Review article

# Genetic determinants of resistance to highly pathogenic avian influenza in chickens

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**Highly pathogenic avian influenza (HPAI) poses a huge threat to poultry production and also introduces an epidemiological risk in the human population. Thus far, HPAI has been controlled mainly through widespread implementation of biosecurity, and in the case of an outbreak, liquidation of flocks and establishment of protection zones. Alternative strategies for combating HPAI include the use of vaccines, genetic modification, and genetic selection for increased general and specific immunity in birds. These kinds of strategies often require identification of the genes involved in the immune response to the pathogen. Many genes have been identified as potentially associated with differences in the response to HPAI between poultry species and between individuals. Thus far, the most attention has been focused on genes taking part in regulating the innate immune response, which is responsible for preventing infection and limiting the replication and spread of the virus. The most commonly mentioned candidates for layer chickens include interferon-stimulated genes (ISGs) and RIG-I-like receptors. Proteins encoded by genes of the BTLN family, defensins, and proteins involved in apoptosis have also been associated with differences in the response to HPAI. Recent years have seen an increasing number of studies on the genetic determinants of individual differences in the response to HPAI in chickens. Data from HPAI outbreaks in the US in the spring of 2015 and Mexico in the years 2012-2016 have enabled a more precise analysis of this problem. A number of**

**genes have been identified as associated with the immune response, but their specific role in determining the survival of birds requires further study. Preliminary results indicate that genetic determinants of resistance to HPAI are highly complex and can vary depending on the virus strain and the genetic line of birds.**

**KEY WORDS: highly pathogenic avian influenza / layer chicken / genetic basis of host resistance**

Highly pathogenic avian influenza (HPAI) has a huge impact on poultry farming, as it causes high bird mortality, necessitates euthanasia of all infected flocks, and creates problems with exports of meat and eggs. Cases of HPAI infection have occurred all over the world, recently including Poland [35]. Many studies have been undertaken to develop strategies to prevent or limit the negative effects of HPAI epidemics in poultry. Currently, the most effective way to fight HPAI is to comply with the principles of biosecurity. When the presence of the virus has been confirmed, all birds are disposed of at the site of infection, and special veterinary supervision is introduced within radii of 3 and 10 km, through the designation of protection zones. However, these measures are extremely expensive, so work on more effective strategies to fight the virus continues. One method is preventive vaccination, which is currently used in some countries [14]. Despite unquestionable advantages, such as reducing the spread of the virus, mortality, and economic losses, this solution has many disadvantages as well [6]. In addition to the limited effectiveness of vaccination, the very high variability of the influenza virus is also problematic and necessitates correct prediction and frequent modification of the vaccine composition. In addition, vaccinations can cause problems with imports and exports of birds, due to difficulties in distinguishing vaccinated birds from those that have been exposed to the virus under natural conditions. To solve this problem, strategies such as DIVA (Differentiating Infected from Vaccinated Animals) have been developed and applied in all European countries, including Poland, but they are not an ideal solution [34].

The influenza virus belongs to the family *Orthomyxovirodae* and consists of single--stranded RNA with negative polarity, a few proteins and a lipid envelope composed of two types of glycoproteins, hemagglutinin (H) and neuraminidase (N), responsible for penetrating the host cell and releasing virus progeny. The main component of the host cell enabling recognition of virus receptors by haemagglutinin is sialic acid, referred to as SA $\alpha$2,3-Gal for avian influenza and SA $\alpha$2,6-Gal for human influenza [29]. Next, the virus enters the cell by endocytosis and is prepared for digestion by lysosomes. However, due to haemagglutinin activation, the viral envelope is fused with the endosomal membrane, and the genetic material of the virus is released into the cell and replicated. The mechanisms responsible for the neutralization and elimination of influenza virus particles and their entry into the cell are the subject of numerous studies conducted all over the world. For example, differences in virulence between HPAI and LPAI (Low Pathogenic Avian Influ-

enza) viruses are believed to be associated with the virus strain and tissue tropism, but also with the specific host response [27]. Recent years have seen increasing research on the genetic determination of immunity, and hence on interspecific and individual differences at the DNA level.

### Genetic modifications

An alternative strategy which is increasingly being considered in the fight against HPAI is genetic modifications. The first successful attempt to create transgenic chickens exhibiting resistance to avian influenza took place in 2011 [24]. Using lentiviral vectors, Lyall et al. [24] introduced a 'hairpin' RNA molecule into the genome of a chicken. The sequence is the binding site of HPAI virus polymerase and has shown the ability to inhibit activity of the viral polymerase in vitro. The modification resulted in chickens that were susceptible to infection, but the amount of virus they shed was significantly reduced, which could potentially significantly reduce the spread of infection in the flock. However, the effect of this modification on the LPAI virus and on the productivity and viability of modified animals in production conditions is not yet known.

Another attempt to obtain chickens resistant to highly pathogenic avian influenza is the method proposed by Byun et al. [5]. In this experiment, chickens were modified to express the 3D8 single-chain variable fragment (scFv), which has high affinity for the nucleoprotein of the influenza virus. The authors showed that the presence of this short scFv insert can also protect the host against a wide range of other viral pathogens. The disadvantage of this solution is the need to strictly control the level of scFv expression, as its uncontrolled increase in the body can lead to degeneration of host cell nucleic acids.

A study by Rohaim et al. [30], published in 2018, describes a method of obtaining transgenic chickens showing stable expression of genes from the chIFIT5 family (IFN--induced proteins with tetratricopeptides repeats 5). Genetically modified birds infected with a clinical dose of the HPAI and Newcastle disease viruses showed increased immunity. Administration of a lethal dose led to the death of some birds, but delayed disease symptoms and reduced mortality and viral shedding after infection. These observations may indicate that stable expression of chIFIT5 genes reduces the severity of clinical symptoms and is an element of the bird's fight against retrovirus infection.

Despite numerous attempts, genetic modifications as a means of combatting the HPAI virus or other pathogens in chickens have not yet become widespread. It is worth noting that apart from the limitations resulting from the technology itself, there are economic, legal and social barriers to the use of genetically modified organisms in farming [23].

**Genetic determination of immunity in chickens**

Genetic determinants of resistance to HPAI in birds result from interspecific, inter-
-breed and individual differences. In recent years, a great deal of attention has been devoted
to interspecific differences, which have been analysed multiple times in birds. Birds of the
order Galliformes, mainly chickens and turkeys, are most susceptible to infection with
the avian influenza virus. For example, some subtypes of the HPAI virus induce a weak
inflammatory response and no symptoms of infection in ducks, while mortality is very high
in domestic chickens [4]. In this case, the interest of the scientific community has primarily
been focused on understanding the nonspecific immune response. It is suspected that inter-
species differences in the response to HPAI between chickens and ducks may be linked to
the lack of or reduced expression of key antiviral genes, such as the RIG-I gene (retinoic
acid-inducible gene-I) [16, 25]. This gene encodes a protein which together with MDA5
(melanoma differentiation-associated gene 5) and LGP2 (laboratory of genetics and phy-
siology 2) belongs to the family of RIG-I-like receptors, and in a broader context to the
group of pathogen-recognizing receptors known as PRR (pattern recognition receptors).
RIG-I-like receptors are intracellular receptors involved in the antiviral response in many
animal species, recognizing viral RNA in the infected cell [15]. Their tasks include initia-
tion of the production of cytokines and interferons activating a further antiviral response.
Barber et al. [1] have shown that the introduction of a duck RIG-I gene into domestic chic-
ken fibroblast cells leads to increased expression of many genes involved in the nonspecific
immune response, such as MX1, PKR, OASL and IFN-β, which in turn reduce the rate of
replication of highly the pathogenic avian influenza virus. Some studies suggest that the
MDA5 gene may compensate for the lack of the RIG-I gene in chickens [22]. Ranaware et
al. [28] report increased expression of genes such as the MDA5, TLR3 and NLRC5 genes
in cells infected with the HPAI virus.

Genes associated with the immune response to HPAI also include interferon-stimulated
genes (ISG), such as MX, OAS, PKR, IFITM and IFIT5. The IFITMs (interferon-induced
transmembrane proteins) are antiviral proteins that impede the penetration of viruses into
host cells, and their role in response to the influenza virus has been well documented in
mammals [3]. The mechanism of action of genes of the IFITM family consists in changing
the lipid composition of the cell membrane and reducing its fluidity, which limits fusion of
the virus envelope with the cytoplasmic membrane [20]. The relationship between HPAI
and this protein family in birds has been confirmed by Smith et al. [33], who sequenced the
transcriptomes of ducks and chickens infected with H5N1 and H5N2 strains. It was shown
that increased expression of the IFITM1, IFITM2 and IFITM3 genes virtually does not
occur in chickens. Only in the case of the IFITM3 gene was a small increase in expression
noted in the initial phase of H5N1 infection, but this was not observed in further stages of
the disease. Unlike chickens, ducks react to H5N1 infection with significantly increased

expression of the IFITM1, IFITM2 and IFITM3 genes in the lungs and ileum. Therefore, one of the reasons for the different response to the HPAI virus observed in these species is believed to be the difference in expression of IFITM genes.

According to the current state of knowledge, one of the main causes of the very high mortality of birds with HPAI infection is abnormal regulation of the host immune response. Ranaware et al. [28] reports that respiratory infection with HPAI H5N1 resulted in significantly increased expression of type I interferons, cytokines, chemokines and genes from the ISG family. For comparison, no such effect was observed in that study for LPAI H9N2. Of particular importance in this case is overproduction of cytokines, or hyper-cytokinaemia, also referred to as a cytokine storm [4]. This phenomenon leads to high expression of pro-inflammatory cytokines and damage to the host's internal organs. It is also worth noting that delayed production of pro-inflammatory cytokines may contribute to increased virulence of HPAI H7N1 in chickens [7]. Kuchipudi et al. [19] observed that gene transcription involving transcription factor STAT-3 may play an important role in differentiating the responses occurring in chickens and ducks. Defence mechanisms against avian influenza in ducks also include high variation in genes encoding β-defensins and receptors belonging to the BTLN (butyrophilin-like) family [13]. The influence of these genes has been observed in mammals, but their effect on birds has not been confirmed. Huang et al. [13] noted that many of these genes were duplicated in duck genomes, which was not observed in chickens. Variants in the number of copies within this family of genes are believed to have a significant impact on the evolution of duck genomes in response to contact with the influenza virus and may determine the host's survival of the infection.

It is suspected that genes involved in the regulation of apoptosis may be responsible for differences in the susceptibility of birds to individual subtypes and strains of the virus. In a study by Kuchipudi et al. [18], duck cells underwent rapid apoptosis after infection with the low pathogenic H2N3 strain, the H1N1 swine flu strain, and the highly pathogenic H5N1 avian influenza strain. Results obtained in vitro on Pekin duck cells that were resistant to infection differed from the results observed in chicken cells, in which apoptosis was slower, with lower DNA fragmentation and activation of the caspase pathway. This resulted in an increased number of infected cells. In addition, when duck lung cells were infected with H5N1, which is lethal for them, similar apoptosis patterns were observed as in chicken cells. These differences are assumed to result from the mechanism of resistance to type A influenza developed in the host, while the loss of the capacity for rapid apoptosis results in increased susceptibility to new H5N1 strains in chickens.

The genetic determinants of inter-breed and individual differences in response to HPAI are poorly understood. The few studies conducted thus far have shown variability between lines and breeds of birds in susceptibility to infection with various HPAI strains [26, 31, 38]. Individual differences are assumed to be mainly associated with

nonspecific immune responses [31]. One of the genes whose polymorphism has been linked to differences in the response to HPAI infection is the MX1 gene, but research results are thus far inconclusive. Some authors suggest that mutations such as S631N may increase the antiviral activity of the MX1 gene [21], although previous studies have not shown such activity in chickens [2]. The S631N mutation has also been shown to be less common in breeds used in commercial farming, which suggests that their antiviral properties may have been lost due to intensive selection [21]. Thus far, in vivo and in vitro studies have not provided a definitive answer regarding the role of the MX1 gene in response to HPAI infection [32, 37].

### Genome association studies

According to the Congressional Research Service (CRS), losses resulting from H5N2 epidemic that broke out in the United States in the spring of 2015 exceeded one billion dollars [12]. Mortality was high, at a level of over 99%, but a few birds survived for several weeks and did not show clinical symptoms [8]. Some of the birds showed a positive antibody level, indicating that they had had contact with the virus, but the infection was asymptomatic. Material acquired during the epidemic was used in research aimed at identifying differences in the genome between birds that survived the infection and a susceptible control group [8, 9, 39]. For biosafety reasons, the biological material for the control group had to be taken from birds from the same lines that had not had contact with HPAI. The research material included a total of 1119 birds (Table 1).

**Table 1**

Number of samples genotyped on a 600 k commercial microarray. Lines 1, 2, 3 and 4 are commercial lines of layers belonging to four breeding companies [8, 9]

| Line | Virus subtype | Location | Group size | | |
| --- | --- | --- | --- | --- | --- |
| | | | control | experimental | total |
| 1 | H5N2 | US Iowa, 2015 | 37 | 44 | 81 |
| 2 | H5N2 | US Iowa, 2015 | 49 | 52 | 101 |
| 3 | H5N2 | US Iowa, 2015 | 45 | 47 | 92 |
| 4 | H5N2 | US Iowa, 2015 | 186 | 104 | 290 |
| 4 | H7N3 | Mexico, 2012 | 95 | 460 | 555 |
| Total | | | 412 | 707 | 1119 |

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All birds from lines 1–4 were genotyped on the Affymetrix 600k commercial microarray [17]. The bioinformatic analysis was a genome-wide association study (GWAS) with a breakdown into lines and virus strains [8, 9]. A total of 420,458 segregating SNP markers were used for the analysis, of which 348,161 SNP markers were selected for samples from Mexico and 340,791 SNP markers for Iowa samples, after quality control. Standard GWAS was performed using linear regression, a mixed model in ASREML [11] and the Bayes B method in Gensel software [10]. Individual markers as well as haplotypes identified as 100,000 adjacent base pairs were analysed. Due to the lack of significant signals during the collective analysis of all samples, it was assumed that the genetic determinants of survival of infection may differ depending on the strain of the virus responsible for the epidemic. The most important results for the Hy-Line 4 commercial line are presented in Table 2.

Four regions associated with the survival of H5N2 infection in Iowa and three regions associated with survival of H7N3 infection in Mexico were identified (Table 2). The greatest variability between the surviving birds and the control group was explained by the region identified for the epidemic in Mexico, which was located on chromosome 1 at 126 Mb. In this region, one of the genes closest to the signal site was the gene coding for neuroligin 4 (NLGN4X), a type 1 membrane protein located most often on the surface of neurons. According to some reports, the avian influenza virus can replicate in nerve cells, and the high efficacy of this process limits the chances of survival of infected individuals [7]. The second significant signal for the same group indicated chromosome 5 at 39 Mb, where there is a gene encoding neurexin 3 (NRXN3), a protein associated with synapse function. Neuroligin belongs to the family of neurexin-binding proteins, which may indicate a functional relationship between these genes. In addition, both genes were located in the vicinity of micro-RNA sequences which play an important role in host–pathogen interactions, including in the case of avian influenza [36].

For samples from birds from Iowa, the strongest signal was identified on chromosome 7 at 28 Mb. The region corresponded with the location of the gene encoding DPP10 (dipeptidyl-peptidase 10), a membrane protein involved in cell communication and the cellular response. The remaining regions located on chromosome 9 and 15 included genes such as BCL6 (B-cell CLL/lymphoma 6) and ZNF639 (zinc finger protein 639), which play an important role in the body's immune response, or MAPK1 (mitogen-activated protein kinase 1), involved in apoptosis.

A study conducted on three lines of chickens that were not of the Hy-Line was also successful in identifying a number of genes that may be important in HPAI infection [9]. The results, however, show that the lines differed in terms of the regions identified, and moreover, no strong signals were obtained that would clearly indicate the association of a single region with survival of HPAI. The study demonstrated that genetic determinants affect the survival of birds during HPAI epidemics, but further testing of the regions identified and candidate genes is required to confirm the results.

**Table 2**

Regions explaining the highest percentage of variance for samples from Mexico (Mexico / H7N3) and Iowa (Iowa / H5N1) according to the Bayes B method [8]

| Chromosome | Bayes B – SNP alleles | | | Bayes B – Haplotypes | | | | |
|---|---|---|---|---|---|---|---|---|
| | window location (Mb) | genetic variance (%) | iterations (%)[1] | genetic variance (%) | location (Mb) | iterations (%)[1] | SNP rs[2] | Logistic model P-value |
| Mexico/H7N3 outbreak | | | | | | | | |
| 1 | 126.0 – 127.0 | 42.55 | 99.5 | 31.29 | 126.1 | 91.2 | 315546485 | 1.91E-12 |
| 5 | 39.0 – 40.0 | 0.23 | 14.0 | 1.15 | 39.3 | 5.9 | 315754877 | 4.15E-06 |
| 12 | 12.0 – 13.0 | 0.54 | 32.8 | 1.31 | 12.5 | 9.6 | 317538164 | 4.19E-05 |
| Iowa/H5N2 outbreak | | | | | | | | |
| 1 | 32.0 – 33.0 | 2.12 | 23.8 | 0.45 | 32.7 | 4.0 | 313567940 | 1.23E-04 |
| 7 | 28.0 – 29.0 | 1.59 | 21.3 | 0.06 | 28.8 | 1.2 | 16605877 | 2.02E-07 |
| 9 | 16.0 – 17.0 | 1.25 | 31.3 | 0.04 | 16.3 | 1.5 | 14677594 | 2.28E-04 |
| 15 | 1.0 – 2.0 | 1.35 | 38.3 | 0.18 | 1.9 | 1.5 | 315054601 | 1.12E-03 |

Regions explaining a high percentage of variance (>0.8%) in the Bayes B model were selected (windowed SNPs or haplotypes)

[1]% of windows with non-zero effect

[2]SNP rs number with lowest P-value (logistic model) within the window

At a later stage of research, the possibility of predicting the survival of birds during an epidemic based solely on their genomic information was analysed [39]. Experimental material from three groups of birds was used: the commercial Hy-Line from the H5N2 outbreak in Iowa in 2015; the commercial Hy-Line from the H7N3 epidemic in Mexico in 2012; and commercial line 1 (Table 1). All SNP polymorphisms were analysed using the Bayes B model and GenSel software. A linear mixed model was fitted to the data, taking into account the fixed effect of the mean and random effects of individual SNP polymorphisms. A posteriori estimates for SNP effects were used to estimate genomic breeding values for survival of HPAI infection in other genotypic birds that were not included in the training dataset.

The potential of genomic prediction was verified by cross-validation with 5 subsets and an ROC curve, which indicates to what extent prediction using the model is better than the random assignment of individuals to groups. The area under the ROC curve was 0.76 for predictions in the case of potential H5N2 infection, 0.71 for H7N3, and only 0.58 where survival was predicted for infection with H5N2 on the basis of estimates of SNP effects obtained for H7N3 infection. Prediction between genetic lines of chickens proved ineffective, with an area of 0.43 under the ROC curve, which is worse than a random classification, yielding a value of 0.5 (Fig.). The results may indicate that the genetic determinants of survival are specific for the virus strain and genetic line of birds. They also confirm that survival of influenza in birds has an important genetic component.

In the autumn of 2017, a project was initiated which sequenced the whole genome for some of the samples from birds previously genotyped on 600k microarrays (Table 1) and an additional 34 samples from birds that had survived another influenza epidemic in Mexico in 2016. The research is carried out with the support of the Egg Industry Center based in Iowa and Hy-Line International. The project is implemented by three research groups: the first under the direction of Dr. Anna Wolc, Dr. Janet Fulton and Dr. Jesus Arango (Hy-Line) in cooperation with Dr. Wioleta Drobik-Czwarno of the Warsaw University of Life Sciences; the second under Jack Dekkers (Iowa State University, USA); and the third headed by Dr. Jacqueline Smith and Prof. Paul Digard (Roslin Institute, Edinburgh, UK). In total, 293 genomic sequences were used. In addition to the sequences that had previously been analysed, the research included samples from 8 chickens that were the only individuals of the 1171 kept on a farm in Mexico to survive the H7N3 infection in 2016. The control group comprised18 birds that DNA analysis had classified as full siblings of individuals that had survived. Readings for all birds were mapped to the reference genome (galGal5), and preliminary detection of single nucleotide polymorphisms (SNPs) and short insertions and deletions (INDEL) was performed as well. Further bioinformatic analysis is currently being performed on regions that differentiate the groups. Candidate genes thus far identified are currently being tested in vitro at the Roslin Institute in Edinburgh.
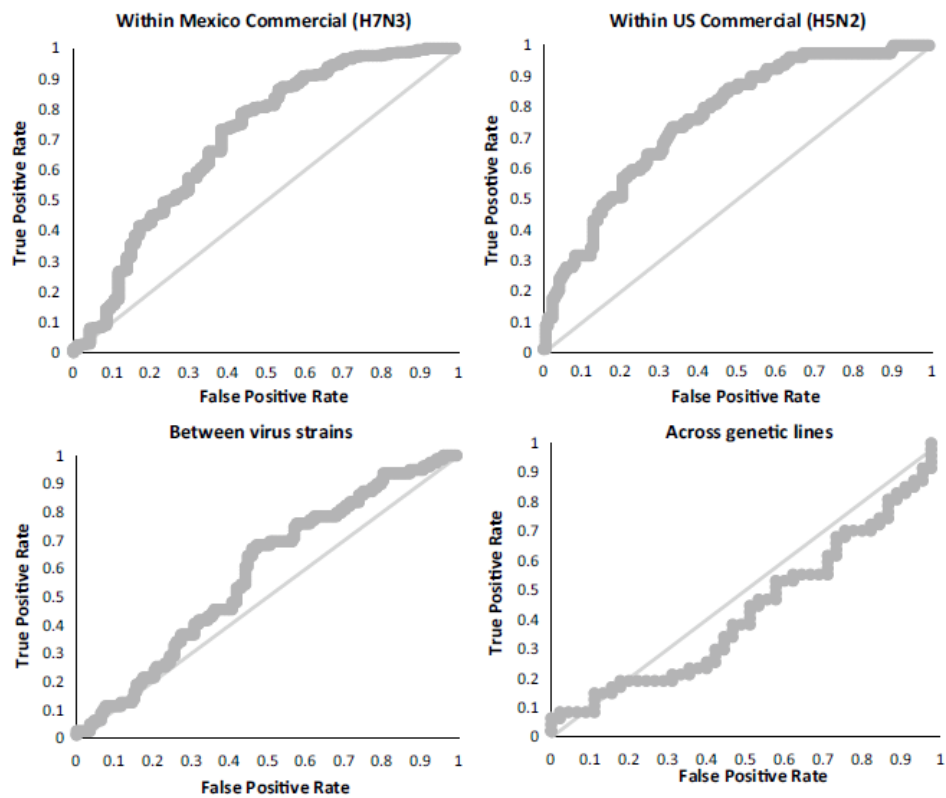
Fig. ROC curves for predicting HPAI survival. Upper left corner – H7N3 Mexico; top right corner – H5N2 Iowa; lower left corner – between H7N3 and H5N2 strains; bottom right corner – between genetic lines [39]

### Conclusion

The genetic determinants of the response to highly pathogenic avian influenza in laying hens are believed to be very complex. Numerous genes have been identified that may be responsible for both the interspecific and individual differences observed, but they do not provide clear answers regarding the mechanisms that determine survival after infection. Numerous studies indicate a significant role of nonspecific immunity. Studies on individual differences among birds surviving the outbreaks on farms in Iowa and Mexico have shown that genetic variation contributes significantly to resistance to and survival of HPAI.

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The research has identified a number of genes known for their connection with immune response, virus replication and nervous system function. However, the results indicate that the signal regions detected in association studies (which include individuals who survived infection and control individuals) differ from one another, which may indicate that resistance and survival depend on the virus strain and genetic line of the birds. Further research is needed to verify the identified regions, genes and variants, as well as the role that specific genes may play in resistance to highly pathogenic avian influenza in laying hens.

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