nx)
4209	H1p, H3g, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A(H3Nx)	A(H1pdm09Nx)	A(H3Nx)
10007	H1p, H3h, H5a, H7a	N1p, N2h	A no subtype [17]	A no subtype	A no subtype	A(H5Nx) [24]	A(H1N1)pdm09	A(H3N2)human
10023	H1p, H3h, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A no subtype	A(H1pdm09Nx)	A(H3humanNx)
10080	H1p, H3h, H5a, H7a	N1p, N1a, N2h	A(H7Nx)	A no subtype [25]	A no subtype	A no subtype	A(H1N1)pdm09	A(H3N2)human
10104	H1p, H1s, H3h, H3s, H5a, H7a	N1p, N2h	A(H7Nx)	A(H1swineNx)	A(H1swineNx)	A(H3swineNx)	A(H1N1) [26]	A(H3N2)human
10115	H1p, H3h, H5a, H7a	N1a, N1s, N2h, N8a	A(H7Nx)	A(HxN1) [27]	A(HxN1) [28]	A(H3Nx) [10]	A(H1N1)pdm09 [29]	A(H3N2)human
10144	H1p, H3g, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A(H3Nx)	A(H1N1)pdm09 [30]	A(H3Nx)
10462	H1p, H1g, H3g, H5a, H7a		A(H7Nx)	A no subtype	A no subtype	A no subtype	A(H1pdm09Nx)	A(H3Nx)
10464	H1p, H1s, H3h, H3s, H5a, H7a	N1p, N1a, N1s, N2h, N2a, N2s, N8a	A(H7Nx)	A no subtype [31]	A(H1N2) [32]	Unknown [33]	A(H1N1)pdm09	A(H3N2)human
10466	H1p, H1s, H3h, H3s, H5a, H7a	N1p, N1a, N2h. N8a	A(H7Nx)	A(H1N1)swine	A(H1N1)swine [34]	A(H3swineNx)	A(H1N1)pdm09	A(H3N2)human
10498	H1p, H3g, H5a, H7a		A(H5Nx) [35]	Unknown	Unknown	A(H3Nx)	A(H1N1)pdm09 [30]	A(H3Nx)
11111	H1p, H3g, H5a, H7a	N1g, N2g	A(H7Nx)	A no subtype	A no subtype	A(H3Nx)	A(H1N1)pdm09 [16]	A(H3N2)
11119	H1p, H3h, H5a, H7a	N1g, N2h, N8a	A(H7Nx)	A(HxN1) [19]	A no subtype	A(H3Nx) [10]	A(H1N1)pdm09 [16]	A(H3N2)human

Colour coding used to categories NAAT subtyping results

Wrong
- Fully negative whereas expected result is positive
- Incorrect subtype
- Correct subtype but incorrect 'likely host species' indication

Not wrong but without subtype
- A no subtype, Unknown, Other

Correct subtype
- H and N subtype correct and 'likely host species' indication correct
- H or N subtype correct and 'likely host species' indication correct
- Negative correct as is expected result

Correct subtype without 'likely host species' indication; generic subtype
- Generic H and N subtype correct
- generic H or N subtype correct

- Laboratory did not receive this specimen

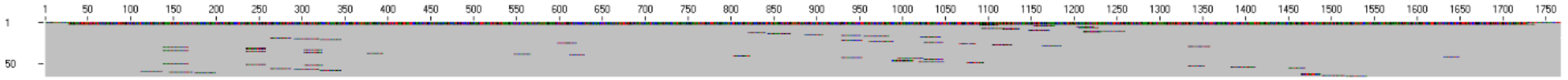
1. a = avian; g = generic; h = human; p = pdm09; pp = pre-pandemic; s = swine; d1-d5 = dilutions of H5N1st; orange shading of Participant ID indicates that this participant uploaded primers and probes information for one or more of the H- and N-subtype NAAT to subtype the panel virus strains and that was used in the analysis; bold letter type of Participant ID indicates laboratories that performed sequencing.
2. H5 NAAT drops off early
3. H3 human and H3 swine NAAT were negative, only N2 generic positive; not clear where H3 and swine come from
4. N1 generic NAAT drops off earlier than H5 NAAT
5. Specimen was positive in N1pmd09 NAAT
6. H1generic positive, seems not to cross-react with H1swine
7. Reported generic type A influenza virus NAAT positive
8. H5 or H7 likely concluded from other dataset as this dataset did not include H5 and H7 subtyping NAAT
9. Concluded from other dataset as for this dataset generic detection of type A influenza virus was negative
10. H3human NAAT positive with H3swine; cross-reactive
11. Likely concluded from other dataset as H5 subtyping NAAT was reported negative
12. Reported Negative whilst generic type A influenza virus detection was positive
13. Likely concluded from other dataset as no H3 NAAT used in this dataset
14. H5 subtyping NAAT drops off
15. H3human NAAT positive with high Ct
16. N1 NAAT generic; 'likely host species' concluded from H-subtyping specificity
17. Has H7 NAAT but negative
18. Laboratory indicated H3 and N2 NAAT generic, but in this panel seems H3N2human specific
19. N1 NAAT generic; without specific H-subtyping 'likely host species' identification not possible
20. N2generic NAAT seems N2human specific as only H3N2human positive
21. H1generic NAAT seems to have specificity for H1swine and H1pdm09

22. N1generic NAAT seems to have specificity for N1pdm09 and N1swine but not N1avian
23. H3generic NAAT seems H3human specific as only H3N2human positive
24. All subtyping NAAT negative; not clear where H5 comes from
25. Specimen was positive in N1avian NAAT with high Ct
26. H- and N-subtyping NAAT seem to be pdm09 specific; reporting error?
27. N1swine NAAT positive
28. N1swine NAAT positive with high Ct, indicating cross-reactivity
29. N1swine NAAT positive; swine probably indicates also pdm09, as is of swine origin
30. Does not test for N-subtype; probably a reporting error
31. H1swine NAAT negative and N1swine NAAT not used
32. H1swine NAAT positive and N2human NAAT positive, but N2swine NAAT negative
33. H3swine NAAT negative, N2swine NAAT not used, N2avian NAAT positive
34. N1 derived from avian influenza virus N1 subtyping NAAT that was positive with high Ct value, indicating cross-reactivity
35. Has H7 NAAT positive and H5 NAAT negative; likely a reporting error
36. Only H5 NAAT positive; generic type A influenza virus NAAT was negative
37. Has N8 NAAT, but negative

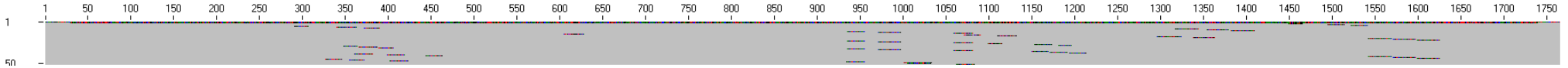
Annex 8. Location of subtyping NAAT primers and probes on the HA and NA genome segments and match with panel viruses by H- and N-subtype

For all sequence alignments a bitmap image is displayed. Full detailed alignments are available on request to ECDC. Primers and probes are per NAAT shown in the order from top to bottom: forward primer(s), probe(s), reverse primer(s).

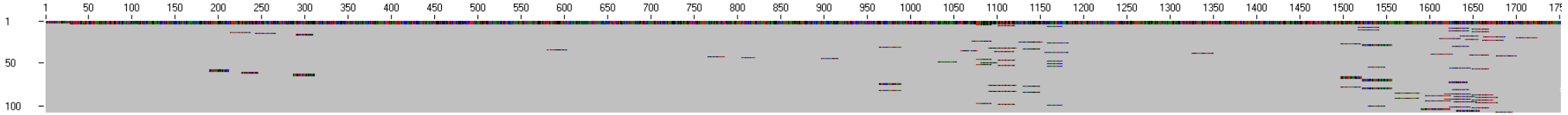
A. H1 subtype HA genome segment. Primers/probes aligned against HA sequences of panel viruses H1N1pdm09, H1N1s and H1N2s as reference.



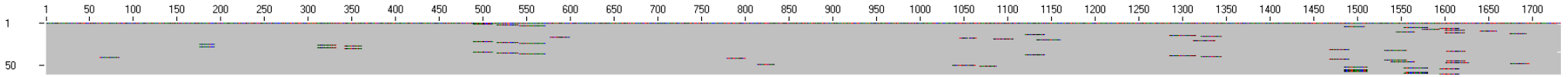
B. H3 subtype HA genome segment. Primers/probes aligned against HA sequences of panel viruses H3N2h and H3N2s as reference.



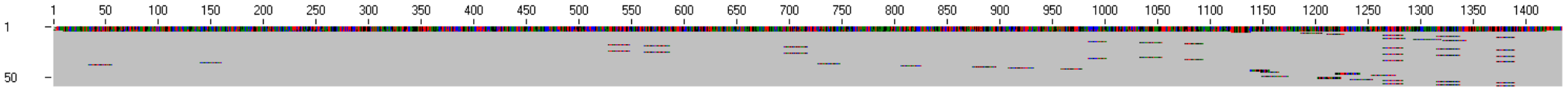
C. H5 subtype HA genome segment. Primers/probes aligned against HA sequences of panel viruses H5N1st, H5N1ew, H5N1bhg and H5N8 as reference.



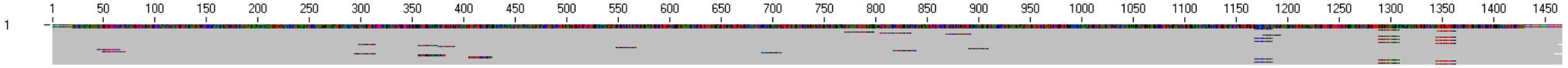
D. H7 subtype HA genome segment. Primers/probes aligned against HA sequence of panel virus H7N2 as reference.



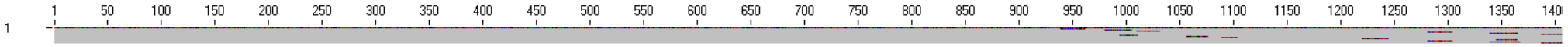
E. N1 subtype HA genome segment. Primers/probes aligned against HA sequences of panel viruses H1N1pdm09, H1N1s, H5N1st, H5N1ew and H5N1bhg as reference.



F. N2 subtype HA genome segment. Primers/probes aligned against HA sequences of panel viruses H3N2h, H1N2s, H3N2s and H7N2 as reference.



G. N8 subtype HA genome segment. Primers/probes aligned against HA sequence of panel virus H5N8 as reference.



Annex 9. Assessment of quality of the reported consensus sequences, by laboratory, virus strain in the panel and genome segment

An assessment of consensus sequence quality of the protein coding reading frames was made by percentage identical nucleotide compared to the panel reference sequences in which gaps in the reported sequences were counted, by percentage coverage of reported nucleotides compared to panel reference sequences and by percentage identical nucleotide relative to the covered region of a segment. The colour scheme is continuous from green to yellow to red with midpoint yellow at 99.7%, indicating ≥99.8% nucleotide identity/coverage, 99.0% - <99.8%, nucleotide identity/coverage and <99.8% nucleotide identity/coverage. Grey background colour of cells means that the laboratory did not receive the panel specimen with the virus on which the data is shown in the table. In all tables, no seq = no sequence uploaded by the participant.

H1N2s

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	100%	100%	100%	100%	100%	32.0%	39.4%	100%	100%	100%	100%	100%	100%	32.1%	39.4%	100%	100%	100%	100%	100%	100%	99.6%	100%	100%
200	100%	100%	100%	100%	100%	99.9%	100%	98.3%	100%	100%	100%	100%	100%	100%	100%	100.2%	100%	100%	100%	100%	100%	99.9%	100%	98.1%
1299	100%	100%	100%	99.8%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%
1643	99.4%	100%	100%	100%	100%	99.7%	99.8%	96.9%	99.8%	100%	100%	100%	100%	100%	100%	97.1%	99.6%	100%	100%	100%	100%	99.7%	99.8%	99.8%
2258	96.4%	100%	100%	100%	100%	99.8%	100%	100%	96.5%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	99.8%	100%	100%
2819	56.9%	93.7%	100%	99.7%	100%	88.8%	98.8%	98.2%	57.0%	93.9%	100%	100%	100%	88.9%	99.0%	98.5%	99.9%	99.8%	100%	99.7%	100%	99.8%	99.8%	99.7%
3442	81.8%	100%	100%	100%	100%	100%	100%	100%	81.9%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
4121	no seq	99.7%	100%	19.8%	100%	46.5%	52.6%	22.1%		99.8%	100%	19.8%	100%	46.7%	52.7%	22.1%		99.9%	100%	99.8%	100%	99.6%	99.8%	99.8%
4209	100%	100%	100%	100%	100%	99.9%	99.8%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	99.8%	100%
10007	100%	100%	100%	100%	99.7%	100%	99.9%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.7%	100%	99.9%	99.9%
10023	95.7%	99.5%	100%	no seq	99.0%	99.9%	99.7%	99.6%	97.4%	100%	100%		99.8%	100%	100%	100%	98.2%	99.5%	100%		99.2%	99.9%	99.7%	99.6%
10080	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10104	100%	100%	100%	100%	100%	99.8%	99.9%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.8%	99.9%	99.9%
10115	100%	100%	100%	100%	100%	100%	99.7%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	99.8%	100%
10144	46.2%	100%	100%	100%	100%	99.8%	98.7%	99.9%	46.3%	100%	100%	100%	100%	100%	98.7%	100%	99.7%	100%	100%	100%	100%	99.8%	100%	99.9%
10462	98.4%	100%	100%	99.3%	100%	99.7%	99.9%	99.4%	98.8%	100%	100%	100%	100%	100%	99.9%	100%	99.6%	100%	100%	99.3%	100%	99.7%	100%	99.4%
10464	91.0%	96.7%	100%	100%	100%	99.8%	100%	100%	91.1%	96.7%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	99.8%	100%	100%
10466	100%	99.7%	100%	100%	99.7%	99.2%	99.6%	99.7%	100%	100%	100%	100%	100%	99.4%	100%	100%	100%	99.7%	100%	100%	99.7%	99.8%	99.6%	99.7%
10498	99.8%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.6%	100%	100%	100%	100%	100%	100%	100%
11111	100%	100%	100%	100%	99.7%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.7%	100%	99.9%	100%
11119	no seq	100%	100%	99.8%	100%	99.7%	98.8%	99.8%		100%	100%	100%	100%	100%	98.9%	100%		100%	100%	99.7%	100%	99.7%	99.9%	99.8%

H5N8

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
200	100%	100%	100%	100%	100%	50.6%	96.6%	78.1%	100%	100%	100%	100%	100%	50.7%	96.7%	78.2%	100%	100%	100%	100%	100%	99.9%	99.9%	99.9%
1299	98.7%	97.7%	100%	98.6%	100%	35.6%	56.4%	57.4%	98.7%	97.7%	100%	98.7%	100%	35.7%	56.5%	57.5%	100%	100%	100%	99.9%	100%	99.7%	99.8%	99.8%
1643	98.2%	100%	100%	100%	100%	42.8%	65.9%	68.9%	98.3%	100%	100%	100%	100%	42.8%	66.0%	69.1%	99.9%	100%	100%	100%	100%	100%	99.9%	99.7%
2258	83.5%	100%	100%	100%	100%	58.3%	56.6%	50.0%	83.8%	100%	100%	100%	100%	58.7%	56.7%	50.0%	99.6%	100%	100%	100%	100%	99.3%	99.9%	99.9%
2819	43.8%	55.2%	99.8%	65.3%	100%	91.1%	69.8%	68.2%	44.1%	55.2%	100%	65.5%	100%	91.4%	69.9%	77.1%	99.4%	100%	99.8%	99.7%	100%	99.7%	99.9%	88.5%
3442	100%	100%	100%	100%	100%	99.3%	99.6%	95.3%	100%	100%	100%	100%	100%	99.4%	99.7%	95.3%	100%	100%	100%	100%	100%	99.9%	99.9%	100%
4121	no seq	no seq	100%	no seq	100%	11.3%	28.5%	25.6%			100%		100%	11.4%	28.5%	25.7%			100%		100%	99.2%	100%	99.8%
4209	87.2%	100%	100%	100%	100%	33.3%	65.3%	57.8%	87.5%	100%	100%	100%	100%	33.4%	65.4%	57.9%	99.7%	100%	100%	100%	100%	99.8%	99.8%	99.8%
10007	100%	100%	100%	100%	100%	63.2%	97.9%	98.5%	100%	100%	100%	100%	100%	63.2%	98.0%	98.6%	100%	100%	100%	100%	100%	100%	99.9%	99.9%
10023	46.1%	80.0%	100%	65.1%	100%	no seq	26.0%	23.5%	46.2%	80.0%	100%	65.2%	100%		26.0%	23.6%	99.8%	99.9%	100%	99.9%	100%		99.8%	99.8%
10080	100%	99.3%	100%	100%	100%	no seq	no seq	no seq	100%	99.4%	100%	100%	100%				100%	99.9%	100%	100%	100%			
10104	98.7%	100%	100%	100%	100%	99.9%	21.2%	100%	98.7%	100%	100%	100%	100%	100%	21.2%	100%	100%	100%	100%	100%	100%	99.9%	99.9%	100%
10115	99.8%	100%	100%	100%	100%	42.6%	70.2%	62.8%	100%	100%	100%	100%	100%	42.7%	70.5%	62.9%	99.8%	100%	100%	100%	100%	99.8%	99.5%	99.8%
10144	52.1%	100%	100%	100%	100%	27.7%	40.7%	49.5%	52.3%	100%	100%	100%	100%	27.7%	40.8%	49.5%	99.5%	100%	100%	100%	100%	100%	99.9%	100%
10462	100%	100%	100%	100%	100%	no seq	no seq	no seq	100%	100%	100%	100%	100%				100%	100%	100%	100%	100%			
10464	92.6%	77.9%	no seq	no seq	no seq	no seq	no seq	no seq	92.7%	78.0%							99.9%	99.9%						
10466	51.8%	83.3%	100%	98.1%	100%	31.2%	37.7%	39.7%	52.1%	83.4%	100%	98.2%	100%	31.2%	37.8%	39.8%	99.4%	99.8%	100%	99.9%	100%	99.9%	99.8%	99.8%
10498	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
11111	100%	100%	100%	100%	100%	37.5%	99.8%	100%	100%	100%	100%	100%	100%	37.5%	99.9%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%
11119	no seq	no seq	100%	51.3%	100%	24.4%	32.1%	46.4%			100%	51.3%	100%	24.5%	32.1%	46.6%			100%	100%	100%	99.6%	99.9%	99.5%

H1N1pdm09

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	100%	100%	100%	100%	100%	no seq	no seq	no seq	100%	100%	100%	100%	100%				100%	100%	100%	100%	100%			
200	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%
1299	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
1643	100%	100%	100%	100%	100%	99.9%	79.9%	96.4%	100%	100%	100%	100%	100%	100%	80.0%	96.5%	100%	100%	100%	100%	100%	99.9%	99.9%	99.9%
2258	100%	100%	100%	100%	100%	82.0%	60.8%	77.7%	100%	100%	100%	100%	100%	82.6%	60.8%	78.0%	100%	100%	100%	100%	100%	99.3%	100%	99.6%
2819	37.4%	62.7%	86.9%	76.6%	99.7%	29.3%	84.7%	77.4%	67.0%	62.8%	87.1%	76.7%	100%	29.3%	84.8%	90.6%	55.8%	99.8%	99.8%	99.9%	99.7%	99.9%	99.9%	85.4%
3442	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
4121	no seq	35.1%	no seq	no seq	no seq	no seq	no seq	no seq		37.8%								92.9%						
4209	99.9%	100%	100%	100%	100%	100%	86.5%	90.3%	100%	100%	100%	100%	100%	100%	86.6%	90.5%	99.9%	100%	100%	100%	100%	100%	99.9%	99.8%
10007	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
10023	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
10080	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
10104	99.9%	100%	100%	99.9%	100%	100%	99.8%	99.8%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	99.9%	100%	100%	99.8%	99.8%
10115	100%	100%	100%	100%	100%	100%	58.7%	99.9%	100%	100%	100%	100%	100%	100%	58.8%	100%	100%	100%	100%	100%	100%	100%	99.8%	99.9%
10144	99.9%	100%	100%	99.9%	100%	67.6%	53.3%	34.7%	100%	100%	100%	100%	100%	67.6%	53.4%	34.9%	99.9%	100%	100%	99.9%	100%	99.9%	99.8%	99.5%
10462	99.7%	99.4%	99.3%	99.3%	99.6%	99.4%	99.5%	99.6%	100%	100%	100%	100%	100%	100%	100%	100%	99.7%	99.4%	99.3%	99.3%	99.6%	99.4%	99.5%	99.6%
10464	94.5%	97.0%	no seq	no seq	no seq	no seq	no seq	no seq	94.6%	97.1%							99.9%	99.9%						
10466	67.1%	100%	100%	99.9%	100%	16.0%	25.6%	30.7%	67.3%	100%	100%	100%	100%	16.1%	25.8%	30.7%	99.8%	100%	100%	99.9%	100%	99.5%	99.3%	99.9%
10498	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
11111	99.9%	100%	100%	100%	100%	100%	58.3%	99.9%	100%	100%	100%	100%	100%	100%	58.4%	100%	99.9%	100%	100%	100%	100%	100%	99.8%	99.9%
11119	99.9%	99.7%	100%	99.9%	100%	68.3%	48.1%	23.6%	100%	100%	100%	100%	100%	68.5%	48.3%	23.9%	99.9%	99.6%	100%	99.9%	100%	99.7%	99.5%	98.7%

H5N1ew

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
200	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
1299	100%	100%	100%	100%	100%	100%	100%	no seq	100%	100%	100%	100%	100%	100%	100%		100%	100%	100%	100%	100%	100%	100%	
1643	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
2258	37.9%	85.4%	no seq	19.1%	98.8%	26.9%	17.5%	21.6%	38.3%	87.9%		19.4%	100%	28.2%	17.8%	22.9%	99.1%	97.1%		98.3%	98.8%	95.5%	98.5%	94.2%
2819	100%	100%	100%	99.9%	100%	98.0%	99.9%	81.8%	100%	100%	100%	100%	100%	99.2%	100%	82.1%	100%	100%	100%	99.9%	100%	98.8%	99.9%	99.7%
3442	100%	100%	100%	100%	100%	99.7%	99.8%	100%	100%	100%	100%	100%	100%	99.8%	99.8%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%
4121	34.1%	37.9%	100%	13.7%	100%	20.1%	17.6%	11.7%	34.3%	38.0%	100%	13.8%	100%	20.1%	17.7%	11.8%	99.5%	99.7%	100%	99.6%	100%	99.9%	99.6%	99.5%
4209	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
10007	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%
10023	98.9%	100%	100%	100%	100%	100%	100%	99.9%	99.9%	100%	100%	100%	100%	100%	100%	100%	99.0%	100%	100%	100%	100%	100%	100%	99.9%
10080	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%
10104	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
10115	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%
10144	99.7%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.7%	100%	100%	100%	100%	100%	99.9%	100%
10462	100%	100%	100%	100%	100%	100%	100%	no seq	100%	100%	100%	100%	100%	100%	100%		100%	100%	100%	100%	100%	100%	100%	
10464	93.4%	100%	no seq	no seq	no seq	no seq	no seq	no seq	93.5%	100%							99.9%	100%						
10466	97.0%	100%	100%	100%	100%	100%	95.7%	99.9%	97.2%	100%	100%	100%	100%	100%	95.8%	100%	99.8%	100%	100%	100%	100%	100%	99.9%	99.9%
10498	99.9%	100%	100%	100%	100%	100%	99.9%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	99.9%	99.9%
11111	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%
11119	99.7%	99.8%	99.8%	100%	100%	100%	100%	99.9%	100%	100.1%	100%	100%	100%	100%	100%	99.9%	99.6%	99.7%	99.8%	100%	100%	100%	100%	100%

H5N1st d1

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	100%	100%	100%	100%	100%	no seq	78.2%	no seq	100%	100%	100%	100%	100%		78.3%		100%	100%	100%	100%	100%		99.9%	
200	100%	99.5%	100%	100%	100%	99.9%	97.5%	99.8%	100%	100%	100%	100%	100%	100%	97.8%	100%	100%	99.5%	100%	100%	100%	99.9%	99.7%	99.8%
1299	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
1643	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
2258	100%	100%	100%	100%	100%	84.1%	100%	80.7%	100%	100%	100%	100%	100%	84.7%	100%	83.0%	100%	100%	100%	100%	100%	99.3%	100%	97.2%
2819	54.4%	78.4%	100%	82.0%	100%	64.8%	93.5%	94.0%	54.4%	78.6%	100%	82.2%	100%	64.9%	93.6%	96.7%	100%	99.8%	100%	99.8%	100%	99.9%	99.9%	97.2%
3442	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
4121	36.4%	37.9%	100%	51.3%	100%	31.6%	23.1%	20.5%	36.5%	38.0%	100%	51.3%	100%	31.7%	23.1%	20.5%	99.7%	99.7%	100%	100%	100%	99.8%	99.9%	99.9%
4209	99.7%	100%	100%	100%	100%	98.2%	79.1%	77.2%	100%	100%	100%	100%	100%	98.2%	79.2%	77.4%	99.7%	100%	100%	100%	100%	100%	99.9%	99.8%
10007	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%
10023	98.5%	91.7%	100%	98.9%	97.8%	99.9%	100%	99.9%	99.9%	91.8%	100%	98.9%	98.2%	100%	100%	100%	98.6%	99.9%	100%	100%	99.6%	99.9%	100%	99.9%
10080	100%	100%	100%	99.8%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%
10104	99.8%	99.8%	100%	100%	100%	99.6%	99.8%	99.5%	100%	100%	100%	100%	100%	100%	100%	100%	99.8%	99.8%	100%	100%	100%	99.6%	99.8%	99.5%
10115	99.7%	100%	100%	100%	100%	72.5%	97.1%	76.7%	100%	100%	100%	100%	100%	72.5%	97.2%	76.7%	99.7%	100%	100%	100%	100%	100%	99.9%	100%
10144	99.7%	100%	100%	100%	100%	60.2%	66.8%	46.1%	100%	100%	100%	100%	100%	60.3%	67.0%	46.5%	99.7%	100%	100%	100%	100%	99.8%	99.7%	99.1%
10462	100%	100%	100%	100%	100%	99.5%	99.5%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.5%	99.5%	99.9%
10464	91.3%	99.9%	no seq	no seq	no seq	no seq	no seq	no seq	91.3%	100%							100%	99.9%						
10466	99.9%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	99.9%
10498	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
11111	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
11119	99.6%	99.7%	100%	100%	100%	60.1%	60.1%	37.1%	100%	100%	100%	100%	100%	60.2%	60.2%	37.2%	99.5%	99.7%	100%	100%	100%	99.9%	99.8%	99.6%

H7N2

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
200	99.9%	100%	100%	100%	100%	100%	33.5%	100%	100%	100%	100%	100%	100%	100%	33.6%	100%	99.9%	100%	100%	100%	100%	100%	99.6%	100%
1299	10.9%	100%	100%	79.9%	100%	99.9%	17.5%	99.8%	11.1%	100%	100%	80.0%	100%	100%	17.6%	99.9%	98.1%	100%	100%	99.9%	100%	99.9%	99.2%	99.9%
1643	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%
2126	100%	99.9%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	99.9%	100%
2258	89.7%	99.9%	100%	99.9%	100%	75.5%	50.9%	37.7%	89.8%	100%	100%	100%	100%	77.2%	51.0%	37.8%	99.9%	99.9%	100%	99.9%	100%	97.8%	99.9%	99.8%
2819	77.4%	75.2%	no seq	57.1%	no seq	48.3%	no seq	27.2%	77.5%	75.3%		57.2%		49.6%		31.1%	99.9%	99.8%		99.9%		97.4%		87.6%
3442	98.8%	99.5%	100%	99.5%	100%	100%	89.4%	99.9%	98.9%	99.6%	100%	99.5%	100%	100%	89.5%	100%	99.9%	99.9%	100%	100%	100%	100%	99.9%	99.9%
4121																								
4209	100%	99.9%	100%	100%	100%	51.4%	70.0%	49.2%	100%	100%	100%	100%	100%	51.5%	70.1%	49.3%	100%	99.9%	100%	100%	100%	99.8%	99.8%	99.8%
10007	100%	99.9%	100%	100%	100%	100%	99.8%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	99.8%	100%
10023	100%	100%	100%	100%	42.6%	no seq	no seq	no seq	100%	100%	100%	100%	42.6%				100%	100%	100%	100%	100%			
10080	100%	100%	100%	100%	100%	100%	no seq	100%	100%	100%	100%	100%	100%	100%		100%	100%	100%	100%	100%	100%	100%		100%
10104	100%	100%	100%	100%	100%	99.9%	99.0%	99.3%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	99.0%	99.3%
10115	100%	99.5%	100%	99.9%	96.8%	99.9%	100%	95.4%	100%	99.9%	100%	100%	97.4%	100%	100%	95.5%	100%	99.6%	100%	99.9%	99.4%	99.9%	100%	99.9%
10144	44.3%	99.7%	100%	100%	100%	22.3%	26.3%	20.6%	45.5%	100%	100%	100%	100%	22.4%	26.4%	20.6%	97.5%	99.7%	100%	100%	100%	99.5%	99.5%	99.9%
10462	99.9%	100%	100%	100%	100%	99.9%	no seq	99.4%	100%	100%	100%	100%	100%	100%		100%	99.9%	100%	100%	100%	100%	99.9%		99.4%
10464	57.5%	69.2%	no seq	no seq	no seq	no seq	no seq	no seq	57.6%	69.3%							99.9%	99.9%						
10466	82.0%	99.8%	100%	100%	100%	21.0%	16.1%	46.0%	82.3%	100%	100%	100%	100%	21.1%	16.2%	46.1%	99.6%	99.8%	100%	100%	100%	99.7%	99.5%	99.9%
10498	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
11111	99.8%	99.9%	100%	100%	100%	100%	78.9%	100%	100%	100%	100%	100%	100%	100%	79.2%	100%	99.8%	99.9%	100%	100%	100%	100%	99.7%	100%
11119	99.5%	99.5%	100%	99.9%	no seq	18.8%	28.6%	20.3%	99.8%	99.9%	100%	100%		18.9%	28.7%	20.4%	99.7%	99.6%	100%	99.9%		99.6%	99.7%	99.5%

H1N1s

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	100%	100%	100%	100%	100%	100%	no seq	17.5%	100%	100%	100%	100%	100%	100%		17.6%	100%	100%	100%	100%	100%	100%		99.5%
200	98.8%	100%	100%	100%	100%	94.1%	99.8%	100%	99.0%	100%	100%	100%	100%	94.7%	100%	100%	99.8%	100%	100%	100%	100%	99.4%	99.8%	100%
1299	97.0%	99.9%	100%	100%	100%	100%	100%	100%	97.0%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
1643	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
2258	100%	100%	100%	100%	100%	99.0%	93.8%	99.9%	100%	100%	100%	100%	100%	99.2%	94.1%	100%	100%	100%	100%	100%	100%	99.8%	99.7%	99.9%
2819	45.2%	62.3%	93.8%	90.9%	no seq	83.8%	59.6%	99.9%	45.2%	62.6%	94.4%	91.0%		83.8%	59.7%	100%	100%	99.6%	99.4%	99.9%		100%	99.9%	99.9%
3442	72.0%	100%	100%	100%	100%	100%	100%	100%	72.1%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
4121	no seq	no seq	no seq	no seq	no seq	8.7%	31.1%	no seq						8.8%	31.2%							99.0%	99.6%	
4209	99.8%	100%	100%	100%	100%	99.9%	94.1%	91.3%	100%	100%	100%	100%	100%	100%	94.2%	91.6%	99.6%	100%	100%	100%	100%	99.9%	99.9%	99.6%
10007	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10023	17.1%	99.7%	100%	97.4%	99.6%	99.8%	no seq	no seq	17.2%	100%	100%	97.5%	100%	99.9%			99.6%	99.7%	100%	99.9%	99.6%	99.9%		
10080	100%	100%	100%	100%	99.8%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.8%	100%	100%	100%
10104	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%
10115	97.0%	99.9%	100%	100%	100%	85.2%	99.9%	99.9%	97.0%	99.9%	100%	100%	100%	85.4%	100%	100%	100%	100%	100%	100%	100%	99.8%	99.9%	99.9%
10144	100%	100%	100%	100%	100%	57.8%	65.5%	52.0%	100%	100%	100%	100%	100%	57.8%	65.6%	52.1%	100%	100%	100%	100%	100%	99.9%	99.9%	99.9%
10462	100%	99.4%	100%	99.4%	99.2%	98.9%	97.0%	99.9%	100%	99.9%	100%	100%	100%	100%	97.4%	100%	100%	99.5%	100%	99.4%	99.2%	98.9%	99.5%	99.9%
10464	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
10466	100%	100%	100%	100%	100%	84.9%	79.4%	69.8%	100%	100%	100%	100%	100%	85.0%	79.5%	69.9%	100%	100%	100%	100%	100%	99.9%	99.9%	99.9%
10498	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
11111	99.8%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.6%	100%	100%	100%	100%	100%	100%	100%
11119	no seq	99.7%	100%	99.9%	99.5%	44.8%	60.2%	48.7%		100%	100%	100%	100%	45.3%	60.4%	48.9%		99.6%	100%	99.8%	99.0%	98.8%	99.6%	99.7%

H3N2h

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	100%	100%	100%	100%	100%	45.8%	no seq	100%	100%	100%	100%	100%	100%	45.8%		100%	100%	100%	100%	100%	100%	99.9%		100%
200	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%
1299	100%	100%	100%	100%	100%	99.9%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	99.9%	100%
1643	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
2258	100%	100%	100%	99.9%	100%	100%	89.7%	99.7%	100%	100%	100%	99.9%	100%	100%	90.3%	99.9%	100%	100%	100%	100%	100%	100%	99.3%	99.8%
2819	71.5%	55.3%	no seq	57.1%	no seq	86.0%	94.5%	100%	71.8%	55.7%		57.2%		86.0%	95.4%	100%	99.6%	99.3%		99.9%		100%	99.1%	100%
3442	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
4121	14.0%	36.1%	no seq	no seq	100%	10.6%	4.0%	13.7%	14.2%	36.2%			100%	10.7%	4.0%	13.8%	98.8%	99.8%			100%	99.1%	98.9%	99.2%
4209	100%	100%	100%	100%	100%	100%	94.7%	99.8%	100%	100%	100%	100%	100%	100%	94.9%	100%	100%	100%	100%	100%	100%	100%	99.8%	99.8%
10007	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%
10023	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%
10080	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10104	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%
10115	100%	100%	100%	100%	100%	100%	99.9%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	99.9%
10144	100%	100%	100%	100%	100%	92.7%	64.8%	69.7%	100%	100%	100%	100%	100%	92.7%	64.9%	70.0%	100%	100%	100%	100%	100%	99.9%	99.9%	99.6%
10462	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%
10464	91.2%	95.3%	no seq	no seq	no seq	no seq	no seq	no seq	91.6%	95.4%							99.6%	99.9%						
10466	99.8%	100%	100%	100%	100%	88.4%	64.0%	78.0%	100%	100%	100%	100%	100%	88.5%	64.0%	78.0%	99.8%	100%	100%	100%	100%	99.9%	100%	100%
10498	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
11111	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%
11119	99.7%	100%	100%	99.7%	100%	68.2%	64.0%	50.4%	100%	100%	100%	99.8%	100%	68.2%	64.1%	50.5%	99.6%	100%	100%	99.9%	100%	99.9%	99.8%	99.8%

H3N2s

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
10104	99.8%	91.9%	86.7%	97.3%	99.2%	25.6%	19.4%	97.7%	100%	100%	100%	100%	100%	30.2%	20.9%	100%	99.8%	91.8%	86.7%	97.3%	99.2%	84.7%	92.9%	97.7%
10466	93.2%	81.4%	100%	99.5%	97.3%	39.0%	50.6%	77.7%	93.3%	81.4%	100%	99.6%	97.4%	39.3%	50.6%	77.8%	99.9%	100%	100%	99.9%	99.9%	99.3%	100%	99.9%
200	99.9%	100%	100%	100%	100%	100%	100%	95.7%	100%	100%	100%	100%	100%	100%	100%	95.8%	99.9%	100%	100%	100%	100%	100%	100%	99.9%
2258	100%	100%	100%	100%	100%	49.8%	59.1%	56.1%	100%	100%	100%	100%	100%	49.9%	59.3%	56.2%	100%	100%	100%	100%	100%	99.8%	99.7%	99.9%
1643	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10007	100%	100%	100%	100%	100%	99.9%	100%	99.8%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	99.8%
10115	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
1299	no seq	99.9%	100%	100%	100%	100%	100%	100%		100%	100%	100%	100%	100%	100%	100%		99.9%	100%	100%	100%	100%	100%	100%
2819	31.4%	34.8%	no seq	28.0%	no seq	52.2%	64.4%	93.9%	37.0%	34.9%		28.3%		52.5%	71.7%	100%	84.8%	99.7%		99.1%		99.5%	89.8%	93.9%
11111	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%
10464	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
4209	99.9%	100%	100%	100%	100%	98.7%	93.0%	98.8%	100%	100%	100%	100%	100%	98.9%	93.1%	99.0%	99.9%	100%	100%	100%	100%	99.8%	99.9%	99.8%
10462	99.8%	100%	100%	99.4%	100%	99.7%	100%	99.8%	100%	100%	100%	100%	100%	99.7%	100%	100%	99.8%	100%	100%	99.4%	100%	100%	100%	99.8%
95	100%	100%	100%	100%	100%	100%	99.9%	no seq	100%	100%	100%	100%	100%	100%	100%		100%	100%	100%	100%	100%	100%	99.9%	
10080	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10144	99.9%	99.9%	100%	99.9%	100%	46.3%	57.8%	56.5%	100%	100%	100%	100%	100%	46.6%	57.9%	56.7%	99.9%	99.9%	100%	99.9%	100%	99.4%	99.9%	99.7%
3442	100%	99.2%	100%	100%	100%	100%	100%	100%	100%	99.3%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%
11119	99.8%	100%	100%	99.9%	100%	99.7%	48.3%	99.8%	99.9%	100%	100%	100%	100%	100%	48.7%	100%	99.9%	100%	100%	99.8%	100%	99.7%	99.1%	99.8%
10023	99.6%	100%	99.6%	100%	97.8%	99.7%	100%	100%	100%	100%	100%	100%	98.2%	100%	100%	100%	99.6%	100%	99.6%	100%	99.6%	99.7%	100%	100%
10498	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
4121	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																

H5N1bhg

Participant ID	A. % identical nucleotides compared to the panel reference sequence								B. % nucleotides covered compared to the panel reference sequence								C. % identical nucleotides relative to the part(s) of the segment covered (A/B)							
	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2	HA	NA	MP	NP	NS	PA	PB1	PB2
95	100%	100%	100%	100%	100%	no seq	no seq	no seq	100%	100%	100%	100%	100%				100%	100%	100%	100%	100%			
200	100%	100%	100%	100%	100%	100%	98.8%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	98.6%	100%
1299	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
1643	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
2258	100%	100%	100%	100%	100%	98.7%	95.5%	96.2%	100%	100%	100%	100%	100%	99.5%	95.6%	97.1%	100%	100%	100%	100%	100%	99.2%	99.8%	99.1%
2819	87.8%	99.7%	100%	100%	100%	75.0%	99.9%	76.0%	88.0%	100%	100%	100%	100%	75.3%	100%	76.1%	99.8%	99.7%	100%	100%	100%	99.6%	99.9%	99.9%
3442	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
4121	36.7%	38.0%	100%	19.1%	98.9%	11.2%	54.5%	13.2%	36.9%	38.0%	100%	19.2%	98.9%	11.3%	54.5%	13.2%	99.6%	100%	100%	99.6%	100%	99.6%	99.9%	99.7%
4209	99.8%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.8%	100%	100%	100%	100%	100%	100%	100%
10007	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10023	98.8%	84.8%	100%	99.8%	99.7%	100%	100%	99.9%	99.9%	84.9%	100%	99.9%	99.8%	100%	100%	100%	98.9%	99.9%	100%	99.9%	99.9%	100%	100%	99.9%
10080	100%	100%	100%	99.6%	99.8%	100%	100%	100%	100%	100%	100%	99.7%	100%	100%	100%	100%	100%	100%	100%	99.9%	99.8%	100%	100%	100%
10104	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%
10115	99.9%	100%	100%	99.9%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.9%	100%	100%	100%	100%
10144	99.7%	100%	100%	99.8%	100%	73.5%	56.0%	39.6%	100%	100%	100%	100%	100%	73.7%	56.4%	39.7%	99.7%	100%	100%	99.8%	100%	99.7%	99.3%	99.8%
10462	100%	100%	100%	99.8%	100%	98.6%	99.3%	100%	100%	100%	100%	100%	100%	98.9%	100%	100%	100%	100%	100%	99.8%	100%	99.7%	99.3%	100%
10464	99.9%	96.8%	100%	100%	100%	100%	100%	100%	100%	96.9%	100%	100%	100%	100%	100%	100%	99.9%	99.9%	100%	100%	100%	100%	100%	100%
10466	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10498	no seq	no seq	no seq	no seq	no seq	no seq	no seq	no seq																
11111	100%	100%	88.2%	84.3%	81.7%	84.9%	87.9%	85.3%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	88.2%	84.3%	81.0%	84.9%	87.9%	85.3%
11119	99.6%	100%	100%	99.8%	99.6%	14.5%	54.5%	18.9%	100%	100%	100%	100%	100%	14.5%	54.5%	18.9%	99.5%	100%	100%	99.8%	99.6%	100%	99.9%	99.8%

Annex 10. Assessment of score for sequence identity for the whole genome and HA plus NA segments only, by laboratory and virus strain in the panel

For each laboratory each virus has been scored: 0 (green) = all genome segments 99.8-100% identical with the reference, 1 (yellow) = at least 1 genome segment less than 99.8% identical with the reference but all more than 99.0% identical, 2 (light red) = at least 1 genome segment less than 99% identical with the reference and 3 (red) = at least 1 genome segment no sequence available. Grey background colour of cells means that the laboratory did not receive the panel specimen with the virus on which the data is shown in the table. The leftmost tables in Annex 9 showing the percentage identical nucleotides in the obtained sequences compared with the full coding region of genome segments have been used for determination of these scores. Subsequently, a cumulative score by laboratory has been calculated by summing the scores by virus. These scores were used for Figure 14. Laboratory 10464 indicated that because of difficulty to sequence the specimens they limited most specimens to Sanger sequencing the HA and NA, resulting in a high score for the whole genome. Scores are shown for the whole genome (A) and HA plus NA genome segments only (B).

A. Whole genome sequence identity score with reference sequence

Participant ID	H1N2s	H5N8	H1N1pdm09	H5N1ew	H5N1st d1	H7N2	H1N1s	H3N2h	H3N2s	H5N1bhg	Cumulative score
95	2	3	3	0	3	3	3	3	3	3	26
200	2	2	0	0	2	2	2	0	2	2	14
1299	0	2	0	3	0	2	2	0	3	0	12
1643	2	2	2	0	0	0	0	0	0	0	6
2258	2	2	2	3	2	2	2	2	2	2	21
2819	2	2	2	2	2	3	3	3	3	2	24
3442	2	2	0	1	0	2	2	0	1	0	10
4121	3	3	3	2	2		3	3	3	2	24
4209	0	2	2	0	2	2	2	2	2	0	14
10007	1	2	0	0	0	0	0	0	0	0	3
10023	3	3	0	2	2	3	3	0	2	2	20
10080	0	3	0	0	0	3	0	0	0	1	7
10104	0	2	0	0	1	1	0	0	2	0	6
10115	1	2	2	0	2	2	2	0	0	0	11
10144	2	2	2	1	2	2	2	2	2	2	19
10462	2	3	1	3	1	3	2	0	1	2	18
10464	2	3	3	3	3	3	3	3	3	2	28
10466	1	2	2	2	0	2	2	2	2	0	15
10498	0	3	3	0	0	3	3	3	3	3	21
11111	1	2	2	0	0	2	0	0	0	2	9
11119	3	3	2	1	2	3	3	2	2	2	23

B. Segments HA plus NA only sequence identity score with reference sequence

Participant ID	H1N2s	H5N8	H1N1pdm09	H5N1ew	H5N1st d1	H7N2	H1N1s	H3N2h	H3N2s	H5N1bhg	Cumulative score
95	0	3	0	0	0	3	0	0	0	0	6
200	0	0	0	0	1	0	2	0	0	0	3
1299	0	2	0	0	0	2	2	0	3	0	9
1643	1	2	0	0	0	0	0	0	0	0	3
2258	2	2	0	2	0	2	0	0	0	0	8
2819	2	2	2	0	2	2	2	2	2	2	18
3442	2	0	0	0	0	2	2	0	1	0	7
4121	3	3	3	2	2		3	2	3	2	23
4209	0	2	0	0	1	0	0	0	0	0	3
10007	0	0	0	0	0	0	0	0	0	0	0
10023	2	2	0	2	2	0	2	0	1	2	13
10080	0	1	0	0	0	0	0	0	0	0	1
10104	0	2	0	0	0	0	0	0	2	0	4
10115	0	0	0	0	1	1	2	0	0	0	4
10144	2	2	0	1	1	2	0	0	0	1	9
10462	2	0	1	0	0	0	1	0	0	0	4
10464	2	2	2	2	2	2	3	2	3	2	22
10466	1	2	2	2	0	2	0	0	2	0	11
10498	0	3	3	0	0	3	3	3	3	3	21
11111	0	0	0	0	0	0	0	0	0	0	0
11119	3	3	1	1	1	1	3	1	0	1	15

Annex 11. Result for subtyping by NAAT and sequencing, by laboratory and panel specimen

The table has been split in two parts, A. all panel viruses with H5 subtype and B. all panel viruses with other H-subtype than H5. Footnotes are listed below part B and are numbered left to right and top to bottom as if both tables were horizontally connected.

A. Viruses in the panel with H5 H-subtype

Participant ID	Reported subtype after NAAT and sequencing [1]							
	H5N1st d1	H5N1st d2	H5N1st d3	H5N1st d4	H5N1st d5	H5N1ew	H5N1bhg	H5N8
95	A(H5N1)	A(H5Nx) [2]	Negative [3]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5Nx) [2]
200	A(H5N1)	A(H5N1) [2]	A no subtype [4]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
1299	A(H5N1)	*Unknown* [5]	*A no subtype* [5]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
1643	A(H5N1)	A(H5N1)	A(H5Nx) [8]	N/A	N/A	A(H5N1)	A(H5N1)	A(H5N8)
2258	A(H5N1)	A(H5Nx) [2]	A(H5N1)	*Unknown* [3]	*Unknown* [3]	A(H5N1)	A(H5N1)	*A(H3N8)avian* [10]
2819	A(H5N1)	A(H3humanNx) [12]	A(H3Nx) [13]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
3442	A(H5N1)	A(H5N1) [16]	Negative [3]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
4121	A(H5N1)	N/A [17]	N/A [18]	N/A	N/A	A(H5N1)	A(H5N1)	A(H5N8) [19]
4209	A(H5N1)	A(H5Nx) [23]	N/A [18]	N/A	N/A	A(H5N1)	A(H5N1)	A(H5N8)
10007	A(H5N1)	A(H5N1)	Negative [3]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10023	A(H5N1)	A(H5Nx) [2]	A(H5Nx) [2]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10080	A(H5N1)	A(H5N1) [16]	A(H5N1) [2]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10104	A(H5N1)	*Negative* [5]	N/A [18]	N/A	N/A	A(H5N1)	A(H5N1)	A(H5N8)
10115	A(H5N1)	A(H5N1) [2]	A(H5N1) [2]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10144	A(H5N1)	A(H5Nx) [23]	*Negative* [25]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10462	A(H5N1)	*Negative* [25]	*Negative* [25]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
10464	A(H5N1)	N/A [26]	N/A [26]	N/A	N/A	A(H5N1)	A(H5N1)	A(H5N8)
10466	A(H5N1)	*Negative* [25]	*Negative* [25]	Negative	N/A	A(H5N1)	A(H5N1)	A(H5N8)
10498	A(H5N1)	N/A [17]	N/A [17]	N/A	N/A	A(H5N1)	N/A [17]	N/A [17]
11111	A(H5N1)	A(H5N1) [30]	A(H5N1)	Negative	Negative	A(H5N1)	A(H5N1)	A(H5N8)
11119	A(H5N1)	A(H5N1) [2]	Negative [3]	Negative	Negative	A(H5N1)	A(H5N1)	A(H5Nx) [2]

B. Viruses in the panel with other H-subtype than H5

Participant ID	Reported subtype after NAAT and sequencing [1]					
	H7N2	**H1N1s**	**H1N2s**	**H3N2s**	**H1N1pdm09**	**H3N2h**
95	A(H7Nx) [2]	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
200	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
1299	*A no subtype* [6]	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine [7]	A(H1N1)pdm09	A(H3N2)human
1643	A(H7N2)	A(H1N1)swine	*A(H1N2)* [9]	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
2258	A(H7N2)	A(H1N1)swine	A(H1N2)swine	*A(H1N2)swine* [11]	A(H1N1)pdm09	A(H3N2)human
2819	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)human [14]	A(H3humanNx) [15]	A(H3N2)human
3442	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
4121		A(H1N1)swine [20]	*A(H3N2)swine* [21]	N/A [22]	A(H1N1)pdm09	A(H3N2)human
4209	A(H7N2)	A(H1N1)swine	*A(H3N2)swine* [24]	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10007	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10023	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10080	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10104	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10115	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10144	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10462	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10464	A(H7N2)	N/A [26]	A(H1N2)swine	N/A [26]	A(H1N1)pdm09	A(H3N2)human
10466	A(H7N2)	A(H1N1)swine	A(H1N2)swine	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
10498	N/A [27]	N/A [26]	A(H1N2)swine	N/A [28]	N/A [29]	N/A [28]
11111	A(H7N2)	A(H1N1)swine	*A(H1N2)* [9]	A(H3N2)swine	A(H1N1)pdm09	A(H3N2)human
11119	A(H7N2)	*A no subtype* [31]	*A no subtype* [32]	*A(H3N2)* [33]	A(H1N1)pdm09	*A(H3N2)* [34]

Colour coding

Fully correct subtype and 'likely host species' indication

H and N subtype correct and 'likely host species' indication correct

Negative correct as is expected result

'likely host species' indication, H-subtype or N-subtype missing

H or N subtype correct with correct 'likely host species' indication

Generic H and N subtype correct, no 'likely host species' indication

Wrong

Fully negative whereas expected result is positive; or reporting Unknown but NAAT negative and no sequences reported

Incorrect or no subtype following NAAT and/or sequencing

Correct H-subtype and/or N-subtype but incorrect 'likely host species' indication

Has sequence for expected subtype but not reported

Assessment not possible as lab did not sequence this specimen

Assessment not possible as lab did not receive this specimen

1. d1-d5 = dilutions of H5N1st; N/A and Negative = not sequenced or no sequences reported; both considered negative (a NAAT result might have been positive though, this is indicated in a footnote then); subtype in italics = reporting error presumed as underlying data allows correct report
2. Conclusion based on NAAT subtyping as no sequence was reported
3. No sequences reported and NAAT was negative
4. Reported sequences of too low quality for assessment and NAAT subtyping negative
5. No sequences reported but H5 NAAT was positive
6. HA sequence reported was H7, NA sequence was N2 and H7 NAAT was positive
7. No HA sequence reported, but all other segments obtained were from swine H3N2 origin
8. H-subtype based on H5 NAAT positive as HA sequence reported was H1pdm09 and no NA sequence reported
9. H1 and N2 sequences reported are of swine origin
10. Reported HA sequence was H5 and subtyping NAAT was H5 positive
11. Reported HA sequence was swine H3
12. Reported HA sequence was H3 human whereas subtyping NAAT was H5 positive
13. Reported HA sequence was H3 human, other reported segments originating from human H3N2 or H1N1pdm09 viruses; subtyping NAAT was negative
14. Reported HA sequence was H3 human and NA sequence swine N2, other reported segments originating from human H3N2 or swine H3N2 origin
15. Reported HA sequence was H3 human whereas reported NA sequence was N1pdm09 and all other reported segments had highest blast hit with sequences from H1N1pdm09 origin; subtyoing NAAT result was H1pdm09Nx
16. No HA sequence reported, NA sequence was avian N1 and NAAT subtyping result was A(H5N1)
17. Not sequenced, NAAT result was A(H5Nx); not a reporting error
18. Not sequenced, NAAT result was negative; not a reporting error
19. No HA and NA sequences reported, all other reported segments orginating from H5N8 virus and H5 subtyping NAAT was positive
20. No HA and NA sequences reported, all other reported segments orginating from swine H1N1 virus
21. No HA sequence reported, NA segment orginating from swine H1N2 virus and H3 subtyping NAAT was negative and H1 subtyping NAAT not done
22. Not sequenced, NAAT result was H3Nx; not a reporting error
23. HA H5 seqeunce reported but no NA sequence reported

24. Reported HA sequence was swine H1
25. No HA (and NA) sequence reported whereas NAAT subtyping result was A(H5Nx) or A(H5N1)
26. Not sequenced, NAAT result was type A influenza virus positive ; not a reporting error
27. Not sequenced, H7 subtyping NAAT was positive; not a reporting error
28. Not sequenced, NAAT result was A(H3Nx); not a reporting error
29. Not sequenced, NAAT result was A(H1N1)pdm09; not a reporting error
30. Reported NA sequence was N1pdm09
31. No HA sequence reported, reported NA sequence was swine N1 and NAAT subtyping was A(HxN1)
32. No HA sequence reported, reported NA sequence was swine N2
33. H3 and N2 sequences reported are of swine origin
34. H3 and N2 sequences reported are of human origin

Annex 12. Clade, subclade and genogroup determination, by laboratory and virus strain in the panel

The table has been split in two parts, A. all panel viruses with avian subtype and B. all panel viruses with swine and human subtype. Footnotes are listed below part B.

A. Viruses in the panel with avian subtype

Participant ID	Reported clade, subclade or H5N1 genotype after sequencing [1]							
	H5N1st d1	H5N1std1_gt	H5N1ew	H5N1ew_gt	H5N1bhg	H5N1bhg_gt	H5N8	H7N2
95	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	Not applicable	Not applicable
200	2.3.4.4b	H5N1/AB	2.3.4.4b	H5N1/C	2.3.4.4b	H5N1/BB	2.3.4.4c	1.1
1299	2.3.4.4b	H5N1/AB	2.3.4.4b	H5N1/C	2.3.4.4b	H5N1/BB	2.3.4.4c	Not done
1643	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4c	Not done
2258	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4	1.1
2819	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4c	1.1
3442	2.3.4.4b	H5N1/AB	2.3.4.4b	H5N1/C	2.3.4.4b	H5N1/BB	2.3.4.4c	1.1
4121	2.3.4.4b	Not done	Not done	Not done	2.3.4.4b	Not done	Not done	
4209	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4c	Not done
10007	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4c	Not done
10023	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4c	Not done
10080	2.3.4.4b	H5N1/AB	2.3.4.4b	H5N1/C	2.3.4.4b	H5N1/BB	2.3.4.4c	1.1
10104	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4c	1.1
10115	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4c	1.1
10144	2.3.4.4b	H5N1/AB	2.3.4.4b	H5N1/C	2.3.4.4b	Not applicable	2.3.4.4c	1.1
10462	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4b	Not done	2.3.4.4c	Not applicable
10464	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4c	1.1
10466	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4c	Not applicable
10498	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	N/A	N/A	N/A	N/A
11111	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4b	Not applicable	2.3.4.4c	Not applicable
11119	2.3.4.4b	Not done	2.3.4.4b	Not done	2.3.4.4b	Not done	Not done	Not done

B. Viruses in the panel with swine and human subtype

Participant ID	Reported clade, subclade or H5N1 genotype after sequencing [1]						
	H1N1s	H1N2s	H3N2s	H1N1pdm09	H1N1pdm09_sc	H3N2h	H3N2h_sc
95	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
200	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
1299	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
1643	**Not done**	**Not done**	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
2258	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
2819	1C.2.2	1C.2.2	**H3human/3C.2a1b.2a.(2a.3a.1)**	**h3-Other-Human-2020**	**Not applicable**	**3C.2a1b.2a.(2a)**	**G.1**
3442	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
4121	Not done	Not done	N/A	Not done	Not done	**Not done**	**Not done**
4209	1C.2.2	**Not done**	1970.1	6B.1A.(5a.2a.1)	C.1.1	**3C.2a1b.2a.(2a.1a)**	G.1.1
10007	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
10023	**Not done**	**Not done**	**Not done**	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
10080	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
10104	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
10115	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	**3C.2a1b.2a.(2a.1a)**	G.1.1
10144	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
10462	1C.2.2	1C.2.2	1970.1	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	**Not applicable**
10464	N/A	1C.2.2	N/A	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
10466	1C.2.2	1C.2.2	1970.1	**Not applicable**	**Not applicable**	3C.2a1b.2a.(2a.1)	G.1.1
10498	N/A	1C.2.2	N/A	N/A	N/A	N/A	N/A
11111	1C.2.2	1C.2.2	**3C.3a1**	6B.1A.(5a.2a.1)	C.1.1	3C.2a1b.2a.(2a.1)	G.1.1
11119	Not done	Not done	**A/HongKong/1/1968**	6B.1A.(5a.2a.1)	**B**	3C.2a1b.2a.(2a.1)	**Not done**

Colour coding

(sub)clade	Incorrect determined (sub)clade
Not done	HA sequence (and other segments for H5N1 genotype) available, but analysis not done
Not applicable	HA sequence (and other segments for H5N1 genotype) available, but analysis not done
Not done	Specimen was sequenced but no HA sequence obtained and/or no full genome obtained for H5N1 virus
Not applicable	Specimen was sequenced but no HA sequence obtained and/or no full genome obtained for H5N1 virus
N/A	Laboratory did not sequence this specimen
	Laboratory did not receive this specimen

1. _sc = subclade; _gt = genotype

Annex 13. Determination of amino acid changes, by laboratory and virus strain in the panel

The tables below show the evaluation of amino acid changes associated with antiviral reduced susceptibility and mammalian adaptation reported by the laboratories participating in the genetic characterisation challenge.

M2-blocker reduced susceptibility amino acid changes

Participant ID	Reported results amino acid marker identification by virus strain [1]														
	H1N2s				H5N8		H1N1p		H1N1s		H3N2h		H3N2s		
	%id_MP	L26I	V27A	S31N	%id_MP	S31N	%id_MP	S31N	%id_MP	S31N	%id_MP	S31N	%id_MP	L26I	S31N
95	100.0%	0	1	1	No sequence	No sequence	100.0%	1	100.0%	1	100.0%	1	100.0%	0	1
200	100.0%	0	0	1	100.0%	No report	100.0%	1	100.0%	1	100.0%	No report	100.0%	No report	No report
1299	100.0%	0	1	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	100.0%	0	1
1643	100.0%	1	1	0	100.0%	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	1
2258	100.0%	No report	No report	No report	100.0%	No report	100.0%	No report	100.0%	0	100.0%	No report	100.0%	No report	No report
2819	100.0%	1	1	1	99.8%	1	86.9%	1	93.8%	1	No sequence	No sequence	No sequence	No sequence	No sequence
3442	100.0%	1	1	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	100.0%	0	1
4121	100.0%	No report	No report	No report	100.0%	No report	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence
4209	100.0%	0	0	0	100.0%	0	100.0%	0	100.0%	0	100.0%	0	100.0%	0	0
10007	100.0%	0	1	1	100.0%	0	100.0%	1	100.0%	1	100.0%	0	100.0%	0	1
10023	100.0%	0	0	1	100.0%	0	100.0%	1	100.0%	0	100.0%	1	99.6%	0	1
10080	100.0%	1	1	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	1
10104	100.0%	1	1	1	100.0%	No report	100.0%	No report	100.0%	0	100.0%	No report	86.7%	0	0
10115	100.0%	0	1	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	100.0%	0	1
10144	100.0%	1	1	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	1
10462	100.0%	0	0	0	100.0%	0	99.3%	0	100.0%	0	100.0%	0	100.0%	0	0
10464	100.0%	0	1	1	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence
10466	100.0%	1	1	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	1
10498	100.0%	Not assessed	Not assessed	Not assessed	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence
11111	100.0%	1	1	1	100.0%	1	100.0%	1	100.0%	0	100.0%	1	100.0%	1	1
11119	100.0%	0	1	1	100.0%	Not assessed	100.0%	1	100.0%	1	100.0%	0	100.0%	0	0

1. Indicated are the mutant amino acids at indicated positions that cause M2-blocker antiviral resistance
%id_MP indicates the percentage of nucleotides in the coding region of the sequence reported by the participant that are identical to the reference panel strain sequence; a lower percentage indicates that nucleotide positions were not precent (shorter or fragmented sequence) rather than high number mismatched nucleotides
Judgement: 1 = correct; 0 = incorrect
'None' in report or specific amino acid change not in the report judged with 0 if sequence was available
'No report', 'Not assessed' or 'No sequence', no judgement made

Polymerase complex (PA and PB2) amino acid changes associated with mammalian adaptation

Participant ID	Reported results amino acid marker identification by virus strain [1]															
	H1N2s							H1N1s				H3N2s				
	%id_PA	PA-409N	%id_PB2	PB2-292T	PB2-607V	PB2-661T	PB2-701N	%id_PA	PA-409N	%id_PB2	PB2-701N	%id_PA	PA-409N	%id_PB2	PB2-701N	
95	32.0%	0	100.0%	0	0	0	1	100.0%	0	17.5%	1	100.0%	0	No sequence	No sequence	
200	99.9%	No report	98.3%	No report	No report	No report	No report	94.1%	0	100.0%	1	100.0%	No report	95.7%	No report	
1299	100.0%	0	100.0%	0	0	0	1	100.0%	0	100.0%	1	100.0%	0	100.0%	1	
1643	99.7%	0	96.9%	0	0	0	1	100.0%	0	100.0%	1	100.0%	0	100.0%	1	
2258	99.8%	No report	100.0%	No report	No report	No report	No report	99.0%	No report	99.9%	No report	49.8%	No report	56.1%	No report	
2819	88.8%	0	98.2%	0	0	0	1	83.8%	0	99.9%	1	52.2%	No report	93.9%	No report	
3442	100.0%	0	100.0%	1	1	0	1	100.0%	0	100.0%	1	100.0%	0	100.0%	1	
4121	46.5%	No report	22.1%	No report	No report	No report	No report	8.7%	No report	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	
4209	99.9%	0	100.0%	0	0	0	0	99.9%	0	91.3%	0	98.7%	0	98.8%	0	
10007	100.0%	No report	99.9%	No report	No report	No report	No report	100.0%	No report	100.0%	No report	99.9%	No report	99.8%	No report	
10023	99.9%	0	99.6%	0	0	0	1	99.8%	0	No sequence	No sequence	99.7%	0	100.0%	0	
10080	100.0%	1	100.0%	1	1	1	1	100.0%	1	100.0%	1	100.0%	1	100.0%	1	
10104	99.8%	1	99.9%	1	0	1	0	99.9%	1	100.0%	0	25.6%	0	97.7%	0	
10115	100.0%	0	100.0%	0	0	0	1	85.2%	0	99.9%	1	100.0%	0	100.0%	1	
10144	99.8%	No report	99.9%	No report	No report	No report	No report	57.8%	No report	52.0%	No report	46.3%	No report	56.5%	No report	
10462	99.7%	0	99.4%	0	0	0	0	98.9%	No report	99.9%	No report	99.7%	0	99.8%	0	
10464	99.8%	0	100.0%	0	0	0	1	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	
10466	99.2%	Not assessed	99.7%	Not assessed	Not assessed	Not assessed	Not assessed	84.9%	Not assessed	69.8%	Not assessed	39.0%	Not assessed	77.7%	Not assessed	
10498	100.0%	No report	100.0%	No report	No report	No report	No report	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	
11111	100.0%	0	100.0%	0	0	0	1	100.0%	0	100.0%	1	100.0%	0	99.9%	1	
11119	99.7%	0	99.8%	0	0	0	1	44.8%	No report	48.7%	No report	99.7%	0	99.8%	1	

1. Indicated are the mutant amino acids at indicated positions that might cause mammalian adaptation
%id_segment indicates the percentage of nucleotides in the coding region of the sequence reported by the participant that are identical to the reference panel strain sequence; a lower percentage indicates that nucleotide positions were not precent (shorter or fragmented sequence) rather than high number mismatched nucleotides
Judgement: 1 = correct; 0 = incorrect
'None' in report or specific amino acid change not in the report judged with 0 if sequence was available
'No report', 'Not assessed' or 'No sequence', no judgement made

Hemagglutinin (HA), non-structural protein 1 (NS1) and nucleoprotein amino acid changes associated with mammalian adaptation

Participant ID	Reported results amino acid marker identification by virus strain [1]													
	H1N2s		H5N1ew		H5N1st		H1N1s				H3N2s		H5N1bhg	
	%id_HA	HA-173N	%id_HA	HA-139P	%id_HA	HA-139P	%id_HA	HA-173N	%id_NS	NS1-215T	%id_HA	HA-175N	%id_NP	NP-33I
95	100.0%	1	100.0%	0	100.0%	0	100.0%	1	100.0%	0	100.0%	1	100.0%	0
200	100.0%	No report	100.0%	1	100.0%	1	98.8%	0	100.0%	0	99.9%	No report	100.0%	0
1299	100.0%	0	100.0%	0	100.0%	0	97.0%	0	100.0%	0	No sequence	No sequence	100.0%	0
1643	99.4%	0	99.9%	0	100.0%	0	100.0%	0	100.0%	0	100.0%	0	100.0%	0
2258	96.4%	No report	37.9%	No report	100.0%	No report	100.0%	No report	100.0%	No report	100.0%	No report	100.0%	No report
2819	56.9%	0	100.0%	0	54.4%	No report	45.2%	0	No sequence	No sequence	31.4%	No report	100.0%	0
3442	81.8%	0	100.0%	1	100.0%	1	72.0%	0	100.0%	0	100.0%	0	100.0%	0
4121	No sequence	No sequence	34.1%	No report	36.4%	No report	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	19.1%	No report
4209	100.0%	0	99.9%	0	99.7%	0	99.8%	0	100.0%	0	99.9%	1	100.0%	0
10007	100.0%	No report	100.0%	0	100.0%	0	100.0%	No report	100.0%	No report	100.0%	No report	100.0%	0
10023	95.7%	0	98.9%	0	98.5%	0	17.1%	0	99.6%	0	99.6%	1	99.8%	0
10080	100.0%	1	100.0%	1	100.0%	1	100.0%	1	99.8%	0	100.0%	1	99.6%	1
10104	100.0%	1	99.9%	0	99.8%	0	100.0%	1	100.0%	1	99.8%	0	100.0%	1
10115	100.0%	0	100.0%	0	99.7%	0	97.0%	0	100.0%	0	100.0%	0	99.9%	1
10144	46.2%	No report	99.7%	0	99.7%	0	100.0%	No report	100.0%	No report	99.9%	No report	99.8%	0
10462	98.4%	0	100.0%	0	100.0%	0	100.0%	No report	99.2%	No report	99.8%	0	99.8%	0
10464	91.0%	0	93.4%	0	91.3%	0	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	100.0%	0
10466	100.0%	Not assessed	97.0%	0	99.9%	0	100.0%	Not assessed	100.0%	Not assessed	93.2%	Not assessed	100.0%	0
10498	99.8%	No report	99.9%	No report	99.9%	No report	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence	No sequence
11111	100.0%	0	100.0%	1	100.0%	1	99.8%	0	100.0%	0	100.0%	0	84.3%	0
11119	No sequence	No sequence	99.7%	0	99.6%	0	No sequence	No sequence	99.5%	0	99.8%	0	99.8%	0

1. Indicated are the mutant amino acids at indicated positions that might cause mammalian adaptation
%id_segment indicates the percentage of nucleotides in the coding region of the sequence reported by the participant that are identical to the reference panel strain sequence; a lower percentage indicates that nucleotide positions were not precent (shorter or fragmented sequence) rather than high number mismatched nucleotides
Judgement: 1 = correct; 0 = incorrect
'None' in report or specific amino acid change not in the report judged with 0 if sequence was available
'No report', 'Not assessed' or 'No sequence', no judgement made

Annex 14. Instructions manual EEIQAP for zoonotic influenza virus for participants

After closure of the EQA new documents were released on:

- Mammalian adaptation markers for avian influenza viruses [3,47])
- Genotyping of European H5N1 avian influenza viruses [50]

The instructions manual shown below is unchanged from the version submitted with the panel to the participants.

European External Influenza Quality Assessment Programme for the assessment of quality of methodologies for Molecular Detection, Subtyping and Genetic Characterization through sequencing of potential zoonotic type A influenza viruses.

For Performance evaluation and research use only

RATIONALE

During the global outbreak of highly pathogenic H5N1 (clade 2.3.4.4b) type A influenza virus in over a 100 million wild birds and poultry in 2022 - 2024, an expanding range of mammals have been found infected. Since increased activity of clade 2.3.4.4b viruses from mid-2021 also at least 12 confirmed human cases with this clade have been reported to the WHO, most after direct contact with affected poultry. This amplified the concern about the enzootic circulation of H5N1 type A influenza virus and transmission to humans. There are also concerns about direct transmission of swine influenza viruses to humans and about possible reassortment, of swine influenza viruses with avian and/or human seasonal influenza viruses e.g. at mixed poultry and swine farms, increasing the risk that viruses develop with enhanced potential for transmission to and between humans. Current external quality assessments (EQA) organised by WHO and other commercial and not-for-profit EQA providers provide challenges for detection of potentially zoonotic avian influenza viruses, but not for potentially zoonotic swine influenza viruses. Hemagglutinin and neuraminidase subtyping is often optional (H-subtyping is included in WHO EQAP studies), if included in the challenge. Sequencing for genetic characterization (clade assignment) and evaluation of markers for enhanced mammalian transmission and virulence and antiviral reduced susceptibility are not, or only for antiviral reduced susceptibility, included in these programmes. Therefore, it is hardly known from objective EQAs what the capabilities of the National Influenza Centres and National Influenza Reference Laboratories in the EU/EEA, Western Balkan and Türkiye are with respect to detection, subtyping and molecular characterization of zoonotic type A influenza viruses. To fill this gap, and to prepare for potential human cases of avian or swine influenza virus infection, on the request of ECDC, the National Institute for Public Health and the Environment (RIVM) with partners in the AURORAE consortium developed an EQA for potentially zoonotic type A influenza viruses. It comprises of two programmes using the same panel specimens, EISNINF_MD24 Molecular Detection and Subtyping and EISNINF_GC24 Genetic Characterization.

WHAT TO DO WHEN YOU RECEIVE YOUR SPECIMENS

1. When you receive your EEIQAP 2024 panel please carefully read all the documents enclosed paying special attention to these instructions.
2. The panel contents should be stored at or below -70°C until required for processing.
3. Confirmation of receipt, and panel condition (whether the specimens are still frozen) should be made immediately by completing the online **Panel Receipt** form on the QCMD website at: http://www.qcmd.org

Submission of Results

Complete the on-line EISNINF_MD24 **Molecular Detection and Subtyping results form** within **7 working days** of receipt of the panel at: http://www.qcmd.org. In addition, answer the on-line questionnaire on used methods for detection and subtyping.
Complete the on-line EISNINF_GC24 **Genetic Characterization results form** within **30 working days** of receipt of the panel at: http://www.qcmd.org. In addition, answer the on-line questionnaire on used methods for sequencing.

SAFETY WARNINGS AND PRECAUTIONS

All specimens are heat inactivated and confirmed to not contain replicating virus by virus isolation using a blind passage for 14 days. Therefore, specimens can be handled at BSL-2 and disposed in accordance with established safety procedures. It is recommended that the panel specimens provided are handled by qualified professionals only and that all staff is trained in current Good Laboratory Practice (cGLP) with particular respect to the handling of potentially infectious biological materials.

STORAGE OF PANEL COMPONENTS

All panel specimens must be **stored at or below -70°C until processing**. During processing, specimens should be thawed once and nucleic acid extracted and further processed for detection and subtyping and sequencing, ideally the same day. After extraction and processing for detection and subtyping and sequencing, nucleic acid extracts are recommended to be stored at -20°C. Any modification of handling or storage conditions is NOT recommended, as it might adversely affect quality of nucleic acid for especially the sequencing part of the EQA. With careful planning it should be feasible to start molecular detection and subtyping and sequencing on the same fresh nucleic acid extract. We understand this might deviate from your normal procedure for treating clinical specimens from.

ACCESSING THE QCMD ITEMS SYSTEM

You should already have an account on the QCMD ITEMS system to submit your results. Your username is your email address and you create a secure password when you first sign in, or you have it already from previous participation. If you have forgotten your password then you can request a new one from the QCMD website. Please note that you will be sent a time-limited link to your email account and clicking this link will enable you to enter a new password. If possible, your laboratory code (e.g. GB999) or email address should be quoted when requesting information from the QCMD Neutral and Executive Office.

PROCESSING SPECIMENS

1. The panel consists of 15 simulated clinical specimens containing **heat-inactivated type A influenza viruses** or no virus.
2. To assure the quality of nucleic acid extracts for the sequencing part of this EQA, thaw the specimens once and extract nucleic acids and perform detection and subtyping and start sequencing, ideally the same day.
3. All 15 specimens should be tested as being collected from patients suspected for zoonotic influenza virus infection in the middle of the human influenza season.
4. All participating laboratories should perform the Molecular Detection and Subtyping component of the EQA. If laboratories perform any form of sequencing, then it is strongly recommended that this part of the EQA is also performed.
5. A summary of what testing and reporting is requested for all 15 specimens:
   - **Molecular Detection** with generic type A influenza virus RT-PCR or other Nucleic Acid Amplification Test (NAAT) that you routinely use in surveillance and investigation of potential zoonotic influenza cases.
   - **Molecular Subtyping** with RT-PCR or other NAAT of the H-subtype, and N-subtype if available, you routinely use in surveillance and investigation of potential zoonotic influenza cases.
   - **Genetic Characterization**: all type A influenza virus positive specimens should be sequenced (HA segment at the minimum; NA and/or whole genome strongly recommended) by Sanger sequencing or Next Generation Sequencing (e.g. Illumina, Nanopore, or other), the technique you routinely use in surveillance and investigation of potential zoonotic influenza cases. You are requested to also report for each specimen the sequences of all segments obtained in one large fasta multiple sequence file for all specimens together.

     Clade assignment human seasonal influenza viruses
     - Clade and genetic (sub)group/clade for human viruses could be determined using the guidance published by Crick/ECDC/WHO Europe, using Nextclade (https://clades.nextstrain.org) or your own analysis tools.

     Clade assignment avian and swine origin influenza viruses

- For H5 avian and H1 and H3 swine origin viruses you can use tools with official nomenclature provided through https://www.bv-brc.org/app/SubspeciesClassification (account needed; for influenza viruses)
- For subtypes for which no official agreed nomenclature exists for clade assignment, but frequently used in papers, the following material is available:
- For H6 avian viruses https://pubmed.ncbi.nlm.nih.gov/37377949;
- For H7 viruses https://pubmed.ncbi.nlm.nih.gov/22345462;
- For H9 avian viruses https://pubmed.ncbi.nlm.nih.gov/31871234;
- For H10 avian viruses https://pubmed.ncbi.nlm.nih.gov/30013138.
- For genotyping of European H5 viruses circulating since 2020 based on all genome segments, guidance is available at the ECDC extranet: https://extranet.ecdc.europa.eu/EISN/Shared%20Documents/Influenza%20virology%20season%20documents/Season%202023-2024/Avian%20influenza%20genotyping (only for ECDC National/Operational Contact Points with password protected access). This guidance is provided by EFSA and is not an official or international nomenclature system, but just a proposal from the EURL to describe genetic diversity and facilitate the communication among laboratories.

Antiviral reduced susceptibility markers

- The amino acid substitutions reducing inhibition to neuraminidase inhibitors can be found in the WHO reference list: https://www.who.int/teams/global-influenza-programme/laboratory-network/quality-assurance/antiviral-susceptibility-influenza/neuraminidase-inhibitor
- The amino acid substitutions reducing inhibition to baloxavir marboxil can be found in the WHO reference list: https://www.who.int/teams/global-influenza-programme/laboratory-network/quality-assurance/antiviral-susceptibility-influenza/polymerase-acidic-protein-inhibitor
- Amino acid substitutions associated with M2 blocker resistance (and for NA and PA inhibitors as well) can be identified through the use of the FluSurver tool (https://flusurver.bii.a-star.edu.sg). Be aware that FluSurver outputs are a guidance and need expert judgement about the applicability of identified markers to the identified virus.

Mammalian adaptation markers

- A list of amino acid changes in H5 viruses associated with adaptation to mammalian replication can be found here: https://www.cdc.gov/flu/pdf/avianflu/h5n1-inventory.pdf (last updated 2012).

General

- Diverse amino acid substitutions can be identified through the use of the FluSurver tool (https://flusurver.bii.a-star.edu.sg). Be aware that FluSurver outputs are a guidance and need expert judgement about the applicability of identified markers to the identified virus. Of course, your own expertise and available tools should guide the genetic characterization of the panel viruses as well.

NOTE OF AWARENESS

1. The specimens in this panel are simulated clinical specimens prepared from cultured and heat-inactivated viruses.
2. Virus growth in cell culture is different from virus replication in the human respiratory airway during an active infection.
3. The necessary inactivation procedure (using mild heat) might affect RNA integrity in such a way that the larger genome segments might be difficult to amplify in certain protocols using universal primers for whole genome segment amplicon sequencing.
4. Therefore, the relationship between the Ct value in virus detection RT-qPCR as a proxy for viral load and genome segments sequencability is different from this relationship seen with fresh clinical specimens.
5. The QC and pretesting of the panel specimens showed that they are suitable to sequence at least the full HA and NA genome segments using Illumina and Nanopore sequencing protocols, including the use of universal primers for whole genome segment amplicon sequencing.
6. Consequently, sequencing of the HA and NA segments for full subtype identification and further characterization to determine the HA clade/subclade/subgroup and likely host species will be considered core results.
7. Sequencing, analysis and reporting results on the H5 genotype (for which all 8 segments are needed) and on amino acid substitutions associated with reduced antiviral susceptibility and mammalian adaptation for the NA and other genome segments is highly recommended, but results will be considered educational results. This means that the results will not be scored, even if they are not provided or incorrect, but are meant to be used for comparison purposes in a report and for your own and Network learning purposes.
8. All communication on results for all components of the EQA except to yourself will be anonymized.

Pre-distribution testing was performed by two independent laboratories using in-house and/or commercially available technologies prior to shipment to validate the content of the panel for its purposes.

Important: Please pay special attention to the safety warnings and precautions section of this manual before

processing any panel specimens. If further technical assistance is required please contact the QCMD office +44 (0) 141 945 6474.

REPORTING RESULTS

General Submission of Results

1. You should log on to ITEMS using your user name (email address) and password at https://items.qcmd.org. If you have forgotten your password then you can reset it from the ITEMS login page.
2. Navigate to the **Programme Dashboard** and ensure that you have selected the year as 2024. There will be a table with a list of programmes for which you are registered including the EISN programmes. Before results can be submitted you should first record Panel Receipt. This will make the Result Return icon active and clicking on this icon will enable you to submit results. Press the **Add New Dataset** button to bring up the respective results return form.
3. A brief questionnaire is part of the result return form of each programme and not a separate entity.
4. You may save incomplete results whilst still inputting data using the **Save Progress** button at the bottom of the webpage. Submitting completed results using the **Submit Completed Form** button at the bottom of the webpage will result in a summary form being created. Please make a copy of this for your records since one will not be sent to you. The main contact for your laboratory will however receive a message via ITEMS and also an email to confirm receipt of the results. Submitted results may be amended any time before the submission deadline.

Important: You **will not** receive an e-mail with details of your submitted results, therefore a copy should be saved for your records.

Submission of Molecular Detection and Subtyping Results

1. Upon completion of influenza virus molecular detection and subtyping, the on-line **EISN Molecular Detection and Subtyping result form** (programme EISN_INF_MD24) should be fully completed within **7 working days** at: http://www.qcmd.org
2. It is crucial that the on-line **Molecular Detection and Subtyping result form** is filled out correctly.
3. Please share primer and probe sequences in one Fasta file with the name 'ITEMS lab code_primers_probes.fst' (e.g. GB999_primers_probes.fst) with for each primer and probe in the Fasta header of the sequence clearly indicated 'A and segment letter(s)' or 'Hnumber' or 'Nnumber', species specificity (generic, human, avian, swine), for primers forward or reverse or probe, separated by underscores, e.g.:
   >A_M_generic_forward_your_primer_code
   sequence
   >H5_avian_probe_your_probe_code
   sequence
   etc.
   After you have chosen the file to upload, scroll down to the bottom and press **Save Progress**. In a window at Primer/Probe FASTA File Upload you now will see your uploaded fasta file as proof that uploading has succeeded.
4. Then, define how many assays you have used for H-subtyping and N-subtyping. You may select between zero and 10 for each. Subsequently you have to define for each assay for which subtype the assay is used from a pick list (choose carefully for species specificity) and add a free text identifier if you use more than one assay for the same subtype. Note that the selected subtype and additional information when taken together must be unique for each assay. The chosen assays are replicated in the names of the result reporting fields for your convenience.
5. Enter your completed results for the panel in the results table. For each positive result on generic influenza type A virus detection and H- and N-subtyping also the Ct value if the used assay generates a Ct value.
6. If a test is not performed, indicate this by selecting 'Not used'. When 'Was Influenza Type A Detected?' is reported negative all subtype assay results are filled by default with 'Not used'. You are allowed to overwrite this default if a subtyping test was used with a Positive or Negative result.
7. Choose the likely species from which the virus originates, the appropriate conclusion for H-subtyping and N-subtyping and a final conclusion on the identified virus, all using pick lists. If you choose 'other', you will be provided a free text field for specification.
8. Complete the extra questions in the Questions tab (scroll to the right under Panel Results using the arrow button).
9. Send your results form by selecting the **Submit Completed Form** button, this is essential to complete the submission process. You will be notified of errors. If this occurs, correct them and press the **Submit Completed Form** button again.
10. At the bottom of the webpage you may use the Print button to save a pdf of your results, or print it as

hardcopy.

11. **Important:** Results must be returned **within 7 working days** to guarantee inclusion in the EEIQAP 2024 final report.

Submission of Genetic Characterization Results

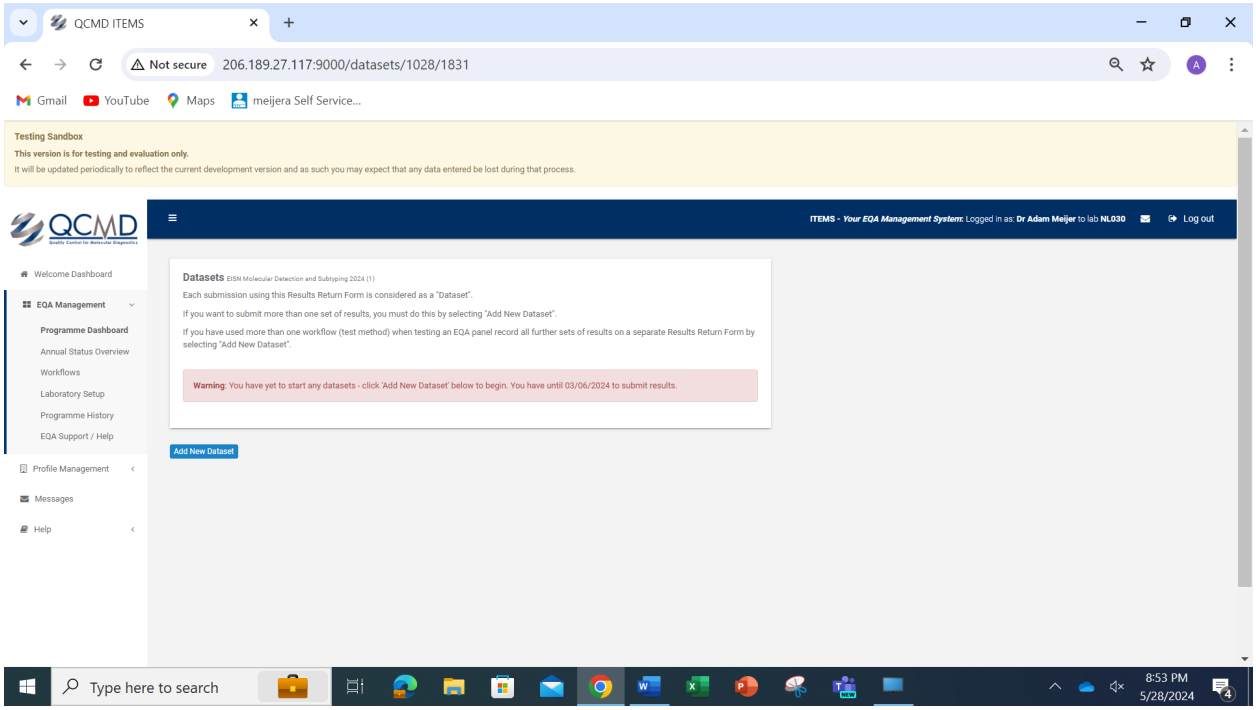
1. Upon completion of Sequencing and Characterization the on-line **EISN Genetic Characterization results form** (programme EISN_INF_GC24) should be fully completed within **30 working days** at: http://www.qcmd.org
2. It is crucial that the on-line **Genetic Characterization result form** is filled out correctly.
3. Please provide obtained sequences for each segment in one Fasta file with the name 'ITEMS lab-code_sequences.fst' (e.g. GB999_sequences.fst). For each sequence's Fasta header clearly indicate the abbreviated ITEMS lab-code, specimen name and segment letter(s), separated by underscores, e.g.:
   > GB999_1_AI24-01_M
   sequence
   > GB999_1_AI24-01_HA
   sequence
   etc.
   After you have chosen the file to upload, scroll down to the bottom and press **Save Progress**. In a window at Sequence FASTA File Upload you now will see your uploaded fasta file as proof that uploading has succeeded.
4. Select if you have results to report for the panel specimen concerned. By default the question 'Was this sample analysed' is checked. If the sample has not been analyzed uncheck this question and result reporting fields will disappear.
5. Enter for each genome segment whether a sequence has been obtained or 'Not sequenced' (e.g. if only the HA was sequenced).
6. Report the likely species from which the virus originates, determined H- and N-subtype and the overall conclusion on the virus detected, all using pick lists. The overall conclusion should be based on your PCR/NAAT detection and subtyping AND your sequencing results.
7. Report the clade and (sub)group/clade or H5 genotype if applicable (picklists are provided for standardized reporting)
8. Report amino acid substitutions identified linked to antiviral reduced inhibition/susceptibility (neuraminidase inhibitors, baloxavir marboxil, M2-blockers) or mammalian adaptation in the free text fields.
9. Reporting of amino acid substitutions identified should be done in the format: [genome segment code]dash[WT amino acid letter][amino acid position][mutant amino acid letter], each separated by semicolon and space. E.g. NA-H275Y; PA-I38V; M-S31N.
10. Complete the extra questions in the Questions tabs (scroll to the right under Panel Results using the arrow button).
11. Send your results form by selecting the **Submit Completed Form** button, this is essential to complete the submission process.
12. At the bottom of the webpage you may use the Print button to save a pdf of your results, or print it as hardcopy.
13. **Important:** Results must be returned **within 30 working days** to guarantee inclusion in the EEIQAP 2024 final report.

Important: All results should be returned as quickly as possible as the **speed of reporting** of molecular virus detection, subtyping and sequence characterization form part of this EEIQAP. This shall provide a measure of the capability of European laboratories for early warning of zoonotic influenza virus infection.

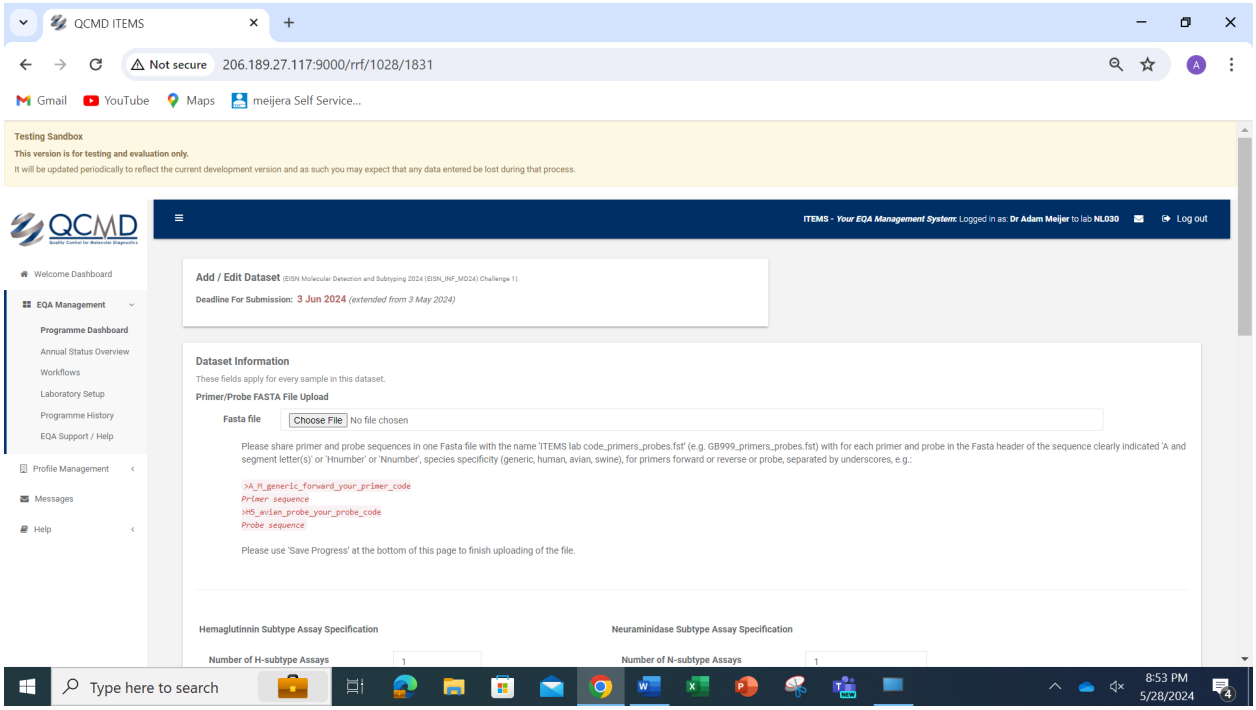
Annex 15. Screenshots of the QCMD ITEMS data entry screens

Shown are the questions and as much as possible the content of pick lists provided for standardised data entry. These screenshots were made on 28 May 2024.

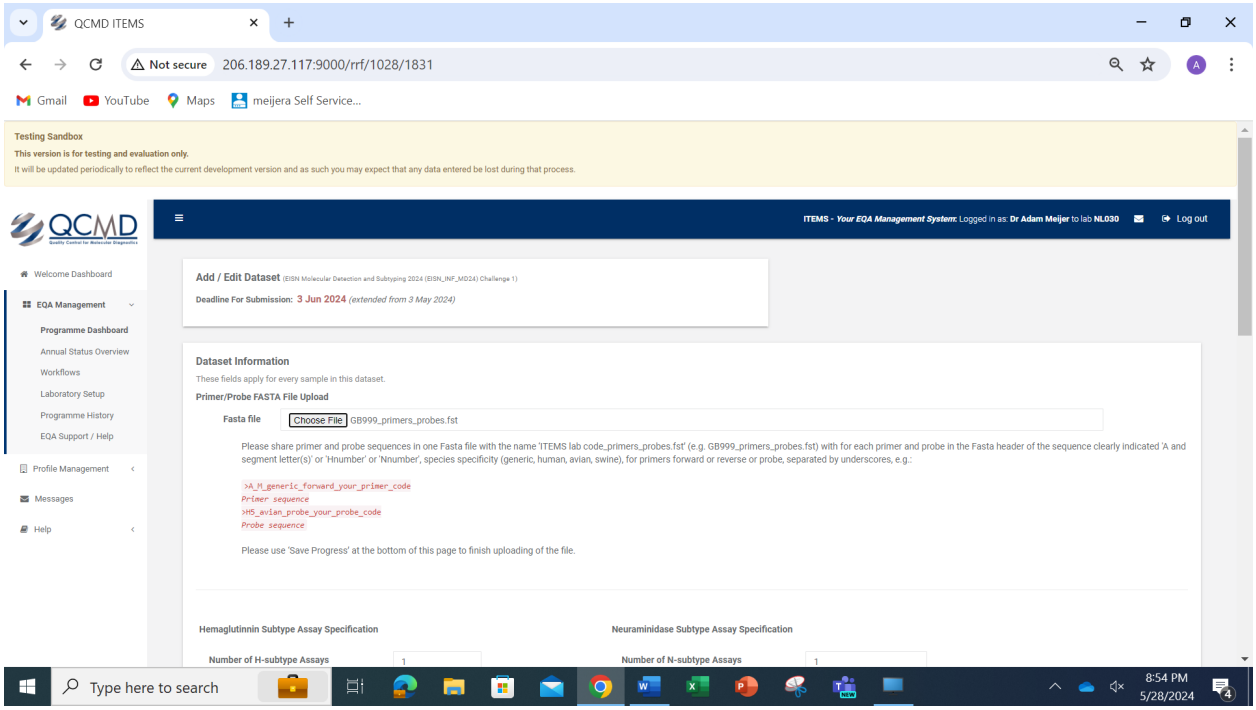
Detection and subtyping programme, Add new dataset.



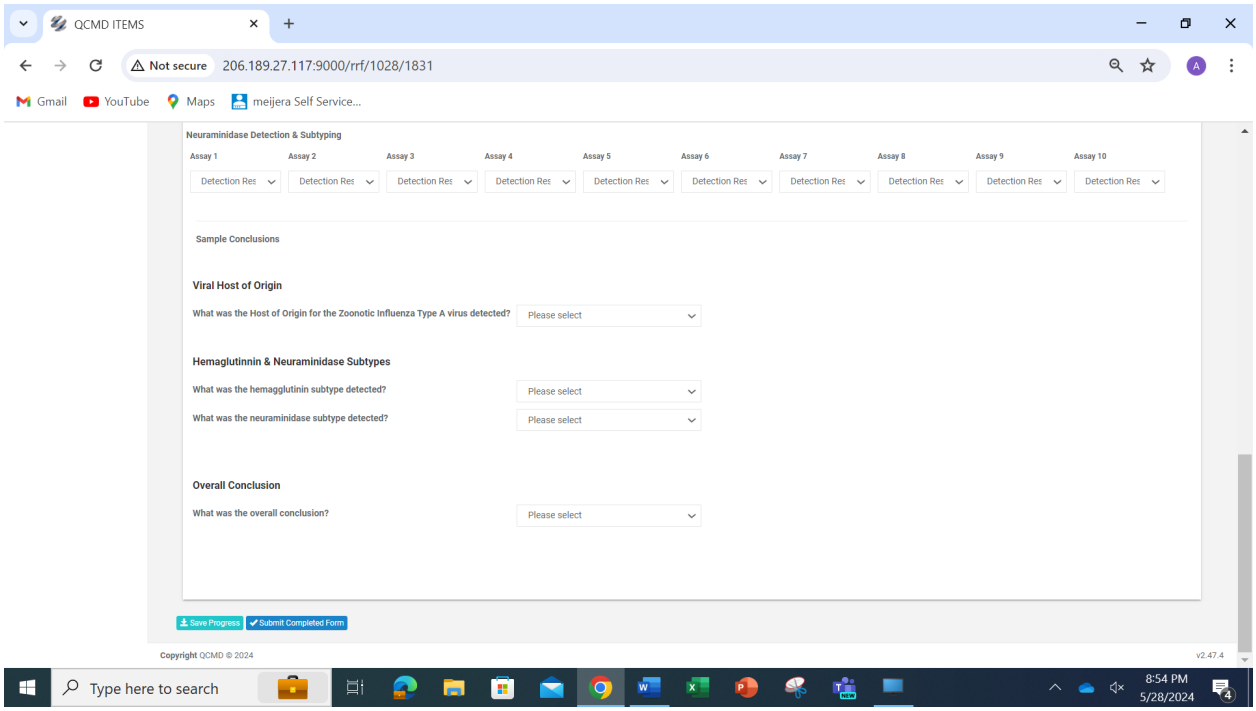
Choose file to upload primers and probes sequences of NAAT used to analyse the panel specimens.



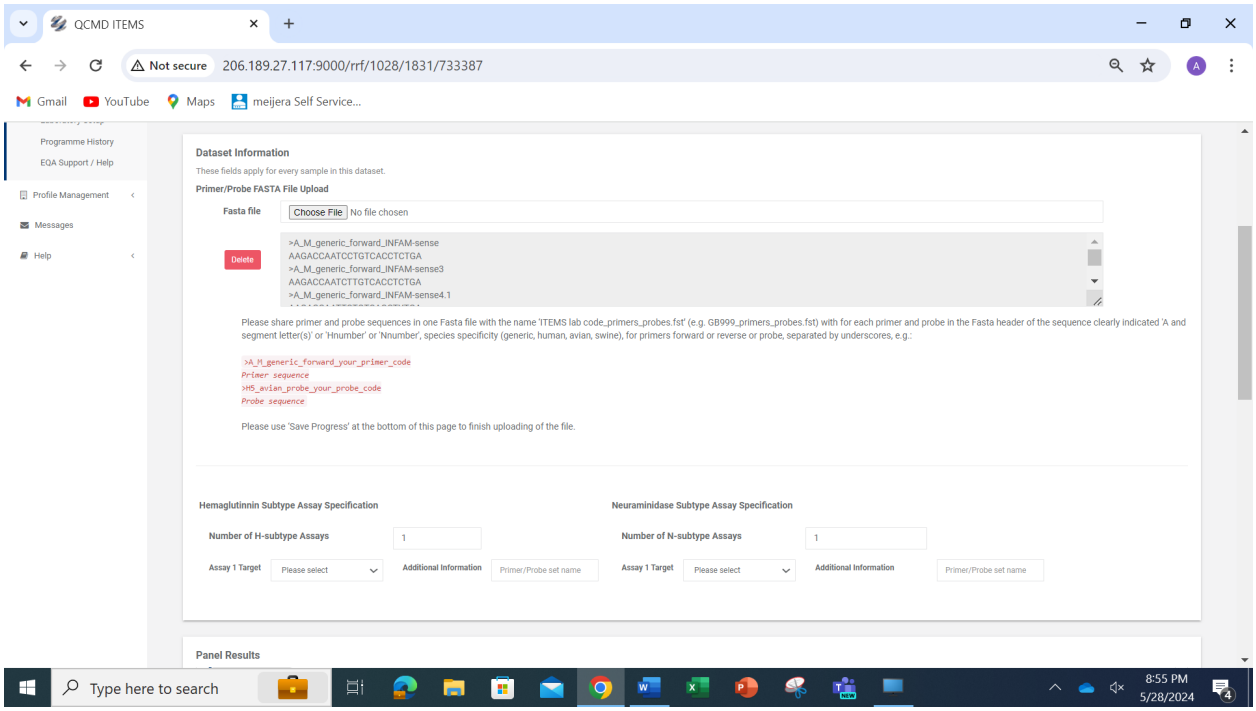
Chosen file shown.



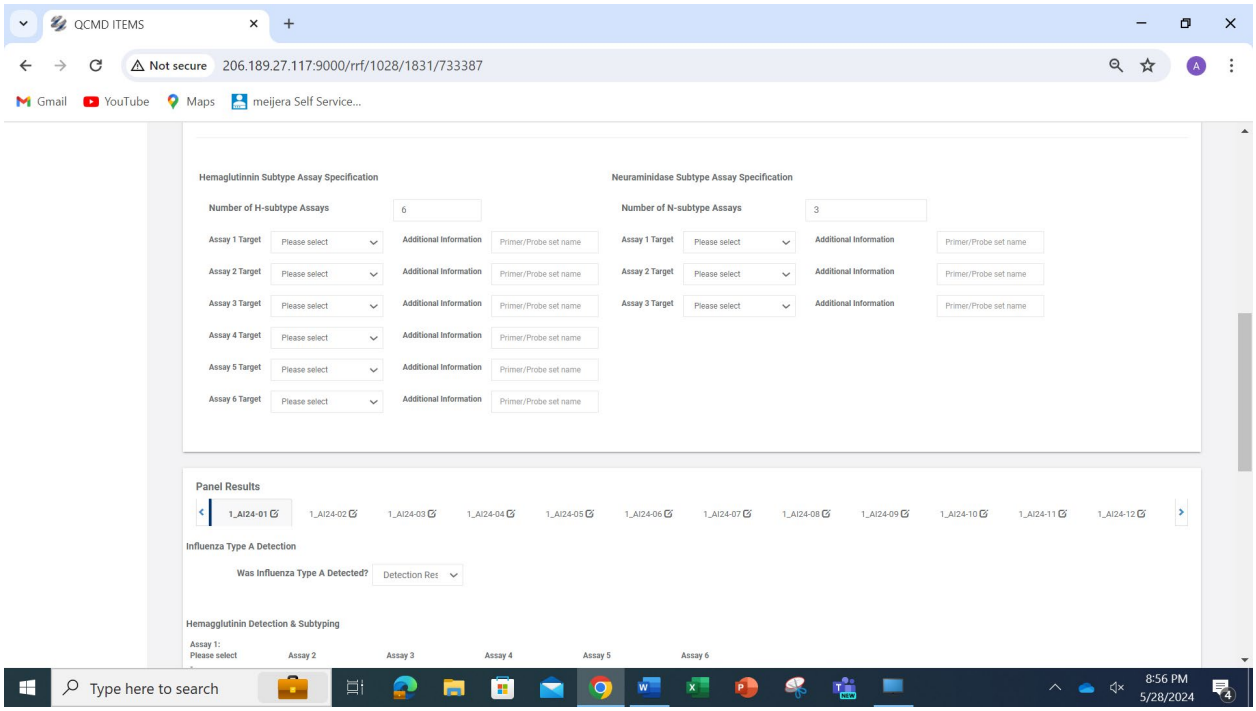
Press Save Progress button to store the chosen file in ITEMS.



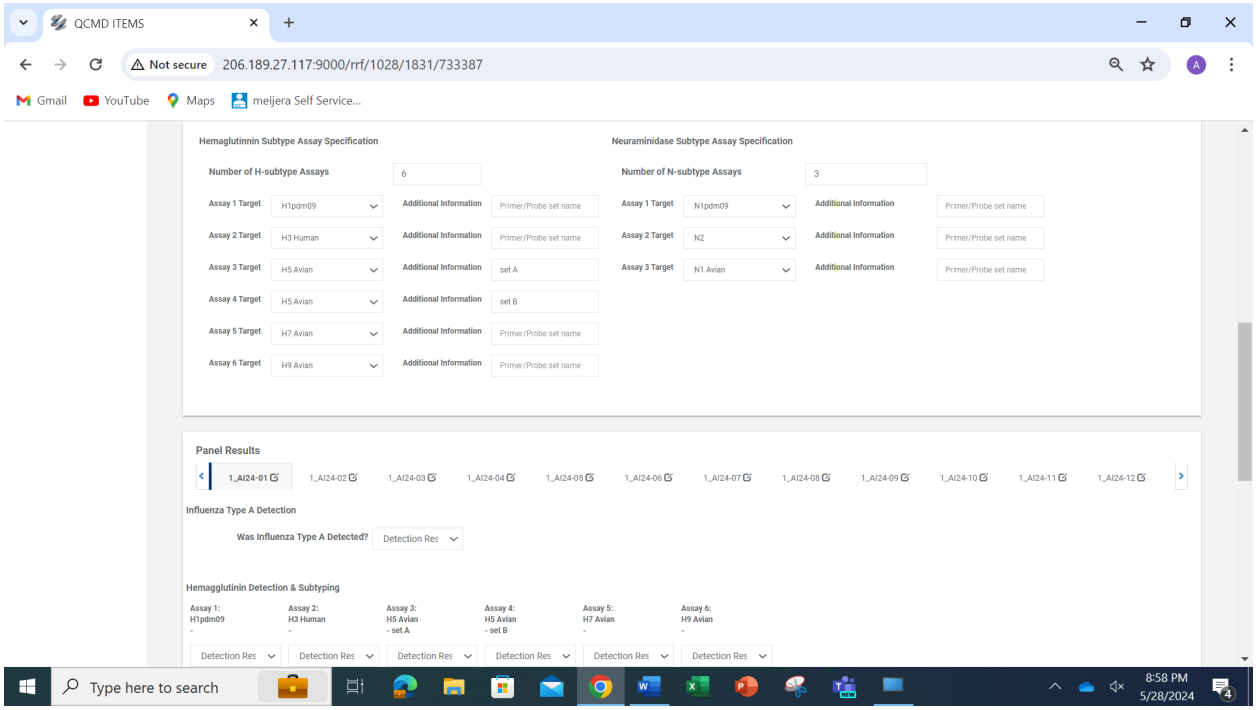
Proof of successful uploaded file with sequences.



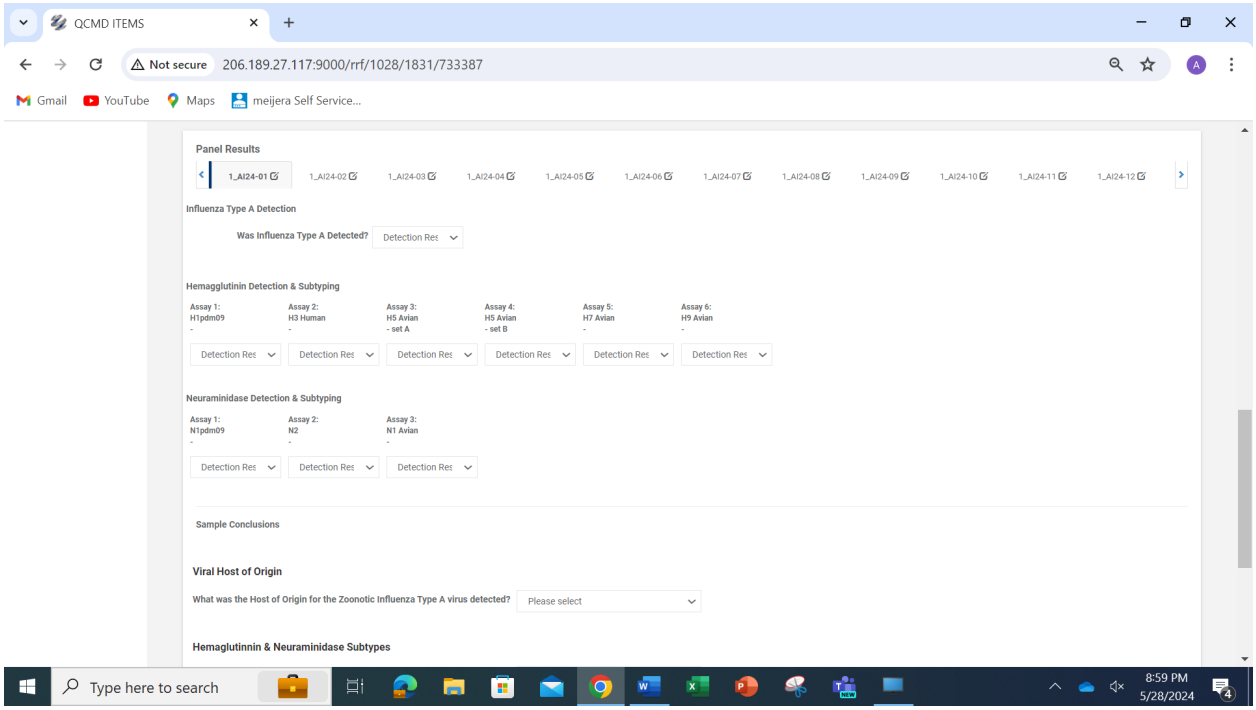
Define number of H- and N-subtyping NAAT used to analyse the panel specimens. Depending on the number chosen fields are provided to define each assay.



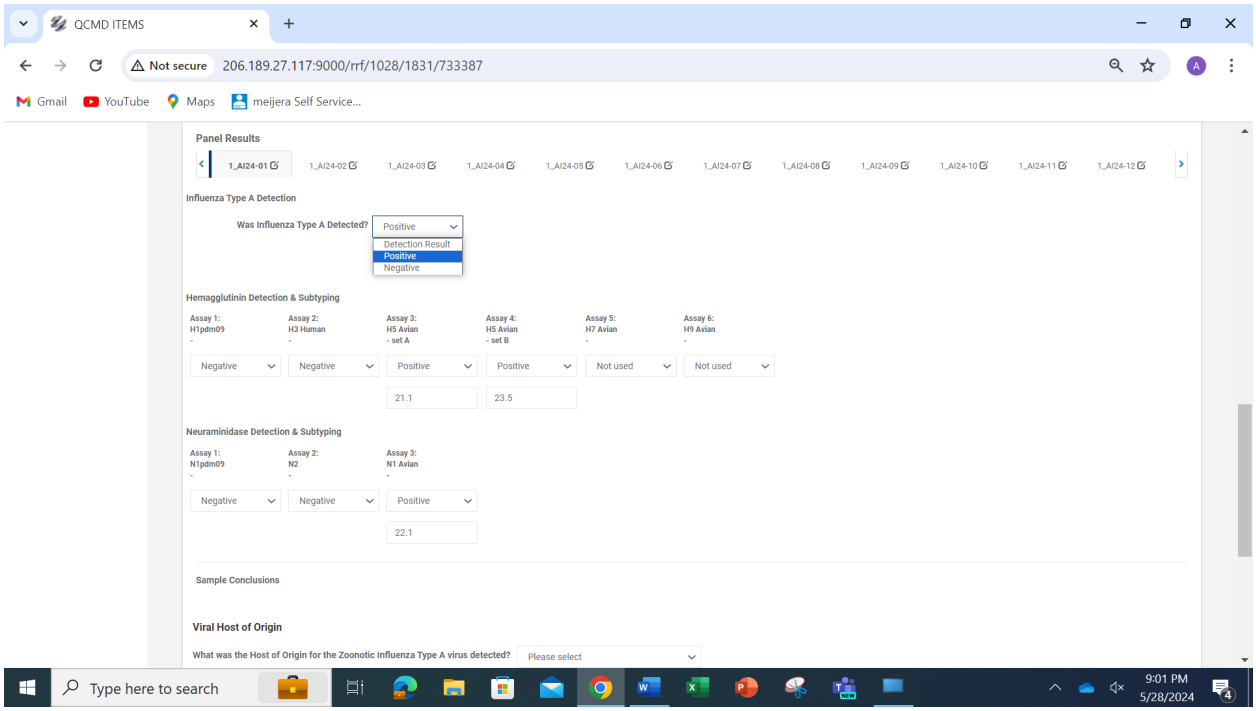
H- and N-subtyping assays defined using predefined options in pick lists and free text additional information.



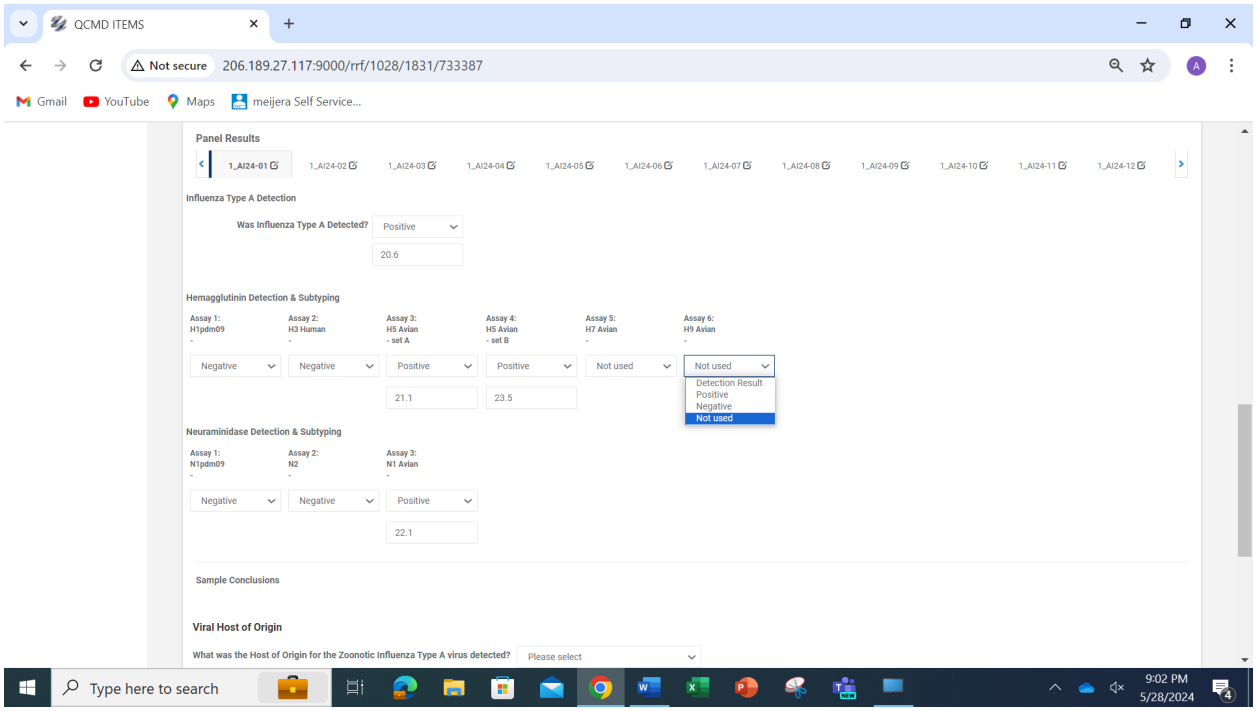
Start of entering test result for detection and subtyping, panel specimen 1. For the H- and N-subtyping assays the definition previously entered is shown for each assay to assist in reporting the results for the used assays. For each of these assays ate least the option 'Not used' should be chosen to pass validation of data entry.



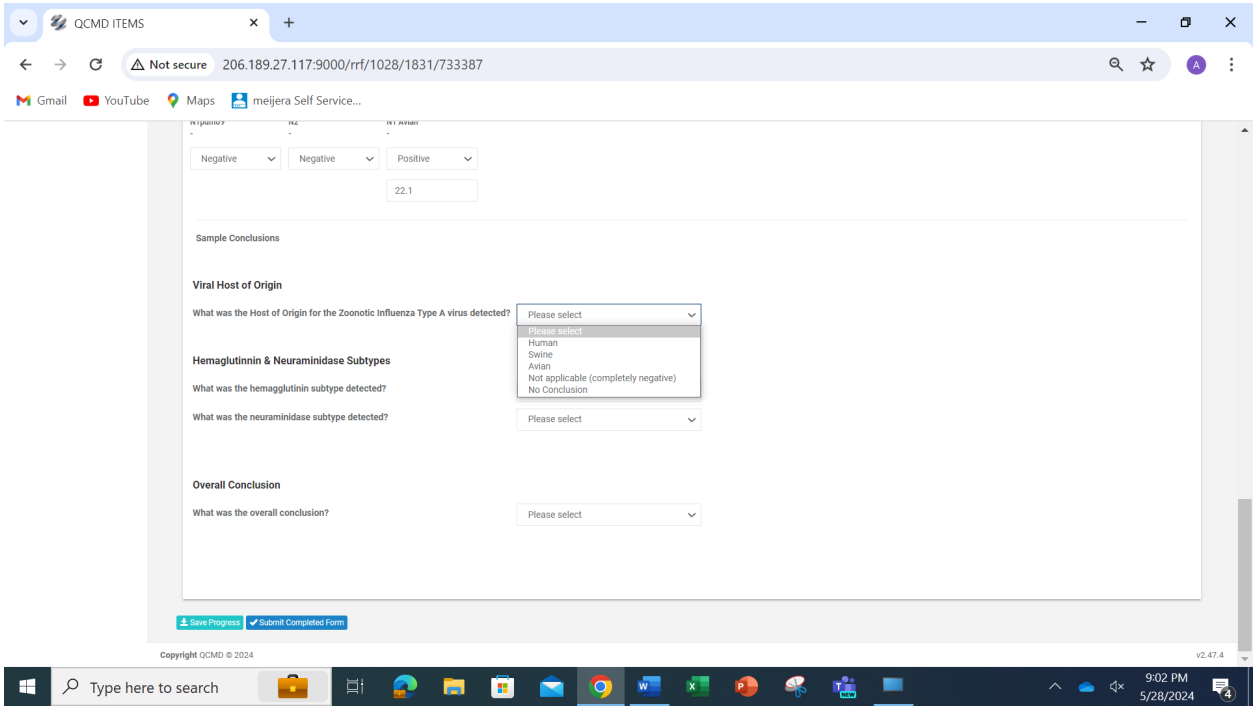
Predefined pick list for reporting the influenza type A detection result.



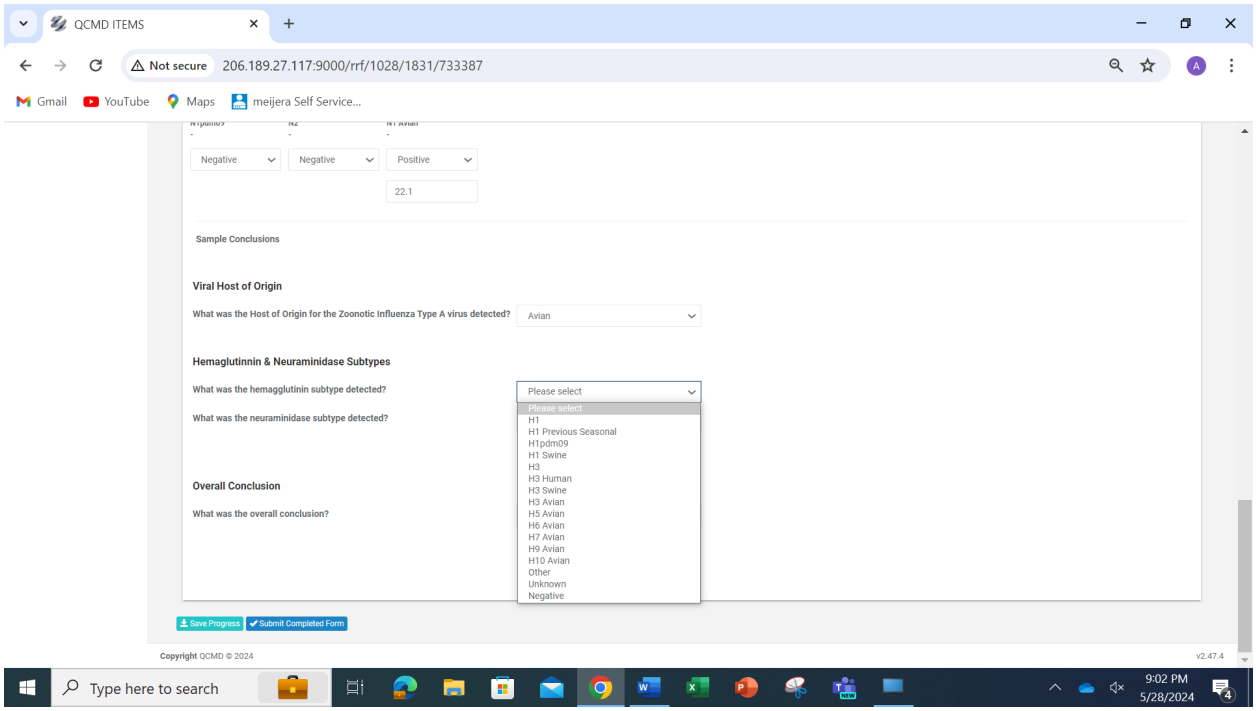
Predefined pick list for reporting the H-subtyping result. When Positive is chosen a field opens to enter the obtained Ct value (if test generates a Ct value). Entering N-subtyping results works the same.



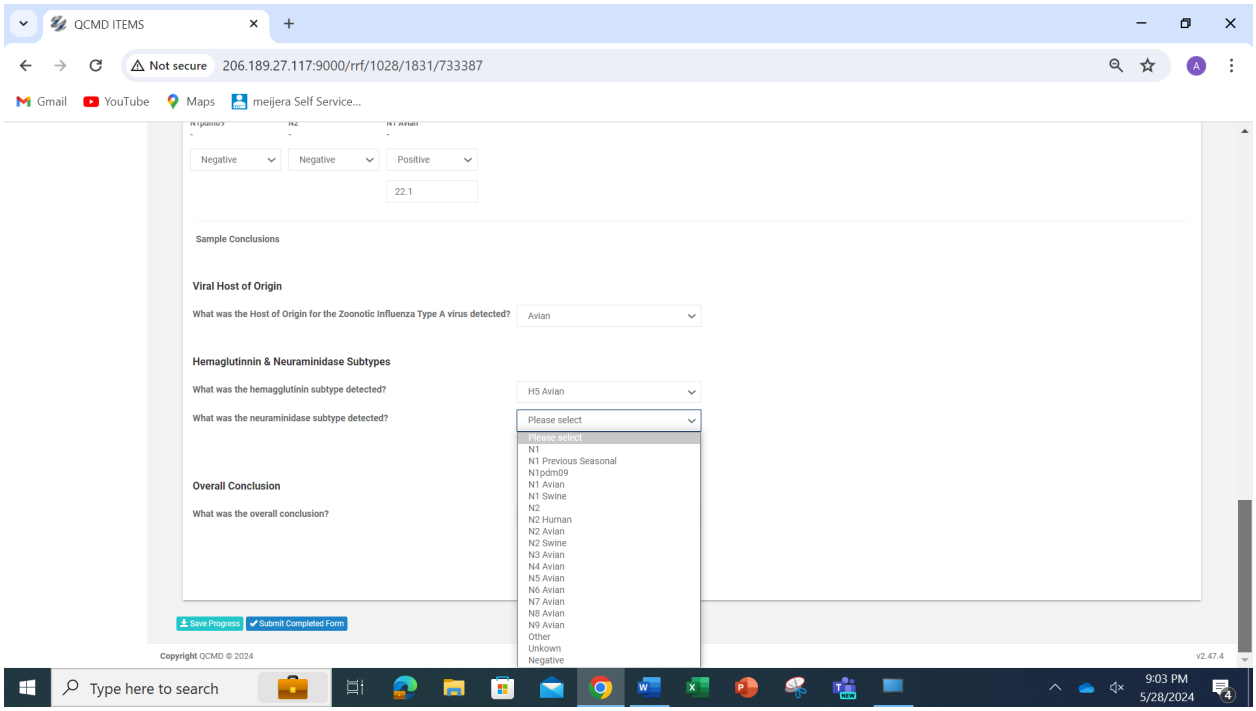
Enter the 'likely host species' identified from the NAAT subtyping results obtained, using predefined pick list.



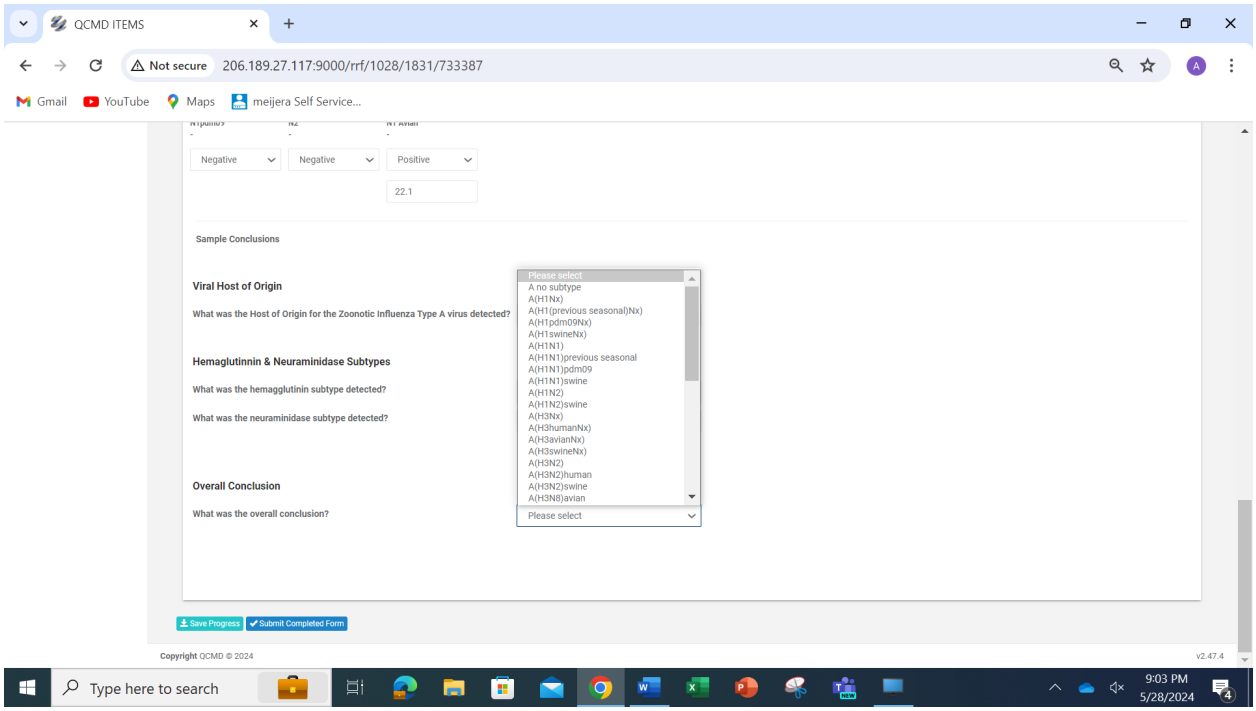
Enter the identified H-subtype using NAAT by selecting the appropriate one from the predefined pick list. If your result is not in the list you can choose 'Other', after which an option is offered to enter free text.



Enter the identified N-subtype using NAAT by selecting the appropriate one from the predefined pick list. If your result is not in the list you can choose 'Other', after which an option is offered to enter free text.

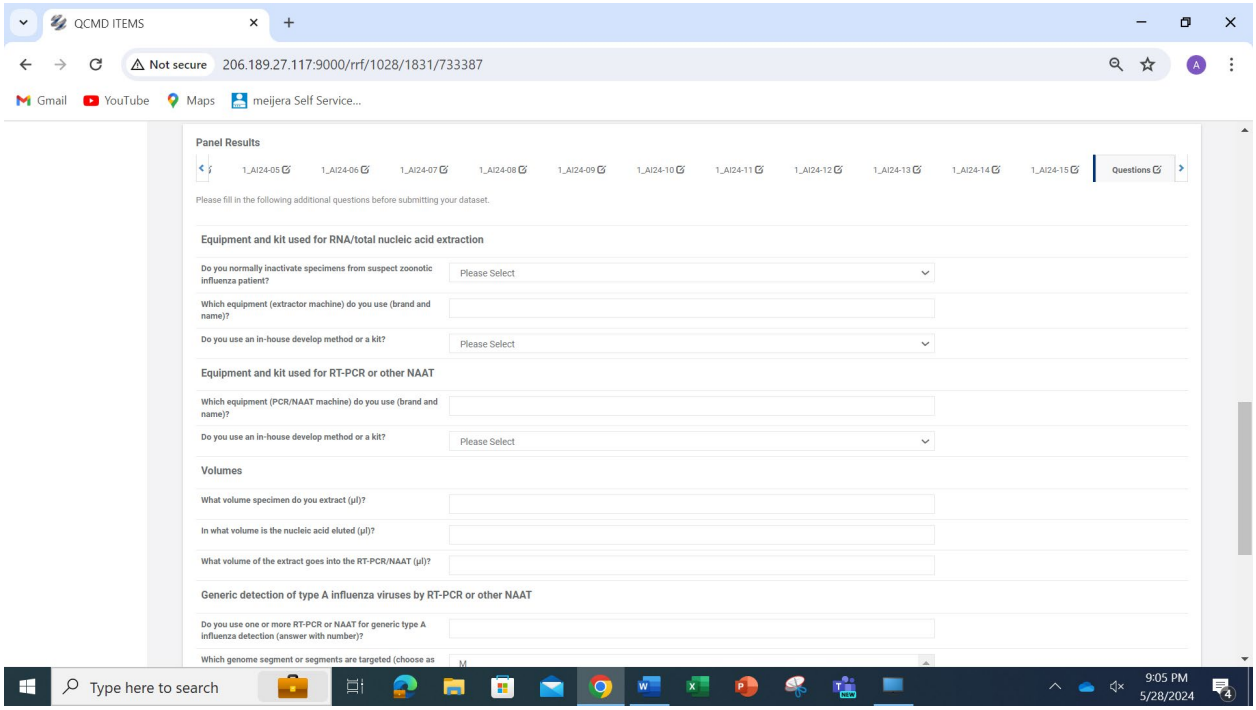


Enter the overall conclusion using NAAT by selecting the appropriate one from the predefined pick list. If your result is not in the list you can choose 'Other', after which an option is offered to enter free text.

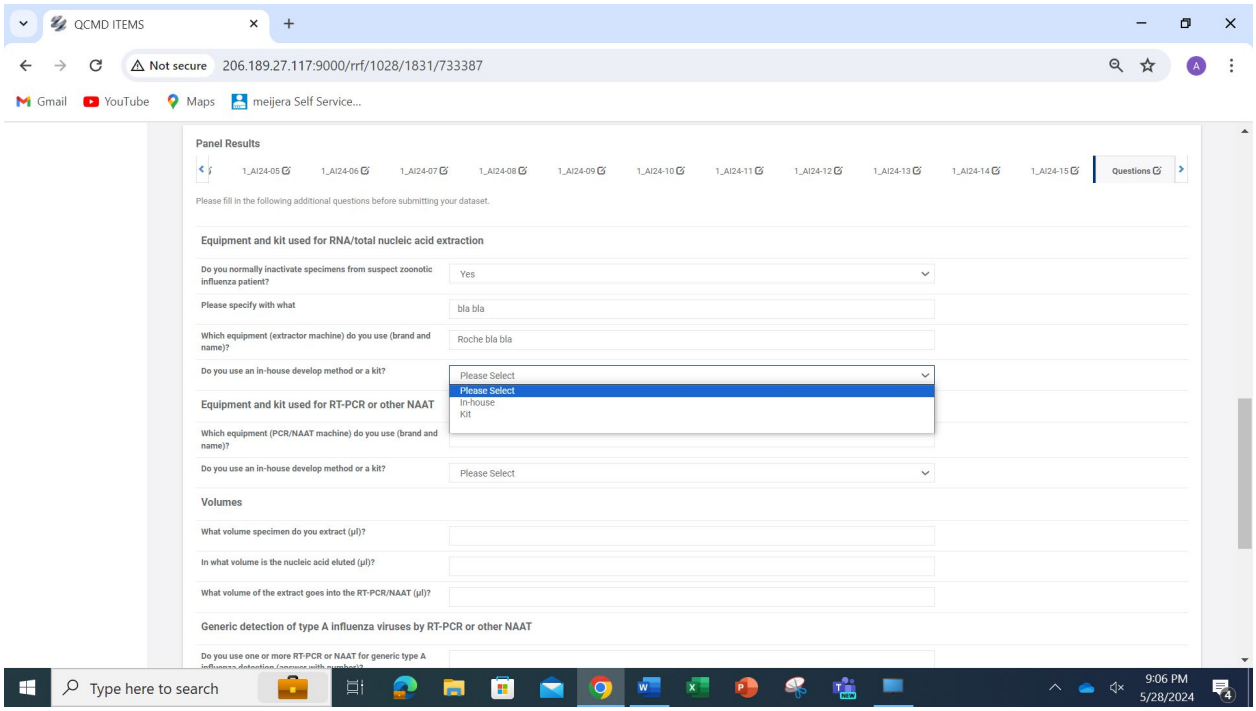


Do the reporting of results similarly for all other specimens in the panel.

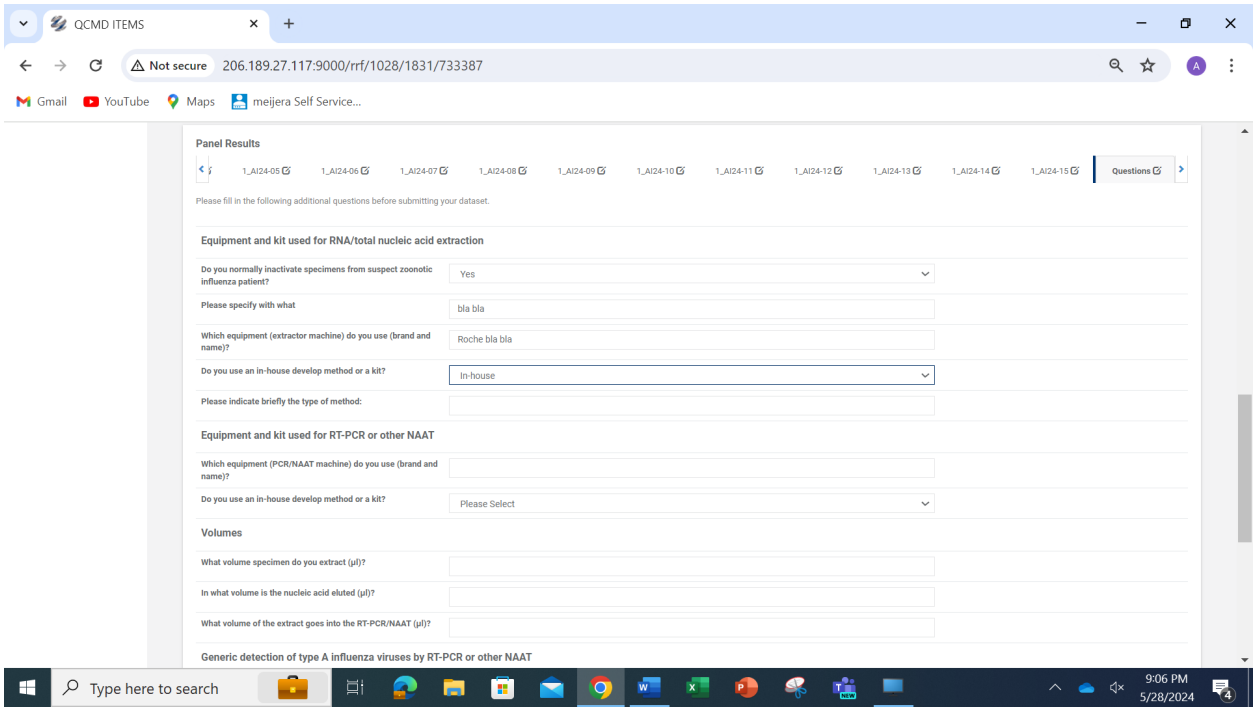
Answer questions on used techniques, where appropriate using one or more options from predefined pick lists or answering in free text.



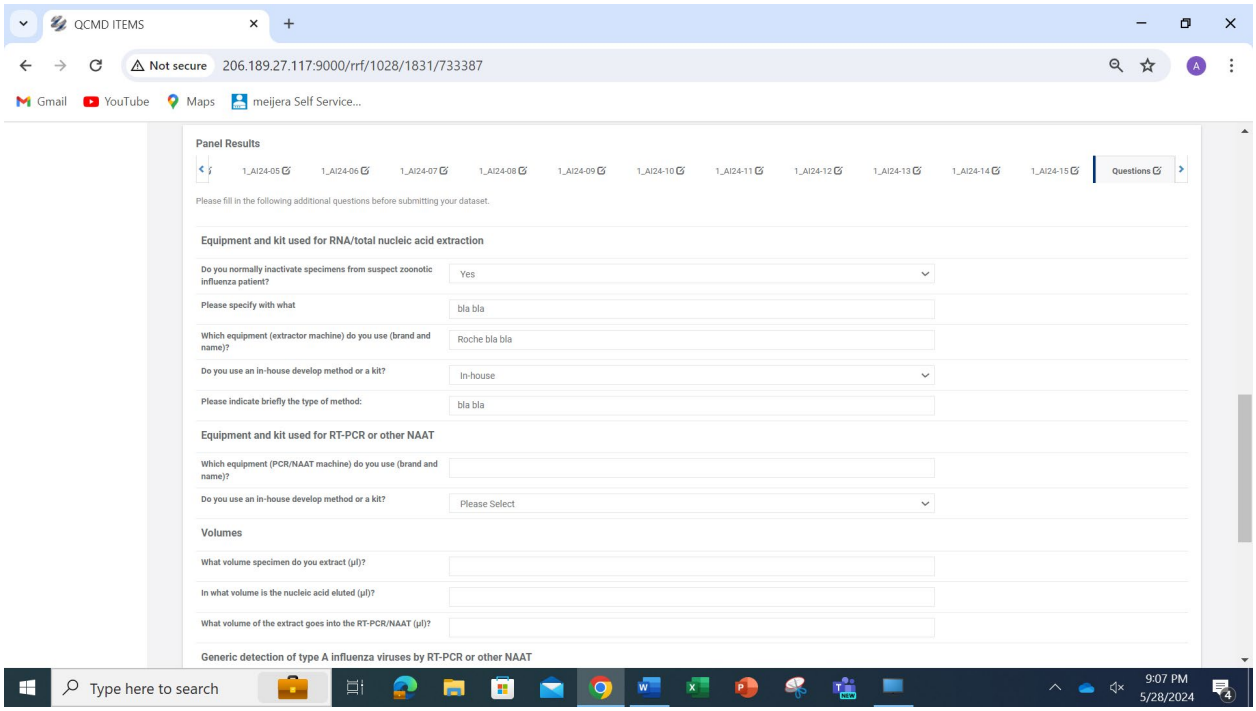
Pick list for In-house or Kit for extraction.



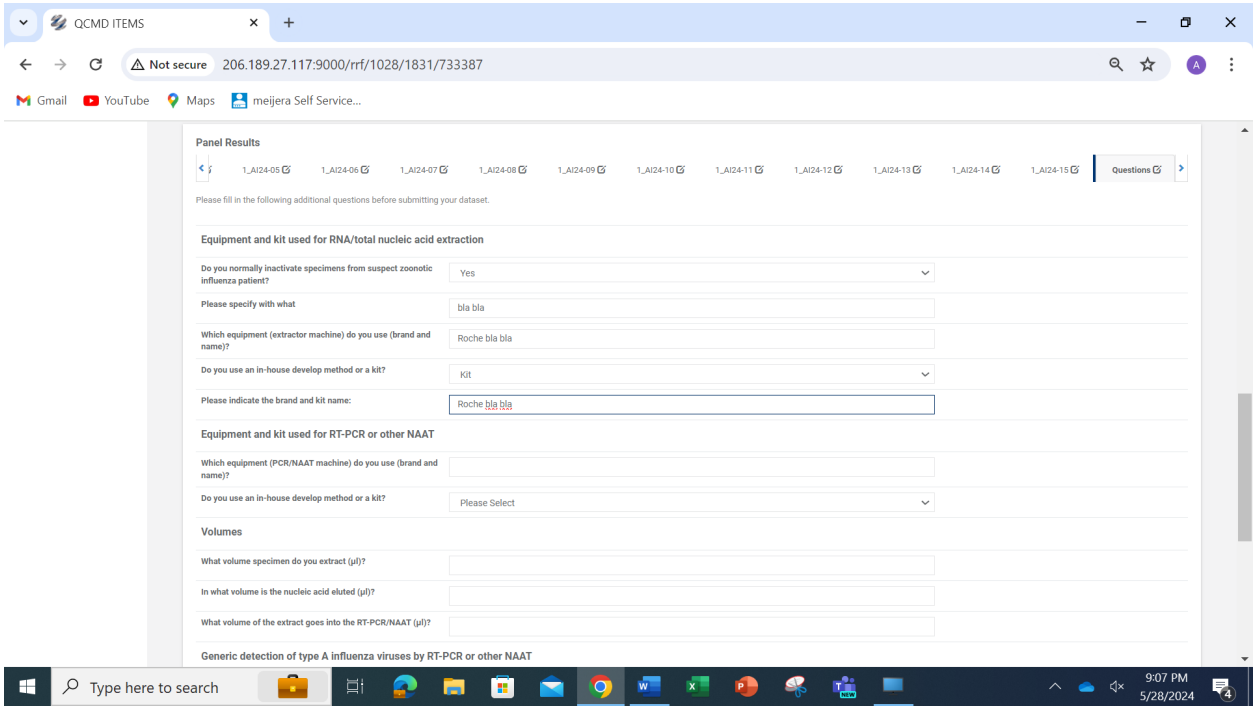
Chosen option.



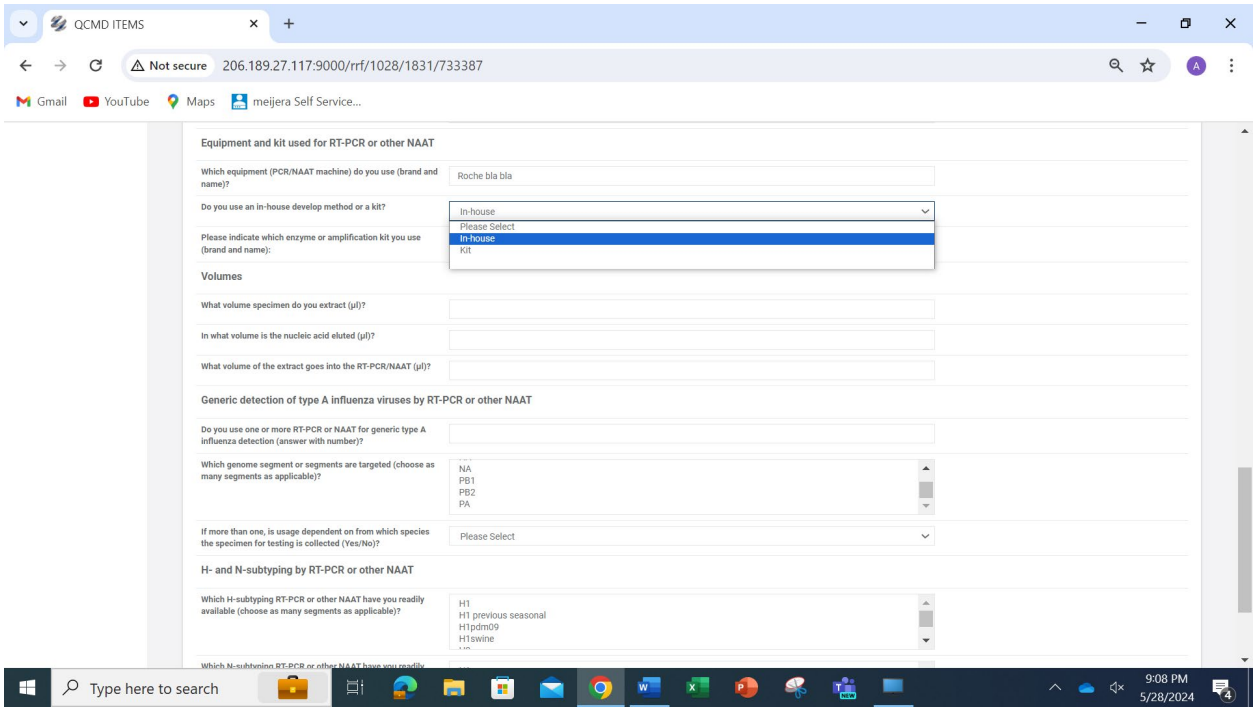
Further filled in questions.



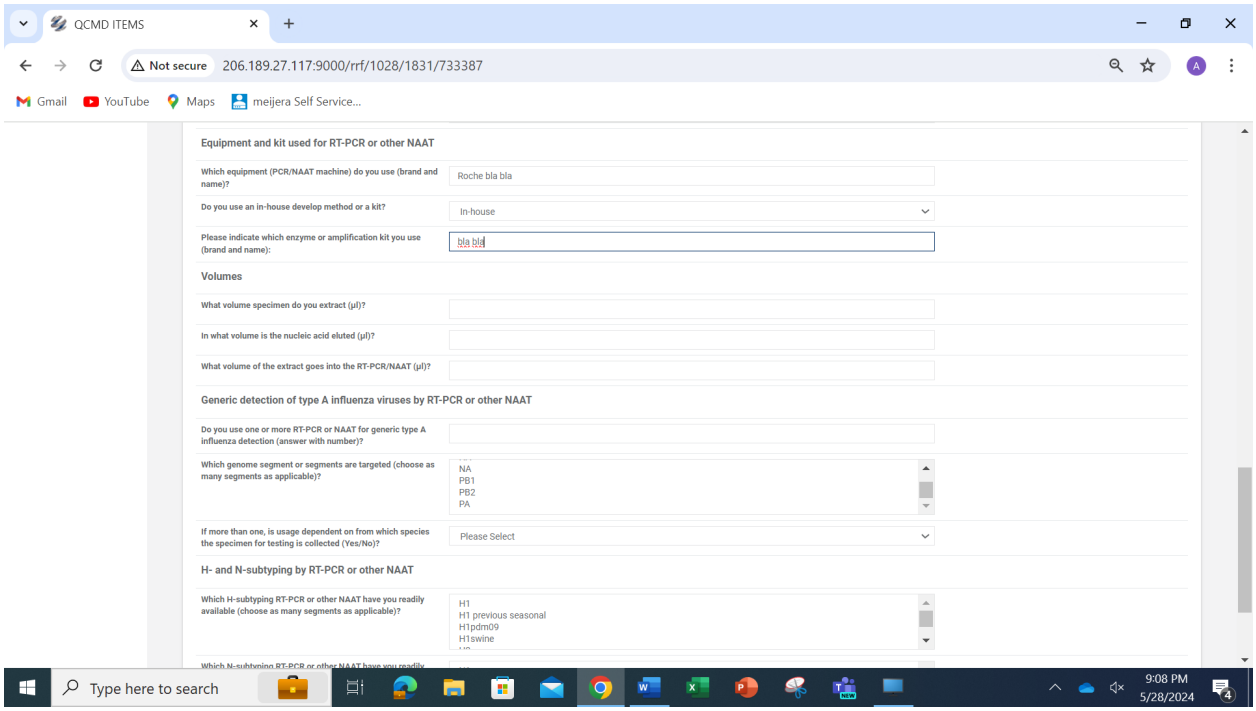
Further filled in questions.



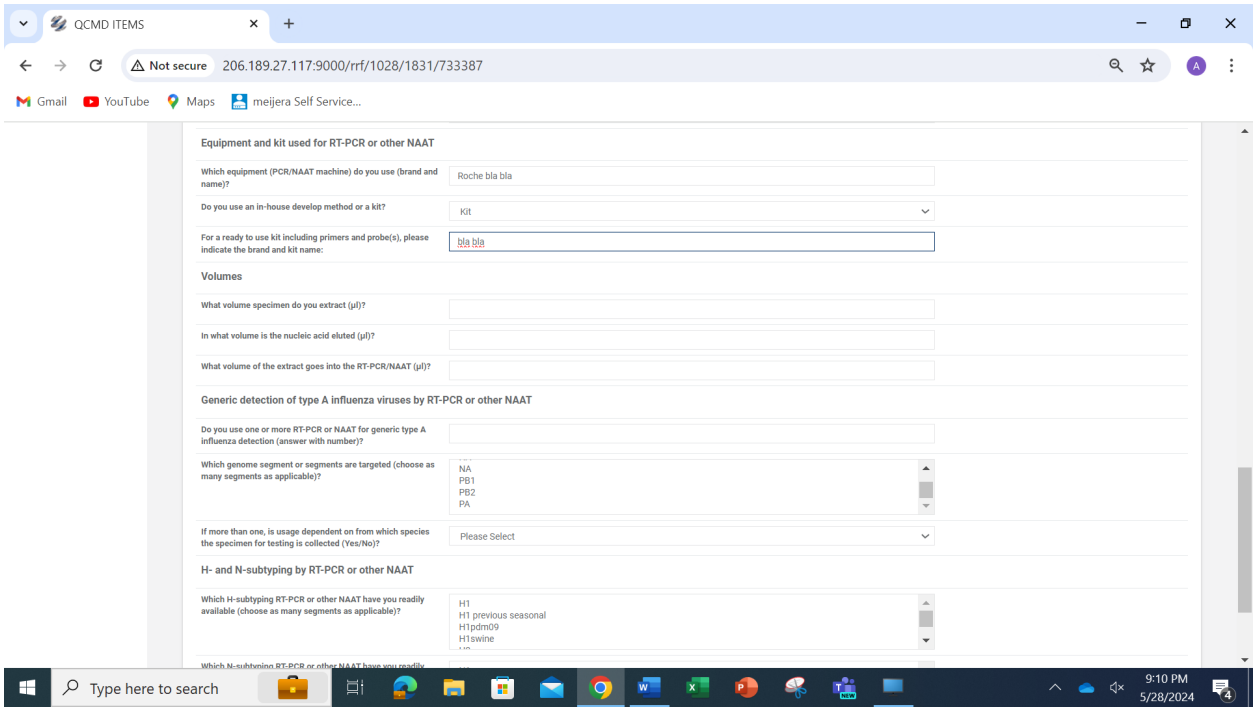
Pick list for In-house or Kit for amplification.



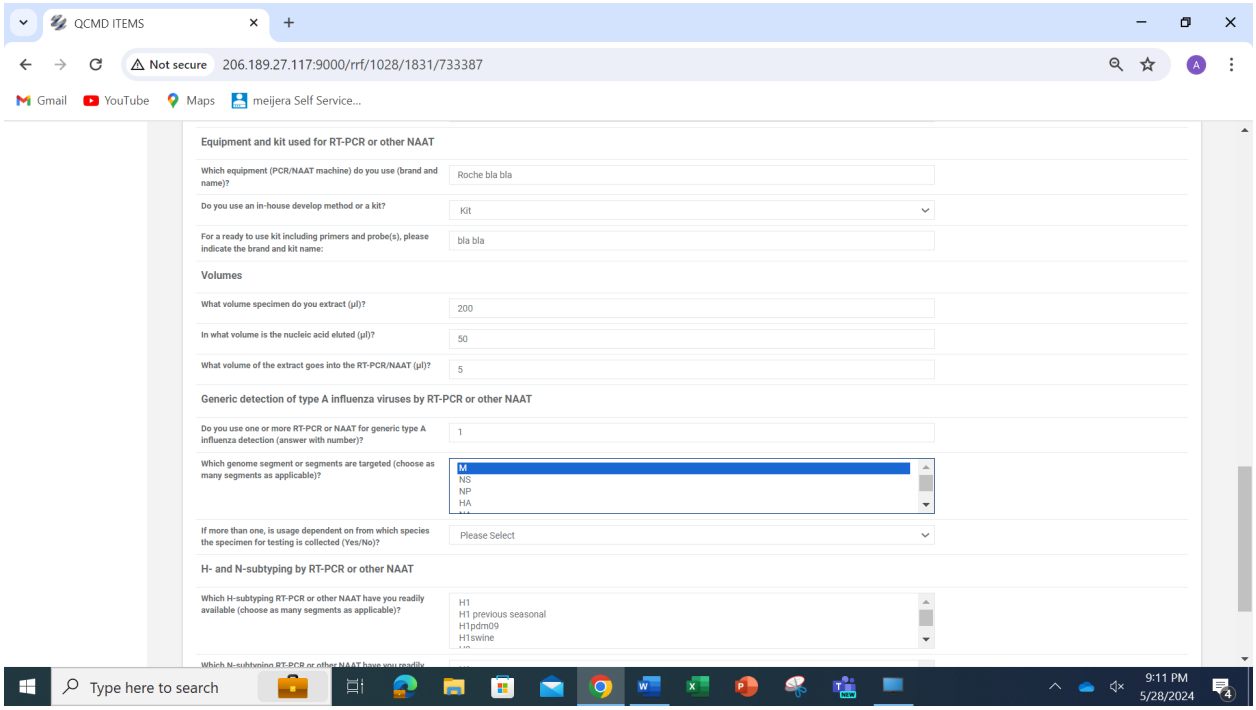
Further filled in information equipment and reagents used for amplification.



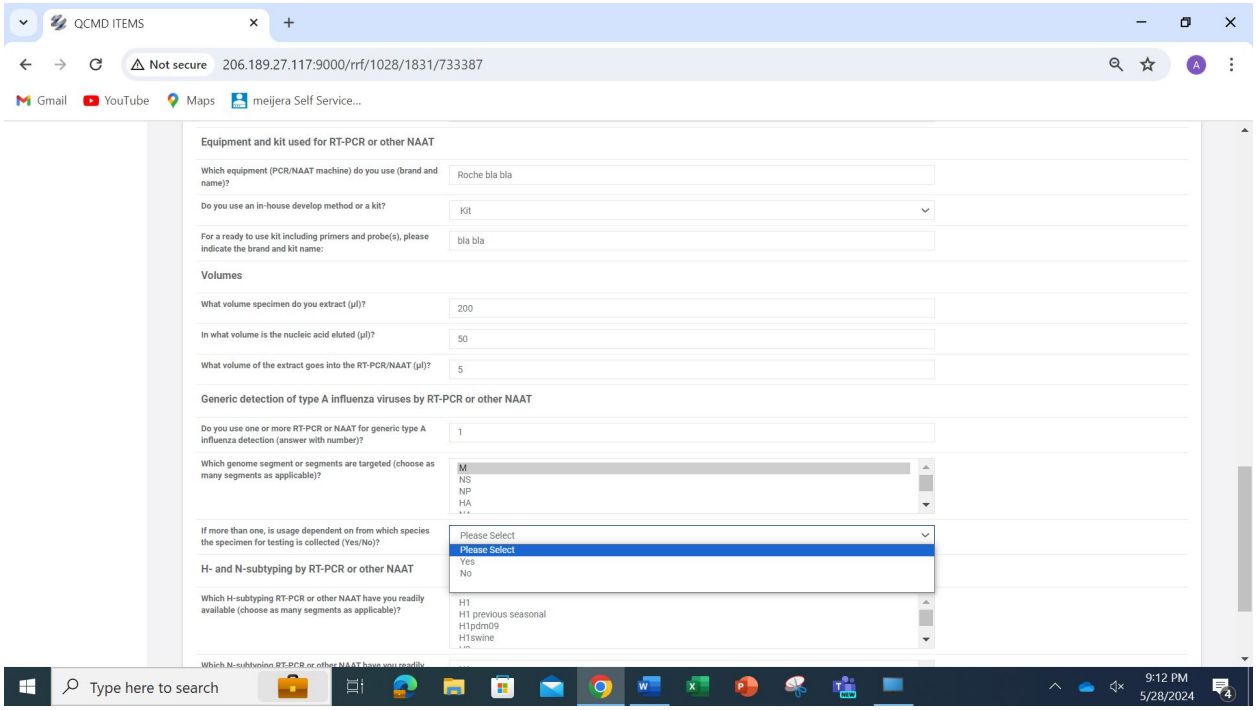
Further filled in information equipment and reagents used for amplification.



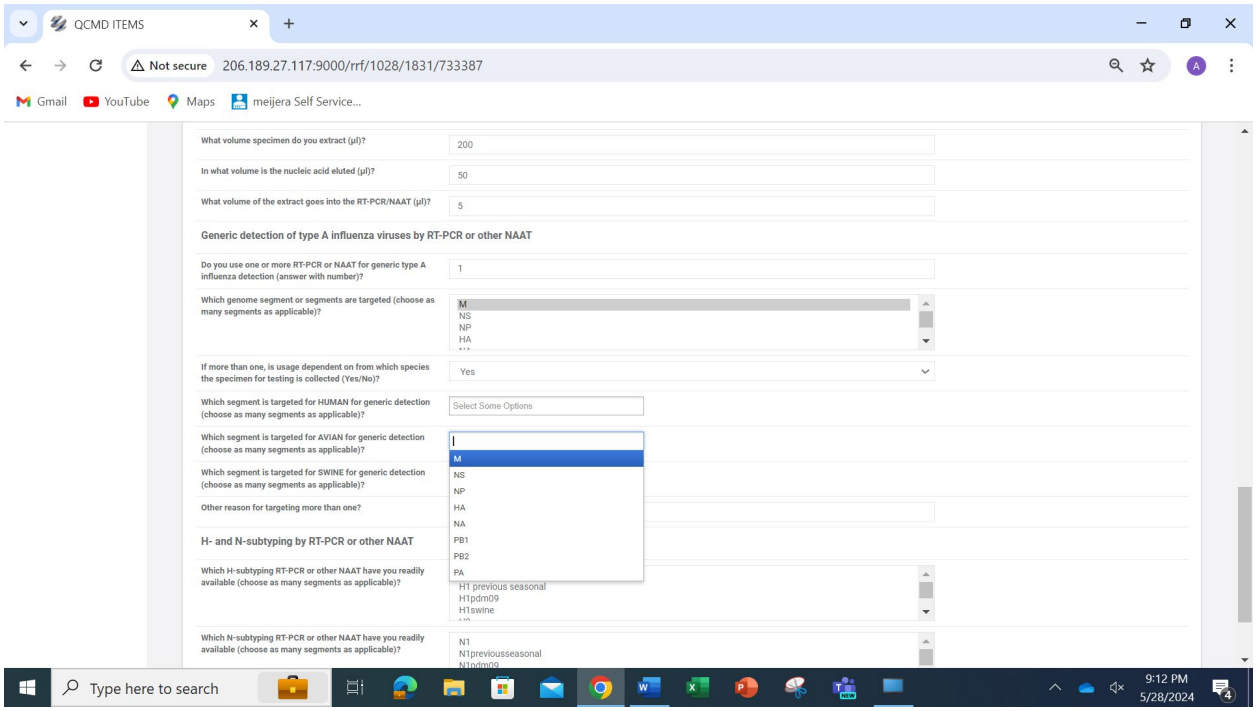
Number of targets and pick list for targets generic detection type A influenza virus NAAT. More than one can be chosen.



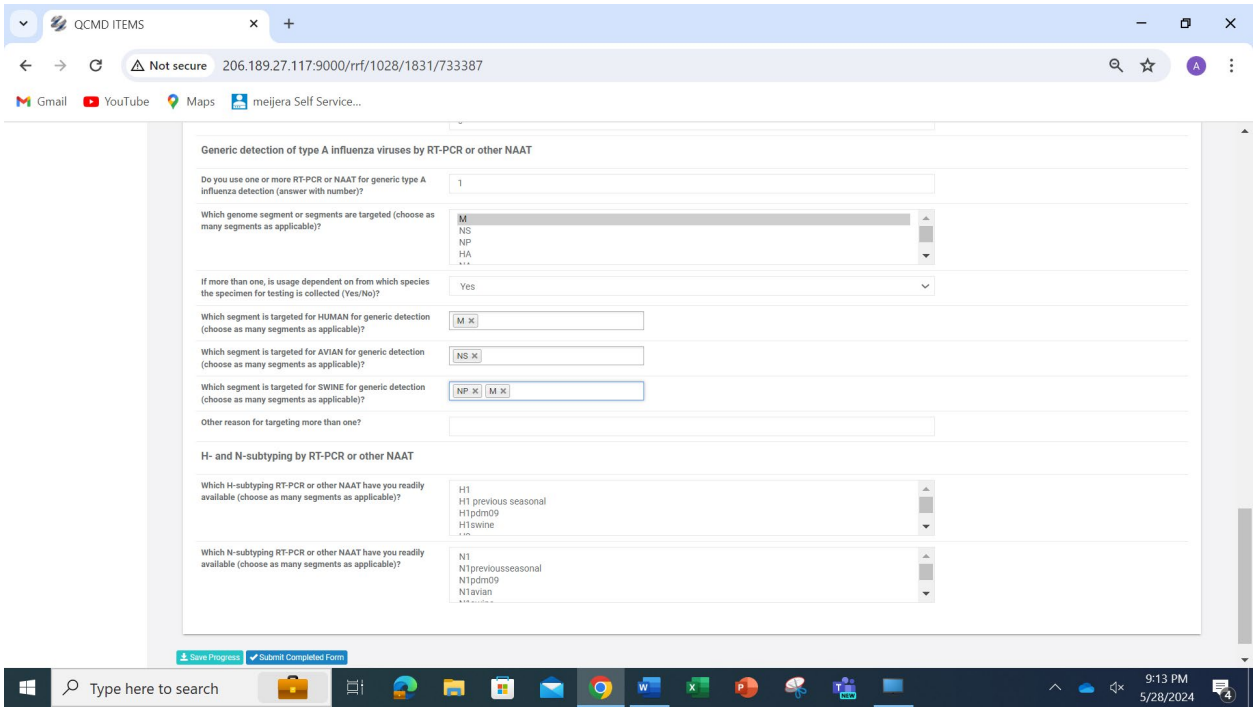
Further specification if more than one target is used for generic detection whether this specific for human, swine, and avian origin viruses.



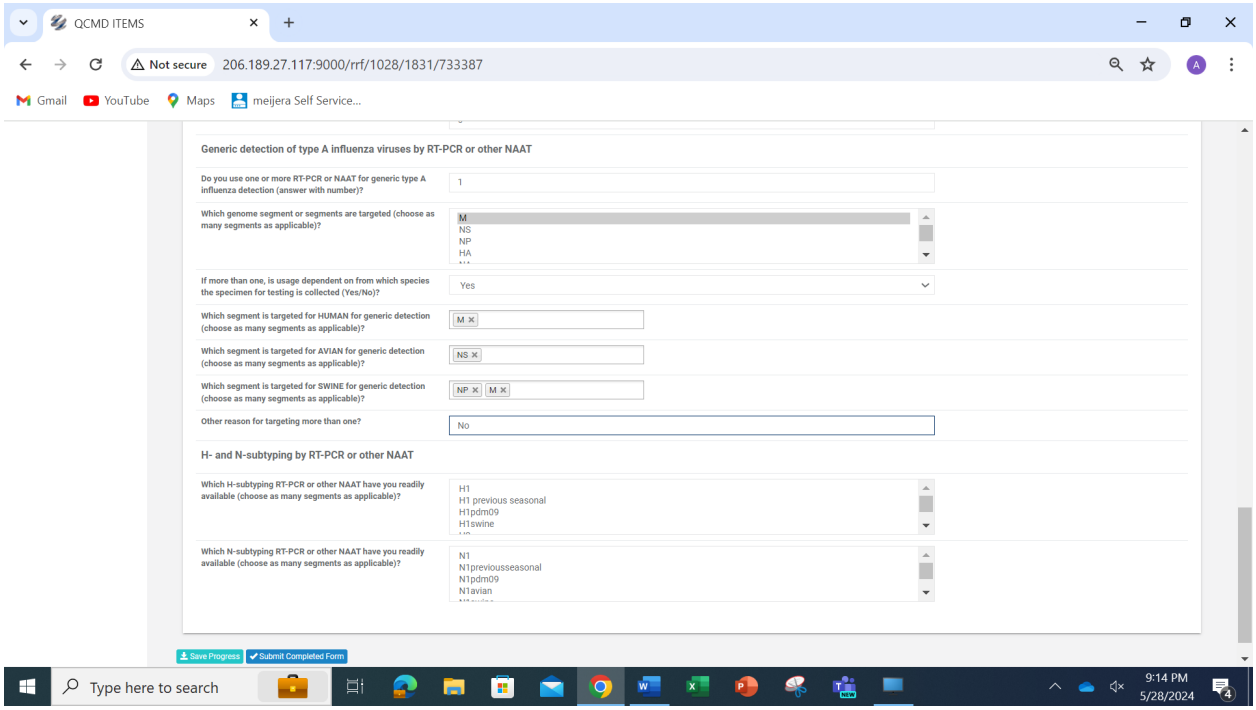
Specification of targets used for human, avian and swine influenza viruses, more than one can be chosen.



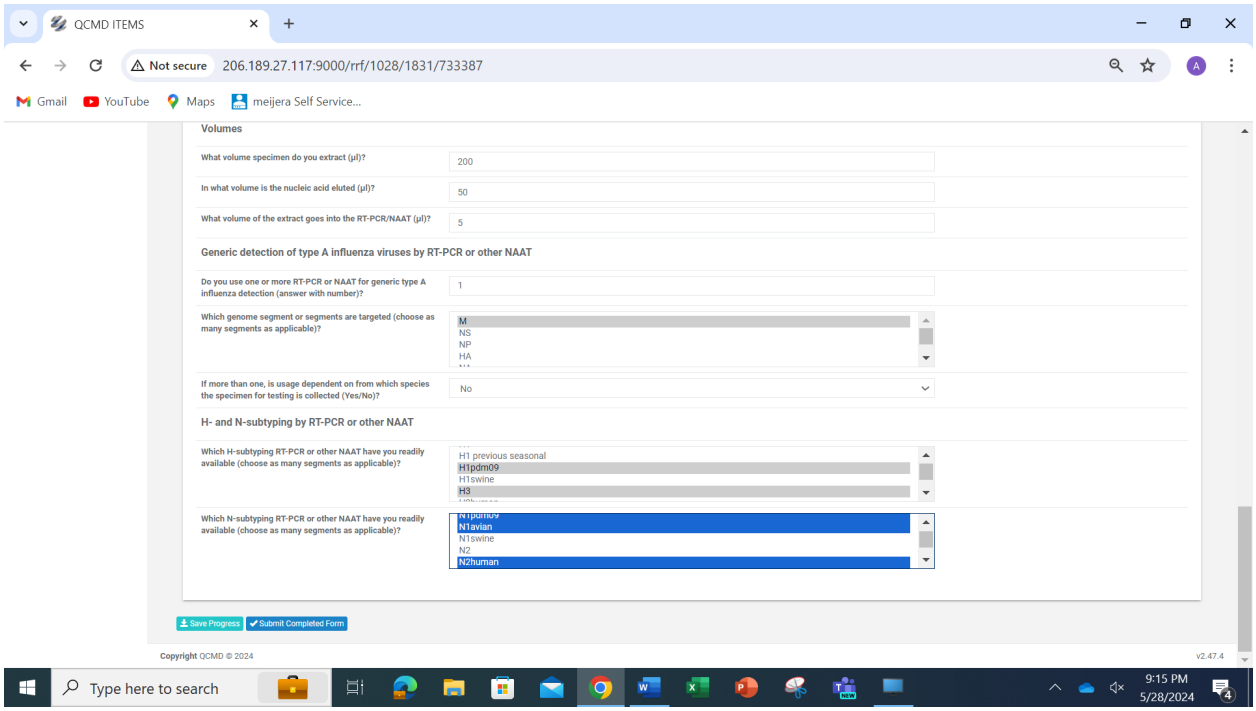
Filled in specifications, including more than one target for in this case swine influenza virus.



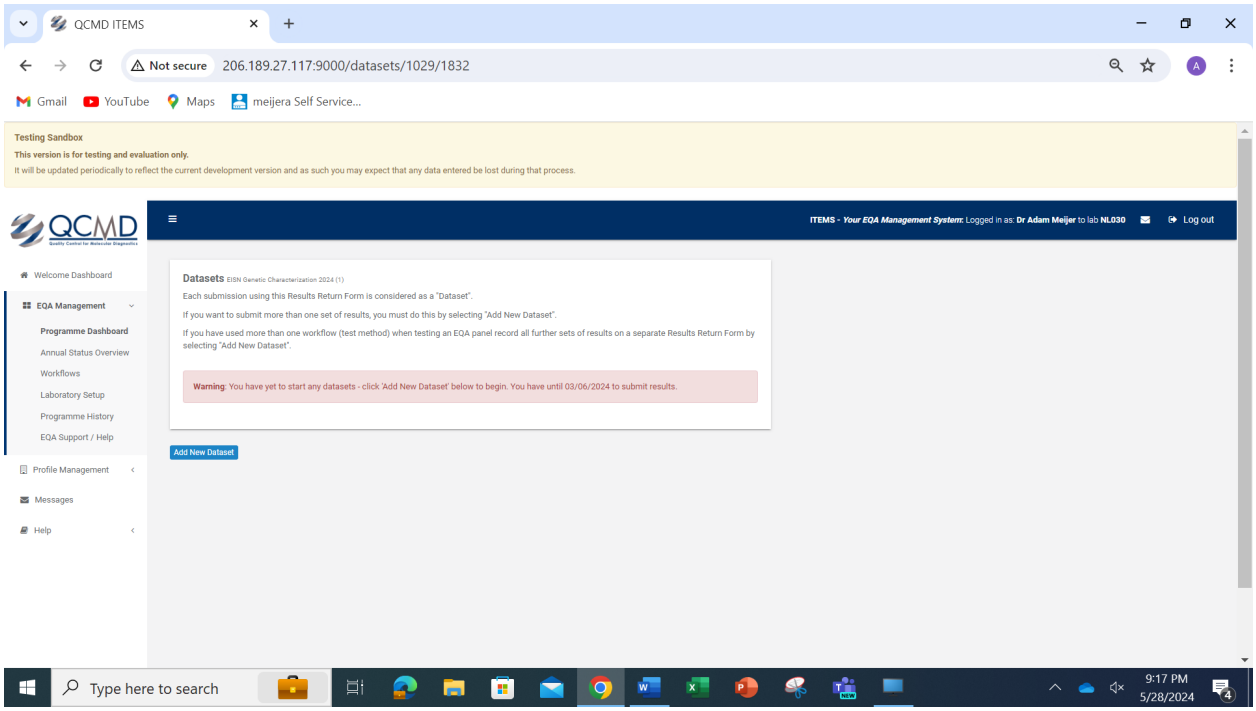
Other reason than differentiation by species for generic detection targets, free text.



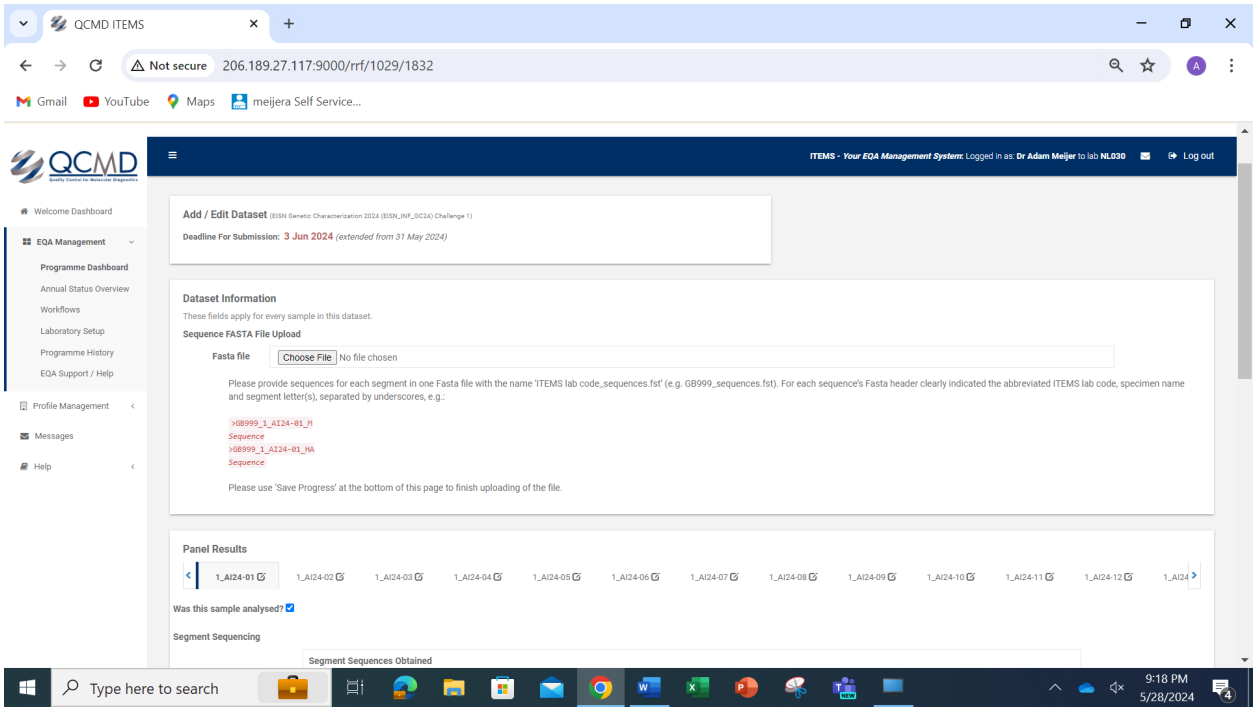
Specification of which H- and N-subtyping NAAT are readily available in the laboratory, multiple can be chosen.



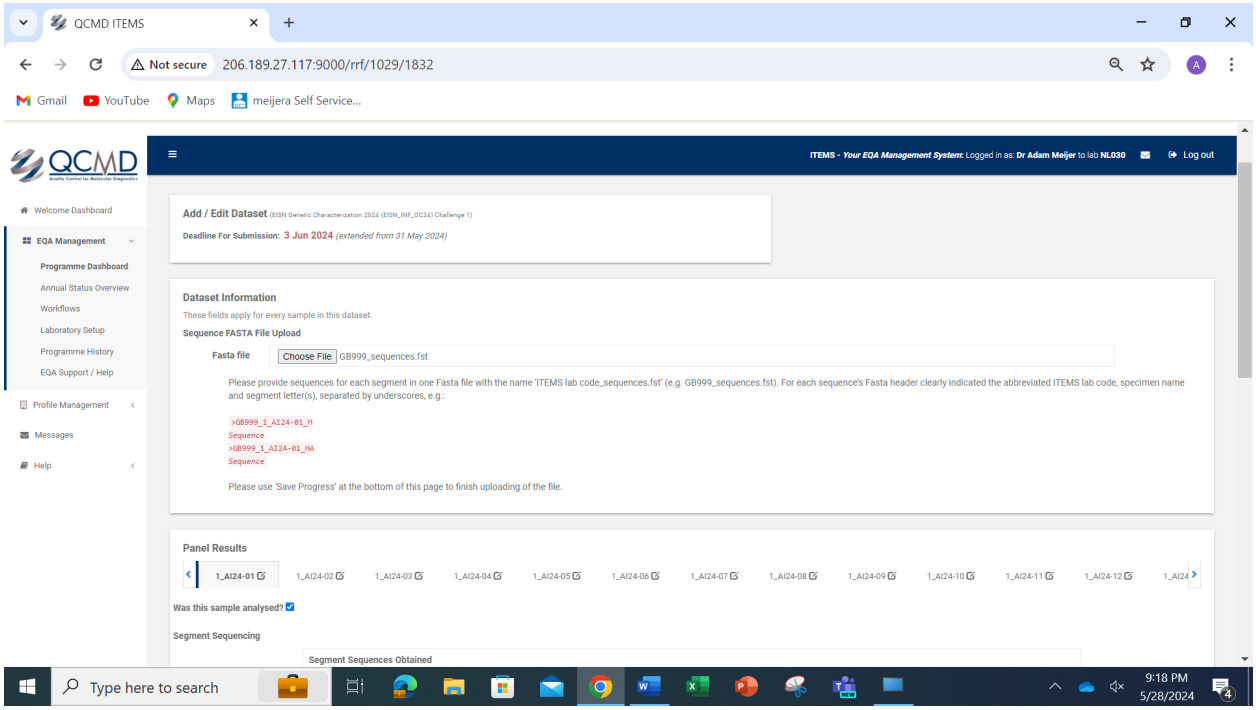
Genetic characterisation programme, Add new dataset.



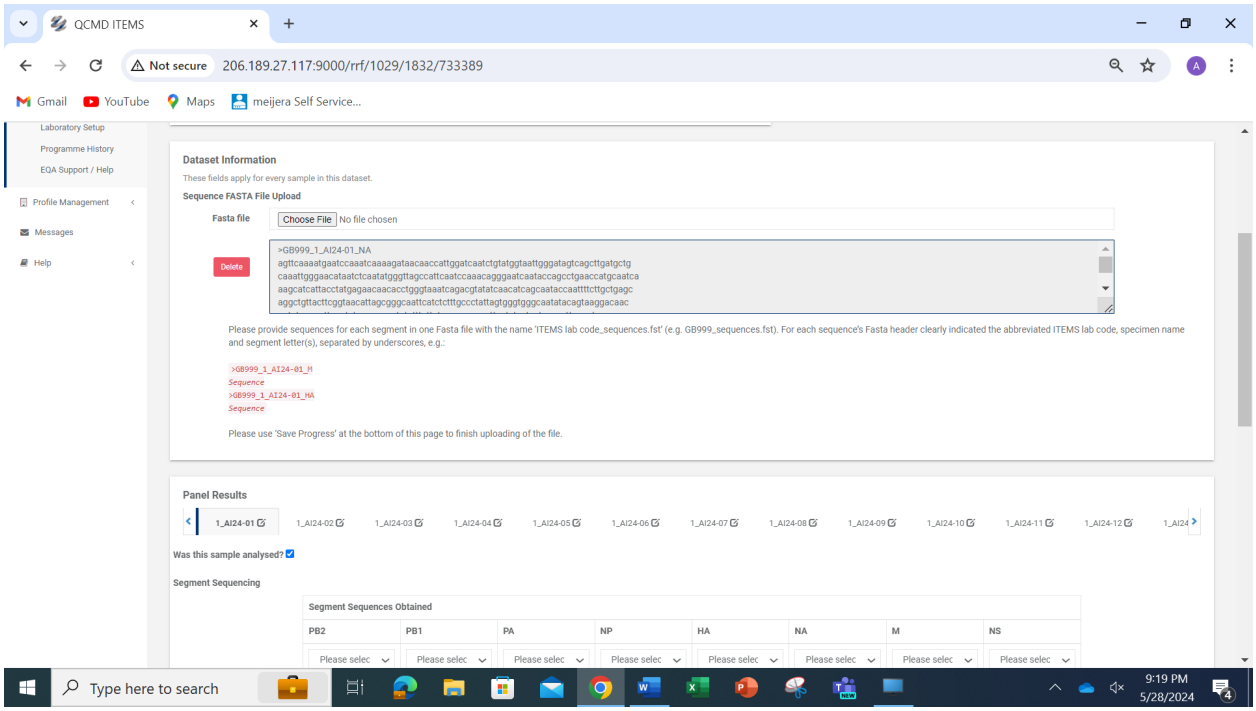
Choose file to upload all obtained sequences of all panel specimens in pre-defined format in one file.



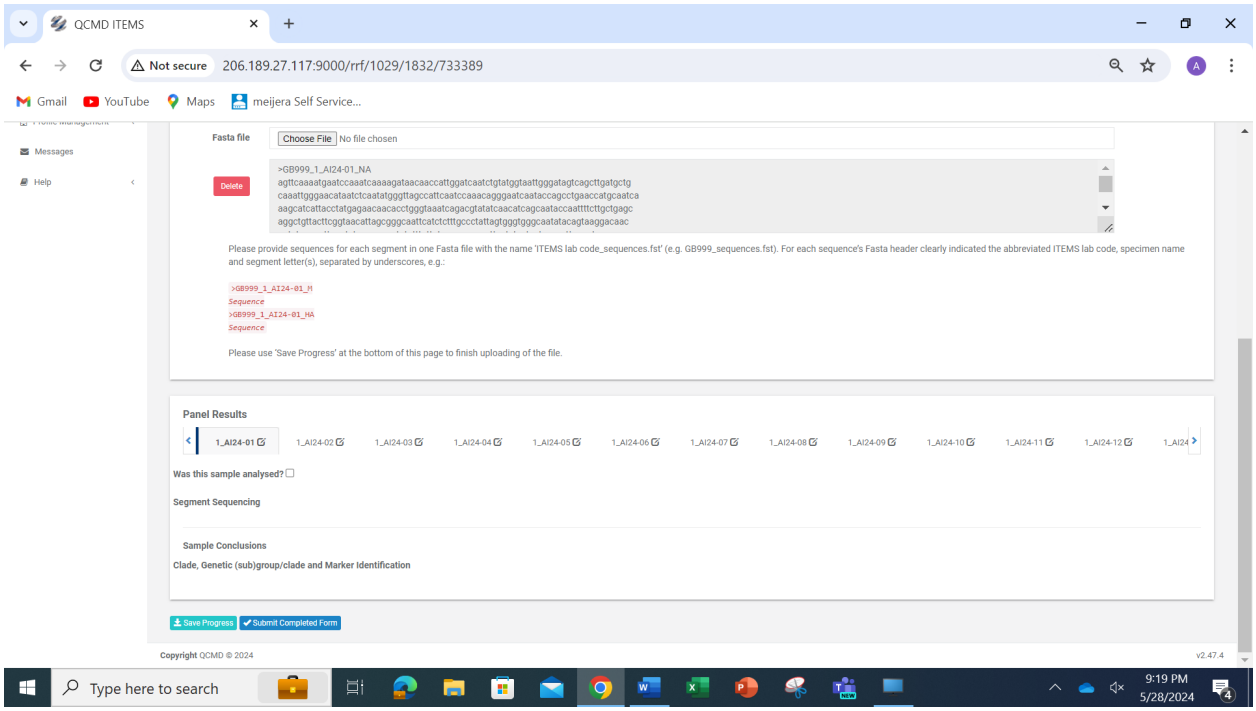
File chosen.



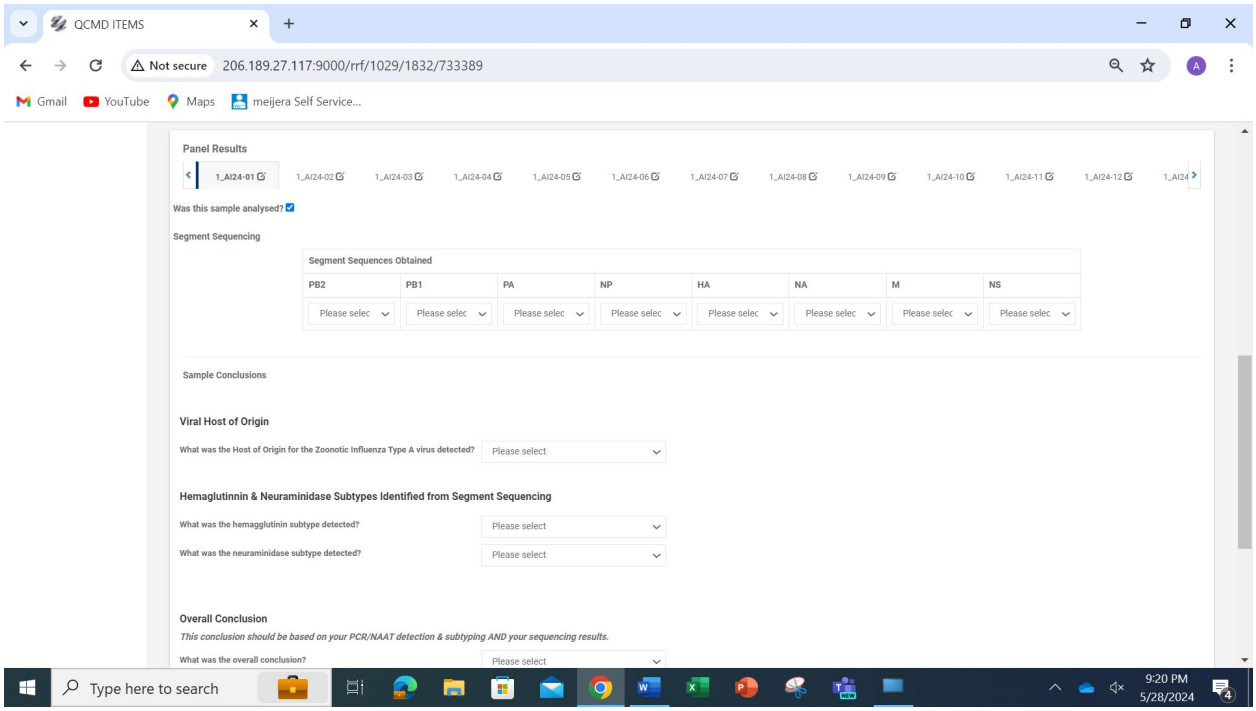
After pressing 'Safe dataset', confirmation of uploaded file is shown.



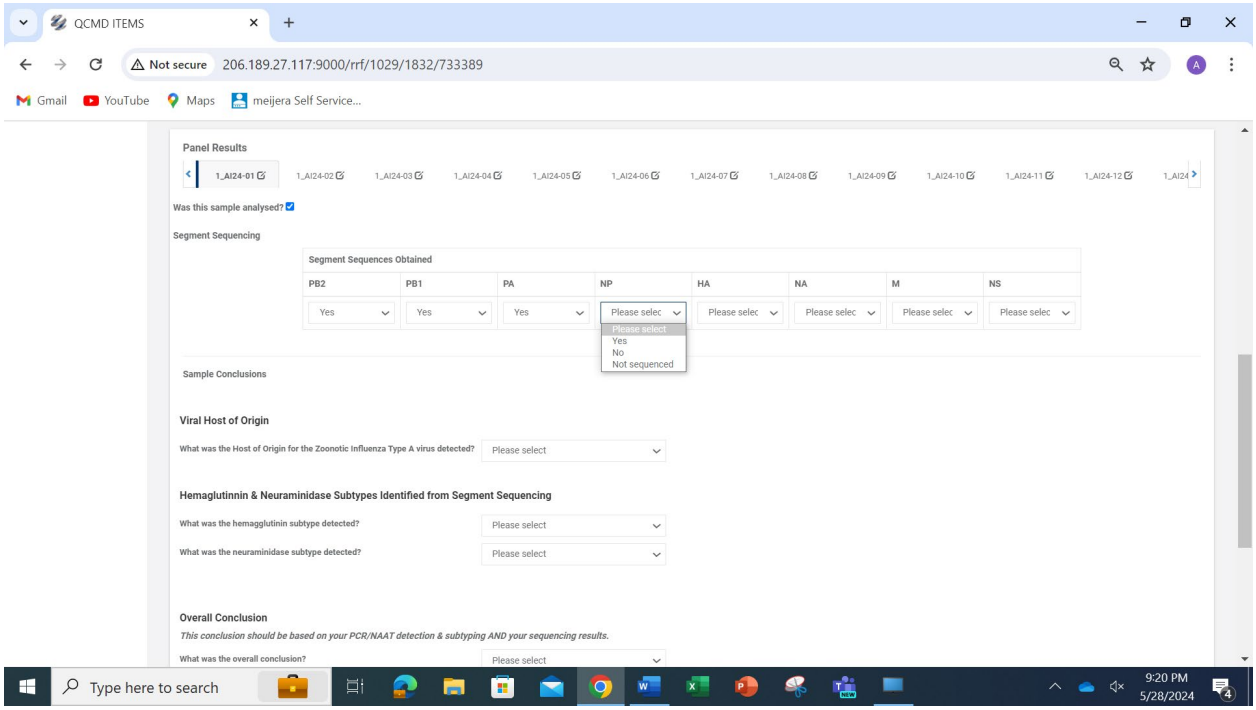
Start reporting of results per specimen in the panel.



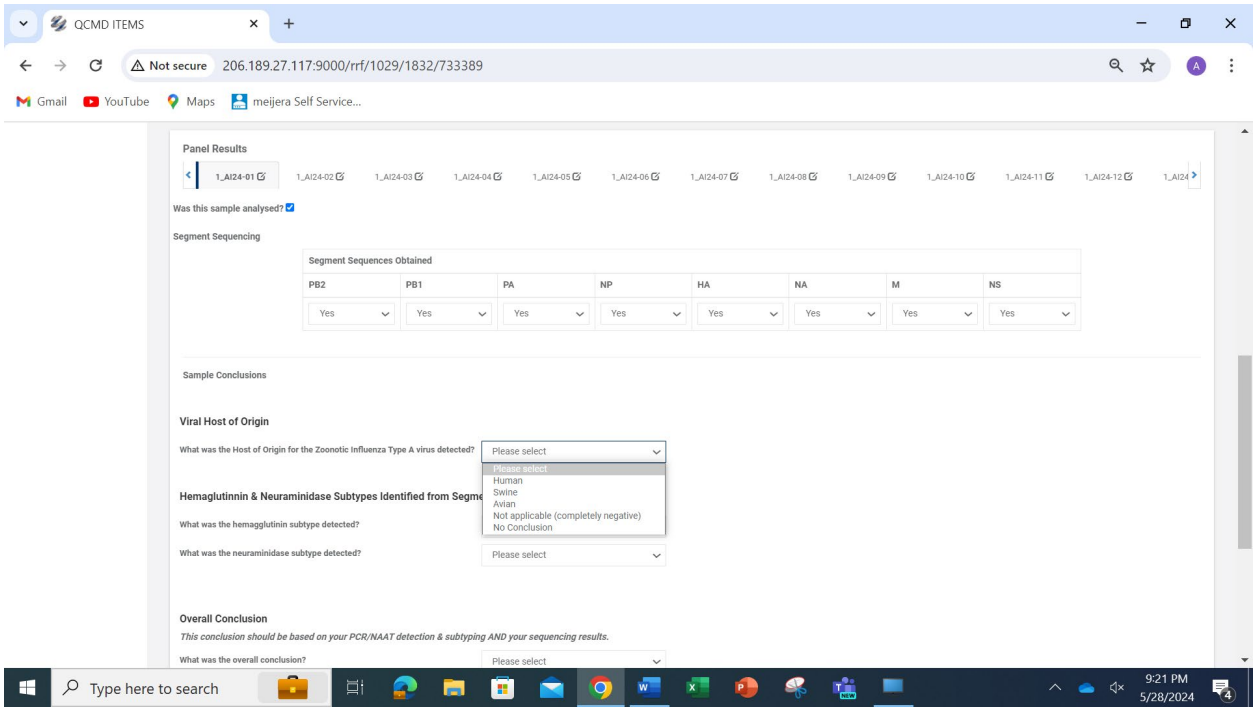
After confirmation whether this specimen is analysed, the data reporting fields are shown.



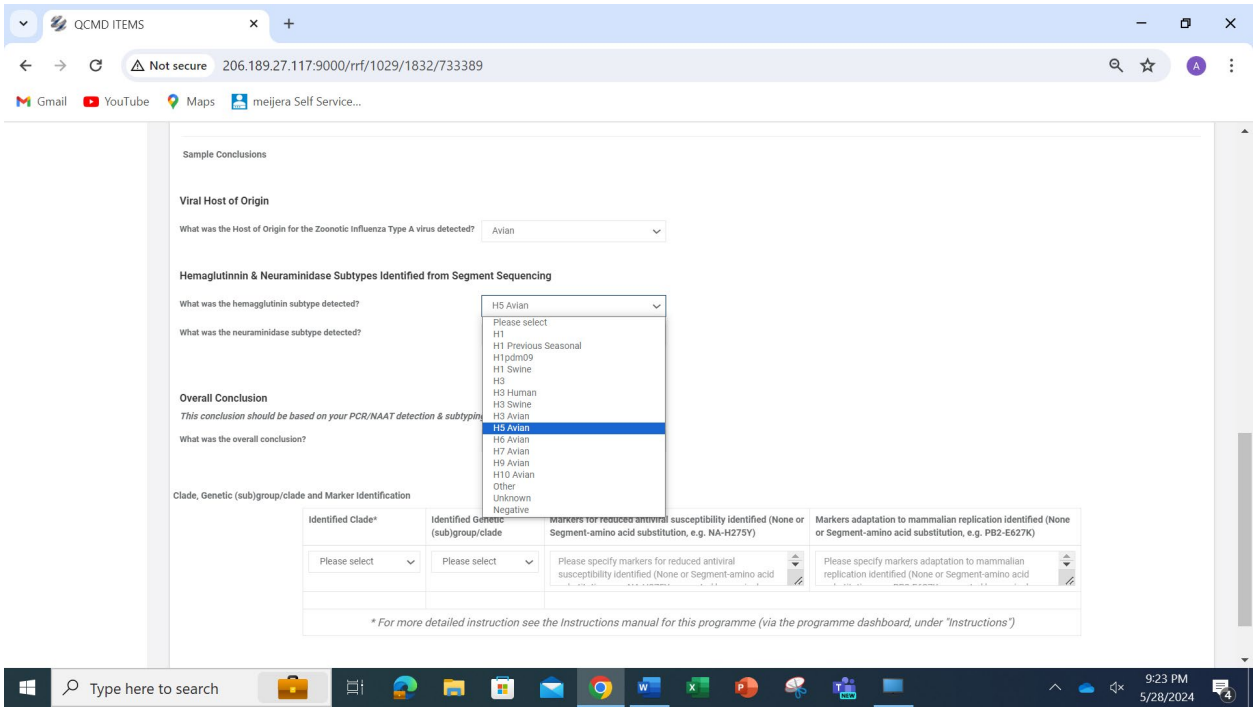
Per genome segment using a pick list is reported whether a sequence was obtained.



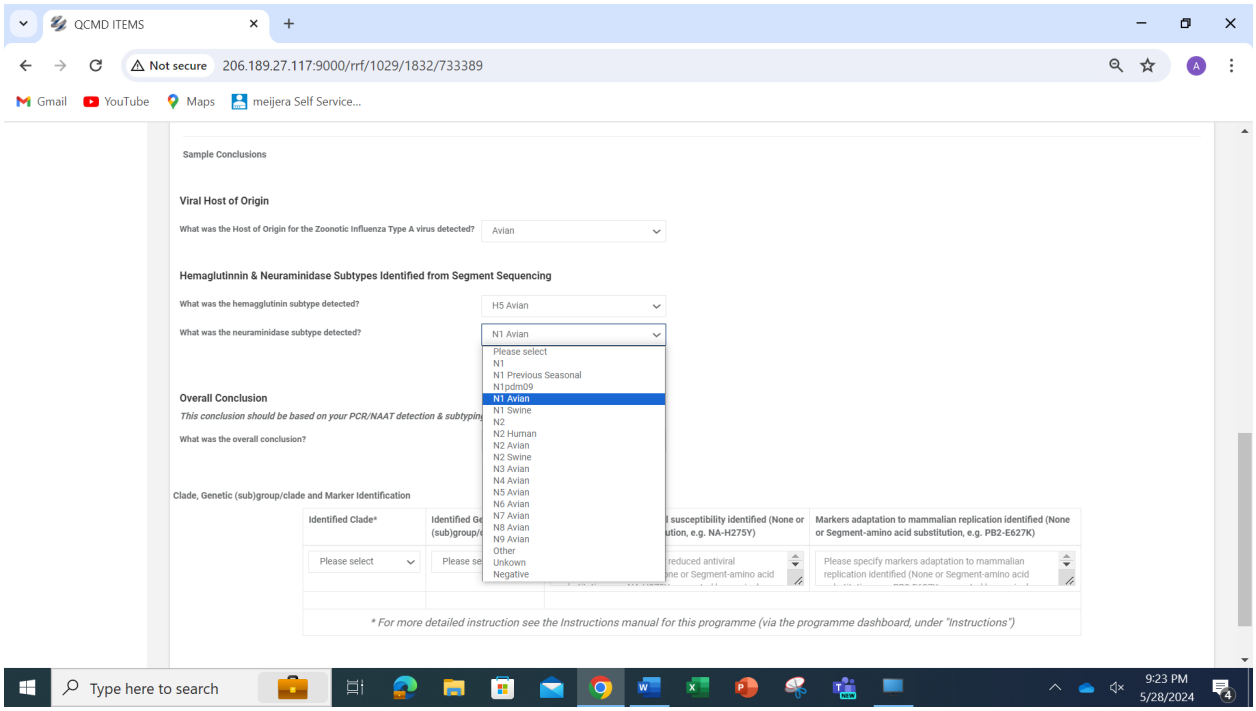
Using a pick list the 'likely host species' of the virus based on obtained sequences is reported.



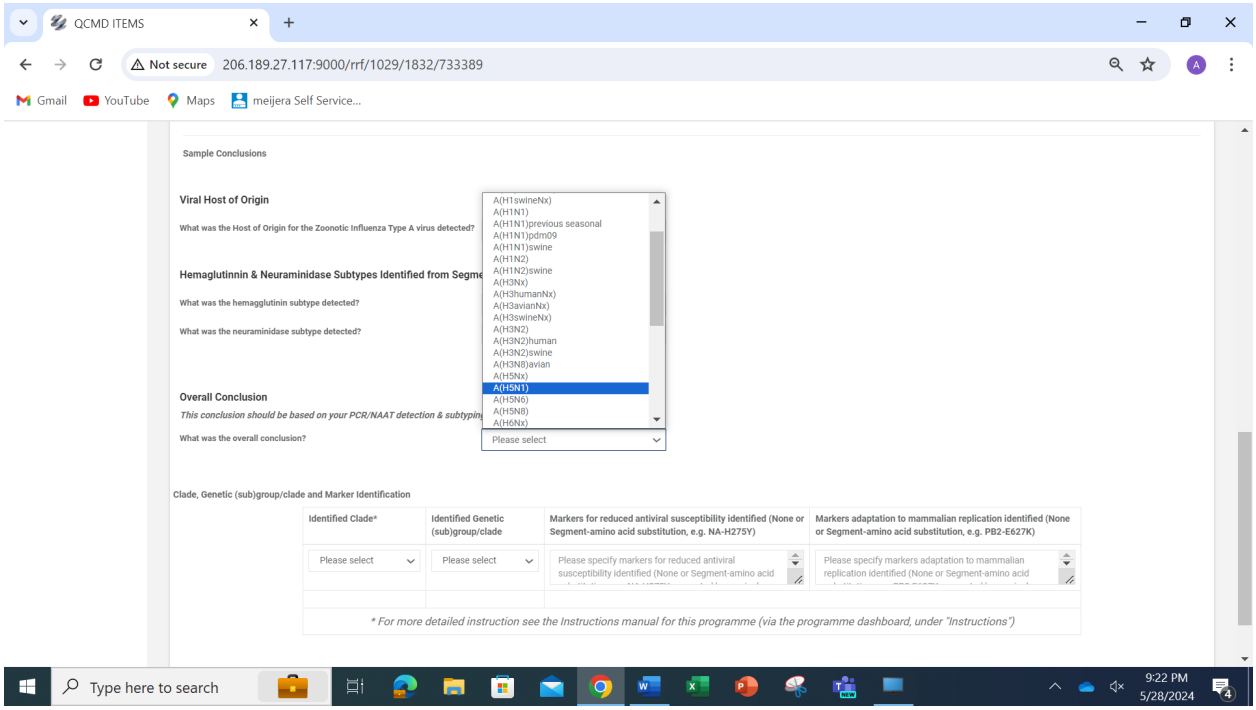
The H-subtype based on the sequencing data is reported using a pre-defined pick list.



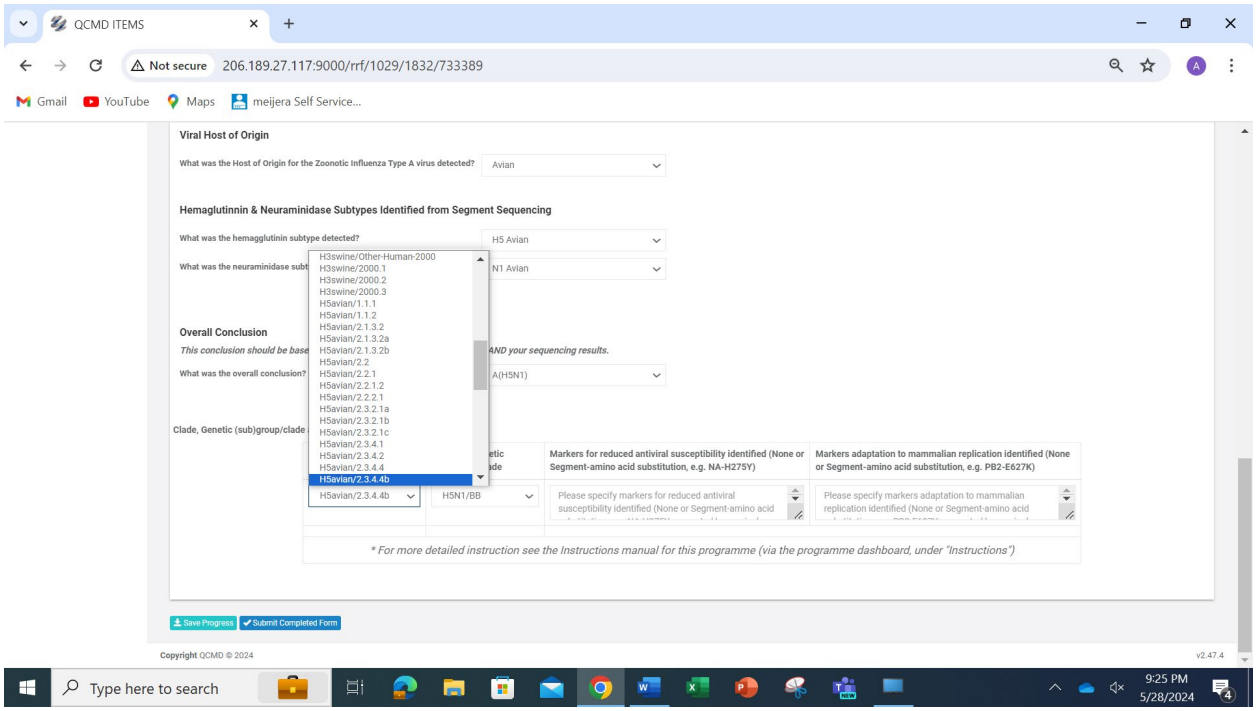
The N-subtype based on the sequencing data is reported using a pre-defined pick list.



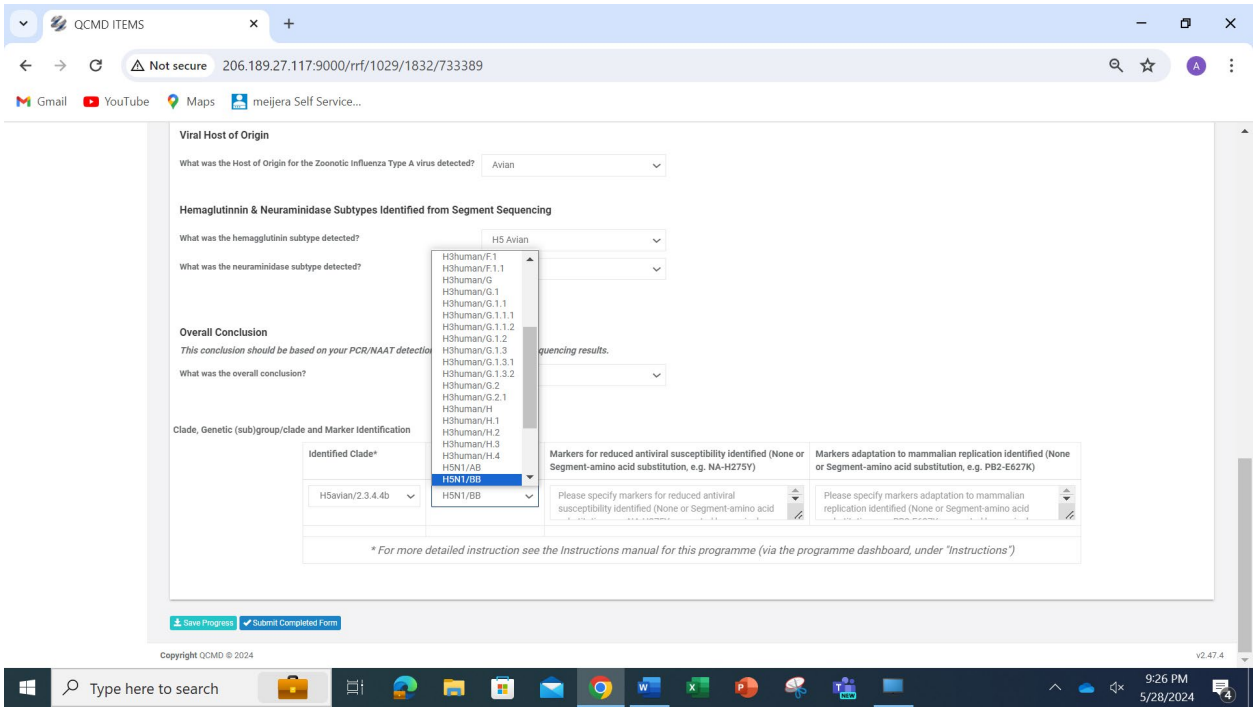
The overall conclusion based on NAAT and sequencing of the H- and N-subtype including 'likely host species' is reported using a pre-defined pick list.



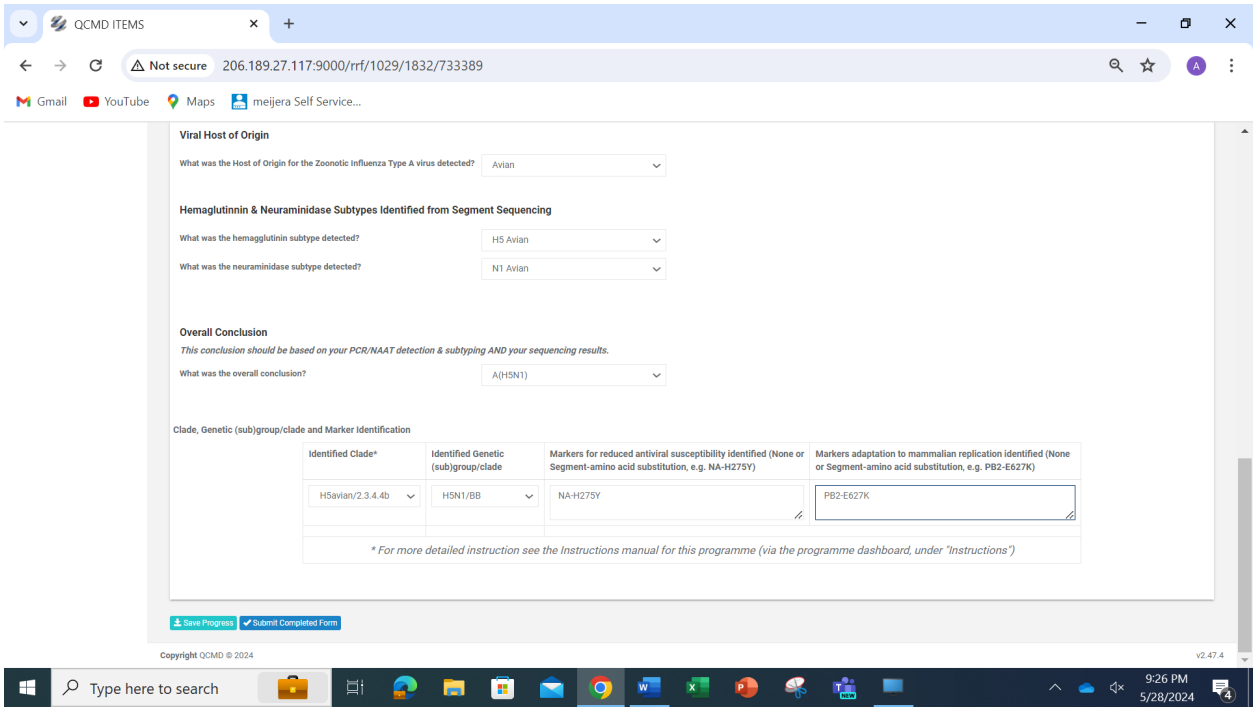
The identified clade of the detected virus is reported using a pre-defined pick list.



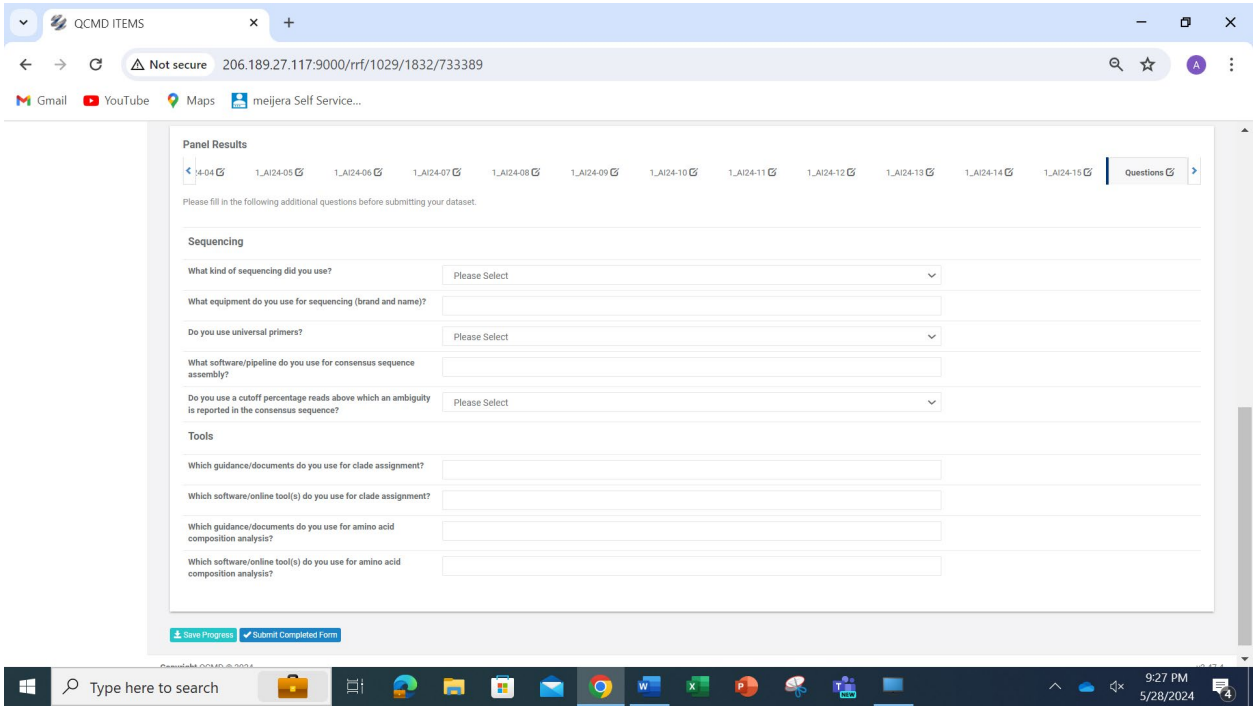
The identified subclade for human influenza viruses or the genotype for H5N1 viruses of the detected virus is reported using a pre-defined pick list.



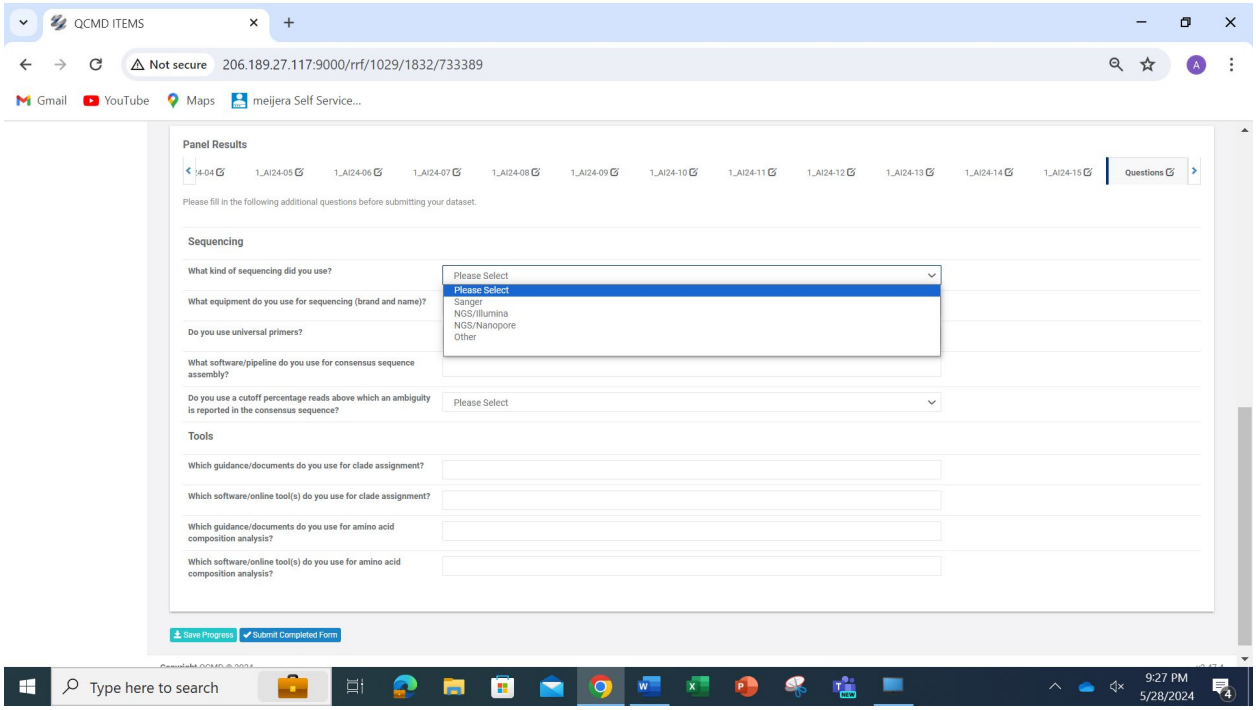
Free text fields are used for reporting of identified amino acid markers for reduced antiviral susceptibility and for mammalian adaptation.



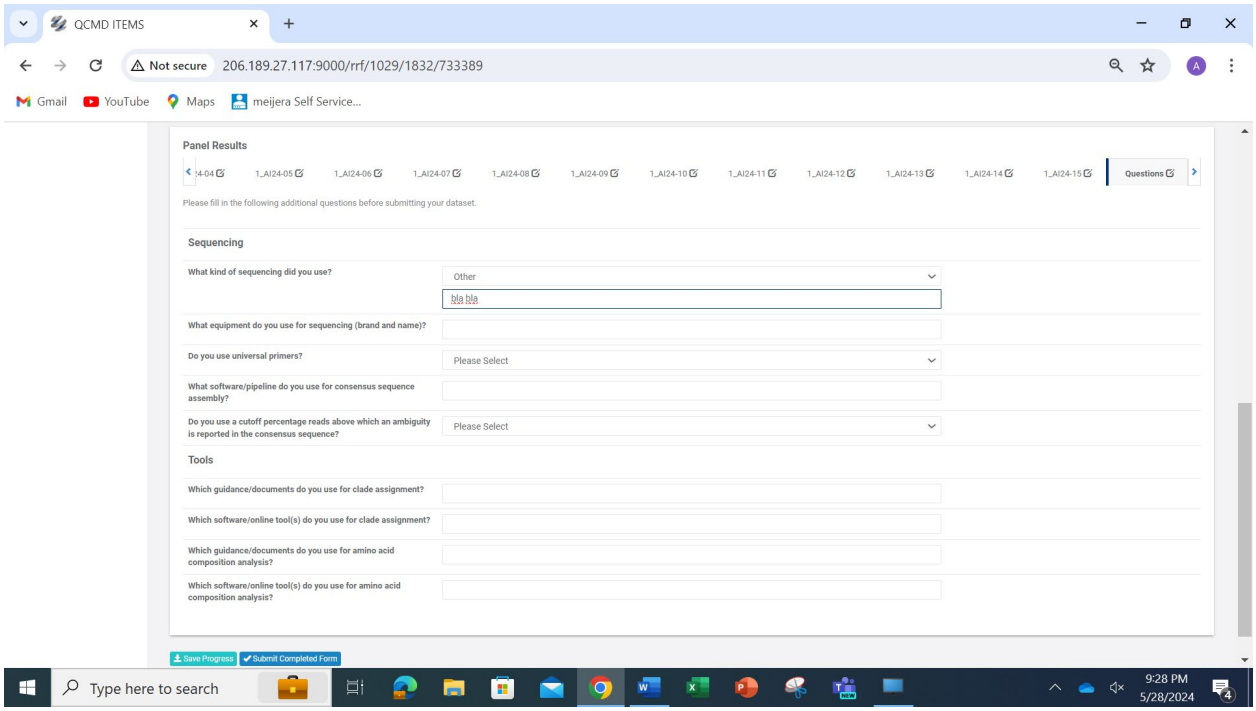
Answer questions on used techniques, where appropriate using one or more options from predefined pick lists or answering in free text.



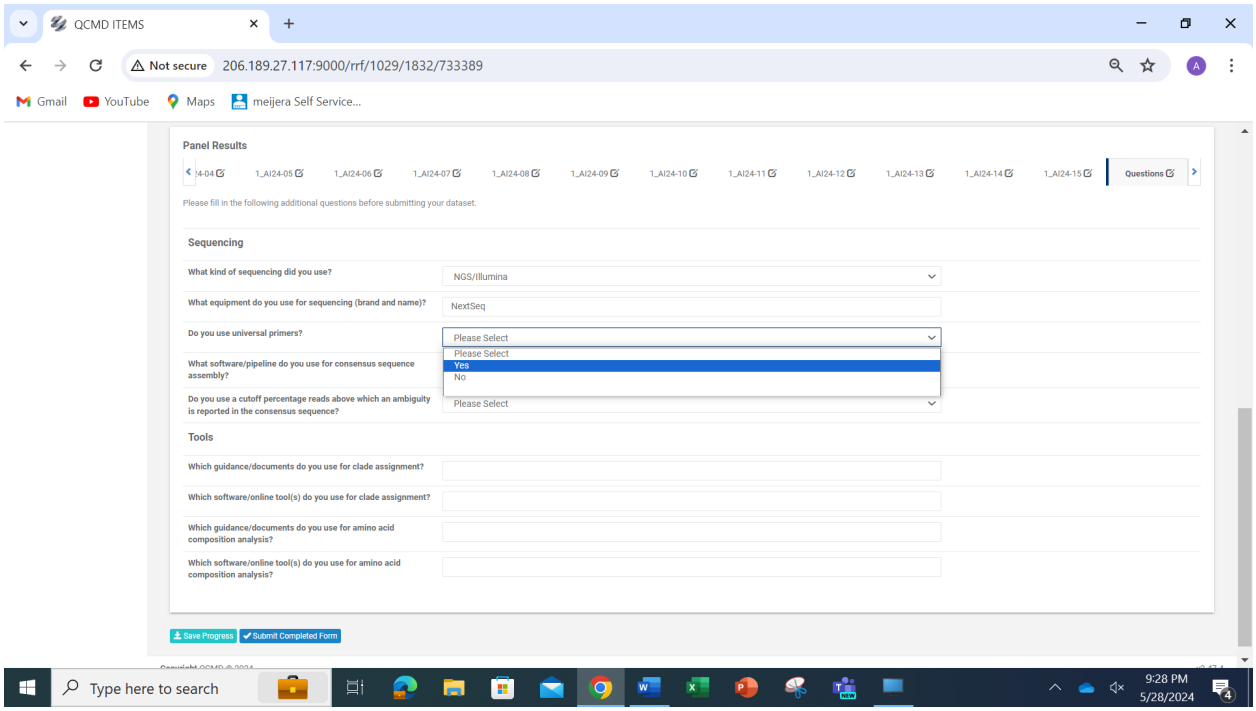
Specify the used technology for sequencing using a predefined pick list. When 'Other' is chosen a free text option is offered to specify 'Other'.



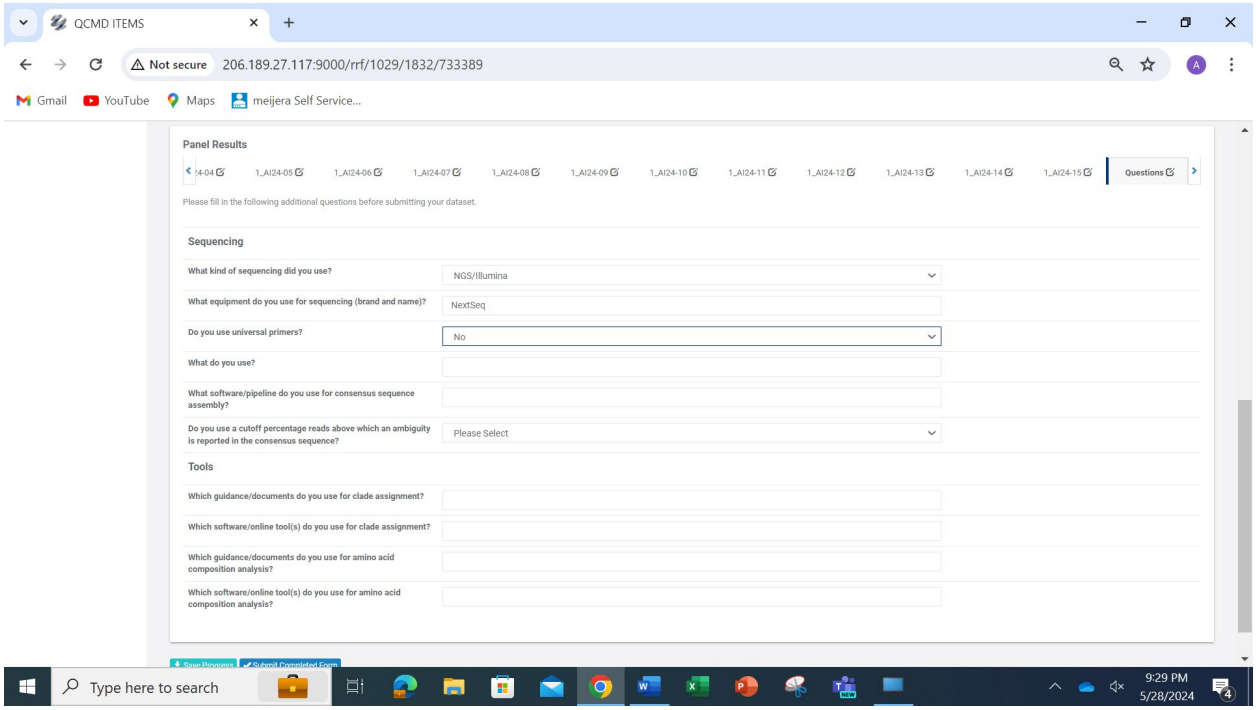
Free text option if 'Other' is chosen.



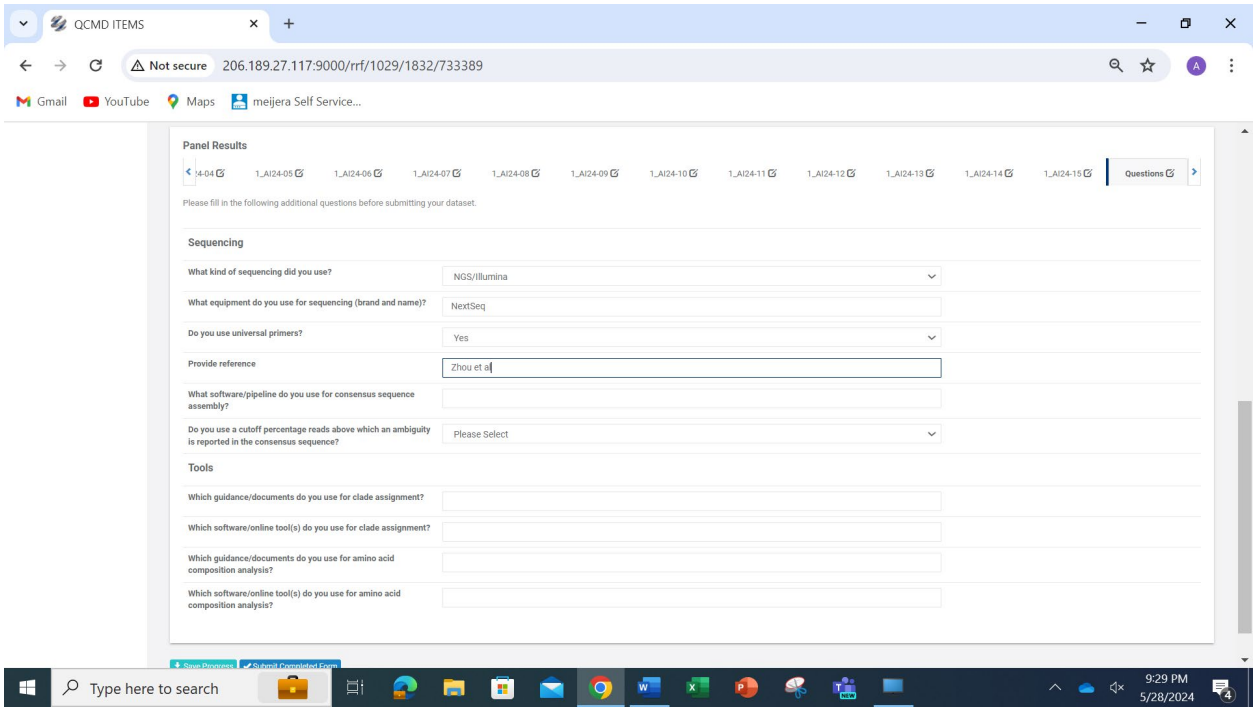
Specify used equipment using a predefined pick list and specify whether universal primers are used for amplicon-based sequencing using a predefined pick list.



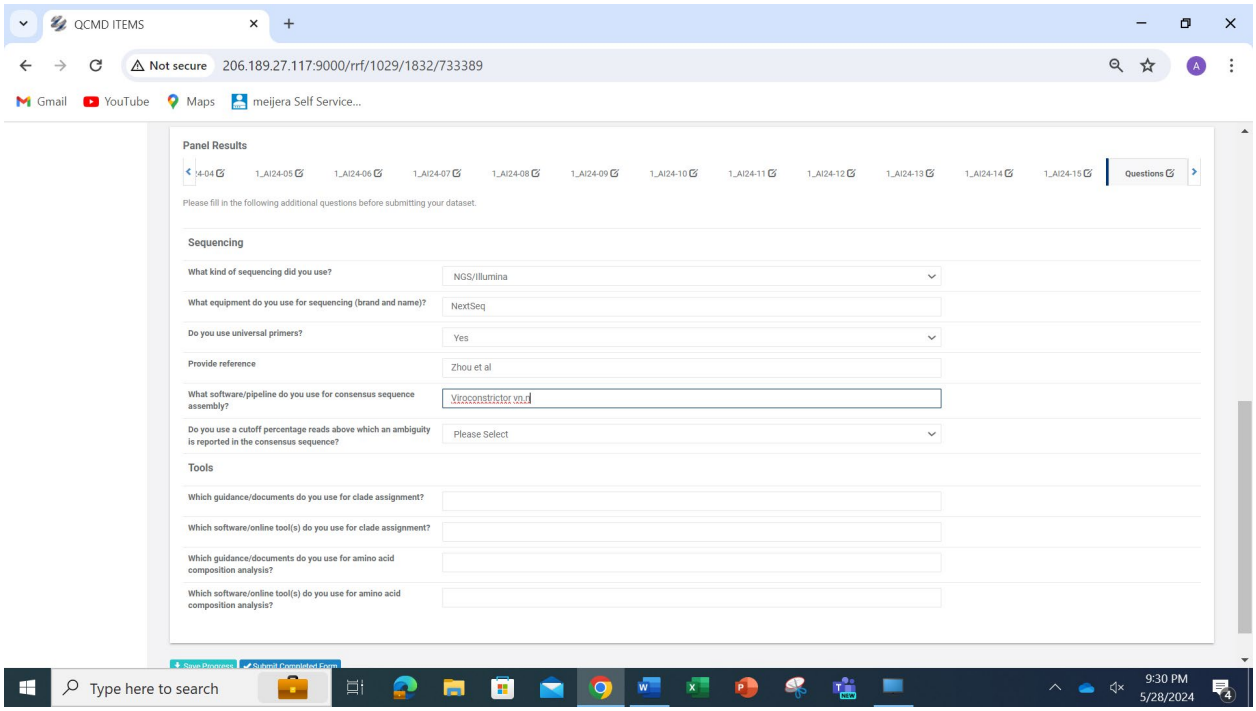
If 'No' is answered on the use of universal primers a free text option is offered to further specify what then is used.



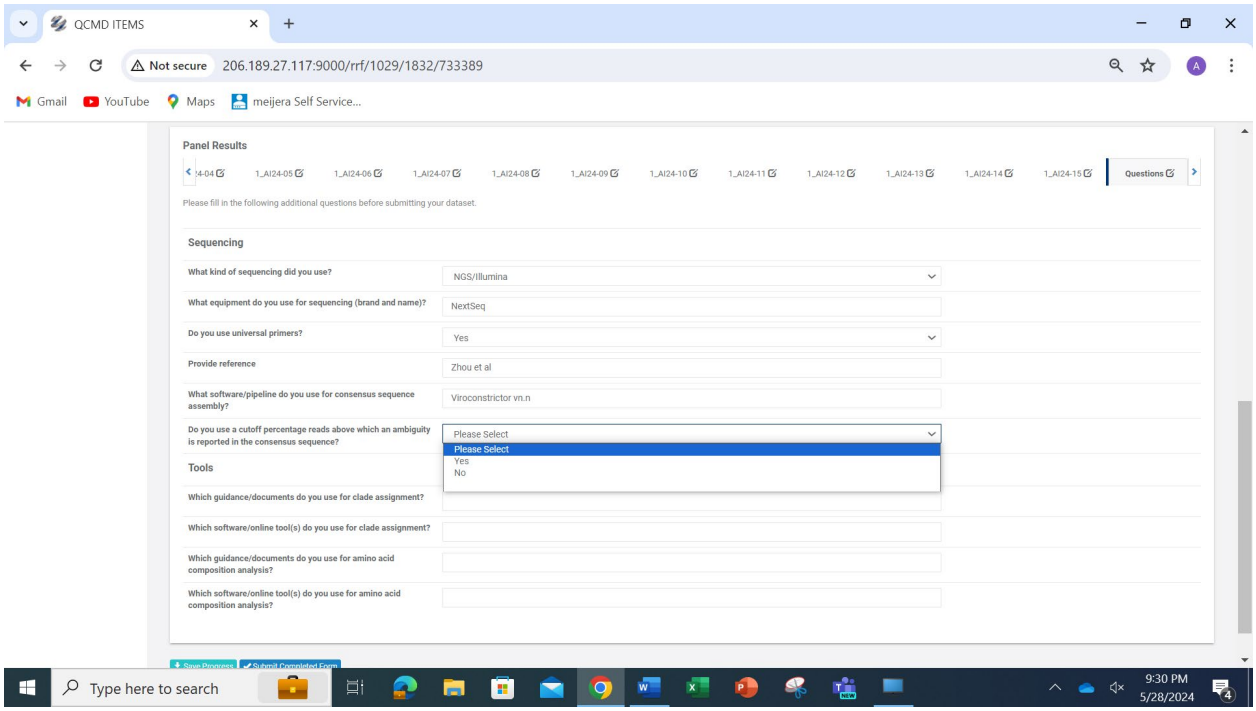
If 'Yes' is answered on the use of universal primers a free text option is offered to provide a reference for the used universal primers.



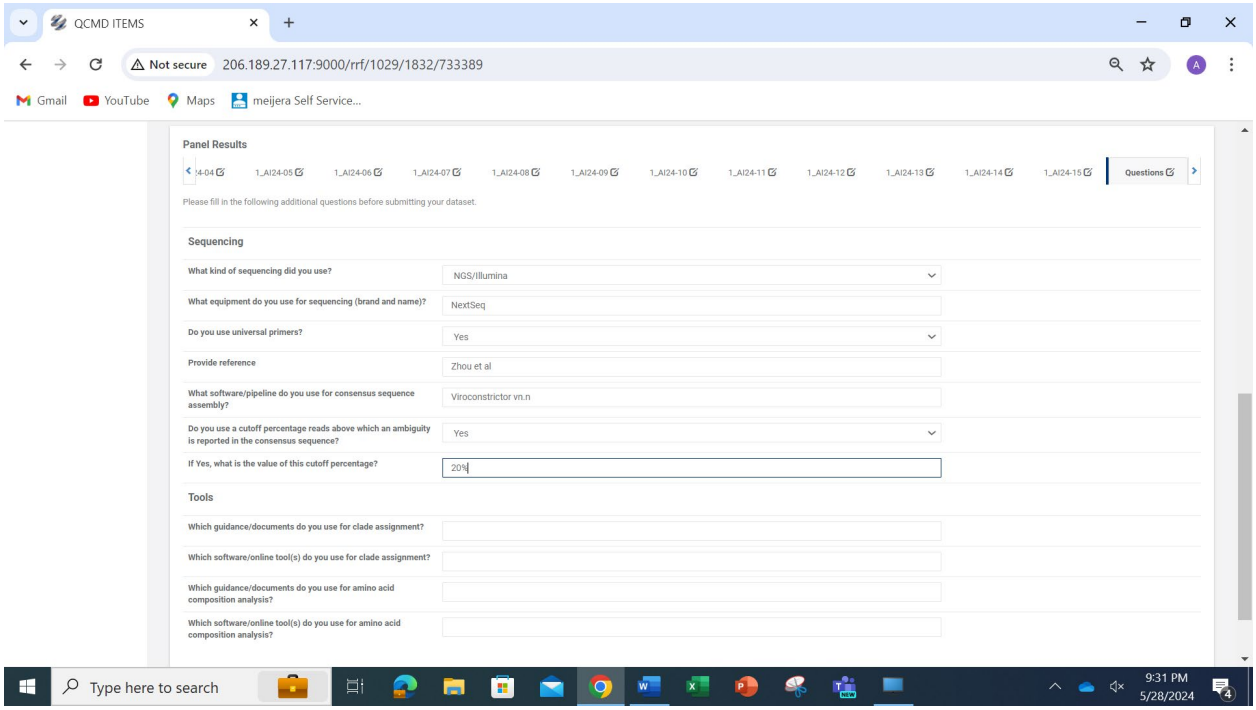
Specify which software/pipeline is used for consensus sequence assembly.



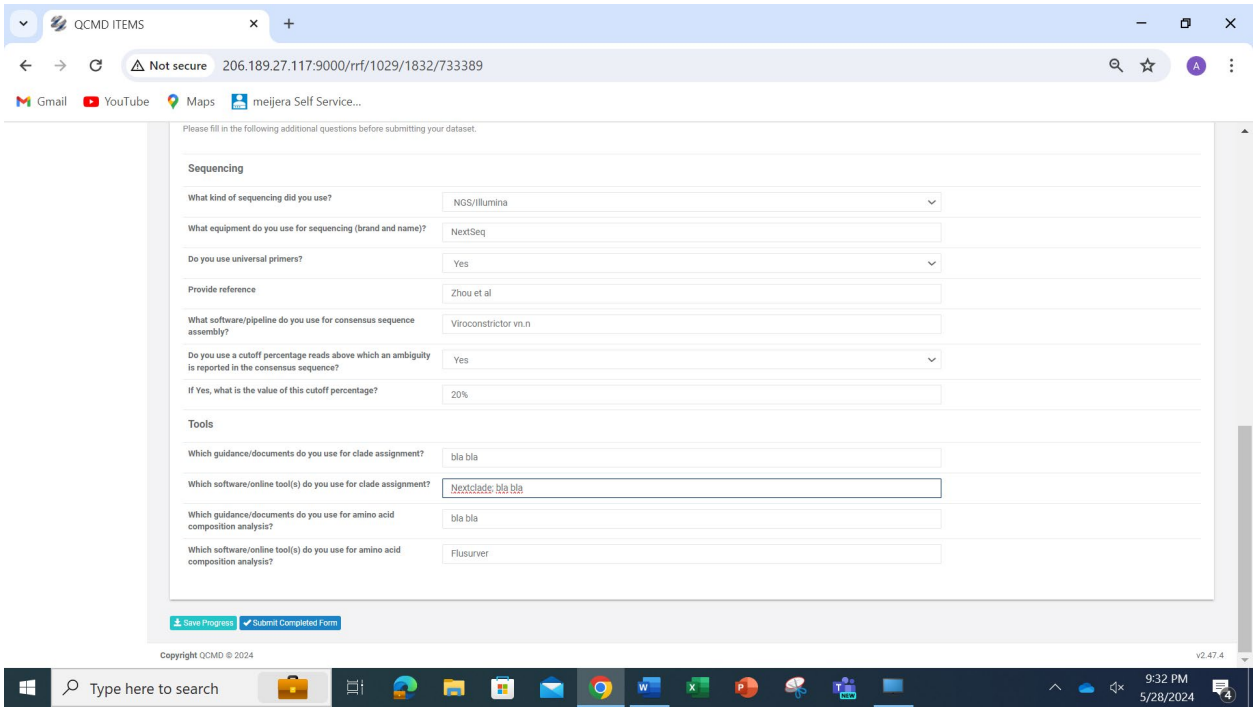
Specify whether a cutoff is used for ambiguity reporting in a consensus sequence.



If 'Yes' an option is offered to specify the used percentage.



Free text answers are offered for specifying guidance and tools used for clade, subclade and genotype assignment and for amino acid marker identification.



European Centre forDisease Prevention and Control (ECDC)

Gustav III:s Boulevard 40
16973 Solna, Sweden

Tel. +46 858 60 10 00
ECDC.info@ecdc.europa.eu

www.ecdc.europa.eu

Publications Office
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