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Application of DNA Marker Technology and Functional Genomics to the Development of Coccidiosis Control Strategy

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INTRODUCTION

Tremendous success in the improvement of commercial chicken production traits including growth, reproduction, and feed efficiency has been accomplished using classical genetic breeding techniques. However, selection of commercial poultry stocks for improved disease resistance using similar breeding techniques has been unsuccessful due to technical difficulties (Gavora, 1990). It is impossible to measure disease-resistant phenotypes without introducing pathogens into chickens, an impractical procedure with significant negative effects on poultry production. Although selection, based on progeny tests may be used to avoid this negative impact, as demonstrated by selection of broiler strains with enhanced antibody responsiveness to *Salmonella enteritidis* (Kaiser et al., 1998), this is a labor-intensive, time consuming and a costly approach. Moreover, lack of a clear understanding of the mechanisms of protective immunity against most avian diseases makes genetic selection of stocks with enhanced disease resistance very difficult. For example, chickens infected with *Eimeria*, the etiologic agent of coccidiosis, mount both humoral and cell mediated immunities, but it is presently unclear whether one or both mechanisms are responsible for protection against reinfection (Lillehoj et al., 1999).

DNA marker technology avoids many of these problems, making it easier to select animals with superior performance for resistance to particular diseases of commercial importance. In the DNA marker approach, phenotypic traits for disease resistance are measured in genetically diverse animals challenged with the pathogen of interest. DNA marker(s) associated with disease resistance are identified in particular genotypes and this information is subsequently used for marker assisted selection (MAS) of breeding stocks. Because of increasing concerns over the drug resistance of coccidia parasites, providing poultry breeders with DNA marker information for MAS to improve resistance to coccidiosis in commercial broiler chickens is urgently needed. This led us to apply DNA marker technology to develop MAS for avian coccidiosis. We hypothesize that chicken genes influencing resistance to coccidiosis can be identified by one or more approaches using DNA marker technology.

DNA marker technology has revolutionized genetic research (Dodgson et al., 1997). DNA markers are classified into two types. Type I markers are designed from sequences of genes with known functions, e.g. genes of the major histocompatibility complex (MHC), whereas type II markers are developed from DNA sequences with unknown functions. Both types usually contain repeated sequences (O'Brian, 1991) and they are based on DNA polymorphisms that can be classified into 4 major types (Dietrich et al., 1998): (1) single nucleotide polymorphisms (SNPs) due to substitutions, deletions or insertions, (2) restriction fragment length polymorphisms (RFLPs), a subtype of SNPs with alteration of restriction enzyme sites and resulting in differences in DNA fragment length after restriction enzyme digestion, (3) random amplified polymorphic DNA (RAPD) produced by polymerase chain reaction (PCR) using randomly selected short oligonucleotide primers, and (4) tandem repeated DNA sequences differing in the number of repeated units.

The most common tandem repeated sequences are simple tandem repeats (STR), e.g., AC dinucleotide repeats, also called microsatellite sequences.

SNPs are the most abundant form of genetic variation in the human genome, comprising 90% of all known polymorphisms (Collins et al., 1998). The density of SNPs is estimated to be approximately 1 per 1,000 base pairs. SNP markers are considered as third generation markers (McKenzie et al., 1998). Although SNPs are mostly bi-allelic and less informative than microsatellite markers, they are more frequent and stable in terms of mutation. Because SNP markers are usually designed based upon genes with known sequences, results obtained from genetic mapping using SNP markers are more meaningful than those obtained using microsatellite markers. SNPs have been used in linkage mapping studies in bovine (Grosse et al., 1999; Lagziel and Soller, 1999) and avian species (Smith et al, 2000). Microsatellite markers are the most widely used markers in genetic mapping because the repeated sequences are highly polymorphic and widely distributed over the genome (Stallings et al., 1991). These markers are co-dominate, multi-allelic and easily detected by PCR, characteristics useful for genetic mapping within pedigrees (Dodgson et al., 1997). The disadvantage of microsatellite markers is their high mutation rate that makes them less useful for mapping loci by linkage disequilibrium (Callen et al., 1993). RAPD markers are dominant and unstable (Dietrich et al., 1998) and therefore rarely used for genetic mapping. RFLPs are relatively rare and the procedure to detect them is tedious and costly. A modified RFLP procedure, amplified fragment length polymorphism (AFLP), uses PCR to reduce the cost and work required for typing (Vos et al., 1995). All of these genetic polymorphisms are usually resolved by gel electrophoresis.

Most of the economically important traits (quantitative traits) of food animals are regulated by multiple genes that manifest different effects and are continuously distributed in the population. The loci affecting these traits are referred to as quantitative trait loci (QTL) (Lynch and Walsh, 1998). With DNA marker technology and statistical methodology, it is possible to map QTL on chromosomes. DNA marker-based methods have had a significant impact on both gene mapping and animal breeding (Dodgson et al., 1997). Genetic mapping using DNA markers that cover the entire genome, with defined intervals between the markers is called whole genome scanning. Candidate genes that potentially affect traits of interest and are positively correlated with QTL can thus be mapped on the genome.

To map QTL efficiently, a linkage map with high marker density is required. Bumstead and Palyga (1992) reported the first DNA marker linkage map of the chicken genome. Currently, more than 1,800 DNA-based genetic markers are available for chicken genotyping (Groenen et al., 2000). A large number of these markers have been mapped to chicken linkage groups (Levin et al., 1993, 1994; Crooijmans et al., 1994, 1995, 1996b; Cheng et al., 1995, 1997; Groenen et al., 1998, 2000). The current chicken linkage map covers more than 95% of the entire genome and provides sufficient marker density for QTL mapping with an average marker interval of less than 20 cM (Groenen et al., 1998). QTL affecting animal growth (Groenen et al., 1997; Van Kaam et al., 1998), feed efficiency (Van Kaam et al., 1999a), carcass traits (Van Kaam et al., 1999b), and resistance to salmonellosis (Hu et al., 1997) or Marek's disease (Vallejo et al., 1997; Xu et al., 1998; Yonash et al., 1999) have been reported.

While both inbred and outbred chickens have been used for these QTL mapping studies, there are several disadvantages of using animals from outbred populations under long-term selection (Ver der Beek, et al., 1995; Lynch and Walsh, 1998). First, loci affecting traits under selection may be present at extremely low frequencies within the population, thus greatly reducing the information content of both markers and QTL. Selection also decreases phenotypic differences between genotypes because alleles with undesirable effects may be reduced in frequency or eliminated during long-term selection. Second, because the linkage phase between QTL and DNA marker may vary

from family to family, the association between QTL and DNA marker must be analyzed separately for each family. Third, statistical analysis is more extensive with outbred populations since up to four independently segregating alleles may exist at the QTL and at the marker. Lastly, the effects of QTL in outbred populations are estimated from genetic variances and they are thus less precise than those using analysis of means in inbred populations. All of these disadvantages result in a significant reduction of QTL detection power. While use of inbred populations is thus more desirable, QTL mapping in outbred populations cannot be completely substituted by inbred populations. QTL detected using inbred line crosses are usually based on fixed differences between lines and their relevance to QTL that segregate within outbred populations is unclear (Lynch and Walsh, 1998). From a practical breeding perspective, such as MAS, the detected QTL in crosses between inbred lines may not be segregating in current commercial populations. Careful considerations of experimental designs for QTL mapping experiments can overcome these disadvantages.

In addition to those listed above, additional factors affect the detection power of QTL mapping (Liu et al, 1996). Trait-associated factors, such as the number and genomic locations of genes affecting the traits, the distribution of gene effects and interactions, and trait heritability are not controllable. Methodology-associated factors, such as the marker density of linkage maps and extent of genomic coverage, as well as statistical methodology could improve QTL mapping as new markers and methods are developed. In contrast, factors such as population types and sampling size may be artificially manipulated to enhance the detection power of QTL mapping.

In general, larger population sizes increase QTL detection power. Two methods have been used to increase population size. Increasing the number of families increases the chance of having at least one informative sibship for every locus (Lynch and Walsh, 1998), while increasing the number of individual family members will create more QTL mapping power (Van der Beek et al., 1995). Unfortunately, the number of samples that can be included in an experiment is usually limited. Knowing the heterozygosity and polymorphism information content (PIC) of test populations is helpful in balancing the number of families and family sizes for QTL mapping experiments. Both heterozygosity and PIC are important considerations for avian genetic mapping given the relatively low heterozygosity of current poultry breeding stocks (Crooijmans et al., 1996a). Van der Beek et al. (1995) proposed 5 family structures, based on full-sib (FS) or half-sib (HS) matings and multiple generations for QTL mapping in outbred populations. According to these authors, FS is more efficient than HS, and FS family structures are considered to be more powerful than HS designs when dominant effects exist (Lynch and Walsh, 1998). It is relatively easier to obtain large FS families from chickens than HS families.

The power of detecting QTL is also dependent on marker density. Higher marker density provides higher detection power until limited by sample size. Shugart and Goldgar (1999) demonstrated by computer simulation that QTL detection power increased as marker density increased. Groenen et al. (1998) suggested that with 100% genome coverage, the preferred distance between adjacent markers is 20 cM or less to map loci affecting quantitative traits in initial genetic mapping studies. Given the size of the chicken genome, approximately 200 evenly spaced markers are needed to cover the entire genome. The distance (m) of 20 cM is equal to 0.165 of the recombination fraction (r) according to Haldane's mapping function, $r = 0.5\ (1 - e^{-2m})$. The power of linkage analysis decreases rapidly when r is greater than 0.3 (Risch, 1991). The effect of r on the analysis power is greater when variances of QTL genotypes are heteroscedastic (Luo et al., 1997). By computer simulation, a 50 cM marker interval was found to be optimal or close to optimum for initial studies in a variety of experimental designs, if experimental cost is a limiting factor (Darvasi and Soller, 1994).

The magnitude of differences between alleles at QTL is another important factor influencing detection power. The larger the differences in allelic effects, the

greater chance to detect the QTL. A common approach used in food animals is recurrent selection to establish two lines or populations that differ widely in the trait(s) of interest. These diverged populations are then crossed to produce F_1, F_2, and backcross populations for mapping studies. The initial selection process can be carried to an extreme (genetic fixation) or near extreme (highly inbred) before crossing to produce the F_1. Linkage disequilibrium between markers and genes affecting traits within families serves as a basis for genetic linkage analysis (Soller and Andersson, 1998). The range of linkage disequilibrium can be up to 20 cM. Diversity in traits may exist not only in those that have undergone long-term artificial selection, but also unintended (nonselected) traits. The diversity in nonselected traits is probably due to genetic association between selected and nonselected traits. For example, in the commercial broiler chickens used in our study, the marker MCW0058 affecting animal growth (selected trait) is 20 cM from the marker LEI0101 affecting coccidiosis resistance (non-selected trait) (see preliminary results).

Statistical analysis of the association between quantitative traits and marker loci is the most complicated step in QTL mapping. Several statistical methods have been developed for QTL detection. Hoeschele et al. (1997) classified these methods into 6 groups: (1) linear regression, (2) maximum likelihood, (3) sib-pair regression analysis, (4) residual or restricted maximum likelihood analysis, (5) exact Bayesian linkage analysis, and (6) approximate Bayesian analysis. Two other approaches are nonparametric mapping (Kruglyak and Lander, 1995) and nonlinear regression (Liu, 1998). These methods can also be grouped into 4 types based on the number of markers included in the analysis: (1) single-marker analysis (Lynch and Walsh, 1998), (2) interval mapping (Lander and Botstein, 1989), (3) composite interval mapping (CIM) (Zeng, 1993), and (4) multiple QTL mapping (MQM) (Jansen, 1993). Single-marker analysis is less powerful than the other methods because QTL genotypic means and positions are confounded and QTL position cannot be precisely determined if the QTL are not located at marker loci. Interval mapping can estimate the QTL location, but it is still subjected to interference by QTL located at other regions of the same chromosome. CIM and MQM include more than one putative QTL and they are less biased.

A large number of computer software packages are available for the analysis of QTL mapping, but most are designed for inbred populations (Manly and Olson, 1999). One commercial program, MapQTL (Van Ooijen and Maliepaad, 1996) is capable of handling data collected from outbred populations. This software can perform nonparametric mapping, interval mapping and MQM. Testing versions of RandomQTL for sib-pair regression (Xu and Atchley, 1995; Xu, personal communication) and REML and Bayesian (Hoeschele et al., 1997) analyses are also available for QTL mapping of outbred populations. In addition, several powerful and well tested software programs originally developed for human genetic studies, such as multiple interval mapping (MIM) (Goldgar, 1990), MAPMAKER/SIBS (Kruglyak and Lander, 1995), and sequential oligogenic linkage analysis (SOLAR) (Almasy and Blangero, 1998) can be used to analyze data from outbred animal populations.

MAPMAKER/SIBS is claimed to be the most powerful QTL mapping software. This program takes full advantage of complete multiple marker loci to compute alleles shared identical by descent (IBD) by sib-pairs. The program is based primarily on the sib-pair approach of Haseman and Elston (1972). The disadvantage of this approach is intensive computation that allows a maximum number of 8 sibs per family, which limits its application in poultry populations. In addition to the relative-pair-based approach, MIM and SOLAR use variance-component linkage analysis methods that can provide reasonable estimates of the magnitude of the effect of detected loci (Almasy and Blangero, 1998). It has been stated that MIM is more powerful than MAPMAKER/SIBS based on computer simulation (Shurgart and Goldgar, 1999). Multipoint linkage analysis, combining variance components increases the ability to locate true linkages and decreases false-positives. MIM is used for pairs

between sibs and allows implementation of difference in linkage maps between sexes. SOLAR extends this capacity to general pedigrees and allows construction of different null hypotheses (models), including sex, hatch, interaction and environmental factors as covariant factors, to test associations between phenotypic variance and marker genotypes. The implementation can greatly increase sensitivity to detect true linkages.

An important component of statistical analysis of the association between marker genotypes and phenotypes is the probability of false positives (α. Although α = 0.05 is a widely acceptable error rate, α = 0.25 may also be acceptable for exploratory QTL experiments (Beavis, 1997). However, these error rates may not be appropriate for determination of significance of differences. Lander and Kruglyak (1995) classified significant levels into two kinds, pointwise and genome-wide. A simulation based on sib-pairs in humans showed that the rate of false positives would be about 1 in 24 in a whole genome scan if a pointwise significant level of 0.05 was used. For α = 0.05 in a whole genome scan, the corresponding P value should be 2 $\times$ 10^{-5} with pointwise analysis in a given experiment. Because this threshold of significance depends on several factors based on experimental designs, permutation has been proposed to set an empirical threshold for each specific experiment (Churchill and Doerge, 1994). In addition, the probability of false positives will be higher if more than one statistical method is used.

The chicken genome comprises 39 pairs of chromosomes, 8 pairs of cytologically distinct chromosomes, one pair of sex chromosomes (Z and W), and 30 pairs of small, cytologically indistinguishable microchromosomes. The size of chicken genome is estimated to be 1.2 billion base pairs (Bloom et al., 1993) and approximately 3,500 to 4,000 cM in genetic length. Therefore, 1 cM is equivalent to approximately 350 kb. There are several high capacity vectors available to clone chicken genomic DNA. These include cosmids (maximum insert size = 30~45 kb), bacteriophage P1 (70~100 kb), P1 artificial chromosomes (PAC, 130~150 kb), bacterial artificial chromosomes (BAC, 120~300 kb), and yeast artificial chromosomes (YAC, 250~400 kb). Among these, BAC is the most attractive vector because it is stable, capable to propagate very large DNA fragments, and easy to manipulate (Sambrook and Russell, 2000).

Recently, two chicken genomic DNA libraries were constructed at the Texas A & M BAC center (http://HBZ.TAMU.EDU/bacindex.html). Both were derived from the Red Jungle Fowl (UCD 001) with the intention of maximizing genetic heterogeneity in expressed clones. The first library was derived from *Hind*III-digested genomic DNA and inserted into the BAC vector pECBAC1. It contains 49,920 clones representing 5.4-times genomic coverage. The average insert size for this library was estimated to be 130 kb. The second library was created from *Bam*HI partial digests of UCD 001 genomic DNA cloned into pBeloBAC11. Its average insert size was estimated to be 150 kb. This library is also maintained at the University of Michigan by Dr. Jerry Dodgson (Coordinator for NAGRP/NRSP-8). For the studies proposed here, both libraries will be used to construct BAC clone contigs covering the chromosomal region of interest.

In addition to DNA marker and cloning technologies, DNA microarray is another revolutionary tool for genomic study of interested traits. By immobilizing thousands of DNA sequences in individual spots on a solid phase, DNA microarray allows simultaneous analysis of a large number of genes in a single step, thereby identifying genes whose expression levels are altered during natural biological processes or experimental treatments or vary due to genetic differences (Eisen and Brown, 1999). In one approach, the sample of interest, such as mRNA isolated from a certain tissue, is used to synthesize cDNA labeled with colored substances (e.g. fluorescent dyes). The labeled cDNA probe is then hybridized to the array and a post-hybridization image is developed. The color density of individual nucleic acid species reflects the relative amount of labeled cDNA hybridized to the DNA immobilized at the known position of the array. By comparing samples tested in well-controlled conditions, change of expression levels of individual genes

can be detected. The DNA sequences immobilized on an array are usually produced by PCR from genes whose sequences are partially or completely known. This technique has been widely used to detect gene mutations and polymorphisms, gene expression profiling, genetic linkage, sequence analysis, and SNP-based tests (McKenzie et al., 1998). While only a small number of chicken genes have been cloned and completely sequenced, more than 5,000 chicken EST from mitogen-activated chicken T cell and macrophage cDNA libraries (Tirunagaru et al., 2000) are currently available for designing DNA microarrays. In addition, more EST sequences will soon be available from Dr. Burnside's and our own laboratory.

Microarray hardware and biochemistry are no longer major challenges with this new technology. Currently, microarray equipment can reliably produce and image more than 10,000 spots on a single microscope slide and three-color fluorescence detection will soon be available (Zhou et al., 2000). However, analyzing microarray data using bioinformatics presents a great challenge due to the large data size and complexity required for precise spot detection. A database infrastructure and sophisticated software are needed to fully analyze the data. Quality control to ensure the reliability of the data analysis is an important component of this process since conclusions based on unreliable data may be misleading.

Reliability of DNA microarray data is defined by reproducibility and accuracy (Bittner et al., 2000). Reproducibility can be accessed by calculating the coefficient of variance between experimental replicates. For example, a ratio of 1:1 in fluorescence intensity from multiple spots printed with identical DNA is expected. Duplicate spots can be used to set confidence intervals to 95-99% as a threshold for establishing differential gene expression. In general, a difference of greater than 2-fold in fluorescence intensity is an acceptable threshold. On the other hand, accuracy of microarray data can be measured by comparing ratios obtained from arrays with those obtained by an independent method. For example, Northern blotting or RT-PCR can be used to confirm genes with differential expression detected by DNA microarray.

Several methods exist to quantify microarray signals and the best method to use is often based on how well each measurement correlates with the amount of DNA probe hybridized to each printed spot. Quantitation can be based on the following signal parameters: total (sum of intensity values of all pixels in a spotted area), mean, median, mode (most likely intensity value), volume (difference between signal mean and background multiplied by signal area), intensity ratio of two colors, or correlation ratio (a ratio between the pixels in two channels by fitting a straight line through a scatter plot of intensities of individual pixels) (Zhou et al., 2000). The best method for a particular experimental design can be determined by analysis of duplicate experiments.

Data normalization and transformation are other important processes to improve the quality of array data (Zhou et al., 2000). Normalization removes differences due to experimental variation and can be achieved by one of the following methods: (1) two color labeling, e.g. with Cy5 and Cy3 for tagging DNA probes against control and treated samples, followed by reversal of the labels, (2) use of an internal control such as a known DNA sequence and its corresponding labeled cDNA probe from distantly related species, or (3) standardization of the data to truly reflect expression values. This step is necessary because of the complexity in manufacturing, DNA labeling, hybridization, scanning, and quantifying microarrays. Transformation, on the other hand, is used to change the variance and distribution property of the data to meet the assumption of statistical analysis, such as homoscedasticity (homologous variance) and normality (normal distribution). Log transformation is commonly used to meet the assumptions.

After normalization, microarray data can be analyzed in several ways dependent on the complexity of the experimental design (Zhou et al., 2000). A scatter plot is the simplest visual analysis method to compare data from two samples. Plotting genes with differential expression will result in them falling outside of the

confidence interval for identity. Principal component analysis is used to reduce complexity (comparisons among more than three samples) to two or three dimensions. For samples collected in time series, parallel coordinate planes can be applied to visualize expression difference corresponding to sampling time. Another commonly used method is cluster analysis to order data by grouping genes with similar expression patterns close to each other, which is useful to predict the functionality of unknown genes. All these analysis methods have been implemented in commercial software. Differences in gene expression detected by DNA microarray have been demonstrated to be highly correlative with the results of Northern blot analysis (Bittner et al., 2000). Once genes with differential expression are identified, they can serve as candidate genes for genetic studies using the SNP technique.

Avian coccidiosis is a major disease costing the U.S. poultry industry over $700 million annually. Avian coccidiosis is caused by several species of *Eimeria* that normally cause an acute and self-limiting disease (Lillehoj and Trout, 1993). *Eimeria* infects the chicken intestinal tract resulting in diarrhea, malabsorption of nutrients, and retardation of growth. Infective parasites are transmitted between animals by oocysts shed in the feces and consumed from the litter by feeding animals. While natural infection with *Eimeria* induces immunity, vaccination procedures on a commercial scale have proven ineffective and disease control is mainly based on prophylactic use of anti-coccidial drugs. Examination of the patterns of disease resistance following experimental infection has suggested 2 separate mechanisms of genetic control of protective immunity to coccidiosis, an innate mechanism following primary infection and acquired immunity following secondary infection (Lillehoj, 1991; Lillehoj et al., 1999). In immune hosts, parasites enter the gut early after infection but are prevented from further development indicating that acquired immunity to coccidiosis may involve mechanisms that inhibit the natural progression of parasite development (Lillehoj and Trout, 1994; Trout and Lillehoj, 1996). Although a direct role of immune effector lymphocytes in inhibiting parasite development has not been proven, $CD8^+$ cytotoxic T cells and interferon-γ (IFN-γ) have been identified as important components of host protection (Lillehoj and Trout, 1996; Lillehoj, 1998). At the genetic level, both MHC-linked genes and non-MHC genes have been implicated in controlling host immune responses to coccidiosis (Lillehoj et al., 1989) although no QTL affecting disease resistance to this disease have been described.

RECENT PROGRESS IN GENOME MAPPING FOR AVIAN COCCIDIOSIS DISEASE RESISTANCE GENE

1. Phenotypic Variations in Immune Response and Disease Resistance among 3 Commercial Broiler Chicken Lines

A local commercial chicken breeder was chosen as our research population because avian coccidiosis and MD are the two major infectious diseases affecting the local poultry industry. The strain of *Eimeria maxima* used in this study was isolated from the Eastern Shore. Chickens from three broiler breeding lines, provided by an Eastern Shore poultry producer, were separately-challenged with the very virulent Marek's disease virus *RB1B* or *E. maxima* oocysts to evaluate their immune responses and disease resistance (Table 1). After MD challenge, mortality and gross lesions were observed to 8 weeks post-challenge. The incidence of MD tumors is reported as the percentage of total birds at the time of challenge. During necropsy, it was noted that Line 3 birds had a higher incidence of proventriculitis. The cause of this inflammation is not known. Chickens from these lines were also injected intramuscularly with 0.5 ml of a 10% suspension of sheep red blood cells (SRBC) at 3 and 4 weeks of age to evaluate general antibody response. Antibody titers were determined using a microhemagglutination assay and are expressed as the mean log2 titer. In summary, variations in host immune responses to MD virus, *E. maxima*, and SRBC were apparent among these 3 lines. Line 3 was identified as the most susceptible to

Table 1. Variations in immune response and disease among 3 commercial chicken lines

Characteristic	Line 1	Line 2	Line 3
Incidence of Marek's disease tumors	8.0 %	8.3 %	33.3 %
Incidence of proventriculitis	−	−	++++
Mortality due to omphalitis, airsacculitis	10%	16%	5%
Primary antibody response to SRBC	2.5 ± 1.3	2.2 ± 1.5	1.2 ± 1.1
Secondary antibody response to SRBC	4.4 ± 0.9	4.2 ± 1.1	3.9 ± 0.8
Oocyst shedding after *E. maxima* infection	2.5×10^6	1.7×10^6	3.3×10^6

coccidiosis and MD and gave the lowest antibody titers to SRBC. To increase phenotypic variation in F_2, Line 3 was crossed to Line 1 and the F_1 chickens were intercrossed to produce F_2 chickens for the QTL mapping experiments.

2. Sex and Dose Effects and Analysis of *Eimeria maxima* Disease Resistance Phenotypic Parameters in Broiler Chickens

To determine the optimal dose for coccidia parasite inoculation and to evaluate genetic resistance or susceptibility in individual chickens, broilers were given 4 different doses of *E. maxima* oocysts. Body weight gain, fecal oocyst shedding, plasma NO_2^- + NO_3^-, plasma carotenoid, and plasma IFN−γ concentrations were measured. The results showed significant dose and sex effects on most parameters tested, as well as interaction between dose and sex in some parameters. Dose effects were mostly linear and quadratic fashions were also observed on some measurements. Measurements from chickens inoculated with 10^4 oocysts displayed the highest correlation coefficients among oocyst shedding, body weight gain, carotenoid, and NO_2^- + NO_3^- concentrations (Zhu et al., 2000).

Three hundred and twenty four F_2 offspring were produced from 12 families for mapping QTL affecting resistance to coccidiosis. These offspring were challenged at ARS facilities with the optimal dose of *E. maxima* oocysts determined above and the five disease−resistance−associated parameters measured. We consider that oocyst shedding is the best parameter to indicate status of resistance or susceptibility to avian coccidiosis. This parameter has been used to measure resistance to coccidiosis in mice. The trait is unique and makes biological sense. This disease reaches its acute phase at day 6 post−infection. Body weight gain in females between days 6 and 9 post−infection (BWG69), i.e. after the acute phase of the disease, showed the highest correlation with oocyst shedding compared with BWG09 and BWG39 (Table 2). When BWG69 was transformed to growth rate (GR69), this parameter displayed a slight increase in the correlation coefficient with oocyst shedding compared to BWG69. These results indicated that oocyst shedding reflects the impact of the disease in females. However, this result was not observed in males. Because larger (male) birds tend to gain more weight than smaller (female) birds, body weight gain of males is much greater than females and the decreased growth in males caused by the disease may be too small compared to overall growth. In contrast, oocyst shedding and plasma carotenoid concentration were significantly correlated in both males and females. Plasma carotenoid concentration was shown to be a more sensitive measurement in avian coccidiosis than

Table 2. Correlation coefficients between oocyst shedding and 5 coccidiosis−associated traits

Trait	Females	Males
BW0	0.07	0.01
BWG09	−0.25	−0.06
BWG39	−0.43*	−0.18
BWG69	−0.57*	−0.16
GR69	−0.62*	−0.16
Carotenoid Cone.	0.57*	0.64*
NO_2 + NO_3^-	0.22	0.02
INF−γ	0.16	0.11

* Statistically significant ($P < 0.05$).

body weight gain (Conway et al., 1990). This parameter has been used as an indicator of intestinal lesion that affects absorption.

3. QTL Mapping of Coccidiosis using Full-sib Families

Seventy-five microsatellite DNA markers were selected based on the consensus chicken genetic linkage map (Groenen et al., 2000) and availability of DNA markers in 4 chicken microsatellite kits provided by the National Animal Genome Research Program. The primary goal of this experiment was to cover the entire chicken genome with 50cM marker intervals to balance experimental cost with detection power. Forty three additional markers then were selected for genotyping in chromosomal areas that displayed potential association between genotype and phenotype, covering 20-30 cM intervals at these regions. Genotypic data collected from F_1 and F_2 chickens of 12 full-sib families were analyzed with CRIMAP version 2.4 (Green et al., 1990) to test the agreement between the linkage map of these families and the chicken consensus map. A minimum LOD ($\log_{10}$ of odds) score of 3.00 was used as the statistically significant threshold for declaring linkage. The linkage results observed were very similar to the chicken consensus genetic linkage map.

QTL analysis was conducted using SOLAR based on the genotypes of the F_1 and F_2 generations and the phenotypes of the F_2 progeny. Hatch and sex factors were statistically significant and were included as covariates in testing the null hypothesis. A LOD of 3.00 was used as the threshold of significance as suggested for genomewise scan at a 10 cM marker interval (Lander and Kruglyak, 1995). A QTL near marker, LEI0101 on chromosome 1 significantly affected oocyst shedding (i.e. increased disease resistance) with a LOD score of 3.14 (Figure 1). The heritability (h_q^2) at this locus was 0.39. Interestingly, a QTL associated with viremia in chickens challenged with MD virus also was mapped near the same marker locus on chromosome 1 (Yonash et al., 1999). In addition, 3 potential loci affecting chicken growth were

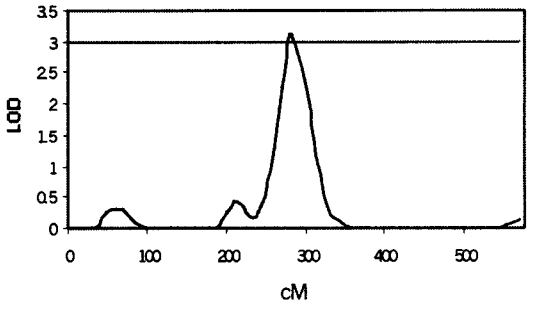

Fig. 1. A locus near marker LE10101 on chromosome 1 shows significant association with reduced oocyst shedding

identified, MCW0058 (LOD = 2.20, h_q^2 = 0.37) and MCW0020 (LOD = 1.84, h_q^2 = 0.30) on chromosome 1, and ADL0142 (LOD = 2.43, h_q^2 = 0.26) on chromosome 6. The MCW0058 marker was previously reported to be significantly associated with chicken growth (Van Kaam et al., 1998). The MCW0058 and LEI0101 markers are 20 cM apart.

4. Expressed Sequence Tags (EST) of a Chicken Intestinal cDNA Library

A supplemental approach to identify chicken genes controlling resistance to coccidiosis will use EST clones analyzed by Southern hybridization on DNA microarray chips. We have established the validity of this approach using a normalized chicken intestinal cDNA library constructed in our laboratory through a service provided by Life Technologies, Inc. (Rockville, MD). The library was prepared from intestinal epithelial cells and lymphocytes at 0, 1, 2, 3, and 4 days post-infection with *Eimeria*. According to the normalization control, the redundancy in this library has been reduced by 37-fold. This library contains 1.87×10^7 transformants with an average insert size of 1.56 kb. Twenty thousand colonies have been robot-picked and Dr. Ashwell (ARS, Beltsville Agricultural Research Center) is in the process of sequencing the clones with an ABI 3700 DNA sequencer. Currently, 6,144 clones have been sequenced. We estimate that 10,000 clones will be sequenced by March 2001 and 20,000 clones sequenced within one year. Based on the sequence information to date, 80% of the clones display high

quality inserts, 50% are unique based on a BLAST search, and redundancy is 25%. Sequence data will be stored using MySQL database server on a Linux 7.0 Dell Server. NCBI BLAST will be installed in the server to perform automatic annotation. The entire system is currently being custom developed by Incogen, Inc. (Clemson, SC). For the experiments proposed below, identified unique cDNA inserts will be used in DNA microarray analysis after annotation.

5. Microarray Analysis of Gene Expression in Non-infected and *Eimeria* infected Chickens

Four hundred fifty clones encoding potential immune response associated genes were prepared in our laboratory and obtained from Drs. Burnside and Keeler (University of Deleware). Arrays were printed on CMT-GAPS coated glass microscope slides (Corning, Rochester, NY). Cy3 and Cy5 labeled cDNA probes were prepared from total RNA isolated from intestinal epithelial cells and lymphocytes of non-infected (control) and *Eimeria* infected SC inbred chickens at days 1, 2, 3, and 4 post-infection. RNA from non-infected chickens was used to make Cy3 labeled cDNA and cDNA from infected chickens was labeled with Cy5. The DNA on separate microarray slides was hybridized to control cDNA mixed with cDNA prepared from one of the 4 days post-infection. Image analysis of normalized scans with ScanAlyze2 (University of Stanford, Stanford, CA) identified a gene at day 1 post-infection and 14 genes at day 2 post-infection whose expressions were increased greater than 2-fold following *Eimeria* infection (Fig. 2). More than half of these genes retained 2-fold or greater increase in expression at day 4 post-infection, including the gene identified at day 1. These data will be further analyzed with a database established using MySQL. Once identified, these genes represent exciting candidates as potential effectors of host immunity to coccidiosis.

CONCLUSIONS AND FUTURE PROSPECT

Chicken meat is a major protein source in the American diet with *per capita* consumption of 77 pounds in 1999 (Delmarva Poultry Industry, http://www.dpichickn.org). By comparison, *per capita* consumption of beef and pork for the same year was 66.1 and 52.3 pounds, respectively. Poultry meat also represents a major U.S. export particularly to developing countries where increasing meat consumption parallels national economic growth. Thus, improving the efficiency of U.S. poultry production will have a positive impact on our national broiler industry profitability and trade competitiveness in international markets both now and in the future.

Infectious diseases are one of the greatest threats to the viability of the food animal industry. Commercial broilers, in particular, have a higher risk of acquiring contagious diseases than other sectors of the food industry due to intensive collective farming practices (Gavora, 1990). Coccidiosis is a poultry disease of

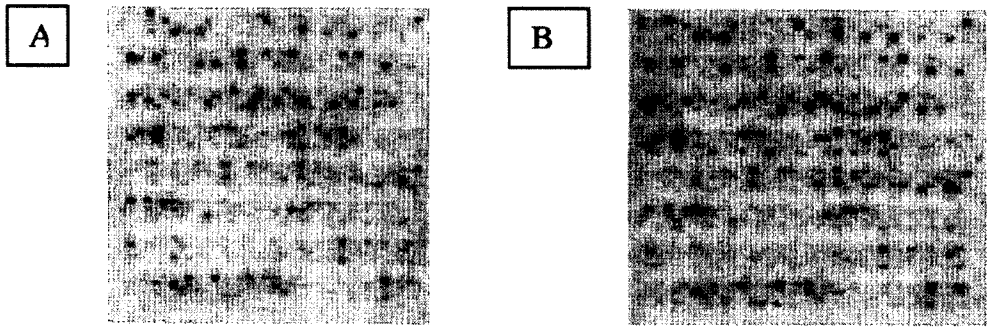

Fig. 2. High-throughput gene profiling to identify gene expression changes following coccidiosis. EST clones were hybridized with RNA from normal (A) and *Eimeria*-infected intestines (B).

substantial economic importance, estimated to cost the U.S. industry greater than $700 million annually. In the absence of efficient vaccines to control this disease and the emergence of new antigenic variants of *Eimeria*, the broiler industry has relied upon prophylactic medication. However, anti-coccidial drugs are expensive and their effectiveness is hindered by widespread parasite drug resistance and the high cost of new drug development (Chapman, 1998). Moreover, consumer concern about drug residues in the food supply may eventually force the industry to eliminate this practice.

Broiler breeders are raised in highly isolated conditions to minimize the spread of infectious diseases. Paradoxically, however, in such environments disease susceptible animals have a better opportunity to survive. If a negative association between selected traits and disease resistance exists, breeding stocks may become more susceptible to diseases in response to selection. To date, no evidence exists to support such a negative association in commercial chickens. However, evidence from experimental turkeys (Saif et al., 1984) and chickens (Gavora, 1990) does suggests a negative association between disease resistance and body weight gain, indicating that disease resistance traits should be taken into account in current breeding programs.

There is evidence for genetic variation in disease resistance in chickens. The average heritability of resistance to specific diseases is 0.25 (Gavora, 1990). Selection of White Leghorn chickens for resistance or susceptibility to avian coccidiosis resulted in a 6-fold difference in mortality rates (Johnson and Edgar, 1982). Improvements in disease resistance by selection were also observed for other diseases (Heller et al., 1992; Kaiser et al., 1998; Gavora, 1990). These studies indicate the potential for improvement of disease resistance in poultry through genetic selection. The QTL located in the vicinity of marker LEI0101 and identified in our laboratory strongly supports this possibility. This marker locus was also found to affect disease resistance to Marek' disease in Dr. Chen's laboratory (ARS, East Lansing, MI). Interestingly, the marker locus MCW0058 also was found to affect growth in two independent experiments. These results strongly suggest that there are QTL affecting disease resistance and growth in these chromosomal regions. To apply MAS on a commercial basis to select for coccidiosis resistant breeding stocks, the tightly linked QTL we have preliminarily identified must be further mapped or, better yet, the genes involved must be cloned. To do so, a series of BAC clone contigs overlapping in the chromosomal region must be constructed to develop a new DNA marker, such as an SNP, to be used for future genetic mapping by classical linkage disequilibrium studies (associate mapping).

Our future strategy include 1).extend marker coverage to 95% of the entire chicken genome (to 20 cM intervals) and thereby more precisely map the identified QTL to within a 2-5 cM interval, 2). enhance the detection power of QTL mapping by increasing the sample size of the resource population, 3). construct, identify, and sequence bacterial artificial chromosome (BAC) clones targeting the chicken chromosomal region containing the coccidiosis resistance associated QTL, and 4). identify candidate gene(s) that enhance resistance to avian coccidiosis using DNA microarray analysis.

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