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Molecular characterization of the hemagglutinin gene of H9N2 avian influenza viruses isolated from broiler flocks in Morocco from 2016 to 2018

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ABSTRACT

Avian influenza viruses of the H9N2 subtype continue to spread in wild birds and poultry worldwide. Infection with H9N2 avian influenza virus was detected for the first time in Morocco in January 2016. In this study, a total of 105 organ and tracheal swab samples from 21 broiler farms in Morocco were collected from July 2016 to October 2018 for H9N2 screening. The suspicion of disease was based on severe respiratory signs such as sneezing, coughing, rales and gasping, while H9N2 virus infection was confirmed by real-time RT-PCR. Hemagglutinin (HA) genes of four isolates were amplified by conventional RT-PCR, sequenced, and aligned for phylogenetic analyses. Among the 21 flocks, 48% (10/21) were qRT-PCR positive for H9, with the cycle threshold values ranging from18.6 to 34.8. The maximum similarity in nucleotide and protein sequences (96-98%) was observed between the Moroccan viruses and an H9 virus isolated from broiler chickens in 2017 in Burkina Faso (A/chicken/BurkinaFaso/17RS93-19/2017) and from a layer chicken in the United Arab Emirates in 2015 (A/chicken/Dubai/D2506/2015). The HA genes revealed the close relationship between the four Moroccan viruses, with 97.9%-99.9% nucleotide identity. Phylogenetic analysis showed that the Moroccan viruses belonged to the G1 lineage, and likely originated from the Middle East, as previously reported in 2016.

Key words: avian influenza; H9N2; molecular evolution; phylogeny; Morocco

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Introduction

Avian influenza viruses (AIV) belong to the Orthomyxoviridae family and the genus Influenza virus A. These viruses are enveloped and contain negative-stranded RNA. Antigenic differences on both viral surface proteins, hemagglutinin (HA) and neuraminidase (NA), led to the classification of AIVs as subtypes. 16 HA subtypes and 9 NA subtypes have been identified in birds and can occur in any of the 144 possible HA and NA combinations (CAPUA and ALEXANDER, 2008; JIANG et al., 2012). The H9N2 low pathogenic avian influenza viruses have been circulating worldwide and are the most prevalent AIV isolated from the poultry industry in the world (UMAR et al., 2016). In addition, H9N2 subtype AIV has also been isolated from other animals, such as partridges, ducks, quail, chukar, pigeons, egret, and swine (XIA et al., 2017; XU et al., 2017; MA et al., 2019). On the basis of the HA gene, H9N2 viruses can be divided into a Eurasian lineage and an American lineage. The Eurasian lineage can be further divided into the A/chicken/Beijing/1/94-like (BJ/94-like), A/quail/Hong Kong/G1/97-like (G1-like) and A/duck/HongKong/Y439/97-like (Y439-like) sublineages (SUN and LIU, 2014). The H9N2 avian influenza virus was isolated from humans for the first time in Hong Kong in 1999 (UYEKI, 2002). Concerns raised that H9N2 may be a potential pandemic candidate prompted a series of molecular and sero-epidemiological surveys around the world (MATROSOVICH et al., 2000). Although no evidence of human-to-human transmission of H9N2 viruses has yet been observed, some H9N2 viruses circulating among poultry have developed human-type receptor specificity, and thus recognize the pattern of sialic acids related to adjacent galactose in conformation α(2, 6); (TOSH et al., 2008; LI et al., 2017).

H9N2 viruses have been widely circulating in the world since their first detection in turkeys in Wisconsin in 1966 (GU et al., 2017). In Asia, H9N2 viruses were regularly isolated from ducks (SHORTRIDGE,1982). Lately, H9N2 viruses have caused epizootics in chickens in many parts of the world: Germany, Italy, Ireland, Iran, Pakistan, Saudi Arabia, United Arab Emirates (UAE), Israel, Jordan, China, South Africa and the United States (ALEXANDER and BROWN, 2000). In Morocco, following the first emergence of this virus in January 2016 (EL HOUADFI et al., 2016), widespread transmission of H9N2 AIV was observed in chickens, but implementation of vaccination helped to control the virus in poultry. H9N2 AIV is, however, currently endemic in all types of poultry production in all regions of Morocco.

In this study, we isolated H9N2 AIV from broiler chickens in different regions of Morocco from 2016 to 2018, and we performed HA gene sequencing and subsequent phylogenetic analyses.

Materials and methods

Sample collection. The samples were collected from January 2016 to December 2018 from 21 broiler farms in different regions of Morocco. A total of 105 organs and tracheal swab samples were collected. Samples from a given farm (n=5) were pooled before further processing so that the study was carried out with 21 pooled samples (1 pool per farm). Table 1 summarizes the information collected from different farms.

Virus isolation. The viruses shown to be positive by real time RT-PCR were isolated in 10-day-oldspecific pathogen free chicken embryonnated eggs. Tissue suspensions were homogenized and centrifuged at 1000 g for 10 min; the supernatant fluids of samples were inoculated into allantoic cavities of five eggs (0.2 mL/embryo). The inoculated embryos were incubated for 48 h at 37 °C then chilled at 4 °C for 4 h before harvesting. The harvested allantoic fluid was clarified by centrifugation, and then stored at -80 °C until use.

RNA extraction. Viral RNA was extracted from 50 µL of the field specimens or infective allantoic fluid using the NucleoSpin® RNA Virus Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

Real time RT-PCR for H9 gene detection. A real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assay was performed with the AgPath-IDTM One-Step RT-PCR Kit on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA), in accordance with the manufacturer's instructions, using primers and probes for generic detection of AIV and the H5, H7 and H9 subtypes, as described by MONNE et al., (2008)

Table 1. Description of the Moroccoflocks tested in this study

Affected farm	Year of isolation	Location	H9 vaccination	Age of flock (days)	CT Value for H9 qRT-PCR detection	Virus identification and accession number
1	2016	Fès-Boulemane	Unvaccinated	20	N	-
2	2016	Casablanca	Unvaccinated	19	31.5	-
3	2016	Marrakech-Tensift- Haouz	Unvaccinated	26	N	-
4	2016	Rabat-Salé-Zemmour-Zaër	Vaccinated	22	27.9	-
5	2016	Doukkala-Abda	Vaccinated	20	N	-
6	2016	Mohammadia	Vaccinated	30	23.8	A/chicken/ Morocco/01/2016 (accession number: KU947112.1)
7	2016	Chaouia-Ourdigha	Vaccinated	33	N	-
8	2016	Taza-Al Hoceima-Taounate	Vaccinated	28	N	-
9	2017	Chaouia-Ourdigha	Unvaccinated	40	31.6	-
10	2017	Fès-Boulemane	Unvaccinated	31	N	-
11	2017	Fès-Boulemane	Unvaccinated	23	18.6	A/chicken/ Morocco/Fès-Boulemane/ SF6/2017 (accession number in process)
12	2017	Casablanca	Unvaccinated	31	19.7	-
13	2017	Marrakech-Tensift- Haouz	Unvaccinated	36	N	-
14	2017	Rabat-Salé-Zemmour-Zaër	Vaccinated	33	N	-
15	2017	Rabat-Salé-Zemmour-Zaër	Vaccinated	30	N	-
16	2017	Oriental	Vaccinated	19	26.8	A/chicken/ Morocco/ Oriental/ SF7/2017 (accession number in process)
17	2018	Fès-Boulemane	Unvaccinated	43	32.5	-
18	2018	Casablanca	Unvaccinated	28	N	-
19	2018	Casablanca	Unvaccinated	36	24.6	A/chicken/ Morocco/ Casablanca / SF8/2018 (accession number in process)
20	2018	Rabat-Salé-Zemmour-Zaër	Vaccinated	29	N	-
21	2018	Oriental	Unvaccinated	20	34.8	-

N - Negative, CT - Cycle Threshold

RT- PCR for N2 subtyping. A pair of primers specific for the N2 subtype was used for PCR amplification (FEREIDOUNI et al., 2008). RT-PCR was performed for subtyping N2 by the one-step protocol using a Superscript III-based one step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. The cycling conditions consisted of 30 min at 50 °C (reverse transcription phase) and then an initial denaturation at 95 °C for 2 min, followed by five touchdown PCR cycles starting with 94 °C for 15 s, 60 °C for 30 s, 68 °C for 1 min, and followed by 30 cycles of 94 °C for 15 s, 54 °C for 15 s, 68 °C for 1 min and a final extension at 68 °C for 5 min.

RT-PCR for HA gene amplification and DNA sequencing. Amplification of the HA gene by RT-PCR was performed using the primers described by (HOFFMANN et al.; 2001). RT-PCR was performed using the Applied Biosystems kit (Life Technologies). The RT-PCR reaction was performed in a 20 µL reaction mixture containing: 2 µL of buffer (10×), 2.5 µL of MgCl2 (25 mmol/L), 2.5 µL of dNTP (10 mmol/L), 0.75 µL of each primer (10 µmol/L), 10.2 µL of sterile water, 0.5 µL of RNAase inhibitor (20 U/µL), 0.3 µL of RT (50 U/µL), and 0.5 µL of Gold Taq polymerase (5 U/µL). Forty cycles at 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 30 s were carried out. A final step at 72 °C for 2 min was added to complete amplification. The predicted size of the product was approximately1700bp.The PCR products were analyzed on 1% agarose gel. PCR products of the expected length were purified with the Nucleospin gel and a PCR cleanup kit (Macherey Nagel), according to the manufacturer's instructions.

The purified RT-PCR products were subjected to Sanger sequencing using the ABI PRISM BigDye terminator cycle sequencing kit (PerkinElmer, Foster City, CA, USA). Assembly and analysis of sequence data were conducted using the BioEdit Software version 5.0.9 (HALL, 1999). Phylogenetic analysis and tree construction for the HA gene were generated using the maximum likelihood (ML) method, with MEGA software Version 5.05 program with the Tamura-Nei model (TAMURA et al., 2013). HA sequences were submitted to GenBank under the accession numbers xxxx to yyyy (numbers to be obtained from GenBank as soon as our submission is dealt with). The sequences obtained here were compared with the first characterized H9N2 sequences from Morocco in the phylogeny (A/chicken/Morocco/SF2-5/2016).

Results

Clinical case history and gross post-mortem lesions. From January 2016 to December 2018, several AI cases appeared in broiler flocks in Morocco in different regions. Clinical presentation started with respiratory depression and distress including nasal discharge, sneezing, coughing, and rales. Other signs included conjunctivitis and watery eyes. We also observed high mortality (30% on average) and a decrease in feed and water intake. The age of the chickens when the symptoms were noticed varied between 19 to 43 days. Table 1 shows the details of the flocks sampled in this study.

Post-mortem examination of dead birds showed typical lesions such as congestive tracheae with bronchial casts. Vaccination history revealed that 40% of flocks were vaccinated with commercially available inactivated vaccines, while 60% were not vaccinated against the disease.

H9 detection by Real time RT-PCR. Real time RT-PCR for H5, H7 and H9 subtypes showed negative results for three negative controls and positive results for each of the three positive controls, with Cycle Threshold (CT) values of 33.24, 34.66 and 26.16 for H5, H7 and H9, respectively. Among the 21 flock samples, 48% (10/21) were positive for H9 with CT values between 18.6 and 34.8. No flock tested positive for the H5 and H7 subtypes (data not shown).

Virus isolation and the full HA gene amplification. Isolation in SPF eggs was attempted for all H9 positive samples from the 10 positive flocks. Conventional RT-PCR analysis revealed that among 10 isolates from field samples positive by real time RT-PCR, full HA PCR products could only be obtained for 4 isolates.

BLAST search analysis of the HA gene. Full HA gene sequences of the four Moroccan isolates were sequenced. The nucleotide sequence and deduced

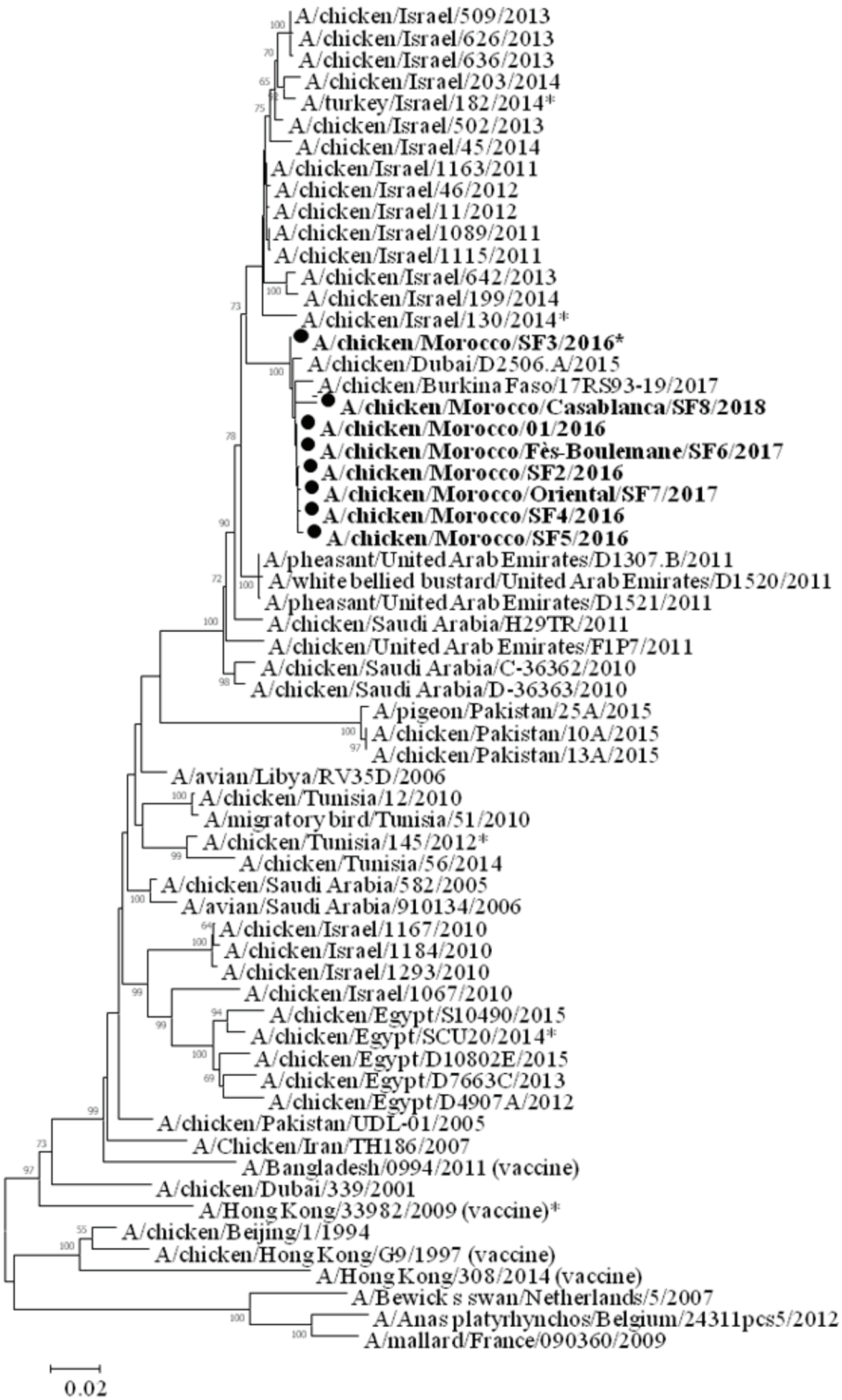

Fig. 1. Phylogenetic relationships of Moroccan isolates and selected reference strains based on HA gene sequences. Numbers along the branches refer to bootstrap values. The nucleotide sequences of Moroccan H9N2 viruses (with black circle shaped symbols) were compared with relevant virus sequences available in the GenBank database. In brief, we selected the first 20 hits by BLAST search, and included WHO recommended vaccine strains, reference viruses, and relevant sequences from neighbouring areas.

amino acid sequences of these H9 isolates were blasted and compared with the reference strain sequences retrieved from GenBank from different regions of the world. The nucleotide sequences of the Moroccan strains were highly similar, and presented 98% nucleotide sequence identity to A/chicken/BurkinaFaso/17RS93-19/2017, an H9N2 virus detected in a broiler chicken in Burkina Faso in 2017 (ZECCHIN et al., 2017). A high similarity in nucleotide and protein sequences (96-98%) was observed between the Moroccan viruses and an H9 virus isolated from a layer chicken in the UAE in 2015, A/chicken/Dubai/D2506/2015.The HA genes revealed a close relationship between the four Moroccan viruses, with 97.9%-99.9% nucleotide identity.

Phylogenetic analyses. Phylogenetic trees were constructed from the nucleotide and deduced amino-acid sequences of the HA glycoprotein genes of Moroccan isolates and non-Moroccan H9 reference strains. The four Moroccan H9 isolates and Moroccan viruses detected in 2016, when the virus was first introduced in the country, clustered together with the Burkina Faso and UAEH9 viruses. They all belonged to the G1 lineage or Lineage A, based on the recent classification (SUN and LIU, 2014). All the viruses were closely related to each other (Fig. 1).

Our results showed the limited genetic evolution of H9N2 HA genes in Morocco over a 2 year period of endemic circulation in the country.

Discussion

Avian influenza virus H9N2 was isolated first in a turkey flock in the USA in 1966. In 1992, the virus appeared in chicken flocks in China, and then the virus spread into many parts of Asia, the Middle East and North Africa.

A recent study based on phylogenetic characteristics classified the virus into 3 distinct lineages: worldwide lineage, Asia-Africa lineage and China lineage (HU et al., 2017). The genetic relatedness of H9N2 isolated in the Middle East and North Africa revealed the presence of two major lineages: lineages A and B. Lineage A represents viruses detected from 1998 to 2016 in all countries of the Middle East and North Africa, whereas, lineage B, represents early viruses isolated between 1998 and 2007 in Saudi Arabia, Iran and Israel (NAGY et al., 2017). Moreover, lineage A contains a widespread panzootic group of H9N2 viruses (P-AIVH9N2) reported in Saudi Arabia in 2005, Libya in 2006, Qatar in 2008, UAE in 2008, Tunisia in 2010, Israel in 2011, and Morocco and Algeria (NAGY et al., 2017).

In the present study, our results highlight the clear link between Moroccan viruses and viruses of the G1 lineage isolated in the Middle East. This finding is in relation to the investigation published by EL HOUADFI et al. (2016). Phylogenetic analyses carried out on H9N2 viruses isolated in Morocco showed that Moroccan H9N2 viruses presented over 90% nucleotide identity with A/pheasant/United Arab Emirates/D1521/2011-like H9N2 virus (EL HOUADFI et al., 2016). These results are corroborated by our finding that the H9N2 virus that affected Morocco may have originated from the UAE, possibly through introduction of falcons for hunting, or through human movements. Phylogenetic analyses of this virus revealed that Burkinabe and Moroccan H9 strains shared 99.2% identity of the HA gene, and that both viruses were very close to the H9N2 detected in Dubai in 2015 (A/chicken/Dubai/D2506/2015).This high similarity can be explained by poultry products trade (hatching eggs, day old chicks), or through movement of poultry professionals (ZECCHIN et al., 2017). While sequence and epidemiological data suggest that the West African virus may have originated from Morocco, the gap in avian influenza surveillance in Africa may bias our interpretation. In 2017, the H9N2 virus spread to Sub-Saharan African countries, primarily to Burkina Faso (ZECCHIN et al., 2017)

Finally, few genetic and phylogenetic differences were observed between the 2016 and 2018 H9N2 viruses in Morocco (Fig. 1), despite vaccination from July 2016 onwards. So far, we can speculate that vaccination coverage may still be low, vaccination may be well conducted, and/or the vaccines used may render vaccine escapes difficult, limiting virus evolution in response to vaccination. However, vaccinated flocks have regularly tested

positive for H9N2 (Table 1), suggesting a more complex scenario. Further studies in the coming years are warranted to follow up the putative selection pressure generated by H9 vaccines on field H9N2 viruses in Morocco.

Conclusion

This study has allowed us to isolate and characterize recent H9N2 influenza viruses in Morocco. Phylogenetic analysis showed that Moroccan H9N2 viruses underwent little genetic evolution over a 2 year period, despite the use of vaccination. The viruses clearly cluster with Middle East G1 lineage strains.

Prevention and control strategies for avian influenza, such as active surveillance, reinforcement of biosecurity, application, rigorous vaccination protocols, border and movement control, are necessary to prevent the further spread of the virus.

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Conflict of interest

The authors declare that there is no conflict of interest that could possibly arise.

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SAŽETAK

Virusi ptičje gripe H9N2 nastavljaju se širiti u peradi i divljih ptica širom svijeta. Infekcija niskopatogenim virusom influence H9N2 prvi je put otkrivena u Maroku u siječnju 2016. godine. U ovom je istraživanju za probir na H9N2 prikupljeno ukupno 105 organa i obrisaka iz dušnika s 21 farme brojlera od srpnja 2016. do listopada 2018. iz različitih regija Maroka. Sumnja na bolest temeljila se na teškim respiracijskim znakovima kao što su kihanje, kašljanje, hropanje i hripanje, a infekcija virusom H9N2 potvrđena je PCR-om obrnute transkripcije u stvarnom vremenu. Sekvencije gena za hemaglutinin (HA) od četiri izolata amplificirane su pomoću RT-PCR qRT-PCR poravnane za filogenetsku i analizu sličnosti aminokiselina. Od 21 uzorka jata 48 % (10/21) bilo je pozitivno na H9 s pragom broja ciklusa u rasponu od 18,6 do 34,8. Maksimalna sličnost u nukleotidnim i proteinskim sekvencijama (96 -98 %) uočena je između marokanskih virusa i virusa H9 izoliranih iz brojlerskih pilića u 2017. u Burkini Faso (A/piletina/BurkinaFaso/17RS93-19) i od kokošjeg pileta u Ujedinjenim Arapskim Emiratima u 2015. (A/piletina/Dubai/D2506/2015). HA geni otkrili su blisku vezu između četiriju virusa, s 97,9 % -99,9 % nukleotidnog identiteta. Filogenetska analiza pokazala je da marokanski virusi pripadaju lozi G1 i vjerojatno potječu s Bliskog istoka, kao što je objavljeno 2016. godine.

Ključne riječi: ptičja gripa; niska patogenost; H9N2; molekularna analiza; filogenija; Maroko