Highly pathogenic avian influenza virus (H7N1) infection in local chicken breeds: pathogenesis and role of Mx gene (A/G 2032 polymorphism) in disease outcome

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Highly-pathogenic avian influenza viruses (HPAIV) produce severe disease in domestic poultry population. Local chicken breeds are generally raised by small-scale farmers in extensive production systems. These systems allow contact with wildlife bird species, which are potential carriers of AIV. Nevertheless, there is a general believe that local breeds are natural resistant to disease after being naturally selected by autochthonous pathogens, food availability and harmful climate. In the present study, we assessed the susceptibility of four common Catalan chicken breeds (Empordanesa, Penedesenca, Catalana del Prat, Flor d'Ametller) and Specific-Pathogen Free (SPF) chicken to HPAIV H7N1 (H7N1/HP). Furthermore, we determined the A/G polymorphism at position 2032 of chicken antiviral-gene Mx by RFLP-PCR techniques. Chickens were intranasally inoculated with 10^5 ELD_{50} of H7N1/HP virus in a final volume of 50 µl and monitored daily during 10 days. The results showed that all local chicken breeds were highly susceptible to H7N1/HP infection, with mortalities that varied from 80 to 100%, compared to the 50% mortality rate present in SPF chicken. Macroscopic lesions typical of HPAIV such as hemorrhagic lesions in internal organs and cutaneous edema and cyanosis were observed. Despite the light microscopic observation of tissues demonstrated the presence of characteristic multifocal necrotic foci associated with inflammatory cells infiltration in all groups, distribution and intensity of lesions as well as amounts of AIV-positive cells varied among the animals of the different local breeds. These suggest the existence of breed-specific host factors affecting H7N1/HP viral replication. Moreover, RFLP-PCR analyses demonstrated a slightly higher % of survival and delayed Mean Death Time in individuals presenting A polymorphism in 2032 position of Mx gene. Considering that Catalan chicken breeds displayed high susceptibility to H7N1/HP virus, those breeds should be closely monitored during HPAIV outbreaks. Host factors such as polymorphisms in antiviral genes are key factors in AIV infection outcome.

Los virus de influenza aviar de alta patogenicidad (IAAP) causan una enfermedad grave en las aves domésticas. Las razas locales de pollos generalmente se crían a pequeña escala en sistemas de producción extensivos. Estos sistemas facilitan el contacto con aves silvestres, potenciales portadoras de virus de influenza aviar. Sin embargo, existe la creencia generalizada de que las razas locales presentan una resistencia natural a las enfermedades al haber sido seleccionadas naturalmente por patógenos autóctonos, la disponibilidad de alimentos y el clima. En el presente estudio, se evaluó la susceptibilidad de cuatro razas locales de pollo, comunes en Cataluña (Empordanesa, Penedesenca, Catalana del Prat, Flor d'Ametller), y de pollos libres de patógenos específicos (SPF), al virus de IAAP H7N1 (H7N1/HP). Además, se determinó el polimorfismo A/G presente en la posición 2032 del gen antiviral Mx mediante técnicas de RFLP-PCR. Los pollos se inocularon por vía intranasal con una dosis de 10^5 ELD_{50} del virus H7N1/HP en un volumen de 50 µl y se monitorizaron diariamente durante 10 días. Los resultados mostraron que

todas las razas de pollo locales evaluadas son altamente susceptibles a la infección por H7N1/AP, con mortalidades que variaron de entre un 80 a un 100%, en comparación con la tasa de mortalidad del 50% detectada en los pollos SPF. Las lesiones macroscópicas observadas fueron las típicas de la infección por IAAP: lesiones hemorrágicas en órganos internos y edema y cianosis cutáneos. Aunque la evaluación microscópica de los tejidos mostró la presencia multifocal de focos necróticos con infiltración de células inflamatorias en todos los grupos, la distribución y la gravedad de las lesiones así como la cantidad de células positivas a IA variaron entre los animales de las distintas razas locales. Esto sugiere la existencia de factores del huésped, específicos de cada raza, que intervienen en la replicación del virus H7N1/AP. Además, los análisis mediante RFLP-PCR mostraron un porcentaje de supervivencia ligeramente superior, así como un tiempo medio de mortalidad más tardío, en los individuos que presentaban el polimorfismo A en la posición 2013 del gen Mx. Teniendo en cuenta que las razas catalanas de pollos mostraron una elevada susceptibilidad al virus H7N1/AP, éstas deberían ser monitorizadas en caso de un brote de IAAP. Factores del huésped como los polimorfismos en genes antivirales son factores clave en el desarrollo de la infección causada por los virus de influenza aviar.

Keywords: Avian influenza; Local chicken breeds; Pathogenesis.

Introduction

Avian influenza (AI) is a viral disease that produce severe illness in domestic poultry population, being one of the main causes of economic losses in avian food-producing industry (Capua & Alexander, 2006). AI viruses (AIV) are classified into low or high-pathogenic depending on hemagglutinin (HA) and neuraminidase (NA) viral surface glycoproteins. At the moment, highly-pathogenic AIV (HPAIV) are limited to AIV carrying H5 and H7 HA subtypes (Reperant et al., 2012).

Wild birds are considered the natural reservoirs of AIV so far (Webster et al., 1992), since they allow the replication of virtually all HA and NA AIV combinations. On October 2016, H5N8 HPAIV belonging to clade 2.3.4.4 was isolated in one wild bird. Since then, this strain has been further identified in wild birds and poultry farms in 22 and 18 Member States from Europe, respectively (European Commission, 2017), demonstrating the pivotal role of wild birds in the dissemination and interspecies transmission of AIV. For that reason, FAO recommends intensified surveillance and improved biosecurity measures, which include avoiding contact of poultry with wild birds and their sub-products (FAO, 2017).

Local chicken breeds are generally raised by small-scale farmers in extensive production systems, which allow close contact with wild birds and their droppings. Consequently, these animals are likely more exposed to AIV. On the other hand, there is a general believe that local chicken breeds are natural resistant to disease, which is associated with the natural selection over the years by autochthonous pathogens, food availability and harmful climate (Minga et al., 2004). However, this assumption is usually a result of empiric experience at field level, since low number of experimental studies addressing the resistance of local breeds to infectious diseases are available.

The resistance to AIV is a complex process that involves both viral and host factors. A non-synonymous G/A polymorphism at position 2032 of chicken Interferon-induced GTP-binding protein Mx1 (Mx) cDNA produces a substitution of serine with asparagine at position 631 of Mx protein. Animals carrying asparagine at this position present higher antiviral activity against vesicular stomatitis virus, Newcastle disease virus and AIV in vitro (Ko et al., 2002).

In the present study, we assessed the pathogenesis of HPAIV H7N1 (H7N1/HP) in four common local chicken breeds from Catalonia and Specific-Pathogen Free (SPF) chicken. Furthermore, the role of of G/A polymorphism at position 2032 of chicken Mx in H7N1/HP infection outcome was evaluated.

Materials and methods

Virus. The virus used in this study was A/Chicken/Italy/5093/1999 H7N1/HP, which was isolated in 1999-2000 during an Italian epidemic (Capua et al., 2000). Virus stocks were produced in 10 days-old SPF embryonated eggs. The allantoic fluid was obtained at 48 hours post-inoculation, filtered and aliquoted at -80ºC until use. Serial ten-fold dilutions of the filtered virus in PBS were used for titration in 10 days-old SPF embryonated eggs. The mean egg lethal dose (ELD50) was determined by Reed and Muench method (Reed and Muench, 1938).

Animals, facilities and experimental infection. 22 two week-old SPF chickens and 26 two week-old chicken of the four different local breeds from Catalonia were used. The local chicken breeds selected in this study were: Catalana del Prat (C. Prat), Penedesenca, Empordanesa and Flor d'ametller (F. Ametller). At arrival, the animals were individually identified and placed in negative-pressured HEPA-filtered isolators present in Biosecurity Level 3 (NBS-3) facilities of Centre de Recerca en Sanitat Animal (Programa de Sanitat Animal, IRTA). Prior to infection, serum samples were obtained from all animals to ensure that they were seronegative to Influenza A virus by an ELISA competition (c-ELISA) test (ID-VET, Montpellier, France). Furthermore, oropharyngeal (OS) and cloacal swabs (CS) were collected from 5 random animals of each group and confirmed to be negative to AIV by one-step quantitative reverse-transcription polymerase chain reaction (qRT-PCR). After 5 days of acclimation, virus was diluted in PBS in order to inoculate intranasally 10^5 ELD50 of H7N1 in a final volume of 0.05 mL. Animals belonging to negative control group were intranasally inoculated with 0.05 mL of sterile PBS. During the experimental procedures, food and water were provided ad libitum.

Clinical signs. All the birds were monitored daily for clinical signs until 10 day post-inoculation (dpi). A standardized OIE clinical scoring system was used (OIE, 2005). Animals with absence of clinical signs were classified as 0. Birds presenting one of the following clinical signs were considered as sick (1) and those showing more than one were considered as severely sick (2): respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head, nervous signs. Animals found dead were scored as 3. Moribund chicken were euthanized with intravenous pentobarbital for ethical reasons and scored as dead. The severity of clinical signs, percentage of mortality and mean death time (MDT) were calculated in each breed.

Pathologic examination and immunohistochemical testing. Three and two animals belonging to H7N1/HP-infected and negative groups of each breed, respectively, were sacrificed at 3 day post-infection (dpi) for pathological studies. A standardized necropsy was performed in order to detect gross lesions. Tissue samples were collected, immersed in 10% formalin for fixation during 48 hours and embedded in paraffin wax. Samples included skin, thymus, pectoral muscle, nasal cavity, trachea, lung, central nervous system (CNS), heart, spleen, liver, kidney, proventriculus, gizzard, pancreas, duodenum, cecum, colon and bursa of Fabricius.

Cut sections of 3 µm (Leica RM2255, Nussloch, Germany) from formalin-fixed, paraffin-embedded tissues were processed, stained with haematoxylin and eosin and then examined under light microscopy. An immunohistochemical (IHC) technique was performed as previously described (Chaves et al., 2011). Briefly, a mouse-derived monoclonal commercial antibody against nucleoprotein (NP) of influenza A virus (ATCC, HB-65, H16L-10-4R5) was used as a primary antibody. The samples were then incubated with biotinylated goat anti-mouse IgG secondary antibody (Dako, immunoglobulins As, Denmark).The antigen–antibody reaction was visualized using the chromogen 3,3'-diaminobenzidine tetrahydrichloride.

RLFP-PCR Mx. Prior to infection**,** total blood in a 1:1 ratio with anticoagulant (Alsever's solution, Sigma-Aldrich, Missouri, USA) was obtained from all animals. Genomic DNA was isolated from 10 µl anticoagulated blood using a standard DNA purification kit (DNeasy Mini Kit, Qiagen, CA, USA), following manufacturer's instructions. To avoid RNA contamination, samples were treated with RNase (RNase A, Qiagen, CA, USA). As described by Sironi et al., (2010), the following primers were used to amplify a 299 pair bases (pb) region of Mx gene: forward 5'-GCACTGTCACCTCTTAATAGA-3' and reverse 5'-GTATTGGTAGGCTTTGTTGA-3'. PCR reaction mixture included 60 ng of genomic DNA, 0.25 µM of each primer, 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 1.5 U of native Taq DNA polymerase (Taq DNA Polymerase, native, ThermoFisher Scientific, Massachusetts, USA) and autoclaved distilled water in a final volume of 25 µl. Mx region was amplified in GeneAmp PCR System 9700 equipment (Applied Byosistems, CA,

USA) as follows: 95°C for 10 min, 35 cycles of 94°C for 1 min, annealing at 53°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 10 min.

5 µl of PCR products were run in a 2% agarose gel in 1X TAE buffer with ethidium bromide (EtBr) to confirm the presence of the band at 299 pb. PCR products were incubated at 37ºC during 16 hours with a restriction enzyme (Hpy8I-MjaIV, 10 U/µL, ThermoFisher Scientific, Massachusetts, USA), following manufacturer's instructions. The restriction enzyme (5'-GTN|NAC-3') cleaves the sequence 2 pb downstream of the Mx polymorphism in presence of guanine (G), whereas the product is not cut in case of an adenine (A) at this position. Digestion products were visualised in a 2.8% agarose gel in 1X TAE buffer with EtBr. Animals were classified in homozygous-resistant allele (AA), heterozygous-intermediate allele (AG) and homozygous-susceptible allele (GG).

Results and Discussion

All local chicken breeds inoculated with H7N1/HP virus presented moderate to severe clinical signs during the experiment. Clinical signs were those typical of HPAIV infection, including severe apathy, prostration, respiratory distress, conjunctivitis, cutaneous edema, cyanosis of the comb and nervous signs (Chaves et al., 2011), although they differed depending on the breed. For instance, specific clinical signs such as cutaneous edema and nervous signs were frequently observed in Penedesenca and C.Prat breeds, respectively, but not in the other breeds.

Accordingly, mortalities varied from 80 to 100% in local chicken breeds, compared to the 50% mortality rate present in SPF chicken. Despite slightly longer MDT were observed in Penedesenca breed, our results demonstrate that all the local chicken breeds tested were highly susceptible to H7N1/HP infection (Table 1).

Table 1. Mortality rate, clinical signs scoring (0-3) and MDT of local chicken breeds and SPF chicken experimentally inoculated with H7N1/HP virus. MDT, mean death time; dpi, day post-infection.

Breed	Mortality rate (%)	Clinical signs scoring	MDT (dpi)
Empordanesa	100	2,19	3,28
Penedesenca	94,4	2,09	4,2
Catalana Prat	100	2,28	3,3
F. Ametller	80	1,89	3,3
SPF	50	0,93	3,8

In agreement with previous studies, gross examination of naturally-dead and euthanized animals revealed the presence of hemorrhages in internal organs and skeletal muscles. Lung consolidation as well as congestion in internal organs were also observed in several animals. Furthermore, some chickens exhibited subcutaneous edema (Image 1A-D) (Chaves et al., 2011; Bertran et al., 2013).

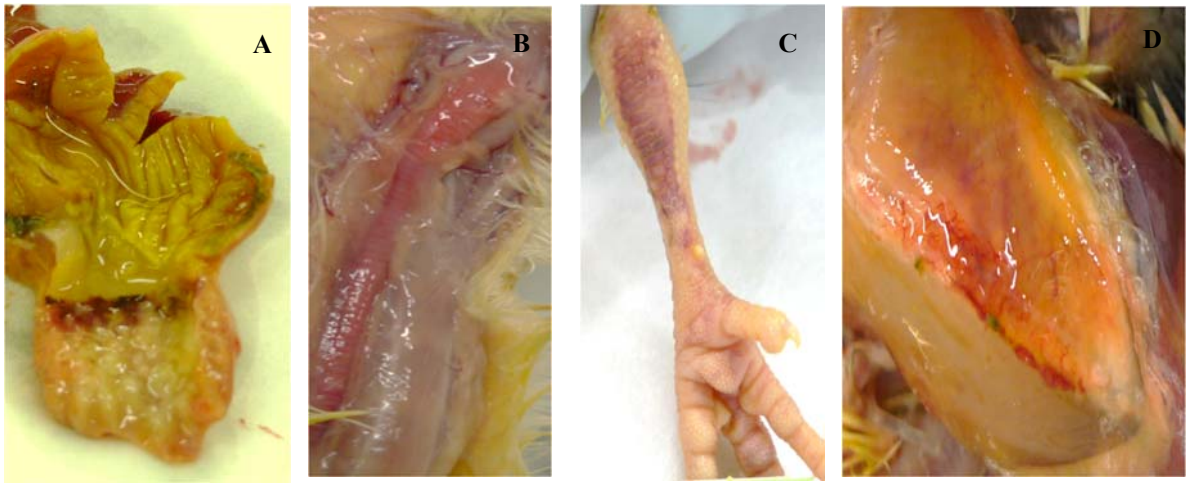

Image 1. Gross lesions observed in local chicken breeds and SPF chicken experimentally inoculated with H7N1/HP. A. Diffuse hemorrhage in gizzard-proventriculus junction. B. Diffuse congestion of the trachea. C. Diffuse hemorrhage in leg skin. D. Subcutaneous edema and diffuse hemorrhage in pectoral muscle.

Microscopic lesions typical of HPAIV infection were observed in all breeds at 3 dpi. Areas of necrosis and hemorrhages with inflammatory infiltrate composed by heterophils, macrophages and lymphocytes were commonly present in collected organs (Image 2A-D). Accordingly, IHQ testing revealed active replication of H7N1/HP virus at 3 dpi in almost all examined organs, demonstrating a systemic dissemination of H7N1/HP virus. In agreement with previous studies, viral replication was mainly observed in heart, CNS, pancreas and skin (Image 2E-H) (Bertran et al., 2011, 2013; Chaves et al., 2011).

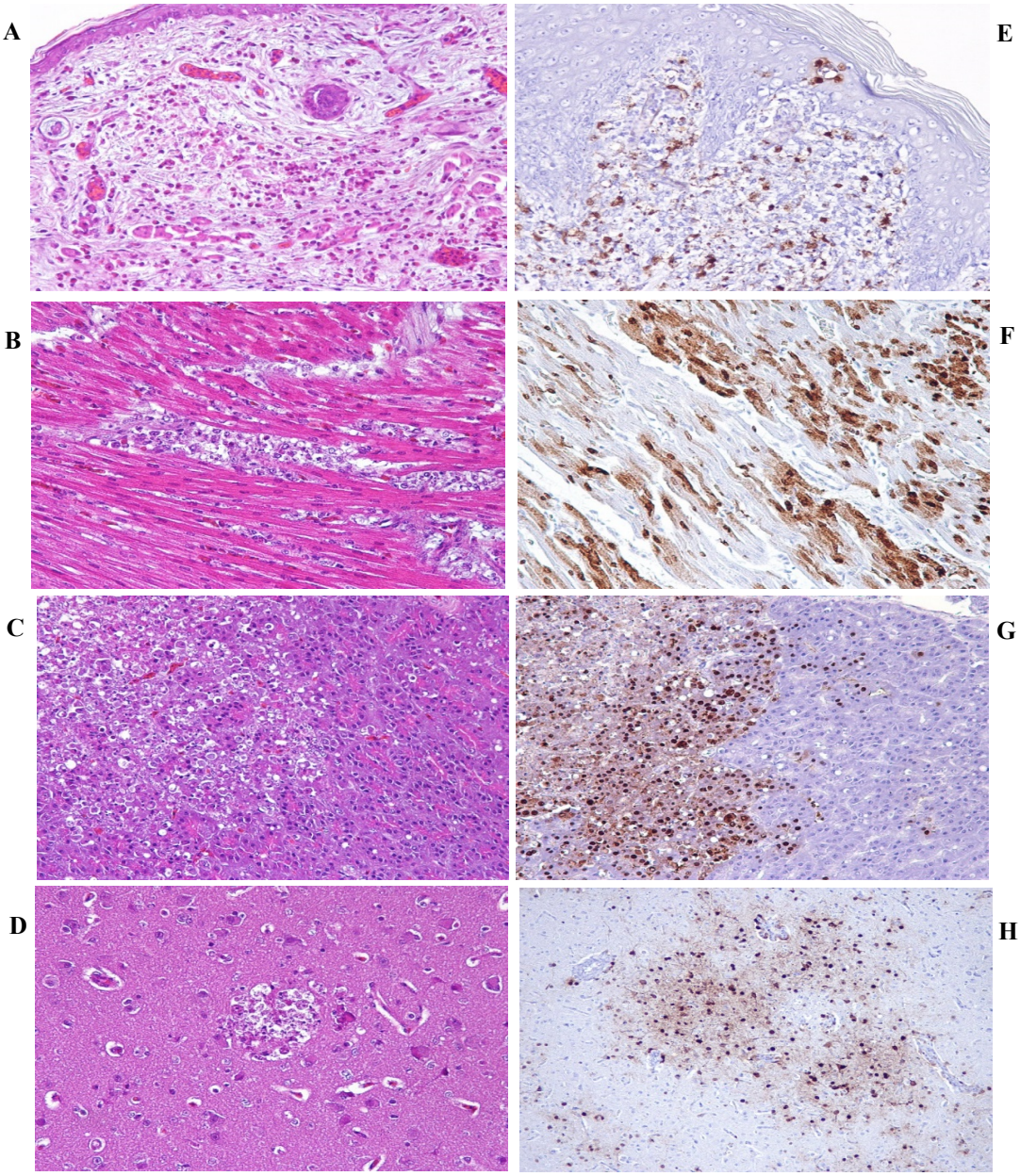

Image 2. Microscopic lesions (A-D) and AIV-IHC staining (E-H) at 3 dpi in several organs of local chicken breeds and SPF chicken experimentally inoculated with H7N1/HP. A. Skin, 20x: diffuse heterophylic infiltration and edema in dermis. B. Heart, 20x: multifocal muscle fiber necrosis. C. Pancreas, 20x: diffuse necrosis of pancreatic acinar cells. D. CNS, 20x: Focal necrosis of neurons and glial cells. E. Skin, 20x: AIV detection in inflammatory cells, endothelial cells and keratinocytes. F. Heart, 20x: AIV detection in muscle fibers. G. Pancreas, 20x: AIV detection in pancreatic acinar cells. H. CNS, 10x: AIV detection in neurons and glial cells. CNS; central nervous system.

Despite typical H7N1/HP microscopic lesions and viral replication were observed in all groups, intensity of lesions as well as distribution of AIV-positive cells varied among the animals of the different local breeds (Table 2).

The highest differences between breeds regarding viral replication were detected in pancreas, heart, CNS, skin and spleen (Table 2). C.Prat and Penedesenca breeds presented the highest amounts of AIV-positive cells in collected tissues, which correlated with the severity of infection. In contrast, lower presence of viral antigen was generally detected in SPF and F.Ametller chickens. The observed variation suggest the existence of breed-specific host factors affecting H7N1/HP replication.

Table 2. AIV-positive cells distribution determined by immunohistochemistry in tissues collected from local chicken breeds and SPF chicken at 3 dpi. In brackets is represented the number of animals presenting AIV-positive cells. +, single positive cells; ++, scattered groups of positive cells; +++, widespread positivity. CNS, central nervous system.

	Tissue							
Breed	**Skin**	**Skeletal Muscle**	**Nasal cavity**	**Lung**	**CNS**	**Pancreas**	**Heart**	**Spleen**
Empordanesa	-	+ (1/3)	+ (1/3)	++ (1/3)	+++ (1/3)	+ (1/3)	+++ (1/3)	+ (2/3)
Penedesenca	++ (1/3)	+ (3/3)	+ (1/3)	+ (2/3)	+++ (3/3)	+++ (3/3)	+++ (3/3)	+ (3/3)
Catalana Prat	++ (3/3)	++ (3/3)	+ (3/3)	+ (2/3)	+++ (3/3)	+++ (3/3)	+++ (3/3)	+++ (3/3)
F. Ametller	+ (2/3)	+ (1/3)	++ (2/3)	+ (2/3)	++ (3/3)	++ (1/3)	++ (3/3)	+ (3/3)
SPF	+ (2/3)	+ (2/3)	+ (1/3)	+ (2/3)	++ (3/3)	+ (2/3)	++ (3/3)	+ (1/3)

	Tissue							
Breed	**Thymus**	**Liver**	**Kidney**	**Provent**	**Gizzard**	**B.Fabricius**	**Small int.**	**Large int.**
Empordanesa	++ (1/3)	++ (1/3)	++ (1/3)	+ (1/3)	++ (1/3)	++ (1/3)	+ (1/3)	+ (1/3)
Penedesenca	+ (2/3)	+ (3/3)	+ (3/3)	+ (3/3)	++ (3/3)	+ (3/3)	+ (1/3)	+ (1/3)
Catalana Prat	++ (1/3)	++ (3/3)	++ (2/3)	++ (3/3)	++ (3/3)	++ (3/3)	+ (2/3)	+ (2/3)
F. Ametller	+ (2/3)	+ (3/3)	+ (3/3)	+ (1/3)	++ (2/3)	+ (1/3)	+ (1/3)	-
SPF	+ (1/3)	+ (3/3)	+ (2/3)	+ (2/3)	++ (3/3)	+ (2/3)	+ (2/3)	-

The host mechanisms underlying resistance to viral diseases remain mostly unknown. However, several studies associate specific alleles in innate immunity-related genes with disease outcome. Mx is a protein that prevents virus-related functions by binding viral components (Verhelst et al., 2013). A specific non-synonymous nucleotide polymorphism at position 2032 of chicken Mx cDNA produces an aminoacid substitution at position 631 (Serine to Asparagine). The presence of an asparagine at position 631 is positively associated with the capacity of Mx gene product to interfere with viral replication (Sironi et al., 2008).

In agreement with previous studies, our results demonstrate that wide differences regarding allelic distribution of Mx gene are present between local breeds (Sulandari et al., 2009). Moreover, broad differences are also observed within breeds (Table 3A), suggesting that Catalan chicken breeds maintain its genetic variability. Conversely, F.Ametller population appears to be virtually fixed for the susceptible allele (94.4 %).

The association of Mx allele with infection outcome revealed a slightly higher percentage of survival and longer MDT in AA individuals (Table 3B), suggesting a potential role of asparagine at position 631 in H7N1/HP infection dynamics. However, Mx allele did not necessary correlated with microscopic findings and viral replication in tissues, demonstrating that HPAIV infection outcome is a result of numerous host and viral factors.

Table 3. A. Allelic average frequencies at position 2032 of chicken Mx cDNA in local chicken breeds and SPF chicken. B. % of survival and MDT of animals experimentally inoculated with H7N1/HP presenting different Mx alleles. AA, resistant allele; AG, heterozygous allele; GG, susceptible allele. Dpi; day post-infection.

A. **Allelic distribution (%)**

Breed	AA	AG	GG
Empordanesa	11,7	47	35,3
Penedesenca	44,4	27,7	27,7
Catalana Prat	29,4	64,7	5,9
F. Ametller	0	5,5	94,4
SPF	75	25	0

B. **Survival (%) and MDT (dpi)**

	AA	AG	GG
Survival	19,2	8,7	13
MDT	4	3,55	3

In conclusion, the data shows that Catalan chicken breeds are highly susceptible to H7N1/HP infection. Since local chicken breeds are usually raised in backyard or extensive production systems that allow contact with wild birds and their droppings, these breeds should be closely monitored during HPAIV outbreaks. The differences in microscopic lesions and viral replication indicate that breed-specific host factors are involved in H7N1/HP infection dynamics. Resistant-allele carriers (AA) in Mx gene present a slightly higher percentage of survival and longer MDT than AG and GG individuals, supporting the idea that host factors are pivotal contributors to the infection outcome.

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