

Monitoring the sensitivity of influenza viruses to oseltamivir, in Romania, from 2016 to 2023

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ABSTRACT

Introduction: influenza is an acute viral disease caused by infection of the upper respiratory tract- difficult to control, which affects a large part of the population. Neuraminidase inhibitors (NAIs), such as oseltamivir and zanamivir, are the antiviral drugs utilized in Romania for the treatment of respiratory infections caused by the influenza viruses. The objective of the study is to monitor and analyze influenza viruses circulating in Romania, in terms of their susceptibility to oseltamivir, with the aim of signaling the emergence of viral strains resistant to antivirals.

Methods: The phenotypic method was performed to evaluate the inhibitory effect of oseltamivir on the neuraminidase enzyme activity of influenza viruses. Genotypic methods (SNP-based assays or sequencing of neuraminidase/whole genome) were used for detecting amino acid substitutions associated with influenza antiviral resistance. The study period is from 2016 to 2023.

Results: from the total number of influenza viruses isolated during the period (833 isolated), 670 strains were characterized antigenically and genetically, and the phenotypic susceptibility to antivirals was performed for 245 isolated viral strains. According to WHO criteria categories antiviral susceptibility based on IC₅₀ data, none of the test results indicated reduced or highly reduced inhibition by oseltamivir.

Conclusion: This monitoring, carried out in the National Influenza Center Romania- Cantacuzino NMMIRD, is extremely important to maintain effective flu control and further development of other antiviral agents.

Keywords: influenza virus neuraminidase, phenotypic method, antivirals

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INTRODUCTION

Influenza is an acute viral disease caused by infection of the upper respiratory tract- difficult to control, which affects a large part of the population.

The annual vaccination could serve as an important line of defence for combating the flu. During epidemics and pandemics, the administration of a new flu vaccine requires a longer period of time, so antiviral drugs play an important role in managing the disease (therapy and prophylaxis).

The need for surveillance on the NAI susceptibility of influenza viruses circulating globally has grown in recent years.

In Romania, the National Influenza Center (NIC) of the Cantacuzino National Medico-Military Institute for Research and Development (NMMIRD) acts as the network's focal point, providing technical advice and support to network members in managing specimen collec-

tion, isolation, antigenic and genetic characterization of influenza virus, and antiviral susceptibility testing.

The purpose of this work is to provide a phenotypic approach for evaluating the inhibitory effect of oseltamivir on neuraminidase enzyme activity of influenza viruses using 2'-(4-Methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA) fluorescent substrate, method which was implemented and validated in NIC Romania (NA-Fluor Influenza Neuraminidase Assay Kit, Applied Biosystems). This method is used to monitor and analyse influenza viruses circulating in Romania in terms of their susceptibility to antiviral medications, with the aim of signaling the emergence of antiviral-resistant strains.

MATERIALS AND METHODS

Methods

Clinical specimens were collected from 2016 to 2023 during the epidemic seasons from patients in sentinel

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units within the national surveillance program according to the National Institute of Public Health (NIPH) methodology [1], as well as from patients in non-sentinel units within the international study – IMOVE (Monitoring vaccine effectiveness during seasonal influenza in the European Union).

Genomic and epidemiological data presented in this study were obtained as part of national surveillance efforts for which ethical approval is not required under Romanian law. However, for the national surveillance system of viral respiratory infections, patients are verbally informed and they give their verbal informed consent to participate in this surveillance.

The international study - IMOVE carried out within the Cantacuzino Institute received local ethical approval from the professional ethics and deontology commission of the Cantacuzino Institute every year.

Nasopharyngeal swabs were collected from people who have presented respiratory diseases that meet the clinical criteria of diseases compatible with influenza (fever $\geq 38^{\circ}\text{C}$, cough, onset in the previous 10 days, absence of other clinical and/or radiological diagnoses), discharged in the transport medium for viruses and sent to NIC Romania, in order to carry out the laboratory diagnosis for influenza viruses.

Positive clinical samples for the molecular detection of RNA specific to influenza viruses were inoculated on a cellular substrate, MDCK or MDCK SIAT1 cell substrate (ECACC 05071502), to obtain isolates of influenza viruses for subsequent antigenic, genetic characterization and antiviral sensitivity testing using neuraminidase inhibition reactions in the presence of oseltamivir [2]. At the beginning of the study, the isolation of the influenza virus was carried out on the MDCK cell line. Later, with the increased variability of the viruses, it was observed that the MDCK-SIAT1 cell line is suitable for the multiplication of A/H3N2 influenza viruses [3]. For the type A/H1N1pdm09 and type B influenza viruses, a good multiplication yield is obtained on the MDCK cell line.

The phenotypic method is performed in two phases. The first step is to determine the influenza virus neuraminidase (NA) activity, which serves to establish an optimal dilution of the influenza virus required for the second step, represented by the neuraminidase inhibition assay in the presence of the antiviral oseltamivir [4].

NA activity was measured using the synthetic substrate 4-methylumbelliferyl- α -D-N-acetylneuraminic acid (4-MUNANA), which is hydrolyzed to produce the fluorescent product 4-methylumbelliferone (4-MU), which can be quantified using a fluorometric reader [5]. The fluorescence intensity is proportional to the NA activity. The antiviral concentrations required for 50% inhibition

of neuraminidase enzyme activity (IC50) are estimated for each virus strain. The IC50 for oseltamivir is given in nanomolar units (nM) [6].

To ensure consistency in the reporting of NAIs susceptibility data, international influenza monitoring organizations (WHO AVWG- antiviral susceptibility expert working group) have defined categories corresponding to reductions in susceptibility to licensed neuraminidase inhibitors, based on their fold differences in inhibition assay (IC50) compared to the median of wild-type viruses from the same type/subtype/ lineage in *normal inhibition -NI*, equivalent to the sensitive strain, *reduced inhibition -RI*, equivalent to the resistant strain and *highly reduced inhibition -HRI*, corresponding to a very resistant strain [7, 8].

Real-time RT-PCR reactions were performed to identify specific oseltamivir resistance mutations H275Y for A/H1N1pdm09 viruses and E119V for A/H3N2 viruses that could not be isolated in cell culture for phenotypic oseltamivir sensitivity analyze [9, 10].

Materials

Influenza strains type A and B were isolated from biological samples using MDCK (Madin-Darby canine kidney) or MDCK-SIAT1 (ECACC 05071502) cell culture (kindly provided by WHO Collaborating Centre for Reference and Research on Influenza Crick Worldwide Influenza Centre);

A commercial kit for testing neuraminidase activity based on fluorescent substrate, produced by Applied Biosystems, was used; Oseltamivir carboxylate was kindly provided by Hoffmann-La Roche Ltd, Switzerland;

The ISIRV-AVG (International Society for Influenza and Other Respiratory Virus Diseases – Antiviral Group) kindly provided reference strains of Influenza virus wild type and variant virus with a mutation in NA: A/H3N2, A/H1N1pdm09, and influenza B for testing resistance to neuraminidase inhibitors;

Influenza virus reference strains and specific antiserum were kindly provided by WHO Collaborating Centre for Reference and Research on Influenza Crick Worldwide Influenza Centre.

Statistical analysis

The optimal dilution for standardization of the antigen (isolated influenza virus) required in the neuraminidase inhibition tests in the presence of NAIs was calculated using Microsoft Excel vw-0574.04_munana_template_external, and the antiviral concentrations required for 50% inhibition of neuraminidase enzyme activity (IC50) was calculated via Microsoft excel vw-0575.04_nai_results_template_external (<https://isirv.org/site/index.php/methodology/nai-assay-ic50>).

Each phenotypic analysis is validated in the presence of reference strains of Influenza virus wild type and variant virus with a mutation in NA, corresponding to the type/subtype of influenza virus tested.

The IC50 value obtained with the recommended programs is reported to the IC50 value of the wild-type reference strain, thus ensuring coherence in the reporting of NAIs susceptibility data.

Viruses isolated with an IC50 value more than the upper limit established as the IC50 mean plus three times the standard deviation are considered outliers and will be genotyped [11].

Box-plot statistical analyses were utilized to assess influenza viral sensitivity monitoring data from six influenza epidemic seasons.

RESULTS

More than 830 influenza A and B strains were isolated on MDCK or MDCK SIAT1 cell culture between 2016 and 2023, with 670 strains being characterized either antigenically or genetically- 307 A/H1N1pdm09, 248 A/H3N2, and 115 of influenza B viruses (71 B/Victoria lineage, 44 B / Yamagata lineage), each season being characterized by the circulation of one or more types of influenza viruses.

From the total number of influenza virus strains isolated, those strains that are geographically representative and come from samples from the beginning, middle, and end of the epidemic season are characterized.

In the period 2016-2023, we performed neuraminidase susceptibility testing on 245 of the influenza strains, 82 of the A/H1N1pdm09 viruses, 101 of the H3N2 viruses, and 62 of the influenza B viruses (Figure 1).

In order to be phenotypically tested, the isolated strains must have a significant multiplication of the influenza vi-

rus that allows the first stage of antigen standardization to be carried out. For example, some samples had an insignificant multiplication, with a hemagglutinin titer of less than 1/16, an inconsistent value for phenotypic testing.

To validate the NA-Fluor assay, all reactions were performed in the presence of reference sensitive wild type (WT) and resistant strains for oseltamivir carboxylate.

In each epidemic season, monitoring the antiviral susceptibility of influenza viruses is performed by considering the mean IC50 values for each type/subtype of influenza virus, identifying resistant viruses and monitoring trends in IC50 data over time.

General analysis of oseltamivir susceptibility data of influenza A/H1N1pdm09 viruses

In the analyzed period, to oseltamivir, all influenza strains A/H1N1pdm09 examined, showed a normal inhibition of neuraminidase activity.

In 2016/2017, one strain of A/H1N1pdm09 subtype was examined, with an IC50 of 0.29nM and a normal inhibition of neuraminidase activity to oseltamivir.

In 2017/2018, in the neuraminidase inhibition assay, 22 viral isolates of the A/H1N1pdm09 subtype were examined, with mean values of IC50 0.41nM. One of the viruses identified during this period, A/Covasna/225506/2018 (EPI1328894-NA Segment Id), had an IC50 of 2.8 nM, which was significantly higher than the upper limit of 2.05 and was considered outlier (Figure 2).

In 2018/2019, 30 viral isolates of the A/H1N1pdm09 subtype were tested, at mean values of IC50 0.37nM. One sample slightly exceeded the upper limit- A/Romania/240712/2019 (EPI1656943- NA Segment Id) IC50 0.68nM.

In 2019/2020, 6 viral isolates of the A/H1N1pdm09 subtype were examined, with mean IC50 values of 0.36nM.

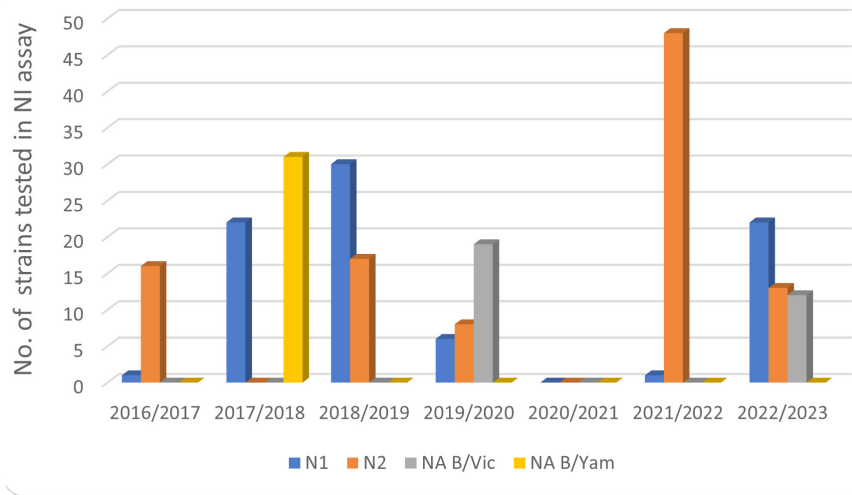


Fig. 1. Annual distributions of influenza strains tested in neuraminidase inhibition assay (2016-2023)

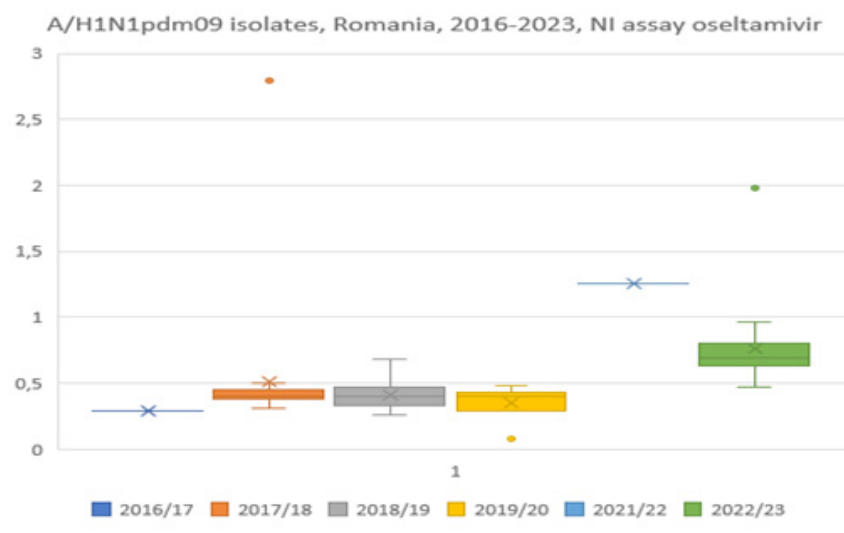


Fig. 2. General analysis of oseltamivir susceptibility data of influenza A/H1N1pdm09 viruses

In 2021/2022, one strain of A/H1N1pdm09 subtype A/Romania/514072/2022 (EPI2115603 NA Segment Id) was examined, with an IC₅₀ value of 1.26nM and a normal inhibition of neuraminidase activity to oseltamivir.

In 2022/2023, 22 viral isolates of the A/H1N1pdm09 subtype were evaluated, with mean values of IC₅₀ 0.76nM. One of the viruses recovered, A/Romania/545221/2023 (EPI2436757 NA Segment Id), had an IC₅₀ of 1.98 nM, which was considered an outlier (Figure2).

General analysis of oseltamivir susceptibility data of influenza A/H3N2 viruses

In the analyzed period, to oseltamivir, all influenza strains A/H3N2 examined, showed a normal inhibition of neuraminidase activity.

In 2016/2017, 16 A/H3N2 virus isolates were examined, with mean values of IC₅₀ 0.16 nM.

In 2018/2019, 17 isolates of the A/H3N2 virus type were examined, with mean values of IC₅₀ 0.21 nM. A sample exceeded this season- A/Romania/239376/2018, IC₅₀ 1.06nM and was deemed an outlier (EPI1343349 HA Segment Id) (Figure 3).

In 2019/2020, 8 A/H3N2 virus isolates were tested, with mean IC₅₀ values of 0.36 nM.

In 2021/2022, 48 A/H3N2 virus isolates were evaluated, with mean values of IC₅₀ 0.35 nM. One sample exceeded the upper limit criteria- A/Romania/514009/2022 IC₅₀ 1.03nM- and was deemed an outlier (EPI2029119 NA Segment Id).

In 2022/2023, 12 A/H3N2 virus isolates were assessed with mean values of IC₅₀ 0.46 nM.

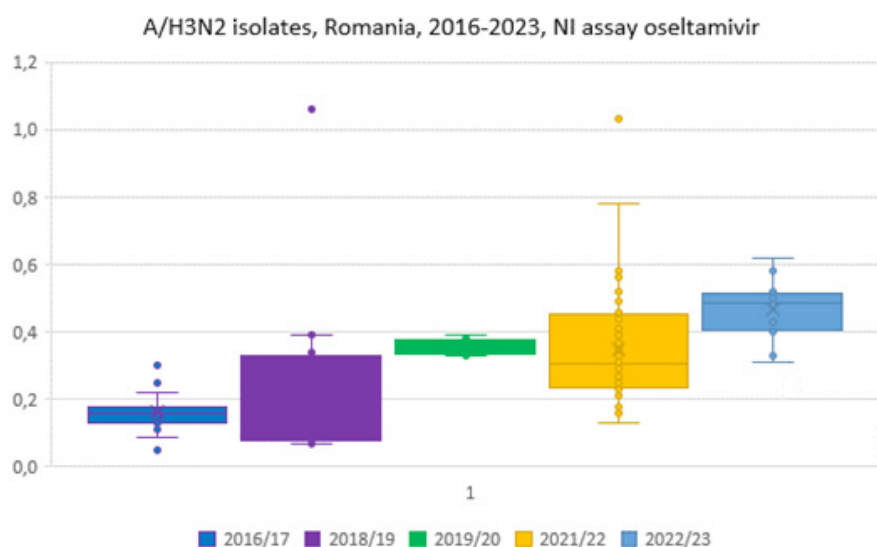


Fig. 3. General analysis of oseltamivir susceptibility data of influenza A/H3N2 viruses

General analysis of oseltamivir susceptibility data of influenza B viruses

In the analyzed period, to oseltamivir, all influenza strains type B examined, showed a normal inhibition of neuraminidase activity.

In 2017/2018, 31 isolates of virus type B were examined, with mean IC₅₀ values of 18.01nM.

In 2019/2020, 19 isolates of virus type B were examined, with mean IC₅₀ values of 25.65nM.

In 2022/2023 neuraminidase inhibition assay was tested, 12 isolates of virus type B, with mean values of IC₅₀ 41.17nM.

Genotypic methods (SNP-based assays or sequencing of neuraminidase/whole genome) were used for detecting amino acid substitutions associated with influenza antiviral resistance. In 2018/2019, 46 samples were tested SNP for H275Y mutation, no amino acid substitution previously associated with (highly) reduced inhibition (AANI=amino acid previously associated with normal inhibition). In 2022/2023, 197 samples were tested (SNP) for detection of H275Y mutation (AANI), one sample (A/Romania/545719/2023) showed amino acid substitution previously associated with reduced inhibition (AARI=amino acid substitution previously associated with reduced inhibition); 8 samples tested for E119V mutation, all AANI.

From a number of 198 samples collected in the 2019/2020 and 2022/2023 seasons, sequenced and phylogenetically analyzed for the NA gene, two samples (A/Romania/256678/2020 and A/Romania/545719/2023) showed the characteristic mutation H275Y, confirmed as resistant to oseltamivir.

DISCUSSION

D1. In the 2016/2017 epidemic season, among the influenza viruses circulating in Romania, the predominant one was the A/H3N2 influenza virus (represented by A/Bolzano/7/2016).

The phenotypically tested samples regarding the sensitivity of influenza viruses to neuraminidase inhibitors in Romania showed a normal inhibition against oseltamivir. In Europe, the percentage of influenza viruses resistant to neuraminidase inhibitors was below 1%, a percentage that also exists at the level of worldwide surveillance. Out of 800 influenza viruses tested by neuraminidase inhibitors at the European level (both type A and type B), only 4 showed resistance: 3 from Poland and 1 in Luxembourg (type A/H3N2 influenza viruses with reduced inhibition by oseltamivir) [12].

D2. In the 2017/2018 season, the A/H1N1pdm09 type viruses (represented by A/Michigan/45/2015) and type B, B/Yamagata lineage (represented by B/Phuket/3073/2013) circulated equally in Romania. In Europe, both type A and type B influenza viruses circulated in equal proportion, and among type A viruses, both subtypes A/H1N1pdm09 and A/H3N2 co-circulated [13].

All the influenza virus isolates of A/H1N1pdm09 subtype, and type B tested showed a normal inhibition of neuraminidase activity to oseltamivir. One of the influenza strains, A/Covasna/225506/2018, was considered an outlier with IC₅₀=2.8nM, and through sequencing the substitution at the T13I, V62I, T72I, S247N, R257K, K265R, D416N were identified. Substitution S247N has been reported in the literature to be related to drug resistance.

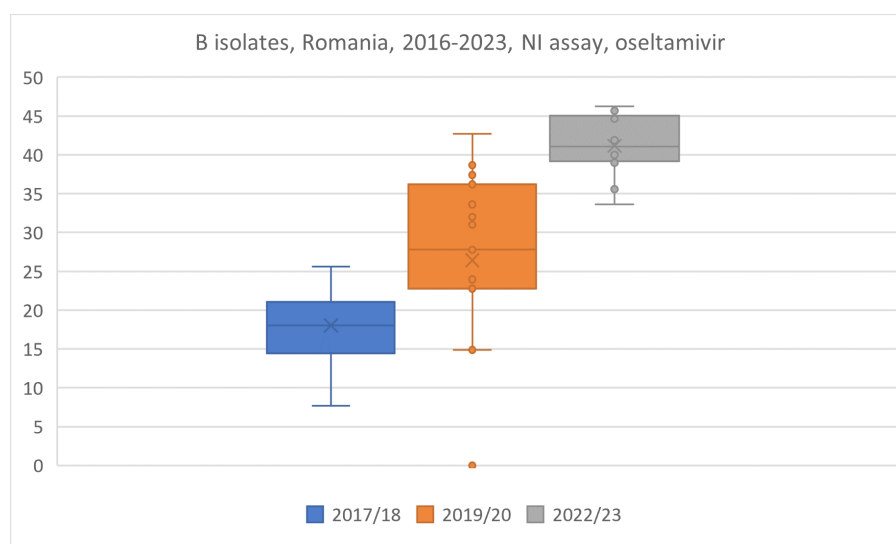


Fig. 4. General analysis of oseltamivir susceptibility data of influenza B viruses

In Europe, there have been very few cases of influenza viruses resistant to one or more of the neuraminidase inhibitors. From 3703 viruses reported by countries to TESSy (The European Surveillance System) on the antiviral susceptibility, 19 A/H1N1pdm09 influenza viruses carried neuraminidase amino acid substitution H275Y and showed HRI by oseltamivir, 2 A/H3N2 viruses carried neuraminidase amino acid substitution R292K and showed RI by oseltamivir and zanamivir, 2 viruses type B showed RI by oseltamivir and zanamivir (with D197N amino acid substitution), while another two B viruses showed RI only to oseltamivir [13].

D3. The 2018/2019 and 2019/2020 seasons were characterized by the majority circulation of the influenza virus type A/H1N1pdm09 type viruses (represented by A/Michigan/45/2015 for 2018/2019 and circulation of all type of influenza viruses - A/H1N1pdm09, A/H3N2, B for 2019/2020 season).

All the influenza viruses isolates showed a normal inhibition of neuraminidase activity to oseltamivir. In 2018/2019 a sample exceeds 1.7 times the upper threshold for that season A/Romania/239376/2018 a/H3N2 type.

In these epidemic seasons, in Europe, the incidence of the circulation of the influenza viruses resistant to NAIs antiviral remained at very low values (below 1%). Among the viruses that showed a reduced inhibition or highly reduced inhibition, viruses type A(H1N1) pdm09 carried NA H275Y amino acid substitution, H295S substitution, Q136K or Q136K/Q and D151D/E amino acid polymorphisms. For A/H3N2 viruses one strain A/Latvia/03-0738053/2019(H3N2) showed RI by zanamivir, sequencing revealed NA D151D/N polymorphism and V165I amino acid substitution and for other A(H3N2) virus (A/Limoges/2326/2019) showed RI by zanamivir associated with NA T148I substitution. Type B viruses with RI to NAIs generally have the D197N amino acid substitution encoded in NA gene. [14].

D4. Following the analysis of the data for monitoring the circulation of influenza viruses in Romania, it is observed that during the period 2016-2023, the values of the concentration of oseltamivir, which inhibits 50% of the neuraminidase activity of type B viruses, increased from mean IC₅₀ of 18.01 nM in season 2017/2018, at mean IC₅₀ 25.65 nM in 2019/2020, respectively 41.17 nM in 2022/2023. These values compared to the value of reference strains of Influenza B virus wild type, lead to classifying the isolated viruses in the category of those with normal inhibition against oseltamivir. (fig. 4).

D5. The methodological limits of our study are determined by the multiplication of the virus that must reach a threshold density of virions, to overcome the relatively

low sensitivity of the hemagglutination method. For example, some samples had an insignificant multiplication, with a hemagglutinin titer of less than 1/16, an inconsistent value for phenotypic testing. Type A influenza viruses that could not be isolated in cell culture for oseltamivir sensitivity analysis (phenotypic) were tested by SNP reactions to identify specific oseltamivir resistance mutations H275Y for viruses type A/H1N1pdm09, respectively E119V for A/H3N2 viruses [9, 10].

D6. Following the continuous monitoring of human influenza viruses, including their reduced sensitivity to antiviral drugs due to mutations in the genome structure, local clusters/incidents of oseltamivir-resistant A/H1N1pdm09 viruses have been reported (Hokkaido, Japan [15], Pennsylvania USA [16], Australia [17], Norway [18]). In specialized studies, the transmission of these viruses in the population was followed and it was demonstrated that influenza viruses type A/H1N1pdm09 which have the H275Y mutation can be transmitted efficiently [19,20].

D7. In the 2020/2021 winter season, characterized by the circulation of the SARS-CoV-2 virus, our laboratory was involved in large-scale testing to identify this virus, but between November 2020 and May 2021, a number of 2182 biological samples were tested for the presence of influenza viruses, all tested samples being negative. Such significant declines in influenza detection in the 2020-2021 season have been reported by surveillance networks in many countries around the world. For example, a study carried out based on epidemiological surveillance data from Canada, showed for influenza A and B, the percentage of tests with positive results during the 2020/2021 season decreased significantly, recorded at a rate of 0.0015 (95% CI: 0.0001–0.0002) and 0.0028 (95% CI: 0.0001–0.0007) times that of pre-pandemic levels, respectively [21].

CONCLUSIONS

The fluorescent assay is a direct, functional enzyme assay that detects both known and new mutations that affect neuraminidase inhibitor drug sensitivity, and in the absence of hemagglutination in A/H3N2 influenza viruses, multiplication of viruses on cell culture could be highlighted by testing neuraminidase activity on the fluorescent substrate helping to increase NIC capability in the surveillance of influenza viruses.

The monitoring of antiviral susceptibility of circulating influenza viruses in Romania serves to detect resistant viral strains. This monitoring carried out in NIC Romania - Cantacuzino NMMIRD, is extremely important to maintain effective flu control and further development of other antiviral agents.

According to WHO criteria categories antiviral susceptibility based on IC50 data, none of the test results indicated reduced or highly reduced inhibition by NAIs oseltamivir, but through genotypic tests, two samples (A/Romania/256678/2020 and A/Romania/545719/2023) showed the characteristic mutation H275Y, confirmed as resistant to oseltamivir.

During the study, the world, implicitly also Romania, faced the COVID-19 pandemic. In the period 2020 - 2021, our laboratory was involved in large-scale testing to identify the SARS-CoV-2 virus (>200.000 samples), but, between November 2020 and May 2021, a number of 2182 biological samples were tested for the presence of influenza viruses, all tested samples being negative.

Such significant declines in influenza detection in the 2020-2021 season have been reported by surveillance networks in many countries around the world.

The circulation of influenza viruses, in a pandemic year, was extremely low to the point of their absence, probably related to the implementation of measures to limit the spread of respiratory infections through measures of hygiene, mandatory wearing of face masks, travel restrictions, important lockdown, closing schools and institutions, workplaces, etc.

ABBREVIATIONS

AANI- amino acid previously associated with normal inhibition

AARI- amino acid substitution previously associated with reduced inhibition

AVWG – Antiviral susceptibility expert working group

CI – Confidence interval

IMOVE – “Integrated Monitoring of Vaccines Effects in Europe” (international study)

ISIRV-AVG- International Society for Influenza and other Respiratory Virus Diseases – Antiviral Group

MDCK- Madin-Darby canine kidney cells

MUNANA – Methylumbelliferyl N-acetylneuraminic acid NA – Neuraminidase

NAIs – Neuraminidase inhibitors

NIC – National Influenza Center

NIPH – National Institute of Public Health (Romania)

NMMIRD – National Medico-Military Institute for Research and Development

RNA – Ribonucleic acid

RT-PCR – Reverse Transcription Polymerase Chain Reaction

TESSy- The European Surveillance System

WHO – World Health Organization

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AUTHORS' CONTRIBUTION

MEM – conceptualization, writing- original draft preparation; formal analysis, data visualization, data curation, writing- review and editing

AEI – data analysis, data visualization

CMC – data analysis

GM – writing- review and editing

ML – writing review and editing, data curation, formal analysis, data management and funding acquisition

CONFLICT OF INTEREST

None to declare.

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