Immunogenetics (2007) 59:687–691
DOI 10.1007/s00251-007-0235-5

BRIEF COMMUNICATION

Low frequency of the Mx allele for viral resistance predates recent intensive selection in domestic chickens

Devanand Balkissoon · Karen Staines · John McCauley · James Wood · John Young · Jim Kaufman · Colin Butter

Received: 19 February 2007 / Accepted: 11 June 2007 / Published online: 4 July 2007

Abstract Avian influenza is a serious threat to the poultry industry and, as the potential source of a human pandemic virus, to public health. Different Mx alleles have been reported to confer resistance or susceptibility to influenza virus replication, and so knowledge of their frequencies is important when considering the potential for improvement of modern commercial flocks. We analysed a range of chicken lines and ancestral breeds for the relevant Mx codon that confers resistance or susceptibility to influenza virus replication. We confirmed the high frequency of the susceptibility allele in contemporary meat-type (broiler) birds compared to egg-laying strains and found this difference is present already in ancestral breeds. We sequenced full-length complementary DNA (cDNA) and noted additional substitutions, which may be associated with the resistance haplotypes. High frequencies of the susceptibility allele could be readily reduced by modern breeding techniques.

Keywords Mx · Interferon · Innate immunity · Inbred strains · Selection · Avian Influenza

D. Balkissoon · K. Staines · J. Young · J. Kaufman · C. Butter (✉)
Institute for Animal Health, Compton,
Newbury, Berkshire RG20 7NN, UK
e-mail: colin.butter@bbsrc.ac.uk

J. McCauley
The National Institute for Medical Research,
The Ridgeway, Mill Hill,
London NW7 1AA, UK

D. Balkissoon · J. Wood
Cambridge Infectious Diseases Consortium (CIDC),
Department of Veterinary Medicine,
University of Cambridge,
CB3 0ES Cambridge, UK

In studies with mice, specific resistance to influenza virus is attributed to Mx protein expression and is independent of other interferon-mediated effects (Staeheli et al. 1986). Mx proteins, part of the dynamin family of large GTPases (Accola et al. 2002), interfere with the replication of RNA viruses (including influenza) by detecting nucleocapsid-like structures (Haller and Kochs 2002) or by inhibiting trafficking or the activity of viral polymerases (Stranden et al. 1993). Previous work showed that the amino acid at position 631 can determine the antiviral activity [to vesicular stomatitis (VSV) and influenza virus] of chicken Mx (Ko et al. 2002) and that cell lines expressing chicken Mx proteins with asparagine at position 631 had higher antiviral activity than those with serine (Ko et al. 2004). In this report, we refer to the serine 631 allele as the susceptibility allele and the asparagine allele as the resistance allele.

Highly skewed Mx allele frequencies have been reported among experimental lines and native Asian breeds of chickens. The frequencies found have been attributed to varying degrees of selection and/or environmental influence (Li et al. 2006). To understand this in more detail, we studied 28 lines of chickens: 1 commercial layer line and 8 experimental lines representing layer ancestors, 11 commercial broiler lines and 4 lines of contemporary breeds representing broiler ancestors and 4 multi-purpose breeds.

Two sets of primers were designed spanning part of the intron prior to exon 14, to within exon 14. The primers were A_forward: CTTGAATAGCAACTCCATACCG; A_reverse: GGTAGGCTTTGTTGAGGTGAC; B_forward: GAATAGCAACTCCATACCGTG; B_reverse: CCAGGT ATTGGTAGGCTTTG. All pairs of forward and reverse primers were used. The PCR protocol was 50 μg genomic DNA, 5 μl 10× polymerase chain reaction (PCR) buffer (Invitrogen, California), 5 μl 2 mM deoxyribonucleotide

Table 1 Codon identity corresponding to position 631 of the Mx protein and frequency distributions of Mx alleles

Line	Total birds	Genotype frequency			Allele frequency	
		N/N AAT/AAT	N/S AAT/AGT	S/S AGT/AGT	AAT	AGT
All purpose breeds						
Brown Leghorn	09	0.56	0.22	0.22	0.67	0.33
Sykes Rhodes	15	0.00	0.47	0.53	0.23	0.77
Rhode Is. Red	09	0.22	0.22	0.56	0.33	0.67
Light Sussex	15	0.00	0.13	0.87	0.07	0.93
Layer stock						
Line Z.1 Layer	15	0.87	0.13	0.00	0.93	0.07
Layer ancestral stock						
Line 7_2	15	1.00	0.00	0.00	1.00	0.00
Line 6_1	13	1.00	0.00	0.00	1.00	0.00
Line 15I	15	0.86	0.07	0.07	0.93	0.07
Line 0	14	0.86	0.07	0.07	0.93	0.07
Line N	15	1.00	0.00	0.00	1.00	0.00
Line P2A	15	0.93	0.07	0.00	0.97	0.03
Line C	28	0.00	0.00	1.00	0.00	1.00
Wellcome Line	15	1.00	0.00	0.00	1.00	0.00
Broiler stock						
Line X.1 Broiler	15	0.00	0.00	1.00	0.00	1.00
Line X.2 Broiler	12	0.00	0.00	1.00	0.00	1.00
Line X.3 Broiler	15	0.00	0.27	0.73	0.13	0.87
Line X.4 Broiler	16	0.00	0.19	0.81	0.09	0.91
Line X.5 Broiler	13	0.00	0.00	1.00	0.00	1.00
Line X.6 Broiler	207	0.00	0.00	1.00	0.00	1.00
Line X.7 Broiler	202	0.00	0.02	0.98	0.01	0.99
Line X.8 Broiler	194	0.00	0.01	0.99	<0.01	>0.99
Line X.9 Broiler	212	0.01	0.21	0.78	0.12	0.88
Line X.10 Broiler	202	0.00	0.00	1.00	0.00	1.00
Line X.11 Broiler	202	0.00	0.00	1.00	0.00	1.00
Broiler ancestral stock						
Indian Game a	8	0.00	0.00	1.00	0.00	1.00
Indian Game b	14	0.00	0.00	1.00	0.00	1.00
Brd. P. Rock a	20	0.00	0.00	1.00	0.00	1.00
Brd. P. Rock b	14	0.00	0.00	1.00	0.00	1.00

Line Z.1 is representative of commercial white egg-laying lines. The Compton inbred lines were derived from White Leghorn layer stock in the USA (lines 6_1, 7_2, 0, 15I, N, P2A) and the UK (line C, Wellcome line). The coefficients of inbreeding for line 6_1, line 7_2, line C were >0.9. The remaining inbred lines had inbreeding coefficients within the range 0.6 to 0.8, and the outbred lines approximately 0.3. The inbred lines were all derived from White Leghorn populations originating as follows: line 6_1 (derived from East Lansing 1939), line 7_2 (East Lansing 1939), line 0 (East Lansing 1979), line 15I (East Lansing 1939), line N (Cornell 1965), Line P2A (Cornell 1965), Wellcome Line (Wellcome Research Lab 1982), line C (Reaseheath/Cambridge 1932). Line X broilers represent individual lines of commercial meat-type chickens. Data for broiler lines X.6–X.11 were obtained from the relevant breeding company. Indian Game and Barred Plymouth Rock (Brd. P. Rock) groups were each sampled from two independent rare-breed collections. The origins of the Plymouth Rock lines were the UK and Norway, and the Indian Game (Cornish) lines were also genetically distinct lines. The Brown Leghorn, Rhode Island Red (Rhode Is. Red) and Light Sussex lines represent traditional all-purpose breeds.

triphosphate (dNTP), 1.5 μl 50 mM $MgCl_2$ (Invitrogen), 10 pmol of each primer and 2.5 U Taq DNA polymerase (Invitrogen) in a total reaction volume of 50 μl. Cycling parameters were 94°C for 2 min and 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final elongation step 72°C for 9 min, in a thermal cycler (DNA Engine®, Bio-Rad, Hercules, CA). PCR product (5 μl) was analysed by electrophoresis to confirm size. The remainder of PCR product was purified using silica membrane technology (QIAquick PCR Purification Kit, Qiagen). Sequencing reactions were set up using the Quickstart kit (Beckman Coulter) and CEQ reaction buffer (Beckman Coulter, Fullerton, CA), then analysed by a CEQ automated sequencer (Beckman Coulter).

We then selected four lines for full-length cDNA sequencing to investigate possible associations between different polymorphisms along the Mx gene. Chick embryo fibroblasts were cultured for 2 days and then stimulated with 1,000 U/ml chick interferon alpha (IFN-α) for 3 h. Reverse transcriptase (RT)-PCR was performed using the primers AAGAGTGGTCGGTGTCGATAA and AGGTTGCTGCTAATGGAGGA and as according to the kit protocol (SuperScript™ III one-step RT-PCR System with Platinum® Taq DNA Polymerase, Invitrogen). The RT-PCR protocol was 0.7 μg RNA, 2× reaction mix (Invitrogen) containing 0.4 mM of each dNTP and 3.2 mM $MgSo_4$, 4 μM of each primer, and SuperScript™ III RT/Platinum® Taq Mix (Invitrogen) in a total volume of 20 μl. Cycling parameters were 55°C for 30 min, 94°C for 2 min, and 40 cycles of 94°C for 15 s, 55°C for 30 s, 68°C for 2 min and a final elongation step 68°C for 8 min, in a thermal cycler (DNA Engine®, Bio-Rad).

PCR product (5 μl) was analysed by electrophoresis to confirm size. Gene-specific primers producing overlapping reads in both strands were used to generate fragments for sequencing by a CEQ automated sequencer (Beckman Coulter). Attention was paid to the possibility of heterozygotes within the sequences using the Beckman Coulter CEQ8000 heterozygote-calling software. The fragments were assembled using the Staden software package and aligned using ContigExpress in VectorNTI (Invitrogen).

The resistance and susceptibility Mx alleles segregated within the less inbred, multi-purpose breeds (Table 1). These breeds, established for over a century, have developed under different selection pressures than the progenitors of the meat-type birds, which have largely lost the resistance allele. The multi-purpose breeds generally have maintained both alleles at comparable frequencies.

Most modern-day white egg layers were derived from the White Leghorn breed (Hillel et al. 2003). The White Leghorn experimental inbred lines analysed in this study were derived from commercial layers. In the USA, the lines 6_1, 7_2, 0, and 15I were derived in 1940, and the line P (the direct ancestor of line P2a line) and line N were derived in 1965. In the UK, the C line was derived in 1928 and the Wellcome line in the 1960s. Therefore, these lines represent the immediate ancestors of the current commercial egg layers. With the exception of line C, the lines all carried the resistance allele predominantly and were fixed in four of these lines. The spatially and temporally distinct origins of the founders of line C could account for this line being homozygous for the susceptibility allele. Either natural selection due to avian influenza or other viruses or hitch-hiking of chicken Mx on nearby genes could account for the preponderance of the resistance allele of Mx in the founder White Leghorn populations.

We determined the genotype and allele frequencies for present-day commercial egg and meat type birds (Table 1). Additional data were obtained from a commercial broiler-breeding company. Commercial egg-layers from the Z.1 layer line predominantly carried the resistance allele (AAT 631N), consistent with the layer ancestor lines. In contrast, commercial broilers (X.1 to X.11) were fixed for the homozygous susceptibility allele in six lines and predominantly carried the susceptibility allele in five other lines. A further six lines of commercial broilers revealed susceptibility allele frequencies of between 0.91 and 1 (data not shown).

We found a high frequency of the susceptibility allele in all commercial broiler lines examined. In previous work, it was speculated that there may be associations of the Mx gene with production traits in broilers (Li et al. 2006). Recently, a number of quantitative trait loci for broiler growth have been identified on chromosome 1 (Wright et al. 2006; Sewalem et al. 2002; Tatsuda and Fujinaka 2001), which are sufficiently close to the Mx gene to suggest that hitch-hiking of a particular Mx allele may be a possibility.

The skewed Mx allele frequencies seen in the present study might be due to recent intensive selection for production traits in modern broiler breeding. To investigate this hypothesis, we analysed the Mx alleles in lines representing the major ancestors of modern commercial broiler lines, generally females derived from Plymouth Rock birds chosen for fast growth and males from white Cornish (Indian Game) birds chosen for short legs, muscular breasts and superior meat qualities (Hillel et al. 2003). All birds analysed from the four independent populations representing these breeds were homozygous for the susceptibility allele of Mx, indicating that precursor breeds had a high circulating frequency of the susceptibility allele.

Based on previous investigations of the evolutionary relationships of Red Jungle Fowl and domesticated chicken breeds, it has been postulated that distinct domesticated lineages exist (Moiseyeva et al. 2003). Modern meat type breeds are thought to be most recently derived probably from game (sporting) breeds. If selection for production characteristics was a factor in accidental selection for Mx-related influenza susceptibility, our data suggest that this was not due to recent intensive selection by commercial breeders but was during or before the establishment of the traditional breeds from which the modern broiler was derived.

To examine whether all the coding variation in Mx co-segregates with the alleles at position 631, the Mx cDNAs of three birds each from lines 6_1, 7_2 and C, and from the Wellcome line were sequenced. In each line, the three sequences were all identical. Single nucleotide polymorphisms were identified at 16 positions, affecting eight exons, in the coding sequence (Table 2). Seven were

Table 2 Polymorphisms in Mx coding sequence in four inbred lines

	Nucleotide position														
	62	122	125	156	351	605	694	696	922	1015	1248	1455	1545	1643	1892	2019
nt substitution	A-G	G-C	C-T	A-T	T-C	G-C	G-A	G-C	A-G	G-A	G-A	C-T	G-A	C-T	G-A	G-A
aa substitution	Q21R	R41P	S42L	–	–	S202T	G232R[a]	-	I308V	A339T	–	–	–	A548V	S631N	–
Line 6_1	G	C	T	T	T	G	A	G	G	A	G	T	A	T	A	A
Line 7_2	A	G	C	A	C	G	G	C	G	A	G	C	G	C	A	G
Line C	G	C	T	T	C	C	G	G	A	G	A	T	A	C	G	G
Wellcome	A	G	C	A	T	G	G	G	G	A	A	T	A	T	A	G
Exon	2	2	2	2	3	5	5	5	7	7	9	11	11	13	14	14

Nucleotide positions and substitutions are shown relative to the coding sequence of reference chicken Mx cDNA, accession number Z23168.
[a] Nucleotide positions 694 and 696 both correspond to codon 232. The substitutions indicated do not occur together in any of the four lines tested or in any of the chicken Mx sequences present in the EMBL database (23rd March 2007). If they occurred together, this would produce a Serine codon.

synonymous. Of the nine non-synonymous single nucleotide polymorphisms (SNPs), three (S202T, I308V and A339T) were distributed between the lines with the same pattern as S631N. Our analysis has identified a new polymorphism, G232R. Considering those SNPs common to our study and that of Ko et al. (2002), the Mx haplotypes of lines 7_2 and C presented new combinations of polymorphic residues as yet untested for antiviral activity. The line 7_2 (B2 haplotype) was the same as an unpublished sequence (accession AB244818).

Ko et al. (2004) studied the antiviral activity of chimaeric chicken Mx molecules. Combining these results with their earlier study (Ko et al. 2002), the N631 allele was shown to confer antiviral activity independently of the alternative residues at any other polymorphic sites, with the single exception of K185R, for which the alternative allele 'R' was not tested. All the haplotypes we studied carried K185.

Reducing the susceptibility of chickens to avian influenza (AI) would minimise the direct zoonotic threat to humans and may be achievable by methods including vaccination, transgenesis and selective breeding. The stratified breeding employed in the poultry industry could be used to introduce higher frequencies of resistance alleles into commercial chickens in as little as 4 years. This short time from great-grandparental stock to production stock, the facts that broilers account for approximately 80% of the worldwide annual production of almost 40 billion chickens (Satterlee 2003) and that their genetics is largely controlled by two breeding companies, make artificial selection for resistance a tenable option.

In summary, it is likely that a high frequency of the susceptibility allele circulated in meat-type birds at least since the establishment of the precursor breeds of modern broiler strains over a hundred years ago. Notwithstanding the possible linkage of the Mx gene with production traits, it is therefore unlikely that selection techniques used in the generation of modern broiler lines resulted in selection for a particular Mx allele. Furthermore, the presence of low levels of the resistance allele in some commercial lines (e.g. broiler lines X.3, X.4, X.7, X.8, X.9 in Table 1) means that there is an opportunity for rapid introduction of the resistance allele of Mx into commercial broilers, which could reduce the burden of infection of poultry and thus contribute to the reduction of the pandemic influenza threat.

Acknowledgements We thank Mr. Shaun Hammon of the Wernlas Collection (Shropshire, UK) and Mr. Michael Figgures of Honeysuckle Farm (Wiltshire, UK) for their kind cooperation with this study. We acknowledge Dr. Pete Kaiser (I.A.H., Compton, UK) and Dr. Paul Hocking (Roslin Inst., Midlothian, UK) for helpful discussion. This work was supported by the Cambridge Infectious Diseases Consortium (C.I.D.C.), University of Cambridge, UK and the Institute for Animal Health, Compton, UK.

References

Accola MA, Huang B, Al Masri A, McNiven MA (2002) The antiviral dynamin family member, MxA, tubulates lipids and localizes to the smooth endoplasmic reticulum. J Biol Chem 277:21829–21835

Haller O, Kochs G (2002) Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. Traffic 3:710–717

Hillel J, Groenen MAM, Tixier-Boichard M, Korol AB, David L, Kirzhner VM, Burke T, Barre-Dirie A, Crooijmans RPMA, Elo K, Feldman MW, Freidlin PJ, Mäki-Tanila A, Oortwijn M, Thomson P, Vignal A, Wimmers K, Weigend S (2003) Biodiversity of 52 chicken populations assessed by microsatellite typing of DNA pools. Genet Sel Evol 35:533–557

Ko JH, Jin HK, Asano A, Takada A, Ninomiya A, Kida H, Hokiyama H, Ohara M, Tsuzuki M, Nishibori M, Mizutani M, Watanabe T (2002) Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res 12:595–601

Ko JH, Takada A, Mitsuhashi T, Agui T, Watanabe T (2004) Native antiviral specificity of chicken Mx protein depends on amino acid variation at position 631. Anim Genet 35:119–122

Li XY, Qu LJ, Yao JF, Yang N (2006) Skewed allele frequencies of an Mx gene mutation with potential resistance to avian influenza virus in different chicken populations. Poultry Sci 85:1327–1329

Moiseyeva IG, Romanov MN, Nikiforov AA, Sevastyanova AA, Semyenova SK (2003) Evolutionary relationships of Red Jungle Fowl and chicken breeds. Genet Sel Evol 35:403–423

Satterlee DG (2003) New vaccine increases broiler breeder chicken production. Louis Agric 46:8

Sewalem A, Morrice DR, Law A, Windsor D, Haley CS, Ikeobi CON, Burt DW, Hocking PM (2002) Mapping of quantitative trait loci for body weight at three, six, and nine weeks of age in a broiler cross. Poultry Sci 81:1775–1781

Staeheli P, Haller O, Boll W (1986) Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. Cell 44:147–158

Stranden AM, Staeheli P, Pavlovic J (1993) Function of the mouse Mx1 protein is inhibited by overexpression of the PB2 protein of influenza virus. Virology 197:642–651

Tatsuda K, Fujinaka K (2001) Genetic mapping of the QTL affecting body weight in chickens using a F2 family. Br Poult Sci 42:333–337

Wright D, Kerje S, Lundstrom K, Babol J, Schutz K, Jensen P, Andersson L (2006) Quantitative trait loci analysis of egg and meat production traits in a red junglefowl × White Leghorn cross. Anim Genet 37:529–534