TECHNICAL REPORT

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This report of the European Centre for Disease Prevention and Control (ECDC) was coordinated by Eeva Broberg.

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Executive summary

During winter 2010–11, an influenza virus rapid detection and culture external quality assessment (EQA) exercise was held for European influenza reference laboratories. This was the second rapid detection and culture EQA panel distributed by the Community Network of Reference Laboratories for Human Influenza in Europe (CNRL) 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 network level as to the capacity and capability for rapid detection by polymerase chain reaction (PCR), influenza virus culture and strain characterisation within a defined reporting timeframe.

All CNRL member laboratories were invited to participate. Thirty four laboratories from 27 European countries participated in the exercise, of which 33 had also participated in the previous EQA in 2008. Each participant received a panel of ten coded samples including influenza A and B viruses that are currently, or have recently circulated in humans, or are antigenically diverse from recent strains and negative samples. Thirty-three participants returned results for rapid detection of influenza viruses using PCR or other methods. Results for influenza virus culture were returned by 30 participants with 26 reporting strain characterisation results.

Despite the inclusion of more challenging test samples, the proportion of laboratories achieving full rapid detection proficiency scores increased in 2010 (76%) compared with 2008 (69%). This represents an excellent achievement as the 2010–11 panel was more demanding with the inclusion of an additional subtype (influenza A(H1N1)pdm09), a new H3 variant (A/Perth/16/2009) and a challenging low viral titre sample. Altogether, the panel was a comprehensive test of sensitivity and specificity, and the laboratories ability to detect both recently circulating and antigenically diverse influenza viruses.

There was a significant improvement in the proportion of laboratories performing influenza A subtyping with only one laboratory returning no subtyping results in 2010–11 compared with six in 2008. Results indicated considerable strength in the network for molecular detection and (sub)typing of influenza viruses and that laboratories have successfully implemented detection methods for A(H1N1)pdm09 virus and increased capacity for subtyping influenza A viruses. A small proportion of false negative errors (9%) indicated that in some cases there may be a low assay sensitivity for detection/typing of low viral titre A(H1N1)pdm09 virus. However the false positive error rate improved indicating laboratories have good systems in place to minimise cross-contamination.

Those laboratories who returned virus culture results performed well, although there was a slight decrease in the proportion of laboratories participating. Some laboratories did not recover virus from the low viral titre sample, highlighting the necessity for optimal cell culture systems and detection methods. Current issues with virus isolation and detection for some influenza subtypes also serve to highlight the need for continual investment in development and training. As the global influenza surveillance system relies on the availability of cultured virus isolates for strain characterisation to inform vaccine selection, it is imperative to support network laboratories in these activities.

The results for strain characterisation showed little change compared to 2008. The majority of participants used haemagglutination inhibition assay (HI) to determine strain identify rather than sequencing, emphasising the need for continued good performance in virus culture to provide virus isolates for HI. Incomplete strain characterisation results indicated key issues including the limited availability of ferret antiserum in terms of volume and strain diversity.

The 2010–11 influenza virus rapid detection and culture EQA demonstrated that the network laboratories have made improvements in rapid detection and subtyping, and good progress in the development and implementation of molecular assays for the A(H1N1)pdm09 virus and for subtyping influenza A viruses. There was little change in performance in culture and strain characterisation. The valuable contribution that network laboratories make in terms of supplying virus isolates and strain characterisation information to the global influenza surveillance network must be supported with ongoing development and training.

Introduction

The European Centre for Disease Prevention and Control (ECDC) is a European Union (EU) agency with a mandate to operate the dedicated surveillance networks and to identify, assess, and communicate current and emerging threats to human health from communicable diseases. 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 assessment schemes.' (Article 5.3, EC 851/2004[1]).

External quality assessments (EQA) are part of quality management systems, and evaluate the performance of laboratories, by an outside agency on material that is supplied specifically for that purpose. ECDC's disease specific networks organise a series of EQAs for EU/European Economic Area (EEA) countries. In some specific networks non-EU/EEA countries are also involved in the EQA activities organised by ECDC. The aim of the EQA is to identify areas for improvement in laboratory diagnostic capacities relevant to surveillance of disease listed in Decision No. 2119/98/EC[2] and to ensure reliability and comparability of results in laboratories from all EU/EEA countries. The main purpose 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
- providing continuing education
- identification of needs for training activities

The European Influenza Surveillance Network (EISN), including the Community Network of Reference Laboratories for Human Influenza in Europe (CNRL) is a dedicated surveillance network to contribute information on the epidemiological and virological surveillance of influenza to the community network as established by Decision 2119/98/EC.

In 2008, a framework contract with CNRL including external quality assessment (EQA) for the influenza virus was put in place for the years 2008–2011. CNRL organises the EQA programme for the national reference laboratories in EU and EEA countries on virus culture and detection, subtyping, antigenic characterisation, molecular typing and antiviral resistance testing for influenza virus.

Objectives

The primary aim of this external quality assessment exercise was to measure individual laboratory performance in the following areas:

- rapid detection by PCR or other tests (within a defined reporting timeframe)
- virus culture (within a defined reporting timeframe)
- virus typing after virus isolation (using HI or PCR)
- influenza A virus subtyping after virus isolation (using HI or PCR)
- virus strain identification (by HI and/or by sequencing)

[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. Available at: http://eur lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32004R0851:EN:HTML

[2] Decision no 2119/98/EC of the European Parliament and of the Council of 24 September 1998 setting up a network for the epidemiological surveillance and control of communicable diseases in the Community. Available at: http://eur-lex.europa.eu/smartapi/cgi/sga_doc?smartapi!celexapi!prod!CELEXnumdoc&lg=EN&numdoc=31998D2119&model=guicheti

Study design

Organisation

The EQA panel was designed by members of the CNRL 'Quality and Training' task group (TG5). The panel was prepared and tested by the Respiratory Virus Unit (RVU) at the Health Protection Agency (HPA), London UK. Further pre-testing was performed by the World Health Organization Collaborating Centre (WHO CC) at the National Institute for Medical Research (NIMR) at Mill Hill, London, UK, and the France South National Influenza Centre (NIC), Lyon, France. The panel contents were distributed to participants frozen on dry ice by specialist courier. Participants submitted results electronically into a web-based database.

Participation

CNRL member laboratories include all EU countries and Norway and Iceland. All influenza laboratory contact points in the CNRL were notified in advance of the EQA exercise. A list of participants in the influenza virus rapid detection and culture EQA can be found in Annex 1. The WHO Regional Office for Europe organised participation for countries not affiliated with CNRL (data not included in this report).

Panel description

The EQA panel consisted of eight samples containing influenza viruses from subtypes that are currently or have recently circulated in humans including influenza A(H1N1)pdm09, former seasonal influenza A H1N1, influenza A H3N2 and influenza B. Two negative samples containing no virus completed the panel of ten samples. Viruses were grown in eggs and diluted to a suitable concentration for testing determined by viral plaque assay and haemagglutination assay. Viruses were aliquoted and stored frozen at -80°C until required. One panel was thawed and pre-tested at the HPA using in-house methods. Panels were sent frozen on dry ice to two independent laboratories for pre-testing. The final panel content was shipped frozen on dry ice by a specialist courier to participants between 29th November and 13th December 2010. The deadline for rapid detection results return was 17th January 2011 and for culture results by 31th January 2011. A web-based database was used by the participants to submit results.

Participant Testing

Participants were asked to test the panel using the standard laboratory protocols normally used by their laboratory for rapid detection, virus culture and antigenic characterisation including PCR, haemagglutination inhibition (HI) and sequencing.

Data reporting

For rapid detection participants were asked to report the influenza type and subtype detected by PCR, or whether a negative result had been obtained. For virus culture and antigenic characterisation, participants were asked to report the virus type and subtype, (or negative) and the strain identification. A questionnaire was used to collect data on culture methods.

Data analysis

The scoring system used for rapid detection was: one point for correct detection of influenza virus A or B; one point for correct typing of influenza virus A or B; one point for correct subtyping of influenza virus A; and three points for correct determination of a negative. The maximum achievable score for rapid detection was 30 points. For virus culture and strain characterisation the scoring system used was: one point for isolation of influenza virus A or B; one point for correct subtyping of influenza virus A or typing of influenza virus B; one point for correct strain identification; and three points for correct determination of a negative. The maximum achievable score for virus culture and strain characterisation was 30 points.

Results

Panel composition and expected results

The EQA panel consisted of eight samples containing various concentrations of influenza virus A or B and two negative samples in virus transport medium (VTM) (Table 1). The panel was multifunctional in that only one aliquot of each panel sample was provided to laboratories and both rapid detection and virus culture tests were performed using the single aliquot. Therefore laboratories received the same panel whether they were performing rapid detection and virus culture or rapid detection only.

Table 1. Panel composition and expected results for the influenza virus rapid detection and culture EQA 2010/11

Panel code	Type	Subtype	Strain designation	Virus titre PFU/ml	Rapid detection expected results	Culture expected results
EISN_INF10-01			Negative			Negative
EISN_INF10-02	A	H1	A/Brisbane/59/2007 (H1)	$1x10^4$	Positive (A, H1)	A/Brisbane/59/2007
EISN_INF10-03	A	H3	A/Wisconsin/67/2005 (H3)	$2x10^4$	Positive (A, H3)	A/Wisconsin/67/2005
EISN_INF10-04	A	H3	A/Perth/16/2009 (H3)	$5x10^3$	Positive (A, H3)	A/Perth/16/2009
EISN_INF10-05	A	H1v	A/California/7/2009 (H1v)	$1x10^2$	Positive (A, H1v)	A/California/7/2009
EISN_INF10-06	B		B/Brisbane/60/2008 (Vic)	$1x10^4$	Positive (B)	B/Brisbane/60/2008
EISN_INF10-07			Negative			Negative
EISN_INF10-08	A	H1	A/New Caledonia/20/1999 (H1)	$2x10^4$	Positive (A, H1)	A/New Caledonia/20/1999
EISN_INF10-09	B		B/Florida/4/2006 (Yam)	$5x10^4$	Positive (B)	B/Florida/4/2006
EISN_INF10-10	A	H1v	A/California/7/2009 (H1v)	$1x10^4$	Positive (A, H1v)	A/California/7/2009

Sample: Unique code for each EISN panel sample. **Strain designation:** Full strain designation for each panel sample. The lineage details for the influenza B virus samples are shown in brackets. Yam – Yamagata lineage; Vic – Victoria lineage. **Type:** Influenza virus type (A or B). **Subtype:** Influenza A virus haemagglutinin subtype. **Virus titre:** PFU/ml – plaque forming units/ml. These values are the mean titre of 3 titrations of each virus in MDCK and MDCK-SIAT1 cells. **Rapid detection and expected results:** Positive / negative influenza virus status for each panel sample; type and subtype of virus. **Culture expected results:** full influenza virus strain designation. H1v refers to influenza A(H1N1)pdm09 virus.

Reporting time and participation

The time taken from receipt of the panel to reporting of rapid detection and culture results is shown in Figure 1. The time period between courier delivery and panel receipt was not considered. Panel distribution was unexpectedly delayed due to severe weather conditions and the reporting times extended due to the holiday period.

For rapid detection, 28out of 34 participants (82.4%) reported a panel receipt and results, and were included in the analysis shown in Figure 1. Of the participants not included (n=6; 26.4%), five returned results but did not return a panel receipt, and a further one did not return a panel receipt or results. An explanation for withdrawal from the EQA was not provided by the one laboratory that did not return results.

For culture, 25 out of 34 participants (73.5%) reported a panel receipt and results, and were included in the analysis shown in Figure 1. Of the participants not included (n=9; 26.4%), five did not return a panel receipt but returned results, three returned a panel receipt but did not return results and a further one did not return a panel receipt or results. In total four laboratories did not return results for culture, although three of these did return rapid detection results. Two laboratories reported that they did not perform culture, one laboratory did not respond to a reminder and one laboratory withdrew from the entire programme as stated above.

Figure 1. Time taken for the return of rapid detection results (a) and culture results (b)

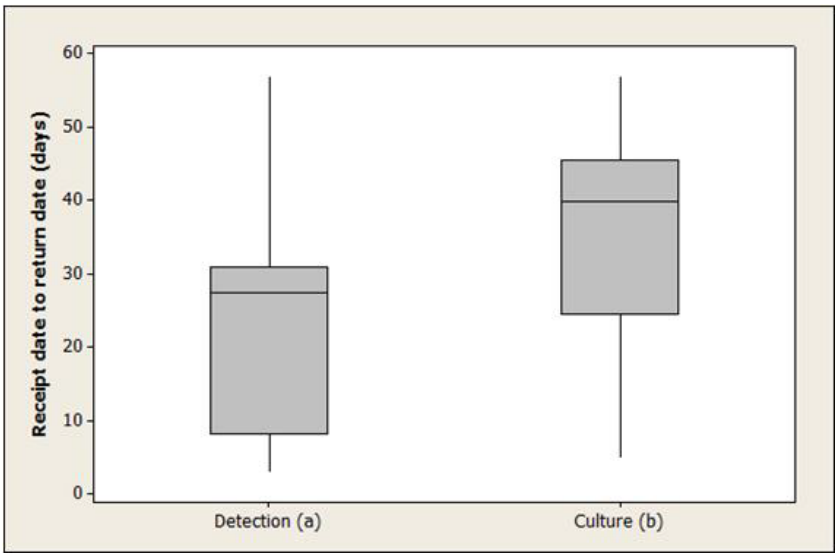

Figure 1. Time taken for return of rapid detection results (a) and culture results (b). Box and whisker plot showing the time taken from receipt of the panel to reporting of results. The number of days required by participants to return results are presented. The date participants reported receipt of 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 whiskers represent the range of values returned and the box represents the first quartile, median and the third quartile.

The due date for return of results was 17th January 2011 for rapid detection and 31st January 2011 for culture. The median time taken for participants to return detection results (from the reported date of receipt) was 27.5 days, and the mean time taken was 22.6 days. The median time taken for participants to return culture results (from the reported date of receipt) was 40days, and the mean time taken was 34.5 days. For detection, the percentage of respondents reporting on time was 85.7% (n=24) and for culture the percentage of respondents reporting on time was 100.0% (n=25).

Results for rapid detection of influenza virus

Thirty-three participants (97.0%) reported results for rapid detection (Table 2). All 33 reported typing for influenza A virus and 32 reported subtyping results for the majority of influenza A samples. One participant did not return results for rapid detection.

Twenty-five out of the 33 participants that returned results achieved a full score (76%). For those participants that did not achieve a full score the scores ranged from 19 to 29 points (Annex 2). The most common reason for lower scores was lack of reporting subtype information. Additionally there were two reported false positive results.

Panel sample EISN_INF10-05 (A/California/7/2009) contained a low viral load. Three participants reported false negatives, and two participants did not report subtype information for this sample.

Table 2. Number and percentage of results reported for rapid detection of influenza virus

Sample	Sample content	Virus titre PFU/ml	Summary of results reported													
			Detection (33/33 returned)					Type (33/33 returned)					Subtype (33/33 returned)				
			Expected result	Correct n	Correct (%)	Incorrect n	Incorrect (%)	Expected result	Correct n	Correct (%)	Incorrect n	Incorrect (%)	Expected result	Correct n	Correct (%)	Incorrect n	Incorrect (%)
EISN_INF 10-02	A/Brisbane/59/2007 (H1)	$1x10^4$	Positive	33	100.0			A	33	100.0			H1	31	93.9	2	6.1
EISN_INF 10-08	A/New Caledonia/20/1999 (H1)	$2x10^4$	Positive	33	100.0			A	33	100.0			H1	30	90.9	3	9.1
EISN_INF 10-10	A/California/7/2009 (H1v)	$1x10^4$	Positive	33	100.0			A	33	100.0			H1	32	97.0	1	3.0
EISN_INF 10-05	A/California/7/2009 (H1v)	$1x10^2$	Positive	30	90.9	3	9.1	A	29	87.9	4	12.1	H1	28	84.8	5	15.2
EISN_INF 10-04	A/Perth/16/2009 (H3)	$5x10^3$	Positive	33	100.0			A	33	100.0			H3	32	97.0	1	3.0
EISN_INF 10-03	A/Wisconsin/67/2005 (H3)	$2x10^4$	Positive	33	100.0			A	33	100.0			H3	32	97.0	1	3.0
EISN_INF 10-06	B/Brisbane/60/2008 (Vic)	$1x10^4$	Positive	33	100.0			B	33	100.0							
EISN_INF 10-09	B/Florida/4/2006 (Yam)	$5x10^4$	Positive	33	100.0			B	33	100.0							
EISN_INF 10-01	Negative		Negative	32	97.0	1	3.0										
EISN_INF 10-07	Negative		Negative	32	97.0	1	3.0										

Sample: Unique code for each EISN panel sample. **Sample content:** Full strain designation for each panel sample. **Virus titre:** PFU/ml – plaque forming units/ml. These values are the mean titre of 3 titrations of each virus in MDCK and MDCK-SIAT1 cells. **Detection:** Number and percentage of laboratories reporting the correct qualitative result for each panel sample. **Type:** Number and percentage of laboratories reporting the correct influenza virus type (A or B) for each panel sample. **Subtype:** Number and percentage of laboratories reporting the correct influenza A virus haemagglutinin subtype. **Incorrect:** Number and percentage of laboratories either reporting negatives incorrectly or not reporting the correct influenza A virus type and haemagglutinin subtype or influenza virus B type. See Table 5 for a description of the incorrect results. H1v refers to influenza A(H1N1)pdm09 virus.

Results for culture of influenza virus

Thirty participants (88.2%) reported results for culture. All 30 reported typing results for influenza virus A and B and 28 reported subtyping results for influenza virus A. Twenty-six out of the 30 reported strain results. Four laboratories did not return culture results. Two of these did not provide this service at the time of this study.

Five out of 30 participants that returned results achieved a full score (17%). For those participants that did not achieve a full score, the scores ranged from 10 to 29 points (Annex 3a-c). Overall the most common reason for lower scores was the reporting of a 'not determined' result. Additionally the majority of participants (n=22/30; 73%) incorrectly reported the strain information for panel sample EISN_INF10-03 (A/Wisconsin/67/2005).

Table 3. Number and percentage of results reported for culture of influenza virus

Sample	Sample content	Virus titre PFU/ml	Summary of results reported													
			Culture (30/30 returned)					Type / subtype (30/30 returned)					Strain (26/30 returned)				
			Expected	Correct		Incorrect		Expected	Correct		Incorrect		Expected	Correct		Incorrect	
				n	(%)	n	(%)		n	(%)	n	(%)		n	(%)	n	(%)
EISN_INF10-02	A/Brisbane/59/2007 (H1)	$1x10^4$	Positive	29	96.7	1	3.3	A, H1	27	90.0	3	10.0	A/Brisbane/59/2007	22	84.6	4	15.4
EISN_INF10-08	A/New Caledonia/20/1999 (H1)	$2x10^4$	Positive	30	100.0	0	0.0	A, H1	28	93.3	2	6.7	A/New Caledonia/20/99	16	61.5	10	38.5
EISN_INF10-10	A/California/7/2009 (H1v)	$1x10^4$	Positive	29	96.7	1	3.3	A, H1	27	90.0	3	10.0	A/California/7/2009	22	84.6	4	15.4
EISN_INF10-05	A/California/7/2009 (H1v)	$1x10^2$	Positive	25	83.3	5	16.7	A, H1	24	80.0	6	20.0	A/California/7/2009	19	73.1	7	26.9
EISN_INF10-04	A/Perth/16/2009 (H3)	$5x10^3$	Positive	28	93.3	2	6.7	A, H3	26	86.7	4	13.3	A/Perth/16/2009	18	69.2	8	30.8
EISN_INF10-03	A/Wisconsin/67/2005 (H3)	$2x10^4$	Positive	29	96.7	1	3.3	A, H3	27	90.0	3	10.0	A/Wisconsin/67/2005	8	30.8	18	69.2
EISN_INF10-06	B/Brisbane/60/2008 (Vic)	$1x10^4$	Positive	27	90.0	3	10.0	B	27	90.0	3	10.0	B/Brisbane/60/2008	23	88.5	3	11.5
EISN_INF10-09	B/Florida/4/2006 (Yam)	$5x10^4$	Positive	30	100.0	0	0.0	B	30	100.0	0	0.0	B/Florida/4/2006	22	84.6	4	15.4
EISN_INF10-01	Negative		Negative	29	96.7	1	3.3										
EISN_INF10-07	Negative		Negative	29	96.7	1	3.3										

Sample: Unique code for each EISN panel sample. **Sample content:** Full strain designation for each panel sample. **Virus titre:** PFU/ml – plaque forming units/ml. These values are the mean titre of 3 titrations of each virus in MDCK and MDCK-SIAT1 cells. **Culture:** Number and percentage of laboratories reporting the ability to culture each panel sample. **Type/subtype:** Number and percentage of laboratories correctly reporting the influenza virus type and haemagglutinin subtype (type only for influenza B viruses. **Strain:** Number and percentage of laboratories reporting the correct influenza virus strain. The exact strain name or "-like" were considered as correct results. **Incorrect:** Number and percentage of laboratories either reporting negatives incorrectly or not reporting the correct influenza virus strain. See Tables 6a-c for a description of the incorrect results. H1v refers to influenza A(H1N1)pdm09 virus.

Summary of overall performance

The number and percentage of participants that recorded rapid detection and culture results for each of the panel samples is summarised in Table 4. The number of participants that returned data sets for both rapid detection and culture was n=30/34 (88%). Strain data was not included in this analysis. Therefore the expected result for culture was taken as the influenza virus type/subtype.

Rapid detection and culture results are presented by individual laboratory in Annexes 2 and 3a–c.

Table 4. Number and percentage of participants reporting correct results for both rapid detection and culture

Sample	Sample content	Summary of results by number of participants (30/34 returned)				
		Virus titre	Rapid detection	Culture	Correct on both	
		PFU/ml	Expected result	Expected result	n	%
EISN_INF10-02	A/Brisbane/59/2007 (H1)	$1x10^4$	A, H1	A, H1	26	86.7
EISN_INF10-08	A/New Caledonia/20/1999 (H1)	$2x10^4$	A, H1	A, H1	26	86.7
EISN_INF10-10	A/California/7/2009 (H1v)	$1x10^4$	A, H1	A, H1	27	90.0
EISN_INF10-05	A/California/7/2009 (H1v)	$1x10^2$	A, H1	A, H1	21	70.0
EISN_INF10-04	A/Perth/16/2009 (H3)	$5x10^3$	A, H3	A, H3	26	86.7
EISN_INF10-03	A/Wisconsin/67/2005 (H3)	$2x10^4$	A, H3	A, H3	27	90.0
EISN_INF10-06	B/Brisbane/60/2008	$1x10^4$	B	B	27	90.0
EISN_INF10-09	B/Florida/4/2006	$5x10^4$	B	B	30	100.0
EISN_INF10-01	Negative		**Negative**	**Negative**	**29**	**96.7**
EISN_INF10-07	Negative		**Negative**	**Negative**	**29**	**96.7**

Sample: Unique code for each EISN panel sample. **Sample content:** Full strain designation for each panel sample. **Virus titre:** PFU/ml – plaque forming units/ml. These values are the mean titre of 3 titrations of each virus in MDCK and MDCK-SIAT1 cells. **Rapid detection:** Expected rapid detection results for each panel sample. **Culture:** Expected culture results (type/subtype) for each panel sample. **Correct on both:** Number and percentage of laboratories reporting the correct results for both rapid detection and culture. H1v refers to influenza A(H1N1)pdm09 virus.

Discussion

The CNRL influenza virus rapid detection and culture EQA panel 2010/11 was distributed to CNRL member laboratories in December 2010. This was the second rapid detection and culture EQA panel distributed by CNRL since EISN was established.

The 2010–11 panel consisted of eight samples containing various concentrations of influenza virus A or B and two negative samples. The viruses for the panel were selected by members of the CNRL 'Quality and Training' Task Group (TG5) and represented examples of the currently circulating influenza A(H1N1)pdm09, influenza A H3N2 and influenza B viruses. Both lineages of influenza B virus (Victoria and Yamagata) were included. Former seasonal H1N1 influenza A viruses were included in addition to the pandemic strain A(H1N1)pdm09 which was incorporated for the first time in this EQA panel. Older strains of former seasonal influenza A H1N1 (A/New Caledonia/20/1999) and influenza A H3N2 (A/Wisconsin/67/2005) were included to test specificity and the laboratories ability to detect antigenically diverse influenza strains. Two dilutions of influenza A(H1N1)pdm09 virus (A/California/7/2009) were included to test sensitivity of detection and culture methodology. Altogether the panel was a comprehensive test of sensitivity and specificity, and laboratories ability to detect both recently circulating and antigenically diverse influenza viruses.

Thirty-three out of 34 (97.1%) participants reported rapid detection results and 30 (88.2%) participants reported culture results. The proportion of participants performing rapid detection increased slightly (2008: n=32), however there was a slight decrease in the proportion returning culture results in 2010/11 (2008: n=31). There were 85.7% of participants who reported detection results on time and 100% reported culture results on time. Panel distribution was delayed by severe weather conditions until early December. To ensure optimum participation, distribution of future panels should be timed at a more convenient time for laboratories such as early autumn before the start of the winter influenza season.

Full scores for rapid detection were achieved by 76% of participants which represents an increase in proficiency over the two years since the last panel (2008 score: 69%). This represents an excellent achievement as the 2010–11 panel was more challenging than the previous panel with the inclusion of the influenza A(H1N1)pdm09 virus at two different concentrations and using a lower virus titre than previously. Sample EISN_INF10-05 had the highest error rate with three (9%) incorrect detection results. A further two laboratories did not return subtyping results for this sample. These false negative errors indicate a lower assay sensitivity for detection/typing of low viral titre A(H1N1)pdm09 virus. Two false positive results were returned compared to three false positives in the previous panel in 2008. Laboratories with these serious errors should examine assay conditions and procedures to optimise sensitivity and for possible sources of contamination. In some cases subtyping was not completed for samples EISN_INF10-02 (n=2; 6%) and EISN_INF10-08 (n=3; 9%) which were both former seasonal H1N1 viruses, suggesting either a sensitivity issue in the subtyping assay or that some laboratories no longer have the capacity to identify this subtype. Only one laboratory did not return any subtyping results, which is a significant performance improvement on 2008 (n=6).

For culture, a score of ≥90% was achieved for nine out of ten samples. A score of 83.3% (n=25) was returned for sample EISN_INF10-05 which was a low viral titre A(H1N1)pdm09 sample indicating a number (n=5) of laboratories were not able to recover this virus in culture. Three laboratories (10%) could not culture virus from sample EISN_INF-06 (B/Brisbane/60/2008). The number of false positive samples for culture was low and unchanged compared to 2008 (n=2), indicating laboratories have good procedures in place to minimise cross contamination between samples.

For combined culture and antigenic characterisation, five out of 30 participants achieved a full score (17%) which was slightly lower compared to the 2008 result of seven out of 31 (23%). The majority of participants determined strain identity by HI assay (90%) rather than sequencing (10%). The correct strain was identified more frequently for the current strains (69.2% - 88.5%) compared to the older strains (30.8% - 61.5%). Key issues for this part of the EQA were lack of strain characterisation (reporting of a strain 'not determined' result) by several participants either for some or all (n=4; 13%) samples. In addition, the majority of participants (n=22/30; 73%) incorrectly reported the strain for the older antigenically distinct H3N2 virus included in panel sample EISN_INF10-03 (A/Wisconsin/67/2005). This is probably due to limited availability of appropriate reagents for strain characterisation by HI, particularly for the older virus strains. The specific post-infection ferret antiserum, required for accurate strain characterisation distributed to CNRL laboratories by the WHO-CC, is a limited resource, and laboratories may not have sufficient antiserum to enable identification of older strains. The inclusion of the older strains in the EQA was intended to test capacity for detecting viruses which are antigenically distinct from currently circulating strains. Viruses identified through surveillance as 'low reactors' with current ferret antiserum should be forwarded to the WHO-CC for detailed characterisation. A future EQA could include an option to report these samples as 'low reactors' for further testing for quality assessment of this process.

Conclusions

- The 2010–11 CNRL rapid detection and culture EQA panel was a challenging panel of recent and older influenza A and B virus strains, including a low viral titre sample, and was a comprehensive test of the CNRL laboratories ability to specifically and sensitively detect both recently circulating and antigenically distinct influenza viruses.
- Thirty four CNRL laboratories from 27 European countries participated in 2010 including 33 who participated in 2008. The unforeseen delay of panel distribution until December 2010 during the influenza season may have influenced laboratories ability to return results or complete testing in time.
- An improvement was noted in rapid detection proficiency since the previous panel and the network continues to maintain a high standard of rapid detection and influenza virus typing/subtyping, with the number of laboratories returning subtyping results showing an increase since the last EQA panel in 2008.
- The majority of laboratories performed antigenic characterisation by HI assay with a small number using sequencing. Incorrect results for characterisation of older strains were possibly due to a lack of appropriate reference antiserum.
- The inclusion of a low virus titre sample was more challenging than samples from the previous EQA and there was a marginally higher rate of false negative results and lack of type/subtype data reported for this sample. This may indicate a lack of sensitivity for rapid molecular detection and recovery in cell culture of low viral titre A(H1N1)pdm09 viruses.
- The network laboratories should be further supported in assay development and improvement through training activities, guidelines and protocol recommendations. Training in molecular methodology including real-time PCR is recommended as this underpins rapid detection and identification of influenza viruses by the European laboratory network. Proficiency in molecular methods also supports development of the network in terms of new technology such as sequencing and genotypic antiviral resistance detection.
- The valuable contribution that network laboratories make in terms of supplying virus isolates and strain characterisation information to the global influenza surveillance network must be supported with ongoing development and training in virus culture and strain characterisation techniques.

Annex 1. List of Participants

Country	Institution
Austria	AKH Wien - Medical Uni of Vienna
Belgium	Institute of Public Health
Bulgaria	Center of Infectious & Parasitic Disease
Czech Republic	National Institute of Public Health
Denmark	Statens Serum Institute
Estonia	Tervisekaltseinspeksioon
Finland	National Institute for Health and Welfare
France	Groupement Hospitalier Est
France	CNR de la Grippe - Institute Pasteur
Germany	Robert Koch Institute
Greece	National Influenza Center for S Greece
Hungary	National Center for Epidemiology
Ireland	University College Dublin
Iceland	Landspitali - University Hospital
Italy	Istituto Superiore di Sanita (NIH)
Lithuania	Nac. Visuomenes Sveikatos Prieziuros Lab
Luxembourg	Laboratoire National de Sante
Latvia	Infectology Center of Latvia
Malta	Mater Dei Hospital
Netherlands	Erasmus Medical Centre
Netherlands	National Institute for Public Health and the Environment (RIVM) (C
Norway	Norwegian Institute of Public Health
Poland	National Institute of Hygiene
Portugal	Lab Nacional de Ref para o VÃ-rus da Gripe
Romania	Cantacuzino Institute
Slovenia	National Institute of Public Health
Spain	Instituto de Salud Carlos III
Spain	Hospital Clinico Universitario: IECSCYL
Spain	Hospital Clinic i Provincial
Sweden	Smittskyddsinstitutet (SMI)
United Kingdom	Royal Group of Hospitals Trust
United Kingdom	Public Health Wales
United Kingdom	National Health Service Greater Glasgow and Clyde
United Kingdom	Health Protection Agency - Microbiology Services

Annex 2. Rapid detection results presented by individual laboratory.

Participant	Overall score	EISN_INF10-02	EISN_INF10-08	EISN_INF10-10	EISN_INF10-05	EISN_INF10-04	EISN_INF10-03	EISN_INF10-06	EISN_INF10-09	EISN_INF10-01	EISN_INF10-07
		A, H1	**A, H1**	**A, H1v**	**A, H1v**	**A, H3**	**A, H3**	**B**	**B**		
1	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
2	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
3a	27	A, H1	A, H1	A, H1v	A, H1 + B	A, H3	A, H3	B	B	Negative	Negative
4	28	A	A	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
5	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
6a	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
7	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
8a	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
9	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
10	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
11	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
12	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
13	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
14	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
15b											
16	30	A, H1	A, H1	A, H1	A, H1	A, H3	A, H3	B	B	Negative	Negative
17	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
18	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
19	27	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	A, H1v	Negative
20	30	A, H1	A, H1v	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
21	29	A, H1	A, H1	A, H1v	A	A, H3	A, H3	B	B	Negative	Negative
22a	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
23	28	A, H1	A	A, H1v	A	A, H3	A, H3	B	B	Negative	Negative
24	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
25	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
26	19	A	A	A	Negative	A	A	B	B	Negative	A, H1
27	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
28	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
29a	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
30a	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
31a	30	A, H1	A, H1	A, H1v	A, H1v	A, H3	A, H3	B	B	Negative	Negative
32	30	A, H1	A, H1	A, H1v	A, H1	A, H3	A, H3	B	B	Negative	Negative
33a	27	A, H1	A, H1	A, H1v	Negative	A, H3	A, H3	B	B	Negative	Negative
34	27	A, H1	A, H1	A, H1v	Negative	A, H3	A, H3	B	B	Negative	Negative

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

Incorrect result

The scoring system used was as follows: one point for correct detection of influenza virus A or B; one point for correct typing of influenza virus A or B; one point for correct subtyping of influenza virus A; three points for correct determination of a negative. The maximum achievable score was 30 points.

The numbering of the laboratories (participants) in this and the following tables are in random and not in alphabetical order.

Annex 3a–c. Culture results presented by individual laboratory.

a: No strain results reported.
b. No results returned.

Incorrect result

The scoring system used was as follows: one point for correct culture of influenza virus A or B; one point for correct subtyping of influenza virus A or typing of influenza virus B; one point for correct reporting of a virus strain; three points for correct determination of a negative. The maximum achievable score was 30 points.
Method: The method used to generate results. HI – antigenic characterisation of samples by HI assay. SEQ: sequencing characterisation of samples.

Annex 3a. Culture results presented by individual laboratory (samples EISN_INF10-02, -08, -08, and -05)

Participant	Method	Overall score	EISN_INF10-02		EISN_INF10-08		EISN_INF10-10		EISN_INF10-05	
			Type/subtype	Strain	Type/subtype	Strain	Type/subtype	Strain	Type/subtype	Strain
			A, H1	A/Brisbane/59/2007 (H1)	A, H1	A/New Caledonia/20/1999 (H1)	A, H1v	A/California/7/2009 (H1v)	A, H1v	A/California/7/2009 (H1v)
1	HI	28	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
2	SEQ	29	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
3	HI	27	A, H1	A/Brisbane/59/2007-like	A, H1	A/Brisbane/59/2007-like	A, H1v	A/California/7/2009-like	A, H1v	A/California/7/2009-like
4	HI	29	A, H1	A/Brisbane/59/2007-like	A, H1	A/New Caledonia/20/1999-like	A, H1v	A/California/7/2009-like	A, H1v	A/California/7/2009-like
5	HI	29	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
6	HI	28	A, H1	A/Brisbane/59/2007-like	A, H1	A/Brisbane/59/2007-like	A, H1v	A/California/7/2009-like	A, H1v	A/California/7/2009-like
7b			0	0	0	0	0	0	0	0
8	SEQ	21	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1	A/California/7/2009	Not determined	Not determined
9	HI	24	A, H1	Not determined	A, H1	Not determined	A, H1v	Not determined	A, H1v	Not determined
10a		22	A, H1	Not determined	A, H1	Not determined	A, H1v	Not determined	A, H1v	Not determined
11	HI	24	A, H1	A/Brisbane/59/2007-like	A, H1	A/New Caledonia/20/1999-like	A, H1v	Not determined	A, H1v	Not determined
12	HI	30	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
13	HI	29	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
14b			0	0	0	0	0	0	0	0
15b			0	0	0	0	0	0	0	0
16b			0	0	0	0	0	0	0	0
17	HI	30	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
18	HI	30	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
19	HI	24	A, H1	A/Brisbane/59/2007	A, H1	A/Brisbane/59/2007	A, H1	A/California/7/2009	A, H1	A/California/7/2009
20	HI	21	A, H1v	Not determined	A, H1v	Not determined	A, H1	Not determined	A, H1	Not determined
21a		16	A	Not determined	A	Not determined	A	Not determined	A	Not determined
22	HI	30	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
23a	HI	20	A, H1	Not determined	A, H1	Not determined	A, H1v	Not determined	A, H1v	Not determined
24	HI	29	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009	A, H1v	A/California/7/2009
25	HI	27	A, H1	A/Brisbane/59/2007-like	A, H1	A/Brisbane/59/2007-like	A, H1v	A/California/7/2009-like	A, H1v	A/California/7/2009-like
26a		10	A	Not determined	A	Not determined	A	Not determined	Not determined	Not determined
27	HI	28	A, H1	A/Brisbane/59/2007-like	A, H1	A/Brisbane/59/2007-like	A, H1v	A/California/7/2009-like	A, H1v	A/California/7/2009-like
28	SEQ	27	A, H1	A/Brisbane/59/2007-like	A, H1	A/New Caledonia/20/1999-like	A, H1v	A/California/7/2009-like	Not determined	Not determined

Participant	Method	Overall score	EISN_INF10-02		EISN_INF10-08		EISN_INF10-10		EISN_INF10-05	
			Type/subtype	Strain	Type/subtype	Strain	Type/subtype	Strain	Type/subtype	Strain
			A, H1	A/Brisbane/59/2007 (H1)	A, H1	A/New Caledonia/20/1999 (H1)	A, H1v	A/California/7/2009 (H1v)	A, H1v	A/California/7/2009 (H1v)
29	HI	30	A, H1	A/Brisbane/59/2007-like	A, H1	A/New Caledonia/20/1999-like	A, H1	A/California/7/2009-like	A, H1	A/California/7/2009-like
30	HI	28	A, H1	A/Brisbane/59/2007	A, H1	A/Brisbane/59/2007	A, H1	A/California/7/2009	A, H1	A/California/7/2009
31	HI	17	Not determined	Negative	A, H1	A/Brisbane/59/2007	A, H1v	A/California/7/2009	Not determined	Not determined
32	HI	27	A, H1	A/Brisbane/59/2007	A, H1	A/New Caledonia/20/1999	A, H1	A/California/7/2009	A, H1	A/California/7/2009
33	HI	26	A, H1	A/Brisbane/59/2007	A, H1	Not determined	Not determined	Not determined	A, H1v	A/California/7/2009
34	HI	25	A, H1	A/New Caledonia/20/1999	A, H1	A/New Caledonia/20/1999	A, H1v	A/California/7/2009-like	Not determined	Not determined

Annex 3b. Culture results presented by individual laboratory (samples EISN_INF10-04, -03, -06, and -09)

Participant	Method	Overall score	EISN_INF10-04		EISN_INF10-03		EISN_INF10-06		EISN_INF10-09	
			Type/subtype	Strain	Type/subtype	Strain	Type/subtype	Strain	Type/subtype	Strain
			A, H3	A/Perth/16/2009 (H3)	A, H3	A/Wisconsin/67/2005 (H3)	B	B/Brisbane/60/2008	B	B/Florida/4/2006
1	HI	28	A, H3	A/Perth/3/2009	A, H3	A/Brisbane/10/2007	B	B/Brisbane/60/2008	B	B/Florida/4/2006
2	SEQ	29	A, H3	A/Perth/16/2009	A, H3	A/Brisbane/10/2007	B	B/Brisbane/60/2008	B	B/Florida/4/2006
3	HI	27	A, H3	A/Brisbane/10/2007	A, H3	A/Brisbane/10/2007-like	B	B/Brisbane/60/2008-like	B	B/Florida/4/2006-like
4	HI	29	A, H3	A/Perth/16/2009-like	A, H3	A/Brisbane/10/2007-like	B	B/Brisbane/60/2008-like	B	B/Florida/4/2006-like
5	HI	29	A, H3	A/Perth/16/2009	A, H3	A/Brisbane/10/2007	B	B/Brisbane/60/2008	B	B/Florida/4/2006
6	HI	28	A, H3	A/Perth/16/2009-like	A, H3	A/Brisbane/10/2007-like	B	B/Brisbane/60/2008-like	B	B/Florida/4/2006-like
7		0	0	0	0	0	0	0	0	0
8	SEQ	21	Not determined	Not determined	Not determined	Not determined	B	B/Brisbane/60/2008	B	B/Florida/4/2006
9	HI	24	A, H3	Not determined	A, H3	Not determined	B	B/Brisbane/60/2008	B	B/Florida/4/2006
10		22	A, H3	Not determined	A, H3	Not determined	B	Not determined	B	Not determined
11	HI	24	A, H3	Not determined	A, H3	Not determined	B	Not determined	B	Not determined
12	HI	30	A, H3	A/Perth/16/2009	A, H3	A/Wisconsin/67/2005	B	B/Brisbane/60/2008	B	B/Florida/4/2006
13	HI	29	A, H3	A/Perth/16/2009	A, H3	A/Brisbane/10/2007	B	B/Brisbane/60/2008	B	B/Florida/4/2006
14		0	0	0	0	0	0	0	0	0
15		0	0	0	0	0	0	0	0	0
16		0	0	0	0	0	0	0	0	0
17	HI	30	A, H3	A/Perth/16/2009	A, H3	A/Wisconsin/67/2005	B	B/Brisbane/60/2008	B	B/Florida/4/2006
18	HI	30	A, H3	A/Perth/16/2009	A, H3	A/Wisconsin/67/2005	B	B/Brisbane/60/2008	B	B/Florida/4/2006
19	HI	24	A, H3	A/Perth/16/2009	A, H3	A/Brisbane/10/2007	B	B/Brisbane/60/2008	B	B/Brisbane/60/2008
20	HI	21	A, H3	Not determined	A, H3	Not determined	Not determined	Not determined	B	B/Florida/4/2006-like
21		16	A	Not determined	A	Not determined	B	Not determined	B	Not determined
22	HI	30	A, H3	A/Perth/16/2009	A, H3	A/Wisconsin/67/2005	B	B/Brisbane/60/2008	B	B/Florida/4/2006

Participant	Method	Overall score	EISN_INF10-04		EISN_INF10-03		EISN_INF10-06		EISN_INF10-09	
			Type/subtype	Strain	Type/subtype	Strain	Type/subtype	Strain	Type/subtype	Strain
			A, H3	A/Perth/16/2009 (H3)	A, H3	A/Wisconsin/67/2005 (H3)	B	B/Brisbane/60/2008	B	B/Florida/4/2006
23	HI	20	A, H3	Not determined	A, H3	Not determined	Not determined	Not determined	B	Not determined
24	HI	29	A, H3	A/Perth/16/2009	A, H3	A/Brisbane/10/2007	B	B/Brisbane/60/2008	B	B/Florida/4/2006
25	HI	27	A, H3	A/Perth/16/2009-like	A, H3	A/Brisbane/10/2007-like	B	B/Brisbane/60/2008-like	B	B/Bangladesh/3333/2007
26		10	A	Not determined	A	Not determined	B	Not determined	B	Not determined
27	HI	28	A, H3	A/Perth/16/2009-like	A, H3	A/perth/016/2009	B	B/Brisbane/60/2008-like	B	B/Florida/4/2006-like
28	SEQ	27	A, H3	A/Perth/16/2009-like	A, H3	A/Wisconsin/67/2005-like	B	B/Brisbane/60/2008-like	B	B/Florida/4/2006-like
29	HI	30	A, H3	A/Perth/16/2009-like	A, H3	A/Wisconsin/67/2005	B	B/Brisbane/60/2008-like	B	B/Florida/4/2006-like
30	HI	28	A, H3	A/Perth/16/2009	A, H3	A/Brisbane/10/2007	B	B/Brisbane/60/2008	B	B/Florida/4/2006
31	HI	17	Not determined	Not determined	A, H3	A/Wisconsin/67/2005	Not determined	Not determined	B	B/Florida/4/2006
32	HI	27	A, H3	A/Victoria/210/2009	A, H3	A/Victoria/210/2009	B	B/Brisbane/60/2008	B	B/Bangladesh/3333/2007
33	HI	26	A, H3	A/Perth/16/2009	A, H3	A/Wisconsin/67/2005	B	B/Brisbane/60/2008	B	B/Florida/4/2006
34	HI	25	A, H3	A/Perth/16/2009-like	A, H3	A/Brisbane/10/2007	B	B/Brisbane/60/2008	B	B/Florida/4/2006

Annex 3c. Culture results presented by individual laboratory (samples EISN_INF10-01, and -07).

Participant	Method	Overall score	EISN_INF10-01		EISN_INF10-07	
			Type/subtype	Strain	Type/subtype	Strain
			Negative	Negative	Negative	Negative
1	HI	28	Negative	Negative	Negative	Negative
2	SEQ	29	Negative	Negative	Negative	Negative
3	HI	27	Negative	Negative	Negative	Negative
4	HI	29	Negative	Negative	Negative	Negative
5	HI	29	Negative	Negative	Negative	Negative
6	HI	28	Negative	Negative	Negative	Negative
7		0	0	0	0	0
8	SEQ	21	Negative	Negative	Negative	Negative
9	HI	24	Negative	Negative	Negative	Negative
10		22	Negative	Negative	Negative	Negative
11	HI	24	Negative	Negative	Negative	Negative
12	HI	30	Negative	Negative	Negative	Negative
13	HI	29	Negative	Negative	Negative	Negative
14		0	0	0	0	0
15		0	0	0	0	0
16		0	0	0	0	0
17	HI	30	Negative	Negative	Negative	Negative
18	HI	30	Negative	Negative	Negative	Negative
19	HI	24	A, H1N1	A/California/07/09	Negative	Negative
20	HI	21	Negative	Negative	Negative	Negative
21		16	Negative	Negative	Negative	Negative
22	HI	30	Negative	Negative	Negative	Negative
23	HI	20	Negative	Negative	Negative	Negative
24	HI	29	Negative	Negative	Negative	Negative
25	HI	27	Negative	Negative	Negative	Negative
26		10	Negative	Negative	A	Not determined
27	HI	28	Negative	Negative	Negative	Negative
28	SEQ	27	Negative	Negative	Negative	Negative
29	HI	30	Negative	Negative	Negative	Negative
30	HI	28	Negative	Negative	Negative	Negative
31	HI	17	Negative	Negative	Negative	Negative
32	HI	27	Negative	Negative	Negative	Negative
33	HI	26	Negative	Negative	Negative	Negative
34	HI	25	Negative	Negative	Negative	Negative