AVIAN DISEASES 63:24–30, 2019

Pathogenicity and Full Genome Sequencing of the Avian Influenza H9N2 Moroccan Isolate 2016

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Received 22 October 2018; Accepted 26 October 2018; Published ahead of print 16 November 2018

SUMMARY. In Morocco in early 2016, a low pathogenic avian influenza virus serotype H9N2 caused large economic losses to the poultry industry, with specific clinical symptoms and high mortality rates on infected farms. Subsequent to the H9N2 outbreak, the causal agent was successfully isolated from chicken flocks with high morbidity and mortality rates, propagated on embryonated eggs, and fully sequenced. The phylogenetic analysis suggested that the Moroccan isolate could have derived from the Middle East isolate A/chicken/Dubai/D2506.A/2015. This study was designed to assess the pathogenicity of the Moroccan isolate H9N2 in experimentally infected broiler and specific-pathogen-free (SPF) chickens. At 22 days of age, one broiler and two SPF chicken groups were inoculated by dropping 0.2 ml of the H9N2 isolate ($10^{7.5}$ EID_{50}/ml) in both nostrils and eyes. Clinically inoculated chickens with H9N2 displayed mild lesions, low mortality rates, and an absence of clinical signs. The H9N2 virus was more pathogenic in broiler chickens and produced more severe tissue lesions compared to SPF chickens. The viral shedding was detected up to 6 days postinoculation (pi) in oropharyngeal and cloacal swabs in infected birds with a maximum shedding in the oropharynges of the broiler group. All experimental chickens seroconverted and registered high hemagglutination inhibition titers as early as day 7 pi. The present study indicates that the H9N2 virus isolated from a natural outbreak was of low pathogenicity under experimental conditions. However, under field conditions infection with other pathogens might have aggravated the disease.

RESUMEN. Estudio de patogenicidad y secuenciación del genoma completo del aislamiento de virus de la influenza aviar H9N2 de Marruecos del año 2016.

En Marruecos, a principios de año 2016, el serotipo H9N2 del virus de la influenza aviar de baja patogenicidad (LPAIV) causó grandes pérdidas económicas en la industria avícola, con signos clínicos específicos y altas tasas de mortalidad en las granjas infectadas. Posterior al brote de H9N2, el agente causal se aisló con éxito de parvadas de pollos con altas tasas de morbilidad y mortalidad, se propagó en huevos embrionados y se secuenció completamente. El análisis filogenético sugirió que el aislado marroquí podría haberse derivado del aislamiento de Medio Oriente (A/pollo/Dubai/D2506.A/2015). Este estudio se diseñó para evaluar la patogenicidad del aislado marroquí H9N2 en pollos de engorde infectados experimentalmente y en pollos libres de patógenos específicos (SPF). A los 22 días de edad, un grupo de pollos de engorde y dos grupos de aves libres de patógenos específicos se inocularon mediante la instilación de 0.2 ml del aislamiento H9N2 ($10^{7.5}$ dosis infectantes de embrión de pollo 50% [EID50] por ml) en ambas fosas nasales y en los ojos. Los pollos clínicamente inoculados con el virus subtipo H9N2 mostraron lesiones leves, bajas tasas de mortalidad y ausencia de signos clínicos. El virus H9N2 fue más patógeno en los pollos de engorde y produjo lesiones tisulares más graves en comparación con las aves libres de patógenos específicos. La excreción viral se detectó hasta seis días después de la inoculación en frotis orofaríngeos y cloacales de aves infectadas con una excreción máxima en la orofaríngeo del grupo de pollos de engorde. Todos los pollos experimentales seroconvirtieron y registraron altos títulos de inhibición de hemaglutinación tan pronto como en el día siete después de la inoculación. El presente estudio indicó que el aislamiento viral H9N2 de un brote natural fue de baja patogenicidad en condiciones experimentales. Sin embargo, en condiciones de campo, la infección con otros patógenos pudo haber agravado la enfermedad.

Key words: isolate, H9N2, SPF, broiler, pathogenicity, shedding, serology

Abbreviations: EID_{50} = egg infectious dose 50; HI = hemagglutination inhibition; LPAIV = low pathogenic avian influenza virus; pi = postinfection; RT-PCR = real time reverse transcription-polymerase chain reaction; SPF = specific-pathogen-free; Q-PCR = quantitative polymerase chain reaction; Q-RT-PCR = quantitative reverse transcriptase-polymerase chain reaction

Influenza is a highly infectious disease of birds caused by influenza A viruses of the family *Orthomyxoviridae*, enveloped particles with an eight-segment negative-sense single-stranded RNA genome (36). The H9N2 subtype virus was first isolated from a turkey in Wisconsin in 1966. This virus has a wide host range and has been isolated from domestic birds, wild birds, pigs, dogs, pets, and monkeys. H9N2 has become endemic in poultry across the Middle East, Far East Asia, and North Africa since the mid-1990s (11,14,18). In Europe, the H9N2 subtype has been detected sporadically in wild birds and poultry; however, in recent years, a number of outbreaks in turkey flocks have been reported in Germany, Italy, England, and Poland (29,31). Molecular genetic analyses of H9N2 isolates during the last two decades revealed a highly evolving and a genetically diverse population. Furthermore, the H9N2 virus has reassorted with other avian influenza viruses to generate multiple novel subtypes. Two distinct lineages of H9N2 have been defined, the North American and the Eurasian lineages (26).

Pathogenicity of the H9N2 virus is variable and depends on the strain, host species, age, and recurrent infections (7,28). The virus has caused large economic losses to the poultry industry around the world. An enormous impact on poultry following outbreaks of H9N2 was reported in the United Emirates in 1999 (1,2), Egypt in 2006 (17,22), and Tunisia in 2009 (35), among other countries.

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In Morocco in January 2016, the national authorities confirmed, for the first time, the spread of H9N2 in broilers, layers, breeders, and turkey flocks from different poultry production areas. Animals showed a severe decrease in daily feed intake with consequent lower weight gain, severe respiratory signs (sneezing, coughing, rales, and gasping), a very rapid increase in mortality rates, and an important drop in egg production.

Phylogenetic analyses showed that the Moroccan isolate belonged to the G1 lineage and likely originated from the Middle East. H9N2 is known to be a low pathogenic avian influenza virus (LPAIV), though the outbreak in Morocco caused specific clinical signs and relevant mortality in infected farms (15).

In this study, we report the molecular characterization of the H9N2 subtype virus strain isolated from a broiler chicken farm in Casablanca, and we describe the results obtained by its experimental infection in both broiler and specific-pathogen-free (SPF) chickens.

MATERIALS AND METHODS

Virus isolation. Specimens were obtained from a farm in Had Soualem, Casablanca province, Morocco. Broiler chickens in this farm suffered from severe respiratory symptoms and when autopsied showed marked lesions: sinusitis, oculonasal discharge, tracheitis with fibrinous exudates in the lumen, strong pulmonary congestion, splenomegaly, and hepatomegaly. Six collected tracheal samples from sick birds were tested positive with a real time reverse transcription-polymerase chain reaction (RT-PCR) -based test for the presence of the RNA genome of the AIV H9 subtype (Ct 13 to 24) with a universal quantitative polymerase chain reaction (Q-PCR) test (3,23).

Virus isolation was performed by inoculation into the allantoic cavities of 9-day-old SPF chicken embryos according the World Organisation for Animal Health (OIE) protocol (2005; 26). The harvested chorio-allantoic fluid was tested for purity, hemagglutination effect, and RT-PCR, and then stored at −80 C for further investigation.

This isolate was confirmed to be an H9 subtype AIV virus using a specific RT-PCR for the H9 subtype. The virus was sent to the OIE reference laboratory IZSVE in Teramo, Italy, which confirmed the presence of the H9N2 virus in the specimen.

The virus was used as an inoculum after an additional passage in SPF embryos. The allantoic fluid inoculum used had a titer of $10^{7.5}$ embryo infective dose (EID_{50}/ml). This inoculum was first tested by RT-PCR for the absence of other common avian pathogens, such as Newcastle disease virus (9), infectious bronchitis virus (5), and *Mycoplasma* (20).

Sequencing. The OIE/Food and Agriculture Organization of the United Nations Reference Laboratory for Avian Influenza (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padua) in Italy performed the RNA purification from the clinical sample with the Nucleospin RNA kit (Macherey-Nagel, Germany). The amplification of the complete influenza A virus genome was obtained with the SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen, CA). Sequencing libraries were obtained using the Nextera DNA XT Sample preparation kit (Illumina) following the manufacturer's instructions and quantified using the Qubit dsDNA High Sensitivity kit (Invitrogen). The libraries were sequenced on the Illumina MiSeq platform, according to the manufacturer's instructions.

Phylogenetic analyses. The sequences of the eight gene segments of the H9N2 viruses from Casablanca were compared to the available sequences in GISAID, and the most related sequences resulted from a BLAST search. MAFFT v. 7 (19) software was used to align the sequences of each gene segment. Maximum likelihood phylogenetic trees were obtained using the best-fit general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites (with four rate categories, Γ4) and a heuristic SPR branch-swapping search (10) in PhyML v3.1. One hundred bootstrap replicates were performed. FigTree v1.4.2 software was used to visualize the phylogenetic trees (http://tree.bio.ed.ac.uk/software/figtree/).

Experimental infection. Broiler chickens were obtained from a commercial hatchery at 1 day of age and SPF eggs from Multi Chemical Industry facilities. A group of 20 broiler chicks (G1) and two groups of SPF chicks (G2 and G3, $n = 20$ each) were reared in two separate controlled environments in ABSL3 of these facilities. None of these animal groups was controlled for other respiratory diseases. At 22 days of age, animals were infected by dropping 0.2 ml of AIV H9N2 isolate ($10^{7.5}$ EID_{50}/ml) in both nostrils and eyes. A day before the infection, animals were cold stressed; chicks were put into cages exposed to fans in a cold room at 14 C for a period of 6 hr.

Clinical signs and lesions. Inoculated chickens were observed daily for clinical signs during 15 days postinoculation (pi) for the presence of respiratory distress, general behavior, change in feed intake, and any other abnormalities. G1 and G2 were euthanatized at 15 days pi ($n = 20$) and G3 at days 5 pi ($n = 7$), 10 pi ($n = 7$), and 15 pi ($n = 6$). Necropsy was performed on each chicken, and lesions were recorded. Lesions of air sacs, trachea, lung and sinus were scored for severity. For trachea, a score of 1 was given for mild inflammation, 2 for congested trachea, and 3 for tracheitis with fibrinous deposits. Similarly, a score of 1 was given for congested lung, 2 for hemorrhagic lung, and 3 for lung with fibrinous deposits. For sinus, a score of 1 was given for mild inflammation and 2 for sinus with fibrinous deposits. A score of 1 was given for thickened air sacs and 2 for air sacs with fibrinous deposits.

Tissues from spleen, liver, trachea, and lung were collected from chickens of each group. The collected tissues were ground with an Ultraturax homogenizer (IKA L005470 T-25) in phosphate-buffered saline with 5% antibiotics. The mixture was then centrifuged, and the supernatant was collected for RT-PCR analysis.

Virus shedding. Oral and cloacal swabs were collected from infected chicken groups at days 3, 6, 9, and 12 pi. Swabs were thoroughly mixed in 1 ml of sterile PBS buffer with 5% antibiotics. Samples were then centrifuged at 2500 rpm for 10 min to remove particles, and supernatants were analyzed with RT-PCR.

Quantitative reverse transcriptase-polymerase chain reaction (Q-RT-PCR). RNA extraction from swabs and collected tissues was carried out with the ISOLATE II RNA Mini Kit (Bioline) according to the manufacturer's instructions.

Real-time RT-PCR for AIV detection was conducted with the superscript III Platinum one-step Q-RT-PCR Kit (Thermo Fisher). The real-time RT-PCR volume was 25 µl, containing 12.5 µl of 2× Reaction Mix, 0.5 µl of ROX Reference Dye (1/10), 1 µl of each primer AIFwd (GAC CRATCC TGT CAC CTC TGA C) and AIRev (AGG GCA TTY TGG ACA AAK CGT CTA, 10 µM), 0.5 µl of probe AIPR (Fam-TGC AGT CCT CGC TCA CTG GGC ACG-Tamra, 10 µM), 0.5µl of superscript III RT/platinum Taq Mix, 6 µl of nuclease-free water, and 3 µl of RNA. The real-time RT-PCR assay was run on the ABI7500 real-time PCR system (Applied Biosystems) with the following cycling conditions: RT at 50 C for 15 min and 95 C for 2 min, followed by 45 cycles of PCR at 95 C for 15 s and 60 C for 1 min (3). For H9 subtype determination-specific primer AIH9 Fwd (ATG GGG TTT GCT GCC)/Rev (TTA TAT ACA AAT GTTGCA CTC TG) and probe AIH9 sets (Fam-TTC TGG GCC ATG TCC AAT GG-Tamra) and probe AIH9 sets for conserved regions in the HA2 subunit of the H9HA gene sequences were used with the same protocol previously described for AI detection (23).

Serology. Serum samples from chickens were collected at 1 day old, at day 1 before infection and at 7 and 15 days pi. The hemagglutination inhibition (HI) test was performed according to the protocol described by Thayer and Beard (34). Serum samples were run on a twofold serial dilution against four hemagglutinating units of antigen (AIV H9N2 isolate). Titers were calculated according to the method described by

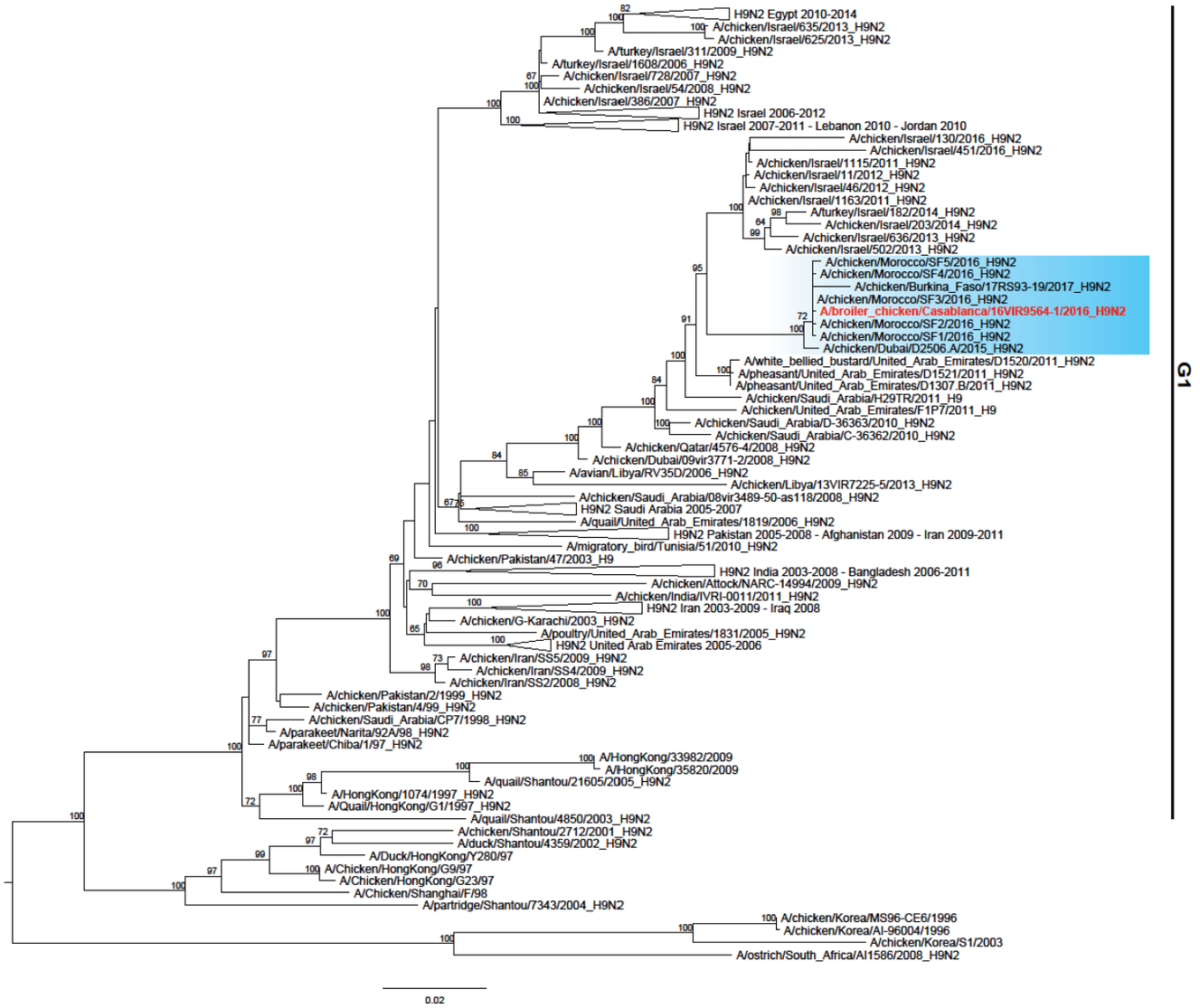

Fig. 1. Maximum likelihood phylogenetic tree of the HA gene. The H9N2 virus from Casablanca is marked in red. The phylogenetic cluster formed by the Moroccan viruses, the H9N2 virus from Burkina Faso, and the H9N2 virusA/chicken/Dubai/D2506.A/2015 are marked in a light blue box. Bootstrap supports higher than 60% are indicated next to the nodes; branch lengths are scaled according to the numbers of nucleotide substitutions per site.

Villegas *et al.* (37). Appropriate controls were included in each test. The titer $\geq 2^5$ was considered as a positive cutoff value (13).

RESULTS

Phylogenetic analyses. Maximum-likelihood phylogenetic tree of the HA gene segment showed that the H9N2 virus isolated in Casablanca belongs to the lineage G1 and clustered with the H9N2 other Moroccan viruses, with the H9N2 virus A/chicken/Burkina_Faso/17RS93-19/2017, and with a H9N2 virus identified in the United Arab Emirates in 2015 (A/chicken/Dubai/D2506.A/2015) (Fig. 1). The phylogenetic trees obtained for all the other gene segments confirmed the clustering observed in the HA phylogeny (Supplemental Fig. 3) (GenBank accession numbers: MG831955, MG831956, MG831957, MG831958, MG831959, MG831960, MG831961, and MG831962).

Clinical signs and gross examination. None of the experimental infected broiler and SPF chickens showed clinical signs of a respiratory disease. Broiler infected birds (G1) registered 17% mortality between days 10 and 12 pi. Autopsied animals showed strong congested lung, thickened air sacs, and fibrinous casts in the abdomen (Table 1; Fig. 2) and for some animals splenomegaly, pale and friable liver, and kidney. The virus has been detected by RT-PCR in 5% of both dead and autopsied animals' tissues.

SPF infected birds of G2 survived during observation period of 15 days. At post mortem, animals showed mucus in the sinuses, mild congested lung, and thickened air sacs (Table 1). No virus has been detected in animals' organs.

Infected SPF chickens of G3 recorded 5% mortality between days 5 and 7 pi. Autopsied animals at days 5, 10, and 15 pi showed congested trachea and lung, thickened air sacs, splenomegaly, and diarrhea. The virus has been detected by RT-PCR in 100% of the

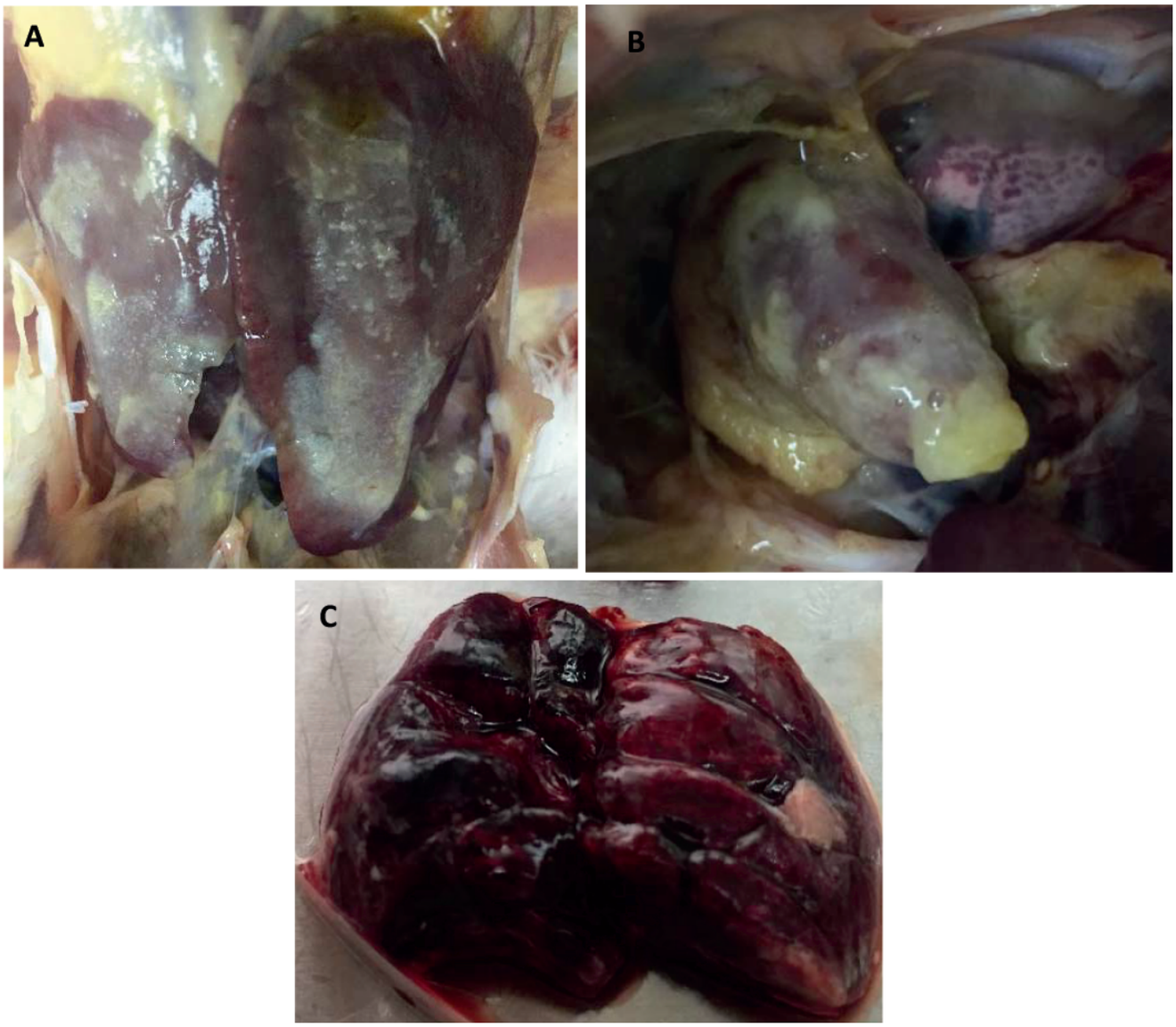

Fig. 2. Macroscopic lesions observed in infected chicken with H9N2 Morocco isolate: (A) and (B) fibrinous casts in the liver and heart, (C) congested lung.

autopsied animals at days 5 and 10 pi, and no virus was detected at day 15 pi.

The percentage of mortality and the scoring was higher for broiler chickens that showed to be more sensitive to H9. Clinical signs and lesions were more pronounced between days 5 and 10 pi (Table 1).

Virus shedding. The virus was detected in infected chickens from days 3 to 6 pi with a maximum shedding in the oropharynx. No virus detected from day 9 pi in cloacal and oropharyngeal swabs (Table 2).

At day 3 pi, the virus was detected in the cloaca of 100% of SPF animals (G2 and G3) with low Ct value (Ct 25) indicating a high number of target nucleic acid in the sample, and in 33% of broiler chickens with lower number of target nucleic acid (Ct 32). From oropharyngeal swabs, the virus was detected from days 3 to 6 pi in the three groups (100% of animals) but with higher target nucleic acid in broiler chickens (Ct 24).

Table 1. Percentage of broiler (G1) and SPF (G2 and G3) infected chickens showing lesions and mean value of the lesional score ($n = 20$ for each group).[A]

	Proportion of infected chickens		
Lesions	G1	G2	G3
Sinusitis	0/20	6/20	0/20
Thickened air sacs	20/20	12/20	7/20
Congested lung	16/20	10/20	17/20
Abdomen fibrin	5/20	0/20	0/20
Scoring	3.0	2.7	1.9

[A]Lesions of air sacs, trachea, lung, and sinus are scored for severity (1 to 3).

Table 2. H9N2 avian influenza virus detection in infected broiler and SPF chicken in cloacal (CL) and oropharyngeal (OR) swabs ($n = 20$ for G1 and G2; for G3 $n = 20$ at day 3, $n = 13$ at days 6 and 9, and $n = 6$ at day 12).

Animal group	Days postinfection											
	3				6				9		12	
	CL	Ct	OR	Ct	CL	Ct	OR	Ct	CL	OR	CL	OR
G1	7/20	32.3 ± 0.7	20/20	24.0 ± 1. 2	10/20	37.7 ± 0.5	20/20	33.6 ± 0.24	0/20	0/20	0/20	0/20
G2	20/20	25.5 ± 0.9	20/20	29.0 ± 0.23	0/20	—	20/20	34.0 ± 1. 5	0/20	0/20	0/20	0/20
G3	20/20	26.0 ± 0.44	15/20	32 ± 0.42	5/13	34 ± 0.5	13/13	33 ± 1. 0	0/13	0/13	0/6	0/6

Serology. At 1 day old, broiler chickens showed maternal antibodies in the blood with a mean titer of 4 $\log_2$ before infection. At day 22, all groups of chickens were negative at HI test. After infection, the mean HI titer in serum collected from G1, G2, and G3 at day 15 pi were 7.6, 7.4, and 7.7 $\log_2$ respectively (Table 3).

DISCUSSION

The H9N2 outbreak that occurred in Morocco in January 2016 was the first influenza outbreak ever reported in the country. Although H9N2 is classified as a low pathogen avian influenza virus, it has caused high mortality (2%–15% in chicken layers and breeders and 10% in turkeys) and specific clinical symptoms in infected Moroccan farms, probably due to recurrent infections or breeding factors. The presence of the virus has been confirmed in nearly 800 farms, causing sharp decrease in egg production (up to 80%) with no complete recovery after 10 wk of infection, which resulted in the increase of egg and broiler prices on the national market (15,24).

The H9N2 serotype has been reported previously in other countries. In the early 2000s, it spread to several countries in the Middle East, including Israel, Saudi Arabia, the United Arab Emirates, and Oman. Then the virus appeared in Egypt and Tunisia in North Africa. In Egypt, H9N2 was first reported in 2006 and in 2010.The virus has infected a wide range of birds in commercial and backyard sectors and has been mostly accompanied and exacerbated by other coinfecting viruses (e.g., IBV, NDV, H5) and bacteria (MG, MS). In Tunisia, the H9N2 virus emerged in 2009, causing several outbreaks affecting 27.7% of poultry flocks (17,24).

Following the H9N2 outbreak in Morocco, the causal agent was isolated and fully sequenced. The strong homology between A/broiler_chicken/Casablanca/16VIR9564-1/2016 and A/chicken/Dubai/D2506.A/2015 (99.5%) and the findings of previous works (38) suggested that the Moroccan isolates could have derived from the Middle East. Possible routes of introduction of the H9N2 virus in Morocco are controversial. It could be due to migratory birds or commercial exchange, or most likely through imported falcons.

Table 3. Mean ± SD values of avian influenza H9 serum antibody titer (HI) in infected groups ($\log_2$).[A]

Group	Days pi		
	0	7	15
G1	2.5 ± 0.18	7 ± 0.11	7.6 ± 0.83
G2	2 ± 0	8.2 ± 0.32	7.4 ± 0.72
G3	2 ± 0	8.0 ± 0.26	7.7 ± 0.76

[A]Titer $\geq 2^5$ is considered as a positive cutoff value.

In our study, the isolated virus was easily propagated on both cells and embryonated SPF eggs showing biological characteristics similar to other IA viruses. We evaluated the pathogenicity of the isolate in broiler and SPF chickens under controlled laboratory conditions. Despite cold stress applied in our experiment, infection of chickens with H9N2 resulted in mild lesions, low mortality rate, and absence of clinical signs. This discrepancy between laboratory and field observations could be explained by recurrent coinfections in the field. In fact, several studies reported a high mortality with varying clinical signs in layers coinfected with H9N2 virus, *Escherichia coli*, *Mycoplasma gallisepticum*, and BI (4,30,36). In addition, it has been reported that pathogenicity of H9N2 could be variable and depends on the strain, host species, and age. In Iran, experimental infection in chickens with H9N2 resulted in a severe infection with 19% mortality (12,25), while, in Pakistan, it was reported that the virus had low pathogenicity and did not cause mortality on its own (4).

Furthermore, in our study, H9N2 virus was more pathogenic in broiler chickens and produced more severe tissue lesions compared to SPF chickens; we emphasize that these animals were not controlled for any other respiratory disease. The same observation has been reported by Gharaibeh (8) showing that the H9 needs recurrent infection to express clinical symptoms (4,33).

In this experiment the viral shedding was detected up to day 6 pi in both broiler and SPF chickens, with a maximum shedding in the oropharynx of broiler chickens. This is in accordance with previous studies on H9N2 shedding, where it was found that the cloacal shedding rate was significantly lower than the tracheal one (11).

Serology was performed to confirm the infection of animal groups with the virulent H9 virus. SPF chickens were initially seronegative contrary to broiler ones that showed maternal antibodies in their blood, probably because they come from infected or vaccinated flocks. We thus consider that infecting them at 22 days of age was sufficient to eliminate maternal antibodies. At day 15 pi, both broiler and SPF chickens showed comparable high antibody titers, similar to what have been observed in other H9N2 experimental infections (6,16,32). However, in other studies lower antibody titers were obtained (4,12), and Li *et al.* reported a delayed seroconversion at day 21 pi (21). This could be explained by experimental conditions and laboratory techniques.

We can therefore conclude that under our experimental conditions, the H9N2 virus isolated from the Moroccan outbreak did not cause severe clinical disease and was low pathogenic to infected chickens. However, lesion scoring, detection of virus shedding by RT-PCR, and serological response could represent an important criteria for a challenge model.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animal experiments were carried out in accordance with the international guidelines for care and handling of experimental animals, as the protocol has been submitted and approved by the internal ethics committee "The Internal Ethics Committee for Animal Experiments, MCI Santé Animale."

Supplemental data associated with this article can be found at http://dx.doi.org//10.1637/AVIANDISEASESJOURNAL-11941-080418.s1.

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ACKNOWLEDGMENTS

All authors have seen and approved the content and have contributed significantly to the work. We gratefully acknowledge the support for this study by MCI (Multi-Chemical Industry) Santé Animale and the OIE Reference Laboratory for Avian Influenza (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, PD, Italy).