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Synopsis

Medical microbiology laboratories in the Netherlands can detect animal type A influenza viruses well. External Quality Assessment 2023

Type A and B influenza viruses can cause severe disease in humans. The type A influenza virus has many subtypes, which also infect birds and swine. In the Netherlands, what are known as 'medical microbiology laboratories' test whether people are infected with an influenza virus. This provides insight into the amount of people infected and whether they were infected by type A or B influenza virus.

The H5 subtype of type A caused a worldwide outbreak of bird flu in 2022, the largest since 2003. The H5 subtype also infected a growing number of mammals, raising the concern about a growing possibility that this virus subtype can infect humans (zoonosis). The same concern exists about swine influenza viruses. The chance of zoonosis might increase further when avian and swine influenza viruses mix with each other. A zoonosis like that could potentially lead to a pandemic. That is why an external quality assessment (EQA) has been carried out to investigate whether the laboratories can detect bird and swine influenza viruses. Fifty laboratories in the Netherlands, Aruba, Bonaire and Curaçao participated.

All laboratories have at least one routine test with which they were able to identify animal influenza viruses in the EQA as *a* type A influenza virus. A number of these tests identified the subtype of the human viruses, but most did not identify the subtype of the avian and swine viruses. Using additional tests, four laboratories identified the H5 subtype of avian influenza viruses. A number of laboratories reported that they are able to perform non-routine tests to identify the subtype.

It is important that medical doctors are aware of the possibility of humans getting infected by an animal influenza virus. If they suspect such an infection in a patient with influenza-like symptoms, further investigation is needed. That is also the case if a laboratory detects a type A influenza virus in a patient that is not of a human subtype. This investigation is conducted by the National Influenza Centre (RIVM and Erasmus MC), which can discriminate between all human and animal influenza viruses.

Keywords: swine flu, avian flu, H5N1, medical microbiology laboratory, National Influenza Centre, molecular diagnostic test, subtyping, influenza

Publiekssamenvatting

Medisch microbiologische laboratoria in Nederland kunnen dierlijke type A griepvirussen goed aantonen. Kwaliteitscontrole 2023

Typen A en B griepvirussen kunnen ernstige ziekte bij mensen veroorzaken. Het type A griepvirus heeft veel subtypen en komt ook bij vogels en varkens voor. In Nederland testen de zogeheten 'medisch microbiologische laboratoria' of mensen besmet zijn met een griepvirus. Dat geeft inzicht in hoeveel mensen besmet zijn met een griepvirus en of het virus van type A of B is.

Het H5 subtype van type A veroorzaakte wereldwijd een vogelgriep uitbraak in 2022, de grootste sinds 2003. Ook raken steeds meer zoogdieren door subtype H5 besmet. De zorg bestaat dat hierdoor de kans groter wordt dat dit virus ook mensen kan gaan besmetten (zoönose). Dat geldt ook voor varkensgriepvirussen. De kans dat mensen besmet raken wordt nog groter als vogel- en varkensgriepvirussen zich met elkaar vermengen. Zo'n zoönose zou een nieuwe pandemie kunnen veroorzaken. Daarom is met een kwaliteitscontrole onderzocht of de laboratoria vogel- en varkensgriepvirussen kunnen opsporen. Vijftig laboratoria in Nederland, Aruba, Bonaire en Curaçao hebben deelgenomen.

Alle laboratoria hebben minimaal één routinetest waarmee ze van de dierlijke griepvirussen in de kwaliteitscontrole konden aantonen dat het om *een* type A griepvirus gaat. Een aantal van deze testen herkende het subtype van menselijke virussen, maar de meeste testen herkenden het subtype van de vogel en varkens virussen niet. Met extra testen herkenden vier laboratoria het H5 subtype van vogelgriepvirussen. Een aantal laboratoria gaf aan speciale, niet-routine testen in te kunnen zetten om het subtype te herkennen.

Het is belangrijk dat artsen zich bewust zijn van een mogelijke besmetting bij mensen met een dierlijk griepvirus. Als artsen zo'n besmetting vermoeden bij een patiënt met griepachtige klachten is verder onderzoek nodig. Dat is ook het geval als een laboratorium bij een patiënt een type A griepvirus aantoont dat geen menselijk subtype is. Dat onderzoek gebeurt bij het Nationaal Influenza Centrum (het RIVM en Erasmus MC), dat alle menselijke en dierlijke griepvirussen kan onderscheiden.

Kernwoorden: varkensgriep, vogelgriep, H5N1, medisch microbiologisch laboratorium, Nationaal Influenza Centrum, moleculair diagnostische test, subtypering, griep

Content

Summary

Background

During the global outbreak of highly pathogenic H5N1 (clade 2.3.4.4b) type A influenza virus in over one hundred million wild birds and poultry in 2022 and 2023, an expanding range of mammals has been found infected. A total of eight infected people has also been reported, most after direct contact with affected poultry. This amplified the existing concern about the enzootic circulation of the H5N1 influenza virus and transmission to humans. Therefore, the need for correct detection and identification of animal influenza A viruses increased. It is important that the influenza molecular diagnostic tests routinely used in medical microbiology laboratories are able to detect animal influenza viruses. After a positive generic test for influenza A virus and reported animal exposure in the clinical history of the patient, rapid subtyping at the National Influenza Centre (NIC) can be requested to identify a patient with animal influenza virus infection, in line with the International Health Regulations of the World Health Organization (WHO). Most commercial Nucleic Acid Amplification Tests (NAATs) for generic detection of influenza A virus have been validated by the manufacturer for animal influenza A virus detection. However, in many cases this validation is not frequently updated for recently circulating or emerging viruses.

Objective

As requested by the Ministry of Health, Welfare and Sport, the primary aim of the current External Quality Assessment (EQA) was to investigate whether the influenza molecular diagnostic tests routinely used in the medical microbiology laboratories in the Netherlands are capable of detecting avian influenza viruses. Due to concerns about the transmission of swine influenza viruses to humans and the possible mixing of swine influenza viruses and avian influenza viruses, the NIC has extended this aim to include (potentially) zoonotic swine influenza viruses. Apart from detecting animal influenza A viruses, the secondary aim of the EQA was to investigate if the laboratories were able to identify the subtype of the detected influenza A viruses, and if so, if they could correctly identify at least the H-subtype.

Material and Methods

To assess the performance of the molecular diagnostic tests, the RIVM produced a panel with various inactivated influenza A viruses; human A(H1N1)pdm09 and A(H3N2), swine A(H1N1)v, A(H1N2)v and A(H3N2) and avian A(H5N1), A(H5N6) and A(H7N2). In the second week of March 2023 this panel was distributed to the 50 medical microbiology laboratories that signed up for this EQA. The laboratories were asked to run their routine diagnostic molecular tests for influenza virus detection on the panel specimens and to report their outcomes using Microsoft Forms. Every reported test was seen as a separate workflow and labs could report multiple workflows. The resulting data was collected in an Excel file and further analysis was performed using Excel and Graphpad Prism.

Results

A total of 120 workflows were reported by 50 different laboratories. Nearly all tests detected the influenza A viruses, including the animal influenza viruses. The exceptions were three commercial kits, in which there seemed to be an issue with the sensitivity for one or two animal viruses, especially the A(H1N1)v zoonotic swine influenza virus, thus generating a negative influenza A virus detection result. This A(H1N1)v containing specimen also produced the highest median Cq value across all viruses in the panel, indicating the relatively low viral load, but was still detectable by most workflows. However, these three commercial kits were capable of detecting two other animal influenza virus containing specimens with a similarly low or even lower viral load. Only 4/50 laboratories (8%) have a test operational to specifically identify avian influenza H5. A higher number of laboratories (20/50; 40%), with 24 workflows, have tests operational that can H-subtype human H3 and/or H1pdm09 viruses. With the exception of two, all the workflows with subtyping capacity identified human H1pdm09, and all workflows identified human H3 influenza virus correctly. According to their Instructions for Use, three commercial assays included H-subtyping of swine H1 and H3 viruses. However, only 2/3 workflows using the same singular assay identified the swine A(H3N2) virus as 'Influenza A H3' while the other two assays did not identify the subtype of the swine A(H3N2) virus. None of the three assays identified the A(H1N1)v (12/12) and A(H1N2)v (5/5) zoonotic swine viruses as 'Influenza A H1'.

Conclusions

Overall, the detection rate of animal influenza A viruses with the molecular diagnostic tests current routinely used in the Netherlands is high. However, further subtyping is necessary in order to know what kind of animal influenza virus is detected. When molecular tests that are capable of subtyping do not identify a human H1(pdm09) or H3 subtype, this is a first sign that there could be an animal influenza virus involved. In that case referral for further investigation at the NIC is required. But, referral to the NIC should also take place in case the patient was recently exposed to infected animals and if the subtyping assay leads to the classification H1(former seasonal), H1(pdm09) or H3. The attending physician needs to be aware of potential exposure to animals and should ask specific questions about it, in order to enhance the identification of a possible case of animal influenza virus infection. Nevertheless, with the current molecular diagnostic tests routinely used by medical microbiology laboratories in the Netherlands, the presence of animal influenza A virus can be reliably detected.

1 Introduction

Current situation regarding H5N1 influenza virus

Since the largest 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 and 2023, an expanding range of mammals has been found to be infected with this H5N1 virus, sometimes with substantial mortality (1, 2, 3). This follows on previous reports of a Spanish farm with transmission of H5N1 in minks in October 2022 (4, 5). Since 2021, 27 wild mammals - especially foxes - in the Netherlands have also been found to be infected with this specific H5N1 virus. Infection was often detected because of strange behaviour due to neurological and/or respiratory disease (6, 7).

Worldwide, eight people with H5N1 clade 2.3.4.4b influenza virus infection have been reported so far, mostly following direct contact with infected poultry. Three people were hospitalised and one case was fatal (8, 9, 10). Historical data of infections with other H5N1 influenza virus clades since 1997 indicate an overall case-fatality rate of over 50% (11). However, it is important to realise that this case-fatality rate is mainly based on the count of hospitalised patients. The actual number of infected individuals is unknown, and therefore the infection-fatality rate is very likely lower.

Notification obligation

In The Netherlands, animal influenza virus infection in humans – by avian or swine influenza virus – is a notifiable group B1 disease (12). This means that in case of a probable infection with animal influenza virus a report must be made to the municipal health service (GGD) and the RIVM National Coordination Centre for Communicable Disease Control (RIVM-LCI). Once the infection with animal influenza virus is confirmed, source finding and contact tracing are started immediately, as part of public health measures.

Indications for diagnostics

In The Netherlands, targeted diagnostics for H5N1 are currently performed by the National Influenza Centre (collaboration of RIVM, Bilthoven and Erasmus MC, Rotterdam). According to the guideline and scenarios, diagnostics are performed for patients who develop respiratory complaints or conjunctivitis after contact with infected birds, including the culling of H5N1-infected poultry farms and other professional encounters with H5N1-infected birds or mammals (12). Prophylactic oseltamivir and vaccination with a human flu vaccine are considered for professionals who encounter avian influenza but have no complaints. When a probable case is detected in regular health care, according to the guideline it is decided in consultation with RIVM-LCI whether additional diagnostics and measures are necessary. As this guideline is about the infection of humans with animal influenza viruses, the same applies when a case of infection with swine influenza virus is suspected or detected in regular healthcare.

Expert consultation avian influenza

In 2022 a Consultation of Experts on Zoonoses was held, after which the Minister of Health, Welfare and Sport assigned RIVM several tasks. These included a check of the molecular diagnostic tests used for diagnosing human influenza cases on their capability to detect influenza viruses of animal origin, both avian and swine (13). Swine influenza viruses included in response to the recent failure of a commercial molecular diagnostic test in a human infection with swine influenza virus in 2020, and the finding of another case of swine influenza virus infection in the national influenza virus surveillance in 2022 (14, 15, 16). In 2023, an additional case of swine A(H1N1)v influenza virus was detected through community-participatory surveillance of acute respiratory infections (17).

Commercial Nucleic Acid Amplification Tests (NAAT)

Because there is not always a suspicion of animal influenza virus in a patient infected with an animal influenza virus, it is important that the molecular diagnostic tests in use can at least detect animal influenza viruses, so that subtyping at the NIC can be requested after a positive generic test for influenza A virus and reported animal exposure in the clinical history of the patient. Commercial NAATs for generic detection of influenza A virus may be validated for animal influenza A virus detection by the manufacturer. This will be stated in the Instructions for Use (IfU). However, often this validation is not regularly updated for recently circulating viruses. It is complicated to check whether the used primers and probes are in principle suitable to detect recent animal and human influenza A viruses, since detailed information on used primers and probes is not included in the IfU and in general the manufacturers of these kits do not share this information. Subtyping of the hemagglutinin genome segment (H-subtyping) is usually not built into commercial assays for influenza virus detection, as there is generally no clinical implication. The most common built-in subtyping for human H1pdm09 and H3 influenza A virus subtypes is relatively rare in commercial NAATs, and the avian H5 influenza A virus subtype is only sporadically included in a commercial NAAT. As a result, using these tests it is hardly possible to identify an H5N1 case or any case of animal influenza virus infection in general, when detecting an influenza A virus without a suspicion of exposure to animals that are potentially infected with influenza virus in the clinical history of the patient.

External Quality Assessment

To address this lack of information on used primers and probes in commercial diagnostic tests, the NIC and the Animal Health Service (Royal GD, Deventer) have prepared an external quality assessment (EQA) ring trial on behalf of the Ministry of Health, Welfare and Sport. In this EQA, generic detection of type A influenza viruses (of human and animal origin) using commercial NAATs and Laboratory Developed Tests (LDT) currently routinely used in medical microbiology laboratories in the Netherlands is being tested. This EQA also includes an assessment for correct H-subtyping, when included in the routinely used commercial kit for influenza virus detection or when available as separate additional LDT. This EQA should provide an answer to the question if the medical microbiology laboratories in the Netherlands can detect animal influenza viruses, in particular the currently circulating H5N1 virus.

2 Materials and methods

2.1 Approach

All medical microbiology laboratories in The Netherlands were invited to participate in the animal influenza EQA through a direct email from the RIVM and a message sent to the members of the Dutch Society for Medical Microbiology (NVMM) in March 2023. Fifty laboratories signed up to participate, including one laboratory from Aruba, one from Bonaire and one from Curaçao.
The animal influenza EQA panel was produced and pre-tested at the RIVM, using LDTs and three commercial NAATs routinely used in surveillance and emergency diagnostics at RIVM. In the second week of March 2023, the panels were distributed to all diagnostic laboratories that signed up for the EQA. Laboratories were given till the first week of May 2023 to report their results. Laboratories using molecular point of care tests (mPOCT) were given the option to test a limited panel to reduce the costs. The limited panel consisted of specimens 1-5 (see Table 3.1).

2.2 Panel preparation and contents

Human and animal viruses were selected to cover recent challenges in human influenza A(H1N1)pdm09 and A(H3N2) detection and avian and swine influenza viruses recently infecting humans or potentially capable of infecting humans (Table 2.1). Viruses were grown on a mixture of MDCK-I and MSCK-SIAT cells, at biosafety level 3 when necessary. Panel specimens were prepared in Minimal Essential Medium (MEM) with Hank's salts and 10,000 HEp2 cells/ml to simulate a clinical specimen. Before preparing the specimens, the viruses were inactivated by heating them for 2.5 hour at 65ºC. Table 2.1 shows the panel composition and the expected results. Extracts for RT-qPCR were obtained by 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. The Cq values shown were determined by in-house 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, which are currently used at RIVM for generic influenza virus type A detection and indicated subtyping. Because primers and probes of the various RT-qPCR assays have a perfect or less than perfect match with the specific viruses, the Cq values cannot be used directly as a proxy to compare the concentration of viruses in the specimens. Therefore, using digital RT-qPCR with the RIVM in-house influenza A matrix gene primers and probes, the number of RNA copies was determined for each panel specimen (Table 2.1).
In the Supplementary material section, additional RIVM results are provided for the three commercial kits that were also tested as a reference for the evaluation of results from participants (Supplementary Table 7.1).

2.3 Dataset and analysis

All participating laboratories were asked to run a molecular detection for influenza A virus for the 10 panel specimens that were sent to them and

to determine at least the H-subtype, if this test was available. Using Microsoft Forms, they reported their data to the RIVM. They had to submit a new form for every workflow. The laboratories were asked to provide information on the workflow that was used. Among other things they were asked which kits they used for RNA extraction and amplification, if they used an in-house method, which equipment they used, and if they were able to do influenza A virus subtyping and if so, for which subtypes. For every workflow the laboratories had to indicate which target gene(s) were used.
After that, results were asked for every panel specimen. For every target gene a positive, negative or inconclusive result (pre-set reporting options) had to be reported, as well as the Cq/RFU values, when available. Furthermore, a final conclusions on the presence or absence of influenza A virus had to be reported and - when applicable - which subtype was identified.
In some workflows a limited panel could be tested, using only the first five specimens of the panel to lower the costs of testing, in particular for expensive mPOCT assays. Therefore, not every panel specimen has the same number of workflows it was tested in.
Finally, the question was asked what the laboratory would do if a detected influenza A virus were from a patient suspected of animal influenza virus infection (free text answer).
The reports in Microsoft Forms were downloaded into an Excel file and further analysis was done using Excel version 2202 and Graphpad Prism version 9.5.1 (733). Before analysis, the data was cleansed. Cleansing included harmonization of commercial kit names and a review of kit IfUs for used target genes and precise result interpretation. If required, a laboratory was contacted to clarify entries or to provide kit information. One laboratory reported four different tests in one form. These were separated into four workflows.
For every workflow a score was calculated by giving 1 point for every correct final result for each of the 10 panel specimens, making 10 points the highest score that could be obtained. An ‘Inconclusive’ final result was given 0.5 point. If a limited panel was tested, the total score of the five specimens was multiplied by 2. No scores were given for subtyping results. The score results for each workflow can be found in Supplementary Figure 7.5.
When commercial kits generated deviant results, the manufacturer was contacted to inform them and to provide them with an opportunity to give a comment for publication. Laboratories with deviant LDT results were also contacted to give a comment for publication.

Table 2.1 Panel contents and expected results animal influenza EQA 2023

Panel coding	Virus[1]	Host	A subtype[2]	Passage history[3]	InfA MP dPCR genome copies per µl specimen	Cq value by host specificity, type/subtype and target gene								
						Generic	Human					Avian		
						InfA MP	H3	N2 292R	N2 119E	H1 pdm09	N1 pdm09 275H	H5 setB	H7 set HA-1	N1
EQA.1_AI23-01	A/Mallard_Duck/Netherlands/2/2019[4]	Avian	H7N2	E2	639	29.06	-	-	-	-	-	-	30.06	
EQA.1_AI23-02	A/Netherlands/11715/2022 MP A12T+C136T[5,6]	Human	H3N2	MDCK-MIX2	1350	27.22	27.80	30.62	31.43	-	-	-	-	
EQA.1_AI23-03	No virus	na	na	na	0	-	-	-	-	-	-	-	-	
EQA.1_AI23-04	A/Netherlands/10370-1b/2020[5,7]	Swine/Human	H1N1v	MDCK-MIX2	137	29.87	-	-	-	-	-	-	-	-
EQA.1_AI23-05	A/Black-headed_Gull/Netherlands/6/2022[4]	Avian	H5N1 d1	MDCK1	335	26.54	-	-	-	-	-	28.67	-	27.66
EQA.1_AI23-06	A/Netherlands/11772/2022 MP C124A+G141A[5,6]	Human	H1N1pdm09	MDCK-MIX2	2220	26.32	-	-	-	26.96	28.86	-	-	-
EQA.1_AI23-07	A/Swine/Netherlands/34973/1999[8]	Swine	H3N2	MDCK-MIX5	145	27.84	-	30.18[9]	-	-	-	-		
EQA.1_AI23-08	A/Domestic Duck/Netherlands/EMC-2/2018[4]	Avian	H5N6	MDCK1	2150	25.64	-	-	-	-	-	24.78	-	
EQA.1_AI23-09	A/Netherlands/11748/2022[5,7]	Swine/Human	H1N2v	MDCK-MIX2	444	26.57	-	-	-	-	-	-	-	
EQA.1_AI23-10	A/Black-headed_Gull/Netherlands/6/2022[4]	Avian	H5N1 d2	MDCK1	20	30.18	-	-	-	-	-	32.63	-	

na means not applicable; '-' means tested with a negative result; grey cells mean not tested.

1. Sequences of the viruses are available from GISAID (gisaid.org) under accession numbers EPI_ISL_14751138, EPI_ISL_717720, EPI_ISL_13429294, EPI_ISL_15544293, EPI_ISL_305416, EPI_ISL_15348505 and EPI_ISL_13429294 respectively, except for A/Mallard_Duck/Netherlands/2/2019 and A/Swine/Netherlands/34973/1999 for which sequences are available on request.
2. H5N1 d2 is a 1:10 dilution of H5N1 d1 but copies/µl were individually determined.
3. E = Egg; MDCK = Madin-Darby Canine Kidney cells; MDCK-MIX = 1:1 mixture of MDCK-SIAT and MDCK-I cells; Number after cell line is the number of passages on the cell line.
4. From the Erasmus MC collection, courtesy of Ron Fouchier and Oanh Vuong. The H5 viruses both have clade 2.3.4.4b HA.
5. From the RIVM collection.
6. Indicated are recently emerging nucleotide mutations in the MP genome segment of these viruses that have led to underreporting in various assays (18, 19)*. The positions of the mutations are relative to the first ATG of M1/M2 genes in the MP genome segment.
7. Originating from recent human infections with swine influenza virus in the Netherlands.
8. From the collection of Royal GD, Deventer, courtesy of Erhard van der Vries and Manon Houben.
9. The genome segment for the neuraminidase of the swine A(H3N2) virus cross-reacts with low fluorescence with primers and probe of the human 292R N2 SNP and subtyping RT-qPCR.

* Another source was personal communication from UKHSA WHO National Influenza Centre, London UK

3 Results

3.1 Aggregated overview

For this EQA 50 laboratories reported a total of 117 workflows (participating laboratories are summarised in Chapter 7.5. One laboratory submitted four workflows in one form; this dataset was split into four separate workflows. This created 120 workflows - two of which were for subtyping only - that were analysed. Table 3.1 provides a summary of the results per panel specimen. Most workflows (109/118; 92%) found the animal and human influenza virus containing specimens correctly positive for influenza virus type A. However, panel specimen 4, with an A(H1N1)v human/swine influenza virus, had the most detection errors, given that 7/118 (6%) reported workflows were not able to detect this virus. Overall, 9/118 (8%) reported workflows had errors. Six reported a negative result for one or two animal influenza virus containing specimens: 1/3 Abbott ID NOW INFLUENZA A & B 2, 2/3 Roche ePLEX Respiratory Pathogen Panel 2, and 3/5 Seegene Allplex RV Essential or Master Assay. For BioMerieux BIOFIRE Respiratory panel 2.1 plus 2/7 returned an inconclusive result for two or three animal influenza virus containing specimens. One of 21 workflows using an LDT reported the human A(H1N1)pdm09 containing virus specimen negative. However, all 118 workflows reported avian influenza virus A(H5N1) and A(H5N6) containing specimens influenza A virus positive. Furthermore, every workflow reported a correct 'influenza A virus negative' final conclusion for the negative (no virus) control panel specimen.
Figure 3.1 shows a visual representation of the reported final conclusion of each workflow. It shows that nearly all reported workflows (92%) were able to detect all influenza viruses that were provided in the panel as an influenza A virus. The exception was panel specimen 4, containing A(H1N1)v influenza virus, for which multiple workflows reported a 'Negative' final conclusion for influenza A virus presence. Although the number of copies in specimen 4 was in the lower range, these workflows did detect influenza A virus in the specimens that contained a similar or even lower number of copies (specimens 7 and 10) (Table 2.1).
More information about the performance of workflows used by each laboratory can be found in Supplementary Figure 7.5, which also includes a score per reported workflow.

3.2 Cq values per target gene

Out of the 120 reported workflows 98 (82%) used an in-house LDT or commercial detection kit that reports Cq values (more information on kits and equipment used can be found in Supplementary Figures 7.1 - 7.4), indicating the presence of a virus by detection of a certain target gene. Figure 3.2 depicts the Cq values for every workflow that reported these values per target gene. The far most common target gene used is the Matrix gene, which is used by a wide variety of kits and all LDTs. Many reported workflows were for results from Cepheid GeneXpert kits (n=42) that included influenza virus A/B detection. These kits use two channels, Flu A1 and Flu A2, for influenza virus type A detection. Flu A1 contains primers and probes against human derived influenza A matrix-, PA- and PB2-genes and Flu A2 contains avian derived primers and

probes against the matrix-gene, but is not limited to detect avian influenza type A viruses only (personal communication, Cepheid). Between the two channels there is great overlap, so when either or both channels produce a PCR curve, a positive result will be given for detection of influenza A virus. For all 42 Cepheid workflows the Flu A1 channel Cq values were reported and for 36/42 the Flu A2 channel Cq values. Two workflows reported result for the Seegene Allplex RV Essential Assay kit, which uses a Flu A channel (according to IfU) which also has the M-gene as target (personal communication, Seegene). Because of outlier results this kit is depicted separately in Figure 3.2. Figure 3.2 shows that the Cq values for panel specimen 3 - the negative control - were all negative. The matrix gene shows a larger spread of Cq values per specimen than the Cepheid targets, but the matrix gene Cq values are derived from a large variety of different commercial kits and LDTs. However, even a closed system like the Cepheid GeneXpert generates a cloud of Cq values with the same specimen, although narrower in spread. All workflows show a similar shift in Cq values between specimens, roughly correlating with the copy number per µl specimen. Supplemental Figure 7.6 shows the correlation between the Cq values and the number of copies per specimen, where panel specimen 4 (H1N1v) had the highest overall Cq value for all target genes (median=33.48, mean=37.14; copy number 137/µl) and panel specimen 8 (H5N6) the lowest (median=26.11, mean=27.43; copy number 2.150/µl).
Furthermore, the Seegene Allplex RV Essential Assay kit (Flu A target gene, actual being the M-gene; personal communication, Seegene) shows some outliers. Higher end Cq values were measured for all panel specimens with this kit in comparison to the results of most other tests (Figure 3.2), while panel specimen 4 (H1N1v) was not detected at all by this kit. Moreover, panel specimen 1 (H7N2) was also not detected by one of the two Seegene Allplex RV Essential Assay workflows. Considered together, these observations indicate that this workflow likely has a sensitivity issue with the detection of certain influenza A viruses.

3.3 Subtyping

Of the 120 workflows, 24 (20%) had a varying capability for subtyping reported by 20/50 (40%) laboratories, and they could indeed subtype the viruses in part of the specimens that were provided on the panel (Table 3.2).
Specimen 2, which is a human A(H3N2) influenza virus strain, was correctly subtyped by 22 workflows that were capable of subtyping H3 (Table 3.2). All six reported commercial kits that include subtyping of H1pdm09 subtyped the human A(H1N1)pdm09 virus correctly. With the exception of one kit none of the commercial kits were able to subtype the animal hemagglutinin subtypes avian H5 or H7 and swine H1 or H3. The latter is correct, as these subtypes are not included in the design of most kits. By design the BIOFIRE kit from BioMerieux should be capable to identify the swine H1 and H3 subtypes. Indeed, 2/3 workflows that used this kit on the A(H3N2) swine influenza virus containing specimen reported it as subtype H3. However, none of seven workflows and none of three workflows using this kit on the A(H1N1)v and A(H1N2)v zoonotic swine influenza viruses included in the panel could identify the

H-subtype of these viruses. Furthermore, the IfU of two commercial kits, Qiagen QIAstat-Dx Respiratory SARS-CoV-2 Panel and Roche ePlex Respiratory Pathogen Panel 2, indicate the capability to subtype swine H1 and H3 viruses. However, none of the workflows using these kits identified the subtypes of swine viruses (Table 3.2). Although the eight in-house LDTs for subtyping subtyped the human H3 containing specimen correctly, two of them were not able to subtype the human H1pdm09 containing specimen, which raised some concerns. All four LDT workflows for H5 subtyping reported by four different laboratories, subtyped the three avian H5 containing panel specimens correctly. Only one of two LDT workflows that included subtyping of avian H7 correctly identified the H7 subtype for the A(H7N2) containing specimen.

3.4 Response from manufacturers and laboratories

The manufacturers of four commercial kits (Abbott, BioMerieux, Seegene and Roche) and five laboratories with deviant results were individually approached for comments on the findings. Eight of them responded.

The one laboratory that had a negative result for panel specimens containing A(H1N1)v and A(H1N1)pdm09 with their LDT for generic influenza A virus detection provided the following explanation: Unfortunately, using our LDT two out of nine virus containing specimens (both H1N1) were not detected. Theoretically the mentioned primer-probe sets should be able to detect all mentioned influenza strains. We have contacted the laboratory from which we have obtained the primer and probe sequences of our currently used LDT. They have informed us that they are aware of the problem, and are currently working on optimalisation of said primers and probes. Fortunately, we also have two commercial PCRs running which were able to detect all virus containing specimens (with good Cq values) in the panel, so for the time being we will only use these commercial PCRs for the detection of influenza virus until the issues with our LDT have been resolved.

Three laboratories submitted a workflow that was capable of subtyping H1, H3 and H5, but did not report a subtype for the A(H1N1)pdm09 containing specimen. These panel specimens were found positive in generic influenza A detection, but no subtype was identified. One lab reported that for their H1pdm09 subtyping PCR the forward primer contained three mismatches and the probe they used had one mismatch with the panel virus, explaining why the subtype was not recognized in their PCR. From the H1 primers and probe information that a second laboratory submitted with their results it became clear that they had specificity for the former seasonal H1 subtype and not for the H1pdm09 subtype, and therefore could not have identified the H1pdm09 subtype. This laboratory confirmed that they indeed included subtyping for former seasonal H1 and not H1pdm09 by omission. The third laboratory answered that the reported results of the Pathofinder RespiFinder® 2Smart kit are based on results up to genus level and not species level and that although this kit does have a test to identify the A(H1N1)pdm09 species, it would be reported only as 'Influenza A positive' to a requester of the test.

The laboratory that failed to subtype the H7 virus in the panel with their H7 subtyping test provided the following explanation: Our laboratory

participated in the External Quality Assessment panel for animal influenza viruses organized by RIVM. All influenza virus containing panel specimens were identified correctly for influenza A virus. In subsequent virus typing by PCR one panel member containing H7N2 virus could not be subtyped. In this case all subtyping assays remained negative (while this panel member was positive for influenza A virus in the detection PCR). Our subtyping assay should be able to correctly subtype H1 (former seasonal), H1pdm09, H3, H5, H7 and H10 viruses. Analysis of genome sequences revealed a minor mismatch in one of the primers that could lead to a slightly lower sensitivity of the subtyping PCR for this H7N2 panel member. In practice, when analysing specimens from clinical cases, such a result (positive result for influenza A detection PCR, but negative result in all virus subtyping PCRs) will be followed by whole genome sequencing. It is therefore unlikely that we would miss an H7N2 infection. Nevertheless, we continuously evaluate, develop and improve our PCR assays with the latest information and will use this result as input to update our assays.

BioMerieux provided the following comment on the observed subtyping of swine H3 and lack of subtyping swine H1 with the BIOFIRE Respiratory Panel 2.1 (plus) kit: The results of the animal influenza EQA panel testing are consistent with expected performance and reporting by the BIOFIRE Respiratory Panel 2.1 (plus) according to the product design and description provided in the instruction for use. The intended use of the device is first and foremost to identify the presence of potential infectious agents in a patient specimen, so that the test results can be used in conjunction with clinical history of the patient (including human and animal contact and/or travel) and the epidemiological status of the region. Results aid in the diagnosis, management, and treatment of the symptomatic patient. Additional targeted testing may be required to monitor or screen for zoonotic transmission events and/or emerging variants within a region or patient population and as part of public health missions for pandemic preparedness.

Roche provided the following comment on the observed lack of detection of the A(H1N1)v swine influenza virus by some users of the ePlex® Respiratory Pathogen Panel 2 kit: Using the sequence information provided (GISAID, EPI_ISL_717720, A/Netherlands/10370-1b/2020), a bioinformatics assessment was done against the primers and probes used in the RP2 Panel assays for influenza. The ePlex RP2 Panel includes a unique assay for the influenza A matrix gene and separate, independent assays for the H1, H1pdm09 and H3 subtypes. This redundancy helps mitigate the risk of a missed result due to (a) new mutation(s). Analysis of the ePlex RP2 influenza A matrix gene assay found 1 mismatch in the forward primer and also 3 mismatches in the capture probe region. This data suggests that amplification will perform as expected, but detection of this specific Eurasian swine-like influenza A strain may be variable due to these mismatches. Additional analysis of the RP2 influenza A H1 assay also has multiple mismatches, suggesting that this strain may not be detected by the RP2 Panel. The ePlex RP and RP2 Panels are designed to be inclusive of as many circulating and recently circulating strains of influenza A in humans. However, as new variants emerge, there may be mutations that are not detected due to mutations in the regions targeted by the ePlex assays.

GenMark and Roche conduct routine influenza surveillance of circulating strains to monitor for inclusivity of new strains in circulation to ensure the test is performing as expected. Furthermore, the risk of transmission of a zoonotic virus into humans and from-human-to-human is closely monitored, to be prepared in case immediate actions are required.

Seegene provided the following comment on the failure to detect specimens 1 (H7N2) and 4 (H1N1v) with the generic influenza A virus target component of several workflows using the Seegene Allplex RV Essential and Master Assays: The IfU show that the assays are validated to detect several animal influenza A virus subtypes, but that emerging animal viruses infecting humans might be missed. Seegene is working on updating the respiratory assays in which these observations will be addressed in the future.

3.5 Follow-up suspect animal influenza case

The question what a laboratory would do if a positive influenza A case proved to be a suspect animal influenza case was answered by 47/50 laboratories; two considered it not applicable and one did not answer the question. Most laboratories (40/47; 85%) answered that they would follow-up with subtyping/sequencing of the detected influenza A virus, either by themselves (5/40) or by the National Influenza Centre (35/40). One out of 40 answered that they would not follow-up, 3/40 that they would first inform RIVM-LCI and the Municipal Health Service before following up with a subtyping action and 3/40 said they would discuss appropriate action with their own staff first. Of the 40 laboratories that would follow-up with subtyping, seven indicated that they would also inform RIVM-LCI and the Municipal Health Service. Two out of these 40 laboratories said they would also inform the staff of Infection Prevention.

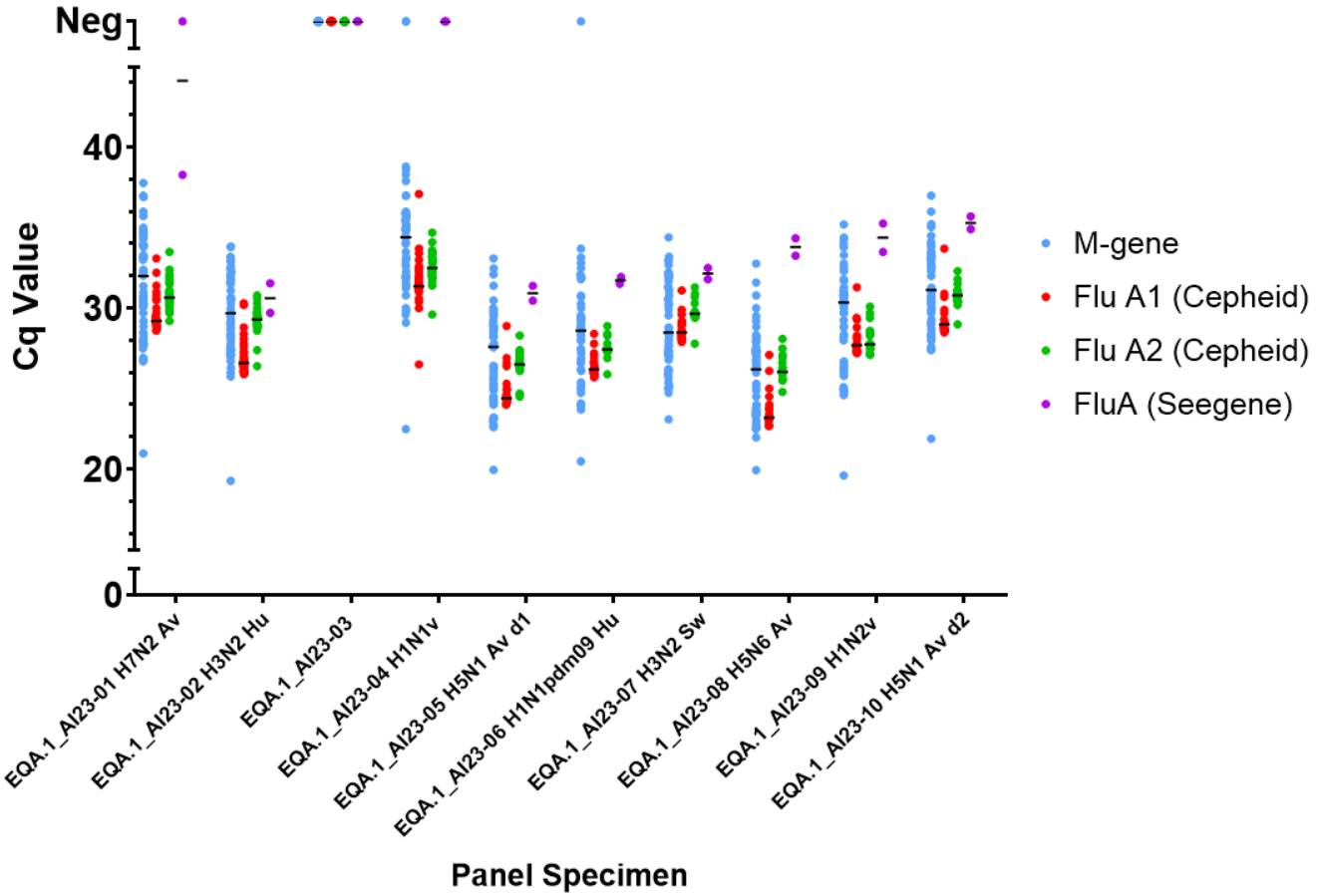
Table 3.1 Summary of reported outcomes per panel specimen

Panel specimen	Content	A subtype	Number of workflows with reported test results	Conclusion workflow detection influenza virus			
				No. Positive	No. Negative	No. Inconclusive	Errors
EQA.1_AI23-01	A/Mallard_Duck/Netherlands/2/2019[1]	H7N2	118	115	1	2	False negative results (n=1) Inconclusive results (n=2)
EQA.1_AI23-02	A/Netherlands/11715/2022 MP A12T+C136T[1]	H3N2	118	118	0	0	None
EQA.1_AI23-03	No virus[1]	None	118	0	118	0	None
EQA.1_AI23-04	A/Netherlands/10370-1b/2020[1]	H1N1v	118	109	7	2	False negative results (n=7) Inconclusive results (n=2)
EQA.1_AI23-05	A/Black-headed_Gull/Netherlands/6/2022[1]	H5N1 d1	118	118	0	0	None
EQA.1_AI23-06	A/Netherlands/11772/2022 MP C124A+G141A	H1N1pdm09	82	81	1	0	False negative results (n=1)
EQA.1_AI23-07	A/Swine/Netherlands/34973/1999	H3N2	82	82	0	0	None
EQA.1_AI23-08	A/Domestic Duck/Netherlands/EMC-2/2018	H5N6	82	82	0	0	None
EQA.1_AI23-09	A/Netherlands/11748/2022	H1N2v	82	80	1	1	False negative results (n=1) Inconclusive results (n=1)
EQA.1_AI23-10	A/Black-headed_Gull/Netherlands/6/2022	H5N1 d2	82	82	0	0	None

1. Panel specimens EQA.1_AI23-01 to EQA.1_AI23-05 were part of the limited panel

A green line represents a correct result, a red line an incorrect result and an orange line an inconclusive result. Panel specimen EQA.1_AI23-06 till EQA.1_AI23-10 were not part of the limited panel, and therefore these boxes remain white. This is also indicated by separating the boxes containing the workflows with a limited or a full panel. At the far right of the figure the panel specimen and corresponding virus are depicted (host: Av = avian; Hu = human; Sw = swine; v = variant, indicating a human infected with swine virus; H5N1 Av d2 is a 1:10 dilution of H5N1 Av d1).

Figure 3.1 Heatmap of reported final conclusion for each workflow.



For every panel specimen the Cq values per workflow are shown. Each dot represents a separate workflow, and each color represents a target gene. At the top of the Y-axis the negative results are shown. The target gene for LDTs and most commercial tests is the M-gene. For the Cepheid GeneXpert tests (personal communication with manufacturer) the target genes for Flu A1 are the acidic polymerase (PA), basic polymerase (P2B) and matrix (M) genes for broad coverage, and for Flu A2 primers and probes designed against avian-derived matrix gene sequences, but not limited to detect avian influenza viruses only. For the Seegene RV Essential Assay kit the FluA target is actually the M-gene (personal communication with manufacturer), but results are shown separately because of deviant results. Only workflows that generate Cq values are shown in this figure (n=98). With the virus names the host is shown: Av = avian; Hu = human; Sw = swine; v = variant, indicating a human infected with swine virus; H5N1 Av d2 is a 1:10 dilution of H5N1 Av d1.

Figure 3.2 Cq values per target gene.

Table 3.2 Overview of panel specimen results with workflows capable of subtyping.

Amplification kit	Number of workflows that used this kit (n=24)	Subtyping integrated in workflow/additional tests[2]	Panel specimen[1] EQA.1_AI23- / Subtype / Limited Panel	01 H7N2	02 H3N2	04 H1N1v	05 H5N1 (d1)	06 H1N1pdm09	07 H3N2 swine	08 H5N6	09 H1N2v	10 H5N1 (d2)
BioMerieux Diagnostics. BIOFIRE® Respiratory panel 2.1 plus	7	H1(former seasonal), H1pdm09, H3 [3]	No	✕	A(H3)	✕	✕	A(H1)pdm09	A(H3)	✕	✕	✕
			Yes	✕	A(H3)	✕	✕	-	-	-	-	-
			No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
			Yes	✕	A(H3)	✕	✕	-	-	-	-	-
			Yes	✕	A(H3)	✕	✕	-	-	-	-	-
			No	✕	A(H3)	✕	✕	A(H1)pdm09	A(H3)	✕	✕	✕
			Yes	✕	A(H3)	✕	✕	-	-	-	-	-
In-house. LDT	8	H1pdm09, H3	No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
		H1pdm09, H3	No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
		H1(former seasonal), H1pdm09, H3, H5, H7	No	A(H7)	A(H3)	✕	A(H5)	A(H1)pdm09	✕	A(H5)	✕	A(H5)
		H1pdm09, H3	No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
		H1pdm09, H3	No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
		H1pdm09, H3, H5	No	✕	A(H3)	✕	A(H5)	✕[4]	✕	A(H5)	✕	A(H5)
		H1pdm09, H3, H5	No	✕	A(H3)	✕	A(H5)	✕[4]	✕	A(H5)	✕	A(H5)
		H1, H3, H5	No	✕	A(H3)	✕	A(H5)	✕[5]	✕	A(H5)	✕	A(H5)
		H1(former seasonal), H1pdm09, H3, H5, H7, H10	No	✕	A(H3)	✕	A(H5)	A(H1)pdm09	✕	A(H5)	✕	A(H5)
Qiagen. QIAstat-Dx® Respiratory SARS-CoV-2 Panel	2	H1(former seasonal), H1pdm09, H3 [3]	Yes	✕	A(H3)	✕	✕	-	-	-	-	-
			Yes	✕	A(H3)	✕	✕	-	-	-	-	-
Roche. ePlex® Respiratory Pathogen Panel 2	3	H1(former seasonal), H1pdm09, H3 [3]	No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
			No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
			Yes	✕	A(H3)	✕	✕	-	-	-	-	-
Seegene. Allplex™ Respiratory Panel 1A	2	H1(former seasonal), H1pdm09, H3	No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
			No	✕	A(H3)	✕	✕	A(H1)pdm09	✕	✕	✕	✕
Siemens. FTD™ Respiratory pathogens 21	1	H1pdm09	No	✕	✕	✕	✕	A(H1)pdm09	✕	✕	✕	✕
Pathofinder. RespiFinder® 2Smart	1	H1pdm09	No	✕	✕	✕	✕	✕[6]	✕	✕	✕	✕

1. Results are shown for each individual reported workflow using the indicated amplification kit. The subtype is the subtype of the panel specimen. If the test did detect a subtype, then the outcome of the test is depicted in the table. A cross (X) means the kit did not detect a subtype, which in 14 cases is considered a deviation from the intended specificity of the test (red cross). A dash (-) means that the panel specimen was excluded from testing (limited panel). Specimen EQA.1_AI23-03 is excluded since it is the negative control.
2. For each individual reported workflow for 'In-house. LTD', the subtyping capability is indicated.
3. Might become positive with swine H1 and H3 subtypes according to Instructions for Use and personal communication with the manufacturers.
4. The laboratory explained that primers and probe were not recently reviewed and updated and therefore likely could not subtype the virus.
5. The laboratory provided primers and probe information showing the specificity of the H1 subtyping was for former seasonal H1 and not for H1pdm09.
6. The laboratory explained that it does not report H1pdm09 subtyping results to the requester for a test, and therefore the result in this EQA was not reported either. However, at RIVM we showed that this commercial assay was capable of subtyping H1pdm09 correctly (Supplementary Table 7.1).

4 Discussion and conclusion

For this EQA, a panel with human and animal type A influenza viruses was distributed to medical microbiology laboratories in the Netherlands, to determine whether the molecular influenza diagnostic tests they routinely use are able to detect animal influenza viruses. The results show that overall the detection of animal influenza viruses is good. The vast majority of used tests found all panel specimens containing human, avian or swine influenza type A viruses positive for *an* influenza virus type A. Five commercial tests did not detect one or two animal influenza viruses, and one LDT did not detect the human A(H1N1)pdm09 virus. All reported workflows detected the avian A(H5) viruses. A decent number of laboratories have commercial tests or LDTs that correctly identified human H1pdm09 and H3 subtypes, only a few have tests that correctly identified avian H5 and only one has a test that correctly identified avian H7. With the exception of the BioMerieux BIOFIRE assay that identified swine H3 with some of the workflows using this assay, none of the tests could identify the subtype of the swine influenza viruses in the panel. Most tests use the Matrix gene as a target for generic type A influenza virus detection, and this appeared to work very well. The Cepheid GeneXpert commercial tests use multiple targets - the Matrix, PA and PB2 genes - in one channel, and another Matrix gene-based amplification in a second channel. All the workflows using the Cepheid GeneXpert tests produced correct results for the influenza A virus containing specimens of the panel, indicating that using multiple target genes can be an effective way for generic detection of type A influenza viruses. Similarly, the BioMerieux BIOFIRE assay, which uses two pan influenza A amplifications, found all influenza A virus containing specimens positive. Including multiple targets in a test or testing strategy potentially prevents failing detection of a virus if this virus has mutations in primers and/or probe sites on one of the target genes.

In particular panel specimen 4, an A(H1N1)v zoonotic swine virus, was difficult to detect for four commercial tests: the Seegene Allplex RV Essential and Master assays, the Abbott ID NOW Influenza A&B 2 and the Roche ePlex Respiratory Pathogen Panel 2. Most of the other molecular tests did detect this virus, but the Cq values were quite high compared to those of the other panel specimen. It is important to mention that the expected Cq values, measured at the RIVM, were also high (Table 2.1), reflecting the relatively low viral load in this specimen. The Cq values reported for this specimen were also the highest compared to the other panel specimens, indicating that sensitivity for this particular virus might be an issue. However, two other animal influenza virus containing specimens with similar or lower target copy numbers were properly detected using these tests. This could be the result of mismatched primers or probes used in these tests. As the sequences of these primers and probes are not released by the manufacturers, the manufacturers have been provided with the virus sequences and were asked to comment on the obtained results. Testing these commercial kits again with a dilution range of this particular virus and some other viruses could provide more clarity about the true limit of detection. Nevertheless, the responses from the manufacturers indicate

that they are aware that emerging viruses can potentially be missed. As a result they follow developments closely to be able to adapt their assays, if deemed necessary.

Several results for some animal influenza viruses were reported as 'Inconclusive'. These were all from workflows that used the BioMerieux BIOFIRE Respiratory Panel 2.1 plus molecular test. This is probably caused by correct use of the IfU, providing an 'Equivocal' result for the indicated virus. This was then translated into 'Inconclusive' in the Microsoft Forms report by design. However, other workflows using the same test reported these viruses as 'Influenza A virus Positive', suggesting the laboratories involved may have had additional arguments to consider the 'Equivocal' results as actually 'influenza A virus Positive'. Similar to the Cepheid GeneXpert tests, the BioMerieux BIOFIRE assay also uses two channels for influenza A virus detection. If only one channel yields a positive result and subtyping is negative, the final conclusion of this test for influenza A virus detection will be 'Equivocal'. This could actually be an advantage, because it could be a first indication that the tested specimen contains an animal influenza virus. BioMerieux BIOFIRE also mentions this in the IfU, indicating that non-human influenza A viruses will generally produce an 'Equivocal' or 'Influenza A (no subtype detected)' result.

The previously indicated issue with the impaired detection of human A(H1N1)pdm09 and A(H3N2) viruses with specific mutations in the matrix gene by several commercial assays (17, 18) was not detected using any of the commercial assays with the viruses containing these mutations that were included in the panel. This is reassuring for the quality of the tests used for the detection of recent human A(H1N1)pdm09 and A(H3N2) influenza viruses.

Not many molecular tests were able to detect the subtype of detected influenza A viruses in the panel specimens. Only 24 out of 120 (20%) workflows were able to perform a subtype identification of detected influenza A viruses. Therefore, most tests can detect the presence of an influenza A virus, but cannot indicate whether it is a human or animal influenza virus subtype. This stresses the importance of sending influenza A virus positive patient specimens to the National Influenza Centre (NIC) for further subtyping and characterization when contact with infected animals is suspected or mentioned in the clinical history of the patient by a GP or an attending physician. This is of particular importance for the monitoring and surveillance of animal influenza viruses that have proved to be able to infect humans. For this purpose, RIVM-LCI (12) has published guidelines and has recently raised awareness of the subject by communication through the Dutch Society for Medical Microbiology (20) and by the publication of a paper targeted at GPs (21).

All tests that were able to do a defined subtype detection produced correct results, except for two A(H1)pdm09 subtyping LDTs and one workflow using a commercial assay that were negative for the specimen containing A(H1N1)pdm09 virus, and one H7 subtyping LDT that was negative with the specimen containing A(H7N2) virus. The inability to subtype the A(H1N1)pdm09 virus was caused by a lack of updated

primers/probe, using H1(former seasonal) instead of H1pdm09 specific primers/probe, and the policy of a laboratory using a commercial assay to not report the subtype to requesters. The failure to subtype the H7 virus was explained by mismatched primer. Although by design the BioMerieux BIOFIRE Respiratory Panel 2.1 plus should be capable of subtyping swine H1 and H3, similar to the Qiagen QIAstat-Dx® Respiratory SARS-CoV-2 Panel and Roche ePlex® Respiratory Pathogen Panel 2, only some of the workflows using the BIOFIRE workflow provided the H3 subtype for the swine H3 virus and none identified the subtype of swine H1 viruses. This may be indicative of the difficulty manufacturers have to keep up with the large variety of Eurasian and North American lineages of swine viruses in the design of primers and probes to identify swine H1 and H3 subtypes. It also implies continuous thorough validation of commercial assays is needed to comply with FDA and CE marking for In Vitro Diagnostic use. Although the BioMerieux BIOFIRE kit identified the H3 subtype of the A(H3N2) swine influenza virus, laboratories can interpret this as a normal, human seasonal H3 influenza virus, since there is no further indication that it could be an animal influenza virus. This carries the risk of missing a patient infected with animal influenza virus, if no further questions are asked about animal exposure in the clinical history of the patient.

Except for the three commercial assays mentioned, other commercial molecular tests that were tested in this EQA are only capable of identifying H1(former seasonal), H1(pdm09) and H3, because avian hemagglutinin variants like H5 and H7 and swine hemagglutinin variants are not included in the design of these tests. Only a couple of LDTs that were reported in this EQA proved capable of identifying avian H5 and H7. Unfortunately, identification of avian or swine influenza virus variants with the commercial kits routinely used in The Netherlands is generally not possible, and when there is no suspicion of animal influenza virus infection it becomes difficult to detect and identify such cases. Therefore, as explained earlier above, awareness in GPs and attending physicians in hospitals and further questioning about possible contact with animals in the clinical history of the patient are imperative.

In conclusion, the current molecular diagnostic tests routinely used in the Netherlands to detect influenza A viruses perform well for animal influenza virus detection. However, in order to know what kind of animal influenza virus is detected further subtyping is necessary. When molecular tests that are normally capable of subtyping human seasonal influenza virus subtypes cannot identify an H1(pdm09) or H3, this is a first sign that it could be an animal influenza virus subtype. These specimens need to be sent to the NIC for further investigation. In addition, to improve the diagnostic strategy to identify a patient infected with an animal influenza virus, GPs and attending physicians in hospitals need to be aware of possible exposure of a patient to animals and must ask specific questions about possible contact with animals.

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7 Supplementary material

7.1 Equipment and kits used

All participating labs were asked to provide information on which kit and equipment was used to generate the reported results. In Supplemental Figures 7.1 – 7.4 this information is summarised.

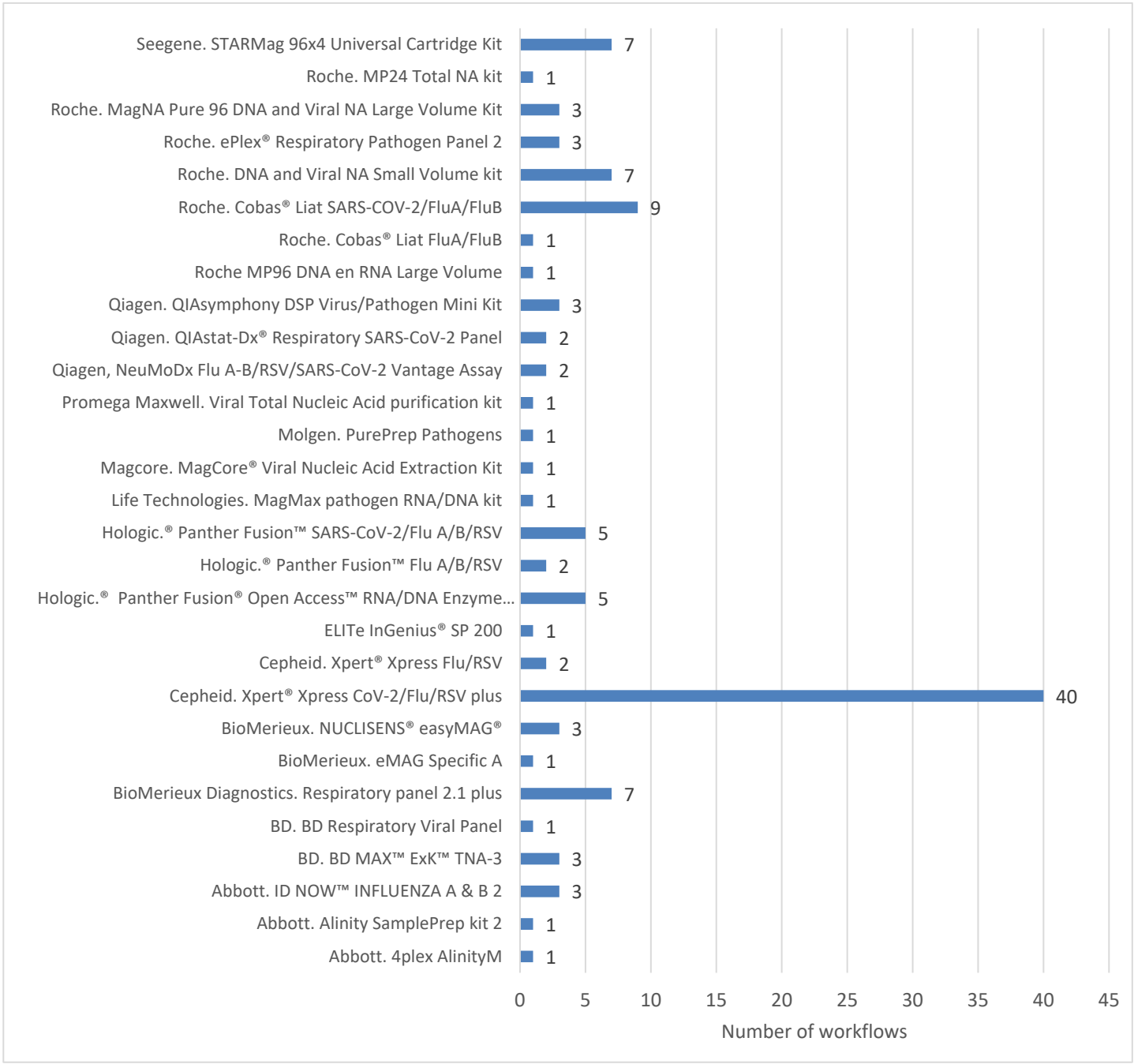


Figure 7.1 The RNA isolation kits used for the reported workflows.

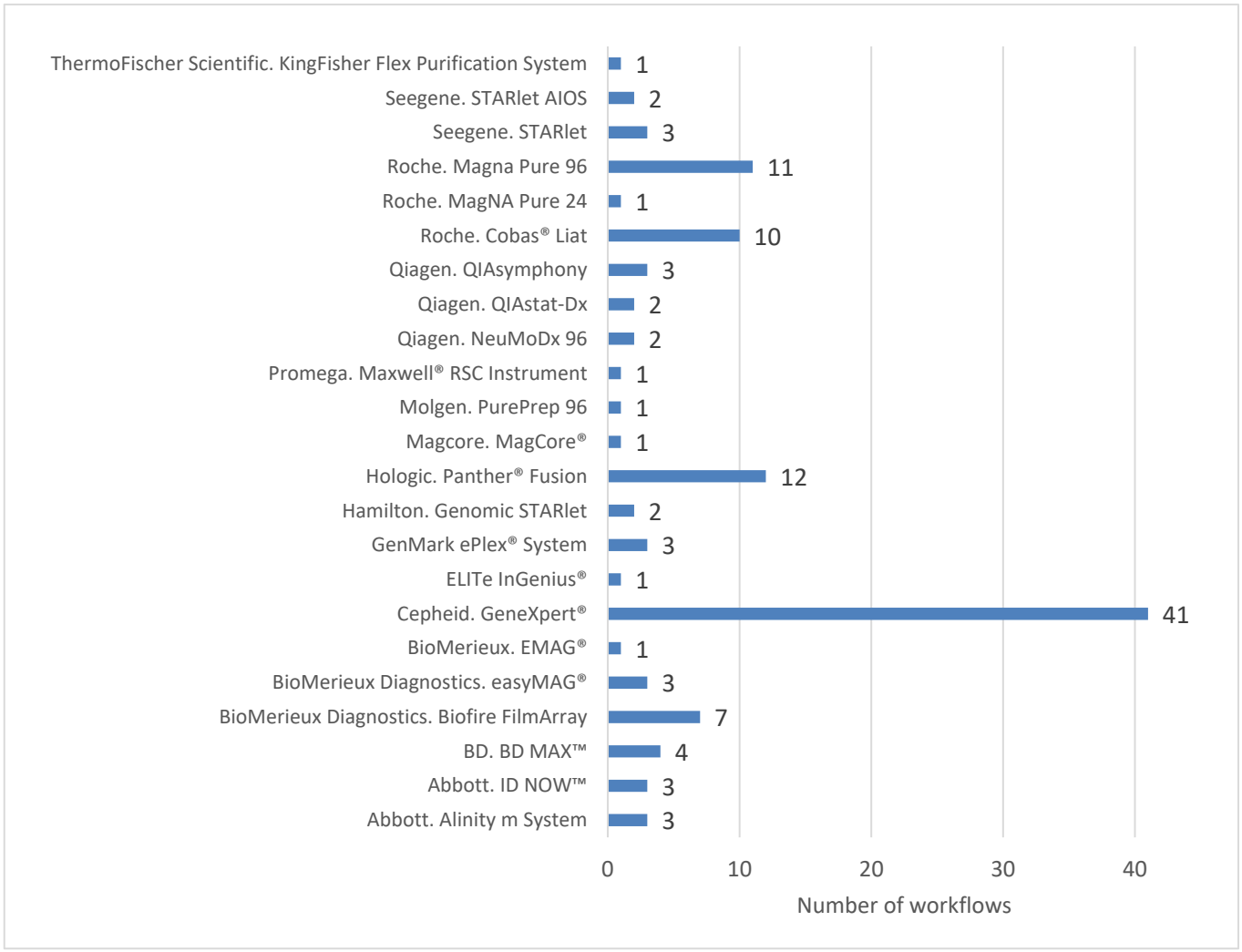
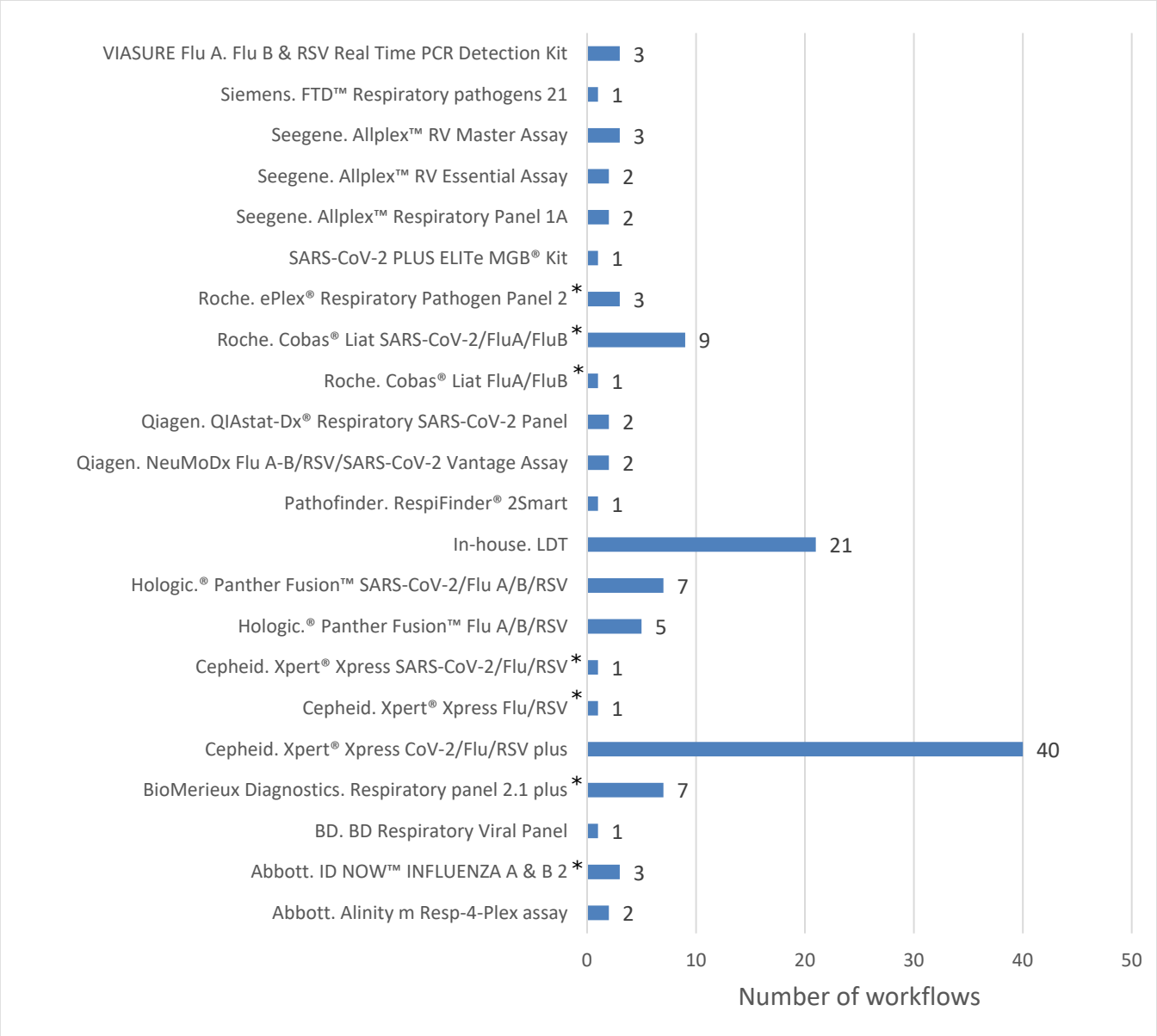


Figure 7.2 The RNA isolation equipment used for the reported workflows.



*Molecular point of care tests (mPOCT)

Figure 7.3 The RNA amplification kits used for the reported workflows.

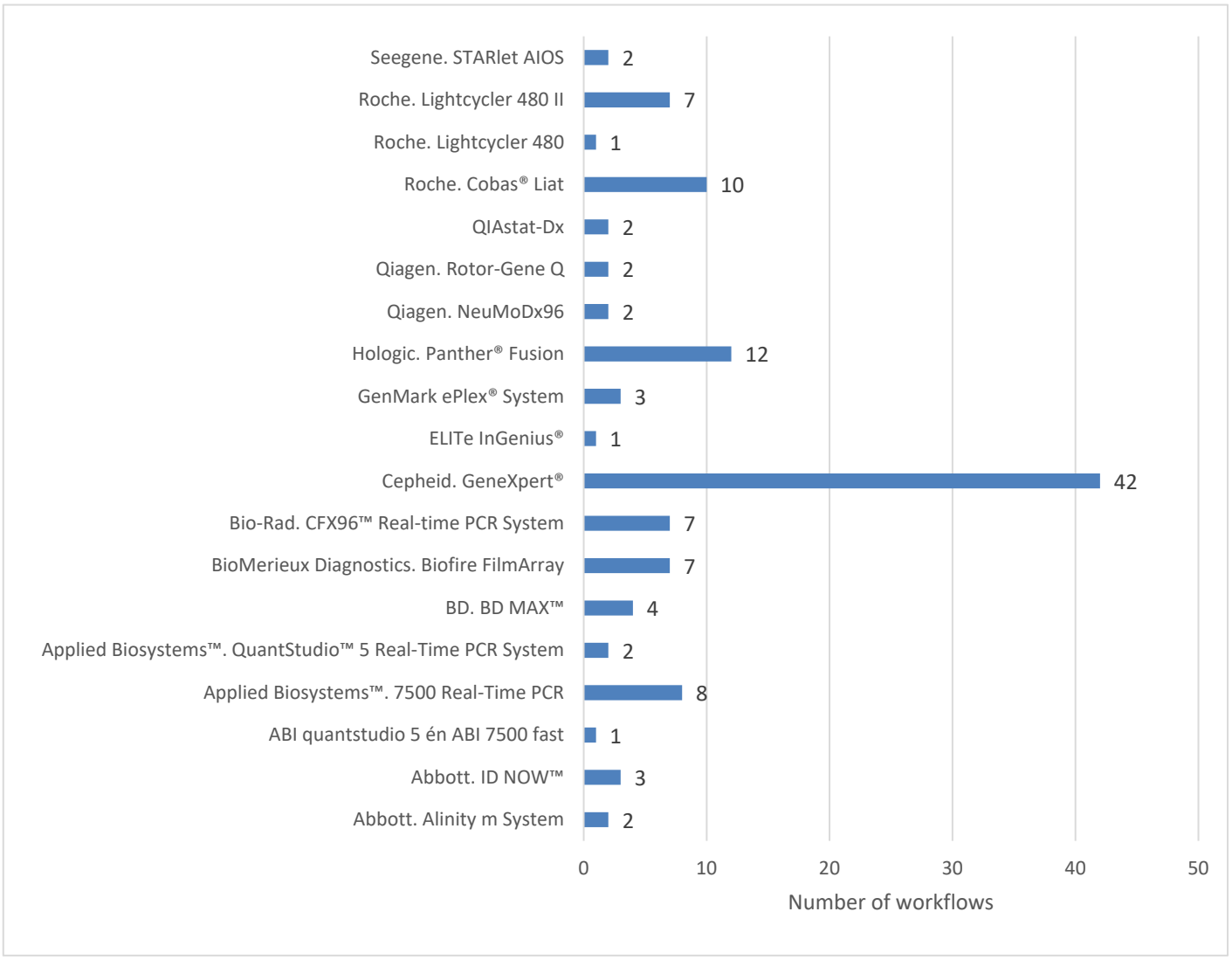
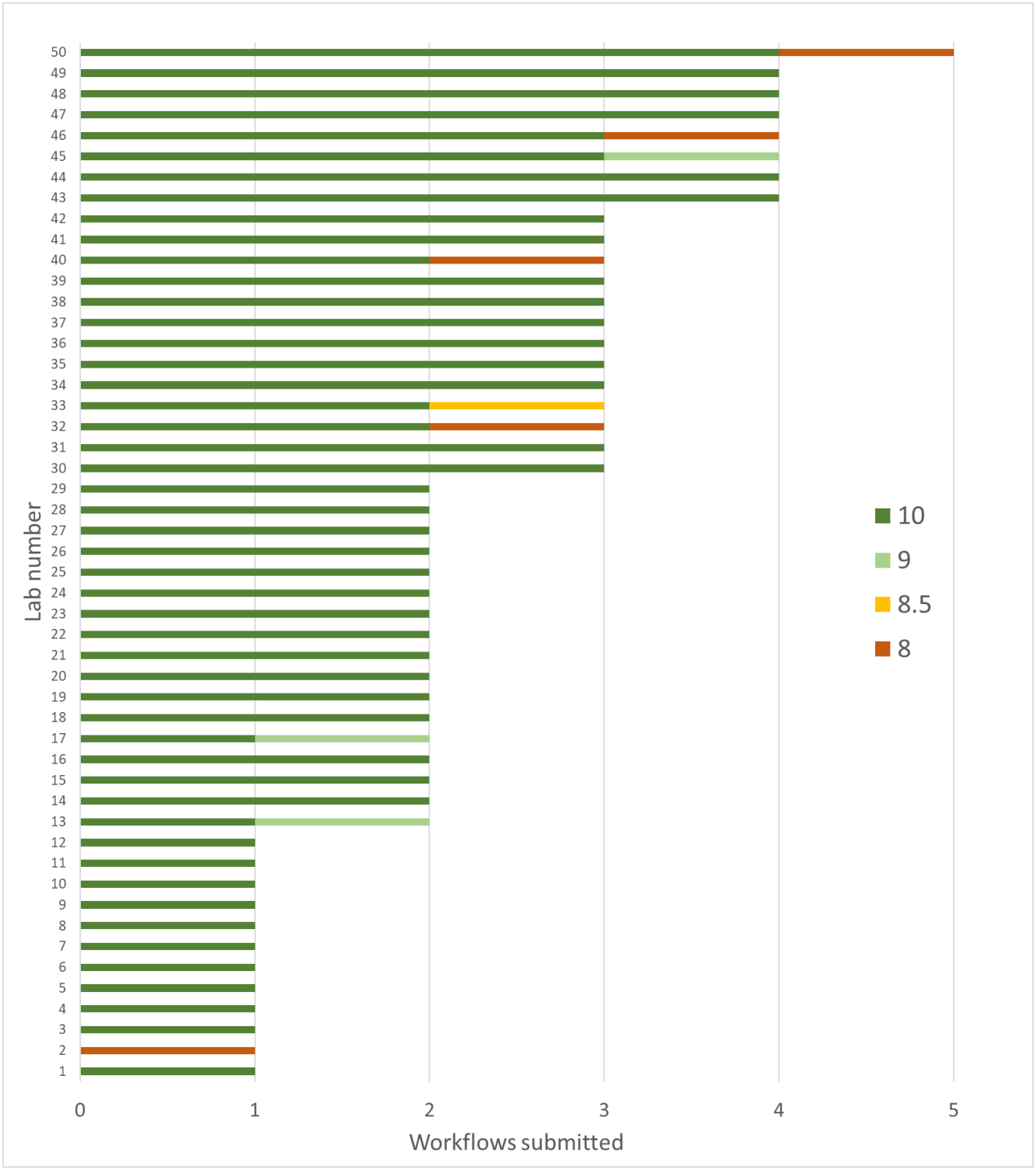


Figure 7.4 The equipment used for RNA amplification for the reported workflows.

7.2 Performance per workflow

In Supplementary Figure 7.5 the number of workflows reported per laboratory is shown, including the score obtained for each workflow for influenza A virus detection; subtyping results were not considered for the scoring. Each laboratory was given an ID number to maintain anonymity.
Only one lab scored an 8 overall for the one workflow which was reported. This was a limited panel, and the kit used was the BIOFIRE Respiratory panel 2.1 plus kit from BioMerieux, for which inconclusive results were reported. However, strictly speaking this is actually correct according to the IfU for an 'Equivocal' result, as is also shown in Supplementary Table 7.1 for the RIVM reference results for this kit. For this workflow, two of seven laboratories reported a final inconclusive result for the same panel specimens (specimens 1, 4 and 9). As mentioned in the Discussion section, this is not necessarily a bad thing, since it can provide a first indication for the detection of an animal influenza virus. Of the other five laboratories using this workflow, two reported 'inconclusive/positive' for the 'target result/final result' and three reported 'positive/positive'. This is not exactly according to the interpretation as dictated by the IfU. However, the laboratories reporting these 'target result/final result' combinations may have used information from additional testing to generate the 'positive' final result, as is also described in the IfU when an 'Equivocal' result is obtained.
Seven other workflows generated a negative result, especially for specimen 4 A(H1N1)v (7/7) and specimens 1 A(H7N2) (1/7), 6 A(H1N1)pdm09 (1/7) and 9 A(H1N2)v (1/7), resulting in a score lower than 10. Of these seven workflows, one used an LDT, one the Abbott ID NOW INFLUENZA A & B 2, two the Roche ePLEX Respiratory Pathogen Panel 2 and three the Seegene Allplex RV Essential or Master Assays. See chapter 3.4 for a response from the manufacturers and laboratories concerning these deviant results.
Nevertheless, most (42/50; 84%) laboratories reported only workflows scoring a 10, indicating that the performance of the tests used to detect animal influenza A virus is good.



Every lab was assigned a number to maintain anonymity.

Figure 7.5 Number of workflows submitted per lab and the scores obtained by these workflows.

7.3 In house RIVM results of commercial kits

Before distributing the panel containing the different viruses, it was tested at RIVM using in-house LTD as 'gold standard' and additionally with three commercial kits as a reference: the BioMerieux BIOFIRE® Repiratory Panel 2.1 plus, the Cepheid GeneXpert and the Pathofinder Respifinder 2Smart assays. The results are shown in supplementary Table 7.1.
The results of the laboratories using the BioFire kit were very similar to the results obtained in-house at RIVM. When inconclusive results were reported (the reporting module option for inconclusive or equivocal results), they were in line with the Equivocal Influenza A readouts we obtained.
For Cepheid GeneXpert workflows the highest Cq values were reported for panel specimen 4, the H1N1v zoonotic swine virus, which is consistent with the results we obtained.
The Pathofinder RespiFinder® 2Smart was only reported once in the EQA. This workflow was able to detect influenza A virus. However, it is also able to do a H1(pdm09) subtype detection, but this was not reported in the work (this is standard practice of the laboratory involved). As our results show, this test detects the H1(pdm09) subtype in panel specimen 6 correctly indeed, besides a successful generic influenza A virus detection for all specimens.

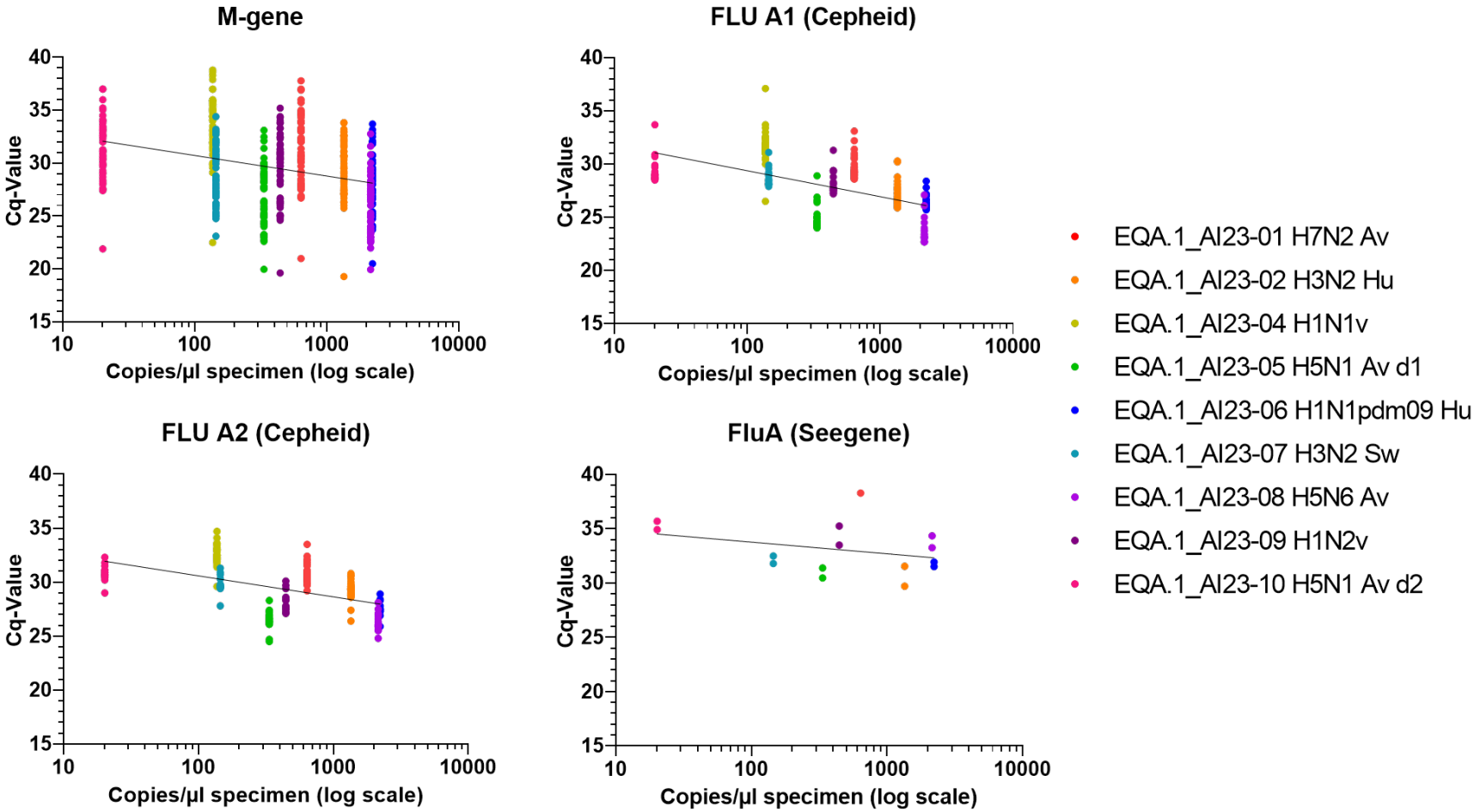
7.4 Correlation Cq value and number of copies/specimen

For the panel, the number of virus genome copies per specimen was determined using digital PCR (dPCR). In Supplementary Figure 7.6 the correlation between the number of copies and the Cq values of all reported workflows is depicted. A linear trendline is shown and indicates a correlation between the number of copies per specimen and the Cq value, despite a considerable bandwidth in Cq values among workflows per specimen. This indicates that the higher the Cq value is, the lower the number of copies in the specimen. However, an absolute correlation between Cq value and number of copies does not exist and is workflow dependent.
Panel specimen 4 with H1N1v virus had the higher number of deviant results. This specimen contained 137 copies/µl, which did not make it the specimen with the lowest number of copies, as this was panel specimen 10 (H5N1 d2, 20 copies/µl). A specimen with a similar number of copies was panel specimen 7 (H3N2 Swine, 145 copies/µl). However, these two specimens were found to have similar or lower Cq values by the workflows that failed to detect A(H1N1)v. This indicates that the number of copies is unlikely to be the source of the deviant results for panel specimen 4.

Table 7.1 Results of three commercial kits evaluated with the EQA panel at RIVM.

Specimen code	Virus	BIOFIRE[1] qualitative readout		GeneXpert[2] Cq value		Respifinder[3] qualitative readout	
		Detected	Equivocal	FLU A1	FLU A2	Influenza A	H1N1pdm09
EQA.1_AI23-01	H7N2	None	Influenza A	29.5	30.8	Positive	Negative
EQA.1_AI23-02	H3N2	Influenza A H3	None	26.4	29.0	Positive	Negative
EQA.1_AI23-03	n/a	None	None	No Cq	No Cq	Negative	Negative
EQA.1_AI23-04	H1N1v	None	Influenza A	31.9	32.9	Positive	Negative
EQA.1_AI23-05	H5N1 d1	Influenza A (no subtype detected)	None	24.5	26.5	Positive	Negative
EQA.1_AI23-06	H1N1pdm09	Influenza A H1-2009	None	26.3	27.4	Positive	Positive
EQA.1_AI23-07	H3N2 swine	Influenza A H3	None	28.7	29.9	Positive	Negative
EQA.1_AI23-08	H5N6	Influenza A (no subtype detected)	None	23.1	26.3	Positive	Negative
EQA.1_AI23-09	H1N2v	None	Influenza A	27.7	27.6	Positive	Negative
EQA.1_AI23-10	H5N1 d2	Influenza A (no subtype detected)	None	29.0	30.8	Positive	Negative

1. BioMerieux Diagnostics. BIOFIRE® Respiratory panel 2.1 plus
2. Cepheid. GeneXpert Xpress CoV-2/FLU/RSV plus
3. Pathofinder. RespiFinder® 2Smart



For every specimen the Cq values are shown as colored dots. On the x-axis the copies/µl specimen are depicted. With a nonlinear fit a trendline was created. With the virus names in the legend the host is shown: Av = avian; Hu = human; Sw = swine; v = variant, indicating human infected with swine virus; H5N1 Av d2 is a 1:10 dilution of H5N1 Av d1.

Figure 7.6 Correlation between Cq values and number of copies per specimen.

7.5 Participating laboratories

List of participating laboratories.

Name laboratory, city/island

Afd Medische Microbiologie. Radboudumc. Nijmegen
Atalmedial. Medisch Microbiologisch Laboratorium. sectie Moleculaire Biologie. Amsterdam
Canisius Wilhelmina ziekenhuis. Medische microbiologie en infectieziekten. Nijmegen
Catharina Ziekenhuis Eindhoven. Algemeen klinisch laboratorium. Eindhoven
CBSL. Tergooi MC. Hilversum
Certe. MMB Friesland en Noordoostpolder. Leeuwarden
Certe. Divisie MMB locatie Groningen/Drenthe. Groningen
Eurofins Medische Microbiologie. Leiden
Eurofins PAMM. Veldhoven
Fundashon Mariadal. Bonaire
Groene Hart Ziekenhuis. Gouda
Klinisch Microbiologisch Laboratorium Afd. Medische Microbiologie LUMC. Leiden
LABHOH/FSLMA. Aruba
Labmicta. Hengelo
Laboratorium voor Medische Diagnostiek. Ziekenhuis Rivierenland. Tiel
LMMI. Isala klinieken. Zwolle
Maasstad Laboratorium Maasstad Ziekenhuis. Rotterdam
MeanderMC. Amersfoort
Medical Laboratory Services. Curaçao
Medisch Microbiologisch en Immunologisch Laboratorium. Rijnstate Ziekenhuis. Arnhem
Medisch Microbiologisch Laboratorium. IJssellandziekenhuis. Capelle aan den IJssel
Medisch Microbiologisch Laboratorium. Ikazia Ziekenhuis. Rotterdam
Medisch Microbiologisch Laboratorium. OLVG Lab. Amsterdam
Medische Microbiologie & Hygiëne en Infectiepreventie. Jeroen Bosch Ziekenhuis. 's-Hertogenbosch
Medische Microbiologie & Infectiepreventie. UMC Groningen. Groningen
Medische Microbiologie en Immunologie. St. Antonius Ziekenhuis. Nieuwegein
Medische Microbiologie en Infectiepreventie. Eurofins Gelre. Apeldoorn
Medische Microbiologie. Noordwest Ziekenhuisgroep. Alkmaar
Medische Microbiologie. UMCU. Utrecht
Medische Microbiologie. Viecuri Medisch Centrum. Venlo
Medische Microbiologie. Ziekenhuis Gelderse Vallei; organisatie: Dicoon. Ede
Medische Microbiologie. Deventer Ziekenhuis. Deventer
Medische Microbiologie. Franciscus Gasthuis en Vlietland. Rotterdam
Medische Microbiologie. Haaglanden Medisch Centrum. Den Haag
Medische Microbiologie. Hagaziekenhuis. Den Haag
Medische Microbiologie, infectieziekten en infectiepreventie (MMI). Maastricht UMC+. Maastricht
Medische microbiologie. Laurentius ziekenhuis. Roermond
Medische Microbiologie. Ziekenhuis St Jansdal. Harderwijk
Medische Microbiologie. Zuyderland Medisch Centrum. Heerlen
Microvida. Laboratorium voor Medische Microbiologie en Immunologie. Tilburg

MMI Adrz. Goes/Vlissingen
MMI Diakonessenhuis Utrecht. Utrecht
Moleculaire technieken. afdeling medische microbiologie en infectiepreventie. AmsterdamUMC. Amsterdam
Mozand B.V. Eindhoven
Regionaal Laboratorium Medische Microbiologie Dordrecht-Gorinchem. Dordrecht/Gorinchem
Reinier Haga MDC. Delft/Zoetermeer
Saltro. Unilabs. Utrecht
Star-shl. Etten-Leur/Rotterdam
Streeklab Haarlem. Haarlem
Unit Klinische Virologie. Viroscience. Erasmus MC. Rotterdam

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