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# Susceptibility of different chicken lines to H7N1 highly pathogenic avian influenza virus and the role of *Mx* gene polymorphism coding amino acid position 631

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ABSTRACT

Five chicken lines were experimentally infected with a HPAI H7N1 virus, to examine the variation in susceptibility to infection. Three lines showed high susceptibility to the virus, while two showed some resistance, with 7 out of 20, and 11 out of 15 birds, respectively, remaining healthy and surviving the experimental infection. Genotyping for the G/A polymorphism at position 2032 of *Mx* cDNA showed that one line was fixed for the G allele, and two were segregating for A and G alleles. Birds in the other two lines were selected to be fixed for the A allele. Statistical analyses indicated that the *Mx* genotype did not affect the clinical status or the time course of infection after viral inoculation.



## Introduction

Mx proteins confer resistance to different viral families, and antiviral activity of *Mx* gene products has been described in several vertebrate species (Lee and Vidal, 2002; Watanabe, 2007). Mx proteins belong to the dynamin family of large GTPases, and have two subcellular localizations: nucleus and cytoplasm (Haller et al., 2007). These proteins are suggested to interfere with viral replication by inhibiting viral polymerases in the nucleus and by binding viral components in the cytoplasm, thus preventing the virus functions (Haller and Kochs, 2002; Pavlovic et al., 1992; Salomon et al., 2007; Turan et al., 2004). *Mx* gene expression is induced by interferon and the gene product has been implicated as major component in resistance to influenza virus in mice (Grimm et al., 2007; Staeheli et al., 1986). Mice expressing the intact *Mx1* gene are protected against human H5N1 influenza virus and pandemic 1918 influenza virus strain, whereas the defective *Mx1* allele is associated with susceptibility (Salomon et al., 2007; Tumpey et al., 2007). Recent data have shown that human and avian influenza A virus strains exhibit different sensitivity to inhibition by murine Mx1 and human MxA proteins, with avian strains being more sensitive than human strains (Dittmann et al., 2008).

Duck Mx was the first avian Mx protein to be characterised, but was not found to have antiviral activity against influenza virus when transfected and expressed in chicken and mouse cells (Bazzigher et al., 1993). Analysis of the chicken *Mx* gene promoter identified an interferon-stimulated response element (ISRE) motif, indicating that chicken *Mx* gene expression is inducible by interferon (Schumacher et al., 1994). The chicken Mx protein is made up of 705 amino acids and, as for duck Mx protein, initial studies revealed no antiviral activity against influenza or other viruses in *in vitro* experiments (Bernasconi et al., 1995). Further sequence analysis of *Mx* cDNA from different chicken breeds (Ko et al., 2002) revealed a total of 25 nucleotide substitutions, 14 of which were non-synonymous. Mouse 3T3 cells, permanently transfected and expressing the *Mx* cDNA isolated from some of these chicken breeds, revealed antiviral activity to vesicular stomatitis virus (VSV) and H5N1 influenza virus. The antiviral activity differed between the *Mx* alleles from different breeds of chicken. Among the 14 possible amino acid variations, only one at position 631 of the chicken Mx protein was shown to affect antiviral activity to VSV. The variant having Asparagine instead of Serine at position 631 conferred the antiviral activity on the transfected cells. The *Mx* cDNA of the chicken Shamo breed, which was shown to confer antiviral activity against H5N1 influenza virus to the transfected cells, carried

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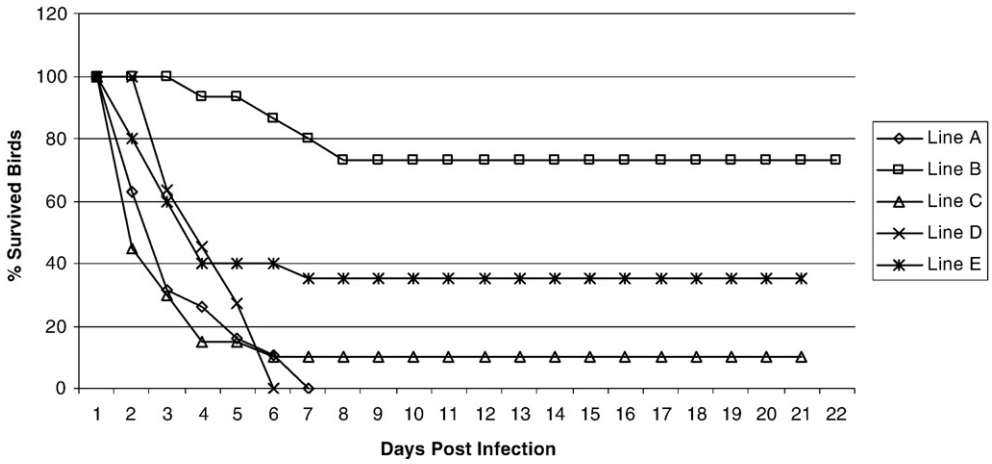


**Fig. 1.** Survival curves for the five chicken lines following experimental challenge (day "0") with HPAI H7N1 influenza virus.

the Asn residue at position 631 too (Ko et al., 2002). The antiviral role in VSV infection of the Asn residue at position 631 was later confirmed *in vitro* using constructed variants of *Mx* cDNA (Ko et al., 2004). Recent *in vitro* data (Benfield et al., 2008) demonstrated that the presence of Asparagine at position 631 of chicken Mx does not protect chicken embryo fibroblasts against infection with an H1N1 influenza virus, and does not suppress viral replication of three different influenza strains in 293T cells transfected with chicken *Mx* (Benfield et al., 2008). The S631N amino acid variation results from a G/A polymorphism at position 2032 of chicken *Mx* cDNA (Acc. No. Z23168). The *Mx* gene is made up of 14 exons, with one exon consisting of the 5′-UTR and 13 protein-coding exons: the polymorphism causing the S631N variation is located in the last exon, and corresponds to the position 1892 of the protein-coding region of the gene.

The frequency of alleles for the *Mx* gene 2032 G/A polymorphism has been investigated in several studies (Li et al., 2006; Seyama et al., 2006; reviewed by Watanabe 2007). Balkissoon et al. (2007) analysed the allelic frequency of the G/A polymorphism at position 2032 in commercial broiler and layer lines and found that the ancestors and modern commercial lines of broilers showed higher frequency of the G allele, whereas most commercial egg-layers and layer ancestor lines have higher frequency of the A allele. The jungle fowl, the supposed progenitor of domesticated chicken, was analysed by Seyama et al. (2006), who found that three Red jungle fowl from Indonesia, one Gray jungle fowl and four Green jungle fowl were homozygous for the G allele, while one Red jungle fowl from Laos carried the A allele. This suggests that the 2032 polymorphism predates chicken domestication.

The present work reports the response of five different chicken lines to H7N1 avian influenza virus following experimental infection and the effect of the *Mx* 2032 G/A polymorphism on the disease outcome.

## Results

### *Clinical observations*

Following experimental challenge of 19 birds of line A, 7 birds died 2 days PI, a further 6 died on day 3 PI, and all had died by day 7 PI. The last 2 birds that died, 7 days PI, were serologically negative indicating that by that time they had not produced antibodies against the virus. Only 4 of the 15 experimentally infected birds of line B died during the trial period, 1 bird on each of days 4, 6, 7 and 8. Eleven chickens survived the full 3 week trial and remained healthy with no clinical symptoms. It should be noted that birds were housed two *per* cage, and that birds that died shared cages with birds that survived. Serological analyses were carried out on the 11 surviving birds at 9, 16 and 22 days PI and all were negative. Twenty birds of line C were experimentally challenged and 11 died at day 2 PI, 3 birds died at day 3 PI, 3 birds died at day 4 PI, and 1 at day 6 PI. Only 2 birds survived the 3 week trial: one remained healthy throughout, showing no clinical symptoms, while the other bird was seriously ill at day 3 PI, then recovered. Serological analyses were carried out on both these birds at days 7, 13 and 21 PI. The bird showing no clinical symptoms was negative at all time points, while the bird that became ill and then recovered was sero-positive from day 13. Eleven birds of line D were experimentally challenged: at day 3 PI 4 birds died, 2 died 4 days PI and all had died by day 6 PI. Finally, 20 birds of line E were inoculated with virus. The first 4 birds died on day 2 PI, 4 birds died on day 3, 4 died on day 4, and 1 bird died on day 7 PI. The other 7 chickens survived the 3 week trial and were serologically negative throughout the trial. The survival profiles for all lines are shown in Fig. 1.

### *Genotyping of Mx gene and statistical analysis*

The genotype for the G/A polymorphism at position 2032 of *Mx* cDNA (Acc. No. Z23168) was determined by sequencing. However, the presence of indels in all the lines analysed meant that for some samples only the forward or the reverse strand could be analysed. Therefore, genotypes were confirmed using a PCR-RFLP protocol (data not shown). Genotyping results and allele frequencies in the five chicken lines are given in Table 1. Line A birds were fixed for the G allele thus having the putatively susceptible Ser residue at position 631 of the protein product. Line B and C birds were segregating for both A and G alleles, with A allele frequencies of 30% and 20%, respectively. Lines D and E were pre-selected to be, as far as possible, fixed for the A allele, although one bird of line E was heterozygous. The clinical status and genotypes of all birds are given in Supplementary Tables 1–5.

Line B showed a high level of resistance to this AI viral strain, with 11 birds surviving the challenge. Seven of the surviving birds were homozygous for the G allele, 3 were heterozygous AG, and only 1 bird

**Table 1**
Allelic and genotypic frequencies of the polymorphism at position 2032 of chicken Mx cDNA in the five chicken lines

| Line | N[a] | Allele frequency | | Genotype frequency | | |
|---|---|---|---|---|---|---|
| | | A | G | AA | AG | GG |
| Line A | 19 | 0.00 | 1.00 | 0.00 | 0.00 | 1.00 |
| Line B | 15 | 0.30 | 0.70 | 0.14 | 0.33 | 0.53 |
| Line C | 20 | 0.20 | 0.80 | 0.05 | 0.30 | 0.65 |
| Line D | 11 | 1.00 | 0.00 | 1.00 | 0.00 | 0.00 |
| Line E | 20 | 0.98 | 0.02 | 0.95 | 0.05 | 0.00 |

Lines D (New Hampshire) and E (White Leghorn) were selected, as far as possible, to be fixed for the A allele.
[a] Number of birds analysed for each line.

**Table 2**
Results of the statistical analysis of the association between clinical status following viral inoculation and either the "genotype" at the 2032 *Mx* cDNA polymorphism or the "line" of chicken

| Source | DF[a] | SS[b] | Mean square | *F* value | Pr>*F* |
|---|---|---|---|---|---|
| Genotype | 2 | 159.105562 | 79.552781 | 1.64 | 0.1998 |
| Line | 4 | 2764.936025 | 691.234006 | 14.29 | <.0001 |

[a] Degrees of freedom.
[b] Sums of squares.

was homozygous for the A allele. Of the 4 birds that died, only 1 was homozygous GG (death at 7 days PI), 2 birds were heterozygous AG (death at 4 and 8 days PI, respectively), and the last bird, which died 6 days PI, was homozygous AA. The line C bird that survived the full 3 week trial period carried the genotype AG, while the bird that fell ill and then recovered was homozygous GG. Six of the birds from this line that died carried the A allele: 1 was homozygous AA (death at day 2 PI), and 5 were heterozygous AG (dead at day 2 or 4 PI). Birds from both lines D and E had been selected to be homozygous for the resistant A allele. However, all line D birds died within 6 days, while for line E, 7 birds survived the 3 week trial. Genotype data and disease status for all individuals in the five chicken lines are given in Supplementary Tables 1–5.

In these data no statistically significant association between response to infection and the *Mx* genotype was detected. However, the effect of chicken line on clinical status (healthy *vs* three levels of disease clinical status) was statistically significant ($p<0.0001$), and the model explained the 44.28% of the observed variation in disease response (Table 2).

## Discussion

The objective of the current study was to examine the possibility that there is natural resistance to avian influenza in chicken, and to assess the role of the *Mx* locus in the phenotypic variation. Earlier work, which carried out an experimental infection of wood ducks and chickens with HPAI H5N1 virus, showed that for chicken different viral doses resulted in different mortality rate (Brown et al., 2007). In the present study, which focused on the response to H7N1, an initial trial was carried out with SPF chickens to investigate the effect of dose on survival (data not shown). A viral dose of $10^5$EID$_{50}$ resulted in 100% death rate in SPF chickens with a mean death time (MDT) of 2.8 days PI. In the study by Brown et al. (2007) where 5 White Leghorn chickens were challenged with similar viral dose, the authors also observed 100% mortality and MDT of 2.0 days PI. However, these authors observed that at a $10^3$EID$_{50}$ dose only 3 out of 5 chickens died with a MDT of 4.3 days PI. In the work presented here this latter dose resulted in 100% mortality but with a MDT of 6.5 days PI, with some birds surviving up to 8 days. Therefore in the present study a $10^3$EID$_{50}$ dose was used as it was considered sufficient to result in infection of all susceptible birds, but not so high that it would overcome natural resistance to the virus.

In the data presented here, variation in susceptibility between lines was detected, with three lines showing high susceptibility and high mortality, and two lines showing different levels of resistance to the virus. For line E, the White Leghorn, 35% of birds survived (7 out of 20), which is similar to the results obtained by Brown et al. (2007) who observed a 40% survival of White Leghorn chickens using a viral dose of $10^3$EID$_{50}$ of a H5N1 AI strain. Line B, a commercial broiler line, showed a higher level of resistance with 73% survival rate. These data suggest that in some chicken lines there is natural resistance to influenza virus infection. The interferon-mediated induction of Mx1 is a principal innate defence mechanism against the avian influenza virus in mice (Grimm et al., 2007; Salomon et al., 2007; Staeheli et al., 1986; Tumpey et al., 2007). Early studies in both duck (Bazzigher et al., 1993) and chicken (Bernasconi et al., 1995) suggested that the avian Mx protein does not have antiviral activity, however, later *in vitro* experiments showed that at least some chicken *Mx* alleles have effective antiviral activity against VSV and H5N1 (Ko et al., 2002). The level of antiviral activity differs between *Mx* alleles, and seems to depend on the presence of Asn at residue position 631 in chicken (Ko et al., 2002). Recent data suggested that alleles with Asn at position 631 of chicken Mx do not inhibit *in vitro* viral replication of 5 influenza strains (Benfield et al., 2008). However, whether this polymorphism is associated with resistance to influenza virus *in vivo* remained to be proven. The present work addressed the effect of genetic variation on disease outcome and assessed whether the *Mx* polymorphism at 2032 of the cDNA had an effect on disease outcome in chickens.

The five chicken lines used in the present study were genotyped at nucleotide 2032 of *Mx* cDNA. The genotype data presented here show that the G allele is present at high frequency in broiler lines: 100% in line A, 70% and 80% in lines B and C, respectively (see Table 1). Balkissoon et al. (2007), also found higher frequencies of the G allele compared to the A allele in broilers and their ancestor lines: the highest frequency of the A allele they identified was in line X.3 Broiler (0.13), which is lower than the frequencies observed in the B (0.30) and C (0.20) lines used in the present study (Table 1). This characteristic distinguishes these two broiler lines, especially line B, from our line A and the other broiler stocks analysed in previous studies. Statistical analysis of the possible association between the *Mx* genotype and disease outcome in the data presented here indicates that alleles at position 2032 of *Mx* gene do not have an effect on the survival of birds following infection with the H7N1 HPAI virus. However, it is possible that polymorphisms at other positions within the *Mx* gene could influence the level of antiviral activity *in vivo*. Balkissoon et al. (2007) identified a non-synonymous polymorphism (G232R) which was not detected by Ko et al. (2002) and which may affect the protein function. One of the alleles analysed by Benfield et al. carried the R residue at position 232 (line C1), nevertheless, none of the alleles conferred protection to embryo fibroblasts against H1N1 virus (Benfield et al. 2008).

The *in vivo* effect of the Mx protein may also depend on the viral strain: Dittmann et al. (2008) recently showed that different human and avian strains of influenza A virus exhibit different sensitivities to inhibition by Mx proteins. The association between the *Mx* 2032 polymorphism and resistance to influenza shown *in vitro* by Ko et al. (2002) was based on a H5N1 strain of virus, whereas a H7N1 strain was used in the study presented here. Rigoni et al. (2007) reported different clinical outcomes in mice infected with 3 different H7N1 HPAI strains isolated in Italy during the 1999–2000 AI outbreak: it is possible that similar variations in the clinical outcomes may occur in chickens when challenged with different strains of AI. Further studies will be necessary to address whether the clinical response differs between strains of virus.

The data presented here shows that, although the polymorphism at position 2032 of the *Mx* gene does not affect survival following infection with this specific viral strain, there does appear to be a genetic basis to the variations in response to influenza infection. In particular, the genetic background of commercial broiler line B, and to a lesser extent line E, the White Leghorn, seems to confer a substantial level of resistance. The level of resistance with respect to the response following higher viral doses than the $10^3$EID$_{50}$ used in this study, and to other viral strains still has to be tested.

## Materials and methods

### *Virus*

The virus strain used was H7N1 HPAI A/chicken/Italy/13474/99, isolated from chicken by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" Brescia, Italy during the 1999–2000 outbreak in Italy.

The viral stock was propagated in 9 to 11 day-old specific pathogen-free (SPF) embryonated chicken eggs. Allantoic fluid was collected and evaluated for haemagglutinin activity and tested in sandwich ELISA using a monoclonal antibody (Mab) specific for influenza virus A nucleoprotein (NPA) (ATCC HB65), according to the protocol by Siebinga and de Boer (1988) or by haemoagglutination test (http://www.oie.int/eng/publicat/en_standards.htm). The virus titre was determined using the method of Reed and Muench (1938) and expressed in $EID_{50}$ (http://www.fao.org/DOCREP/005/AC802E/ac802e0w.htm). The virus stock was stored at −70 °C, diluted in MEM and titred before each study.

*Animals*

The five chicken lines used in the present work were broiler (commercial Lines A, B, C), one experimental White Leghorn line (Line E), and one experimental New Hampshire line (Line D). Birds from the latter two lines were selected to be homozygous for the A allele at position 2032 of *Mx* cDNA.

Eggs of each line were hatched and the chicks were maintained in pathogen-free conditions, fed *ad libitum*. At 7–10 weeks of age, groups of 11–20 chickens *per* line (depending on hatching and survival rate) were moved into a bio-containment (BSL3) laboratory for the viral challenge and two birds *per* cage were housed in 80×80 cm cages. Before experimental infection, blood samples were collected from each bird into EDTA vacutainers and kept for genetic analyses.

*Animal trial*

Each group of chickens was tested for exposure to AIV before the experimental challenge, by competitive ELISA for antibodies against influenza virus A nucleoprotein (NPA) by using the Mab anti-NPA ATCC HB65 (de Boer et al., 1990). All chickens were negative.

Birds were infected by intra-tracheal infection with an experimental dose of $10^{3\pm0.25}$ $EID_{50}$/bird. Previous trial infections of SPF chickens showed that at this dose all susceptible birds died between day 4 and 8 PI, at higher doses all birds died within 3 days PI (data not shown). After the challenge, chickens were monitored daily and their clinical status scored daily as follows: score "0" clinically healthy, "1" ill bird, displaying one specific clinical symptom (depression, respiratory disorders, diarrhoea, cyanotic combs and wattles, head and neck oedema, or neurological signs), "2" seriously ill bird, showing at least two of the specific symptoms, "3" dead.

The serological analyses were repeated on each bird surviving the experimental challenge to detect antibodies against AI virus A nucleoprotein starting from day 7 PI.

*Genotyping of Mx gene*

Genomic DNA was extracted from whole blood collected before the viral challenge, using the Puregene DNA Kit (Qiagen). The genotype at 2032 G/A polymorphism was determined by direct sequencing using primers designed based on the genomic sequences available in the Ensembl database (ENSGALG00000016142). PCRs were carried out in a total volume of 15 μl, with 20 ng of genomic DNA as template, 6 pmol of each forward and reverse primer (F: 5′-GGTTAGCAGAGAGAGGGAGA-3′; R: 5′-AGGTTGCTGCTAATGGAGGA-3′), 1.5 μl of 10× buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U of AmpliTaq Gold (Applied Biosystems). The PCR protocol was as follows: 95 °C for 10 min, 35 cycles of 94 °C for 1 min, annealing at 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. PCR products were analysed by electrophoresis through a 2% agarose gel in 1× TAE buffer, and stained with Ethidium Bromide. The 611 bp amplicons were purified using ExoSAP-IT (USB Corporation). Sequencing reactions were performed with BigDye terminator chemistry (Applied Biosystems), and sequences resolved using an ABI PRISM 3730 DNA Analyzer (Applied Biosystems), according to the protocols from the manufacturer. Electropherograms were visualised using Chromas 1.45 (http://www.technelysium.com.au/chromas.html). Genotypes were confirmed with PCR-RFLP: samples were amplified using the following primer pair: F: 5′-GCACTGTCACCTCTTAATAGA-3′, and R: 5′-GTATTGG-TAGGCTTTGTTGA-3′. Amplicons were cleaved with the restriction enzyme Hpy8I (Fermentas), which cuts 2 bp downstream from the 2032 polymorphism in the presence of the G allele (no cut in case of A allele).

*Statistical analysis*

The association between the response to influenza virus infection and *Mx* genotype was analysed using four inter-dependent variables: clinical score, time course of disease, genotype at the *Mx* locus (AA, AG, or GG), and the chicken "line" (A, B, C, D, or E). An index was created based on the highest clinical score and the day the score was reached. Birds missing information on one or more conditions (e.g., birds passed from "healthy" at day 1 PI to "dead" at day 2 PI) were assigned a negative score to stress the rapid onset of the disease. The following statistical model was used to assess effects of various factors on the observed phenotypes:

$$y_{ij} = \mu + L_i + G_i + e_{ij},$$

where $y$ is the phenotype for either the clinical score or the time course of the disease, $\mu$ was the population mean, $L$ was the effect of the line (A, B, C, D, or E), $G$ was the effect of the genotype (GG, AG, AA) and $e$ was the random residual. General linear models (proc GLM) and logistic regression (proc LOGISTIC) of SAS (SAS Institute, Cary NC, USA) were used to analyse the association between the 2032 *Mx* genotype and the chicken line, with clinical status and time course of the disease. Results were further confirmed using the LIFETEST procedure of SAS program.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.07.022.

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