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Severe sensitivity loss in an influenza A molecular assay due to antigenic drift variants during the 2014/15 influenza season☆

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ABSTRACT

The 2014–2015 influenza season in Belgium was dominated by the circulation of 2 influenza A(H3N2) subgroups: 3C.2a and 3C.3b. Analysis of 166 nasopharyngeal aspirates, collected in patients with respiratory illness at the start of the epidemic season, showed a decreased sensitivity for the detection of influenza A(H3N2)/3C.2a using a commercially available multiplex assay. Gene sequencing of the matrix protein showed a point mutation (C163T) leading to a mismatch with the assay probes.

1. Introduction

Influenza virus is an important cause of respiratory illness, annually causing serious morbidity and mortality in both adults and children (Poehling et al., 2006; Reed et al., 2014). Rapid and accurate diagnosis is important for patient management, including discontinuation of antibiotics, initiation of antiviral therapy, and infection control measures. Diagnosis solely based on clinical symptoms can be challenging, due to their nonspecific nature such as fever, cough, headache, and malaise (Dugas et al., 2015). Therefore, diagnostic tests can be helpful. These tests should focus on virus detection rather than antibody response (Kumar and Henrickson, 2012). The use of viral cell culture, the traditional gold standard, is abandoned in most clinical laboratories because it can take up to 14 days to obtain the final result. It is replaced by antigen detection tests and/or nucleic acid amplification tests (NAATs). Antigen detection tests, based on direct fluorescence or the immunochromatographic principle, yield results within 10 minutes to 1 hour and are widely used. However, sensitivities vary between different manufacturers and patient populations (Chartrand et al., 2012). Despite the fact that the latest generation of these tests, some with a digital read-out, provide acceptable results (Chartrand et al., 2012; Peaper and Landry, 2014), a higher sensitivity and specificity are attained with NAAT. Consequently, the latter have become the new gold standard (Kumar and Henrickson, 2012). Recently, multiplex molecular assays have reached the market, allowing a syndrome based approach by the detection of multiple respiratory pathogens, both viruses and bacteria, in a single test (Peaper and Landry, 2014; Salez et al., 2015). The latest evolution in influenza testing is the emergence of "rapid NAAT", combining the speed and flexibility of an antigen test and the superior sensitivity and specificity of a molecular assay. Some of these test are easy to perform and can even be used in a point-of-care setting (Dunn and Ginocchio, 2015).

Influenza A is classified in different subtypes based on its surface glycoproteins neuraminidase (NA) and hemagglutinin (HA). During influenza replication, minor genetic changes in the NA or HA appear and accumulate, leading to so-called antigenic drift. Antigenic shift variants can arise following reassortment of NA and HA genes, occasionally resulting in pandemics. Even previously infected or vaccinated individuals have very little or no immunity to these new strains (Treanor, 2010).

Genetic variation should be taken into account when primers and probes are designed for molecular assays, since a mismatch (e.g., due to a point mutations) could lead to false-negative results (Steensels et al., 2013). Therefore, most molecular assays target conserved gene sequences of the influenza A virus such as the matrix (M) or nucleoprotein

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genes (Peaper and Landry, 2014; Steensels et al., 2013; Yang et al., 2014). The World Health Organization advises to target the M gene region 144 to 251 (start codon of the M1 gene was designated 1) (Ward et al., 2004; WHO, 2009).

At the start of the influenza season 2014–2015, we presumed a decreased sensitivity for the detection of influenza A using our routine molecular detection method. Samples of several clinically suspect cases of influenza did not yield positive PCR results or showed small but distinct peaks below the threshold of the assay.

2. Materials and methods

2.1. Samples and setting

From December 11, 2014, until February 3, 2015, 184 consecutive nasopharyngeal aspirates from patients presenting with respiratory illness in our acute secondary care hospital were analyzed. Samples were stored straight at −80 °C immediately after testing. Eighteen samples were excluded for further analysis due to insufficient sample volume, resulting in 166 analyzed samples.

2.2. Methods

All 166 samples were tested using RespiFinder RG (Qiagen, manufactured by PathoFinder, Maastricht, The Netherlands) and Xpert Flu/RSV XC (Cepheid, Sunnyvale, CA, USA). Both are CE-IVD marked; Xpert Flu/RSV XC is also Food and Drug Administration cleared. A third in-house molecular method was performed on a subset of 23 samples to validate the Xpert Flu/RSV XC assay.

Automated nucleic acid extraction of nasopharyngeal aspirates, executed prior to RespiFinder RG and in-house PCR, was performed on the easyMag extraction system (bioMérieux, Marcy l'Etoile, France) using the generic protocol with off-board lysis (200-μL input and 100-μL elution volume).

RespiFinder RG, a multiplex molecular assay for the detection of 22 respiratory pathogens, including influenza A, influenza A H1N1pdm09, and influenza B, was used as the routine test method. For influenza A, the M gene is targeted. The test was executed according to the manufacturer's instructions using the Rotor-Gene Q (Qiagen, Hilden, Germany) and melt-curve analysis. Briefly, the assay consists of a preamplification step that combines reverse transcriptase and multiplex target amplification PCR, followed by a probe hybridization step, a probe ligation step, and a probe amplification step. An internal amplification control provided by the manufacturer was added to monitor the whole process from extraction to detection (Dabisch-Ruthe et al., 2012).

Xpert Flu/RSV XC is a rapid PCR assay in which extraction; amplification; and detection of influenza A, influenza B, and respiratory syncytial virus take place within a single-use disposable cartridge that is placed in the random access GeneXpert platform (Cepheid). Sample preparation was executed as indicated in the package insert, 600-μL nasopharyngeal aspirate was added to 3-mL universal transport medium (Cepheid), and 300-μL prepared sample was then transferred into the cartridge. Per the package insert for influenza A, the following genes are targeted: M, polymerase basic protein 2, and polymerase acidic protein. Exact sequences are not disclosed. From January 12, 2015 on, this test was used in parallel to the RespiFinder RG due to the suspicion of false-negative results. Samples obtained between December 11, 2014, and January 12, 2015, were analyzed retrospectively by Xpert Flu/RSV XC.

The in-house molecular assay is a multiplex assay for the detection of influenza A and B. For influenza A, 2 different regions in the M gene are targeted by using primers and probes recommended by the Centers for Disease Control and Prevention (CDC) and published by Steensels et al. (2013), CDC (n.d.), and Schulze et al. (2010). The assay was executed on a pilot sample set (n = 23), using the Applied Biosystems 7500 FAST Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA), to validate results obtained with the Xpert Flu/RSV XC. This sample set consisted of all 16 samples designated negative by RespiFinder RG but positive by Xpert Flu/RSV XC obtained between January 12, 2015, and February 3, 2015, plus 7 randomly selected samples with concordant results in both assays. Five microliter RNA extract was added to 20-μL master mix, containing primers, probes, and Taqman Fast Virus 1-step master mix (Thermo Fisher Scientific).

HA (nucleotides 1–1541) and partial M gene sequencing were performed by the Belgian National Reference Center for Influenza (NRC Influenza) and PathoFinder, respectively, both using Sanger sequencing. A consensus phylogenetic tree of the HA sequences was made using MEGA 4 (The Biodesign Institute, Tempe, AZ, USA). Sequences of reference strains were obtained from GISAID's EpiFlu Database (accession numbers shown in Fig. 1).

3. Results

The results of the Xpert Flu/RSV XC (n = 23) were concordant with results of the in-house PCR. The positive results obtained by Xpert Flu/RSV XC in 16 samples and designated as negative by RespiFinder RG were confirmed to be positive. Furthermore, 2 samples that were positive with both Xpert Flu/RSV XC and RespiFinder and 5 that were negative in both assays were analyzed. Hence, true positives were defined as positive with Xpert Flu/RSV XC. False negatives were samples that were negative by RespiFinder RG but showed to be positive when analyzed using the Xpert Flu/RSV XC.

Analysis of 166 nasopharyngeal aspirates showed that 60 samples were positive for influenza, of which 58 (35%) were influenza A and 2 (1%) influenza B. Influenza A H1N1pdm09 was present in 7 of these 58 (12%) samples. All influenza A H1N1pdm09 and influenza B were detected by both RespiFinder RG and Xpert Flu/RSV XC.

Out of 58 influenza A–positive samples, 25 (43%) were reported to be negative by the RespiFinder RG, despite low cycle threshold (Ct) values encountered using Xpert Flu/RSV XC (median Ct 21.1, range: 17.1–33.9) and in-house PCR (subset of 16 samples, median Ct 21.6, range: 18.0–35.8). Results are presented in Table 1.

HA gene sequencing was performed on a subset of 13 samples positive in the Xpert FLU assay but negative (n = 11) or positive (n = 2) in the RespiFinder RG assay. In Fig. 1, a phylogenetic tree of HA sequences is presented showing 26 influenza A(H3N2) viruses circulating in Belgium during the 2014–2015 season, reference strains from around the world (including the vaccine strain A/Texas/50/2012) and the 13 strains from our lab. Sequencing analysis revealed that all 13 strains belonged to influenza A(H3N2) subgroup 3C.2a, represented by influenza A(H3N2)/Hong Kong/5738/2014 (Fig. 1).

Since the 2014/15 influenza season was dominated by the circulation of influenza A(H3N2) viruses belonging to subgroup 3C.2a and 3C.3b (NRC Influenza, unpublished data), 4 strains belonging to the influenza A (H3N2) subgroup 3C.3b were sent to us by the NRC Influenza to verify RespiFinder RG sensitivity for this subgroup of circulating A(H3N2) strains. Positive results were found for all 4 strains for both Xpert FLU/RSV XC and RespiFinder RG assays.

Matrix protein gene sequencing of 10 false-negative and 5 true (but weakly)–positive strains showed a point mutation (C163T) leading to mismatch with the assay probes.

4. Discussion

The influenza A virus is extremely subjective to genetic variation. In 2014, 3 influenza A(H3N2) subgroups have emerged, 1 in subdivision 3C.2 and 3C.2a and 2 in 3C.3, 3C.3a, and 3C.3b (European Centre for Disease Prevention and Control, 2015). In Belgium, the 2014/15 influenza season was dominated by the circulation of influenza A(H3N2) viruses belonging to subgroups 3C.2a and 3C.3b (NRC Influenza, unpublished data), while the A/Texas/50/2012 strain used in the 2014/15 northern hemisphere vaccine belongs to genetic subgroup 3C.1 (Flannery et al., 2015). Compared with A/Texas/50/2012, subdivision 3C.2 is defined

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