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# Integrated Genomic Approaches to Enhance Genetic Resistance in Chickens

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The chicken has led the way among agricultural animal species in infectious disease control and, in particular, selection for genetic resistance. The generation of the chicken genome sequence and the availability of other empowering tools and resources greatly enhance the ability to select for enhanced disease resistance via genetic markers and to understand more deeply the biological basis of host resistance. In this review, we discuss how integrated genomic approaches are able to identify specific genes and genetic markers associated with disease resistance, give select examples of contemporary work involving various genomic strategies to identify disease resistance genes, and finish by giving some final thoughts on predicted applications in the near future.

**Keywords**
chicken, disease, genetic resistance, quantitative trait loci (QTLs), genomics, expression profiling

**Disciplines**
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# Integrated Genomic Approaches to Enhance Genetic Resistance in Chickens

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## Abstract

The chicken has led the way among agricultural animal species in infectious disease control and, in particular, selection for genetic resistance. The generation of the chicken genome sequence and the availability of other empowering tools and resources greatly enhance the ability to select for enhanced disease resistance via genetic markers and to understand more deeply the biological basis of host resistance. In this review, we discuss how integrated genomic approaches are able to identify specific genes and genetic markers associated with disease resistance, give select examples of contemporary work involving various genomic strategies to identify disease resistance genes, and finish by giving some final thoughts on predicted applications in the near future.

**Quantitative trait loci (QTLs):** regions containing one or more genes that account for genetic variation of a complex trait

**Marker-assisted selection (MAS):** a method to select for traits using genetic markers



## INTRODUCTION

Modern agriculture has been very successful in meeting the growing demands of consumers for high-quality, safe, and affordable products. This trend is particularly evident in the poultry industry. As determined by the Food and Agriculture Organization (1), worldwide per capita consumption of poultry meat has gone from 2.87 kg per year in 1961 to 12.62 kg per year in 2007, a more than fourfold increase during this period. Furthermore, this dynamic growth trend is projected to continue, with poultry overtaking pork as the main meat consumed worldwide within the next five years.

Presently, several major issues confront the poultry industry in meeting the growing demands of consumers. Control of infectious diseases and food safety is certainly at or near the top of the list. Avian influenza, *Salmonella*, and *Campylobacter* are just a few of the pathogens well known to the public that harm the poultry industry through bird morbidity and mortality, reduced public confidence, and/or lost market accessibility via trade restrictions. Disease outbreaks, or the potential for them to occur, are enhanced by both high-density and free-range chicken rearing, reduced genetic diversity from continued industry consolidation, and government restrictions on chemoprophylactics to control pathogens. Changes in animal husbandry and new vaccines have helped to alleviate some of the problems; however, improved or alternative control measures are still needed to address current diseases and impede emerging threats.

The field of genomics offers a very exciting avenue for solving or ameliorating many of these issues. Although still in its formative years, by identifying quantitative trait loci (QTLs) and genes that control heritable traits of agricultural importance, it is possible to use refined knowledge about existing biodiversity to genetically select for birds with superior agricultural traits, such as improved disease resistance, via marker-assisted selection (MAS). For infectious diseases, MAS would eliminate the exposure risk to elite flocks associated with handling a hazardous pathogen, which traditionally is needed to select for more disease-resistant birds. The release of the chicken genome sequence in 2004 (2) and additional improvements since then toward finishing the assembly have only increased the power of this discipline. The ultimate goal is to address the long-standing, major biological question of how genetic variation explains the observed phenotypic (i.e., connecting genotype-to-phenotype) variation.

This review provides information on the emergence of modern molecular genetics in poultry and gives specific examples of how the employment of integrated approaches is yielding results with high potential for enhancing genetic resistance to three different pathogens, while also preserving appropriate levels of production in commercial poultry. Although the focus is on chickens and genetic resistance to disease in intensive production systems, the strategies, opportunities, and challenges should be applicable to other species, including those with no or draft genome assemblies, which is becoming increasingly common owing to significant gains in next-generation sequencing technologies.

## POULTRY BREEDING AND DISEASE CONTROL

Poultry production has a long history of embracing and applying innovations to meet consumer demands and improve profitability. Many key developments, especially since the 1950s, have resulted in the highly efficient and specialized industry that exists and operates today. Of particular note are the tremendous advancements in poultry breeding that have resulted in commercial chickens with greater genetic potential. Specifically, the primary breeding companies, which are responsible for genetic improvements in poultry, have been able to continually select for birds with superior production and other agronomic traits. For example, a 2001 commercial broiler had an average body weight of 3,946 g at 56 days of age, compared with 809 g for its 1957 counterpart

(3). Utilizing feeds typical for years 1957 and 2001, it was shown that 85–90% of this 4.87-fold improvement is accounted for by genetics; the remaining 10–15% is due to nutrition. These genetic gains are multiplied through the vertically integrated system of hatcheries, producers, and growers to the consumer, with the net result being greater efficiency at all levels and lower commodity prices. Other traits show similar gains, such as feed conversion rate (FCR), in which commercial birds require 1.8 g or less of feed to gain 1 g of body weight; in comparison, FCR is ~3.5 and 8 for pigs and cattle, respectively (4, 5). In short, poultry is the most affordable source of animal-derived protein, and its breeding methods are considered models for other animals.

Disease control and prevention was another critical factor in the dramatic growth of the poultry industry. With the widespread distribution of poultry and high-intensity rearing, infectious diseases could be spread and transmitted rapidly, which resulted in considerable economic losses. Consequently, the demand by growers (and consumers, for zoonotic pathogens) forced the industry to control specific diseases, which has been implemented through a combination of biosecurity, chemoprophylactics, and vaccines as well as selection for genetic resistance, the focus of this review.

**Single-nucleotide polymorphisms (SNPs):** the most prevalent type of polymorphism and genetic marker

**Linkage disequilibrium (LD):** the nonrandom association of alleles at two or more loci

## POULTRY GENETICS IN THE POST–GENOME SEQUENCE ERA

Just prior to the release of the chicken genome sequence in 2004, there was great excitement in the field of molecular genetics for chickens and other animal agricultural species (e.g., cattle, swine, sheep). The main cause was that, armed with molecular genetic maps composed of several thousand markers (mainly PCR-based microsatellite markers), scientists then felt they had the ability to survey the entire genome and identify QTLs that could account for most genetic variation observed for traits, including multifactorial ones such as disease resistance. Numerous studies, in chicken and other species, indicated strongly that these QTLs are real and account for a significant portion of the genetic variation. Thus, there was great hope that the identification of the underlying genes and causative polymorphisms would soon be in hand for use in animal breeding through MAS.

The advent of the genome sequence of red jungle fowl (2) only strengthened this expectation. Not only did scientists have the "blueprint" for the chicken and knowledge of where most of the genes lie in the genome, but newer discoveries and technologies continued to enhance the power of genomics. Specifically, by sample sequencing three other diverse chickens (commercial broiler, Chinese Silkie, and White Leghorn), a Beijing Genome Institute–led consortium identified approximately three million single-nucleotide polymorphisms (SNPs), which provided several orders of magnitude more genetic markers (6). This identification, combined with the ability to genotype first thousands (7) then tens to hundreds of thousands of SNPs (8), it was economically feasible to genotype thousands of genetic markers for QTL scans. As a result, with the ability to scan the entire genome with hundreds to thousands of markers, 3,162 QTLs have been identified in chicken from 158 publications (9).

Despite this success, it soon became apparent that the QTL mapping resolution was insufficient for MAS for two main reasons: First, unless the individual QTL effects are large and the extent of linkage disequilibrium (LD) very small, it is almost impossible to fine-map a QTL down to 5 cM or less; with ~23,000 genes and ~3,000 cM in the chicken genome, on average, each cM of the chicken genome encompasses ~7 genes. Second, sufficient mapping resolution requires small LD blocks, which in turn require more genetic markers. This can be achieved easily by using resource populations that have accumulated more recombination events, such as advanced intercross lines or commercial populations. However, increasing the number of genetic markers also requires increasing the number of measured animals; theoretically, the number of phenotyped animals should equal or exceed the number of genetic markers. Although commercial poultry-breeding

companies have the resources to measure tens of thousands of birds for many traits, this is often not true for traits related to disease resistance. Therefore, although possible, it is unlikely that a purely genetics-driven approach will be powerful enough to map a QTL down to the single gene level, let alone the causative mutation. The inability to achieve mapping resolution sufficient to identify individual genes and to account for the majority of the genetic variation is not unique to chicken, as evidenced by similar issues in human genetics, to which even greater genomic resources and efforts have been devoted (10).

In addition, the inability to fine-map QTLs and results from other genomic approaches in chicken and other species has led to numerous discussions questioning whether large-effect QTLs truly exist. Prior to the identification of QTLs, the "infinitesimal model," originated by Fisher (11), postulated that traits were controlled by many loci of relatively small effect. If the infinitesimal model is true, then it would make implementing MAS for complex traits much more difficult than originally anticipated. However, the chicken major histocompatibility complex (MHC) and other loci with large effects have been identified and undergone commercial selection, suggesting a more balanced view of biological reality, which likely includes genes of both small and large effects. Fortunately, several existing tools can augment purely genetics-driven efforts. Those that explore the biology at the genome-wide scale (i.e., functional genomics) include but are not limited to the following.

**Major histocompatibility complex (MHC):** a 242-kb region in the chicken genome containing 46 genes, many of which are key immune regulators and are in high linkage disequilibrium

**Expression quantitative trait loci (eQTLs):** loci and candidate genes with *cis*- and *trans*-acting genetic influences on gene expression

**Allele-specific expression (ASE):** a genomic method to identify genes with *cis*-acting regulatory elements from RNA sequencing data sets

## RNA Expression Profiling

Differences in gene expression (when, where, how much) are thought to be major contributors to phenotypic variation. Typically through microarrays or RNA sequencing, one can identify genes that are differentially expressed between two or more samples. For disease challenges, this is a powerful approach to determine what genes and associated pathways are altered when the host encounters a specific pathogen. Because RNA is being measured, information on when and where to isolate the RNA is relevant when using animals, and these factors are not always clear at the beginning of a study. A major limitation of this approach is the limited annotation of genes in the chicken genome, especially those involved in the immune system, although this is improving.

## Expression Quantitative Trait Loci

Although RNA expression profiling can provide substantial insights into biological functions and pathways associated with complex traits such as disease resistance, it cannot distinguish the genetic basis for this variation in gene expression, because expression of all the genes downstream from the causative variant potentially could be perturbed. To identify the genetic basis for expression variation, two approaches have been employed. The first is known as genetical genomics, or eQTL (expression QTL). In this method, transcript abundance levels are treated as quantitative traits for QTL mapping. This allows molecular genetics to be combined with transcript profiling, usually through microarrays. As a result, elements (eQTLs) that control the expression of genes with heritable expression variation can be identified. These eQTLs are further defined as *cis* or *trans* based on the genomic position of the eQTL relative to the gene it regulates. Thus, *cis* eQTLs are believed to identify genes that account for a portion of the genetic basis. Unfortunately, eQTLs suffer from the same mapping resolution issues inherent to QTL mapping.

## Allele-Specific Expression Screens

An alternative to eQTL is allele-specific expression (ASE). With ASE, each allele is measured for genes that are heterozygous as judged by a marker polymorphism (e.g., a SNP). When allelic

imbalance or differential expression is observed, then a polymorphic *cis*-acting element must be present for that gene, "since allelic variation is by definition reflective of *cis*-acting influence" (12, p. 452). In fact, this is a primary strength of the ASE approach. As both alleles are present in a diploid cell, if the alleles respond differentially, then this must be a result of a *cis*-acting effect. Key advantages of ASE over eQTL are that: (*a*) separation of an overall gene expression signal into allelic components significantly increases sensitivity and adds power; (*b*) because both alleles of the gene are in the same cell of an individual, monitoring the expression of each allele controls for issues such as differences in cellular composition, sampling, RNA quality, or environmental effects; and (*c*) genes influenced by *cis*-acting elements are readily identified because they must account for some or all of the allelic imbalance. The final and most crucial advantage of the ASE approach is that, because genetic factors that influence transcriptional regulation in *cis* are generally in close proximity to the gene itself, identification of a *cis*-acting regulatory element essentially identifies a specific gene or locus that contains the polymorphism leading to the allelic imbalance. In other words, identification of a SNP exhibiting ASE essentially identifies a high-confidence candidate gene (genetic factor) with expression differences that may account for the complex trait.

The rationale for integrating both genetics and functional genomic approaches is that the strengths of each system can be combined to yield results of higher confidence. Given the large volume of data produced by genomics, each method provides an additional screen to limit the number of targets to verify and characterize in future experiments. The following examples represent the primary efforts that have employed integrative genomic approaches to identify specific disease-resistance genes as well as to provide fundamental biological information.



## MAREK'S DISEASE

### Rationale for Enhanced Genetic Resistance

Marek's disease (MD) is a T cell lymphoma disease of domestic chickens induced by Marek's disease virus (MDV), a naturally oncogenic, highly contagious, and cell-associated α-herpesvirus (13, 14). The disease is characterized by a mononuclear infiltration of the peripheral nerves, gonads, irises, various viscera, muscles, and skin. Partial or complete paralysis is a common symptom of MD owing to accumulation and proliferation of tumor cells in peripheral nerves. During the 1960s, as the industry converted to high-intensity rearing, MD generated tremendous economic losses. Since the 1970s, MD has been controlled by vaccination and improved animal husbandry. However, even with vaccines, estimated annual losses worldwide from MD owing to meat condemnation and reduced egg production are $1–2 billion (15). Although vaccination prevents the formation of lymphoma and other MD symptoms, it does not prevent MDV infection, replication, or horizontal spread (16). Moreover, even though available vaccines protect chickens against the disease, MD still remains a threat owing to increasingly frequent outbreaks caused by highly virulent strains of MDV combined with the incomplete immunity that is elicited by vaccination (17–19). Thus, to break the cycle of MDV evolving to higher virulence, knowledge supporting the development of new strategies for control of MD is needed. For this reason, increasing genetic resistance to MD is highly desirable.

### Pre–Genome Sequence Efforts

Genetic differences in resistance to fowl paralysis, assumed to be MD, have been reported for 70 years (20). Although genetic resistance to MD is complex and controlled by many genes, the MHC

(also known in the chicken as the B complex because of its linkage with the B blood group) is clearly a major locus. Prior to modern genomics, the MHC was shown to be associated with MD resistance (21). Cole's (22) selection of the B-G (class IV) locus through blood-typing resulted in dramatically different MD incidences (7% and 94% in Cornell lines N and P, respectively), which clearly demonstrates that it is possible to enhance genetic resistance to MD given the right selective pressure and without the need for DNA-based markers. Because no differences between the two lines are observed during the first week of infection, MHC resistance may result from factors other than the number or type of target cells present early after infection (23, 24).

In addition to the MHC, other genetic factors exert a major influence on MD resistance. For example, Avian Disease and Oncology Laboratory (ADOL) inbred line 6 and 7 chickens share the same B*2 haplotype (25) but differ greatly in resistance to MD. In contrast to MHC-controlled resistance, non-MHC genetic resistance can be related to the number of target cells. Spleen and thymus cells from line 6 birds are infected with less MDV than similar cells from line 7 (26, 27). The sizes of the primary lymphoid organs (thymus and spleen) and the number of lymphocytes in line 6 chickens are also significantly smaller than in line 7 chickens (28–30).

Apart from a few candidate gene studies [e.g., *Rfp-Y* (31), vitamin D receptor (32)], most efforts to identify MD resistance genes have used genome-wide QTL scans with microsatellite markers. Two studies utilized ADOL lines 6 (MD resistant) and 7 (MD susceptible). Vallejo et al. (33) and Yonash et al. (34) identified 14 QTLs (7 significant and 7 suggestive) where, collectively, the QTLs explained up to 75% of the genetic variance. Interestingly, by measuring not only disease incidence but also disease-related traits, the QTLs could be grouped by trait type. Some QTLs were associated almost exclusively with viremia levels and the remaining QTLs with disease, survival, tumor incidence, nerve enlargement, and other disease-associated traits, which suggests that disease resistance occurs at least at two levels: initial viral replication and cellular transformation. Similarly, Bumstead (35) mapped a single significant QTL on chromosome 1 that had conserved synteny with the mouse CMV1 locus, which controls resistance to murine cytomegalovirus, another herpesvirus. *Ly49H* is the causative gene for CMV1 resistance, and the encoded protein is a receptor on natural killer cells that interact with MHC class I (36).

Two other studies used commercial layer (egg-type) lines that allowed for larger populations and industrially relevant results. Using microsatellites genotyped on DNA pools from selected individuals, McElroy et al. (37) identified 17 markers of the 81 screened to be associated with length of survival post-MDV infection. Heifetz et al. (38) identified 15 QTLs on two consecutive backcross (BC) populations; however, only 5 of the QTLs were common to both BC populations. The second BC hatch showed an MHC association, although the B*2 allele was unexpectedly found to confer susceptibility. The interaction of the MHC with other background genes had been observed previously (39).

To complement the QTL scans, gene expression profiling using microarray technology has been integrated. The rationale is that gene expression profiling will identify genes and pathways involved in MD resistance, which, combined with genetic mapping, can reveal positional candidate genes (40). In other words, positional candidate genes are those that have a genetic association and are identified as being relevant through gene expression analyses. Gene profiling has been conducted to identify differentially expressed genes between MD-resistant and -susceptible lines after MDV challenge (40, 41); among MHC-congenic lines of chickens following inoculation with different MD vaccines; in chicken embryo fibroblasts (CEF) infected with MDV (42); and in CEF transformed with *Meq*, the likely MDV oncogene (43). Several genes and pathways are consistently associated with either MD resistance or MDV infection, and the results suggest that chickens with immune systems that are more stimulated by MDV infection are more susceptible. Because

MDV is thought to infect only activated lymphocytes, chickens with immune systems that are more responsive may present more targets for MDV to infect and later transform.

MDV-chicken protein-protein interactions provided additional evidence that genes are associated with MD resistance. Niikura et al. (44) screened for MDV-chicken protein-protein interactions using a two-hybrid screen confirmed by an in vitro binding assay and identified nine such interactions. Of particular interest were growth hormone (GH1) (45), stem cell antigen 2 (SCA2) (46), and MHC class II β chain (B-LB), because the transcripts for each gene were differentially expressed between MD-resistant and -susceptible birds following MDV infection, and there is an association with MD resistance. Also, *GH1* allele frequencies have changed in response to selection for MD resistance (47), which supports growth hormone as an MD-resistance gene. Novel upregulation of both MHC class II, following MDV infection (48), and vitamin D receptor (VDR), which modulates MHC class II cell surface expression and is associated with MD resistance (32), further supports MHC class II β chain as a candidate gene for MD resistance.

Protein profiling using mass spectrometry, a powerful technique that can query the proteome, was conducted on UA-01 cells, a MDV-transformed cell line (49). Prior work had indicated that MD tumors overexpress CD30 and, thus, might be a natural model for human T cell lymphomas (50). Bioinformatic analysis of the data indicates that MD tumor cells have a pattern that is consistent with other tumors and are probably derived from regulatory T cells. More importantly, the prometastatic integrin and ERK/MAPK signaling pathways were predominant, which suggests that these pathways are important for transformation and migration of MD tumors. Similar studies by Liu et al. (51) have cataloged the spectrum of MDV proteins expressed.

## Post–Genome Sequence Efforts

Given the difficulty of purely genetics-driven approaches to identify high-confidence candidate genes, alternative efforts have been employed. MacEachern et al. (52) incorporated a genome-wide ASE screen for chicken non-MHC genes that respond to MDV infection. In this study, using an RNA sequencing data set, SNPs were first identified, and then the ratio of the two alleles in uninfected and MDV-infected animals was determined. If the expression ratio changed in response to viral infection, it indicated that there was a *cis*-acting regulatory element affecting the expression of the gene, thus identifying a genetic element in the gene containing the SNP.

In brief, ADOL lines 6 (MD resistant) and 7 (MD susceptible) were intermated to produce $F_1$ progeny. Half of the progeny were challenged with MDV at 2 weeks of age. At 1, 4, 7, 11, 13, and 15 days postinfection (dpi), 12 birds from each treatment group were euthanized, and RNA from the spleen was isolated. To get a genome-wide and unbiased survey of all the expressed genes and an indication of ASE, replicate RNA pools from a single time point (4 dpi) were sequenced using an Illumina GA. This resulted in $\geq$ 11 million mapped reads per treatment group with a total of $>$ 1.7 Gb surveyed. Statistical analysis revealed that 5,360 (in 3,773 genes) of the 22,655 high-quality SNPs identified exhibited statistically significant allelic imbalance; gene expression was detected for 12,696 genes. To validate and extend the results, 1,536 selected SNPs were screened on RNA samples from all 456 $F_1$ birds using Illumina GoldenGate arrays. Allelic imbalance was confirmed in 861 (70%) of the 1,233 working assays. Infection was found to greatly impact the expression of alleles from these genes over time, and significant differences in ASE were detected between infected and uninfected individuals at all time points. The identified genes and pathways (e.g., cell proliferation, apoptosis) are consistent with what is thought to be the case for MD genetic resistance. Thus, it was concluded that ASE is a powerful approach to identify regulatory variation responsible for differences in transcript abundance. Experiments are currently underway to

answer the major question, which is whether the SNPs showing ASE are also associated with MD genetic resistance.

Smith et al. (53) also profiled gene expression in line 6 and 7 birds following MDV infection. RNA was isolated at 2, 3, and 4 dpi to concentrate on the innate immune response. As is typical for microarray-based studies, several genes and pathways were altered in comparing the two lines of chickens. Most interesting, however, was the enrichment of HIC1 binding sites in the promoters of genes that were repressed in response to viral infection. HIC1 is a transcription factor that drives antitumor mechanisms. This would suggest that MDV is actively suppressing antitumor mechanisms. This type of analysis would not have been possible without the chicken genome sequence. Furthermore, by conducting association analyses in MD resource populations, it was shown that IRG1 has a potential role in MD susceptibility.

A direct approach to identify MD resistance genes and pathways takes advantage of the fact that MDV *Meq*, the viral oncogene, is also a bZIP transcription factor. Thus, one can employ chromatin immunoprecipitation using antibodies directed against Meq, followed by next-generation sequencing, to identify the regions in the chicken genome to which Meq binds. Integrating DNA microarrays to compare cells expressing Meq with those that do not theoretically allows one to identify all the genes directly regulated by Meq. Furthermore, motif analysis of the Meq-bound sites provides opportunities to account for ASE between lines 6 and 7. Preliminary results using this strategy have been reported (54).

To summarize, through a combination of genetic and functional genomic approaches, a large number of candidate genes have been identified, which confirms the multigenic nature of MD genetic resistance. Experiments are under way to determine if MAS based on thousands of candidate genes and SNPs can select for and against MD resistance as well as to provide information on how transferable the genetic markers are across various populations (H.H. Cheng, manuscript in progress).

## *Salmonella*

**Rationale for enhanced genetic resistance.** The importance of *Salmonella* as both a poultry and a food-safety pathogen, along with the recognition early in the twentieth century of a heritable basis for host resistance to *Salmonella* (55), has resulted in a long history of investigation into the genetics and genomics of resistance to *Salmonella* in chickens. Depending upon the bacterial species, *Salmonella* can either be highly pathogenic or cause virtually no response in the host. *Salmonella enterica* serovars Gallinarum and Pullorum, both of which cause systemic salmonellosis, lack flagella and therefore are not recognized effectively by Toll-like receptor 5 (TLR5) such that there is no strong inflammatory response to help limit the infection to the gut (56). The inflammatory reaction induced by *Salmonella* Typhimurium and *Salmonella* Enteritidis (SE) often limits the infection to the gastrointestinal tract, where it may establish a carrier state and become a potential source of poultry-product contamination.

Birds with subclinical salmonellosis may remain in production flocks and transmit the zoonotic bacteria into the food chain (57). Chicks infected with *Salmonella* upon hatching can be colonized persistently, and the bacteria can infect table or hatching eggs laid by adult hens (58). SE accounts for over three-fourths of the cases of food-borne salmonellosis (59). Chicken consumption is a major risk factor in SE infections (60). Over one million cases of human infection with *Salmonella* species occur each year in the United States alone (61). In addition, reduced growth and reproductive performance can occur as a result of microbial infection, even at subclinical levels (62).

Poultry resistance to *Salmonella* has provided a durable example of the integration of diverse genetic and genomic approaches to elucidate the host genes, networks, and QTLs associated with

resistance. The essential first stage in such studies is to determine that a genetic basis does indeed underlie the traits of interest; this establishes the feasibility of identifying the specific genetic components controlling resistance and thereby the potential to enhance host resistance by genetic or genomic selection. Complex disease phenotypes often are divided into well-defined components of the response, each of which typically has higher heritability than the total response. Heritability of chick survival after *Salmonella* challenge ranged from 0.14 to 0.62 (63); number of bacteria in internal organs, 0.02–0.29 (64); cecal carrier state, 0.06–0.20 (65); and spleen and cecal contamination, 0.13–0.47 and 0.24–0.53, respectively (66).

The estimated heritabilities of parameters of response to *Salmonella*, as well as the differences between distinct genetic lines of chickens (67), strongly suggest that there is partial genetic control of most response phenotypes and, therefore, that genetic selection to improve resistance to *Salmonella* carrier state and salmonellosis is feasible. Genetic selection can be based on variation in genomic structural or expression-level variation (63, 68–70). However, studies to date suggest that many genes are associated with genetic control of response to *Salmonella*, and the effect of most individual genes is rather small (see 71–75).



**Candidate gene studies: structural polymorphisms.** Strategies used to identify the genetic control of resistance to *Salmonella* have spanned the spectra from gene-centric to genome-wide and from variation in structure to variation in expression. They have also capitalized on the strengths of using varied population structures, including inbred and congenic lines, diverse genetic populations, and outbred commercial lines. Outbred populations possess extensive phenotypic diversity for traits such as morphology, behavior, and disease susceptibility, predominantly owing to underlying genetic diversity. Understanding the relationship between DNA sequence polymorphism and the variability observed for complex traits will increase opportunities for predicting disease risk and also, in the case of livestock, allow selective breeding programs to maximize improvement for the trait of interest. Both commercial and inbred lines of chickens differ in innate immune responses to pathogen challenge. This correlates with disease resistance, and the differential responses are under genetic control (67, 76–78).

Early candidate gene studies on the molecular basis of *Salmonella* resistance in chickens effectively used a comparative approach by examining the role of major loci that control resistance of mice to *Salmonella* Typhimurium infection. One-third of the differential resistance in mortality after *Salmonella* infection in a BC of inbred chicken lines was associated with variation in the natural resistance–associated macrophage protein 1 (*NRAMP1*, now termed *SLC11A1* as a member of the solute carrier gene family) and tenacin C (*TNC*, which was used as a marker for the nearby lipopolysaccharide, or *LPS*, locus, known to control response to LPS in mice) (79). Successful investigation of these biological candidate genes led the way to identification of other positional and biological candidate genes. Located near the TNC locus is *TLR4* (formerly *LPS*), which encodes the receptor that binds LPS, a major component of gram-negative bacterial membranes such as *Salmonella*. *TLR4* was associated with response to *Salmonella* in chickens (80,81). Associations with both *SLC11A1* and *TLR4* have subsequently been demonstrated across a wide range of *Salmonella*-response phenotypes and chicken populations (72–75).

Many biological candidate genes have been associated with host response to *Salmonella*. A major biological candidate gene region, the MHC, was selected for investigation because of its association with many other disease-resistance traits. Once again, specialized experimental populations served as an effective target of study; B-complex (MHC) congenic lines revealed differences in mortality and morbidity after *Salmonella* challenge (82), and polymorphism in MHC class I genes was associated with other *Salmonella*-response traits in experimental line crosses (83, 84).

Other candidate genes have been identified by their hypothesized roles in important pathways of host response to *Salmonella*. Cytokines are essential communication molecules secreted by immune system cells and other tissues. They serve a primary role in modulating pro- and anti-inflammatory responses and in directing appropriate adaptive immune responses. Genetic variants in cytokines and related genes have been associated with numerous *Salmonella*-resistance phenotypes (85–87). The antimicrobial β-defensin peptides serve in the innate immune response against bacteria (88). Structural variants in several β-defensin genes were associated with bacterial load in the cecal content or spleen tissue after *Salmonella* challenge (89). Apoptosis, or programmed cell death, is an important functional feature of the immune system, and genes in apoptotic pathways (*CASP1* and *IAP1*) have been associated with *Salmonella* persistence in internal organs in both experimental crosses and commercial broilers (85, 90).

Experiments that test associations between candidate genes and *Salmonella* resistance can seldom reject the possibility that the causal gene could be a nearby, rather than the specific, gene tested because of LD between the tested marker and nearby genes. Therefore, additional supporting lines of evidence, including verification in independent populations, either by gene expression data or with genome-wide QTL scans, add confidence to the detected gene-resistance phenotype associations. In addition to studies of tissues from animals infected with *Salmonella*, insights into resistance mechanisms can be gained by intensive analysis of isolated, relevant cell types and in vitro systems. For *Salmonella* infections, heterophils and macrophages are key responder cell types.

**Expression variation: targeted genes to global arrays.** Gene-targeted studies of mRNA expression changes associated with *Salmonella* infection in chickens have focused on three main gene families: TLRs, cytokines, and β-defensins. The TLRs are pattern-recognition receptors (PRRs) that initiate immune response after detection of pathogen-associated molecular patterns. After SE challenge, *TLR4* expression differed between chicken lines with different levels of resistance to *Salmonella* (91). Diverse genetic lines of chickens exhibit different expression patterns of up- and downregulation of *TLR2*, *TLR4*, and *TLR5* in the spleen in response to *Salmonella* infection (92). Downregulation, rather than the expected upregulation, of *TLR5*, which recognizes bacterial flagella, supposedly has the beneficial effect of protecting host cells from overstimulation (93). Expression of *TLR15*, an avian-unique TLR, differed in heterophils isolated from broiler breeder chicken lines that differ for resistance to *Salmonella* (94).

*Salmonella* infection induces expression of multiple chemokines, cytokines, and their receptors in a variety of chicken tissues. After *Salmonella* infection, distinct chicken breeds express different profiles of expression in the spleen or cecum, including *IL10*, *IL12A*, *IL12B*, *IL18*, *CCLi2*, and *CXCLi2*, which may explain some of the general breed differences in immune response (95, 96). Comparing four lines of broilers, including two parental lines and their $F_1$ crosses, the heterophils from the two phenotypically resistant lines, after isolation and treatment with SE, had higher levels of expression of the proinflammatory cytokines *IL6*, *IL8*, and *IL18* and lower levels of the anti-inflammatory *TGFB4* than the two susceptible lines (97). Responses to SE of heterophils from lines that had undergone commercial selection for meat or egg production differed from those of heterophils from an unimproved line, which had increased expression of pro- (*IL6* and *GM-CSF*) and anti-inflammatory (*IL10* and *TGFB4*) cytokines (98). Macrophages isolated from blood of resistant and susceptible lines of chickens produced cytokines with different kinetics and levels, including more rapid and higher levels of *IL18* in the resistant line, which suggests that Th1 adaptive immunity is important in protective responses (99). Although studies conducted with a variety of genetic lines, challenge species of *Salmonella*, timing, and cells or tissues show some

variation in results, *IL1α*, *TNFα*, *IFNG*, *IL12*, *IL15,* and *IL18* generally appear to be associated with a protective role, and *IL4* and *IL10* with inhibition of host defenses against *Salmonella*.

Because antimicrobial peptides are an important component of the innate immune response to pathogens, the rapid expression of avian β-defensin (AvBD) genes after infection is likely important in resistance. Expression of AvBD genes in leukocytes was increased significantly within 6 h in response to *Salmonella* Typhimurium infection of broilers (100). Inbred chicken lines differing in cecal bacterial carriage showed marked differences in expression of AvBD1 and AvBD2, with higher expression in the line with the lower bacterial carriage (91). *Salmonella* infection interfered with AvBD2 expression in the cells from a susceptible, but not a resistant, line (101). Collectively, these studies reinforce the concept of an important role of the β-defensins in *Salmonella* resistance in chickens.

Although it is rapidly being replaced by sequencing-based technologies, large-scale expression profiling using microarrays has been a powerful tool to identify genes and biological pathways associated with *Salmonella* infection phenotypes. A broad picture of the transcriptional differences that occur in response to *Salmonella* infection, or between resistant and susceptible phenotypes, can serve to direct future, targeted studies of gene function to identify the causal genes of *Salmonella* resistance. Both immune-centric and global expression microarrays have been used. Immune-centric arrays are produced by selective placement of probes from immune system genes or are produced from tissues relevant to the immune response. This approach increases the likelihood that a large percentage of the included elements will be involved in response to infection with *Salmonella.* Global arrays broadly represent all gene categories and have the potential to discover novel pathways not identified previously with resistance phenotypes. Global arrays can also serve as consistent platforms across studies of diverse physiological traits and therefore help to integrate information about gene networks that interconnect many biological functions.

Studies have been conducted on *Salmonella* response and a variety of tissues or cell types using immune-centric microarrays. Both infection status and genetic line have major impacts on differences in gene expression. In a study of intestinal tissue from two broiler lines, genes of the innate immune system and wound healing were upregulated after *Salmonella* infection (102). The lines, however, differed in expression of genes involved in inflammation, acute phase response, fibrinogen system, and actin-polymerization pathways. In a related study, line-specific responses to *Salmonella* included genes related to T cell activation and macrophage function (103). Transcriptional profiles of the HD11 chicken macrophage cell line after SE infection were assayed on a 5K microarray generated from activated macrophages/monocytes (104). The chemokine ah294 (CCL5 or RANTES) had the highest expression, and *IL6* and antiapoptotic genes were also upregulated. Genes associated with cell proliferation, adhesion, and transcription were downregulated.

Using a chicken 13K cDNA global array with transcripts derived from 24 tissue and cell sources (105), many cytokines, chemokines, and genes related to apoptosis and T cell functions were significantly differentially expressed in spleen between SE-inoculated and noninoculated chicks, as well as between chicks with high and low bacterial burden (106). With the same microarray, diverse genetic lines of birds were shown to preferentially use different biological systems in their early, innate response to *Salmonella* infection. One line predominantly used immune response; another line, apoptosis and nonimmune cellular responses; and the third line, immune defense mechanisms (S.J. Lamont & H. Zhou, unpublished data).

Heterophils from relatively resistant and relatively susceptible commercial broiler lines exposed to SE in vitro were evaluated for transcriptional profile using a 44K global array (107). The susceptible line had more downregulated immune-function genes than the resistant line. Immune-related genes that were upregulated in the resistant line included members of the TLR-signaling

pathway and genes that activate T helper cells. Analysis of the chicken HD11 macrophage cell line after stimulation with *Salmonella*-derived endotoxin, using the same 44K array, showed that the *NFKBIA*, *IL1B*, *IL8,* and *CCL4* genes were induced. Expression of the intracellular PRR *NLRC5* (a NOD-like receptor family member) was also induced, which signified the first demonstration of its potential function in the response to *Salmonella* in chicken macrophages (108).

Although varied experimental designs and microarray platforms have generated varied results, some consistent pictures emerge from use of this experimental approach regarding important pathways associated with response to *Salmonella*. Strong candidates for genetic control of *Salmonella* in poultry, as identified through large-scale transcriptional profiles, include TLR, cytokine, and antimicrobial β-defensin genes, as well as genes involved in T cell function and apoptosis.

**QTLs: from genomic regions to genes.** Identification of QTLs for response to *Salmonella* has progressed over time, from the use of low-density microsatellite markers; through moderate-density SNP panels (8); to the current, routine genotyping of over one-half million SNPs. The increasing marker density allows increasingly fine mapping of the QTL location, which facilitates identification of the specific causal genes or nucleotides underlying the QTL.

The most prominent example of QTL mapping is the identification of SAL1. Mariani et al. (109) identified a significant linkage between spleen colonization with *Salmonella* and genetic markers on chromosome *5* and named the QTL SAL1. Subsequent fine-mapping of the SAL1 region revealed two strong functional candidates for *Salmonella* response: *AKT1* (protein kinase B, or PKB) and CD27 binding protein (*SIVA*) (76). Fine-mapping of heterophil functional response to *Salmonella* in a highly advanced intercross strongly supported the SAL1 QTL position containing *AKT1* and *SIVA* and suggested heterophil function as a specific mechanism to explain the host-resistance properties that map to this region (110).

Many QTL regions associated with resistance to *Salmonella* carrier state, antibody response, or salmonellosis have been identified through genome scans located widely throughout the genome (69, 77, 111–114). This large QTL number supports the highly polygenetic nature of the host response, but QTLs with effects as large as 37% of the phenotypic variance in carrier state have been identified (111). Some QTLs are in regions known to contain immune-response genes, such as the MHC (111), or in regions in which previous studies have identified associations of gene SNPs with *Salmonella*-response phenotypes (82, 83). Locations of QTLs have also been verified from experimental to commercial populations (115), which demonstrates the effectiveness of using experimental populations to identify QTLs of value in commercial application. QTLs associated with response to both SE and *Escherichia coli* have also been identified (116), suggesting that some QTLs are associated with general properties of response to bacteria, which may make them especially useful in selection for improved resistance. The convergence of multiple lines of evidence for genomic control of host resistance to *Salmonella*, using independent populations and different strategies, strengthens confidence in the true existence of these QTL associations and encourages detailed study of these genomic regions to identify the causal mutations.

## *Campylobacter*

**Rationale for enhanced genetic resistance.** *Campylobacter* is the leading cause of acute enteritis in humans, and infections may be complicated by severe sequelae, including inflammatory neuropathies and reactive arthritis. In the United Kingdom, there were 65,000 laboratory-confirmed cases of human infection in 2009 (a 17% rise over the preceding year), and eight times as many

cases are estimated to be unreported (117). It is thought that 20–30% of cases result from handling or consuming contaminated broiler meat, and up to 80% of cases are attributable to the chicken reservoir as a whole (118). *Campylobacter* was detected in 65% of chicken samples on retail sale in the United Kingdom during 2007–2008 (119), and a pressing need exists for strategies to reduce entry of *Campylobacter* into the food chain. That on-farm control of *Campylobacter* is required to reduce the incidence of zoonosis is widely acknowledged.

*Campylobacter* may be a part of the normal avian gut flora owing to the asymptomatic carriage of the bacteria (i.e., essentially a commensal); therefore, at first consideration resistance to colonization would seem unlikely. However, increasing evidence suggests that, rather than being a commensal of the chicken, *Campylobacter* is actually a very accomplished pathogen. *Campylobacter jejuni* can colonize up to $10^8$ colony-forming units per gram of intestinal content, has the ability to invade the intestinal mucosa (120), and generates an antibody response during colonization (121). *Campylobacter* induced proinflammatory cytokines and chemokines in a chick epithelial cell model (122), and infection of chickens with *Campylobacter* induced a rapid influx of heterophils (the avian functional equivalent of the mammalian neutrophil) into the gut and the production of proinflammatory cytokines and chemokines in the intestinal epithelium (123). Such data indicate that *Campylobacter* can induce typical innate immune responses, although these are time- and magnitude-limited compared with similar responses induced by *Salmonella* serovars. The surface of *Campylobacter* is decorated with a plethora of carbohydrate moieties that may enable it to evade activation of avian innate immunity and thereby colonize poultry by stealth. Bacterial pathogens associated with enteric fever use such strategies (124), and it is noteworthy that *C. jejuni* can sometimes be found in deep muscle and visceral organs. *C. jejuni* may evade detection by avian TLR5, because mutant strains lacking genes responsible for glycosylation of the flagella elicit stronger proinflammatory cytokine responses in chick ceca and exhibit defects in intestinal persistence relative to the parent strain (125). *Salmonella* Typhimurium strains expressing the *C. jejuni* proteins CjaA or Peb1A elicit protection against intestinal colonization of chickens by *C. jejuni* (126), which suggests that immune control of *C. jejuni* in the avian intestines may be feasible.

Host variability in *Campylobacter* colonization of the chicken intestine was observed in young chicks over twenty years ago (127), and resistance to cecal colonization by *C. jejuni* is significantly influenced by the chicken host lineage (128). Boyd et al. (128) examined the ability of *C. jejuni* to colonize the intestines of four different inbred lines for 2–3 weeks postinoculation on the day of hatch. There was a consistent ten- to one hundred–fold difference between the four inbred lines in the number of *C. jejuni* present in the cloaca or in the ceca; the greatest differences were detected between line N, which carried relatively high bacterial levels, and line $6_1$, which carried relatively low numbers of bacteria. The MHC apparently was not a major factor in determining the resistance. The difference in numbers of colonizing bacteria was observed as early as 24 h after challenge and was still present at the end of the experiment. The effect appears independent of bacterial strain and age of bird; it was evident with both strain 14N and strain 81176 in newly hatched line $6_1$ versus line N chicks (128) and in birds challenged at three weeks of age with strain 11168H. Reciprocal BC experiments between lines N and $6_1$ revealed that the difference in bacterial numbers was heritable (128), which suggests the possibility of identifying the genes responsible by genetic mapping and candidate gene analysis.

**Genome-wide and functional genomic scans.** Despite associations between several *Campylobacter* genes and colonization (129), little is known about host gene associations with resistance to *Campylobacter* colonization. In our recent study (P. Kaiser, J. Howell & M. Fife, unpublished data), we used lines $6_1$ (resistant) and N (susceptible) in a BC experimental design. A total of 1,243

SNP markers, fully informative for the two lines, were used in a genome-wide screen for the identification of QTLs associated with levels of *Campylobacter* gut colonization in the first few dpi. Analysis of log-transformed cecal bacterial levels between the parental lines revealed a significant difference on all four dpi ($P < 0.05$). Four QTLs were identified through analysis of the BC ($F_1$xN) population. These included one genome-wide significant QTL on chromosome 11 at ~12 Mb and a QTL on chromosome 7 at ~28 Mb, which was highly significant at the chromosome-wide level. Two further QTLs on chromosomes 12 and 27, at ~18 Mb and ~1 Mb, respectively, were also significant at the chromosome-wide level.

Four QTLs for resistance to colonization with *Salmonella* Typhimurium were identified in a similar BC between lines $6_1$ and N (77). Three of them were at different genomic locations than the four QTLs identified in this study; the fourth was at the distal end of chromosome 12, overlapping significantly with the QTL identified at the distal end of chromosome 12 in this study. The 1-LOD-drop intervals of the two chromosome 12 QTLs cover approximately 3.5 Mb, although the QTLs could extend to the end of chromosome 12 (20.5 Mb). This is a comparatively gene-poor region of the chicken genome, with only 75 genes annotated, as well as one microRNA and one novel small nucleolar RNA. Among these there are several interesting candidate genes, including some involved in signaling leading to transcription and others with a more intriguing potential role in resistance to bacterial infection. For example, interleukin-1 receptor-associated kinase-like 2 (*IRAK2*) is one of two serine/threonine kinases that associate with the IL1 receptor upon stimulation, and it plays a role in upregulation of NF-κB. NF-κB signaling has a role in the induced innate responses to both *Salmonella* and *Campylobacter* infection in mammals, driving production of the proinflammatory cytokines IL1β, IL6, IL8, and TNFα. Infection of chickens with *Salmonella* (130, 131) or *Campylobacter* (121, 123) drives the production of IL1β, IL6, and IL8, presumably through NF-κB. Other genes in this region whose products have a role in signaling are *PP4R2* (serine/threonine-protein phosphatase 4 regulatory subunit 2); *RYBP* (RING1 and YY1 binding protein), a repressor protein for transcription factors; and *LRIG1* (leucine-rich repeats and immunoglobulin-like domains 1), which interacts with receptor tyrosine kinases of the EGFR family. Caveolin 3 (*CAV3*) plays a crucial role in endocytosis but also provides a scaffold for signaling molecules. Leiomodin 3 (*LMOD3*) is an actin filament nucleator. Finally, *GHRL* encodes the peptide hormones ghrelin and obestatin, which in mammals are thought to be produced in the cells lining the stomach and small intestine (132). Though the influence of obestatin on *Campylobacter* is unknown, the stress-related catecholamine hormone noradrenaline activates growth, motility, and invasion by *C. jejuni* (133), and it is plausible that other examples of such interkingdom signaling exist. Polymorphisms in any of the genes identified could influence susceptibility to colonization by either *Salmonella* or *Campylobacter*.

Li et al. (134–136) used the complementary approach of whole-genome gene expression analysis by microarray to compare responses to *Campylobacter* infection between two parental commercial lines of chickens that also differ in resistance to *Campylobacter* colonization (137). They demonstrated differential responses between the lines and between infected and noninfected birds. In the spleen, more genes were differentially expressed in response to infection in the resistant line than in the susceptible line. Specifically, genes for lymphocyte activation, differentiation, and humoral responses were upregulated in the resistant line, whereas genes for regulation of erythrocyte differentiation, hemopoiesis, and RNA biosynthesis were all downregulated in the susceptible line. An interaction analysis between genetic lines and treatment demonstrated distinct defense mechanisms between lines: the resistant line upregulated genes involved in apoptosis and cytochrome c release from mitochondria, whereas the susceptible line responded by down-regulating genes involved in both functions (137).

## CONCLUDING THOUGHTS: CHALLENGES AND OPPORTUNITIES

Steady progress clearly has been made in identifying the genetic determinants of resistance for several poultry diseases as well as in gaining a greater understanding of the underlying biology. This progress will continue using the methods mentioned, as well as new ones, which are difficult to predict though they are usually adopted readily and have major impact. Of particular interest is the implementation of genomic selection, in which genetic markers evenly spaced throughout the genome are able to capture the majority of the genetic variation for complex traits (138). Owing to its promise, all major poultry-breeding companies are evaluating this new and exciting method, which is equal, if not superior, to best linear unbiased prediction, the current state-of-the-art method for breeding (139, 140; H.H. Cheng, unpublished data). Although selection for enhanced disease resistance is one of the most discussed advantages for genomic selection, many scientific (e.g., analysis) and logistical (e.g., SNP chip cost) problems must be resolved before it can be implemented on a routine basis.

It is also becoming apparent that biology is much more complex than long thought. Fields such as epigenetics, RNA modification, alternative splicing, and microRNAs indicate that complete biological knowledge will require in-depth information at multiple levels. Thus, efforts must be made to annotate the chicken genome at a level similar to that of human and other model organisms (141). Similarly, the interplay of nutrition and gut microbes is becoming increasingly important in understanding not only gut function but also overall host immune responses (142). These metagenomic studies offer the potential to identify specific determinants of environmental factors for complex traits, may partially explain the growth-promoting action of antibiotics, and provide new and fertile avenues for enhancing disease resistance.

In closing, it is an extremely exciting period for biologists. New findings and technologies are bringing closer the ever-elusive goal of bridging from genotype to phenotype. Ultimately, combining existing and new technologies should allow for precise and economical genetic selection of poultry for enhanced disease resistance, which will lead to enhanced animal welfare, productivity, and food safety.

### SUMMARY POINTS

- The poultry industry needs to enhance genetic resistance for infectious diseases to enhance animal health and welfare, for profitability, to maintain consumer confidence, and as a means to augment current animal husbandry and vaccines.
- With the chicken genome sequence and powerful tools (e.g., high-density SNP chips, next-generation sequencing), the field of genomics can identify genes and genetic markers that can be used to increase genetic resistance or improve vaccinal response to specific pathogens.
- Owing to the difficulty of identifying specific genes that account for disease resistance using QTL-mapping efforts alone, integrated genomic approaches (e.g., transcript profiling to provide candidate genes for use in association studies) are required.
- Owing to its simplicity and power, allele-specific expression (ASE) screening is one of the more promising approaches to identify candidate genes. It is especially amenable for disease resistance studies because it compares RNA derived from two states only (uninfected versus infected birds).
- Integrated efforts to identify disease resistance for Marek's disease, *Salmonella*, and *Campylobacter* have successfully identified many genes and other strong candidates as well as provided information on the biological pathways involved in the response to the pathogens.

## FUTURE ISSUES

- If breeding values of markers for disease resistance are determined, then genomic selection of unchallenged individuals using markers spaced throughout the genome will become an attractive method for poultry-breeding companies to select for enhanced disease resistance.
- Given the power and precision of next-generation sequencing, enormous data sets will be produced that will provide increasing insights into complex traits such as disease resistance. However, there will be significant bioinformatic challenges to properly handling and analyzing the data.
- To address the increasing complexity of disease resistance, the majority of chicken genes must be experimentally annotated using functional data from chickens.
- New technologies, like those developed in the past (e.g., PCR, automated sequencing, microarrays), will continue to play important but unpredictable roles in genomics.
- As always, phenotype remains key and is often the rate-limited step. Many disease problems in the poultry industry are not caused by single pathogens but rather are syndromes caused by multiple pathogens and exacerbated by stress or nutritional challenges. Defining which phenotypes to measure in these complex challenges to quantify disease resistance will require a deeper understanding of their pathology.



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