# MOLECULAR SURVEILLANCE OF INFLUENZA VIRUSES IN IASI COUNTY

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**Abstract:** Influenza viruses type A and B are responsible for epidemics often associated with increased rates of morbidity and mortality. As a result of their high genetic diversity, accurate diagnosis of influenza is difficult. Rapid and sensitive molecular assays play an important role in the diagnosis and surveillance of this disease. The aim of this study was to implement a qRT-PCR technique in the Microbiology Laboratory of the Regional Public Health Center Iasi that allows the identification of influenza types A and B, as part of the national surveillance program. We have included in the study 50 clinical specimens from Iasi County collected beginning with surveillance week 50/2015 until week 20/2016. PCR assay revealed, that 13 samples (26%) were positive for influenza A virus, which were subsequently identified as A (H1N1)pdm09 in National Institute of Research "Cantacuzino" Bucharest. Only one specimen (2%) was positive for influenza B virus. The highest number of positive cases was recorded in week 7/2016. The results showed a correlation between national reports and our study group regarding the predominant circulation of A (H1N1)pdm09 influenza virus. Data obtained sustain the importance of molecular assays for rapid diagnosis and epidemiological surveillance of circulating influenza viruses in order to update influenza vaccine formula each year.

## INTRODUCTION

Influenza is an acute respiratory disease caused by influenza types A, B and C viruses, belonging to the *Orthomyxoviridae* family. Types A and B of influenza viruses are responsible for epidemics often associated with increased rates of morbidity and mortality. They are negative-strand RNA viruses with a segmented genome. Influenza type A viruses can further be classified into subtypes according to differences in the two surface proteins: haemagglutinin (HA) (18 subtypes) and neuraminidase (NA) (11 subtypes) (Tong et al.,2013, World Health Organization. Global Influenza Surveillance Network, 2011). Antigenic structure of human influenza viruses undergoes changes as a result of mutations (antigenic drift), associated with epidemics or genomic reassortment (antigenic shift), a major genotypic change that characterizes only influenza virus types A, leading to pandemics (World Health Organization. Global Influenza Surveillance Network, 2011).

As a result of high viral diversity and rapid changes caused by mutations, accurate diagnosis of influenza is difficult and complex (Zheng et al., 2016). Surveillance of circulating influenza viruses is essential in ensuring an optimal prophylaxis. The development of rapid and sensitive molecular assays, used directly on clinical samples, that allow for timely and accurate detection of influenza play an important role in the diagnosis and surveillance of influenza viruses and can greatly influence patient management (Ellis and Zambon, 2002).

Reverse-transcription polymerase chain reaction (RT-PCR) can reverse transcribe viral RNA in complementary DNA (cDNA) which is then amplified and detected. Gel electrophoresis is used for cDNA detection in conventional RT-PCR, while in real time (qRT-PCR), fluorescent dyes (e.g. SYBR green – non-specific binding to cDNA) or fluorophore labelled oligonucleotide probes (TaqManR probe – specific binding) are being used. Other qRT-PCR assays use "minor groove binding" probes, fluorescence resonance energy transfer (FRET) probes, molecular beacons or Scorpion probes with specific binding on target. All of these methods can be used to identify influenza viruses and to determine the degree of relatedness between different types and subtypes. Type identification is based on detection of genes that encode highly conserved structures such as internal virus proteins or non-structural proteins. Genes encoding HA and NA proteins are detected for subtype identification. Alternative methods for detection of amplicons are Restriction Fragment Length Polymorphism (RFLP); genetic sequence analysis or probe hybridization (e.g. microarrays assay that can be used for screening and type/subtype identification of influenza viruses) (Sengupta et al.,2003, World Health Organization. Global Influenza Surveillance Network, 2011).

The aim of this study was to implement a qRT-PCR technique in the Microbiology Laboratory of the Regional Public Health Center Iași that allows the identification of influenza types A and B, as part of the national surveillance program of influenza-like illness (ILI) and severe acute respiratory infections (SARI). This method allows us to determine

the type of influenza viruses circulating in Iasi County in comparison to the national pattern and to evaluate the morbidity in influenza cases and acute respiratory viral infections. All data collected in Europe allowed WHO to make recommendations on the composition of influenza vaccines each year according to laboratory results from previous season.

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## MATERIALS AND METHODS

Molecular detection of influenza viruses' type was performed following an "in house" multiplex qRT-PCR technique adapted from Ward et al. (Ward et al., 2004). Quality control was assessed using laboratory grown stocks of A/H3N1: A/Switzerland/971529/3/2013, A/H1N1: A/California/7/2009, B/Phuket/3073/2013, B/Brisbane/60/08, which were supplied by National Institute of Research "Cantacuzino" Bucharest.

We have included in the study 50 clinical specimens (49 nasal and throat swabs and one tracheobronchial aspirate) from Iași County. Sample collection for diagnosis in the Regional Public Health Center Iași began in surveillance week 50 (December 2015) and continued until week 20 (May 2016) of the sentinel surveillance program.

Since successful influenza virus diagnosis depends upon sample quality and the conditions under which it is stored and transported, we have recommended that the specimens should be collected within 3 days of the onset of disease and then stored and transported using a suitable medium (e.g. Virocult). Viral ARN was isolated from 200 μl of sample using *RNeasy® Plus Mini* kit *(Qiagen)*.

The reaction mix was prepared at a 25 μl final volume, using *Rotor Gene Multiplex RT-PCR* kit *(Qiagen)*, primers and TaqMan probes *(Eurogentec)* (Table 1) and 5 μl of purified template RNA (Table 2). For each target gene we have prepared a 20x primer/probe mix, as follows: 12μM FluA_Fwd + 12μM FluA_Rev + 8μM FluA probe and respectively 8μM FluB_Fwd + 8μM primer FluB_Rev + 4μM FluB probe.

Amplification and detection of influenza virus types A and B was performed using *Rotor Gene Q (Qiagen)* (Table 3). Negative template controls (NTCs) and positive controls (PCs) were included in each run.

Table 1. Primer and probe sequences for detection of type A and B influenza viruses (adapted from Ward et al., 2004)

| Primer/Probe sequence (5'-3') | Amplified gene |
|---|---|
| FluA_Fwd: AAG ACC AAT CCT GTC ACC TCT GA | M1 (104bp) |
| FluA_Rev: CAA AGC GTC TAC GCT GCA GTC C | |
| FluA Probe: FAM TTT GTG TTC ACG CTC ACC GTG CC BHQ1 | |
| FluB_Fwd: GAG ACA CAA TTG CCT ACC TGC TT | M (92bp) |
| FluB_Rev: TTC TTT CCC ACC GAA CCA AC | |
| FluB Probe: ROX AGA AGA TGG AGA AGG CAA AGC AGA ACT AGC BHQ2 | |

Table 2. Reaction mix for detection of type A and B influenza viruses

| Reaction mix | Volume/reaction | Final concentration |
|---|---|---|
| 2x*Rotor-Gene Multiplex RT-PCR Master Mix* | 12,5 μl | 1x |
| 20x type A primer/probe mix | 1,25 μl | 0,6 μM FluA_Fwd<br>0,6 μM FluA_Rev<br>0,4 μM SondaFluA |

| 20x type B primer/probe mix | 1,25 µl | 0,4 µM FluB_Fwd<br>0,4 µM FluB_Rev<br>0,2 µM SondaFluB |
|---|---|---|
| *Rotor Gene RT Mix* | 0,25 µl | - |
| *RNase-free water* | 4.75 µl | - |
| TOTAL | 20 µl | - |

Table 3.Thermal cycling conditions for detection of type A and B influenza viruses

| Number of cycles | Step | Time | Temperature |
|---|---|---|---|
| 1 | revers transcription | 15 min | 50°C |
| 1 | PCR initial activation step | 5 min | 95°C |
| 40 | denaturation | 15 sec | 95°C |
| | primers annealing/ elongation | 15 sec | 60°C |

## RESULTS AND DISCUSSIONS

The samples collected from Iași County belonged to patients diagnosed mainly with SARI (31 cases), the rest being ILI (19 cases). Twenty four SARI patients had received antibiotic therapy prior to investigation and only two had also been treated with Oseltamivir. Only one patient had been vaccinated against influenza virus. The average age of the patients was 27.92, with a minimum age of 7 months and a maximum of 67 years (Fig. 1). Most ILI and SARI cases were registered in the age group 15-49 years, but only SARI was reported in infants < 1 year and elderly > 65 years (Fig. 2). The number of women was slightly higher (27) than men, with no significant differences between the two groups regarding age or clinical diagnosis (Fig. 1). Seven of 16 women diagnosed with SARI had associated cardiovascular, pulmonary or metabolic diseases, while only three out of 15 men with SARI had associated comorbidities. Most of the samples were collected from sporadic cases.
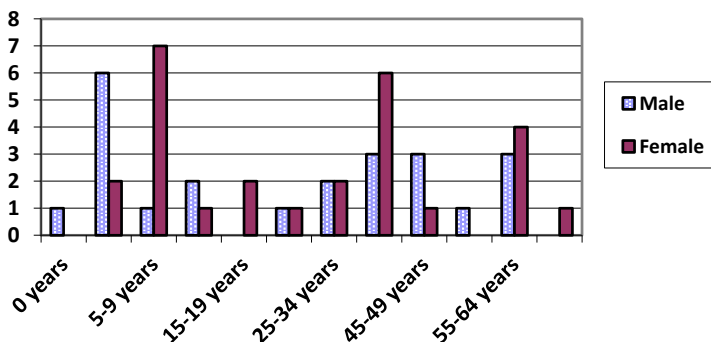


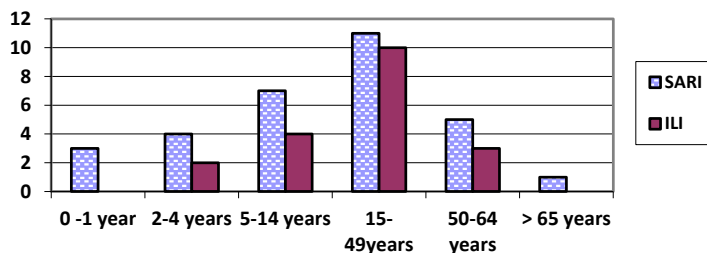Figure 1. Characteristics of the study group by age and gender

Figure 2. The number of SARI and ILI cases investigated

PCR assay revealed, that of the 50 samples analyzed from Iaşi, 13 (26%) were positive for influenza A virus. The positive samples were detected in both ILI (6 cases) and SARI patients (7 cases). All samples positive for influenza A virus were sent for detection of subtype to the National Institute of Research "Cantacuzino" Bucharest. All were identified as A (H1N1)pdm09. Only one specimen (2%) was positive for influenza B virus and it belonged to a 9 year old girl diagnosed with ILI.

The highest number of samples collected was in the months of January (15 samples) and February (16 samples) (Fig. 3), but positive cases were recorded starting week 6/2016 and February was the month when the peak of positive cases was detected (week 7/2016) (Table 4). Influenza B virus was detected in week 8/2016 (Table 4).
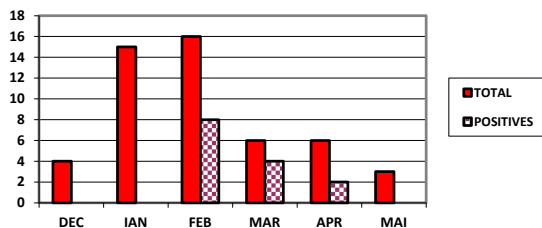


Figure 3. Monthly distribution of collected samples

Table 4. Weekly distribution of number of specimens tested and the results

| Surveillance week | Total no. | No. of positives | Influenza virus type |
|---|---|---|---|
| 51 (14-20.12.2015) | 3 | 0 | - |
| 52 (21-27.12.2015) | 1 | 0 | - |
| 53 (28.12.2015-03.01.2016) | 0 | 0 | - |
| 1 (04-10.01.2016) | 2 | 0 | - |
| 2 (11-17.01.2016) | 3 | 0 | - |
| 3 (18-24.01.2016) | 7 | 0 | - |

| 4 (25-31.01.2016) | 3 | 0 | - |
|---|---|---|---|
| 5 (01-07.02.2016) | 3 | 0 | - |
| 6 (08-14.02.2016) | 4 | 3 | A |
| 7 (15-21.02.2016) | 5 | 4 | A |
| 8 (22-28.02.2016) | 4 | 1 | B |
| 9 (29.02-06.03.2016) | 2 | 1 | A |
| 10 (07-13.03.2016) | 1 | 1 | A |
| 11 (14-20.03.2016) | 2 | 2 | A |
| 12 (21-27.03.2016) | 1 | 0 | - |
| 13 (28.03-03.04.2016) | 1 | 1 | A |
| 14 (04-10.04.2016) | 2 | 1 | A |
| 15 (11.04-17.04.2016) | 3 | 0 | - |
| 16 (18.04-24.04.2016) | 0 | 0 | - |
| 17 (25.04-01.05.2016) | 0 | 0 | - |
| 18 (02.05- 08.05.2016) | 1 | 0 | - |
| 19 (09.05-15.05.2016) | 2 | 0 | - |
| 20 (16.05-22.05.2016) | 0 | 0 | - |
| TOTAL | 50 | 14 | 13A, 1B |

Data analysis showed that none of the positive cases had been vaccinated and only two of them received Oseltamivir. Patients with laboratory confirmed diagnosis were aged between 18 months and 64 years, 8/14 being women. A third of the positive cases had associated cardiovascular, pulmonary and / or metabolic diseases. Most were sporadic cases and only three came from familial outbreaks.

Influenza represents an important cause of mortality and hospitalization in many parts of the world. Although there are settings in which its impact on human health is uncertain, this is probably a result of a lack of virological surveillance. According to World Health Organization (WHO), global surveillance is essential for a better understanding, prevention and control of influenza (World Health Organization. WER. 2016).

The qRT PCR method we used for type detection and surveillance of circulating influenza viruses was successfully introduced in our Laboratory and the results were confirmed by supplemental tests for subtype performed at National Institute of Research "Cantacuzino" Bucharest, who also provided the influenza type A and B panel for external quality assessment.

The ability of influenza virus to cause epidemics and pandemics led to the WHO decision to create the international network Global Influenza Surveillance and Response System (GISRS) to coordinate the diagnosis, treatment, prevention and surveillance of influenza cases. GISRS provides recommendations for the annual composition of influenza vaccines, information regarding antiviral susceptibility and emergence of new pandemic strains and updated laboratory protocols (World Health Organization. WER. 2016). Recommended molecular protocols for RT-PCR (conventional and real time) tests have been updated by WHO for influenza type A viruses (matrix gene), A (H1N1)2009 virus (H1 gene), influenza B lineage, seasonal influenza viruses and influenza A (H5N1) viruses and also for sequencing (World Health Organization, 2014).

Although molecular methods ensure rapid results, they cannot replace virus isolation necessary for antigenic characterization (Ellis and Zambon, 2002). The gold standard for influenza

diagnosis is represented by virus isolation (in cell culture or embryonated eggs) and identification based on antigen - antibody reaction (e.g. hemagglutination inhibition assay HAI or immunofluorescence). Although several days are required for results, viral cultivation is indispensable for influenza vaccine production (World Health Organization. Global Influenza Surveillance Network, 2011). Immunofluorescence antibody staining is a rapid technique that uses monoclonal antibodies against influenza viruses. This method can be applied on isolates and clinical specimens, but WHO recommends the association with other laboratory tests to improve sensitivity and specificity (World Health Organization. Global Influenza Surveillance Network, 2011). Zheng et al. reports a new, simple and rapid technique for influenza virus detection, using glycan-functionalized gold nanoparticles (gGNPs), a method based on the specific binding of viruses to the glycans and the subsequent aggregation of gGNP probes on the viral surface, allowing the differentiation of influenza strains, including the human and avian circulating strains (Zheng et al., 2016). Serological tests (HAI, microneutralization, enzyme-linked immunosorbent assay) evaluate immune response and can be useful for virus identification and for determining the efficacy of vaccination. They are applicable when viral isolation is not possible, due to lack of facilities, late diagnosis (after viral shedding is over) or the clinical specimens cannot be obtained (World Health Organization. Global Influenza Surveillance Network, 2011).

In temperate regions, epidemics of influenza occur almost every year, though the onset and peak of activity may vary from one season to the next. In the northern hemisphere, activity begins to increase in late autumn, with periods of peak typically occurring between December and March and lasting for 6–8 weeks. The severity of illness can vary substantially from one year to another, depending on the circulating types, subtypes and strains, as well as the level of protective antibodies in the general population. Pandemics occur infrequently at irregular intervals and affect all age groups (World Health Organization. Global Influenza Surveillance Network, 2011).

Increased circulation of influenza viruses is associated with increases in acute respiratory illnesses, hospitalization rate and deaths, resulting from pneumonia or exacerbation of existing cardiopulmonary conditions. The highest rates of seasonal influenza-related illness occur among school-age children (sometimes exceeding 30%), when compared to adults (1% - 15%). However, the highest rates of associated hospitalizations occur among: children under 2 years of age, patients with chronic medical conditions, patients aged 65 or older and pregnant women (World Health Organization. Global Influenza Surveillance Network, 2011).

According to the Declaration of the WHO Regional Office for Europe, influenza virus A (H1N1) that caused the pandemic in 2009 (called "swine flu" in the past) - continued to circulate as a seasonal virus along with influenza A (H3N2) and influenza B. Seasonal influenza virus A (H1N1) has not changed significantly from the 2009 pandemic virus. Since its circulation in 2009, influenza A (H1N1) has been known for causing severe illness in young, healthy adults, in contrast with influenza virus A (H3N2), which causes severe disease and deaths especially in the elderly (Declarația Biroului Regional OMS, 2015).

During 2015-2016 influenza season, in most European countries, 56% of detected influenza viruses were type A, 86% of them being subtype A (H1N1)pdm09. The number of laboratory-confirmed influenza cases peaked in weeks 5–7/2016. In the northern hemisphere, since week 9/2016, influenza B viruses have predominated in samples from sentinel sources, 96% of those that were ascribed to a lineage belonged to B/Victoria lineage (European Centre for Disease Prevention and Control, 2016).

Similar to other European regions, in the 2015-2016 season, in Romania there was a predominant circulation of influenza viruses type A (83.7%). Virological surveillance revealed 1.526 influenza viruses (38.5% more compared to the previous season – 1.102). Of these, 1.174 were detected during ILI surveillance and were confirmed as influenza virus type A (1064) subtype A (H1N1)pdm09 (954, 89.66%) and subtype A (H3N2) (66, 6.20%), 44 (4.13%) being non-subtypable and also influenza virus type B (110). Other 352 viruses were detected during SARI surveillance and were in majority type A (213) (Institutul Național de Sănătate Publică, 2016). In Iași County all type A viruses were identified as A (H1N1)pdm09, and only one type B virus was detected. Data from sentinel surveillance, including our results, supported the need to include A/California/7/2009 (H1N1)pdm09-like virus in influenza vaccine for 2016-2017 season.

In Romania, the highest percentage positive for influenza virus A (H1N1)pdm09 (70.6%) was registered two weeks later than in other European countries (week 9/2016) while influenza virus type B was predominant in week 14/2016 in samples from sentinel sources (European Centre for Disease Prevention and Control. Latest surveillance data).

In Iași County, the highest number of positives cases was detected in week 7/2016 and type B virus was registered in week 8/2016, earlier than on national level but similar to the rest of Europe. This situation can be explained by migration of people during the winter holiday.

In Romania, there had been 4263 ILI cases, most of which were registered in the age group 15-49 years (40.6%) (Institutul Național de Sănătate Publică, 2016)**,** which is similar to data recorded from Iași (10 /19 ILI cases belonged to this age group). Also, the national surveillance for SARI reported 444 cases, with the highest positivity rate of A (H1N1)pdm09 confirmed cases in the age group 50-64 years (32%) (Institutul Național de Sănătate Publică, 2016). In Iași, SARI positive cases had an equal distribution among age groups: 5-14 years (2 cases), 15-49 years (2 cases) and 50-64 years (2 cases), but this difference can be explained by the small size of our study group.

## CONCLUSIONS

The use of Real Time RT-PCR technique allowed us to rapidly identify the circulating types of influenza viruses in our region. The results showed a correlation between national reports and our study group regarding the predominant circulation of A (H1N1)pdm09 influenza virus. However, in Iași, the peak of positive cases was detected earlier than at national level and no A (H3N2) subtype was detected. Data obtained sustain the importance of molecular assays for diagnosis and epidemiological surveillance of circulating influenza viruses in order to support the selection of most prevalent strains as references for annual influenza vaccine.

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