TECHNICAL REPORT

External quality assessment scheme for antiviral susceptibility detection in influenza viruses for the Community Network of Reference Laboratories for Human Influenza in Europe

2010/2011

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External quality assessment (EQA) scheme for antiviral susceptibility detection in influenza viruses for the Community Network of Reference Laboratories for Human Influenza in Europe (CNRL)

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This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Eeva Broberg (ECDC), and produced by Catherine Thompson, Kate Howell, Praveen Sebastian-Pillai and Maria Zambon, Health Protection Agency, London, UK, on behalf of the members of the 'Quality and Training' and 'Antiviral' task groups and the Community Network of Reference Laboratories for Human Influenza in Europe (CNRL).

Catherine Thompson, Angie Lackenby and Maria Zambon (Health Protection Agency, London, UK), and Adam Meijer (RIVM, Bilthoven, Netherlands) contributed to the design, planning and analysis of the exercise. The panel was produced by the Respiratory Virus Unit, Health Protection Agency, London, UK.

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Contents

Abbreviations

CNRL	Community Network of Reference Laboratories for Human Influenza in Europe
EISN	European Influenza Surveillance Network
EQA	External quality assessment
GISRS	Global Influenza Surveillance and Response System
HA	Haemagglutinin
HPA	Health Protection Agency
IC_{50}	50% inhibitory concentration
MUNANA	2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate
NA	Neuraminidase
NAI	Neuraminidase inhibitors
NIC	National Influenza Centre
NIMR	National Institute for Medical Research
PCR	Polymerase chain reaction
RIVM	National Institute for Public Health and the Environment
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
WHO	World Health Organization
WHO-CC	World Health Organization Collaborating Centre for Reference and Research on Influenza

Executive summary

The first European external quality assessment (EQA) exercise for antiviral susceptibility detection in influenza viruses was carried out during winter 2010/11. The objectives of the exercise were to a) offer participants an independent mechanism to check performance, and b) provide information on performance of antiviral susceptibility testing at the network level.

Twenty different laboratories from 16 European countries (see Annex 1) participated in the exercise. Each participant received a panel of ten coded samples, including recent influenza A and B viruses containing substitutions known to confer resistance to antiviral drugs. Participants tested the viruses using the antiviral susceptibility testing methodology currently used in their laboratories. Although there was good participation from CNRL member laboratories (~60%), many laboratories chose not to participate, suggesting there is further potential for development within the network.

All twenty participating laboratories returned good results from the genotypic detection of the H275Y mutation in influenza A(H1N1)pdm09 virus (90% correctly reported consensus result), demonstrating that these laboratories have rapidly and successfully implemented the new assays since the emergence of the pandemic virus in April 2009. The proficiency for detection of a mixture of resistant and sensitive A(H1N1)pdm09 viruses was lower, although the majority of laboratories (79%) did identify resistance in this sample. Analysis suggested that sequencing methods may have some limitations regarding mixture identification.

Fewer laboratories (55–75%) performed genotypic tests for other influenza (sub)types. The priority for expansion of these assays within the network would depend on the risk of resistance emergence. Fifteen laboratories returned good results for amantadine resistance detection. The widespread resistance and limited use of these drugs suggests this is a lower priority for capacity development.

Twelve laboratories reported phenotypic testing results, while eight laboratories did not, possibly due to limited resources or lack of technical support for this test. Areas of difficulty included influenza A(H1N1)pdm09 mixture analysis, analysis of NAI-resistant H3N2 and influenza B virus. Variation between genotypic and phenotypic susceptibility testing was observed, suggesting that results achieved with different assays are not easily compared.

Participants were asked to provide an interpretation of variations in the results for genotypic and phenotypic testing in terms of virus susceptibility to antiviral drugs. This was a challenging aspect of the exercise, leading to different interpretations for similar results. The interpretations of genotypic test results varied widely (20–95% correct match to consensus), compared with the interpretations of phenotypic results, which were more consistent but still showed some variation (67–100% correct match to consensus). As there is no widely accepted definition of resistance or reduced susceptibility, results are often subject to individual interpretation. Furthermore, different countries use locally defined baseline values for phenotypic testing, which could lead to varying interpretations. One of the goals of the EQA was to gain insights into these variations, which could inform work towards harmonisation of interpretation of antiviral data and reporting of results.

Provision of guidelines on the interpretation of resistance mutations and phenotyping results would help to improve consistency. Other strategies towards achieving consistency between laboratories include the harmonisation of baseline values and the provision of a reference set of viruses. A reference panel of influenza A and B viruses for assessment of resistance to neuraminidase inhibitors has been developed and is available from the *isirv* Antiviral Group. The technical challenges of implementing phenotype testing should be taken into account for future activities towards the development of the network, which may be best served by a supranational phenotype testing service.

Future capacity building should focus on the development of widespread capability for genotypic and phenotypic detection of oseltamivir resistance in A(H1N1)pdm09 and other human influenza viruses. Although global oseltamivir resistance is currently at a low level, it is acknowledged that there is real potential for the emergence of oseltamivir resistance in A(H1N1)pdm09 viruses.

The results of this exercise will be used to guide the development of the network and promote harmonisation of antiviral susceptibility testing, data interpretation and reporting through the implementation of recommendations for further improvement and additional training activities.

1 Introduction

The European Centre for Disease Prevention and Control (ECDC) is a European Union agency with a mandate to identify, assess, and communicate current and emerging threats to human health from communicable diseases, as well as operate dedicated disease surveillance networks. Within its mission, ECDC shall 'foster the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents which may threaten public health. The Centre shall maintain and extend such cooperation and support the implementation of quality assurance schemes.' (Article 5.3, EC 851/2004[1])

External quality assessment (EQA) schemes are an integral part of quality assurance: the performance of laboratories is assessed through an external evaluator and with material that is supplied specifically for this purpose. ECDC's disease-specific networks organise a series of EQA for EU/EEA countries, with the aim to identify needs for improvement in laboratory diagnostic capacity relevant to the surveillance of diseases listed in Decision No 2119/98/EC, and to ensure the reliability and comparability of results from laboratories in all EU/EEA countries. The main objectives of external quality assessment schemes include:

- assessment of the general standard of performance ('state of the art');
- assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration);
- evaluation of individual laboratory performance;
- identification and justification of problem areas;
- provision of continuing education; and
- identification of training needs.

WHO-recognised National Influenza Centres (NICs) in the European Union are part of the Community Network of Reference Laboratories for Human Influenza in Europe (CNRL) and the European Influenza Surveillance Network (EISN). Virological surveillance activities are supported by the CNRL Coordination Group and CNRL Task Groups composed of experts from member laboratories. The objectives of CNRL are to provide high-quality reference services for human influenza surveillance, early warning and pandemic preparedness in Europe [5, 6]. One of the ways this can be achieved is by Europe-wide harmonisation and standardisation of laboratory methods and monitoring through quality assessment exercises.

In recent years there has been increased clinical use of antiviral drugs against influenza. The neuraminidase inhibitors (NAI) oseltamivir and zanamivir were developed by structure-based drug design to mimic the natural substrate of NA, sialic acid. The close similarity to the natural substrate was predicted to limit the emergence of resistance. The adamantanes class of M2 inhibitors have been available longer, and resistance is now widespread.

Emergence of antiviral resistance is closely monitored through virological surveillance. Very few cases of resistance were found during clinical trials and post-licensure surveillance. Mutations in the NA gene associated with NAI resistance were described from the few cases of clinical resistance and *in vitro*-generated resistance that emerged. In 2007, naturally occurring resistance to oseltamivir due to a histidine-to-tyrosine mutation at position 275 in the N1 NA (H275Y) was observed in former seasonal A(H1N1) viruses, and the resistant virus rapidly spread worldwide [44]. NAI resistance in other influenza A subtypes and influenza B has not been widely observed.

During the 2009 H1N1 pandemic, oseltamivir was widely used for treatment and control of spread. A small number of A(H1N1)pdm09 viruses resistant to oseltamivir due to the H275Y mutation have been observed since the emergence of the virus; this situation is closely monitored by CNRL and the WHO Global Influenza Surveillance and Response System (GISRS).

Widespread clinical use of oseltamivir has created a demand for antiviral susceptibility testing to monitor the potential emergence of resistant viruses in treated patients. Genotyping tests based on SNP PCR or sequencing have been developed, particularly for A(H1N1)pdm09 virus to screen for the H275Y mutation [7]. Genotyping tests for resistance mutations in other influenza A subtypes and influenza B are also performed in some laboratories [1, 3]. The H275Y mutation in the H1N1 subtype is sufficiently well-characterised that laboratory tests can be targeted to detection of this single mutation. This is not true of the H3N2 subtype and influenza B, where several mutations have been identified that generate resistance or reduced susceptibility and a broader testing strategy is required. The gold standard phenotyping test requires cultured viruses and is not as widely performed [8]. Phenotyping tests (IC_{50} assay) can detect changes in NAI susceptibility (resistance or reduced susceptibility) due to known and unknown mutations in influenza A and B viruses. Standard baseline values for interpretation of IC_{50} assay data as sensitive, showing reduced susceptibility, or resistant have not been defined. Laboratories frequently use in-house data to determine baseline values for interpretation of IC_{50} values, and this lack of standardisation can lead to inter-laboratory variation in definitions of resistance.

[1] Regulation (EC) no 851/2004 of the European Parliament and of the Council of 21 April 2004 establishing a European Centre for Disease Prevention and Control

The increased use of laboratory diagnostics for detection of influenza antiviral resistance requires that the quality of testing is monitored. This EQA was designed to provide laboratories in the European network with an independent mechanism to check the quality of results and performance of tests currently implemented in their laboratories. It was also designed to provide insights into the performance of different techniques used for the detection of antiviral resistance in influenza viruses in European laboratories, thus helping CNRL to determine training priorities and produce guidelines on the harmonisation of interpretation of antiviral data and the reporting of results.

This report presents the results of the first European-level EQA for the detection of antiviral resistance in influenza viruses. By providing an EQA scheme and encouraging laboratories to participate the organisers hope to ensure high-quality results which will then be reported as part of the virological surveillance data.

2 Objectives

The objectives of this quality assessment exercise were:

- to design an EQA scheme utilising a panel of influenza A and B viruses with known mutations in the NA or M2 genes conferring resistance to NAI and adamantanes, respectively; and to include sensitive controls, for genotypic and phenotypic antiviral susceptibility characterisation, by CNRL laboratories that already have antiviral susceptibility testing in place;
- to provide participants in the EQA exercise with an independent mechanism to assess the performance of the influenza antiviral susceptibility testing methodology used in their laboratories;
- to gain insights into the performance of different techniques used for influenza antiviral susceptibility testing in European laboratories, thus helping CNRL to determine training priorities and produce guidelines on the harmonisation of interpretation of antiviral data and the reporting of results.

3 Study design

3.1 Organisation

The influenza virus antiviral susceptibility detection EQA was the first European (and global) level exercise of its kind. The EQA was proposed by the CNRL Coordination Group and designed by the CNRL task groups TG3 'Antiviral' and TG5 'Quality and Training'. The EQA panel was prepared and tested by the Respiratory Virus Unit at the Health Protection Agency (HPA), London UK. Further pre- testing was performed by the WHO-CC at the National Institute for Medical Research (NIMR) at Mill Hill, London, UK, and the Influenza Laboratory at the National Institute for Public Health and the Environment (RIVM), Netherlands. All panel samples were distributed to each participating laboratory by specialist courier frozen on dry ice. Participants submitted results electronically to a web-based database.

3.2 Participation

CNRL member laboratories include all EU countries as well as Norway and Iceland. All influenza laboratory contact points in the CNRL were notified in advance of the EQA exercise. Laboratories with any form of genotypic and/or phenotypic testing in place for any influenza subtype were expected to participate. Laboratories that did not undertake any antiviral susceptibility testing could choose not to participate in the EQA exercise. These laboratories were asked to indicate their usual method of referral of specimens or viruses for antiviral susceptibility testing and the expected turnaround time for results.

3.3 Panel description

The EQA panel consisted of ten samples containing inactivated influenza viruses from subtypes that are currently or have recently circulated in humans including A(H1N1)pdm09, former seasonal H1N1, H3N2 and influenza B. The viruses carried known mutations conferring resistance to antiviral drugs used to treat influenza infection, including oseltamivir, zanamivir and the adamantanes. Sensitive control viruses for each influenza subtype were also included. The selected viruses carried well characterised mutations conferring resistance that have been identified in the clinic or during clinical trials of the antiviral drugs.

Viruses were grown in MDCK or MDCK-SIAT1 cells and the HA titre determined. Viruses were diluted to a suitable concentration for testing and inactivated by a validated method which retains neuraminidase (NA) activity and RNA integrity [2].Viruses were aliquoted and stored frozen at −80 °C until required. One panel was thawed and pre-tested at the HPA using in-house genotypic and phenotypic methods. Panels were sent frozen on dry ice to two independent laboratories for pre-testing. The final panel content were shipped frozen on dry ice by specialist courier to participants between 29 November and 13 December 2010. The deadline for results return was 31 January 2011, and a web-based database was used by the participants to submit results.

3.4 Participant testing

Participants were asked to test the panel using the standard laboratory protocols normally used by their laboratory to characterise antiviral susceptibility. Genotypic methods included SNP detection by PCR, pyro-sequencing and partial or full-length gene sequencing. Phenotypic methods included fluorescent IC_{50} assay using the MUNANA substrate or the chemiluminescent NA-STAR assay. The virus subtype was identified on the sample description allowing the laboratory to select panel samples for genotypic testing according to the availability of subtype specific protocols in the laboratory. Laboratories were asked to identify the presence of mutations commonly associated with antiviral resistance, measure IC_{50} values and provide an interpretation of the results, namely whether the virus was resistant (R), sensitive (S) or showed reduced susceptibility (RS) to the antiviral drugs oseltamivir, zanamivir and the adamantanes.

3.5 Data reporting

For genotyping, testing participants were asked to describe which SNPs had been evaluated and which mutations had been detected in either the NA or M2 gene. An interpretation of the genotype with regard to susceptibility to oseltamivir, zanamivir and adamantanes was requested. Data was collected on the type of assay and equipment used for PCR, pyro-sequencing and sequencing, the region of the gene analysed, the method or SOP used (if published), the nucleic acid extraction method, the use of controls and/or standards and whether the samples were tested singly or in duplicate. An example of the genotyping results return sheet and questionnaire can be found in Annex 2.

For phenotypic testing, participants were asked to provide the sample dilution used for testing and for the IC_{50} values determined in assays with oseltamivir and zanamivir. An interpretation of the phenotype with respect to susceptibility to oseltamivir and zanamivir was requested. As standard baseline values and interpretations have not been defined, the interpretation provided would be dependent upon the statistical methods and baseline values used for data analysis in each laboratory. Data was collected on the method, equipment, controls used, whether NA activity was determined prior to testing, and the statistical methods used for data analysis. An example of the phenotyping results return sheet and questionnaire can be found in Annex 3.

4 Results

4.1 Panel composition and expected results

The influenza type, subtype, strain characterisation and antiviral susceptibility profile with associated mutations in NA or M2 genes for each sample in the EQA panel are shown in the expected results table (Table 1). Amino acid numbering corresponding to the relevant NA subtype is used throughout.

Table 1. Panel composition and expected results for the influenza virus antiviral resistance detection EQA 2010

Panel Code	Influenza type	Influenza subtype	Strain designation (*prototype strain*)	NA gene identity	M2 gene identity	Sensitive	Resistant
EISN_AV10-01	A	A(H1N1) pdm09	A/California/7/2009	H275	S31N	OS[2], ZN[3]	AM[4]
EISN_AV10-02	A	A(H1N1) pdm09	A/England/1434/2009 (*A/California/7/2009-like*) A/California/7/2009	H275Y mixture	S31N	ZN	AM, OS
EISN_AV10-03	A	H3N2	A/Okayama/23/2004[1]	R292	S31	AM, OS, ZN	
EISN_AV10-04	A	H1N1 (former seasonal)	A/England/313/2008 (*A/Brisbane/59/2007-like*)	H275	S31	AM, OS, ZN	
EISN_AV10-05	A	H3N2	A/Okayama/23/2004[1]	R292K	S31	AM	OS, ZN
EISN_AV10-06	A	H3N2	A/Lisbon/1/2008		V27A, S31N	OS, ZN	AM
EISN_AV10-07	B		B/England/137/2006	I221		OS, ZN	
EISN_AV10-08	A	H1N1 (former seasonal)	A/England/26/2008 (*A/SolomonIslands/3/2006-like*)	H275Y	S31	AM, ZN	OS
EISN_AV10-09	A	A(H1N1) pdm09	A/England/1434/2009 (*A/California/7/2009-like*)	H275Y	S31N	ZN	AM, OS
EISN_AV10-10	B		B/England/MH48/2006	I221T		ZN	OS

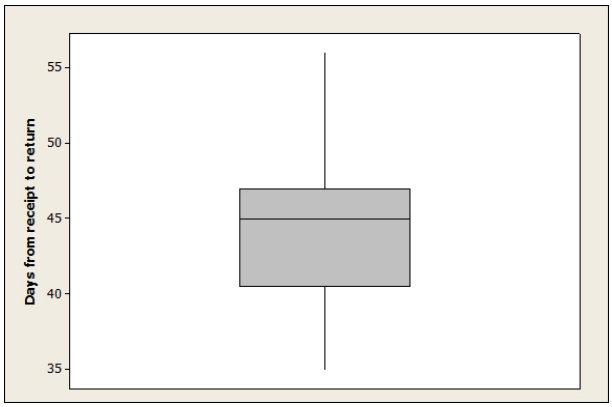
[1] *Plaque-purified variants*
[2] *OS: oseltamivir*
[3] *ZN: zanamivir*
[4] *AM: adamantanes (includes amantadine and rimantadine)*

The influenza subtype was identified in the description of each panel sample so that participants could target subtype-specific tests appropriately. The strain designation was provided to laboratories in the expected results letter which was distributed when testing was complete. The strain designation together with the complete gene sequences would allow laboratories to check specificity of primers and probes used in testing.

4.2 Reporting time and participation

The time taken from receipt of the panel to reporting of results is shown in Figure 1. The time period between courier delivery and panel receipt was not considered. Panel distribution was unexpectedly delayed and the reporting times extended due to the holiday period. For antiviral analysis, 14/24 (58.3%) reported a panel receipt, and results were included in the analysis shown in Figure 1. Of the participants not included (n=10; 41.7%), six did not return a panel receipt but returned results, and four did not return a panel receipt or results. The due date for return of results was 31 January 2011. The median time taken for participants to return results (from the reported date of receipt) was 45 days, and the mean time taken was 44 days. The percentage of respondents reporting on time was 93% (n=13).

Figure 1. Time taken for return of antiviral results



Box and whisker plot showing the time taken from receipt of the panel to reporting of results. The number of days required by participants to return antiviral results is presented. The date participants reported receipt of the panel samples was considered as the start date (courier date not considered) (see Annex 4).

Twenty-four laboratories received the panel, and 20 laboratories returned full or partial results for genotyping analysis. Twelve laboratories returned results for phenotype analysis. The number of laboratories returning genotyping analysis for the NA and M2 genes varied according to the influenza type/subtype (Table 2). Phenotype analysis was performed for all subtypes by the 12 laboratories that undertook this testing.

Table 2. Overview of number of laboratories reporting results by subtype

Influenza type/subtype	NA genotyping (N=20)	M2 genotyping (N=20)
A(H1N1) pdm09	19–20 (95–100%)	14–15 (70–75%)
A/H1N1 (formerseasonal)	15 (75%)	13–14 (65–70%)
A/H3N2	11–13 (55–65%)	13–14 (65–70%)
B	11–12 (55–60%)	NA

NA: Not applicable

4.3 Genotypic testing for NA gene substitutions

Participants were asked to screen for mutations in the NA gene that confer resistance to oseltamivir and/or zanamivir using the genotypic methods currently available in their laboratory, to report the NA gene SNP identity, and to give an interpretation of the results. The following NA gene mutations were included: H275Y mutation in influenza A H1N1 which confers resistance to oseltamivir; R292K mutation in H3N2 which confers resistance to oseltamivir and zanamivir; I221T mutation in influenza B which confers resistance to oseltamivir.

Table 3 shows a summary of the number of laboratories returning results for each panel sample, the substitutions identified in the NA gene by the participants, the number and percentage results matching the consensus, and the non-consensus results. NA genotyping results by participating laboratory are shown in Annex 5.Results matching the consensus NA gene substitution ranged from 47% to 100%. Sample EISN_AV10-02 which contained a 60:40 mixture of sensitive and resistant A(H1N1)pdm09 viruses proved the most difficult sample to analyse, with nine participants (47%) correctly reporting a mixture in this sample, six participants reporting the H275Y substitution, and four laboratories reporting no substitution. All participants correctly identified the H275Y substitution in sample EISN_AV10-09 although two laboratories reported a mixture in this sample. A comprehensive analysis of results can be found in Annex 13.

Table 3. Results for genotypic analysis of NA gene substitutions

Panel code	Influenza type/subtype	NA gene SNP identity	Number of laboratories returning results	Number of laboratories reporting consensus (%)	Non-consensus results reported (number of reports)
EISN_AV10-01	A(H1N1)pdm09	H275	20	15 (75%)	H275Y (1); other mutations (2); ns (2)
EISN_AV10-02	A(H1N1)pdm09	H275Y mixture	19	9 (47%)	none(4); H275Y(6)
EISN_AV10-03	A/H3N2	R292	11	11 (100%)	
EISN_AV10-04	A/H1N1 (formerseasonal)	H275	15	13 (87%)	H275Y(1); N21S(1)

Panel code	Influenza type/subtype	NA gene SNP identity	Number of laboratories returning results	Number of laboratories reporting consensus (%)	Non-consensus results reported (number of reports)
EISN_AV10-05	A/H3N2	R292K	13	12 (92%)	no mutation (1)
EISN_AV10-06	A/H3N2		11	10 (91%)	other mutation (1)
EISN_AV10-07	B	I221	11	7 (64%)	R152K, G402S (2); other(2)
EISN_AV10-08	A/H1N1 (former seasonal)	H275Y	15	15 (100%)	
EISN_AV10-09	A(H1N1)pdm09	H275Y	20	18 (90%)	H275Y mixture (2)
EISN_AV10-10	B	I221T	12	9 (75%)	G402S(2); none (1)

ns = Not specified

4.4 Genotypic testing for M2 gene substitutions

Participants were asked to screen for mutations in the M2 gene that confer resistance to adamantanes (amantadine and rimantadine) using the genotypic methods currently available in their laboratory, to report the M2 gene SNP identity and to give an interpretation of the results. The following M2 gene mutations were included: S31N mutation in influenza A(H1N1) which confers resistance to adamantanes; V27A and S31N mutation in H3N2 which confers resistance to adamantanes. Influenza B viruses are not susceptible to the adamantanes so this analysis was not relevant for samples EISN_AV10-07 and EISN_AV10-10.

Table 4 shows a summary of the number of laboratories returning results for each panel sample, the substitutions identified in the M2 gene by the participants, the number and percentage results matching the consensus, and the non-consensus results. M2 genotyping results by participating laboratory are shown in Annex 6.

The percentage of correct results ranged from 69% to 93%. The main determinant of amantadine resistance is the S31N substitution in the M2 gene. In the majority of cases the correct result was reported although there were a few instances where the S31N substitution was not detected. The sample with the lowest score was EISN_AV10-06, which contained two substitutions V27A and S31N associated with resistance to adamantanes. Nine (69%) participants reported both V27A and S31N mutations, two participants reported S31N only, one participant reported V27A only, and one participant reported another mutation. A comprehensive analysis of results can be found in Annex 13.

Table 4. Results for genotypic analysis for M2 gene substitutions

Panel code	Influenza type/subtype	M2 gene SNP identity	Number of laboratories returning results	Number of laboratories reporting consensus (%)	Non-consensus results reported (number of reports)
EISN_AV10-01	A(H1N1)pdm09	S31N	15	13 (87%)	none (2)
EISN_AV10-02	A(H1N1)pdm09	S31N	15	13 (87%)	none (2)
EISN_AV10-03	A/H3N2	S31	13	11 (85%)	S23N (1); S14N, N22S, V42I, K47R, G80S (1)
EISN_AV10-04	A/H1N1 (former seasonal)	S31	13	12 (92%)	L36V (1)
EISN_AV10-05	A/H3N2	S31	14	11 (79%)	S23N (1); S14N,N22S,V42I,K47R, G80S (1); ns (1)
EISN_AV10-06	A/H3N2	V27A, S31N	13	9 (69%)	S31N (2); V27A (1); V13A (1)
EISN_AV10-07	B	na	na	na	na
EISN_AV10-08	A/H1N1 (former seasonal)	S31	14	13 (93%)	L36V (1)
EISN_AV10-09	A(H1N1)pdm09	S31N	14	10 (71%)	none (3); ns (1)
EISN_AV10-10	B	na	na	na	na

na: not applicable; ns: not specified

4.5 Results of phenotypic testing

Twelve (60%) of the 20 CNRL laboratories that participated in the EQA performed phenotypic testing and returned IC_{50} values (nM) and corresponding susceptibility phenotype interpretations (Figure 2). Laboratories tested for susceptibility to both oseltamivir (12/12; 100%) and zanamivir (11/12; 92%). Phenotypic testing was performed by in-house fluorescence IC_{50} assay using the MUNANA substrate (nine laboratories; 75%) and chemiluminescence

IC_{50} assay using the NA-*Star* Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems) (three laboratories; 25%). NA activity was determined prior to IC_{50} assay by nine (75%) participants. A comprehensive analysis of results can be found in Annex 13.

4.6 Interpretation of influenza antiviral susceptibility

Participants were required to make an interpretation of their results in terms of virus susceptibility to antiviral drugs. The number and percentage of interpretations that fully matched the consensus interpretation defined for each sample were determined for sensitive phenotype (Table 5) and resistant phenotype (Table 6).

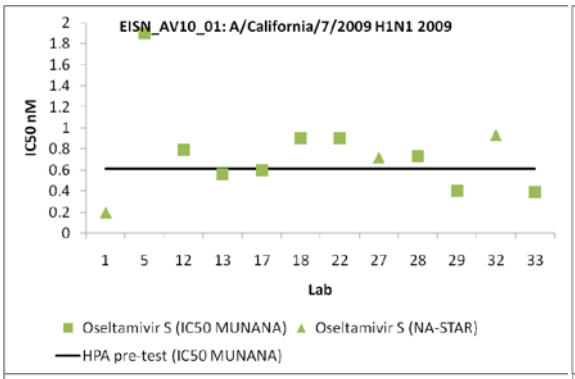
Interpretation of genotyping results by participating laboratory are shown in Annexes 7 and 8. Interpretation of phenotyping results by participating laboratory are shown in Annexes 9 and 10.

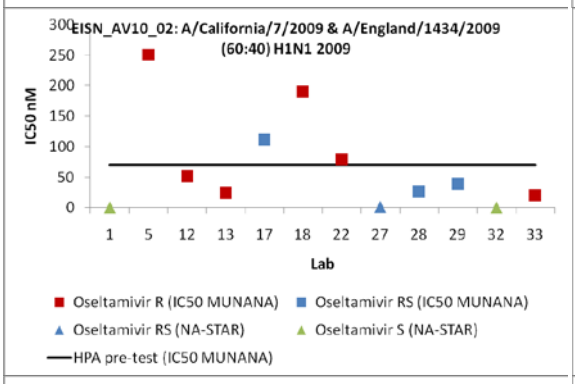
The baseline values used by participating laboratories for interpretation of IC_{50} assay results with oseltamivir and zanamivir, respectively, are shown in Annexes 11 and 12.

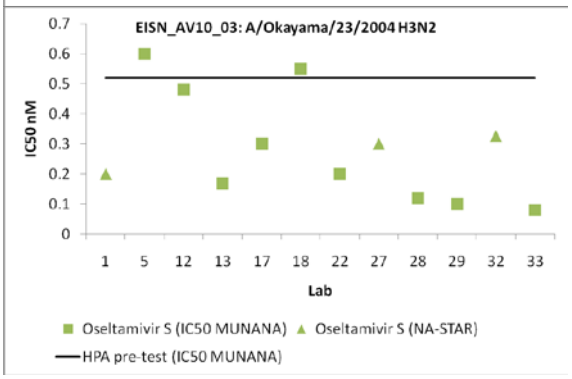
Annex 13 provides a comprehensive analysis of genotyping and phenotyping results as well as interpretations for each sample ordered by influenza type/subtype.

Figure 2. Phenotypic testing for oseltamivir and zanamivir susceptibility: IC_{50} values by laboratory

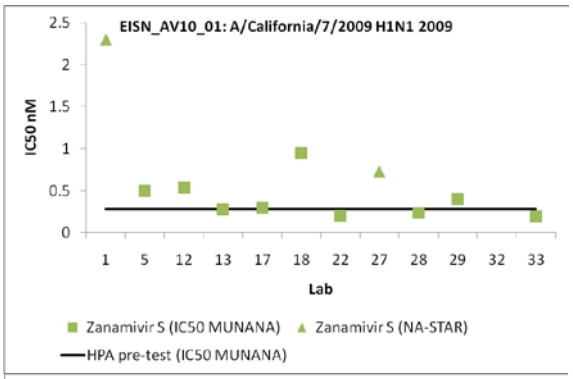
Oseltamivir

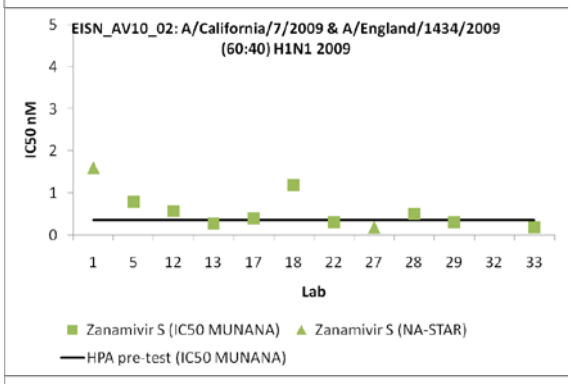


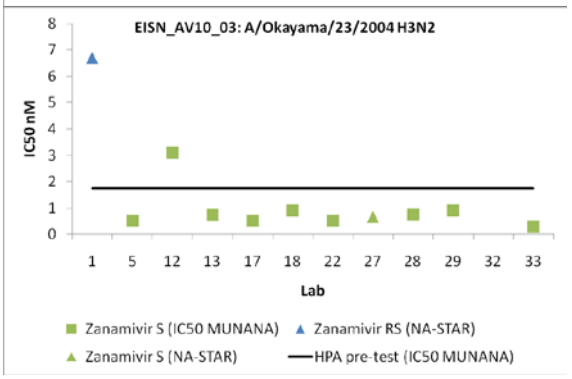




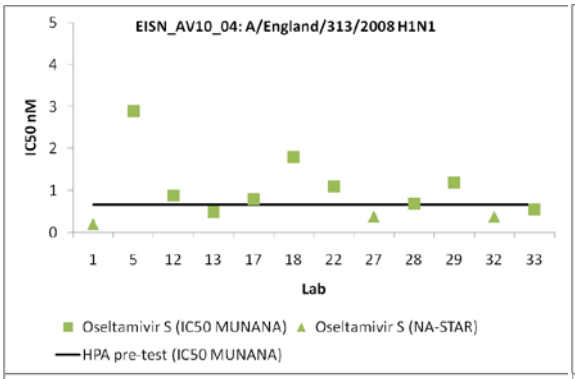
Zanamivir

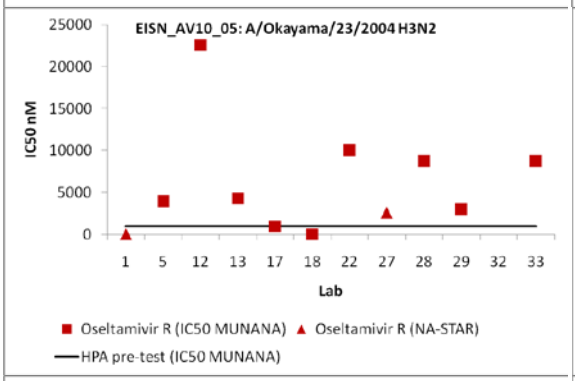


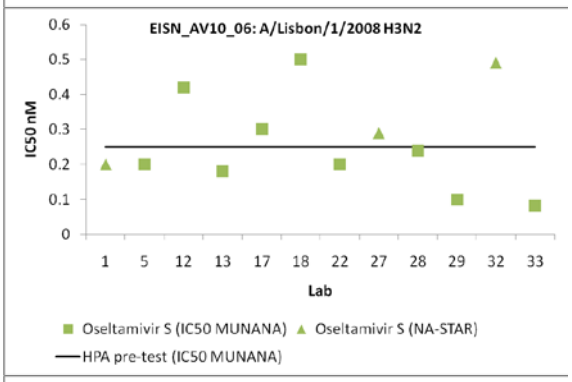


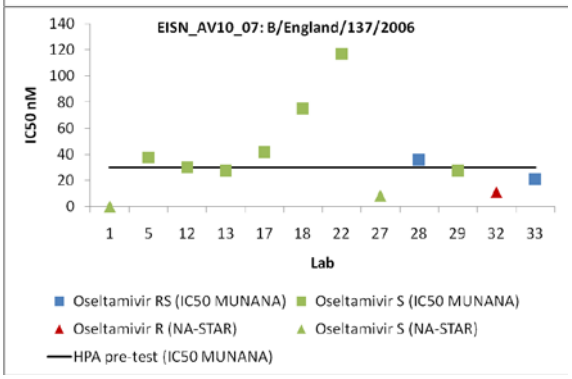


Oseltamivir

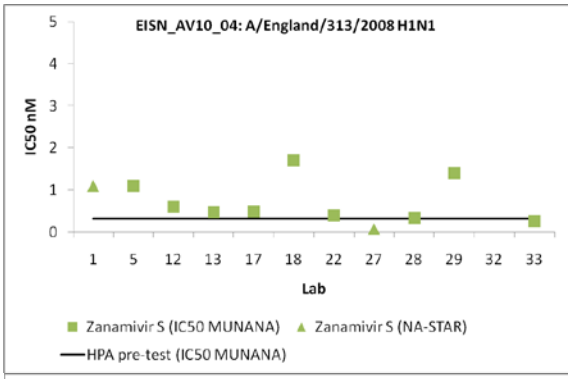


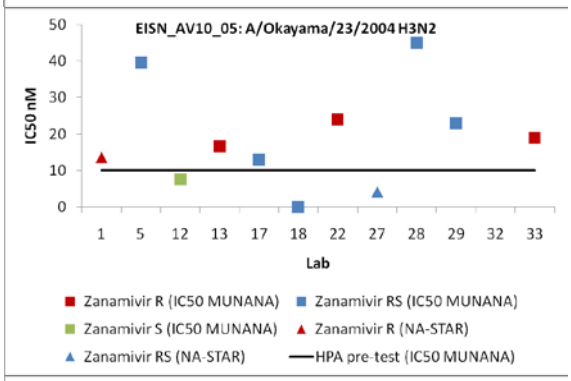


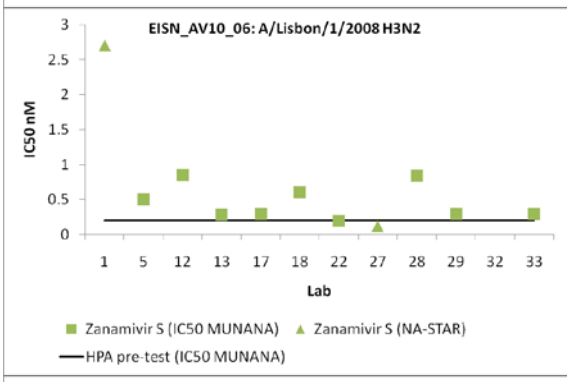


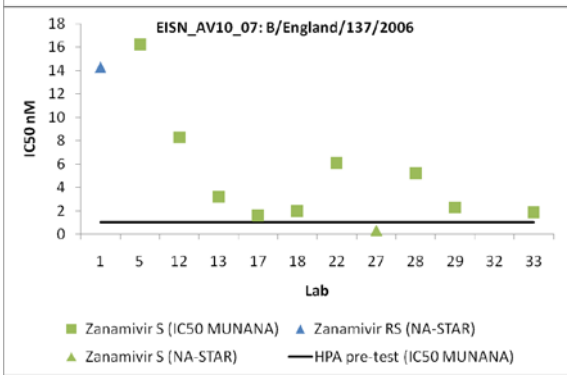


Zanamivir



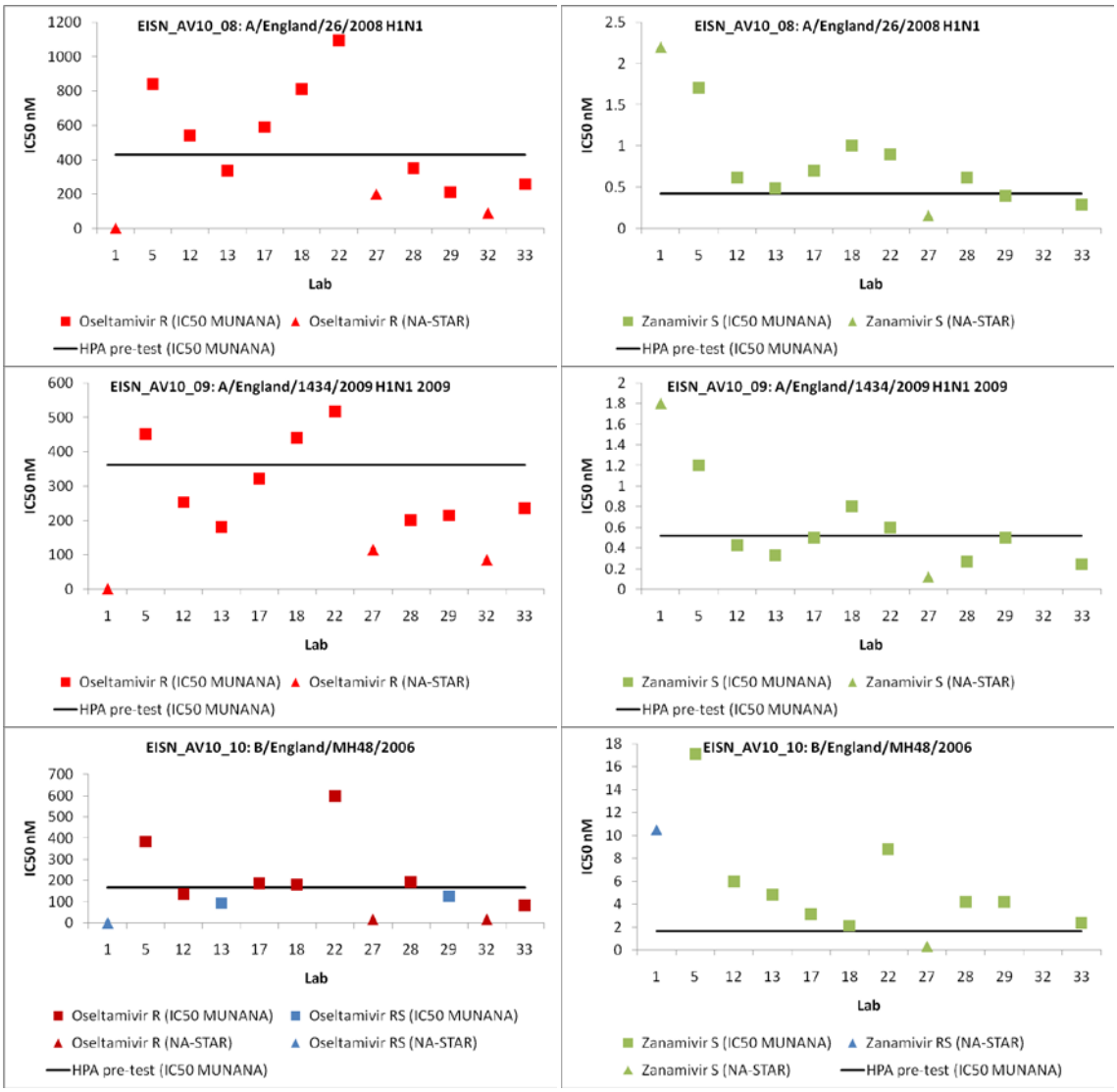






Oseltamivir

Zanamivir



The IC_{50} values (nM) reported by each laboratory are shown for oseltamivir and zanamivir separately for each sample. The IC_{50} value obtained in pre-testing using fluorescent MUNANA substrate is indicated (black line). Laboratories using the in-house IC_{50} assays with the fluorescent MUNANA substrate (□) or chemiluminescence assay using NA-Star substrate (Δ) are indicated. The interpretation of antiviral susceptibility phenotype provided by the laboratory for each IC_{50} value are shown as sensitive (S) (green), reduced susceptibility (RS) (blue), or resistant (R) (red).

Table 5. Number and percentage of influenza antiviral sensitivity interpretations

Sample	Sample content	Sensitive	Summary of results reported							
			Genotypic (20/24 returned)				Phenotypic (12/24 returned)			
			Consensus		Non-consensus		Consensus		Non-consensus	
			n	(%)	n	(%)	n	(%)	n	(%)
EISN_AV10-01	A/California/7/2009 (H1N1v)	Oseltamivir/zanamivir	10	50.0	10	50.0	11	91.7	1	8.3
EISN_AV10-02	A/England/1434/2009 (H1N1v) + A/California/7/2009 (H1N1v)	Zanamivir	8	40.0	12	60.0	11	91.7	1	8.3
EISN_AV10-03	A/Okayama/23/2004 (H3N2) §	Adamantanes/oseltamivir/zanamivir	8	40.0	12	60.0	10	83.3	2	16.7
EISN_AV10-04	A/England/313/2008 (H1N1)	Adamantanes/oseltamivir/zanamivir	9	45.0	11	55.0	11	91.7	1	8.3
EISN_AV10-05	A/Okayama/23/2004 (H3N2) §	Adamantanes	12	60.0	8	40.0	10	83.3	2	16.7
EISN_AV10-06	A/Lisbon/1/2008 (H3N2)	Oseltamivir/zanamivir	9	45.0	11	55.0	11	91.7	1	8.3
EISN_AV10-07	B/England/137/2006	Oseltamivir/zanamivir	6	30.0	14	70.0	8	66.7	4	33.3
EISN_AV10-08	A/England/26/2008 (H1N1)	Adamantanes/zanamivir	8	40.0	12	60.0	11	91.7	1	8.3
EISN_AV10-09	A/England/1434/2009 (H1N1v)	Zanamivir	9	45.0	11	55.0	11	91.7	1	8.3
EISN_AV10-10	B/England/MH48/2006	Zanamivir	4	20.0	16	80.0	10	83.3	2	16.7

Sample: *Unique code for each EISN panel sample.*
Sample content: *Full strain designation for each panel sample.*
Sensitive: *Antiviral sensitivities for each panel sample.*
Genotypic: *Number and percentage of laboratories reporting the full consensus interpretation of genotypic results for each panel sample.*
Phenotypic: *Number and percentage of laboratories reporting the full consensus interpretation of phenotypic results for each panel sample. Adamantanes were not considered when determining the number and percentage of consensus interpretations for the phenotypic results.*

H1N1v refers to influenza A(H1N1)pdm09 virus.

Table 6. Number and percentage of influenza antiviral resistance interpretations

Sample	Sample content	Resistant	Summary of results reported							
			Genotypic (20/24 returned)				Phenotypic (12/24 returned)			
			Consensus		Non-consensus		Consensus		Non-consensus	
			n	(%)	n	(%)	n	(%)	n	(%)
EISN_AV10-01	A/California/7/2009 (H1N1v)	Adamantanes	11	55.0	9	45.0	12	100.0	0	0.0
EISN_AV10-02	A/England/1434/2009 (H1N1v) + A/California/7/2009 (H1N1v)	Adamantanes/oseltamivir	5	25.0	15	75.0	10	83.3	2	16.7
EISN_AV10-03	A/Okayama/23/2004 (H3N2)		19	95.0	1	5.0	11	91.7	1	8.3
EISN_AV10-04	A/England/313/2008 (H1N1)		18	90.0	2	10.0	12	100.0	0	0.0
EISN_AV10-05	A/Okayama/23/2004 (H3N2)	Oseltamivir/zanamivir	7	35.0	13	65.0	10	83.3	2	16.7
EISN_AV10-06	A/Lisbon/1/2008 (H3N2)	Adamantanes	10	50.0	10	50.0	12	100.0	0	0.0
EISN_AV10-07	B/England/137/2006		16	80.0	4	20.0	8	66.7	4	33.3
EISN_AV10-08	A/England/26/2008 (H1N1)	Oseltamivir	13	65.0	7	35.0	12	100.0	0	0.0
EISN_AV10-09	A/England/1434/2009 (H1N1v)	Adamantanes/oseltamivir	10	50.0	10	50.0	12	100.0	0	0.0
EISN_AV10-10	B/England/MH48/2006	Oseltamivir	11	55.0	9	45.0	12	100.0	0	0.0

Sample: *Unique code for each EISN panel sample.*
Sample content: *Full strain designation for each panel sample.*
Resistant: *Antiviral resistances for each panel sample (includes resistant and reduced susceptibility).*
Genotypic: *Number and percentage of laboratories reporting the full consensus interpretation of genotypic results for each panel sample.*
Phenotypic: *Number and percentage of laboratories reporting the full consensus interpretation of phenotypic results for each panel sample. Adamantanes were not considered when determining the number and percentage of consensus interpretations for the phenotypic results.*

H1N1v refers to influenza A(H1N1)pdm09 virus.

Note for Tables 5 and 6: Consensus results were those that fully matched the expected interpretation. Non-consensus results include incorrect results, results that partially matched the interpretation, and non-responders. The number and percentage of consensus and non-consensus interpretations is based on the total number of participants for genotyping (20 laboratories) and phenotyping (12 laboratories). Some laboratories did not return results for every sample.

5 Discussion

All 20 laboratories that participated were able to screen for the H275Y mutation in influenza A(H1N1)pdm09 viruses and the results returned were of a good standard. This demonstrates that laboratories have successfully implemented these new assays since the emergence of the pandemic virus in April 2009. The number of laboratories returning genotyping results for other subtypes of influenza A, including former seasonal H1N1 and H3N2 and influenza B was lower (11 to 15 laboratories dependent on (sub)type) suggesting that only laboratories that have performed antiviral testing for some time have these assays in place. Detection of a low frequency of oseltamivir resistance (H275Y) in circulating influenza A(H1N1)pdm09 viruses and the absence of resistance in currently circulating influenza A(H3N2) and influenza B viruses suggests that capacity building within the network should be focused on genotyping assays for detection of H275Y substitutions in influenza A(H1N1)pdm09 viruses.

The number of laboratories returning results for each panel sample varied by influenza type/subtype, with 100% of the laboratories returning results for A(H1N1)pdm09 samples, and a lower proportion for former seasonal H1N1 (75%), H3N2 (55–65%) and influenza B (55–60%) (Tables 2 and 3). This was due to the participation of laboratories that only performed A(H1N1)pdm09 antiviral testing, perhaps due to recent implementation of antiviral testing following the 2009 pandemic.

The EQA objectives were to encourage the participation of all laboratories with any form of antiviral testing in place, and the virus (sub)type was provided on the panel sample to allow laboratories to target testing appropriately. The lower rate of return for some samples compared to A(H1N1)pdm09 demonstrates that laboratories with limited antiviral testing in place were not excluded from participation.

The NA genotyping results were of a good standard with few significant errors. Sample EISN_AV10_02 which was a mixture of influenza A(H1N1)pdm09 viruses (60% H275:40% H275Y) had the lowest overall score. Although the majority of laboratories identified this as resistant (H275Y), only 47% identified this sample as a mixture, and four laboratories failed to detect the H275Y substitution. Methods used by laboratories that did not identify the mixture included partial or full gene sequencing (80%) and direct identification of H275Y by SNP PCR (20%), suggesting that sequencing methods may have some limitations regarding mixture identification. Lower scores were obtained for identification of the I221T mutation in influenza B viruses (64–75%), and other mutations such as R152K and G402S were reported for these samples instead. Antiviral resistance mutations in influenza B are less frequently observed than in influenza A and perhaps less well known.

Many laboratories also screened for mutations in the M2 gene conferring resistance to adamantanes (65-75% participants dependent on subtype). The majority of laboratories correctly identified SNPs in the M2 gene, although two laboratories consistently failed to report the S31N mutation in A(H1N1)pdm09 viruses. A lower score (69%) was achieved for sample EISN_AV10_06 which contained a dual mutation in M2 (V27A, S31N) suggesting that awareness of other mutations that can confer adamantane resistance could be improved. All influenza A(H1N1)pdm09 viruses currently in circulation are resistant to adamantanes and clinical use of these drugs is low, so further implementation of M2 gene screening assays would not be the first priority for the network, although it is valuable that a number of laboratories have this capability.

Laboratories were asked to provide an interpretation of antiviral drug susceptibility associated with the SNP identified. This was a challenging aspect of the exercise. Not unexpectedly, there was some variation in the interpretations provided, particularly for sample EISN_AV10_02 containing A(H1N1)pdm09 virus (60:40 mixture H275Y), where interpretations of resistance and reduced susceptibility to oseltamivir (and in one case resistance to zanamivir) were returned for similar genotyping results. Some participants did not provide an interpretation of their genotyping results or provided an incomplete interpretation.

The majority of participants provided a coherent interpretation of their results, with few exceptions and some differences in interpretation of resistance/reduced susceptibility as described above for A(H1N1)pdm09 virus H275Y mixture, and also for zanamivir susceptibility of H3N2 virus (R292K). Although zanamivir susceptibility was requested for all samples, this can only be inferred from an H275Y genotyping result and should be confirmed with phenotypic testing if possible. Approximately 50% of the participants did not provide an interpretation of zanamivir susceptibility for genotyping results. In a few cases an incorrect interpretation of zanamivir susceptibility was provided, including an interpretation of zanamivir resistance when the H275Y mutation was identified in H1N1 viruses. For the R292K mutation in H3N2 viruses there was considerable variability in the interpretation of zanamivir susceptibility by the different laboratories which interpreted it as sensitive, reduced susceptible or resistant to zanamivir. Similarly there was varied interpretation of the effect of I221T mutation on zanamivir susceptibility in influenza B viruses. Guidelines on the significance of specific substitutions in NA and M2 for influenza antiviral susceptibility, the interpretation of genotyping results, and the expected interpretation based on characterisation of currently circulating strains would be a useful resource for the network, particularly for laboratories that are new to testing.

Twelve laboratories reported phenotypic testing (IC_{50} assay) results, determining susceptibility to oseltamivir and zanamivir, and eight laboratories did not return phenotypic testing results. This could be due to limited resources or lack of technical support for implementing these tests which can be technically difficult to perform and analyse. One laboratory performed phenotypic testing with only one drug (oseltamivir), while the reason for not testing with zanamivir was not stated.

The majority (75%) of participants used in-house fluorescence (MUNANA) assays for IC_{50} testing, although three (25%) laboratories used a chemiluminescence kit. Chemiluminescence kits for IC_{50} testing are useful for laboratories with little experience in this area or which only perform limited testing, as in-house fluorescence assays can be difficult and time-consuming to implement. However, the sensitivity of chemiluminescence assays may be lower than in-house fluorescence assays for detection of mixtures of resistant and sensitive viruses.

The range of IC_{50} values reported was quite wide, indicating there is considerable laboratory-to-laboratory variation for phenotype testing. This may be due to differences in assay protocols between laboratories. Variation between the two assay methods was observed with IC_{50} values generated by chemiluminescence assay lower than those generated by fluorescence assay, suggesting that the comparison of results from different assays is not a straightforward process. Conversely, one laboratory using the chemiluminescence kit consistently reported IC_{50} values for zanamivir which were notably higher than average. Both laboratories that incorrectly reported sample EISN_AV10_02 as sensitive to oseltamivir by genotyping were using the chemiluminescence kit for phenotypic testing and found it to be oseltamivir sensitive using this method also.

Implementation of phenotypic testing in a laboratory needs to be adequately resourced and supported with training in laboratory methods, data analysis and reporting. One strategy towards achieving consistency between laboratories would be the harmonisation of baseline values and the provision of a reference set of viruses. A reference panel of influenza A and B viruses for assessment of resistance to neuraminidase inhibitors has been developed and is available from the ISIRV Antiviral Group. The panel can be used for validation, standardisation and quality control of genotypic and phenotypic assays and will facilitate comparison of data between laboratories.

Phenotyping results were interpreted coherently and matched the consensus result, with the exception of samples containing the A(H1N1)pdm09 mixture, resistant H3N2 and influenza B viruses. Interpretations that did not match the consensus were occasionally made and were associated with baseline values not given, comparatively lower baseline values, and use of the chemiluminescence assay. Corresponding genotyping results were correctly interpreted. Sample EISN_AV10_05 which contained an oseltamivir- and zanamivir-resistant H3N2 virus produced a wide range of interpretations of IC_{50} value for zanamivir susceptibility, including sensitive, reduced susceptible and resistant. Several participants commented that the NA activity of sample EISN_AV10_05 was low, due to the presence of the R292K mutation in the NA, and this may have affected the results.

For samples containing influenza B virus, a wide range of IC_{50} values with oseltamivir were reported with varying interpretations. Laboratories in different countries use locally defined baseline values which gives rise to varying interpretations for the same sample. IC_{50} values for influenza B virus were consistently 10 times higher than for influenza A, although they remain clinically sensitive to oseltamivir. This difference may have contributed to false interpretations of resistance or reduced susceptibility in these samples, particularly if laboratories have little in-house influenza B data for comparison. As samples containing influenza B virus were associated with lower scores in both genotypic and phenotypic testing, a specific focus on influenza B antiviral testing as part of a future training course would be of benefit.

Antiviral testing by genotypic and phenotypic methods is underpinned by key tasks of the influenza reference laboratory including typing and subtyping using molecular methods and virus culture. The number of laboratories which returned genotyping results was lower for other influenza (sub)types (55–75%) compared with A(H1N1)pdm09 (100%). Robust molecular technology coupled with the ability to type, subtype and often sequence influenza viruses is necessary for genotyping. Phenotypic testing results were returned by 60% participants. IC_{50} assays require virus isolates and are therefore dependent upon successful culture of influenza viruses. This can be technically demanding due to evolving changes in the virus receptor interaction, which can affect the rate of virus isolation in cells or eggs. Laboratory support and training in these key tasks is essential to ensure continued high quality antiviral resistance testing and counter the potential risk of resistance or the development of resistance to current and future antiviral agents.

6 Conclusions

Capacity for the detection influenza virus antiviral susceptibility among participating EU/EEA influenza reference laboratories varies from a single test for oseltamivir resistance in A(H1N1)pdm09 virus to full genotypic and phenotypic characterisation of influenza A and B viruses for susceptibility to neuraminidase inhibitors and adamantanes. Participation in the exercise provided laboratories with an independent mechanism to check the performance of their assays, and allowed them to identify areas of underperformance in need of improvement.

All 20 participating laboratories returned results of a high standard for genotypic detection of the H275Y mutation in influenza A(H1N1)pdm09 virus, demonstrating that these laboratories are fully capable to detect oseltamivir resistance in A(H1N1)pdm09 viruses. The proficiency for detecting a mixture of resistant and sensitive A(H1N1)pdm09 viruses was lower, although the majority of laboratories did identify resistance in this sample. Analysis suggested that sequencing methods may have some limitations regarding mixture identification. Capacity building within the network should be focused on genotyping assays for detection of H275Y substitutions in influenza A(H1N1)pdm09 viruses.

Not all participants performed IC_{50} assays, although the results returned by these laboratories were of a high standard. Areas of greatest difficulty were analysis of the influenza A(H1N1)pdm09 mixture, oseltamivir and zanamivir resistant H3N2, and influenza B virus. Implementation of phenotypic testing in a laboratory needs to be adequately resourced and supported with training in laboratory methods, data analysis and reporting. Ongoing training in data analysis and interpretation is important to maintain a high standard of competency. When planning the future development of the network and scheduling associated training priorities, planners and developers need to clarify priorities first: is it necessary that every laboratory is capable of phenotypic analysis, or is a more regional approach to phenotypic analysis sufficient.

Participating laboratories were asked to provide an interpretation of both genotypic and phenotypic testing results in terms of virus susceptibility to antiviral drugs, which proved to be challenging. Results varied widely, with different interpretations for similar results. Guidelines for the interpretation of genotyping results would be a useful resource for the network. In future EQA exercises, the list of interpretations should be similar to those used for clinical diagnosis or guidance and conform to current antiviral testing guidelines.

The results of the exercise will be used to promote harmonisation of antiviral susceptibility testing, data interpretation and reporting in the European network through implementation of recommendations for improvement and training activities. The use of harmonised baselines and/or provision of reference viruses for IC_{50} assays would facilitate comparison of results from different laboratories across the network.

Recommendations arising from the experience of designing, producing and analysing the first influenza virus antiviral resistance detection EQA exercise will be used to inform future programmes. Future provision of EQA for influenza antiviral resistance detection is required to ensure a continued high standard of testing for European surveillance and to support laboratories implementing new genotyping or phenotyping methods.

7 Future work and developmental needs

- The most significant error was the failure to detect the H275Y mixture (Table 3, Annex 13). Guidelines and recommended assays for detection of H275Y should be made available to all CNRL laboratories particularly to guide those that underperformed in this area or that are setting up new assays. Underperforming laboratories may require further expert follow up discussions.
- Development of capability for genotypic detection of oseltamivir resistance in A(H1N1)pdm09 viruses should be the focus of future capacity building. Although global oseltamivir resistance is currently at a low level it is acknowledged that there is real potential for emergence of oseltamivir resistance in A(H1N1)pdm09 viruses.
- Participants were asked to provide an interpretation of the genotyping and phenotyping results with regard to antiviral susceptibility. These were not scored but were collected to provide an overview of the range of interpretations provided for each mutation or IC_{50} value. Considerable differences in interpretation were seen. Guidelines on the diagnostic interpretation of results would be a useful resource for the network. In a future EQA exercise suitable interpretations, particularly for genotyping results, could be more clearly defined.
- To facilitate comparison of data between laboratories in the CNRL network, efforts should be made to harmonise methodology and interpretation of results. Harmonised baseline values together with guidelines on the normal range of IC_{50} values for sensitive and resistant influenza A and B viruses would improve consistency of interpretation.
- Provision of a reference panel of sensitive and resistant influenza A and B viruses for use as a laboratory standard would facilitate comparison of IC_{50} data across the network.
- In July 2011, ECDC and the CNRL held an Antiviral Susceptibility Surveillance Training Course in London, UK, to provide training in genotyping and IC_{50} assay methods and analysis for laboratories that participated in the EQA exercise. Consideration should be given to future training activities to support laboratories implementing phenotypic testing following this training course. Follow up training on data analysis would be of benefit to laboratories new to IC_{50} testing. Training activities will be required in the future to maintain high quality testing as genotyping assays are necessarily adapted due to the constant evolution of influenza virus.
- The experience of designing, producing and analysing the first influenza virus antiviral susceptibility detection EQA exercise can be used to form the basis of planning for future exercises in Europe. These should include improvements to the reporting form so interpretations of resistance are more clearly defined and are in line with recommendations from the CNRL Antiviral Task Group and WHO; greater capture of methodological detail in the questionnaire; encouragement of laboratories to return IC_{50} graphs and sequence data for analysis supported with appropriate web based reporting. The timing of panel distribution should be reviewed. Introduction of an aspect of timeliness of test completion and reporting could be considered for a future exercise.

8 References

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8. Zambon M, Hayden FG; Global Neuraminidase Inhibitor Susceptibility Network. Position statement: global neuraminidase inhibitor susceptibility network. Antiviral Res. 2001 Mar;49(3):147-56.

Annex 1. List of participants

Country	Institution
Austria	AKH Wien – Medical University Vienna, Vienna
Denmark	Statens Serum Institute, Copenhagen
Finland	National Institute for Health and Welfare, Helsinki
France	Groupement Hospitalier Est, Bron, Lyon
France	CNR de la Grippe – Institute Pasteur, Paris
Germany	Robert Koch Institute, Berlin
Greece	National Influenza Center for Southern Greece, Athens
Hungary	National Center for Epidemiology, Budapest
Ireland	University College Dublin, Dublin
Italy	Istituto Superiore di Sanita (NIH), Rome
Netherlands	Erasmus MC, Rotterdam
Netherlands	RIVM (CIb), Bilthoven
Norway	Norwegian Institute of Public Health, Oslo
Portugal	National Institute of Health Dr. Ricardo Jorge, Lisbon
Romania	Cantacuzino Institute, Bucharest
Spain	Instituto de Salud Carlos III, Madrid
Spain	Hospital Clinic i Provincial, Barcelona
Sweden	Smittskyddsinstitutet (SMI), Solna
United Kingdom	West of Scotland Specialist Virology Centre, Glasgow
United Kingdom	HPA – Microbiology Services, London

Annex 2. Genotypic testing report form and questionnaire

Genotypic testing report form

Data was requested for the following fields:

- Neuraminidase gene SNPs evaluated
- Mutations found in neuraminidase
- If mixtures detected, % of each SNP
- Other mutations detected
- M2 gene SNPs evaluated
- Mutations found in M2 gene
- Other mutations detected
- Interpretation of resistance genotype
- Further remarks

Participants were requested to upload sequence trace files/pyrograms.

Genotypic testing methods questionnaire

1. Which genotypic method has been used for antiviral resistance detection?
    a. Cycle sequencing
        i. PCR amplification
            1. Platform provider
            2. Model
            3. Type of assay
            4. Region of gene targeted
            5. If in-house assay, please state publication or SOP
        ii. Sequencing reaction
            1. Platform provider
            2. Model
            3. Type of assay
            4. Region of gene targeted
            5. If in-house assay, please state publication or SOP
    b. SNP PCR
        i. SNP PCR reaction
            1. Platform provider
            2. Model
            3. Type of assay
            4. Region of gene targeted
            5. If in-house assay, please state publication or SOP
    c. Pyro-sequencing
        i. PCR amplification
            1. Platform provider
            2. Model
            3. Type of assay
            4. Region of gene targeted
            5. If in-house assay, please state publication or SOP
        ii. Pyro-sequencing reaction
            1. Platform provider
            2. Model
            3. Type of assay
            4. Region of gene targeted
            5. If in-house assay, please state publication or SOP
        iii. Pyro-sequencing analysis (SQA or SNP)?
2. Nucleic acid extraction method used
3. Were external controls/standards used with this dataset (yes/no)?
4. Controls/standards comment
5. Did you perform this analysis singly, in duplicate or other?

Annex 3. Phenotypic testing report form and questionnaire

Phenotypic testing report form

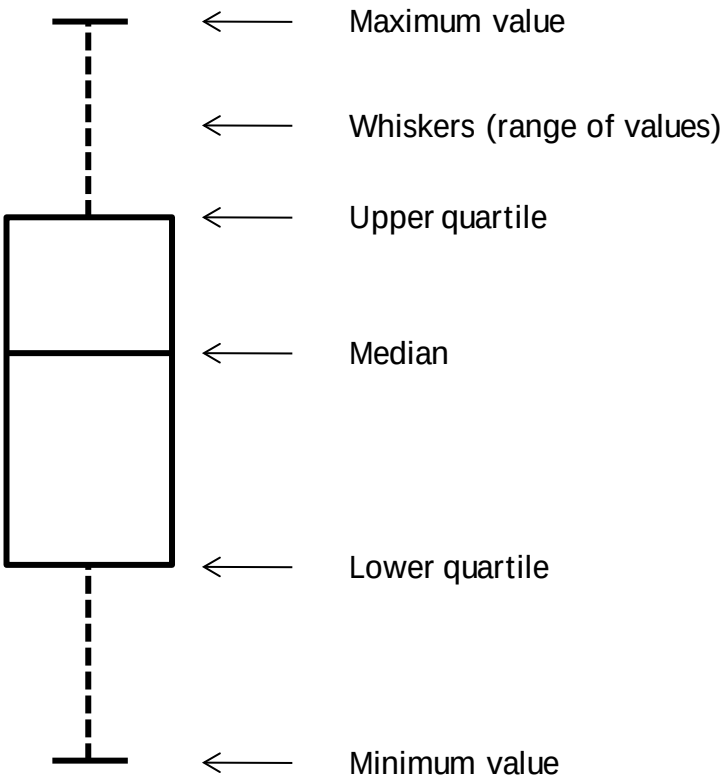
Data was requested for the following fields:

- Sample dilution used for test
- Phenotypic results (IC_{50} value) for oseltamivir
- Phenotypic results (IC_{50} value) for zanamivir
- Interpretation of phenotype
- Further remarks

Phenotypic testing methods questionnaire

1. Which phenotypic method has been used for antiviral resistance detection?
2. If in-house assay used, please state publication or SOP.
3. Provider of plate reader?
4. Model of plate reader?
5. Substrate addition (NA STAR only)? (Manual addition/automatic injection?)
6. Range of drug dilutions
   a. oseltamivir
   b. zanamivir
7. Was NA activity determined prior to IC_{50}? (Yes/no)
8. Comment on NA activity
9. Software used for data analysis
10. Which statistical method was used to define resistance?
    (box and whisker plots/median absolute deviation/>10-fold from mean/other)
11. Were external controls/standards used with this dataset? (Yes/no)
12. Controls/standards comment
13. Did you perform this analysis singly, in duplicate, or other?

Annex 4. Schematic box and whisker plot



A schematic box and whisker plot (also known as a box plot), where the whiskers represent the range of values from minimum to maximum, and the box represents the first (lower), median and third (upper) quartile.

Annex 5. NA genotyping results by laboratory

A. NA genotyping results by laboratory: samples EISN_AV_10-01 to EISN_AV_10-04

Participant	EISN_AV10-01		EISN_AV10-02			EISN_AV10_03		EISN_AV10_04	
	NA mutations	Other NA mutations	NA mutations	Other NA mutations	Mixtures	NA mutations	Other NA mutations	NA mutations	Other NA mutations
	H275		H275Y mixture		60:40	R292		H275	
1	None[1]		None			None[2]		None[1]	
2	None[1]		H275Y		35/65	None[2]		None[1]	
3	None[1]		None						
4	None[1]		None			None[2]		None[1]	
5	None[1]		H275Y			None[2]		None[1]	
6	None[1]		H275Y		70/30	None[2]		None[1]	
7	None[1]		H275Y					None[1]	
8	None[1]		H275Y		53/47	None[2]		None[1]	
9	None[1]		H275Y						
10	None[1]		H275Y		64.2% 275Y	None[2]		None[1]	
11	None[1]							None[1]	
12	H275Y		H275Y						
13					60/40 C/T				
14	None[1]		275HY					None[1]	
15		V108I	H275Y	V108I	50/50	None[2]	None		N21S
16	None[1]		H275Y			None[2]			
17			H275Y		75/25				
18		D354G	H275Y	D354G				None[1]	
19	None[1]		None			None[2]		None[1]	
20	None[1]		H275Y		Less than 50%	None[2]		None[1]	

[1]*Result presumed to be H275 as interpretation says 'sensitive to oseltamivir/zanamivir'.*

[2]*Result presumed to be R292 as interpretation says 'sensitive to oseltamivir/zanamivir'.*

B. NA genotyping results by laboratory: samples EISN_AV_10-05 to EISN_AV_10-08

Participant	EISN_AV10-05			EISN_AV10-06		EISN_AV10-07		EISN_AV10-08		
	NA mutations	Other NA mutations	Mixtures	NA mutations	Other NA mutations	NA mutations	Other NA mutations	NA mutations	Other NA mutations	Mixtures
	R292K			R292		I221		H275Y		
1	R292K			None[2]			V205I, N32	H275Y	D354G	
2	R292K			None[2]		None[3]			H275Y	
3								H275Y		
4	R292K			None[2]				H275Y		
5	R292K			None[2]		None[3]		H275Y		
6	R292K			None[2]			R152K, G402S, D198N	H275Y		
7								H275Y		
8	R292K			None[2]		None[3]		H275Y		
9										
10	R292K			None[2]		None[3]		H275Y		
11						None[3]		H275Y		

12										
13										
14	R292K			None[2]		None[3]		H275Y		
15	R292K	none			D147N/I2		V204I/N3	H275Y	N21S /D354	
16	R292K					None[3]				
17	R292K									
18								H275Y	D354G	
19	None			None[2]			G402S	H275Y	D354G	100%
20	R292K		100%	None[2]				H275Y		

[2]*Result presumed to be R292 as interpretation says 'sensitive to oseltamivir/zanamivir'.*

[3]*Result presumed to be I221 as interpretation says 'sensitive to oseltamivir/zanamivir'.*

C. NA genotyping results by laboratory: samples EISN_AV_10-09 to EISN_AV_10-10

Participant	EISN_AV10-09			EISN_AV10-010	
	NA mutations	Other NA mutations	Mixtures	NA mutations	Other NA mutations
	H275Y			I221T	
1	H275Y	N248D		I221T, other	P89S, N341
2	H275Y			I221T	
3	H275Y				
4	H275Y				
5	H275Y			I221T	
6	H275Y			R152K, I221T, G402S	D198N
7	H275Y				
8	H275Y		15/85	I221T	
9	H275Y				
10	H275Y			I221T	
11	H275Y			G402S	
12	H275Y				
13			10/90 C/T		
14	H275Y			I221T	
15	H275Y	V106I/V1		None	
16	H275Y			I221T	
17	H275Y			I221T	
18	H275Y				
19	H275Y			G402S	
20	H275Y		100%		

Annex 6. M2 genotyping results by laboratory

A. M2 genotyping results by laboratory: samples EISN_AV_10-01 to EISN_AV_10-04

Participant	EISN_AV10-01		EISN_AV10_02		EISN_AV10_03		EISN_AV10_04	
	M2 mutations	Other M2 mutations	M2 mutations	Other M2 mutations	M2 mutations	Other M2 mutations	M2 mutations	Other M2 mutations
	S31N		S31N		S31		S31	
1	S31N	V28I, N20S	S31N	V28I, N20S		S23N		L36V
2	S31N		S31N		None[1]		None[1]	
3	None		None					
4	S31N		S31N		None[1]		None[1]	
5	S31N		S31N		None[1]		None[1]	
6	S31N		S31N		None[1]		None[1]	
7								
8	S31N		S31N		None[1]		None[1]	
9								
10	S31N		S31N		None[1]		None[1]	
11	S31N		S31N		None[1]		None[1]	
12								
13								
14	S31N		S31N		None[1]		None[1]	
15	None	none	None	none		S14N/N22S/V42I/K47R/G80S	None[1]	none
16	S31N		S31N		None[1]		None[1]	
17	S31N		S31N					
18								
19	S31N		S31N		None[1]		None[1]	
20	S31N		S31N		None[1]		None[1]	

[1]*Result presumed to be S31.*

B. M2 genotyping results by laboratory: samples EISN_AV_10-05 to EISN_AV_10-09

Participant	EISN_AV10-05		EISN_AV10_06		EISN_AV10_08		EISN_AV10_09	
	M2 mutations	Other M2 mutations	M2 mutations	Other M2 mutations	M2 mutations	Other M2 mutations	M2 mutations	Other M2 mutations
	S31		S31N		S31		S31N	
1		S23N	V27A, S31N			L36V	S31N	N20S
2	None[1]		V27A, S31N	V27A, S31N	None[1]		S31N	
3					None[1]		None	
4	None[1]		V27A, S31N		None[1]			
5	None[1]		V27A, S31N		None[1]		S31N	
6	None[1]		V27A, S31N		None[1]		S31N	
7								
8	None[1]		V27A	31N	None[1]		S31N	
9								
10	None[1]		S31N		None[1]		S31N	
11	None[1]		V27A, S31N		None[1]		S31N	
12								
13								
14	None[1]		V27A	S31N	None[1]		S31N	
15		S14N/N22S/V42I/K47R/G80S		V13A	None[1]		None	
16	None[1]		V27A	S31N	None[1]		S31N	
17								
18								
19	None[1]		V27A		None[1]		S31N	
20	None[1]		S31N		None[1]		None	

[1]Result presumed to be S31.

Annex 7. Interpretation of genotyping results for sensitivity to antiviral drugs

Participant	EISN_AV10-01	EISN_AV10-02	EISN_AV10-03	EISN_AV10-04	EISN_AV10-05	EISN_AV10-06	EISN_AV10-07	EISN_AV10-08	EISN_AV10-09	EISN_AV10-10
	Oseltamivir/ zanamivir	Zanamivir	Adamantanes/ oseltamivir/ zanamivir	Adamantanes/ oseltamivir/ zanamivir	Adamantanes	Oseltamivir/ zanamivir	Oseltamivir/ zanamivir	Adamantanes/ zanamivir	Zanamivir	Zanamivir
1	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Amantadine	Oseltamivir, zanamvir	Oselatamivir, zanamivir	Zanamivir, amantadine	Zanamivir	
5a	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir, adamantanes	Oseltamivir, zanamivir, adamantanes	Adamantanes	Oseltamivir, zanamivir	Oselatamivir, zanamivir	Zanamivir, adamantanes	Zanamivir	Zanamivir
6										
7b										
8	Oseltamivir	Oseltamivir	Oseltamivir, adamantanes	Oseltamivir, adamantanes	Adamantanes	Oseltamivir		Adamantanes		
9b										
10	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Amantadine	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir, amantadine	Zanamivir	
11	Oseltamivir, zanamivir	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Amantadine	Oseltamivir, zanamivir		Zanamivir, amantadine	Zanamivir	
12	Oseltamivir			Oseltamivir						
13	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Amantadine	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir, amantadine	Zanamivir	Zanamivir
14b										
15b										
16	Oseltamivir									
17a	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Zanamivir, amantadine	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir, amantadine	Zanamivir	Zanamivir
18	Oseltamivir		Amantadine	Oseltamivir, amantadine	Amantadine		Oseltamivir	Amantadine		
19	Oseltamivir									
20	Oseltamivir									
22	Oseltamivir, zanamivir		Amantadine	Oseltamivir, zanamivir, amantadine	Amantadine	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir, amantadine	Zanamivir	
27										
28				Adamantanes	Adamantanes			Adamantanes		
29	Oseltamivir, zanamivir	Zanamivir							Zanamivir	Zanamivir
31				Oseltamivir						
32	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir		Amantadine		
33	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir, amantadine	Adamantanes	Oseltamivir, zanamivir		Amantadine, zanamivir	Amantadine, zanamivir	

a: Combined dataset (more than one set of data returned by participant)
b: No results returned.

Interpretation partially matches consensus, or no interpretation provided
Incorrect interpretation
Not tested

Annex 8. Interpretation of genotyping results for resistance to antiviral drugs

Participant	EISN_AV10-01	EISN_AV10-02	EISN_AV10-03	EISN_AV10-04	EISN_AV10-05	EISN_AV10-06	EISN_AV10-07	EISN_AV10-08	EISN_AV10-09	EISN_AV10-10
	Adamantanes	Adamantanes/ oseltamivir			Oseltamivir/ Zanamivir	Adamantanes		Oseltamivir	Adamantanes/ oseltamivir	Oseltamivir
1	Amantadine	Amantadine			Oseltamivir, zanamivir	Amantadine		Oseltamivir	Oseltamivir, amantadine	Oseltamivir, zanamivir
5a	Adamantanes	Oseltamivir (RS), adamantanes			Oseltamivir, zanamivir (RS)	Adamantanes		Oseltamivir	Oseltamivir, amantadine	Oseltamivir
6										
7b										
8	Amantadine	Adamantane			Oseltamivir	Adamantanes	Not performed	Oseltamivir	Oseltamivir	
9b										
10	Amantadine	Oseltamivir, amantadine			Oseltamivir, zanamivir	Amantadine		Oseltamivir	Oseltamivir, amantadine	Oseltamivir, zanamivir (RS)
11	Amantadine	Oseltamivir, zanamivir, amantadine			Oseltamivir, zanamivir	Amantadine	Oseltamivir, zanamivir	Oseltamivir	Oseltamivir, amantadine	Oseltamivir, zanamivir
12		Oseltamivir						Oseltamivir	Oseltamivir	
13	Amantadine	Oseltamivir, amantadine			Oseltamivir, zanamivir	Amantadine	Amantadine	Oseltamivir	Oseltamivir, amantadine	Amantadine, oseltamivir (RS)
14b										
15b										
16		Oseltamivir	Resistance profile not analysed	Resistance profile not analysed					Oseltamivir	
17a	Amantadine	Oseltamivir (RS), Amantadine			Oseltamivir	Amantadine		Oseltamivir	Oseltamivir, Amantadine	Oseltamivir
18	Amantadine	Amantidine				Amantadine		Oseltamivir	Oseltamivir, amantadine	Oseltamivir
19		Oseltamivir							Oseltamivir	
20		Oseltamivir (RS)		Oseltamivir				Oseltamivir	Oseltamivir	
22	Amantadine	Oseltamivir (RS), amantadine			Oseltamivir, Zanamivir	Amantadine		Oseltamivir	Oseltamivir, Amantadine	Oseltamivir
27										
28		Oseltamivir, Amantadine			Oseltamivir	Amantadine			Oseltamivir, amantadine	Oseltamivir
29		Oseltamivir (RS)			Oseltamivir, zanamivir (RS)				Oseltamivir	Oseltamivir
31		Oseltamivir (RS)						Oseltamivir	Oseltamivir	
32	Amantadine	Amantadine				Amantadine	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir, amantadine	Oseltamivir, zanamivir
33	Amantadine	Oseltamivir, amantadine			Oseltamivir, zanamivir (RS)	Amantadine		Oseltamivir	Oseltamivir	

a: Combined dataset (more than one returned by participant).
b: No results returned.

Interpretation partially matches consensus, or no interpretation provided
Incorrect interpretation
Not tested

Annex 9. Interpretation of phenotyping results for sensitivity to antiviral drugs

Participant	EISN_AV10-01	EISN_AV10-02	EISN_AV10-03	EISN_AV10-04	EISN_AV10-05	EISN_AV10-06	EISN_AV10-07	EISN_AV10-08	EISN_AV10-09	EISN_AV10-10
	Oseltamivir/ Zanamivir	Zanamivir	Adamantanes/ oseltamivir/ zanamivir	Adamantanes/ oseltamivir/ zanamivir	Adamantanes	Oseltamivir/ zanamivir	Oseltamivir/ zanamivir	Adamantanes/ zanamivir	Zanamivir	Zanamivir
1	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Oseltamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Oseltamivir	Zanamivir	Zanamivir	
5a	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir
6b										
7b										
8b										
9b										
10b										
11b										
12	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir
13	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir
14b										
15b										
16b										
17a	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir
18	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir
19b										
20b										
22	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir
27	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir
28	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir	Zanamivir
29	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir
31b										
32*	Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir	indeterminate	Oseltamivir				
33	Oseltamivir, zanamivir	Zanamivir	Oseltamivir, zanamivir	Oseltamivir, zanamivir		Oseltamivir, zanamivir	Zanamivir	Zanamivir	Zanamivir	Zanamivir

a: Combined dataset (more than one returned by participant).
b: No results returned.
** Did not test with zanamivir.*

Interpretation partially matches consensus, or no interpretation provided
Incorrect interpretation
Not tested

Annex 10. Interpretation of phenotyping results for resistance to antiviral drugs

Participant	EISN_AV10-01	EISN_AV10-02	EISN_AV10-03	EISN_AV10-04	EISN_AV10-05	EISN_AV10-06	EISN_AV10-07	EISN_AV10-08	EISN_AV10-09	EISN_AV10-10
	Adamantanes	Adamantanes/ oseltamivir			Oseltamivir/ zanamivir	Adamantanes		Oseltamivir	Adamantanes/ oseltamivir	Oseltamivir
1			Zanamivir (RS)		Oseltamivir, zanamivir		Zanamivir (RS)	Oseltamivir	Oseltamivir	Oseltamivir (RS), zana-mivir (RS)
5a		Oseltamivir			Oseltamivir, zanamivir (RS)			Oseltamivir	Oseltamivir	Oseltamivir
6b										
7b										
8b										
9b										
10b										
11b										
12		Oseltamivir			Oseltamivir			Oseltamivir	Oseltamivir	Oseltamivir
13		Oseltamivir			Oseltamivir, zanamivir			Oseltamivir	Oseltamivir	Oseltamivir (RS)
14b										
15b										
16b										
17a		Oseltamivir (RS)			Oseltamivir, zanamivir			Oseltamivir	Oseltamivir	Oseltamivir
18		Oseltamivir			Oseltamivir, zanamivir			Oseltamivir	Oseltamivir	Oseltamivir
19b										
20b										
22		Oseltamivir			Oseltamivir and zanamivir			Oseltamivir	Oseltamivir	Oseltamivir
27		Oseltamivir			Oseltamivir, zanamivir (RS)			Oseltamivir	Oseltamivir	Oseltamivir
28		Oseltamivir			Oseltamivir, zanamivir (RS)		Oseltamivir (RS)	Oseltamivir	Oseltamivir	Oseltamivir
29		Oseltamivir			Oseltamivir, zanamivir (RS)			Oseltamivir	Oseltamivir	Oseltamivir (RS)
31b										
32*					indeterminate		Oseltamivir	Oseltamivir	Oseltamivir	Oseltamivir
33		Oseltamivir			Oseltamivir, zanamivir		Oseltamivir (RS)	Oseltamivir	Oseltamivir	Oseltamivir

a: Combined dataset (more than one returned by participant).
b: No results returned.
** Did not test with zanamivir.*

Interpretation partially matches consensus, or no interpretation provided
Incorrect interpretation
Not tested

Annex 11. Baseline values used by participating laboratories for interpretation of IC_{50} assay results with oseltamivir

Participant	A/H1N1 former seasonal			A(H1N1)pdm09			A/H3N2			Influenza B		
	Geometric mean (nM)	Minor outlier threshold	Major outlier threshold	Geometric mean (nM)	Minor outlier threshold	Major outlier threshold	Geometric mean (nM)	Minor outlier threshold	Major outlier threshold	Geometric mean (nM)	Minor outlier threshold	Major outlier threshold
1												
5	1.13 (2007/08)	2.93	3.83	1.18 (2009/10)	3.68	4.93	0.40 (2008/09)	0.8	1.0	76 (2010/11)	136	166
12	1.36 [SD IC_{50}:0.70][1] (2007/08 H275)	3.46[2]	13.6[3]	0.46 [SD IC_{50}:0.23][1] (2009/10)	1.15[2]	4.6[3]	0.39 [SD IC_{50}:0.16][1] (2008/09)	0.87[2]	3.9[3]	13.84 [SD IC_{50}:3.18][1] (2007/08)	23.38[2]	138.4[3]
13												
17	4.04 (2007/08 season)	13	55	0.95 (2010/11 season)	1.9nM	3.81nM	0.65nM (2008/09 season)	1.52	3.0	26.3 (2007/08 season)	51	89
18												
22	2.3	3.6	4.8	2.8	3.5	4.4	0.67	1.2	1.7	30	50	70
27	0.44		10x mean[4]	0.3		10x mean[4]	0.26		10x mean[4]	4.5		10x mean[4]
28	0.85	3.20	6.83	0.79	1.49	2.38	0.25	1.51	3.34	13.6	25.42	38.02
29												
32	0.49	0.93	4.9	0.48	0.81	4.8	0.26	0.41	2.6	6.13	8.29	10.45
33				0.270	0.41	0.52	0.102	0.28	0.43	9.41	16.2	21.7

[1]Geometric mean (SD: standard deviation)
[2]Geometric mean IC_{50} + 3SD
[3]Geometric mean IC_{50} x10
[4]10x geometric mean (nM) for subtype

Annex 12. Baseline values used by participating laboratories for interpretation of IC_{50} assay results with zanamivir

Participant	A/H1N1 old seasonal			A(H1N1)pdm09			A/H3N2			Influenza B		
	Geometric mean (nM)	Minor outlier threshold	Major outlier threshold	Geometric mean (nM)	Minor outlier threshold	Major outlier threshold	Geometric mean (nM)	Minor outlier threshold	Major outlier threshold	Geometric mean (nM)	Minor outlier threshold	Major outlier threshold
1												
5	0.76 (2007/08)	2.1	2.69	0.51 (2009/10)	1.61	2.16	0.45 (2008/09)	1.03	1.32	12.6 (2010/11)	25	32
12	1.54 [SD IC_{50}:0.71][1] (2007/08 H275)	3.67[2]	15.4[3]	0.44 [SD IC_{50}:0.24][1] (2009/10)	1.16[2]	4.4[3]	1.13 [SD IC_{50}:0.74][1] (2008/09)	3.35[2]	11.3[3]	10.79 [SD IC_{50}:4.98][1] (2007/08)	25.73[2]	107.9[3]
13												
17	1.74 (2007/08 season)	2.5	7.1	0.43 (2010/11 season)	0.62	0.84	0.33 (2008-9 season)	1.24	1.9	6.94 (2007/08 season)	21	53
18												
22	1.33	2.1	2.8	1.02	1.66	2.38	0.74	1.12	1.5	7	11.9	17.8
27	0.9		10x mean[4]	0.2		10x mean[4]	0.64		10x mean[4]	1.8		10x mean[4]
28	0.68	2.84	6.74	0.45	0.92	1.54	0.74	1.56	2.22	5.62	20.0	50.49
29												
32	0.49	0.93	4.9	0.48	0.81	4.8	0.26	0.41	2.6	6.13	8.29	10.45
33				0.182	0.42	0.61	0.255	0.46	0.63	2.81	4.30	5.51

[1]Geometric mean (SD: standard deviation)
[2]Geometric mean IC_{50} + 3SD
[3]Geometric mean IC_{50} x10
[4]10x geometric mean (nM) for subtype

Annex 13. Comprehensive analysis of results by influenza subtype

Influenza A(H1N1)pdm09

Sample EISN_AV10-01. This sample contained influenza A(H1N1)pdm09 A/California/7/2009. This strain is sensitive to the neuraminidase inhibitor drugs oseltamivir and zanamivir. It is resistant to the adamantanes due to the presence of the S31N substitution in the M2 gene.

NA genotyping. 15/20 laboratories correctly reported no mutations conferring a change in susceptibility in the NA gene. 1/20 laboratories reported the H275Y mutation in this sample. 4/20 laboratories did not specify whether a mutation had been detected. Two laboratories reported additional NA mutations: V108I and D354G.

Genotyping methods used for sample EISN_AV10-01 were full-length NA gene sequencing (6/20), partial NA gene sequencing (6/20), direct identification of H275Y (6/20), or both full-length gene sequencing and direct identification of H275Y (2/20). The laboratory which reported the H275Y mutation in this sample used direct identification by SNP PCR.

M2 genotyping. 15/20 laboratories returned results for M2 genotyping of sample EISN_AV10-01. 13/15 laboratories correctly identified the S31N substitution in this sample. 2/15 laboratories did not report any mutation in the M2 gene.

Genotyping methods used for sample EISN_AV10-01 were full-length M2 gene sequencing (8/15), partial M2 gene sequencing (5/15) and SNP analysis (2/15), including analysis of L26F, V27A, V27D, A30T, S31N and G34E substitutions. The laboratories which did not report any mutations in the M2 gene used full-length gene sequencing (1) and partial gene sequencing (1).

Interpretation. Laboratories were informed that this sample contained A(H1N1)pdm09 virus and were expected to screen for the H275Y mutation. The expected interpretation was sensitive (oseltamivir, zanamivir) or resistant (amantadine). 16/20 laboratories provided an interpretation of oseltamivir sensitivity, including the laboratory which reported H275Y, suggesting a data entry error. 10/20 laboratories also interpreted the sample as zanamivir sensitive, thus fully matching the consensus interpretation. 11/15 laboratories described the sample as amantadine resistant, and 4/15 laboratories did not comment on amantadine susceptibility. 4/20 laboratories did not provide an interpretation of sample susceptibility to any antiviral drugs. Two laboratories that did not provide interpretations remarked that 'Needs confirmation by phenotypic tests (it lacks the H275Y substitution, yet has the D354G mutation usually found in resistant viruses)' and 'A: 180-1329 has S31N substitution in M2 associated with adamantane resistance, and no substitutions detected in NA associated with neuraminidase inhibitor resistance'.

Phenotypic testing. 12/12 laboratories reported this sample as oseltamivir sensitive (<0.2–1.9 nM). 11/12 laboratories tested for zanamivir susceptibility and interpreted their IC_{50} values as sensitive to zanamivir (0.197–2.3 nM).

Sample EISN_AV10-02. This sample contained a mixture of two influenza A(H1N1)pdm09 viruses A/California/7/2009 and A/England/1434/2009 (60:40). A/California/7/2009 is sensitive to the neuraminidase inhibitor drugs oseltamivir and zanamivir. A/England/1434/2009 is sensitive to zanamivir and resistant to oseltamivir due to the presence of the H275Y mutation in the neuraminidase (NA) gene. Both A/California/7/2009 and A/England/1434/2009 are resistant to the adamantanes due to the presence of the S31N substitution in the M2 gene.

NA genotyping. 19/20 laboratories returned a NA genotyping result for this sample. 9/19 laboratories detected a mixture in sample H275Y. The mixture was proportionally 60% H275 and 40% H275Y (also described as 60/40 C/T). The percentage of mixtures detected covered the following range: 'less than 50%, 35/65, 50/50, 53/47, 60/40 C/T, 64.2% 275Y, 70/30, and 75/25'. 10/19 laboratories did not report a mixture, with six reporting H275Y and four reporting no mutation.

Genotyping methods used for sample EISN_AV10-02 were full-length NA gene sequencing (5/19), partial NA gene sequencing (6/19), direct identification of H275Y (6/19) and both full-length gene

sequencing and direct identification of H275Y (2/19). The laboratories which identified mixtures used full-length NA gene sequencing (4/9), direct identification of H275Y (3/9) or both full-length gene sequencing and direct identification of H275Y (2/9). The laboratories that did not detect the presence of H275Y at all used full (1) or partial (3) gene sequencing.

M2 genotyping. 13/15 laboratories correctly identified the S31N substitution in this sample. 2/15 laboratories did not identify a mutation associated with resistance in this sample.

Interpretation. Of the 9/20 laboratories that detected a mixture, 5/9 interpreted this as reduced susceptibility (35/65, 64.2% 275Y, 60/40 C/T and 75/25), 2/9 interpreted this as resistant to oseltamivir and sensitive to zanamivir (53/47, less than 50%), and 1/9 interpreted this as resistant to oseltamivir and zanamivir (70/30). One laboratory did not provide an interpretation of resistance phenotype. Of the 10/19 laboratories that did not detect a mixture, one laboratory provided an interpretation of reduced susceptibility for oseltamivir (also reported D354G mutation), five reported the sample as oseltamivir resistant, three reported it as oseltamivir sensitive and one did not provide an interpretation. 12/15 laboratories interpreted the sample as amantadine resistant. 3/15 did not provide an interpretation of amantadine resistance.

Phenotypic testing. 6/12 laboratories reported this sample as oseltamivir resistant (20–251 nM). 4/12 laboratories interpreted the IC_{50} value as reduced susceptibility to oseltamivir (1.14–111.2 nM). 2/12 laboratories returned a result of oseltamivir sensitive (<0.2–0.038 nM). Both of these laboratories were using the NA-Star kit for phenotypic testing. 11 laboratories reported zanamivir sensitivity (0.18–1.6 nM).

Sample EISN_AV10-09. This sample contained influenza A(H1N1)pdm09 virus A/England/1434/2009. This strain is sensitive to zanamivir and resistant to oseltamivir due to the presence of the H275Y mutation in the NA gene. It is resistant to adamantanes due to the presence of the S31N substitution in the M2 gene.

NA genotyping. Using genotyping methods 18/20 (90%) laboratories correctly identified the H275Y substitution in this sample. 2/20 (10%) laboratories identified mixtures (15/85 and 10/90 C/T) indicating the assay used had detected a low level of H275 (10–15%) in the sample. One laboratory specified 100% H275Y. Two laboratories reported additional NA mutations which were N248D and V106I.

Genotyping methods used for sample EISN_AV10-09 were full-length NA gene sequencing (4/20), partial NA gene sequencing (8/20), direct identification of H275Y (6/20), both full-length gene sequencing and direct identification of H275Y (2/20). The laboratories which identified mixtures used direct identification of H275Y (2).

M2 genotyping. 10/14 (71%) laboratories correctly identified the S31N substitution in this sample. 4/14 (29%) laboratories reported no mutation in the M2 gene. One laboratory reported an additional substitution N20S.

Genotyping methods used for sample EISN_AV10-09 were full-length M2 gene sequencing (7/14), partial M2 gene sequencing (5/14) and SNP analysis (2/14), including analysis of L26F, V27A, V27D, A30T, S31N, G34E substitutions.

Interpretation. 17/20 laboratories correctly interpreted the sample as oseltamivir resistant. 1/20 laboratory incorrectly reported oseltamivir and zanamivir resistance. The laboratories that reported mixtures reported the samples as oseltamivir resistant. 10/14 laboratories correctly identified the sample as amantadine resistant. 3/14 laboratories did not provide an interpretation of amantadine resistance, and 1/14 laboratory falsely identified the sample as amantadine sensitive (no mutation in M2 reported). 2/20 laboratories did not provide an interpretation of susceptibility for any drug.

Phenotypic testing. All 12 laboratories that did phenotypic testing found this sample to be resistant to oseltamivir (1.4–517 nM). The 11 laboratories that performed IC_{50} assays with zanamivir found it to be sensitive (0.12–1.8 nM).

Influenza A H1N1 (former seasonal)

Sample EISN_AV10-04. This sample contained A/England/313/2008 which is an former seasonal H1N1 virus (*A/Brisbane/59/2007-like)* sensitive to oseltamivir, zanamivir and adamantanes.

NA genotyping. 13/15 (87%) laboratories reported no resistance mutations. 1/15 laboratories did not specify a result but provided an incorrect interpretation of oseltamivir resistance (H275Y) using an SNP PCR method. 1/15 laboratories reported another mutation N21S (no interpretation provided).

M2 genotyping. 12/13 laboratories reported no mutations associated with resistance for this sample. One laboratory reported another mutation L36V (interpreted as sensitive).

Interpretation. The expected interpretation for this sample was sensitive to oseltamivir, zanamivir and amantadine, and nine laboratories returned this result. Two laboratories reported that the sample was sensitive to oseltamivir and amantadine, two laboratories reported oseltamivir sensitivity only, and one laboratory reported amantadine sensitivity only. One laboratory provided a false interpretation of oseltamivir resistant. One laboratory did not provide an interpretation.

Phenotypic testing. All 12 laboratories that did phenotypic testing found this sample to be sensitive to oseltamivir (<0.2–2.9 nM). The 11 laboratories that performed IC_{50} assays with zanamivir found it to be sensitive (0.8–1.7 nM).

Sample EISN_AV10-08. This sample contained A/England/26/2008, a former seasonal H1N1 virus (*A/SolomonIslands/3/2006-like*) resistant to oseltamivir due to the H275Y mutation in the NA gene, sensitive to zanamivir, and sensitive to adamantine.

NA genotyping. 15/15 laboratories identified the H275Y mutation. One laboratory provided an interpretation of oseltamivir resistance but did not identify the mutation. Other mutations identified in this sample included D354G (3) and N21S (1).

M2 genotyping. 13/14 laboratories reported no mutations associated with resistance. One laboratory identified another mutation (L36V) and interpreted this as amantadine sensitive.

Interpretation. The expected interpretation for this sample was oseltamivir resistant, zanamivir sensitive, amantadine sensitive; eight laboratories returned this interpretation, two did not comment on zanamivir, and three returned oseltamivir resistant only (M2 gene not analysed). One laboratory reported amantadine sensitive only (NA gene not analysed). One laboratory incorrectly interpreted the sample as resistant to both oseltamivir and zanamivir. Two laboratories did not provide an interpretation.

Phenotypic testing. All 12 laboratories that did phenotypic testing found this sample to be resistant to oseltamivir (2–1094 nM). The 11 laboratories that performed IC_{50} assays with zanamivir found it to be sensitive (0.16–2.2 nM).

Influenza A H3N2

Sample EISN_AV10-03. This sample contained influenza A H3N2 A/Okayama/23/2004 plaque purified variant sensitive to oseltamivir and zanamivir (NA R292) and adamantanes (M2 S31).

NA genotyping. 11/11 (100%) laboratories correctly reported no resistance mutations in NA in this virus.

M2 genotyping. 11/13 (85%) laboratories correctly reported no resistance mutations in M2 in this virus. Two laboratories reported other mutations including S23N (1) and S14N/N22S/V42I/K47R/G80S (1).

Interpretation. The expected interpretation for this sample was sensitive to oseltamivir, zanamivir and adamantanes. 9/11 laboratories that performed NA genotyping interpreted the sample as oseltamivir sensitive, and 8/9 also stated zanamivir sensitive. 11/13 laboratories that performed M2 genotyping interpreted the sample as adamantine sensitive. One laboratory reported ‘no resistance found’. One laboratory did not provide an interpretation.

Phenotypic testing. 12/12 laboratories reported oseltamivir sensitivity (0.1 to 0.6 nM) and 10/12 reported zanamivir sensitive (0.284 to 3.1 nM). One laboratory that used the NA-Star kit reported reduced susceptibility to zanamivir (6.7nM). One laboratory did not test for zanamivir susceptibility.

Sample EISN_AV10-05. This sample contained influenza A H3N2 A/Okayama/23/2004 plaque purified variant resistant to oseltamivir and zanamivir (NA R292K) and sensitive to adamantanes (M2 S31).

NA genotyping. 12/13 laboratories correctly identified the R292K mutation in the NA gene. One laboratory reported no mutations in the NA gene.

M2 genotyping. 11/14 laboratories reported no M2 mutations associated with resistance. 2/14 laboratories reported other mutations S23N (1) and S14N/N22S/V42I/K47R/G80S (1). 1/14 indicated the analysis had been performed but did not report mutations or interpretations.

Interpretation. The expected interpretation for this sample was resistance to oseltamivir/zanamivir and sensitivity to adamantanes. 11/13 laboratories reported oseltamivir resistance; 1/13 falsely reported oseltamivir sensitivity (no mutations in NA reported); 1/13 did not provide an interpretation. 12/14 laboratories interpreted the sample as adamantine sensitive. The laboratory which reported other mutations (S14N/N22S/V42I/K47R/G80S) did not provide an interpretation of amantadine susceptibility. The interpretation of zanamivir susceptibility was more varied. 5/13 laboratories provided an interpretation of zanamivir resistance; 3/13 indicated reduced susceptibility to zanamivir; 2/13 indicated zanamivir sensitivity (including one laboratory which reported no resistant mutation); and 3/13 did not provide an interpretation of zanamivir susceptibility.

Phenotypic testing. 11 laboratories reported this sample as resistant to oseltamivir (42 to >10000nM), and one laboratory reported an indeterminate result for oseltamivir (IC_{50} value not reported). Four laboratories reported zanamivir resistance (13.6 to 24nM). Six reported reduced susceptibility for zanamivir (4.11 to 45). One laboratory reported sensitivity to zanamivir (7.57nM), and one reported indeterminate result for zanamivir (IC_{50} value not reported).

Sample EISN_AV10-06. This sample contained influenza A H3N2 virus A/Lisbon/1/2008 sensitive to oseltamivir and zanamivir and resistant to amantadine due to the V27A and S31N mutations in the M2 gene.

NA genotyping. 10/11 laboratories reported no resistance mutations in this sample, and 1/11 reported other mutations (D147N/I2).

M2 genotyping. 9/13 laboratories reported both the V27A and S31N mutations; 2/13 reported S31N only; 1/13 reported V27A only; and 1/13 reported V13A.

Interpretation. 12/13 laboratories interpreted the sample as amantadine resistance. 10/11 interpreted the sample as oseltamivir sensitive, and 9/11 also reported zanamivir sensitivity. One laboratory did not provide an interpretation of resistance (but reported other mutations).

Phenotypic testing. 12/12 laboratories found this sample sensitive to oseltamivir (0.1 to 0.5 nM), and 11 found it sensitive to zanamivir (0.12 to 2.7). One laboratory did not test for zanamivir susceptibility.

Influenza B

Sample EISN_AV10-07. This sample contained influenza B B/England/137/2006 sensitive to both oseltamivir and zanamivir. The adamantanes are not effective against influenza B and were not considered in the analysis.

NA genotyping. 7/11 laboratories reported no resistance mutations. 2/11 reported other mutations including V205I, N32 (1) (interpreted as sensitive) and V204I/N3 (1) (no interpretation). 2/11 reported mutations R152K, G402S (1) and G402S (1), which were interpreted as oseltamivir and zanamivir resistant.

Interpretation. 7/11 laboratories interpreted the results as sensitive to oseltamivir, and six of these also indicated sensitivity to zanamivir. One laboratory reported that no resistance was found. 2/11 incorrectly reported oseltamivir and zanamivir resistance. 1/11 did not provide an interpretation. One laboratory also reported amantadine resistance.

Phenotypic testing. 8/12 laboratories correctly interpreted the sample as sensitive to both oseltamivir and zanamivir. In total, 9/12 reported sensitivity to oseltamivir (IC_{50} >0.2 to 117nM), two reported reduced susceptibility to oseltamivir (20.8, 36nM), and one reported resistance to oseltamivir (11nM). The laboratory that reported oseltamivir resistance used a chemiluminescence assay and had comparatively low baseline values (Annexes 11 and 12). Ten laboratories reported sensitivity to zanamivir (0.34 to 16.2nM). One laboratory reported reduced susceptibility to zanamivir (14.3). One laboratory did not test for zanamivir susceptibility.

Sample EISN_AV10-10. This sample contained B/England/MH48/2006 which is resistant to oseltamivir due to the I221T mutation in the NA gene and sensitive to zanamivir. The adamantanes are not effective against influenza B and were not considered in the analysis.

NA genotyping. 12/20 laboratories performed NA genotypic analysis. 9/12 laboratories identified the I221T mutation, and 2/9 reported additional mutations including P89S, N341, R152K, G402S, and D198N. 3/12 laboratories did not identify the I221T mutation, and two identified a G402S mutation instead. One laboratory commented that a poor sequence had been obtained so an interpretation was not possible.

Interpretation. 10/11 laboratories interpreted the sample as resistant to oseltamivir, and 1/11 interpreted it as showing reduced susceptibility to oseltamivir. 3/11 interpreted it as zanamivir resistant; 1/11 interpreted it as showing reduced susceptibility to zanamivir. 4/11 interpreted it as zanamivir sensitive; and 3/11 did not comment on zanamivir susceptibility. One laboratory reported amantadine resistance.

Phenotypic testing. 3/12 reported reduced susceptibility to oseltamivir (range 0.2 to 125nM) and 9/12 reported oseltamivir resistance (16.17 to 598nM). The three laboratories that reported reduced susceptibility to oseltamivir did not use baseline values for the interpretation of results. One laboratory reported reduced susceptibility to zanamivir (10.5nM), and 10 reported zanamivir sensitivity (0.34 to 17.1nM). One laboratory did not test for zanamivir susceptibility.