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ABSTRACT Understanding the genetic evolution of A(H1N1)pdm09 and H3N2 viruses can help better select strains to be included in the annual influenza vaccine. There is little information on their evolution in Jordan so this study investigated the genetic and antigenic variability of A(H1N1)pdm09 and H3N2 viruses in Jordan by performing phylogenetic and genetic analyses of the HA and NA genes of A(H1N1)pdm09 and H3N2 viruses between 2011 and 2013. The full HA and NA genes of 16 H1N1-positive samples obtained in our study and 21 published HA sequences and 20 published NA sequences from Jordanian viruses that were available on online gene databases were analysed. For H3N2, we generated 20 HA and 19 NA sequences and included 19 published HA and NA sequences each in the analysis. Jordanian H1N1 viruses had mutations that are characteristic of antigenic group 6 while H3N2 virus mutations belonged to group 3. No markers of resistance to oseltamivir were detected. The individual mutations are described in detail.
Épidémiologie moléculaire et mutations des virus A(H1N1)pdm09 et H3N2 en Jordanie

RÉSUMÉ La compréhension de l'évolution génétique des virus A(H1N1)pdm09 et H3N2 permet de mieux sélectionner les souches devant être ajoutées au vaccin antigrippal annuel. Peu de renseignements sont disponibles sur les mutations des virus saisonniers de la grippe A(H1N1)pdm09 et H3N2 en Jordanie. Afin de remédier à ce problème et d'étudier les variations génétiques et antigéniques des virus A(H1N1)pdm09 et H3N2, nous avons procédé à des analyses génétiques et phylogénétiques des gènes de l'hémagglutinine (HA) et de la neuraminidase (NA) de ces virus, sur la période 2011-2013 en Jordanie. L'analyse a porté sur les séquences complètes des gènes de l'HA et de la NA de 16 échantillons positifs au virus H1N1 prélevés dans le cadre de cette étude, ainsi que sur 21 séquences publiées de l'HA et 20 séquences publiées de la NA, issues de virus jordaniens disponibles sur les bases de données de gènes en ligne. Pour le virus H3N2, nous avons généré 20 séquences de l'HA et 19 de la NA, et avons également inclus dans l'analyse 19 séquences publiées de l'HA et 19 de la NA. Les virus H1N1 jordaniens présentaient des mutations caractéristiques du groupe antigénique 6, tandis que les virus H3N2 appartenaient au groupe 3. Aucun marqueur de résistance à l'oseltamivir n'a été détecté. Les mutations individuelles sont décrites en détail.

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Introduction
Influenza viruses are considered one of the most common causes of respiratory infection among humans and they are associated with high morbidity and mortality (1). Influenza A viruses are classified based on the antigenic properties of the 2 surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) (2). Alterations in these proteins are key determinants of the pathogenicity of influenza viruses and their ability to avoid the host immune response.

The influenza pandemic of 2009 was caused by a novel strain of influenza A(H1N1)pdm09, which was first identified in Mexico and then spread globally. It had a massive impact on the health and economy of all affected regions (3, 4). Genetic analysis confirmed that it was a triple reassorted virus, with polymerase basic (PB)1 from viruses of human origin, HA, nucleoprotein (NP) and non-structural (NS) genes from classical swine virus, PB2 and polymerase A (PA) from North American avian viruses and NA and matrix (M) from Eurasian swine avian-like viruses (5,6). In Jordan, the first 2 confirmed case of influenza strain A(H1N1)pdm09 was reported on 17 June 2009 (7).

The A(H1N1)pdm09 virus gradually replaced the seasonal H1N1 virus and has co-circulated with H3N2 and influenza B virus since 2010. H3N2 viruses have been circulating in humans since 1968 (8). Molecular analysis and antigenic characterization of circulating A(H1N1)pdm09 strains and H3N2 viruses has classified them into genetic group and subgroup (9,10).

Understanding the evolution of A(H1N1)pdm09 and H3N2 viruses is essential for studying diversification, determining genetic and antigenic relations and choosing vaccine strains to be included in the annual influenza vaccine. As there is little information on the evolution of seasonal influenza A(H1N1)pdm09 and H3N2 viruses in Jordan, we performed a phylogenetic analysis and genetic analysis of the HA and NA genes of A(H1N1)pdm09 and H3N2 viruses in Jordan between March 2010 and March 2013.

Methods
Samples

Nasal and throat swabs were collected as part of a large prospective cohort study of 3168 children < 2 years of age who presented with fever and/or respiratory symptoms to a Government hospital in Amman, Jordan, in 2010–2013. Of those, 71 children tested positive for influenza A infection. Details of the cohort study design have been published previously (11,12).

RNA extraction and PCR
Amplification and sequencing of HA and NA genes
RNA was extracted from original swabs using QIAGEN viral extraction kit (Qiagen, Hilden, Germany). One-step real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed with Invitrogen Superscript One-Step RT-PCR and a Platinum Taq kit (Invitrogen, Carlsbad, California, USA), with full-length primers, as described previously (13). The PCR products were separated by 1% agarose gel electrophoresis. Amplicons of the appropriate sizes were subsequently excised from the gel and purified with a QIAGEN gel extraction kit (Qiagen). The purified PCR products were used directly for cycle sequencing reactions in a BigDyeR Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and were further amplified for 26 cycles at 95 °C, 30 s; 50 °C, 15 s; and 60 °C, 4 min. The reaction product was purified by exclusion chromatography on CentriSep columns (Princeton Separations, Adelphia, NJ, USA). The recovered materials were sequenced in an ABI 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). Sequences were generated with SeqMan DNA Lasergene 7 software (DNASTAR, Madison, Wisconsin, USA). The sequences were submitted to GenBank under accession numbers KU933838–KU933908.

**Sequence analysis and phylogenetic tree construction**

DNA Lasergene 7 and BioEdit 7.0 (14) were used for multiple sequence alignment and genomic signature analysis with the ClustalW algorithm (15). The full genes of 16 H1N1-positive samples obtained in this study, in addition to 21 published HA sequences and 20 published NA sequences from Jordanian viruses that were available on GenBank and the Global Initiative on Sharing All Influenza Data (GISAID), were included in the analysis. For H3N2, we generated 20 HA and 19 NA sequences and included 19 published HA sequences and 19 published NA sequences in the analysis.

MEGA 6 was used for phylogenetic tree construction by applying the neighbour-joining method with Kimura's 2-parameter distance model and the reliability of phylogenetic inference at each branch node was estimated by the bootstrap method with 1000 replications (16). Sequence group designation was according to WHO nomenclature (17).

**Results**

**H1N1**

The results of phylogenetic analysis of the HA and NA genes from H1N1 viruses from Jordan are shown in Figures 1 and 2. A/California/07/09 was used as the root for the tree.

Phylogenetic analysis of the HA gene of pandemic H1N1 viruses revealed that the viruses in
Jordan in 2011–2013 viruses belonged to three antigenic groups: 5, 6 and 7 (Figure 1). Antigenic group 6 included isolates from 2012–2013. Seven isolates belonged to group 6.C, which is characterized by mutations V234I, K283E and E172K. Six viruses from 2012 belonged to antigenic group 6.A, which is characterized by mutations H138R and V249L. One isolate from 2012 (A/Jordan/B1713/2012) belonged to antigenic group 7, which had mutation A197T. One isolate (A/Jordan/A0088/2011) from 2011 belonged to antigenic group 5, which shares two mutations, V234I and K283E, with group 6.C.

The genetic analysis (Table 1) showed that all the viruses in this study had one mutation at antigenic site Sb (S185T) and one at antigenic site Ca1 (S203T). Viruses belonging to antigenic group 6.A had one mutation at antigenic site Ca2 (H148R). One virus had mutation L161I in antigenic site Sa. No changes in the glycosylation sites were detected.

Phylogenetic analysis of the NA gene from pandemic H1N1 viruses revealed that the 2011–2013 viruses belonged to 2 antigenic groups, groups 5 and 6 (Figure 2). Analysis of the antiviral susceptibility of Jordanian strains was based on the presence of mutations encoding amino acid substitutions at key residues that confer resistance to NA inhibitors. None of these viruses displayed oseltamivir-resistant clinical markers at positions D198N, I222R, H274Y, N294S.

**H3N2**

The results of phylogenetic analysis of the HA and NA genes from H3N2 viruses from Jordan during 2011–2012 are shown in Figures 3 and 4. A/Brisbane/10/2007 was used as the root for the tree. Phylogenetic analysis of H3 showed that the viruses belonged to the A/Victoria/361/2011 genetic clade and fell into 2 antigenic groups: 3 and 6. Viruses in group 3 had mutations V223I and D158N and were further divided into 2 groups, 3B and 3C. One isolate belonged to antigenic group 6, which contains mutations D53N, Y94H, I230V and E280A. Two viruses from 2011 had 3 common mutations, G49A, K92R and V347M, and were grouped together.

(Figure 3 - Figure 4)

The H3N2 viruses in our study accumulated 16 mutations as compared with the reference vaccine strain A/Perth/16/2009 (Table 2). The mutations included 4 antigenic site A mutations (I140M, R142G, K144N and N145S), 1 antigenic site B mutation (A198S/P), 4 antigenic site C mutations (D53N, N278K, E280A/P/S and N312S), 4 antigenic site D mutations (T212A,
S214I/V, V223I and I230V) and 3 antigenic site E mutations (T48A/I, K62E/I and Y94H).

At the potential N-linked glycosylation sites of H3N2 viruses, amino acid 45 (SSS) was mutated to (NSS) in 4 strains, which created an additional glycosylation site. Two isolates lacked the glycosylation site at amino acid 63. Most isolates lost a glycosylation site at amino acid 402 in the NA gene (Table 3).

Discussion

All the influenza H1N1 viruses detected in the world after pandemic 2009 have been related to A(H1N1)pdm09. Genetic variations have been observed in circulating A(H1N1)pdm09 viruses, but they remain antigenically similar to the A/California/07/2009 vaccine strain (18). Most isolates of A(H1N1)pdm09 in our study belonged to antigenic subgroups 6A and 6C. In the case of A(H3N2), all viruses from the 2011–2012 season sequenced in the study belonged to the A/Victoria/208/2011-like lineage, and most fell into antigenic subgroup 3B. Similar results were found in isolates in neighbouring Lebanon (19). The Jordanian isolates closely resembled the WHO-designated reference strains A/Stockholm/18/2011 and A/England/259/2011, with amino acid substitutions in HA1/HA2 (N145S and V223I A198S, N312S and D158N) (20).

Patterns of antigenic site variation were observed by amino acid alignment of the HA genes of A(H1N1)pdm09 and H3N2 viruses. In A(H1N1)pdm09, amino acid mutations were detected at four antigenic sites (Sa, Sb, Ca2 and Ca1), while A(H3N2) showed mutation at antigenic sites (A, B, C, D, and E). N-Glycosylation affects not only the antigenic and functional properties of surface proteins but can also provide an opportunity for immune escape (21,22). The 3 glycosylation sites (residues 146, 367 and 402) on the head of the NA monomer encoded by H3N2 strains are located around the enzymatic active site (23). We observed that most isolates lost a glycosylation site at 402 in the NA gene of A(H3N2).

In the 2014–2015 season, antigenic drift of subtype H3N2 decreased vaccine effectiveness. The H3N2 strain of the 2014–2015 influenza vaccine was A/Texas/50/2012, which belongs to antigenic subgroup 3C.1 (24). In September 2014, the WHO consultation and information meeting on the composition of influenza virus vaccines indicated the emergence of 2 new genetic subgroups, 3C.2a and 3C.3a, of A(H3N2) viruses containing antigenic drift viruses of previously circulating strains (25). WHO recommended that the H3N2 component be updated with an A/Switzerland/9715293/2013-like (antigenic subgroup 3C.3a) virus for the 2015–2016 vaccine for the Northern Hemisphere (26).
Antigenic and genetic analyses are invaluable in guiding effective influenza vaccine selection and control measures. The results of this study contribute to understanding the genetic and antigenic evolution of A(H1N1)pdm09 and H3N2 viruses in Jordan. Further investigation is required to better understand how genetic and antigenic diversity and how to utilize it for vaccine strain selection.

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References


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