

Influenza virus epidemiological surveillance in Argentina, 1987–1993, with molecular characterization of 1990 and 1993 isolates

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ABSTRACT

This report describes findings from epidemiological surveillance of influenza virus in two cities in Argentina (Mar del Plata and Córdoba) from 1987 to 1993. It includes information on reporting and serologic characterization of isolated influenza viruses. In addition, determination was made of the nucleotide sequences of the HA1 subunits of five type A (subtype H3) viral strains isolated in the epidemics of 1990 and 1993. The incidence of illness, type of viruses isolated, and H gene sequences were similar to what has been reported from other parts of the world during the same period. The H3 strains isolated in the 1990 and 1993 seasons were somewhat removed in their molecular characteristics from the strains the World Health Organization recommended for vaccines for those years, and appeared closer to the strains recommended for vaccination in subsequent seasons.

Influenza viruses have a casing and carry their genetic information in segments of single chain RNA with negative polarity (1). Of the three known serologic types—A, B, and C—only the

first two types have epidemiological relevance in human disease (2). Type A viruses are classified into subtypes according to their reactivity with antisera against the two principal viral surface antigens, hemagglutinin (H) and neuraminidase (N). Altogether 14 H subtypes (H1–H14) and 9 N subtypes (N1–N9) have been described. The combinations H1N1, H2N2, and H3N2 have been found in humans, the rest only in other animal species (3, 4). Influenza virus is distinguished by its ability to generate antigenic variants that elude the host immune response primed by a previous infection. The mechanisms that produce these variants are: 1) acquisition of a new subtype of H or N (antigenic shift), and 2)

accumulation of amino acid substitutions in the H and/or N protein, as a result of errors introduced by the viral polymerase during replication of the viral genome (antigenic drift) (5).

Hemagglutinin plays a crucial role in the pathogenesis of infection, mediating viral attachment to the host cell and the fusion of membranes that precedes release of the viral genome into the target cell cytoplasm (6). H is a glycoprotein that is synthesized as a polypeptide and subsequently undergoes proteolytic cleavage into two subunits, HA1 and HA2, which are linked by disulfide bonds. HA1 contains the site that attaches to cell membrane receptors, as well as five antigenic areas with sites where neutralizing antibodies at-

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tach (7). These are the areas of greatest amino acid variability between isolates (8).

Because of influenza's health impact, in 1947 the World Health Organization created an international surveillance network for purposes of isolating and characterizing the viral strains that circulate in populations. Thanks to these efforts, supplemented by contributions from additional laboratories, we know that type A virus subtype H3N2 and type B have circulated in the human population from 1968 to the present, and type A subtype H1N1 has also circulated since 1977 (2, 3, 4, 9). With respect to type A subtype H3 virus found in humans, it is known to evolve in nature through progressive accumulation of mutations along a central evolutionary line with short lateral branches (5, 7, 10, 11). This study was undertaken to add to the existing information on the strains found in South America. It describes the results of epidemiological surveillance for influenza virus in the Argentine cities of Mar del Plata and Córdoba from 1987 to 1993. Data are presented on disease notification, serologic characterization of influenza viral isolates, and the sequence of the HA1 subunit in 5 isolates of type A subtype H3N2 from the epidemics of 1990 and 1993.

Clinical samples were obtained and processed in the "Dr. Juan H. Jara" National Institute of Epidemiology in Mar del Plata (NIE) and the "Dr. J.M. Vanella" Institute of Virology in Córdoba (IVC), both of which are World Health Organization National Collaborating Centers. The patients providing samples were receiving treatment for acute respiratory infection (ARI) in hospitals and health centers in the two cities. The IVC also worked up some samples from employees of an automobile factory that serves as a sentinel surveillance site. Virus isolation from nasal and pharyngeal washes taken from patients with influenza-like upper tract ARI was attempted by inoculation

into embryonic chicken eggs. Nasopharyngeal aspirates from patients with lower tract ARI were analyzed by indirect immunofluorescence, and isolation was attempted from those that reacted positively with virus-specific antisera.

In Mar del Plata, influenza incidence rates were obtained from the weekly reports of influenza morbidity in a selection of health centers that serve approximately 120 000 persons. These data were provided by the Eighth Sanitary Region of the Ministry of Health of the Province of Buenos Aires. In Córdoba, rates were taken from the illness registry of an automobile factory with about 5 000 employees. Incidence data were analyzed using the "endemic corridor" method, using two standard deviations as the threshold for the presence of an epidemic (12).

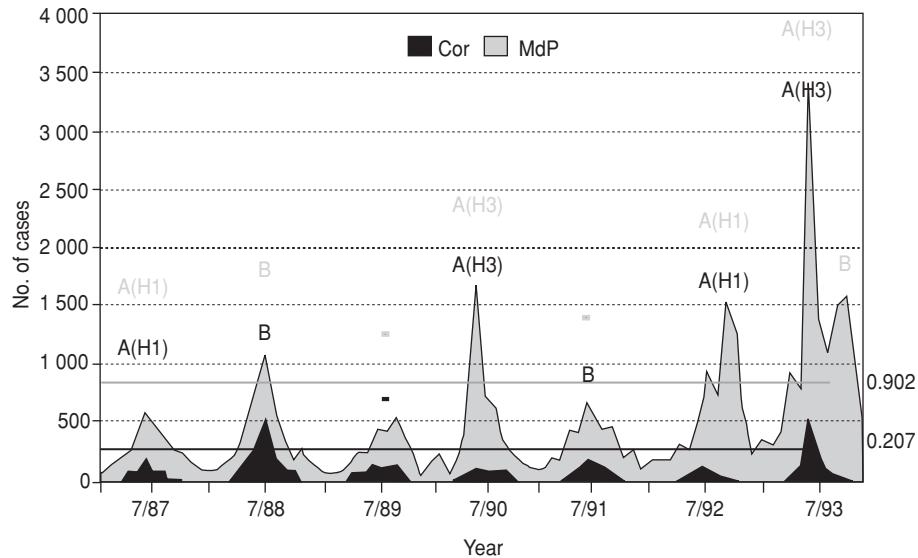
Three oligonucleotides that include conservative sequences from the region that encodes the HA1 subunit of influenza virus, subtype H3 were used. Their names, entire sequences in the direction 5'-3', strings shared with the HA gene (in capitals), and the positions of the shared sequences on the HA gene are: i) *1bHAH3*, ggaattcagaattCT-*TCCCGGAAATGACAACA*, nucleotides 84-102; ii) *2bHAH3*, ggatcccgatc-*cGTTTCTCTGGTACATTCCGCATCC*, nucleotides 1056-1033, and iii) *393HAH3*, GCCTCCCTTAGGTCAC-*TAGTTGCCT*, nucleotides 393-417. The strings in lowercase do not correspond to the HA gene and include targets for restriction enzymes.

A 100 µL aliquot of allantoic liquid (hemagglutinin titer >1/80) was treated with proteinase K in the presence of 1% SDS and the proteins were extracted with a solution of phenol:chloroform. Nucleic acids in the sample were precipitated in the presence of 0.1 M NaCl and ethanol, and resuspended in 20 µL water (13). The first cDNA strip was synthesized in a 50 µL reaction, with 5 µL of genomic RNA, 200 ng of oligonucleotide *1bHAH3*, and 40 U of AMV reverse transcriptase, under conditions described elsewhere

(14). To synthesize the second strand of cDNA and run the polymerase chain reaction, 5 µL of the previous reaction was used, diluted to 100 µL in a solution containing 1 µg of initiator *1bHAH3*, 1 µg of initiator *2bHAH3*, and 2.5 U *ampli-Taq* polymerase (Perkin Elmer) using the buffer supplied by the manufacturer. The mixture underwent 25 cycles of denaturation (94 °C/60 sec), hybridization (37 °C/60 sec), and polymerization (72 °C/90 sec). The resulting amplification products (between 3 and 5 µg) were isolated on agarose gel with a low melting point and ligated in the presence of the vector pGEM-T following the steps recommended by the manufacturer. *Escherichia coli* DH5 cells were transformed with the ligation mixture and those containing the HA gene were identified through hybridization with ³²P-labeled oligo *393 HAH3* (13). The recombinant plasmids were sequenced using the three above-mentioned oligonucleotides with the T7 Sequencing Kit (Pharmacia-Biotech) and ³⁵S-α-dATP. In some cases sequencing was performed directly on the PCR product using the fmol DNA Sequencing System (Promega). The products of the sequencing reactions were analyzed by electrophoresis in denaturing polyacrylamide gels and autoradiography of the dried gel. Between 250 and 330 nucleotides were determined per sequencing reaction.

Antigenic analysis was accomplished using the hemagglutination inhibition test with goat antisera. The nucleotide and amino acid sequences of the HA1 subunits were compared with those previously published. Concordances were established with the Clustal V program (15). The phylogenetic tree (Figure 2) was constructed using the same program, which applies a neighbor-joining algorithm, including corrections for multiple substitutions (16). Positions with gaps were not used in distance measurements. In the interpretation, vertical lines do not represent distances be-

FIGURE 1. Influenza reporting in Córdoba (Cor) and Mar del Plata (MdP), Argentina, 1987–1993^a



^a Epidemiological surveillance of influenza in Mar del Plata and Córdoba, 1987–1993. **Mar del Plata:** Reported clinical cases and epidemic threshold among a population estimated at 120 000 persons. **Córdoba:** Reported clinical cases and epidemic threshold among 5 000 factory employees. Also shown are the type and subtype of virus isolated in each year.

tween sequences, and are only included for clarity. The sum of the lengths of horizontal branches separating two sequences is proportional to their degree of dissimilarity.

Figure 1 presents data on influenza reporting in Mar del Plata and Córdoba in 1987–1993. In Mar del Plata the epidemic threshold of 902 cases per week was surpassed in 1988, 1990, and 1992, with strains B, A(H3), and A(H1) circulating, respectively, and in 1993, when there was a double epidemic wave with H3 and B type viruses circulating. In Córdoba the threshold of 207 cases per week was surpassed in 1988 with type B and in 1993 with virus H3.

Type A (subtypes H1 and H3) and type B viruses were isolated during the study period. The same types and subtypes were isolated in both cities in each season; in the years 1990 and 1993, they were primarily subtype H3. The two cities are situated 1 200 km

apart, Mar del Plata on the Atlantic coast and Córdoba in the interior of the country. The fact that the viral types and subtypes as well as the epidemic curves correspond suggests that the data may be extrapolated to a large area of Argentina. Moreover, the levels of virus circulation and the types and subtypes of virus isolated coincide with the patterns described in other regions of the world during the same period (17).

Because of the primary importance of type A, strain H3N2 in 1990 and 1993, our antigenic analysis compared these strains with the prototypes from the preceding and following years. In 1990, the Mar del Plata strains were antigenically similar to Shanghai/11/87 (the source of the 1990 vaccine). Characterization by the CDC demonstrated similarities with the reference strains England/427/88 (MdP/3) and Shanghai/16/89 (MdP/1, 5, and 12), and poor reactivity with Beijing/353/

89 (the source of the 1993 vaccine). The strains from Córdoba, with the exception of Córdoba/2966, were found to be dissimilar to Shanghai/11/87 and more reactive with Beijing/353/89; characterization by the CDC showed similarities with England/427/88 (Córdoba 2965 and 2966) and Shanghai/16/89 (Córdoba 2953 and 2956). The 1993 strains from both cities reacted similarly with antisera against Beijing/353/89 and Beijing/32/92 (the vaccine sources for 1993 and 1994, respectively), with the exception of strain A/MdP/13/93, which was dissimilar to Beijing/353/89.

To take the analysis a step further, we cloned and sequenced the region of the viral gene that codes for the HA1 subunit in 5 strains of subtype H3 isolated during the epidemic periods of 1990 and 1993. The strains selected for this analysis were A/Córdoba/2953/90, A/Córdoba/3105/93, A/Córdoba/3068/93, A/MdP/13/93, and A/MdP/21/93. To obtain cDNA corresponding to the HA1 subunit, purified RNA was used with synthetic oligonucleotides that act as initiators in the synthetic reaction. The cDNA obtained was cloned with a plasmid vector and its nucleotide sequence determined using the procedures detailed earlier in the section describing materials and methods. The corresponding amino acid sequences were deduced, which in every case included the sequence between positions 31 and 302 of the HA1 subunit. The amino acid variations observed among the analyzed strains and between the analyzed strains and the vaccine source strains A/Beijing/353/89 and A/Beijing/32/92 are shown in Table 1.

When influenza virus isolated from mammals grows in an avian host, variants are selected with HA molecular changes that affect amino acids 137, 145, 156, 186, 226, 248, and 276 of the HA1 subunit (18). Since all the strains analyzed in this study grew in chicken embryos, it is probable that the sequences observed differ from the orig-

TABLE 1. Positions of amino acid substitutions in the sequenced strains of influenza viruses, Argentina, 1990 and 1993

| Strain | Position of amino acid substitutions ^a | | | | | | | | | | | | | | | | | |
|-----------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| A/Beijing/353/89 | S | S | E | K | E | S | F | I | I | R | E | N | L | Y | S | T | G | T |
| A/Córdoba/2953/90 | • | • | G | N | K | • | • | V | S | • | • | • | Q | • | • | • | E | • |
| A/Córdoba/3105/93 | N | • | G | N | • | • | • | • | • | • | • | • | Q | • | • | N | • | N |
| A/Beijing/32/92 | • | D | G | N | K | L | V | • | S | • | D | S | Q | • | • | N | • | T |
| A/Mar del Plata/13/93 | • | D | K | N | K | L | • | • | S | S | D | S | Q | H | G | N | • | N |
| A/Córdoba/3068/93 | N | D | K | N | K | L | • | • | S | S | D | S | Q | • | • | N | • | N |
| A/Mar del Plata/21/93 | • | D | T | N | K | L | • | • | S | S | D | S | Q | • | • | N | • | N |
| | | | | * | * | | | * | | | | * | | | | | | * |

^a Amino acid differences in the HA1 subunit (positions 31–302) between the vaccine source strains A/Beijing/353/89, A/Beijing/32/92, and the five strains sequenced in the present study. Positions marked with an asterisk are those that are known to acquire alterations when virus is grown in chicken embryo. Dots indicate conformity with the amino acid of the strain Beijing/353/89.

inal isolates in those positions. For this reason, those positions were not included in the following comparisons between strains. In the case of the four strains isolated in 1993, three (A/Córdoba/3068/93, A/MdP/13/93, and A/MdP/21/93) were very closely related, differing by only a few amino acids. The other strain isolated in that year (A/Córdoba/3105/93), although it is also related to the other three, presents a greater number of substitutions (between 8 and 11 depending on the strain with which it is compared). The degree of difference detected between the Argentine strains in 1993 is similar to that observed during the same season in other regions of the world (10, 11).

Similar to observations made in previous studies, many of the amino acid substitutions that distinguish the Argentine strains from the vaccine source strain A/Beijing/353/89 are located in the antigenic sites of the subunit HA1

(for example, the changes in positions 189, 190, and 193 are in antigenic site B, and changes in position 54 are in the vicinity of site C). This indicates that immune pressure is the principal factor in determining the virus' evolution in nature (6).

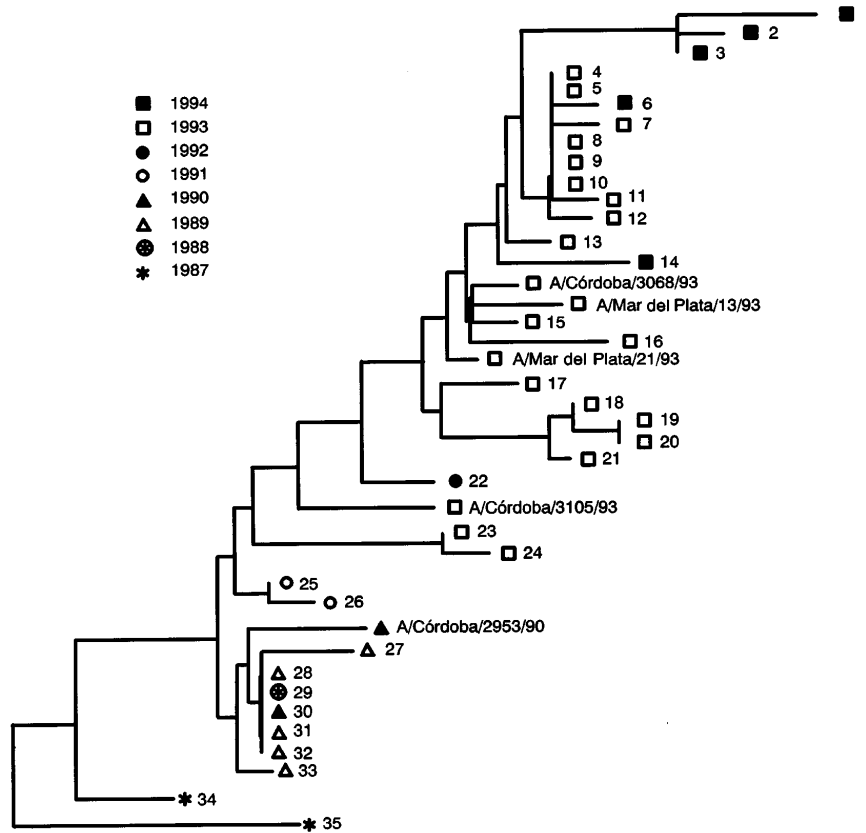
To relate the lineage of the Argentine strains to isolates from other parts of the world a phylogenetic tree (Figure 2) was constructed using the amino acid sequences found in the strains isolated between 1987 and 1994. The structure of the phylogenetic tree is robust, as its global topology does not alter when a tree is constructed from the corresponding nucleotide sequences (data not shown). The strains A/Córdoba/3105/93 and A/Córdoba/2953/90 derive from the same branch as the vaccine source strain A/Beijing/353/89, and in fact only differ from that strain by three amino acid substitutions. The rest of the Argentine strains derive from the same

branch as the vaccine source strain A/Beijing/32/92, with which they share substitutions in the positions 133, 157, 190, and 193.

The Argentine strain isolated in 1990 was found in a subgroup of the tree that also contains all of the strains isolated in the years 1988, 1989, and 1990. The Argentine strains from 1993 are found in the part of the tree with isolates from 1992, 1993, and 1994. The isolate A/Córdoba/3105/93 is found near the strains isolated in early 1993 in England and Scotland while the other three strains are found closer to A/Madrid/252/93 and A/Guangdong/25/93.

In summary, molecular characterization of the Argentine strains leads to the conclusion that they are genetically similar to strains isolated contemporaneously elsewhere in the world. However, the fact that three of the strains isolated in 1993 are very closely related to the antigenic variants A/Madrid/252/93

FIGURE 2. Phylogenetic tree of the HA1 subunit of human influenza virus (H3) isolated between 1987 and 1994^a



^a The tree was prepared using the sequences of amino acids between positions 31 and 302 of the HA1 subunit without considering positions 137, 145, 156, 186, 248, and 276, which are associated with adaptations of the virus when grown in chicken embryos. In addition to the strains sequenced in this study, the following strains were included: 1. A/England/67/94; 2. A/England/68/94; 3. A/Hong Kong /1/94; 4. A/Scotland/160/93; 5. A/Scotland/174/93; 6. A/England/7/94; 7. A/England/286/93; 8. A/England/328/93; 9. A/England/347/93; 10. A/England/269/93; 11. A/Scotland/173/93; 12. A/England/284/93; 13. A/England/247/93; 14. A/Hong Kong/2/94; 15. A/Madrid/252/93; 16. A/Guangdong/25/93; 17. A/Shangdong/9/93; 18. A/England/289/93; 19. A/England/471/93; 20. A/Scotland/142/93; 21. A/England/346/93; 22. A/Beijing/32/92; 23. A/England/1/93; 24. A/Scotland/2/93; 25. A/Hawaii/01/91; 26. A/South Dakota/01/91; 27. A/Guangdong/39/89; 28. A/Beijing/4/89; 29. A/England/427/88; 30. A/Indiana/01/90; 31. A/Singapore/12/89; 32. A/Singapore/13/89; 33. A/Beijing/353/89; 34. A/Shanghai/11/87; 35. A/Wyoming/3/87.

and A/Guangdong/25/93 suggests that the virus that circulated in Argentina in the 1993 season may differ antigenically from the primary variant encountered elsewhere.

These results underline the importance of epidemiological surveillance of influenza and the molecular characterization of the virus strains isolated in each country, in order to select the

most appropriate vaccine in each season. In addition, the analysis of strains from different geographic areas is important to understand the global pattern of evolution of the virus.

REFERENCES

1. Lamb RA. Genes and proteins of influenza viruses. In: Krug RM. *The influenza viruses*. New York and London: Plenum Press; 1989: pp. 1-88.
2. Kilbourne ED. The epidemiology of influenza. In: Kilbourne ED. *Influenza*. New York and London: Plenum Publishing Corporation; 1987: pp. 255-289.
3. Smith FI, Palese P. Variation in influenza virus genes. In: Krug RM. *The influenza viruses*. New York and London: Plenum Press; 1989: pp. 319-359.

4. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev* 1992;56:152-179.
5. Cox NJ, Bender CA. The molecular epidemiology of influenza virus. *Semin Virol* 1995; 6:359-370.
6. Wharton SA, Weis W, Skehel JJ, Wiley DC. Structure, function and antigenicity of the hemagglutinin of influenza virus. In: Krug RM. *The influenza viruses*. New York and London: Plenum Press; 1989: 153-188.
7. Wiley DC, Skehel JJ. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Ann Rev Biochem* 1987;56:365-394.
8. Ina Y, Gojobori T. Statistical analysis of nucleotide sequences of the hemagglutinin gene of human influenza A viruses. *Proc Nat Acad Sci USA* 1994;91:8388-8392.
9. World Health Organization. *Respiratory viruses: Report of a WHO Scientific Group*. Geneva: WHO; 1969. (Technical Report 408).
10. Cox N, Xu X, Bender C, Kendal A, Regnery H, Hemphill M, et al. Evolution of hemagglutinin in epidemic variants and selection of vaccine viruses. In: Hannoun C, Kendal AP, Klenk HD, Ruben FL. *International Conference on Options for the Control of Influenza II*. Amsterdam; New York: Elsevier Science Publishers; 1993; pp. 223-230.
11. Ellis JS, Chakraverty P, Clewley JP. Genetic and antigenic variation in the hemagglutinin of recently circulating human influenza A (H3N2) viruses in the United Kingdom. *Arch Virol* 1995;140:1889-1904.
12. Stroup DF, Williamson DF, Dean AD, Haddad S, Basha N, Rapose W. Statistical Software for Public Health Surveillance. Atlanta, Georgia: Centers for Disease Control and Prevention; 1994.
13. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning, a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
14. Portela A, Melero J, Martínez C, Domingo E, Ortín J. Oriented synthesis and cloning of influenza virus nucleoprotein cDNA that leads to its expression in mammalian cells. *Virus Res* 1985;4:69-82.
15. Higgins DG, Bleasby AJ, Fluchs R. CLUSTAL V: Improved software for multiple sequence alignment. *Comput Appl Biosci* 1992;8:189-191.
16. Saitu N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406-425.
17. *Wkly Epidemiol Rec* 63 (44), October 28, 1988; 64 (50), December 15, 1989; 65 (46), November 16, 1990; 66 (50), December 13, 1991; 67 (50), December 13, 1992; 68 (45), November 5, 1993.
18. Rocha EP, Xu X, Hall HE, Allen JR, Regnery HL, Cox NJ. Comparison of 10 influenza A (H1N1 and H3N2) haemagglutinin sequences obtained directly from clinical specimens to those of MDCK cell- and egg-grown viruses. *J Gen Virol* 1993;74:2513-2518.

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RESUMEN

Vigilancia epidemiológica de los virus de gripe en la Argentina, 1987-1993, y caracterización molecular de los aislados en 1990 y 1993

En este informe se describen los resultados de la vigilancia epidemiológica de virus de gripe en dos ciudades de la Argentina (Mar del Plata y Córdoba) de 1987 a 1993. Se incluye información acerca de la notificación y la caracterización serológica de los virus aislados. Además, se determinaron las secuencias de nucleótidos de las subunidades HA1 de cinco cepas tipo A (subtipo H3) aisladas durante las epidemias de 1990 y 1993. La incidencia de enfermedad, los tipos de virus aislados y las secuencias genéticas H fueron similares a las notificaciones del mismo período en otras partes del mundo. En sus características moleculares, las cepas H3 aisladas en las estaciones de 1990 y 1993 se distinguían un poco de las cepas que la Organización Mundial de la Salud recomendó incluir en las vacunas de esos años y se parecían más a las cepas recomendadas para vacunación en estaciones subsecuentes.