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Molecular Determinants of Drug Resistance and Mutation Patterns in Influenza Viruses Circulating in Poland Across Multiple Epidemic Seasons: Implications for Vaccination Strategies

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Data Collection B
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Background: According to the WHO, up to 650 000 people die each year from seasonal flu-related respiratory illnesses. The most effective method of fighting the virus is seasonal vaccination. However, if an infection does occur, antiviral medications should be used as soon as possible. No studies of drug resistance in influenza viruses circulating in Poland have been systematically conducted. Therefore, the aim of the present study was to investigate the drug resistance and genetic diversity of influenza virus strains circulating in Poland by determining the presence of mutations in the neuraminidase gene.

Material/Methods: A total of 258 clinical specimens were collected during the 2016-2017, 2017-2018, and 2018-2019 epidemic seasons. The samples containing influenza A and B were analyzed by RT-PCR and Sanger sequencing.

Results: Differences were found between the influenza virus strains detected in different epidemic seasons, demonstrating the occurrence of mutations. Influenza A virus was found to be more genetically variable than influenza B virus ($P<0.001$, Kruskal-Wallis test). However, there was no significant difference in the resistance prevalence between the influenza A subtypes A/H1N1/pdm09 (4.8%) and A/H3N2/ (6.1%). In contrast, more mutations of drug-resistance genes were found in the influenza B virus ($P<0.001$, chi-square test). In addition, resistance mutations appeared en masse in vaccine strains circulating in unvaccinated populations.

Conclusions: It seems important to determine whether the influenza virus strains tested for drug resistance as part of global influenza surveillance are equally representative of viruses circulating in populations with high and low vaccination rates, for all countries. Our results suggest that countries with low levels of influenza immunization may constitute reservoirs of drug-resistant influenza viruses.

Keywords: Sequence Analysis, RNA • Influenza A virus • Influenza B virus • Poland

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Background

The evolution of the influenza virus is most pronounced in the case of surface glycoproteins, but it also affects each of the segments. The accumulation of molecular changes is possible by means of different mechanisms: (a) point mutation (antigenic drift); (b) genetic reassortment (antigenic shift); (c) defective-interfering particles; and (d) RNA recombination [1,2]. These changes may take place alongside other antigenic drift or antigenic shift changes [3]. Mixing of segments from different strains (antigenic shift) gives rise to the possibility of 256 different genotypes [4]. Viral copies that are resistant to one or more drugs – such as neuraminidase inhibitors used to treat or prevent influenza – will be selected for replication in populations using these drugs [5]. Anti-influenza medication should be given as early as possible, preferably within 48 hours from the onset of symptoms. In addition to increasing the drug's effectiveness, this also reduces the emergence of resistant viruses [6]. There are 3 levels of detecting viral drug resistance: genotypic – detected by sequencing the viral genome and identifying mutants associated with drug resistance; phenotypic – viral drug resistance is determined by measuring resistance at different drug concentrations in vitro; and clinical – based on measuring the response to a drug administered to humans and animals [7]. In Poland, oseltamivir is commercially available for the treatment and prevention of influenza. Its advantage is that it can be used in the full spectrum of age and risk groups [8], but also has a higher risk of developing resistance [9]. Prolonged exposure to oseltamivir during sustained viral replication, suboptimal doses (eg, once-daily prophylactic dosing in people with active infection), and treatment interruptions are reported as risk factors for the development of resistance [10]. By inhibiting neuraminidase activity, oseltamivir prevents the spread of influenza virus from infected cells to healthy ones. However, if the amino acid sequence of influenza neuraminidase proteins is altered, oseltamivir may lose its ability to bind to neuraminidase and inhibit the function of neuraminidase proteins, causing resistance to neuraminidase inhibitors [11,12].

Resistance to inhibitors of neuraminidase is most often associated with a single substitution in a neuraminidase gene. In the influenza A virus, common mutations include H275T in N1 and E119I/V, R292K, N294S, and E119V + I222V in N2 [13,14]. In the influenza B virus, common mutations include D198N/E and R152K [15]. These substitutions prevent oseltamivir from inhibiting neuraminidase activity and cause the mutant virus to spread to healthy cells in patients taking oseltamivir. Both hemagglutinin and neuraminidase are susceptible to neuraminidase inhibitors. Research using chemiluminescence shows that influenza A is more sensitive to drugs than influenza B. Overall resistance to antiviral drugs in viruses isolated worldwide between 2004 and 2008 was low (0.2%). However, in the 2006-2007 epidemic season in the United States, resistance to oseltamivir A/H1N1/ was reported at 0.7%, while in the following season, it was already 10 times higher, at 7.1% [16]. In Norway, in the 2007-2008 epidemic season, resistance due to the H274Y mutation in A/H1N1/ was 67% [17], while in Japan, in the 2008-2009 epidemic season, resistance due to this same mutation was almost 100% [18]. Global influenza surveillance estimated drug resistance to oseltamivir to be <1.5% in the 2009-2010 influenza season [19]. In 2011, in Singapore and Australia, the number of A/H1N1/pdm09 strains resistant to oseltamivir and zanamivir, with 2 mutations, H274Y and S246N, increased by 10% and 30%, respectively [20]. Drug resistance to oseltamivir raises concerns about the emergence of the H274Y mutant among the highly pathogenic avian influenza virus strains; therefore, it is important to monitor drug resistance among influenza viruses in each country. The aim of the present study was to investigate the drug resistance in influenza virus strains circulating in Poland, and to demonstrate their diversity, by determining the occurrence of mutations in the neuraminidase gene.

Material and Methods

Clinical Samples

The research included samples from 3 influenza epidemic seasons (2016-2017, 2017-2018, and 2018-2019), obtained in Poland by the National Influenza Center at the National Institute of Public Health NIH – NRI. Clinical samples collected as part of the global virological and epidemiological surveillance of influenza in Poland (GISRS) were used for this study. Samples included nasal and throat swabs from patients with clinical signs of influenza-like illness, defined as those with a sudden onset of illness, fever >38°C, and cough or sore throat. Samples from patients aged from 1 to 89 years were examined. A total of 258 clinical specimens were analyzed: 91 samples from patients infected with the A/H1N1/pdm09 virus; 105 samples from patients infected with the A/H3N2/ virus; and 62 samples from patients infected with influenza B virus. The samples were stored at -80°C until the time of testing.

Nucleic Acid Extraction of Viral Genetic Material

The viral RNA was isolated using the Qiagen QIAamp Viral RNA Mini Kit (Hilden, Germany). Each time, according to the manufacturer's instructions, a 50 µL solution containing RNA was obtained from a 140 µL solution of dissolved clinical material [21].

Real-Time RT-PCR for Influenza Type/Subtype Determination

A quantitative real-time RT-PCR (qRT-PCR) analysis was performed on the purified RNA extracted from the collected clinical

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Table 1. Sequences of primers used in the RT-PCR for the detection of selected fragments of genes associated with resistance to anti-influenza drugs.

Primer	Sequence 5'→3'	Postion (nt)/ (amplicon size)	GenBank	Annealing temperature (°C)
Subtype A/H1N1/pdm09				
N11+	CAG GGA GCA AAA GCA GGA GTT YA	34-1038	EF486248	63
N11-	TTR GTY CTY CCK ATC CAA ACA CCA	(1005bp)		
N12+	GCA AGY GCW TGY CAT GAT GG	509-1335	GU050351	63
N12-	TAC TTG TCA ATG GTR AAY GGC A	(827bp)		
Subtype A/H3N2/				
N21+	CAG GGA GCA AAA GCA GGA GTR AA	25-883	EU625366	58
N21-	TTG TCT CTG CAG ACA CAT CTG AC	(859bp)		
N22+	TGT ACA GTA GTA ATG ACT GAT GG	732-1390	CY131287	58
N22-	AGC TTA TAT AGG CAT GAG ATT GA	(659bp)		
Type B /lineages Victoria i Yamagata/				
NB1S	AGC AGA AGC AGA GCA TCT TC	123-1028	JQ340369	56
NB1A	TGC TGT GTA ACT GTT ATC TCT AC	(906bp)		
NB2S	GAA CAC AAG AAA GTG CCT GC	741-1533	LC409195	56
NB2A	AGT AGT AAC AAG AGC ATT TTT CAG	(793bo)		

specimens to determine the type/subtype or lineage of influenza virus. The reactions were carried out in capillaries using a Roche Light Cycler 2.0 thermal cycler. The positive control was RNA isolated from the reference viruses used for the vaccine in the analyzed epidemic season, as recommended by the WHO. RNase-free water was used as the negative control. Primer and probe sequences were those recommended by the WHO [22]. Positive influenza samples with CT≤35 after subtyping were then sequenced [23].

Conventional RT-PCR to Detect Mutations in the Neuraminidase Gene

To detect mutations in the neuraminidase gene, samples were analyzed by means of RT-PCR. Reactions were performed in tubes using a BioRad Thermal Cycler C1000, with a Qiagen OneStep RT-PCR Kit. The reagents were: 10 µL of 5X RT-PCR buffer, 0.6 µM of each of the primers, 400 µM of dNTPs, 2 µL of enzyme mix, 100 ng of template, and RNAse-free water to make a total volume of 50 µL (according to the manufacturer's instructions). The primers were designed specifically for each influenza A and influenza B subtype [24,25]. The reaction conditions were established experimentally, taking into account the melting points of the individual primers (**Table 1**).

Amplification involved an initial reverse transcription step at 50°C for 30 min followed by initial denaturation at 95°C for 15 min, 35 cycles of: denaturation at 94°C for 1 min; annealing for 1 min at 63°C for A/H1N1/pdm09, at 58°C for A/H3N2/, and at 56°C for influenza type B; extension at 72°C for 1 min; and a final extension step at 72°C for 10 min.

Gel Electrophoresis and Isolation of PCR Products

The electrophoretic separation of the PCR products obtained and their isolation from the gel allowed a higher concentration of DNA and its desalting, which has a positive effect on the quality of the sequencing reaction [26]. Agarose gel with a concentration of 1.9% was used with Midori Green Advance nucleic acid dye [27]. Electrophoresis was carried out for 90 minutes at 80 volts. The extraction of PCR products from the gel was carried out on columns according to the manufacturer's instructions (A&A Biotechnology, Gdańsk, Poland). DNA concentration was checked using the NanoDrop One apparatus (ThermoFisher, Waltham, USA).

Sanger Sequencing of Samples with Detected Mutations

Sequencing was carried out using a DTCS Quick Start Kit (Beckman Coulter, Brea, USA), according to the manufacturer's

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Table 2. Summary of results related to the detected mutations in the influenza viruses tested.

Parameter	A/H1N1/ pdm09	A/H3N2/	B	Statistical significance of differences in selected parameters
Number of samples	36	100	57	–
% of samples with mutations	86%	89%	72%	–
Median number of mutations in the sample	19	4	4	For A/H1N1/pdm09 significantly more than for each of the others ($p<0.001$), no differences between A/H3N2/ and B ($p>0.05$)
% of samples in which mutations carrying drug resistance were detected	50%	22%	47%	For A/H3N2/ significantly less than for A/H1N1/pdm09 ($p=0.002$) and for B ($p<0.001$), no differences between A/H1N1/pdm09 and B ($p>0.05$)
Number of mutations carrying drug resistance/1 sample tested	1.1	0.3	1.7	For A/H3N2/ significantly less than for A/H1N1/pdm09 ($p=0.004$) and for B ($p<0.001$), no differences between A/H1N1/pdm09 and B ($p>0.05$)
% of mutations carrying drug resistance	5.4%	6.3%	19.1%	For B significantly more than for the others ($p<0.001$), no differences between A/H1N1/pdm09 and A/H3N2/ ($p>0.05$)

instructions, with a C1000 BioRad Thermal Cycler, in the laboratory of the Virology Department in the National Institute of Public Health NIH-NRI. The analysis was performed using the GenomeLab GeXP by Beckman Coulter [28,29]. Each type of influenza virus was analyzed in a separate 96-well plate to minimize the possibility of sample contamination [30].

Sequence Analysis

The analysis of nucleotide sequences was carried out using the following software: MEGA 6 [31], CLC Main Workbench. The resulting protein sequences were then analyzed using BioEdit version 7.0.5.3 [32] and Clustal W [33] to identify resistance-associated mutations, also using the FluSurver bioinformatics tool. The FluSurver application was also used to create figures representing mutations.

Statistical Analysis

Descriptive statistics methods, including box-plots and statistical tests, were used in the results analysis. The statistical significance of differences between the tested virus strains in terms of the prevalence of detected mutations, including those conferring resistance, was assessed using the chi-square test. The same test was used to compare mutation rates in 2 age groups: ≤14 years of age and ≥15 years of age. The Mann-Whitney test was used to compare the distribution of the number of mutations between 2 groups (based on virus subtypes and age categories,), while the Kruskal-Wallis test was used when larger numbers of groups were compared. (In this case, after showing statistically significant differences between 2 groups, to identify different pairs, Dunn's post-hoc test was used.)

A significance level of 0.05 was used for all the statistical tests. The analyses were performed using SPSS 12.0 PL statistical software.

Results

Real-Time RT-PCR

Influenza A virus was found in 91 samples, which contained the A/H1N1/pdm09 virus genetic material, and 105 samples, which contained the A/H3N2/ virus genetic material. Influenza B virus was found in 62 samples, of which 91.3% were the Yamagata lineage of influenza B virus.

Sanger Sequencing of Samples

The influenza viruses were sequenced. Results were obtained from 193 clinical specimens: 36 samples were from patients infected with the A/H1N1/pdm09 virus; 100 samples were from patients infected with the A/H3N2/ virus; and 57 samples were from patients infected with the influenza B virus.

Description of Detected Mutations

The percentage of influenza A sequences with mutations in the neuraminidase gene was high: 86.1% (31/36) for A/H1N1/pdm09, 89% (89/100) for A/H3N, 71.9% (41/57) for influenza

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Table 3. Percentage distribution of other properties at the site of detected mutations in influenza viruses in Poland.

Properties at the mutation site	Influenza virus subtype A/H1N1/pdm09	Influenza virus subtype A/H3N2/	Influenza virus type B
Viral oligomerization interface	34%	36%	31%
Drug binding site	3%	4%	8%
Ligand binding site	34%	43%	41%
Antibody binding site	11%	7%	13%
Host protein binding site	2%	2%	0.3%
Point mutation/escape mutation	8%	4%	4%
Virulence	0.5%	0.2%	0.3%
N-glycosylation	6%	4%	2%

B (**Table 2**). Regarding mutations conferring drug resistance, they were found in 50% (18/36), 22% (22/100) and 47.4% (27/57) of samples of influenza A/H1N1/pdm09, influenza A/H3N2, and influenza B, respectively. Mutations conferring drug resistance among influenza viruses were detected in 35% of all the clinical specimens.

Statistical Analysis of Mutations

No statistically significant differences in resistance prevalence were detected between subtypes A/H1N1/pdm09 (5.4%) and A/H3N2/ (6.3%) (chi-square test). In the case of the influenza B virus, the frequency of drug-resistant mutations among the overall detected mutations was significantly higher (19.1%) than in the influenza A virus samples (5.8%) ($P<0.001$, chi-square test). However, when comparing the total number of mutations detected in each subtype/type of the influenza virus, greater genetic diversity of the A/H1N1/pdm09 virus was confirmed compared with the A/H3N2/ and B viruses ($P<0.001$, Kruskal-Wallis test; and Dunn's test for pairwise comparison, $P<0.001$). The average number of mutations conferring drug resistance was 1.1 per sample for A/H1N1/pdm09, 0.3 for A/H3N2/, and 1.7 for type B. The differences within the 3 compared groups were statistically significant ($P<0.001$, Kruskal-Wallis test); for A/H3N2/ there were significantly fewer mutations associated with drug resistance than for A/H1N1/pdm09 and for B (Dunn's test, $P=0.004$ and $P<0.001$, respectively). The percentage of samples in which drug-resistance mutations were detected differed significantly for the influenza A virus (29%) vs for the influenza B virus (47%) ($P=0.017$, chi-square test). When comparing the distribution of the number of detected mutations in viruses sampled from children up to 14 years of age and in people aged 15 or older, no differences were found for any type of the influenza virus (Mann-Whitney test).

Types/Properties of Other Mutations Detected

In addition to drug-resistance mutations, other types of mutations were also detected in the influenza A and B viruses, as shown in **Table 3**.

a) Mutation model referred to as the viral oligomerization interface (**Figure 1**).
b) The drug-binding site is shown in **Figure 2**.
c) The ligand-binding site is shown in **Figure 3**.
d) The antibody-binding site is shown in **Figure 4**.
e) The host protein-binding site is shown in **Figure 5**.
f) Point mutations/escape mutations and virulence.
g) N-linked glycosylation. A necessary condition for this reaction is the presence of asparagine within the consensus sequence asparagine-X-serine/threonine, where X is any amino acid except proline [34] (**Figure 6**).

Discussion

Resistance to anti-influenza drugs in Europe is seen at different levels. The highest level of neuraminidase inhibitor resistance in the 2008 A/H1N1/ virus strain was found in Norway, where resistance was seen in 70% of the viruses, while in France the percentage was 17%. By contrast, resistance was detected in less than 10% of the viruses in the UK, the Netherlands, and Germany. In turn, neither Austria nor France reported any drug-resistant strains at that time [35]. Drug resistance prevalence in Poland in the 3 analyzed epidemic seasons, taking into account all the samples analyzed, was 33%. Anti-influenza drugs – neuraminidase inhibitors – do not replace vaccination, but these constitute an effective method for preventing post-influenza complications. Neuraminidase inhibitors also prove useful when a new strain of the influenza virus appears in the population that is significantly different from the viruses that were used to make the vaccine for a given epidemic season.

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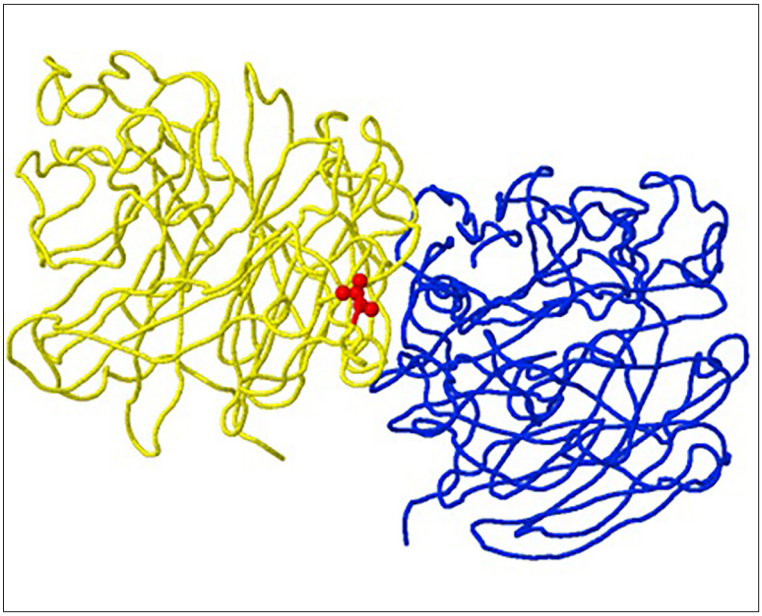

Figure 1. Structure of neuraminidase A/Brisbane/02/2018 (A/H1N1/pdm09) with the R77G mutation detected in clinical sample number 145, as an example of a "viral oligomerization interface" mutation. The location of the mutation corresponds to position 77 of the B subunit chain (yellow backbone) and is 5 Ångström (A) from the A subunit oligomeric chain (blue backbone) in the A/H1N1/pdm09 neuraminidase gene (*created using the FluSurver mutations app – https://gisaid.org/database-features/flusurver-mutations-app/*).

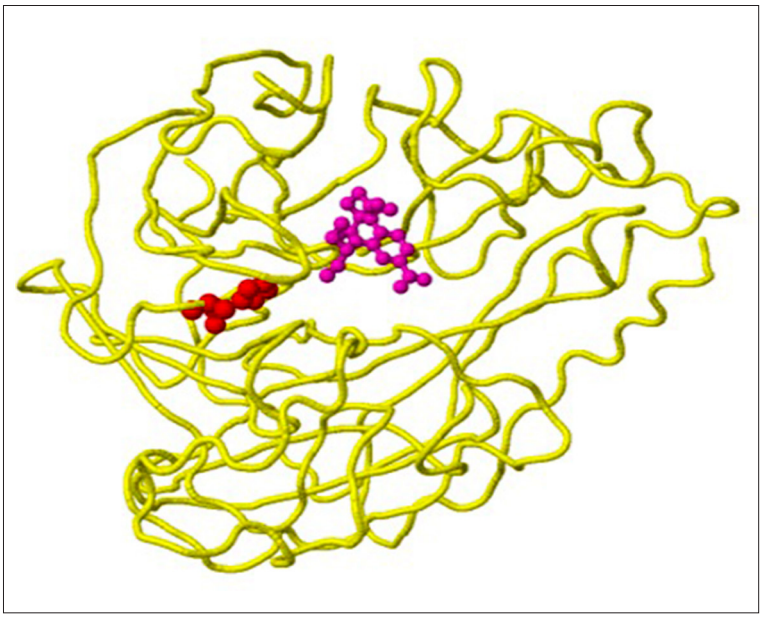

Figure 2. Structure of the neuraminidase of the virus B/Phuket/3073/2013 with the D356H mutation detected in clinical sample number 203, as an example of a mutation with the property of drug binding. The location of the mutation corresponds to position 224 of the A chain (yellow backbone) and is 5 Ångström (A) from the drug-binding site (*created using the FluSurver mutations app – https://gisaid.org/database-features/flusurver-mutations-app/*).

Neuraminidase inhibitors play an essential role as these target the highly conserved neuraminidase active site [36].

Studies of viruses from the 2016-2017, 2017-2018, and 2018-2019 seasons showed that almost all of the detected infections with drug-resistant strains could be prevented by vaccination. Among the tested samples with detected mutations associated with drug resistance, both the subtype A/H1N1/pdm09 and the influenza B viruses were vaccine strains. The presented data demonstrate the importance of influenza vaccination in terms of limiting the spread of infections and the emergence of drug-resistant strains. This also confirms the accuracy of strain selection for vaccine development in the seasons studied.

Prior to the introduction of neuraminidase inhibitors in 1999, drug resistance rates were estimated to be <1% [37]. The first reports of influenza viruses resistant to neuraminidase inhibitors come from the 2007-2008 epidemic season [38]. When WHO influenza expert Frederick Hayden announced that a single oseltamivir-resistant mutation (H275Y substitution in neuraminidase) had been discovered in Europe, it caused a wave of concern. One of the best tools to fight the flu might no longer be effective. In Japan in the 2007-2008 epidemic season, resistant viruses belonging to clade 2B A/Brisbane/59/2007 were detected; these viruses had originated from the European cluster [39]. Therefore, it can be concluded that Europe was the cradle of this mutant variant. It has also been concluded that

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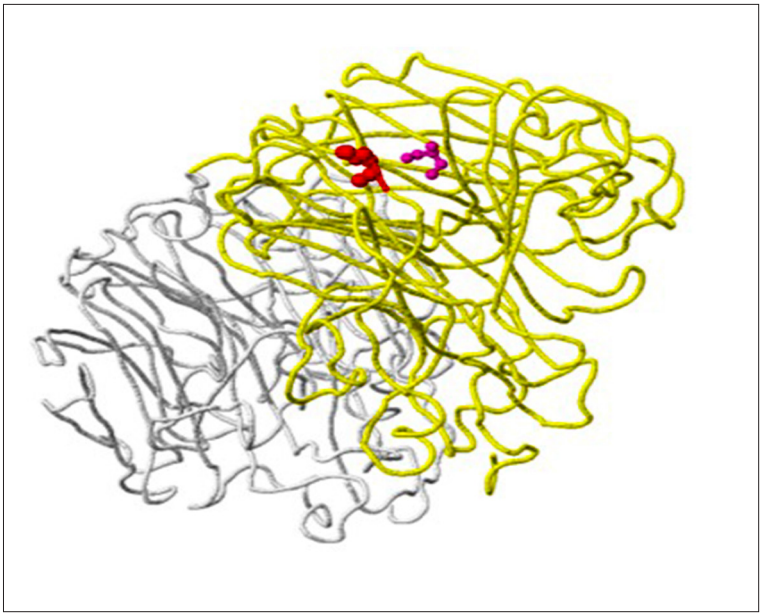

Figure 3. Structure of the neuraminidase virus A/SingaporeINFIMH-16-0019/2016(A/H3N2/) with the D356H mutation detected in clinical sample number 25, as an example of a ligand-binding mutation. Mutation site 356 is shown in red on the A chain (yellow backbone), which is 5 Ångström (A) from the ligand-binding site (*created using the FluSurver mutations app – https://gisaid.org/database-features/flusurver-mutations-app/*).

Figure 4. An example of a mutation site related to antibody-binding properties: the K369N mutation in the neuraminidase gene of virus A/Brisbane/02/2018 (A/H1N1/pdm09) in clinical sample number 770. The location of the mutation (red) corresponds to position 369 on the viral N chain (yellow backbone) and is 5 Ångström (A) from the heavy chain (H) antibodies (purple skeleton) (*created using the FluSurver mutations app – https://gisaid.org/database-features/flusurver-mutations-app/*).

resistance is largely due to the spread of a single variant [40]. Now, more than 10 years after this discovery, knowledge about not only the phenomenon of drug resistance, but also new anti-influenza drugs, vaccinations, or the genetics of the influenza virus itself, has significantly increased through the work of scientists and medical doctors [41,42]. Assessment of the susceptibility of currently circulating influenza viruses to antivirals is an important element of influenza virological surveillance required by the WHO, which the National Influenza Centers are trying to fulfill [43]. The WHO regularly updates the list of detected mutations [44]. As part of influenza surveillance, susceptibility to neuraminidase inhibitors is routinely assessed phenotypically, using a neuraminidase inhibition assay on selected samples. PA cap-dependent endonuclease, neuraminidase, and M2 gene sequence analysis is used to identify known markers of molecular resistance to all 3 classes of antiviral drugs [45]. However, it is important for scientists to also have information about other types of mutations that may be helpful in designing new drugs. A way to alleviate the problem of drug resistance is to design drugs that target core structural components or enzymatic active sites of viral proteins, because mutations in these residues likely produce structurally unstable or inactive proteins. In the future, the main way to combat influenza infection may be to select combinations of drugs with different mechanisms of action in response to drug failure due to viral mutations [46]. In the present study, we detected different

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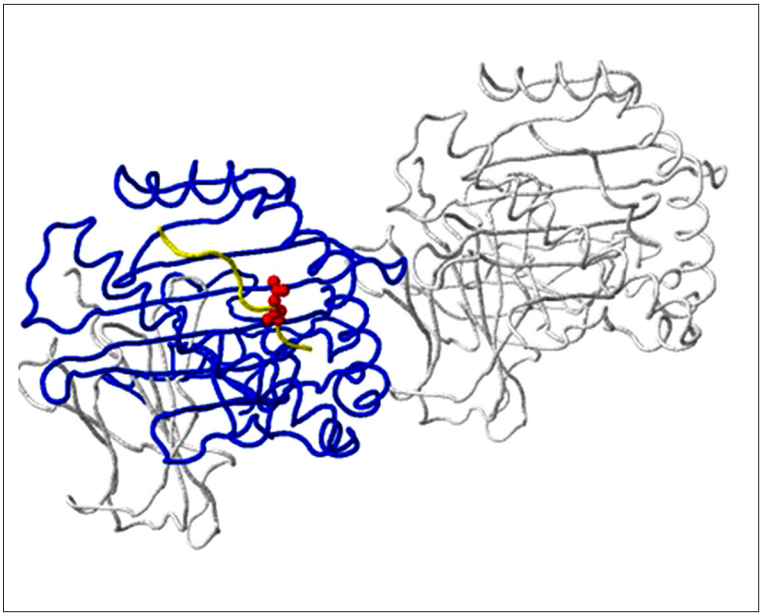

Figure 5. Host protein-binding site exemplified by the G451R mutation in the neuraminidase gene of virus A/SingaporeINFIMH-16-0019/2016 (A/H3N2/) in clinical sample number 229. The location of the mutation corresponds to position 452 of the viral F chain (yellow backbone) of the neuraminidase gene and is 5 Ångström (A) from the D-chain of a non-viral protein (blue backbone), in this case the MHC class I antigen. The white backbone represents the other chains found in the structure (*created using the FluSurver mutations app – https://gisaid.org/database-features/flusurver-mutations-app/*).

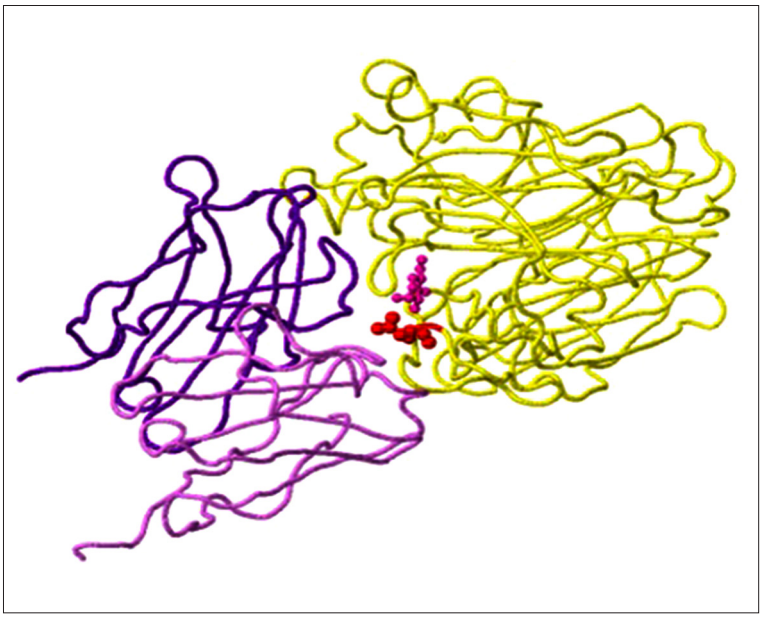

Figure 6. N-glycosylation on the example of the detected N329S mutation in the neuraminidase gene of virus A/SingaporeINFIMH-16-0019//2016 (A/H3N2/) in clinical sample number 30598. The mutation site corresponds to position 329 of the viral N chain (yellow backbone) and is located 5 Ångströms (A) from the ligand, the antibody heavy (H) chain (purple backbone), and the antibody light (L) chain (light purple backbone) (*created using the FluSurver mutations app – https://gisaid.org/database-features/flusurver-mutations-app/*).

types of mutations, which are described in the results section. The highest percentage of mutations detected in all types of influenza viruses occurred in genes related to ligand-binding and oligomerization properties of the virus. The viral oligomerization interface is an area responsible for the stability of neuraminidase oligomerization. A better understanding of the biological functions of neuraminidase may facilitate an alternative design for antiviral drugs to combat influenza virus infection. Notably, escape mutations occurred 644 times worldwide (6.89% prevalence). In the present study, such mutations were found 3 times in the influenza B virus and twice in the influenza A virus. They evade the host's immune system response by no longer being effectively recognized by neutralizing antibodies. This is possible, for example, by producing antigen proteins that are similar to host proteins [47]. Based on published information, N-linked glycosylation may be important in adhesion with host cells or the surrounding environment, and may play a role in antigenicity [48]. Recent studies have shown that many viral proteins, especially structural proteins, are glycosylated during the viral infection cycle. The N-glycans of the viral glycoproteins have multiple functions, which include promotion of expression, transport, fusion, binding to cell surface receptors, and prevention of antibody neutralization [49,50].

In Brazil, studies were conducted in 2017-2019 that confirmed the presence of adamantane resistance markers M2: S31N in

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most of the viruses tested, in line with what has been reported around the world. No increased resistance of viruses to neuraminidase inhibitors has been reported. Despite this, this situation can change rapidly, and resistant-strain surveillance is a priority. This highlights the importance of neuraminidase inhibitors in the treatment of influenza infections [51]. During the COVID-19 pandemic, measures taken to limit the spread of SARS-CoV-2 led to a substantial decrease in global influenza activity in the 2020-21 and 2021-22 epidemic seasons [52]. Data from WHO influenza surveillance in 2022 revealed laboratory-confirmed cases of influenza well above the historical average [53]. Analysis of drug-resistant influenza viruses carried out in Germany in 2019-2022 showed that resistance to influenza antiviral drugs may develop in the absence of selection pressure, spontaneously even, in untreated patients. Circulation and wider spread of such viral quasi-species, carrying smaller, resistant gene variants, can lead to the failure of an antiviral drug or even an entire class of drugs [54]. In Poland, the National Influenza Center has not yet performed studies from the post-pandemic period. The number of samples collected in the country in the 2020-2021 and 2021-2022 seasons did not allow for detailed analyses. The CDC reports that antiviral susceptibility patterns changed very little during the 2020-2021 and 2021-2022 seasons. Only a very small number of viruses were found to be resistant to oseltamivir [55].

The use of anti-influenza drugs for treatment depends on the policy of a given country. The available neuraminidase inhibitor in Poland is oseltamivir [56]. Japan has been one of the largest consumers of neuraminidase inhibitors in the world [57]. However, in recent years, the statistics have changed, and now the United States is ahead of Japan in neuraminidase inhibitor use. According to media information provided by F. Hoffmann-La Roche, in the period between January and December 2014, the United States accounted for 71.5% of the global consumption of neuraminidase inhibitors, Japan accounted for 11.8%, Europe accounted for 7.7%, and the rest of the world accounted for 9% [58]. Due to the percentage of the adult population that is vaccinated in Japan, which was 19.17% in 2011; 17.17% in 2012; and 42.86% in patients with immunodeficiency, it can be presumed that the high use of anti-influenza vaccines in the local population did not affect the development of resistance among viruses, because this phenomenon should be eliminated by vaccination. A similar situation also applies to the United States, as mentioned previously, where 36.2% of the population has been vaccinated against influenza [59]. In Poland, a very small part of the population gets vaccinated against influenza – in the analyzed epidemic seasons, vaccination in the population ranged from 3.3 to 3.9% [60,61]. In the previously discussed countries, in the corresponding epidemic seasons, the percentage of the population that was vaccinated was on average 42.2% in the USA [62], and similarly in Japan, about 40% [63].

Contrary to what was observed in Japan [64], we observed a higher frequency of mutations within the neuraminidase gene in the influenza A strains in Poland. Moreover, unlike the observations from Japan, all the strains detected in the present study were vaccine strains. These data suggest that the observed differences in drug resistance mutation incidence in influenza A and B viruses may depend on the level of vaccination of the human population from which the tested influenza virus isolates were obtained. Therefore, it seems important to determine whether the strains of influenza viruses tested for drug resistance as part of global influenza surveillance are representative of high and low vaccination rates of the population to a similar extent for all countries. Our results suggest that countries with low levels of influenza immunization may constitute reservoirs of drug-resistant influenza viruses. Drug resistance studies of influenza virus strains in other countries with a low level of vaccination in their population should be conducted to assess the scale of the risk of new drug-resistant strains arising from the lack of influenza vaccination.

An interesting study was conducted in the United States which estimated that for every 100 children under the age of 15 hospitalized for cardiopulmonary diseases during the flu season, 3 to 9 courses of antibiotic therapy were prescribed each year. The study encompassed 19 years of observations using the collected data and calculated the incidence of influenza in each given period. It turned out that 10-30% of excessive use of antibiotics during the winter period may be due to influenza [65]. A meta-analysis conducted in 2019 showed that influenza vaccination reduced the duration of antibiotic therapy among healthy adults by 28.1% and moderately in children from 6 months to 14 years of age [66]. This suggests that the use of prophylaxis in the form of vaccination works synergistically with treatment, and taking advantage of this synergy may be an approach for reducing the development of antiviral resistance. Vaccination can reduce the evolution of resistant variants, while drugs can subsequently be used against strains for which vaccination has not provided adequate protection. The broad use of neuraminidase inhibitors, coupled with a low level of vaccination of the population, may serve to create selection pressure driving the evolution of resistant strains. The present study is the first of this type conducted in Poland. It would be helpful to extend this research by employing phenotype-based drug resistance tests and comparing the results with the mutations detected using the genotypic method.

Conclusions

All viruses in which mutations were detected were the same strains as those in the seasonal influenza vaccine in use during the examined epidemic season.

- The influenza A virus was more genetically diverse.

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- The influenza B virus mutated more slowly than the influenza A virus, but the frequency of mutations in regions associated with drug resistance was higher among the influenza B strains.
- Drug resistance mutations are rampant in vaccine strains circulating in unvaccinated populations.
- It is worth conducting drug-resistance studies of the influenza virus strains in other countries with a low level of vaccination in their populations, to assess the scale of the risk of new drug-resistant strains arising in association with a lack of influenza vaccination.

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Declaration of Figures Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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