CASE REPORT

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Acute respiratory distress syndrome (ARDS) in the course of influenza A/H1N1v infection – genetic aspects

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Abstract

Influenza is a contagious respiratory disease caused by viruses belonging to the family *Ortomyxoviridae*. Among the influenza viruses type A, B and C, the A type virus shows the most pathogenic potential. Its surface receptor glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are characterized by high antigenic variation, thus a host organism cannot develop permanent resistance. The case is described of a male patient with severe acute respiratory distress syndrome in the course of influenza A/N1H1v infection, confirmed by virological molecular analysis. During diagnostic procedures based on the MSSCP genotyping it was observed that the WHO recommended RT-PCR kits and/or procedure of sample collection from patients for molecular investigation could lead to false positive A/H1N1 pandemic strain detection because of the co-amplification during the RT-PCR fragments of the human genome.

Key words

A/H1N1v influenza, complications, respiratory failure, MSSCP genotyping

INTRODUCTION

The A/H1N1v virus is a new virus to which humans are not yet resistant, and its incidence rates may be higher than those for the seasonal influenza [1, 2]. Unlike seasonal influenza, novel influenza A/H1N1 usually affects subjects below the age of 65, especially pregnant women, obese persons, as well as patients with comorbidities, including asthma, chronic bronchitis, neoplasms, and previous transplants [3, 4, 5]. Less commonly, influenza may evolve within a very short time into acute respiratory distress syndrome (ARDS) [6, 7, 8, 9]. The presented report describes a fatal case history of ARDS in the course of influenza A/H1N1v infection.

CASE REPORT

A 60-year-old male patient with dyspnoea, dry cough and chest pains on deep breathing was admitted to the Department of Internal Medicine of the Independent Public Clinical Hospital in Lublin, Poland. The patient had already undergone treatment because of spinal pain in the Department of Rehabilitation and then in the Department of Dermatology because of severe lesions. After a lower respiratory tract infection occurred, the patient was transferred to the Department of Internal Medicine where, after a physical examination, X-ray and laboratory results, infection of the lower respiratory tract excluding lung involvement was diagnosed. However, two days later, a sudden decrease was observed in his health. The dyspnoea symptoms became severe and the patient was unable to speak fluently. Furthermore, repeated examinations revealed scattered parenchymal infiltrates affecting nearly half of both the lungs. With diagnosed acute respiratory distress syndrome, the patient was transferred to the Intensive Care Department. After continuous deterioration of the patient's respiratory functions and negative blood and urine test, collected swabs were sent to the Military Institute of Hygiene and Epidemiology, Biological Threat Identification and Countermeasures Centre for analysis for the presence of A/N1H1v.

Performed laboratory tests were positive for the presence of A/H1N1 virus (Fig. 1). Due to this fatal case, the Biological Threat Identification and Countermeasures Centre Laboratory decided to send the RT-PCR products to the BioVectis laboratory to confirm swine influenza infection, and to exclude possible co-infection with other respiratory viruses, using the Multitemperature Single Strand Conformation Polymorphism (MSSCP method) [10]. For the purpose of the MSSCP analysis, RT-PCR products obtained with a previously confirmed case of A/H1N1 infection (collected in the Department of Internal Medicine, University Hospital, Lublin, Poland) was added.

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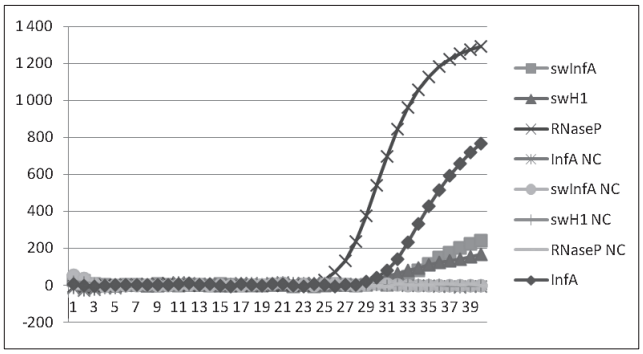

Figure 1. Real time RT PCR results for A/H1N1 virus detection.
1 – RNaseP – Ribonuclease P;
2 – InfA – Influenza type A virus;
3 – swInfA – swine Influenza A virus;
4 – swH1 – swine haemagglutinin gene;
5 – RNaseP NC – Ribonuclease P negative control;
6 – InfA NC – Influenza type A virus negative control;
7 – swInfA NC – swine Influenza A virus negative control;
8 – swH1 NC – swine haemagglutinin gene negative control.

MATERIALS AND METHOD

Swabs. Swabs from nose and throat were collected according to the procedure recommended by the WHO National Influenza Centres (National Institute of Hygiene- Warsaw). Sterile plastic dry swabs (made from of synthetic materials) were used to take biological samples form surface of both tonsils and back wall of the throat, avoiding touching oral mucosa. After breaking-off the end of the chip, the swab was placed carefully into a test tube for transportation. In order to take a sample from the nares, the patient's head was tilted back, and a swab taken from a nostril. The end of the chip was broken off nearby a cap and placed into the same transport test-tube. Subsequently, a swab was taken in the same way from the other nostril. Sterile saline (ca. 1 ml) was added to the tube to immerse all three swabs. The vertically positioned tubes were sent to the laboratory in cooled boxes.

RNA isolation. QIAamp Viral RNA Mini Kit (Qiagen) was used for RNA isolation. Briefly, 140 µl of sample were chemically lysed in RNA protecting environment according to the manufacturer's procedure. The material was bound to silica micro-columns and purified with washing buffers. Eluted RNA solution (60 µl) was stored at -80 °C for further analysis.

RT real time PCR. RNA obtained was used immediately in qualitative RT real time PCR [11]. IScript One-Step RT-PCR Kit for Probes (Bio-Rad) was used for 'one step' type reverse transcription and amplification in a CFX96 Real-Time System (Bio-Rad). A USA-CDC panel of specific primers pairs and dual-labeled hydrolysis probes, recommended by the WHO, was used in the study.
- The InfA primers and probe set were designed for the universal detection of type A influenza viruses.
- The swInfA primers and probe set were designed to specifically detect all swine influenza A viruses [12].
- The swH1 primers and probe set were designed to specifically detect swine H1 influenza (Tab. 1).

Additional human RNaseP primers and probe set served as an internal control of assay. 5 µl of the sample were used as a template for RT real time PCR in a final volume of 20 µl of reaction mixture with a concentration of 0.75 µM of primers and 0.1875 µM of probes. Reverse transcription at 50 °C for 10 min initiated the run, followed by denaturation/activation at 95 °C for 5 min and 40 cycles of denaturation at 95 °C for 15 s and combined annealing and extension at 55 °C for 30 s, with fluorescence measurement at the end of extension. An increase in fluorescence signal observed in each of them indicated specific products amplification.

Table 1. Sequence of the primers and probe used in RT real time PCR.

	Sequence
InfA F	GACCRATCCTGTCACCTCTGAC
InfA R	AGGGCATTYTGGACAAAKCGTCTA
InfA probe	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ1
swInfA F	GCACGGTCAGCACTTATYCTRAG
swInfA R	GTGRGCTGGGTTTTCATTTGGTC
swInfA probe	FAM-CYACTGCAAGCCCA"T"-BHQ1-ACACACAAGCAGGCA
swH1 F	GTGCTATAAACACCAGCCTYCCA
swH1 R	CGGGATATTCCTTAATCCTGTRGC
swH1 probe	FAM-CAGAATATACA"T"-BHQ1-CCRGTCACAATTGGARAA
RP F	AGATTTGGACCTGCGAGCG
RP R	GAGCGGCTGTCTCCACAAGT
RP probe	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1

MSSCP-based minor variant enrichment procedure. The RT-PCR products were analyzed by the MSSCP method at strictly controlled (to ± 0.2°C) gel temperature in dedicated equipment: DNA*Pointer*® System/BioVectis (Warsaw, Poland) as described by Kaczanowski et al [13]. The RT-PCR products were heat denatured and resolved as ssDNA conformers on a 9% polyacrylamide gel in native conditions (TBE buffer) at 3 different temperatures during one run. Subsequently, DNA bands were visualized by silver nitrate staining (SilverStain DNA Kit, BioVectis, Warsaw, Poland). Fragments of the MSSCP gel containing bands of interest were cut out, ssDNA was eluted and re-amplified using primers and PCR conditions, as described above. For subsequent DNA Sanger sequencing [14], 1/10 vol of PCR products were used (3730×l DNA Analyzer, Applied Biosystems, Carlsbad, CA, USA).

RESULTS

In the presented study, 2 respiratory samples collected from patients with influenza symptoms in Poland during January 2011 were analyzed. Both samples were collected (marked 1 and 2 in Fig. 2) in the Department of Internal Medicine, University Hospital, Lublin, Poland. The samples were sent to the Biological Threat Identification and Countermeasures Centre Laboratory. Performed real time PCR tests were positive for the presence of A/H1N1 virus (Fig. 1). In the first case, after introduction of Oseltamivir combined with antibiotic therapy, the patient was recovered completely. In the second case the acute respiratory distress syndrome of fatal course developed. It was then decided to perform confirmation analysis using MSSCP method. In MSSCP method the haemagglutinin gene fragments between nucleotides 125 and 302 were amplified by the

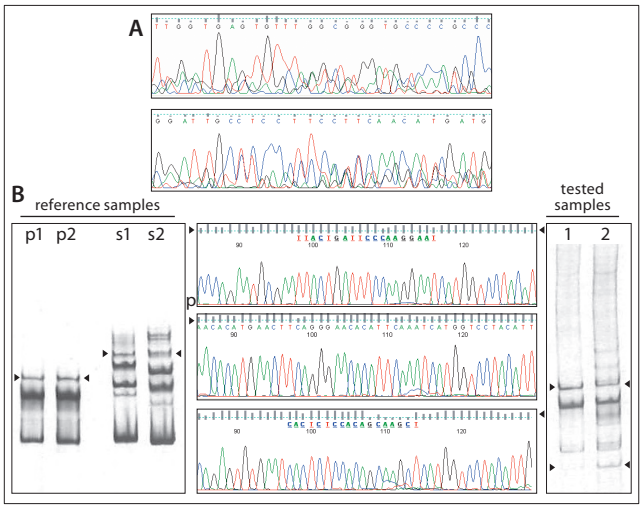

Figure 2. Identification of diverse genetic variants in clinical samples obtained from AH1N1 virus-infected patients by MSSCP method and sequencing.
Sample 1 – previously confirmed case of AH1N1 infection collected in the same Department.
Sample 2 – described above fatal case.
(A): DNA sequence reads of clinical samples after direct Sanger sequencing (samples 1, 2) prior to MSSCP analysis. DNA sequences are highly heterogenic and are not suitable for reliable diagnosis of A/H1N1v infection.
(B): Sanger sequencing of two clinical isolates (samples 1 and 2) after MSSCP-based minor variant enrichment. PCR products of hemagglutinin gene amplification obtained from pandemic A/H1N1v (p1 – A/Mexico/4486/09, p2 – A/England/195/09) and seasonal (s1 – A/Fukushima/141/2006, s2 – A/New Caledonia/20/1999) strains of influenza virus A/H1N1 were denatured and separated in 9% polyacrylamide gel using MSSCP method under optimal electrophoretic conditions. DNA bands were visualized with silver stain. ssDNA bands indicated by arrows, representing pandemic (p, red) and seasonal (s, black) strains (left panel), as well as DNA bands obtained from samples 1 and 2 separation (right panel), were cut out from the gel, DNA was recovered and sequenced. Strain-specific regions are indicated by letters above the histograms. Representative chromatograms for one pandemic and seasonal bands are shown. DNA sequencing of clinical samples identified the presence of pandemic (p, red) sequences proving the presence of A/H1N1v infection. Furthermore, the presence of additional PCR product, corresponding to human chromosome 21q, section 15/105 (h, blue) was revealed.

RT-PCR method, as described in Materials and Method. Afterwards, PCR products were directly sequenced to verify the genetic profile of influenza virus. Highly heterogenic DNA chromatograms were obtained (Fig. 2A). This suggested the presence of minor genetic variants in these samples, which could not be distinguished via Sanger sequencing. To verify this assumption and to identify possible minor genetic variants, the MSSCP-based minor genetic variants enrichment procedure was performed on these samples (5). DNA fragments obtained from influenza seasonal (s) and pandemic (p) A/H1N1 strains, as well as clinical samples, were denatured and subjected to MSSCP separation at strictly controlled gel temperature profile during the electrophoresis. The optimum MSSCP electrophoretic conditions were 15 – 10 – 5°C, in which electrophoretic patterns of ssDNA fragments from seasonal (s) and pandemic (p) A/H1N1 strains were easily distinguishable (Fig. 2B, left panel). In clinical samples, a genetic profile comparable with pandemic strains was found. Additionally, other DNA bands which could be attributed to co-infections with a seasonal A/H1N1 strain or representing another unknown genetic variant, were detected (Fig. 2B, right panel).

All electrophoretically separated bands representing putative pandemic and seasonal strains, respectively (Fig. 2B), were cut out from the MSSCP gel, ssDNA fragments from these gel pieces were recovered, and DNA sequenced. The sequences obtained revealed clear sequencing histograms (no double peaks) and confirmed the presence of pandemic influenza variants (black arrow) in the 2 analyzed samples (Fig. 2B). Representative chromatograms for one pandemic and seasonal bands are shown. Additional DNA bands (blue arrow) were diagnosed as human DNA, corresponding to human chromosome 21q, section 15/105 (according to BLAST database). Detailed DNA sequences found in the tested material are summarized in Table 2.

DISCUSSION

The pandemic strain A/H1N1v infection was primarily detected in the patient's nasal swabs by RT-PCR test. [15] However, due to the development of ARDS in the fatal case, an additional molecular method – MSSCP genotyping – was used to confirm the sample composition at the molecular level. Direct Sanger sequencing of the RT-PCR product resulted in highly heterogenic histograms, suggesting the presence of different sequences among amplified PCR product in the analyzed samples. This assumption was supported by a previously described co-infection with pandemic and seasonal strains of A/H1N1v in patients infected with flu virus in the 2009 pandemic season [16, 17, 18] [X- XI]. MSSCP analysis confirmed the obtained RT-PCR results and diagnosis of the pandemic strain of A/H1N1v infection. Interestingly, the source of an additional product visualized by the above-mentioned, heterogenic chromatograms, was also identified (Fig. 2A).

According to the BLAST database, the PCR mixture contained human DNA, corresponding to human chromosome 21q, section 15/105. In is noteworthy that human DNA was efficiently amplified with the same primers as those designed for A/H1N1v detection. Since the material for DNA analysis was obtained from the nose, it is possible that the swabs contained human cells. From the point of

Table 2. DNA sequences obtained from Sanger sequencing of DNA bands after MSSCP-based enrichment procedure. DNA region distinctive for pandemic and seasonal A/H1N1 virus strains is shown.

sample/ band no.	A/H1N1v strain	DNA sequence
p1/2	pandemic (ref)	5'TATGCAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAG-CTCATGGTCCTACATTGTATACC3'
s1/2/3	seasonal (ref)	5'TATGTCTACTAAAAGGATAGCCCCACTACAATTGGGTAATTGCAGCGTTGCCGGATGGATCTTAGGAAACCCAGAATGCGAATTACTGATTTCCAAGGAA-TCATGGTCCTACATTGTAA3'
1/1, 1/2, 2/3, 2/4	pandemic	5'TATGCAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAG-CTCATGGTCCTACATTGTTAA3'
1/3, 2/1, 2/2, 2/5	–	5'TCTTCATGACTTAATCACCTCTTAGTAGACTCCACCTCTCGATGCTGTTGCTTTTGGGAAAAGTTTCCAACACATGAACTTCAGGGAACACATTCAAATCA-TGGTCCTACATTGTAA3.

view of the presented report, this case points out important problem of false-positive results. It be can assumed that in the case of the absence of A/H1N1 virus, the RT-PCR technique could still detect a specific product amplified from human but not virus DNA, resulting in the patient's being included into unnecessary treatment. This issue should be seriously considered in the case of RT-PCR diagnosis of influenza and other viruses [19, 20]. The presented study indicates that the additional method should be available to verify RT-PCR results in patients suspected of A/H1N1 infection.

CONCLUSIONS

The above scrutiny of the relevant tests clearly exemplifies an important problem in the differentiation between pandemic, seasonal, and especially mixed influenza infection, being an effect of the RT-PCR technique. Furthermore, the human DNA also could interfere with RT-PCR, which could lead to obtaining false positive results. As a result, there is a justified concern regarding this procedure as it could still detect an explicit product extracted from human but not virus DNA, resulting in the patient's undergoing a needless treatment. Therefore, it should not be the only predicament taken into consideration for the detection of influenza and other viruses. The above research identifies the thesis that further technique should be accessible to authenticate RT-PCR results in patients suspected of A/H1N1 infection.

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