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INFLUENZA A AND B VIRUSES IN THE POPULATION OF VOJVODINA, SERBIA

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Abstract - At present, two influenza A viruses, H1N1pdm09 and H3N2, along with influenza B virus co-circulate in the human population, causing endemic and seasonal epidemic acute febrile respiratory infections, sometimes with life-threatening complications. Detection of influenza viruses in nasopharyngeal swab samples was done by real-time RT-PCR. There were 60.2% (53/88) positive samples in 2010/11, 63.4% (52/82) in 2011/12, and 49.9% (184/369) in 2012/13. Among the positive patients, influenza A viruses were predominant during the first two seasons, while influenza B type was more active during 2012/13. Subtyping of influenza A positive samples revealed the presence of A (H1N1)pdm09 in 2010/11, A (H3N2) in 2011/12, while in 2012/13, both subtypes were detected. The highest seroprevalence against influenza A was in the age-group 30-64, and against influenza B in adults aged 30-64 and >65.

Key words: Influenza, acute respiratory infections, epidemic, pandemic, real-time PCR, seroprevalence

INTRODUCTION

Influenza viruses are classified into three genera within the Orthomyxoviridae family: *Influenzavirus A*, *Influenzavirus B,* and *Influenzavirus C*. Influenza A and B viruses are important human pathogens causing *substantial morbidity and mortality worldwide*. They usually cause endemic and epidemic flu, self-limited, acute febrile respiratory infection. However, severe disease and life-threatening complications such as pneumonia, which is associated with increased rates of hospitalization and death, may occur in elderly persons, infants, and patients with chronic medical conditions (Taubenberger and Morens, 2008). Influenza C virus sporadically causes a mild respiratory illness, has no epidemic potential, and does not have severe public health impact.

The genome of influenza A and B viruses consists of eight single-stranded, negative-sense RNA segments, encoding up to 11 proteins. Influenza A viruses are divided into subtypes, based on the antigenic properties of surface glycoprotein hemagglutinin (H) and neuraminidase (N). There are 16 different subtypes of H and 9 of N. All known subtypes, in various combination, circulate in aquatic bird and can infect a range of mammal species (pigs, horses, cats, seals), but only some of these subtypes have been identified in humans. Subtypes H1N1, H2N2 and H3N2, were associated with seasonal epidemics and three major pandemics of the twentieth century (1918, 1957 and 1968, respectively). In the spring of 2009, a new influenza A (H1N1)pdm09 virus emerged and caused the first influenza pandemic in more than 40 years. Occasional transmissions of

avian H5N1, H7N7 and H9N2 viruses to humans have also been recorded (Taubenberger and Morens, 2010). There are no influenza B virus subtypes. The influenza B virus almost exclusively infects humans, although it has also been isolated from seals.

Influenza A viruses show rapid genetic evolution, notably in the gene-coding surface glycoproteins (Lavenu et al., 2006; Taubenberger and Morens, 2010). Antigenic drift is a process of continuous change in the amino acid sequences of the antigenic portions of H and N. It results from the accumulation of point mutation during viral replication leading to new virus strains, causing annual winter outbreaks. Antigenic shift is an abrupt, major change in which the virus acquires a new H subtype antigenically novel to humans, usually by genetic reassortment with another influenza A virus. Most people have no immunity to the new subtype, so the virus infects up to 50% of the population and quickly spreads, causing a pandemic (Taubenberger and Morens, 2010). Influenza B viruses undergo antigenic drift less rapidly than influenza A viruses. They are associated with less frequent and less severe epidemics than influenza A viruses, and they have not caused pandemics (WHO GISN, 2011).

A number of tests can be applied in the diagnosis of influenza: conventional viral cell culture, rapid cell culture (shell vials), direct or indirect immunofluorescence assays, molecular assays and serological testing. Serological testing is recommended for retrospective diagnosis and seroepidemiological surveillance. Molecular tests, such as real-time RT-PCR, can be used for fast diagnosis of acute influenza infections, as well as for the detection and investigation of outbreaks (WHO GISN, 2011).

MATERIALS AND METHODS

ELISA test

In order to determine the seroprevalence of influenza A and B, serum samples from patients suspected of having respiratory virus infection, were collected from January 2011 to May 2013 in the Center of Virology of the Institute of Public Health of Vojvodina. Specimens were tested for immunoglobulin G antibodies (IgG) against influenza A and B viruses, using commercially available enzyme-linked immunosorbent assays (ELISA), (Euroimmune, Lubecq, Germany). For the presence of IgG antibodies against influenza A and B viruses, a total of 353 and 271 patients were assayed in 2011, 398 and 280 in 2012, 333 and 309 in 2013, respectively. Patient ages ranged from younger than 1 year to 83 years.

Testing, calculation and interpretation of results were performed strictly following the instructions of the manufacturer. Samples were tested using an automated ELISA system for microtiter plates, Euroimmune Analyzer I-2P. Results were evaluated semiquantitatively by calculating the ratio of the extinction value of patient samples over the extinction value of the calibrator 2, which was included in the test. Results were considered positive if the ratio was equal to or greater than 1.1, intermediate if the ratio was between 0.8 and 1.1, and negative if the ratio was less than 0.8. Positive results for IgG anti-influenza antibodies confirmed past infection with this viruses. Borderline results were inconclusive and, according to the instructions of manufacturer, those samples should be retested after 7 days.

Statistical analysis

Differences in the anti-influenza IgG antibody prevalence between three seasons were calculated by Fisher's statistical test.

Real-time RT-PCR

Nasopharyngeal swab samples were obtained from sentinel and hospitalized patients with influenza-like illness and transferred to the World Health Organization (WHO) National Influenza Center in the Institute of Public Health of Vojvodina in Novi Sad. A total of 88, 82, and 369 specimens were collected, during the influenza seasons 2010/11, 2011/12, and 2012/13, respectively.

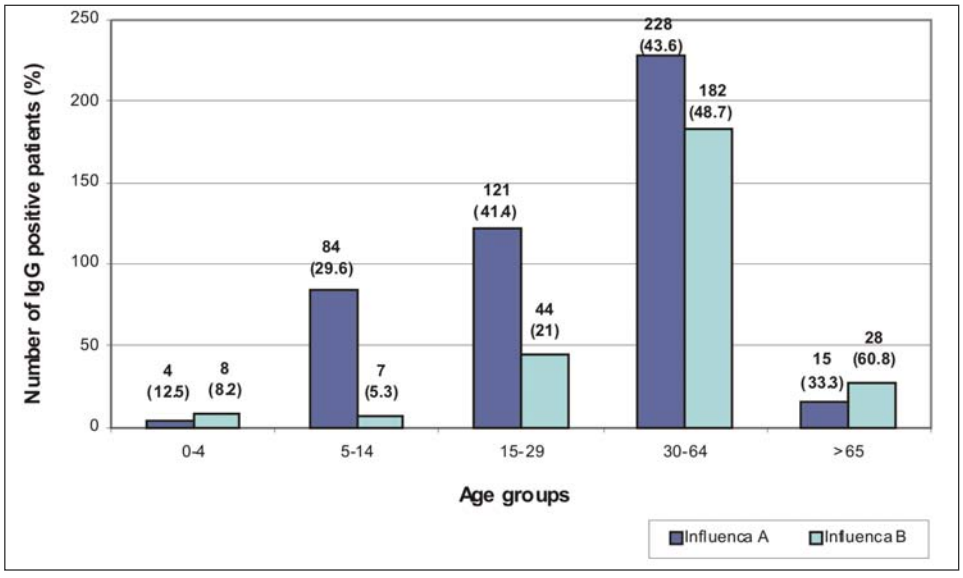

Fig 1. Age distribution of anti-influenza A and B IgG positive patients

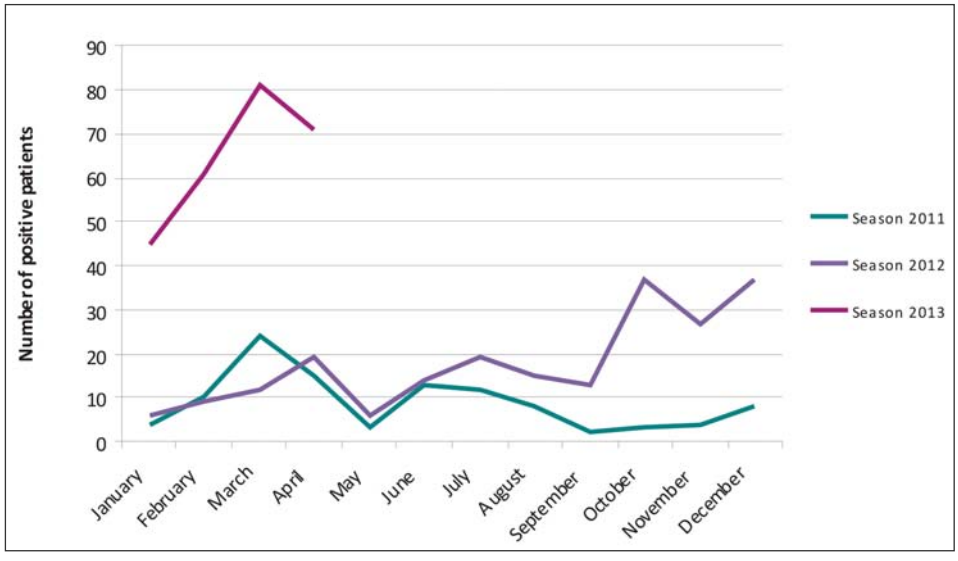

Fig 2. Seasonal distribution of seroprevalence of anti-influenza A virus IgG antibodies

Samples were processed in accordance with WHO Global Influenza Surveillance Network recommended protocols (WHO GISN, 2011). Viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Germany). Influenza detection and subtyping was done by singleplex real-time RT-PCR assays recommended by the WHO (CDC, 2009). Reverse transcription and amplification were performed using one-step AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, USA), and oligonucleotide primer and probe sets designed for detection of universal influenza A, pandemic influenza A (H1N1)pdm09, seasonal influenza A (H1N1), A (H3N2), avian influenza A (H5N1) and influenza B viruses, provided by Centers for Disease Control and Prevention (CDC). Negative and positive tem-

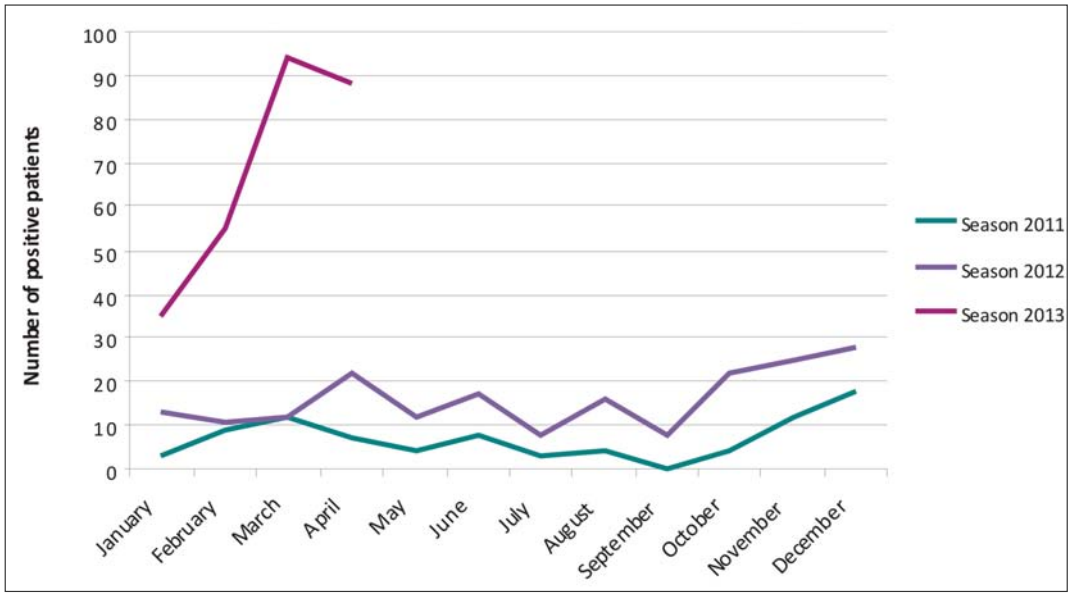

Fig 3. Seasonal distribution of seroprevalence of anti-influenza B IgG antibodies

plate controls for all primer/probe sets were included in each run. The human RNase P gene primer and probe set served as an internal positive control for human RNA. PCR was performed on Applied Biosystems 7500 real-time thermocycler.

Results were analyzed using Applied Biosystems 7500 Software and the interpretation of data was done according to WHO guidelines (WHO GISN, 2011).

RESULTS

Results of serological investigation for influenza A and B are summarized in Table 1. The prevalence of antibodies against influenza A and B viruses were not significantly different in the same year. The proportions of IgG-positive patients for influenza A and B in 2011 were 30% (106/353) and 34.3% (93/271), in 2012 53.8% (214/398) and 69.3% (194/280), and in 2013 77.2% (257/333) and 85.4% (264/309), respectively. However, each year, seroprevalences of antibodies to influenza A and B viruses were extremely significantly higher than in the previous year ($P<0.0001$).

Seroprevalences against influenza varied by age group (Fig. 1), with adults aged 30-64 having the highest seroprevalence for influenza A (43.6%), and adults aged 30-64 and >65 for influenza B (48.7% and 60.8%, respectively). The lowest influenza A age-specific seroprevalence of 12.5% was in the age-group of 0-4 years, while the lowest prevalence of influenza B antibodies (5.35) was in the age-group 5-14 years.

Figs. 2 and 3 show the seasonal distribution of anti-influenza A- and B IgG-positive patients during 2011, 2012 and for the first four months of 2013. A substantial increase in seroprevalence of both viruses was detected at the beginning of 2013.

The results of real-time RT-PCR testing are summarized in Table 2. Influenza viruses were detected by real-time RT-PCR in 60.2% (53/88) of specimens taken from patients during 2010/11, 63.4% (52/82) in 2011/12 and 49.9% (184/369) in 2012/13. Among the positive patients, infections with influenza A viruses were much more prevalent than infections due to influenza B viruses, throughout the seasons 2010/11 (84.9% vs. 15.1%) and 2011/12 (96.1% vs 3.9%). In the 2012/13 season, viruses of type A and

Table 1. Results of ELISA testing for IgG anti-influenza A and B antibodies per year

	Samples tested in 2011 (%)	Samples tested in 2012 (%)	Samples tested in 2013 (%)
Influenza A virus			
Positive	106 (30)	214 (53.8)	257 (77.2)
Negative	205 (58.1)	137 (34.4)	49 (14.7)
Borderline	42 (11.9)	47 (11.8)	27 (8.1)
Total	353	398	333
Influenza B virus			
Positive	93 (34.3)	194 (69.3)	264 (85.4)
Negative	150 (55.4)	83 (29.6)	39 (12.6)
Borderline	28 (10.3)	3 (1.1)	6 (2)
Total	271	280	309

Table 2. Distribution of influenza viruses in patients tested by real-time RT-PCR by season

	Positive patients (%)		
	Season 2010/11	Season 2011/12	Season 2012/13
Influenza A virus	45 (84.9)	50 (96.1)	87 (47.3)
A (H1N1)pdm09	45 (100)	-	61 (70.1)
A (H1N1)	-	-	-
A (H3N2)	-	50 (100)	22 (25.3)
Unsubtypeable	-	-	4 (4.6)
Influenza B	8 (15.1)	2 (3.9)	97 (52.7)
Positive/analyzed	53/88 (60.2)	52/82 (63.4)	184/369 (49.9)

type B were about equally distributed among positive patients (47.3% vs. 52.7%).

Subtyping of influenza A-positive samples during the first two seasons, revealed the presence of only one subtype, A (H1N1)pdm09 in 2010/11, and A (H3N2) in 2011/12. In the 2012/13 winter season, among the influenza A-positive patients there were 61 (70.1%) influenza (H1N1)pdm09 positive, 22 (25.3%) influenza A (H3N2) positive, and in 4 (4.6%) cases it was not possible to determine the subtype of detected influenza A virus.

Seasonal A (H1N1) and avian (H5N1) subtypes were not identified in any sample, during the given periods of time.

DISCUSSION

Serological testing is not convenient for early diagnosis of acute influenza virus infections, but it may be very useful for retrospective diagnosis and seroepidemiological surveillance.

In this study, the observed IgG prevalences against influenza A virus varied, depending on the year, from 30% to 77.2%, and against influenza B virus from 34.3% to 85.4%. However, in the same year, prevalences against both viruses were similar. Data from a seroepidemiological study conducted by Sauerbrei et al. (2009) showed that the prevalence of IgG antibodies against influenza A was 99,4% and against influenza B 56.7% in a population of healthy adult blood-donors in Germany. In another investigation, a high number of IgG-positive for influenza A (93.8%), and a lower number of influenza B positive (42.1%), were also observed among healthy pregnant women in Germany (Wutzler et al., 2009).

After the decline of maternal antibodies during the first year of life, the prevalence of antibodies

against influenza viruses increases during life time. In accordance with this, our serological investigation revealed lower antibody prevalences for influenza A and B in children aged up to 14 years compared to adults. Compared to the results of some other investigations, the prevalences detected in this study were generally lower. Sauerbrei et al. (2009) recorded very high seroprevalences of influenza A in children (82%) by the age of 12 years and in adults (99.4). Our results could be explained by the low influenza vaccination rate in the population of Vojvodina. It is obvious that annual influenza immunization may improve protection against influenza virus infections during epidemics.

A substantial increase in the seroprevalence of influenza A and B viruses was detected at the end of 2012 and beginning of 2013. This was probably a consequence of the early beginning of the 2012/13 winter influenza season, and the high activity of both viruses in our Province, as in some other European countries (WHO, 2013).

The results of real-time RT-PCR testing show that during the first two seasons, influenza A viruses were predominant, while influenza B viruses circulated at low levels. During the 2010/11 and 2011/12 influenza seasons, influenza A types were responsible for 84.9% (45/88) and 96.1% (50/82) of influenza virus infections, respectively. Only 15.1% (8/88) infections in the first, and 3.9% (2/82) in the second season, were due to influenza B type. In 2010/11, the situation was similar in other European countries, as well as in America and most parts of Africa and Asia (WHO, 2011), but in 2011/12 the activity of influenza B type increased in Africa, as well as in Eastern and South Asia, and Canada (WHO, 2012). During the 2012/13 season, influenza B viruses circulated in many countries and in some of them were the predominant influenza viruses (WHO, 2013). Similarly, the activity of influenza B type also increased in Vojvodina. Infections with influenza B type were detected in more than half (52.7%, 97/184) of positive patients, while influenza A infections were confirmed in a slightly smaller percentage (47.3%, 87/184).

Historically, influenza pandemics have been associated with a replacement of the previously circulating influenza A subtype, as in the 1918 pandemic virus was A (H1N1). In 1957 it was replaced with A (H2N2), which circulated in humans until 1968, when it was replaced by A (H3N2). In 1977, influenza A (H1N1) was reintroduced, and since then it has been co-circulating with A (H3N2) in humans. Influenza A (H1N1)pdm09 virus emerged in spring 2009 and became the dominant influenza virus around the world, causing a pandemic.

One year after the pandemic, during the 2010/11 influenza season, influenza A (H1N1)pdm09 virus was the only detected influenza A subtype in the Province of Vojvodina. In the same period, this subtype was by far the most common influenza virus in Europe, while influenza A(H3N2) subtype was rare (WHO, 2011). However, in contrast to the pattern observed during the 2009 pandemic, in many parts of the world, A (H1N1)pdm09 co-circulated with other influenza viruses and was no longer the predominant influenza subtype. For example, in North America, influenza A (H1N1)pdm09 co-circulated with influenza A (H3N2) and influenza B viruses.

Data from this study show that in 2011/12, the only detected influenza A subtype was A (H3N2). During that season, the predominant virus varied widely in different European countries, but overall influenza A (H3N2) was the most commonly reported (WHO, 2012). The situation was similar for Central Asia, South America and Africa. In North America, the distribution of influenza A subtypes varied, so Mexico reported almost exclusively influenza A (H1N1)pdm09, while in the USA, influenza A (H3N2) was the most active influenza virus (WHO, 2012).

The dominant influenza A virus subtype during the season 2012/13 was A (H1N1)pdm09, which was detected in 70.1% (61/87) of influenza A-positive samples. The presence of influenza A (H3N2) subtype was revealed in 25.3% (22/87) of positive samples. A similar distribution of influenza A subtypes was observed in some countries in Europe, Africa,

Asia, Central and South America, while in others, and in most parts of North America, influenza A (H3N2) was predominant (WHO, 2013). Out of 87 influenza A-positive samples, 4 (4.6%) could not be subtyped. These samples were sent to WHO Collaborating Centers for Research on Influenza (CCRI) in London, as per WHO recommendation that all unsubtypeable influenza A specimens should be immediately sent for further characterization to one of the six WHO CCRI. Influenza viruses constantly change their surface glycoproteins that are responsible for receptor binding and antigenic properties of a virus. This process can result in the emergence of a virus with different host-range, tissue tropism, virulence, or antiviral drug resistance. This is why the characterization of all unsubtypeable, potentially novel, influenza viruses is of great importance.

Seasonal influenza A (H1N1) subtype was not detected in this study. Reduction in seasonal influenza A (H1N1) activity was the most obvious effect of the 2009 pandemic, while seasonal influenza A (H3N2) continued to circulate. At present, it seems that emerging pandemic influenza A (H1N1) pdm09 virus has replaced the previously circulating seasonal A (H1N1). It is not fully understood why pandemic influenza viruses replace existing seasonal influenza A subtypes and strains. Results from some studies show that heterosubtypic immunity – short-lived immunity which is cross-protective against different influenza A subtypes – is able to inhibit reinfection by any new strain in animal models (Grebe et al., 2008). It is possible that during pandemics, a substantial fraction of the global population is infected with the new virus and is then transiently immune to infection with the previously circulating subtypes. This leaves a critically low number of susceptible individuals, leading to the extinction of seasonal influenza strains (Blyth et al., 2010).

Although avian influenza A (H5N1) subtype was not detected in this study, continued monitoring of the occurrence of human infection with this virus is critically important to assess its pandemic potential. The case fatality rate associated with human infections with the highly pathogenic H5N1 avian subtype can reach nearly 60%, causing great concern that adaptation of this virus to human-to-human transmission could cause a devastating pandemic (Taubenberger and Morens, 2008).

Endemic and epidemic influenza virus infections continue to be a major health threat. The rapid, continuous and unpredictable nature of influenza viral evolution makes vaccine strategies and pandemic planning difficult. The recent emergence of the swine-origin influenza A (H1N1)pdm09 subtype and the ongoing highly pathogenic avian influenza A (H5N1) epizootic, associated with a growing number of human "spill-over" infections, has heightened the importance of rapid and accurate identification and subtyping of influenza viruses for surveillance, outbreak management, diagnosis and treatment.

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