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Seroepidemiological study of pandemic influenza H1N1 following the 2009–2010 wave in Greece

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ABSTRACT

Knowledge of seroprevalence rates against 2009 pandemic H1N1 virus will assist vaccination recommendations and the preparation of the health-care system during subsequent years. This study was conducted in Greece during June–August 2010 to estimate the seroprevalence rate against pandemic H1N1 virus. Persons presenting in 29 health-care facilities across the country were studied. Seroprevalence was estimated employing a virus-free ELISA that specifically recognizes 2009 H1N1 virus antibodies in human sera. Sera collected from 2005 to April 2009 were also used to estimate pre-pandemic seroprevalence rates. A total of 954 persons were studied. The overall seroprevalence rate was 28.5% (95% confidence interval = 25.6–31.3%). Age-specific rates were 34.2% in persons 0–4 years, 36.3% in persons 5–19 years, 25.0% in persons 20–39 years, 23.4% in persons 40–59 years, and 31.8% in persons ≥60 years. The highest rates were recorded in the Regions of Ionian Islands (67%) and Epirus (42.9%), while the lowest (8.4%) in the Region of Thessaly. Age-specific attack rates of infection during 2009–2010 were 28.8% in persons 0–4 years, 32.5% in persons 5–19 years, 14.3% in persons 20–39 years, 19.1% in persons 40–59 years, and 14.4% in persons ≥60 years. Multivariate analysis revealed that Region of residence and caring for children <5 years were associated with increased risk for seropositivity. Urbanity, personal and family characteristics, working in a health-care facility or in a school, history of pandemic H1N1 vaccination or history of influenza-like illness during 2009–2010 were not associated with increased risk for seropositivity.

1. Introduction

Influenza A viruses constantly evolve and cause seasonal epidemics and irregularly pandemics. By definition a pandemic influenza virus is genetically distinct from circulating seasonal influenza viruses, causes sustained human-to-human transmission and clinical disease, and to which the majority of population is susceptible [1]. These criteria were met by the pandemic influenza A H1N1 virus (hereafter referred to as pandemic H1N1 virus), which derived by a unique re-assortment of swine, avian, and human influenza gene segments. The pandemic H1N1 virus was first identified in North America in April 2009 and spread worldwide, necessitating the declaration of the first influenza pandemic in the 21st century on June 11, 2009 [1,2]. As of August 1, 2010 more than 214 countries have reported laboratory-confirmed cases including over 18,449 deaths to World Health Organization [3]. In Greece the first laboratory-confirmed case was detected on May 18, 2009. Following a moderate wave during July–August 2009, Greece entered the winter wave in late October 2009, with a peak during November 23–29, 2009, followed by a steady decline [4]. Overall,

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18,230 laboratory-confirmed cases and 149 deaths were recorded [Hellenic Center for Disease Control and Prevention; unpublished data].

Although it was almost certain that the pandemic H1N1 virus would circulate and predominate during 2010–2011 and subsequent seasons [5–7], there was considerable uncertainty regarding transition to the next interpandemic phase in terms of timing and cumulative burden of infections. Knowledge of the immunity level of a population is crucial in order to determine the magnitude of pandemic H1N1 virus circulation during 2010–2011, and thus the development of vaccination recommendations, the better preparation of the health-care system, and overall our understanding of the pandemic epidemiology. In a nationwide seroprevalence study conducted in New Zealand following its "winter" wave, wide age- and ethnic-specific variations were found, stressing the need for serosurveys at the country level [8]. This was crucial for Greece since only 3.2% of the population and 11.8% of high-risk groups were vaccinated against the pandemic H1N1 virus as of March 2010 [Hellenic Center for Diseases Control and Prevention; unpublished data]. We present the results of a prospective, nationwide study conducted during June–August 2010 to estimate seropositive rates against pandemic H1N1 virus and to investigate factors influencing the risk for seropositivity. This research was based on a novel ELISA that specifically recognizes 2009 H1N1 influenza virus antibodies in human serum samples using synthetic peptides derived from specific amino acid sequences on hemagglutinin (HA) and neuraminidase (NA) antigens of the novel pandemic H1N1 influenza virus [9].

2. Methods

2.1. Sample size

With a 30–35% expected seroprevalence rate against the pandemic H1N1 virus, we estimated that a sample of 912 persons was required. This estimation was based on a 90% study power and 95% confidence interval (CI) (STATA).

2.2. Enrolment of participants

In June 2010 the Hellenic Center for Disease Control and Prevention communicated with the directors of 5 tertiary-care hospitals in 3 cities (Athens, Thessaloniki, and Patras) and 35 health-care centers in rural areas across the country. The directors of 5 hospitals and 24 health-care centers agreed to participate, and were requested to enroll a pre-defined number of persons (40–120 per hospital and 20–60 per health-care center). Health-care facilities and number of enrolled persons were selected based on geographic and population criteria. Persons were selected randomly among persons referred for blood testing, irrespective of demographic characteristics, underlying diseases, history of vaccination against the pandemic H1N1 virus, history of influenza-like illness (ILI) or diagnosis of pandemic H1N1 by a physician during 2009–2010, or reason for blood testing. Consent was requested by participants or, in case of children, by their parents or guardians. Attention was requested in order to enroll persons across all age groups. The protocol was approved by the Hellenic Center for Diseases Control and Prevention Board of Directors.

2.3. Data collection and sera sampling

The following data were collected anonymously using one standardized form per subject: residential address, age, sex, profession, number and age of household members, caring of children <5 years, history of vaccination against the pandemic H1N1 virus, and history of ILI or diagnosis of pandemic H1N1 by a physician during 2009–2010. The survey and sera sampling were conducted concomitantly and prospectively from June 25 through August 29 2010.

2.4. Laboratory diagnosis

Sera were sent to the 2nd Southern Greece Influenza Reference Center (University of Athens, Department of Microbiology). IgG specific antibodies were estimated by a described previously virus-free ELISA that allows the determination of titers of antibodies specifically directed against the swine-origin influenza A/California/14/2009 (H1N1) [9]. The immunoassay is based on the use of synthetic peptides (H1-pep and N1-pep; Meridian Life Science, Inc., Memphis, TN) corresponding to amino acid sequences, which are identified on hemagglutinin (HA) and neuraminidase (NA) of H1N1 swine-origin influenza A/California/14/2009, respectively [10]. Synthetic H1 and N1 peptides derived from the seasonal viruses influenza A/Brisbane/59/2007 (H1N1) and influenza A/Georgia/20/2006 (H1N1), respectively, were purchased (Meridian Life Science, Inc.), were used for the immunization of rabbits and the production of antibodies capable of recognizing the different amino acid sequences. These specific rabbit antisera that recognize H1-pep and N1-pep peptides were used as positive controls and for normalization of readings among different ELISA plates. No cross reactions were observed between antisera from rabbits that were immunized with synthetic pandemic H1N1 and seasonal H1N1 peptides, respectively. The cut-off value for anti-peptide ELISA was calculated as mean normal human serum binding units plus 3 standard deviations. Samples were considered positive when the corrected optical density was above the cut-off.

Further, residual sera collected from 377 persons for diagnostic purposes in 5 laboratories from 2005 through April 2009, were tested for pandemic H1N1 IgG antibodies in order to estimate pre-pandemic immunity. Age was available for these samples. Control human serum samples were used in all assays.

2.5. Definitions

ILI was defined as acute onset of fever (>38.0 °C) and cough or another respiratory symptom. Seroprevalence rate against the pandemic H1N1 virus was defined when a serum sample was positive for IgG antibodies against the synthetic peptides H1-pep and/or N1-pep. Attack rate refers to the percentage of persons infected during 2009–2010. Given that only 3.2% of the population was vaccinated and that several months elapsed between the end of the 2009–2010 wave and sera collection, attack rate was calculated as follows: seroprevalence rate minus pre-pandemic immunity. Rural and urban areas were defined as a population of <2000 residents and ≥2000 residents, respectively [11].

2.6. Statistical analysis

In order to estimate the seroprevalence rate against the pandemic H1N1 virus of the population of Greece, weighting was conducted according to Region (10 Regions), age (5 age groups), and urbanity (2 categories). Greece was divided in 100 strata of same Region, age, and urbanity. The current study covered 90 of 100 strata with a population of 10,598,307 residents, compared to a total of 10,934,097 residents in Greece. The weighs that were used for each stratum h were:

$$W_h = \frac{N_h}{N} \times \frac{n}{n_h}, \quad h = 1, \ldots, 90$$

where $n = 954$ is the sample size, n_h the number of tested persons per stratum h, N_h the population of the stratum, and $N = 10{,}598{,}307$.

Table 1
Characteristics of tested persons.

Characteristic	Number of tested persons (n = 954)
Age, years	
0–4	46 (4.8%)
5–19	158 (16.6%)
20–39	284 (29.8%)
40–59	247 (25.9%)
≥60	218 (22.9%)
Male gender	401 (42.1%)
Region of residence	
Attica	350 (36.6%)
Sterea Ellada	62 (6.5%)
Peloponnesus	99 (10.4%)
Ionian Islands	18 (1.9%)
Epirus	16 (1.7%)
Thessaly	67 (7.0%)
Macedonia	217 (22.8%)
Thrace	33 (3.4%)
Aegean	45 (4.8%)
Crete	47 (4.9%)
Type of residence	
Rural	231 (24.3%)
Urban	723 (75.7%)
Health-care facility employer	214 (22.5%)
Teacher–professor in a school	8 (0.9%)
Mean number of cohabitants (SD)	2.4 (1.9)
Mean number of cohabitants <18 years (SD)	0.7 (0.9)
Caring for children <5 years	149 (15.6%)
Vaccination against the pandemic H1N1 virus	112 (11.7%)
History of influenza-like illness	178 (18.6%)
Influenza diagnosed by a physician	65 (6.8%)

SD: standard deviation.

In order to estimate the pre-pandemic seroprevalence rate, weighing was conducted according to age (5 age groups). Greece was divided in 5 strata of same age. The weights that were used for each age stratum h were:

$$W_h = \frac{N_h}{N} \times \frac{n}{n_h}, \quad h = 1, 2, 3, 4, 5$$

where n = 377 total sample size, n_h the number of tested persons per stratum h, N_h the population of the stratum, and N = 10,934,097. 95% CI was calculated (normal distribution approximation). The 2001 census was used in these analyses.

Multiple logistic regression analysis (forward selection) was applied to examine the relation between seroprevalence rates and the characteristics of participants. The results were reexamined using χ^2-test for the categorical variables and t-test for the continuous variables. p-Values of 0.05 or less were considered statistically significant (two-sided). Statistical analysis was performed using STATA.

3. Results

A total of 972 persons were enrolled; 18 were excluded due to missing data, therefore the study group comprised 954 persons. Table 1 shows their characteristics.

3.1. Estimation of seroprevalence rate against the pandemic H1N1 virus in Greece

The overall seroprevalence rate against the pandemic H1N1 virus was estimated at 28.5% (95% CI = 25.6–31.3%), which means that 3,116,217 residents of Greece (95% CI = 2,799,129–3,422,372) were seropositive.

Table 2 shows seroprevalence rates per age group and Region. Age-specific seroprevalence rates were 34.2% in subjects 0–4 years, 36.3% in subjects 5–19 years, 25.0% in subjects 20-39 years, 23.4% in subjects 40–59 years, and 31.8% in subjects ≥60 years (p-value = 0.025). In terms of residence, the highest seroprevalence rates were recorded in a small town in Northeastern Greece (68.42%) and a village in the Ionian Island of Corfu (63.3%), while the lowest in the Aegean Island of Karpathos (0%). Overall, the highest rates were recorded in Ionian Islands (67%) and Epirus (42.9%), while the lowest (8.4%) in Thessaly (p-value < 0.001) (Fig. 1). The most populated Regions of Attica and Macedonia (35.6% and 22.1% of Greek population, respectively) accounted for the majority of seropositive persons (43.1% and 22.7%, respectively). However the relative ratio of seropositive persons to residents was disproportionate among Regions, ranging from 0.31 in Thessaly to 2.31 in Ionian Islands. Urbanity, gender, underlying and family characteristics, working in a health-care facility or a school, history of vaccination against the pandemic H1N1 virus or ILI were not associated with increased risk for seropositivity (Tables 3 and 4).

Table 2
Estimation of pandemic H1N1 seroprevalence rates per age group and Region.

Characteristic	Seroprevalence[a] (n = 271) (28.5%)	95% CI
Age (years)		
0–4	16 (34.2%)	20.0–48.4
5–19	57 (36.3%)	28.7–43.9
20–39	71 (25.0%)	20.0–30.1
40–59	58 (23.4%)	18.0–28.7
≥60	69 (31.8%)	25.6–38.0
Region of residence		
Attica	117 (33.4%)	28.5–38.4
Sterea Ellada	10 (15.3%)	6.1–24.6
Peloponnesus	36 (36.5%)	26.8–46.1
Ionian Islands	12 (67.0%)	42.9–91.1
Epirus	7 (42.9%)	15.9–70.0
Thessaly	6 (8.4%)	1.6–15.3
Macedonia	62 (28.3%)	22.3–34.3
Thrace	8 (24.2%)	8.7–39.7
Aegean	8 (17.1%)	5.7–28.5
Crete	7 (15.1%)	4.5–25.7

CI: confidence interval.
[a] Against the pandemic H1N1 virus.

Table 3
Serology status against the pandemic H1N1 virus per categorical characteristics.

Characteristic	Seropositive[a] (n = 271) (28.5%)	p-Value
Gender		
Male	123 (30.7%)	NS
Female	148 (26.8%)	
Type of residence		
Rural	73 (31.7%)	NS
Urban	198 (27.4%)	NS
Health-care facility employer		
Yes	51 (23.8%)	NS
No	220 (29.7%)	
Teacher–professor in a school		
Yes	4 (50.0%)	NS
No	267 (28.2%)	
Caring for children <5 years		
Yes	55 (37.0%)	0.013
No	216 (24.0%)	
Vaccination against pandemic H1N1		
Yes	34 (30.4%)	NS
No	237 (28.2%)	
History of influenza-like illness		
Yes	60 (33.9%)	NS
No	211 (27.2%)	
Influenza diagnosed by a physician		
Yes	16 (24.6%)	NS
No	255 (28.6%)	

NS: not significant.
[a] Against the pandemic H1N1 virus.

Fig. 1. Seroprevalence rates against pandemic H1N1 per Region of Greece.

Multivariate regression analysis revealed that Region of residence (Wald statistic p-value < 0.001; χ^2 statistic p-value = 0.001) and caring for children <5 years (Wald statistic p-value < 0.019; odds ratio = 1.598; χ^2 statistic p-value = 0.013) were statistically significantly associated with increased risk for seropositivity.

3.2. Estimation of attack rates of pandemic H1N1 infection during 2009–2010 in Greece

The baseline pandemic H1N1 seroprevalence rate was estimated at 9.2% (35 of 377 sera; 95% CI = 6.2–12.1%). Age-specific baseline

Table 4
Serology status against the pandemic H1N1 virus per continuous characteristics.

Characteristic	Seropositive[a] (n = 271) (28.5%)	Seronegative[a] (n = 683) (71.5%)	p-Value
Mean age, years (SD)	39.2 (24.4)	40.5 (22.5)	NS
Mean no. of cohabitants (SD)	2.5 (2.1)	2.4 (1.9)	NS
Mean no. of cohabitants <18 years (SD)	0.6 (1.0)	0.7 (0.9)	NS

NS: not significant; SD: standard deviation; no.: number.
[a] Against the pandemic H1N1 virus.

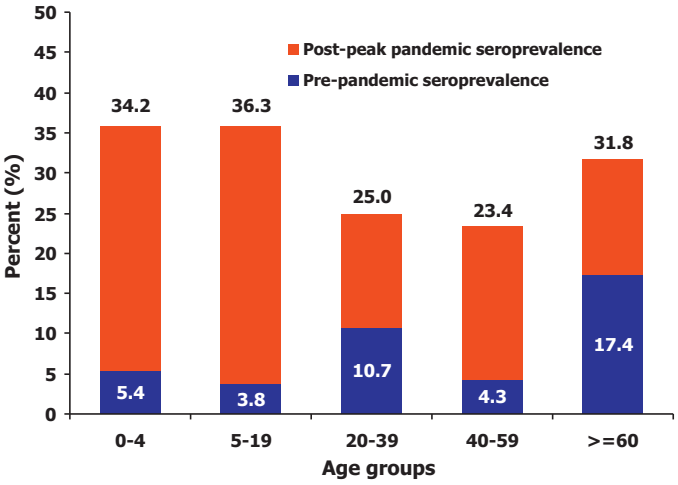

Fig. 2. Pre- and post-peak pandemic seroprevalence rates against pandemic H1N1 virus per age group.

seroprevalence rate was 5.4% (1 of 18 sera; 95% CI = 0–16.9%) in persons 0–4 years, 3.8% (2 of 64 sera; 95% CI = 0–8.6%) in persons 5–19 years, 10.7% (12 of 115 sera; 95% CI = 5.0–16.5%) in persons 20–39 years, 4.3% (4 of 95 sera; 95% CI = 0.1–8.4%) in persons 40–59 years, and 17.4% (15 of 85 sera; 95% CI = 9.2–25.6%) in persons ≥60 years. Age-specific attack rates of pandemic H1N1 infection during 2009–2010 were 28.8% in the 0–4 age-group, 32.5% in the 5–19 age group, 14.3% in the 20–39 age group, 19.1% in the 40–59 age group, and 14.4% in the ≥60 years age group (Fig. 2).

4. Discussion

Seasonal influenza constitutes a leading cause of morbidity, mortality, and utilization of health-care services globally, mainly among persons of extreme age and persons with underlying diseases [12]. However, influenza A viruses are notorious for their continuous in-field genetic interplay among avian species, swine, and humans, leading to an endless variety of new viruses and the unpredictable onset of pandemics. Historically influenza pandemics demonstrate signature features, including increased transmission driven by lack of population immunity, shifts of the highest mortality rates to younger populations compared to seasonal influenza, multiple waves of varying severity lasting for a total of 2–5 years, and geographic variability in impact [13–17].

This a nationwide study conducted in Greece to investigate seroprevalence rates and factors associated with seropositivity following the 2009–2010 pandemic wave. At this point, it should be noted that seroconversion rates seen in Greece could not be directly compared with other studies done around the world since the principle of our novel enzyme immunoassay is different from haemagglutination inhibition or virus neutralization assays. Our findings indicate that the pandemic H1N1 virus circulated widely in the community, building antibody levels in more than one every four residents in Greece. In the only Southern hemisphere nationwide seroprevalence study conducted among 1156 persons, based on age and ethnic standardization it was estimated that 29.5% of New Zealand residents were immune three months after the 2009 peak [8]. In contrast, a 14% age-weighted seroprevalence rate was revealed among 710 residents of Beijing, China one month after the 2009 peak [18]. Such differences are attributed to differences in pre-existing immunity or virus spread, as well as differences in diagnostic cut-offs and overall performance of employed tests. Timing of testing should also be considered when comparing data from different studies; in all the abovementioned studies data refer to their 2009–2010 "winter" pandemic wave. Similar to the United States and England where a significant proportion of persons >60 years had pre-existing antibody titers compared to younger persons [19,20], a 9.2% pre-pandemic seroprevalence against pandemic H1N1 virus was detected in Greece increasing with age. However, no or negligible pre-existing immunity was detected in Beijing, China [15] and Pune, India [21]. Geographic variations are attributed to heterogeneous exposure to antigenically related influenza A subtypes in the past. The assay method used in the present study may also account for the higher background rates noted. In our study, attack rates were higher among children and adolescents, groups with very low baseline seroprevalence rates, and lower among persons ≥60 years, resulting in the equalization of seropositive levels across age groups. Similar to our results, in Beijing, China, people ≥60 years were less likely to get infected during the 2009 pandemic compared to younger persons [18]. Up to 43.4% attack rates among school-aged children were also found in New Zealand [8], England [20], India [21], and Hong Kong [22] whereas as many as 70% of school-aged children in London had been infected by the end of the second (2009–2010) wave [23]. These findings are consistent with age-specific patterns during seasonal influenza epidemics, where attack rates are highest among children who subsequently spread infections to adults [24], and are explained by the higher viral load in respiratory secretions, transmissibility, and social contacts in children and adolescents. In our study, caring for children <5 years was significantly associated with seropositive status. Health-care workers experienced no increased risk compared to general population, and this is attributed to the wide use of personal protective equipment during the pandemic [25]. Similarly, very low (6.5%) seroconversion rates were detected in hospital staff in Singapore following its single 2009 pandemic wave [26]. In our study, vaccination against pandemic H1N1 was not associated with increased seroprevalence rates. Currently there is little published data on the persistence of specific antibody following pandemic H1N1 vaccination but in studies with seasonal influenza vaccines, antibody titers have been shown to drop below seroprotective levels after 4–6 months [27,28]. It is not defined if pandemic H1N1 vaccination actually results in antibody response against the two synthetic peptides used in our assay, as the natural H1N1 infection does. Since the present study was performed several months after the end of the vaccination period, the design of our study was not to detect seroconversion due to vaccination.

Wide spatial differences in seroprevalence rates were noted across Greece, with the highest burden of infections recorded in Ionian Islands and Epirus (nearby Regions in Northwestern Greece) and the lowest in Thessaly (Central Greece). Surveillance data show that laboratory-confirmed pandemic H1N1 illness rates per 100,000 population also varied among Regions [4], the highest rates being found in the island of Crete (>300 laboratory-confirmed cases per 100,000 population) followed by Ionian Islands, Epirus, Attica, and Aegean Islands (201–300 laboratory-confirmed cases per 100,000 population) [4]. Discordance between laboratory-confirmed illness rates and seroprevalence rates yielded in the current study at the regional level may be attributed to different performance characteristics of rRT-PCR among reference centers participating in the laboratory surveillance system, as well as differences in referring persons for testing or the small numbers tested in some regions. Nonetheless, great geographic heterogeneity in terms of infections and fatalities was disclosed in past pandemics [14,16] and was also found in the current [20], and is probably explained by variations in transmission factors including population density, movement and connectivity, and small seasonal changes in the effective transmission rate ("seasonal forcing") [15,29,30]. However, given that the Regions of Epirus and Thessaly have among the lowest population densities in Greece (36.55 and 52.73 residents/km^2, respectively), it appears that factors other than population density account for the wide spatial differences

in our study. Further, epidemiological and animal models indicate that absolute humidity and temperature may also influence spread of influenza virus [31,32], however the regional differences in seroprevalence rates encountered in our study could not be explained by relative humidity data (data not shown).

In conclusion, the pandemic H1N1 virus circulated widely in Greece during 2009–2010, however broad Region-specific differences were noted. As experienced in Europe [33], the virus predominated in Greece during 2010–2011 as well, however at lower levels compared with 2009–2010 [Hellenic Center for Disease Control and Prevention; unpublished data]. Overall, our study provides valuable data to understand the epidemiology of the 2009 pandemic and to plan future intervention strategies, and highlights the significance of seroprevelance studies at the country level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.06.098.

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