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GENOMIC VARIABILITY AND NEURAMINIDASE INHIBITOR DRUG SUSCEPTIBILITY PATTERNS OF INFLUENZA A/H1N1PDM09 STRAINS CIRCULATING IN MOROCCO DURING THE 2015-2016 SEASON

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ABSTRACT

Information regarding the genomic variability, and susceptibility profile to antiviral drug resistance are scarce for influenza A/H1N1pdm09 strains circulating in Morocco. The aims of this study are to survey genetic changes in the hemagglutinin (HA) and neuraminidase (NA)genes and to phenotypically assess the antiviral drug resistance (AVR) to neuraminidase inhibitors (NAIs) drugs-related mutations.

During the 2015-2016 flu season, 1189 nasopharyngeal swabs samples were collected from influenza-like illness (ILI) and severe acute respiratory illness (SARI) patients in eight sentinel sites distributed throughout the country. Reactive samples by rRT-PCR were inoculated on MDCK cell-line for virus isolation. Sequencing and phylogenetic analysis of HA and NA genes were performed to evaluate the genetic diversity of Moroccan A/H1N1pdm09 strains. AVR screening against two NAIs (oseltamivir and zanamivir) was performed on MDCK-propagated viruses using the functional neuraminidase inhibition assay.

Among those 1,189 specimens, 185 were A/H1N1pdm09, 59 A/H3N2, and 30 influenza B. Ninety-six of the 185 A/H1N1pdm09 propagated strains had a HA titer of at least 1:16 and were subjected to NAIs assay. Phylogenetic analysis of HA and NA genes revealed that Moroccan A/H1N1pdm09 isolates clustered with the predominate genetic group 6B.1. Several amino acid changes in the antigenic site were identified: S203T, S189T, K163Q, and S162N (N1 numbering), as well as a unique S185T in the receptor-binding domain. The NA assay showed no increased resistance (normal inhibition) against both NAI drugs in all tested isolates.

This study confirms that A/H1N1pdm09 circulating strains in Morocco have undergone genetic variability and dynamic evolution. Surveillance of viral genetic evolution is important for disease management and appropriate public health interventions during influenza outbreaks

Keywords: Influenza, Hemagglutinin; Neuraminidase; Neurminidase Inhibitors

INTRODUCTION

Human influenza is an acute respiratory infection transmitted via direct contact and respiratory droplets **[1]** causing respiratory illness and is responsible for significant mortality and morbidity during seasonal epidemics **[2, 3]**.

Indeed, seasonal influenza triggered 5 to 20% of the world's population causing severe disease in 3 to 5 million people and the death of 290,000 to 650,000 people worldwide every year **[4]**.

Two main types of seasonal influenza viruses were identified to be the cause of illness among human being: The flu virus type A, linked to seasonal epidemics in the winter months and global pandemics, and the flu B virus associated with seasonal epidemics, but not typically with pandemic **[5]**.

Despite the seasonal circulation of flu viruses, sporadic and unpredictable pandemic occurs every 3 to 5 decades, enabling the introduction of influenza A virus strain, antigenically different from previous evolving strains **[6]**.

Accumulation of minor mutations within the antibody-binding sites on the two main viral surface proteins hemagglutinin (HA) and neuraminidase (NA) (antigenic drift), or major changes due to genetic reassortment (antigenic shift) can result in the emergence of antigenically distinct viruses and be responsible of seasonal epidemics or pandemics, respectively **[7, 8]**.

In April 2009, a novel A/H1N1 virus emerged and spread rapidly; It spread to all continents in less than nine weeks becoming the first pandemic of the 21st century **[9]**.

The so called A/H1N1pdm09 virus had a complex combination of gene segments from avian, swine, and human influenza viruses **[10]**. Since 2010, the A/H1N1pdm09 strain replaced the seasonal A/H1N1 and became the endemic strain **[11]**. Upon the onset of the A / H1N1pdm09 virus, molecular determinants unrecognized by human cells seemed to favor the transmission and the establishment of the AH1N1viruses **[10]**.

This had directed research towards the study of the antigenic sites which are keys to population immunity and viral fitness.

Similarly, monitoring of the susceptibility of influenza viruses to antiviral drugs particularly neuraminidases inhibitors (NAIs) is necessary to allow rapid detection of resistant viruses and prevention of further transmission **[8]**.

In fact, mutations associated with NAI resistance occur at the eight catalytic sites or 11 residues of the sialic acid binding pocket (SA) framework of Neuraminidase. These mutations are responsible for a decrease in the level of interaction of Neuraminidase and the binding pocket of sialic acid **[12]**. Understanding the diversity and epidemiology of the virus is essential to devising strategies, both for the control of viral spread and in overcoming drug-resistance **[13]**.

The WHO recommends that health authorities should monitor respiratory disease activity and the {Citation}circulation of the influenza A/H1N1pdm09 virus during the post-pandemic period to assess important genetic, antigenic, and functional changes associated with antiviral drug resistance.

In 2012-2013, the World Health Organization (WHO) Global Influenza Surveillance and Response System (GISRS) Expert Working Group for Antiviral Susceptibility Surveillance conducted global surveillance the susceptibility of influenza A and B viruses to NAIs.

In Morocco, INAs were used for the first time in 2009 as a preventive and curative approach in the management of swine flu and for the care of people at risk. Since then, a more widespread use of NAIs, more particularly Oseltamivir, has been observed at the national level. However, to our knowledge, no study has been undertaken to assess the epidemiological situation of the profiles of susceptibility or resistance to antivirals of influenza viruses circulating in Morocco.

The aims of this study were to establish genetic changes in the hemagglutinin (HA) and neuraminidase (NA) genes and to phenotypically assess the antiviral drug resistance (AVR) to neuraminidase inhibitors (NAIs) drugs-related mutations of the A/H1N1 pdm09 evolving in Morocco.

2. MATERIALS AND METHODS

Study population

Out-patients with ILI symptoms, attending the eight sentinel site (health centers) around the counrty , and in-patients with SARI, admitted in eight University and Regional hospitals were enrolled in the framework on the virological surveillance of influenza. The inclusion criteria for ILI patients were sudden onset of fever (> 37.8 °C) with more than two of the following symptoms: cough, rhinorrhea, myalgia, arthralgiaand diarrhea; The inclusion criteria for SARI patients were requierment for hospitaliazation for the following symptoms : history or measured fever of ≥ 38 °C, cough, onset within the last 10 days, difficulty in breathing with chest in-drawing for children under 5 years of age, cough, rhinorrhea, myalgia, arthralgiaand diarrhea.

Collection and transport of clinical specimens

Oropharyngeal (OP) and Nasopharyngeal (NP) swabs specimens along demographic information were collected at the sentinel surveillance sites from enrolled patients presenting ILI and SARI symptoms. The swabs were placed into collection tubes containing 3ml of viral transport media (VTM). The specimens were packaged using a standard triple packaging system at the sentinel sites and shipped in cool boxesto the national influenza center (NIC)within 48 to 72 hours.

RNA extraction and real-time RT-PCR

Viral RNA was extracted from 200 µL nasopharyngeal swab supernatant of the original clinical sample for initial typing and subtyping/lineage detection of influenza A or B, using the automatic extraction method (iPrepTM Purelink Virus Kit; Thermo Fisher Scientific Carlsbad, CA 92008 USA).

For sequencing analysis, RNA was purified from isolates using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Screening of influenza viruses from original clinical specimens for initial typing as Type A or B. and subtyping PCR for A(H1N1)pdm09 or A(H3N2) and differentiation for B/Victoria or B/Yamagata lineages was performed usning the CDC Human Influenza Virus Real-time RT-PCR Detection and Characterization kits provided by the CDC in Atlalta as part of a material transfer agreement that ensures privacy and non-publication.

According to the kit providers, amplification curve with a Cycle threshold of less than 37 were considered nucleic acid detected for any target assayed **[14]**.

Virus isolation

Viral isolation was performed by inoculating 100 µl of samples confirmed positive for A/H1N1pdm09 into two wells of a 24-well plate with 70–90% confluent monolayers Madin–Darby Canine Kidney (MDCK) cell line. After 30 min of adsorption at room temperature, 2 mL per well of minimum essential medium (MEM) supplemented with 0.18% Bovine Serum Albumin (BSA) Fraction V, 50 units/ml of penicillin, 50 µg/ml of streptomycin (Invitrogen, Carlsbad, CA) and 2 mg/ml of TPCK trypsin were added. Cells were then incubated in sealed bags at 35 °C. Viral replication was assessed by the HA assay. Viral isolates showing HA titers ≥ 16 up to two passages were selected for further sequencing and phenotyping analysis **[15]**.

Genome Sequencing and phylogenetic analysis

Conventional PCRs targeting HA and NA genes were performed on the nucleic acid extracts from cell culture isolates. PCR products were purified and sequenced using the ABI Big Dye Terminator, version 3.1 (Applied Biosystems, Cat No 4337455, Foster City, USA) and M13 primers (CDC protocol unpublished) on an ABI 3130XL sequencer. The sequences of HA and NA genes were assembled, edited, and aligned using Sequencher® (Gene Codes Corporation, MI, USA). The phylogenetic analysis of the HA and NA genes were constructed using Mega 7.0 software by the neighbour-joining method with bootstrap analysis of 1000 replicates.

The nucleotide sequence genes of HA and NA from this study have been submitted to the Global Initiative on Sharing Avian Influenza Data (GISAID).

Neuraminidase (NA) inhibition assay and IC50 analysis,

The NAI phenotypic assay was performed to assess the susceptibility of the Moroccan A/H1N1pdm09 circulating strains to two NAIs drugs, oseltamivircarboxylate (Hoffmann–La Roche (Basel, Switzerland) and zanamivir (GlaxoSmithKline; Uxbridge, United Kingdom) using a fluorescent enzyme inhibition assay based on a previously published CDC protocol **[16]**. Two viral strains controls, A/Perth/265/2009 (sensitive) and A/Perth/261/2009 (resistant) by the NAI's were included to validate each assay.

Neuraminidase inhibition activity reciprocal to the fluorescence emitted by each drug concentration was used to calculate the overall median inhibitory concentration (IC50) using JASPER V1.2 curve-fitting software **[16]**. Interpretation of the IC50 was evaluated using the WHO AVWG criteria based on the fold change of IC50 values compared with drug-sensitive reference virus IC50 values. For influenza A viruses, a <10 fold increase in IC50 represents normal inhibition, and a 10–100 fold increase represents reduced inhibition, while a >100 fold increase is highly reduced inhibition **[17]**.

Statistical analysis:

Proportions, means and all statistical analyses were performed using the Epi-info version 7.1 software developed by the CDC (CDC; Atlanta, USA).

For IC50 values calculation, JASPR curve-fitting software (Meck Alderich). Fold changes in IC50 dassessed by comparing IC50 of test viruses to the mean IC50 (outliers excluded) by respective drug and influenza type/subtype.

Ethical consideration:

The protocol was approved by the Ministry of Health as part of the tools used forconducting the virological surveillance of influenza and other respiratory viruses with epidemic potential. Participants identity remain anonymous, and therefore did not require an assessment of the ethics committee or IRB approval. Verbal consent was obtained from all patients.

3. RESULTS

Influenza virological surveillance

From September 2015 to August 2016, a total of 1189 specimens were collected from ILI and SARI patients, referred to National Influenza Centre; Virology Department, National Institute of Hygiene, Morocco. Using the real time RT-PCR, 23 % (n=274) of all samples were flu positive. The influenza A/H1N1pdm09 represented the majority of circulating viruses during the season 2015-2016 (15,6% , n= 185). Influenza A/H3N2 viruses accounted for 5% (n=59), whereas influenza B strains represented 2,5% (n= 30) of the tested samples (**Table1**).

Phylogenetic characterization

The phylogenetic trees of the HA and NA gene of A/H1N1pdm09 were constructed using sequences from both the Moroccan isolates and public databases (the Global Initiative on Sharing Avian Influenza Data (GISAID)) (**Table 2**). Phylogenetic analysis of the 10 HA and NA genes of the 2009 Pandemic A/H1N1 viruses circulating during 2015/2016 season demonstrate that the Moroccan isolates clustered with the strains of antigenic group 6B.1, the group that emerged at the end of 2016. High sequence identities were observed for HA and NA in influenza A/ H1N1pdm09, displaying 85 and 99 % nucleotide identities, respectively, with the A/H1N1pdm 09 Northern Hemisphere WHO vaccine strain A/California/07/2009 in the 2017-2018 season (**Figure 1, Figure 2**).

Amino acid variations of HA gene.

Compare to the California strain of 2009 (A/California/7/2009), a significant amino acid change was identified. Using N1 numbering, P83S, S203T and I321V have been found to be consistent in A/H1N1pdm09 of 2009-2010 strains from the initial outbreak. Substitutions of D97N and S185T are characteristic of genetic group 6. K163Q, A256T, and K283E define group 6B, while S84N, S162N and N216T define subclade 6B1. Among the specific amino acid substitutions, four were located in antigenic sites: S203T in Ca and S185T in Sb domains, while K163Q and S162N were found in the Sa domain of HA gene **[18]**. Analysis of the receptor-binding domain (RBD63-286) revealed amino acid variations of S185T in the 190 helix (184–195) regions of predicted antigenic sites of all HA sequences reported during the study season, while, the 130-loop (131–138) and 220-loop (218–228) structure were found to be highly conserved. In contrast, several minor changes were observed at positions A256T and K283E in HA1 and E374K, S451N, and E499K in the HA2 polypeptide. The notable features in the HA gene sequence of A/Fes/231/2016 were polymorphisms at residues 127 and 222 of HA1 **(Table 3)**. The HA D222G substitution is reportedly associated with severe disease **[19]**. All Moroccan viruses carried nine potential sites of N-glycosylation motifs in HA1 (10, 11, 23, 87, 162, 276, 287 and 154, 213 in HA2). S162N marker which is specific to the subclade 6B1 viruses, creates a new potential N-glycosylation motif within the Sa antigenic site.

Amino acid variations of NA gene.

Globally, H275Y and N295S mutations in the NA polypeptide have been associated with multiple drug resistance in Influenza A **[20]**. These two mutations were not detected in any of the nine isolates analyzed in this study. However, several other amino acid substitutions like N44S, N200S, V241 and N369K in genetic group 6 viruses; I34V L40I I321V N386K and K432E in genetic group 6B viruses; and V13I, K264I, N270K, and I314E in genetic group 6B.1 were detected. The 11 framework (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, E425) and eight functional (R118, D151, R152, R224, E276, R292, R371, Y406) residues comprising the NA catalytic site [8] were conserved among all nine viruses (**Table 4**). The Moroccan A/H1N1pdm09 viruses circulating during 2015-2016 carried eight conserved potential N-glycosylation motifs in the NA (position 42, 50, 58, 63, 68, 88, 146, and 235).

Phenotypic analysis

A total of 100 A/H1N1pdm09 virus samples had a titre at least > 1:8 in the haemagglutination and were subjected to the NAI assay. Using the standardised definitions for reporting NAI susceptibility data **[17]**, the NAI test showed that all influenza A/H1N1pdm09 viruses tested in this study were sensitive to both drugs oseltamivir and zanamivir (**Table 5**).

Table 1: Demographic characteristics and influenza prevalence among patients with ILI and SARI, Morocco, 2015–2016

Characteristic	ILI (n = 624) No. (%)	SARI (n = 565) No. (%)	Total (n = 1189) No. (%)
Age			
0–23 months	38 (6.1)	327 (57.9)	365 (30.7)
2–5 years	62 (9.9)	62 (11.0)	124 (10.4)
6–15 years	68 (10.9)	20 (3.5)	88 (7.4)
16–49 years	296 (47.4)	63 (11.2)	359 (30.2)
50–64 years	100 (16.0)	41 (7.3)	141 (11.9)
≥ 65 years	42 (6.7)	28 (5.0)	70 (5.9)
Unknown	18 (2.9)	24 (4.3)	42 (3.5)
Gender			
Female	352 (56.4)	244 (43.2)	596 (50.1)
Male	272 (43,6)	321 (56,9)	593 (49,9)
Influenza subtype			
A (H1N1) pdm09	124 (19.9)	61 (10.8)	185 (15.6)
A (H3N2)	51 (8.2)	8 (1.4)	59 (5.0)
B	24 (3.8)	6 (1.1)	30 (2.5)
Negative	425 (68.1)	490 (86.7)	915 (77.0)

ILI =influenza-like illness; SARI = severe acute respiratory illness

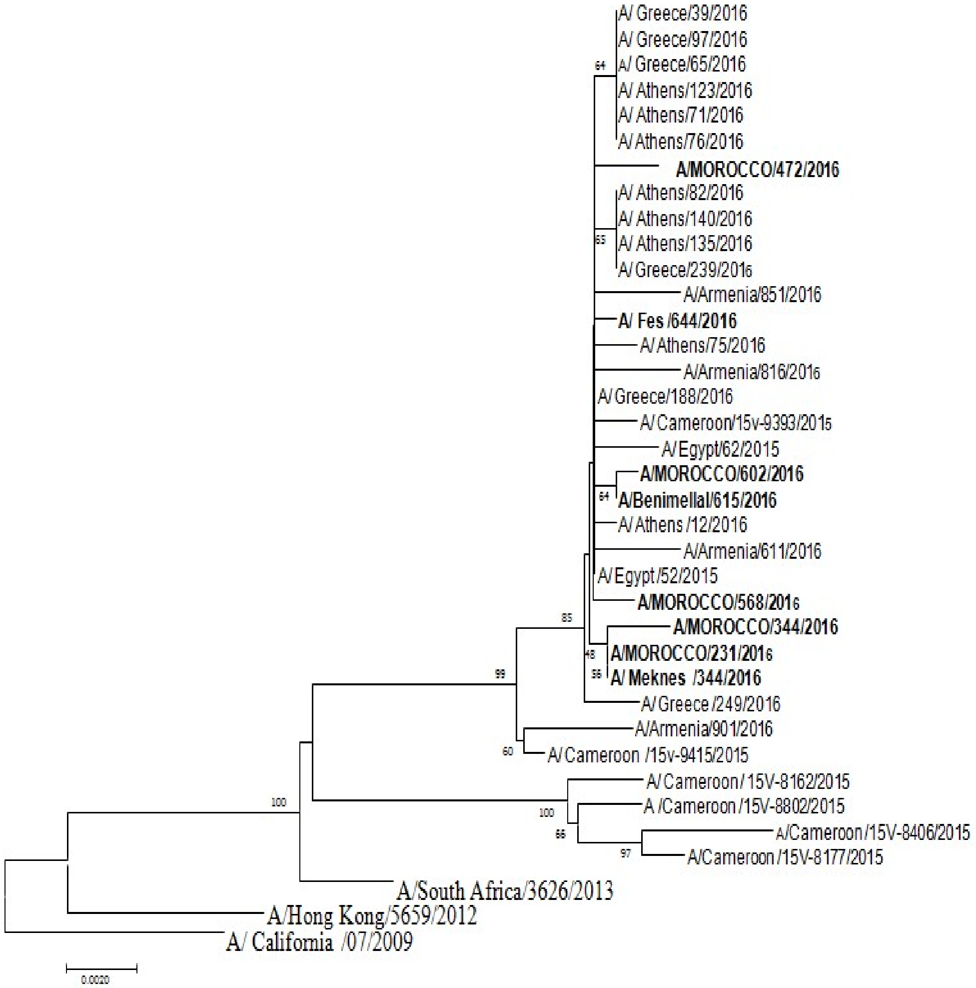

Figure 1: Phylogenetic relationships of the HA of A/H1N1pdm09 viruses isolated in Morocco compared to vaccine strain, A/California/7/09.The tree was constructed with the Neighbour-Joining method with bootstrap analysis of 1000 replicates using Mega7 software

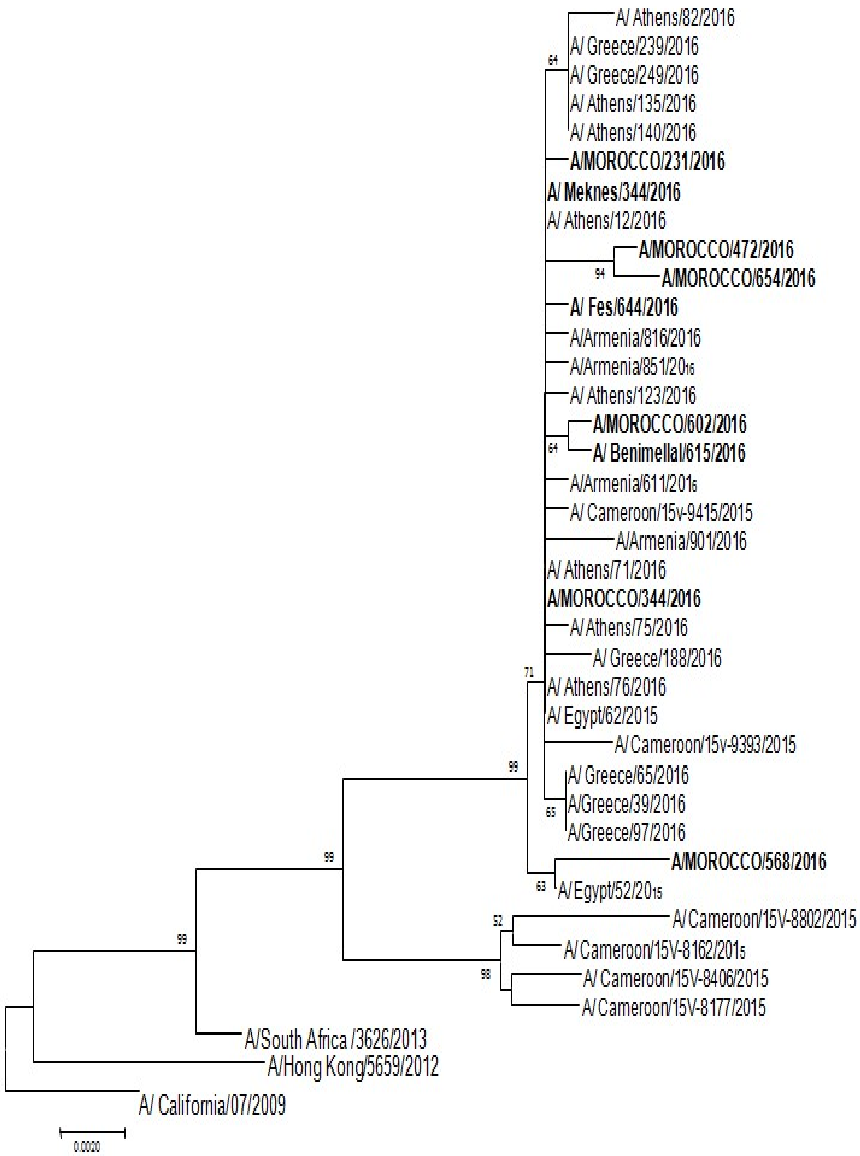

Figure 2: Phylogenetic relationships of the NA of A/H1N1pdm09 viruses isolated in Morocco compared to vaccine strain, A/California/7/09.The tree was constructed with the Neighbour-Joining method with bootstrap analysis of 1000 replicates using Mega7 software

Table 2: GISAID accession numbers for the haemagglutinin sequences (segment 4) and neuraminidase sequences (segment 6) of Moroccan influenza A/H1N1 09pdm strains

A/H1N12009pdm	accession of HA sequences	accession of NA sequences	Submitting laboratory
A/Morocco/ 11/2009	**EPI316932**	**EPI316933**	**National Institute of Hygiene**
A/Morocco/ 51/2009	**EPI316935**	**EPI316936**	**National Institute of Hygiene**
A/Morocco/ 49/2009	**EPI316938**	**EPI316939**	**National Institute of Hygiene**
A/Morocco/ 231/2016	**EPI729910**	**EPI729911**	**National Institute of Hygiene**
A/Morocco/ 602/2016	**EPI729912**	**EPI729913**	**National Institute of Hygiene**
A/Morocco/ 344/2016	**EPI748990**	**EPI749891**	**National Institute of Hygiene**
A/Benimellal/ 614/2016	**EPI748820**	**EPI748821**	**Crick Worldwide Influenza center**
A/Fes/ 644/2016	**EPI748906**	**EPI748907**	**Crick Worldwide Influenza center**
A/Meknes/ 344/2016	**EPI730811**	**EPI737407**	**Crick Worldwide Influenza center**
A/Morocco/ 472/2016	**EPI921259**	**EPI921270**	**National Institute of Hygiene**
A/Morocco/ 568/2016	**EPI921376**	**EPI921377**	**National Institute of Hygiene**
A/Morocco/ 654/2016		**EPI921448**	**National Institute of Hygiene**

Table 3: Comparison of amino acid sequences of HA gene of A/H1N1pdm09 Morocco viruses with reference strain A/California/7/09, showing the specific substitutions

Amino Acids HA	83	84	97	162	163	185	203	216	222	256	283	321	374	451	499
A/california/07/2009	P	S	D	S	K	S	S	I		A	K	I	E	S	E
A/Morocco/ 11/2009	S	.	.	.	.	.	T	.		.	.	V	.	.	.
A/Morocco/ 51/2009	S	.	.	.	.	.	T	.		.	.	V	.	.	.
A/Morocco/ 49/2009	S	.	.	.	.	.	T	.		.	.		K	.	.
A/Morocco/ 231/2016	S	N	N	N	Q	T	T	T		T	E	V	K	N	K
A/Morocco/ 602/2016	S	N	N	N	Q	T	T	T		T	E	V	K	N	K
A/Morocco/ 344/2016	S	N	N	N	Q	T	T	T		T	E	V	K	N	K
A/Benimellal/ 614/2016	S	N	N	N	Q	T	T	T		T	E	V	K	N	K
A/Fes/ 644/2016	S	N	N	N	Q	T	T	T		T	E	V	K	N	K
A/Meknes/ 344/2016	S	N	N	N	Q	T	T	T		T	E	V	K	N	K
A/Morocco/ 472/2016	S	N	N	N	Q	T	T	T		T	E	V	K	N	K
A/Morocco/ 568/2016	S	N	N	N	Q	T	T	T		T	E	V	K	N	K

Table 4: Comparison of amino acid sequences of NA gene of A/H1N1pdm09 Morocco viruses with reference strain A/California/7/09, showing the specific substitutions

Amino Acids NA	13	34	40	44	106	200	241	248	264	270	314	321	351	369	386	432
A/california/07/2009	V	I	L	N	V	N	V	N	V	N	I	I	Y	N	N	K
A/Morocco/ 11/2009	.	.	.	.	I	.	.	D	.	.	.	.	F	.	.	.
A/Morocco/ 51/2009	.	.	.	.	I	.	.	D	.	.	.	.	F	.	.	.
A/Morocco/ 49/2009	.	.	.	.	I	.	.	D	.	.	.	.	F	.	.	.
A/Morocco/ 231/2016	I	V	V	S		S	I	D	I	K	M	V	F	K	K	E
A/Morocco/ 602/2016	I	V	V	S		S	I	D	I	K	M	V	F	K	K	E
A/Morocco/ 344/2016	I	V	V	S		S	I	D	I	K	M	V	F	K	K	E
A/Benimellal/ 614/2016	I	V	V	S		S	I	D	I	K	M	V	F	K	K	E
A/Fes/ 644/2016	I	V	V	S		S	I	D	I	K	M	V	F	K	K	E
A/Meknes/ 344/2016	I	V	V	S		S	I	D	I	K	M	V	F	K	K	E
A/Morocco/ 472/2016	I	V	V	S		S	I	D	I	K	M	V	F	K	K	E
A/Morocco/ 568/2016	I	V	V	S		S	I	D	I		M		F	K	K	E
A/Morocco/ 654/2016	I	V	V	S		S	I	D	I	K	M	V	F	K	K	E

Table 5: NAI susceptibility of influenza A/H1N1pdm09 isolates by fluorescentNAI assay

NA inhibitors	origin of specimens	IC50(nM)			
		No. Analyzed (n)	Median [range]	Mean ± SD	NAI Sucseptibility (folder change)
Oseltamivir®	ILI	56	1,29 [0,12 - 1,96]	1,31 ± 0,27	S(1)
	SARI	40	1,43 [1,10 - 1,70]	1,45 ± 0,16	S (1)
	Total	96	1,35 [0,12 - 1,96]	1,37 ± 0,24	S(1)
Zanamir®	ILI	56	0,51 [0,15 - 1,59]	0,54 ± 0,27	S(1)
	SARI	40	0,53 [0,12 - 0,91]	0,50 ± 0,17	S(1)
	Total	96	0,51 [0,12 - 1,59]	0,53 ± 0,23	S(1)

4. DISCUSSION

In Morocco, the first laboratory-confirmed A/H1N1pdm09 case was identified on 10 June 2009 in a 19-year-old woman travelling from Canada to Morocco **[21]**. In order to limit the communal spread, the Ministry of Health instituted an infection control plan, which included social distancing, early isolation of cases, and antiviral prophylaxis for close contacts of case patients. During the initial phase of the 2009 pandemic, antiviral drugs were prescribed for all patients with confirmed infection and close contacts. Thereafter, the drugs were recommended for persons with suspected influenza virus infections who were at high risk for severe disease. Over the six seasons since the 2009 A/H1N1pdm emergence, very little virological surveillance of the 2009 A/H1N1pdm in

Morocco has been published. In order to understand the genetic changes and antiviral drug susceptibility of the 2009 A/H1N1pdm, phylogenetic analysis and amino acid modification of the 2015-2016 influenza viruses were examined.

The phylogenetic analysis of HA and NA genes demonstrated that circulating strains belonged to subclade 6B1. This subclade diverges genetically by the mutations they harbored compared to the prototype A/California/07/2009 strain. Among specific amino acid substitutions in HA genes sequences, four were located in antigenic sites: S203T in Ca and S185T in Sb domains along with K163Q and S162N were found in the Sa domain. Substitution S185Twas located in the RBS/190 helix **[22]**. It has been reported that substitutions near or in the RBS can influence the antigenic proprieties of A/H1N1pdm09 strains **[23]**. Certain amino acid substitutions such as D222G are associated with severe disease and poor outcome **[24]**; only one of the Moroccan strains, A/Fes/231/2016, analyzed here displayed this mutation, thus a more accentuated surveillance is recommended. All of the HA genes sequences analyzed in this study present the I321V polymorphism. In contrast to the HA D222G mutation, the effect of retaining isoleucine at this position on disease severity has not been clearly demonstrated **[13]**. Substitutions such E374 and S451N were also present in all the analyzed Moroccan strains. In addition to the variation in the antigenic sites of HA sequences; analysis was also done for number of N-glycosylation sequons in HA. Subclade 6B.1 viruses have an additional N-glycosylation motif. S162N substitution, specific to latter subclade generated a new potential N-glycosylation motif at residues162-164 of HA within the SA antigenic site. Evolutionary studies of A/H1N1strains indicate that the number of N-glycosylation site in HA is relatively conserved, but it appears to increase as influenza viruses evolve **[25]**. The acquisition of N-linked glycosylation sites on the HA protein is thought to play a beneficial role for the virus by shielding antigenic sites from interaction with antibodies **[26]**. In addition, the number and location of glycosylation sites on HA may alter neutralization antibody recognition **[27]**. NA active sites at catalytic and framework residues were also examined, and showed all the viruses isolates had conserved catalytic and framework residues. The isolates from Morocco are found to have the characteristic amino acid substitutions V241I and N369K. It has been documented that amino acid modification of V241I and N369K would improve protein stability in neuraminidase gene which would possibly improve oseltamivir-resistance and virus fitness **[28]**. Furthermore, all strains examined in this study carried the N248D substitution. It has been suggested that mutation at the residue 248 alters the central part of an antibody recognition site on NA **[29]**. Located near H275, this N248D mutation may be associated with oseltamivir resistance **[30]**. In Morocco, limited information is available on the drug resistant profiles of circulating A/H1N1pdm09 strains. To evaluate the impact of amino acid substitution to neuraminidase inhibitor susceptibility particularly oseltamivir and zanamivir, phenotypic assay of the representatives isolates (N=100) was performed. All studied viruses showed normal inhibition to both drugs. The decision by the Moroccan Ministry of Health to use NAIs, especially oseltamivir, as the treatment and control for persons with suspected influenza virus infections that were at high risk for severe disease is still recommended and effective.

CONCLUSION

Findings from the present study confirm that influenza A/H1N1pdm09 strains of subclade 6B.1 predominated in Morocco during the 2015/2016 season, as they remained antigenically closely related to the vaccine strain, despite some genetic changes located at the antigenic sites.

Furthermore, Oseltamivir-resistance related mutations were not detected during the study season. Accumulation of sporadic amino acid substitutions and active dynamic of variation in N-glycosylation sites in the genes of external glycoprotein HA and NA confirm the genetic instability of A/H1N1pdm09 viruses evolving during this season.

Our observation underscores the importance of continued genetic surveillance of A/H1N1pdm09 strains to monitor influenza susceptibility to antiviral drugs. It is also crucial to monitor the virus evolutionary changes in different geographical areas to assist with vaccine strain selection. Influenza surveillance ultimately will help public health in establishing clinical management and therapeutic guidelines.

Author Contributions:

“Conceptualization, methodology FE. IC, AR, TB, YB and HO.;validation, FE, HO and IC formal analysis, FE. ZR, HI, AB IC, SB.; investigation, FE, HO, IC, AB, and IC.; datacuration, FE, HO AB IC; writing—original draft preparation, FE, HO; writing—review and editing, EF, HO.; supervision, FE, HO, IC.; project administration, HO TB.; funding acquisition, HO TB.

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