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Phylogenetic analysis and assessment of the pathogenic potential of the first H9N2 avian influenza viruses isolated from wild birds and Lagoon water in Tunisia

Imen Larbi [a,*], Kais Ghedira [b], Marwa Arbi [a], Gary David Butcher [c], Natalia Rego [d], Hugo Naya [d,e], Halima Tougorti [a], Jihene Lachhab [a], Imen EL Behi [a], Jihene Nsiri [a], Abdeljelil Ghram [a]

[a] Laboratory of Epidemiology and Veterinary Microbiology, LR19IPT03, Institut Pasteur de Tunis, University of Tunis El Manar, Tunis, Tunisia
[b] Laboratory of Bioinformatics, Biomathematics and Biostatistics, LR16IPT09, Institut Pasteur de Tunis, University of Tunis El Manar, Tunis, Tunisia
[c] College of Veterinary Medicine, University of Florida, Gainesville, FL, United States
[d] Bioinformatics Unit, Institut Pasteur de Montevideo, Mataojo, Montevideo, Uruguay
[e] Departmento de Producción Animal y Pasturas, Facultad de Agronomía, Universidad de la República, Uruguay

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ABSTRACT

H9N2 avian influenza virus (AIV) has been isolated from various species of wild birds and domestic poultry worldwide. It has been reported since the late 1990s, that H9N2 AIV has infected humans as reported in some Asian and North African countries. This subtype has already been circulating and constituting a serious threat to the poultry industry in Tunisia back in 2009. To investigate zoonotic potential and pathogenicity of H9N2 AIV in chickens and mice in Tunisia, five strains have been isolated during the period from 2014 to 2018. Samples were withdrawn from several wild bird species and environment (Lagoon water) of Maamoura and Korba Lagoons as well as Kuriat Island. Phylogenetic analyzes demonstrated that the isolated H9N2 strains belonged to the G1-like sublineage and were close to AIV H9N2 poultry viruses from North Africa, West Africa and the Middle East. All strains carried in their hemagglutinin the residue 226 L, which is an important marker for avian-to-human viral transmission. The hemagglutinin cleavage site has several motifs: PSKSSR/G, PARSSR/G and HARSSR/G. The neuraminidase showed S372A and R403W substitutions that have been previously detected in H3N2 and H2N2 viruses that were reported in human pandemics. Many mutations associated with mammalian infections have been detected in internal proteins. Pathogenicity evaluation in chickens showed that GF/14 replicates effectively in the lungs, tracheas, spleens, kidneys and brains and that it was transmitted among contact chickens. However, GHG/18 replicates poorly in chickens and has not an efficient transmission in contact chickens. GF/14 and GHG/18 could not kill mice though they replicated in their respiratory tract and caused a significant body weight loss ($p < 0.05$). This study highlights the importance of H9N2 AIV monitoring in both migratory birds and the environment to prevent virus transmission to humans.

1. Introduction

Avian Influenza virus (AIV) belongs to Orthomyxoviridae family and has a segmented, single-stranded RNA genome of negative polarity. The genome has a size of about 13.5 kb and is made up of 8 segments; the polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural (NS) (Lamb and Choppin, 1983). The virus is classified into several subtypes based on its surface glycoproteins, hemagglutinin and neuraminidase. Wild birds are the natural hosts of all avian influenza A viruses (AIV), except for H17N10 and H18N11, that have only been found in bats (Wu et al., 2014). Thus far, all 16 HA and 9 NA subtypes of AIV are reported in the wild birds of the orders Anseriformes and Charadriformes (Fouchier et al., 2005). H9N2 is the most prevalent LPAI in poultry worldwide (Peacock et al., 2019). In general, low pathogenic avian influenza (LPAI) H9N2 virus infection in wild birds induces subclinical to mild disease. H9N2 virus poses a threat to commercial poultry industry and human health through mutations and reassortments (Salomon and Webster, 2009). The first human infection with H9N2 virus was reported from Hong Kong in 1998

* Corresponding author.
E-mail address: imen.larbi@pasteur.tn (I. Larbi).

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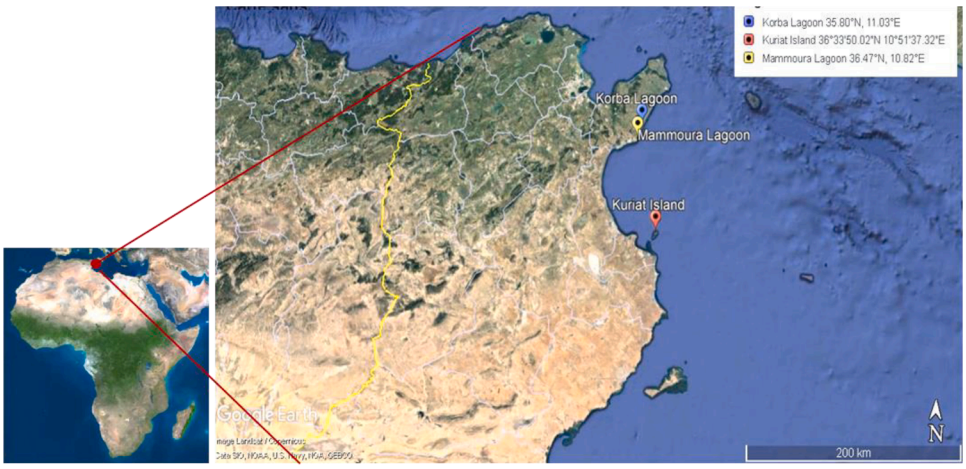


Fig. 1. Location of sample collection in Tunisia.

Table 1
Summary table of flock sample history.

Host abbreviation /year of isolation	Host	Birds status	Samples collected	Site of collection	Accession number of the studied Tunisian H9N2 Viruses
GF/14	Greater Flamingo	healthy birds	TCS	Korba Lagoon	MW375808 - MW375815
GF/15	Greater Flamingo	healthy birds	TCS	Korba Lagoon	MW356853 - MW356860
BWS/18	Black-winged Stilt	dead birds	T,L,I,S, K,B,Li,H	Mammoura Lagoon	MT609882 - MT609889
GHG/18	Gray-headed Gull	dead birds	T,L,I,S, K,B,Li,H	Kuriat Island	MW375771 - MW375778
EN/18	Lagoon Water	/	Lagoon water	Mammoura Lagoon	MT607623 - MT607630

(T) Trachea, (L) lungs, (I) intestine, (S) spleen, (K) kidney, (B) brain, (Li) liver and (H) heart, (TCS) Tracheal/cloacal swab.

(Peiris et al., 1999). The high degree of similarity between avian and human viruses indicates that replication in humans is minimal. This is expected since patients would likely have contracted an infection from poultry exposure as there was no evidence of human to human transmission (Bridges et al., 2000). During the last decades, H9N2 AIVs have been circulating widely in the poultry population and became endemic across Asia, the Middle East and North and West Africa. They are causing considerable production and economic losses (Gaidet et al., 2007; Sun and Liu, 2015).

In Tunisia, since 2009, AIV subtype H9N2 became endemic and it is causing significant production and economic losses to the industrial poultry sector (Tombari et al., 2011). However, AIV data isolated from wild birds and the environment are not characterized. It is important to determine the evolution of H9N2 viruses in the wild bird population in Tunisia. Wild birds play a significant role in the dissemination of AIVs through long distance migration especially that Tunisia is an important migration flyway, and that there are near the wetlands poultry farms with substandard biosecurity programs.

To understand the evolution and molecular features of H9N2 AIV isolated from wild birds in Tunisia, we carried out virus surveillance in greater flamingo (*Phoenicopterus roseus*), gray-headed gulls (*Chroicocephalus crirrocephalus*), and black-winged stilt (*Himantopus himantopus*) as well as in the environment (Lagoon water) from three humid zone including in the Ramsar Wetland (korba Lagoon, Mammoura Lagoon and Kuriat Island) during 2014–2018. A full-length genome sequencing of five H9N2 strains was processed and two AIV H9N2 viruses were characterized in chicken and mouse models.

2. Materials and methods

2.1. Site and sample collection

Korba and Maamoura Lagoons are located in the north-east of Tunisia (36°33′50.02, 10°51′37.32) and (36.47°, 10.82°), respectively. These wetlands are part of the Ramsar site of Lagoons located in Eastern Cap Bon. Korba Lagoon is the only permanent water located all along the coast. It provides a valuable habitat that welcomes breeding water or migratory birds. Korba Lagoon extends over 9 km and communicates with the Mediterranean Sea through several openings. It also occupies a prominent place insofar as it provides a critical migratory bird stopover and nesting habitat along the migratory route.

As for Maamoura Lagoon, it extends over 7 km and communicates with the Mediterranean Sea through several openings. Kuriat Island is located in the center-east of Tunisia (35.80°, 11.03°) and spans 270 hectares. It offers a valuable stopover habitat for of migratory birds (Fig. 1).

As part of a project, realized during 2014–2015 that aimed at identifying and describing the first successful nesting of flamingos, regular visits were carried out in cooperation with agents from the Tunisian Ministry of Agriculture. Tracheal and cloacal swabs were withdrawn from two healthy young Greater flamingos ($n = 2$) (*Phoenicopterus roseus*), during ringing operation.

In 2018, as a part of national program of AIV surveillance in wild birds, we investigated the death of wild birds. Given the constraints encountered in the field, one black winged stilt *(Himantopus himantopus)* ($n = 1$) in Mamoura Lagoon and nine gray-headed gull *(Chroicocephalus crirrocephalus)* ($n = 9$) in Kuriat Island and organ samples were taken. Water from Maamoura Lagoon was also collected as environment sample (5 surface sample pooled) ($n = 1$). All samples ($n = 13$) were placed in cryotubes containing viral transport medium (PBS-glycerol, Sigma®, antibiotics and antifungi) containing 10,000 units/mL penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL, transported to the Laboratory at 4 °C, andstored at −80 °C until used.

2.2. AIV subtyping and virus isolation

Viral RNA was extracted from swabs and organ by the TRIzol method (Invitrogen, Carlsbad, CA, USA). The detection of influenza A was performed using real time RT-PCR by targeting M gene. Positive samples

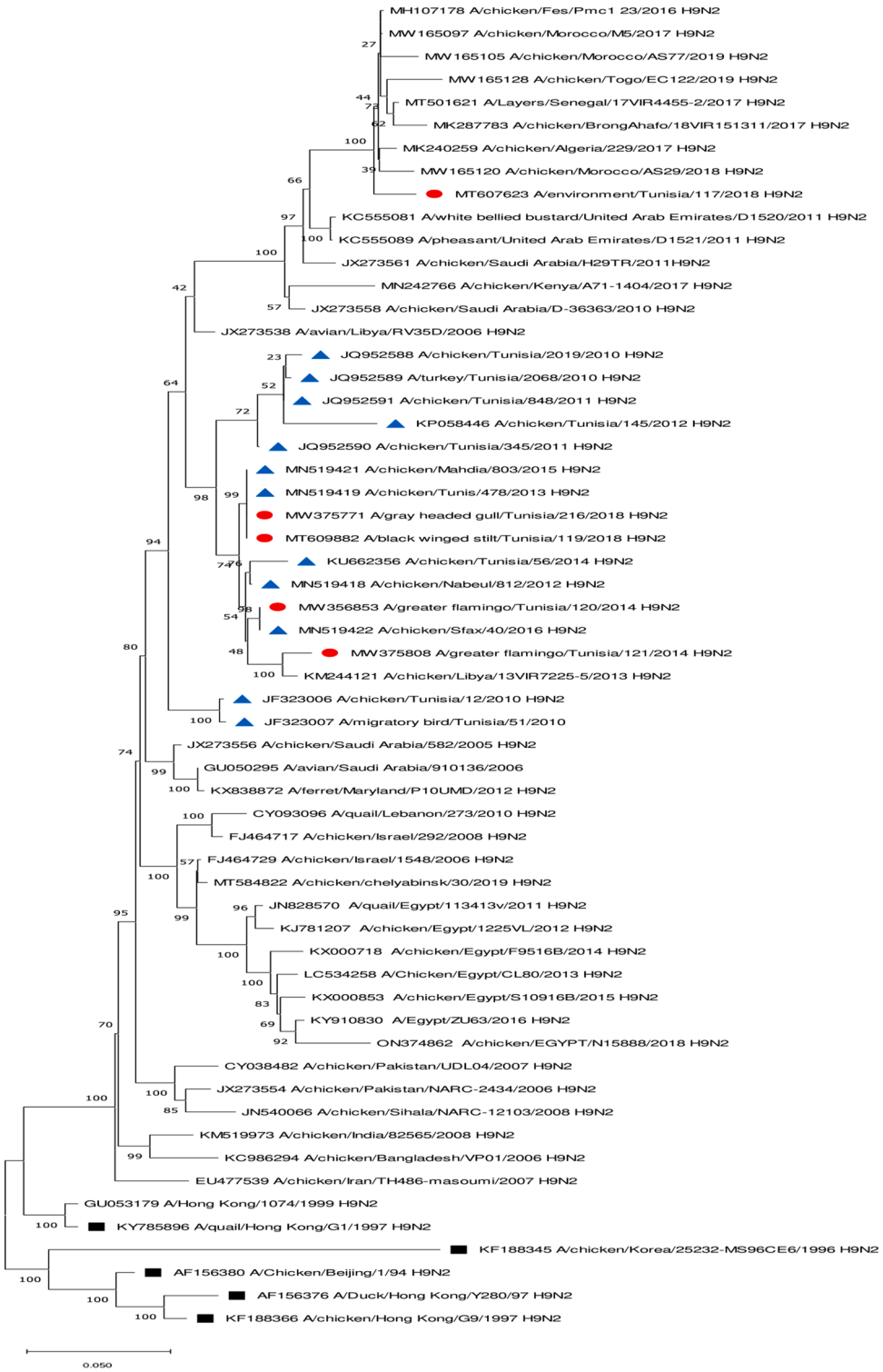
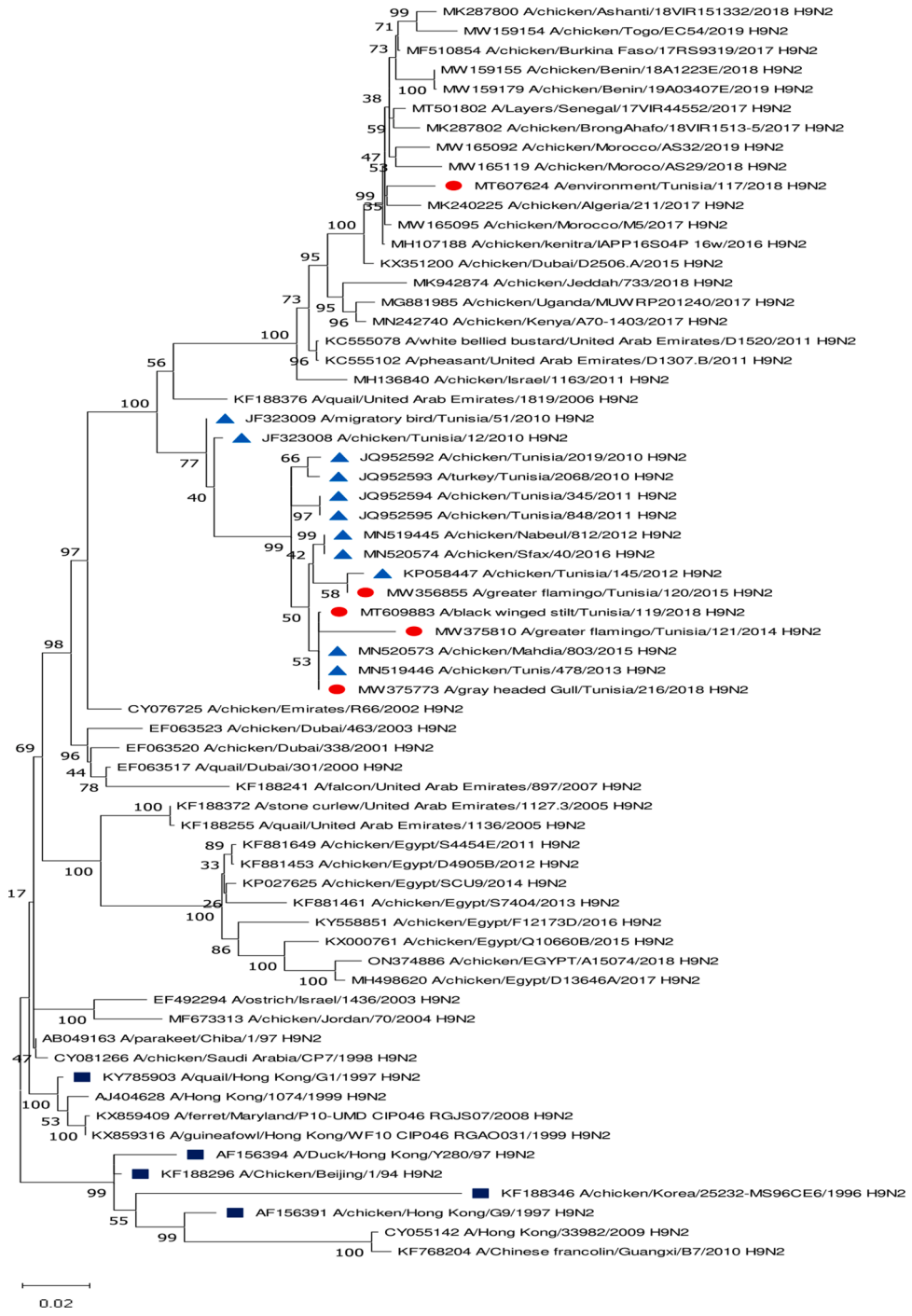


Fig. 2. Phylogenetic trees of the gene segments HA (A), NA (B), PB1 (C), PB2 (D), PA (E), NP (F), M (G,) and NS (H), with evolutionary relationship between the studied viruses (red circles) and the previous Tunisian poultry viruses (bleu triangles), and the reference AIVs from Gen Bank (black squares).

were then subtyped by real time RT-PCR for H9, H5 and H7 genes (Capua and Alexander, 2009; Shabat et al., 2010; Tombari et al., 2013). All real time RT-PCR were performed using AgPath-ID One-Step RT-PCR (Applied Biosystems, UK).

The swab and the organ supernatant that were AIV positive, were used to isolate the virus in 9-to-11 day-old specific pathogen free (SPF) embryonated chicken eggs (Lohmann Ltz, Germany) though allantoic cavity. After incubation of the eggs for 96 h at 37 °C, allantoic fluids were harvested (WOAH Terrestrial Manual., 2015) and the viral titer was determined by haemagglutination assays using standard procedures (WOAH Terrestrial Manual., 2015). The virus stock was stored at −80 °C. The 50% egg infectious dose (EID50) was determined by serial dilutions of the virus in SPF embryonated eggs (Lohmann Ltz, Germany), and the value was calculated using the method described by Reed and



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Muench (1938).

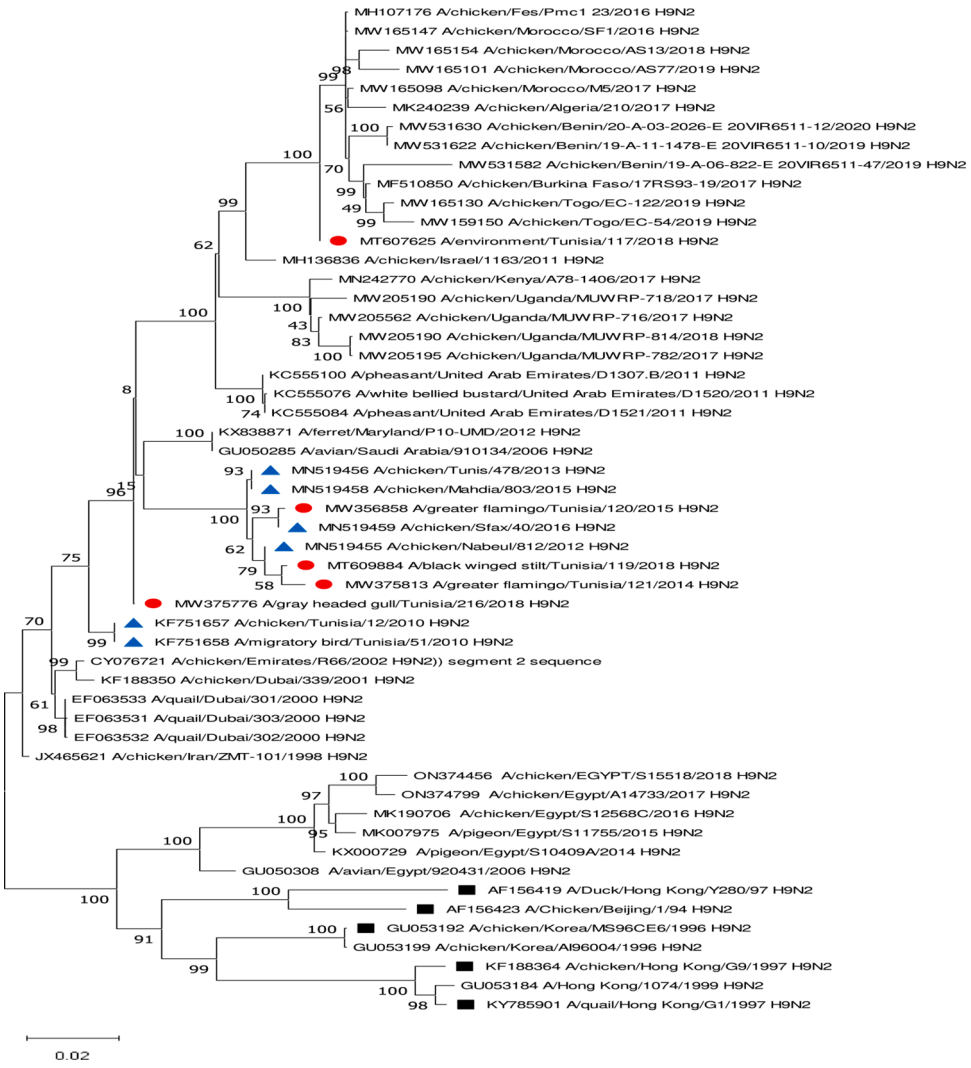
Out of the thirteen tested samples, eleven were positive by rRT-PCR for M and H9 genes. Only five viruses ($n = 5$) were isolated successfully in embryonnated eggs and characterized.

2.3. Full genome amplification

The full-length genome amplification of the studied viruses was carried out by multisegment RT-PCR (MRT-PCR) (Zhou et al., 2009) using a SuperScript III One-Step RT-PCR System Kit (Invitrogen, USA). The MRT-PCR reaction was performed, as follows: 45 °C for 60 min, 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 45 °C for 30 s, and 68 °C for 3 min, and an extension step at 68 °C for 10 min.The MRT-PCR products were purified by the eCube PCR Purification kit (Phile Korea, Korea) following the manufacturer's instruction.

2.4. Sequencing and phylogenetic analysis

The quality and quantity of genomic dsDNA were determined using a Qubit® 3.0 Fluorometer (Invitrogen, USA). A sequencing library was prepared from 1 ng of dsDNA using a Nextera XT DNA Library Preparation Kit (Illumina, USA). The quality and length of the library was evaluated using an Agilent 2100 Bioanalyzer System (Agilent, USA) employing a High Sensitivity DNA Kit (Agilent, USA). Paired-end sequencing was performed using an Illumina Miseq sequencer (Illumina, USA) and a Miseq Reagent Kit V2 (Illumina, USA). Read quality



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was assessed using the FastQC program (Andrews, 2010) about 200 MB of data (2 × 300 bp paired-end reads) was produced for each sample. The *de novo* and mapping of reads was performed using the IVA program (Hunt et al., 2015). Nucleotide and aa sequences alignment was carried out using ClustalW in BioEdit Sequence Alignment Editor v7.2.6. The Blast software program (https: //blast. ncbi.nlm.nih.gov/Blast.cgi) was used to determine the sequence similarity of the Tunisian viruses. Phylogenetic trees were constructed by Maximum likelihood method (ML) in MEGA X 10.1.8 program (Kumar et al., 2018), using the General Time Reversible (GTR) nucleotide substitution model. The nucleotide sequences of all studied viruses are available in GenBank database, under accession numbers: MW375808-MW375815; MW356853-MW356860; MT609882-MT609889; MW375771-MW375778; MT607623-MT607630 (Table 1).

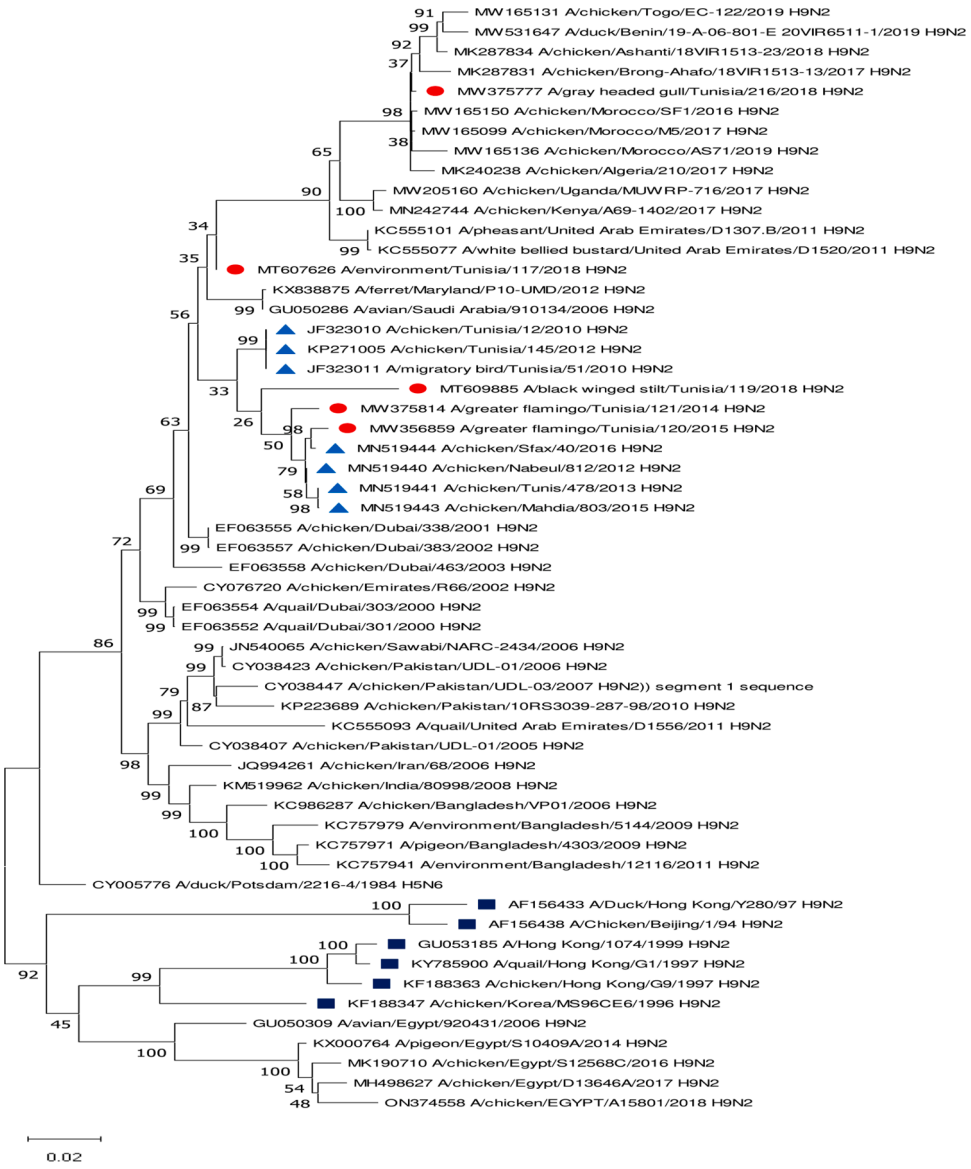
2.5. Animal manipulation and ethics statement

All animal studies have been approved by the Bio-Medical Ethics Committee, Institut Pasteur de Tunis (IPT) and were done in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Experimental Animals. All experiments involving live viruses and animals and viruses were carried out in biosafety level 2+ laboratory (ABSL2) facilities in IPT (reference: 2015/33/I/LRAAIPT/03).

2.6. Pathogenicity and transmission in chickens

To determinate the intravenous pathogenicity index (IVPI) for GF/14 and GHG/18, six-week-old SPF white leghorn chickens were intravenously inoculated with 10^6 (EID50) in 0.1 ml. All chickens were monitored for clinical signs of disease for 10 days (WOAH Terrestrial Manual., 2015).

In order to evaluate the susceptibility of GF/14 and GHG/18 to be transmitted between chickens, two groups of six 6-week-old SPF white leghorn chickens ($n = 6$) were intranasally inoculated with 10^6 (EID50) in 0.2 ml of the selected virus. In addition, a negative control group of six chickens was inoculated with sterile PBS. After 24 h of infection, three contact chickens were introduced in the cage of each infected group. All chickens were monitored daily for clinical signs and mortality over 14 days. Oropharyngeal (OP) and cloacal (CL) swabs were collected on 1, 3, 5, 7, 9, 11 and 13 day post-inoculation (dpi) to detect viral shedding. To investigate virus replication, three infected chickens from each group were euthanized on 3 dpi and lungs, trachea, brain, spleen and kidneys were collected and processed for viral analysis (WOAH Terrestrial Manual., 2015). All swabs and tissues were titrated for virus titers in eggs. Sera were collected 14 dpi for the study of seroconversion by hemagglutination inhibition (HI) test. HI tests were performed using standard procedure (WOAH Terrestrial Manual., 2015). The homologous H9N2 virus was used as antigen in the HI test.



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2.7. Pathogenicity in mice

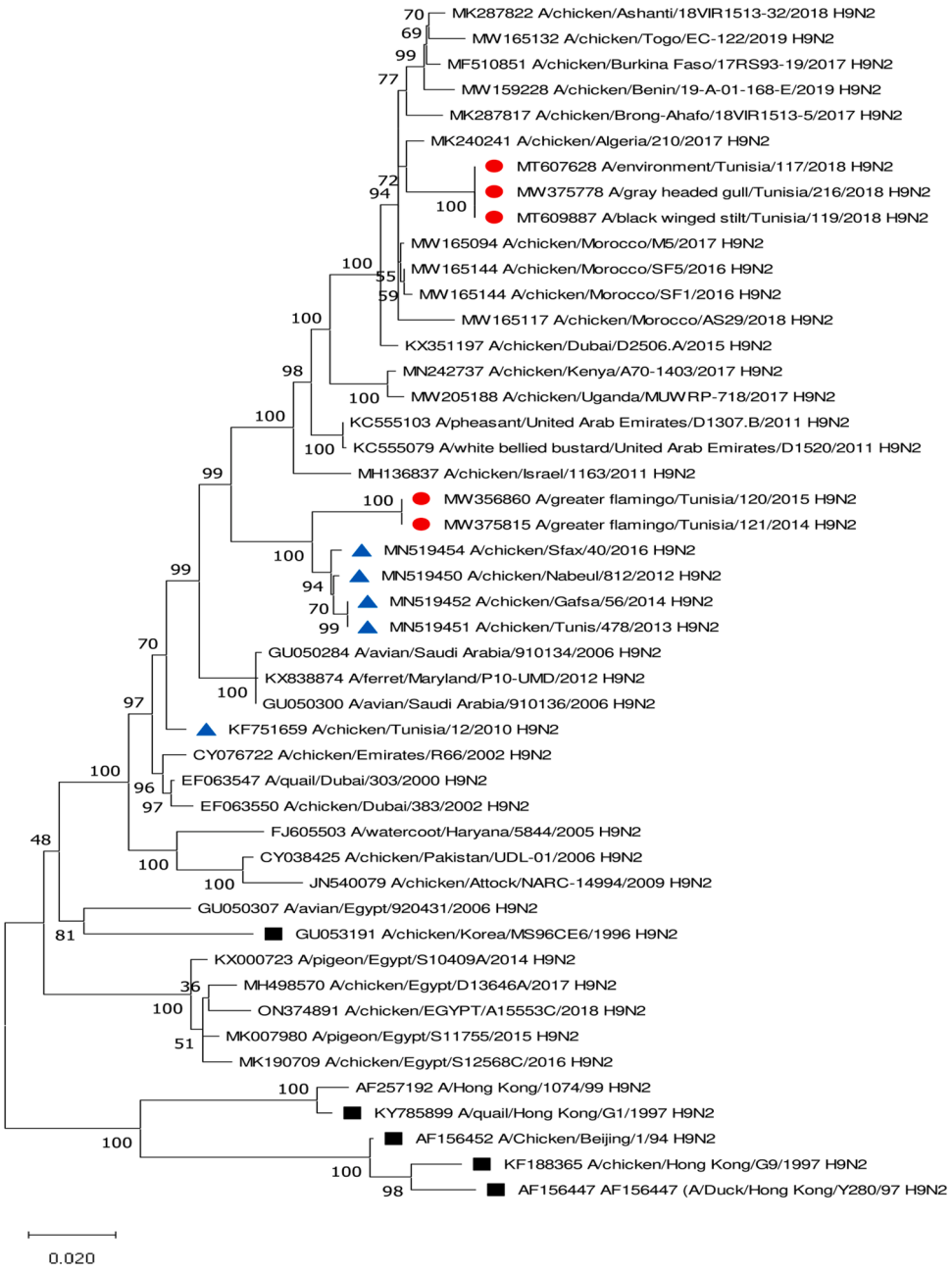
To test the pathogenicity of H9N2 strains GF/14 and GHG/18 in a mammal model, forty-two ($n = 42$) 6–8 weeks old BALB/c female mice (IPT, Tunisia) were divided into three groups, each group was composed of fourteen mice. Two groups were anesthetized with isoflurane and inoculated intranasally with 50 µl of GF/14 or GHG/18 (10^6 EID50). The control group was inoculated with the same volume of sterile PBS. All mice were observed during 14 days for weight loss, inappetence, respiratory distress and mortality. Three mice from each group were euthanized on days 3, 6, 8 post-infection, and samples of turbinate, lung, brain, kidney, liver, spleen and intestine were collected for virus titration in eggs.

3. Results

3.1. Phylogenetic analysis

The phylogenetic analysis of HA and NA genes showed that all studied AIV H9N2 viruses ($n = 5$) grouped in the G1 sub-lineage of the Eurasian lineage. The Tunisian H9N2 viruses isolated from wild birds, GF/14, GF/15, BWS/18, and GHG/18 were closely related to each other, with nucleotide identity of 98.97% and 99% for HA and NA, respectively. However, the Tunisian H9N2 virus isolated from Lagoon water EN/18 exhibited the lowest percentage identity (91.75–90.3%) for HA and NA, respectively. Besides, the precited four H9N2 viruses were closely related to Tunisian chicken AIV H9N2 identified previously in 2010–2016 (similarities of 96.32% to 99.06% and 97.73 to 100% for the HA and NA genes, respectively (Tombari et al., 2011; Arbi et al., 2020). However, EN/18 showed a lower percentage of similarity to previous isolated Tunisian chicken AIV H9N2 for the HA (similarities of 67 to 91%) and NA (similarities of 90.84 to 91.41%) (Arbi et al., 2020; Tombari et al., 2011). The studied wild bird AIV H9N2 studied clustered with AIV H9N2 isolated from Libya 2015 (Kammon et al., 2015), with genetic identity values of 97.95 and 98.51% for HA and clustered with the United Arab Emirates (UAE) AIV H9N2 with genetic identity (93.54 to 94.83%) for NA (Wernery et al., 2013). The Lagoon water EN/18 clustered with AIV H9N2 isolated from the different geographic regions: Morocco 2016 (El Houadfi et al., 2016), Algeria 2017 (Barberis et al., 2020), (UAE) 2013 (Wernery et al., 2013), Senegal and Burkina Faso 2017 (Zecchin et al., 2017) (Fig. 2A.B).

Phylogenetic analysis of the six genes (PB2, PB1, PA, NP, M, and NS)



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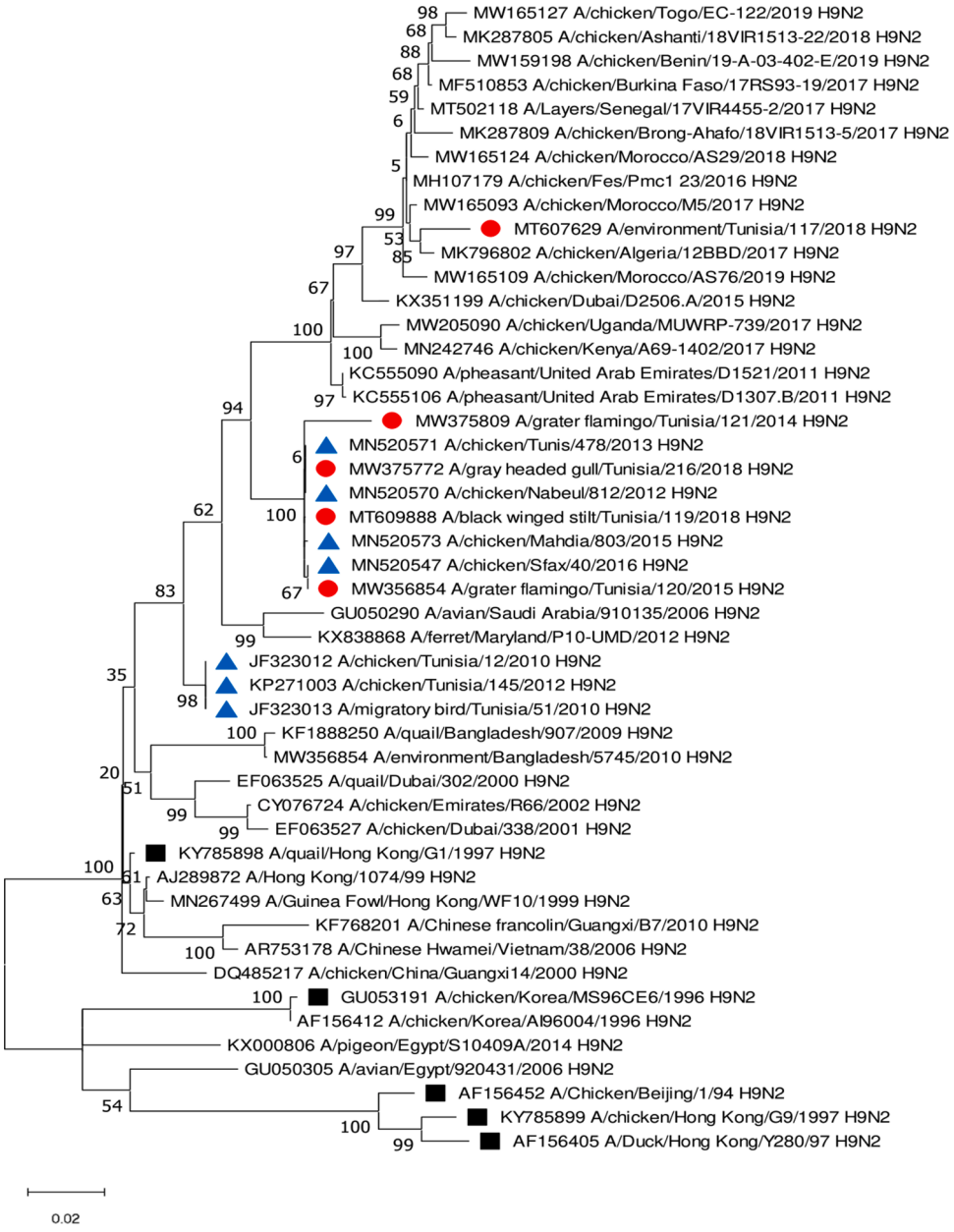
of the studied Tunisian H9N2 viruses belonged to the G1 sub-lineage and were related to each other with nucleotide identities of 94–99%. EN/18 remained the most distinct virus. The five AIV H9N2 viruses isolated from wild birds and environment shared nucleotide sequence identity with chicken Tunisian AIV H9N2 viruses isolated previously (2010–2016) (Arbi et al., 2020) with percentage identity values of 94%, 94%, 92–93%, 94%, 95%, and 97–98% for PB2, PB1, PA, NP, M, and NS, respectively.

The PB2, PB1, PA, NP, M, and NS genes of GF/15, GF/14, GHG/18 and BSW/18 showed high nucleotide identities and phylogenetic relationship with those reported in the UAE, and Saudi Arabia, of at least 95.76%, 96.98%, 98.76%, and 94.65%, respectively. EN/18 showed high nucleotide identities and a close phylogenetic relationship with AIV H9N2 isolated in Morocco (El Houadfi et al., 2016), Algeria (Barberis et al., 2020), Togo, Ghana (Awuni et al., 2019) and Burkina Faso (Zecchin et al., 2017), already grouped within the G1 sub-lineage (Fig. 2C–F).

3.2. Molecular characteristics

Sequence analysis of the HA protein showed that these H9N2 AIV viruses carried four amino acid sequences motifs at the cleavage site: PSRSSR/GLF for (GF/15), PARSSR/GLF for GHG/18 and BWS/18, HARSSR/GLF for EN/18, and PSKSSR/GLF for GF/14), indicating low pathogenicity. All H9N2 viruses harbor HA markers of Human-like 2,6, sialic acids binding preference (Q234L and Q235I) and two H9N2 viruses GF/15 and GF/14 had the A198T substitution, which has been associated with mammalian tropism of H9N2 viruses (Table 2) (Wan and Perez, 2007). All studied H9N2 viruses showed seven potential glycosylation sites N-X-T/S (X is an amino acid other than P) at positions: 29–31 (NST), 105–107 (NGT), 141–143 (NVT), 289–300 (NST), 305–307 (NIS), 492–494 (NGT), and 551–553 (NGS).

Analysis of NA protein sequences revealed mutations in amino acid residues out of three loops that interact directly with sialic acid. On the loops carrying the amino acids 367–370–372, the three Ser (S) were



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substituted with KLA. Furthermore, D and Y substitutions were found at residues 401 and 406, respectively (Zhang et al., 2011).The R371, A372, N402, and E425 amino acid substitutions were detected at the framework site. Also, the amino acid sequence of the NA protein shows the presence of residues L370, I392, and I427, which provide the ability for H9N2 viruses to replicate in mouse cells without killing them (Li et al., 2005).

The PB1 of all the studied H9N2 viruses showed residues 13P, 207 K, and 436Y that are associated with AIV adaptation to mammals (Gabriel et al., 2007). Gene analysis of PB2 protein demonstrated that amino acid 251 K was identified for GF/15. This substitution is reported to increase viral replication and pathogenicity of H1N1 viruses (Cai et al., 2020). The PA protein of the five studied viruses showed mutation S409N that have been reported in highly pathogenic AIVs (H1N1pdm09, H7N9 novel, H2N2, H5N1, H3N2) (Liu et al., 2016; Shaw et al., 2002) that enhance the fitness of the virus in humans. The NP protein revealed an E392D substitution only in viruses BWS/18 and GHG/18, commonly observed in human-associated H9N2 viruses (Jakhesara et al., 2014).

The M1 protein of the studied H9N2 viruses showed mutation located at position 15 (M1), 30D (M1), 215A (M1), and 55F (M2) (Chen et al., 2006; Shaw et al., 2002; Wang et al., 2018). The M2 protein of all studied viruses showed the A30S and S31N mutations observed for the first time in wild birds and the environment in Tunisia and are involved in amantadine resistance (Ilyushina et al., 2005). The NS protein of all studied viruses had the ^{227}GSEV^{230}PDZ ligand (PL) C-terminal motif. This motif causes the virus to have low pathogenicity in mice (Kim et al., 2015; Koçer et al., 2014) (Table 3).

3.3. Pathogenicity and transmission in chicken and mouse models

Two H9N2 strains GF/14 and GHG/18 were selected for pathogenicity and transmission studies in chicken and mouse models, based on their collection year and the genetic signature of their cleavage site which is the molecular modulator of avian influenza viruses. In fact, GF/14 is the first detected H9N2 isolated in 2014 with cleavage site motif "PSKSSRG" reported for the first time. GHG/18 represents the most

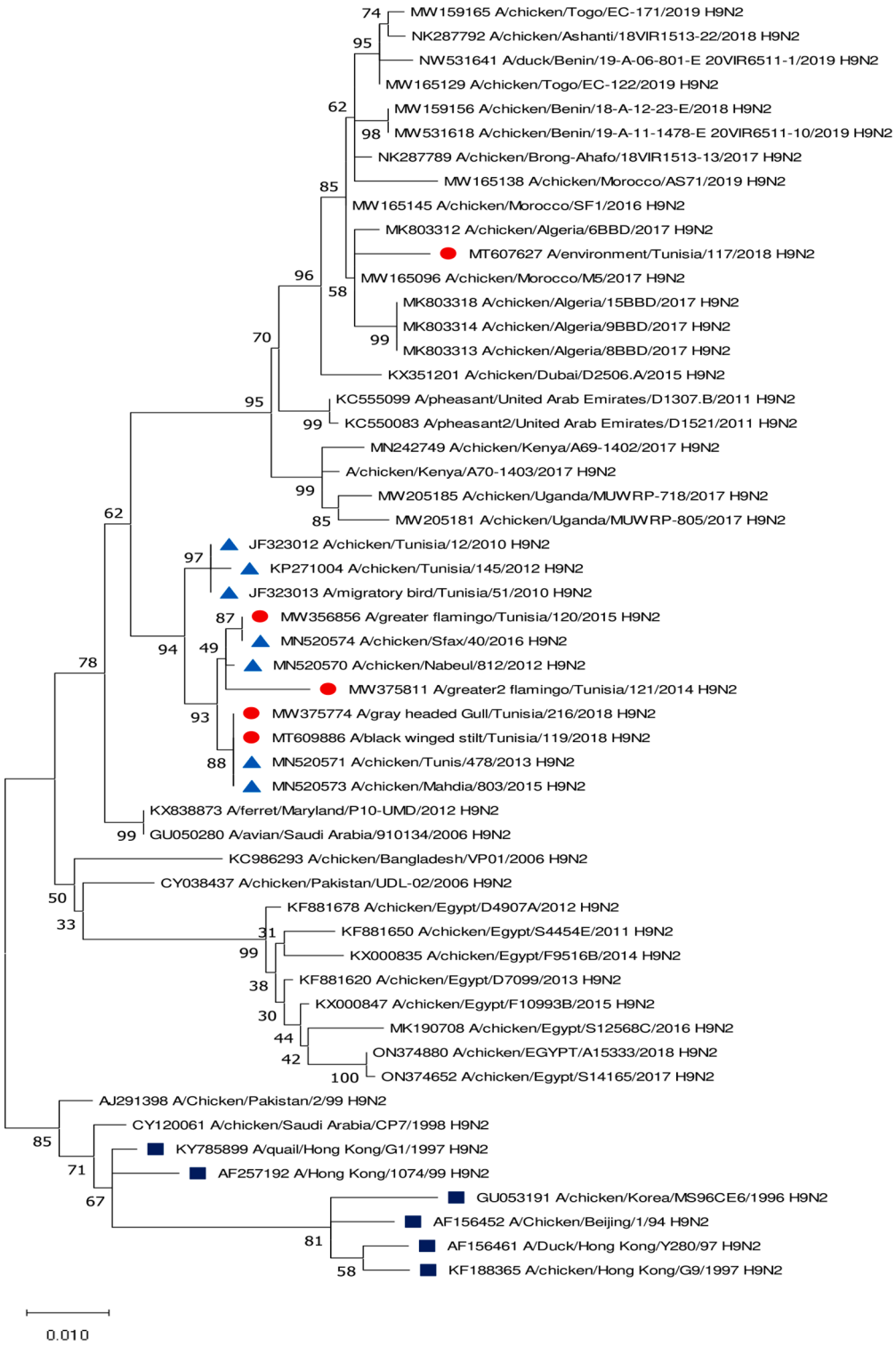


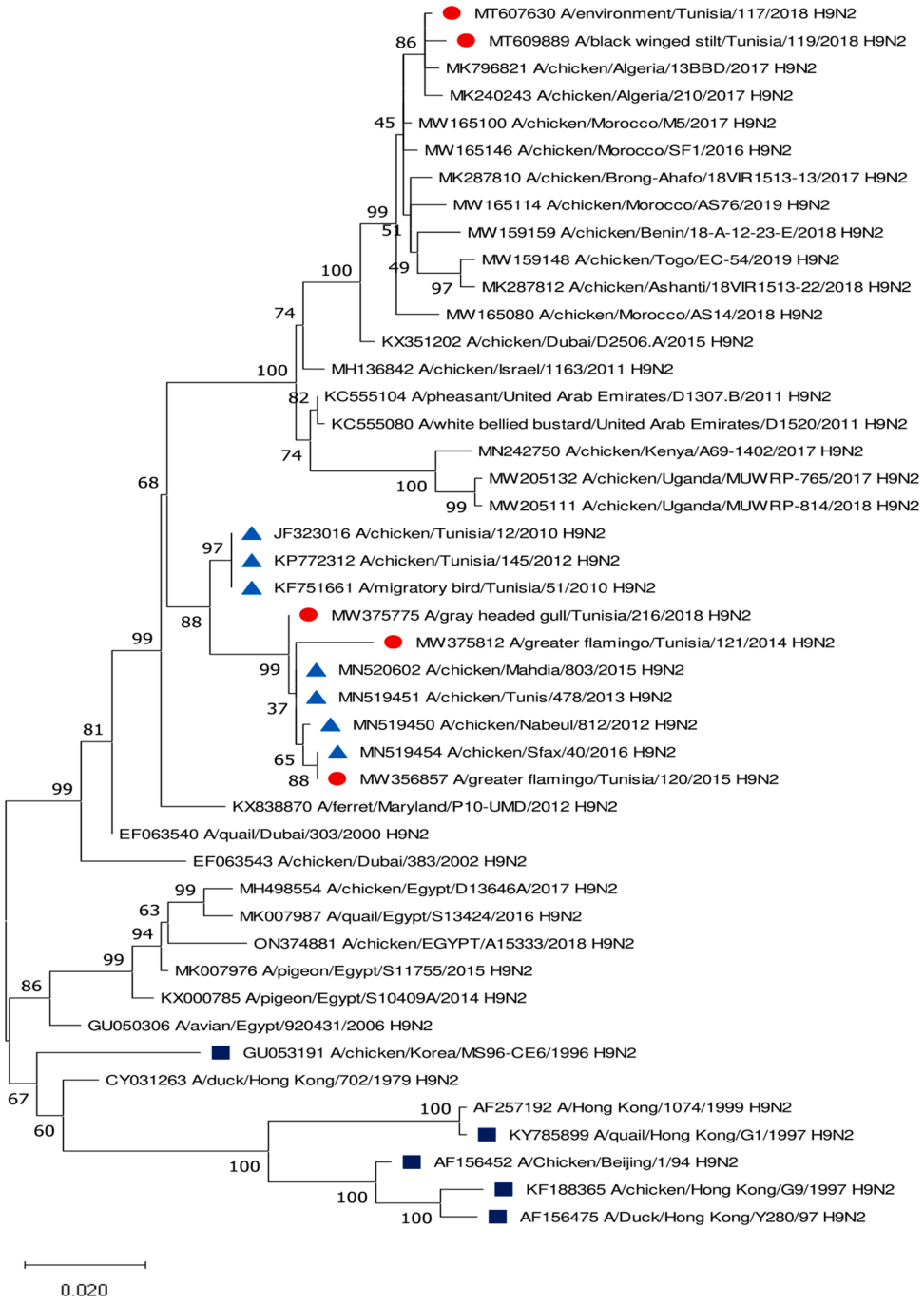
Fig. 2. (*continued*).

recent strain isolated in 2018 and harbors a cleavage site "PARRSSRG" which is identical to BWS/18 and GF/15 strains.

3.3.1. *Pathogenicity and transmission of H9N2 in chickens*

The (IVPI) values were 0.43 for (GF/14), and 0.30 for (GHG/18). Four chickens infected with AIV (GF/14) showed mild clinical signs (general depression, dyspnea, nasal discharge, swollen head and reluctance to move), from 3dpi until 7dpi. For GHG/18 virus infected group, 3 chickens showed mild clinical signs (dyspnea and nasal discharge) from 3dpi until 6dpi and then recovered. No weight loss or mortality was observed.

To test the pathogenicity and transmission of the isolated viruses, chickens were challenged with either GF/14 or GHG/18 H9N2 viruses by intranasally inoculation. To assess virus replication in the organs of inoculated chickens, lungs, trachea, spleen, kidneys and brain were collected on 3 dpi. The results showed that the GF/14 virus could be detected from all organs tested where mean titers were: 2.52 log10EID_{50}/0.1 mL in lung, 2.23 log10EID_{50}/0.1 ml in trachea, 2



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log10EID$_{50}$/mL in spleen, 1.61 log10EID$_{50}$/0.1 mL in kidney,1.94 log10EID$_{50}$/0.1 mL in brain. GHG/18 virus replicated in the trachea and lung and the mean titers were 1.65 and 2.17 log10EID$_{50}$/0.1 ml (Table 4).

To measure the virus shedding, oropharyngeal (OP) and cloacal (CL) swabs samples were collected. The virus was detected in the oropharyngeal swab samples for all groups. Chickens infected with GF/14 shed virus via OP route from 1 to 7 dpi with titers ranging from $10^{1.97}$ to $10^{2.65}$ EID$_{50}$/0.1 ml, whereas contact chickens shed virus from 3 to 11 dpi with titers ranging from $10^{1.83}$ to $10^{2.83}$ EID$_{50}$/0.1 ml. Chickens infected with GHG/18 shed virus via the OP route from 1 to 5 dpi with titers ranging from $10^{1.68}$ to $10^{1.83}$ EID$_{50}$/0.1 ml, whereas contact chickens shed virus from 5 to 7 dpi with titers ranging from $10^{1.83}$ to $10^{2.15}$ EID$_{50}$/0.1 ml. Chickens infected with GF/14 shed virus via the CL route from 5 to 13 dpi with titers ranging from $10^{1.78}$ to $10^{2.83}$ EID$_{50}$/0.1 ml, whereas chickens infected with GHG/18 shed virus via the CL route for 5 to 7dp with titers ranging from $10^{1.83}$ to $10^{2.15}$ EID$_{50}$/0.1 ml. No clinical disease signs of respiratory disease or mortality were observed in the infected and contact chickens during the observed period. All of the inoculated and contact chickens seroconvert at 14 dpi. Sera collected from chickens infected with GF/14 virus showed 6.5 ± 1.0 log$_2$ HI titer against a homolgous virus, the contact chicken showed 6.4 ± 0.6 log$_2$ HI titer. Sera collected from chicken infected with GHG/18 virus had 6.2 ± 0.9 log$_2$ HI titer, the contact chickens showed 6.0 ± 0.7 log$_2$ HI (Table 5).

3.3.2. *Pathogenicity of H9N2 in mice*

No noticeable clinical symptoms were present, and all the mice

Table 2
Genetic analysis of amino acid sequence of the HA and NA protein of wild birds and environment viruses.

Virus	HA cleavage	Receptor binding site (RBS)	NA HB active sites	Glycosylation sites	
		166 191 198 202 203 234 235	366–373	399–406	431–433
GF/14	PSKSSR ↓G	N H T L Y L I	IKKDLRA	DSDDWSGY	PKE
GF/15	PARSSR ↓G	N H T L Y L I	IKKDLRA	DSDDWSGY	PKE
BWS/18	PARSSR ↓G	N H A L Y L I	IKKDLRA	DSDDWSGY	PKE
GHG/18	PARSSR ↓G	N H A L Y L I	IKKDLRA	DSDNWSGY	PKE
EN/18	HARSSR↓G	N H A L Y L I	IKKDLRA	DSDNWSGY	PKE

survived for 14 days. Both GF/14 and GHG/18 were detected in the turbinate and the lungs. However, they were not detected in the livers, spleen, intestine, kidneys and brain of the inoculated mice. The infected mice with GF/14 and GHG/18 had higher viral titers in the lungs (2.8 ± 0.43 log10 EID_{50}–3.24 ± 0.5 log10 EID_{50}) than in the turbinate (2.1 log10 EID_{50}–2.65 log10 EID_{50}) respectively, at 3dpi. Additionally, mice inoculated with GF/14 showed viral titers at 8 dpi in the lungs and turbinate (1.76 log10 EID_{50} – 1.45 log10 EID_{50}) (Table 6). However, no viral titers were detected in the group inoculated with GHG/18 at 8dpi. Both GF/14 and GHG/18 induced a significant body weight loss ($p < 0.05$) from 1dpi to 14dpi (Fig. 3).

4. Discussion

AIV H9N2 has been distributed worldwide. Wild birds play an important role in the genetics and evolution of AIV viruses (Li et al., 2005). In Tunisia AIV H9N2 became endemic in poultry farms and is causing significant economic losses to the industrial poultry sector. The reasons of the persistence of AIV H9N2 remain unclear due to the absence of large populations of waterfowl that can constitute an aquatic reservoir, as is the case in Asia or in The Nile Valley (Couacy-Hymann et al., 2012). However, the epidemiology, genetics and evolution of H9N2 AIV in wild birds and environment in Tunisia are not elucidated. To understand the evolution and molecular features of H9N2 AIV isolated from wild birds and environment in Tunisia, we carried out surveillance of AIV H9N2 in wild birds and in environment from three humid zones of the Ramsar Wetland (korba Lagoon, Mammoura Lagoon and Kuriat Island) during 2014–2018. We report the identification of H9N2 viruses from multiple species of wild birds (greater flamingos, gray-headed gull, black-winged stilt) and environment (Lagoon water) in Tunisia. Our study allowed us to provide evidence that Falconiformes and Phoenicopteriformes probably played a role in the infection and transmission of H9N2 AIV in Tunisia.

The phylogenetic analysis showed that the five AIV H9N2 viruses isolated from wild birds and environment belong to the G1 lineage. It was reported that The H9N2 G1-like have the ability to replicate in human alveolar epithelial cells (Lee et al., 2010) and have infected humans causing mild disease symptoms since the early 1990s (Lin et al., 2000; Peiris et al., 1999).

We noticed that some segments (PA and PB2) of H9N2 isolated viruses from wild birds and environment clustered in different group from those previously isolated from industrial poultry. For the other segments (HA, NA, NP, M, PB1 and PB2), the viruses isolated from environment are found distant from the other strains of wild birds and poultry. These findings may indicate, probably, that H9N2 virus has been transmitted from poultry to wild birds through environment contamination and then circulated between avian multispecies by direct or indirect contact. Tunisia is located in two wild birds' migratory flyways: the Black sea Mediterranean flyway and the East Africa West Asia flyway (Nagy et al., 2017). Consequently, Tunisia remains at high risk for potential high

Table 4
Replication in the chicken of GF/14 and GHG/18 (H9N2).

	Virus replication on 3 DPI($log_{10}EID_{50}$/0.1 mL)[1]				
Viruses	Lung	Trachea	Spleen	Kidney	Brain
GF/14	2,52 ± 0,5	2,23 ± 0,5	2 ± 0,15	1,61 ± 0,6	1,94±0,5
GHG/18	2,17 ± 0,3	1,65 ± 0,5	-	-	-

At 3 days Post-Inoculation (DPI), organs samples were collected from three birds experimentally infected with GF/14, GHG/18. The lungs, trachea, spleen, kidneys, and brain of the three taken chickens were collected for virus titer in eggs.

[1] A value of 1.5 was assigned if the virus was not detected from the undiluted sample in three embryonated chicken eggs. Virus titers are expressed as means ± standard deviation in $log_{10}EID_{50}$/0.1 mL of tissue.

- Not detected.

Table 3
Molecular characterization of H9N2 viruses isolated from wild birds and environment during 2014–2018.

Protein	Sites	GF/14	GF/15	BWS/18	GHG/18	EN/18	Function[1]
HA	198	T	T	A	A	A	Situated in RBS
	234	L	L	L	L	L	Confers the ability to bind α2,6 sialic receptors of human epithelial cells (Wan and Perez, 2007)
NA	72	T	T	T	T	T	Gives H9N2 the ability to replicate in mouse cells (Li et al., 2005)
	393	D	I	I	I	I	Gives H9N2 the ability to replicate in mouse cells (Li et al., 2005)
	470	I	I	I	I	I	Gives H9N2 the ability to replicate in mouse cells (Li et al., 2005)
	331	N	N	N	N	S	S331N creates the glycosylation site 331NSS33
	402	D	N	N	N	N	D402N creates the glycosylation site 402NWS404
PB2	249	E	A	E	D	D	Located in the cap-binding region
	251	R	K	R	R	R	Increase viral replication and pathogenicity of H1N1 (Cai et al., 2020)
PB1	538	D	D	D	D	D	Important for virulence and pathogenicity (Cai et al., 2020)
	678	S	S	S	S	S	Important for virulence and pathogenicity (Cai et al., 2020)
PA	S409N	N	N	N	N	N	Could enhance viral fitness in humans(Shaw et al., 2002)
NP	M136	M	M	M	M	M	Markers of human-associated H9 viruses (Chen et al., 2006)
M1	V15I	I	I	I	I	I	Mammalian host-specific markers (Chen et al., 2006)
	N30D	D	D	D	D	D	Increased virulence in mice (Chen et al., 2006)
	T215A	A	A	A	A	A	Increased virulence in mice (Chen et al., 2006)
M2	A30S	S	S	S	S	S	Provides viral resistance to Amantadine (Ilyushina et al., 2005)[Ilyushina et al., 2005]
	S31N	N	N	N	N	N	Provides viral resistance to Amantadine (Ilyushina et al., 2005)
NS1	F103L	L	L	L	L	L	Adaptive genetic determinant of growth and virulence in humans (Dankar et al., 2011)
	G184	G	G	G	G	G	Contribute to cleavage and binding to the specific polyadenylation factor(Steidle et al., 2010)
	P42S	S	S	S	S	S	Increased virulence in mammals (Dankar et al., 2011)
	V149A	A	A	A	A	A	Increased virulence in mammals (Steidle et al., 2010)

[1] The function of each site is described in brief, together with the related references.

Table 5
Virus titers in chicken swabs.[1]

Viruses	Group	Route	Virus titer ($log_{10}EID_{50}$/0.1 mL, mean+ SD)[2] 1 Day	3 Days	5 Days	7 Days	9 Day	11 Days	13 Days	HI titer (log_2, mean ± SD)
GF/14	Inoclulated	OP	1.97 ± 0.3 (6/6)	2.33 ± 0.3 (6/6)	2.65 ± 0.44 (3/3)	2.56 ± 0.50 (2/3)	ND (0/3)	ND (0/3)	ND (0/3)	6.5 ± 1.0 (3/3)
		CL	ND (0/3)	ND (0/3)	2,78 ± 0.50 (2/3)	2.83 ± 0.85 (2/3)	2.71 ± 0.85 (2/3)	1.89 ± 0.43 (1/3)	1.78 ± 0,32 (1/3)	
	Contact	OP	ND (0/3)	2.83 ± 0.3 (6/6)	2.70 ± 0.81 (1/3)	2.81 ± 0.5 (2/3)	2.80 ± 0.5 (2/3)	1.83 ± 0.56 (1/3)	ND (0/3)	6.4 ± 0.6 (3/3)
		CL	ND (0/3)	ND (0/3)	2.64 ± 0.14 (2/3)	2.75 ± 0.43 (2/3)	1.77 ± 0,45 (2/3)	ND (0/3)	ND (0/3)	
GHG/18	Inoclulated	OP	1.83 ± 0,25 (6/6)	1.78 ± 0,32 (5/6)	1.68 ± 0,13 (2/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	6.2 ± 0.9 (3/3)
		CL	ND (0/6)	ND (0/6)	2,15 ± 0,51 (2/3)	1,83 ± 0,38 (2/3)	ND (0/3)	ND (0/3)	ND (0/3)	
	Contact	OP	ND (0/3)	ND (0/3)	1,73 ± 0.45 (2/3)	1,96 ± 0.43 (3/3)	ND (0/3)	ND (0/3)	ND (0/3)	6.0 ± 0.7 (3/3)
		CL	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	

[1] oropharyngeal (OP) and cloacal (CL) swabs.
[2] A value of 1.5 was assigned for calculations if the virus was not detected from the undiluted sample in three embryonated chicken eggs. Virus titers are expressed as mean ± standard deviation in $log_{10}EID_{50}$/0.1 mL of tissue.
- Not detected.

Table 6
Virus titer in turbinate (Tb) and lung (L) of infected mice at the indicated time.

Virus	GF/14		GHG/18	
	Tb	L	Tb	L
3dpi	2.65 ± 0,63 (3/3)	3.24 ± 0.5 (3/3)	2.1 (3/3)	2.8 ± 0.43 (3/3)
6dpi	2.12 ± 0,49 (2/3)	2.23 (2/3)	1.52 ± 0,6 (1/3)	1.7 (1/3)
8dpi	1.45 (1/3)	1.76 (1/3)	-	-

The average virus titer was expressed as log10EID50/ml+- SD (positive number/total number).
- indicate that virus was not detected.

pathogenic avian influenza (HPAI) introductions. Areas such as Maamoura Lagoon, where wetland birds rest during their migration, might be highly contaminated with AIV from bird feces. Rohani et al. (2009) reported that water plays a significant role in the spread of pathogenic viruses, such as avian influenza viruses (AIV).

Analysis of molecular characteristics showed that four Tunisian AIV viruses GF/15, BSW/18, GHG/18, and EN/18 possess a monobasic cleavage site, which is characteristic of H9N2 viruses with low pathogenicity isolated from the Middle East (Wang et al., 2018). These four viruses shared the same HA1/HA2 cleavage site motif (^{335}RSSR/GLF^{341}), while one virus from greater flamingo GF/14 exhibited the dibasic pattern at the cleavage site (^{335}KSSR/GLF^{341}) motif reported for the first time in Tunisia. Soda et al. (2011) reported that this substitutions increase pathogenicity of AIV H9N2 in chicken. This is typical for low-pathogenic AIV to poultry and was confirmed by the chicken intravenous pathogenic test (IVPI). The receptor-binding site (RBS) of the studied H9N2 Tunisian AIV viruses carried the 234 L (236 L in H3 numbering) and 235I markers (Wan and Perez, 2007). This molecular marker has been observed for the first time in Tunisian AIV H9N2 in wild birds, and environment viruses. In this regard, Matrosovich et al. (2008) reported that residue 234 L is typical of human pandemics AIV H2 and H3 subtypes. Neuraminidase for Tunisian viruses was substituted in the HB site, similar to other H9N2 avian viruses isolated from Asia and the Middle East during human H2N2 and H3N2

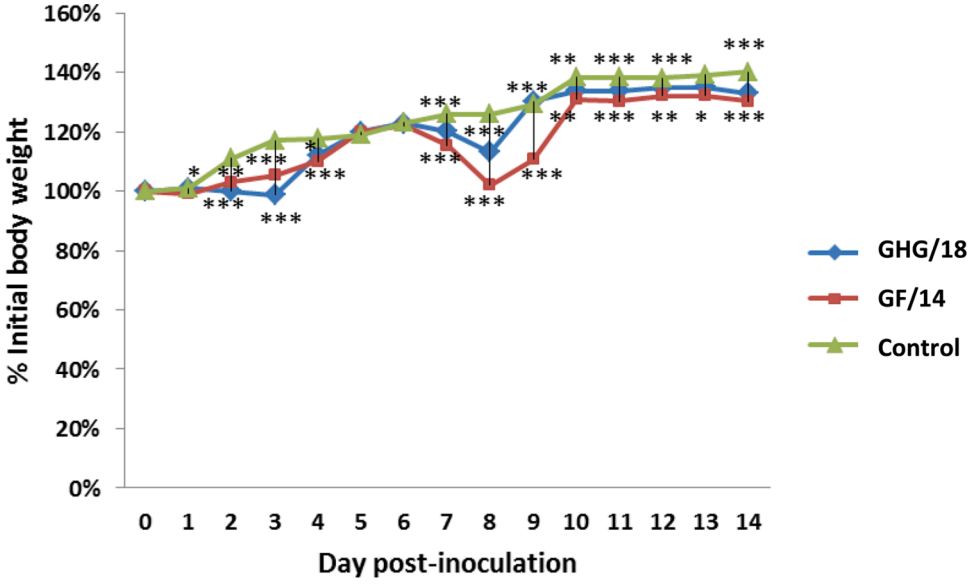

Fig. 3. Mean changes in body weight of mice infected with H9N2 viruses.

pandemics that have the ability to bind to the α2,6 receptor. In the viruses isolated in this work, the substitution of W to S occurred and led to the emergence of a new site of glycosylation 331NSS334. The mutation in NA created two glycosylation sites inside and near the HB site that might prevent antibodies from binding. Matrosovich et al. (2008) reported that two mutations, S372A and R403W, enhanced the cross of the species barrier and the adaptation to a mammalian host. These two mutations were detected in Asia and the Middle East in H2N2 and H3N2 subtypes that caused human pandemics. The studied H9N2 AIV were characterized by accumulation of new adaptive mutations in M2, PA and NP which were previously shown to contribute to viral fitness and adaptation to mammalian hosts (Ilyushina et al., 2005; Jakhesara et al., 2014; Liu et al., 2016). In addition, PA amino acids of GF/14 and GF/15 were characterized by the appearance of mutations I407V, which is characteristic of H1N1 are also rare in other avian influenza viruses (Liu et al., 2016), associated with mammals, specifically, residues that are important for changing the range of avian to humans hosts. The studied H9N2 AIV contained on NS1 protein a serine in position 42 and alanine in position 149. In a study using strains from ostriches in China, these two motifs would found to participate in the increased virulence in mammals (Davidson et al., 2010).

In the pathogenicity study, both GF/14 and GHG/18 were able to replicate directly in inoculated chickens and also transmitted to naïve chickens. In terms of viral shedding, all chicken inoculated with GF/14 and GHG/18 shed titers virus at low titers via the OP and OC route. Chrzastek et al. (2021) and Spickler et al. (2008) studies described that LPAI viruses as mainly targeting the respiratory, limitedly targeting the gastrointestinal tract and usually associated with poor cloacal virus shedding in infected birds. It was noticed that there are different patterns in oropharyngeal shedding among the infected groups, where the GF/14 virus produced later oropharyngeal virus shedding on dpi 1 to till 7 compared to GHG/18, which produced CL shedding from 1 to 5dpi. Variation in virus shedding onset and the duration of virus shedding was described in previous studies with LPAI viruses (Spickler et al., 2008). In addition, GF/14 replicated in the extrapulmonary organs, including the brain of chickens. The replication of H9N2 virus in the brain has been already reported (Kye et al., 2021; Song et al., 2019). After inoculation of BALB/c mice, the Tunisian H9N2 caused a body weight loss and merely replicated in the mice tract respiratory organs. Guo et al. (2020) demonstrated the ability of H9N2 virus isolated from chickens in China to infect mice lung causing tissue damage and body weightloss. Liu et al. (2014) also reported that mice challenged with duck-origin H9N2 virus became infected and after a few passages in mice the H9N2 virus acquired more virulence.

This study reported, for the first time, the full length genome sequencing of five H9N2 strains isolated from wild birds and the environment in Tunisia. Here, we also demonstrated that these viruses harboring human-like markers have the ability to be air-born transmitted between chickens and infect efficiently mice. Hence, it is necessary to conduct additional studies assessing avian to human transmission of H9N2 in occupationaly workers in Tunisia.

Declarations ethical approval and consent to participate

All experiments were conducted under biosafety level 2 conditions. All animal studies have been approved by the Bio-Medical Ethics Committee of the Institut Pasteur de Tunis (IPT), and were done in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Experimental Animals laboratory (reference: 2015/33/I/LRAAIPT/03).

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CRediT authorship contribution statement

Imen Larbi: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Kais Ghedira:** Data curation. **Marwa Arbi:** Data curation. **Gary David Butcher:** Methodology, Writing – review & editing. **Natalia Rego:** Software, Validation. **Hugo Naya:** Software, Validation. **Halima Tougorti:** Data curation. **Jihene Lachhab:** Visualization. **Imen EL Behi:** Visualization. **Jihene Nsiri:** Visualization. **Abdeljelil Ghram:** Methodology, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability

No data was used for the research described in the article.

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