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Original Article

Retrospective serological survey of influenza viruses in backyard pigs from Mexico City

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Background In the present study, we analyzed the presence of antibodies to four different influenza viruses (pH1N1, hH1N1, swH1N1, and swH3N2) in the sera of 2094 backyard pigs from Mexico City. The sera were obtained between 2000 and 2009.

Objectives The aim of this study was to perform a retrospective analysis of the 2000–2009 period to determine the seroprevalence of antibodies against pH1N1, hH1N1, swH1N1, and swH3N2 viruses in sera obtained from backyard pigs in Mexico City.

Methods Antibody detection was conducted with hemagglutination inhibition assay (HI) using four influenza viruses. We used linear regression to analyze the tendency of antibody serum titers throughout the aforementioned span.

Results We observed that the antibody titers for the pH1N1, swH1N1, and swH3N2 viruses tended to diminish over the study period, whereas the antibodies to hH1N1 remained at low prevalence for the duration of the years analyzed in this study. A non-significant correlation ($P > 0{\cdot}05$) between antibody titers for pH1N1 and swH1N1 viruses was observed (0·04). It contrasts with the significance of the correlation (0·43) observed between the swH1N1 and swH3N2 viruses ($P < 0{\cdot}01$).

Conclusions Our findings showed no cross-antigenicity in the antibody response against the same subtype. Antibodies against pH1N1 virus were observed throughout the 10-year study span, implying that annual strains shared some common features with the pH1N1 virus since 2000, which would then be capable of supporting the ongoing presence of these antibodies.

Keywords backyard swine systems, influenza viruses, seroprevalence pH1N1-hH3N2-swH1N1-swH3N2.

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Introduction

In Mexico, three main swine-farming systems co-exist: intensive, semi-intensive, and backyard systems.[1] Swine backyard farming systems are characterized by lack of technology, they are rustically implemented and regional products are used in their construction. It is frequent to find there other species, such as dogs, cats, cattle, sheep, poultry, wild birds, and noxious fauna.[2] The swine influenza virus (SIV) is one of many viral agents implicated in respiratory disorders in pigs.[3] It is present in endemic form in swine farms worldwide in intensive as well as backyard production systems.[4,5] The influenza A virus (H1N1) was first isolated from pigs in 1930.[6] It belongs to the *Orthomyxoviridae* family, which includes two further types B and C.[7] Wild aquatic birds are considered natural reservoirs for the different subtypes of influenza A viruses (IAV).[8,9] It is believed that pigs serve as "mixing vessels" for the genetic reassortment of viral segments between the human and avian influenza viruses.[10] Since 1977, two subtypes of influenza A viruses, H1N1 and H3N2, have been seasonally present in the human population.[11]

There are several reports that confirm the presence of IAV (pH1N1) in swine herds on every continent. In each case, it is believed that the herds were infected as a consequence of human-to-pig transmission.[12] Consequently, circulating IAV in pigs may have origins related to H1N1 and H3N2 human strains. In the past decades, there have been several reports of

sporadic cross-transmission from swine and avian reservoirs to the human population.[13] Recently, it has been demonstrated that sera from certain pigs infected by European lineage influenza swine viruses cross-react with antibodies directed against the influenza virus pH1N1 and against swine viruses from North America, suggesting that pigs in Europe might have partial immunity against the pH1N1 A virus.[12,14] The aim of the present study was to perform a retrospective analysis of the 2000–2009 period to determine the seroprevalence against pH1N1, hH1N1, swH1N1, and swH3N2 viruses in backyard pigs from Mexico City.

Material and methods

Serological samples

Sera obtained from 2094 backyard pigs in Mexico City were used for this study. Samples collected during the period from 2000 to 2009 were remitted for diagnosis to the Departamento de Medicina y Zootecnia de Cerdos at the Facultad de Medicina Veterinaria y Zootecnia UNAM as part of Aujeszky disease-monitoring program in Mexico (Modificacion a la Norma Oficial Mexicana NOM-007-ZOO-1994, Campaña Nacional, contra la Enfermedad de Aujeszky).[15] Sampled pigs were randomly chosen, and they were all healthy animals. Serological samples were sent to the diagnosis laboratory along the whole year and coming from different areas of Mexico City (Figure 1). All the samples were taken from pig backyard systems, these production units are characterized by a low number of pigs that cohabit closely with human populations. Sera were stored at −20°C prior to use in the hemagglutination inhibition (HI) assay.

Virus strains

The following influenza viruses strains were used as antigens: seasonal human influenza (hH1N1) A/Mexico/INER1/2000 (H1N1) (GenBank access number: JN086908), pandemic influenza (pH1N1) A/Mexico/LaGloria-3/2009 (H1N1) (GenBank access number: CY077595), classical swine influenza (swH1N1) A/swine/New Jersey/11/76 (H1N1) (GenBank access number: K00992) and triple reassortant (swH3N2) A/swine/Minnesota/9088-2/98 (H3N2) (GenBank access number: AF153234). Viruses were inoculated into the allantoic cavity of 9-day-old specific pathogen-free chicken embryos and incubated at 37°C. The allantoic fluid was harvested 72 h after inoculation and titrated for the hemagglutination (HA) test with chicken erythrocytes at 0·5%. All procedures were performed in the biosafety level-3 laboratory of the Departamento de Medicina y Zootecnia de Cerdos-UNAM.

Hemagglutination inhibition assay

We used the standard procedure established by the World Organization for Animal Health (OIE)[16] with the following modifications: we standardized to eight hemagglutinating units (HAU). Sera were heat inactivated at 56°C and adsorbed with kaolin and chicken erythrocytes at 5%. Twofold serial dilutions were made from 1:40 through 1:5120, and serum titers were considered positive when they at a level equal or higher than 1:80.

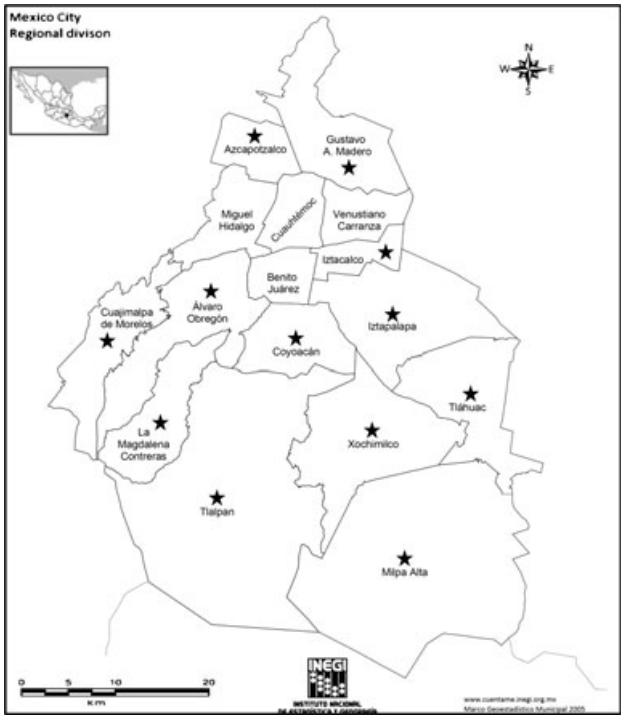

Figure 1. Sampling sites in Mexico City. Stars represent the regions from which pig sera were obtained (modified from INEGI, 2005; http://cuentame.inegi.org.mx/mapas/pdf/entidades/div_municipal/dfdeleg.pdf).

Statistical analysis

Antibody sera titers were transformed using a $\log_2$ transformation. We used linear regression to analyze the trend in reactive serum titers across the years. All statistical analyses were performed using the statistical software package JMP® 9.0 software (SAS Institute Inc., Cary, NC, USA).

Results

We analyzed the sera collected from 2094 backyard pigs from Mexico City. We processed 8376 (HI) tests in sera obtained from 2000 to 2009 to determine the prevalence of antibodies directed against four different influenza viruses: pH1N1, hH1N1, swH1N1, and swH3N2. The swH1N1 virus showed the highest seroprevalence (74%), followed by the swH3N2 (24·2%), pH1N1 (17·8%), and hH1N1 (1·3%) viruses (Table 1).

The boroughs that provided more samples were Azcapotzalco, Tlahuac, Xochimilco, Tlalpan and Milpa Alta with 402, 402, 395, 344 y 321 sera respectively. Among those that

Table 1. Seroprevalence and total number of positive samples against four different influenza viruses detected in backyard pigs during the years 2000–2009

Year	*n* sera	pH1N1 (+)	hH1N1 (+)	swH1N1 (+)	swH3N2 (+)
2000	250	72 (28·8)	0 (0)	217 (86·8)	158 (63·2)
2001	250	42 (16·8)	2 (0·8)	202 (80·8)	67 (26·8)
2002	250	24 (9·6)	5 (2)	171 (68·4)	99 (39·6)
2003	250	67 (26·8)	2 (0·8)	167 (66·8)	56 (22·4)
2004	154	57 (37)	5 (3·2)	134 (87)	69 (44·8)
2005	165	52 (31·5)	3 (1·8)	113 (68·4)	28 (16·9)
2006	52	1 (1·9)	1 (1·9)	12 (23)	1 (1·9)
2007	250	6 (2·4)	7 (2·8)	227 (90·8)	14 (5·6)
2008	223	10 (4·4)	1 (0·4)	188 (84·3)	11 (4·9)
2009	250	42 (16·8)	3 (1·2)	120 (48)	5 (2)
Total	2094	373 (17·8)	29 (1·3)	1551 (74)	508 (24·2)

Numbers in parenthesis indicate percentage of seroprevalence.

had higher seroprevalence of hH1N1 were Tlahuac with 1·9%, and Azcapotzalco to pH1N1, swH1N1 and swH3N2 with 25·6, 82·3 and 35·8% respectively.

In the cases where antibodies were observed against the four subtypes, subtype swH1N1 was the one that most often showed the highest titer ($n = 1204$), when compared to the other subtypes. Subtype hH1N1 showed the higher titer in the fewest number of occasions ($n = 11$) (Table 2).

When antibody titers were simultaneously detected for two subtypes, it was observed that subtypes swH1N1 and swH3N2 were the most frequently presented ($n = 468$). In 257 occasions of these 468, subtype swH1N1 depicted a higher titer than swH3N2 (107). When antibody titers were simultaneously detected for three subtypes, the ones presenting the highest frequency were pH1N1, swH1N1, and swH3N2 ($n = 114$). From these subtypes, swH1N1 was present in more occasions (40) followed by swH3N2 (32), and lastly by pH1N1 (12) (Table 3). There was only one serum with antibodies against the four subtypes, and the highest titer in this case was for pH1N1.

Table 2. Number of sera per year with the highest titers against each viral subtype

Year	*n* sera	pH1N1	hH1N1	swH1N1	swH3N2
2000	250	27	0	153	11
2001	250	16	0	166	13
2002	250	3	3	124	39
2003	250	37	2	108	22
2004	154	14	0	72	29
2005	165	28	1	73	8
2006	52	1	1	12	0
2007	250	4	1	213	3
2008	223	8	0	180	1
2009	250	33	3	103	3
Total	2094	171	11	1204	129

The sera from pigs that were analyzed for subtype showed hH1N1 positive frequency and the average titers were low compared to other subtypes; however, against swH1N1, swH3N2 and pH1N1 they presented high values; pH1N1 reached the highest titers as compared to other subtypes. The frequency and average titers for each subtype are presented in Table 4.

Although not too graphically evident (Figure 2), a negative association for swH1N1 across the years was detected using regression analysis. The linear regression coefficient of log swH1N1 per year was negative ($-0{\cdot}175$) ($P < 0{\cdot}0001$). We ran the same analysis but excluding the 2006 data and found that this coefficient was essentially the same ($-0{\cdot}167$) ($P < 0{\cdot}0001$). Hence, we report the analysis using the full data set.

Figure 3 shows the mean antibody titer values for each virus per year. The highest antibody titers were observed for the swH1N1 virus, which showed a mean titer value of 444·6 in the year 2000 and 75·4 in 2009. The resulting antibody titers were analyzed by linear regression throughout the study period. These results showed that antibody titers against the swH1N1 virus tend to decrease by approximately 9% each year. The mean titer values for antibodies reactive to the swH3N2 virus were 189 and 15·9 in the first and last year, respectively. The antibody titers for the swH3N2 virus also showed a decrease across the study period, exhibiting a more

Table 3. Number of sera in which antibodies against two and three subtypes were observed simultaneously, and number of cases in which each subtype showed the highest titer

Subtypes simultaneously observed	Number of times	Number of cases with the highest titer by subtype
Two		
pH1N1-hH1N1	6	4-0*
pH1N1-swH1N1	282	92-120*
pH1N1-swH3N2	119	36-61*
swH1N1-swH3N2	468	257-107*
swH1N1-hH1N1	19	13-2*
hH1N1-swH3N2	7	1-4*
Three		
pH1N1-hH1N1-swH1N1	6	3-0-0*†
pH1N1-hH1N1-swH3N2	1	1-0-0†
pH1N1-swH1N1-swH3N2	114	7-40-32*†
hH1N1-swH1N1-swH3N2	6	0-4-1*

*Lacking samples correspond to same antibody titers.
†Subtype pH1N1 was considered as higher titer, when it exceeded at least two dilutions with respect to the other subtypes.

Table 4. Frequency of the sera titer and average of the antibodies titer of the four influenza subtypes

Titers	pH1N1	hH1N1	swH1N1	swH3N2
<40	1604	2006	273	1356
40	117	59	270	230
80	110	23	495	174
160	131	5	463	146
320	82	1	309	83
640	30	-	171	59
1280	16	-	113	46
2560	4	-	-	-
Average of the titer of antibodies*	283·96	102·06	300·86	315·90

*As positive were considered only those above a 1:80 titer.

pronounced decreasing trend than that observed for swH1N1 antibody titers. Our results show that the titer of antibodies directed against the hH1N1 virus remained consistently low during the study period. For the pH1N1 virus, serological values were positive in all years tested, with the highest number observed in the year 2000 (72) and the lowest number observed in the year 2006 (1). A similar pattern was observed for the swH1N1and swH3N2 viruses, for which the highest titers were obtained in the first year(s) followed by a decreasing trend over time. Regression analysis of the $\log_2$-transformed data (with and without year 2006) showed that antibody titers for pH1N1 ($\beta = -149$), swH1N1 ($\beta = -0{\cdot}174$) and swH3N2 ($\beta = -0{\cdot}254$) viruses tended to decrease across the years ($P < 0{\cdot}0001$), whereas antibody titers for hH1N1 ($\beta = 0{\cdot}008$) virus maintained a low and constant seroprevalence, with no trend over time ($P = 0{\cdot}324$).

Regardless of whether all the sera or only positive sera samples were considered, there were no significant antibody correlations between different subtypes ($P > 0{\cdot}05$), with the exception of the correlation between the swH1N1 and swH3N2 subtypes (table 5).

Discussion

The classical swine influenza (swH1N1) A/swine/New Jersey/11/76 (H1N1) virus differs from the triple reassortment H1N1 virus, a new American lineage that is currently circulating among pigs, as a consequence of the antigenic drift common to influenza viruses. Because the influenza RNA polymerization complex lacks an error correction system, these viruses show a high degree of mutation (approximately 1×10^{-3} to 8×10^{-3} substitutions/year), which leads to an accumulation of point mutations during replication.[17] These mutations replace amino acids in the antigens of surface glycoproteins and lead to selective advantages of mutated viral strains due to the ability to evade preexisting immunity, a process known as 'antigenic drift'.[18] Furthermore, due to the segmental nature of the genome, reassortment of genetic material occurs periodically when the genetic material of the H and N proteins is exchanged in a cell infected by at least two IAV. This process can lead to a "genetic shift", which also allows for the pre-existing immunity of the population to be evaded.[19] Prior to 1998, the surface antigens of classical H1N1 influenza viruses in swine remained relatively stable, whereas substantial antigenic drift of H1 occurred in humans. This divergence has created a substantial antigenic gap between classical swine H1N1 and human seasonal H1N1 viruses. Thus, pigs have become a reservoir of influenza viruses, with the potential to cause an outbreak of major respiratory disease in humans, possibly resulting in a pandemic.[13,20,21] Genetically, the influenza A virus (pH1N1) is very similar to both the classic swine virus and the North American lineage triple reassortment that has circulated in the United States of subtype H1N1 swine over the past 10 years, occasionally infecting humans during the same period.[21,22]

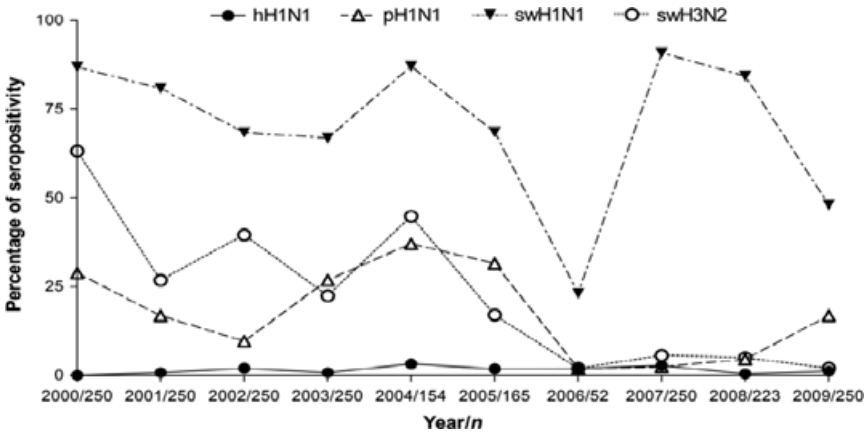

Figure 2. Percentages of seropositivity against four different influenza viruses in backyard pigs during the period 2000–2009.

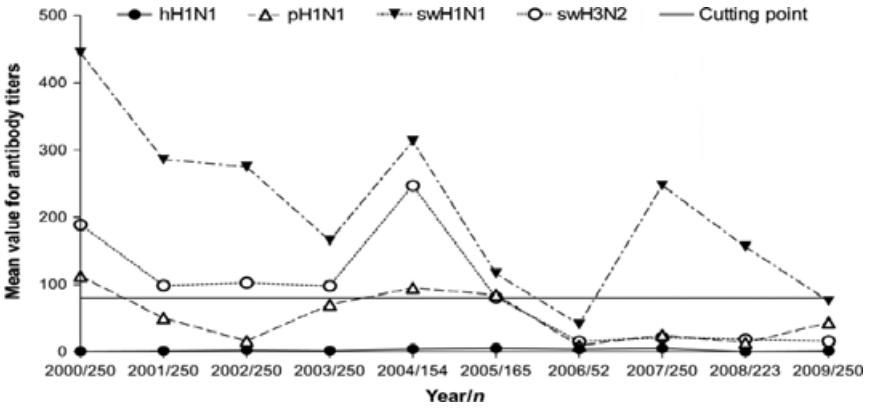

Figure 3. Mean value for antibody titers against four different influenza viruses along a 10-year period.

Table 5. Correlations between antibody titers against different combinations of influenza subtypes, for positive sera versus all sera samples

Sera	pH1N1-hH1N1	pH1N1-swH1N1	pH1N1-swH3N2	swH1N1-swH3N2	swH1N1-hH1N1	hH1N1-swH3N2
Positive	0·20	−0·03	0·05	0·13*	−0·41	−0·38
All	0·06	0·04	0·12	0·43*	−0·01	0·03

*$P < 0·01$.

Seroprevalence for the four subtypes was found at higher proportion in the sites with larger agricultural and farming activities. The number of obtained samples in these sites was higher, because swine farms are more abundantly there. In the sites where the activity is lower due to urbanization, a lower seroprevalence was observed. These data are similar to observations made in other geographical regions.[23]

Our analysis of antibody titers from the 2094 sera tested revealed distinct patterns among the four different influenza viruses. For instance, antibody titers of pH1N1, swH1N1, and swH3N2 tended to decline over time. Antibody titers against pH1N1 were identified in the first years, but they diminished across the years studied. Antibody titers against subtype hH1N1 never exceeded the antibody titers of the other analyzed subtypes. The presence of titers against the pH1N1[24] virus and porcine influenza virus[25] is similar to that reported by other authors. The swine influenza swH1N1 and swH3N2 viruses used for serological testing were isolated 35 and 13 years ago, respectively. Given that the probable antigenic variation incurred during the intervening time period could have accounted for the negative serologic association, for example it seems plausible to assume that if more recent virus strains had been used, specific antibody reactivity may have been greater.

No significant correlation between antibody titers of the different viral strains was observed, except in the case of swH1N1 and swH3N2 viruses. This correlation might be expected because these viruses are endemic to most production models, including intensive and backyard systems.[25,26]

The origin of the hemagglutinin of pH1N1 virus is from classic swine H1N1 virus, that is, genetically similar to this protein, but it is antigenically distinct.[27] In this study, a specific recognition is given for each of the subtypes analyzed.

Our results suggest that a virus with similar antigenic properties to that of the 2009 pandemic pH1N1 virus was circulating in the Mexican swine population in 2000.

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Conflict of interests

The authors declare no conflicts of interest.

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