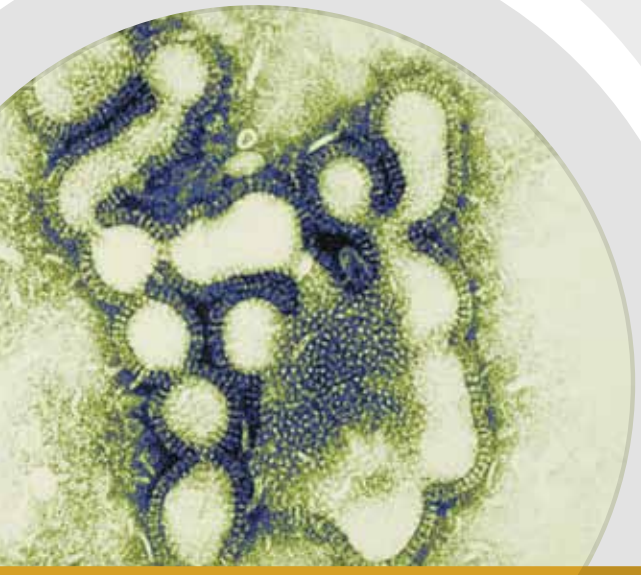

TECHNICAL REPORT

External quality assessment for influenza antiviral susceptibility for the European Reference Laboratory Network for human influenza

2013

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This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Cornelia Adloch and Eeva Broberg, and produced by Ian Harrison with assistance from Angie Lackenby and Catherine Thompson, on behalf of the European Reference Laboratory Network for Human Influenza (ERLI-Net).

Angie Lackenby, Public Health England, London, UK, and Adam Meijer, RIVM, Bilthoven, Netherlands, together with the antiviral task group designed the panel and contributed to the planning of the exercise. The panel was produced by the Respiratory Virus Unit, Public Health England, London, UK.

Data services were provided by Quality Control for Molecular Diagnostics (QCMD), Glasgow, UK. Analysis of the data was conducted by QCMD and Ian Harrison.

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Abbreviations

AANI	No amino acid substitution previously associated with highly reduced inhibition
AARI	Amino acid substitution previously associated with reduced inhibition.
AAHRI	Amino acid substitution previously associated with highly reduced inhibition
AV	Antiviral
AV13	Influenza antiviral susceptibility external quality assessment panel of ERLI-Net
EISN	European Influenza Surveillance Network
EQA	External quality assessment
ERLI-Net	European Reference Laboratory Network for Human Influenza
HRI	Highly reduced inhibition
IC50	50% inhibitory concentration
MUNANA	2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate
NAI	Neuraminidase inhibitors
NI	Normal inhibition
PCR	Polymerase chain reaction
PHE	Public Health England
QC	Quality Control
QCMD	Quality Control for Molecular Diagnostics
RI	Reduced inhibition
RIVM	National Institute for Public Health and the Environment, the Netherlands
SNP	Single nucleotide polymorphism
WHO	World Health Organization

Executive summary

During autumn 2013, an influenza virus antiviral (AV) susceptibility external quality assessment (EQA) was held for European reference laboratories. This was the second AV EQA panel distributed by the European Reference Laboratory Network for Human Influenza (ERLI-Net), and its predecessor organisation, since the European Influenza Surveillance Network (EISN) was established in 2008. The objectives of the exercise were to both provide participants with an independent mechanism to check performance and to provide information at the level of the whole network as to capacity and capability for phenotypic and/or genotypic AV testing and accurate reporting into The European Surveillance System (TESSy).

Twenty-five laboratories from 20 European countries participated in the exercise while 11 laboratories opted out as they did not perform assays capable of determining antiviral susceptibility, representing 69% of the network laboratories. Each participant received a panel of seven coded samples containing recent influenza viruses with substitutions known to confer reduced or highly reduced inhibition by neuraminidase inhibitor class of antiviral drugs. Participants tested the viruses using the antiviral susceptibility testing methodology currently used in their laboratories.

Twenty-four of the 25 (96%) laboratories participating in the EQA undertook genetic characterisation. Depending on the laboratory and the sample involved, techniques varied from pyrosequencing, single nucleotide polymorphism PCR's to direct sequencing of the neuraminidase gene. As a consequence, the interpretation that could be made about the antiviral susceptibility of the virus varied based on the technique used. In light of issues identified through the routine reporting of antiviral susceptibility data to TESSy, strict marking criteria were applied to the interpretation of genotypic results. Despite this, all 24 participating laboratories returned results for the three influenza A(H1N1)pdm09 samples, with 82% correctly interpreting results for oseltamivir and 78% correctly interpreting results for zanamivir susceptibility. A detailed assessment of interpretive issues identified will inform future training.

Seventeen of the laboratories (68%) returned phenotypic results. Of these, 14 (82%) of the laboratories reported no errors in any of the samples, while the remaining three laboratories accounted for four incorrect results. A mixed sample consisting of two influenza A(H1N1)pdm09 viruses, one with normal inhibition and one with highly reduced inhibition, accounted for three of the four incorrect answers.

Overall results for the exercise were broadly encouraging. There was an increase in the number of laboratories using phenotypic testing and an improvement in the average score achieved with this technique compared to the previous panel (97% compared to 92% in 2010). The proficiency for genotypic testing also improved, highlighted by the detection of amino acid substitution previously associated with highly reduced inhibition (AAHRI) from within a mixed sample also containing sequences with no amino acid substitutions previously associated with highly reduced inhibition (AANI). There were 87.5% of laboratories identifying the AAHRI genotype in this sample compared with 79% in 2010. This underlines the general improvement in the technical ability of network laboratories and suggests that interpretation and reporting of genotypic results is the area where training and support should now be targeted.

Introduction

Influenza virus is a highly contagious acute respiratory disease that can spread rapidly and widely causing high levels of morbidity and mortality. 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 neuraminidase (NA) natural substrate sialic acid. The close similarity to the natural substrate was predicted to limit the emergence of resistance.

Emergence of antiviral resistance is closely monitored through virological surveillance. Very few cases of resistance were found during clinical trials and post-licensure surveillance. Amino acid substitutions in the NA gene associated with highly-reduced inhibition (HRI) or reduced inhibition (RI) to NAI were described from the few cases of clinical resistance. In 2007, naturally occurring oseltamivir HRI due to a histidine-to-tyrosine amino acid substitutions at position 275 in the N1 NA (H275Y) was observed in former seasonal A(H1N1) viruses, and the oseltamivir HRI virus rapidly spread worldwide [1]. The neuraminidase inhibitors resistance in other influenza A subtypes and influenza B has not been widely observed. During the 2009 H1N1 pandemic, oseltamivir was commonly used for treatment and post-exposure prophylaxis. A small number of A(H1N1)pdm09 viruses with HRI by oseltamivir, due to the H275Y substitution, have been observed since the emergence of this virus. The situation is closely monitored by ERLI-Net and the WHO Global Influenza Surveillance and Response System.

The H275Y substitution in the A(H1N1) subtype is the most common mutation and the only single polymorphism unequivocally considered to confer clinical resistance to oseltamivir in the absence of compensatory or secondary amino acid substitutions. Other RI pathways have, however, been described for A(H1N1) viruses so the absence of H275Y does not preclude RI via alternative amino acid substitutions. Whereas the H275Y substitution is relatively easily targeted by laboratory assays, the situation for A(H3N2) subtype and influenza B is different, where several substitutions have been identified that generate RI [2]. For these viruses a broader testing strategy is required. The gold standard phenotyping test requires cultured viruses and is therefore not as widely performed [31]. Phenotyping tests (IC_{50} assay) can detect changes in NAI susceptibility (RI/HRI) due to known and unknown amino acid substitutions in influenza A and B viruses.

The present report contains the results of the influenza virus antiviral susceptibility EQA, designed and prepared by the ERLI-Net coordination team and funded by ECDC. The EQA was distributed in September 2013 by Public Health England (PHE), Quality Control for Molecular Diagnostics (QCMD) was subcontracted by the ERLI-Net coordination team to collate the results and perform initial analyses. Twenty-five laboratories participated in the antiviral susceptibility EQA.

This is the second influenza antiviral (AV) EQA for the EISN ERLI-Net associated laboratories in the EU/EEA [4]. As with all other aspects of influenza surveillance, it is essential that the correctness of influenza antiviral susceptibility testing methods is assessed through effective quality control (QC). An integral part of QC is EQA, which provides a means of externally evaluating individual laboratory performance and performance of the network as a whole.

Objectives

The specific objectives of the 2013 EISN influenza antiviral susceptibility testing EQA panel was to measure individual laboratory performance in the following areas:

- to test participants' ability to detect nucleotide mutations in the NA gene resulting in amino acid substitutions known to confer RI/HRI by NAI in a panel of influenza A and B viruses by genotypic and/or phenotypic antiviral susceptibility methods
- 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 ERLI-Net and ECDC to determine training priorities and produce guidelines on the harmonisation of interpretation of antiviral data and the reporting of results
- to test the interpretation of results and its implications for the quality of data uploaded into TESSy.

The overall aim of EQA testing is to aid:

- assessment of the general standard of performance ('state of the art')
- assessment of the effects of analytical procedures (method principles and techniques)
- evaluation of individual laboratory performance
- identification and justification of problem areas
- providing continuing education in terms of testing against samples of known status and enabling comparisons with other laboratories
- identification of needs for training activities

Study Design

Organisation

The EQA panel was designed by staff from PHE together with members of the ERLI-Net Coordination Team (ECDC contractors) and the ERLI-Net antiviral task group. The panel was prepared and tested by the Respiratory Virus Unit at PHE, London UK. Further pre- testing was performed by the World Health Organization Collaborating Centre for Reference and Research on Influenza at the National Institute for Medical Research at Mill Hill, London, UK, and the France South National Influenza Centre, Lyon, France. The panel contents were distributed to participants frozen on dry ice by specialist courier.

Participation

All influenza laboratory contact points in the ERLI-Net were notified in advance of the EQA exercise and were offered the option to opt out if they did not perform any antiviral susceptibility test in the laboratory. A list of participants in the influenza virus antiviral susceptibility EQA can be found in Annex 4.

Panel description

Seven simulated clinical samples were provided, containing inactivated influenza A(H1N1)pdm09, influenza A(H3N2) and influenza B viruses. Samples contained a viral load that allows direct measurement of IC_{50} without a virus culture step. Virus type and subtype were provided for each sample to guide selection of appropriate tests and analysis. Viruses were inactivated with Triton X-100, aliquoted and stored frozen at -80°C until dispatched. One panel was thawed and pre-tested at the PHE using in-house 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 (DHL) on the 24th September 2013 and all were received within two days. The deadline for reporting results was within 28 days of receipt of the panel.

Participant testing

Laboratories were expected to use only those genotypic and/or phenotypic method(s) they had in place for antiviral susceptibility testing to characterise this AV EQA panel. Laboratories were not penalised for reporting 'not tested' results on those samples in the panel for which no suitable methods were available for testing. Laboratories were scored on their final interpretation of the results obtained (normal, reduced or highly reduced inhibition-based on genotypic and/or phenotypic data or no interpretation possible for this drug from the tests performed).

Data reporting

For genotypic characterisation, participants were asked to report the predicted amino acid substitutions based on nucleotide sequence detected in the sample and their interpretation of the impact on NAI susceptibility (amino acid substitutions associated with (highly) reduced inhibition (AARI, AAHRI), or AANI). For phenotypic characterisation, participants were asked to report the IC_{50} value measured and their interpretation of the data (reduced, highly reduced or normal inhibition (RI, HRI, NI)). For both testing methods, participants were asked to specifically report if an interpretation was not possible from the methodology used, or if a negative result was obtained. QCMD managed web-based reporting tool collected data on the genotypic and phenotypic methods.

Data analysis

The World Health Organization phenotypic classification definitions are shown in Annex 1 [5]. The phenotypic scoring system used was as follows: a maximum of one point was awarded for each sample analysed irrespective of whether the participating laboratory tested for oseltamivir and zanamivir or oseltamivir alone. One point was deducted for each incorrect answer. No distinction was made between samples that were classified as HRI and samples that were classified as RI. The maximum achievable score for phenotypic characterisation was seven points. The genotypic scoring system awarded one point for a 'correctly interpreted' result when this was defined as results gained when an appropriate test had been used with appropriate interpretation. Where present, amino acid substitutions associated with reduced inhibition must be correctly identified and reported. The maximum achievable score for genotypic characterisation was 14 points.

Results

Panel composition and expected results

The influenza type, subtype, strain characterisation and antiviral susceptibility profile with associated amino acid substitutions in the NA protein for each sample in the EQA panel are shown in the panel composition table (Table 1). Amino acid numbering corresponding to the relevant NA subtype is used throughout.

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.

Table 1. Panel composition

Sample code	Subtype*	Strain designation	Mutation	Oseltamivir interpretation	Zanamivir interpretation
EISN_AV13-01	A(H3N2)	A/Galicia/RR9911/2012	E119V	Reduced inhibition	Normal inhibition
EISN_AV13-02	A(H3N2)	A/Galicia/15/2012	None	Normal inhibition	Normal inhibition
EISN_AV13-03	A(H1N1) pdm09	A/England/428+356 mix	275Y (mixed H/Y)	Reduced/ highly reduced inhibition †	Normal inhibition
EISN_AV13-04	A(H1N1) pdm09	A/England/356/2013	275Y	Highly reduced inhibition	Normal inhibition
EISN_AV13-05	A(H1N1) pdm09	A/England/428/2013	None	Normal inhibition	Normal inhibition
EISN_AV13-06	B-Vic	B/Lyon/CHU/12.88/2011	None	Normal inhibition	Normal inhibition
EISN_AV13-07	B-Vic	B/Lyon/CHU/15.216/2011	I221L	Highly reduced inhibition	Reduced inhibition

** Participants were provided with the subtypes of samples. † Reduced susceptibility to oseltamivir as a result of a mixture of virus with normal and highly reduced inhibition by oseltamivir. 'Mutation' - amino acid substitutions associated with (H)RI.*

Reporting time and participation

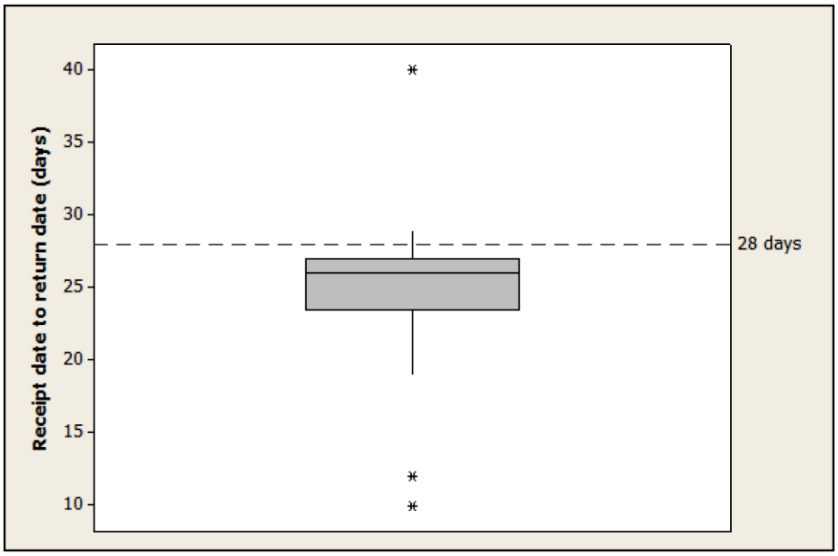
A graphical comparison of the return times for antiviral susceptibility results is presented in Figure 1. The results summarise the time taken from receipt of the panel to reporting of antiviral susceptibility results. The time period between courier delivery and panel receipt was not considered.

For this programme, 24 of 29 participants (82.8%) reported a panel receipt and results, and were included in the analysis shown in Figure 1. An additional participant did not report a panel receipt but did return results, two further participants reported a panel receipt but did not return results and two participants neither reported a panel receipt nor returned results. Eleven laboratories did not participate in the AV13 panel.

The median time taken for participants to return results (from the reported date of receipt) was 26 days (range 10 to 40 days), and the mean time taken was 25 days. Participants were expected to return their results within 28 days of receipt which twenty-two of the 24 laboratories achieved (91%).

The number of laboratories participating in the EISN-AV13 antiviral susceptibility EQA increased in 2013 from 2010, when the first quality assessment panel of this type was performed. The total number of participants increased from 20 to 25 with the number using phenotypic characterisation increasing from 12 to 17 laboratories. The number of laboratories using genotypic characterisation also increased from 20 to 24 (Figure 2).

Figure 1. Time taken to return results



The numbers of days taken by participants to return their antiviral susceptibility results are presented. The date participants reported receipt of the panel samples was considered as the start date (courier date not considered). These data are presented as a box and whiskers plot, where the box represents the 1st quartile, median and the 3rd quartile and the whiskers represent the range of values within 1.5 interquartile ranges of the box. Outlier values are represented as individual points.

Figure 2. Laboratories performing antiviral susceptibility testing (2010 & 2013)

	2010		2013	
2013 Participant ID	Antiviral - Phenotypic	Antiviral - Genotypic	Antiviral - Phenotypic	Antiviral - Genotypic
75	1			
95				
112				
117				
200				
207				
1159				
1174				
1262				
1299				
1323				
1402				
1433				
1456				
1515				
1534				
1643				
1649				
2001				
2125				
2126				
2253				
2258				
2270				
2271				
2274				
2275				
2276				
2277				
2278				
2306				
2820				
3442				
4208				
4209				
4213				
	12	20	17	24
	20		25	

Participating laboratories are identified by a unique anonymised participant ID code. Green shading indicates participation in AV13 and/or AV10 panel. Grey shading indicates non-participation in the panel (i.e. no results returned or no panel requested). Black shading indicates non-participation in EQA exercise. Participation in the EISN 2010 and 2013 EQA is shown.

Results of phenotypic testing

Seventeen of the 25 ERLI-Net laboratories (68%) that participated in the AV13 panel used phenotypic testing and determined the susceptibility of the EQA panel against oseltamivir. Fifteen of the 17 participants also determined the susceptibility of the EQA panel to zanamivir (Figure 2A). Analysis of the phenotypic results presented by individual laboratories (Table 2B) shows that one sample (AV13-03) was wrongly characterised by three laboratories, representing 75% of the incorrect results in the panel. This sample contained a mixture of two A(H1N1)pdm09 viruses, one with NI and the other with HRI phenotype.

Two fundamental assay platforms can be used for phenotypic testing; chemiluminescence substrate based assays (e.g. NA-Star) and fluorescent substrate based assays (e.g. MUNANA). To determine if either of the assay platforms produced a more reliable phenotypic characterisation, the average score achieved from each type of assay technology was compared. Figure 3 shows that the average phenotypic score achieved with the MUNANA based technology (n=14) was 9.8 out of a maximum 10. With the NA-Star chemiluminescence technology (n=3) the average score was 9 out of 10. Sample AV13-03 containing a mixture of NI and HRI phenotypes accounted for all of the NA-Star mistakes. Annex 2A and 2B depict the raw IC_{50} (nM) data for each sample and the interpretation of antiviral susceptibility phenotype (green – NI; red – RI/HRI).

The average score achieved in 2013 from phenotypic testing increased from 9.2 in 2010 to 9.7 out of a maximum of 10 (Figure 4).

Table 2A. Number and percentage of correctly interpreted phenotypic results

Sample code	Phenotypic interpretation		Summary of phenotypic results reported to QCMD					
			Oseltamivir interpretation			Zanamivir interpretation		
			Total results	Correct results		Total results	Correct results	
	Oseltamivir	Zanamivir		n	%		n	%
EISN_AV13-01	RI	NI	17	17	100.0	15	15	100.0
EISN_AV13-02	NI	NI	17	17	100.0	15	15	100.0
EISN_AV13-03	HRI/RI	NI	17	14	82.4	15	15	100.0
EISN_AV13-04	HRI	NI	17	17	100.0	15	15	100.0
EISN_AV13-05	NI	NI	17	17	100.0	15	15	100.0
EISN_AV13-06	NI	NI	17	16	94.1	15	15	100.0
EISN_AV13-07	HRI	RI	17	17	100.0	15	15	100.0

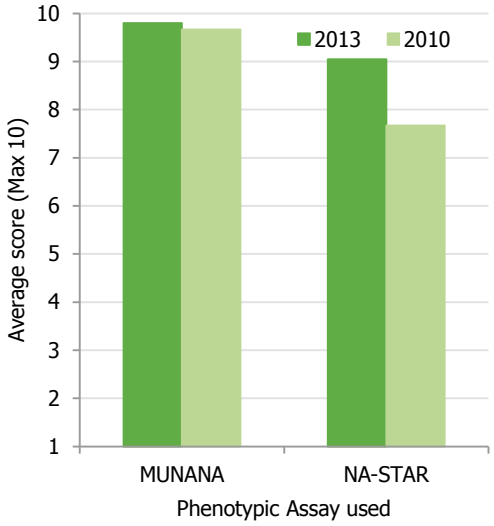
HRI: Highly reduced inhibition, RI: Reduced inhibition, NI: Normal inhibition

Table 2B. Phenotypic results presented by individual laboratory

Participant ID	EISN_AV13-01 (RI/NI)		EISN_AV13-02 (NI/NI)		EISN_AV13-03 (RI/NI)		EISN_AV13-04 (HRI/NI)		EISN_AV13-05 (NI/NI)		EISN_AV13-06 (NI/NI)		EISN_AV13-07 (HRI/RI)		Score
	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir	
75	HRI	NI	NI	NI	RI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
95	RI	NI	NI	NI	NI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	6
200	HRI		NI		HRI		HRI		NI		NI		HRI		7
1159	RI	NI	NI	NI	RI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
1174	HRI	NI	NI	NI	RI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
1299	HRI	NI	NI	NI	HRI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
1323	HRI	NI	NI	NI	RI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
1402	RI	Not determined	NI	Not determined	NI	Not determined	RI	Not determined	NI	Not determined	RI	Not determined	HRI	Not determined	5
1456	HRI	NI	NI	NI	HRI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
1534	RI	NI	NI	NI	RI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
1643	HRI	NI	NI	NI	RI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
2001	HRI	NI	NI	NI	RI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
2126	HRI	NI	NI	NI	HRI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
2271	HRI	NI	NI	NI	HRI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7
2276	RI	NI	NI	NI	Not determined	NI	HRI	NI	NI	NI	NI	NI	HRI	HRI	6
3442	HRI	NI	NI	NI	HRI	NI	HRI	NI	NI	NI	NI	NI	HRI	HRI	7
4213	RI	NI	NI	NI	RI	NI	HRI	NI	NI	NI	NI	NI	HRI	RI	7

Participating laboratories are identified by a unique anonymised participant ID code. Oseltamivir and zanamivir interpretations are presented per panel sample for each laboratory that returned results. Shaded cells indicate incorrect or incompletely correct results. Blacked out cells indicate no result was submitted. The scoring system used was as follows: a maximum of one point was awarded for each sample, irrespective of whether the participating laboratory tested for oseltamivir and zanamivir, or oseltamivir alone. One point was deducted for each incorrect answer.

Figure 3. Comparison of phenotypic assay platforms



Phenotypic scores were segregated based on which assay substrate was used; chemiluminescence (NA-Star) or fluorescence (MUNANA). Average score out of 10 are shown.

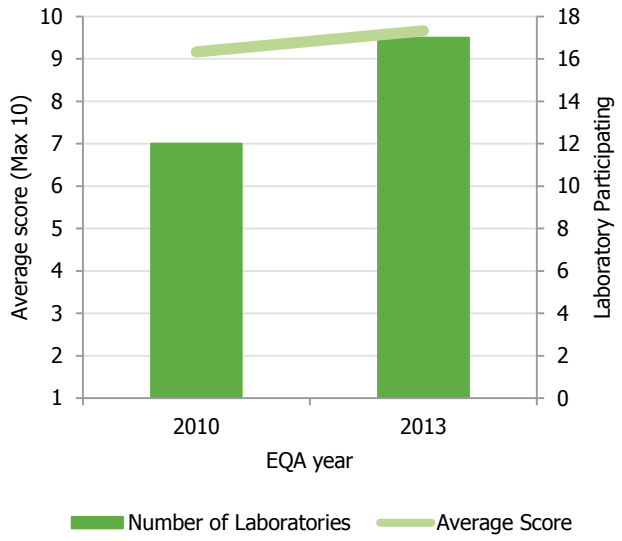
Genotypic testing for NA gene substitutions

Twenty four laboratories participated in at least one aspect of the genotypic characterisation (96% of participants in the AV13 panel). Of these, all participants attempted analysis of influenza A(H1N1)pdm09 whereas fewer laboratories characterised influenza B and influenza A(H3N2) samples.

Correct genotypic characterisation was defined as results gained when an appropriate test had been used with appropriate interpretation. Where present, amino acid substitutions associated with reduced inhibition must be correctly identified and reported. Therefore an appropriate assay, result and interpretation were required before being considered correct. Participants were also not given a score if a result was over interpreted, for example the detection of H275 in an A(H1N1)pdm09 sample by single nucleotide polymorphism (SNP) PCR can only be interpreted as 'no interpretation' for oseltamivir susceptibility as we do not know what other amino acid substitutions might be present. Laboratories using only pyrosequencing or SNP PCR are therefore more limited in what interpretation they can draw from their results. Furthermore, participants were asked to identify the amino acid substitutions that they considered important for antiviral susceptibility.

Table 3 shows that the number of laboratories correctly interpreting oseltamivir susceptibility varied from 56% to 92% while for zanamivir it varied from 63% to 83%. Annex 3A to 3C show the raw data presented by individual laboratory with the technique used to analyse the sample and any amino acid substitutions associated with (highly) reduced inhibition (AARI, AAHRI) or whether the sample contains no amino acid substitution previously associated with highly reduced (AANI).

Figure 4. Phenotypic characterisation: improvements since 2010



The number of laboratories participating in phenotypic characterisation in 2010 and 2013 are shown along with the average score (out of 10) achieved.

A summary of the genotypic characterisation results depicting the number of correctly and incorrectly analysed samples is shown in Figure 5. Very strict marking has been used in this analysis. Fifteen laboratories have returned results for both oseltamivir and zanamivir for each sample in the panel. Due to fundamental differences in the expected results return in the 2010 panel, a direct comparison is not possible. Figure 6 depicts the ability of laboratories to detect NAI associated nucleotide mutation in a mixed wild type/mutant population compared to a similar sample in the 2010 panel. The number of laboratories returning results increased from 19 to 24 while the proportion of laboratories detecting 275Y within a mixed wild-type population increased from 79% to 88%. The proportion of laboratories using either sequencing or SNP/pyrosequencing based technologies is shown in Figure 7. Roughly equal numbers of participants use sequencing as use SNP/pyrosequencing for the A(H1N1)pdm09 samples whereas the number using SNP/pyrosequencing reduces from 16 to 7 and 5 for A(H3N2) and B samples respectively.

Table 3. Number and percentage of correctly interpreted genotypic results

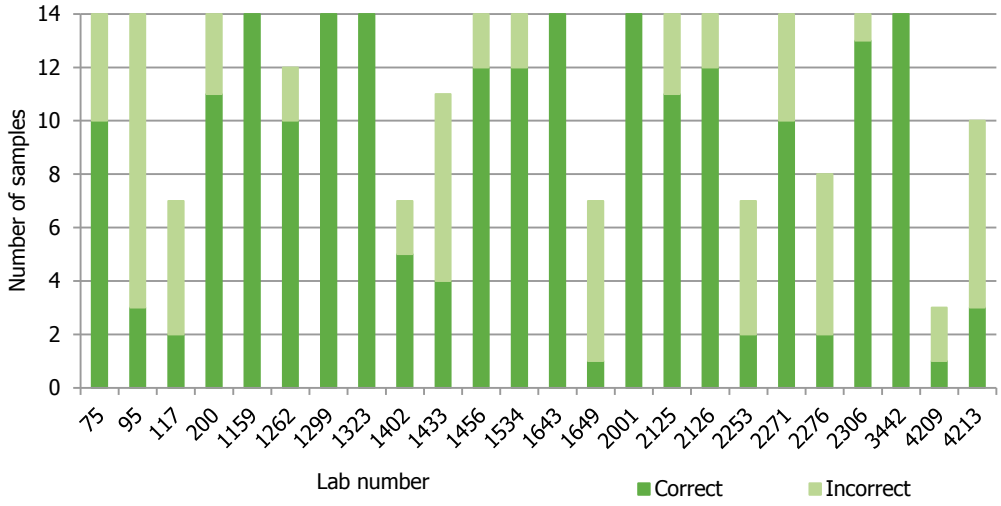
Sample code	Genotypic interpretation		Summary of genotypic results reported to QCMD					
			Oseltamivir interpretation			Zanamivir interpretation		
			Total results	Correct results		Total results	Correct results	
	Oseltamivir	Zanamivir		n	%		n	%
EISN_AV13-01	AARI	AANI	22	17	77.3	18	14	77.8
EISN_AV13-02	AANI	AANI	20	14	70.0	16	13	81.3
EISN_AV13-03	AAHRI/AARI	AANI	24	20	83.3	18	14	77.8
EISN_AV13-04	AAHRI	AANI	24	22	91.7	18	13	72.2
EISN_AV13-05	AANI	AANI	24	16	66.7	18	15	83.3
EISN_AV13-06	AANI	AANI	21	13	61.9	17	12	70.6
EISN_AV13-07	AAHRI	AARI	23	13	56.5	19	12	63.2

A correct result for the genotypic interpretation is defined as results gained when an appropriate test has been used with appropriate interpretation. Where present, amino acid substitutions associated with reduced inhibition must be correctly identified. AAHRI: Amino acid substitution previously associated with highly reduced inhibition. AARI: Amino acid substitution previously associated with reduced inhibition. AANI: No amino acid substitution previously associated with highly reduced inhibition.

Summary of overall performance

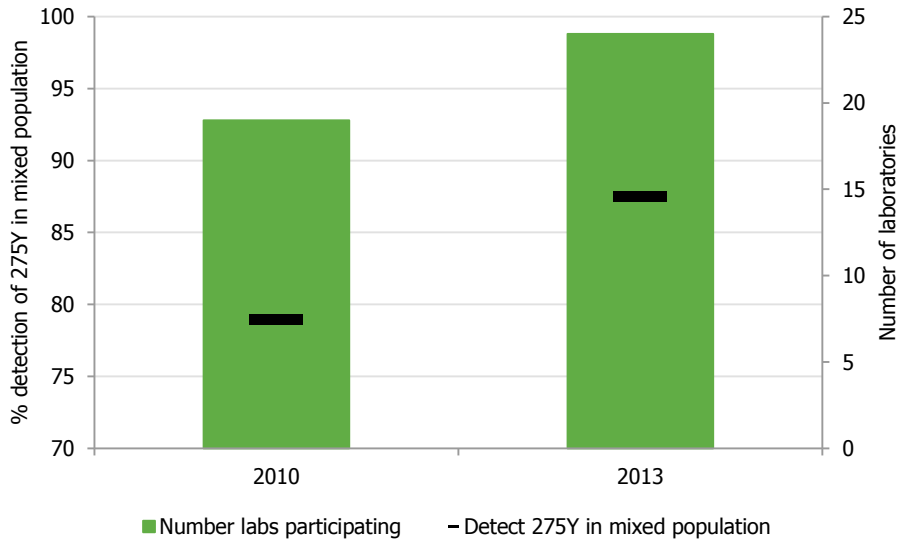
Of the 25 participants who returned results, eight (32%) returned genotypic results, one (4%) returned phenotypic results only and 16 (64%) returned both. A summary of the overall performance is shown in Table 4. Two A(H3N2) samples were analysed for zanamivir susceptibility with 19 laboratories registering the correct interpretation and only one recording an incorrect result. All zanamivir susceptibility interpretations for the three A(H1N1)pdm09 viruses were correctly interpreted whereas a total of seven reports on the two influenza B(Vic) viruses were incorrect. For oseltamivir susceptibility the number of incorrect interpretations was 4, 6, 7 for Influenza A(H3N2), A(H1N1)pdm09 and B(Vic) respectively. In total 84.6% of the oseltamivir results were correct and 9.7% incorrect, for zanamivir 72.6% of results were correct compared to 4.6% incorrect.

Figure 5. Number of correctly/incorrectly reported genotypic results, presented by laboratories



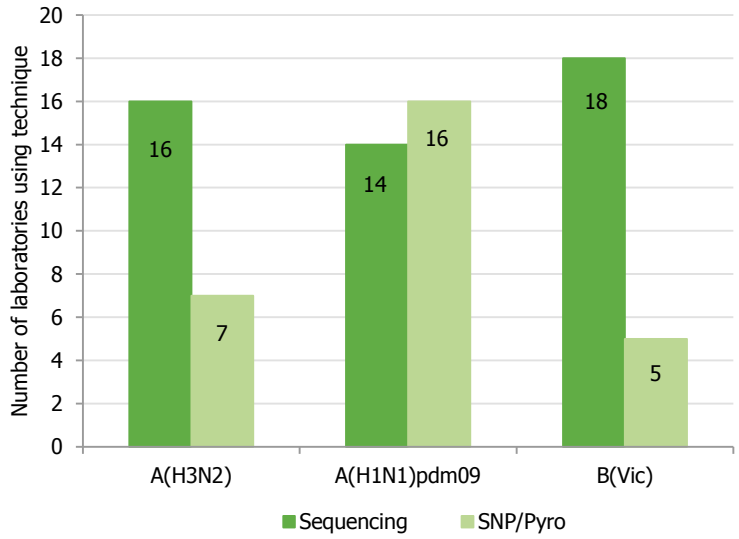
The numbers of correctly and incorrectly interpreted genotypic results recorded by each laboratory are shown for the AV13 panel.

Figure 6. Detection of 275Y in mixed populations; 2010 vs 2013



Ability of laboratories to detect the H275Y amino acid substitution in samples of mixed genotype was compared from 2010 to 2013. The number of participants in each year and the average score are shown.

Figure 7. Use of sequencing compared to SNP/pyrosequencing



The number of laboratories using either sequencing or SNP/pyrosequencing to obtain genotypic results in the AV13 panel is shown. Methods are separated by virus type/subtype.

Conclusion

Overall the 2013 EQA showed a reasonable level of competence among participating laboratories for antiviral susceptibility testing. Twenty-five laboratories participated, which represents 69% of the network laboratories invited to join the EQA exercise. This exercise has highlighted areas which would benefit from additional training to improve the overall quality of network results.

The panel contained a range of samples encompassing strains with amino acid substitutions affecting NAI susceptibility most likely to be encountered during surveillance. This included influenza A(H1N1)pdm09, A(H3N2) as well as B(Victoria). It also included a range of possible phenotypes represented by samples with NI, RI and HRI by both oseltamivir and zanamivir. As participants had the option to use phenotypic and/or genotypic testing, all possible techniques for antiviral susceptibility testing were available in the AV13 EQA panel.

Seventeen laboratories used phenotypic testing on the EQA panel, 96.6% of oseltamivir results and 100% of zanamivir results were correct. Of the four incorrect results, two may be attributed to the use of the chemiluminescence-based substrate on a sample with mixed population of NI and HRI, a possible test sensitivity issue that has previously been described [5]. The other two incorrect results may be avoided in future EQA exercises following some additional training. Overall the number of laboratories using phenotypic testing has increased since 2010 and the average score achieved has also increased (Figure 2B).

Table 4. Summary of overall performance

Sample code	Strain designation	Mutation	Interpretation		Summary of results reported to QCMD (n=25)											
					Oseltamivir interpretation						Zanamivir interpretation					
					Correct		Incorrect		Not tested		Correct		Incorrect		Not tested	
			Oseltamivir	Zanamivir	n	%	n	%	n	%	n	%	n	%	n	%
EISN_AV13-01	A/Galicia/RR9911/2012	E119V	RI	NI	19	76.0	3	12.0	3	12.0	19	76.0	1	4.0	5	20.0
EISN_AV13-02	A/Galicia/15/2012	None	NI	NI	21	84.0	1	4.0	3	12.0	19	76.0	0	0.0	6	24.0
EISN_AV13-03	A/England/428+356 mix	275Y (mixed H/Y)	HRI/RI	NI	20	80.0	5	20.0	0	0.0	19	76.0	0	0.0	6	24.0
EISN_AV13-04	A/England/356/2013	275Y	HRI	NI	24	96.0	1	4.0	0	0.0	18	72.0	0	0.0	7	28.0
EISN_AV13-05	A/England/428/2013	None	NI	NI	25	100.0	0	0.0	0	0.0	19	76.0	0	0.0	6	24.0
EISN_AV13-06	B/Lyon/CHU/12.88/2011	None	NI	NI	21	84.0	2	8.0	2	8.0	18	72.0	2	8.0	5	20.0
EISN_AV13-07	B/Lyon/CHU/15.216/2011	I221L	HRI	RI	18	72.0	5	20.0	2	8.0	15	60.0	5	20.0	5	20.0

Sample Code: Panel sample codes for the samples distributed to participants. Strain Designation: The influenza virus strain designation of individual panel samples. Mutation: Amino acid substitution conferring antiviral reduced or highly reduced inhibition in individual panel samples. Interpretation: Expected antiviral susceptibility status. Oseltamivir interpretation: Number and percentage of all oseltamivir susceptibility results (genotypic and phenotypic) correctly and incorrectly interpreting the panel sample. Zanamivir interpretation: Number and percentage of all zanamivir susceptibility results correctly and incorrectly interpreting the panel sample.

Several laboratories encountered difficulties in amplifying full length/large NA gene PCR products, as is specified in the WHO recommended protocol for NA gene sequencing. Alternative/modified protocols generating smaller amplicons were more successful. These technical difficulties may have been a result of reduced RNA integrity, possibly following the Triton X-100 inactivation of virus during panel preparation. Concern has been raised that the quality of data being uploaded into TESSy could be compromised by the over-interpretation of genotypic data. For this reason, very strict marking rules were employed this year to highlight to participants where over interpretations have previously been made. Considering these two factors it is therefore encouraging that overall 72.4% of oseltamivir and 74.3% of zanamivir genotypic interpretations were correct, with future training this should improve further. Results for influenza A(H1N1)pdm09 showed the highest accuracy which is not surprising as these viruses have the best characterised resistance pathway. It was also encouraging that participants' ability to detect nucleotide mutations associated with highly reduced inhibition within a mixed population has improved since the last panel was circulated (Figure 6).

Three training courses were held in November 2010, November 2011 and October 2013 covering the techniques of sequencing and bioinformatics tools. The ability to sequence the NA gene is fundamental to accurate interpretation of NAI susceptibility; especially in non-A subtypes where SNP and pyrosequencing assays are constrained by more limited interpretations. A full list of all training offered and attended can be found in Annex 5.

Discussion

The number of participants performing AV testing increased in 2013 from the previous panel in 2010. Results were returned within the expected timeframe, with a median of 26 days for data submission (range 10 to 40 days). Laboratories will have worked to the time-frame provided for this exercise, however, the ability of EISN to detect influenza antiviral resistance in a timely manner in community and hospitalised specimens is an important aspect of the public health response.

Compared to 2010, the average score for phenotypic tests improved from 9.2/10 to 9.7/10 and the number of laboratories performing this test also increased. Training in antiviral susceptibility was provided to 15 laboratories in July 2011, which may help to explain this increase.

The aim of the first antiviral susceptibility EQA in 2010 differed from that of 2013 which makes it difficult to compare the global genotypic results between the two exercises. In 2010, the focus was on evaluating whether participating laboratories could apply genotypic methods to detect amino acid substitutions affecting NAI susceptibility. In 2013, the focus shifted to evaluating the interpretation of the genotypic results; particularly whether participating laboratories had a sound knowledge of the limitations, especially of those methods targeting SNPs as opposed to full segment length sequencing.

Common to both the 2010 and 2013 exercises was the inclusion of a sample containing a mixture of oseltamivir HRI (H275Y) and NI viruses. More laboratories returned results for this sample and a greater proportion detected the H275Y amino acid substitution (79% in 2010 to 88% in 2013). This is an important improvement in participating laboratories capability as samples with a (highly) reduced inhibited virus quasispecies are the more common scenario, if laboratories are receiving samples for analysis from treated patients as part of their surveillance program.

Due to the technical difficulties encountered with NA gene sequencing, which may have been a result of the manner in which the panel was prepared, consideration in future should be given to whether inactivation of the antiviral EQA panel is required, and if so, whether other inactivation methods may be preferable. Both issues have previously been discussed at length; inactivation of samples was preferred as the risk associated with introducing live HRI viruses into many European laboratories were considered too great. High viral load preparations would be required to avoid culturing the samples, which would introduce logistical difficult with shipping. However, as many laboratories now maintain HRI viral stocks for phenotypic assays the risk associated with live HRI samples could be considered to have reduced. If inactivation is still preferred, several alternative methods could be considered. The WHO EQAP has used both gamma irradiation and BPL treatment with varying consequences on the ability to sequence the full NA gene. As laboratories have the option of using phenotypic assays, the inactivation process must preserve NA activity [6] which poses a limitation on many alternative inactivation techniques.

Recommendations

Phenotypic characterisation:

- Twinning funds are available, and laboratories should consider applying for those, if additional training either in laboratory methods or data interpretation would be of benefit.
- A further training course in phenotypic characterisation may help increase the number of laboratories using this technique (currently 17).

A theoretical training course should be held based on genotypic data interpretation and reporting into TESSy:

- The use of appropriate assay; SNP based assays may be suitable for screening of A(H1N1)pdm09, as the majority of HRI viruses have H275Y, or A(H3N2) as the majority of HRI viruses have R292K or E119V. SNP is less appropriate for influenza B viruses due to the number of different amino acid substitutions which have been described to impact NAI susceptibility, all of which occur infrequently.
- How to interpret genotypic data and how to avoid over-interpretation; where SNP based data or partial sequencing data is used, interpretations for wild-type viruses are not possible.
- How to report data; if reporting a HRI/RI virus then the associated amino acid substitutions must also be reported as soon as possible, especially in situations when sequencing is performed by a third party.

The requirement for inactivation of the antiviral EQA panel should be reviewed.

References

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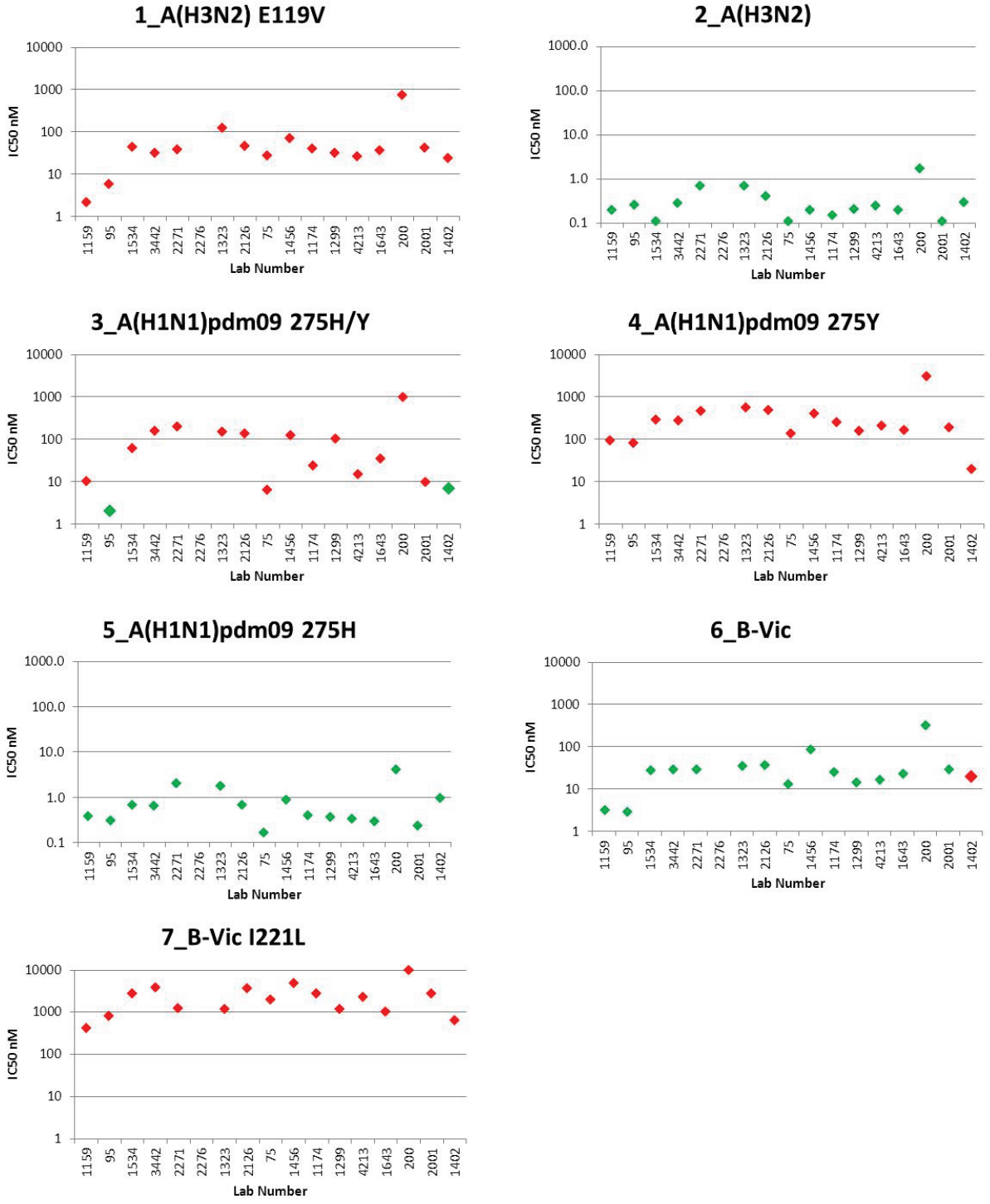
6. Jonges M, Liu WM, Vries E. van der, Jacobi R, Pronk I, Boog C, Koopmans M, Meijer A, and Soethout E. (2010). Influenza Virus Inactivation for Studies of Antigenicity and Phenotypic Neuraminidase Inhibitor Resistance Profiling. J. Clin. Microbiol. *48*, 928–940.

Annex 1. Definition of phenotypic classification

Category	Fold IC_{50} change *	
	Type A viruses	Type B viruses
Normal inhibition (NI)	< 10	< 5
Reduced inhibition (RI)	10-100	5-50
Highly reduced inhibition (HRI)	>100	>50

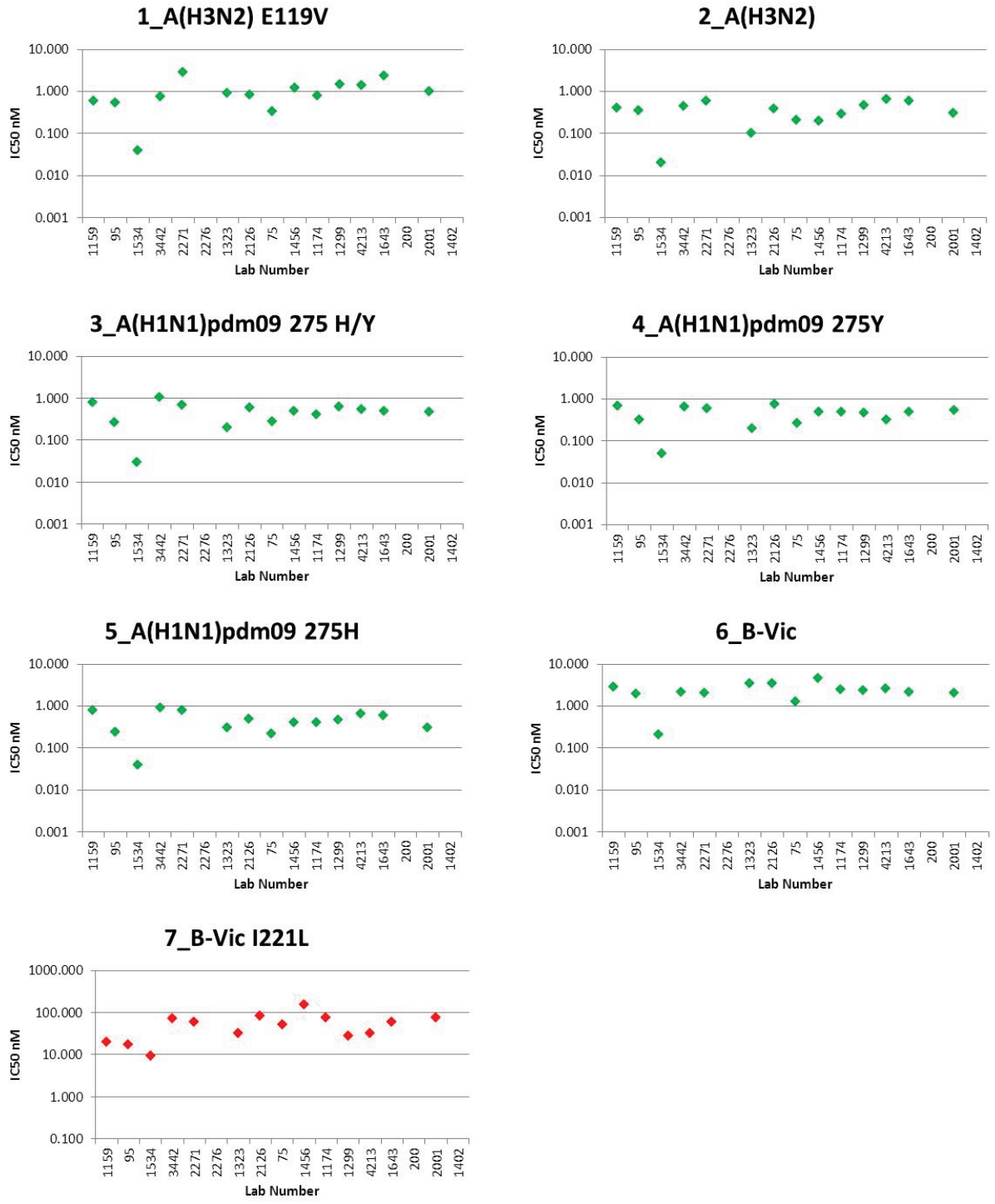
** Fold change against the median/mean IC_{50} for the (sub)type previous or current season after removal of obvious outliers, or against a known wild type virus of the same subtype.*

Annex 2A. Oseltamivir phenotypic IC_{50} results presented by laboratories.



Individual laboratories IC_{50} (nM) for each sample of the AV13 panel is shown. Interpretation based on the IC_{50} in comparison to controls is also indicated by the colour of the data point (red; RI/HRI or green; NI) is also indicated.

Annex 2B. Zanamivir phenotypic IC_{50} results presented by laboratories.



Individual laboratories IC_{50} (nM) for each sample of the AV13 panel is shown. Interpretation based on the IC_{50} in comparison to controls is also indicated by the colour of the data point (red; RI/HRI or green; NI) is also indicated.

Annex 3A. Genotypic results presented by individual laboratories

Participant ID	Genotypic testing method	EISN_AV13-01 (AARI/AANI)			EISN_AV13-02 (AANI/AANI)		
		NA Mutation	Oseltamivir	Zanamivir	NA Mutation	Oseltamivir	Zanamivir
75	Sequencing	E119V	AAHRI	AANI	None	No interpretation	No interpretation
95	SNP/Pyro [119]	119V	AARI	AANI	none	AANI	AANI
117	SNP/Pyro [275]	None	AANI	Not tested	None	AANI	Not tested
200	Sequencing	119V	AAHRI	AANI		AANI	AANI
1159	Sequencing	119V	AAHRI	AANI		AANI	AANI
1262	Sequencing	119V	AAHRI	AANI			
1299	Both	119V	AAHRI	AANI	119E	AANI	AANI
1323	Sequencing	119V	AAHRI	AANI		AANI	AANI
1402	Sequencing	119V; 292R	AAHRI	Not tested	119E; 292R	AANI	Not tested
1433	Sequencing	R292; N294; E119	AANI	AANI	E119V; D151; R292; N294	AAHRI	AANI
1456	Sequencing	119V	AAHRI	AANI		AANI	AANI
1534	Sequencing	E119V	AARI	AANI	none	AANI	AANI
1643	Sequencing	119V	AAHRI	AANI		AANI	AANI
1649	SNP/Pyro [275]	Negative	No interpretation	Not tested	Negative	No interpretation	Not tested
2001	SNP/Pyro [119;292]	119V	AAHRI	No interpretation	none	No interpretation	No interpretation
2125	Sequencing	119V	AARI	AARI	none	AANI	AANI
2126	SNP/Pyro [119]	119V	AARI	No interpretation	119E	No interpretation	No interpretation
2253	SNP/Pyro [275]	NO	AANI	Not tested	NO	AANI	Not tested
2271	Sequencing	119V	AAHRI	AANI	119E	AANI	AANI
2276							
2306	Sequencing	119V	AAHRI	AANI	negative	AANI	AANI
3442	Sequencing	119V	AAHRI	AANI		AANI	AANI
4209			Not tested	Not tested		Not tested	Not tested
4213	Sequencing		No interpretation	No interpretation		Not tested	Not tested

Participating laboratories are identified by a unique anonymised participant ID code. Participants' genotypic assay types are categorised as 'Sequencing', 'partial gene sequencing', 'SNP/Pyro' or both SNP and sequencing 'Both'. Oseltamivir and Zanamivir interpretations are presented per panel sample for each laboratory that returned results. Blacked out cells indicate no result was submitted. Scores per sample are indicated to the right of each drug interpretation column

Annex 3B. Genotypic results presented by individual laboratories

Participant ID	Genotypic testing method	EISN_AV13-03 (AA(H)RI/AANI)				EISN_AV13-04 (AAHRI/AANI)			EISN_AV13-05 (AANI/AANI)		
		NA Mutation	Ratio H/Y	Oseltamivir	Zanamivir	NA Mutation	Oseltamivir	Zanamivir		Oseltamivir	Zanamivir
75	SNP/Pyro [275]	H275Y	50 / 50	AAHRI	No interpretation	H275Y	AAHRI	No interpretation	None	No interpretation	No interpretation
95	SNP/Pyro [275]	275H / Y	50 / 50	AARI	AANI	275Y	AAHRI	AANI	none	AANI	AANI
117	SNP/Pyro [275]	H275Y		AAHRI	Not tested	H275Y	AAHRI	Not tested	None	AANI	Not tested
200	Sequencing	275H/Y		AAHRI	AANI	275Y	AANI	No interpretation		AANI	AANI
1159	Sequencing	275H/Y	Mix H/Y	AAHRI	AANI	275Y	AAHRI	AANI		AANI	AANI
1262	Sequencing	275H/Y	Mix H/Y	AAHRI	AANI	275Y	AAHRI	AANI		AANI	AANI
1299	Both	275HY	70 / 30	AAHRI	AANI	275Y	AAHRI	AANI	275H	AANI	AANI
1323	Both	275H/Y	60 / 40	AARI	AANI	275Y	AAHRI	AANI		AANI	AANI
1402	Both	275H; 275Y	50 / 50	AAHRI	Not tested	275Y	AAHRI	Not tested	275H	AANI	Not tested
1433	Sequencing	H275Y; N294	Mix H/Y	AAHRI	Not tested	H275Y; N294	AAHRI	Not tested	H275; N294	AANI	Not tested
1456	Sequencing	275Y		AAHRI	AANI	275Y	AAHRI	AANI		AANI	AANI
1534	Both	275H/Y	64 / 36	AAHRI	AANI	275Y	AAHRI	AANI	none	AANI	AANI
1643	Sequencing	275H/Y	Mix H/Y	AAHRI	AANI	275Y	AAHRI	AANI		AANI	AANI
1649	SNP/Pyro [275]	Negative		AANI	Not tested	275Y	AARI	Not tested	Negative	AANI	Not tested
2001	SNP/Pyro [275]	275H/Y	Mix H/Y	AAHRI	No interpretation	275Y	AAHRI	No interpretation	275H	No interpretation	No interpretation
2125	Sequencing	H275Y	50 / 50	AAHRI	AANI	275Y	AAHRI	AANI	none	AANI	AANI
2126	SNP/Pyro [275]	275H/Y	70 / 30	AAHRI	No interpretation	275Y	AAHRI	No interpretation	275H	No interpretation	No interpretation
2253	SNP/Pyro [275]	H274Y		AAHRI	Not tested	H274Y	AAHRI	Not tested	NO	AANI	Not tested
2271	SNP/Pyro [275]	275H/Y	50 / 50	AAHRI	AANI	275Y	AAHRI	AANI	275H	AANI	AANI
2276	Both			AAHRI	AANI		AAHRI	AANI		AANI	AANI
2306	Sequencing	negative		AANI	AANI	275Y	AAHRI	AANI	negative	AANI	AANI
3442	Both	275H/Y	Mix H/Y	AAHRI	AANI	275Y	AAHRI	AANI		AANI	AANI
4209	SNP/Pyro [275]	wild type		AANI	Not tested	Y274	AARI	Not tested	wild type	AANI	Not tested
4213	SNP/Pyro [275]	275H/Y	80 / 20	AARI	AANI	275Y	AAHRI	AANI		AANI	AANI

Annex 3C. Genotypic results presented by individual laboratories

Participant ID	Genotypic testing method	EISN_AV13-06 (AANI/AANI)			EISN_AV13-07 (AAHRI/AARI)		
		NA Mutation	Oseltamivir	Zanamivir	NA Mutation	Oseltamivir	Zanamivir
75	Sequencing	none	No interpretation	No interpretation	I222L	AAHRI	AARI
95	SNP/Pyro [ ]	none	AANI	AANI	None	AANI	AANI
117	SNP/Pyro [275]	None	AANI	Not tested	None	AANI	Not tested
200	Sequencing	402S	AANI	AARI	221L;402S	AAHRI	AARI
1159	Sequencing		AANI	AANI	221L	AAHRI	AARI
1262	Sequencing		AANI	AANI	221L	AANI	AANI
1299	Sequencing	221I	AANI	AANI	221L	AAHRI	AARI
1323	Sequencing		AANI	AANI	222L	AAHRI	AARI
1402	Sequencing	152K; 197D	AAHRI	Not tested	152K; 197D	AAHRI	Not tested
1433	Sequencing	152K 198N I221	AAHRI	AAHRI	152K 198N I221L	AAHRI	AAHRI
1456	Sequencing		No interpretation	No interpretation	222L	AAHRI	AARI
1534	Sequencing		AANI	AANI		AAHRI	AARI
1643	Sequencing		AANI	AANI	221L	AAHRI	AARI
1649	SNP/Pyro [275]	Negative	No interpretation	Not tested	Negative	No interpretation	Not tested
2001	Sequencing		No interpretation	No interpretation	I221L	AAHRI	AARI
2125	Sequencing	none	AANI	AANI	None	AANI	AANI
2126	SNP/Pyro [152;198;222]	152R;198D;222I	No interpretation	No interpretation	152R;198D;222R	AAHRI	AARI
2253	SNP/Pyro [275]	NO	AANI	Not tested	NO	AANI	Not tested
2271	Sequencing	221I	AANI	AANI	221L	AAHRI	AARI
2276	Sequencing					AANI	AANI
2306	Sequencing	negative	AANI	AANI	221L	AAHRI	AARI
3442	Sequencing		AANI	AANI	221L	AARI	AARI
4209			Not tested	Not tested		Not tested	Not tested
4213	Sequencing		Not tested	Not tested	221L	AARI	AANI

Annex 4. List of participating laboratories

Country	City	Organisation
Austria	Vienna	AKH Wien - Medical Uni of Vienna
Belgium	Brussels	Institute of Public Health
Czech Republic	Prague	National Institute of Public Health
Denmark	Copenhagen	Statens Serum Institute
Finland	Helsinki	National Institute for Health and Welfare (THL)
France	Paris	CNR de la Grippe - Institute Pasteur
France	Bron Lyon	CNR Virus Influenza - HCL Lyon
Germany	Berlin	Robert Koch Institute
Greece	Athens	National Influenza Center for S Greece
Hungary	Budapest	Országos Epidemiológiai Központ
Ireland	Dublin	University College Dublin
Italy	Rome	Istituto Superiore di Sanita (NIH)
Latvia	Riga	National Microbiology Reference Laboratory ,NIC of Latvia
Lithuania	Vilnius	National Public Health Surveillance Laboratory
Netherlands	Bilthoven	RIVM
Netherlands	Rotterdam	Erasmus MC
Norway	Oslo	Norwegian Institute of Public Health
Portugal	Lisbon	Instituto Nacional de Saúde Doutor Ricardo Jorge
Romania	Bucharest	Cantacuzino Institute
Spain	Majadahonda, Madrid	Instituto de Salud Carlos III
Spain	Valladolid	Hospital Clinico Universitario
Spain	Barcelona	Hospital Clinic i Provincial
Sweden	Solna	Folkhälsomyndigheten
United Kingdom	London	Publich Health England
United Kingdom	Glasgow	Gartnavel General Hospital

Annex 5. Participating laboratories and training received from ERLI-Net

	VIRGIL	Influenza surveillance	Influenza antiviral susceptibility surveillance	Virus culture	Virus culture	Sequencing and bioinformatics tools	Sequencing and bioinformatics tools	Sequencing and bioinformatics tools
		Jun-10	Jul-11	Nov-12	Apr-13	Nov-10	Nov-11	13-Oct
75	2006; 2007							x
95								x
117								
200			xx	x				
1159		x	x		x		x	
1174			x		x	x		
1262	2006; 2007	x	x		x		x	x
1299								
1323	2006							
1402		x	x	x				
1433		x	x	x				
1456	2006 (2); 2007	x					x	
1534	2006 (2)				x			
1643	2007		x	x		x		
1649	2007						x	x
2001	2006 (2); 2007						x	
2125	2007	x				x		
2126								
2253	2007	x				x		x
2271	2006; 2007 (2)		x	x		x	x	x
2276	2007	x	x		x	x		
2306			x	x			x	
3442	2006 (2); 2007 (2)	x	x	x			x	x
4209		x	x	x				
4213	2006; 2007						x	x

X indicated attendance at an ERLI-Net training course