Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company’s public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Protocols

The validation of a real-time RT-PCR assay which detects influenza A and types simultaneously for influenza A H1N1 (2009) and oseltamivir-resistant (H275Y) influenza A H1N1 (2009)

Susan Bennett*, Rory N. Gunson, Alasdair MacLean, Rhona Miller, William F. Carman

West of Scotland Specialist Virology Centre, A Member of the UK Clinical Virology Network, Gartnavel General Hospital, Great Western Road, Glasgow G12 0YN, United Kingdom

ABSTRACT

Influenza A H1N1 (2009) was declared by the World Health Organisation (WHO) as the first influenza pandemic of the 21st century. Rapid detection of influenza A and differentiation of influenza A H1N1 (2009) and seasonal influenza A is beneficial. In addition the rapid detection of antiviral resistant strains of influenza A H1N1 (2009) would be useful for clinicians to allow for change to an effective treatment at a much earlier stage if resistance is found. It was the aim of this study to develop a real-time RT-PCR that can detect all influenza A viruses and type simultaneously for influenza A H1N1 (2009) and oseltamivir resistant (H275Y) influenza A H1N1 (2009). This multiplex assay will allow laboratories to screen respiratory samples for all types of influenza A, influenza A H1N1 (2009) virus and oseltamivir resistant (H275Y) influenza A H1N1 (2009) virus in a rapid and cost effective format, ensuring that typing methods for seasonal and avian viruses are used on a smaller subset of samples. Since most virology laboratories already offer a molecular service for influenza A this assay could easily be implemented into most areas at little cost therefore increasing local access to resistance testing.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The World Health Organisation (WHO) received the first report of an oseltamivir-resistant influenza A H1N1 (2009) virus in July 2009 (WHO, 2009a). Sequence analysis revealed that the resistance was associated with a histidine to tyrosine substitution in the viral neuraminidase (N1) at position 275. Since this discovery a total of 304 oseltamivir-resistant influenza A H1N1 (2009) (H275Y mutant) cases have been reported to the Global Influenza Surveillance Network (GISN) (WHO as of 30.09.10) (WHO, 2010). Of these cases, 28% have been in immunocompromised patients who have received prolonged oseltamivir treatment, 33% have been associated with oseltamivir treatment (WHO, 2010). Several cases have also been associated with chemoprophylaxis (MMWR CDC, 2009a) and there have also been cases found in individuals who had not received treatment (Chen et al., 2009). Despite the increasing number of cases there has been two reports of person to person transmission of resistant virus (Gulland, 2009; WHO, 2009a). There has been one report of a community cluster of oseltamivir resistant cases (Le et al., 2010) however there has been no evidence of ongoing circulation of the virus in the community. Furthermore, the resistance strain has not been shown to cause altered or more severe infection.

Being able to detect rapidly the emergence of resistant virus is important as it allows clinicians to change rapidly patient treatment to a more effective drug (all cases bar one remain sensitive to zanamivir (WHO, 2010)). Such information also informs infection control procedures and public health surveillance.

Numerous methodologies are available for the detection of antiviral drug resistance in influenza viruses. Phenotyping assays are the gold standard for oseltamivir resistance testing (Zambon and Hayden, 2001). However, this method is time consuming, expensive and is only available at specialist centres. Newer methods such as pyrosequencing are rapid and sensitive in detecting H275Y but few laboratories are equipped with these machines (CDC, 2009b).

A group in Rotterdam developed recently a real-time PCR based method for the detection of H275Y (Personal Communication with M. Schutten). This method was shown to be sensitive, rapid and, unlike the aforementioned methods, uses a chemistry available to most diagnostic laboratories.

The incorporation of this H275Y resistant typing assay into an established duplex assay (Gunson et al., 2010) that detects influenza A while typing simultaneously all that are H1N1 (2009) positive is described. The new assay will allow rapid diagnosis, typing and resistance testing of respiratory samples in real time.
Table 1

| Virus target | Target gene | Forward primer sequence | Reverse primer sequence | Probe sequence | Publication |
|--------------|-------------|-------------------------|-------------------------|---------------|------------|
| Influenza A  | Matrix      | aagacaagacactacgctccctt | tctagygtgcagctcycgst   | FAM-tyscctacgcgtcggcagtg-MGB | Gunson et al. (2010) |
| H1N1         | N1          | gttacatcaacacacactcctt | gagaggaacctcgcctaat    | HEX-tgcxgctcgcgtcggcagtg-BQQ | Gunson et al. (2010) |
| H1N1 H275Y   | N1          | cagctgaatggtcctccataa  | tggacacacatgccatgctcag | Cy5-attTAAtgAgaatg-BQQ          | van der Vries et al. (2010) |

* The H275Y probe contains a locked nucleic acid (LNA). LNA nucleotides are in upper case, DNA nucleotides are in lower case and LNA nucleotide complementary to the predicted single nucleotide polymorphism (SNP) is italics.

The influenza A assay was manufactured by Applied Biosystems. The H1N1 and H1N1 H275Y assays were manufactured by TIB MOLBIOL.

2. Materials and methods

2.1. Development of FLUA/N1/H275Y triplex assay

The universal influenza A and influenza A H1N1 (2009) N1 assays were developed in-house and are published previously (Gunson et al., 2010). The universal influenza A assay is a widely used assay that targets the matrix region of the virus. Participation in various External Quality Assessment (EQA) schemes has shown this assay to detect influenza A viruses from humans and animals with high sensitivity (Gunson et al., 2010). The N1 assay is specific for influenza A H1N1 (2009). The H275Y resistance assay was developed by a group in Rotterdam and was supplied as personal communication (by M. Schutten). This allelic discrimination assay was designed to distinguish between the cytosine (oseltamivir sensitive) and the thymidine (oseltamivir resistant) mutation at the nucleotide position 823 in the N1 gene of influenza A H1N1 (2009) that gives rise to the antiviral resistance. The probe of the H275Y resistance assay was modified by the developers of the assay by adding two nucleotides (TT) at the 5' end at a later date and published (van der Vries et al., 2010). It should be noted that we did not use the adapted probe. The N1 probe from this paper was also not used in this study as there was a well validated N1 probe in use in the laboratory (Gunson et al., 2010).

The primer probe concentrations for each assay were optimised individually using in-house protocols (Gunson et al., 2003, 2006). The H275Y resistance assay used the primers at an optimised concentration of 1 μM and the probe at 0.2 μM in a 15 μl reaction volume. The universal influenza A H1N1 (2009) duplex used the primers at an optimised concentration of 1 μM and the probes at 0.2 μM in a 15 μl reaction volume (Gunson et al., 2010). The triplex used the primers at an optimised concentration of 1 μM, universal influenza A and H1N1 (2009) probe at 0.2 μM, and the H275Y probe at 0.3 μM in a 15 μl reaction volume (all primers and probes are shown in Table 1).

For all assays, one-step rRT-PCR was performed on 6 μl of RNA extract with the Platinum One-step qRT-PCR kit (Invitrogen) on an ABI Prism 7500 SDS real-time platform (Applied Biosystems). The following thermal profile was used: a single cycle of reverse transcription for 15 min at 50 °C, 2 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation followed by 40 amplification cycles of 8 s at 95 °C and 34 s at 60 °C each (annealing-extension step). Thresholds were set manually for analysis.

2.2. Samples used for the assessment of the triplex assay

The specificity of the triplex assay was assessed using 2 WHO panels (2008, 2009). Both panels contained seasonal influenza A H1N1, H3N2, and avian H5N1 viruses. The 2009 panel also contained influenza A H1N1 (2009). A pool containing the following components present in the pool were present at Ct values of between 26 and 31).

The endpoint detection limit of the universal influenza A and H1N1 (2009) components of the triplex was directly compared to the published duplex using a dilution series of an influenza A H1N1 (2009) clinical sample and clinical samples containing seasonal H1N1 and H3N2 viruses. Each dilution was tested in duplicate. These were carried out to ensure that the addition of the H275Y assay to the universal influenza A H1N1 (2009) duplex did not result in a reduction in endpoint detection limit of the universal influenza A and H1N1 (2009) components.

The endpoint detection limit of the H275Y component of the triplex was compared directly to the H275Y single assay using a dilution series of influenza A H1N1 (2009) H275Y positive clinical sample. Each dilution was tested in duplicate. This was carried out to ensure that the addition of the H275Y assay to the universal influenza A H1N1 (2009) duplex did not result in a reduction in the endpoint detection limit of the H275Y component.

The new triplex assay was also directly compared to the universal influenza A H1N1 (2009) duplex assay on 281 clinical samples. All the samples were respiratory specimens sent to the laboratory between 21st October 2009 and 26th October 2009 and were taken from patients with a clinical diagnosis of influenza A H1N1 (2009).

To assess inter-assay variability (reproducibility/long-term precision) positive extraction run controls for FLUA, N1 and H275Y were monitored over 20 PCR runs. To assess intra-assay variability (repeatability/short-term precision) a positive extraction run control for FLUA, N1 and H275Y were tested in 20 wells on the same PCR run.

Finally the ability of the triplex assay to detect minor populations of the oseltamivir resistant influenza A virus in samples containing a mixture of resistant and sensitive viruses was assessed. To do this similar concentrations of H275Y and wild-type influenza A H1N1 (2009) RNA controls were mixed and then diluted the H275Y positive RNA further in wild-type (WT) RNA. Diluting resistant virus in wild-type virus represents decreasing populations of resistant RNA in a mixed strain sample, allowing to predict the approximate level which minor populations of resistant RNA could be detected.

All clinical samples were extracted on the Qiagen MDx using the QIAamp viral RNA kit (Qiagen, Crawley, UK) according to manufacturer’s instructions.

3. Results

3.1. Specificity

The triplex was evaluated using 2 WHO influenza A quality control panels. The universal influenza A component of the triplex assay detected successfully all the samples that contained influenza A subtypes. The H1N1 (2009) component of the assay correctly detected the 3 influenza A H1N1 (2009) samples present in the 2009 panel (Table 2). The H275Y component of the assay did not
Table 2
Evaluation of the specificity of the triplex assay using 2008 (part 2) and 2009 WHO panels.

| Type | FluA N1 | H275Y | FluA N1 | H275Y |
|------|---------|-------|---------|-------|
| 2008 sample | FLUA N1 | FLUA N1 | H275Y | H275Y |
| 11  | H5      | 24.45 | N       | 22.76 | N       | N |
| 12  | H5      | 24.05 | N       | 21.38 | N       | N |
| 13  | H5      | 21.20 | N       | 19.40 | N       | N |
| 14  | H1      | 23.20 | N       | 21.64 | N       | N |
| 15  | H5      | 22.35 | N       | 20.37 | N       | N |
| 16  | H5      | 23.20 | N       | 21.38 | N       | N |
| 17  | H5      | 23.09 | N       | 20.74 | N       | N |
| 18  | H3      | 22.50 | N       | 21.10 | N       | N |
| 19  | –       | N      | N       | N      | N       | N |
| 20  | –       | N      | N       | N      | N       | N |

| 2009 sample | FLUA N1 | H275Y | FLUA N1 | H275Y |
| 1  | H1      | 24.25 | N       | 22.5   | N       | N |
| 2  | H5      | 25.16 | N       | 22.2   | N       | N |
| 3  | –       | N      | N       | N      | N       | N |
| 4  | H5      | 21.86 | N       | 23.27  | N       | N |
| 5  | H3      | 25.41 | N       | 24.17  | N       | N |
| 6  | H5      | 23.84 | N       | 24.09  | N       | N |
| 7  | H5      | 22.15 | N       | 23.15  | N       | N |
| 8  | H5      | 23.02 | N       | 25.33  | N       | N |
| 9  | H5      | 23.39 | N       | 25.62  | N       | N |
| 10 | FLUA    | 24.90 | N       | 24.17  | N       | N |
| 11 | H5      | 21.30 | N       | 23.61  | N       | N |
| 12 | H1N1 (2009) | 19  | 19.55 | 20.64  | 20.98  | N |
| 13 | H1      | 22.52 | N       | 22.38  | N       | N |
| 14 | H5      | 20.33 | N       | 26.38  | N       | N |
| 15 | H5      | 19.01 | N       | 27.37  | N       | N |
| 16 | H1N1 (2009) | 23.27 | 23.68 | 24.74  | 24.69  | N |
| 17 | H5      | 23.11 | N       | 25.55  | N       | N |
| 18 | H3      | 23.23 | N       | 29.06  | N       | N |
| 19 | H5      | 21.88 | N       | 23.76  | N       | N |
| 20 | H5      | 19.04 | N       | 27.41  | N       | N |

The endpoint detection limit of the universal influenza A and the H1N1/2009 component of the triplex assay in comparison to the seasonal H1N1 and H3N2 viruses (Table 3). When testing the dilution series of the influenza A H1N1 (2009) sample, the influenza A component of the triplex detected down to the 10^-5 dilution in 1 out of 2 occasions whereas the influenza A component of the triplex detected down to the 10^-6 dilution in 1 out of 2 occasions. The H1N1 (2009) components in the duplex and triplex both detected down to the 10^-5 dilution. The H275Y component of the triplex did not detect any samples in the dilution series as positive (Table 3).

When testing the dilution series of the seasonal H1N1 and H3N2 clinical samples, the influenza A component of the duplex assay detected the 10^-4 dilution of the H1N1 dilution series whereas the influenza A component of the triplex detected down to the 10^-5 dilution in 1 out of 2 occasions. The influenza A component of the duplex and triplex detected the 10^-4 dilution of the H3N2 dilution series. The H1N1 (2009) and H275Y components of the triplex did not detect any of the samples in the dilution series as positive (Table 3).

The endpoint detection limit of the universal influenza A and the H1N1/2009 component of the triplex assay in comparison to the seasonal H1N1 and H3N2 viruses (Table 3). When testing the dilution series of the influenza A H1N1 (2009) sample, the influenza A component of the triplex detected the 10^-5 dilution in 1 out of 2 occasions whereas the influenza A component of the triplex detected down to the 10^-6 dilution in 1 out of 2 occasions. The H1N1 (2009) components in the duplex and triplex both detected down to the 10^-5 dilution. The H275Y component of the triplex did not detect any samples in the dilution series as positive (Table 3).

3.2. Endpoint detection limit

The endpoint detection limit of the universal influenza A and the H1N1/2009 component of the triplex assay in comparison to the duplex assay was assessed using serial dilutions of clinical samples containing influenza A H1N1 (2009) and seasonal H1N1 and H3N2 viruses (Table 3). When testing the dilution series of the influenza A H1N1 (2009) sample, the influenza A component of the duplex detected the 10^-5 dilution in 1 out of 2 occasions whereas the influenza A component of the triplex detected down to the 10^-6 dilution in 1 out of 2 occasions. The H1N1 (2009) components in the duplex and triplex both detected down to the 10^-5 dilution. The H275Y component of the triplex did not detect any samples in the dilution series as positive (Table 3).

3.3. Prospective testing of clinical samples

The triplex was then compared to the duplex assay on 281 clinical samples taken from patients with a clinical diagnosis of influenza A H1N1 (2009). In total, 148 were positive by both assays and there were 7 discrepancies. Of these discrepancies 3 were positive by the duplex but negative by the triplex (1 influenza A positive only and 2 H1N1 positive only), 4 were positive by the duplex but negative by the triplex (2 influenza A positive only and 2 H1N1 positive only). All of these samples that were discrepant had high discordance.

Table 3
Dilution series of oseltamivir resistant (H275Y) influenza A H1N1 (2009) RNA comparing the endpoint detection limit of the H275Y component of the triplex and single H275Y assays.

| Assay | FLUA N1 | H275Y | FLUA N1 | H275Y |
|-------|---------|-------|---------|-------|
| RNA   | H1N1 (2009) | H1N1 | H3N2 | H1N1 | H3N2 |
| Probe | FLUA N1 | FLUA N1 | H275Y | FLUA N1 | H275Y | FLUA N1 | H275Y | FLUA N1 | H275Y |
| –1    | 25.62 | 23.37 | 28.22 | N     | 27.47 | N     | 23.45 | 23.24 | N     | 27.0 | N     | 26.71 | N     | N     |
| –2    | 29.28 | 27.06 | 31.46 | N     | 31.57 | N     | 27.42 | 26.85 | N     | 30.61 | N     | 30.35 | N     | N     |
| –3    | 32.42 | 29.14 | 35.09 | N     | 35.49 | N     | 30.25 | 29.83 | N     | 34.58 | N     | 31.96 | N     | N     |
| –4    | 36.78 | 33.45 | 39.72 | N     | 38.14 | N     | 34.82 | 33.03 | N     | 39.73 | N     | 38.52 | N     | N     |
| –5    | 38.12/N | 37.52 | N     | N     | N     | N     | 37.07 | 36.74 | N     | 39.79/N | N     | N     | N     | N     |
| –6    | N     | N     | N     | N     | N     | N     | 39.54/N | N     | N     | N     | N     | N     | N     | N     |
| –7    | N     | N     | N     | N     | N     | N     | N     | N     | N     | N     | N     | N     | N     | N     |

FLUA – Universal influenza A probe; N1 – H1N1 (2009) probe; H275Y – H1N1 (2009) H275Y probe.
N – negative.
Table 5
Inter-assay and intra-assay variability of the triplex.

| Probe          | Mean Ct | SD  | Max   | Min   | CoV |
|----------------|---------|-----|-------|-------|-----|
| Inter-assay    | FLUA    | 28.04 | 0.40  | 29.25 | 26.82 | 0.0142 |
|                | N       | 26.61 | 0.37  | 27.72 | 25.50 | 0.0139 |
|                | H275Y   | 27.13 | 0.37  | 28.21 | 26.01 | 0.0136 |
| Intra-assay    | FLUA    | 31.87 | 0.29  | 32.73 | 31.01 | 0.009  |
|                | N       | 33.62 | 0.69  | 35.89 | 31.75 | 0.020  |
|                | H275Y   | 28.75 | 0.15  | 29.19 | 28.31 | 0.005  |

FLUA – Universal influenza A probe; N – H1N1 (2009) probe; H275Y – H1N1 (2009) H275Y probe.
SD – standard deviation. Max and min are ±3 standard deviation.
CoV – coefficient of variation.

Table 6
Detection of minor populations of H275Y using triplex assay.

| %   | FLUA Ct | N1 Ct | H275Y Ct |
|-----|---------|-------|----------|
| WT  | 100     | 28.96 | 28.52    | N        |
| H275Y | 100   | 27.13 | 28.05    | 29.48    |
| 1:2 | 50      | 28.15 | 28.34    | 29.97    |
| 1:4 | 25      | 28.51 | 28.40    | 30.72    |
| 1:8 | 12.5    | 29.16 | 28.60    | 32.34    |
| 1:10| 10      | 29.31 | 28.66    | 32.56    |
| 1:20| 5       | 29.48 | 28.59    | 32.89    |
| 1:40| 2.5     | 29.89 | 28.62    | 33.91    |

FLUA – Universal influenza A probe; N1 – H1N1 (2009) probe; H275Y – H1N1 (2009) H275Y probe.
N – negative.
WT – wild-type.
The first column details the RNA present in the sample tested, WT and H275Y RNA tested individually then H275Y diluted 1:2; 1:4; 1:8 and so in WT RNA. The % refers to the percentage oseltamivir resistant RNA present in the sample.

3.4. Inter- and intra-assay variability

The inter-assay variability was assessed by monitoring positive run controls over 20 PCR runs and intra-assay variability by testing a positive control in 20 wells on one PCR run (Table 5). The results suggest that there is little inter-assay variability as the Ct of each component was similar over 20 different runs with low standard deviation (SD) and coefficient of variation (CoV) values. The results also suggest that the intra-assay variability of the assay is also good for each component as little variation was observed when the positive control was repeatedly tested. It should be noted that for H1N1 the control used was weaker than that of the other components of the assay and so was nearer the endpoint detection limit (see Tables 3 and 4) of this test which is likely to be the cause of the slightly higher SD and CoV values. Overall these results suggest that the triplex assay is precise and robust.

3.5. Detection of minor populations of H275Y

Finally the ability of the triplex assay to detect minor populations of H275Y was assessed by testing dilutions of H275Y RNA diluted in WT RNA. The results suggest that there is reliable detection of the H275Y to a dilution of 1:40 (Table 6). This represents a minor population of 2.5%.

4. Discussion

In the present study, a multiplex assay which detects influenza A and simultaneously types those that are influenza A H1N1 (2009) and highlights whether the virus contains the H275Y mutation was developed.

Adding the H275Y discrimination assay to the published duplex assay was shown to have no effect on the performance of any of the individual components. This was shown using several dilution series, a number of EQA panels and on prospective clinical samples taken from patients with clinically diagnosed H1N1 (2009). Multiplexing the methods had no detrimental effect on the Ct values of each component and slightly improved the performance of each component in the triplex. The multiplex was also precise and robust as shown by assessment of inter-assay and intra-assay variability of the individual components of the assay.

Multiplexing these different test components offers several advantages during future outbreaks of this virus. As mentioned in the previous publication multiplexing the universal assay and the H1N1 (2009) typing assay will allow laboratories to screen respiratory samples for influenza A H1N1 (2009) virus in a rapid and cost effective format, ensuring that typing methods for seasonal and avian viruses are used on a smaller subset of samples. The incorporation of the H275Y resistance assay is achieved at little extra cost (Gunson et al., 2008). Since most virology laboratories already offer a molecular service for influenza A this assay could be easily implemented into most areas therefore increasing local access to resistance testing.

Access to a local service offers several clinical advantages over the current system which relies upon samples being referred to specialist centres. For example this assay allows the detection of resistant virus while the patient is being monitored for the presence of influenza A. Consequently clinicians can change to an effective treatment at a much earlier stage than currently achievable (WHO, 2009b).

As mentioned above, a number of the reports of H275Y resistant virus were found in patients with no history of receiving oseltamivir. As a result this assay would also prove useful in the surveillance of oseltamivir resistant influenza A H1N1 (2009) strains in the community (Lackenby et al., 2008).

During the 2007–2008 seasonal influenza A (H1N1) season the oseltamivir-resistant strain became the dominant strain circulating in the community, therefore it is possible that this could happen again with the 2009 pandemic strain in the next influenza season (Harvala et al., 2010).

It is important to note that this assay only detects the H275Y mutation in influenza A H1N1 (2009) virus and will not detect other mutations associated with resistance such as E119V and N295S. Consequently if resistance is still suspected in an influenza A positive patient that has tested H275Y negative then other oseltamivir resistant mutations should be considered and samples should be sent for specialist testing (Collins et al., 2009).

References

Chen, H., Cheung, C.L., Tai, H., Zhao, P., Chan, J.F.W., Cheng, V.C.C., Yuen, K.Y., 2009. Osel tamivir-resistant 2009 Pandemic influenza A (H1N1) virus, Hong Kong, China. Emerg. Infect. Dis. 15 (12).

Collins, P.J., Hairea, L.F., Lina, Y.P., Liua, J., Russell, R.J., Walker, P.A., Martin, S.R., Daniels, R.S., Gregory, V., Skehel, J.J., Gamblin, S.J., Hay, A.J., 2009. Structural basis for oseltamivir resistance of influenza viruses. Vaccine 27, 6317–6632.

CDC. 2009a. Pyrosequencing assay to detect H275Y mutation in the neuraminidase of the novel A (H1N1) viruses. http://www.who.int/csr/resources/publications/swineflu/NA_DetailedPyrosequencing_200909513.pdf.

CDC. 2009b. Oseltamivir-resistant 2009 Pandemic influenza A (H1N1) virus infection in 2 summer campers receiving prophylaxis–North Carolina. 2009/58, (35), pp. 969–972.

Gulland, A., 2009. First cases of spread of oseltamivir resistant swine flu between patients are reported in Wales. Br. Med. J. 339, b4975.

Gunson, R.N., Gillespe, G., Carman, W.F., 2003. Optimisation of PCR reactions using primer chessboarding. J. Clin.Virol. 26 (3), 369–373.

Gunson, R.N., Collins, T.C., Carman, W.F., 2006. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. J. Clin. Virol. 35, 355–367.
Gunson, R.N., Bennett, S., MacLean, A., Carman, W.F., 2008. Using multiplex real-time PCR in order to streamline a routine diagnostic service. J. Clin. Virol. 43 (4), 372–375.

Gunson, R.N., MacLean, A., Davies, E., Bennett, S., Miller, R., Carman, W.F., 2010. Development of a multiplex real-time RT-PCR that allows universal detection of influenza A viruses and simultaneous typing of influenza A/H1N1/2009 virus. J. Virol. Meth. 163 (2), 258–261.

Harvala, H., Gunson, R., Simmonds, P., Hardie, A., Bennett, S., Scott, F., Roddie, H., McKnight, J., Walsh, T., Rowney, D., Clark, A., Brenner, J., Aitken, C., Templeton, K., 2010. The emergence of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus amongst hospitalised immunocompromised patients in Scotland, November–December. Euro Surveill. 15 (14), 1.

Lackenby, A., Hungnes, O., Dudman, S.G., Meijer, A., Paget, W.J., Hay, A.J., Zambon, M.C., 2008. Emergence of resistance to oseltamivir among influenza A (H1N1) viruses in Europe. Euro Surveill. 13 (1–3), 1–2.

Le, Q.M., Wertheim, H.F., Tran, H.D., van Doorn, H.R., Nguyen, T.H., Horby, P., 2010. A community cluster of oseltamivir-resistant cases of 2009 H1N1 influenza. N. Engl. J. Med. 362 (1), 86–87.

Schutten, M., 2009. Personal Communication.

van der Vries, E., Jonges, M., Herfst, S., Maaskant, J., Van der Linden, A., Guldemeester, J., Aron, G.I., Bestebroer, T.M., Koopmans, M., Meijer, A., Fouchier, R.A.M., Osterhaus, A.D.M.E., Boucher, C.A., Schutten, M., 2010. Evaluation of a rapid molecular algorithm for detection of pandemic influenza A (H1N1) 2009 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase. J. Clin. Virol. 47, 34–37.

WHO, 2009a. Weekly Epidemiological record 30th October, vol 84, 44, 453–468. http://www.who.int/wer/2009/wer8444/en/index.html.

WHO, 2009b. Oseltamivir resistance in immunocompromised hospital patients. Pandemic (H1N1) 2009 briefing note 18. http://www.who.int/csr/disease/swineflu/notes/briefing_20091202/en/print.html.

WHO, 2010. Pandemic (H1N1) 2009 – Update 115. http://www.who.int/csr/don/2010_08_27/en/index.html.

Zambon, M., Hayden, F.G., 2001. Position Statement: global neuraminidase inhibitor susceptibility network. Antivir. Res. 49, 147–156.