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DISTRIBUTION OF RESPIRATORY INFECTION VIRUSES IN 2019-2020 SEASON AND DETERMINATION OF OSELTAMIVIR RESISTANCE OF INFLUENZA VIRUSES

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Abstract

Objective: This study aimed to determine the seasonal distribution of the factor data of viral agents determined by the multiplex PCR method in routine practice in patients admitted to the hospital with upper respiratory tract infection symptoms and to detect oseltamivir resistance in viruses that are positive for Influenza A (H1N1).

Methods: Nasopharyngeal swab and bronchoalveolar lavage samples of 354 patients between the ages of 0-94 who were admitted to the Sakarya University Sakarya Training and Research Hospital with symptoms of upper respiratory tract infection between 12 September 2019 and 19 February 2020 were studied with the multiplex PCR method. Oseltamivir resistance was investigated in 11 samples selected from Influenza A (H1N1) positive samples by Sanger sequence analysis method.

Results: One or more respiratory viruses were identified in 233 (66%) of 354 respiratory samples evaluated. Of these, 64 (27.5%) are Influenza A/H1N1, 4 (1.7%) are Influenza A/H3N2, 24 (10.3%) are Influenza B, 64 (27.5) are Respiratory. Syncytial Virus A/B (RSV A/B), 58 (24.9%) Rhinovirus/Enterovirus, 18 (7.7%) Adenovirus, 12 (5.2%) Human Metapneumo Virus (hMPV)), 10 (4.3%) Bocavirus, 4 (1.7%) Parainfluenza 1, 7 (3%) Parainfluenza 3, 3 (1.3%) Parainfluenza 4, 5 (2.1%) were found to be Coronavirus HKU1 and 13 (5.6%) were found to be Coronavirus NL63. More than one factor was detected simultaneously in 45 of 233 positive samples (19.3%). Oseltamivir resistance was not found in any of the 11 influenza A/H1N1 positive samples.

Conclusion: No oseltamivir resistance was observed in any of the Influenza A/H1N1 positive samples evaluated in this study. Periodic analysis of influenza A/H1N1 strains for oseltamivir resistance is necessary to guide empirical treatment.

Keywords: *Influenza, oseltamivir, drug resistance, sequence analysis.*

Introduction

The causative microorganism of flu is the influenza virus. Some kinds of influenza virus, which show different clinical pictures, are easily transmitted from person to person and caused seasonal epidemics and pandemics, especially in winter months. While people infected with influenza may receive hospitalization and treatment every year, this may cause mortality in some high-risk groups.[1]

In the United States of America (USA), average 23,483 influenza-related deaths occurred annually in the adult age group between 1976 and 2007.[2] Antigen detection must be performed to distinguish influenza from other respiratory pathogens and to make a definitive diagnosis. Reverse transcriptase-Polymerase Chain Reaction (RT-PCR) method of viral RNA detection in clinical samples is most commonly used in the diagnosis of influenza.[3, 4]

It is recommended to start antiviral treatment within 48 hours in patients who are hospitalized or at high risk of complications due to respiratory viruses, especially pandemic influenza A/H1N1. In most of the studies, it has been observed that antiviral treatment started in the first 48 hours at the onset of the disease increases the survival rate, and it has been determined that the frequently preferred antiviral is oseltamivir, a neuraminidase inhibitör. [5] However, between 2007 and 2009, oseltamivir resistance caused by the H274Y 26 mutation was observed in all seasonal influenza A (H1N1) viruses. In 2009, Influenza A/H1N1 viruses were replaced by the pandemic Influenza A 2009 H1N1 virus, which is generally sensitive to oseltamivir and has oseltamivir resistance at a rate of approximately 1% worldwide. However, the H274Y mutation, which increased remarkably (approximately 24%) in 2011, is expressed as the conversion of the histidine amino acid to tyrosine (H274Y), located at position 274 of the NA (N1) protein, and is the genetic marker of oseltamivir resistance.[6] The increase in this resistance mobilized all the countries of the world against the resistance problem and antiviral resistance studies were initiated.

In this research, it was aimed to examine the distribution of viral factors in patients who applied to our hospital with upper respiratory tract infection symptoms in the 2019-2020 season and to investigate drug resistance to oseltamivir, the most commonly used antiviral, in patient samples detected to be Influenza A positive.

Methods

Approval was received for this study from Sakarya University Non-Interventional Research Ethics Committee (permission dated 23.01.2020 and numbered 62). Within the scope of the study, viral agents were investigated in the samples taken from nasopharyngeal swab and bronchoalveolar lavage of 354 patients between the ages of 0-94 who applied to Sakarya University Training and Research Hospital with upper respiratory tract infection symptoms between 12.09.2019-19.02.2020.

Multiplex PCR

The distribution of viral agents in the samples was examined using the Respiratory Panel test (QIAstat-Dx®-Qiagen, Germany). Pathogens that can be detected by the multiplex PCR method: Influenza A, Influenza A subtype H1N1 /2009, Influenza subtype H1, Influenza subtype H3, Influenza B, Coronavirus 229E, Coronavirus HKU1, Coronavirus NL63, Coronavirus OC43, Parainfluenza virus 1, Parainfluenza virus 2 , Parainfluenza virus 3, Parainfluenza virus 4, Respiratory Syncytial virus A/B, Human Metapneumovirus A /B (hMPV), Adenovirus, Bocavirus, Rhinovirus/Enterovirus, *Mycoplasma pneumoniae, Legionella pneumophila, Bordetella pertussis*.

Virus Cell Culture

Eleven randomly selected samples from 64 patient samples detected as positive for Influenza A (H1N1) were sent to the National Reference Microbiology Laboratory. Madin-Darby Canine Kidney MDCK-SIAT (Modified sialic acid receptors) cells were used for influenza virus isolation from clinical samples. The classical cell culture method was applied[7]. Samples taken into microtubes from the flasks where growth (CPE) was observed were taken into the extraction process. Viral genomic RNA was isolated by taking 400 µl of the grown culture medium using the Qiagen EZ1 Virus Mini Kit v2.0 and EZ1 Advanced extraction robot (Qiagen, Germany) according to the manufacturer's directions. Conventional PCR was used to sequence the extraction product.

Investigation of Drug Resistance

QiagenOne Step RT PCR kit was used for amplification of the 1410 bp long neuraminidase gene with primers N1F1, N1R1099, N1F401 and NARUc. Amplicons were run in 1.5% agarose gel at 120 V and a 1410 bp band was observed. These bands were determined to be the H1N1 virus. The materials used are as follows:

- 5x Reaction buffer (10 µl)
- dNTP (2 µl)
- 10 µM Forward Primer (1,5 µl)
- 10 µM Reverse Primer (1,5 µl)
- Enzyme mixture (Enzyme Mix) (2 µl)
- RNase inhibitor (optional) (0,5 µl)
- Template RNA (10 µM)
- RNase-free water (22,5 µl).

ABI 3500 Genetic Analyzer (Applied Biosystems, USA) sequencer was used to investigate drug resistance. Oseltamivir resistance was investigated in the neurominidase gene by comparing the obtained H1N1 strains with reference strains. The sequencing process was completed in 3 parts; purification of PCR products, cycle sequence reaction and purification of cycle sequence products.

Purification of PCR products

PCR products were purified with Agencourt AMPure XP (cat. No: A63881) according to the manufacturer's instructions.

Cycle Sequence Reaction

The reaction was prepared with the BigDye Terminator v3.1 Cycle Sequencing Kit (4337454). The materials used are as follows:

- 5x sequencing buffer 2 µl
- Primer F or R (5 µM) 2 µl
- Big dye Terminator 2 µl
- DNA Template 2 µl
- Total volume 10 µl

Purification of Cycle Sequence Products

Axygen AxyPrep MAG DyeClean-Up Kit (Cat.No: MAG-DYECL-250) according to the manufacturer's instructions.

Reference strains used; A /California/7/2009, A/Bayern/69/2009, A/Lviv/N6/2009, A/Astrakhan/1/2011, A/St Petersburg/27/2011, A/St Petersburg/100/2011, A/Hong Kong/5659/2012, A/South Africa /3626/2013, A/Slovenia/2903/2015, A/Israel/Q-504/2015, A/Michigan/45/2015, A/Norway/3433/2018,

A/Switzerland/3330/2017, A/Ireland/84630/2018, A/Switzerland/2656/2017, A/Paris/1447/2017, A/Slovenia/2903/2015, A/Hong_Kong/110/2019.

Statistical Analysis

In our study, the results were evaluated with frequency and percentage calculations using descriptive statistics data.

Results

Of the patients included in the study, 244 (68.9%) were children and 110 (31.1%) were adults. While no respiratory viral pathogen was detected in 121 (34%) of the 354 patient samples examined, at least one viral agent was identified in 233 (66%). Distribution of viruses in samples with positive results; 64 (27.5%) Influenza A/H1N1, 4 (1.7%) Influenza A/H3N2, 24 (10.3%) Influenza B, 64 (27.5%) RSV A/B, 58 (24%) Rhinovirus/Enterovirus, 18 (7.7%) Adenovirus, 12 (5.2%) Human Metapneumo Virus (hMPV), 10 (4.3%) Bocavirus, 4 (1.7%) Parainfluenza 1, 7 (3%) Parainfluenza 3, 3 (1.3%) Parainfluenza 4, 5 (2.1%) Coronavirus HKU1 and 13 (5.6%) Coronavirus NL63 (Figure 1). RSV A/B and Rhinovirus/Enterovirus were more common than respiratory factors other than Influenza. The reason for this is that samples were collected mostly from pediatric patients at that time, and these virus types were especially common between the ages of 0-5.

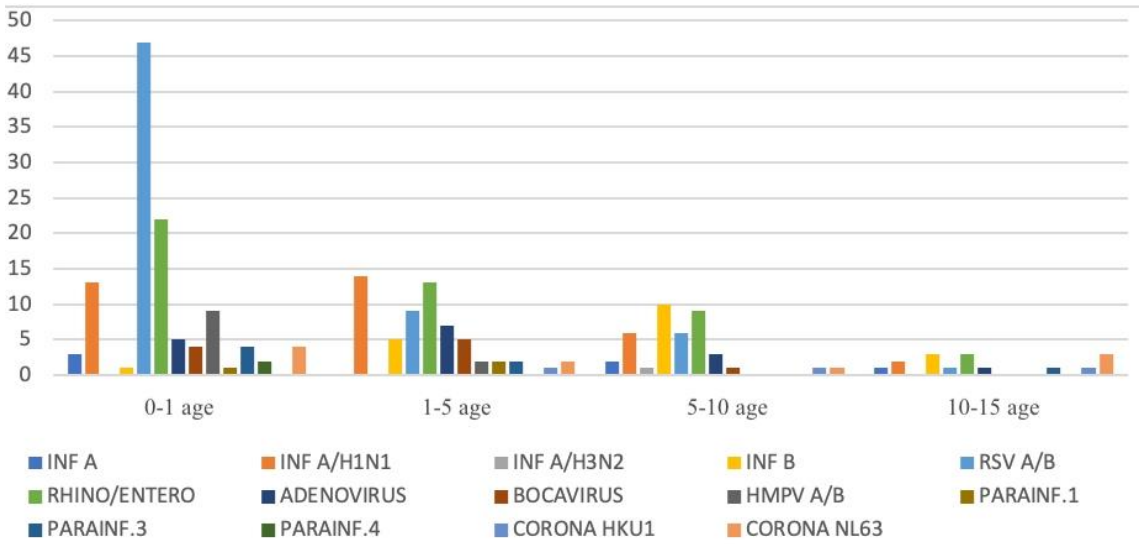

Figure 1. Distribution of respiratory viruses in children by age in the 2019-20 winter season (INF A: Influenza A virus, RHINO/ENTERO: Rhinovirus/Enterovirus, PARAINF: Parainfluenzavirus, CORONA: Coronavirus, INF B: Influenza B virus, HMPV A/B: Human Metapneumovirus A/B, RSV A/B: Respiratory syncytial virüs A/B)

Flu and similar symptoms increase especially in the winter season. This situation is observed with an increase in the months between September-February, when samples are collected, and December-January-February, when the most frequent applications are made. The distribution of viruses according to weeks in the winter season is shown in Figure 2.

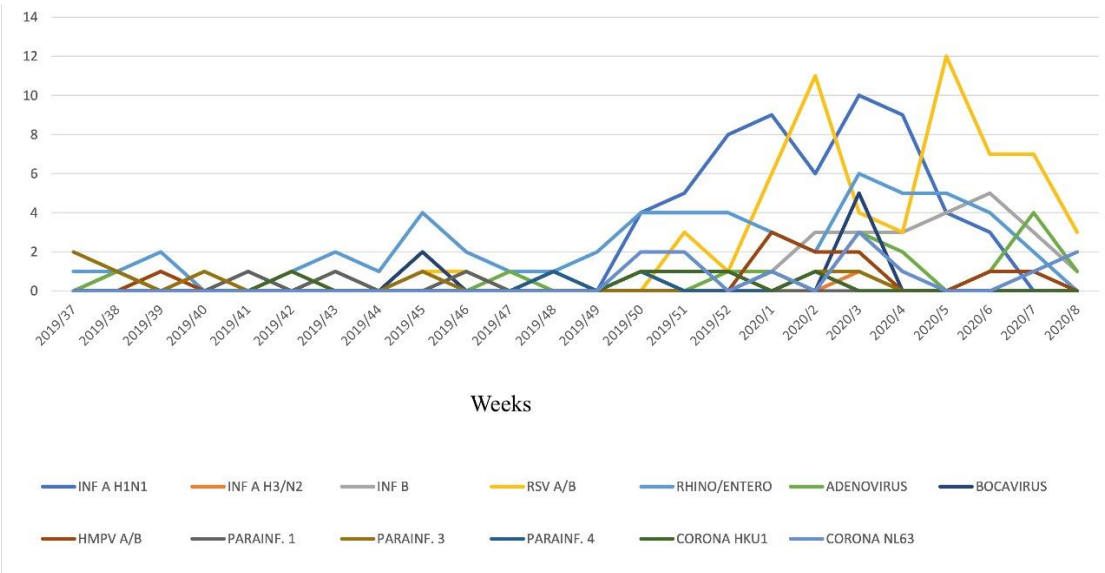

Figure 2. Distribution of respiratory viruses by weeks in the 2019-20 winter season (INF A: Influenza A virus, RHINO/ENTERO: Rhinovirus/Enterovirus, PARAINF: Parainfluenzavirus, CORONA: Coronavirus, INF B: Influenza B virus, HMPV A/B: Human Metapneumovirus A/B, RSV A/B: Respiratory syncytial virüs A/B)

According to the multiplex PCR results from patient samples, the distribution of influenza viruses detected is as follows: 64 (27.5%) Influenza A/H1N1, 4 (1.7%) Influenza A/H3N2, 24 (10.3%) Influenza B. When the distribution of influenza serotypes by months is examined, an increase is seen in December and January. In particular, influenza A is the more common type than others.

Oseltamivir Resistance Results in H1N1 Isolates

Eleven randomly selected samples among Influenza A positive samples isolated from child and adult patients were examined for oseltamivir drug resistance with sequence. Virus isolation was performed by viral cell culture. Amplicons from the grown cultures were run on a 1.5% agarose gel at 120 V and a 1410 bp band was observed and the samples were determined to be positive for Influenza A H1N1 virus (Figure 3).

Discussion

In studies investigating the causes of respiratory tract infections, it has been shown that molecular tests used in the diagnosis of these infections are useful and the detected agents are generally of viral origin.[7-10] These tests can detect even very low amounts of microorganisms and can detect microorganisms that cannot be produced by conventional methods. Additionally, it can detect many agents simultaneously and quickly.[11]

In the study where the nasopharyngeal aspirates of 155 children under the age of 9 who were hospitalized with signs of lower respiratory tract infection were examined, 14 respiratory tract agents were investigated and the agent was detected in 103 (66%) of 155 patients.[12] In a similar study, at least one agent was detected in 67 (42%) of 160 pediatric patients.[13] Sancakli et al.[14] reported that they detected a viral agent at a rate of 67.8% in their study. In the studies of Ozdamar and Turkoglu[11], this rate was given as 87%. In our study, viral agents were identified in 233 of 354 patients (65.8%). In our study, the detection rate of viral agents in cases followed up with a diagnosis of acute respiratory tract infection was found to be similar to previous studies.

In the study of Bicer et al.[12] the most frequently detected agent in respiratory tract infections in children was RSV (32.0%), followed by Adenovirus (26.2%) and Parainfluenza viruses. In the study of Akcali et al.[13] the most common was RSV (61%), followed by Rhinovirus (36%), while in the study conducted by Sancakli et al.[14] the most common was Rhinovirus (26%), followed by RSV (10%). In the studies of Ozdamar and Turkoglu [11], the most common agent was Rhinovirus, second was Adenovirus, and RSV was third.

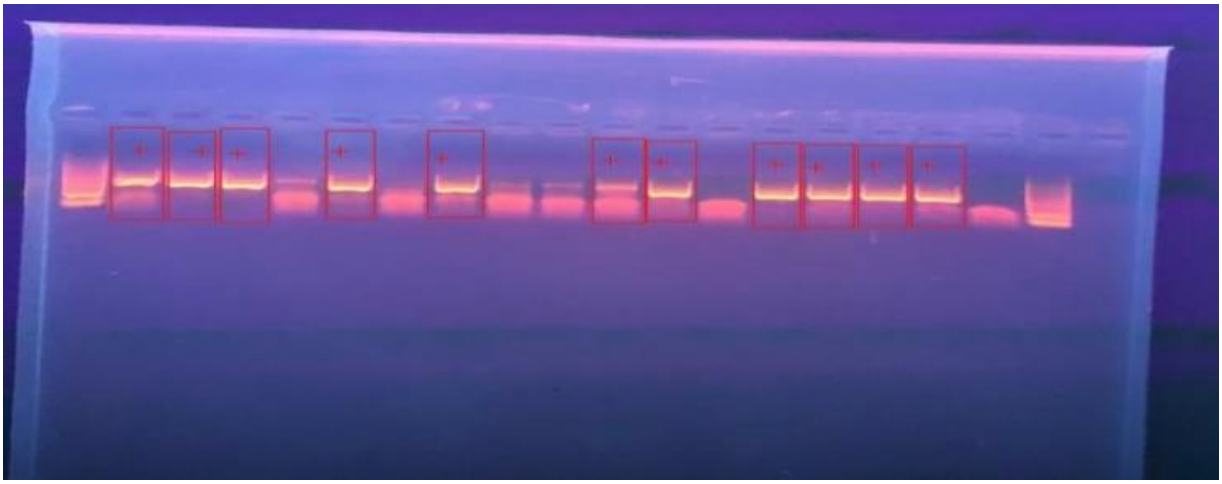

Figure 3. Agarose gel electrophoresis result

In our study, in children (0-15 years old): RSV A/B (23%), Rhinovirus/Enterovirus (17%), Influenza A (16%), Influenza A/H1N1 (13%), Influenza B (7%), Adenovirus (6%), Bocavirus (4%), hMPV A/B (4%), Coronavirus NL63 (4%), Parainfluenza virus 3 (3%), Influenza A/H3N2 (2%), Parainfluenza virus 1 (%1), Parainfluenza virus 4 (1%), Coronavirus HKU1 (1%) were detected.

In the diagnosis of respiratory tract viral infections, molecular methods such as multiplex PCR enable testing of multiple agents simultaneously and can detect more than one agent in a patient. Infection occurring with a new virus in addition to the existing virus is described as double-triple infection or co-infection.[11] Bicer et al.[12] detected coinfection in 20% of the patients in their study, identified three pathogens simultaneously in only three of them, and did not detect a relationship between the severity of the disease and coinfection. In the study of Ozdamar and Turkoglu[11], the coinfection rate was determined as 20% and three agents were detected together in three of them. In our study, multiple viral agents were found in 45 (19.3%) of the children. The most common agents were Rhinovirus/Enterovirus and RSV.

Seasonal influenza viruses are viruses that circulate among humans and cause infections every year. It can cause mild to severe disease and even death, especially in some high-risk individuals.[15] The severity of regularly occurring influenza virus epidemics may vary depending on different gene regions in the virus. The latest example of influenza virus epidemics is Influenza A (H1N1).[16]

Influenza A and B are the two main types that cause seasonal flu. Among these, influenza A viruses have the ability to cause pandemics depending on the differences they create in their surface proteins. According to WHO data, the period in which the incidence of influenza increases worldwide is between December and April, while in Turkey this period covers December and March. In our study, it was found that Influenza A and B positivity was highest in December-January.

In our study, it is seen that influenza has a seasonal distribution feature; Influenza A positivity was clustered in December and January. Bicer et al.[12] reported the frequency of this virus as 12.6% without distinguishing the influenza subtype. Gulen et al.[17] gave the prevalence of influenza as 20-

36% in subtype A and 1-4% in subtype B. In this study, the distribution of Influenza viruses; 27.5% is Influenza A/H1N1, 1.7% is Influenza A/H3N2 and 10.3% is Influenza B.

Antiviral drugs are drugs that act directly on viruses to prevent them from multiplying. M2 inhibitors cannot be used in the prophylaxis and treatment of influenza because they are not effective against influenza B and influenza A viruses have developed resistance to this drug. Today, NA inhibitors, which are known to have low resistance levels, are preferred. Oseltamivir, one of these antivirals, is recommended by WHO because it is effective against influenza and has no serious side effects.

After the introduction of antivirals, resistance monitoring against NA inhibitors began. NA inhibitor resistance was investigated in a study conducted with 2364 influenza A strains isolated worldwide between 2004 and 2008. As a result, H275Y oseltamivir resistance mutation was detected in six A/H1N1 isolates and E119V oseltamivir resistance mutation was detected in one A/H3N2 isolate.[18]

In the resistance study conducted with 388 strains known to be pandemic H1N1 virus, H275Y mutation was detected in four samples.[19] In a study conducted with 4968 pandemic influenza A/H1N1 viruses collected in the United States, oseltamivir resistance was detected in 59 of the samples.[20]

In a study conducted in Turkey during the post-pandemic period oseltamivir resistance was investigated in 233 samples in which pandemic influenza A/H1N1 virus was detected, and no resistance was found.[21]. In the thesis study by Oksuz[6], 131 strains determined to be pandemic influenza A/H1N1 were examined for oseltamivir resistance, and no resistance was reported.

In our study, Influenza A (H1N1) was detected as positive in 64 of 354 patient samples, and resistance to oseltamivir, the most commonly used antiviral, was investigated. No resistance was found in any of the isolates.

Limitations

This study has some limitations. Firstly, sequence analysis was performed on 11 samples selected from these strains due to the fact that they are pandemic agents in the H1N1 subtype of Influenza A and can cause more severe infections and for economic reasons. Secondly, the study covers the period just before the COVID-19 pandemic and cannot be continued during the pandemic.

Conclusion

As a result, periodic analysis of Influenza A/H1N1 strains for oseltamivir resistance is important in directing empirical treatment. In patients presenting with influenza-like symptoms, starting oseltamivir treatment only with clinical prediction, if necessary, may be important for the treatment of influenza infection and prevention of complications.

Conflict of interest

The authors have no conflicts of interest to disclose.

Compliance with Ethical Statement

Approval was received for this study from Sakarya University Non-Interventional Research Ethics Committee (permission dated 23.01.2020 and numbered 62).

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The authors declared that no financial support was received for this paper.

Author's Contributions

M.A., H.T., B.E.: Study idea/Hypothesis; M.A., H.T., F.B., Y.C.: Design; H.T., E.G., T.K.: Data Collection; H.A.T., T.K.: Biological Material Collection; H.T., M.A.: Analysis; M.A., E.P.K.K, F.B., Y.C., M.K.: Literature review; F.B., Y.C., M.K., B.E., E.G., M.A.: Writing; M.A., T.K., H.A.T., H.T.: Critical review.

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